Investigating the association between diet-induced “leaky gut” and the development of prediabetes

By

Nosipho Rosebud Dimba

(218006885)

Submitted as a dissertation component in fulfilment of the requirements for the degree of Master of Medical Science in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal.

Supervisor: Dr. A. Khathi

Co-supervisors: Dr. PS. Ngubane

Department of Human Physiology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

University of Kwa-Zulu Natal

April 2023

PREFACE

High-calorie diets have been shown to dysregulate the gut microbiota composition and to be implicated in the development of type-2 diabetes (T2D). This condition is often preceded by prediabetes. Prediabetes is an intermediate state that occurs between normal glucose regulation and T2D. Various complications, including increased intestinal permeability (IP), are seen in T2D. However, it remains unknown whether increased IP begins in the prediabetic state. Prolonged consumption of diets with low carbohydrates and high unsaturated fats have shown improved glucose tolerance. The effect of this diet on intestinal permeability has not yet been investigated. In our laboratory, a prediabetic rat model was developed using a high-fat high, carbohydrate diet, which mimics the human condition of prediabetes. In this study, this diet-induced prediabetic rat model was used to investigate the changes in gut microbiota and the association between prediabetes and markers associated with intestinal permeability. In addition, to evaluate the effect of a low carbohydrate and high unsaturated fat diet on the concentration levels of markers associated with a leaky gut and glucose homeostasis. The experimental work described in this dissertation was conducted at the University of Kwa-Zulu Natal, Westville Campus, Durban, South Africa. This was conducted under the supervision of Dr. Andile Khathi and co- supervised by Dr. Phikelelani Ngubane.

DECLARATION

I, **Nosipho Rosebud Dimba**, hereby declare that the dissertation entitled:

"**Investigating the association between diet-induced "leaky gut" and the development of prediabetes**" 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.R Dimba **Signature** ___ ___ **Date:** 21 April 2023

Supervisor: Dr A Khathi **Signature**_____ **Date:** 21 April 2023

Co-Supervisor: Dr P.S Ngubane **Signature** _____ **Date:** 21 April 2023

PLAGIARISM DECLARATION

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

MASTERS DEGREE IN MEDICAL SCIENCES 2023

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
2. I have used the Vancouver convention for citation and referencing. Each contribution to, and quotation in, this dissertation from the works of other people has been attributed and has been cited and referenced.
3. This dissertation is my own work.
4. I have not allowed and will not allow anyone to copy my work with the intention of passing it off as his or her own work.

Signature 

PRESENTATION

Symposium Presentation

Dimba, NR., Mzimela, N., Mosili, P., Ngubane, P., Khathi, A. INVESTIGATING THE ASSOCIATION BETWEEN DIET-INDUCED "LEAKY GUT" AND THE DEVELOPMENT OF PREDIABETES. School of Laboratory Medicine and Medical Sciences Research Symposium **30 November 2022**. University of KwaZulu-Natal, Durban, South Africa.

DEDICATION

This work is dedicated to God, my supportive grandmother and my late mom.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Dr. Khathi for his continuous support, guidance, patience, motivation, enthusiasm, and immense knowledge he has shown to me and my project this year. I could not have imagined having a better supervisor for my master's project.

To my co-supervisor, Dr. Ngubane, I would like to thank him for his continual support and all his work behind the scenes with my supervisor.

My sincere thanks also go to Mr. Dennis Makhubela for technical support, the College of Health Sciences, School of laboratory medicine and medical science, and the UKZN Biomedical Resource Unit for providing me with the necessary facilities for the research. To the National Research Foundation, thank you for your financial support throughout this project.

To my mentor Palesa, I thank you for your kindness, constant encouragement throughout my research, working with me before deadlines, and always making up time for me if I need help or guidance.

I would like to thank the endocrinology group for their moral support.

Last but not least, to my caring, loving, and supportive grandmother, your prayers and encouragement are much appreciated when times get rough.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADA	American Diabetes Association
BRU	Biomedical Research Unit
CRP	C-reactive protein
DI	Dietary intervention
ELISA	Enzyme linked immunosorbent assay
HBA1c	Glycated haemoglobin
HFHC	High fat high carbohydrate
IDF	International Diabetes Federation
IFABP	Intestinal fatty-acid binding protein
IFG	Impaired fasting glucose
IFN-γ	Interferon gamma
IGT	Impaired glucose tolerance
IL-6	Interleukin 6
IP	Intestinal permeability
IRS-1	Insulin receptor subtrate-1
JNK-1	Jun N-terminal kinases
LCHUF	Low carbohydrate high unsaturated fat
LPS	Lipopolysaccharides
MLCK	Myosin-light chain kinase
NF-KB	Nuclear factor kappa B
NPD	Non-prediabetes
OGTT	Oral glucose tolerance test
PD	Prediabetes
SCD14	Soluble CD14
T2DM	Type 2 diabetes Mellitus
TNF-α	Tumor necrosis factor-alpha
UKZN	University of Kwazulu-Natal
WHO	World Health Organisation

STUDY OUTLINE

This dissertation is presented in manuscript format, consisting of 4 chapters. Chapter 1 provides a background of the research from other articles, a literature review, aims, objectives, and the hypothesis of the study. Chapter 2 contains a prologue and the first study in manuscript form, which seek to investigate changes in gut microbiota composition and the association between prediabetes and markers associated with intestinal permeability. This study further investigated if there could be any links between intestinal permeability and the development of prediabetes. This work is authored by NR. Dimba under the supervision of Dr A. Khathi, co-supervised by Dr P.S. Ngubane and has been formatted and submitted for publication in the "Journal of Experimental Clinical Endocrinology and Diabetes" according to journal's guidelines. Chapter 3 contains a prologue and the second research study in manuscript form, which sought to investigate the effect of a low carbohydrate and high unsaturated fat diet on glucose homeostasis and concentration levels of markers associated with a leaky gut. The manuscript is authored by NR. Dimba under the supervision of Dr A. Khathi and has been formatted and submitted for publication in the "Journal of Nutrition and Diabetes" according to journal's guidelines. Chapter 4 is the synthesis of the study with the aim of linking the two studies and the conclusion as well as the appendix which consists of the ethical clearance, abstract and the journal's guidelines to authors.

ABSTRACT

Introduction

Type 2 diabetes (T2D) is the most common type of diabetes mellitus, which is reported to be associated with life-threatening co-morbidities. This condition is characterized by hyperglycaemia due to a defect of the insulin receptor, and its often preceded by prediabetes. Chronic consumption of a calorie diets is the primary cause of T2D, and this diet has been associated with altered intestinal permeability in diabetic patient. However, it remains unknown whether increased intestinal permeability complications begin in the prediabetic state. Previous studies done in our laboratory developed a high-fat high carbohydrate (HFHC) diet-induced prediabetic animal model, using male Sprague Dawley rats. This model was found to mimic the human condition of prediabetes. In this model, the animals develop prediabetes after 20 weeks of ingesting a HFHCdiet. Using this HFHC diet-induced animal model of prediabetes, this study sought to investigate the changes on gut microbiota and the association between prediabetes and markers associated with intestinal permeability. Furthermore, this study sought to investigate changes in concentration level of markers associated with a leaky gut and glucose homeostasis, following change to a low carbohydrate, high unsaturated fat (LCHUF) diet.

Method and Materials

12 male Sprague Dawley rats (3 weeks old) were randomly assigned into the non-prediabetic group and diet-induced prediabetic group (n=6). Group A animals were exposed to a standard diet with normal drinking water for 20 weeks, and group B animals were exposed to a HFHC diet supplemented with 15% fructose for a period of 20 weeks. After 20 weeks, the American Diabetes Association criteria for diagnosis of prediabetes was used to diagnose prediabetes in all animals. The fecal samples were analyzed to measure the gut microbiota composition of *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* in both animal groups. Furthermore, blood glucose, plasma insulin, serum zonulin, plasma lipopolysaccharide (LPS), soluble CD14 (sCD14), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), and intestinal fatty-acid binding protein (IFABP) concentrations were measured. The first manuscript measured all these parameters at 20 weeks.

In the second manuscript, 12 male Sprague Dawley rats were used and fed a HFHC diet for 20 weeks. The prediabetic animals were subdivided into two groups. Group A animals remained on the HFHC diet (the prediabetic control group), while the other 6 animals in group B were switched to a low carbohydrate, high unsaturated fat (LCHUF) diet. Group B was then categorized as the prediabetic group that had dietary intervention (PD+DI). All animals were then maintained on their respective diets and monitored for further 12 weeks. The fecal samples were analyzed to measure the gut microbiota composition of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in both animal groups. Furthermore, blood glucose, glycated haemoglobin (HbA1c), serum zonulin, plasma LPS, sCD14, TNF-

α, IL-6, CRP,

and IFABP concentrations were measured. The second manuscript measured all these parameters at 32weeks.

Results and Discussion

Prolonged consumption of a HFHC diet results in the development of prediabetes. This was evidenced by a significant increase in fasting blood glucose and plasma insulin in the prediabetic animals compared to the non-prediabetic animals. The HFHC diet also showed to dysregulate the gut microbiota causing gut dysbiosis which enhances translocation of endotoxins from the gut lumen into the bloodstream that elicits an inflammatory response. In the prediabetic group (PD), there was a reduction in the *Firmicutes* levels and an increase in *Bacteroidetes* and *Proteobacteria* compared to the non-prediabetic group (NPD). Serum zonulin, plasma sCD14, TNF-α, IL-6, CRP, and IFABP concentrations in the PD group were increased compared to the NPD group, while plasma LPS concentrations were similar. The low-grade inflammation that is observed in the prediabetic state is suggested to further progresses onset of prediabetes. However, to reverse prediabetes and to combat a leaky gut problem, the second manuscript illustrated that switching to a LCHUF diet can effectively improve glucose homeostasis thus reverse prediabetes. This was evidenced by a significant decrease in fasting blood glucose and HbA1c concentration. These results were accompanied by a decrease of *Firmicutes* and an increase of *Bacteroidetes* and *Proteobacteria* suggesting that a LCHUF diet effectively improved gut microbiome composition. This caused the release of serum zonulin and its effect on disassembling the tight junctions to decrease. This was evidenced by a decrease in plasma LPS and sCD14 concentration. In addition, we also observed a decrease in plasma TNF-α, IL-6, CRP, and IFABP indicating another beneficial effect of this diet on reducing intestinal inflammation, and risks of insulin resistance.

Conclusion

Taken together, these results suggest that chronic consumption of the HFHC diet may be associated with the dysregulation of gut microbiota, leading to increased intestinal permeability. This could be associated with chronic subclinical inflammation that have been reported to result in the development of insulin resistance in the pre-diabetic stage. In addition, a LCHSF diet markedly improved intestinal permeability as well as the glucose regulation.

CHAPTER 1: LITERATURE REVIEW

1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycaemia due to impaired insulin secretion or insulin action (1, 2). Type 2 diabetes mellitus (T2DM) is characterized by hyperglycaemia due to a diminished insulin sensitivity (3). Diabetes mellitus resulted in the death of approximately 1.5 million people in 2019 and, T2DM estimated for 90-95% of those deaths (1, 3). According to a previous study, more than 550 million people will have prediabetes by 2040 (4). Various complications including increased intestinal permeability are seen in T2DM (De Kort et al., 2011, Mabuza et al., 2019). The onset of T2DM is often preceded by a long-lasting, asymptomatic condition known as prediabetes which is a state of intermediate hyperglycaemia which occurs between normoglycemia and T2DM (5, 6). This condition is characterized by impaired glucose tolerance, impaired fasting glucose, and moderate elevation in glycated haemoglobin (7).

Increased intestinal permeability, also known as "leaky gut," is a condition in which the lining of the intestines becomes more permeable, allowing translocation of endotoxin lipopolysaccharides (LPS), peptidoglycan (PG), bacterial fragments, gut bacteria, and antimicrobial compounds from the mucosa into the circulation triggering an immune response (8). According to recent literature, high-calorie diets damage the intestinal mucosal barrier, enterocytes as well the permeability of the entire intestine causing intestinal leakage in T2D patients (9). The high-calorie diets cause gut dysbiosis by altering the maintained microbiota diversity in the gut, resulting in a reduction of firmicutes like *Lactobacillus*, *Bacillus*, and *Clostridium* which protect the intestinal lining causing an increase in zonulin expression. Zonulin is a marker that is associated with a leaky gut that has been shown in loosened-up tight junctions. The leakage in leaky gut causes an inflammatory response that play a role in many diseases, including diabetes, obesity, autoimmune and gastrointestinal diseases (10). However, it is unknown whether there are changes in intestinal permeability (IP) in the pre-diabetic state and what role this may play in the development of insulin resistance. A study conducted in our laboratory developed a high-fat high carb (HFHC) diet-induced prediabetic animal model which mimics the human condition of prediabetes (11). Using this animal model, the first manuscript of this study sought to investigate changes in intestinal permeability and in the development of diet-induced prediabetes.

Previous studies have shown prediabetes to be reversible (12). The management of prediabetes relies heavily on lifestyle modification that include moderate exercise and dietary intervention to low calorie diets (12, 13). Several types of diets have been postulated to possess ameliorative effects and these include the banting, atkins and ketogenic diet (14, 15). All these diets involve the use of low amount of carbohydrate and high amount of unsaturated fats (16). The effect of such diets however, on intestinal permeability in the prediabetic state remains unknown. In the second manuscript of this study, we

evaluated the effect of a low carbohydrate and high unsaturated fat (LCHUF) diet on markers associated with intestinal permeability and glucose homeostasis in a diet-induced prediabetic rat model.

1.1 Intestinal permeability

In the stomach, the intestinal tract is lined by a single layer of epithelial cells, which form a part of the dynamic and semi-permeable gut barrier (17). These cells are highly specialized in allowing specific molecules to pass through the aqueous pore. Smaller molecules pass by the transcellular pathway, while the larger molecules pass by the paracellular pathway as they vary in size (8, 17). Intestinal permeability is an essential component of the intestinal barrier in which this barrier is formed by tight junction proteins that seal the paracellular space between adjacent cells and compromised of physical (mucus and the epithelial cells), biochemical (bile salts, enzymes, antibacterial proteins), immunological (IgA and immune cells), and microbial components (the microbiota) (18). All these factors maintain the proper functioning of the intestinal barrier (18). The tight junctions form an essential part of the intestinal barrier, and they are responsible for the adherent of intestinal epithelial cells to another. These junctions are made up of transmembrane proteins, including adhesions, claudins, tricellulin, coupling adhesion molecules (JAM), angulins, and occluded zonules (ZO) that are attached to the cytoskeleton of the actin cytoskeleton. Interaction of the transmembrane maintains the overall tight junction and barrier integrity and controls the movement of molecules across the cell space within the paracellular pathway (19).

Intestinal epithelial cells function to absorb nutrients while also preventing translocation of toxins, harmful bacteria (microorganism), and dietary antigens from the mucosal barrier into the blood circulation (20). The intestine also consists of what is known as the gut microbiome or microbiota, representing a diversity of microorganisms, including bacteria, fungi, archaea, viruses, and eukaryotic cells; all these components live inside the intestinal tract in both human and animals (21). The intestinal microbiota's function in many processes, including the production of crucial vitamins, minerals, and nutrients, the breakdown and absorption of nutrients, prevention of colonization by pathogens, as well as support in bone development, and promote better sleep, (22). Firmicutes being one of the dominant phyla in the gut, they also function to protect the intestinal barrier thus prevent inflammation by inhibiting NF-kB and IFN-*y* production (23). They achieved this function by fermenting carbohydrate into variety of short chain fatty acid like butyrate (24). Butyrate maintains the tight junction and strengthen the intestinal barrier. The imbalance of the gut microbiota or any defect in the tight junction epithelial cells with the intestinal barrier could lead to gut complications such as increased intestinal permeability (19, 25).

Previous studies have shown that it is not only the high caloric diet or hyperglycaemic state characterized in T2DM patients that affects intestinal permeability but several factors such as gut microbiota modification, mucus layer alteration, epithelial damage, and other lifestyle choices such as

overconsumption of alcohol (26, 27). The above-mentioned factors in combination with HFHC diet and hyperglycaemia have been shown to contribute to even further changes of IP (27, 28).

The various factors that change intestinal permeability result in a condition known as increased intestinal permeability or leaky gut in which the gut epithelium wall's tight junctions loses their integrity, allowing increased toxic compounds such as lipopolysaccharide (LPS) or LPS-containing bacteria, peptidoglycan, luminal antigens, and partially large undigested food from the lumen to translocate into the bloodstream or to the tissues beneath the gut (8). The toxic products from the microbiota that are released induce inflammation in the adipose tissue and impact the gut microbiota, which leads to several disorders associated with the digestive tract, such as peptic ulcer disease, inflammatory bowel, irritable bowel syndrome, and celiac disease (8). These toxic compounds, when primarily found in the bloodstream, have been associated with intestinal permeability through the activation of macrophages and pro-inflammatory cytokines.

1.2 Markers associated with leaky gut and microbial translocation

1.2.1 Zonulin

Zonulin was initially identified as an endogenous human analogue of zonula occludens toxin (Zot), a bacterial enterotoxin produced by the intestinal bacterium Vibrio cholerae (Wang et al., 2000). Zonulin, a 47kDa protein, functions to regulate leakiness in the gut by opening and closing spaces between the intestinal lining cells, the tight junctions, to allow the absorption of nutrients and other beneficial molecules into the intestine (Sturgeon and Fasano, 2016). In healthy individuals, zonulin levels increase but contingent on which factor is responsible for triggering its release. It can play a role in the pathogenesis of the autoimmune disease such as sepsis, celiac disease, multiple sclerosis (29)

Two main factors identified to increase the serum level of zonulin in the gut:

1.2.1.1 Bacterial colonization

Gut dysbiosis results when the imbalance between beneficial and harmful bacteria transforms the gut community. In this community, the abundance of harmful bacteria increases in the stomach (22), and this condition is usually caused by overconsumption of a high-fat diet (22). Some of these harmful bacteria induce increased zonulin expression, which leads to this protein providing a protective mechanism by weakening the tight junctions to reduce/eliminate bacteria (30). Symptoms like diarrhea, constipation, bloating, fatigue, and abdominal pain are associated with an increased serum level of zonulin, which causes a leaky gut and allowing, even more, larger molecules or toxins to pass through the tight junctions and induce low-grade systemic inflammatory that shown to worsen the tight junction integrity even further (22).

1.2.1.2 Gluten

The second factor is gluten and two more of its component's gliadin and glutenin found in rye and wheat, which promotes the release of zonulin and causes intestinal diseases (30). These proteins are insoluble/ not easily digested due to high amounts of proline and glutamine which induces changes in the intestinal barrier, thus result into loss of tight junction integrity (31) .

The effect of zonulin on increased intestinal permeability is mediated by activation of epidermal growth factor receptor (EGFR) through proteinase-activated receptor 2 (PAR_2) as well as G protein-coupled receptor PAR_2, which transactivates EGFR (32). When these two receptors are activated, they reduce transepithelial resistance, which means that intestinal permeability increases (32). Zonulin loosens the gut barrier integrity, followed by translocation of microbial antigen and endotoxin, triggering an innate and adaptive immune response, causing activation of proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-*y* (IFN-*y*). Activation of these cytokines further damages the intestinal cell, and microvilli resulting in nutrient absorption deficiency (22).

Increased intestinal permeability with elevated zonulin levels has been associated with inflammatory bowel disease, celiac disease, multiple sclerosis, and diabetes (30). In manuscript one of the study, serum zonulin will be the first marker used in measuring intestinal permeability in a prediabetic state. In manuscript two, this marker will also be measured after inducing dietary intervention of a LCHUF diet assess changes in zonulin concentration.

1.2.2 Lipopolysaccharides (LPS)

LPS are significant components of Gram-negative bacteria's outer membrane; they contribute to this bacteria's structural integrity (33). LPS is comprised of a distal polysaccharide (also termed O-antigen) and a non-repeating "core" oligosaccharide region that is anchored in the outer bacterial membrane by a lipophilic, carbohydrate lipid moiety termed lipid A (34). Gut microbiotas consist of most of this endotoxin kept within bacteria cells and released only after destruction or replication of the bacteria (34). LPS function to protect the membrane from any chemical attacks, induce a robust immune response in humans, and increase the cell membrane's negative charge to assist in stabilizing the entire structure.

Under normal circumstances, lipopolysaccharides are present in the intestinal lumen and not to have any harmful effects or trigger an immune response within the lumen. In humans, low plasma levels of LPS get translocated from the lumen into the circulation, which does not affect the health of the individual (35). Plasma LPS increases only after the consumption of a high caloric/ fat diet in a healthy individual (36). However, evidence suggests that higher concentration of LPS present in the circulation of patients with T2DM or with obesity results in increased intestinal permeability or in diseases associated with the digestive tract (37). The increase of LPS resulting in the pathophysiology's state before can be attributed to the amount of fat consumed or "excessive intake" of high caloric/fat diet and

persistent hyperglycaemia. T2D patients have shown to have increased plasma levels of LPS (37) due to long-term hyperglycaemia. Due to this, LPS will be used in manuscript one as one of the markers associated with leaky gut and bacterial translocation. In manuscript two, this marker will also be measured after inducing dietary intervention of a LCHUF diet to assess changes in LPS concentration.

1.2.3 Soluble CD14

Soluble CD14(sCD14) is a co-receptor for LPS, and it an activation marker for monocytes and other mononuclear cells released after stimulation (38). Upon translocation of LPS from the lumen into the blood, this toxin induces secretion of sCD14 from the immune cells (39). A previous study speculated that high levels of this co-receptor in the blood indicate LPS exposure (39). Therefore, sCD14 in diet-induced prediabetic rat's models may be used as a marker in manuscript one to evaluate whether there is LPS exposure in the blood circulation. In manuscript two, this marker will also be measured after inducing dietary intervention of a LCHUF diet to observe any changes in LPS exposure.

1.2.4 Intestinal fatty acid-binding protein (IFABP)

Intestinal fatty acid-binding protein (IFABP) is an intracellular protein that is found in the epithelial cells of the mucosal layer of the small and large intestine tissues (40). According to literature, high levels of IFABP in the circulation only get translocated when two enterocyte and the mucosal tissue is damaged (41). Hence, IFABP was considered as the potential marker of the intestinal damage. Increased serum levels of this protein have been shown to be associated with minor intestinal diseases such as coeliac disease (42). However, it remains unknown whether IFABP concentration is elevated in prediabetic stage. Hence, IFABP becomes another potential marker in manuscript one that will be measured to assess whether features such as intestinal barrier, enterocyte and the tight junction are compromised in the prediabetic stage. In manuscript two, this marker will also be measured after inducing dietary intervention of a LCHUF diet to further assess intestinal barrier state.

1.2.5 C-reactive protein (CRP)

Increased intestinal permeability have been shown as of the risk factors for chronical intestinal diseases because it triggers chronic inflammation. C-reactive protein being an inflammation marker, it was reported to be associated with zonulin (43). A previous study investigated patients with T2D that had healthy and unhealthy metabolic profiles (44). Patients with unhealthy metabolic profile shown to have high levels of PG, LPS-binding protein and C-reactive protein compared to those with healthy metabolic profiles (44). They speculated that these results indicate that there is a certain risk of developing chronic intestinal disease in patients with T2D (44). However, it has never been discovered whether CRP is elevated during the prediabetic stage. In manuscript one, CRP will be one of the markers measured to examine if there's any chronical inflammation during the prediabetic state. In manuscript two, this marker will also be measured after inducing dietary intervention of a LCHUF diet to assess any changes to an inflammatory response.

1.3 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases that results in hyperglycaemia due to defects in insulin secretion, insulin action or both (1, 2). DM is classified into two types, there is type 1 diabetes (T1D), which is an insulin-dependent type, and the most common type is type 2 diabetes (T2DM) which is non-insulin-dependent. T1D results from cellular-mediated autoimmune destruction of beta-cells (islet of Langerhans), thereby impairing the release of insulin hormone that is required to make glucose enter cells for energy (2). In contrast, T2DM is characterized by hyperglycaemia, an increase or high blood glucose in the bloodstream due to a defect of the insulin receptor (3). In T2DM, the body cells resist the insulin effect, which drives glucose from the blood into the interior of the cells. Glucose that is not used up by the cells or muscles accumulates in the circulation and further worsens the type 2 diabetic complications such as increased intestinal permeability (9).

1.3.1 Intestinal permeability (IP) in T2D

Patients with T2D have long-term hyperglycaemia, which is considered as one of the factors that damage the intestinal barrier, epithelial cells and the overall intestinal permeability (9). Chronic consumption of a high calorie diet has been shown as a secondary factor not only in the progression of T2DM but also leads to the dysregulation of the intestinal permeability (27, 28). In the gastrointestinal tract, chronic consumption of a high caloric/fat diet causes an increase in satiety by stimulating the secretion of orexigenic hormones such as glucagon-like peptide-1 and cholecystokinin (5). These two hormones limit the rate at which food passes through the digestive tract by delaying gastric emptying and decreasing motility. Since there is more food that is kept in the stomach, the body degrades it into smaller glucose to allow for absorption by the intestinal epithelial cells (45).

Excess glucose circulates into the blood and further increases the hyperglycaemic state that has already been characterized in T2DM. Inside the gut, long-term hyperglycaemia and high levels of saturated fats promote growth and provide nourishment to harmful bacteria. Such effects shift the maintained equilibrium or imbalances of the gut's microbiome and cause these harmful bacteria to become overwhelmed and outcompete the essential microbe resulting in gut dysbiosis (22, 46). Some of the harmful bacteria induce increased zonulin expression disrupting the tight junctions, leading to markers associated with increased intestinal permeability, zonulin and LPS being translocated from the mucosal barrier, resulting in cross-reaction with host tissues to enter the bloodstream (37). All these substances disrupt the protective features on the epithelial cells, resulting in thickening of the mucus that traps harmful microbial products, destroying epithelial cells, producing an increase of gram-negative bacteria, causing a decrease in transepithelial resistance and finally disorganizing of tight junction proteins, which leads to an increase IP (18).

1.3.2 LPS effect in the circulation in T2D

Hyperglycaemia has been shown to cause changes in the microbiota by shifting the maintained equilibrium of essential and harmful bacteria resulting in gut dysbiosis, as previously discussed. This dysbiosis has been shown to dysregulate the intestine's integrity by increasing zonulin expression, causing an increased intestinal permeability which further enhances the translocation of toxins and microbial products, specifically LPS and peptidoglycan, due to loss of tight junction proteins such as ZO-1 (30). LPS is translocated from the intestinal lumen into the circulation by a mechanism facilitated by chylomicrons synthesized from the intestinal epithelial cells in response to HFHC/ hyperglycaemia from T2DM (17). Toll-like receptors form part of the transmembrane pattern-recognition receptor which provides an essential function in the recognition of microbial and in the control of the immune response. LPS have been shown to serve as a ligand for toll-like receptor 4 (TLR-4) which is present on immune cell membrane surfaces like macrophages, monocytes, and others such as endothelial cells and adipocytes (47). The binding of LPS to TLR-4 triggers an inflammatory process resulting in the release of various cytokines from these immune cells such as TNF-α, IFN-γ, and IL-6 in the lumen (37). TNF-α, during an inflammatory process, augments paracellular permeability by removing transmembrane proteins such as claudin-1 from tight junctions, increasing claudin-2 expressing and enhancing occluding degradation (48). In contrast, IFN-*y* changes in cytoskeletal rearrangement as well as changes in the tight junction protein expression and localization; this effect increases intestinal permeability (49). Furthermore, these cytokines in the circulation have been shown to cause peripheral insulin resistance in the liver, muscle, and adipose tissues by increasing inflammation, activating JNK1 and NF-kB, which results in serine phosphorylation of insulin receptor substrate-1 leading to insulin resistance (50). The leakage of LPS also targets the pancreas triggering inflammation and dysfunction of the pancreatic β cells causing insulin secretory defects. All these effects further worsen the hyperglycaemic state in T2DM (50). In manuscript one, a measure of cytokines such as IL-6, TNF-α, and INF-y, could serve as a parameter in determining increased intestinal permeability. In manuscript two, these cytokines will also be measured after inducing dietary intervention of a LCHUF diet to assess any changes to an inflammatory response and intestinal permeability state.

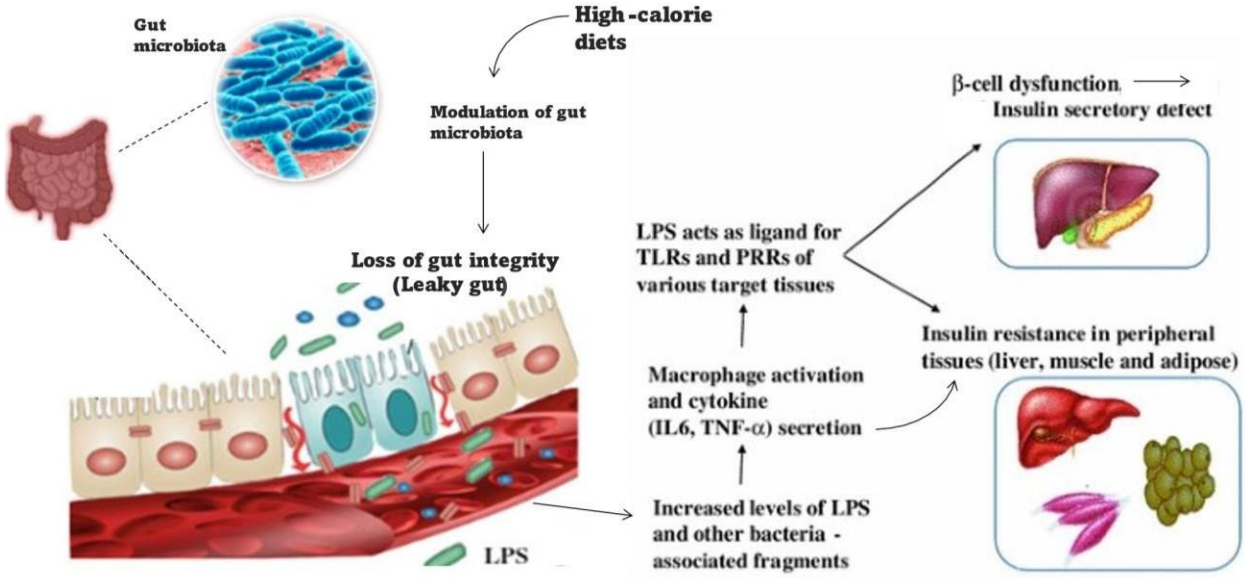

Figure 1: The association of zonulin, lipopolysaccharides, inflammatory markers, and insulin resistance.

Adapted from (37).

1.4 Prediabetes

Prediabetes is characterized as intermediate hyperglycaemia, in which fasting glucose levels in the blood are above the homeostatic range but below the threshold for diagnosis (4). It is correlated with the simultaneous presence of insulin resistance and or pancreatic β cell dysfunction way before glucose changes is detectable (4, 51). The increase in the prevalence of prediabetes has led to scientists predicting that greater than 470 million people will have prediabetes in 2030 (4). According to the World Health Organization (WHO), prediabetes criteria is defined by two measurement parameters; impaired fasting glucose (IFG) which can be further defined as fasting plasma glucose of 6.1 – 6.9 mmol/L, and impaired glucose tolerance (IGT) which is defined as postprandial or 2hr plasma glucose of 7.8-11.0 mmol/L after ingestion of 75g of oral glucose load or a combination of both based on a 2hr oral glucose tolerance test (OGTT) (52). The America Diabetes Association has the same cut-off value of IGT; however, it has lowered the cut-off levels for IFG with a parameter of about 5.6-6.9 mmol/L, and in addition to that, a haemoglobin A1c (HbA1c) based criteria of a level of 5.7%-6.4% used as diagnostic criteria for prediabetes (4, 53). As previously discussed in T2D, various complications have been studied that begin in prediabetes, for example, immune dysfunction, cardiovascular disease, and immune response dysregulation (54, 55). Yet, for the state of intestinal permeability, it is still unknown whether dysregulation of its barrier begins in the prediabetic stage. This will be investigated in manuscript one of the study. Approaches to reversing prediabetes have gradually increased recently, which include dietary intervention with low-carb diets such as banting, Atkins, and keto. All these types of diets have been shown to manage prediabetes, however, the effect of these diets on intestinal

permeability in the prediabetic state remains unknown and this will be investigated in manuscript two of the study.

1.5 Risk factors leading to prediabetes and T2D

Dietary fat, saturated fat, and a high-calorie diet have been identified as risk factors that precede the onset of prediabetes and its progression to T2D (56, 57). Other than diet, there are other risk factors including obesity (58). All these types of diets along with a lack of physical activity have been shown to be associated with obesity (59). These two risk factors result in an increase of free fatty acid in the circulation, which causes an increase in blood glucose levels (60). To compensate for hyperglycaemia, the pancreas β-cells secrete insulin to lower blood glucose which leads to exhaustion and dysfunction of β-cells in the pancreas (61). Eventually, this effect causes the development of insulin resistance through the reduction of insulin receptors as well as decreasing glucose transport which subsequently leads to T2D (62). Patients with obesity tend to have accumulated adipose tissues (60). These tissues cause increased secretion of pro-inflammatory cytokines into the bloodstream and further damage the pancreatic β-cells, causing exacerbation of hyperglycaemia (63).

1.5.1 Effect of obesity on intestinal barrier

Excessive consumption of a high-calorie diet increases the metabolic activity of the gut microbiota, which is to extract calories and store them in adipose tissues for later use (64). The intake eventually leads to the overload and enlargement of adipocytes and also provides energy for bacterial growth and proliferation (64). This results in an alteration of the intestinal barrier function, leading to increased intestinal permeability and favouring of bacterial endotoxin translocation (LPS) to the circulation (65). Endotoxemia and overgrowth of adipocytes activate or increase the secretion of pro-inflammatory cytokines, which subsequently cause chronic low-grade inflammatory state and insulin resistance observed in people with obesity (66).

1.5.2 Effect of diet on the intestine barrier

The western diet has also been reported as a risk factor for altering the intestinal barrier and inducing intestinal inflammation (67). The diet compromises high levels of saturated fat, trans-fatty acid, refined sugars, and high-sweetened refined sugars that had been shown to shift the maintained microbiota composition (68). The imbalance of the gut microbiome dysregulates the intestinal barrier thus causing activation of an immune response upon translocation of the toxins and bacterial antigens (8). Activation of an inflammatory response causes the secretion of various cytokines which results in chronic inflammation that damages the enterocytes and epithelial cells of the intestine (40).

Previous studies have shown that avoiding a western-style and gluten-rich diet might improve gut permeability and thus restore disrupted tight junctions (31, 40). Another study used prebiotic components and dietary fiber to avoid gut dysbiosis, thus preventing a leaky gut (69, 70). Prebiotic dietary fiber stimulates the growth of beneficial species. They also function to improve the immune

system, reduce protein fermentation/ pathogenic bacterial composition, increase calcium absorption, and thus help to keep intestinal integrity intact. (40, 71). Consumption of a diet supplemented with probiotics has also been shown to be able to prevent increased intestinal permeability (72). This is through maintaining microbiota diversity, improving the integrity of the tight epithelial junction, and protecting against mucosal injury (72). They achieve all these functions by enhancing the expression of a junction-associated protein (ZO-1), which causes a reduction of the leaky gut by also enhancing the junctional complexes (73). Another possible way is by activating a cell signalling pathway that can reduce increased intestinal permeability by improving intestinal barrier integrity (73).

Zinc supplements have grown in popularity in recent years because of the expanding scientific research demonstrating their beneficial effects on gut healing via strengthening intestinal barrier function (74). These supplements in diet have been shown to improve the tight junction by increasing the expression of occluding and ZO-1 proteins (75). Furthermore, these supplements protect against bacterial toxins-induced intestinal dysfunction (76).

A diet supplemented with glutamine has also been discovered to combat leaky gut (40). The glutamine effect is through the enterocyte proliferation and cells lining the small intestine (77). These cells regulate mucosal epithelial tight junction proteins, reduce pro-inflammatory signalling pathways, and protect the epithelial cell against apoptosis cellular stressors (40, 77). However, the effect of a diet supplemented with glutamine, zinc, prebiotics, and probiotics on improving the gut is still debatable, and additional information is required for further scientific research.

1.6 Prediabetes management/ interventions

Over the past few years, possible approaches to reversing prediabetes have been investigated to reduce or prevent T2D progression and its complications. Dietary interventions, including a diet low in carbohydrates and high in unsaturated fat, reported to manage prediabetes (78). The intensive lifestyle modifications also improved insulin sensitivity, reduced cardiovascular risks, and decreased glycemia (12). The use of metformin was also reported useful in restoring normal glucose regulation in patients with prediabetes (79). However, this pharmacological intervention has been shown to be less effective since patients typically disregarded dietary intervention/ physical activities and became overly reliant on pharmacological intervention, and this affected the effectiveness of metformin (80). Recent studies conducted in our lab investigated compounds that will remain effective even in the absence of dietary modifications. These studies used plant-derived oleanolic acid (OA) and ruthenium Schiff base complex as alternative treatments (54, 81). Both compounds had a beneficial effect on preventing the progression of prediabetes to T2D by restoring insulin sensitivity and regulating glucose homeostasis without the use of diet intervention (54, 81). But this study is more focused on a LCHUF diet.

There are various types of diets have been used in the management of prediabetes these include banting, Atkins, and the ketogenic diet (14, 15). All these diets involve the use of a low amount of carbohydrates

and a high amount of unsaturated fats (16). Banting, Atkins, and the keto diet have been shown to be beneficial for people with obesity, diabetes, or prediabetes (14). In the body this diet plan causes the body to get into the metabolic state of ketosis and eventually the body gets adapted to primarily burning fat as the main source of fuel instead of sugar from carbohydrates (82). This results in fat loss while preserving muscle mass thus reducing the secretion of insulin hormone in pancreatic β-cells (83). Atkins also involves the consumption of a large amount of proteins and restricting carbohydrates to 20 grams daily (84). It starts with a very specific phase and then goes to a maintained phase, which allows more range of nutrients to be absorbed by the body (84). According to the literature, all these types of diets have shown a beneficial effect on regulating blood glucose by improving insulin sensitivity, insulin resistance, and glycated haemoglobin (HbA1c) levels (85-87). They aid in weight loss by decreasing triglycerides and they also reduce the risk of cardiovascular diseases by increasing good high-density lipoprotein cholesterol levels (88, 89). Therefore, manuscript two of the study sought to investigate the effect of a low carbohydrate high unsaturated fat (LCHUF) diet on markers associated with intestinal permeability and glucose homeostasis in a diet-induced prediabetic rat model.

1.7 HFHC diet-induced animal model

Rats first became an important animal to use in research around 1906. More specifically, in Medical Research, scientists use these animals to develop medical procedures, investigate pathogenesis, complication and test possible therapeutic agents to treat global burden disorders such as type 2 diabetes (90). This use of a HFHC diet in an animal model is due to the high current consumption of takeout food with high-fat and high carbohydrates and drinks containing high fructose levels in a majority of the world's population today individual (91). This lifestyle leads to numerous diseases such as obesity, cardiovascular disease, T2DM, and more specifically, its preceded state, prediabetes (27, 28). A previous study conducted in our laboratory developed a HFHC diet-induced animal model that induced a prediabetic state which mimics human condition of prediabetes with its complications (11). Male Sprague Dawley rats were used in developing this model as they have shown in T2DM animal models, to have genetic disposition to exhibit a great distribution in HFHC diet-induced body weight gain, which resembles the genetic heterogeneity of T2DM in humans (92, 93). They were divided into two groups; then, after 20 weeks, changes in glucose homeostasis resembling prediabetes in human condition were observed, and IFG and IGT parameters were measured applying ADA criteria (11, 81). In addition, the study also investigated the cardiovascular function in the pre-diabetic state, investigating whether the risk factors related to cardiovascular disease are related to T2DM, and the results indicate that the risk of cardiac complications in the pre-diabetic state is increased (11, 81). Other studies have shown that an animal-fed HFHC diet develops prediabetes and various metabolic derangements, including increased glycated haemoglobin (HbA1c), impaired glucose tolerance, and elevated plasma ghrelin

levels (81). After rats were fed a HFHC diet, the above changes eventually led to diabetes associated with renal and cardiovascular complications.

A diet containing high-calories and fats has been reported as the primary factor in the development of prediabetes (57). In T2D patients this diet compromises the intestinal barrier function, leading to increased intestinal permeability (27). Therefore, as part of manuscript one, the effect of high-fat high carbohydrate (HFHC) diet-induced prediabetes on intestinal permeability will be investigated on this study. Management and intervention for reversing prediabetes include lifestyle modification (moderate exercise), and dietary and or pharmacological intervention (80, 85). Hence, manuscript two sought to investigate the effect of a low carbohydrate high unsaturated fat diet on markers associated with intestinalpermeability and glucose homeostasis in a diet-induced prediabetic rat model.

2. Aim

To investigate the effect of high-fat high carbohydrate (HFHC) diet-induced prediabetes on intestinal permeability in male Sprague Dawley rats. In the second study, we sought to evaluate the effect of a low carbohydrate and high unsaturated fat diet on the concentration levels of markers associated with intestinal permeability. In addition, investigate the association of the changes in these markers with changes in glucose homeostasis.

3. Objectives

The objectives of the entire study are separated into two studies that are presented as manuscripts.

Objectives of Manuscript 1 were:

- To determine microbial levels of *Firmicutes*, *Proteobacteria* and *Bacteroidetes* in fecal stool
- To determine the effect of diet-induced prediabetes on glucose homeostasis by measuring fasting blood glucose concentration and plasma insulin concentration
- To examine the OGT test following induction of prediabetes with HFHC diet
- To measure serum zonulin concentration to investigate the integrity of the intestinal barrier
- To measure plasma LPS and sCD14 concentration to determine bacterial translocation
- To evaluate the level of secretion of inflammatory markers at pre-diabetic stage
- To measure plasma IFABP concentration to determine intestinal damage

Objectives of Manuscript 2 were:

- To determine microbial levels of *Firmicutes*, *Proteobacteria* and *Bacteroidetes* in fecal stool
- To determine the effect of a LCHUF diet on glucose homeostasis by measuring fasting bloodglucose concentration and plasma glycated haemoglobin (HbA1c) concentration

- To measure serum zonulin concentration in pre-diabetic rats to investigate the integrity of the intestinal barrier
- To measure plasma LPS and sCD14 concentration in pre-diabetic rats to determine bacterial translocation.
- To investigate if pro-inflammatory cytokines concentration decreases in in pre-diabetic stage.
- To measure serum IFABP concentration in prediabetic rats to determine intestinal damage.

4. Hypotheses

During the prediabetic stage, there will be changes in the concentration of markers associated with leaky gut and those of bacterial translocation. The effect of a low carbohydrate and high unsaturated fat (LCHUF) diet intervention will be effective in improving intestinal permeability so as the glucose regulation.

Null hypotheses: There is no significant association between diet-induced leaky gut and the development of prediabetes. A LCHUF diet intervention has no effect on the concentration levels of markers associated with a leaky gut.

5. References

1. Association AD. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2010;33(Supplement_1):S62-S9.
2. Van Belle TL, Coppieters KT, Von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiological reviews. 2011;91(1):79-118.
3. DeFronzo RA, Ferrannini E, Groop L, Henry RR, Herman WH, Holst JJ, et al. Type 2 diabetes mellitus. Nature reviews Disease primers. 2015;1(1):1-22.
4. 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.
5. Alhabeeb H, AlFaiz A, Kutbi E, AlShahrani D, Alsuhail A, AlRajhi S, et al. Gut Hormones in Health and Obesity: The Upcoming Role of Short Chain Fatty Acids. Nutrients. 2021;13(2).
6. Hostalek U. Global epidemiology of prediabetes - present and future perspectives. Clinical Diabetes and Endocrinology. 2019;5(1).
7. Ghody P, Shikha D, Karam J, Bahtiyar G. Identifying prediabetes–Is it beneficial in the long run? Maturitas. 2015;81(2):282-6.
8. Derikx JP, Luyer MD, Heineman E, Buurman WA. Non-invasive markers of gut wall integrity in health and disease. World J Gastroenterol. 2010;16(42):5272-9.
9. Thaiss CA, Levy M, Grosheva I, Zheng D, Soffer E, Blacher E, et al. Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection. Science. 2018;359(6382):1376-83.
10. Camilleri M. Leaky gut: mechanisms, measurement and clinical implications in humans. Gut. 2019;68(8):1516-26.
11. Luvuno M, Mbongwa H, Khathi A. Development of a novel prediabetes animal model using a high fat high carbohydrate diet: implications for type 2 diabetes. PloS one. 2017;13:8-14.
12. Perreault L, Færch K. Approaching Pre-diabetes. Journal of Diabetes and its Complications. 2014;28(2):226-33.
13. Zinman B, Harris SB, Neuman J, Gerstein HC, Retnakaran RR, Raboud J, et al. Low-dose combination therapy with rosiglitazone and metformin to prevent type 2 diabetes mellitus (CANOE trial): a double-blind randomised controlled study. The Lancet. 2010;376(9735):103-11.

14. Brouns F. Overweight and diabetes prevention: is a low-carbohydrate-high-fat diet recommendable? Eur J Nutr. 2018;57(4):1301-12.
15. Noakes TD, Windt J. Evidence that supports the prescription of low-carbohydrate high-fat diets: a narrative review. Br J Sports Med. 2017;51(2):133-9.
16. Dorans KS, Bazzano LA, Qi L, He H, Chen J, Appel LJ, et al. Effects of a Low-Carbohydrate Dietary Intervention on Hemoglobin A_{1c}. JAMA Network Open. 2022;5(10):e2238645.
17. De Kort S, Keszthelyi D, Masclee AAM. Leaky gut and diabetes mellitus: what is the link? Obesity Reviews. 2011;12(6):449-58.
18. De Santis S, Cavalcanti E, Mastronardi M, Jirillo E, Chieppa M. Nutritional Keys for Intestinal Barrier Modulation. Frontiers in Immunology. 2015;6.
19. Liu Z, Li N, Neu J. Tight junctions, leaky intestines, and pediatric diseases. Acta Paediatrica. 2007;94(4):386-93.
20. Wang L, Llorente C, Hartmann P, Yang A-M, Chen P, Schnabl B. Methods to determine intestinal permeability and bacterial translocation during liver disease. Journal of Immunological Methods. 2015;421:44-53.
21. Nehra V, Allen JM, Mailing LJ, Kashyap PC, Woods JA. Gut microbiota: modulation of host physiology in obesity. Physiology. 2016;31(5):327-35.
22. Wisniewski PJ, Dowden RA, Campbell SC. Role of Dietary Lipids in Modulating Inflammation through the Gut Microbiota. Nutrients. 2019;11(1):117.
23. Hamer HM, Jonkers DM, Bast A, Vanhoutvin SA, Fischer MA, Kodde A, et al. Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. Clinical Nutrition. 2009;28(1):88-93.
24. Huang Y, Shi X, Li Z, Shen Y, Shi X, Wang L, et al. Possible association of Firmicutes in the gut microbiota of patients with major depressive disorder. Neuropsychiatric disease and treatment. 2018;14:3329.
25. Sanz Y, Olivares M, Moya-Pérez Á, Agostoni C. Understanding the role of gut microbiome in metabolic disease risk. Pediatric Research. 2015;77(1-2):236-44.
26. Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke J-D, Serino M, et al. Intestinal permeability – a new target for disease prevention and therapy. BMC Gastroenterology. 2014;14(1).
27. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2010;299(2):G440-G8.
28. Devaraj S, Dasu MR, Jialal I. Diabetes is a proinflammatory state: a translational perspective. Expert review of endocrinology & metabolism. 2010;5(1):19-28.
29. Wang W, Uzzau S, Goldblum SE, Fasano A. Human zonulin, a potential modulator of intestinal tight junctions. Journal of cell science. 2000;113(24):4435-40.
30. Sturgeon C, Fasano A. Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. Tissue Barriers. 2016;4(4):e1251384.
31. Drago S, El Asmar R, Di Pierro M, Grazia Clemente M, Sapone ATA, Thakar M, et al. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. Scandinavian journal of gastroenterology. 2006;41(4):408-19.
32. Tripathi A, Lammers KM, Goldblum S, Shea-Donohue T, Netzel-Arnett S, Buzza MS, et al. Identification of human zonulin, a physiological modulator of tight junctions, as prehaptoglobin-2. Proc Natl Acad Sci U S A. 2009;106(39):16799-804.
33. Derikx JP, Luyer MD, Heineman E, Buurman WA. Non-invasive markers of gut wall integrity in health and disease. World journal of gastroenterology: WJG. 2010;16(42):5272.
34. Raetz CRH, Whitfield C. Lipopolysaccharide Endotoxins. Annual Review of Biochemistry. 2002;71(1):635-700.
35. Ghoshal S, Witta J, Zhong J, De Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. Journal of Lipid Research. 2009;50(1):90-7.

36. Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. The American journal of clinical nutrition. 2007;86(5):1286-92.
37. Jayashree B, Bibin YS, Prabhu D, Shanthirani CS, Gokulakrishnan K, Lakshmi BS, et al. signify novel biomarkers of proinflammation in patients with type 2 diabetesIncreased circulatory levels of lipopolysaccharide (LPS) and zonulin Molecular and Cellular Biochemistry. 2014;388(1-2):203-10.
38. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. AIDS (London, England). 2015;29(10):1263.
39. Sandler NG, Koh C, Roque A, Eccleston JL, Siegel RB, Demino M, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. Gastroenterology. 2011;141(4):1220-30. e3.
40. Al-Ayadhi L, Zayed N, Bhat RS, Moubayed NMS, Al-Muammar MN, El-Ansary A. The use of biomarkers associated with leaky gut as a diagnostic tool for early intervention in autism spectrum disorder: a systematic review. Gut Pathogens. 2021;13(1).
41. Adriaanse M, Tack G, Passos VL, Damoiseaux J, Schreurs M, Van Wijck K, et al. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. Alimentary pharmacology & therapeutics. 2013;37(4):482-90.
42. Funaoka H, Kanda T, Fujii H. [Intestinal fatty acid-binding protein (I-FABP) as a new biomarker for intestinal diseases]. Rinsho Byori. 2010;58(2):162-8.
43. van Hemert S, Verwer J, Schütz B. Clinical studies evaluating effects of probiotics on parameters of intestinal barrier function. 2013.
44. Hoshiko H, Feskens EJM, Oosterink E, Ariens RMC, Mes JJ, De Wit NJW. Identification of leaky gut-related markers as indicators of metabolic health in Dutch adults: The Nutrition Questionnaires plus (NQplus) study. PLOS ONE. 2021;16(6):e0252936.
45. Buckley ST, Bækdal TA, Vegge A, Maarbjerg SJ, Pyke C, Ahnfelt-Rønne J, et al. Transcellular stomach absorption of a derivatized glucagon-like peptide-1 receptor agonist. Science translational medicine. 2018;10(467).
46. Gummesson A, Carlsson LMS, Storlien LH, Bäckhed F, Lundin P, Löfgren L, et al. Intestinal Permeability Is Associated With Visceral Adiposity in Healthy Women. Obesity. 2011;19(11):2280-2.
47. Creely SJ, McTernan PG, Kusminski CM, Fisher fM, Da Silva N, Khanolkar M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. American Journal of Physiology-Endocrinology and Metabolism. 2007;292(3):E740-E7.
48. Amasheh M, Fromm A, Krug SM, Amasheh S, Andres S, Zeitz M, et al. TNFα-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFκB signaling. Journal of Cell Science. 2010;123(23):4145-55.
49. Bruewer M, Utech M, Ivanov AI, Hopkins AM, Parkos CA, Nusrat A. Interferon-γ induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. The FASEB Journal. 2005;19(8):923-33.
50. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nature Reviews Immunology. 2008;8(12):923-34.
51. Bansal N. Prediabetes diagnosis and treatment: A review. World journal of diabetes. 2015;6(2):296.
52. Organization WH. Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia: report of a WHO/IDF consultation. 2006.
53. Monnier VM, Sell DR, Genuth S. Glycation products as markers and predictors of the progression of diabetic complications. Annals of the New York Academy of Sciences. 2005;1043(1):567-81.
54. Mabuza LP, Gamede MW, Maikoo S, Booysen IN, Ngubane PS, Khathi A. Cardioprotective effects of a ruthenium (II) Schiff base complex in diet-induced prediabetic rats . Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. 2019;Volume 12:217-23.

55. Mzimela NC, Ngubane PS, Khathi A. 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. Autoimmunity. 2019;52(1):27-36.
56. Ble-Castillo JL, Aparicio-Trapala MA, Juárez-Rojop IE, Torres-Lopez JE, Mendez JD, Aguilar-Mariscal H, et al. Differential effects of high-carbohydrate and high-fat diet composition on metabolic control and insulin resistance in normal rats. International journal of environmental research and public health. 2012;9(5):1663-76.
57. Tuso P. Prediabetes and lifestyle modification: time to prevent a preventable disease. The Permanente Journal. 2014;18(3):88.
58. Control CfD, Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. Atlanta, GA: US department of health and human services, centers for disease control and prevention. 2011;201(1):2568-9.
59. Huang BW, Chiang MT, Yao HT, Chiang W. The effect of high-fat and high-fructose diets on glucose tolerance and plasma lipid and leptin levels in rats. Diabetes, Obesity and Metabolism. 2004;6(2):120-6.
60. Algoblan A, Alalfi M, Khan M. Mechanism linking diabetes mellitus and obesity. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. 2014:587.
61. Kasuga M. Insulin resistance and pancreatic β cell failure. The Journal of clinical investigation. 2006;116(7):1756-60.
62. Yaturu S. Obesity and type 2 diabetes. Journal of Diabetes Mellitus. 2011;01(04):79-95.
63. Karpe F, Dickmann JR, Frayn KN. Fatty acids, obesity, and insulin resistance: time for a reevaluation. Diabetes. 2011;60(10):2441-9.
64. Frazier TH, DiBaise JK, McClain CJ. Gut microbiota, intestinal permeability, obesity-induced inflammation, and liver injury. Journal of Parenteral and Enteral Nutrition. 2011;35:14S-20S.
65. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007;56(7):1761-72.
66. Guo S, Al-Sadi R, Said HM, Ma TY. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. Am J Pathol. 2013;182(2):375-87.
67. Leech B, McIntyre E, Steel A, Sibbritt D. Risk factors associated with intestinal permeability in an adult population: A systematic review. International Journal of Clinical Practice. 2019;73(10):e13385.
68. 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.
69. Patnode ML, Beller ZW, Han ND, Cheng J, Peters SL, Terrapon N, et al. Interspecies competition impacts targeted manipulation of human gut bacteria by fiber-derived glycans. Cell. 2019;179(1):59-73. e13.
70. Lau WL, Tran T, Rhee CM, Kalantar-Zadeh K, Vaziri ND. Diabetes and the Gut Microbiome. Seminars in Nephrology. 2021;41(2):104-13.
71. Carlson JL, Erickson JM, Lloyd BB, Slavin JL. Health effects and sources of prebiotic dietary fiber. Current developments in nutrition. 2018;2(3):nzy005.
72. L Madsen K. Enhancement of epithelial barrier function by probiotics. Journal of Epithelial Biology and Pharmacology. 2012;5(1).
73. Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, et al. Health benefits of probiotics: a review. International Scholarly Research Notices. 2013;2013.
74. Wang X, Valenzano MC, Mercado JM, Zurbach EP, Mullin JM. Zinc supplementation modifies tight junctions and alters barrier function of CACO-2 human intestinal epithelial layers. Digestive Diseases and Sciences. 2013;58(1):77-87.
75. Miyoshi Y, Tanabe S, Suzuki T. Cellular zinc is required for intestinal epithelial barrier maintenance via the regulation of claudin-3 and occludin expression. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2016;311(1):G105-G16.

76. Skrovanek S, DiGuilio K, Bailey R, Huntington W, Urbas R, Mayilvaganan B, et al. Zinc and gastrointestinal disease. World journal of gastrointestinal pathophysiology. 2014;5(4):496.
77. DeMarco VG, Li N, Thomas J, West CM, Neu J. Glutamine and barrier function in cultured Caco-2 epithelial cell monolayers. The Journal of nutrition. 2003;133(7):2176-9.
78. Yang, Liang, Balakrishnan, Belobrajdic, Feng, Zhang. Role of Dietary Nutrients in the Modulation of Gut Microbiota: A Narrative Review. Nutrients. 2020;12(2):381.
79. Perreault L, Kahn SE, Christophi CA, Knowler WC, Hamman RF, Group DPPR. Regression from pre-diabetes to normal glucose regulation in the diabetes prevention program. Diabetes care. 2009;32(9):1583-8.
80. Evert AB, Boucher JL, Cypress M, Dunbar SA, Franz MJ, Mayer-Davis EJ, et al. Nutrition therapy recommendations for the management of adults with diabetes. Diabetes care. 2014;37(Supplement_1):S120-S43.
81. 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.
82. Paoli A, Bosco G, Camporesi EM, Mangar D. Ketosis, ketogenic diet and food intake control: a complex relationship. Front Psychol. 2015;6:27.
83. Moreno B, Bellido D, Sajoux I, Goday A, Saavedra D, Crujeiras AB, et al. Comparison of a very low-calorie-ketogenic diet with a standard low-calorie diet in the treatment of obesity. Endocrine. 2014;47(3):793-805.
84. Fields H, Ruddy B, Wallace MR, Shah A, Millstine D. Are Low-Carbohydrate Diets Safe and Effective? Journal of Osteopathic Medicine. 2016;116(12):788-93.
85. Tay J, Luscombe-Marsh ND, Thompson CH, Noakes M, Buckley JD, Wittert GA, et al. Comparison of low- and high-carbohydrate diets for type 2 diabetes management: a randomized trial. Am J Clin Nutr. 2015;102(4):780-90.
86. Wang LL, Wang Q, Hong Y, Ojo O, Jiang Q, Hou YY, et al. The Effect of Low-Carbohydrate Diet on Glycemic Control in Patients with Type 2 Diabetes Mellitus. Nutrients. 2018;10(6).
87. Yancy WS, Jr., Foy M, Chalecki AM, Vernon MC, Westman EC. A low-carbohydrate, ketogenic diet to treat type 2 diabetes. Nutr Metab (Lond). 2005;2:34.
88. Mansoor N, Vinknes KJ, Veierød MB, Retterstøl K. Effects of low-carbohydrate diets v. low-fat diets on body weight and cardiovascular risk factors: a meta-analysis of randomised controlled trials. Br J Nutr. 2016;115(3):466-79.
89. Wylie-Rosett J, Aebersold K, Conlon B, Isasi CR, Ostrovsky NW. Health effects of low-carbohydrate diets: where should new research go? Curr Diab Rep. 2013;13(2):271-8.
90. Srinivasan K, Ramarao P. Animal model in type 2 diabetes research: An overview. Indian Journal of Medical Research. 2007;125(3):451.
91. Panchal SK, Poudyal H, Iyer A, Nazer R, Alam A, Diwan V, et al. High-carbohydrate high-fat diet–induced metabolic syndrome and cardiovascular remodeling in rats. Journal of cardiovascular pharmacology. 2011;57(1):51-64.
92. Kleinert M, Clemmensen C, Hofmann SM, Moore MC, Renner S, Woods SC, et al. Animal models of obesity and diabetes mellitus. Nature Reviews Endocrinology. 2018;14(3):140.
93. King AJ. The use of animal models in diabetes research. British Journal of Pharmacology. 2012;166(3):877-94.

CHAPTER 2: MANUSCRIPT 1

Prologue

The consumption of high-calorie diets has been associated with increased intestinal permeability, which is also known as a leaky gut. Increased intestinal permeability dysregulates the intestinal barrier that prevents the entry of pathogenic bacteria and toxic luminal substances, leading to a systemic inflammation and to the release of pro-inflammatory molecules. This systemic inflammation is believed to contribute to insulin resistance, which is a key characteristic of prediabetes and T2D. The consumption of high calorie diets has also been shown to lead to the development of prediabetes. However, we do not know if there is an association between the increased intestinal permeability and the development of prediabetes. Nevertheless, it is crucial to acknowledge that research in this field is still evolving, and the precise nature of the relationship between increased intestinal permeability and prediabetes remains to be fully understood. Therefore, further studies are necessary to establish a definitive cause-and-effect relationship and gain a deeper understanding of underlying mechanisms involved. Hence, in this study we sought to investigate if there could be any links between intestinal permeability and the development of prediabetes in a diet-induced prediabetic animal model.

The manuscript in chapter 2 is titled "**Investigating the association between diet-induced "leaky gut" and the development of prediabetes**".

The current manuscript is currently under review in **the Journal of Experimental Clinical Endocrinology and Diabetes** and has been formatted according to the journal's guidelines to authors (see Appendix 2).

Investigating The Association Between Diet-Induced "Leaky Gut" And The Development Of Prediabetes

Nosipho R. Dimba[1,2], Nhlakanipho Mzimela[1], Palesa Mosili[1], Phikelelani S. Ngubane[1], Andile Khathi[1].

1. School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville, South Africa ,4000
2. Corresponding Author

Department of Human Physiology

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Westville, South Africa, 4000

Phone number: 062 948 8290/ (031) 260 7585

Fax: (27) (31) 260 7132

Email address: 218006885@stu.ukzn.ac.za

Word Count: Abstract = 276, manuscript = 4596

Abstract

Chronic consumption of a high-calorie diet compromises the gut microbiota and the integrity of the intestinal wall, which causes translocation of bacterial lipopolysaccharides into the blood. This elicits secretion of pro-inflammatory cytokines, which result in inflammation. However, it has not been investigated how a high-fat high carbohydrate diet affects intestinal permeability and whether or not this plays a role in the development of prediabetes. This study investigated the effects of HFHC diet-induced prediabetes on gut microbiota and intestinal permeability in male Sprague Dawley rats. The animals were randomly assigned into the non-prediabetic group (NPD) and diet-induced prediabetic group (PD) (n=6) for a period of 20 weeks. After 20 weeks, the fecal samples were analyzed to measure the gut microbiota level of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in both animal groups. Furthermore, blood glucose, OGT response, plasma insulin, serum zonulin, plasma LPS, soluble CD14, tumor necrosis factor-alpha, interleukin-6, C-reactive protein, and intestinal fatty-acid binding protein concentrations were measured. The fecal samples revealed that in the PD group, there was a reduction in the *Firmicutes* levels and an increase in *Bacteroidetes* and *Proteobacteria* compared to NPD. Blood glucose and insulin concentration were significantly increased in the PD group by comparison to NPD. The OGT response in the PD group resulted in increased blood glucose concentration. Serum zonulin and plasma sCD14 concentrations in the PD group were increased compared to NPD, while plasma LPS concentrations were similar. An increase in plasma TNF-α, IL-6, CRP, and IFABP concentrations in PD was observed compared to NPD. Taken together, these results suggest that chronic consumption of the HFHC diet may be associated with the dysregulation of gut microbiota, leading to increased intestinal permeability.

Keywords: Pre-diabetes, high-fat high carbohydrate, gut microbiota, zonulin, lipopolysaccharides, intestinal permeability

Introduction

Type 2 diabetes (T2D) has become a major concern in healthcare worldwide (1). According to the International Diabetic Federation (IDF), 463 million people around the world have T2D, while it's complications are estimated to have killed nearly 4 million people aged 20-79 years in 2019 (2). High-calorie diets and sedentary lifestyles are recognized as some of the leading causes of this condition (3). The small intestine is home to a wide variety of microorganisms that play a role in the digestion of food as well as in maintaining the integrity of the intestinal wall (4). The intestinal barrier and the intestinal epithelium cells function to prevent the translocation of toxins, luminal antigens, and partially undigested food into circulation (5).

Chronic consumption of a high-calorie diet has been shown to cause gut dysbiosis by altering the microbial diversity in the gut, resulting in a reduction of *Firmicutes* like *Lactobacillus, Bacillus,* and *Clostridium,* which all protect the intestinal lining (6, 7). This results in decreased protection of the lining of the gut and increase in gut permeability which is characterized by increased serum zonulin concentrations (8, 9). Zonulin is a leaky gut marker that has been shown to loosen intercellular tight junctions between epithelial cells in the digestive tract (10). Therefore, upregulation of zonulin causes translocation of lipopolysaccharides (LPS), peptidoglycan, intestinal fatty acid-binding protein (IFABP), and soluble CD14 (sCD14) a co-receptor that gets secreted from the immune cells (11). All these substances and toxins elicit an inflammatory response leading to the secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interferon-*y* (IFN-*y*) in the lumen, as well as an inflammatory marker called C-reactive protein (CRP) (12). This further compromises the intestinal epithelial barrier.

Prediabetes is a condition where blood glucose concentrations are above normal but below the detectable threshold of T2D (13). This condition is characterized by impaired glucose tolerance (IGT), elevated glycated haemoglobin (HbA1c), and impaired fasting glucose (IFG) (14, 15). There are no studies that have been done to investigate the association between intestinal permeability and prediabetes. Previous studies done in our laboratory developed a high-fat high carbohydrate (HFHC) diet-induced prediabetic animal model, using male Sprague Dawley rats. This model was found to mimic the human condition of prediabetes (16, 17). In this model, the animal develop prediabetes after 20 weeks of ingestion of a high fat high carbohydrate diet (17, 18). Using this HFHC diet-induced animal model of prediabetes, this study sought to investigate the changes on gut microbiota and the association between prediabetes and markers associated with intestinal permeability. We further investigated if there could be any links between intestinal permeability and the development of prediabetes.

Materials and Methods

Drugs and Chemicals

All chemicals and reagents were sourced from standard pharmaceutical suppliers and were of analytical grade. The kits components were as follows; concentrated biotinylated detection antibody, biotinylated detection antibody diluent, substrate reagent, stop solution, concentrated wash buffer, standards micro enzyme-linked immunosorbent assay (ELISA) plate, reference standard, concentrated avadin-horseradish peroxide (HRP), HRP diluent (Elabscience product purchased from Biocom Africa, Centurion, South Africa).

Animal and housing

Male Sprague-Dawley rats (150-180g), 3 weeks old bred for this study were kept in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal. All animal procedures and housing conditions were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (ethics no: AREC/00003627/2021). The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), relative humidity (55±5%), and illumination (12 h light/dark cycle, lights on at 07h00). The level of noise was maintained at less than 65 decibels and the animals were allowed access to food and fluids *ad libitum*. The animals were allowed to acclimatize to their new environment for one week while consuming standard rat chow and water before they were exposed to a high-fat high-carbohydrate (HFHC) experimental diet (18).

Induction of prediabetes

Using Luvuno *et al*., 2017 established laboratory protocol, prediabetes was induced in Male Sprague-Dawley rats. Briefly, the animals were divided into two diet groups, group A and B (n=6 per each group). Group A animals were exposed to a standard diet with normal drinking water for 20 weeks and group B animals were exposed to a HFHC diet supplemented with 15% fructose for a period of 20 weeks (18). The composition of a HFHC diet was customized as follows; carbohydrates (55% Kcal/g), fats (30% Kcal/g), and proteins (15% Kcal/g). After 20 weeks the American Diabetes Association criteria for diagnosis of prediabetes was used to diagnose prediabetes in all animals. Briefly, all animals that would exhibit fasting blood glucose concentrations of 5.6 to 7.1 mmol/L and oral glucose tolerance test (OGTT) 2-h glucose concentration of 7.8 to 11.0 mmol/L were considered prediabetic.

Experimental procedures

Sample collection and Analysis

Prior to sampling the rats were placed in individual tecniplast metabolic cages. The fecal samples were then collected from both groups of rats that were fed a standard diet with distilled water and HFHC diet supplemented with 15% fructose at the beginning and end of the experimental period (20 weeks). Once obtained, fecal samples were placed into a sterile zip-lock bag. The samples were then transported to Inqaba Biotechnology laboratory in cooler boxes containing ice packs for further analysis. Upon arrival,

the samples were pre-processed for DNA extraction and stored in a Bio Ultra freezer at -80 °C (Labtech, Umhlanga, South Africa).

DNA extraction, pooling, and quantification

Microbial DNA from the samples was extracted using a previously described protocol (19). This was done using the Qubit 1x dsDNA HS Assay Kit kit (Zymo Research, Inqaba Biotec™). Briefly, the fecal samples were lysed by bead beating in a ZR BashingBead™ lysis matrix filled with buffer and placed in a bead beater (TissueLyser LT, Qiagen®) prior to DNA extraction. The eluted DNA samples were quantified using Qubit® Fluorometer 4.0 (Invitrogen, Thermo Fisher Scientific Inc) and stored at -20°C for downstream molecular application (19). For 16S rRNA sequencing, DNA samples extracted from the fecal samples were sequenced. The samples were pooled according to each animal from the non-prediabetic (NPD) and prediabetic (PD) group. Each pooled DNA sample of the faeces constituted of an equimolar mixture of two samples obtained from the same animal. Thereafter, the pools were quantified, then normalized for Illumina sequencing.

Amplification of bacterial 16S rRNA and sequencing

The bacterial 16S rRNA gene was amplified using M13-tailed target-specific primer with 5' block. PCR Forward Primer: 5′ GTA AAA CGA CGG CCA GT(N) 3′ and PCR Reverse Primer: 5′ CAG GAA ACA GCT ATG AC(N) 3′ (Integrated DNA Technologies, Whitehead Scientific). The samples were processed aseptically to minimise contamination. We added two non-template controls consisting of nuclease free water as well as PCR and sequencing laboratory reagents in place of experimental DNA template were incorporated in the amplification and sequencing steps and processed alongside the test samples. Library preparation was performed according to the standard instructions of the 16S SMRTbell® prep kit 3.0 (PacificBiosciences, California, USA). Indexed amplicons were quantified using Qubit® High Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific) and the sizes of the amplicons were visualised using the 4200 TapeStation (Agilent Technologies, Germany). The normalized libraries were pooled for sequencing, denatured to single strand using NaOH, then PhiX (10%) was added to the library. Libraries were then sequenced using the 16S SMRTbell® prep kit 3.0 (PacificBiosciences, California, USA).

Oral glucose tolerance test (OGT) response

An oral glucose test was conducted in both groups of animals, this test was conducted following carbohydrate loading. The OGT response of all animals was monitored in the animals using our established laboratory protocol (20). Briefly, after an 18 h fasting period, glucose was measured (time 0) followed by loading with a monosaccharide syrup (glucose; 0.86 g/kg, p.o.) 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

(21). Glucose concentrations were measured using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). Glucose concentrations were measured at 15, 30, 60, and 120 minutes following carbohydrate loading.

Blood collection

All the animals were anaesthetized at the end of 20-week period 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 3 minutes. Blood was taken by a cardiac puncture while the rats were unconscious and then injected into individual pre-cooled heparinized containers. After that, the blood was centrifuged for 15 minutes at 4°C, 503 g (Eppendorf centrifuge 5403, Germany). Plasma was collected and stored at -70 °C in a Bio Ultra freezer (Snijers Scientific, Holland) for biochemical analysis.

Biochemical analysis

Plasma lipopolysaccharides (LPS) and serum zonulin concentrations were measured at Lab 24 (Mt. Edgecombe, Durban). Plasma insulin, soluble CD14 (sCD14), intestinal fatty acid-binding protein (IFABP), C-reactive protein (CRP), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) concentrations were measured using their respective rat-sandwich ELISA kits from Elabscience (Wuhan, China) as per manufacturer's instructions. For all kits, the micro-plate provided was coated with antibodies specific to standards and the plasma samples of 100 µL were added into each well of the plates. The plate with the standards and plasma samples was incubated for 90 minutes and 37 ℃. After this, 100 µL of biotinylated detection Ab was pipetted respectively into each well of insulin, sCD14, TNF-α, IL-6, CRP and IFABP. This was followed by another 90 minutes incubation period at 37 ℃ then the unbound components were washed away using the wash buffers. The wash step was carried out three times in total.

In each micro-plate, 100 µL of avidin–horseradish peroxidase (HRP) conjugate was added, which was then incubated for 30 minutes at 37 °C. The HRP solution was discarded from the plate, and the washing procedure was repeated 5 times with 350 µL of wash buffer in each well. Each well received 90 µL of substrate reagent and the plates were then incubated for 15 minutes at 37 °C. In each well, 50 µL of stop solution was added in the same manner as the substrate solution step. A nano spectrophotometer from BMG Labtech (Ortenburg, Germany) was used to measure optical density at 450 nm. Insulin, sCD14, TNF-α, IL-6, CRP and IFABP concentrations in the samples were extrapolated from their respective standard curves.

Statistical Analysis

Statistical analysis was conducted using Graph Pad InStat Software (version 5.00, Graph Pad Software, Inc., San Diego, California, USA). All data went under a normality test with the relevant parametric and non-parametric tests used to further analyze the data. A student t test was used to determine statistical differences between two independent groups. All data was expressed as means and $\pm$ S.E.M and a value of $p < 0.05$ was considered statistically significant.

Results

Gut microbiota composition

By comparison with the non-prediabetic group, the prediabetic animals that were fed a HFHC diet showed a significant decrease in *Firmicutes* and a significant increase in pathogenic phyla (*Bacteroidetes* and *Proteobacteria*) ($p < 0.0001$; Table 1) after 20 weeks.

Table 1: Displays the gut microbiota composition (%) of both groups; non-prediabetic (NPD) and prediabetic (PD) at the end of the experimental period. The values are presented as standard error of mean ± SEM.

	WEEK 20	
	GROUPS	
Gut Microbiota Composition	**NPD (Std diet, %)**	**PD (HFHC diet, %)**
Firmicutes	77.5 ± 4.6	64.1 ± 2.4 * * *
Bacteroidetes	5.2 ± 0.7	12.4 ± 0.9 * * *
Proteobacteria	10.2 ± 0.7	13.1±0.7 * * *

* * * = $p < 0.0001$ denotes comparison with NPD.

Food intake and fluid intake

Table 2 displays food intake and fluid intake monitored at week 10 and 20 of the experimental period.

Table 2: Food intake and fluid intake of rats in week 10 and 20 following ingestion of a normal diet and HFHC diets (n=6, per group).

	WEEK 10	
	GROUPS	
	NPD	**PD**
FOOD INTAKE (g)	13.00 ± 0.58	9.98 ± 0.41
FLUID INTAKE (mL)	20.00 ± 2.00	21.00 ± 3.50
	WEEK 20	
	GROUPS	
	NPD	**PD**
FOOD INTAKE (g)	18.35 ± 0.47	12.55 ± 0.32
FLUID INTAKE (mL)	40 ± 5.00	32.33 ± 1.45

Body weight

Table 3 showed the body weight gain of the NPD and PD animal groups from week 0 to week 20 of the experimental period. There was no significant effect of a HFHC diet supplemented with 15% fructose on body weight gain.

Table 3: The body weight measured during the experimental period of 20 weeks in both the NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM.

	BODY WEIGHT (g)	
	GROUPS	
Time (Weeks)	**NPD (g)**	**PD (g)**
0	173 ± 5.71	179.9 ± 3.57

1	250 ± 6.01	187.7 ± 3.66
4	294.7 ± 5.52	197.3 ± 6.65
8	360 ± 6.42	282.7 ± 7.98
12	417.7 ± 11.16	276 ± 7.10
16	398 ± 10.73	294.3 ± 8.20
20	443.3 ± 11.67	335.3 ± 9.32

Fasting blood glucose

Figure 1 shows fasting blood glucose concentration in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in fasting blood glucose concentration in the PD group ($p < 0.05$, Figure 1).

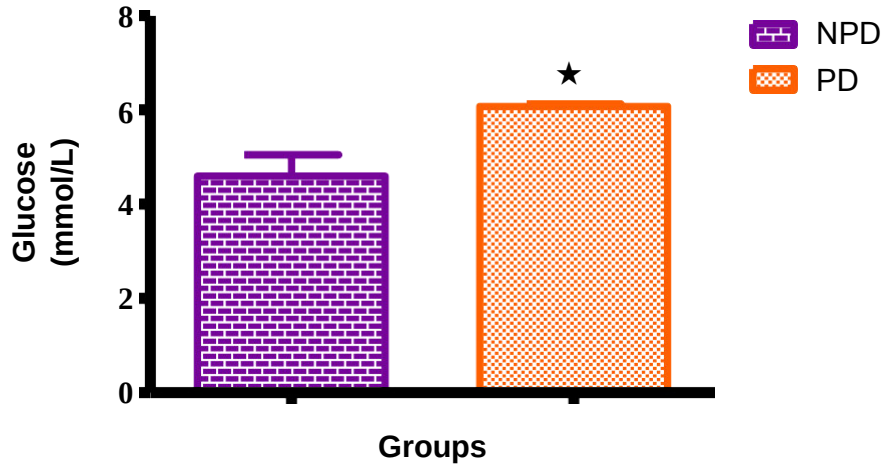

Figure 1: The fasting blood glucose concentration in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * = $p < 0.05$ denotes comparison with NPD.

OGT response

The OGT response of the NPD and PD group were monitored. Blood glucose concentration of the PD group was lower than NPD group before loading with glucose at time 0 ($p < 0.05$). Following glucose loading, blood glucose concentration in the PD group increased significantly than NPD group during the first 30 minutes ($p < 0.05$). Interestingly after 30 minutes blood glucose concentration in PD group did not return to normal range 2-h later of OGT test as shown in figure 2.

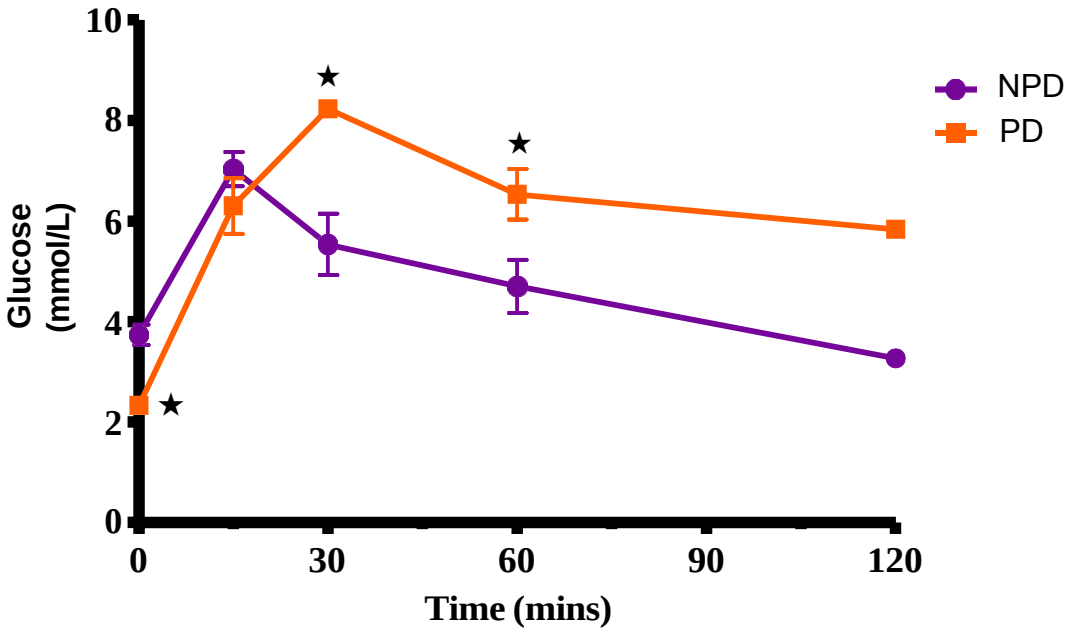

Figure 2: Oral glucose tolerance response of NPD and PD rat (n=6, per group) at the end of the experimental period. The values are presented as standard error of mean ± SEM. * = $p < 0.05$ denotes comparison with NPD.

Plasma insulin

Figure 3 shows plasma insulin concentration in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in plasma insulin concentration in the PD group ($p < 0.05$, Figure 3).

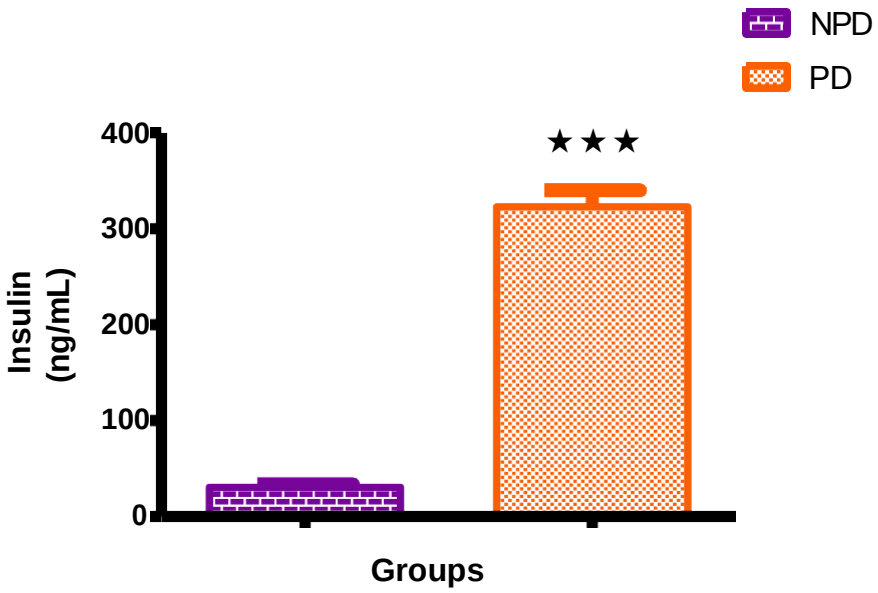

Figure 3: Plasma insulin in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * * * = p < 0.0001 denotes comparison with NPD.

Serum zonulin

Figure 4 shows serum zonulin concentration in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in serum zonulin concentration in the PD group (p =< 0.0001, Figure 4).

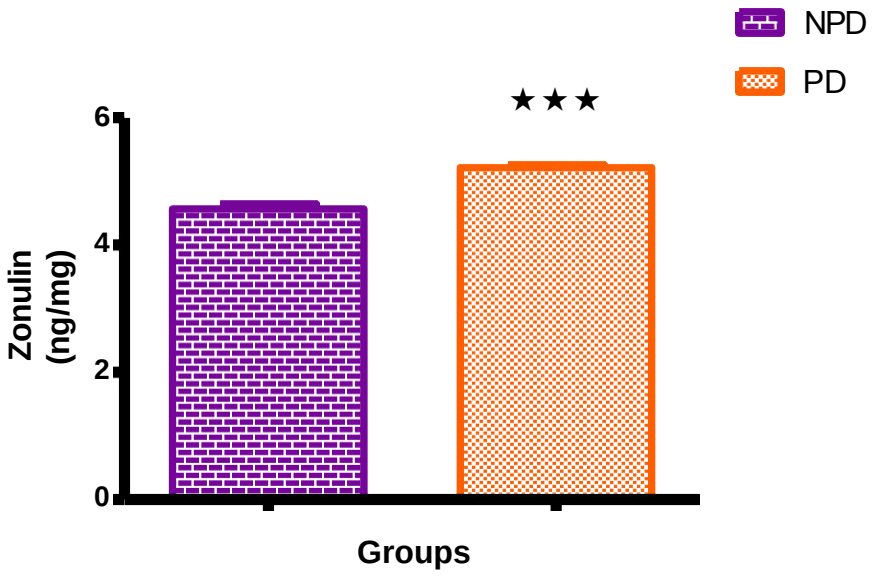

Figure 4: Serum zonulin in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * * * = p < 0.0001 denotes comparison with NPD.

Plasma lipopolysaccharide

Figure 5 shows plasma lipopolysaccharides concentration in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). The result (Fig.5) showed that there were no significant (p = < 0.0001) changes to plasma LPS concentration in both NPD and PD group.

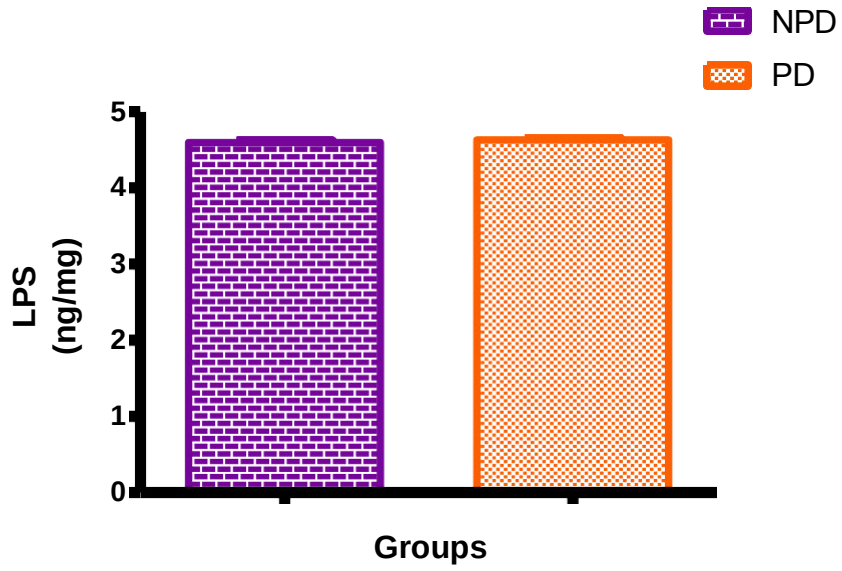

Figure 5: Plasma Lipopolysaccharides (LPS) in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM.

Plasma soluble CD14

Figure 6 shows plasma soluble CD14 concentration in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in plasma soluble CD14 concentration in the PD group (p =< 0.0001, Figure 6).

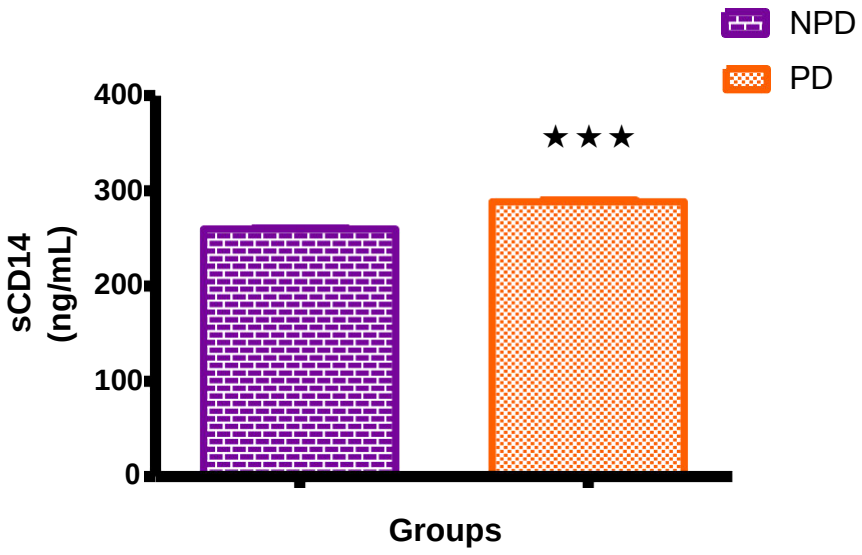

Figure 6: Soluble CD14 in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * * * = $p < 0.0001$ denotes comparison with NPD.

Plasma tumour necrosis factor

Figure 7 shows plasma tumor necrosis factor (TNF-α) measured in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in plasma TNF-α concentration in the PD group ($p < 0.05$); figure 7.

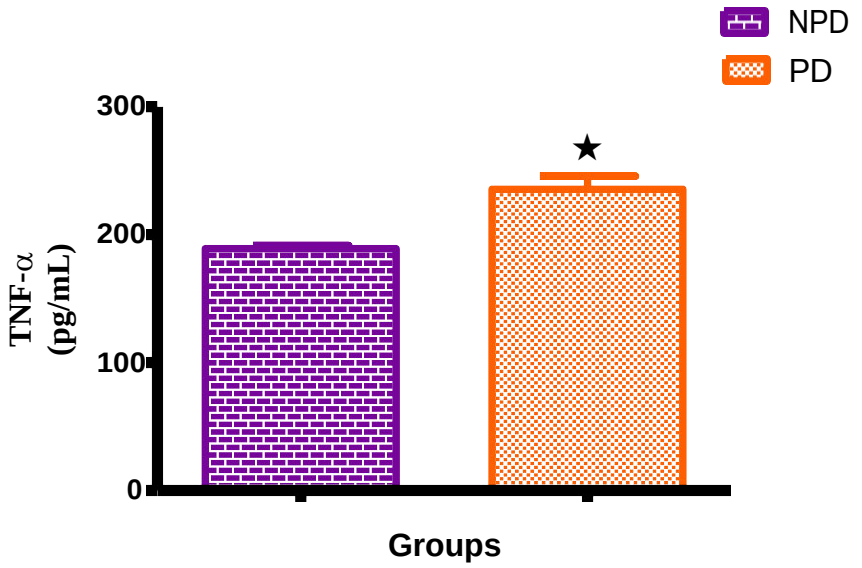

Figure 7: Tumour necrosis factor (TNF-α) concentration in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * = $p < 0.05$ denotes comparison with NPD.

Plasma inteleukin-6

Figure 8 shows interleukin-6 (IL-6) measured in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in plasma IL-6 concentration in the PD group ($p < 0.05$); figure 8.

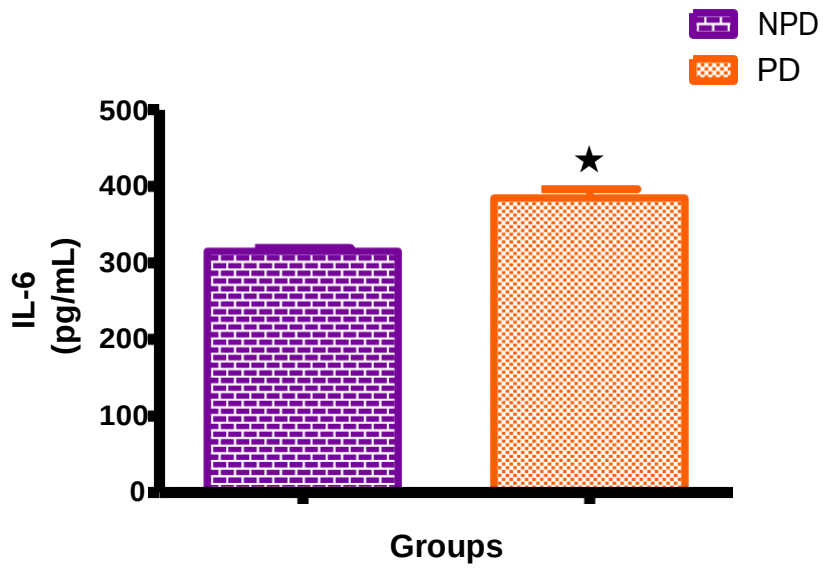

Figure 8: Inteleukin-6 concentration in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * = $p < 0.05$ denotes comparison with NPD.

Plasma c-reactive protein (CRP)

Figure 9 shows plasma c-reactive protein (CRP) concentration in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in plasma CRP concentration in the PD group ($p = < 0.0001$, Figure 9).

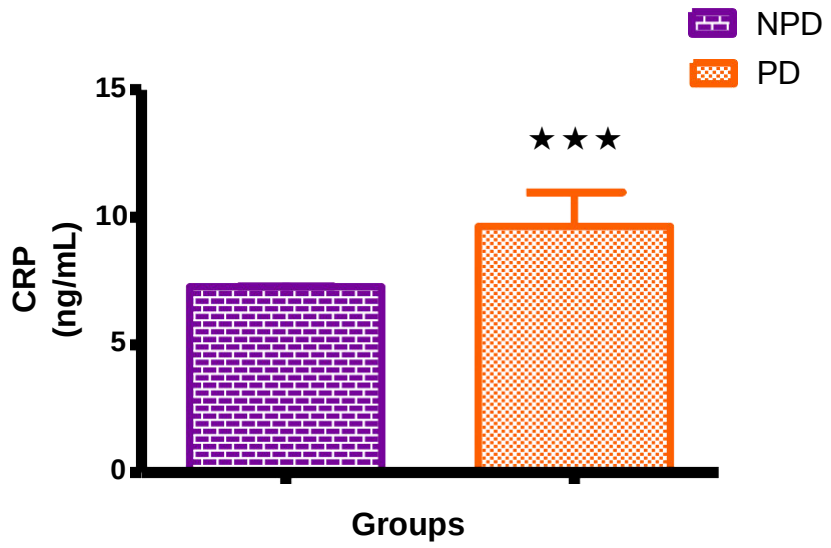

Figure 9: C-reactive protein (CRP) in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * * * = p < 0.0001 denotes comparison with NPD.

Plasma intestinal fatty acid-binding protein

Figure 10 shows plasma intestinal fatty-acid binding protein (IFABP) concentration in the non-prediabetic (NPD) and prediabetic (PD) at the end of the 20-week experimental period (n=6, per group). By comparison with NPD, there was a significant increase in plasma IFABP concentration in the PD group (p = < 0.0001, Figure 10).

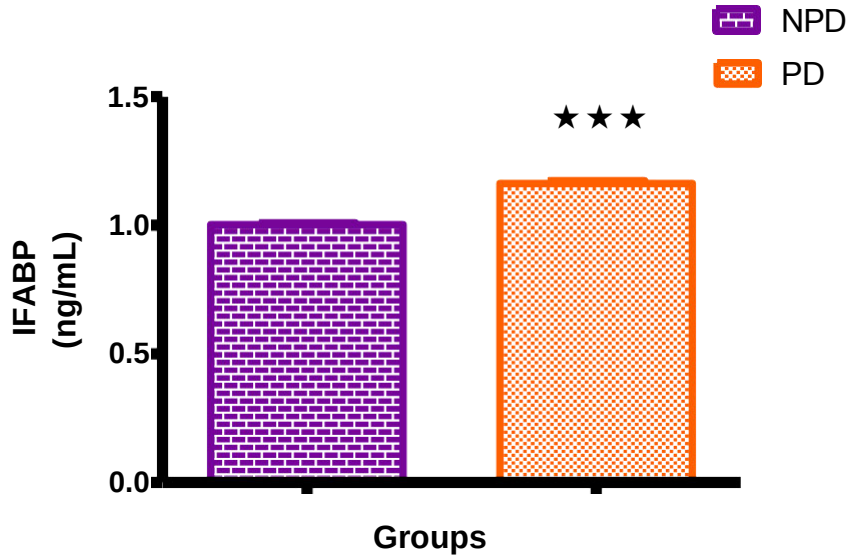

Figure 10: Intestinal fatty acid-binding protein (IFABP) in NPD and PD rat (n=6, per group). The values are presented as standard error of mean ± SEM. * * * = $p < 0.0001$ denotes comparison with NPD.

Discussion

The role of the intestinal mucosal barrier and enterocytes is to provide the first line of defense in the gastrointestinal tract by preventing the translocation of luminal antigen, peptidoglycan, endotoxin LPS, and other foreign substances into the circulation (22, 23). They also promote the absorption of nutrients from the ingested food (12). Studies have shown that the food that we eat influence the growth of specific bacteria within the gut which may affect intestinal permeability (24, 25). In recent studies, increased intestinal permeability has been associated with the development of metabolic conditions such a type 2 diabetes (23, 26, 27). We have previously reported that many complications seen in T2DM begin in the prediabetic state (28). However, no studies have been conducted to investigate if intestinal permeability is compromised during the prediabetic state.

In our laboratory, we have created a HFHC diet-induced animal model of prediabetes that has been shown to mimic the human condition (18). In this model, the animals develop prediabetes after 20 weeks of ingestion of a HFHC diet (18). In this study, blood glucose concentration failed to return to baseline following a 2-h OGT test, the results suggest that there is the presence of impaired glucose tolerance. This further suggests that ingestion of a HFHC diet results in the development of prediabetes. Moreover, a significant increase in fasting blood glucose was observed in the prediabetic animals compared to the non-prediabetic animals. The findings of the present study coincided with a previous study which, reported that prediabetes is associated with elevated blood glucose concentration (29). In addition to these observations, the prediabetic animals also had a significant increase in plasma insulin in comparison to the non-prediabetic animals, suggesting that the pancreatic beta-cell increased insulin secretion to compensate high blood glucose concentration at this stage. However due to chronic consumption of a HFHC diet, blood glucose continues to be elevated which could result in exhaustion of the pancreas (30). Using this HFHC diet-induced animal model of prediabetes, this study sought to investigate the changes in gut microbiota and the association between prediabetes and markers associated with intestinal permeability. We further investigated if there could be any links between intestinal permeability and the development of prediabetes.

Chronic consumption of a high-caloric diet promotes growth and provides nourishment to pathogenic bacteria (24, 25). As a result of these effects, the intestinal flora becomes imbalanced, allowing harmful bacteria to overwhelm and outcompete beneficial bacteria resulting in gut dysbiosis (12, 31). In this

study gut dysbiosis was observed in the prediabetic group as there was a significant reduction of *Firmicutes* when compared to the non-prediabetic group. The role of the *Firmicutes* in the gut is to protect the intestinal barrier (4). We also observed a significant increase of *Bacteroidetes* and *Proteobacteria* in the prediabetic group by comparison to the non-prediabetic group. These two phyla play a role in maintaining balance in the gut (4, 32). However, in high amounts they tend to be pathogenic thus aggravate inflammation in the intestine (4, 32). The differences in animal groups could be attributed to the different diets that they were consuming during the 20-week period. In this study there was a mix of high carbohydrates (HC) and high fats (HF) which mimics human eating preferences and habits. Increased consumption of HC/ HF diets stimulated growth of pathogenic bacteria which may have outcompeted beneficial bacteria. These findings suggest that chronic consumption of a HFHC results in gut dysbiosis which could leave the intestinal barrier compromised.

A biomarker that is routinely used to assess intestinal permeability is serum zonulin (33). Zonulin is a 47kDa protein that regulates intestinal leakage by modulating intracellular tight junctions, which then allows the movement of nutrients, water, and other useful molecules into the cells (10). Increased levels of serum zonulin serve as an indicator of increased intestinal permeability (34). The current study showed an elevated serum concentration of zonulin in the prediabetic group which was kept on a HFHC diet by comparison with the non-prediabetic group that was kept on a standard diet. These findings are in agreement with previous studies where chronic and excessive consumption of high-caloric diets was shown to increase zonulin concentration in the circulation (6, 23). Taken together with the results on the intestinal flora, these results may suggest that chronic consumption of the HFHC diet dysregulates the intestinal composition which leads to reduced protection of the gut leading to disruption of the tight junctions resulting in increased intestinal permeability.

Lipopolysaccharide (LPS) has been shown to be increased in patients with impaired intestinal permeability due to dysregulation of the tight junction (35). LPS forms an important part of the outer membrane (OM) of Gram-negative bacteria, and it functions to protect the membrane from any chemical attacks (36). In humans, it triggers a strong immune response and increases the negative charge of the cell membrane, which helps to stabilize the entire structure (37). Evidence suggests that high concentration of LPS are seen in T2D as well as in diseases associated with the digestive tract (38). In this study, there was no significant difference in plasma LPS concentrations observed between the two diet groups. These results may be explained by the findings of another recent study that showed that LPS has a half-life of 2-4 minutes in rat models (39). Therefore, LPS is rapidly detoxified and cleared in the circulation by the liver, mediated by Kupffer cells, hepatocytes, and sinusoidal endothelial cells (39). This could explain why there were no significant differences in the plasma levels of LPS in both groups. Most endotoxin LPS is bound with lipoproteins in the circulation, having the highest affinity for HDL. LPS-lipoprotein complexes have been shown to have little or no stimulatory effects on

cytokine production in acute conditions (39). In contrast, when this occurs for sustained periods, it can result in chronic sub-clinical levels of pro-inflammatory cytokines.

Soluble CD14(sCD14) is a co-receptor for LPS, and it is an activation marker for monocytes and other mononuclear cells released after stimulation (11). Upon translocation, LPS induces secretion of sCD14 from the immune cells which further activates an immune response to clear foreign substances (40). A previous study speculated that high levels of this co-receptor in the blood indicate LPS exposure (40). The findings of the present study showed increased sCD14 concentration in the prediabetic group by comparison with the non-prediabetic group. This suggest that even though LPS concentration were shown to be unchanged, there could have been increased LPS as shown by the increased sCD14 seen in the prediabetic stage.

Studies have also shown that when there is increased intestinal permeability, pro-inflammatory cytokines such as IL-6, IFN-*y* and TNF- α, are elevated (41). This may be caused by an increase in plasma LPS or other toxic compounds in the circulation (42). In the present study, it was observed that there were elevated concentrations of TNF-α and IL-6 in the prediabetic group in comparison to the non-prediabetic group. A previous study showed that TNF-α may further cause disruption of the intestinal barrier and this was shown to be mediated by myosin-light chain kinase (MLCK) (43). Activating this kinase reduces tight junction permeability in *vitro* and in *vivo,* while other cytokines increase intestinal permeability by changing the tight junction protein expression and localization (43, 44). This suggests that during increased intestinal permeability the body might be constantly in a state of activated immune response which induces sub-clinical chronic inflammation. This sub-clinical chronic inflammation has been postulated to cause peripheral insulin resistance in the liver, muscle, and adipose tissue thus resulting in the development of prediabetes. Activation of JNK-1 and NK-Fβ result in serine phosphorylation of insulin receptor substrate-1 leading to insulin resistance which also lead to insulin secretory defects due to pancreatic beta cell dysfunction (45).

Another marker of inflammation that was investigated was C-reactive protein (CRP). CRP is an inflammatory marker whose concentration has been shown to be positively correlated with increased zonulin concentrations (46). In this study, we observed that the concentration of plasma CRP increased significantly in the prediabetic group at the end of the 20-week experimental period. This supported our speculation that the sub-clinical inflammation observed in the prediabetic state may be a result of increased intestinal permeability. To further, evaluate these findings intestinal fatty acid-binding protein (IFABP) level was measured. IFABP is an intracellular protein that is found in the epithelial cells of the mucosal layer of the small and large intestine tissues (47). According to Adriaanse *et al.*, 2013, high levels of IFABP in the circulation only get translocated when two enterocytes and the mucosal tissue is damaged (48). In addition, this protein has been shown to be associated with minor intestinal diseases

such as coeliac disease (49). The result of this study showed that there was an increase in IFABP concentration during the prediabetic stage. This further supported our earlier observations.

Conclusion

The results obtained from the current study confirmed that chronic consumption of the HFHC diet resulted in dysregulation of the gut microbiota. This was accompanied by increased gut permeability which was further accompanied by sub-clinical increases in markers of systemic inflammation. We speculate that increases in systemic inflammation could result in the development of peripheral insulin resistance which is implicated in the development of prediabetes. Taken together, the results propose a possible explanation of why chronic consumption of a HFHC diet could lead to the development of prediabetes.

Conflict of Interest

The authors declare no conflict of interest.

Future recommendations

In future studies, the histology of the intestinal lining should be studied to assess the effect of a HFHC and inflammation on the integrity of the intestinal barrier.

Acknowledgments

The authors would like to acknowledge the Biomedical Resource Units of KwaZulu-Natal for their technical assistance, as well as the National Research Foundation (NRF) South Africa for their funding.

References

1. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H, Al Kaabi J. Epidemiology of Type 2 Diabetes – Global Burden of Disease and Forecasted Trends. Journal of Epidemiology and Global Health. 2019;10(1):107.
2. Mutyambizi C, Pavlova M, Hongoro C, Groot W. Inequalities and factors associated with adherence to diabetes self-care practices amongst patients at two public hospitals in Gauteng, South Africa. BMC Endocrine Disorders. 2020;20(1).
3. Fareed M, Salam N, Khoja AT, Mahmoud AM, Ahamed M. Life style related risk factors of type 2 diabetes mellitus and its increased prevalence in Saudi Arabia: A brief review. International Journal of Medical Research & Health Sciences. 2017;6(3):125-32.
4. Huang Y, Shi X, Li Z, Shen Y, Shi X, Wang L, et al. Possible association of Firmicutes in the gut microbiota of patients with major depressive disorder. Neuropsychiatric disease and treatment. 2018;14:3329.
5. Alizadeh A, Akbari P, Garssen J, Fink-Gremmels J, Braber S. Epithelial integrity, junctional complexes, and biomarkers associated with intestinal functions. Tissue Barriers. 2022;10(3):1996830.
6. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2010;299(2):G440-G8.

7. Devaraj S, Dasu MR, Jialal I. Diabetes is a proinflammatory state: a translational perspective. Expert review of endocrinology & metabolism. 2010;5(1):19-28.
8. Liu Z, Li N, Neu J. Tight junctions, leaky intestines, and pediatric diseases. Acta Paediatrica. 2007;94(4):386-93.
9. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.
10. Sturgeon C, Fasano A. Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. Tissue Barriers. 2016;4(4):e1251384.
11. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. AIDS (London, England). 2015;29(10):1263.
12. Wisniewski PJ, Dowden RA, Campbell SC. Role of dietary lipids in modulating inflammation through the gut microbiota. Nutrients. 2019;11(1):117.
13. Ghody P, Shikha D, Karam J, Bahtiyar G. Identifying prediabetes–Is it beneficial in the long run? Maturitas. 2015;81(2):282-6.
14. 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.
15. Nathan DM, Davidson MB, DeFronzo RA, Heine RJ, Henry RR, Pratley R, et al. Impaired fasting glucose and impaired glucose tolerance: implications for care. Diabetes care. 2007;30(3):753-9.
16. Luvuno M, Khathi, A., and Mabandla, M. V. Voluntary ingestion of a high-fat high-carbohydrate diet: a model for prediabetes. Masters of medical science dissertation University of KwaZulu-Natal; 2017.
17. 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.
18. Luvuno M, Mbongwa H, Khathi A. Development of a novel prediabetes animal model using a high fat high carbohydrate diet: implications for type 2 diabetes. PloS one. 2017;13:8-14.
19. Mtshali K, Khumalo ZTH, Kwenda S, Arshad I, Thekisoe OMM. Exploration and comparison of bacterial communities present in bovine faeces, milk and blood using 16S rRNA metagenomic sequencing. PLOS ONE. 2022;17(8):e0273799.
20. Musabayane C, Tufts M, Mapanga R. Synergistic antihyperglycemic effects between plant-derived oleanolic acid and insulin in streptozotocin-induced diabetic rats. Renal failure. 2010;32(7):832-9.
21. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. Journal of pharmacology & pharmacotherapeutics. 2010;1(2):87.
22. Wang L, Llorente C, Hartmann P, Yang A-M, Chen P, Schnabl B. Methods to determine intestinal permeability and bacterial translocation during liver disease. Journal of Immunological Methods. 2015;421:44-53.
23. Thaiss CA, Levy M, Grosheva I, Zheng D, Soffer E, Blacher E, et al. Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection. Science. 2018;359(6382):1376-83.
24. Daniel H, Gholami AM, Berry D, Desmarchelier C, Hahne H, Loh G, et al. High-fat diet alters gut microbiota physiology in mice. The ISME journal. 2014;8(2):295-308.
25. Rohr MW, Narasimhulu CA, Rudeski-Rohr TA, Parthasarathy S. Negative Effects of a High-Fat Diet on Intestinal Permeability: A Review. Advances in Nutrition. 2019.
26. De Kort S, Keszthelyi D, Masclee AAM. Leaky gut and diabetes mellitus: what is the link? Obesity Reviews. 2011;12(6):449-58.
27. Tai N, Wong FS, Wen L. The role of gut microbiota in the development of type 1, type 2 diabetes mellitus and obesity. Reviews in Endocrine and Metabolic Disorders. 2015;16(1):55-65.

28. Mabuza LP, Gamede MW, Maikoo S, Booysen IN, Ngubane PS, Khathi A. Cardioprotective effects of a ruthenium (II) Schiff base complex in diet-induced prediabetic rats . Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. 2019;Volume 12:217-23.
29. 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.
30. Huang BW, Chiang MT, Yao HT, Chiang W. The effect of high-fat and high-fructose diets on glucose tolerance and plasma lipid and leptin levels in rats. Diabetes, Obesity and Metabolism. 2004;6(2):120-6.
31. Gummesson A, Carlsson LMS, Storlien LH, Bäckhed F, Lundin P, Löfgren L, et al. Intestinal Permeability Is Associated With Visceral Adiposity in Healthy Women. Obesity. 2011;19(11):2280-2.
32. Shin N-R, Whon TW, Bae J-W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in biotechnology. 2015;33(9):496-503.
33. Seethaler B, Basrai M, Neyrinck AM, Nazare J-A, Walter J, Delzenne NM, et al. Biomarkers for assessment of intestinal permeability in clinical practice. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2021;321(1):G11-G7.
34. Kim AS, Ko H-J. Plasma concentrations of zonulin are elevated in obese men with fatty liver disease. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. 2018;Volume 11:149-57.
35. Guo S, Al-Sadi R, Said HM, Ma TY. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. Am J Pathol. 2013;182(2):375-87.
36. Derikx JP, Luyer MD, Heineman E, Buurman WA. Non-invasive markers of gut wall integrity in health and disease. World J Gastroenterol. 2010;16(42):5272-9.
37. Raetz CRH, Whitfield C. Lipopolysaccharide Endotoxins. Annual Review of Biochemistry. 2002;71(1):635-700.
38. Jayashree B, Bibin YS, Prabhu D, Shanthirani CS, Gokulakrishnan K, Lakshmi BS, et al. signify novel biomarkers of proinflammation in patients with type 2 diabetesIncreased circulatory levels of lipopolysaccharide (LPS) and zonulin Molecular and Cellular Biochemistry. 2014;388(1-2):203-10.
39. Yao Z, Mates JM, Cheplowitz AM, Hammer LP, Maiseyeu A, Phillips GS, et al. Blood-Borne Lipopolysaccharide Is Rapidly Eliminated by Liver Sinusoidal Endothelial Cells via High-Density Lipoprotein. The Journal of Immunology. 2016;197(6):2390-9.
40. Sandler NG, Koh C, Roque A, Eccleston JL, Siegel RB, Demino M, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. Gastroenterology. 2011;141(4):1220-30. e3.
41. Hawa MI. Immune and metabolic changes leading to diabetes: Queen Mary University of London; 2012.
42. Amasheh M, Fromm A, Krug SM, Amasheh S, Andres S, Zeitz M, et al. TNFα-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFκB signaling. Journal of cell science. 2010;123(23):4145-55.
43. Shen L, Black ED, Witkowski ED, Lencer WI, Guerriero V, Schneeberger EE, et al. Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. Journal of cell science. 2006;119(10):2095-106.
44. Bruewer M, Utech M, Ivanov AI, Hopkins AM, Parkos CA, Nusrat A. Interferon-γ induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. The FASEB Journal. 2005;19(8):923-33.
45. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nature Reviews Immunology. 2008;8(12):923-34.
46. Hoshiko H, Feskens EJM, Oosterink E, Ariens RMC, Mes JJ, De Wit NJW. Identification of leaky gut-related markers as indicators of metabolic health in Dutch adults: The Nutrition Questionnaires plus (NQplus) study. PLOS ONE. 2021;16(6):e0252936.

47. Al-Ayadhi L, Zayed N, Bhat RS, Moubayed NMS, Al-Muammar MN, El-Ansary A. The use of biomarkers associated with leaky gut as a diagnostic tool for early intervention in autism spectrum disorder: a systematic review. Gut Pathogens. 2021;13(1).
48. Adriaanse M, Tack G, Passos VL, Damoiseaux J, Schreurs M, Van Wijck K, et al. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. Alimentary pharmacology & therapeutics. 2013;37(4):482-90.
49. Funaoka H, Kanda T, Fujii H. [Intestinal fatty acid-binding protein (I-FABP) as a new biomarker for intestinal diseases]. Rinsho Byori. 2010;58(2):162-8.

BRIDGE

The first manuscript displayed that chronic consumption of a high-fat high carbohydrate (HFHC) diet imbalances the gut microbiota composition thus increasing markers associated with a leaky gut. This was evidenced by a significant increase in pathogenic phyla (*Bacteroidetes* and *Proteobacteria*) and a significant decrease in essential bacteria (*Firmicutes*). In addition, serum zonulin, plasma lipopolysaccharides, soluble CD14, tumor necrosis factor-alpha, interleukin-6, C-reactive protein, and intestinal fatty acid-binding protein were significantly increased in the prediabetic state. This resulted in subclinical chronic inflammation which was suggested to further compromise the intestinal barrier and thus progress the onset of prediabetes. Observing the effect of a HFHC diet in glucose homeostasis and markers associated with a leaky gut, led to the second study which sought to investigate the effect of a low carbohydrate high unsaturated fat (LCHUF) diet in concentration levels of markers associated with a leaky gut and glucose homeostasis.

CHAPTER 3: MANUSCRIPT 2

Prologue

Chronic consumption of a high-fat high carbohydrate (HFHC) diet results in the development of prediabetes. In the previous study in chapter 2, we established that the HFHC diet dysregulates the gut microbiota composition, which results in a leaky gut. In the prediabetic state, the subclinical chronic inflammation that was observed was suggested to further compromise the intestinal barrier thus contributing to insulin resistance. Prediabetes, an intermediary that precedes T2D, has become a therapeutic target over the years to reduce the prevalence of metabolic disorders. This condition has been reported to be reversible through lifestyle modifications that include moderate exercise and dietary intervention involving low amounts of carbohydrates. Therefore, this study sought to investigate the effect of a low carbohydrate and high unsaturated fat diet on glucose homeostasis and concentration levelsof markers associated with a leaky gut.

The manuscript in chapter 3 is titled "**Investigating the effect of a low carbohydrate, high unsaturated fat diet on concentration levels of markers associated with a leaky gut and the association with glucose homeostasis**".

The current manuscript is currently under review in the journal of **Nutrition and Diabetes** and has been formatted according to the journal's guidelines to authors (see Appendix 3).

Investigating The Effect of A Low Carbohydrate, High Unsaturated Fat Diet On Concentration Levels Of Markers Associated With A Leaky Gut And The Association With Glucose Homeostasis

Nosipho R. Dimba[1,2], Nhlakanipho Mzimela[1], Andile Khathi[1].

1. School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville, South Africa ,4000
2. Corresponding Author

Department of Human Physiology

School of Laboratory Medicine & Medical Sciences

University of KwaZulu-Natal

Private Bag X54001

Westville, South Africa, 4000

Phone number: 062 948 8290/ (031) 260 7585

Fax: (27) (31) 260 7132

Email address: 218006885@stu.ukzn.ac.za

Word Count: Abstract =319, manuscript = 4127

Abstract

Background: A high-fat high carbohydrate (HFHC) contribute to the development of prediabetes. This condition precedes the onset of type 2 diabetes (T2D). Hence it has become a therapeutic target to prevent metabolic disorders such as T2D. Several approaches such as lifestyle modifications that include moderate exercise and dietary intervention have been reported to effectively reverse prediabetes. This study investigated the effect of a low carbohydrate, high unsaturated fat (LCHUF) diet on glucose homeostasis and concentration levels of markers associated with a leaky gut in male SpragueDawley rats.

Methods: 12 male Sprague Dawley rats ingested a HFHC diet for a period of 20 weeks. After 20 weeks, the prediabetic animals were subdivided into two groups to further investigate the effects of diets for additional 12 weeks. Group 1 was the prediabetic control group (PD) that remained on a HFHC diet and group 2 was prediabetic group that switched to a LCHUF diet (PD+DI). At the end of 32 weeks, the fecal samples were analyzed to measure the gut microbiota composition of *Firmicutes, Bacteroidetes,* and *Proteobacteria* in both animal groups. Furthermore, blood glucose, glycated haemoglobin (HbA1c), serum zonulin, plasma LPS, soluble CD14, tumor necrosis factor-alpha, interleukin-6, C-reactive protein, and intestinal fatty-acid binding protein concentrations were measured.

Results: In the PD+DI group, there was a significant increase in the *Firmicutes* levels and a decrease in *Bacteroidetes* and *Proteobacteria* compared to the PD group. Blood glucose and HbA1c concentration were significantly decreased in the PD+DI group in comparison to the PD group. Serum zonulin and plasma sCD14 concentrations in the PD+DI group decreased compared to the PD group, while plasma LPS concentrations were similar. A decrease in plasma TNF-α, IL-6, CRP, and IFABP concentrations in the PD+DI was observed compared to the PD group.

Conclusion: Taken together, these results suggest that a LCHUF diet effectively improved glucose homeostasis, gut microbiota composition and beneficially reduced risk of insulin resistance observed in prediabetic state.

Keywords: Pre-diabetes, high-fat high carbohydrate, low carbohydrate high unsaturated fat, gut microbiota, zonulin, lipopolysaccharides, intestinal permeability

Introduction

According to Diabetes Excellence Centre and Endocrinology, Africa currently has 12.1 million people with diabetes mellitus, and this number is expected to almost double by the year 2023 (1). The prevalence of type 2 diabetes (T2D) has increased rapidly and has been shown to be often preceded by prediabetes (2). Prediabetes is an intermediate state that occurs between normal glucose regulation and T2D (3). It is diagnosed using specific parameters such as impaired fasting glucose, impaired glucose tolerance, or glycated haemoglobin (HbA1c) levels (4, 5). Prediabetes has become a therapeutic target to prevent metabolic disorders such as T2D (6, 7).

The intestinal barrier comprises tight junctions formed between enterocytes and integral proteins such as zonulin (8, 9). This protein functions to regulate leakiness in the gut by opening and closing spaces between tight junctions (10). Under normal dietary conditions, gut microbiota composition function to maintain the intestinal barrier, preventing the entry of toxic luminal substances from the lumen into the lamina propria (11). However, in diabetic patients, overconsumption of a high-fat diet has been shown to enhance the dysregulation of the intestinal barrier (12). In addition, this diet induces chronic-low inflammation in various organs (12, 13). A study conducted in our laboratory showed that chronic ingestion of a high-fat-high carbohydrates diet results in prediabetes (14). This diet-induced prediabetes has further been associated with changes in intestinal permeability integrity (Dimba et al., 2023). The findings from the study conducted by Dimba and colleagues revealed increased levels of markers associated with a leaky gut, such as zonulin, endotoxin lipopolysaccharide (LPS), and soluble CD14 (sCD14) (Dimba et al., 2023). High levels of zonulin cause translocation of endotoxins (LPS), which increased secretion of sCD14 and pro-inflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) interferon-*y* (IFN-*y*)) (15, 16). These cytokines in response to the translocation, stimulate inflammation which decreases insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscles (11). In addition, pro-inflammatory cytokines cause activation of nuclear factor kappa B (NF-kB) which in turn activates serine residue of insulin receptor substrate-1 that causes more inflammation leading to the development of insulin resistance seen in the prediabetic stage (17, 18).

Several studies have shown improved glucose tolerance with continued ingestion of a diet with low carbohydrates and high unsaturated fats (19-21). The effect of this diet on intestinal permeability has not yet been investigated. Therefore, the current study sought to investigate the effect of a low carbohydrateand high unsaturated fat diet on the concentration levels of markers associated with a leaky gut. We further investigated the association of the changes in these markers with changes in glucose homeostasis.

Materials and Methods

Animal and housing

Male Sprague-Dawley rats (150-180g), 3 weeks old bred for this study were kept in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal. All animal procedures and housing conditions were approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (ethics no: AREC/00003627/2021). The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), relative humidity (55±5%), and illumination (12 h light/dark cycle, lights on at 07h00). The level of noise was maintained at less than 65 decibels and the animals were allowed access to food and fluids *ad libitum*. The animals were allowed to acclimatize to their new environment for one week while consuming standard rat chow and water before they were exposed to a high-fat, high-carbohydrate (HFHC) experimental diet (14).

Induction of prediabetes

Male Sprague-Dawley were randomly divided into two diet groups, group A (n=6) and group B (n=6). Experimental prediabetes was induced in animals using a protocol previously described by Luvuno et al., 2017. Briefly, both groups (A & B) of animals were exposed to a HFHC diet supplemented with 15% fructose for a period of 20 weeks. After 20 weeks, the American Diabetes Association criteria for diagnosis of prediabetes was used to diagnose prediabetes in all animals. Briefly, all animals that would exhibit fasting blood glucose concentrations of 5.6 to 7.1 mmol/L, and glycated haemoglobin of 5.7-6.4% were considered prediabetic. Group A animals remained on the HFHC diet, while the other 6 animals in group B were switched to a low carbohydrate, high unsaturated fat (LCHUF) diet. Group B wasthen categorized as the prediabetic group that had dietary intervention (PD+DI). All animals were thenmaintained on their respective diets and monitored for further 12 weeks, as Figure 1 shows below.

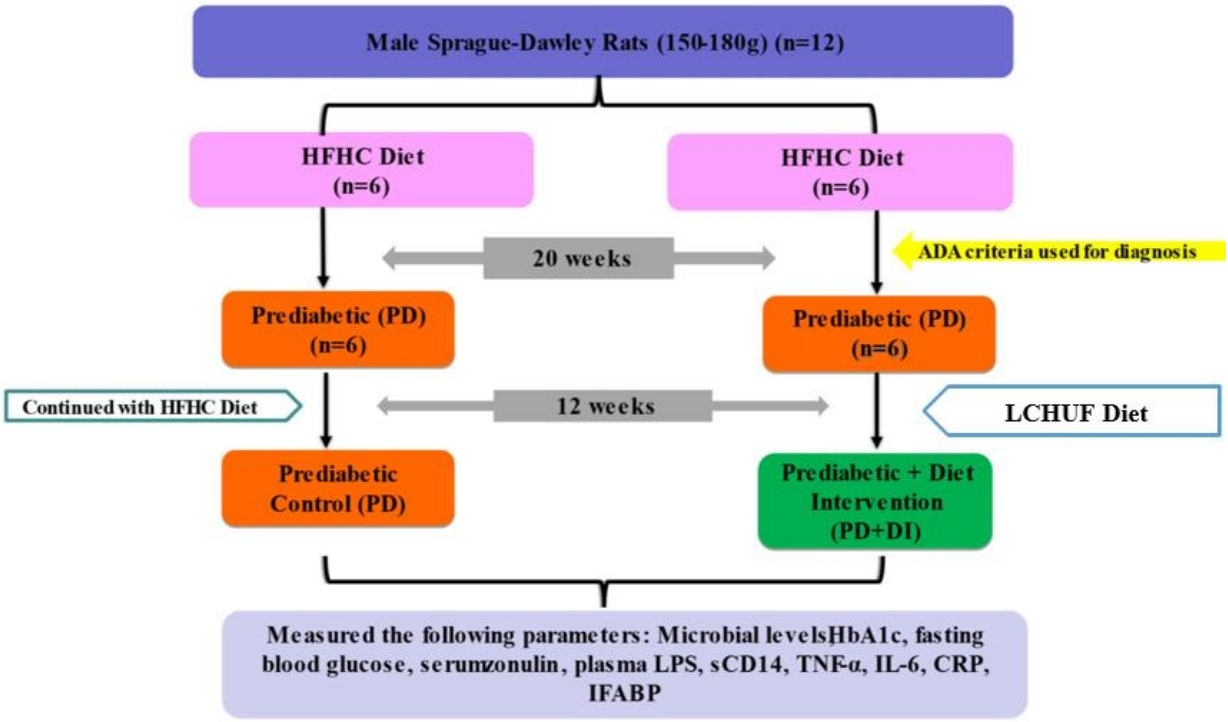

Figure 1: Schematic diagram showing the experimental design.

Experimental procedures

Sample collection and Analysis

Prior to sampling the rats were placed in individual tecniplast metabolic cages. The fecal samples were then collected from both groups of rats that were fed a HFHC diet supplemented with 15% fructose and the group that had dietary intervention at the end of the experimental period (32 weeks). Once obtained, fecal samples were placed into a sterile zip-lock bag. The samples were then transported to Inqaba Biotechnology laboratory in cooler boxes containing ice packs for further analysis. Upon arrival, the samples were pre-processed for DNA extraction and stored in a Bio Ultra freezer at -80 °C (Labtech, Umhlanga, South Africa). Microbial DNA from the samples was extracted using a previously described protocol (22). Each pooled DNA sample of the faeces constituted of an equimolar mixture of two samples obtained from the same animal. Thereafter, the pools were quantified, then normalized for Illumina sequencing.

Amplification of bacterial 16S rRNA and sequencing

The bacterial 16S rRNA gene was amplified using M13-tailed target-specific primer with 5' block. PCR Forward Primer: 5′ GTA AAA CGA CGG CCA GT(N) 3′ and PCR Reverse Primer: 5′ CAG GAA ACA GCT ATG AC(N) 3′ (Integrated DNA Technologies, Whitehead Scientific). The samples were processed aseptically to minimise contamination. We added two non-template controls consisting of nuclease free water as well as PCR and sequencing laboratory reagents in place of experimental DNA

template were incorporated in the amplification and sequencing steps and processed alongside the test samples. Library preparation was performed according to the standard instructions of the 16S SMRTbell® prep kit 3.0 (PacificBiosciences, California, USA). Indexed amplicons were quantified using Qubit® High Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific) and the sizes of the amplicons were visualised using the 4200 TapeStation (Agilent Technologies, Germany). The normalized libraries were pooled for sequencing, denatured to single strand using NaOH, then PhiX (10%) was added to the library. Libraries were then sequenced using the 16S SMRTbell® prep kit 3.0 (PacificBiosciences, California, USA).

Fasting glucose

An established laboratory methodology was used to conduct the OGTT on both groups to obtain fasting glucose measurements. Briefly, animals were fasted at the end of the 32 weeks for 12 hours and their blood was collected using a tail prick method. Glucose concentrations were measured using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom) (23).

Blood collection

For an experimental period of 32 weeks, all the animals were anaesthetized 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 3 minutes. Blood was collected by cardiac puncture while the rats were unconscious and then injected into individual pre-cooled heparinized containers. After that, the blood was centrifuged for 15 minutes at 4°C, 503 g (Eppendorf centrifuge 5403, Germany). Plasma was collected and stored at -80 °C in a Bio Ultra freezer (Snijers Scientific, Holland) for biochemical analysis.

Biochemical analysis

Fasting blood glucose, plasma lipopolysaccharides (LPS), zonulin, and tumor necrosis factor-α (TNF-α) concentrations were measured at Lab 24 (Mt. Edgecombe, Durban). Glycated haemoglobin (HbA1c), soluble CD14 (sCD14), intestinal fatty acid-binding protein (IFABP), C-reactive protein (CRP), and interleukin-6 (IL-6) concentrations were measured using their respective rat-sandwich ELISA kits from Elabscience (Wuhan, China) as per manufacturer's instructions.

Statistical Analysis

All data were expressed as means $\pm$ S.E.M. Statistical analysis was performed with a Graph Pad InStat Software (version 5.00, Graph Pad Software, Inc., San Diego, California, USA) using a student t test to determine statistical differences between two independent groups. A value of $p < 0.05$ was considered statistically significant.

Results

Gut microbiota composition

By comparison with the prediabetic control group, the prediabetic animals that were switched to a LCHUF diet (PD+DI) showed a significant increase in *Firmicutes* and a significant decrease in pathogenic phyla (*Bacteroidetes* and *Proteobacteria*) ($p < 0.0001$; Table 1) after 12 weeks.

Table 1: Gut microbiota composition in PD (HFHC) and PD + DI (LCHUF) diet fed animals (n-6, pergroup) at week 32 of the experiment.

	WEEK 32	
	GROUPS	
Gut Microbiota Composition	**PD (HFHC diet, %)**	**PD + DI (LCHUF diet, %)**
Firmicutes	63.1 ± 0.9	76.1 ± 1.0 * * *
Bacteroidetes	12.8 ± 0.6	5.6 ± 0.3 * * *
Proteobacteria	13.6 ± 0.6	11.3 ± 0.5 * * *

* * * = $p < 0.0001$ denotes comparison with PD

By comparison with the prediabetic control group, the prediabetic animals that were switched to a LCHUF diet (PD+DI) showed a significant decrease in fasting blood glucose and glycated haemoglobin (HbA1c) ($p < 0.0001$; Table 1) after 12 weeks.

Blood glucose and HbA1c

Table 2: Blood glucose and glycated haemoglobin of rats in week 32 following ingestion of a HFHC and LCHUF diets (n=6, per group).

	WEEK 32	
GROUPS	**PD (HFHC Diet)**	**PD + DI (LCHUF Diet)**
Blood Glucose (mmol/L)	6.72 ± 0.12	5.4 ± 0.08 * * *

HbA1c (%)	6.1 ± 0.07	5.3 ± 0.05 * * *

* * * = p < 0.0001 denotes comparison with PD

Serum zonulin

Figure 2 shows serum zonulin concentration levels measured in both groups: PD and PD+DI after 12 weeks. The prediabetic animals that were switched to a LCHUF diet (PD+DI) showed a significant decrease in serum zonulin by comparison with the prediabetic control (PD) ($p < 0.0001$).

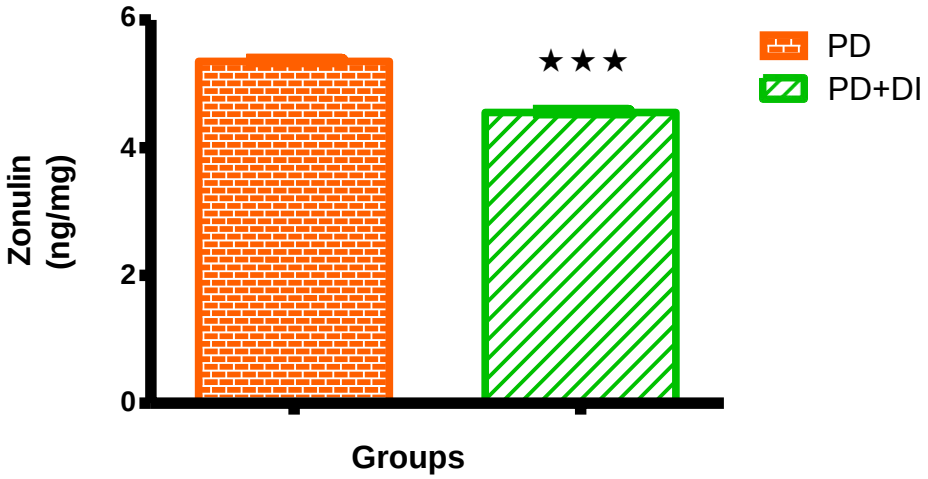

Figure 2: Serum zonulin concentration in the prediabetic control (PD) and prediabetic group with dietary intervention (PD+DI) at the end of the experimental period (32 weeks). The values are presented as the standard error of mean SEM (n=6, per group). * * * = p < 0.0001 denotes comparison with PD.

Plasma lipopolysaccharide (LPS)

Figure 3 shows plasma lipopolysaccharide concentration levels measured in both groups: PD and PD+DI after 12 weeks. The result (Fig.3) showed that there were no significant changes to plasma LPS concentration in prediabetic control (PD) and the prediabetic group that was switched to a LCHUF diet (PD+DI).

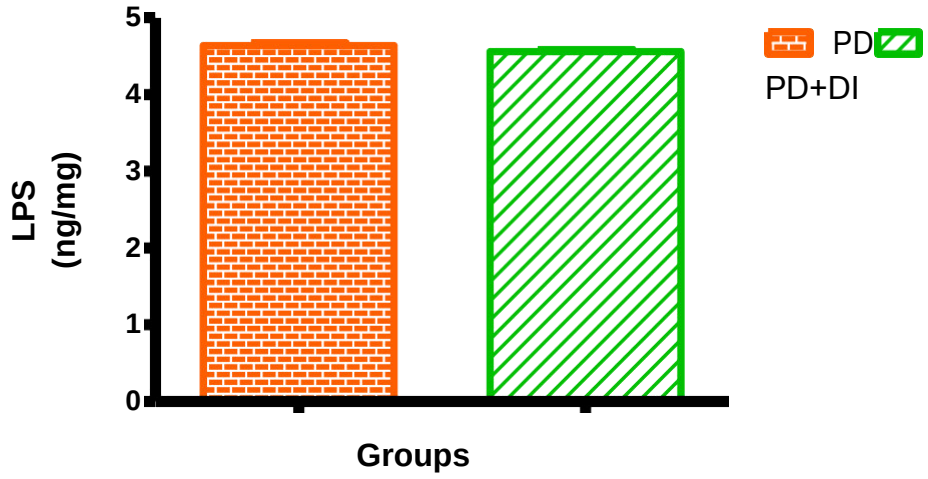

Figure 3: Plasma lipopolysaccharide concentration in the prediabetic control (PD) and prediabetic group with dietary intervention (PD+DI) at the end of the experimental period (32 weeks). The values are presented as the standard error of mean SEM (n=6, per group).

Plasma soluble CD14 (sCD14)

Figure 4 shows plasma soluble CD14 concentration levels measured in both groups: PD and PD+DI after 12 weeks. The prediabetic animals that were switched to a LCHUF diet (PD+DI) showed a significant decrease in plasma sCD14 by comparison with the prediabetic control (PD) ($p < 0.0001$).

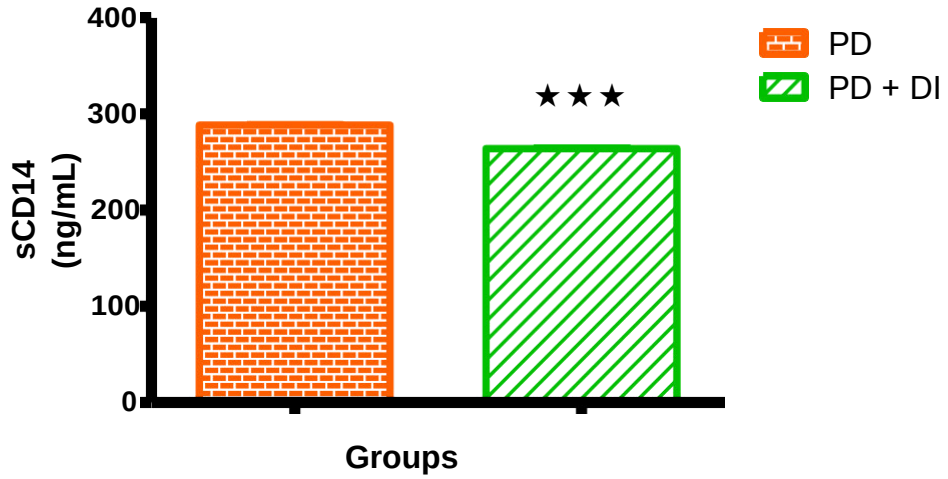

Figure 4: Plasma soluble CD14 concentration in the prediabetic control (PD) and prediabetic group with dietary intervention (PD+DI) at the end of the experimental period (32 weeks). The values are presented as the standard error of mean SEM (n=6, per group). * * * = $p < 0.0001$ denotes comparison with PD.

Plasma tumor necrosis alpha (TNF-α)

Figure 5 shows plasma tumor necrosis alpha concentration levels measured in both groups: PD and PD+DI after 12 weeks. The prediabetic animals that were switched to a LCHUF diet (PD+DI) had a significant decrease in plasma TNF-α when compared with the prediabetic control (PD) ($p < 0.0001$).

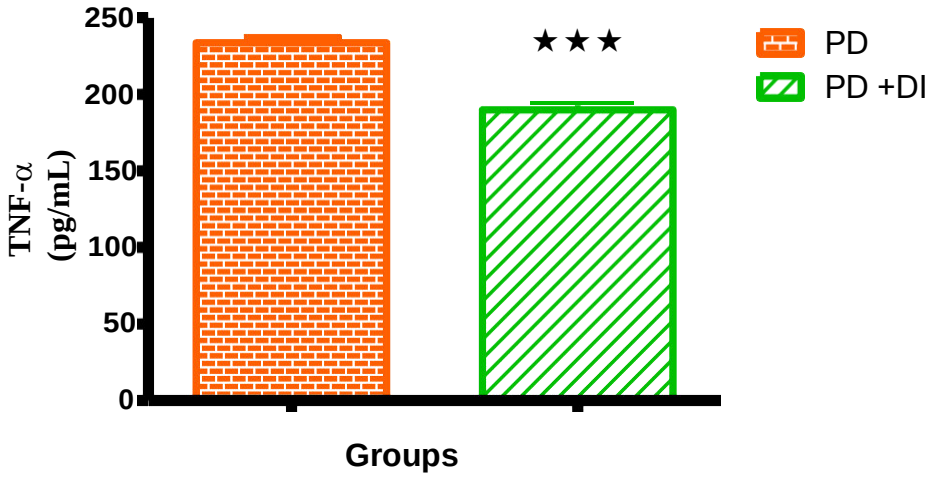

Figure 5: Plasma tumor necrosis alpha concentration in the prediabetic control (PD) and prediabetic group with dietary intervention (PD+DI) at the end of the experimental period (32 weeks). The values are presented as the standard error of mean SEM (n=6, per group). * * * = $p < 0.0001$ denotes comparison with PD.

Plasma interleukin-6 (IL-6)

Figure 6 shows plasma interleukin-6 concentration levels measured in both groups: PD and PD+DI after 12 weeks. The prediabetic animals that were switched to a LCHUF diet (PD+DI) had a significant decrease in plasma IL-6 when compared with the prediabetic control (PD) ($p < 0.0001$).

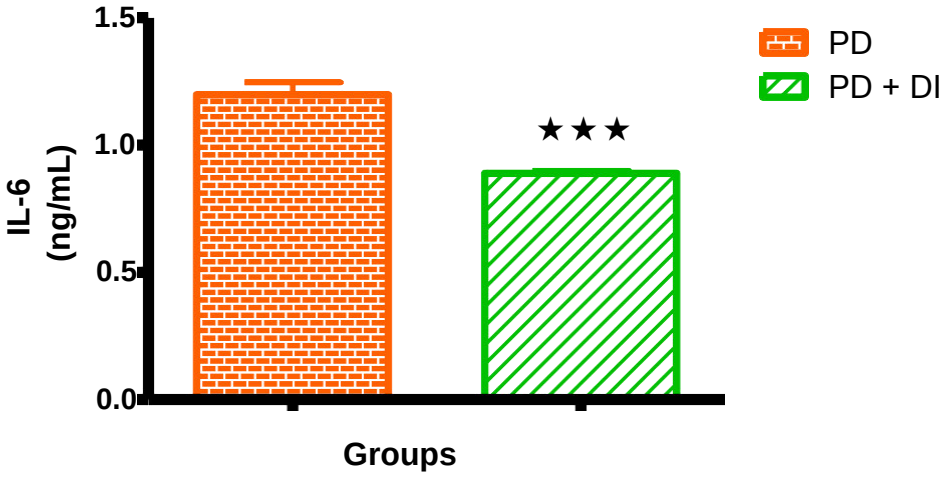

Figure 6: Plasma interleukin-6 (IL-6) concentration in the prediabetic control (PD) and prediabetic group with dietary intervention (PD+DI) at the end of the experimental period (32 weeks). The values are presented as the standard error of mean SEM (n=6, per group). * * * = $p < 0.0001$ denotes comparison with PD.

Plasma c-reactive protein (CRP)

Figure 7 shows plasma C-reactive protein concentration levels measured in both groups: PD and PD+DI after 12 weeks. The prediabetic animals that were switched to a LCHUF diet (PD+DI) showed a significant decrease in plasma sCD14 by comparison with the prediabetic control (PD) ($p < 0.0001$).

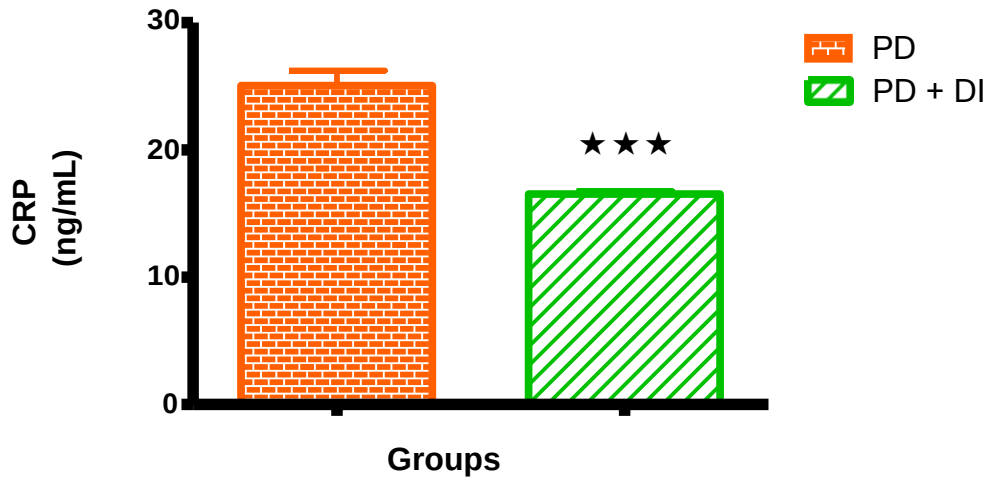

Figure 7: Plasma c-reactive protein (CRP) in the prediabetic control (PD) and prediabetic group with dietary intervention (PD+DI) at the end of the experimental period (32 weeks). The values are presented as the standard error of mean SEM (n=6, per group). * * * = $p < 0.0001$ denotes comparison with PD.

Plasma intestinal fatty-acid binding protein (IFABP)

Figure 8 shows plasma intestinal fatty-acid binding protein concentration levels measured in both groups: PD and PD+DI after 12 weeks. The prediabetic animals that were switched to a LCHUF diet (PD+DI) had a significant decrease in plasma IFABP by comparison with the prediabetic control (PD) ($p < 0.0001$).

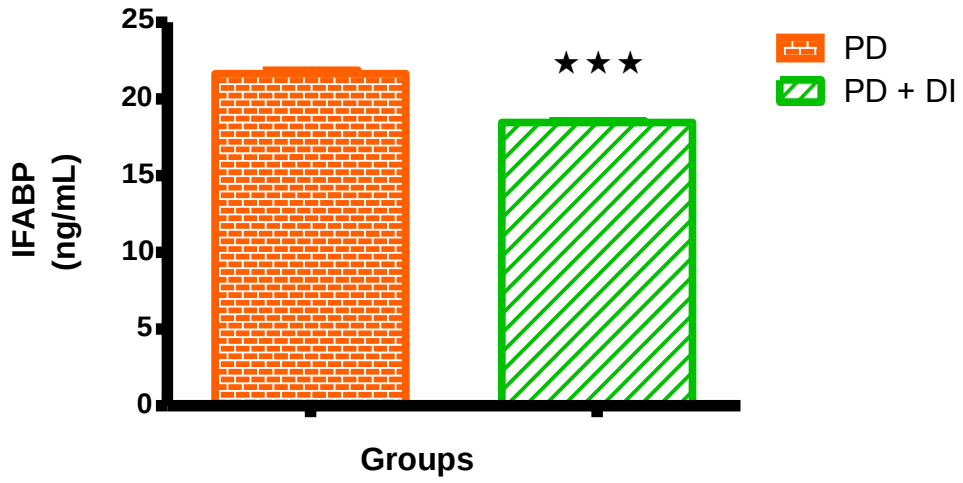

Figure 8: Plasma intestinal fatty acid-binding protein (IFABP) concentration in the prediabetic control (PD) and prediabetic group with dietary intervention (PD+DI) at the end of the experimental period (32 weeks). The values are presented as the standard error of mean SEM (n=6, per group). * * * = $p < 0.0001$ denotes comparison with PD.

Discussion

A diet high in fats and carbohydrates has been shown to result in gut dysbiosis which subsequently disrupts the intestinal barrier integrity, thus contributing to the development of a leaky gut (Dimba et al., 2023). It is well known that a leaky gut leads to metabolic disorders such as prediabetes (8). Prediabetes is a long-lasting condition that often precedes the onset of type 2 diabetes mellitus (T2DM) (3). However, studies have indicated that prediabetes is reversible (4, 24). In these studies, they employed several strategies, such as lifestyle modifications that include moderate exercise and dietary intervention (DI), which effectively reduced the incidence of T2D (24, 25). A study conducted in our laboratory developed a high-fat, high carbohydrate (HFHC) diet-induced prediabetic animal model, which mimicked the human condition of prediabetes (14, 26). In this model, we found that chronic consumption of a HFHC diet dysregulates the composition of the gut microbiota, which causes an imbalance between beneficial and unfavorable bacteria leading to what is known as gut dysbiosis (Dimba et al., 2023). Furthermore, chronic consumption of this diet causes the translocation of pathogens or toxins across the blood, which triggers an inflammatory response that further compromises the intestinal lining and other organs (Dimba et al., 2023). In this study, we used this model to investigate changes in the concentration level of markers associated with a leaky gut following a change in diet to a low carbohydrate, high unsaturated fats (LCHUF) diet for 12 weeks. We further investigated the concomitant effect on glucose homeostasis.

Consuming a HFHC diet over an extended period of time can cause persistently higher blood glucose levels, which may be related to the insulin resistance observed in prediabetes (Dimba et al., 2023). Insulin resistance is a common condition in which one body's cells become less effective in taking up blood glucose (27). In this study, the effect of a LCHUF diet on glucose homeostasis was investigated in a HFHC diet-induced animal model. A significant decrease in fasting blood glucose was observed in animals that changed their diet to a LCHUF in comparison to those that remained on a HFHC diet. Thefindings suggest that a LCHUF diet had a beneficial effect on blood glucose regulation. Moreover, decreased glycated haemoglobin (HbA1c) concentration in the prediabetic group that was switched to a LCHUF diet in comparison to the prediabetic control group was observed in this study. This observation along with a decrease in fasting blood glucose concentration suggests that a LCHUF diet might have improved insulin sensitivity. In the prediabetic state, the cells are reported to have not completely lost their sensitivity to insulin however, it is a slow progression that occurs due to the impairment of the insulin signalling pathway (2). Therefore, considering a lower HbA1c and fasting blood glucose there is reason to believe that a LCHUF diet may prove more efficacious in reversing or delaying the onset of prediabetes and its progression to T2D.

The gut microbiota is composed of different bacterial phylum, including *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Tenericutes* (28, 29). They all play an essential role in the intestinal homeostasis of a host. They provide defense against harmful microbes, break down toxic compounds, improve the immune system, and allow the absorption of ions and vitamins (16, 30, 31). According to a previous study, the HFHC diet is one of the major factors influencing the composition of the gut microbiota as previously discussed (16, 25, 32). However, a low carbohydrate diet has been shown to re-establish healthy gut microbiota thus stimulate gut health (33). The findings reported by Attaye et al., 2021 correlate with this study, which showed a significant increase of *Firmicutes* and a decrease of *Bacteroidetes* and *Proteobacteria* in the prediabetic group that had dietary intervention in comparison to the prediabetic control group that continued with HFHC diet. The phylum *Firmicutes* provide a protective function of the intestinal barrier and protect the intestine from inflammation (30). In contrast, pathogenic phylum including *Bacteroidetes* and *Proteobacteria* exacerbate inflammation in the gut and contribute to the onset of metabolic disorders (34, 35). We speculate that a reduction of pathogenic phyla and upregulation of *Firmicutes* improved normal intestinal homeostasis thus reducing inflammation in the gastrointestinal tract.

In this study, the effect of a low carbohydrate high unsaturated fat diet on zonulin, a protein that can reversibly regulate IP by modulating tight junctions was investigated (36). According to existing literature, this protein gets triggered or get stimulated on epithelium cells by pathogenic bacteria, gluten, or, more specifically, its two components, gliadin, and glutenin (10, 37). In addition, a recent study from our laboratory reported that high levels of harmful bacteria trigger zonulin expression and these findings agree with the observations of the previous literature (Dimba et al., 2023). In the present study,

we observed a significant decrease in serum zonulin concentration in the prediabetic group that was switched to a LCHUF diet in comparison to the prediabetic control group that remained on a HFHC diet. These findings observed in the current study further support previous research, which reported that a low carbohydrate diet might promote the proliferation of beneficial species and improve gut permeability by keeping the intestinal barrier intact (33). Moreover, the result suggests that a LCHUF diet can effectively reduce pathogenic bacteria colonization thereby decreasing the expression of zonulin.

Increased zonulin expression upon chronic consumption of a high-fat high, carbohydrate diet causes a leaky gut (Dimba et al., 2023). It also enhances the translocation of toxic substances such as lipopolysaccharides (LPS), peptidoglycan, and food components from the lumen into the bloodstream (12, 38). In the current study, LPS, a marker associated with increased IP, was observed. LPS is a bacterial endotoxin derived from gram-negative bacteria species (39). In this study, there was no significant difference in plasma LPS concentrations observed between the prediabetic group that was switched to a LCHUF diet and the prediabetic control group. A possible reason for the observed result may be because a LCHUF diet effectively reduced the expression of zonulin, which improved the integrity of the tight junction and reduced toxins such as LPS from being translocated into the lamina propria. However, to further understand if these animals indeed had any changes in concentration levels of plasma LPS in their circulation, we measured sCD14 a co-receptor of LPS (40). According to Shive et al., 2015, the secretion of sCD14 from immune cells is induced by LPS, hence its high plasma levels were speculated to indicate LPS exposure (41, 42). We found a significant decrease in plasma sCD14 in the prediabetic group that was switched to a LCHUF diet in comparison to the prediabetic control group that remained on a HFHC diet. We, therefore, speculate that a decrease in the secretion of sCD14 from the immune cells is due to less LPS exposure in the bloodstream.

Studies have shown that the innate and adaptive immune systems are constantly activated by circulating toxins and luminal antigens in the blood (36, 39). However, this protective mechanism has been shown to negatively impact or further compromise the intestinal barrier and extraintestinal organs in the prediabetic state (Dimba et al., 2023). According to the literature, bacterial endotoxins and antigens in the circulation induce a release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interferon-*y* (IFN-*y*) and c-reactive protein (CRP) (8). TNF-α increases paracellular permeability by binding to TNF-receptor 2 which activate the myosin light chain in intestinal epithelial cell (43). This activation removes transmembrane proteins such as claudin-1 from tight junctions thus promoting occluding degradation (44, 45). The potential mechanism through which IFN-y modulates epithelial tight junction barrier is by rearranging cytoskeletal, protein expression, and localization of tight junctions (46). Furthermore, these cytokines are responsible for the pathophysiological effect of clinical and subclinical inflammation, which is suggested as a mediator of insulin resistance (27, 47, 48). They result in insulin resistance by activating the c-Jun-N-terminal (JNK)

and inhibitor kappa B kinase (IKKβ) (49-51). This activation results in serine phosphorylation of insulin receptor-1 (IRS-1) and insulin receptor-2 (IRS-2), which subsequently interferes with the insulin signaling pathway (49-51). Therefore, a decrease in plasma LPS concentration observed in this study correlates with the observed decreased concentration levels of the plasma TNF-α, IL-6, and CRP in the prediabetic group that was switched to a LCHUF diet in comparison to the prediabetic control group that remained on a HFHC diet. These observations suggest that the immune response eventually gets less triggered or activated as the leakiness of the bacterial toxins across the gut into circulation reduces.

Increased intestinal permeability and low-grade inflammation have been shown to be associated with many intestinal disorders, including irritable bowel syndrome, inflammatory bowel disease, and celiac disease (15). Patients which suffered from these disorders were reported to have high levels of pro-inflammatory cytokines (TNF-α, IL-6, IFN-y) and intestinal fatty acid-binding protein (IFABP) (52-54). IFABP is a useful marker of intestinal damage, as it is mainly expressed in epithelial cells of the small intestine mucosal layer (52, 55). According to the literature, increased concentrations of IFABP only get translocated into the circulation following enterocyte and epithelial cell damage (40, 55, 56). A study conducted in our laboratory using diet-induced prediabetic rat models showed a significant increase concentration level of plasma IFABP in the prediabetic state (Dimba et al., 2023). This study speculated that the intestinal barrier, enterocytes, and tight junctions were compromised during this stage, and these animals were certainly at risk of developing chronic disease (Dimba et al., 2023). Notably, in the current study, the prediabetic group that was switched to a LCHUF diet showed a significant decrease in plasma IFABP concentration compared to the pre-diabetic control group that remained on a HFHC diet. These findings might indicate that the pre-diabetic animals that had dietary intervention have a lower risk of developing the intestinal disease as the inflammatory response gets less triggered, supported by the previously discussed result of decreased plasma LPS, TNF-α, IL-6, and CRP concentration (25). This may have allowed the epithelial integrity to be restored by replacing and regenerating any damaged cells with new ones from the intestinal crypt (57). However, a recent study showed that there is no short-term cure for maintaining normal intestinal flora (40). So, depending on the situation, it may take up to three to six months to completely develop an adequate intestinal barrier, but only if dietary interventions are carefully implemented (40).

Conclusion

In this study, a low carbohydrate, high unsaturated fat (LCHUF) diet effectively improved glucose homeostasis in the pre-diabetic state. Dietary changes were made to target a leaky gut and to reduce the incidence of T2D. The effect of a LCHUF diet markedly improved gut microbiome composition, evidenced by a decrease of *Firmicutes* and an increase of *Bacteroidetes* and *Proteobacteria*. These findings reduce the release of serum zonulin and its effect of dissembling the tight junctions. This is evidenced by a decrease in plasma LPS and sCD14 concentration. In addition, these results were accompanied by a decrease in plasma TNF-α, IL-6, CRP, and IFABP indicating another beneficial

effect of this diet on reducing intestinal inflammation, risk of insulin resistance, and intestinal disorders in diet-induced pre-diabetic rats.

Future recommendations

In future studies, the histology of the intestinal lining and mucosal tight junctions, which were postulated to be modulated by elevated zonulin in the pre-diabetic state should further be validated on whether they are fully restored after switching to a LCHUF diet.

Acknowledgments

The authors would like to acknowledge the Biomedical Resource Units of KwaZulu-Natal for their technical assistance, as well as the National Research Foundation (NRF) South Africa for their funding.

Author Contributions

NRD and NM contributed to the study design, conducted the experiments, collected, analyzed and interpreted data, as well as being involved in writing the manuscript. NRD and NM were also involved in the study design, interpretation of data and editing of the manuscript. AK was involved in the conceptualization of the study, study design, analysis, and interpretation of data, writing and editing of the manuscript as well as provide funding. All authors have read and approved submission of the final manuscript.

Competing Interests

The authors declare no conflict of interest.

Data Availability Statement

The datasets presented in this study are included in the article and available on reasonable request from the corresponding author.

References

1. Wagenaar P, Hardy G. Cde watch: 2012 update from the centres for diabetes excellence: summaries. South African Journal of Diabetes and Vascular Disease. 2012;9(4):183-8.
2. Grundy SM. Pre-diabetes, metabolic syndrome, and cardiovascular risk. Journal of the American College of Cardiology. 2012;59(7):635-43.
3. 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.
4. Bansal N. Prediabetes diagnosis and treatment: A review. World journal of diabetes. 2015;6(2):296.
5. Organization WH. Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia: report of a WHO/IDF consultation. 2006.
6. Galaviz KI, Weber MB, Suvada K, Gujral UP, Wei J, Merchant R, et al. Interventions for Reversing Prediabetes: A Systematic Review and Meta-Analysis. American Journal of Preventive Medicine. 2022;62(4):614-25.
7. Sallar A, Dagogo-Jack S. Regression from prediabetes to normal glucose regulation: state of the science. Experimental Biology and Medicine. 2020;245(10):889-96.
8. De Kort S, Keszthelyi D, Masclee AAM. Leaky gut and diabetes mellitus: what is the link? Obesity Reviews. 2011;12(6):449-58.
9. De Santis S, Cavalcanti E, Mastronardi M, Jirillo E, Chieppa M. Nutritional keys for intestinal barrier modulation. Frontiers in immunology. 2015;6:612.
10. Sturgeon C, Fasano A. Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. Tissue Barriers. 2016;4(4):e1251384.
11. Tung Y-T, Zeng J-L, Ho S-T, Xu J-W, Lin IH, Wu J-H. Djulis Hull Improves Insulin Resistance and Modulates the Gut Microbiota in High-Fat Diet (HFD)-Induced Hyperglycaemia. Antioxidants. 2021;11(1):45.
12. Thaiss CA, Levy M, Grosheva I, Zheng D, Soffer E, Blacher E, et al. Hyperglycemia drives intestinal barrier dysfunction and risk for enteric infection. Science. 2018;359(6382):1376-83.
13. Rohr MW, Narasimhulu CA, Rudeski-Rohr TA, Parthasarathy S. Negative Effects of a High-Fat Diet on Intestinal Permeability: A Review. Advances in Nutrition. 2019.
14. Luvuno M, Mbongwa H, Khathi A. Development of a novel prediabetes animal model using a high fat high carbohydrate diet: implications for type 2 diabetes. PloS one. 2017;13:8-14.
15. Fukui H. Increased Intestinal Permeability and Decreased Barrier Function: Does It Really Influence the Risk of Inflammation? Inflammatory Intestinal Diseases. 2016;1(3):135-45.
16. Wisniewski PJ, Dowden RA, Campbell SC. Role of dietary lipids in modulating inflammation through the gut microbiota. Nutrients. 2019;11(1):117.
17. Baker RG, Hayden MS, Ghosh S. NF-κB, inflammation, and metabolic disease. Cell metabolism. 2011;13(1):11-22.
18. Mzimela NC, Ngubane PS, Khathi A. 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. Autoimmunity. 2019;52(1):27-36.
19. Cai L, Yin J, Ma X, Mo Y, Li C, Lu W, et al. Low-carbohydrate diets lead to greater weight loss and better glucose homeostasis than exercise: a randomized clinical trial. Frontiers of Medicine. 2021;15(3):460-71.
20. Russell WR, Baka A, Björck I, Delzenne N, Gao D, Griffiths HR, et al. Impact of Diet Composition on Blood Glucose Regulation. Critical Reviews in Food Science and Nutrition. 2016;56(4):541-90.
21. Tricò D, Moriconi D, Berta R, Baldi S, Quinones-Galvan A, Guiducci L, et al. Effects of Low-Carbohydrate versus Mediterranean Diets on Weight Loss, Glucose Metabolism, Insulin Kinetics and β-Cell Function in Morbidly Obese Individuals. Nutrients. 2021;13(4):1345.

22. Mtshali K, Khumalo ZTH, Kwenda S, Arshad I, Thekisoe OMM. Exploration and comparison of bacterial communities present in bovine faeces, milk and blood using 16S rRNA metagenomic sequencing. PLOS ONE. 2022;17(8):e0273799.
23. Musabayane C, Tufts M, Mapanga R. Synergistic antihyperglycemic effects between plant-derived oleanolic acid and insulin in streptozotocin-induced diabetic rats. Renal failure. 2010;32(7):832-9.
24. Perreault L, Færch K. Approaching Pre-diabetes. Journal of Diabetes and its Complications. 2014;28(2):226-33.
25. Yang, Liang, Balakrishnan, Belobrajdic, Feng, Zhang. Role of Dietary Nutrients in the Modulation of Gut Microbiota: A Narrative Review. Nutrients. 2020;12(2):381.
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. Soumya D, Srilatha B. Late stage complications of diabetes and insulin resistance. J Diabetes Metab. 2011;2(9):1000167.
28. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.
29. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. The ISME journal. 2011;5(2):220-30.
30. Huang Y, Shi X, Li Z, Shen Y, Shi X, Wang L, et al. Possible association of Firmicutes in the gut microbiota of patients with major depressive disorder. Neuropsychiatric disease and treatment. 2018;14:3329.
31. Ruiz L, Delgado S, Ruas-Madiedo P, Sánchez B, Margolles A. Bifidobacteria and their molecular communication with the immune system. Frontiers in Microbiology. 2017;8:2345.
32. 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.
33. Attaye I, van Oppenraaij S, Warmbrunn MV, Nieuwdorp M. The Role of the Gut Microbiota on the Beneficial Effects of Ketogenic Diets. Nutrients. 2021;14(1).
34. Hiippala K, Jouhten H, Ronkainen A, Hartikainen A, Kainulainen V, Jalanka J, et al. The potential of gut commensals in reinforcing intestinal barrier function and alleviating inflammation. Nutrients. 2018;10(8):988.
35. Shin N-R, Whon TW, Bae J-W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in biotechnology. 2015;33(9):496-503.
36. Fasano A. Intestinal permeability and its regulation by zonulin: diagnostic and therapeutic implications. Clinical Gastroenterology and Hepatology. 2012;10(10):1096-100.
37. Clemente MG, De Virgiliis S, Kang J, Macatagney R, Musu M, Di Pierro M, et al. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. Gut. 2003;52(2):218-23.
38. Jayashree B, Bibin YS, Prabhu D, Shanthirani CS, Gokulakrishnan K, Lakshmi BS, et al. signify novel biomarkers of proinflammation in patients with type 2 diabetesIncreased circulatory levels of lipopolysaccharide (LPS) and zonulin Molecular and Cellular Biochemistry. 2014;388(1-2):203-10.
39. Derikx JP, Luyer MD, Heineman E, Buurman WA. Non-invasive markers of gut wall integrity in health and disease. World J Gastroenterol. 2010;16(42):5272-9.
40. Al-Ayadhi L, Zayed N, Bhat RS, Moubayed NMS, Al-Muammar MN, El-Ansary A. The use of biomarkers associated with leaky gut as a diagnostic tool for early intervention in autism spectrum disorder: a systematic review. Gut Pathogens. 2021;13(1).
41. Sandler NG, Koh C, Roque A, Eccleston JL, Siegel RB, Demino M, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. Gastroenterology. 2011;141(4):1220-30. e3.

42. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. AIDS (London, England). 2015;29(10):1263.
43. Su L, Nalle SC, Shen L, Turner ES, Singh G, Breskin LA, et al. TNFR2 Activates MLCK-Dependent Tight Junction Dysregulation to Cause Apoptosis-Mediated Barrier Loss and Experimental Colitis. Gastroenterology. 2013;145(2):407-15.
44. Amasheh M, Fromm A, Krug SM, Amasheh S, Andres S, Zeitz M, et al. TNFα-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFκB signaling. Journal of cell science. 2010;123(23):4145-55.
45. Turner JR. Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. The American journal of pathology. 2006;169(6):1901-9.
46. Bruewer M, Utech M, Ivanov AI, Hopkins AM, Parkos CA, Nusrat A. Interferon-γ induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. The FASEB Journal. 2005;19(8):923-33.
47. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. American journal of physiology-endocrinology and metabolism. 2001.
48. Ragheb RF, Medhat AM. Mechanisms of fatty acid-induced insulin resistance in muscle and liver. 2009.
49. Coope A, Torsoni AS, Velloso LA. Mechanisms in endocrinology: metabolic and inflammatory pathways on the pathogenesis of type 2 diabetes. European Journal of Endocrinology. 2016;174(5):R175-R87.
50. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, et al. Serine phosphorylation of insulin receptor substrate 1 by inhibitor κB kinase complex. Journal of Biological Chemistry. 2002;277(50):48115-21.
51. Navarro-González JF, Mora-Fernández C, De Fuentes MM, García-Pérez J. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. Nature Reviews Nephrology. 2011;7(6):327-40.
52. Lau E, Marques C, Pestana D, Santoalha M, Carvalho D, Freitas P, et al. The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity. Nutr Metab (Lond). 2016;13:31.
53. Sanchez-Munoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. World J Gastroenterol. 2008;14(27):4280-8.
54. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. Gastroenterology. 2011;140(6):1756-67.
55. Funaoka H, Kanda T, Fujii H. [Intestinal fatty acid-binding protein (I-FABP) as a new biomarker for intestinal diseases]. Rinsho Byori. 2010;58(2):162-8.
56. Adriaanse M, Tack G, Passos VL, Damoiseaux J, Schreurs M, Van Wijck K, et al. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. Alimentary pharmacology & therapeutics. 2013;37(4):482-90.
57. Krafts KP. Tissue repair. Organogenesis. 2010;6(4):225-33.

CHAPTER 4: SYNTHESIS AND CONCLUSION

Type 2 diabetes mellitus (T2DM) is a major public health concern worldwide (1). The increased prevalence of this condition has been shown to be positively correlated with chronic consumption of high-calorie diets (2). The onset of type 2 diabetes (T2D) is often preceded by a long-lasting, asymptomatic condition known as prediabetes (3). Various complications, including intestinal permeability (IP), also known as a leaky gut, are seen in T2D (4). Increased IP is a condition in which the intestinal lining becomes more permeable, allowing the translocation of toxins and partially undigested food from the lumen into the lamina propria (5). Diets high in either dietary fats or carbohydrates have been considered a contributing factor to the dysregulation of the intestinal barrier and its tight junctions (6, 7). It has been postulated that this is driven by a dysregulation in gut microbiota (8). These types of diets cause an overgrowth of harmful bacteria that increases inflammation in the digestive tract (9). The increase of harmful bacteria results in decreased diversity of good bacteria that function to protect the intestinal barrier against inflammation, which leaves the intestinal permeability compromised (8, 10). However, there are no studies that have been done to investigate the association between intestinal permeability and the development of prediabetes. Hence, the first manuscript of this study aimed to investigate the effect of a high-fat high carbohydrate diet on gut microbiota composition and the association between prediabetes and markers associated with a leaky gut. Previous studies have shown prediabetes to be reversible (11). The management of prediabetes relies heavily on lifestyle modification that includes moderate exercise and dietary interventions that involve the use of a low amount of carbohydrates and a high amount of unsaturated fats (11, 12). However, the effect of such diets on intestinal permeability in the pre-diabetic state remains unknown. Therefore, the second manuscript of this study sought to investigate the effect of a low carbohydrate, high unsaturated fat (LCHUF) diet on the concentration level of markers associated with a leaky gut and its concomitant effect on glucose homeostasis.

In our laboratory, we have created a HFHC-diet induced animal model of prediabetes that has been shown to mimic the human condition (13). In this model, the animals developed prediabetes after ingesting a HFHC diet for 20 weeks (13). In the first manuscript of this study, this was evidenced by a significant increase in fasting blood glucose concentration in the pre-diabetic group (PD) in comparison to the non-pre-diabetic group (NPD). These results were accompanied by a significant increase in plasma insulin in the PD group compared to the NPD group. These findings suggest that the pancreatic beta-cells increased insulin secretion to compensate for high blood glucose concentration in the pre-diabetic state (3, 14). However, due to chronic consumption of a HFHC diet, blood glucose continues to be elevated in the circulation, which could result in pancreas exhaustion as the condition progresses to T2D (3).

It has been shown previously that increased consumption of a high-calorie diet not only contributes to the progression of T2D but can also compromises intestinal permeability (10, 15). A high-calorie diet negatively impacts the gut microbiome by enriching the gut with gut-disrupting bacteria resulting in gut dysbiosis. An increase of gut-disrupting bacteria modulates intestinal permeability by altering the intestinal epithelial cells and tight junctions resulting in the translocation of toxin from the lumen to the circulation, which induces inflammation that further compromise the intestinal barrier (10, 16). In this study, we observed a significant increase in pathogenic phyla (*Bacteroidetes and Proteobacteria*) and a significant decrease in *Firmicutes* in the PD group in comparison to the NPD group. This suggests that a HFHC diet may have effectively shifted the maintained gut microbiome, which caused pathogenic bacteria to become overwhelmed and outcompete the essential bacteria (*Firmicutes*) that protect the intestinal barrier (17). The gut dysbiosis observed in this study could leave the intestinal barrier compromised, evidenced by a significant increase in serum zonulin concentration in the PD group compared to the NPD group. Zonulin is a family protein that reversibly regulates intestinal permeability by modulating intercellular tight junctions (18). This protein is used as a biomarker to measure the disruption of the tight junctions (19). The increased concentration of serum zonulin suggests increased permeability in the PD group, which could possibly result in the translocation of endotoxins, luminal antigens, and antimicrobial substances.

To evaluate if there had been increased translocation of endotoxin in the PD group, a biomarker called lipopolysaccharide was measured in this study. We observed no significant change in plasma LPS concentration in the PD group compared to the NPD group. This observation correlates with the findings of a previous study that reported that LPS has a half-life of 2-4 minutes in the blood. It acts as a strong stimulator of an immune response (20). Therefore, it is rapidly detoxified and cleared by the liver(20). So, another way to observe whether there had been increased translocation, soluble CD14 (sCD14), a co-receptor of LPS, which in high levels is reported to indicate LPS exposure was measured (21, 22). The diet-induced pre-diabetic animal group had a significant increase in sCD14 in comparison to the NPD group. This suggests that there was increased translocation of endotoxins as evidenced by the sCD14 elevation during the pre-diabetic state, although plasma LPS concentration has been shown to be unchanged in both groups.

Increased translocation of endotoxins has also been associated with low-grade inflammation, which has been shown in previous studies to be associated with prediabetes and T2D (23, 24). We found a significant increase of pro-inflammatory cytokines such as tumor necrosis-alpha (TNF-α), interleukin 6 (IL-6), and C-reactive protein (CRP) in the PD group in comparison to the NPD group. These results may have been caused by the translocation of plasma LPS into the blood, which exacerbates inflammation (23, 25). The sub-clinical chronic inflammation observed in this state has also been postulated to cause insulin resistance, thus contributing to the development of T2D (26). We further observed a significant increase in plasma intestinal fatty acid-binding protein (IFABP) in the PD group

in comparison to the NPD group. IFABP is an intracellular protein found in epithelial cells, which in high levels is speculated to indicate enterocyte and mucosal tissue damage and it is associated with several intestinal disorders such as coeliac disease and irritable bowel syndrome (27-29). We speculate that the pre-diabetic animals may have a higher risk of developing minor intestinal disease supported by the observed result of increased plasma IFABP. For the first time, this chronic ingestion of a HFHC diet dysregulates the gut microbiota composition, thus increasing markers associated with a leaky gut which results in a subclinical chronic inflammation that further progresses the onset of prediabetes.

The animals in the first manuscript developed prediabetes after ingesting a HFHC diet for 20 weeks. They were also reported with a dysregulated gut microbiome. After 20 weeks, the pre-diabetic animals were subdivided into two groups; the pre-diabetic control group continued ingesting the HFHC diet. The other pre-diabetic group switched to a low carbohydrate high unsaturated fat (LCHUF) diet. Both groups were further observed for 32 weeks. Observing the effect of a HFHC diet in the first manuscript resulted in a need to investigate the effect of other types of diets on glucose homeostasis and markers associated with a leaky gut. Banting, Atkins, and the ketogenic diets involve the use of a low amount of carbohydrates and a high amount of unsaturated fats (30). They all have been used in previous studies,and they were reported to manage prediabetes and its complications (31, 32). Therefore, in the second manuscript, a low carbohydrate high unsaturated fat (LCHUF) diet was used to investigate its concomitanteffect on glucose homeostasis. We further investigated changes in the concentration level of markers associated with increased intestinal permeability. The pre-diabetic group that was switched to a LCHUF diet showed a significant decrease in fasting blood glucose and glycated hemoglobin in comparison tothe pre-diabetic control that remained on the HFHC diet for additional 12 weeks. These findings suggest that a LCHUF diet effectively improved blood glucose regulation and insulin sensitivity, which causedcells to effectively take up glucose. Prediabetes in this study was specifically targeted to reduce or delaythe onset of metabolic disorders such as T2D. Therefore, with the observed results there is a reason to believe that a LCHUF diet effectively reversed prediabetes.

This led to the next observation in this study which was to look at the effect of a LCHUF diet on gut microbiota composition and markers associated with microbial translocation. It was found that the pre-diabetic group that was switched to a LCHUF diet showed a significant decrease in pathogenic phyla (*Bacteroidetes and Proteobacteria)* in comparison to the pre-diabetic control group that remained on a HFHC diet. We also observed a significant increase in *Firmicutes* in the pre-diabetic group that had dietary intervention (LCHUF diet) compared to the pre-diabetic control group that remained on a HFHC diet. We speculate that the reduction of pathogenic phyla and upregulation of *Firmicutes* improved normal intestinal homeostasis, thus reducing the permeability of the intestine. This was further evidenced by a significant decrease in serum zonulin concentrations in the pre-diabetic group that was switched to a LCHUF diet by comparison with the pre-diabetic control group that remained on a HFHC diet. The observed result correlates with a previous study that showed that a low carbohydrate diet

promotes the proliferation of beneficial species, thus improving intestinal integrity (33). These results confirmed that a LCHUF diet effectively reduced pathogenic bacteria colonization seen by a decrease in serum zonulin concentration.

A significant decrease in serum zonulin may have indicated that the translocation of toxic substances and luminal antigens across the lumen was reduced. However, in this study, there were no significant changes in plasma LPS in both group. A possible reason for the observed result may be that the integrity of the tight junction improved in the pre-diabetic state. This led to a need to further investigate whether there were any changes in the concentration level of plasma LPS. According to a previous study, soluble CD14 (sCD14) a co-receptor of LPS is secreted from the immune cell, and its high levels are speculated to indicate LPS exposure (21, 22). We found a significant decrease in plasma sCD14 in the pre-diabetic group that was switched to a LCHUF diet in comparison to the pre-diabetic control group that remained on a HFHC diet. We, therefore, speculate that a decrease in the secretion of sCD14 is due to less LPS exposure in the circulation.

In the first manuscript, the pro-inflammatory cytokines such as tumor necrosis-alpha (TNF-α), interleukin (IL-6), and C-reactive protein (CRP) were significantly increased in the pre-diabetic state (Dimba et al., 2023). The pro-inflammatory cytokines were reported to be triggered by circulating endotoxins and other microbial compounds in the bloodstream (Dimba et al., 2023). However, in the second manuscript, we observed a significant decrease in concentration levels of plasma sCD14 and LPS, which correlates with the observed significant decrease of plasma TNF-α, IL-6, and CRP in the pre-diabetic group that was switched to a LCHUF diet in comparison to the pre-diabetic control group that remained on a HFHC diet. These findings suggest that the immune system is less triggered as fewer toxins are being translocated into the blood.

IFABP is a useful marker of intestinal damage, as it is mainly expressed in epithelial cells of the small intestine mucosal layer (29, 34). According to the literature, increased concentrations of IFABP only get translocated into the circulation following enterocyte and epithelial cell damage (27-29). To investigate intestinal damage at this state, IFABP was measured. The pre-diabetic group that was switched to a LCHUF diet showed a significant decrease in plasma IFABP concentration in comparison to the pre-diabetic control group that remained on a HFHC diet. We, therefore, speculate that diet-induced pre-diabetic animal models have a lower risk of developing intestinal disease supported by a decrease in pro-inflammatory cytokines. For the first time, a LCHUF diet effectively delayed the onset of prediabetes and thus prevented metabolic disorders such as T2D. In addition, this diet improved gut microbiota composition and positively changed the concentration levels of markers associated with increased intestinal permeability, and also prevented a leaky gut condition.

The findings in the first manuscript showed that chronic consumption of a high-fat high carbohydrate (HFHC) diet results in the development of prediabetes. This was evidenced by a significant increase in

fasting blood glucose and insulin concentration. The findings in manuscript two showed that switching from a HFHC diet to a low carbohydrate high unsaturated fat (LCHUF) diet can effectively reverse or delay the onset of prediabetes. This was evidenced by a significant decrease in fasting blood glucose and glycated haemoglobin (HbA1c) concentration. Both these studies also observed the effect of diets (HFHC/LCHUF) on the concentration level of markers associated with a leaky gut. In the first manuscript, the pre-diabetic animal model was reported with increased intestinal permeability as one of the complications that begin during this stage. This observation showed that gut microbiota composition plays a significant role in the development of a leaky gut so as in the progression of prediabetes to T2D. In the second manuscript, significant changes in concentration levels of markers associated with a leaky gut were observed following a change to a LCHUF diet. The findings observedfurther support that gut microbiota composition is one factor that is mostly affected by diets (HFHC/ LCHUF).

As shown in figure 1 (Barnette et al., 2020), chronic consumption of a HFHC diet results in the development of prediabetes, and insulin resistance is observed during this state. The HFHC diet is shown to also cause gut dysbiosis (↓ Firmicutes, ↑ Proteobacteria, ↑Bacteroidetes), which causes increased intestinal permeability shown by increased serum zonulin concentration. This enhances the translocation of plasma LPS thus inducing secretion of plasma sCD14. LPS in the blood increase expression of pro-inflammatory cytokines (TNF-α, IL-6, CRP) as well as increased IFABP resulting in a subclinical chronic inflammation that further compromises the gut increasing the risk of developing prediabetes. In the pre-diabetic animal model, switching diet from a HFHC diet to a LCHUF diet for an additional 12 weeks decreased fasting blood glucose and HbA1c concentration, which improved insulin sensitivity and glucose homeostasis → reversed prediabetes. A LCHUF diet effectively proliferated beneficial bacteria and prevented gut dysbiosis (↑ Firmicutes, ↓ Proteobacteria, ↓ Bacteroidetes). Decreased pathogenic phyla reduced serum zonulin concentration which kept the intestinal barrier and its tight junction intact. This caused less leakiness of toxic substances in circulation. This was evidenced by a significant decrease in plasma LPS, sCD14, and pro-inflammatory cytokines (TNF-α, IL-6, CRP) and IFABP. These findings reduced inflammatory response, enterocyte/ mucosal tissue damage as well as the risk of developing prediabetes.

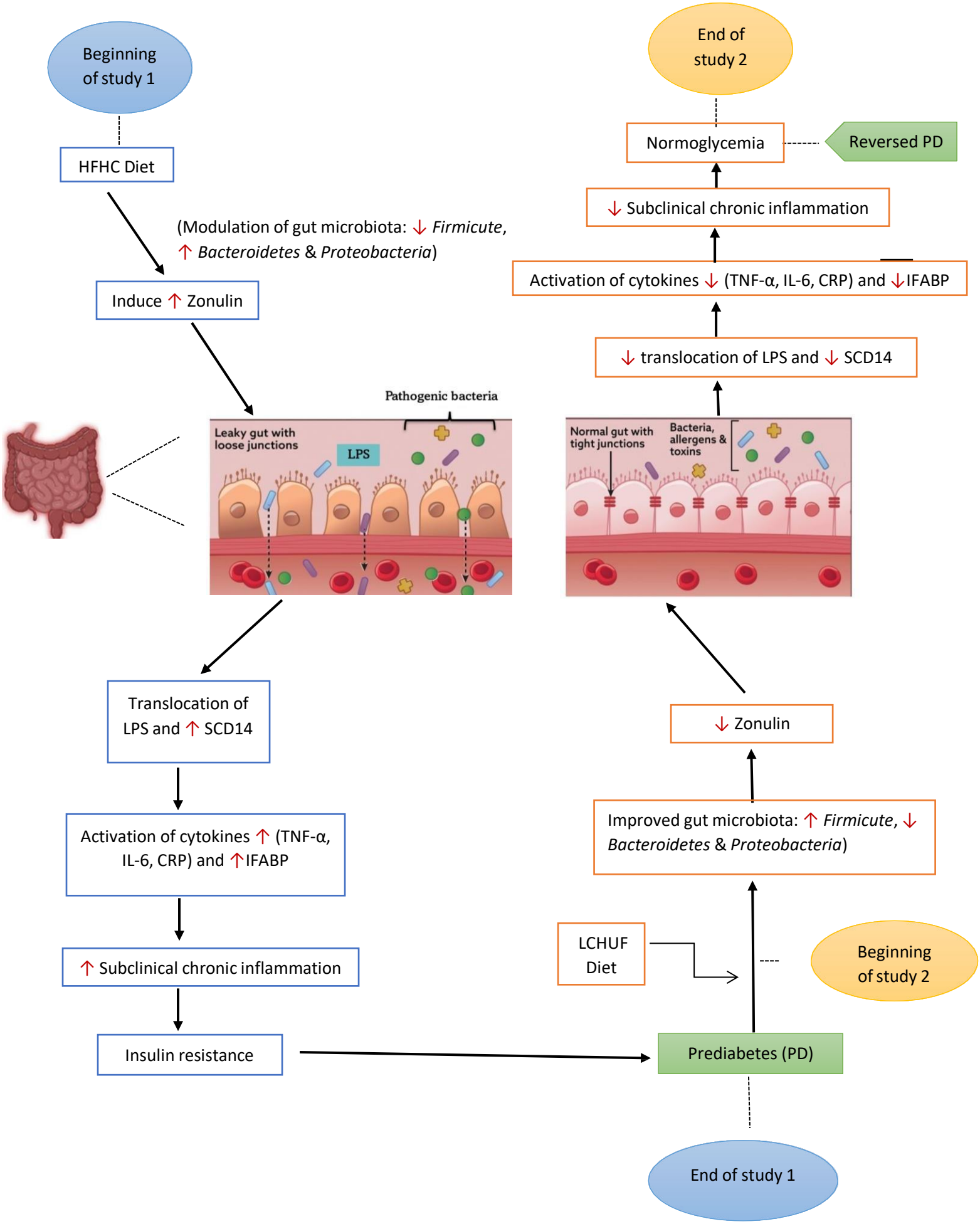

Figure 1: Diagram illustrating the effect of diets (HFHC/ LCHUF) on glucose homeostasis and gut microbial composition adapted from Barnette et al., 2020.

Conclusion

In conclusion, the first manuscript showed that chronic ingestion of a HFHC diet dysregulates the gut microbiota composition, thus increasing markers associated with a leaky gut, resulting in a subclinical chronic inflammation that further progresses the onset of prediabetes. The second manuscript showed that the most effective way of delaying or reversing the onset of prediabetes or combating a leaky gut problem is through ingesting a LCHUF diet. This diet effectively improved blood glucose homeostasis and maintained balance in the gut microbiome. Taken together, the findings of this dissertation show how the type of diets that are consumed influence the gut microbiota, which in turn influences intestinal permeability. The changes in intestinal permeability influence the entry of pathogens, which determinesthe amount of chronic inflammation in the body. This in turn, affects the sensitivity of peripheral tissuesto insulin.

Shortfalls and future studies

In the first manuscript, we investigated the effect of a HFHC diet on glucose homeostasis and markers associated with a leaky gut. We established that chronic consumption of a HFHC dysregulates the gut microbiota composition, thus increasing the translocation of endotoxins in the circulation. This results in subclinical inflammation that further progresses prediabetes. In the second manuscript, we investigated changes in concentration levels of markers associated with a leaky gut and glucose homeostasis following a change in diet to a LCHUF. We established that switching from a HFHC diet to a LCHUF diet can effectively improve balance in the gut microbiome and reverse prediabetes. In both manuscripts, we observed that gut microbiota composition is mainly affected by the type of diets consumed, which then progresses all these complications that were previously discussed and observed. Both manuscripts could have looked at the histology of the intestinal lining however, due to budget constraints this was not possible. Therefore, it is recommended that in future studies, the histology of the intestinal lining be studied to assess the effect of a HFHC and inflammation on the integrity of the intestinal barrier. We also recommend histology sections of the intestinal integrity and mucosal tight junctions, which were postulated to be modulated by elevated zonulin in the pre-diabetic state to be further validated on whether they are fully restored after switching to a LCHUF diet.

References

1. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H, Al Kaabi J. Epidemiology of Type 2 Diabetes – Global Burden of Disease and Forecasted Trends. Journal of Epidemiology and Global Health. 2019;10(1):107.
2. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nature reviews endocrinology. 2018;14(2):88-98.
3. 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.
4. De Kort S, Keszthelyi D, Masclee AAM. Leaky gut and diabetes mellitus: what is the link? Obesity Reviews. 2011;12(6):449-58.
5. Derikx JP, Luyer MD, Heineman E, Buurman WA. Non-invasive markers of gut wall integrity in health and disease. World J Gastroenterol. 2010;16(42):5272-9.
6. de La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2010;299(2):G440-G8.
7. Devaraj S, Dasu MR, Jialal I. Diabetes is a proinflammatory state: a translational perspective. Expert review of endocrinology & metabolism. 2010;5(1):19-28.
8. Wan Y, Wang F, Yuan J, Li J, Jiang D, Zhang J, et al. Effects of dietary fat on gut microbiota and faecal metabolites, and their relationship with cardiometabolic risk factors: a 6-month randomised controlled-feeding trial. Gut. 2019;68(8):1417-29.
9. Liu J, Su X, Lu J, Ning J, Lin M, Zhou H. PM2. 5 induces intestinal damage by affecting gut microbiota and metabolites of rats fed a high-carbohydrate diet. Environmental Pollution. 2021;279:116849.
10. Rohr MW, Narasimhulu CA, Rudeski-Rohr TA, Parthasarathy S. Negative Effects of a High-Fat Diet on Intestinal Permeability: A Review. Advances in Nutrition. 2019.
11. Perreault L, Færch K. Approaching Pre-diabetes. Journal of Diabetes and its Complications. 2014;28(2):226-33.
12. Zinman B, Harris SB, Neuman J, Gerstein HC, Retnakaran RR, Raboud J, et al. Low-dose combination therapy with rosiglitazone and metformin to prevent type 2 diabetes mellitus (CANOE trial): a double-blind randomised controlled study. The Lancet. 2010;376(9735):103-11.
13. Luvuno M, Mbongwa H, Khathi A. Development of a novel prediabetes animal model using a high fat high carbohydrate diet: implications for type 2 diabetes. PloS one. 2017;13:8-14.
14. Huang BW, Chiang MT, Yao HT, Chiang W. The effect of high-fat and high-fructose diets on glucose tolerance and plasma lipid and leptin levels in rats. Diabetes, Obesity and Metabolism. 2004;6(2):120-6.
15. Daniel H, Gholami AM, Berry D, Desmarchelier C, Hahne H, Loh G, et al. High-fat diet alters gut microbiota physiology in mice. The ISME journal. 2014;8(2):295-308.
16. Wisniewski PJ, Dowden RA, Campbell SC. Role of dietary lipids in modulating inflammation through the gut microbiota. Nutrients. 2019;11(1):117.
17. Huang Y, Shi X, Li Z, Shen Y, Shi X, Wang L, et al. Possible association of Firmicutes in the gut microbiota of patients with major depressive disorder. Neuropsychiatric disease and treatment. 2018;14:3329.
18. Sturgeon C, Fasano A. Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases. Tissue Barriers. 2016;4(4):e1251384.
19. Seethaler B, Basrai M, Neyrinck AM, Nazare J-A, Walter J, Delzenne NM, et al. Biomarkers for assessment of intestinal permeability in clinical practice. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2021;321(1):G11-G7.
20. Yao Z, Mates JM, Cheplowitz AM, Hammer LP, Maiseyeu A, Phillips GS, et al. Blood-Borne Lipopolysaccharide Is Rapidly Eliminated by Liver Sinusoidal Endothelial Cells via High-Density Lipoprotein. The Journal of Immunology. 2016;197(6):2390-9.

21. Sandler NG, Koh C, Roque A, Eccleston JL, Siegel RB, Demino M, et al. Host response to translocated microbial products predicts outcomes of patients with HBV or HCV infection. Gastroenterology. 2011;141(4):1220-30. e3.
22. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. AIDS (London, England). 2015;29(10):1263.
23. Amasheh M, Fromm A, Krug SM, Amasheh S, Andres S, Zeitz M, et al. TNFα-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFκB signaling. Journal of cell science. 2010;123(23):4145-55.
24. Hawa MI. Immune and metabolic changes leading to diabetes: Queen Mary University of London; 2012.
25. Creely SJ, McTernan PG, Kusminski CM, Fisher fM, Da Silva N, Khanolkar M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. American Journal of Physiology-Endocrinology and Metabolism. 2007;292(3):E740-E7.
26. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nature Reviews Immunology. 2008;8(12):923-34.
27. Adriaanse M, Tack G, Passos VL, Damoiseaux J, Schreurs M, Van Wijck K, et al. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. Alimentary pharmacology & therapeutics. 2013;37(4):482-90.
28. Al-Ayadhi L, Zayed N, Bhat RS, Moubayed NMS, Al-Muammar MN, El-Ansary A. The use of biomarkers associated with leaky gut as a diagnostic tool for early intervention in autism spectrum disorder: a systematic review. Gut Pathogens. 2021;13(1).
29. Funaoka H, Kanda T, Fujii H. [Intestinal fatty acid-binding protein (I-FABP) as a new biomarker for intestinal diseases]. Rinsho Byori. 2010;58(2):162-8.
30. Dorans KS, Bazzano LA, Qi L, He H, Chen J, Appel LJ, et al. Effects of a Low-Carbohydrate Dietary Intervention on Hemoglobin A_{1c}. JAMA Network Open. 2022;5(10):e2238645.
31. Brouns F. Overweight and diabetes prevention: is a low-carbohydrate-high-fat diet recommendable? Eur J Nutr. 2018;57(4):1301-12.
32. Noakes TD, Windt J. Evidence that supports the prescription of low-carbohydrate high-fat diets: a narrative review. Br J Sports Med. 2017;51(2):133-9.
33. Attaye I, van Oppenraaij S, Warmbrunn MV, Nieuwdorp M. The Role of the Gut Microbiota on the Beneficial Effects of Ketogenic Diets. Nutrients. 2021;14(1).
34. Lau E, Marques C, Pestana D, Santoalha M, Carvalho D, Freitas P, et al. The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity. Nutr Metab (Lond). 2016;13:31.

APPENDICES

Appendix 1: Ethical Clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

25 January 2022

Dr Andile Khathi (13246)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Dr Kathi,

Protocol reference number: AREC/00003627/2021
Project title: Use of stored samples collected under AREC/024/018D for supervised student research.

Full Approval – Research Application

With regard to your revised application received on 18 November 2021, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

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 24 January 2023.

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

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee
/kr

cc BRU Manager: Dr Jaca

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 **Facsimile**: +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix 2: Manuscript 1 Journal Guide

Thieme

Instructions for Authors

Editors-in-Chief
Endocrinology
Prof. Dr. med. Martin Reincke
Universität München
Medizinische Klinik und Poliklinik IV
Ziemssenstr. 1, 80336 München
martin.reincke@med.uni-muenchen.de

Diabetes
Prof. Dr. med. Karsten Müssig
Klinik für Innere Medizin, Niels-Stensen-Kliniken
Franziskus-Hospital Harderberg,
Alte Rothenfelder Str. 23
49124 Georgsmarienhütte
Karsten.Muessig@niels-stensen-kliniken.de

Editorial Office
Sabine Klee
Hamilton Services GmbH
Landwehrstr. 9, 80806 München
Tel.: 089/907793614
eced.editorialoffice@thieme.de

Scope

Experimental and Clinical Endocrinology & Diabetes accepts manuscripts in English in the fields of endocrinology and diabetology from clinical and laboratory research. Special attention is given to obesity, bone metabolism and dyslipidemia. The journal publishes original papers, reviews, mini-reviews and commentaries. Abstracts from national and international meetings submitted by the organizers will also be considered for publication upon request.
Manuscripts are received with the explicit understanding that they have not been published elsewhere and are not under simultaneous consideration by any other publication.

Preprint Server Statement

Experimental and Clinical Endocrinology & Diabetes encourages the submission of manuscripts that have been deposited in an initial draft version in preprint repositories such as Research Square, arXiv, and medRxiv. Drafts of short conference abstracts or degree theses posted on the website of the degree-granting institution, and draft manuscripts deposited on authors' or institutional websites are also welcome. All other prior publication is forbidden.
During submission, authors should (1) note use of the preprint repository in the cover letter, (2) state what adjustments and/or updates the draft has undergone between deposition and submission and (3) cite the preprint, including the DOI, as a reference in the manuscript.
After submission to the journal, and until a final decision has been made, authors are discouraged from depositing versions of their manuscript as preprints. Upon publication authors should add a link from the preprint to the published article. Twelve months after publication, authors can update the preprint with the accepted manuscript.

Manuscript Submission

All manuscripts must be submitted exclusively via online submission at http://mc.manuscriptcentral.com/eced

Submissions of hardcopy manuscripts will not be accepted. Please refrain from sending manuscripts via e-mail. For submission of all manuscripts, please follow the instructions on the online submission system. Before submission, keep ready full metadata of all manuscripts (title, short running title, authors' names including affiliations and addresses, list of keywords and abstract). Figures should be uploaded separately as *.tif or *.jpg files (resolution: colored and black-white bitmaps: 300 dpi; diagrams and line drawings: 600 dpi minimum). Tables should be uploaded in a separate Word file (not as a *.jpg file). The legends to the figure and table including Arabic numerals should be entered in the appropriate fields during the file upload. Please note that figures and tables should not be integrated into the main document, but a list with the legends of the figures and tables should be included here. Authors are responsible for the correctness of the manuscripts and the list of references.

A. Original Articles

Original papers should deal with investigations and results of high scientific value which have not been published previously.
Authors are asked to follow the outline set below: Page 1: a) title, b) short running title (limit: 40 characters), c) name of the author (no titles or academic grades) and address of the institute(s) where the investigations have been carried out. Should the address of the author at the time of publication differ from the one stated in the paper, the current address should be stated in a footnote, d) complete mailing address of corresponding author including telephone and telefax numbers and e-mail addresses. Page 2: a) an abstract containing not more than 250 words with no abbreviations, b) keywords (3–6 without repeating words in the title). Page 3 and onwards: a) introduction also indicating the aim of the study, b) materials and methods, c) results: double presentation of data in the form of text, tables or figures should be avoided, d) discussion and conclusions, e) list of references, f) legends of tables and figures.
3. References a) *Text:* Citations and references should be numbered consecutively using square brackets in the order in which they are mentioned in the text, followed by any tables or legends. Please do not alphabetize references and bibliographics. Do not use footnotes and hyperlinks. If authors are mentioned in the text, only the first author should be given followed by "et al." whenever the reference has three or more authors. Example: " ...protein concentrations were determined according to Lowry et al. [12]. b) List of References: References should be given as plain text. Do not use fields in MS Word, as these are difficult to process later. The references should be listed in numbered order according to the sequence they appear in the text. All authors or groups of authors of each publication should be mentioned. The name of the author(s) should be followed by the full title of the paper, name of the journal in which it has been published (abbreviations according to Index Medicus viz. PubMed/Medline), year of publication, volume, first and last page. Abstracts and supplements have to be clearly marked. Chapters from books have to be cited as follows: author(s), title of chapter, title of book, editor(s), place of publication, publisher, year of publication, first and last page of the chapter. **Please note that the journal's reference style is covered by Endnote: https://www.thieme.de/journal-authors**

Examples:

9 Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin-phenol reagent. J Biol Chem 1951; 193: 265–275
10 Kerner W, Pfeiffer EF. The artificial pancreas. In: Samols E, ed. The endocrine pancreas. New York: Raven Press, 1991: 441–456

Original papers should not exceed 6 printed pages, including references, tables, figures and legends. One printed page equals approx. 630 words. Small tables/figures (sized 1/4 of a page) reduce the number of words by approx. 150 words per table/figure, large tables/figures (sized 1/2 of a page) reduce the number of words by approx. 300 words per table/figure. Please do not use more than one blank space between words and sentences. A maximum of 4 figures and 3 tables is allowed. Longer manuscripts will be subject to editing and a page charge of € 180 per printed page (including 19% VAT) starting with the seventh printed page.

B. Reviews, Mini-Reviews and Meta-Analyses

Reviews are normally published by invitation only. Reviews deal with previous research on a certain topic and serve to summarize the current state of the art. Their structure varies from an original paper according to the nature of the review. Reviews covering basic research should take a causal and mechanistic approach, whereas reviews dealing with clinical topics should focus on therapeutic relevance. They should not exceed 8 printed pages, including a maximum of 100 references. All reviews will be peer reviewed. **Please refrain from submitting uninvited reviews.** If you plan to submit a review, please contact the editors first, explaining in your letter to the editor why this review is unique and suited to advance the field.
Mini-Reviews summarize the main findings only and give a brief outline. They should not exceed 3 printed pages.
Meta-Analyses will only be considered if they make a substantial contribution to the field.

C. Methods and Techniques

This section focusses on papers covering novel methods and or substantial improvements on established, proven techniques in endocrinology and diabetes research. The aim is to provide researchers with new, innovative tools that will help them better conduct their research, hence practical relevance is of utmost importance to papers published in this section.
Original articles covering recent technical and/or methodological developments or innovations are accepted. Methods must be accurately described and validated and there should ideally be an application to a specific question that the new technique addresses better than other, older methods. Methods must be described in detail so that other researchers can use this method for their own research.

For formal requirements please refer to the instructions for original articles above.

D. Commentaries

Commentaries are usually invited. They aim at commenting on subjects with a strong impact upon experimental endocrinology and diabetology or they refer to a published article directly.

E. Letters to the Editor

This section has been introduced in order to encourage the authors in a free exchange of ideas. The opinions presented will not necessarily reflect the opinions of the Editors.

Publication of manuscripts immediately upon acceptance

Experimental and Clinical Endocrinology & Diabetes offers its authors the option to have their manuscripts published immediately upon acceptance (if the publication preference Open Access is chosen, the manuscript gets published only after payment of the APC has been confirmed).

This means that the unedited, unformatted version of the manuscript as it stands after peer review is published online, with a DOI. Authors wishing to make use of this service will be asked to upload "clean" versions of their manuscripts after every revision; this is a precondition for this service, as is the confirmation that the Copyright Transfer Agreement (CTA) will be signed upon receipt.

This service is offered for Original articles, Reviews, Mini-Reviews, Methods and Techniques and Meta-Analyses.

Implications of "accepted manuscript" publication

Once the paper has been accepted, the last clean version of the manuscript, including all metadata entered during submission (title, abstract, author affiliations etc.), becomes the first version of the article to be published online. This means that no changes can be made to the submitted clean version as this version will be published as the "Accepted Manuscript", should it be accepted. Changes by the authors will only be possible subsequently in the proofs from the typesetters for correction. This means in detail:

- For all authors, the affiliation information entered **during submission** will be published.
- If an author is already in the system, please use "Edit" to update the address information if necessary.
- To facilitate the entry of co-author information, please use the "Quick Fill" option if applicable.
- The order of authors entered during submission will be the order of authors on the "Accepted Manuscript".
- All authors named under step "Authors & Institutions" agree to the publication and signing of the CTA.
- The conflict of interest and funding information will be published as entered at the step "Details & Comments".
- Clinical trial information will be published as entered at the step "Details & Comments".

Instructions and further information are available during the submission process and upon request to the Editorial Office.

Abbreviations

Abbreviations should only be used when necessary, e.g., for procedures (ANOVA), long chemical names (ATP), or other expressions used throughout your paper. See below for the full list of abbreviations that do not need to be defined.

Supporting Information

To keep articles as concise and at the same time as informative as possible, authors are encouraged to submit part of their tables and figures as Supporting Information (SI). The following type of data will be published as SI: high-resolution halftone and color illustrations, and tables summarizing data that are not essential but useful to the understanding of an article. Tables and figures provided as SI must be referred to in the manuscript as follows: Table 1S and Figure 1S. SI has to be submitted as a separate file.

Clinical Trials

Experimental and Clinical Endocrinology & Diabetes supports trial registration. All trials reported must be registered at an official trial registry recognised by the International Committee of Medical Journal Editors, such as ClinicalTrials.gov (www.clinicaltrials.gov) or any of the primary registries on the World Health Organization's International Clinical Trial Registry Platform (www.who.int/ictrp).

Conflict of Interest

A statement concerning the conflicts of interest of all authors is mandatory.

English Language

It is in the authors' best interest that manuscripts be proofread by a native English speaker. We recommend language editing by a professional editing service such as Enago. Use this link to get a 15% discount on their services: www.enago.com/thieme/

Proofs and Reprints as PDF File

Galley proofs will be sent to the corresponding author as a PDF file. The corresponding author receives a PDF file of the published article free of charge.

Reproduction of Colour Figures

Figures are automatically reproduced in black and white in print and online. Should you want your figures in colour, you will be charged € 440 for the first colour figure and € 80 for any further figure (including 19% VAT).

IMPORTANT COPYRIGHT INFORMATION FOR AUTHORS

The publishers hold the copyright on all material appearing in *Experimental and Clinical Endocrinology & Diabetes*. A Copyright Transfer Agreement will be sent to the corresponding author together with the galley proofs. The agreement must be completed and returned to the publishers before the article can be published.

All your submitted figures, tables and videos must be original work. They must be created fully by you and/or co-authors for the purpose of this publication. We cannot accept any material that has already been published in books, magazines or electronic products of other providers, including websites. We may also not publish any material to which a third party additionally has rights of use (e.g. your employer).

Please do NOT pay any license fees (e.g. for "RightsLink"/ Copyright Clearance Center) for any such material. Even the standard license agreements of "Creative Commons" cannot be recognized as proof that the material may be used.

If you are including any material that is not strictly text, you are required to provide the following information in the cover letter on submission:

- Confirmation that all figures, images, illustrations, tables and videos are original work created fully by you and/or co-authors for the purpose of this publication
- Confirmation that every component of illustrations that combine more than one element (e.g. pictograms, images, etc.), are your and/or co-authors' own work
- Confirmation that you and/or co-authors hold all rights of use for every submitted figure, table and video and no third party holds rights of use

To summarize which material may not be used in your submission:

- Please do not use images that have already been published in books, magazines or electronic products (including websites)
- Please do not use images to which a third party additionally has rights of use (e.g. your employer)
- Please do not use even a small part of a third party image. Such images are not free from copyright protection even if they have been altered using a graphic editor
- Please do not use screenshots of third-party material (e.g. third-party websites, publications, etc.)
- Please do not use any industry photography
- Please do not use logos of institutions, manufacturers or any other branding

Please contact the Editorial Office if you have any questions regarding the use of illustrations.

In order to ensure that your manuscript meets the formal requirements of the publishers, please consult Author's Guidelines for different Types of Articles available at

https://www.thieme.de/de/autorenlounge/journals-158774.htm

Research Ethics

For all research involving humans, subjects must have given their informed consent. Research on animals must have been approved by the local ethics committee.

Appendix 3: Manuscript 2 Journal Guide: Nutrition & Diabetes

Guide to Authors

Article Type Specifications

Article: An Article is a substantial, in-depth, novel research study of interest to the readership of the journal. The structure an Article should follow is detailed below.
Specifications: Structured abstract (Background/Objectives; Subjects/Methods; Results; Conclusions) max. 300 words; Main body of text (excluding abstract, tables/figures, and references) not to exceed 4,000 words; Max 6 tables or figures; Max 60 references

Brief Communication: These are studies that fall short of the criteria for full Articles (e.g. preliminary experiments limited by sample size or duration, or novel hypotheses). Apart from including an abstract, there is no obligation to divide the text into sections.
Specifications: Unstructured abstract max. 200 words; Main body of text (tables/figures, and references) not to exceed 1,500 words; Max 2 tables or figures; Max 20 references

Review Article: A Review Article is an authoritative, balanced survey of recent developments in a research field**.** Review Articles should incorporate a) a review of previously published literature from the past 5-10 years, describing the pros and cons of these studies, b) the authors opinion on how to approach the issue/situation being discussed, c) the authors thoughts on what is necessary to move the field forward in the future. Review Articles are regularly commissioned, however pre-submission enquiries are also welcome. Please contact the editorial office.
Specifications: Unstructured abstract max. 200 words; Main body of text (excluding abstract, tables/figures, and references) not to exceed 7,500 words; Max 8 tables or figures; Max 120 references

Mini-Review: Mini-reviews should follow the guidelines provided for a full review but can focus on a more niche topic/ provide a much more succinct survey of recent developments in the field and on what is necessary to move the field forward in the future.
Specifications: Unstructured abstract max. 150 words; Main body of text (excluding abstract, tables/figures, and references) not to exceed 3,000 words; Max 6 tables or figures; Max 50 references

Correspondence: Correspondence provides readers with a forum for comment on papers published in a previous issue of the journal or to address new issues relevant to the research community.
Specifications: No abstract required; Main body of text (tables/figures, and references) not to exceed 500 words; Max 2 tables or figures; Max 10 references

Editorial: By Editor invitation only. Proposals for Editorials may be submitted; authors should only send an outline of the proposed paper for initial consideration.Please contact the editorial office to propose an idea.
Specifications: No abstract required; Main body of text (tables/figures, and references) not to exceed 1,000 words; Max 2 tables or figures; Max 10 references

Technical Report: Technical Reports are articles that address areas of more methodological interest. The contents of these Reports must have the same level of scientific rigour expected of an Article.
Specifications: Structured abstract max. 300 words; Main body of text (excluding abstract,

tables/figures, and references) not to exceed 2,500 words; Max 4 tables or figures; Max 25 references

Preparation of Articles

House Style: Authors should adhere to the following formatting guidelines

- Text should be double spaced with a wide margin.
- All pages and lines are to be numbered.
- Do not make rules thinner than 1pt (0.36mm).
- Use a coarse hatching pattern rather than shading for tints in graphs.
- Colour should be distinct when being used as an identifying tool.
- Spaces, not commas should be used to separate thousands.
- At first mention of a manufacturer, the town (and state if USA) and country should be provided.
- *Statistical methods:* For normally distributed data, mean (SD) is the preferred summary statistic. Relative risks should be expressed as odds ratios with 95% confidence interval. To compare two methods for measuring a variable the method of Bland & Altman (1986, Lancet 1, 307–310) should be used; for this, calculation of P only is not appropriate.
- *Units:* Use metric units (SI units) as fully as possible. Preferably give measurements of energy in kiloJoules or MegaJoules with kilocalories in parentheses (1 kcal = 4.186kJ). Use % throughout.
- *Abbreviations:* On first using an abbreviation place it in parentheses after the full item. Very common abbreviations such as FFA, RNA, need not be defined. Note these abbreviations: gram g; litre l; milligram mg; kilogram kg; kilojoule kJ; megajoule MJ; weight wt; seconds s; minutes min; hours h. Do not add ‘s’ for plural units. Terms used less than four times should not be abbreviated.
- *People friendly language: Nutrition & Diabetes* would like to encourage its authors to use people friendly language in the articles published in the journal. Thus, we encourage authors to use terms such as 'people with overweight or obesity' in manuscripts submitted to the journal.

Cover Letter: Authors should provide a cover letter that includes the affiliation and contact information for the corresponding author. Authors should briefly discuss the importance of the work and explain why it is considered appropriate for the diverse readership of the journal. The cover letter should confirm the material is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration. If the manuscript has been previously considered for publication in another journal, please include the previous reviewer comments, to help expedite the decision by the Editorial team. Please also include a Competing Interests statement - see Editorial Policies for more details.

Title Page: The title page should contain:

- Title of the paper - brief, informative, of 150 characters or less and should not make a statement or conclusion

- Running title – should convey the essential message of the paper in no more than 50 characters. Should not contain any abbreviations
- Full names of all the authors and their affiliations, together with e-mail address of the corresponding author. If authors regard it as essential to indicate that two or more co-authors are equal in status, they may be identified by an asterisk symbol with the caption 'These authors contributed equally to this work' immediately under the address list.

Large Language Models (LLMs), such as ChatGPT, do not currently satisfy our authorship criteria. Notably an attribution of authorship carries with it accountability for the work, which cannot be effectively applied to LLMs. Use of an LLM should be properly documented in the Methods section (and if a Methods section is not available, in a suitable alternative part) of the manuscript.

Abstract: Articles must be prepared with an unstructured abstract designed to summarise the essential features of the paper in a logical and concise sequence.

Graphical Abstracts (optional): A graphical abstract, which summarizes the manuscript in a visual way, is designed to attract the attention of readers in the table of contents of the journal. Files should be uploaded as a 'Figure' and be labelled 'Graphical abstract'. A standard file format (.tiff, .eps, .jpg, .bmp, .doc, or .pdf.) should be used, and the graphic should be 9 cm wide x 5 cm high when printed at full scale and a minimum of 300 dpi. All graphical abstracts should be submitted with a white background and imagery should fill the available width, whenever possible. Colour graphical abstracts are encouraged and will be published at no additional charge. Textual statements should be kept to a minimum.

Introduction: The Introduction should assume that the reader is knowledgeable in the field and should therefore be as brief as possible but can include a short historical review where desirable.

Materials/Subjects and Methods: This section should contain sufficient detail, so that all experimental procedures can be reproduced, and include references. Methods, however, that have been published in detail elsewhere should not be described in detail. Authors should provide the name of the manufacturer and their location for any specifically named medical equipment and instruments, and all drugs should be identified by their pharmaceutical names, and by their trade name if relevant.

Results: The Results section should briefly present the experimental data in text, tables or figures. Tables and figures should not be described extensively in the text.

Discussion: The Discussion should focus on the interpretation and the significance of the findings with concise objective comments that describe their relation to other work in the area. It should not repeat information in the results. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take.

Acknowledgements: These should be brief, and should include sources of support including sponsorship (e.g. university, charity, commercial organisation) and sources of material (e.g. novel drugs) not available commercially.

Author Contributions: Authors must include a statement about the contribution of each author to the manuscript (see Editorial Policies page for details regarding authorship). The statement can be up to several sentences long, describing the tasks of individual authors referred to by their initials. See example below:
MAJ was responsible for designing the review protocol, writing the protocol and report, conducting

the search, screening potentially eligible studies, extracting and analysing data, interpreting results, updating reference lists and creating 'Summary of findings' tables. SBM was responsible for designing the review protocol and screening potentially eligible studies. She contributed to writing the report, extracting and analysing data, interpreting results and creating 'Summary of findings' tables. DIH conducted the meta-regression analyses and contributed to the design of the review protocol, writing the report, arbitrating potentially eligible studies, extracting and analysing data and interpreting results. NAL contributed to data extraction and provided feedback on the report. FRT and RAL provided feedback on the report.

Competing Interests: Authors must declare whether or not there are any competing financial interests in relation to the work described. This information must be included at this stage and will be published as part of the paper, but should also be noted in the cover letter. Please see the Competing Interests definition in the Editorial Policies section for detailed information.

Data Availability Statement: An inherent principle of publication is that others should be able to replicate and build upon the authors' published claims. *Nutrition & Diabetes* adheres to Springer Nature's Data Policy Type 3. This means that a submission to the journal implies that materials described in the manuscript, including all relevant raw data, will be freely available to any researcher wishing to use them for non-commercial purposes, without breaching participant confidentiality. It also means that a Data Availability Statement (see here for more details) must be included as part of your manuscript.

References: Only papers directly related to the article should be cited. Exhaustive lists should be avoided. References should follow the Vancouver format. In the text they should appear as numbers starting at one and at the end of the paper they should be listed (double-spaced) in numerical order corresponding to the order of citation in the text. Where a reference is to appear next to a number in the text, for example following an equation, chemical formula or biological acronym, citations should be written as (ref. X). Example "detectable levels of endogenous Bcl-2 (ref. 3), as confirmed by western blot".

All authors should be listed for papers with up to six authors; for papers with more than six authors, the first six only should be listed, followed by *et al.* Abbreviations for titles of medical periodicals should conform to those used in the latest edition of Index Medicus. The first and last page numbers for each reference should be provided. Abstracts and letters must be identified as such. Papers in press may be included in the list of references.
Personal communications can be allocated a number and included in the list of references in the usual way or simply referred to in the text; the authors may choose which method to use. In either case authors must obtain permission from the individual concerned to quote his/her unpublished work.

Examples:
Journal article: Neidlein, S, Wirth, R, Pourhassan, M. Iron deficiency, fatigue and muscle strength and function in older hospitalized patients. Eur J Clin Nutr. 2020; 75:456–463.
Journal article by DOI: Kurotani K, Shinsugi C, Takimoto H. Diet quality and household income level among students: 2014 National Health and Nutrition Survey Japan. Eur J Clin Nutr. 2020; https://doi.org/10.1038/s41430-020-00794-1.
Journal article, in press: Gallardo RL, Juneja HS, Gardner FH. Normal human marrow stromal cells induce clonal growth of human malignant T-lymphoblasts. Int. J Cell Cloning (in press).
Complete book: Atkinson K, Champlin R, Ritz J, Fibbe W, Ljungman P, Brenner MK (eds). Clinical Bone Marrow and Blood Stem Cell Transplantation. 3rd ed. Cambridge University Press, Cambridge,

2004.
Chapter in book: Coccia PF. Hematopoietic cell transplantation for osteopetrosis. In: Blume KG, Forman SJ, Appelbaum FR (eds). Thomas' Hematopoietic Cell Transplantation. 3rd ed. Blackwell Publishing Ltd, Malden, 2004. pp 1443–1454.
Abstract: Abstracts from the 2020 Annual Scientific Meeting of the British and Irish Hypertension Society (BIHS). J Hum Hypertens 34; 2020; 1–20
Website: Kassambara A. rstatix: pipe-friendly framework for basic statistical tests. 2020. https://rpkgs.datanovia.com/rstatix/.
Online Document: Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

Tables: Tables should only be used to present essential data; they should not duplicate what is written in the text. All tables must be editable, ideally presented in Excel. Each must be uploaded as a separate workbook with a title or caption and be clearly labelled, sequentially. Please make sure each table is cited within the text and in the correct order, e.g. (Table 3). Please save the files with extensions .xls / .xlsx / .ods / or .doc or .docx. Please ensure that you provide a 'flat' file, with single values in each cell with no macros or links to other workbooks or worksheets and no calculations or functions.

Figure Legends: These should be brief, specific and appear on a separate manuscript page after the References section.

Figures: Figures and images should be labelled sequentially and cited in the text. Figures should not be embedded within the text but rather uploaded as separate files. The use of three-dimensional histograms is strongly discouraged unless the addition of the third dimension is important for conveying the results. Composite figures containing more than three individual figures will count as two figures. All parts of a figure should be grouped together. Where possible large figures and tables should be included as supplementary material.
Detailed guidelines for submitting artwork can be found by downloading our Artwork Guidelines. Using the guidelines, please submit production quality artwork with your initial online submission. If you have followed the guidelines, we will not require the artwork to be resubmitted following the peer-review process, if your paper is accepted for publication.

Graphs, Histograms and Statistics
Plotting individual data points is preferred to just showing means, especially where N<10
If error bars are shown, they must be described in the figure legend
Axes on graphs should extend to zero, except for log axes
Statistical analyses (including error bars and p values) should only be shown for independently repeated experiments, and must not be shown for replicates of a single experiment
The number of times an experiment was repeated (N) must be stated in the legend

Supplementary Information: Supplementary information is material directly relevant to the conclusion of an article that cannot be included in the printed version owing to space or format constraints. The article must be complete and self-explanatory without the Supplementary Information, which is posted on the journal's website and linked to the article. Supplementary Information may consist of data files, graphics, movies or extensive tables.

Please submit supplementary figures, small tables and text as a single combined PDF document. Tables longer than one page should be provided as an Excel or similar file type. Please refer to the

journal's Data Policies, outlined in the Editorial Policies section of these guidelines for additional options for such files, and which provides guidance on alternatives to supplementary files for data deposition, linking, preservation, and storage.

For optimal quality video files, please use H.264 encoding, the standard aspect ratio of 16:9 (4:3 is second best) and do not compress the video. Important: Supplementary information is not copyedited, so please ensure that it is clearly and succinctly presented, that the style and terminology conform to the rest of the manuscript, and that any tracked-changes or review mark-ups are removed.

Authors should submit supplementary information files in the FINAL format as they are not edited, typeset or changed, and will appear online exactly as submitted. When submitting Supplementary Information, authors are required to:

- Include a text summary (no more than 50 words) to describe the contents of each file.
- Identify the types of files (file formats) submitted.

Please note: We do not allow the resupplying of Supplementary Information files for style reasons after a paper has been exported in production, unless there is a serious error that affects the science and, if by not replacing, it would lead to a formal correction once the paper has been published. In these cases we would make an exception and replace the file; however there are very few instances where a Supplementary Information file would be corrected post publication.

Video summaries: Authors are welcome to include a video summary of their submission in order to support and enhance their scientific research. Files should be uploaded as a 'video' and be labelled 'Video abstract'.
Please take note of the technical requirements listed below.
Technical requirements:
The maximum file size of a video should not exceed 25 GB. An audio track is required, and video and audio streams must be in the correct order (video before audio). To ensure streamed video playout in HD in an acceptable quality, the following minimum requirements are recommended:
Resolution - At least 480p. If no HD is available: 1024 x 576 (PAL 16:9) respectively 768 x 576 (PAL 4:3)
Aspect ratio - Standard 16:9 or acceptable 4:3
Video bitrate - 5.000 to 10.000 Kbit/s
Audio bitrate - 320 Kbit/s, stereo, 44,1 KHz
Sound - AAC

Tips for presentation:
1. The video should introduce the topic of the article, highlight the main results and conclusions, discuss the current status and potential future developments in the field
2. Write your script and practise first – explain any obscure terminology
3. Film in a quiet room against a plain (white if possible) background and ensure there is nothing confidential in view
4. Avoid using background music
5. Include figures, slides, video clips of the experiment, etc. to help explain your methods and results. Please try to include a mixture of you talking to the camera and slides – it is nice for viewers to see your face at times
6. Keep figures simple; don't show raw data and ensure any text is legible. Do not include lots of small text or data that won't be legible in a small video player that's the size of a smartphone screen.

7. Please do not use images, music, or insignia in your video for which you do not own the copyright or have documented permission from the copyright holder.
Files will be viewed by the editorial office for quality; however the onus for creating, uploading and editing the video falls on the author.

Subject Ontology

Upon submission authors will be asked to select a series of subject terms relevant to the topic of their manuscript from our subject ontology. Providing these terms will ensure your article is more discoverable and will appear on appropriate subject specific pages on nature.com, in addition to the journal's own pages. Your article should be indexed with at least one, and up to four unique subject terms that describe the key subjects and concepts in your manuscript. Click here for help with this.

Nutrition & Diabetes (*Nutr. Diabetes*) ISSN 2044-4052 (online)

Appendix 4: Abstract of CHS symposium 2022 oral presentation

SCHOOL OF LABORATORY MEDICINE AND MEDICAL SCIENCES RESEARCH DAY

30 NOVEMBER 2022

SENATE CHMABER, WESTVILLE CAMPUS

INSTRUCTIONS:

- Please forward your abstracts electronically to dudhrajhp@ukzn.ac.za by **11 Nov 2022**.
- Participants should indicate their preference of presentation type (oral or poster).

CRITERIA FOR THE ACCEPTANCE OF ABSTRACTS:

- Abstracts are open to all postgraduate and post-doc students, developmental lecturers and credentialing staff in the School of Laboratory Medicine and Medical Sciences.
- Abstracts must adhere to the format indicated on the template provided.
- It is the author's responsibility to submit an abstract that is free of spelling and grammatical errors.
- All submissions must have ethical clearance.
- Abstracts will be reviewed by the scientific committee based on the following:
  (1) Clear background/statement of the problem.
  (2) Appropriateness of the methodology/study design to achieve the objectives.
  (3) Clear presentation/significance of the results
  (4) Relevance of the research findings; are the conclusions supported by the results.
  (5) Novelty of the study.

GUIDELINES:

Use Arial 11 point font throughout.

- The abstract must have a title: IN BOLD TYPE AND UPPER CASE (capitals).
- All authors must be listed: Surname followed by initials; do not include titles. (bold and underline the presenter's name).
- Author affiliations must be shown: Department, Centre or Unit; Use * and # symbols to match affiliation with author.

  Example: **Taylor, M.**[*], Suleman, F.[#]
  [*]Department of Public Health; [#] Discipline of Pharmaceutical Sciences

- Abstract Layout: Please fit into the frame. **250 words max**.
  - **Background/Aim(s):** Clearly state the purpose(s) of the study.
  - **Methods:** Clearly state how your study was conducted, sample selection, tools and instruments used
  - **Results:** Present your results in a logical sequence

- **Discussion/Conclusion:** Emphasize new and important aspects of the study and conclusions that are drawn from them.

PLEASE FILL IN ALL PRESENTER DETAILS
Presenter Information

Surname: Dimba	**First Name: Nosipho**
Research Theme: Prediabetes	**Tel: 0629488290**
E-Mail: 218006885@stu.ukzn.ac.za	**Date: 24 November 2022**

ABSTRACT TEMPLATE

Only abstracts that have strictly adhered to this template will be considered.
250 words max.

TITLE, AUTHOR AND AFFILIATION	**INVESTIGATING THE ASSOCIATION BETWEEN DIET-INDUCED "LEAKY GUT" AND THE DEVELOPMENT OF PRE-DIABETES** <u>Dimba, N.R</u>. *, Khathi, A.*, Ngubane, P.S.*, Mosili, P.* ***Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of Kwa-Zulu Natal, Westville, South Africa

ABSTRACT & BODY

Chronic consumption of a high-fat high carbohydrate diet compromises the intestinal permeability in type 2 diabetes. Increased intestinal permeability also known as a leaky gut causes the translocation of bacterial lipopolysaccharides (LPS), luminal antigens, and other toxic substances into the blood. Such elicits an immune response and secretion of pro-inflammatory cytokines which result in inflammation. However, it has not been investigated if increased intestinal permeability occurs during pre-diabetes. This study investigated the effects of HFHC diet-induced pre-diabetes on intestinal permeability in male Sprague Dawley rats. The animals were randomly assigned into non-pre-diabetic group and diet-induced pre-diabetic group (n=6) for a period of 20 weeks. After 20 weeks, blood glucose, plasma insulin, serum zonulin, plasma LPS, and soluble CD14, were measured. Furthermore, plasma tumor necrosis factor-alpha, interleukin-6, C-reactive protein, and intestinal fatty-acid binding protein concentrations were also measured. Blood glucose and insulin concentration were significantly increased in the pre-diabetic group (PD) by comparison to the non-pre-diabetic group (NPD). Serum zonulin and sCD14 concentrations in the PD group were increased compared to the NPD group while plasma LPS concentrations were similar. An increase in TNF-α, IL-6, CRP, and IFABP concentrations in PD compared to the NPD group was observed. Taken together, these results suggest that chronic consumption of HFHC diet may be associated with disruption of the intestinal permeability leading to leaky gut. This also could lead to chronic subclinical inflammation that may result in the development of the observed insulin resistance in pre-diabetes. This study could provide an alternative connection between high-calorie diets and the development of pre-diabetes.

Keywords: Pre-diabetes, high-fat high carbohydrate, zonulin, lipopolysaccharides, intestinal permeability

Research Theme: Prediabetes

Ethics Number: AREC/00003627/2021

Please tick the appropriate box

	Poster Presentation
	Oral Presentation

*Please follow the guidelines as abstracts will be reproduced exactly as submitted.
*The Title, Author affiliations and abstract must fit into the boxes above.