THE ASSOCIATION BETWEEN INCRETIN HORMONES CONCENTRATION AND THE DEVELOPMENT OF DIET-INDUCED PREDIABETES.

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

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PREFACE

Prolonged consumption of a diet high in carbohydrates and fats has been shown to lead to the development of insulin resistance and the onset of type 2 diabetes mellitus (T2DM). T2DM has been shown to be preceded by an intermediate hyperglycemic condition known as prediabetes. Prediabetes is characterised by glucose levels that are above normal but below the diabetes threshold, and studies have demonstrated that the complications associated with T2DM begin in the prediabetic state. Impaired incretin peptides in T2DM have been shown to maintain the abnormal concentrations of blood glucose and insulin resistance. However, the changes and association of incretin peptides in the development of prediabetes have not been characterised. A diet-induced prediabetes animal model that mimics the human condition of prediabetes was established in our laboratory. In the development of this animal model, we investigated the changes and the association of incretin peptides. When the animal model was developed and prediabetes had been induced, we further investigated the effect of a low carbohydrate, high fat diet on incretin peptides levels and observed their role in the reversal of prediabetes. Incretin hormone levels could potentially serve as biomarkers for early detection and risk stratification for prediabetic individuals. Identifying the biomarkers could lead to the development of targeted drugs or treatments that aim to modulate these hormone levels to prevent or treat prediabetes effectively. This knowledge can be particularly useful for individuals with high-risk dietary habits or those seeking to make dietary modifications to reduce their prediabetes risk.

The experimental work described in this dissertation was conducted at the University of KwaZulu Natal, Westville Campus, Durban, South Africa. All work was conducted under the supervision of Dr. Andile Khathi and co-supervised by Mr. Aubrey Sosibo.

DECLARATION

I, Mr. Nhlakanipho Mzimela declare.

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institutions for purposes of obtaining an academic qualification, whether by myself or any other party.
2. The dissertation is my own investing and work from other researchers is rephrased and referenced, or if their exact words are used, they are quoted and referenced.
3. Dr. Khathi and Mr. AM Sosibo supervised the research done in this study.

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PLAGIARISM DECLARATION

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ACKNOWLEDGEMENTS

Words cannot express my gratitude to my supervisor Dr. Andile Khathi and my co-supervisor Mr Aubrey Sosibo for their patience and guidance throughout this journey. It was a pleasure working with you, and I hope this is our first of many studies together. I would also like to thank Mr. Dennis Makhubela (Bab' Dennis) for his technical assistance.

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LIST OF ABBREVIATIONS

Abbreviation	Name
ADA	American Diabetes Association
AgRP	Agouti-related protein
BBB	Blood Brain Barrier
BRU	Biomedical Research Unit
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and amphetamine-regulated transcript
CCK	Cholecystokinin
DPP-4	Dipeptidyl-peptidase 4
ELIZA	Enzyme linked immunosorbent assay
GH	Growth hormone
GHS-R	Growth hormone secretagogues receptor
GHSs	Growth hormone secretagogues
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-Like Peptide 1
GLUT-4	Glucose transporter type 4
HbAc1	Glycated hemoglobin
HFHC	High fat and high carbohydrate
HOMA	Homeostatic model assessment
IR	Insulin resistance
LEP-R	Leptin receptor
LH	Lateral hypothalamus
ND	Normal diet (standard diet)
NPD	Non-prediabetes
NPDC	Non-prediabetes control
NPY	Neuropeptide Y
PD	Prediabetes
POMC	Pro-opiomelanocortin
PYY	Peptide YY
SEM	Standard error of mean
T2DM	Type 2 diabetes Mellitus.
UKZN	University of KwaZulu-Natal.
WHO	World Health Organization

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STUDY OUTLINE

This dissertation is presented in a manuscript format consisting of 4 chapters; chapter 1: a literature review. Chapter 2: prologue, abstract and manuscript 1. Chapter 3: prologue, abstract and manuscript 2, chapter 4: synthesis and conclusion. Chapter 1 details the study's introduction in the form of a literature review, the key aims and objectives as well as the justification of the study. Chapter 2 comprises the prologue and abstract of manuscript 1, which further details the link between incretins and the development of prediabetes. In addition, the work in chapter 2 has been submitted for publication and is currently under review in the “Canadian Journal of Physiology and Pharmacology”. Chapter 3 presents manuscript 2, which looked at the effect of a low carbohydrate, high unsaturated fat diet on incretin levels on diet-induced prediabetic rats. This work is currently under review in the journal "Endocrinologia, Diabetes y Nutricion”. Chapter 4 consists of the synthesis and conclusion. Mzimela N authored the work presented in this study, supervised by Dr A Khathi and Mr. A Sosibo.

ABSTRACT

Background

The increase in the prevalence of type two diabetes mellitus(T2DM) is attributed to unhealthy lifestyles and high-calorie diets. T2DM is a chronic metabolic condition characterised by impaired insulin function and high blood glucose concentration. Prediabetes is an intermediate hyperglycemic condition that frequently occurs before the onset of T2DM. This condition is characterised by a gradual reduction of insulin sensitivity by insulin receptors in insulin-dependent cells, frequently followed by significantly high plasma glucose levels. In this condition, the blood glucose concentration is insufficient to diagnose T2DM. Studies have looked at how incretin peptides affect the pathology of T2DM. However, the link between incretin peptide levels and the onset of prediabetes remains unknown. Additionally, the effect of a low carbohydrate, high unsaturated fat diet on incretin levels during the reversal of prediabetes has not been established. Thus, this study aimed to assess the role of incretin levels in the emergence of prediabetes and the effect of a low carbohydrate, high unsaturated fat diet on incretin levels during the reversal of prediabetes.

Methods

The first study was conducted using 24 male Sprague-Dawley rats, divided into two groups given a standard rat diet (NPD) (=12), while the other group was given a high-fat high carbohydrate (HFHC) (n=12) diet. Six animals from each group were sacrificed at week 10 and week 20, and blood was collected for biochemical analysis at each time interval. After 20 weeks, the HFHC fed group was found to be prediabetic and were therefore named the prediabetic group (PD). At week 10, the NPD group had the following mean measurements for the NPD and HFHC groups respectively: Glucose (4.3mmol/L and 5.9mmol/L), Insulin (40.26 and 118.32pmol/L), HbAc1 (4.9 and 5.15%), GIP (9.308 and 12.91pmol/L),GLP-1 (18.53 and 15.73pmol/L), Leptin(1.92 and 1.08mmol/L), and Ghrelin (122.1 and 186.5pmol/L). At week 20, the PD group had the following mean measurements for the NPD and PD groups: Glucose (4.4mmol/L and 7.35mmol/L), Insulin (41.18 and 159.42pmol/L), HbAc1 (4.7 and 6.65%), GIP (10.03 and15.1pmol/L),GLP-1 (21.52 and 6.73pmol/L), Leptin (2.16 and 0.78mmol/L), Ghrelin (124.2and 210.63pmol/L).

After 20 weeks of pre-diabetes induction, the second study began with 18 male Sprague-Dawley rats. Group A continued with the standard diet and was used as a non-prediabetic control (NPDC) (n=6). The pre-diabetic group B (n=12) was split into two experimental groups. One of the groups continued a HFHC diet and served as the pre-diabetic control group (PD)(n=6). In contrast, the other group had a diet intervention where the diet was changed to a low carbohydrate-high unsaturated fats diet (PD+DI) (n=6). All groups were then maintained on their respective diets for a further 12 weeks. At week 32, the PD+DI group had the following mean measurements: Glucose (5.367mmol/L), Insulin (188.5ng/ml), HbAc1 (4.62%), GIP (24.08pg/ml) , GLP-1, Leptin (1.267ng/ml) , and Ghrelin (17.09pg/ml).

Results

In the first study, after 20 weeks, the HFHC diet resulted in moderate hyperglycaemia, elevated plasma insulin, elevated HbA1c and insulin resistance in the PD group compared to the NPD group. There were also significantly increased GIP and ghrelin concentrations with significantly low GLP-1 and leptin concentrations in the PD group compared to the NPD group. Interestingly, at week 10, there was moderate hyperglycaemia, elevated plasma insulin, elevated HbA1c and insulin resistance in the HFHC group. There were also significant GIP and ghrelin levels with significantly low GLP-1 and leptin concentrations, but there was no prediabetes.

In the second study, there were significantly reduced blood glucose levels, plasma insulin levels, HOMA-IR index, and HbA1c in the PD+DI group compared to the PD group. In the PD+DI group, there are significantly reduced GIP and ghrelin levels with significantly increased GLP-1 and leptin concentrations compared to the PD group. However, when the PD-DI group is compared to the NPDC group, there is no significant difference in all measured parameters.

Conclusion

The first study's findings show that chronic ingestion of a HFHC diet causes dysregulation of incretin hormones from week 10, while prediabetes was only diagnosed at week 20. This dysregulation of incretin hormones precedes the onset of prediabetes and may trigger chronic insulin stimulation, leading to prediabetes development. In the second study, we observed the effect of diet on incretins as they play a significant role in developing and reversing pre-diabetes. Chronic consumption of a HFHC diet led to elevated blood glucose and insulin concentration, resulting in abnormal concentrations of incretins. The abnormal incretins then maintained this state of hyperglycemia and hyperinsulinemia, resulting in prediabetes. Chronic consumption of a LCHF by the pre-diabetic rats led to reduced concentrations of incretins, which could have led to a reduced HbA1c and eventually to the reversal of pre-diabetes. The results of this study suggest that incretin concentrations preceded the development of prediabetes and may even have a role in its development as well as its reversal.

Chapter 1: Literature Review

1. Introduction

Glucose is a carbohydrate we get from the food we eat and is used for energy generation (1). Blood glucose concentration is maintained within a homeostatic range via a process known as glucose homeostasis (2). Insulin and glucagon are hormones secreted by islet cells of the pancreas to help maintain the balance of blood glucose concentration within the homeostatic range of 3.5-5.5mmol/L (2).

In the case of increased glucose concentration in a healthy individual above the homeostatic range, insulin is secreted shortly after the ingestion of food (2). It stimulates the uptake of glucose as well as the conversion of glucose into glycogen by the liver for storage (3). Insulin facilitates this process by binding to the insulin receptors on membranes of skeletal muscle and adipose tissue, triggering the exocytosis of glucose transporter type 4 (GLUT4) to the cell membrane (4). The binding of insulin prevents the mobilization of GLUT4; this allows for the removal of glucose from the blood into the cell, which is used for energy generation through glycolysis (4). This exocytosis of GLUT4 in muscle cells is also triggered during exercise resulting in reduced blood glucose levels (5). When the blood glucose concentration falls below the homeostatic range, glucagon is secreted. Once secreted, it binds to the glucagon receptors in the cell membrane (6). This triggers a cascade of reactions that eventually shuts down glycolysis in the adipose tissues and promotes glycogenolysis and gluconeogenesis in the liver cells (7). Glycogen is then converted into glucose and released into the bloodstream, increasing the blood glucose concentration (7).

There are other hormones involved in regulating glucose homeostasis known as incretin hormones. Incretins are hormones secreted in the gut that enhance insulin secretion and contribute significantly to postprandial insulin release (8). The incretin peptides account for up to 60% of postprandial insulin release. The "incretin effect" is a significant factor in maintaining postprandial glucose homeostasis (9). The incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are associated with appetite-regulating hormones ghrelin and leptin (10, 11). Leptin and ghrelin work closely related to insulin and glucagon to maintain glucose homeostasis (8, 12). A disturbance in glucose homeostasis results in many health problems over time, such as type 2 diabetes mellitus (T2DM) (13). During T2DM, glucose remains in the blood and doesn't enter the cells causing health problems such as fatigue, hyperphagia, and excessive thirst (13). An intermediate hyperglycemic condition known as prediabetes was found to precede the onset of T2DM (14). The disturbances related to insulin during prediabetes are well defined, however, changes in incretin homeostasis are unknown. This raised the question of whether the disturbances in incretin hormones also present and what effect a low carbohydrate, high unsaturated fat diet has on the incretin levels of a prediabetic animal model.

2. Incretin hormones

Incretins are hormones secreted in the gut and aid in maintaining glucose homeostasis by enhancing insulin secretion as they contribute significantly to overall postprandial insulin release (8). The incretin hormones work together to produce the incretin effect, which is a two- to three-fold increase in insulin secretion in response to oral glucose administration compared to intravenous glucose administration (8). Incretin peptides include glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), associated with ghrelin and leptin.

2.1. The Physiological Role of GIP on Glucose Homeostasis

GIP is a 42-amino-acid peptide secreted by the enteroendocrine K-cells in the duodenum and proximal jejunum in response to the presence of glucose in the stomach (15). GIP is an inhibitory peptide that plays a role in insulin secretion via binding to the beta-pancreatic cells (16). The release of insulin into the blood occurs before glucose absorption to prepare for the incoming blood glucose concentration elevation (17).

In response to an empty stomach of a healthy individual, the hormone ghrelin is secreted from the stomach (18). Ghrelin is a gut hormone that induces food ingestion leading to the entrance of glucose in the stomach (18). The enteroendocrine K-cells are stimulated by glucose in the stomach and secrete the glucose-dependent peptide GIP into the blood (17). GIP acts on the G-protein coupled receptors in the beta cells of the pancreatic islets (19). The binding of GIP to its receptor increases the intracellular cyclic adenosine monophosphate (cAMP) concentration, leading to the activation of adenylate cyclase, protein kinase A, and phospholipase C/protein C cascades, respectively (19). This promotes an influx of calcium ions and insulin exocytosis (19). The production of insulin leads to an inhibition of ghrelin and leptin secretion (18) (20). Leptin is a hormone secreted in the gut that increases insulin sensitivity, which aids in energy balance; it suppresses hunger and reduces fat storage in adipocytes (21).

When glucose enters the bloodstream, the beta-pancreatic cells detect increased blood glucose concentration above the homeostatic range and secrete even more insulin to lower blood glucose within the homeostatic range (3). Increasing GIP concentration moderately inhibits gastric acid secretion and gastrointestinal movement, this inhibitory feedback mechanism controls the transit of a meal to optimize nutrient digestion and absorption and is known as the "ileal break" (22). The incretins are rapidly degraded by the ubiquitous enzyme dipeptidyl peptidase 4 (DPP-4) (22). DPP-4 works by cleaving alanine and proline residues in the second position of the N-terminus in peptide chains (19). GIP has a substitution of L-alanine for D-alanine residue at position 2; this makes GIP resistant to the action of DPP-4, enhancing its incretin effect (19). Although GIP is still inactivated, its inactivation occurs slower than GLP-1, giving GIP a half-life of 5 to 7 minutes (19).

2.2. The Physiological Role of GLP-1 on Glucose Homeostasis

Glucagon-like peptide 1 is a 30-amino acid peptide hormone produced by the intestinal epithelial endocrine L-cells (23). GLP-1 is rapidly degraded and inactivated by the enzyme DPP-4 before it leaves the stomach, supporting the notion that GLP-1 activities are communicated via GLP-1 receptor expressing sensory neurons in the intestine and liver (24). The primary functions of GLP-1s are to stimulate insulin secretion (acting as an incretin hormone) and inhibit glucagon secretion, thereby reducing postprandial blood glucose concentration (25). Leptin's interaction with other incretin hormones is needed to stimulate short-term satiation. Leptin has been shown to cause satiation by interacting with GLP-1 and its receptor antagonist (26). Leptin receptors have been discovered in GLP1-secreting endocrine L cells and neurons, and leptin has been shown to promote GLP-1 release in L cells. GLP-1 plays a vital role in regulating satiety and food intake (26).

The lateral hypothalamus (LH) contains the glucagon-like peptide-1 receptor (GLP-1R), which binds the GLP-1 activating a series of reactions that are required for average body weight, food intake, and food reinforcement management (25). Reduced GLP-1R expression in the LH enhanced weight gain, hyperphagia, and minor glucose tolerance deficits. Like GIP, GLP-1 also functions as an enterogastrone and part of the ileal brake mechanism by inhibiting gastrointestinal motility and secretion (25). GLP-1 secretion is thought to play a role in developing obesity, while exaggerated secretion is believed to cause postprandial reactive hypoglycemia (27). GLP-1 receptor agonists are currently being studied for T2DM due to these activities (27). The stimuli and molecular mechanisms that cause GLP-1 to be produced in response to meal ingestion are still being explored.

2.3. The Physiological Role of Ghrelin on Glucose Homeostasis

Ghrelin is a 28-amino-acid peptide that was discovered to be the first circulating hunger hormone (28, 29). The ghrelin receptor, also known as the growth hormone secretagogue receptor (GHS-R), is a G protein-coupled receptor that binds growth hormone secretagogues (GHSs), such as ghrelin (30). Ghrelin acts in the brain to regulate food intake, body weight, adiposity, and glucose metabolism. GHSR is thought to play a role in maintaining energy homeostasis and body weight (31). Ghrelin concentration in the blood rises during fasting and falls during feeding. Ghrelin is primarily synthesized in the stomach and increases growth hormone production (GH) (32). A-like cells in the stomach's oxyntic glands generate and secrete this peptide hormone's octanoylated form (Acyl ghrelin) (32). GHSR1a and 1b receptors are widely distributed and can be detected in various body and brain regions, including tumours and metastasis (33). This wide distribution of ghrelin and its receptors means that ghrelin plays many physiological roles.

In the hypothalamus, particularly in the arcuate nucleus, there are appetite stimulator neurons, namely neuropeptide Y (NPY), that promote food intake and Agouti-related protein (AgRP); which prolongs feeding (32). During a hypoglycaemic state, ghrelin is secreted from the stomach, increasing the circulating peripheral ghrelin that directly crosses the brain-blood barriers as a saturable transport

system and acts on the hypothalamic arcuate nucleus to activate and increase the synthesis of NPY and AgRP neurons, thus promoting an increased appetite and food intake and overall increase in blood glucose (31).

An increased insulin secretion inactivates the AgRP neurons due to increased blood glucose simultaneously with an increased leptin secretion (34). The capacity of ghrelin-producing cells to perceive foods directly or gut hormones (insulin, glucagon-like-peptide 1 (GLP-1), peptide YY (PYY), and cholecystokinin (CCK) released following a meal may be one proposed mechanism for suppressing ghrelin production by food (33). Ghrelin has been found to inhibit glucose-stimulated insulin release, while insulin has been shown to reduce ghrelin secretion (18).

2.4. The Physiological Role of Leptin on Glucose Homeostasis

Leptin is a 167-amino-acid protein produced predominantly by white adipose tissue and absorbed into circulation. Leptin is secreted after an increased insulin secretion in a hyperglycaemic state and increases insulin sensitivity by activating the insulin receptor substrate-1 found in the brain (20, 35, 36). Leptin aids in energy balance by suppressing hunger and reducing fat storage in adipocytes (21). Longform leptin receptor-b (LEPR-b) is found in both the peripheral and central nervous systems, with the hypothalamus on the arcuate nucleus (ARC) being the primary site of expression (32). The ARC is the first-order centre that senses peripheral metabolic signals and other brain areas (32).

After increasing insulin secretion, leptin is secreted by white adipose tissues and absorbed into the circulation (37). In the hypothalamus, it crosses the brain-blood barriers as a saturable transport to the hypothalamic arcuate nucleus, where it binds to LEPR-b (37). The binding of leptin stimulates neurons in the ARC, and the stimulated neurons, such as pro-opiomelanocortin (POMC), cocaine and amphetamine-regulated transcript (CART) neurons, operate as appetite inhibitors that increase satiety (20, 35, 36). When leptin acts on the hypothalamic neurons, it has been found to deactivate the NPY and AgRP appetite stimulator neurons and therefore suppress appetite and food intake, inducing satiety (37). The incretin hormones' mechanisms of action are disturbed in conditions such as TDM2 (20, 38) (39). The changes to incretins in T2DM are well-defined; however, the changes during prediabetes are still unknown.

3. Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is a metabolic disorder characterised by hyperglycemia due to insulin resistance (40). This can be due to resistance of insulin receptors developed over time, mainly from an inactive lifestyle and diet high in fats and carbohydrates (41). Insulin resistance refers to a condition whereby insulin is secreted into the blood but cannot effectively bind to its receptors on the cell membranes.

The ineffective binding of insulin means glucose cannot be allowed inside cells because the GLUT4, which allows glucose in the cell, will not be embedded in the plasma membrane (42). An oral glucose tolerance test (OGTT), a fasting glucose test, a postprandial glucose test, or glycated haemoglobin (HbA1c) test can all be used to diagnose T2DM (43). When an individual's fasting blood glucose concentration is greater than 7 mmol/L with postprandial glucose concentration greater than 11.1 mmol/L and glycated haemoglobin concentration greater than 6.5 percent, that individual is diagnosed with T2DM (44). T2DM is associated with several microvascular and macrovascular complications, such as renal failure, heart disease and bone weakness (45, 46).

3.1 Changes in GIP levels in T2DM

Due to hyperglycemia, there are high concentrations of the hormone gastric inhibitory peptide since it's a glucose-dependent peptide (38). GIP secretion is triggered after a meal because of the increase in blood glucose concentration, but it is secreted constantly in T2DM due to a high blood glucose concentration (38). If a person with diabetes mellitus consumes food high in carbohydrate and fats content, their blood glucose, together with GIP and glucagon-like peptides, will further increase, inhibiting gastric acid secretion and absorption in the small intestines and reducing the gut motility (47). Due to the Inhibition of gastric emptying and gastric acid secretions, there are low motilin and ghrelin concentration. These hormones help induce muscle peristalsis in the digestive tract and signal hunger, which increases gut motility (48, 49). This blockage might lead to bloating and constipation.

The reduced gut motility results in a build-up of food in the small intestines, and the Inhibition of absorption results in an overgrowth of bacteria in the small intestines (49). The excess bacteria trigger an increase in zonulin concentration, a protein that regulates the permeability of the tight junctions between cells in the digestive tract wall and increases the washout of bacteria (50). The washout of the excess bacteria causes pain and leads to diarrhea. The increase in zonulin secretion increases the size and permeability of the tight junctions, allowing foreign entities like bacteria and digested food particles to enter the extracellular membrane, causing inflammation, and leading to the immune changes observed in T2DM (50). The Increased concentration of GIP is responsible for the irregularities observed in T2DM.

3.2. Changes in GLP-1 levels in T2DM

GLP-1 (glucagon-like peptide-1) is an incretin hormone that causes glucose-dependent insulin production, glucagon suppression, delayed stomach emptying, and reduced caloric intake (25). In T2DM, DPP4 expression is elevated during adipose tissue differentiation and is linked to an increased secretion rate from adipose tissue in obesity (BMI) (39). The pathway of GLP-1 is disturbed in T2DM; GLP-1 is being actively degraded by the increased circulating DPP-4 enzyme as fast as it is secreted, meaning it brings about no significant changes (39).

Oral glucose stimulates the release of GLP-1, whereas intravenously delivered glucose does not affect plasma GLP-1 concentrations (51). Glucagon-like peptide-1 is a glucose-lowering peptide that provides the basis for two new types of glucose-lowering drugs for type 2 diabetic individuals: incretin mimetics and inhibitors of protease DPP-4 (27).

3.3. Ghrelin and Leptin levels in T2DM

During a hypoglycaemic state or when the stomach is empty, ghrelin is secreted into the blood, increasing the circulating peripheral ghrelin that directly crosses the brain-blood barriers as a saturable transport system (52). Ghrelin binds to the GHS-R and therefore activates the hypothalamic arcuate nucleus, which increases the synthesis of NPY and AgRP neurons, promoting an increased appetite and food intake (31). After a meal, increased blood glucose stimulates insulin secretion from the pancreatic beta cells (53). As mentioned above, leptin increases after insulin secretion, secreted by white adipose tissues, increases circulation, and crosses the brain-blood barriers as a saturable transport to the hypothalamic arcuate nucleus, where it binds leptin receptor-b (LEPR-b) (37). Its binding stimulates the synthesis of appetite inhibitors and increases its synthesis, which reduces appetite and food intake by activating the satiety centre (Ventromedial hypothalamus) (20, 54). Giving an individual a feeling of satisfaction by reducing appetite and food intake reduces ghrelin secretion after a meal, thus regulating glucose homeostasis (55).

However, in T2DM, this pathway is impaired due to hyperlipidaemia and hyperinsulinemia; leptin resistance occurs because of increased leptin concentration for an extended period (20). Due to insulin resistance, the cells cannot access glucose from the blood, and they constantly transmit signals to the hypothalamus to increase food intake (56). The excitation of the vagus nerve stimulates ghrelin secretion in the stomach, increasing appetite and food intake (57). Due to leptin resistance, satiety is not induced, and appetite and food intake are not inhibited, so the individual is constantly hungry (20).

A condition known as prediabetes has been discovered to precede T2DM (58). The main difference between T2DM and prediabetes is that T2DM has a higher blood glucose concentration, meaning its effects are more intense than prediabetes (58). This raised whether the complications mentioned above brought upon the incretins, ghrelin and leptin are also present in the prediabetic state.

4. Prediabetes

Prediabetes is a high-risk state characterised by intermediate hyperglycaemia (59). It occurs when the blood glucose is higher than normal but it's not high enough to diagnose type 2 diabetes mellitus (59). According to studies, prolonged consumption of food high in carbohydrates and fat content is linked to an increased risk of developing prediabetes (60). Other risk factors for prediabetes are inactive lifestyles, lack of exercise, obesity, and family history (59, 61). Long durations of inactivity can impair normal blood glucose homeostasis, resulting in obesity, cardiovascular disease, and the risk of prediabetes (61). Individuals at risk of developing T2DM have one or more prediabetic conditions, such as impaired

fasting glucose (IFG) of 5.5- 6.9 mmol/L, impaired glucose tolerance (IGT) of 7.8-11.1 mmol/L, and impaired glycated haemoglobin (Hb1Ac) concentration of 5.7-6.4 percent (62). Glycated haemoglobin determines how much haemoglobin has become bound to glucose in the past months (63).

A study to observe the changes in immune cell concentration during the progression of HFHC dietinduced prediabetes was conducted in our lab, using male rats to mimic the human conditions (64). They were fed a high-fat, high-carbohydrate diet for 20 weeks to induce prediabetes. Results were collected using blood samples after 12 weeks, and proof of inflammation was confirmed by decreasing blood neutrophils and eosinophils (64). However, this study focused solely on changes in immune cell concentration during prediabetes. Pathophysiologies in the small intestine, such as celiac disease and chronic inflammation, undoubtedly contribute to immune changes during prediabetes.

5. Management of T2DM and prediabetes

As the prevalence of T2DM rises worldwide, it's now more important to prevent prediabetes from progressing to T2DM. A large portion of prediabetic individuals progresses toward the development of T2DM because they are unaware that they have it (65). Type 2 diabetes mellitus has no known cure; it can be kept under control with exercise, a change in diet and medication (66). The association between incretins on T2DM has been established, and several studies have been conducted to check their effects on T2DM. However, this is not known for the prediabetic state. This raises the question of whether the complications associated with the incretins experienced in T2DM begin in the prediabetic state. This work is significant because if the incretins are linked to the development of prediabetes, the findings could develop a new marker for detecting prediabetes. The findings of this study could also help reverse prediabetes and prevent T2DM.

6. HFHC animal model of prediabetes

The average person's diet is high in saturated fats and carbohydrates, with little in the way of vegetables and fruits. As a result, the HFHC diet supplemented with 15% fructose is used in animal models to mimic the average person's diet is good (67). According to studies, the combined effect of an HFHC diet causes the most severe symptoms, including hyperglycaemia, hypercholesterolemia, and increased inflammatory mediators (41). Additionally, daily consumption of sugars, refined high-calorie foods, high saturated fats, and high-sweetened refined sugars has been linked to the development of prediabetes in studies (41). The prediabetes symptoms in individuals are best described using this dietinduced prediabetic animal model (67). Since the concentration of the incretin hormones is affected by diet, changing from a HFHC diet to a healthy diet could reduce the GIP. Therefore, the insulin in the blood could also be reduced, which could reverse prediabetes.

7. Aim

This study sought to investigate the association of incretins, ghrelin & leptin in the development of dietinduced prediabetes as well as look at their role in the reversal of prediabetes.

8. Objectives

8.1. Manuscript 1.

To assess the association between incretin peptides levels and the development of prediabetes by investigating the following:

- Glycated haemoglobin concentration
- Fasting Blood Glucose
- Insulin concentration
- GIP concentration
- GLP-1 concentration
- Ghrelin concentration
- Leptin concentration

8.2. Manuscript 2.

To explore the effects of a low carbohydrate, high unsaturated fat diet in incretin peptides levels, to attempt to reverse prediabetes and its effects by investigating the following:

- GIP concentration
- GLP-1 concentration
- Ghrelin concentration
- Leptin concentration
- Insulin concentration
- Glycated haemoglobin concentration

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Chapter 2: Manuscript 1

Investigating the effect of incretin peptide levels in the development of diet-induced prediabetes.

Prologue 1

Chronic consumption of high-caloric diets has been associated with the development of a chronic metabolic condition known as type 2 diabetes (T2DM). T2DM is preceded by prediabetes which is a state of intermediate hyperglycaemia. Up to 70% of people with prediabetes will, in the long run, develop T2DM. Incretins have been shown to contribute to the progression from prediabetes to T2DM however the role of incretin concentrations in the development of T2DM has not been elucidated. Hence the study sought to assess the link between incretin concentrations and the development of prediabetes using our laboratory developed HFHC diet-induced prediabetes animal model.

The manuscript in chapter 2 titled **"Investigating the effect of incretin peptide levels in the development of diet-induced prediabetes".** is authored by N. Mzimela, NR Dimba, A.M Sosibo, P.S Ngubane and A Khathi.

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Investigating the effect of incretin peptide levels in the development of diet-induced prediabetes.

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Abstract

Prediabetes is a chronic metabolic condition characterised by a gradual reduction of insulin sensitivity by insulin receptors in insulin-dependent cells, frequently followed by increased plasma glucose levels, which are still below the threshold for a type 2 diabetes mellitus diagnosis. Studies already conducted have looked at how incretin peptides affect the pathology of type 2 diabetes mellitus. On the other hand, the link between incretin peptide levels and the onset of prediabetes remains unknown. Thus, this study aimed to assess the role that incretin peptide levels play in the emergence of prediabetes. This study was conducted using 24 male Sprague-Dawley rats, divided into two groups given a standard rat diet (NPD) (=12), while the other group was given a high-fat high carbohydrate (HFHC) (n=12) diet. Six animals from each group were sacrificed at week 10 and week 20, and blood was collected for biochemical analysis at each time interval. The chronic ingestion of a HFHC diet for 10 weeks resulted in significantly increased GIP ($p=0.0001$), GlP-1 ($p=0.0019$), leptin ($p=0.0001$) and ghrelin ($p=0.0001$) concentrations. After 20 weeks, there were further increased incretin hormone concentrations; there were significantly higher concentrations of GIP, GLP-1, Leptin, and ghrelin. The dysregulation of incretins was first observed at week 10, while prediabetes was only diagnosed at week 20. This dysregulation of incretin hormones precedes the onset of prediabetes and may trigger chronic insulin stimulation which may lead to the development of prediabetes. The results of this study suggest that the dysregulation of incretin concentrations may not only precede the development of prediabetes but may even have a role in its development.

Keywords: Type two diabetes mellitus, incretins, insulin resistance.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic disorder resulting in increased blood glucose concentration over time (68). However, a state of intermediate hyperglycaemia known as prediabetes often precedes the onset of T2DM (69). In this condition, there is elevated blood glucose concentration, but they are not high enough for the diagnosis of T2DM (70). Prediabetes is characterised by a steady decrease in responsiveness to insulin-by-insulin receptors in insulin-dependent cells of the liver, adipose tissue, and skeletal muscle (70).

In reaction to an empty stomach in a healthy individual, A-like cells in the stomach's oxyntic glands secrete a hormone known as ghrelin (32). Ghrelin acts in the hypothalamus to stimulate appetite and induce food intake, leading to the entrance of glucose into the stomach (32). The entrance of glucose in the stomach inhibits ghrelin secretion and triggers the enteroendocrine K cells of the duodenum jejunum and throughout the small intestines to secrete glucose-dependent insulinotropic polypeptide (GIP), its release is dependent on the rate of absorption of glucose into the blood (32, 71). GIP is secreted simultaneously with the hormone glucagon-like peptide 1 (GLP-1) produced by the enteroendocrine L cells, and together they are known as the incretin peptides (10). GLP-1's primary functions are stimulating insulin secretion and inhibiting glucagon secretion, reducing postprandial blood glucose concentration, and inducing satiety by binding to its receptors in the hypothalamus (25). Insulin secretion triggers the release of the hormone leptin, it then is transported via a saturable transporter system across the blood-brain barriers (BBB) to leptin or obesity receptors (LEP-R/OB-R) for activation to induce satiety (72). Leptin also stimulates the secretion of GLP-1 from the endocrine L-cells of the small intestines to induce satiety and reduce adipocyte fat storage (11).

In conditions such as T2DM, the efficiency of GLP-1 is impaired as it is actively degraded by the dipeptidyl peptidase 4 enzyme (DPP-4); this enzyme is produced on the surface of most cell types and is linked to immune regulation, signal transduction, and apoptosis (73). However, the secretion of GIP is increased since it is secreted in the presence of glucose (74). Over-secretion of GIP causes a further increase in insulin concentration by constantly promoting insulin secretion further, thus promoting hyperinsulinemia (74). Due to hyperlipidemia and hyperinsulinemia, there is increased inhibition of ghrelin and elevated leptin concentration (75, 76). There is also the development of leptin resistance due to increased plasma leptin concentrations for extended periods (20). This results in impaired satiety, thus resulting in individuals displaying hyperphagia (20). Hyperinsulinemia also promotes lipogenesis in the liver resulting in increased synthesis of lipids (77). This increased lipogenesis plays a significant role in T2DM complications such as heart attacks, stroke, and kidney failure (77). Because of their functions in the regulation of glucose, GIP and GLP-1 have been used as therapeutic targets for the treatment of T2DM (78). DPP4 inhibitors, GIP, and GLP-1 receptor agonists (RA) were discovered because of research into these medicinal targets (78, 79). The dual GIP and GLP-1 RAs show significant

effects on body weight loss and glycemic control, suggesting their therapeutic applications in the treatment of T2DM (78).

Several studies have shown the role of incretin levels on fasting glucose, impaired glucose tolerance, and that complications observed in T2DM often begin during prediabetes (58, 78, 130, 131). While several other studies have demonstrated moderate insulin resistance and hyperinsulinemia in the prediabetic state, there have been no studies showing the role of incretins in the development of prediabetes. Therefore, this study aims to characterize the association between incretin hormone concentration and the development of prediabetes in a diet-induced animal model of prediabetes.

2. Materials and Methods

2.1 Chemicals and drugs

All chemicals and reagents used were purchased from standard pharmaceutical suppliers and were of analytical grade.

2.2 Animals

24 male Sprague-Dawley rats were used in the current study, and the procedure was conducted according to the institutional guidelines of UKZN, Animals Research Ethics Committee (Ethics number: AREC/00003627/2021). The animals were fed and stored at the University of Kwa-Zulu Natal's biomedical research unit (BRU). The animals were maintained in standard laboratory conditions. There was a constant temperature of 25°C, the lighting was cycled between dark and light every 12 hours, the noise was kept below 65 decibels, and they were allowed uncontrolled access to food and water.

2.3.1. Induction of prediabetes

The animals were randomly assigned to the following diet groups; Prediabetes was induced by allowing the animals to feed on the HFHC diet for 20 weeks, as previously described (64, 80). Glucose tolerance was evaluated 5 days after the 20 weeks of induction with a well-known, established laboratory protocol, the oral glucose tolerance test (OGTT), to determine prediabetes according to the American Diabetes Association criteria (81). The rats with fasting blood glucose (FBG) of more than 5.6 mmol/L were considered prediabetic and grouped further for pharmacological studies. The animals fed the standard diet were also tested and found to be normoglycemic without prediabetes.

2.3.2. Experimental design

The twenty-four (24) animals were given one week to acclimatize and separated into two groups. Group 1(n=12) and group 2(n=12). Group 1 and Group 2 were further divided into 2 groups each. They both had a normal diet (ND) group (n=6) which was each given a standard diet with normal drinking water and they both had a high fat high carbohydrates diet group (HFHC) (n=6) which was each given a high fat, high-carbohydrate diet with drinking water supplemented with 15% fructose (AVI Products (Pty)

Ltd, Waterfall, South Africa). The composition of the high-fat, high-carbohydrate diet was customized as follows: Carbohydrates (55% KCAL/G), fats (30%KCAL/G), and proteins (15%KCAL/G). At week 10, all the animals in group 1 were sacrificed, while nothing was done to group 2. The animals from group 2 were maintained on their respective diets for another 10 weeks. At week 20, the animals from group 2 were sacrificed.

2.3.3. Blood collection and Termination

At weeks 10 and 20, the animals were anaesthetized with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodepoort, South Africa) via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, Durban, South Africa) for ±2 minutes. Blood was collected from all the animals using cardiac puncture, and the collected blood was then injected into individual heparinized EDTA containers. Some of this blood was used for glycated haemoglobin measurements. The remainder of the blood was centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes. Plasma was collected and stored at -80 °C in a Bio Ultra freezer (Snijders Scientific, Holland) until ready for biochemical analysis.

2.4. Biochemical Analysis

Plasma GIP, GLP-1, insulin, leptin and ghrelin concentrations were determined using specific ELISA kits in accordance with the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China). The kits included a micro-ELISA plate that was coated with antibodies specific to each of the parameters measured. The protocols for GLP-1, insulin and leptin were the same as they all required sandwich ELISA. Ghrelin required competitive ELISA, and the procedure was still the same except for the incubation periods, as ghrelin required less time. Standards and samples were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min at 37 °C. This was followed by adding the relevant biotinylated detection antibody (100 µl). After 60 min incubation at 37 °C, avidin–horseradish peroxidase conjugate (100 µl) was added to each microplate well. After a further 30 min incubation at 37 °C, the unbound components were washed away using the wash buffer provided.
Substrate solution (100 µl) was added to each microplate well, and after 15 min incubation at 37 °C, the stop solution (50 µl) was added. Optical density was measured using a nano spectrophotometer 47 (BMG Labtech, Ortenburg, Germany) at 450 nm. The concentrations of each parameter in the samples were extrapolated from a standard curve.

2.5. HOMA2-IR index

The homeostatic model assessment (HOMA) is a recognized method for measuring insulin resistance (IR) using fasting glucose and insulin. Two variants, known as HOMA1-IR or HOMA2-IR, make up the HOMA. The updated HOMA2-IR index was the choice model since it produced a more accurate index than the original HOMA1-IR that Matthews and Cols released in 1985. An average HOMA-IR value is within the range of 0.5 to 1.4. Insulin sensitivity is at its peak when the index value is less than

1.0. While index values over 1.9 indicate the onset of insulin resistance, and index values over 2.9 indicate substantial insulin resistance, respectively. HOMA-IR was calculated according to the formula: fasting plasma insulin (μU/ml) x fasting plasma glucose (mmol/L)/22.5.

2.6. Statistical analysis

Data expressed as mean ± S.E.M, using GraphPad Prism Instant software (version 5) to perform the statistical analysis. A student t-test was used to test for statistical significance of the obtained data from the control and the experimental group. P value<0.05 was considered to be significant.

3. Results

3.1. Plasma Glucose

The plasma glucose concentrations were measured at week 10 (n=12), halfway to prediabetes induction and at week 20 (n=12) when prediabetes is induced. The findings showed that the HFHC group and the PD group had significantly higher plasma glucose concentrations than the ND and NPD groups.

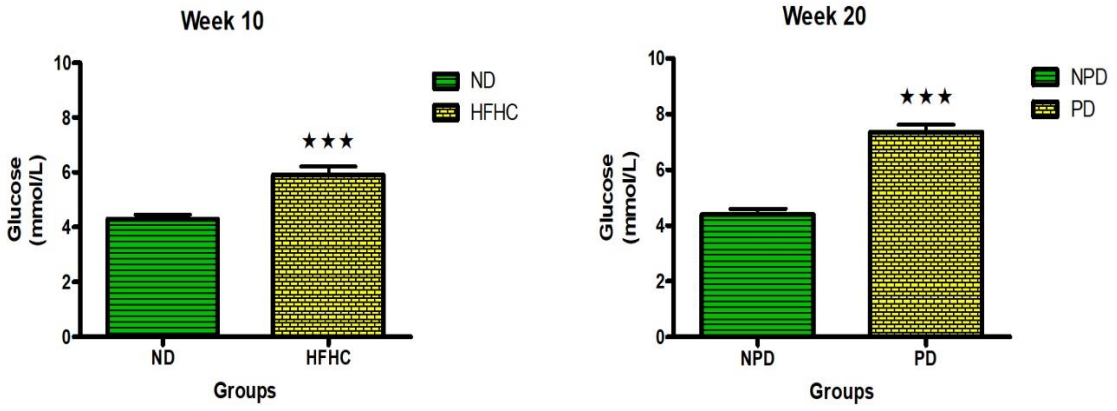

Figure 1: Plasma glucose concentrations in the standard and HFHC diet-fed animals for 10 and 20 weeks. Values are presented as mean ± SEM (n=6). ★$p < 0.05$ denotes comparison with the standard diet group; ★★★p=0.0009 and ★★★p=0.0001 respectively.

3.2. Plasma Insulin

The plasma insulin concentrations were measured at week 10 (n=12), halfway to prediabetes induction and at week 20 (n=12) when prediabetes is induced. The findings showed that the HFHC group and the PD group had significantly higher plasma insulin concentrations than the ND and NPD groups.

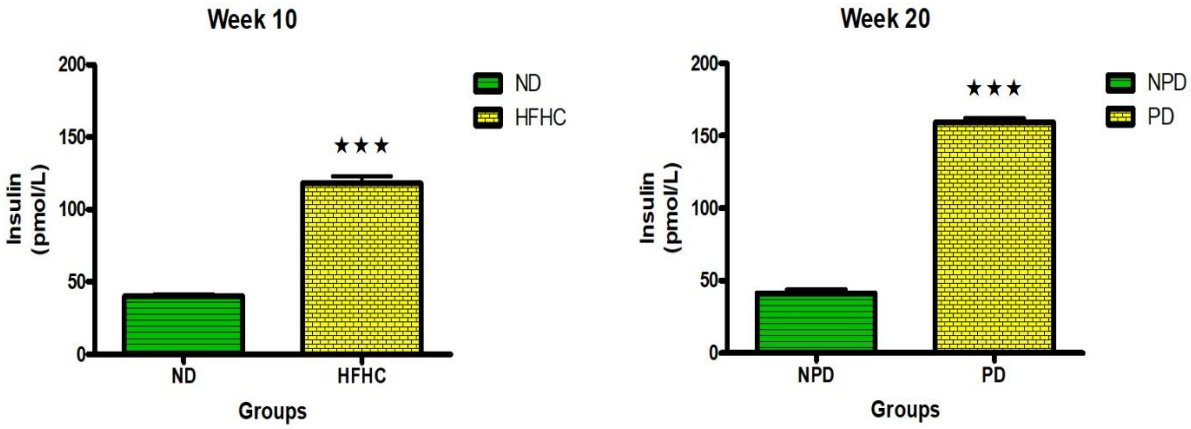

Figure 2: Plasma insulin concentrations in the standard and HFHC diet-fed animals for 10 and 20 weeks. Values are presented as mean ± SEM (n=6). ★$p < 0.05$ denotes comparison with the standard diet group; ★★★p=0.0001.

3.3. HOMA2-IR Index

The HOMA2-IR index was calculated in the ND and HFHC groups at 10 weeks. The HOMA2-IR value of the ND group at 10 weeks was within the insulin-sensitivity range (<1.0). By comparison with the ND group, the HFHC group showed a higher HOMA2-IR value, which was above the early IR range (>1.9) but lower than the overt IR range (>2.9). (★ = IR range > 1.9). The HOMA2-IR value of the NPD group at 20 weeks was within the insulin-sensitivity range (<1.0). By comparison with the NPD, the PD group showed a higher HOMA2-IR value, which was above the early IR range (>1.9) and the overt IR range (>2.9) (★ = IR range > 1.9).

GROUPS	SEM Glucose	SEM Insulin	**HOMA2-IR**	**p value Glucose**	**p value Insulin**
ND (10 weeks)	0.15	1.41	0.73	0.0001	0.0001
HFHC (10 weeks)	0.31	4.66	2.27★		
NPD (20 weeks)	0.20	2.69	0.75	0.0009	0.0001
PD (20 weeks)	0.28	2.85	3.17★		

Table 1: Displays the SEM, p value and the HOMA2-IR index calculated at weeks 10 and 20 using the HOMA2 calculator in the NPD and PD groups (n=6 per group).

3.4. Plasma GIP

The plasma GIP concentrations were measured at week 10 (n=12), halfway to prediabetes induction and at week 20 (n=12) when prediabetes is induced. The findings showed that the HFHC group and the PD group had significantly higher plasma GIP concentrations than the ND and NPD groups.

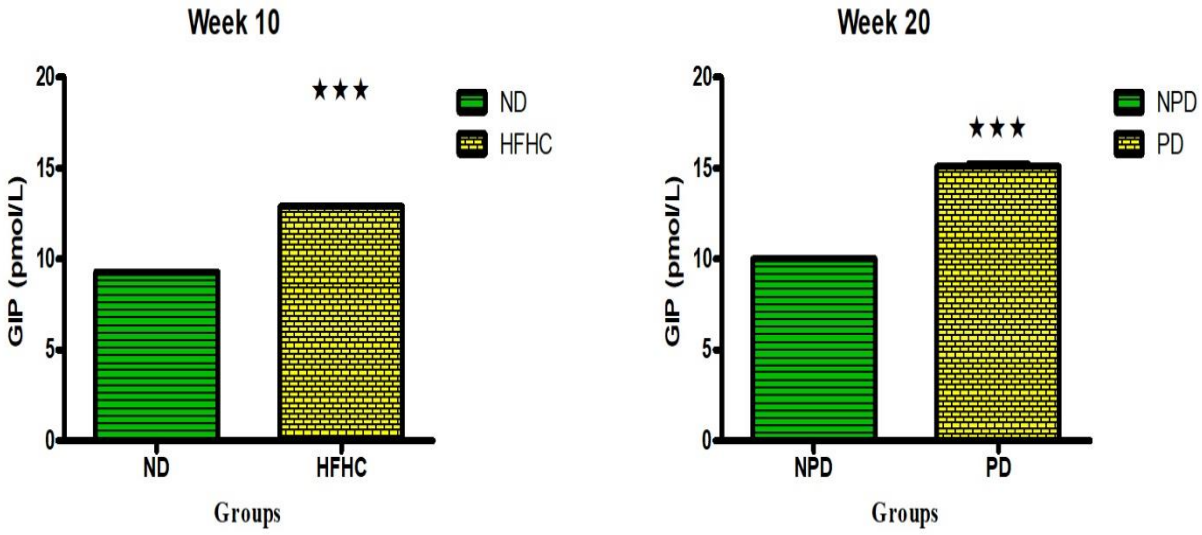

Figure 3: Plasma GIP concentrations in the standard and HFHC diet-fed animals for 10 and 20 weeks. Values are presented as mean ± SEM (n=6). ★$p < 0.05$ denotes comparison with the standard diet group; ★★★p=0.0001.

3.5. Plasma Leptin

The plasma leptin concentrations were measured at week 10 (n=12), halfway to prediabetes induction and at week 20 (n=12) when prediabetes is induced. The findings showed that the HFHC group and the PD group had significantly lower plasma leptin concentrations than the ND and NPD groups.

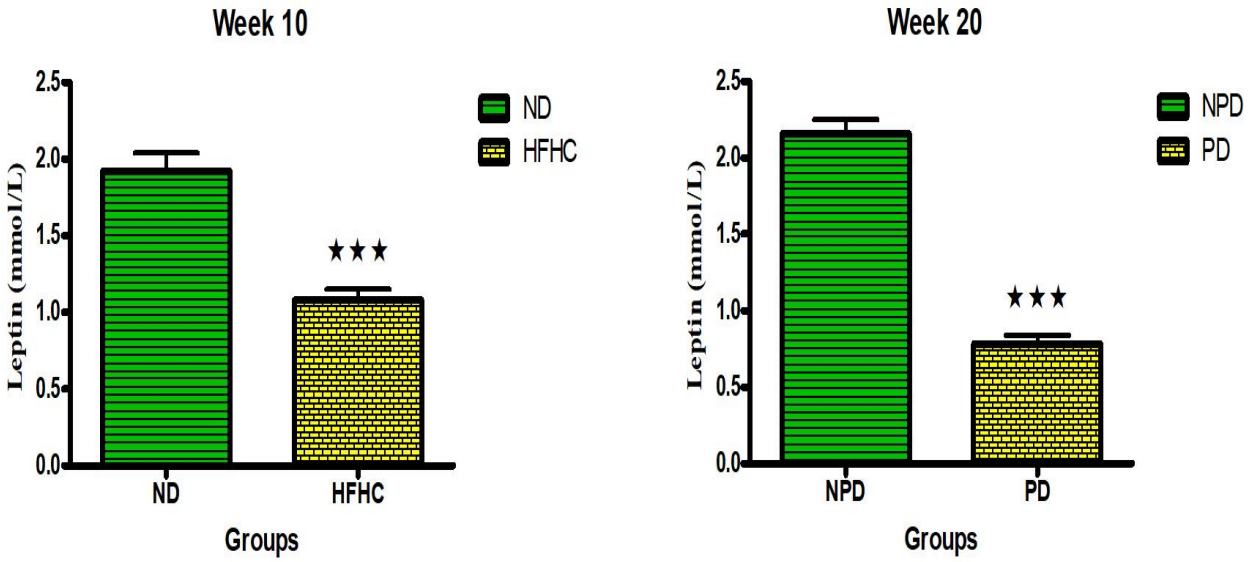

Figure 4: Plasma leptin concentrations in the standard and HFHC diet-fed animals for 10 and 20 weeks. Values are presented as mean ± SEM (n=6). ★$p < 0.05$ denotes comparison with the standard diet group; ★★★p=0.0001.

3.6. Plasma GLP-1

The plasma GLP-1 concentrations were measured at week 10 (n=12), halfway to prediabetes induction and at week 20 (n=12) when prediabetes is induced. The findings showed that the HFHC group and the PD group had significantly lower plasma GLP-1 concentrations than the ND and NPD groups.

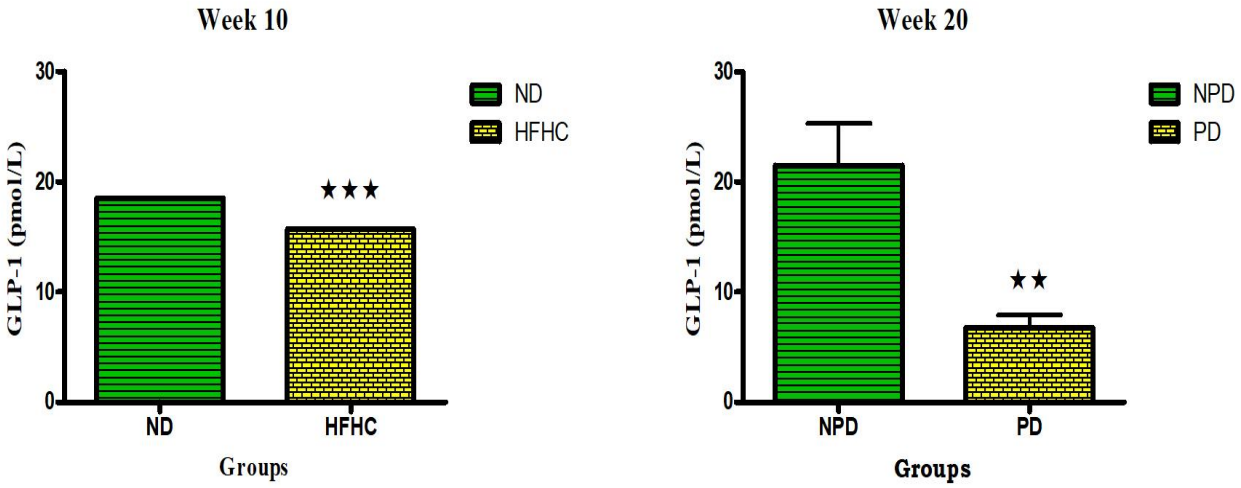

Figure 5: Plasma GLP-1 concentrations in the standard and HFHC diet-fed animals for 10 and 20 weeks. Values are presented as mean ± SEM (n=6). ★$p < 0.05$ denotes comparison with the standard diet group; ★★★p=0.0001 and ★★p=0.0019.

3.7. Plasma Ghrelin

The plasma ghrelin concentrations were measured at week 10 (n=12), halfway to prediabetes induction and at week 20 (n=12) when prediabetes is induced. The findings showed that the HFHC group and the PD group had significantly higher plasma ghrelin concentrations than the ND and NPD groups.

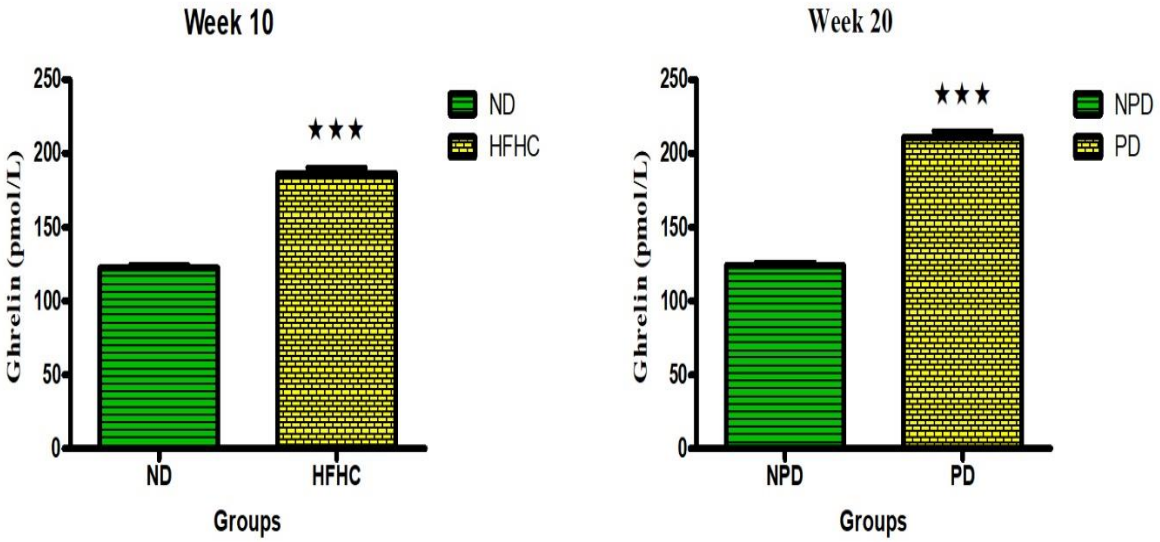

Figure 6: Plasma ghrelin concentrations in the standard and HFHC diet-fed animals for 10 and 20 weeks. Values are presented as mean ± SEM (n=6). ★$p < 0.05$ denotes comparison with the standard diet group; ★★★p=0.0001.

3.8. Plasma Glycated Hemoglobin (HbA1c)

The plasma HbAc1 concentrations were measured at week 10 (n=12), halfway to prediabetes induction and at week 20 (n=12) when prediabetes is induced. The findings showed that the HFHC group slightly increased with no significant difference compared to the ND group. The PD group had a significantly higher glycated hemoglobin concentration than the NPD group.

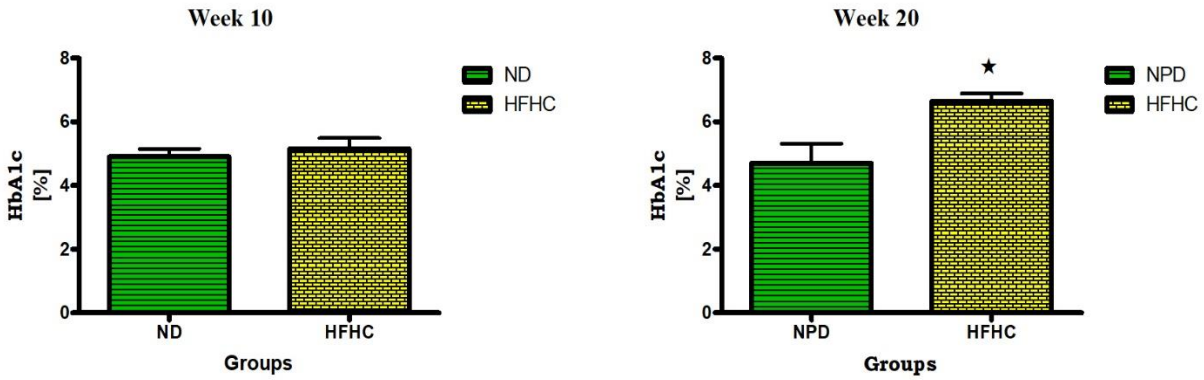

Figure 7: Plasma HbAc1 concentrations in the standard and HFHC diet-fed animals for 10 and 20 weeks. Values are presented as mean ± SEM (n=6). ★$p < 0.05$ denotes comparison with the standard diet group; p= 0.5667 and ★p= 0.0141 respectively.

4. Discussion

Studies have shown that subjecting rats to a chronic HFHC diet and drinking water supplemented with fructose for a period of 20 weeks leads to moderate insulin resistance and the development of prediabetes (81, 82). This diet-induced animal model of prediabetes has been shown to mimic the natural progression of prediabetes as it displays moderate hyperglycaemia and moderate insulin resistance (82). Incretin peptides associated with the appetite-regulating hormones work together and aid in glucose homeostasis. The role of incretin in the pathology of type 2 diabetes mellitus has been established. However, minimal work has been done in assessing the link between incretin peptide levels and the development of prediabetes. Therefore, this study sought to characterize the association between incretin peptide concentrations and the development of prediabetes in a diet-induced animal model of prediabetes.

The homeostatic model assessment (HOMA) is a technique for evaluating β-cell function and insulin resistance (IR) using fasting blood glucose and insulin concentrations (83). Elevated blood glucose and insulin concentrations lead to an increased HOMA-IR index, blood glucose concentration is regulated by insulin (83). After food intake, the blood glucose concentration increases, and insulin secretion is stimulated in the pancreatic β-cells (4). Insulin reduces blood glucose concentration by binding to its receptors, therefore, promoting the movement of glucose from the blood into tissue cells (4). However, in conditions like prediabetes and T2DM, there is a dysregulation of insulin caused by a reduced insulin sensitivity (42, 84). Several factors, such as chronic ingestion of high-calorie diets and a sedentary lifestyle, have been implicated in the development of prediabetes. In prediabetes, there is elevated insulin and blood glucose observed in figure 1 and 2 respectively; this resulted in increased HOMA-IR index values as seen in table1.

In this study, we speculate that the elevation of blood glucose and insulin at 10 weeks may be a direct response to the prolonged ingestion of the HFHC diet which was then sustained for a further 10 weeks leading to overt insulin resistance. The American Diabetes Association (ADA) recommends an additional assessment of glycated hemoglobin (HbAc1) in the diagnosis of prediabetes. The HbA1c test measures the amount of blood glucose attached to hemoglobin (85). Hemoglobin is an iron-rich protein in the blood and also transports oxygen and carbon dioxide for respiration (86). Sustained high plasma glucose concentrations lead to glucose-mediated non-enzymatic glycation of hemoglobin which results in a high HbA1c value (85). Glucose in the bloodstream spontaneously links to hemoglobin, and the amount combined with this protein is proportional to how much glucose is in the bloodstream at the time (87). The red blood cells in the human body only last 8-12 weeks (approximately 120 days) before they need to be replaced; this means HbA1c can be used to reflect the average blood glucose concentration over that period, offering a valuable longer-term assessment of blood glucose control (87, 88). The current study, in figure 3 we observed that after 20 weeks of HFHC diet consumption there was a significant increase in HbA1c, indicating developed prediabetes in our animals. Interestingly, after 10 weeks of HFHC diet consumption, there was no statistical difference in HbA1c values in the

HFHC diet-fed animals compared to those kept on a standard diet. In this study, the glucose and insulin results suggested that prediabetes had been developed at week 10, but this was not the case according to ADA criteria which require the additional HbA1c test. While the diagnostic criteria of prediabetes are not the same across the various international organizations, the clinical significance of HbA1c values in monitoring glucose homeostasis is strongly supported by a multitude of studies (89, 90).

The stomach secretes ghrelin in a fasting state into the blood (31). Increased circulating peripheral ghrelin concentration moves to the brain in the hypothalamus and activates the hypothalamic arcuate nucleus, promoting an increased appetite and food intake (52). After ingesting food, elevated blood glucose concentrations result in insulin and leptin secretion (53). Insulin reduces blood glucose, and leptin reduces appetite and food intake; this reduces ghrelin secretion, thus regulating glucose homeostasis (55). However, in T2DM, this pathway is impaired due to IR and hyperlipidaemia, the cells cannot access glucose from the blood, and they constantly transmit signals to the hypothalamus to stimulate increased food intake (56). Ghrelin is secreted in the stomach, and it stimulates the appetite centres in the hypothalamus to increase appetite, hence, the development of chronic hyperphagia (57). After 20 weeks, when prediabetes was induced in the PD group, in figure 4 we found elevated ghrelin levels. This suggests that the hyperphagia observed in T2DM develops from prediabetes as increased ghrelin concentrations induce increased appetite and food intake. However, after 10 weeks of the HFHC diet, before the induction of prediabetes, we found elevated ghrelin concentration with increased blood glucose, which could mean early onset IR. This mimics the human condition as humans develop prediabetes between the ages of 25-45 years. Due to chronic consumption of the HFHC diet, it could be that the body adapts to elevated blood glucose levels over long periods. This could mean that if the blood glucose concentration is reduced, higher concentrations of ghrelin are secreted to induce food intake to increase the blood glucose concentration to the abnormal new normal level, resulting in increased secretion of insulin by the incretin peptides.

GIP is a glucose-dependent peptide; this means its secretion is stimulated by the presence of glucose (91). The presence of glucose in the stomach after a meal stimulates the secretion and the release of this peptide into the bloodstream from the endocrine cells of the small intestines (91). After secretion, GIP induces insulin secretion from the pancreas to reduce the blood glucose concentration. Once there is a low blood glucose concentration, GIP secretion is inhibited, and GIP is degraded by DPP-4 (91). In conditions such as T2DM, where there are significantly elevated blood glucose concentrations, the pathway of GIP secretion is impaired (92). GIP is constantly secreted because of the high blood glucose concentration; the high GIP concentrations are responsible for hyperinsulinemia; GIP constantly induces insulin secretion (92). The animal used in this study were fed a HFHC diet every day for 20 weeks, there were significantly higher concentrations of GIP in the HFHC group compared to the ND group as observed in figure 5. Elevated insulin levels accompany increased GIP concentrations, possibly due to chronic secretion by the pancreatic beta cells. Interestingly at 10 weeks, before the induction of prediabetes, we found significantly high GIP concentrations in the HFHC group. The elevated GIP

levels could be due to the increased blood glucose as GIP is glucose-dependent, increased blood glucose over time stimulates the continued secretion of GIP. The elevated concentration of GIP over a long period could then be responsible for hyperinsulinemia due to continued stimulation of insulin secretion.

In the case of an increase in blood glucose concentration, insulin is secreted and released into the bloodstream (93). Insulin binds to its receptors and is degraded in the liver, adipose tissues and skeletal muscle to allow for the entrance of glucose, thereby reducing its amount in the blood (93). In conditions like T2DM, where there is high glucose concentration, the insulin signalling pathway is disturbed (94). As blood glucose concentration increases, GIP secretion increases, and the pancreatic β cells are forced to overly work to release even more insulin, intending to reduce the blood glucose concentration (94). A prolonged increase in insulin secretion negatively affects the insulin receptors as they gradually lose their sensitivity to insulin (94). The reduced sensitivity by the insulin receptors disturbs the insulin signaling cascade, leading to insulin resistance (93). There were indeed elevated insulin concentrations at 20 weeks in the PD group compared to NPD; this was accompanied by the increased HOMA-IR value, which confirmed the presence of overt insulin resistance in prediabetes. The current literature supports our findings, there is increased insulin concentration in the prediabetic group, which is due to hyperglycaemia and the developing IR present in prediabetes (83). However, after 10 weeks of HFHC diet consumption, before the onset of prediabetes there is significantly high insulin concentration (hyperinsulinemia) in the HFHC group compared to the ND group as seen on figure 2; the high insulin concentration is possibly due to chronic secretion by the elevated GIP concentration. There were elevated insulin concentrations over a long period due to increased blood glucose and GIP. The chronic secretion of insulin leads to hyperinsulinemia, which could be the reason for the onset of insulin resistance shown by the elevated HOMA-IR value in table 1. GIP could be responsible for the sustained hyperinsulinemia from 10 weeks to the induction of prediabetes. GIP could, therefore, be responsible for the onset of insulin resistance observed after 10 weeks. Kanemaru *et al.* published a systematic review and confirmed that prolonged high concentrations of GIP induce insulin resistance (95). Therefore, the findings suggest that GIP might play a significant role in the onset and progression of prediabetes to type 2 diabetes mellitus.

Increased blood glucose concentration has been shown to promote the secretion of leptin into the bloodstream by the adipose tissues, and this is suggested to be due to the increase in insulin which stimulates leptin secretion (96). After secretion by white adipose tissues, leptin increases circulation and crosses the BBB as a saturable transport to the hypothalamic arcuate nucleus, where it binds to LEPR-b (97). Leptin binding promotes the activation of appetite suppressor neurons, thus inducing satiety and boosting energy expenditure by activating the satiety centre (Ventromedial hypothalamus)
(97). Thus, leptin acts as an afferent signal in a negative feedback loop that keeps the body fat reserves steady. Leptin secretion is stimulated by insulin in the adipose tissues (98). In conditions like T2DM, the leptin signaling pathway is disturbed (20). Due to the developing IR and hyperinsulinemia, stimulation of the adipose tissues to secrete leptin is diminished. Our findings on figure 6 show

significantly decreased leptin concentrations in the PD group compared to the NPD group after 20 weeks; this could be due to the overt IR due to elevated GIP and ghrelin concentrations. However, after 10 weeks of HFHC diet consumption, the PD group had a significantly reduced leptin concentration; this could be due to the onset of IR and severely increased ghrelin concentrations. Wang *et al.* conducted a study to establish the relationship between IR and leptin in prediabetes; they found that IR is associated positively with diminished leptin concentrations (99). The reduced leptin concentration also means diminished secretion of GLP-1 and decreased satiety which is observed in this study.

GLP-1 is secreted simultaneously with GIP immediately after food consumption and is secreted by the small intestines' endocrine cells, which induce insulin secretion from the pancreas (10). GLP-1 has receptors in the hypothalamus, and once secreted, it induces satiety by binding to the receptors mentioned above (25). Unlike GIP, GLP-1 is degraded faster by dipeptidyl-peptidase 4 (DPP-4) shortly after its secretion due to the presence of the induced insulin secreted into the bloodstream (73). In conditions like T2DM, GLP-1 secretion is diminished due to leptin resistance and GLP-1 degradation is increased to hyperinsulinemia and DPP-4 (73). Increased plasma DPP-4 is associated with increased blood glucose; it degrades the reduced concentration of GLP-1 shortly after secretion (73). Satiety is, therefore, not induced via the GLP-1 pathway. In the current study, on figure 7 there were severely low levels of GLP-1 in the PD group, possibly due to the reduced Leptin concentration and increased DPP4. However, at 10 weeks, we also observed a significant decrease in GLP-1 in the prediabetic group compared to the non-prediabetic control, this could be due to the onset of IR and the reduced concentration of leptin present. Hira *et al.* conducted a study to find out what GLP-1 does. They found that it was reduced in prediabetic subjects; therefore, the current literature supports the results obtained in this study (100). Both the leptin and GLP-1 satiety-inducing pathways are disturbed. Therefore, there is diminished satiety in prediabetes.

Conclusion

In this study, we observed that the prolonged consumption of a HFHC diet results in abnormal incretin concentrations. This is evidenced by elevated ghrelin and decreased plasma leptin levels. This increases food intake and blood glucose levels, resulting in increased GIP and reduced plasma levels of GLP-1. The increased GIP levels may then cause chronic insulin stimulation and can result in hyperinsulinemia, which can result in insulin resistance. Hence, chronic consumption of the HFHC diet increases blood glucose and insulin. The dysregulated plasma incretin concentrations then maintain these abnormal concentrations leading to the onset of insulin resistance and later prediabetes. As a result, the incretins are linked to the onset and progression of prediabetes. This could further suggest that incretins play a role in the development of prediabetes to T2DM.

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Author Contributions

NM and NRD contributed to the study design, conducted the experiments, collected, analyzed, and interpreted data, as well as being involved in writing the manuscript. AMS and PSN were 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.

Conflict of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

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

Ethics Approval and Consent to Participate

The animal study protocol was reviewed and approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, South Africa (AREC/00003627/2021).

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Chapter 3: Manuscript 2

Investigating the effect of a low carbohydrate, high unsaturated fats diet on diet-induced prediabetes.

Prologue 2

In the first manuscript we found that the abnormal incretin concentrations not only precede, but they also play a role in the development of prediabetes. In this study, we sought to further explore the involvement of incretins in prediabetes by observing how subjecting our prediabetic animal model with a low carbohydrate, high unsaturated fat diet would affect them.

The manuscript in chapter 2 titled **"Investigating the effect of a low carbohydrate, high fat diet in diet-induced prediabetes"** is authored by N. Mzimela, NR Dimba, A.M Sosibo, and A Khathi.

This manuscript has been submitted and is currently under review in the Endocrinologia, Diabetes y Nutricion journal.

Investigating the effect of a low carbohydrate, high fat diet in diet-induced prediabetes.

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Abstract

Type 2 diabetes mellitus is a chronic metabolic disease that results from a loss of sensitivity to insulin by-insulin receptors in insulin dependent cells. In this condition, there are chronically increased insulin and blood glucose concentrations. An intermediate hyperglycemic condition known as prediabetes was found to precede the onset of T2DM. Prediabetes is a chronic metabolic condition that has been shown to be reversable using dietary intervention and has become a therapeutic target to prevent T2DM. However, the effect of a low carbohydrate, high unsaturated fat (LCHF) diet on incretin levels during prediabetes has not been established. Hence, this study aims to characterise the effect of a LCHF on incretin levels in a diet-induced prediabetic animal model. This study was conducted using 18 male Sprague-Dawley rats, divided into two groups, group A was given a standard rat diet (SD) (n=6), while group B was given a high-fat high carbohydrate (HFHC) (n=12) diet. The animals were given their respective diet for a total of 20 weeks. After 20 weeks, pre-diabetes was induced in group B. Group A continued with the standard diet and was used as a non-prediabetic control (NPDC) (n=6). The pre-diabetic group B (n=12) was split into two experimental groups. One of the groups continued a HFHC diet and served as the pre-diabetic control group (PD)(n=6), while the other group had a diet intervention where the diet was changed to a low carbohydrate-high unsaturated fats diet (PD+DI) (n=6). All groups were then maintained on their respective diets for a further 12 weeks. Chronic consumption of the HFHC diet by the PD group led to elevated blood glucose (p=0.0010) and insulin concentration (p=0.0001), resulting in abnormal concentrations of GIP (p=0.0010), GLP-1 (0.0199), Leptin (p=0.0001) and Ghrelin (p=0.8647). The abnormal incretins then maintained this state of hyperglycemia and hyperinsulinemia, resulting in pre-diabetes. Chronic consumption of the LCHF by the PD+DI group led to reduced concentrations of GIP (p=0.0010), GLP-1 (0.0199), Leptin (p=0.0212) and Ghrelin (p=0.8112), which could have led to a reduced HbA1c and eventually to the reversal of pre-diabetes.

Keywords: incretins, prediabetes, low carbohydrates, high unsaturated fat diet.

1. Introduction

The global prevalence of type two diabetes mellitus (T2DM) has increased rapidly in recent years and is one of the leading causes of morbidity and mortality worldwide (101). T2DM is often preceded by a condition known as pre-diabetes (102). According to the American Diabetes Association (ADA), prediabetes is 4 by impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and elevated glycated hemoglobin (HbA1c) concentration (81). Pre-diabetes is a long-lasting condition resulting from prolonged consumption of high-calorie diets and a sedentary lifestyle (103). Literature evidence shows that a diet rich in saturated fat and refined carbohydrates is strongly linked to the development of pre-diabetes. (64, 80, 129). This was demonstrated in a recent study in our laboratory, where we produced an animal model of diet-induced pre-diabetes by exposing rats to chronic consumption of a high-fat, high-carbohydrate (HFHC) diet (129). The findings of that study revealed that chronic ingestion of a HFHC diet is directly related to a change in incretin levels, which may play a role in the emergence of mild insulin resistance and pre-diabetes (129).

It has been demonstrated that incorporating moderate exercise into the management of pre-diabetes may benefit pre-diabetic patients in improving glucose tolerance, but these effects are short-lived as they are often associated with poor patient compliance (104). Literature evidence shows that incorporating a low-carbohydrate and high-unsaturated fat diet may alleviate the moderate insulin resistance observed in pre-diabetes (104). Hence, dietary intervention has become a target to reduce the incidence of T2DM. Several randomized controlled trials (RCT) studies indicated that lifestyle interventions that seek to reduce caloric intake or increase physical activity could delay or prevent the onset of T2DM (105, 106). Under normal physiological conditions, gut-derived incretin hormones such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are secreted shortly after the detection of glucose in the stomach (107). They induce insulin secretion before glucose is absorbed into the blood to prepare for increased blood glucose concentration (108). The incretin effect explains how oral glucose consumption evokes more robust insulin secretory responses than intravenous glucose despite yielding the same glycemia levels (109). GIP and GLP-1 mediate this action, which is consistently deficient in type 2 diabetes patients due to impaired incretin release (109). However, a previous study in our laboratory shows how chronic ingestion of a HFHC diet is associated with increased/decreased incretin hormones, leading to the development of insulin resistance observed in pre-diabetes (64). The incretin hormones are influenced by diet because they are glucose-dependent polypeptides produced by the enteroendocrine cells (107). The effect of incretins on glucose homoeostasis is well understood, and incretin-based medicines are among the most promising novel treatments for type 2 diabetes (107, 108, 109). Incretins work closely with the appetite-controlling hormones ghrelin and leptin to regulate blood glucose concentration (110). Ghrelin and leptin regulate appetite and work in opposition to each other; ghrelin is secreted just before a meal and increases appetite to induce food intake, while leptin is secreted when there is increased blood glucose and reduces

appetite (111). A higher glucose-content diet stimulates a higher secretion of the incretin hormones and, therefore, a constant secretion of insulin, resulting in diabetes mellitus (112).

Previous papers have examined the effects of low carbohydrates and high unsaturated fats on glucose homeostasis in T2DM and found them to be beneficial in its management (109, 113). However, the effects of this type of diet on incretin levels in the pre-diabetic state remain unknown. In this study, we sought to investigate the effects of a low carbohydrate and high unsaturated fats diet on incretin hormones and the association with glucose homeostasis.

2. Methods and Materials

2.1. Chemicals and drugs

All chemicals and reagents used were purchased from standard pharmaceutical suppliers and were of analytical grade.

2.2. Animals and housing

This study used 18 male Sprague-Dawley rats (150-180 g). The animals were housed in a room with a 12-hour light/12hour dark cycle, at room temperature (25°C), for the duration of the study. The animals in each group had access to food and water. The Animal Research Ethics Committee approved all animal procedures and housing conditions of the University of KwaZulu-Natal (Ethics number: AREC/00003627/2021). The animals were monitored for pain, discomfort and distress using the criteria listed in the university's Animal Research Ethics Committee's humane endpoint document.

2.3.1. Induction of pre-diabetes

The animals were randomly assigned to the following diet groups; Group A (n=6): rats that consumed a standard diet with drinking water (NPDC). Group B (n=12); rats that consumed a high fat, high carbohydrate diet (HFHC) with drinking water supplemented with 15% fructose (AVI Products (Pty) Ltd, Waterfall, South Africa) (83). Pre-diabetes was induced by allowing the animals to feed on the HFHC diet for 20 weeks, as previously described (64, 80). Glucose tolerance was evaluated 5 days after the 20 weeks of induction with a well-known, established laboratory protocol, the oral glucose tolerance test (OGTT), to determine pre-diabetes according to the American Diabetes Association criteria (81). Group A was found to be non-diabetic while group B was found to be pre-diabetic. The animals in group B were then grouped further for pharmacological studies. The animals fed the normal diet were also tested and found to be normoglycemic and without pre-diabetes.

2.3.2. Experimental design

After 20 weeks of pre-diabetes induction, group A continued with the standard diet and was used as a non-prediabetic control (NPDC) (n=6). The pre-diabetic group B (n=12) was split into two experimental groups. One of the groups continued a HFHC diet and served as the pre-diabetic control group (PD)(n=6), while the other group had a diet intervention where the diet was changed to a low

carbohydrate-high unsaturated fats diet (PD+DI) (n=6). All groups were then maintained on their respective diets for a further 12 weeks.

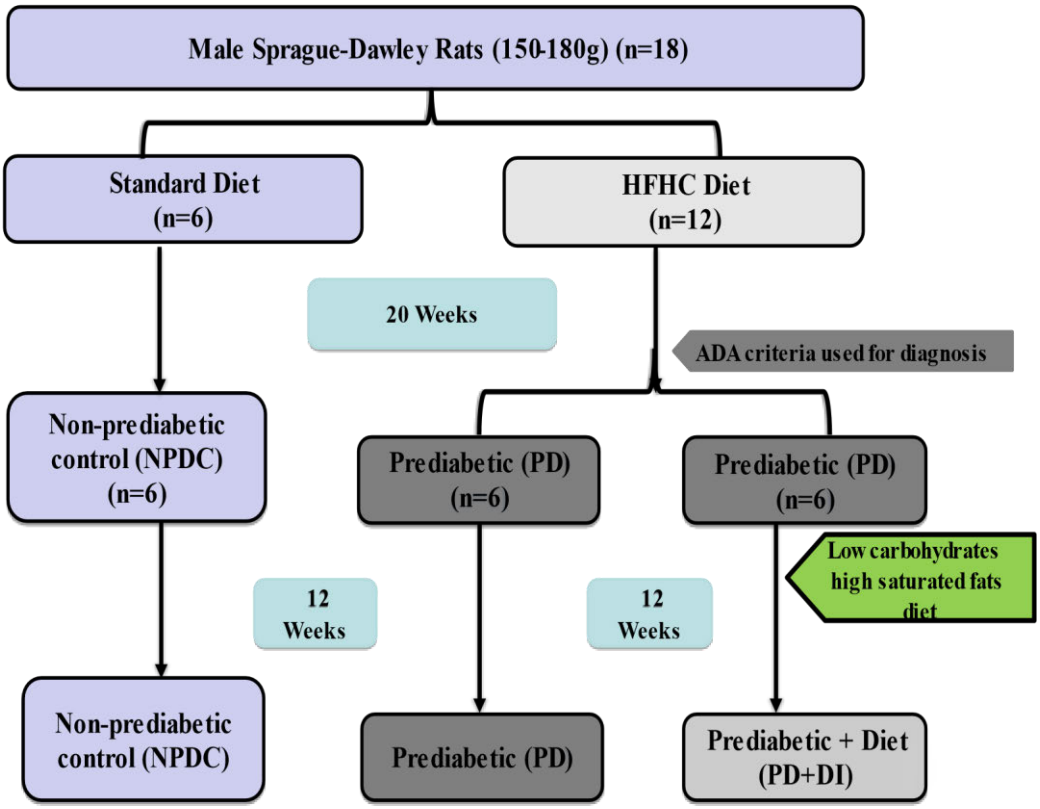

Figure 1. Schematic diagram showing the experimental design.

2.3.3. Blood collection and tissue harvesting

All animals were anaesthetized with Isofor (100 mg/kg)) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) using a gas anesthetic chamber (Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa) and allowed to inhale for 3 minutes. Blood was collected by cardiac puncture and injected into pre-cooled heparinized containers. The collected blood was injected into individual heparinized and EDTA containers and then centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 minutes. Plasma was collected and stored at -80 °C in a Bio Ultra freezer (Snijders Scientific, Holland) until ready for biochemical analysis.

2.4 Biochemical analysis

Plasma GIP, GLP-1, insulin, leptin and ghrelin concentrations were determined using specific ELISA kits in accordance with the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China). The kits included a micro-ELISA plate that was coated with antibodies specific to each of the parameters measured. The protocols for GLP-1, insulin and leptin were the same as they all required

sandwich ELISA. Ghrelin required competitive ELISA, and the procedure was still the same except for the incubation periods, as ghrelin required less time. Standards and samples were pipetted into the appropriate wells of the micro-ELISA plate and incubated for 90 min at 37 °C. This was followed by adding the relevant biotinylated detection antibody (100 µl). After 60 min incubation at 37 °C, avidin–horseradish peroxidase conjugate (100 µl) was added to each microplate well. After a further 30 min incubation at 37 °C, the unbound components were washed away using the wash buffer provided. Substrate solution (100 µl) was added to each microplate well, and after 15 min incubation at 37 °C, the stop solution (50 µl) was added. Optical density was measured using a nano spectrophotometer 47 (BMG Labtech, Ortenburg, Germany) at 450 nm. The concentrations of each parameter in the samples were extrapolated from their respective standard curves.

2.5. HOMA2-IR index

The homeostatic model assessment (HOMA) is a recognized method for measuring insulin resistance (IR) using fasting glucose and insulin. Two variants, known as HOMA1-IR or HOMA2-IR, make up the HOMA. The updated HOMA2-IR index was the choice model since it produced a more accurate index than the original HOMA1-IR that Matthews and Cols released in 1985. An average HOMA-IR value is within the range of 0.5 to 1.4. Insulin sensitivity peaks when the index value is less than 1.0. While index values over 1.9 indicate the onset of insulin resistance, and index values over 2.9, indicate substantial insulin resistance, respectively. HOMA-IR was calculated using fasting plasma insulin (µU/ml) x fasting plasma glucose (mmol/L)/22.5.

2.6 Statistical analysis

Data were reported as mean ± SEM. GraphPad Prism Software (version 5) was used to perform statistical analysis. The differences between non-diabetic, pre-diabetic and dietary intervention pre-diabetic groups were analyzed using One-way analysis of variance (ANOVA) followed by Tukey-Kramer. Values of $P< 0.05$ show statistical significance between the compared groups. Correlation analysis was also performed using a scatter chart from Pearson's comparative analysis. A value of $r>0.7$ or $r<-0.7$ was considered a strong association.

3. Results

3.1. Plasma GIP

The plasma GIP concentrations were measured at the end of the experimental period at 32 weeks. The findings showed that the PD group had significantly higher plasma GIP levels than the NPDC group (p=0.0010) and the PD+DI group. The plasma GIP concentration of the PD+DI group was slightly lower with no significant difference than that of the NPDC group (p= 0.7914) and significantly lower than the PD group (p=0.0010).

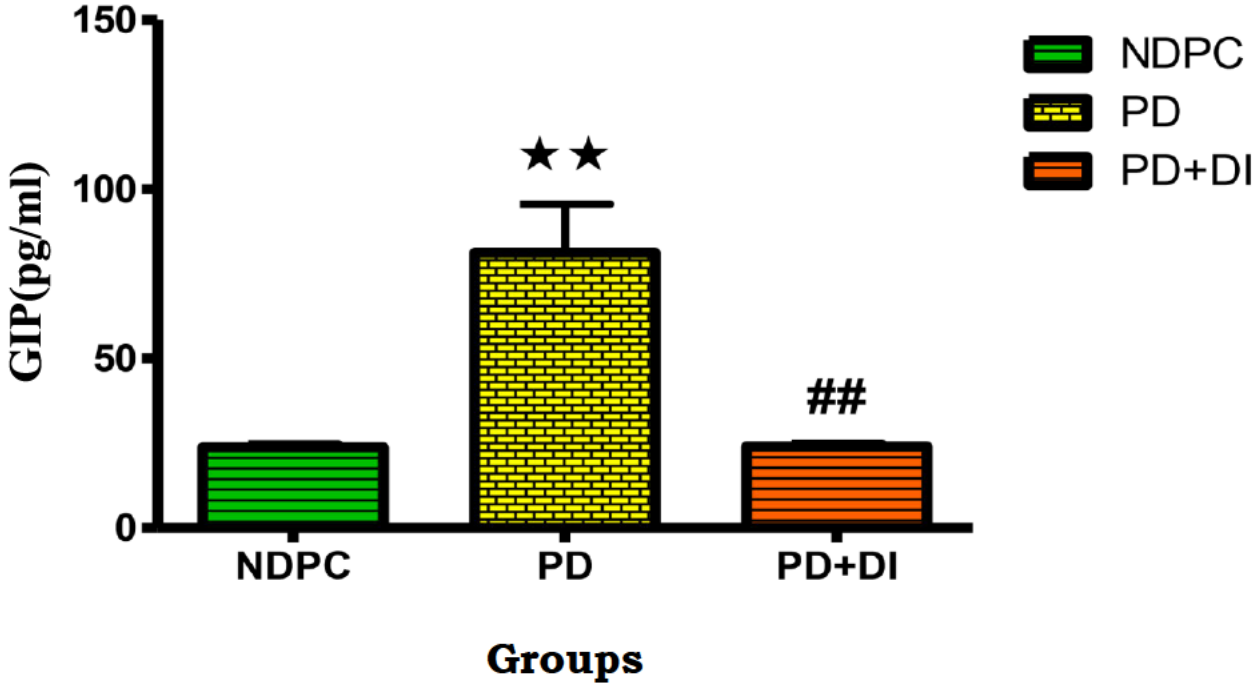

Figure 2: Plasma GIP concentrations in non-prediabetic control animals (NPDC), untreated pre-diabetic control animals (PD), as well as pre-diabetic animals that had a dietary intervention (PD +DI) for 12 weeks. Data expressed as means ± SEM (n=6). ★★$p< 0.05$ denotes comparison with NPDC group; ##$p< 0.05$ denotes comparison with PD group.

3.2. Plasma GLP-1

The plasma GLP-1 concentrations were measured at the end of the experimental period at 32 weeks. The findings showed that compared to the NPDC group (p=0.0019) and the PD+DI group, the PD group had significantly lower plasma GLP-1 levels. The plasma GLP-1 concentration of the PD+DI group was lower than that of the NPDC group (p=0.6111) and higher than that of the PD group (p=0.0199).

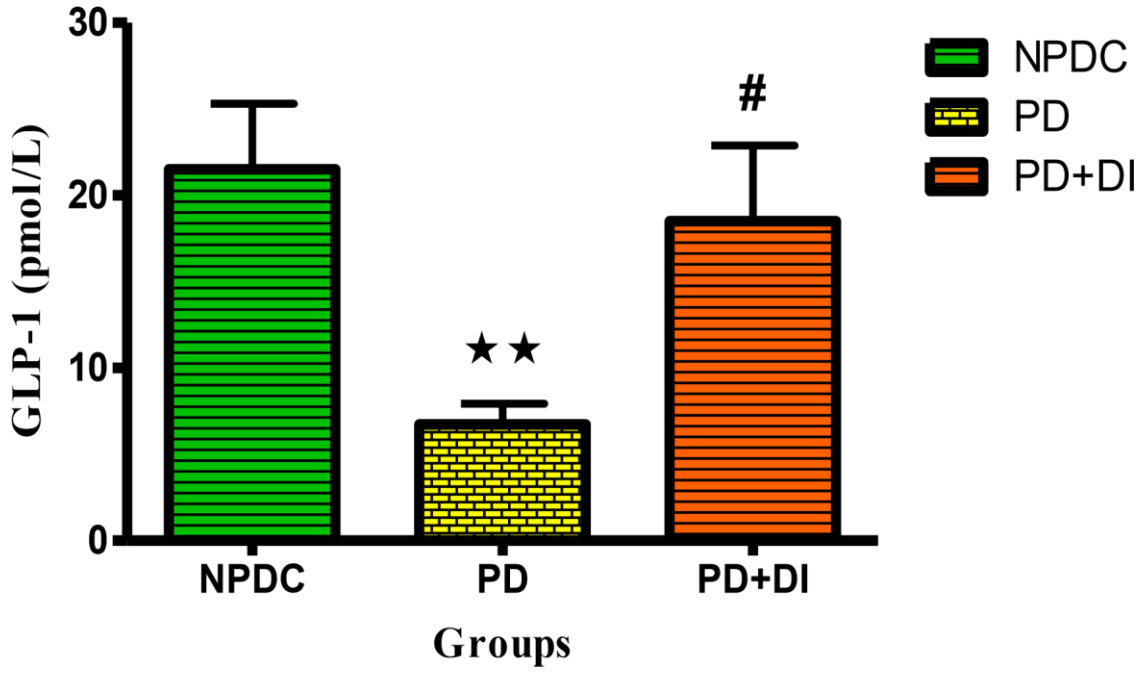

Figure 3: Plasma GLP-1 concentrations in non-prediabetic control animals (NPDC), untreated pre-diabetic control animals (PD), as well as pre-diabetic animals that had a dietary intervention (PD +DI) for 12 weeks. Data expressed as means ± SEM (n=6). ★★$p < 0.05$ denotes comparison with NPDC group; #$p < 0.05$ denotes comparison with PD group.

3.3. Plasma Insulin

The plasma insulin concentrations were measured at the end of the experimental period at 32 weeks.

The results demonstrated that the PD group had significantly higher plasma insulin levels than the NPDC group (p=0.0001) and the PD+DI group. The PD+DI group's plasma insulin concentration was higher than the NPDC group (p=0.2661) and significantly lower than the PD group (p=0.0066).

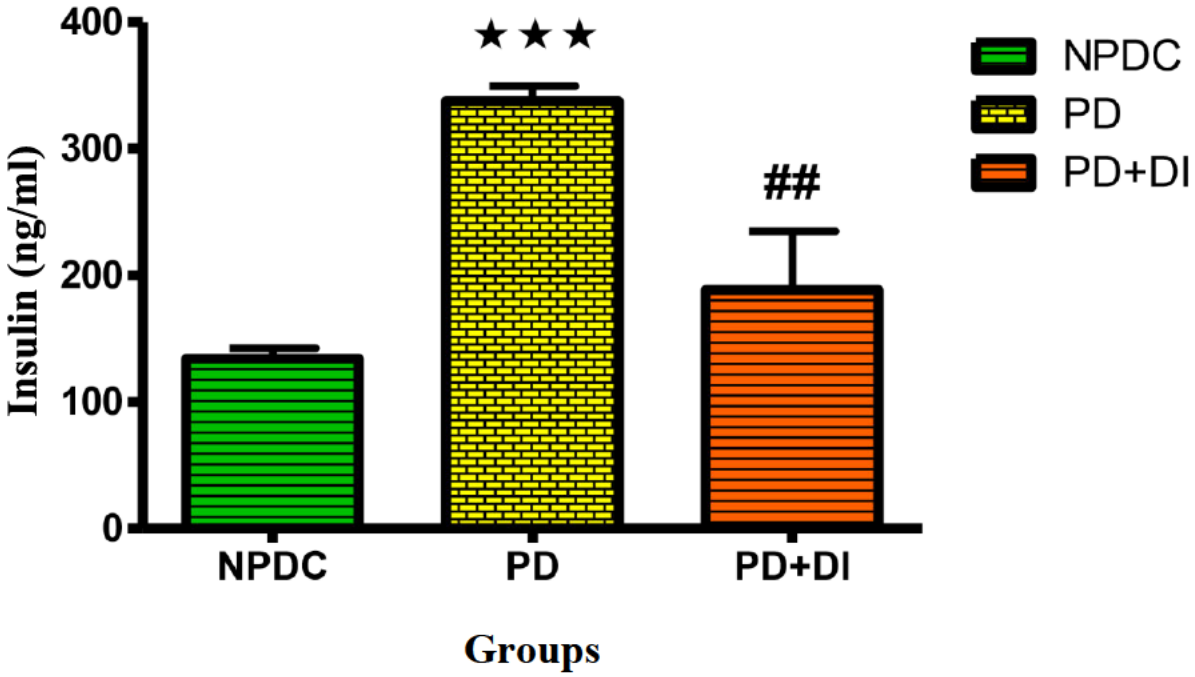

Figure 4: Plasma insulin concentrations in non-prediabetic control animals (NPDC), untreated prediabetic control animals (PD), as well as pre-diabetic animals that had a dietary intervention (PD +DI) for 12 weeks. Data expressed as means ± SEM (n=6). ★★★$p < 0.05$ denotes comparison with NPDC group; ##$p < 0.05$ denotes comparison with PD group.

3.4. Plasma Leptin

The plasma leptin concentrations were measured at the end of the experimental period at 32 weeks. The results demonstrated that the PD group had significantly lower plasma leptin levels than the NPDC group (p=0.0001) and the PD+DI group. The PD+DI group's plasma leptin concentration was higher than the PD group (p=0.0212) and significantly lower than the NPDC group (p=0.2295).

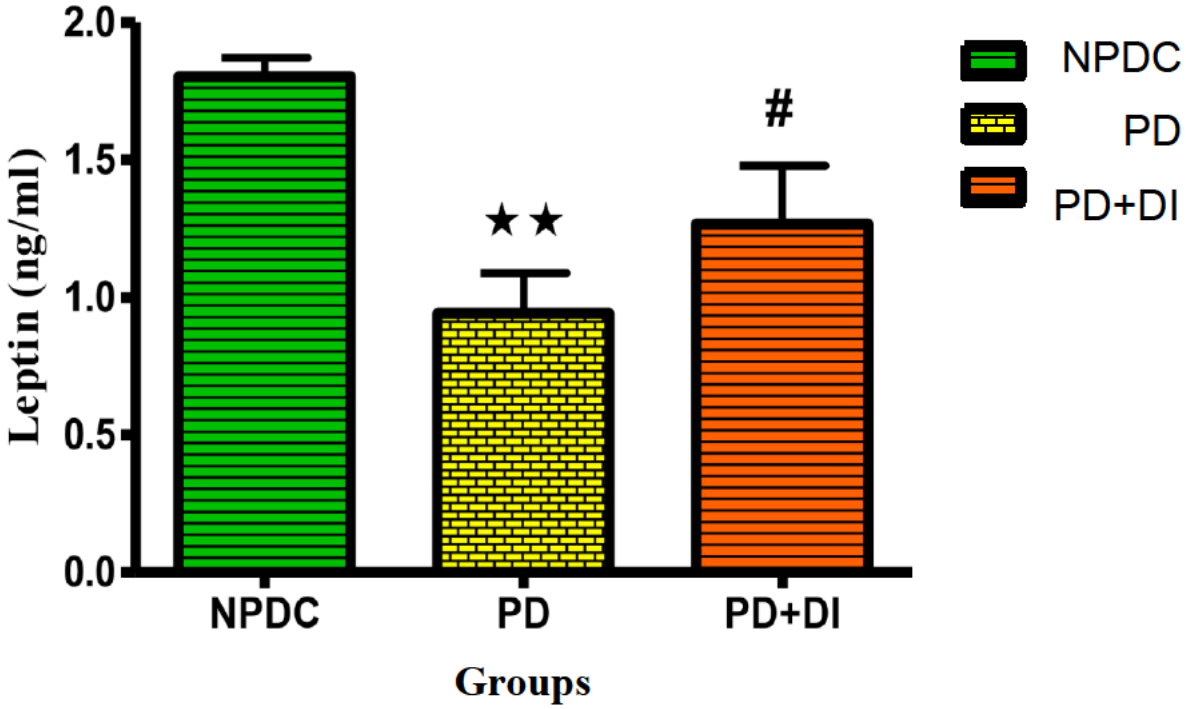

Figure 5: Plasma leptin concentrations in non-prediabetic control animals (ND), untreated pre-diabetic control animals (PD), as well as pre-diabetic animals that had a dietary intervention (PD +DI) for 12 weeks. Data expressed as means ± SEM (n=6). ★★$p < 0.05$ denotes comparison with NPDC group; #$p < 0.05$ denotes comparison with PD group.

3.5. Plasma Ghrelin

The plasma Ghrelin concentrations were measured at the end of the experimental period at 32 weeks.

The results demonstrated that the PD group had significantly higher plasma ghrelin levels than the NPDC group (p=0.8647) and the PD+DI group. The PD+DI group's plasma ghrelin concentration was slightly higher than the NPDC group (p=0.8112) and significantly lower than the PD group (p=0.4644).

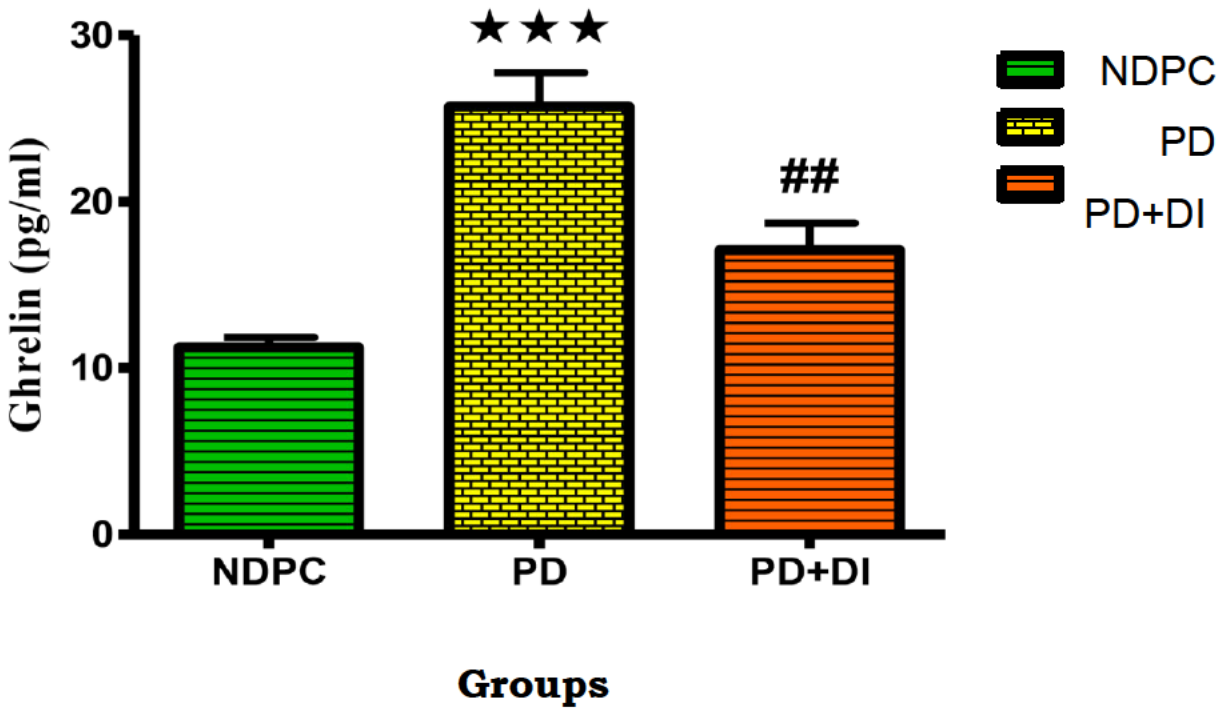

Figure 6: Plasma GLP-1 concentrations in non-prediabetic control animals (NPDC), untreated prediabetic control animals (PD), as well as pre-diabetic animals that had a dietary intervention (PD +DI) for 12 weeks. Data expressed as means ± SEM (n=6). ★★★$p < 0.05$ denotes comparison with NPDC group; ##$p < 0.05$ denotes comparison with PD group.

3.6. Plasma Glucose

The plasma glucose concentrations were measured at the end of the experimental period at 32 weeks.

The results demonstrated that the PD group had significantly higher plasma glucose levels than the NPDC group (p=0.0010) and the PD+DI group. The PD+DI group's plasma glucose concentration was significantly lower than both the NPDC group (p=0.0010) and the PD group (p<0.0001).

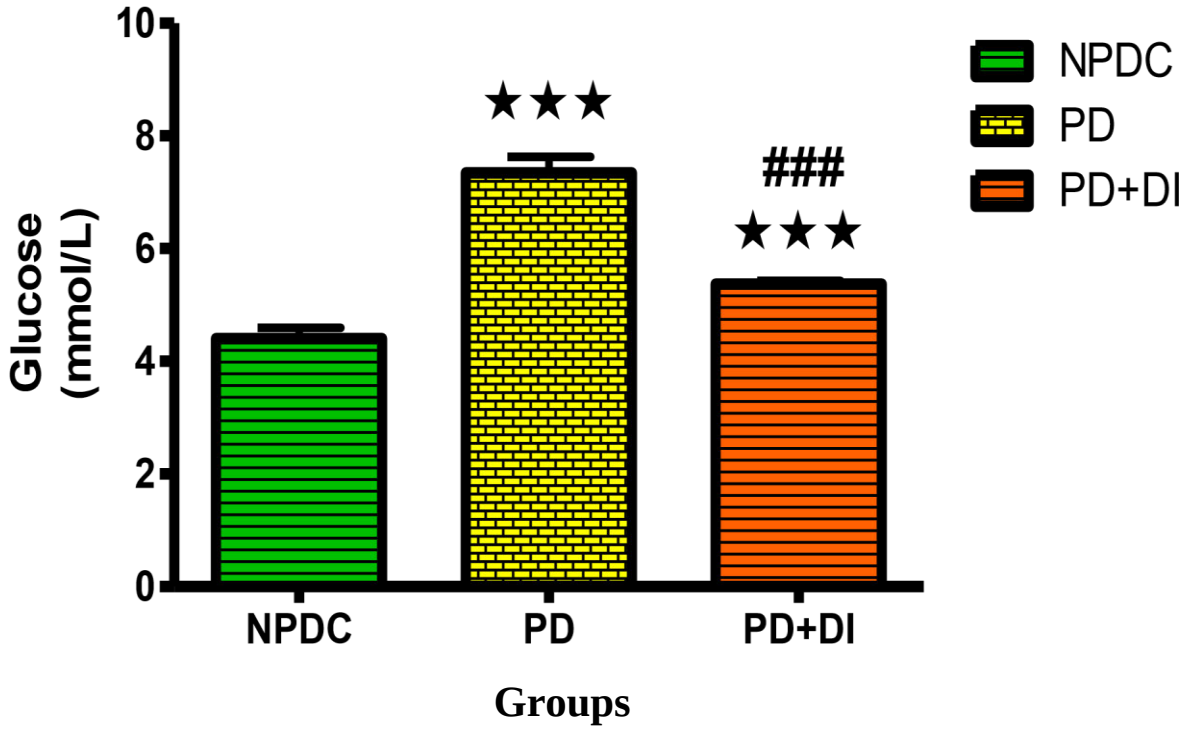

Figure 7: Plasma glucose concentrations in non-prediabetic control animals (NPDC), untreated prediabetic control animals (PD), as well as pre-diabetic animals that had a dietary intervention (PD +DI) for 12 weeks. Data expressed as means ± SEM (n=6). ★★★$p < 0.05$ denotes comparison with NPD group; ###$p < 0.05$ denotes comparison with PD group.

3.7. Plasma Glycated Hemoglobin (HbA1c).

The plasma HbA1c concentrations were measured after pre-diabetes induction at week 20 and the end of the experimental period at 32 weeks. The results demonstrated that the PD group had significantly higher HbA1c levels than the NPDC group (p=0.0010) and the PD+DI group. The PD+DI group's HbA1c levels were slightly lower with no significance compared to the NPDC Group (p=0.0010) and significantly lower than the PD group (p<0.0001).

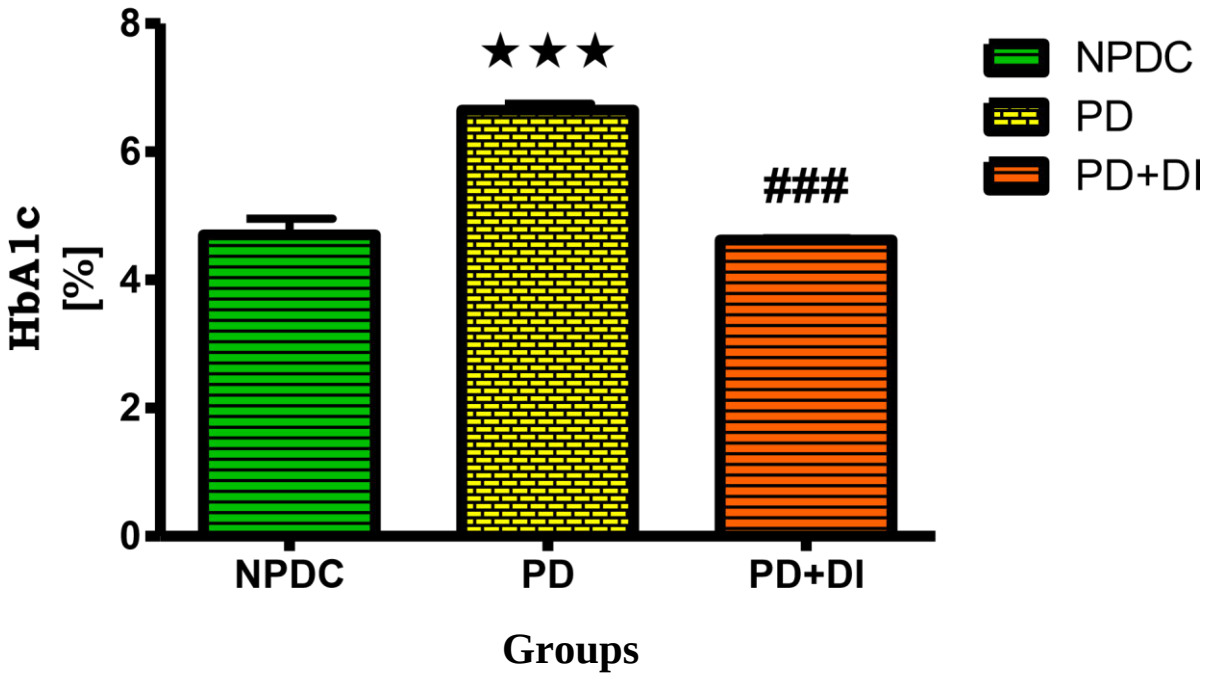

Figure 8: Plasma HbA1c concentrations in non-prediabetic control animals (NPDC), untreated prediabetic control animals (PD), as well as pre-diabetic animals that had a dietary intervention (PD +DI) for 12 weeks. Data expressed as means ± SEM (n=6). ★★★p < 0.05 denotes comparison with NPD group; ###p< 0.05 denotes comparison with PD group.

3.8. HOMA2-IR Index

The HOMA2-IR index was calculated in the NPD, PD and PD + DI groups at 32 weeks. The HOMA2IR value of the NPDC group was above the insulin-sensitivity range (<1.0) and below the early IR range (>1.9). By comparison with the NPDC group, the PD group showed a higher HOMA2-IR value, which was above the early IR range (>1.9) and the overt IR range (>2.9). The HOMA2-IR value of the PD + DI group was above the insulin-sensitivity range (<1.0) and below the early IR range (>1.9). (★ = IR range > 1.9).

GROUPS	**SEM GLUCOSE**	**SEM INSULIN**	**p value Glucose**	**p value insulin**	**HOMA2-IR**
NPDC	0.20000	8.465	0.0010	0.0001	1.08
PD	0.28000	11.74	0.0001	0.0066	2.99★
PD + DI	0.06455	46.37	0.0010	0.2661	1.58

Table 1: Displays the HOMA2-IR index calculated at week 32 using the HOMA2 calculator in the NPDC, PD and PD + DI groups (n=6 per group).

4. Discussion

Pre-diabetes is a long-lasting condition characterised by moderate hyperglycemia that often precedes the onset of type 2 diabetes mellitus. Literature evidence shows that a diet rich in saturated fat and refined carbohydrates is strongly linked to pre-diabetes development. About 5–10% of people with prediabetes go on to develop T2DM annually (58). Recently, several studies have shown that pre-diabetes is reversible, while the exact mechanism involved in the reversal of pre-diabetes has not yet been established (114, 115, 116). The pre-diabetic state has been identified as a target to reduce the onset of T2DM.

Several studies have shown that continued ingestion of a low-carbohydrate, high-fat diet is able to reverse the pre-diabetic state. The studies postulated that the reduced carbohydrate content in the LCHF diet yields a low glucose concentration that is absorbed into the bloodstream during digestion (117). The body is promoted to enhance fat oxidation to meet energy needs with reduced carbohydrate intake (117, 118, 119). Therefore, this results in a reduction in glycated hemoglobin levels and an improvement in glucose tolerance (118, 120). Our laboratory produced a rat model of diet-induced pre-diabetes by allowing chronic consumption of a high-fat, high-carbohydrate (HFHC) diet (81). According to a recent study from our laboratory, prolonged consumption of the HFHC diet leads to abnormal incretin concentrations, which may lead to the development of insulin resistance and the onset of pre-diabetes (129). To advance from the previous study, we sought to investigate the possibility of reversing pre-diabetes primarily using a LCHF diet to influence the incretin peptides.

Previous studies have established that chronic consumption of a high-fat and calorie diet causes elevated blood glucose concentrations for prolonged periods, resulting in the development of pre-diabetes (83). In pre-diabetes, there are high blood glucose concentrations for extended periods, and these elevated blood glucose concentrations are not high enough to be diagnosed as T2DM (83). A recent study in our laboratory established that abnormal concentrations of incretins precede pre-diabetes development, and incretins play a significant role in its development (129). Chronic HFHC diet consumption for a prolonged period leads to increased glucose absorption and chronic secretion of GIP and GLP-1 (121). GLP-1 degradation is increased as the dipeptidyl peptidase-4 enzyme concentration is also increased under high glucose conditions; this results in a severely reduced GLP-1 concentration (121). The elevated GIP constantly induces insulin secretion, resulting in elevated insulin over long periods (107). Over time, the insulin receptors lose their sensitivity to insulin and become resistant to insulin due to being overworked in the process of reducing the blood glucose concentration (83). The onset of insulin resistance results in fewer cells that receive glucose, and fewer cells receive glucose and send signals to the hypothalamus to suppress appetite (122). Therefore, there are reduced leptin concentrations and increased ghrelin concentrations; cells which do not receive glucose send signals to the hypothalamus to induce food intake by increasing appetite, which increases the secretion of ghrelin (75, 76). The increased secretion of ghrelin inhibits the leptin hormone's secretion, thus reducing satiety (75, 76). Leptin plays a role in the secretion of GLP-1; therefore, the secretion of GLP-1 is also impaired, which

further reduces its concentration (129). The incretins maintain these abnormalities, which leads to pre-diabetes, which can be confirmed by increased glycated hemoglobin concentration (129).

In the current study, we intervened by subjecting our pre-diabetic animal model to 12 weeks of LCHF diet consumption to reverse the effects of pre-diabetes. After 12 weeks, the significantly reduced blood glucose concentration confirmed the reversal of pre-diabetes. We speculate that the chronic consumption of a LCHF diet allowed for the absorption of a low amount of new glucose entering the bloodstream, which could have resulted in reduced blood glucose which could reduce the HbA1c concentration. The HbA1c concentration was indeed reduced to within the non-prediabetic range, indicating a restoration of glucose homeostasis.

Several hormones play a role in glucose homeostasis. GIP is a glucose-dependent peptide secreted shortly after meal consumption due to glucose in the stomach and its absorption into the bloodstream (107). It induces insulin secretion to reduce the upcoming blood glucose (107, 108). There are increased GIP concentrations in pre-diabetes due to prolonged elevated blood glucose concentration (74). Increased GIP concentration results in chronic insulin secretion, which causes hyperinsulinemia and later insulin resistance (74, 123). After chronic consumption of the LCHF diet, there are significantly reduced concentrations of GIP. We postulated that the secretion of GIP was reduced due to reduced blood glucose, which could also mean reduced plasma insulin concentrations. There were indeed significantly reduced plasma insulin concentrations after prolonged consumption of a LCHF diet. Taken together, the reduced blood glucose, insulin and HbA1c indicate improved insulin sensitivity. The homeostatic model assessment (HOMA) is a recognized method for measuring insulin resistance (IR) using fasting glucose and insulin (83, 124). HOMA-IR is calculated according to the formula: fasting plasma insulin (µU/ml) x fasting plasma glucose (mmol/L)/22.5 (83, 124). Using this method, we determined overt insulin resistance in the PD group, which fed on the HFHC diet for 32 weeks. After prolonged consumption of the LCHF, there was indeed improved insulin sensitivity in the PD + DI group.

In pre-diabetes, there are reduced leptin concentrations due to the developing insulin resistance (111, 125); The cells in tissues such as skeletal muscle does not receive glucose and send signals into the hypothalamus to increase appetite and food intake, which results in increased ghrelin concentrations (72). Increased ghrelin concentrations inhibit leptin secretion; therefore, there are reduced leptin concentrations (72). After chronic consumption of a LCHF diet, there was a significantly reduced ghrelin concentration and a significantly increased leptin concentration. We speculated that after the movement of glucose into the cells, the cells sent signals into the hypothalamus to inhibit appetite and induce satiety which could have resulted in a reduced ghrelin secretion and an increased leptin concentration. Leptin plays a role in the secretion of GLP-1; increased leptin could result in improved GLP-1 concentration (11). GLP-1 is secreted simultaneously with GIP after the entrance of glucose into the stomach and its absorption in the small intestines; there are reduced GLP-1 concentrations in prediabetes due to the increase of circulating DPP4 enzyme, which speeds up its degradation (73). The

prolonged blood glucose increases the circulating DPP4 enzyme (73). There are increased GLP-1 concentrations after chronic consumption of the LCHF diet. We speculate that due to reduced blood glucose, there could be reduced concentrations of the DPP4 enzyme, which could have allowed for a reduced degradation of GLP-1. Using the information gathered from the previous study, we speculated that the incretins abnormalities amelioration preceded the reversal of pre-diabetes as it was established that they precede pre-diabetes development (129).

Conclusion.

In this study, we observed the effect of diet on incretins as they play a significant role in developing and reversing pre-diabetes. Chronic consumption of a HFHC diet led to elevated blood glucose and insulin concentration, resulting in abnormal concentrations of incretins. The abnormal incretins then maintained this state of hyperglycemia and hyperinsulinemia, resulting in pre-diabetes. Chronic consumption of a LCHF by the pre-diabetic rats led to reduced concentrations of incretins, which could have led to a reduced HbA1c and eventually to the reversal of pre-diabetes.

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Author Contributions

NM and NRD contributed to the study design, conducted the experiments, collected, analyzed, and interpreted data, as well as being involved in writing the manuscript. AMS and PSN were 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.

Conflict of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

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

Ethics Approval and Consent to Participate

The animal study protocol was reviewed and approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, South Africa (AREC/00003627/2021).

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Chapter 4: Synthesis and Conclusion

Synthesis

Pre-diabetes is a condition of intermediate hyperglycemia that often precedes the onset of type 2 diabetes mellitus (T2DM) (70). In this condition, we observe chronically elevated blood glucose concentrations that are not high enough for a diagnosis of T2DM (70, 81). Several studies report that often life-threatening complications associated with T2DM begin in the pre-diabetic state (69, 78). While T2DM can be managed, it has no known cure, while pre-diabetes has been shown to be reversible (126, 127). Therefore, pre-diabetes has become a therapeutic target to prevent the onset of T2DM (70). Incretins are hormones secreted in the gut that and aid in maintaining glucose homeostasis by enhancing insulin secretion as they contribute significantly to overall postprandial insulin release (8). Incretin peptides include glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), associated with ghrelin and leptin. Hyperglycemia in T2DM has been observed to impair the normal functioning of incretins, resulting in abnormal incretin concentrations (20, 73). Furthermore, abnormal incretins have been shown to play a role in the pathogenesis of T2DM (20). However, the changes in incretins in the pre-diabetic state have not been established. Hence, the first manuscript of this study aimed to investigate the effect of incretin concentrations in the development of diet-induced pre-diabetes. The Second manuscript of this study sought to investigate the effect of a low carbohydrate, high unsaturated fat (LCHF) diet on incretin concentrations in a diet-induced pre-diabetic animal model as this diet has been shown to be beneficial in the management of prediabetes.

Incretin peptides are associated with the appetite-regulating hormones, together they aid in glucose homeostasis. Several studies have shown that abnormal incretin concentrations play a role in the progression of the pathology of T2DM (20, 121). However, minimal work has been done in assessing the link between incretin peptide levels and pre-diabetes development. In our laboratory, a pre-diabetic animal model mimicking the human pre-diabetic state has been developed (64, 82). Previous studies on this model have established that pre-diabetes is induced in male Sprague Dawley rats after 20 weeks of a chronic high-fat, high-carbohydrate diet consumption (82, 83). This model has been used to observe changes that occur in the pre-diabetic state. However, no work had been done to explore the changes in incretin concentration and their possible involvement in the development of pre-diabetes as well as the effect of a low carbohydrate, high unsaturated fat diet in incretin levels of a prediabetic animal model. This led to manuscript 1, where we induced pre-diabetes using a HFHC diet to observe the involvement of incretins in the development of pre-diabetes, and manuscript 2.

The first manuscript separated twenty-four (24) animals separated into two randomized groups: group 1 (n=12) and group 2 (n=12). Group 1 was kept on a standard rat diet (SD) while group 2 was kept on the experimental high-fat, high carbohydrates diet (HFHC) (n=6). The animals were maintained on their respective diets for 20 weeks. After 10 weeks being on their respective diets, 6 animals were sacrificed from each group and biochemical analysis was done. The rest of the animals continued in the study for

an additional 10 weeks while continuing their respective diets. At week 20, all the remaining animals in each group were sacrificed and biochemical analysis was carried out.

In line with previous studies, the results of this study showed that ingestion of a HFHC diet for 20 weeks resulted in elevated plasma glucose and insulin levels and increased HbA1c and HOMA-IR compared to the SD group. These results suggested that the rats fed the HFHC diet had indeed developed pre-diabetes (PD) while those on the standard (SD) diet remained non-pre-diabetic. Additionally, at week 20, the results depicted significantly higher concentrations of ghrelin and GIP with significantly reduced leptin and GLP-1 concentrations, suggesting a derangement in incretin homeostasis during the prediabetic state.

Of interest to this study, at 10 weeks, the results showed that ingestion of a HFHC diet resulted in elevated plasma glucose but other markers such as, plasma insulin, HbA1c and HOMA-IR showed that the animals had not developed prediabetes. Furthermore, at 10 weeks, the results showed significantly high plasma ghrelin, and GIP concentrations as well as significantly reduced leptin and GLP-1 thus suggesting a derangement in incretin homeostasis before the onset of pre-diabetes. The chronic consumption of the HFHC diet could have led to hyperglycemia which may have led to constant secretion of GIP and GLP-1 due to the presence of glucose in the stomach and its absorption. We speculate that due to hyperglycemia, there could have been an increase in circulating DPP-4 concentration, leading to increased degradation and reduced concentrations of GLP-1. Chronic GIP secretion could be responsible for hyperinsulinaemia due to the constant induction of insulin secretion; This may have led to the development of insulin resistance. Increased circulating ghrelin could be attributed to the presence of insulin resistance. Tissue cells with insulin-resistant receptors could not have been able to receive glucose. They could have constantly sent signals to the hypothalamus to induce food intake, which could have resulted in the constant secretion of ghrelin. Increased levels of ghrelin have been shown to lead to reduced leptin concentrations (76); Which could be the reason for the significantly reduced levels of leptin. Increased ghrelin concentration could have led to increased food intake, further increasing blood glucose. Additionally, the reduced leptin concentrations could have also been due to the reduced number of tissue cells that receive glucose and send signals to the hypothalamus to induce satiety, leading to leptin secretion. Leptin also plays a role in the secretion of GLP-1 (128); This could mean a reduction in GLP-1 secretion. This study, for the first time suggests that chronic ingestion of a HFHC could possibly dysregulate plasma incretins and that if this is maintained for extended periods, could have led to the development of pre-diabetes. The findings of this manuscript suggests that incretins play a role in the development of pre-diabetes.

To advance on this knowledge, the second manuscript sought to investigate the possibility of reversing pre-diabetes primarily using a LCHF diet to influence the incretin peptides as their concentrations are directly affected by diet. In a previous study, it has been postulated that the reduced carbohydrate content in the LCHF diet yields a low glucose concentration that is absorbed into the bloodstream during digestion (117). For this study, we had six (6) non-prediabetic animals that were kept on a standard rat

diet. These animals served as the non-prediabetic control (NPC) group. We also induced prediabetes in twelve (12) male Sprague Dawley rats using the HFHC diet. After induction, the animals were split into two experimental groups (n=6 in each group). One of the groups continued a HFHC diet and served as the pre-diabetic control group (PD). The other group was changed to a low carbohydrate-high unsaturated fats diet (PD+DI). Both groups were then maintained on their respective diets for an additional 12 weeks. Following the additional 12 weeks, all three groups were sacrificed, and biochemical analysis was performed.

By comparison to the non-prediabetic control, the elevated plasma glucose, insulin, HOMA-IR index, and HbA1c in the PD group suggested that there was overt insulin resistance thus confirming that the prediabetes had progressed. However, by comparison to the prediabetic control group, the PD+DI group had improved incretin concentration, reduced plasma insulin, reduced blood glucose, and reduced HbA1c concentrations suggesting a reversal of prediabetes. These findings were in agreement with several studies that have shown that continued ingestion of a low-carbohydrate, high-fat diet is able to reverse the pre-diabetic state. These studies postulated that the reduced carbohydrate content in the LCHF diet yields a low glucose concentration that is absorbed into the bloodstream during digestion (117). The body is promoted to enhance fat oxidation to meet the energy needs with reduced carbohydrate intake (117, 118, 119). Therefore, this results in a reduction in glycated hemoglobin levels and an improvement in glucose tolerance (118, 120).

Based on the findings of manuscript 1, which showed that changes in incretin levels precede changes in the onset of prediabetes, we suggest that chronic consumption of the LCHF diet could have resulted in the reversal of prediabetes, in part, by modulating incretin levels. In pre-diabetes, GIP is responsible for the constant secretion of insulin, a reduced GIP secretion could therefore result in reduced stimulation of insulin secretion which could allow for the circulating insulin to be degraded over time without a lot of new insulin being secreted into the bloodstream. The gradual degradation of insulin could be responsible for reduced blood glucose as it facilitates its absorption into tissue cells. The reduced blood glucose could have led to a reduced plasma DPP-4, resulting in the increased plasma GLP-1 observed after 12 weeks. The absorption of blood glucose into tissue cells could have also resulted in the secretion of leptin and inhibition of ghrelin induced by tissue cells sending signals into the hypothalamus to inhibit food intake and induce satiety. The reduced blood glucose, plasma insulin, and HOMA-IR index suggest improved insulin sensitivity. After 12 weeks, the significantly reduced blood glucose concentration and reduced HbA1c confirmed the reversal of pre-diabetes. Therefore, for the first time, this study suggests that incretins play a significant role in the mechanism to reverse pre-diabetes.

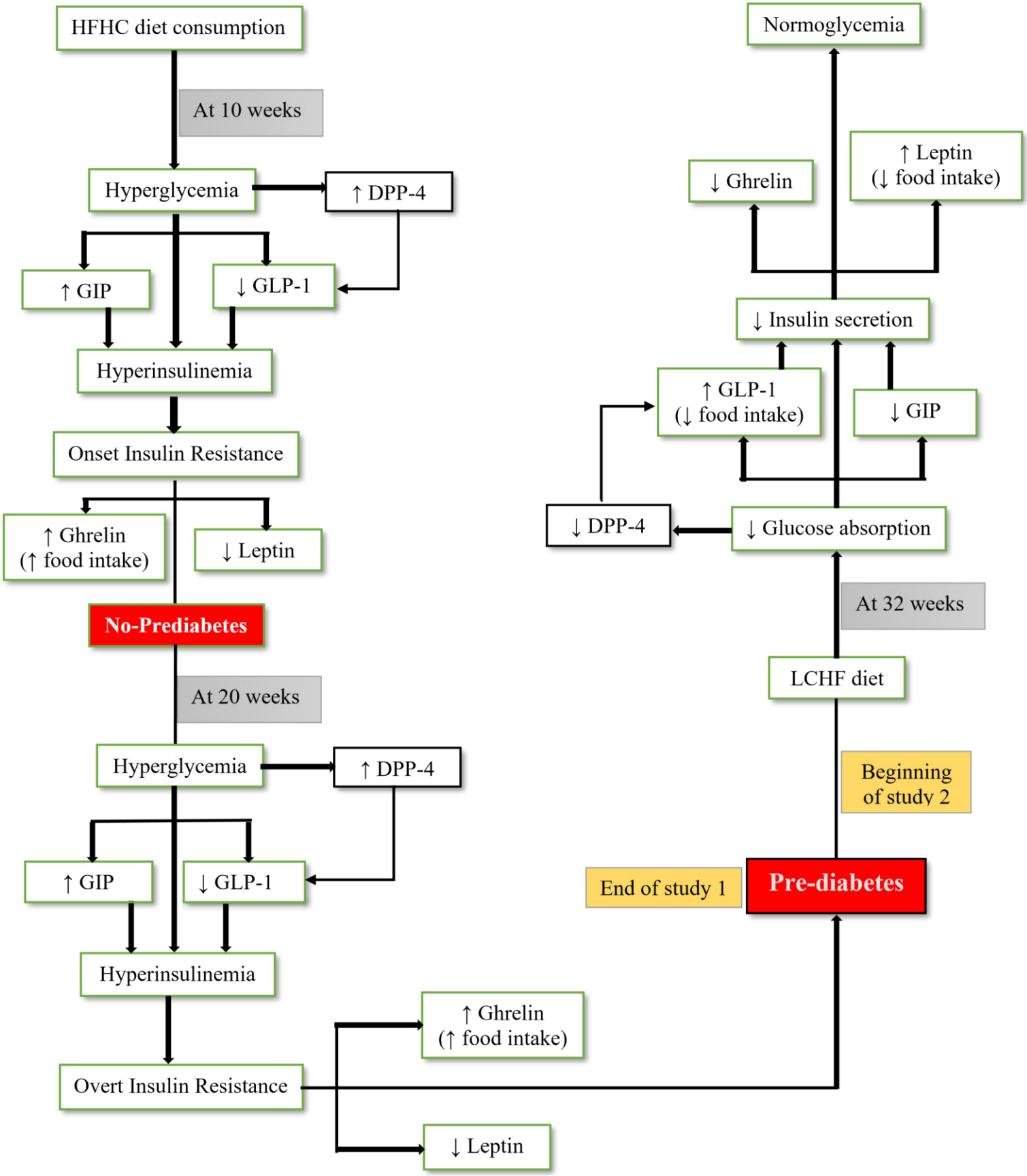

Figure 1: Diagram illustrating the role of incretins in developing and reversing pre-diabetes.

Conclusion

Taken together, the findings of this study revealed that incretin concentrations are indeed influenced by diet. Hence, the dysregulation of incretin levels may contribute to the onset of pre-diabetes. This study suggested, for the first time, that incretin abnormalities precede and significantly contribute to pre-diabetes development. This research also suggested that incretins significantly impact the reversal of pre-diabetes. The research may help identify specific incretin hormone concentration patterns or changes that are associated with the development of diet-induced prediabetes. Identifying biomarkers can aid in targeted interventions and preventive measures for at-risk individuals. This may open a new avenue of drugs in the management of prediabetes that seek to modulate the functioning of incretin levels.

Shortfalls and Future Studies

In manuscript 1, we looked at the involvement of incretins in the development of diet-induced prediabetes. This study could have further looked at the DPP-4 enzyme pathway, as it plays a major role in the regulation of incretin levels; however, due to budget constraints, this was not possible. Therefore, future studies should investigate the involvement of DPP-4 in the development of prediabetes. Some studies show conflicting results to our study showing normal, increased, and decreased GLP-I and GIP levels in individuals with prediabetes which highlights, we therefore recommend more studies on incretins, specifically in animal models without any confounding factors.

In manuscript 2, we looked at the effect a LCHF diet has on incretin levels, hoping for a possibility of reversing pre-diabetes primarily using the LCHF diet to influence the incretin peptides. This study could have looked at the incretin levels at the halfway point into the reversal of prediabetes. This could have shown conclusively whether changes to incretin levels precede the reversal of prediabetes. Therefore, future studies should investigate the effect of a LCHF diet on the incretin concentrations at 6 weeks, halfway to the reversal of prediabetes to conclusively show their involvement in the reversal of prediabetes.

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APPENDICES

Appendix 1: AREC Ethics Approval Letter

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)
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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix 2: Manuscript 1 journal guide: Canadian Journal of Physiology and Pharmacology

Prepare your manuscript.

Format and style Manuscript text must: be in English or French be double-spaced be single-column include page numbers include continuous line numbers (before acceptance only) be 8.5 x 11 inches in page size (or ISO A4)

follow this order: title page, abstract, keywords, body text (Introduction, Materials and methods, Results, Discussion), acknowledgements, references, tables, figure captions, figures, appendices

Abbreviations and acronyms

Define abbreviations and acronyms when they are first mentioned in the text.

Footnotes

In body text, try to avoid footnotes. If unavoidable, cite footnotes using superscript Arabic numbers (^{1,2,3}), in order of appearance (starting with the title page), and include the footnote at the bottom of the page on which it is cited. Do not include footnotes in the reference list.

In tables, cite footnotes using symbols (in the order ★, †, ‡, §, ‖, ¶, #) or superscript lowercase italic letters (^{a,b,c}).

Mathematical expressions

Identify equations by calling out with numbers in parentheses placed flush with the left margin (for the *Canadian Journal of Physics*, place on the right).

A letter or symbol should represent only one entity and be used consistently throughout the paper.

Each variable (including those representing vectors, matrices, and tensors) must be clearly identified and defined in the text.

Supply complex equations in an editable format by using LaTeX or a math editor (MathType).

Supply simple, inline equations in Word, without using MathType. Insert symbols from Word's

"Symbol" palette, using "normal text" or "Symbol" fonts only. Insert symbols using MathType ONLY if they cannot be found in the "Symbol" palette under one of those two fonts.

Reporting guidelines

Study reporting guidelines can help authors report their work transparently and accurately. We encourage their use. Up-to-date guidelines can be found at the EQUATOR Network, where authors can search or consult the GoodReports wizard to identify which guideline(s) to use. A completed copy of the guideline checklist may be submitted with the manuscript as a Supplementary file.

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Spelling should follow that of *Webster's Third New International Dictionary* or the *Oxford English Dictionary*. Authors are responsible for consistency in spelling.

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Use SI units of measure (Système international d'unités). If non-SI units are used, at first mention, supply the equivalent in SI units in parentheses.

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Title

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List all author names on the title page: check author order, spelling, capitalization, initials, and hyphens.

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Provide the abstract in the manuscript text file on page 2, after the title page. Authors must supply their abstract in English and/or French. (Note that CSP plans to accommodate author-provided abstract translations in other languages in the future.)

The abstract should be a single paragraph that summarizes the article. It helps readers decide whether to keep reading. Briefly describe the study rationale, objectives, methods, findings, and impact. Use keywords (and their plain-language synonyms) in the abstract to optimize search engine discovery. Do not include headings, reference citations, tables, figures, or acronyms.

Keywords

List keywords after the abstract. Good keywords are common to your field and accurately describe your topic. Use keywords in the title, abstract, and manuscript text to optimize search engine discovery. (Consider what words you would enter in a search box to find your work online.)

Graphical abstract

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Plain language summaries

(Optional) At acceptance, authors are encouraged to submit a plain language summary of their article to increase the reach of their research. For guidelines and submission instructions, see Writing a Plain

Main body of the article

Introduction

In 1–3 paragraphs, explain the study rationale and objective(s).

What is the problem, and why is it important?

What is known on the topic? Establish context: provide background, briefly review the key literature, and mention existing gaps or controversies in the field.

What is the overall aim of the study? State your research question, hypotheses, and predictions.

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Describe what you did (and how you did it) clearly and comprehensively enough for the study to be replicated.

Include as appropriate the study design; primary and secondary outcome measures; computational, experimental, and statistical methods; materials; locations.

Avoid long descriptions of known procedures; provide relevant references instead.

Specify materials used (e.g., laboratory or field equipment, chemicals, biologic materials) and their sources (provide company name, city, and country in parentheses).

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Information about Ethics Approvals should be reported in the Materials and methods section (usually the first paragraph).

For studies involving human participants: name the institutional ethics review committee that approved the study; and confirm that participants gave informed consent before participating in the study.
For studies involving animals:

name the animal care review committee that approved the study;

name the animal care guidelines that were followed (must be in line with the Canadian Council on Animal Care guidelines or the Guide for the Care and Use of Laboratory Animals);

confirm that research involving endangered species was conducted in accordance with all applicable laws; and if permits were required for fieldwork, supply the research permit and (or) license numbers.

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confirm that research involving collection of specimens was conducted in accordance with all applicable laws, guidelines, regulations; and if permits were required for fieldwork, supply the research permit and (or) license numbers.

Results

Report results that are directly relevant to your research question. Raw data and other observations may be submitted with the manuscript as Supplementary files or uploaded to a recognized data repository.

Use subheadings, tables, and figures to organize and communicate your findings.

Begin sections and (or) sentences with high-level observations, followed by statistical data.

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Cite each figure and table, and name them in order of first appearance in the text (Figure 1, Figure 2, Table 1, Figure 3, Table 2, etc.).

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SUBMIT ARTICLE

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For general matters of style, this journal uses for biological terms, *Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers* (8th ed., 2014)), published by the Council of Science Editors.

Nomenclature

Nomenclature should follow the rules established by international authoritative bodies.

Abstract

Abstracts should not exceed 200 words for all article types.

Length per manuscript type

Article type	Description	Specifications (word, table/fig, reference limits)
Article that reports new and original research.	A completed, definitive study	A maximum of 10,000 words including references, tables and figures is suggested for this article type. Editors or reviewers may request changes to the length and/or structure of the article at their discretion.
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("undecided" is not an acceptable answer); what data will be shared; what additional, related documents will be available (e.g., study protocol, statistical analysis plan); when the data will become available and for how long; and

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Appendix 3: Manuscript 2 Journal guide: Endocrinología, Diabetes y Nutrición

Guide for Authors

Types of articles

The Journal contains the following sections:

Original articles

Original clinical, translational, or experimental research papers covering any aspect related to endocrinology, nutrition, diabetes, or metabolism. The recommended designs are analytical in the form of observational studies (cross-sectional, case-control studies, cohort studies) following the STROBE methodology and randomized controlled trials following the CONSORT methodology and registered in a public database. The article should have a maximum length of 4000 words, not including references, abstract, and figure legends. It will be structured in the following sections: Introduction, Material and methods, Results, and Discussion. Subsections are only allowed in the Material and Methods and Results sections. The abstract (in Spanish and English) can have a maximum of 250 words and must be structured. The bibliography must be limited to a maximum of 30 references. A maximum of 6 tables and/or figures in total will be accepted. It will be included the main findings section, together with the abstract, no more than 80 words, divided into 4 or 5 sentences. Authors are also encouraged to provide a graphical abstract of the article.

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Systematic reviews using PRISMA or MOOSE criteria are recommended. It is recommended that the review protocol is registered in a public database and that the reference number is included in the publication. The article should have a maximum length of 5000 words, excluding literature references, abstract and figure legends. It should be structured in the following sections: Introduction, Material and Methods, Results and Discussion. There is no limit to the number of literature references (provided they are justified). A maximum of 6 tables and/or figures in total will be accepted. If necessary, they can be complemented with supplementary tables/figures.

Case reports

A case report is another type of review. It is an update on a topic related to a clinical case. This type of manuscript can be commissioned or submitted on the authors' own initiative. They will be written in English, will have a maximum length of 2500 words, and an unstructured abstract of 150 words, the corresponding keywords, and up to 50 literature references. Up to 3 figures and/or tables will be accepted. It will be structured as follows: description of the case, management (and supporting evidence), areas of uncertainty, guidelines and conclusion and recommendations.

Scientific letters

Descriptions may be included of one or more clinical cases of exceptional observation that represent a significant contribution to understanding the pathophysiology or other aspects of endocrine diseases. They may also report research experiences or results which, because of their characteristics, are not sufficiently important to be published as an original article. Maximum length of the text will be 1000 words, and it should not be structured in sections. It should not be accompanied by an abstract either. One figure or one table will be accepted. No more than 10 literature references should be included.

There should be no more than five authors.

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Letters to the Editor may refer to studies published in the Journal or express opinions on a current issue. They may not exceed 1000 words and 1 Figure or 1 Table and a maximum of 10 literature references will be accepted. There should be no more than 4 authors.

Other sections

The journal includes Editorials commissioned by the Editorial Board. Authors who wish to contribute to any of these sections should contact the journal's Editors beforehand. *Endocrinología, Diabetes y Nutrición* will also publish clinical guidelines and consensus documents on the diagnosis or treatment of diseases, provided they are promoted directly by the SED and SEEN or by working groups. These documents must be made according to the indications recommended by the SED and SEEN (www.seen.es) and follow the AGREE methodology for clinical guidelines. The guidelines prepared jointly with other scientific societies may be published in Endocrinology, Diabetes and Nutrition, and in parallel in the publications of the other scientific societies, provided that the regulations established by the SED-SEEN and the AGREE methodology have been followed. In this case, the correspondence author will attach a cover letter stating the Societies that endorse the Document (attaching the endorsement of each Society), as well as the journals to which the consensus will be sent. The journal will publish the guideline or the consensus document accepted by the Editorial Committee as an Executive Summary, whose maximum length will be 3000 words, and an unstructured abstract that will have a maximum of 150 words. It will be accompanied by tables and figures that are considered convenient to help interpret the information in a clear and concise manner and it will be sent in English. The title will begin with the following sentence: "Executive summary of the guideline/consensus document…". The full consensus document will be published online as additional material to the Executive Summary and/or as a supplement to the journal. The objective of the publication of the Executive Summary is to offer readers a synthesis of the consensus document, but with enough detail and clarity to understand the full document's scope and most relevant points. A maximum of 6 executive summaries per year will be published in the first issue of each year, with priority being given to those produced jointly by the two societies. Papers must be submitted to the board(s) of the corresponding

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All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

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30 NOVEMBER 2022
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- Abstracts are open to all postgraduate and post-doc students, developmental lecturers and credentialing staff in the School of Laboratory Medicine and Medical Sciences.
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- It is the author's responsibility to submit an abstract that is free of spelling and grammatical errors.
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  (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.
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- 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
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Presenter Information

Surname: Mzimela	**First Name: Nhlakanipho**
Research Theme: Prediabetes	**Tel: 0655035416**
E-Mail: 218006756@stu.ukzn.ac.za	**Date: 23 November 2022**

ABSTRACT TEMPLATE

Only abstracts that have strictly adhered to this template will be considered.
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TITLE, AUTHOR AND AFFILIATION

THE ASSOCIATION BETWEEN INCRETIN PEPTIDES LEVELS AND THE DEVELOPMENT OF DIET-INDUCED PREDIABETES.

Mzimela, N.*; Khathi, A.*; Ngubane, P.S.*; Sosibo, A.M*.
*Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville, South Africa

ABSTRACT BODY:

Prediabetes is a chronic metabolic condition that frequently occurs before type 2 diabetes mellitus develops. This condition is characterized by a gradual reduction of insulin sensitivity by insulin receptors in insulin-dependent cells, which is frequently followed by an increase in plasma glucose levels. These glucose levels, however, are still below the diabetes threshold. Pre-diabetic patients are more prone to have issues with immune dysregulation and diabetic nephropathy. Studies already conducted have looked on how incretin peptides affect the pathology of type 2 diabetes mellitus. The link between incretin peptide levels and the onset of prediabetes, on the other hand, remains unknown. Thus, the purpose of this study is to assess the role that incretin peptide levels play in the emergence of prediabetes. This study was conducted using male Sprague-Dawley rats, divided into the normal group (control) and the HFHC group (prediabetic control). The groups were given their diet accordingly for 20 weeks to induce prediabetes. After that, the animals were sacrificed, and blood was collected for final glucose readings. The findings of this study show that long-term use of HFHC or an unhealthy diet causes abnormal incretin concentrations, which trigger ongoing insulin stimulation. According to this study, incretin peptides contribute favorably to the onset of prediabetes. Therefore, GIP could be employed as therapeutic target and biomarker for early diagnosis of prediabetes.

Keywords: Type two diabetes mellitus, incretins, insulin resistance, prediabetes.

Research Theme: Prediabetes

Ethics Number: AREC/00003627/2021

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√	Oral Presentation

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