Investigating the effects of diet-induced pre-diabetes on calcium homeostasis in male Sprague Dawley rats

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PREFACE

Diets comprised with a surplus of carbohydrate and fat content have shown to lead to the onset of type 2 diabetes mellitus (T2DM). Insulin resistance or insufficiency causes T2DM; this state is preceded by pre-diabetes. Although pre-diabetes has been described as asymptomatic, studies have demonstrated that complications associated with T2DM begin in this stage. T2DM has shown to disturb calcium homeostasis by inducing changes to calciotropic hormones and calcium-regulating organs. Furthermore, altered levels of calciotropic hormones in T2DM have shown to further exacerbate insulin resistance and hyperglycaemia. However, the changes to calcium homeostasis in the pre-diabetic state have not been characterised. A pre-diabetic rat model that mimics the human condition of pre-diabetes was established using a high-fat high-carbohydrate diet. This diet-induced pre-diabetic rat model was used to investigate the changes to calciotropic hormones, the association of calciotropic hormones with glucose parameters as well as to evaluate the changes to the functioning of calcium-regulating organs. The experimental work described in this dissertation was conducted at the University of Kwa-Zulu Natal, Westville Campus, Durban, South Africa. All work was conducted under the supervision of Dr. Andile Khathi and co-supervised by Dr. Phikelelani Ngubane.

DECLARATION

I, Karishma Naidoo hereby declare that the dissertation entitled:

"Investigating the effects of diet-induced pre-diabetes on calcium homeostasis in male Sprague Dawley rats." is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

Student: Karishma Naidoo **Signature_______** **Date: 11 Jan 2022**

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Co-Supervisor: Dr Phikelelani Ngubane **Signature______** **Date: 11 Jan 2022**

PLAGIARISM DECLARATION

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

MASTER'S DEGREE IN MEDICAL SCIENCES 2022

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's
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DEDICATION

This work is dedicated to God, my supportive parents, sister and my late best friend Medusa.

ACKNOWLEDGEMENTS

To my supervisor Dr. Khathi: It was a great pleasure to work with such an astonishing academic. Thank you for your patience and assistance in guiding me throughout this journey. You are a great academic, mentor and friend, thank you Dr. for all your efforts and motivation.

To my co-supervisor Dr. Ngubane: Thank you for your guidance and support.

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To my family: Thank you for believing in me and allowing me to further my studies. To my parents, Mrs S. Naidoo and Mr. T Naidoo, I have only gotten this far in my life because of all the values and support you all have given to me. Thank you mum and dad for teaching me to fight for my dreams and to never lose hope. To my sister Derushka, I am grateful to have you in my life; you have always motivated me to be resilient.

To my best friend, Medusa: You will forever be a part of me and live on in my heart.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AREC	Animal Research Ethics Committee
AUC	Area Under Curve
BRU	Biomedical Research Unit
Ca^{2+}	Calcium ion
cDNA	Complementary DNA
CO_2	Carbon dioxide
DM	Diabetes Mellitus
ELISA	Enzyme-linked Immunosorbent Assay
FBG	Fasting blood glucose
FFA	Free fatty acid
GADPH	Glycerideal dehyde-3-phosphate dehydrogenase
GLUT-4	Glucose transporter type 4
HbA1c	Glycated haemoglobin
HCD	High carbohydrate diet
HFD	High fat diet
HFHC	High-fat high-carbohydrate
HOMA-IR	Homeostatic model assessment for insulin resistance
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
KK	Kuo Kondo
M-CSF	Macrophage colony-stimulating factor
mRNA	Messenger ribonucleic acid
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
PD	Pre-diabetes
PMCA	Plasma membrane Ca^{2+} ATPase
PTH	Parathyroid hormone

RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SEM	Standard error of means
T2DM	Type 2 diabetes mellitus
TALH	Thick ascending limb of Henle
TRPV5	Transient receptor potential vanilloid 5
TRPV6	Transient receptor potential vanilloid 6
UKZN	University of KwaZulu-Natal
VDR	Vitamin D receptor

STUDY OUTLINE

The current dissertation is in manuscript format and is divided into four chapters; chapter 1: literature review, chapter 2: prologue, abstract and manuscript 1, chapter 3: prologue, abstract and manuscript 2, chapter 4: synthesis and appendices. Chapter 1 comprises an introduction to the study, literature review and rationale/justification of the study in addition to the aims and objectives. Chapter 2 of this dissertation presents the first study that is in manuscript form which seeks to investigate the effects of diet-induced pre-diabetes on calciotropic hormones in male Sprague Dawley rats. This work is authored by K. Naidoo, supervised by Dr. A. Khathi and co-supervised by Dr. P.S. Ngubane. Furthermore, this manuscript has been formatted and submitted to the Journal of Experimental Clinical Endocrinology and Diabetes according to the journal guidelines for authors. Chapter 3 of this dissertation presents the second study that is written in manuscript form that soughts to determine the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs in male Sprague Dawley rats. This manuscript is authored by K. Naidoo, supervised by Dr. A. Khathi and co-supervised by Dr. P.S. Ngubane and has been formatted and submitted to the Journal of Endocrine Pathology according to the guidelines of the journal. Chapter 4 constitutes the synthesis of the study and appendices.

ABSTRACT

Background

Diabetes mellitus (DM) affects over 400 million people worldwide with 90-95% being type 2 diabetes mellitus (T2DM) in South Africa. T2DM is positively correlated with the chronic consumption of a high caloric diet, often preceded by pre-diabetes. Pre-diabetes is a long-term intermediate stage of hyperglycaemia which is usually asymptomatic. One of the key aetiologies for the complications of physiological systems seen in T2DM has been found to be the chronic intake of high caloric diets. However, dysregulation of these physiological systems seen in T2DM have been reported to begin in pre-diabetes. Calcium homeostasis has been demonstrated to be one of the body's mechanisms that is disrupted in T2DM, leading to changes in calciotropic hormone levels and the functioning of calcium-regulating organs. Altered levels of calciotropic hormones in diabetes have been shown to increase the risk of developing insulin resistance and hyperglycaemia. Furthermore, disrupted functioning of calcium-regulating organs in diabetes impairs their responsiveness to calciotropic hormones. A pre-diabetic rat model was utilized in our laboratory to explore numerous systems and mechanisms in the body, including glucose homeostasis, the cardiovascular system, and immunity, using a high-fat high-carbohydrate diet to induce pre-diabetes. However, there is a paucity in literature elucidating the changes to calcium homeostasis in pre-diabetes. Hence, the present study aimed to investigate the effects of diet-induced pre-diabetes on calcium homeostasis by looking at calciotropic hormones and the functioning of calcium-regulating organs.

Materials and Methods

Twelve male Sprague-Dawley rats were randomly divided into 2 groups (n=6, each group) whereby the first group: non-pre-diabetic (NPD) group was subjected to standard rat chow and the second group: pre-diabetic (PD) group was subjected to a high-fat high-carbohydrate (HFHC) for 20 weeks. At week 20, the American diabetes association criteria (ADA) were employed for pre-diabetes diagnosis. Plasma was collected for biochemical analysis to measure glucose, insulin, glycated haemoglobin (HbA1c) and the homeostatic model assessment for insulin resistance (HOMA-IR) in addition to urine and plasma calcium concentrations. This was accompanied by measurement of plasma parathyroid hormone (PTH), calcitonin, vitamin D, 1,25-dihydroxyvitamin D3 (calcitriol), osteocalcin and deoxypyridinoline via enzyme linked immunosorbent assay (ELISA). Correlation analysis of calciotropic hormones with HbA1c and HOMA-IR were performed. Furthermore, small intestine and kidney tissue were harvested after the experimental period for analysis of gene expression. Renal expressions of transient receptor potential vanilloid 5 (TRPV5), 1-alpha hydroxylase along with intestinal expressions of vitamin D receptor (VDR) and calbindin-D_{9k} were measured via reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

Results and discussion

The HFHC diet resulted in moderate hyperglycaemia, elevated plasma insulin, elevated HbA1c and insulin resistance in the PD group by comparison to the NPD group. In the first study, there were increased calciotropic hormone concentrations; plasma PTH, calcitonin, calcitriol and vitamin D in addition to elevated urine calcium and unchanged plasma calcium in the PD group by comparison to NPD. This suggested that elevated calciotropic hormone concentrations in pre-diabetes may compensate for changes to plasma calcium. Furthermore, plasma PTH and calcitonin levels were positively correlated with HbA1c but not insulin resistance in the PD group. Plasma calcitriol concentrations were negatively correlated with HbA1c in the PD group. Altered levels of calciotropic hormones in pre-diabetes may exacerbate the moderate hyperglycaemia in pre-diabetes. In the second study, plasma fasting glucose, insulin, OGT response and HOMA-IR were higher in PD group compared to the NPD. It was observed that normal plasma calcium levels in the pre-diabetic group were accompanied by an upregulation in renal TRPV5, 1-alpha hydroxylase, intestinal VDR and calbindin-D9K expression in addition to increased plasma osteocalcin and decreased urine deoxypyridinoline. Calcium-regulating organs may have responded to disturbed calcium homeostasis by promoting increased intestinal calcium absorption, renal calcium reabsorption in addition to decreasing bone resorption and increasing bone formation.

Conclusion

The findings suggest that normocalcaemia is maintained in the pre-diabetic state due to compensation from calciotropic hormones and calcium-regulating organs. However, altered levels of calciotropic hormones in pre-diabetes may play a role in the onset of hyperglycemia in T2DM. Due to the cumulative evidence produced in study 1 and study 2, we accept the hypothesis which states that during the pre-diabetic state there will be changes to calciotropic hormones and calcium-regulating organs indicative of disturbed calcium homeostasis.

CHAPTER 1: LITERATURE REVIEW

1. Introduction

A chronic state of hyperglycaemia caused by insulin insufficiency or insulin resistance is known as type 2 diabetes mellitus (T2DM) [1]. Based on the total number of diabetes mellitus (DM) cases, T2DM accounts for 90% of the cases in South Africa [2]. T2DM is anticipated to affect 642 million people worldwide by 2040, according to the International Diabetes Federation (IDF) [2]. Pre-diabetes precedes T2DM, with blood glucose levels higher than the homeostatic range but below the threshold for clinical diabetes diagnosis [3]. It is shown that fasting glucose levels and glucose tolerance are impaired due to early insulin resistance in this stage [4]. Diabetes and pre-diabetes are becoming more common as people consume more high-caloric foods and live unhealthy lifestyles [5]. About a third of the population is pre-diabetic and this condition generally goes unnoticed [1]. Pre-diabetes is anticipated to affect 482 million individuals globally by 2040, according to the IDF [2]. Obesity has shown to be a contributing factor in the development of insulin resistance and pre-diabetes [6]. Alterations to calcium homeostasis have been linked to abnormal blood glucose levels, insulin resistance, beta (β)-cell dysfunction and obesity [6, 7].

Calciotropic hormones such as parathyroid hormone (PTH), calcitonin, and calcitriol, are responsible for maintaining calcium homeostasis [7]. These calciotropic hormones act on calcium-regulating organs namely, the intestine, kidney and bone [7]. Studies have shown that calcium homeostasis is disturbed in T2DM [8, 9]. Several studies have shown changes to plasma calcium levels, calciotropic hormones, calcium transporters, bone turnover, intestinal absorption and the renal reabsorptive capacity of calcium in T2DM individuals [10, 11]. Obesity has shown to pose as a double burden in the development of pre-diabetes and calcium homeostatic dysfunction [4, 12]. Metabolic failure associated with obesity such as dysregulated adipokine levels and proinflammatory mediators have shown to play a role in calcium homeostatic dysfunction [13, 14]. Studies have shown that obesity disturbs calcium homeostasis by promoting secondary hyperparathyroidism, vitamin D deficiency and hypercalcaemia [15, 16]. Optimal levels of calcium are essential for proper functioning of insulin-responsive tissue, insulin secretion, nerve function, bone mineralization and hormone communication [17]. Processes that take place within the body that depend on calcium would become impaired if the calcium homeostasis is interrupted [17]. Studies conducted in our laboratory have developed a diet-induced pre-diabetic animal model which depicts the human condition of pre-diabetes [18, 19]. Several investigations using this model have shown that changes in T2DM frequently begin during the pre-diabetic stage [19, 20]. While the alterations that occur to calcium homeostasis in the diabetic state have been well documented, the changes that occur to calcium homeostasis in pre-diabetes are not known [21, 22]. Hence, this study sought to investigate the changes to calciotropic hormones and calcium-regulating organs in the pre-diabetic state using a diet-induced pre-diabetic rat model.

2. Calcium homeostasis

Calcium is the fifth most common element in the human body with majority stored as hydroxyapatite in bone [10]. The minority of calcium is found extracellular in free form, bound to protein and a small percentage bound to anions in plasma [7]. The normal serum calcium concentration is 2- 2.5 mmol/L and the normal urinary calcium concentration is 15-20 mmol/L [23]. Derangements to plasma calcium levels lead to conditions such as hypocalcaemia or hypercalcaemia [9]. Calcium is responsible for many physiological processes such as neuromuscular transmission, muscle contraction and nerve function [10]. It acts as a co-factor during blood coagulation and is responsible for the release of neurotransmitters and hormones [24]. PTH, calcitonin and calcitriol act on the intestine, kidney and bone to regulate plasma calcium levels [25]. The following section describes the physiological role of calciotropic hormones in the maintenance of calcium homeostasis.

2.1. Calciotropic hormones

Plasma calcium concentration is controlled by calciotropic hormones to ensure that there is proper calcium absorption in intestine, its storage in bones and kidney elimination of excess calcium [26]. There are three main calciotropic hormones involved in the regulation of blood calcium levels, namely PTH, calcitonin and active vitamin D [10].

2.1.1. The physiological role of PTH

The 84-amino-acid peptide generated by the chief cells of the parathyroid glands and circulates in the blood for 2-3 minutes is known as parathyroid hormone (PTH) [27]. It is produced in response to a low calcium concentration in the blood, with a normal range of 10-65 pg/mL [7]. The primary role of PTH is to increase blood calcium levels by promoting increased bone resorption, intestinal calcium absorption and renal calcium reabsorption [28].

PTH concentration rises in response to low blood calcium levels, which stimulates bone resorption [29]. It reduces the metabolic activity of osteoblasts (bone-forming cells) while activating osteoclasts (bone-resorbing cells) to cause bone to break down and calcium to enter circulation [25]. Parathyroid hormone is indirectly responsible for promoting an increase in intestinal calcium absorption by activating hepatic 25-hydroxylase and renal 1-alpha hydroxylase to produce calcitriol [30]. Calcitriol promotes an increase in intestinal calcium absorption by increasing the production of calcium transport proteins such as calbindin-D_{9k} [30]. PTH increases calcium reabsorption from the filtrate in the distal convoluted tubule of the kidney [28]. Due to the various actions of PTH on calcium-regulating organs, it may be used as a marker to study calcium homeostasis.

2.1.2. The physiological role of calcitonin

The parafollicular cells of the thyroid glands produce a 32 amino acid peptide known as calcitonin which circulates throughout the blood for 10.2-37.8 minutes [31]. Calcitonin is a hormone that is secreted in response to elevated blood calcium levels and functions in opposition to PTH [32]. The main function of calcitonin is to lower blood calcium levels by decreasing calcium resorption in the bones, calcium absorption in the intestine and reabsorption from kidneys [27].

In response to high blood calcium levels, calcitonin concentration increases which decreases bone resorption [26]. It causes osteoclasts to contract which exposes high-affinity calcium-binding sites thus reducing the release of calcium into the blood [32]. It also causes the proliferation of osteoblasts which promotes bone mineralization [27]. Intestinal calcium absorption takes place in the active transcellular route and the passive paracellular route [32]. Calcitonin inhibits calcium absorption at the transcellular route which reduces serum calcium concentration [31]. It also inhibits calcium reabsorption at the distal convoluted tubule in the kidney by reducing permeability into tissue resulting in increased calcium urinary output [27]. Due to the various actions of calcitonin on calcium-regulating organs, it may be used as a marker to study calcium homeostasis.

2.1.3. The physiological role of vitamin D

Vitamin D is obtained from supplementation, diet and sunlight [33]. The normal serum vitamin D concentration is between 9.7- 41.7 mg/mL and it is converted to its active form when stimulated by PTH or low blood calcium [28]. It is hydroxylated in the liver to calcifediol through the action of 25-hydroxylase followed by hydroxylation in the kidney through the action of 1-alpha hydroxylase to 1,25-dihydroxyvitamin D3 also known as calcitriol [33]. The basic function of active vitamin D is to raise blood calcium levels by promoting increased bone resorption, intestinal calcium absorption, and kidney calcium reabsorption [7].

Calcitriol enhances calcium release from the bone by causing osteoblasts to secrete osteoclast differentiation factor, which stimulates osteoclast activity [30]. While calcitriol is required for bone production, it is also required for bone resorption, without it, the levels of calcium within the blood are too low for normal bone formation [34]. Calcitriol also increases the production of calcium transport proteins such calbindin-D_{9k}, which promotes an increase in intestinal calcium and phosphorus absorption [30]. It functions by increasing calcium reabsorption in the renal distal tubule, which reduces calcium loss in the urine [7]. Due to the various actions of calcitriol on calcium-regulating organs, it may be used as a marker to study calcium homeostasis. The following section describes the role of calcium-regulating organs in the maintenance of calcium homeostasis.

2.2. Calcium-regulating organs

Calciotropic hormones primarily target the small intestine, bone and kidney [9]. Some of the processes that take place within these organs include gastrointestinal calcium absorption, renal calcium reabsorption and deposition into or removal of calcium from bone [35]. The following section describes the molecular mechanisms of intestinal calcium absorption, calcium reabsorption in the kidneys and bone resorption.

2.2.1. The role of the intestine in calcium homeostasis

Calcium absorption in the intestine is crucial for calcium homeostasis to be maintained and is stimulated by PTH and calcitriol [7]. The ileum absorbs 88% of calcium, the duodenum absorbs 8%, and the jejunum absorbs 4% [36]. Intestinal epithelial cells absorb calcium through two major pathways, namely the transcellular and paracellular pathways [37]. Transcellular calcium absorption is dependent on hormonal regulation and occurs through three steps as depicted in Fig. 1 below [38].

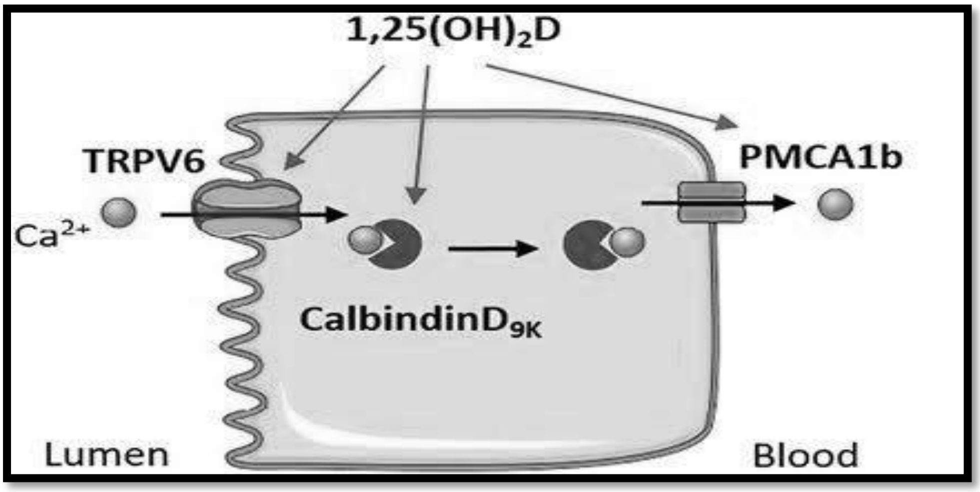

Figure 1: Mechanism of calcium absorption in the small intestine mediated by calcitriol adapted from Corbeels *et al.,* 2018 [38]

Firstly, calcium enters the enterocyte with aid from vitamin D through calcium channels in the apical membrane, such as transient receptor potential vanilloid subfamily member 6 (TRPV6) [37]. Thereafter, calbindin-D_{9k} a calcium-binding protein binds to calcium and diffuse calcium across the cytoplasm, followed by the extrusion of calcium via the plasma membrane Ca^{2+} ATPase (PMCA) dependent pump across the basolateral membrane into circulation [39]. This process is regulated by calcitriol which activates genes responsible for increasing the expression of calcium transporters, calbindin-D as well as increases the activity of the Ca^{2+} -ATP dependent pump as per Fig.2 below [40].

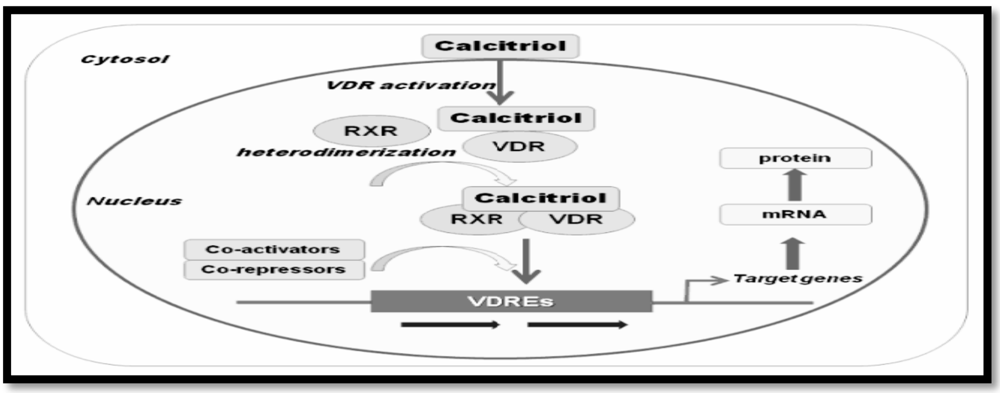

Figure 2: Mechanism of VDR action at target cells mediated by calcitriol adapted from Dominguez *et al.*, 2021 [40]

The vitamin-D receptor (VDR) is responsible for calcitriol's biological function [41]. The VDR is found in abundance in the kidney, small intestine and bone [39]. The VDR is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily [23]. Calcitriol is the natural ligand to VDR and through interacting with it, it has both genomic and non-genomic effects [42]. Low plasma calcium concentration stimulates the binding of calcitriol to VDR which enters the nucleus forming a heterodimer with retinoid X receptor (RXR) [39]. This complex regulates gene transcription by interacting with response elements in target promoters [39]. The non-genomic functions of the vitamin D receptor are mediated by the membrane-associated vitamin D receptor, which is activated by calcitriol through various confirmations [37]. The second major route is the paracellular pathway, which allows calcium to transverse the lateral intercellular space and is dependent on an electrochemical gradient [37]. However, since transcellular calcium transport is influenced by calciotropic hormones, it was evaluated in this study. Calcium transport in the intestine is essential in the conservation of plasma calcium levels and any deleterious effect to calcium transporters or VDR may impede calcium absorption [37]. Furthermore, renal calcium reabsorption also occurs via the paracellular and transcellular routes.

2.2.2. The role of the kidney in calcium homeostasis

The kidney plays an important role in expelling and retaining calcium in the body [43]. The renal glomerulus filters roughly 50% of plasma calcium, with the renal tubules reabsorbing 99% of the filtered calcium [9]. Renal calcium reabsorption occurs via the paracellular and transcellular pathways in the proximal tubules, thick ascending limbs of Henle (TALH) and distal tubules [43] The paracellular pathway is controlled by the extent of concomitant sodium reabsorption, whereas the transcellular pathway is regulated by calciotropic hormones [43]. When plasma calcium levels are high, the calcium-sensing receptor in the TALH senses the disturbance and inhibits renal calcium reabsorption [43]. However, low plasma calcium levels stimulate PTH and calcitriol mediating an increase in renal calcium reabsorption [43]. Calcium enters through the TRPV5 channels which thereafter binds to calcium-binding proteins and diffuses into the cytoplasm [43]. Thereafter, calcium is extruded into the bloodstream through the calcium-ATPase and sodium-calcium exchanger [43]. Since transcellular calcium transport is influenced by calciotropic hormones, it was evaluated in this study. Furthermore, the final step in the synthesis of calcitriol occurs in the kidney [9]. Renal 1-alpha hydroxylase is an enzyme that catalyses' the conversion of 25-hydroxyvitamin D to calcitriol and is found primarily in the proximal tubule [9]. In the renal distal tubule, calcitriol interacts with vitamin D receptor (VDR) to impact the transcription of genes involved in the upregulation of calcium transport proteins, such as transient receptor potential vanilloid subfamily member 5 (TRPV5) and calbindin-

D_{28k} [43]. When calcitriol is no longer needed, the enzyme 24-hydroxylase which is located in calcium-regulating organs catabolizes calcitriol to its inactive form [39]. Calcium transport in the kidney is essential in the conservation of plasma calcium levels and any deleterious effect to renal 1-alpha hydroxylase or calcitriol may impede renal calcium reabsorption [10].

2.2.3. The role of the bone in calcium homeostasis

The main storage site for calcium is the bone and it maintains calcium homeostasis by allowing for the release and uptake of calcium under the influence of calciotropic hormones [7]. Old, weakening bone is removed through bone resorption and replaced by new bone through bone formation in the process of bone turnover [44]. The bone resorption rate should be equivalent to the bone production rate in healthy, mature bone [44]. When bone resorption exceeds bone formation conditions such as osteoporosis and bone weakness develop [17]. Low levels of plasma calcium stimulate the release of PTH which in turn stimulates the release of calcitriol [9]. Both these hormones promote bone resorption by increasing the activity of osteoclasts [44]. The breakdown of hydroxyapatite causes calcium to be released during bone resorption [9]. When PTH and calcitriol bind to their receptor, they cause a change to the genomic activity of bone-forming cells directing them to promote resorption through the secretion of a variety of cytokines [45]. Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) are two key cytokines that bind to the receptor activator of nuclear factor kappa B on osteoclasts and stimulate their activity [7]. Activated osteoclasts degrade bone by secreting hydrochloric acid and proteases which promote the release of calcium from bone [36]. However, high levels of plasma calcium decrease PTH and stimulate the release of calcitonin [44]. Calcitonin decreases osteoclastic activity and the formation of new osteoclasts resulting in decreased bone resorption [7]. During bone resorption markers such as deoxypyridinoline are released and during bone formation markers such as osteocalcin are released into circulation [17]. Studies have used plasma osteocalcin as a marker of bone formation due to its strong association with bone formation [46, 47]. Furthermore, plasma osteocalcin is the preferred marker of bone formation compared to other bone formation markers due to its greater sensitivity in the detection of low bone formation rates and minimal within-person variation [48]. In addition, studies have widely accepted the measurement of urine deoxypyridinoline as the preferred marker of bone resorption because it is not influence by diet and is not extensively metabolised by the liver [49, 50]. Therefore, the levels of osteocalcin and deoxypyridinoline may serve as indicators of bone turnover. The following section describes type 2 diabetes mellitus; its diagnosis, prevalence and associated complications.

3. Type 2 diabetes mellitus (T2DM)

Type 2 diabetes is associated with hyperglycaemia and is caused by either insulin insufficiency or insulin resistance [1]. Approximately one in eleven of the world’s population are diagnosed with diabetes with majority been T2DM [51]. An oral glucose tolerance test (OGTT) , a fasting glucose test, post-prandial glucose test or glycated haemoglobin (HbA1c) test can be used to diagnose pre-diabetes [50]. Individuals are diagnosed with T2DM when fasting blood glucose (FBG) levels are ≥ 7 mmol/L, glucose concentrations in the postprandial state are ≥ 11.1 mmol/L and glycated haemoglobin concentrations are ≥ 6.5% [51]. Renal failure, heart disease, and bone weakness are among the microvascular and macrovascular problems associated with T2DM [52, 53]. Over the last few decades, it has been noticed that in the diabetic condition there is derangement to calcium homeostasis, particularly relating to changes in calciotropic hormone secretion and poor functioning of calcium-regulating organs [17, 50, 54]. Thus, in this study we are interested in investigating whether the above-mentioned changes occur in the pre-diabetic state. The following section describes the changes to calciotropic hormones in T2DM.

3.1. Effects of T2DM on calciotropic hormones

There have been several studies that have shown changes to calciotropic hormones in T2DM [10, 35, 55]. Alterations to calciotropic hormone concentrations may be due to pathological changes that occur to the organs that synthesis these hormones or as a compensatory response to disturbed plasma calcium levels in T2DM [8]. Furthermore, derangements to calciotropic hormone levels may exacerbate hyperglycaemia and insulin resistance in diabetes [8]. The section below describes the effects of T2DM on plasma PTH, calcitonin and vitamin D concentrations.

3.1.1. Effects of T2DM on PTH

Some studies have shown that plasma PTH concentrations in type 2 diabetic individuals were significantly higher as compared to non-diabetic individuals [56, 57]. The high PTH levels in T2DM were suggested to be a compensatory response to hypocalcaemia, vitamin D deficiency and kidney diseases [56, 57]. Studies have reported that increased PTH secretion may contribute to the maintenance of normocalcaemia in T2DM [56, 57]. However, the increased secretion of PTH in response to renal dysfunction in diabetes may lead to hyperparathyroidism [58]. Furthermore, studies have shown increased bone breakdown and decreased bone formation in diabetes [59, 60]. It was stated that increased PTH-induced bone resorption as a result of compensation contributes to bone weakness [17]. On the contrary, other studies have reported decreased plasma PTH concentration in patients with T2DM when compared to non-diabetics [34, 61]. Studies showed that hyperglycaemia in T2DM inhibits the secretion of PTH due to the accumulation of reactive oxygen species in the parathyroid glands [34, 62]. Low PTH concentration results in reduced bone turnover, decreased intestinal calcium absorption and decreased calcitriol production in the kidney [26]. Hypocalcaemia is commonly a consequence of PTH and calcitriol deficiency [26]. Studies have shown that low plasma PTH levels and the prevalence of hypocalcaemia in T2DM patients were associated [26, 35].

Interestingly, studies have shown that PTH concentrations in plasma were positively associated with hyperglycaemia and insulin resistance in T2DM [50, 63]. It was stated that elevated plasma PTH concentration in diabetes may worsen and contribute to the development of hyperglycaemia [17]. Elevated parathyroid hormone has shown to decrease insulin dependent glucose transport thereby inducing insulin resistance [55]. Reduced insulin receptor substrate (IRS-1) and glucose transporter type 4 (GLUT-4) protein expressions, as well as enhanced phosphorylation of IRS-1 on serine307, were seen as a result of increased plasma PTH [55]. Furthermore, elevated PTH levels have shown to increase plasma glucose levels by stimulating hepatic gluconeogenesis and glycogenolysis [55]. These cellular events in adipocytes may underlie the association of high plasma PTH levels with hyperglycaemia and insulin resistance. Hence, the changes to plasma PTH levels and its association with glycated haemoglobin and insulin resistance in pre-diabetes were investigated in this study.

3.1.2. Effects of T2DM on calcitonin

Some studies have shown no significant change to plasma calcitonin concentration in patients with T2DM when compared to non-diabetics [64, 65]. Another study has shown that plasma calcitonin concentrations in T2DM rats were significantly higher as compared to non-diabetic rats [66]. Studies have stated that high calcitonin levels may be a recovery mechanism against bone loss and elevated PTH levels induced by hyperglycaemia [31, 66]. In T2DM-induced hyperparathyroidism, the increased calcitonin compensates for hypercalcaemia by decreasing osteoclastic bone resorption while activating osteoblasts to promote bone mineralization [26]. Furthermore, calcitonin inhibits intestinal absorption and kidney reabsorption of calcium which serves to decrease plasma calcium concentration [31]. However, pathologically excessive calcitonin secretion in T2DM may be involved in the significant reduction of blood calcium levels observed in some T2DM individuals [31].

Furthermore, studies have shown that plasma calcitonin levels were positively correlated with HbA1c and insulin resistance in T2DM [55, 67]. It was shown that increased calcitonin concentration in diabetes may lead to insulin resistance and hyperglycaemia [23]. High levels of plasma calcitonin impair insulin sensitivity in adipose and muscular tissue [23]. Calcitonin enhances calcium entry inside the cell thus increasing intracellular calcium concentration [31]. This triggers calcium release from depots that inhibit insulin-stimulated mobilization of glucose transporter type 4 (GLUT-4) to the membrane of the cell [43]. Furthermore, studies have shown that calcitonin promotes hyperglycaemia by intensifying hepatic gluconeogenesis and glycogenolysis [60, 65]. These cellular events in skeletal muscle cells and adipocytes may underlie the association of high plasma calcitonin levels with HbA1c and insulin resistance. Hence, the changes to plasma calcitonin and its association with HbA1c and insulin resistance in the pre-diabetic state were investigated in this study.

3.1.3. Effects of T2DM on vitamin D

Studies have shown significantly lower vitamin D and calcitriol concentrations in type 2 diabetic humans and rats as compared to the non-diabetic group [33, 68]. These studies have reported that impaired intestinal vitamin D absorption and increased adipose tissue vitamin D sequestration may account for the decreased plasma vitamin D and calcitriol concentrations seen in T2DM patients [69, 70]. Furthermore, renal dysfunction in T2DM has shown to downregulate renal 1-alpha hydroxylase expression and reduces the kidney's responsiveness to PTH, accounting for a decrease in calcitriol production [69, 70]. A deficiency in plasma calcitriol reduces renal calcium reabsorption and absorption of calcium in the intestine [30]. This is evidenced by previous studies that have reported low calcitriol levels with increased urinary excretion of calcium in T2DM [69, 70].

According to studies, vitamin D deficiency has been associated with increased glycated haemoglobin and insulin resistance in T2DM patients [39, 55]. Furthermore, previous studies have found that elevated plasma calcitriol levels were associated with hyperglycemia and insulin resistance [39, 55]. A deficiency of vitamin D leads to impaired ability of the pancreatic beta-cells to secrete insulin in response to glucose [33]. It also contributes to insulin resistance by increasing the calcium concentration in insulin-responsive tissue which decreases GLUT-4 activity [42]. Furthermore, vitamin D and calcitriol have shown to ameliorate hepatic glucose metabolism and lipid metabolism as well as promote pancreatic islet survival [69, 70]. Studies reported that the changes to vitamin D and calcitriol may worsen and contribute to hyperglycaemia in T2DM [69, 70]. Hence, the changes to plasma vitamin D, calcitriol and its association with pre-diabetes were examined in this study. The following section describes the effects of T2DM on the functioning of calcium-regulating organs.

3.2. Effects of T2DM on calcium-regulating organs

Several investigations have indicated that T2DM causes alterations in calcium-regulating organs [10, 35, 55]. The functioning of calcium-regulating organs in T2DM is disturbed, evidenced by intestinal calcium malabsorption, renal calcium wasting and bone deterioration [8]. The section below describes the effects of T2DM on intestinal calcium absorption, renal calcium reabsorption and bone turnover.

3.2.1. Effects of T2DM on intestinal calcium absorption

Previous studies have shown reduced calcium absorption in the intestines of T2DM patients [71, 72]. This occurred concurrently with decreases in plasma calcitriol levels, cytoplasmic calcium-binding proteins such as calbindin-D_{9k} and intracellular vitamin D receptor in enterocytes [17, 73]. Studies have stated that hyperglycaemia in T2DM has shown to downregulate intestinal calcium-binding proteins contributing to impaired intestinal calcium absorption [17, 73]. Efficient intestinal calcium absorption is dependent on adequate plasma calcitriol and vitamin D levels [74]. Studies have shown that abnormal vitamin D metabolism in T2DM impairs the ability of the intestine to absorb sufficient calcium [9, 75]. Impaired intestinal calcium absorption contributes to the development of hypocalcaemia [9, 75]. High levels of fructose commonly found in westernized diets make people susceptible to intestinal calcium malabsorption [72]. Diets that contain high fructose have shown to decrease active intestinal calcium transport and the levels of calcitriol [71, 72]. High fructose has shown to decrease intestinal calcium transport by downregulating the expression of VDR and calbindin-D_{9k} [74]. Furthermore, high dietary fructose has shown to increase 24-hydroxylase and decrease 1-alpha hydroxylase expression [39]. It was reported that fructose may have impaired intestinal calcium absorption by enhancing the catabolism of calcitriol while impairing its synthesis [71]. Furthermore, the physiological actions exerted by calcitriol are determined by its interaction with the VDR [41]. A reduction in intestinal VDR expression in diabetes can alter the sensitivity of enterocytes to calcitriol and reduce intestinal calcium absorption [39]. However, some studies have shown an upregulation in the expression of intestinal VDR and calbindin-D_{9k} expression in T2DM [74, 76]. An increase in intestinal calcium absorption was found to compensate for hypocalcaemia in diabetes patients [39]. Since, intestinal calbindin-D_{9k} and VDR participate in intestinal calcium transport; they may serve as markers of intestinal calcium absorption [74, 76]. While all these changes have been noted in T2DM, this is not known for the pre-diabetic state. Hence, the expression of intestinal VDR and calbindin-D_{9k} expression in the pre-diabetic state was investigated in this study.

3.2.2. Effects of T2DM on renal calcium reabsorption

Renal calcium wasting is a result of long-term hyperglycemia and T2DM-related disruption of the metabolism of calciotropic hormones [8, 17]. The mechanisms responsible for renal calcium wastage in diabetes have been extensively reviewed in literature [77, 78]. It was shown that a specific renal tubular defect in calcium reabsorption and disturbances to vitamin D metabolism led to calcium loss [77, 78]. Previous studies have shown decreased renal calcium reabsorption in T2DM which occurred concurrently with decreases in renal TRPV5 expression and renal 1-alpha hydroxylase [79, 80]. It was stated that hyperglycaemia in T2DM has shown to impair renal calcium reabsorption by downregulating the expressions of calcium transport channels and proteins [77, 78]. In addition, it was shown that the diabetic kidney may be resistant to the effects of calcitriol on the regulation of calcium channels [79]. Furthermore, renal dysfunction in T2DM has shown to impair calcitriol production in the kidney by downregulating renal 1-alpha hydroxylase expression [79]. Calcitriol deficiency impairs renal calcium reabsorption which leads to renal calcium wastage [81]. However, interestingly other studies have shown an increase in renal calcium reabsorption evidenced by an upregulation in renal TRPV5 and 1-alpha hydroxylase expression in T2DM [77, 78]. It was reported that the simultaneous increase in renal TRPV5 and 1-alpha hydroxylase suggested a compensatory response to renal wastage and thus may serve as a marker for the detection of renal calcium wasting [77, 78]. While changes to these markers have been noted in T2DM, this has not been investigated in the pre-diabetic state. Hence, the expression of renal TRPV5 and 1-alpha hydroxylase in the pre-diabetic state was investigated in this study.

3.2.3. Effects of T2DM on bone turnover

Several studies have shown that bone resorption exceeds the rate of formation in type 2 diabetes patients [82, 83]. This was evidenced by elevated urinary deoxypyridinoline concentrations and decreased plasma osteocalcin concentrations [59, 60]. Hyperglycaemia induces reactive oxygen species (ROS) production in osteoblasts [50]. This promoted apoptosis of osteoblasts, depressed bone mineralization and differentiation of osteoblasts [84]. Furthermore, studies have shown that during T2DM there is an increased demand to conserve plasma calcium levels as a result there is increased bone resorption [59, 60]. In addition, hyperparathyroidism in T2DM has shown to promote excessive bone resorption despite normal plasma calcium levels [59, 60]. Excessive breakdown of bone and depressed formation leads to bone weakness and increased risk of fractures [85, 86].

However, other studies have shown decreased bone resorption followed by increased bone formation in T2DM [82, 87]. This was evidenced by decreased urinary deoxypyridinoline concentrations and increased plasma osteocalcin concentrations [82, 87]. These studies have stated that hyperinsulinemia in T2DM has anabolic effects on bone [48]. Insulin has shown to promote formation of bone and inhibit resorption of bone by promoting osteoclast differentiation into osteoblasts [48]. Furthermore, studies have shown associations between increased plasma osteocalcin levels with hyperglycaemia and insulin resistance [88, 89]. It was shown that elevated plasma osteocalcin in diabetes ameliorates hyperglycaemia and insulin resistance [87]. Osteocalcin increases insulin sensitivity in muscle, stimulates insulin secretion in the pancreas promotes pancreatic beta-cell proliferation and adiponectin secretion in adipose tissue [87]. While all these changes have been noted to calcium-regulating organs in T2DM, studies have not elucidated the changes that occur to the functioning of these organs during the pre-diabetic state. Hence, bone turnover was measured in this study by determining the concentration of osteocalcin and deoxypyridinoline in the pre-diabetic state. T2DM is often preceded by pre-diabetes; the following section describes pre-diabetes and the high-fat high-carbohydrate (HFHC) diet-induced model of pre-diabetes.

4. Pre-diabetes

Pre-diabetes is frequently undetected and occurs before the development of T2DM [2]. It is an intermediate hyperglycaemia due to glucose tolerance and the fasting glucose concentrations being impaired [2]. It is a condition where the glucose concentrations are greater than the homeostatic range but not high enough to be classified as diabetes [21]. The global prevalence of pre-diabetes in 2017 was 352.1 million which is expected to increase by 8.3% in 2045 [90]. Individuals at risk of developing T2DM have one or more characteristics of pre-diabetes, such as 5.5-6.9 mmol/L impaired fasting glucose, 7.8-11.1 mmol/L impaired glucose tolerance (IGT) and 5.7-6.4 percent glycated haemoglobin (Hb1Ac), according to the American Diabetes Association (ADA) [90]. The HbA1c test measures the amount of haemoglobin that is irreversibly attached to glucose [4]. The Hb1Ac test is used for the diagnosis of pre-diabetes as it provides the average blood glucose levels over the past two to three months [50]. Insulin resistance and dysfunction of pancreatic beta-cells precedes pre-diabetes diagnosis [21].

Obesity, sedentary lifestyle and diets rich in saturated fat and carbohydrates has shown to promote the development of moderate hyperglycaemia [90]. High caloric diets have shown to increase triacylceride and free fatty acid exposure to insulin-dependent tissue promoting insulin resistance [50]. In the insulin-resistant state, standard plasma insulin would fail to stimulate a response in the insulin-targeted peripheral tissue [91]. As a result, β-cells of the pancreas respond by secreting more insulin in order to counteract the elevated glucose levels [92]. When the β-cells are unable to release enough insulin to compensate for the insulin resistance, blood glucose levels begin to fluctuate [91]. This leads to hyperinsulinemia and glucotoxicity which creates an unfavourable environment leading to alterations in β-cell function [93]. Obesity contributes to the development of pre-diabetes but also causes a disturbance to calcium homeostasis [94]. Obesity is defined by hypertrophied adipocytes with a dysregulated adipokine secretion profile, increased inflammatory cell recruitment and impaired metabolic homeostasis, which leads to insulin resistance and calcium homeostasis disturbances [95]. Obesity is caused by an imbalance between food intake and energy expenditure, resulting in an increased accumulation of adipose tissue [95]. Proinflammatory substances released by adipose tissue can make the body less sensitive to the insulin it generates by altering the functioning of insulin-responsive cells and their ability to respond to insulin, contributing to the development of pre-diabetes [96]. Previous studies have linked obesity to disturbances to calcium homeostasis by promoting vitamin D insufficiency, secondary hyperparathyroidism and hypercalcaemia [15, 97]. According to studies, vitamin D is sequestered in adipose tissue due to its hydrophobic nature, resulting in decreased plasma vitamin D bioavailability [98, 99]. Furthermore, studies have also shown that plasma PTH levels increase to compensate for low plasma calcium levels induced by decreased circulating vitamin D levels [98, 99]. Vitamin D inhibits PTH secretion from the parathyroid glands, hence a deficit of vitamin D could lead to excessive PTH secretion in humans [99]. Furthermore, obesity-associated increases in adipokines and proinflammatory mediators have been linked to increased bone resorption [100]. As a result, obese patients have greater plasma calcium levels while also having higher plasma triglyceride levels than non-obese people [100]. Interestingly, current studies have shown that obesity was linked to interferences with intestinal calcium absorption [100, 101]. Free fatty acids have been demonstrated to create insoluble calcium soaps that are unabsorbable and hence contribute to reduced intestinal calcium absorption [102]. These previous findings have shown that pre-diabetic individuals are faced with the double burden of obesity which makes them susceptible to disturbances to calcium homeostasis.

Furthermore, pre-diabetes is a risk factor for the development of overt T2DM as well as several comorbidities related to T2DM [90]. Pre-diabetes has been linked to a 15% greater risk of acquiring cardiovascular diseases over a 10-year follow-up period, according to studies [103]. Furthermore, a study found that pre-diabetic patients had a three-fold increased risk of myocardial infarction than people who are normally glucose tolerant [103]. Microvascular problems seen in T2DM, such as retinopathy, neuropathy, and nephropathy, have been described in the pre-diabetic state as well [3]. A study had estimated the prevalence of microalbuminuria among patients with pre-diabetes to be 15.5% [90]. Since T2DM-associated complications begin before the onset of diabetes mellitus (DM), it is essential that the pre-diabetic condition is more extensively looked at to prevent some of the common complications experienced in T2DM. Furthermore, there is insufficient research done in the pre-diabetic state especially with regards to the changes to calcium homeostasis. Hence, this study sought to establish the changes to calcium homeostasis using a HFHC-diet-induced pre-diabetic animal model.

5. High-fat high-carbohydrate (HFHC) diet-induced model of pre-diabetes

Animal models are commonly used in diabetes research with rodents mostly used in this regard [104]. Animal models have been crucial in the study of physiological and pathophysiological states in research [5, 104]. Animal models have been found to resemble human disease conditions and are thus widely used to research physiological systems and disease states in humans [105]. Genetically induced models and diet-induced models are two types of animal models used to study T2DM [104, 106]. The Zucker fatty rat, the Kuo Kondo (KK) mouse and the db/db mouse are genetically engineered animal models that acquire T2DM symptoms as a result of a satiety hormone deficiency that leads to hyperphagia and obesity [104, 106]. Diet-induced animal models of T2DM include obesogenic diets which contain high calories, fat, sugar and carbohydrate content [104, 106]. Studies have shown that these diets induce T2DM by promoting insulin resistance, obesity and dyslipidemia [5, 104]. Male Sprague Dawley rats are employed in diabetes studies due to their genetic variability, which matches the human condition of T2DM [5, 104]. Furthermore, male Sprague Dawley rats were preferred over the use of female rats in diabetes research due to several factors [107]. Male rats have a more stable hormonal profile in comparison to female subjects [108]. Due to change of hormones during the menstrual cycle, female rats were not used [108]. This study focused on pre-diabetes which includes the early development of insulin resistance. Male rats tend to develop more pronounced insulin resistance whilst females show a greater loss of insulin release and beta cell mass [109]. Furthermore, female rats develop obesity over a longer duration of time than male rats [109].

Studies have demonstrated that diet-induced diabetes models are better suited as a model for pre-diabetes in humans because of their strong link with obesity [5, 104]. Several studies have found that a high fat diet (HFD) and high carbohydrate diet (HCD) can cause pre-diabetes, as evidenced by moderate hyperglycemia and an aberrant lipid profile [5, 104]. Obesity induced by diets high in fat and carbohydrates have shown to be associated with metabolic failure, multiple endocrine alterations and changes in the concentration of circulating hormones[110]. Obesity-induced metabolic failure promotes cellular senescence through cell-cycle arrest and proinflammatory reaction [111]. Several studies have shown that excess adiposity has been linked to disturbed to calcium homeostasis through reduced circulating vitamin D levels [98, 99]. Metabolic failure associated with obesity has shown to decrease the synthesis of vitamin D due to cell senescence [96]. The intake of HFHC diets highlights the double burden of obesity and calcium homeostatic dysfunction [96]. Furthermore, studies have shown that inducing pre-diabetes with a combination of both high fat and high carbohydrate content is an efficient way to induce pre-diabetes and best describes the clinical manifestations of the human condition of pre-diabetes [5, 104]. Interestingly, studies have confirmed that the combined effect of surplused fat and carbohydrates added to diets supplemented with 15% fructose presented the most severe symptoms such as hyperglycaemia, insulin resistance, hypercholesterolaemia and higher inflammatory mediators levels in comparison to each diet used separately [5, 106]. Sprague Dawley rats fed HFHC supplemented with 15% fructose had deranged fasting glucose concentrations and glucose tolerance after week 20, according to several studies conducted in our laboratory [5, 104]. Furthermore, studies in our laboratory using this model have shown that many of the complications associated with T2DM begin in the pre-diabetic state [5, 104]. This model was used to study renal function and the renin-angiotensin aldosterone system (RAAS) and found that renal dysfunction and activated RAAS begins in the pre-diabetic state [19-22, 112]. Furthermore, another study using this model had shown that immune changes in T2DM begin in the pre-diabetic state [5, 104]. This warrants that use of the HFHC-diet induced pre-diabetic animal model in this study to investigate the changes to calcium homeostasis in the pre-diabetic state.

6. Basis of the study

Bidirectional calcium fluxes, which occur in the intestine, bone and kidney, regulate calcium homeostasis [17]. As the prevalence of T2DM increases worldwide, there is an urgency to stop T2DM development. Pre-diabetes is asymptomatic and as a result many pre-diabetic individuals progress towards the development of T2DM and develop its associated complications because they are unaware that they have pre-diabetes [90]. Research on pre-diabetes is important as it is a reversible stage and may aid in the prevention of T2DM [90]. Several studies have shown that T2DM disturbs calcium homeostasis by disrupting the functioning of calciotropic hormones and calcium-regulating organs; however, this is not known for the pre-diabetic state [10, 11]. Furthermore, altered levels of calciotropic hormones have shown to exacerbate hyperglycaemia as well as lead to pathological changes to calcium-regulating organs. Investigating calcium homeostasis in the pre-diabetic state may help researchers to understand whether these changes begin before overt T2DM. By understanding the changes that occur to calcium homeostasis in pre-diabetes, we may be able to target and prevent the processes that contribute to T2DM-related complications. Novel therapeutic drugs may be created to alleviate conditions such as hypocalcaemia, osteoporosis and insulin resistance by targeting processes involved in calcium homeostasis. This study utilized the HFHC diet to induce intermediate hyperglycaemia in rats in order to study calcium homeostasis. The HFHC was chosen based on the similarity to the metabolic manifestation of the human condition of pre-diabetes. Hence, this study was conducted based on that no studies have addressed the changes to calcium homeostasis during diet-induced pre-diabetes. The markers chosen to be investigated in this study are based on literature findings mentioned above and their involvement in maintaining calcium homeostasis. Findings, therefore, will possibly reveal whether changes to calcium homeostasis begin in the pre-diabetic state. This study is divided into two manuscripts, one which focuses on the changes to calciotropic hormones and the other on the changes to the functioning of calcium-regulating organs.

7. Aim

To investigate the effects of diet-induced pre-diabetes on calciotropic hormones and the functioning of calcium-regulating organs in a pre-diabetic rat model, in attempt to ellucidate the changes to calcium homeostasis in the pre-diabetic state.

8. Objectives

Study 1.

- To measure HbA1c, plasma insulin and glucose, to determine insulin resistance
- To measure plasma and urinary calcium concentration in the pre-diabetic (PD) and non-pre-diabetic (NPD group), to determine the body's overall calcium status
- To measure plasma parathyroid hormone, calcitonin, calcitriol and vitamin D concentrations in the PD and NPD group, to determine the levels of calciotropic hormones
- To determine the association between calciotropic hormone levels with HbA1c and HOMA-IR

Study 2.

- To measure plasma fasting glucose, insulin and glucose tolerance, to determine glucose homeostasis
- To measure plasma and urine calcium in the PD and NPD group, to determine calcium status
- To quantify bone turnover by-products (deoxypyridinoline and osteocalcin) in the PD and NPD group, to evaluate bone function
- To determine the expression of intestinal calbindin-D_{9k} and VDR mRNA expression in the PD and NPD group, to evaluate intestinal calcium transport function
- To determine the expression of renal 1-alpha hydroxylase mRNA expression in the PD and NPD group, to evaluate kidney function
- To determine the expression of renal TRPV5 mRNA expression in the PD and NPD group, to evaluate kidney calcium transport

9. Hypothesis

During the pre-diabetic state there will be changes to calciotropic hormones and calcium-regulating organs, indicative of disturbed calcium homeostasis

10. Laboratory methods

All laboratory methods involved in this study are detailed in the respective manuscripts within the dissertation.

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CHAPTER 2: MANUSCRIPT 1

PROLOGUE

The habitual consumption of high-caloric diets have been associated with the development of type 2 diabetes (T2DM). The failure of a multitude of organ systems and physiological functions, including calcium homeostasis, has been related to T2DM. The same high caloric diets have shown to instigate the development of pre-diabetes, a moderate hyperglycaemic state which precedes the onset of T2DM. Furthermore, derangements observed in T2DM such as insulin resistance and hyperglycaemia have shown to be linked to disturbances to calciotropic hormones. Thus, a thorough assessment of previous literature was carried out to determine the effects of T2DM on calcium homeostasis with regards to the changes to calciotropic hormones. However, there is no scientific findings that show the changes to calciotropic hormones in the pre-diabetic state. Therefore, chapter 2 was conducted to investigate the effects of diet-induced pre-diabetes on calciotropic hormones to establish the physiological functions in a diet-induced pre-diabetic animal model.

The manuscript in Chapter 2 is titled **"Investigating the effects of diet-induced pre-diabetes on calciotropic hormones in male Sprague Dawley rats"** and is authored by K Naidoo, Ngubane PS and Khathi A.

The manuscript is currently under review in the **Journal of Experimental Clinical Endocrinology and Diabetes** and has been formatted according to the journals guidelines for authors. See Appendix 2

Investigating the effects of diet-induced pre-diabetes on calciotropic hormones in male Sprague Dawley rats

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Abstract

Calcium homeostasis is disturbed emanating from altered calciotropic hormone concentrations in type 2 diabetes mellitus (T2DM), a condition preceded by pre-diabetes. Disrupted calcium homeostasis has shown to promote insulin resistance and hyperglycaemia. However, the changes to calciotropic hormones in the pre-diabetic state is not known yet. Hence, this study investigated the effects of diet-induced pre-diabetes on calciotropic hormones in a pre-diabetic rat model. Furthermore, this study also sought to determine the association of calciotropic hormones with glycated haemoglobin (HbA1c) and insulin resistance during pre-diabetes. Male Sprague Dawley rats (n=12) were randomly assigned to one of the two groups (n=6, per group): pre-diabetic (PD) and non-pre-diabetic (NPD). Fasting blood glucose (FBG), insulin, HbA1c and the homeostatic model assessment for insulin resistance (HOMA-IR) in addition to urine calcium, plasma calcium, parathyroid hormone (PTH), calcitonin, vitamin D and calcitriol concentrations were analysed at week 20. Correlation analysis was performed to examine the associations of calciotropic hormones with HOMA-IR and HbA1c. The results demonstrated increased concentrations of HbA1c, FBG, insulin and HOMA-IR in the PD group by comparison to NPD. Furthermore, plasma PTH, calcitonin, urine calcium, calcitriol and vitamin D levels increased along with unchanged plasma calcium concentrations in the PD group by comparison to NPD. Plasma PTH and calcitonin levels were positively correlated with HbA1c but not with HOMA-IR in the PD group. In addition, plasma calcitriol levels were negatively correlated with HbA1c in the PD group. These observations suggest that calcium homeostasis is disturbed in diet-induced pre-diabetes but the body compensates for the changes by inducing an increase in calciotropic hormone levels. Furthermore, pre-diabetes may promote the development of hyperglycemia in T2DM through altering calciotropic hormone levels.

Keywords: Calcium homeostasis, calciotropic hormones, high-fat high carbohydrate diet, pre-diabetes

Introduction

Pre-diabetes is a state that exists between normoglycaemia and T2DM, and it occurs before T2DM develops [1]. This condition is characterised by impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and elevated glycated haemoglobin (HbA1c) concentrations [2]. Pre-diabetes and type 2 diabetes are linked to sedentary lifestyles and the consumption of high-fat and high-carbohydrate diets [3]. The International Diabetes Federation (IDF) estimates that by 2030 over 578 million people worldwide are expected to have diabetes, while it is further anticipated that over 470 million people would be pre-diabetic by 2050 [4]. A positive correlation has been noted between altered calcium homeostasis, abnormal blood glucose levels, insulin resistance and β-cell dysfunction [2].

Calcium homeostasis is regulated by calciotropic hormones, namely parathyroid hormone (PTH), calcitonin and 1,25-dihydroxyvitamin D3 also known as calcitriol which act on the intestine, kidney and bone [5]. Increased renal calcium reabsorption, intestine absorption and breakdown of bone allow plasma calcium levels to be conserved as a result of these interactions [6]. Several studies have shown that calcium homeostasis is disturbed in T2DM and have used calciotropic hormones as markers to study the overall calcium status of the body [7, 8]. Studies have shown changes to plasma calcium, urinary calcium and calciotropic hormone levels in T2DM individuals [9, 10]. Alterations to calciotropic hormone concentrations may occur due to pathological changes that occur to the organs which synthesis these hormones or as a compensatory response to disturbed plasma calcium levels in T2DM [11, 12]. Furthermore, studies have shown that derangements to calciotropic hormone levels may exacerbate insulin resistance in the diabetic state [6, 7]. Optimal intracellular levels of calcium are required for the adequate functioning of pancreatic beta (β)-cells as well as insulin-responsive tissue such as the liver and skeletal muscles [13]. There are various processes that are dependent on calcium in the body that would be impaired by calcium homeostasis being interrupted [13].

In our laboratory, a pre-diabetic model of the human condition of pre-diabetes was created by feeding rats a high-fat high-carbohydrate diet [14, 15]. Several investigations employing this model have found that various complications associated with T2DM begin in the pre-diabetic stage [15, 16]. In the diabetic state, the changes to calcium homeostasis and the association of calciotropic hormones with glycated haemoglobin (HbA1c) and insulin resistance have been well documented [9, 10]. However, these changes have not yet been investigated during the pre-diabetic state. Hence, this study aimed to investigate the effects of diet-induced pre-diabetes on calciotropic hormones in a pre-diabetic rat model. Furthermore, this study also sought to determine the association of calciotropic hormones with glycated haemoglobin (HbA1c) and insulin resistance in a pre-diabetic rat model.

Materials and Methods

Animals and housing

This study employed male Sprague-Dawley rats (150-180g) which were bred and housed at the University of KwaZulu-Natal Biomedical Research Unit (BRU). Male rats were selected due to their more stable hormonal profile in comparison to female subjects. The animals were kept under standard laboratory conditions, which included a constant temperature of 22±2°C, a carbon dioxide (CO2) content of <5000 p.m, a relative humidity of 55 ± 5% and illumination (12 hour light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels. The animals were allowed access to food and fluids *ad libitum*. The Animal Research Ethics Committee of the University of KwaZulu-Natal (ETHICS#: AREC/024/018D) approved all animal experimentation. The animals were allowed to acclimatize to their new environment for 1 week while consuming standard rat chow and tap water before exposure to the experimental diets [14]. The procedures involving animal care followed the University of KwaZulu-Natal's institutional guidelines for animal care.

Induction of pre-diabetes

For an experimental period of 20 weeks, rats were randomly divided into two groups (n=6, per group) and fed their respective diets. Experimental pre-diabetes was induced in the animals using a previously described protocol by Luvuno et al. 2017 [14]. To induce pre-diabetes, one group was fed a high-fat high-carbohydrate (HFHC) diet supplemented with 15% fructose enriched water (AVI Products (Pty) Ltd, Waterfall, South Africa), whereas the other group was fed a standard rat chow and tap water. The animals were evaluated for pre-diabetes after 20 weeks using the American Diabetes Association (ADA) criteria. Animals with a fasting blood glucose concentration of 5.6 to 6.9 mmol/L, oral glucose tolerance test (OGTT) 2-h glucose concentration of 7.8 to 11.0 mmol/L and a glycated haemoglobin concentration of 5.7 to 6.4% were regarded as pre-diabetic. The animals that were fed the standard diet were also tested at week 20 to confirm normoglycaemia.

Experimental design

This study comprised of two groups, namely a non-pre-diabetic (NPD) group and a pre-diabetic (PD) group (n=6, in each group). The NPD group consisted of animals which consumed the standard rat chow for 20 weeks and did not have pre-diabetes, while the PD group consisted of animals which consumed the HFHC diet for the same number of weeks and were diagnosed with pre-diabetes.

Urine and blood collection

At the end of the experimental period, all animals were housed individually in Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa) for a 24-hour urine collection period. Thereafter, the urine samples were centrifuged (Eppendorf centrifuge 5403, LGBW Germany) at 1000 g for 20 minutes at 4 °C. The supernatants were then frozen at -80°C in a Bio Ultra freezer

(Labotec, Umhlanga, South Africa). Thereafter, the animals were anaesthetized with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) for 3 minutes via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, South Africa). Blood was collected by cardiac puncture while the rats were unconscious and then injected into individual pre-cooled heparinized containers. The blood was centrifuged (Eppendorf centrifuge 5403, LGBW Germany) for 15 minutes at 4 °C, 503 g. Plasma was isolated from blood and stored in a Bio Ultra freezer (Labotec, Umhlanga, South Africa) at -80 °C until biochemical analysis, as previously described by Luvuno et al., 2018 [19]. Of note, plasma and urine samples were obtained from a previous study (ETHICS#: AREC/024/018D).

HOMA-IR index

Insulin resistance was calculated from fasting blood glucose and insulin levels using the homeostatic model assessment (HOMA) [20]. The HOMA-IR index was calculated using the HOMA2 Calculator v2.2.3 program [21]. Insulin sensitive is values <1.0, early insulin resistance is values >1.9, and significant insulin resistance is values >2.9.

Biochemical analysis

An autoanalyzer (IDEXX VetLab station, Hoofddorp, Netherlands) was used to determine the concentration of calcium in the plasma and urine. The glycated haemoglobin (HbA1c), plasma insulin, parathyroid hormone (PTH), calcitonin, vitamin D and 1,25-dihydroxyvitamin D3 concentrations were measured using separate specific ELISA kits according to the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China). Micro-ELISA plates were coated with antibodies as part of the standard experimental protocol in the ELISA kits. The plasma samples were pipetted into the appropriate wells, followed by the immediate addition of the appropriate biotinylated detection antibody (50 µl). The samples were then incubated for 45 minutes at 37°C, after which the unbound components were washed away with the supplied wash buffer. After washing, the wells were filled with 100 µl of Avidin-horseradish peroxidase (HRP), which was incubated at 37°C for 30 minutes. After removing the unattached components with a second wash, the substrate reagent (90 µl) was applied to the wells. This was followed by a 15-minute incubation period at 37°C. Finally, a stop solution (50 µl) was applied to the micro-wells to stop the reaction and allow for appropriate measurements. The optical density at 450 nm was determined using a nano-spectrophotometer (BMG Labtech,Ortenburg,Germany). Glycated haemoglobin, insulin, PTH, calcitonin, vitamin D, and 1,25-dihydroxyvitamin D3 concentrations in the samples were extrapolated from their respective standard curves.

Statistical analysis

The mean ± standard error of the mean (SEM) were used to represent the data. Statistical comparisons were performed with Graph Pad InStat Software (version 5.00, Graph Pad Software, Inc., San Diego, California, USA). The student t test was used to determine statistical differences between two independent groups. Pearson's correlation test was used to determine the association of calciotropic hormones with HOMA-IR and HbA1c. A value of $p < 0.05$ was considered statistically significant. A coefficient value between ± 0.70 and ± 1.0 was considered strong.

Results

Glycated haemoglobin

Glycated haemoglobin concentrations were measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig.1) that the concentration of glycated haemoglobin was significantly (p=< 0.0001) higher in the PD group as compared to the NPD.

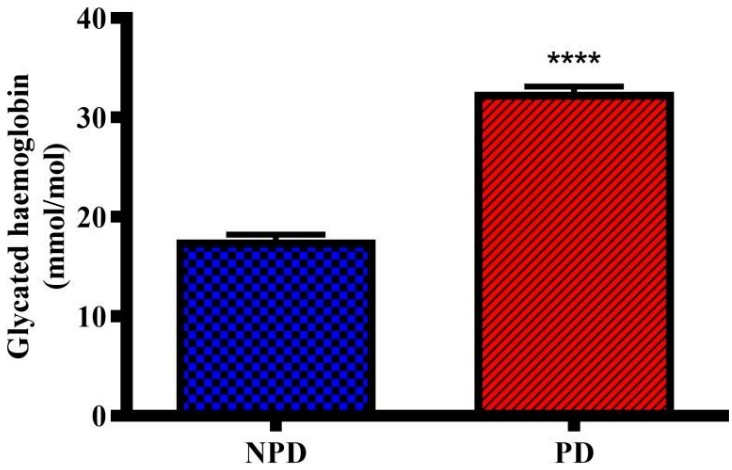

Figure 1: Concentrations of glycated haemoglobin in the non-pre-diabetic (NPD) and pre-diabetic (PD) groups (n=6 per group). The values are depicted as a mean± SEM. ****= $p < 0.0001$ when compared to NPD

Fasting plasma glucose, insulin and HOMA-IR

Fasting plasma glucose concentrations, insulin concentrations and HOMA-IR were measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Table 1) that the concentrations of fasting glucose (p=< 0.0001) and insulin (p=< 0.0001) in plasma were significantly higher in the PD group as compared to the NPD. Furthermore, the PD group had significantly (p=<0.0001) higher HOMA-IR value compared to the NPD group, which was in the range of significant insulin resistance (>2.9), whereas the NPD group HOMA-IR value was within the insulin-sensitive range (1.0).

Table 1: Concentrations of plasma glucose, insulin and HOMA-IR indices in the non-pre-diabetic (NPD) group and pre-diabetic group (PD) (n=6, per group). The values are depicted as mean ± SEM. ****= $p<0.0001$ when compared to NPD

Groups (n=6)	Plasma glucose (mmol/L)	Plasma insulin (ng/ml)	HOMA-IR values
NPD	4.40 ± 0.20	3.47 ± 0.12	0.68±0.05
PD	6.72 ± 0.12****	10.87 ± 0.06****	3.24±0.06****

Plasma and urinary calcium from 24-hour urine samples

Plasma and urinary calcium concentrations were measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig.2) that the concentration of calcium in plasma was not significantly (p=0.0959) changed in the PD group as compared to the NPD. The concentration of calcium in urine was significantly (p= <0.0001) higher in the PD group as compared to the NPD.

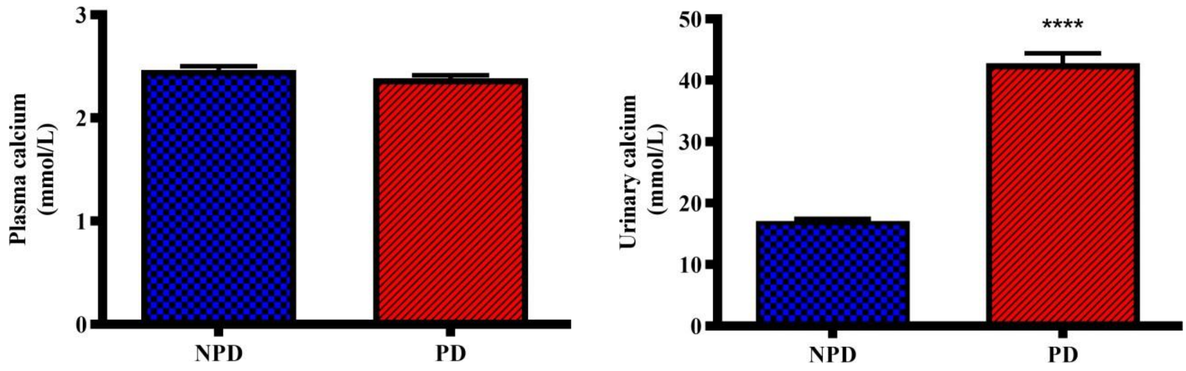

Figure 2: Concentrations of plasma and urine calcium in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group). Values are depicted as mean ± SEM. ****= p < 0.0001 when compared to NPD

Plasma PTH and calcitonin

Plasma parathyroid hormone (PTH) and calcitonin concentrations were measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig.3) that the concentrations of PTH (p=< 0.0001) and calcitonin (p=< 0.0001) in plasma were significantly higher in the PD group as compared to the NPD.

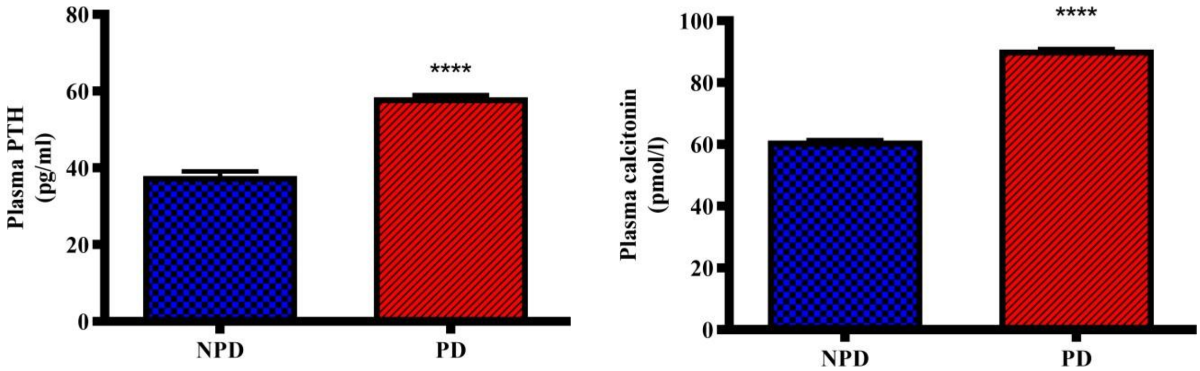

Figure 3: Concentrations of plasma PTH and calcitonin in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group).Values are depicted as mean ± SEM. ****= p < 0.0001 when compared to NPD

Plasma vitamin D and 1, 25-dihydroxyvitamin D3

Plasma vitamin D and 1, 25-dihydroxyvitamin D3 concentrations were measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig.4) that the concentrations of vitamin D (p=0.0001) and 1, 25-dihydroxyvitamin D3 (p= 0.0311) in plasma were significantly higher in the PD group as compared to the NPD.

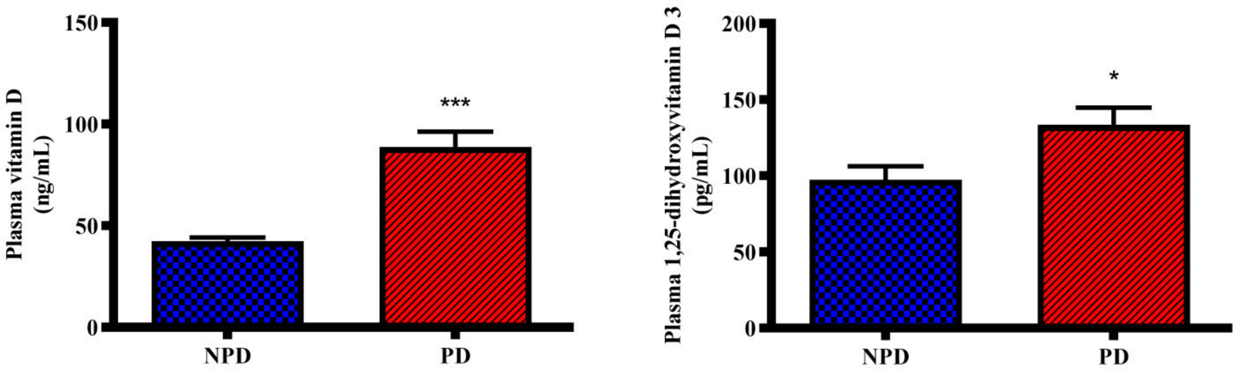

Figure 4: Concentrations of plasma vitamin D and 1, 25-dihydroxyvitamin D3 in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group). Values are depicted as mean ± SEM. *=p<0.05, *** = p < 0.001 when compared to NPD

Correlation between metabolic parameters and calciotropic hormone levels

Pearson's correlation analyses were performed between metabolic parameters and calciotropic hormone levels in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Table 2) that the concentrations of PTH (r= -0, 29; p= 0.58), calcitonin (r= -0.70; p=0.12) and 1,25-dihydroxyvitamin D3 (r= 0.71; p= 0.11) in plasma were not associated with insulin resistance in the PD state. However, the concentrations of PTH (r=0.82; p=0.02) and calcitonin (r=0.95; p=0.004) in plasma were positively correlated with HbA1c in the PD state. Furthermore, the concentration of 1, 25-dihydroxyvitamin D3 in plasma was negatively correlated (r= - 0.93; p= 0.007) with HbA1c in the PD state.

Table 2: Pearson's rank correlation between metabolic parameters and calciotropic hormone levels in the non-pre-diabetic (NPD) and pre-diabetic (PD) group (n=6, per group). *= p < 0.05, ** = p<0.01

Metabolic Parameters		**PTH (pg/ml)**	**Calcitonin (pmol/l)**	**1,25-dihydroxyvitamin D 3 (pg/ml)**
HOMA-IR values	**NPD**	r= 0.55 p= 0.26	r= - 0.37 p= 0.48	r= 0.18 p= 0.73
	PD	r= -0.29 p= 0.58	r= -0.70 p= 0.12	r= 0.71 p= 0.11
HbA1c (mmol/mol)	**NPD**	r= -0.13 p= 0.81	r= 0.54 p= 0.27	r= -0.64 p= 0.17
	PD	r= 0.82 p= 0.02 *	r= 0.95 p= 0.004 **	r= - 0.93 p= 0.007 **

Discussion

Calcium homeostasis is regulated by calciotropic hormones which act on the bone, kidney and intestine [6]. Several studies have shown that calcium homeostasis is disturbed in type 2 diabetes mellitus (T2DM), a condition that is often preceded by pre-diabetes [12, 22]. Furthermore, disrupted calcium homeostasis has shown to promote the onset of hyperglycaemia and insulin resistance [23]. Pre-diabetes was induced in rats in our laboratory using a well-established protocol that included the chronic intake of a high-fat high-carbohydrate (HFHC) diet [14, 15]. These studies have shown that complications in T2DM such as cardiovascular disorders, immune dysregulation and renal failure begin in the pre-diabetic state [15, 16]. The changes to calcium homeostasis that are associated with T2DM have been well-characterised; however, there is paucity in information for the pre-diabetic state [24, 25]. Therefore, in this study the effects of diet-induced pre-diabetes on calciotropic hormones were investigated. Furthermore, this study sought to also investigate the association of calciotropic hormones with glycated haemoglobin and insulin resistance in the pre-diabetic state.

Pre-diabetes is a metabolic disorder characterised by early insulin resistance and blood glucose levels that are over the homeostatic range [3]. Physiologically, increased glucose concentrations promote insulin release and increase insulin in circulation, allowing peripheral tissue to absorb glucose [2]. This accounts for plasma glucose concentrations been conserved within the homeostatic range in normal glucose tolerant (NGT) individuals [26]. After plasma glucose levels become conserved, plasma insulin levels return towards the homeostatic range [27]. Furthermore, glucose levels are not constantly elevated in NGT individuals to promote increased glycation of haemoglobin and insulin resistance [27]. However, in the pre-diabetic state there is increased glucose concentration, insulin secretion, HbA1c and early insulin resistance by comparison to normal glucose tolerant individuals [2, 15]. During pre-diabetes, plasma insulin fails to stimulate a response in insulin-targeted peripheral tissue such as skeletal muscle [20]. The accumulation of plasma glucose results in enhanced glycation with haemoglobin [28]. As a result, pancreatic β-cells respond by secreting a greater quantity of insulin to overcome the high glucose concentrations resulting in compensatory hyperinsulinemia [28]. In this study, the fasting glucose concentration, plasma insulin concentration, HbA1c and HOMA-IR index were significantly higher in the PD group than the NPD group. These results corroborated with previous findings that have shown elevated plasma glucose, insulin, HOMA-IR and HbA1c in pre-diabetic patients by comparison to normal glucose tolerant individuals [29, 30]. This study further validates that the consumption of the HFHC diet promotes the development of pre-diabetes, as seen by the impaired fasting glucose, insulin, HbA1c and HOMA-IR value in the range of insulin resistance. This suggests that the body's ability to use glucose in insulin-dependent tissues has been disturbed [16]. The excessive intake of dietary fats, which has shown to increase triacylglycerides levels, may have contributed to insulin resistance [31]. The increased exposure of triacylglycerides to insulin-dependent peripheral tissue induces insulin resistance [31]. Consequently, pancreatic beta (β)-cells may have produced more insulin to compensate for the elevated plasma glucose that is found in insulin-resistant tissue [30]. Calcium homeostasis has been found to be disrupted by elevated plasma glucose concentrations and insulin resistance in T2DM [22, 32].

Calcium is needed for a variety of bodily functions, including signal transmission, secretion of hormones and mineralization of bone [22]. Plasma calcium levels are regulated by PTH, calcitonin and 1,25-dihydroxyvitamin D3 also known as calcitriol which act on the bone, kidney and small intestine [6]. When plasma calcium levels fall below normal, the chief cells of the parathyroid gland produce PTH, which stimulates calcitriol synthesis [6, 22]. The parafollicular cells of the thyroid gland on the other hand, are triggered to release calcitonin when plasma calcium levels rise above normal [6, 22]. Some studies have shown that plasma calcium concentrations are within the homeostatic range in type 2 diabetic patients [33, 34]. These studies have stated that plasma calcium levels do not change significantly due to calciotropic hormones maintaining a constant plasma calcium concentration, despite variations in calcium excretion [33, 34]. In this study, the PD group displayed no significant change to the plasma calcium concentrations by comparison to the NPD group. These results coincided with prior literature that showed no significant change in calcium levels in plasma among T2DM patients [33, 34]. However, these results contradicted other studies that have found either hypocalcaemia or hypercalcaemia among T2DM patients [7, 25]. The possible reason for no significant change to plasma calcium levels in the PD group of this study may have been due to calciotropic hormones compensating for the changes to plasma calcium concentration. The significant alterations to plasma calcium levels in studies on T2DM patients may have been due to the failure of the body to compensate for the changes to plasma calcium levels [32, 35]. In the current study, it was observed that calciotropic hormones had compensated for changes to plasma calcium levels which may have occurred as a result of pre-diabetes.

The concentrations of plasma calcium are kept within the homeostatic range by calciotropic hormones [23]. Calcium levels in the plasma control the secretion of PTH, calcitriol and calcitonin [36]. Parathyroid hormone with aid from calcitriol increase plasma calcium levels by promoting an increase in calcium absorption in intestine, renal calcium reabsorption and bone resorption whereas calcitonin promotes the opposite [13]. Previous research has shown increased concentrations of PTH and calcitonin in plasma of type 2 diabetic individuals [37, 38]. According to studies the increased PTH concentration may have compensated for the increased calcium loss in urine and the increased plasma calcitonin concentration may have protected the body against the adverse effects of PTH oversecretion [13, 39]. The harmful effects associated with elevated PTH secretion include hypercalcaemia and bone loss [23]. In this study, the plasma PTH and calcitonin concentrations in the PD group were significantly higher than the NPD group. These findings validated prior research that has shown elevated levels of PTH and calcitonin in plasma of T2DM patients [11, 40]. In the PD group of this study, the elevated plasma PTH and calcitonin concentrations may have been ascribed to the coordination between PTH and calcitonin, in order to stabilize plasma calcium levels. Additionally, PTH levels in plasma may have risen to compensate for the increased urine calcium loss. This may lead to secondary hyperparathyroidism which has shown to be associated with impaired glucose tolerance, decreased insulin sensitivity and increased risk of T2DM [41]. Plasma calcitonin concentration may have increased to compensate for the elevated plasma PTH levels in the PD group.

The kidneys, bone and small intestine regulate urinary calcium homeostasis under the influence of PTH and calcitriol [7]. Nearly 98% of filtered calcium by the kidneys is reabsorbed back into the bloodstream via the renal tubules, which helps to maintain plasma calcium levels [13]. About 60-70% of filtered calcium is reabsorbed in the kidney's proximal tubule [13]. The remaining calcium is reabsorbed along the ascending limb of Henle and distal convoluted under the influence of PTH [13]. It is estimated that less than 2% of filtered calcium is lost in urine due to renal calcium reabsorption [13]. Type 2 diabetes mellitus promotes renal calcium wastage by altering renal functioning, calcium transport mechanisms and damaging renal tubular cells [7, 32]. Furthermore, compensatory hyperinsulinemia as a consequence of insulin resistance has shown to inhibit renal calcium reabsorption in T2DM patients [42, 43]. In this study, the urinary calcium levels in the PD group were significantly higher by comparison to NPD. Furthermore, the urinary calcium levels in the PD group were above the homeostatic range of 15 mmol/L-20 mmol/L, indicative of hypercalciuria [44]. These findings coincide with recent studies that have shown increased urine calcium levels in T2DM patients [45, 46]. Renal impairment is present in the pre-diabetic state, according to studies conducted in our laboratory [16, 19]. The elevated urinary calcium concentration in the PD group may have been due to impaired renal regulatory function induced by pre-diabetes. Furthermore, high glucose levels in the PD state may have directly damaged mechanisms of calcium transport in the renal tubule [47]. In addition, studies in human and rodent models have shown that increased urinary calcium levels were associated with elevated plasma insulin levels [7, 10]. Compensatory hyperinsulinemia as a consequence of insulin resistance has shown to inhibit renal calcium reabsorption [10]. Hyperinsulinemia-induced by pre-diabetes may have promoted increased urinary calcium loss by inhibiting renal calcium reabsorption. Additionally, increased renal calcium load may have also contributed to the higher concentrations of calcium in the urine of the PD group. Diets that have content high in protein have been demonstrated to cause metabolic acidosis, which is manifested by the elevated loss of calcium in urine [48]. The increased renal acid load contributes to bone breakdown and consequently promote hypercalcaemia [48]. Therefore, the kidneys may try to compensate for the increased plasma calcium by excreting it into urine.

Vitamin D is a fat-soluble vitamin that plays a significant role in regulating whole body calcium homeostasis within the endocrine system [49]. Vitamin D is the precursor molecule needed for calcitriol synthesis [49]. Calcitriol is vitamin D in its hormonally active form responsible for increasing plasma calcium concentrations [49]. Previous studies have found reduced plasma vitamin D and calcitriol levels among T2DM individuals [49, 50]. These studies have shown that impaired renal reabsorption of vitamin D binding proteins, impaired intestinal vitamin D absorption and increased sequestration of vitamin D by adipose tissue may be responsible for decreased plasma vitamin D and calcitriol concentrations in T2DM [39, 43]. Furthermore, studies have shown that calcitriol production is impaired due to renal damage, low plasma PTH levels and PTH resistance in T2DM [47, 51]. In this study, the PD group had significantly higher plasma vitamin D and calcitriol levels by comparison to the NPD group. These results contrasted previous findings which have shown decreased plasma vitamin D and calcitriol in T2DM individuals [49, 50]. The PD group in this study was fed a diet high in saturated fats and carbohydrate content by comparison to the standard diet. Diets that contain high fat content have shown to stimulate bile secretion and consequently enhanced intestinal vitamin D absorption [52, 53]. It is plausible that the increased dietary fat content in the HFHC diet may have promoted an increase in vitamin D absorption, accounting for the higher plasma vitamin D levels in the PD group. In addition, the increased calcitriol levels in the PD group may have been a compensatory response to reduced plasma calcium levels and elevated plasma PTH levels. Elevated plasma PTH levels induce an increase in renal-1 alpha hydroxylase expression [54]. Renal1-alpha hydroxylase catalyses' the conversion of calcifediol to calcitriol, promoting an increase in plasma calcitriol levels as seen in this study [54].

Previous studies have shown that calciotropic hormones participate in the regulation of glucose homeostasis by modulating effects on gluconeogenesis, glycogenolysis and insulin-signaling [23, 39]. Studies have shown that plasma PTH and calcitonin levels were positively correlated with hyperglycaemia and insulin resistance in T2DM [40, 47]. Conversely, previous studies have shown that calcitriol levels in plasma were inversely correlated with hyperglycemia and insulin resistance [12, 40]. Elevated PTH and calcitonin levels were found to promote insulin resistance and hyperglycaemia by disrupting the insulin signaling pathway, increasing hepatic glycogenolysis and gluconeogenesis [40]. In contrast, calcitriol has been shown to improve the sensitivity to insulin and tolerance of glucose [8]. In this study, plasma PTH and calcitonin levels were positively correlated with HbA1c but not with insulin resistance in the PD state. Furthermore, plasma calcitriol levels were negatively correlated with HbA1c in the PD group. These results corroborated with previous findings that reported that plasma PTH, calcitonin and calcitriol were associated with HbA1c [39, 40]. However, the lack of association between plasma PTH, calcitonin and calcitriol with insulin resistance contrasted previous studies that have reported associations between these hormones with insulin resistance [23, 32]. These observations may suggest that the elevated plasma PTH and calcitonin levels due to disrupted calcium homeostasis may contribute to the elevated HbA1c concentrations in the PD state. Elevated plasma PTH and calcitonin levels from disrupted calcium homeostasis may stimulate gluconeogenesis and glycogenolysis [55]. It may be speculated that early insulin resistance and beta dysfunction in the PD state may create an unfavourable environment, where increased glucose production stimulated by elevated PTH and calcitonin levels may not be compensated for in a pre-diabetic individual. This may lead to a vicious cycle where disrupted calcium homeostasis in the PD state may promote the development of hyperglycaemia in T2DM. The significant negative correlation between plasma calcitriol and HbA1c in the PD state may suggest a protective role of calcitriol against intermediate hyperglycaemia in the PD state.

The findings of this study may serve of clinical importance in the early detection of type 2 diabetes mellitus. These results may increase our understanding of calcium homeostasis and may provide a possible insight into potential target sites in the treatment of overt T2DM and its associated complications. Furthermore, these findings have elucidated the mechanism of calcium homeostasis in a pre-diabetic rat model. Due to the similarity in the genetic variability of Sprague Dawley rats and humans, these findings may provide an understanding of calcium homeostasis in pre-diabetic humans. Since the current study indicated that some of the complications associated in T2DM such as hyperparathyroidism and hypercalciuria begins in the pre-diabetic state, it is worth translating this research to human studies as a future prospective.

It was evident that hypercalciuria is present in the PD state, an indication of disturbed calcium homeostasis. In addition, plasma calcium levels may have been conserved due to elevated plasma PTH, calcitonin, vitamin D and calcitriol levels in the PD state. Furthermore, plasma PTH and calcitonin levels were positively correlated with HbA1c but not with insulin resistance in the PD state.

In addition, plasma calcitriol levels were negatively correlated with HbA1c in the PD state. Although calciotropic hormones try to maintain calcium homeostasis in pre-diabetes, elevated levels of PTH and calcitonin may promote the development of hyperglycaemia in T2DM.

Conclusion

Taken together, the effects associated with diet-induced pre-diabetes on calciotropic hormones include elevated plasma PTH, calcitonin, calcitriol and vitamin D concentrations. This was accompanied by elevated urine calcium and unchanged plasma calcium levels. Collectively, these observations may suggest that calcium homeostasis is disturbed in the PD state but calciotropic hormones compensate for the changes to plasma calcium levels. Furthermore, altered levels of calciotropic hormones in the PD state may contribute to the development of hyperglycaemia and not insulin resistance in T2DM.

Conflict of interest

I declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Future recommendations

In future, the exact mechanisms in which calciotropic hormones contribute to the development of hyperglycaemia needs to be investigated in the PD state.

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Bridge

The first manuscript illustrated that high-fat high-carbohydrate diet-induced pre-diabetes was associated with disturbed calcium homeostasis. This was evidenced by the elevated urinary calcium, plasma PTH, calcitonin, vitamin D, calcitriol along with unchanged plasma calcium levels. In order for calciotropic hormones to maintain plasma calcium levels they have to act on calcium-regulating organs namely, the intestine, kidney and bone. Furthermore, the unchanged plasma calcium concentration and altered levels of calciotropic hormones in the PD group in manuscript 1 prompted us to investigate the changes to calcium-regulating organs. In addition, the functioning of calcium-regulating organs have shown to be disturbed in T2DM. Furthermore, these organs may not respond to calciotropic hormones. Altered functioning of calcium-regulating organs may be disturbed in the pre-diabetic state. This hypothesis led to the second study which sought to investigate the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs with regards to markers associated with intestinal calcium absorption, renal calcium reabsorption and bone turnover.

CHAPTER 3: MANUSCRIPT 2

PROLOGUE

Disrupted functioning of calcium-regulating organs in T2DM has shown to contribute to disturbances to calcium homeostasis. In the previous study in chapter 2, we established that calcium homeostasis was disturbed however calciotropic hormones compensated for the changes to plasma calcium in the pre-diabetic state. Interestingly, the dysregulation of calciotropic hormones in T2DM have shown to further worsen the functioning of calcium-regulating organs. It has been noted throughout the last decade that the ability of calcium-regulating organs to compensate for changes to calcium homeostasis is compromised and the influence of calciotropic hormones on these organs have led to complications such as osteoporosis in the diabetic state. Pre-diabetes, an intermediary state between normoglycaemia and T2DM, has been reported to be caused by a diet high in fats and carbohydrates. Complications seen in T2DM have been shown to begin in the pre-diabetic state. However, it is not known whether the functioning of calcium-regulating organs in the pre-diabetic state is disturbed. Subsequently, manuscript 2 explores the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs namely the kidney, intestine and bone.

The manuscript is titled **"Investigating the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs in male Sprague Dawley rats: Effects on selected markers"** and is authored by K Naidoo, Ngubane PS and Khathi A.

The manuscript is currently under review in the **Journal of Endocrine Pathology** and has been formatted according to the journals guidelines for authors. See Appendix 2

Investigating the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs in male Sprague Dawley rats: Effects on selected markers

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Abstract

Derangements to the functioning of calcium-regulating organs have been associated with type 2 diabetes mellitus (T2DM), a condition preceded by pre-diabetes. Type 2 diabetes has shown to promote renal calcium wastage, intestinal calcium malabsorption and increased bone resorption. However, the changes to the functioning of calcium-regulating organs in pre-diabetes are not known. Subsequently, the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs in a rat model for pre-diabetes was investigated in this study. Male Sprague Dawley rats were separated into two groups (n=6, each group): non-pre-diabetic group and a diet-induced pre-diabetic group for 20 weeks. After the experimental period, postprandial glucose and HOMA-IR were analysed in addition to plasma and urinary calcium concentrations. Gene expressions of intestinal vitamin D (VDR), intestinal calbindin-D_{9k}, renal 1-alpha hydroxylase and renal transient receptor potential vanilloid 5 (TRPV5) expressions in addition to plasma osteocalcin and urinary deoxypyridinoline concentrations were analysed at week 20. The results demonstrated significantly increased concentrations of postprandial glucose, HOMA-IR and urinary calcium in addition to unchanged plasma calcium levels in the PD group by comparison to NPD. Renal TRPV5, renal 1-alpha hydroxylase, intestinal VDR and intestinal calbindin-D_{9k} expressions were increased in the PD group by comparison to NPD. Furthermore, plasma osteocalcin levels were increased and urine deoxypyridinoline levels were decreased in the PD group by comparison to NPD. These observations may suggest that calcium-regulating organs compensate for the changes to calcium homeostasis by inducing increased renal calcium reabsorption, increased intestinal calcium absorption and decreased bone resorption followed by increased bone formation.

Keywords: Calcium-regulating organs, reabsorption, resorption, high fat high carbohydrate

Introduction

Urban lifestyle and the chronic consumption of diets that contain high fat and carbohydrate content have shown to promote the onset of type 2 diabetes mellitus (T2DM), a condition which is preceded by pre-diabetes [1]. Pre-diabetes is a stage of moderate hyperglycemia in which the glycemic parameters are slightly higher than the homeostatic range but below the threshold for clinical diabetes diagnosis [1]. Insulin resistance and beta (β)-cell dysfunction are associated with pre-diabetes [2]. According to the International Diabetes Federation (IDF), 352 million individuals worldwide were diagnosed with pre-diabetes in 2017, while it is further projected that by 2045 the prevalence of pre-diabetes is expected to increase 8.3% [2]. While T2DM is associated with micro-and macrovascular complications, studies have also shown that T2DM compromises calcium homeostasis by disturbing the functioning of calcium-regulating organs, such as the kidney, bone and intestine [3, 4].

Fluxes of calcium between the small intestine, bone and kidney are controlled by parathyroid hormone (PTH), calcitonin and calcitriol [5]. Calcium is released into the bloodstream and removed from the bloodstream as needed through calcium-regulating organs [4]. The kidneys regulate urinary calcium excretion, the bone acts as a reservoir for calcium and the small intestine is responsible for calcium absorption [6]. Several studies have shown physiological changes to calcium-regulating organs in T2DM individuals [7-9]. Type 2 diabetes mellitus promotes impaired intestinal calcium absorption, renal calcium wasting and bone deterioration [7, 10]. Furthermore, it also leads to dysregulation of calciotropic hormones, thereby worsening the already impaired functioning of calcium-regulating organs [6]. Calcium homeostasis is important since calcium is responsible for the modulation of many important processes [4]. Calcium is involved in bone mineralization, hormone secretion, nervous system modulation and muscle tone [5]. Calcium-dependent processes within the body would be impaired if calcium homeostasis was disrupted as well as conditions such as hypocalcaemia and osteoporosis may develop [4].

Previous studies in our laboratory led to the development of a pre-diabetic animal model induced by a high-fat high-carbohydrate (HFHC) diet, which closely resembles the human pre-diabetic state [11, 12]. Numerous studies using this model have shown that various complications associated with T2DM begin in the pre-diabetic condition [12, 13]. While the changes to the functioning of calcium-regulating organs have been well documented in the diabetic state, these changes have not yet been investigated during the pre-diabetic state [14, 15]. Hence, the aim of this study was to elucidate the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs in male Sprague Dawley rats.

Materials and Methods

Animals and Housing

This study employed male Sprague-Dawley rats (150-180g) which were bred and housed at the University of KwaZulu-Natal Biomedical Research Unit (BRU). Male rats were selected due to their more stable hormonal profile in comparison to female subjects. The animals were kept under standard laboratory conditions, which included a constant temperature of 22±2°C, a carbon dioxide (CO2) content of <5000 p.m, a relative humidity of 55 ± 5% and illumination (12 hour light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels. The animals were allowed access to food and fluids *ad libitum*. The Animal Research Ethics Committee of the University of KwaZulu-Natal (ETHICS#: AREC/024/018D) approved all animal experimentation. The animals were allowed to acclimatize to their new environment for 1 week while consuming standard rat chow and tap water before exposure to the experimental diets [11]. The procedures involving animal care followed the University of KwaZulu-Natal's institutional guidelines for animal care.

Induction of pre-diabetes

For an experimental period of 20 weeks, rats were randomly divided into two groups (n=6, per group) and fed their respective diets. Experimental pre-diabetes was induced in the animals using a previously described protocol by Luvuno et al. 2017 [11]. To induce pre-diabetes, one group was fed a high-fat high-carbohydrate (HFHC) diet supplemented with 15% fructose enriched water (AVI Products (Pty) Ltd, Waterfall, South Africa), whereas the other group was fed a standard rat chow and tap water. After 20 weeks, the American Diabetes Association (ADA) criteria was used to diagnose pre-diabetes whereby the criteria to define prediabetes include impaired fasting glucose (IFG) with fasting plasma glucose levels of 5.6 to 6.9 mmol/L, impaired glucose tolerance (IGT) with plasma glucose levels of 7.8 to 11.0 mmol/L 2-hour postprandial, or a glycated haemoglobin (hba1c) of 5.7 to 6.4%. The animals that were fed the standard diet were also tested at week 20 to confirm normoglycaemia.

Experimental design

This study comprised of two groups, namely a non-pre-diabetic (NPD) group and a pre-diabetic (PD) group (n=6, in each group). The NPD group consisted of animals which consumed the standard rat chow for 20 weeks and did not have pre-diabetes, while the PD group consisted of animals which consumed the HFHC diet for the same number of weeks and were diagnosed with pre-diabetes.

Oral glucose tolerance (OGT) response

At week 20, an oral glucose tolerance test (OGTT) was conducted following glucose loading, to determine the glucose tolerance response of animals subjected to the chronic ingestion of the HFHC diet. The OGT responses were monitored in the animals according to a well-established protocol [16].

Briefly, after a 12 hour fast, glucose levels were measured (time, 0 min) in all animals. Thereafter, the animals were loaded with glucose (glucose; 0.86 g/kg) through an oral gavage (18-gauge gavage needle, 38mm long curved with 21/4 mm ball end). To measure glucose concentration, blood was collected using the tail-prick method [17]. Glucose concentrations were measured by a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom). The glucose concentrations were measured at 15, 30, 60, and 120 minutes following glucose loading.

Urine collection, blood collection and tissue harvesting

At the end of the experimental period, all animals were housed individually in Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa) for a 24-hour urine collection period. Thereafter, the urine samples were centrifuged (Eppendorf centrifuge 5403, LGBW Germany) at 1000 g for 20 minutes at 4 °C. The supernatants were then frozen at -80°C in a Bio Ultra freezer (Labotec, Umhlanga, South Africa). Thereafter, the animals were anaesthetized with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) for 3 minutes via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, South Africa). Blood was collected by cardiac puncture while the rats were unconscious and then injected into individual pre-cooled heparinized containers. The blood was centrifuged (Eppendorf centrifuge 5403, LGBW Germany) for 15 minutes at 4 °C, 503 g. Plasma was isolated from blood and stored in a Bio Ultra freezer (Labotec, Umhlanga, South Africa) at -80 °C until biochemical analysis, as previously described by Luvuno et al., 2018 [11]. Following blood collection, the kidney and small intestine were removed and placed in pre-cooled Eppendorf containers and snap-frozen in liquid nitrogen before storage in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at − 80 °C. Of note, plasma, urine, kidney and intestinal tissue were obtained from a previous study which had ethical approval (ETHICS#: AREC/024/018D).

HOMA-IR index

Insulin resistance was calculated from fasting blood glucose and insulin levels using the homeostatic model assessment (HOMA) [18]. The HOMA-IR index was calculated using the HOMA2 Calculator v2.2.3 program [19]. Insulin sensitive is values <1.0, early insulin resistance is values >1.9, and significant insulin resistance is values >2.9.

Biochemical analysis

Plasma calcium, urinary calcium and creatinine concentrations were measured with an autoanalyzer (IDEXX VetLab station, Hoofddorp, Netherlands). The plasma insulin, plasma osteocalcin and urinary deoxypyridinoline concentrations were measured using separate specific enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Elabscience and Biotechnology, Wuhan, China).

Quantitative real-time PCR

The ReliaPrep tissue Miniprep system was used to extract ribonucleic acid (RNA) from the collected kidney and small intestinal tissue (Promega, USA). The purity and concentration of RNA was determined by Nanodrop 2000 (Thermo Scientific, Roche, South Africa). For conversion to complementary DNA (cDNA), a purity ratio (A260/A280) of 1.7-2.1 was considered acceptable. The GoTaq®2-Step RT-qPCR System (Promega, USA) as a cDNA synthesis kit was used to produce reverse transcription reactions using 2 μg of total RNA as described by the manufacturer.

According to the manufacturer's instructions on the ROCHE light cycler system, the ROCHE light cycler SYBR Green I master mix was utilized for amplification. Table 1 below shows the primer sequences (Metabion, Germany) utilized in this work. The cycling conditions were as follows: pre-incubation at 95°C for 60s, followed by a 3-step amplification of 45 cycles at 95°C for 15s, 60°C for 30s, and 72°C for 30s. Melting was executed at 95°C for 10s, 65°C for 60s and 97°C for 1s. Furthermore, cooling was achieved at 37°C for 30s. The housekeeping gene employed was glyceraldehyde-phosphate dehydrogenase (GAPDH). The $2^{-\Delta\Delta Ct}$ relative quantication method was used to represent gene expression values.

Table 1: List of primers used in this study

Gene of interest	Sequence
TRPV5	Forward: 5'-TGTGAGCCATTTGTAGGTCAG-3' Reverse: 5’-GAGGTTGTGGGAACTTCGA-3’
CYP27B1 **(1-alpha hydroxylase)**	Forward: 5'-CACCCATTTGCATCTCTTCC -3' Reverse: 5'-GATGGATGCTCCTCTCAGGT -3'
VDR	Forward: 5′-GTGACTTTGACCGGAACGTG-3′ Reverse: 5’- ATCATCTCCCTCTTACGCTG -3’
S100G **(Calbindin-D_{9k})**	Forward: 5'CCCGAAGAAATGAAGAGCATTTT-3' Reverse: 5'-TTCTCCATCACCGTTCTTATCCA-3'
GAPDH	Forward: 5'-AGTGCCAGCCTCGTCTCATA-3' Reverse: 5'-GATGGTGATGGGTTTCCCGT-3'

Statistical analysis

The mean ± standard error of the mean (SEM) were used to represent the data. Statistical comparisons were performed with Graph Pad InStat Software (version 5.00, Graph Pad Software, Inc., San Diego, California, USA). The student t test was used to determine statistical differences between two independent groups. Correlation analysis was performed using Pearson’s correlation analysis.

Statistical significance was considered as a p value of less than 0.05. A coefficient value between ± 0.70 and ± 1.0 was considered strong.

Results

Oral glucose tolerance test (OGTT)

The OGTT and Area under curve (AUC) was measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig. 1) that the concentration of FBG in the PD group was significantly (p=0.0020) higher at time 0 as compared to the NPD group. Glucose concentrations in the PD group were significantly (p=0.0386) higher in the PD group at 120 minutes after glucose loading as compared to NPD. Furthermore, the AUC for the PD group was significantly (p=<0.0001) higher in the PD group as compared to the NPD group.

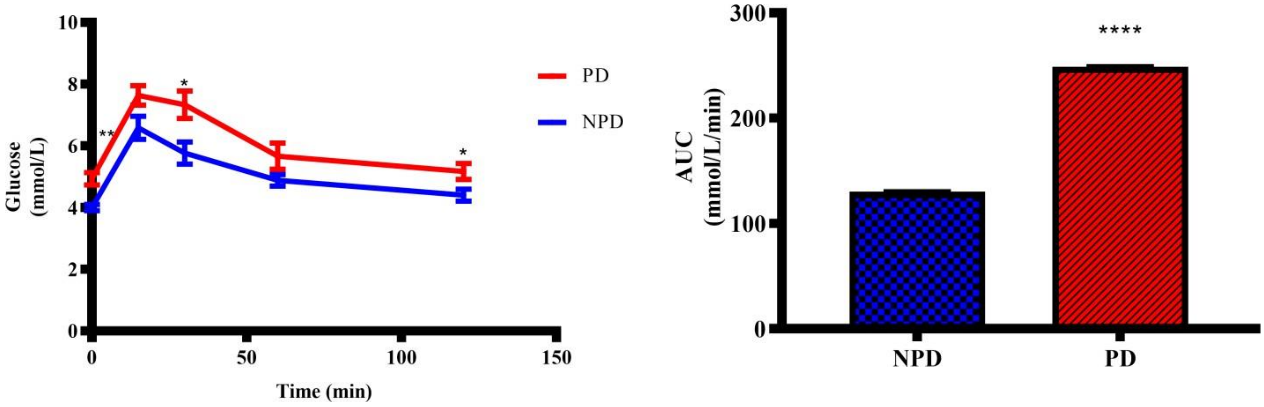

Figure 1: The OGT response and AUC values in the non-pre-diabetic (NPD) group and pre-diabetic group (PD) (n=6, per group). Values are depicted as mean ± SEM. *=p<0.05, **=p<0.01, ****= p<0.0001 when compared to NPD

Homeostatic model assessment for insulin resistance (HOMA-IR)

The HOMA-IR values were calculated from the concentrations of fasting plasma glucose and insulin after the experimental period (n=6, per group). It was evident (Table 2) that the concentrations of fasting glucose (p=< 0.0001) and insulin (p=< 0.0001) in the plasma were significantly higher in the PD group as compared to the NPD group. The PD group had significantly (p=<0.0001) higher HOMA-IR value compared to the NPD group, which was in the range of significant insulin resistance (>2.9), whereas the NPD group HOMA-IR value was within the insulin-sensitive range (<1.0).

Table 2: Concentrations of plasma glucose, insulin and HOMA-IR indices in the non-pre-diabetic (NPD) group and pre-diabetic group (PD) (n=6, per group). Values are depicted as mean ± SEM. ****= p<0.0001 when compared to NPD

Groups	Plasma glucose	Plasma insulin	HOMA-IR values

(n=6)	(mmol/L)	(ng/ml)	
NPD	4.40 ± 0.20	3.47 ± 0.12	0.68±0.05
PD	6.72 ± 0.12****	10.87 ± 0.06****	3.24±0.06****

Plasma calcium and urinary calcium from 24-hour urine sample

Plasma and urinary calcium concentrations were measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig.2) that the concentration of calcium in plasma was not significantly (p=0.0959) changed in the PD group as compared to NPD. The concentration of calcium in urine were significantly (p= <0.0001) higher in the PD group as compared to NPD.

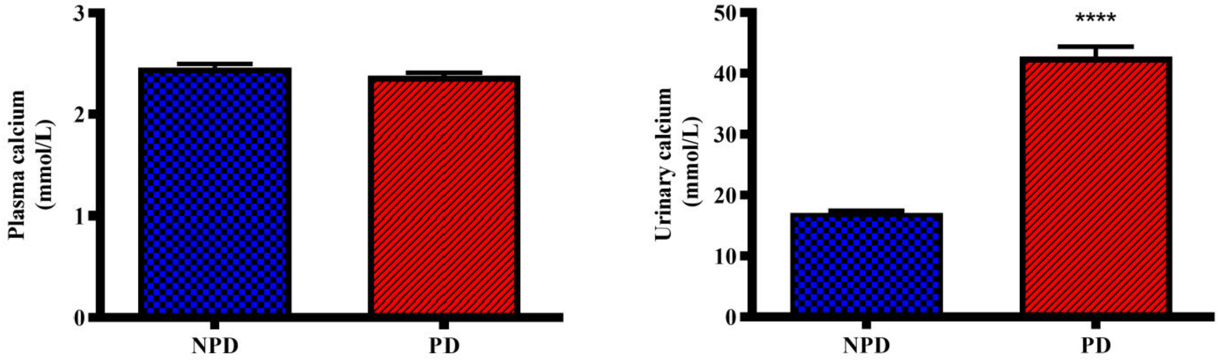

Figure 2: Concentrations of plasma and urinary calcium in the non-pre-diabetic (NPD) group and pre-diabetic group (PD) (n=6, per group). Values are depicted as mean ± SEM. ****= p<0.0001 when compared to NPD

Plasma osteocalcin and urine deoxypyridinoline

Plasma osteocalcin and urine deoxypyridinoline concentrations were measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig. 3) that the concentration of osteocalcin in plasma was significantly higher (p= 0.0002) in the PD group as compared to NPD. The concentration of deoxypyridinoline in urine was significantly (p=<0.0001) lower in the PD group as compared to the NPD group. It was also evident (Supplementary Table 1) that osteocalcin levels in plasma were positively correlated (r=0.87, p=0.02) with HOMA-IR in the PD state.

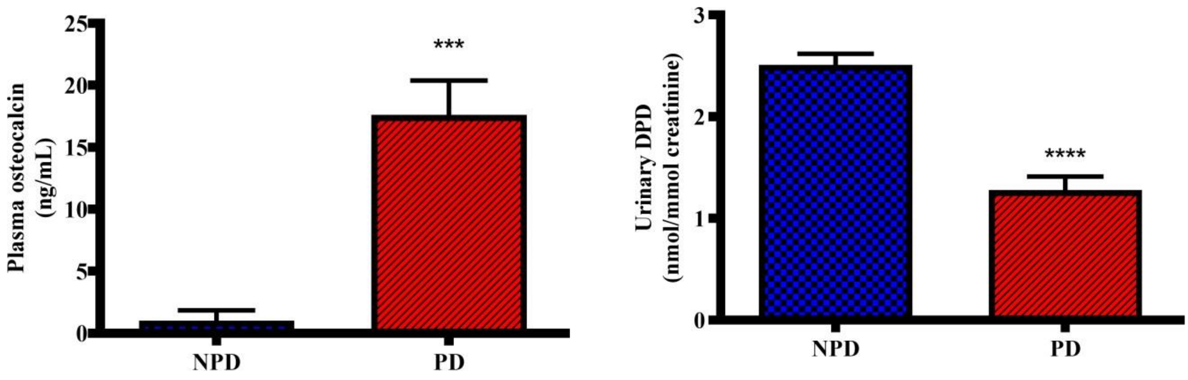

Figure 3: Concentrations of plasma osteocalcin and urine deoxypyridinoline in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group).Values are depicted as mean ± SEM. *** = $p < 0.001$, ****=$p<0.0001$ when compared to NPD

Renal TRPV5 mRNA

Renal Transient receptor potential cation channel subfamily V5 (TRPV5) gene expression was measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig 4) that the relative expression of renal TRPV5 was significantly (p=<0.0001) increased by 3.89-fold in the PD group relative to the NPD group.

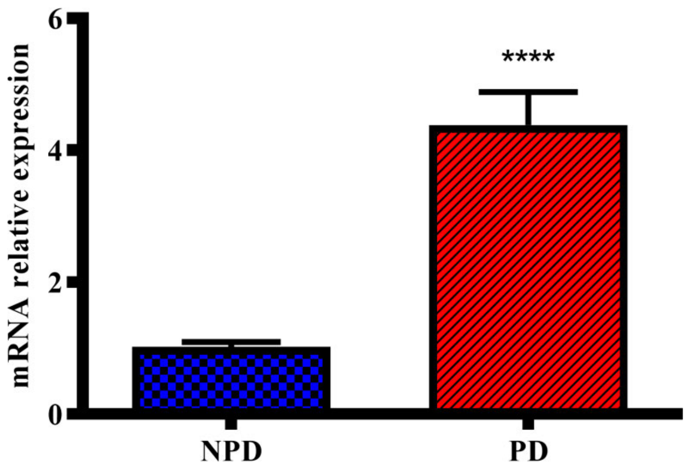

Figure 4: Renal TRPV5 gene expression in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group).Values are depicted as mean ± SEM. **** = $p < 0.0001$ when compared to NPD

Renal 1-alpha hydroxylase mRNA

Renal 1-alpha hydroxylase gene expression was measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig 5) that the relative expression of 1-alpha hydroxylase was significantly (p=<0.0001) increased by 10.96-fold in the PD group relative to the NPD group.

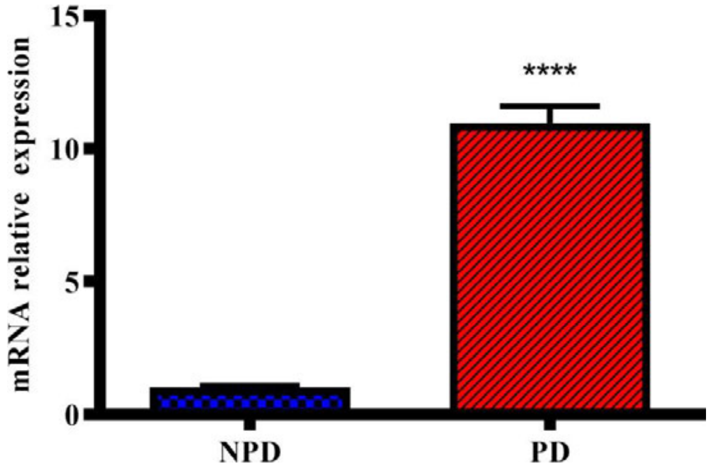

Figure 5: Renal-1 alpha hydroxylase gene expression in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group).Values are depicted as mean ± SEM. **** = $p < 0.0001$ when compared to NPD

Intestinal VDR mRNA

Intestinal vitamin D receptor (VDR) gene expression was measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig 6) that the relative expression of intestinal VDR was significantly (p=<0.0001) increased by 5.55-fold in the PD group relative to the NPD group.

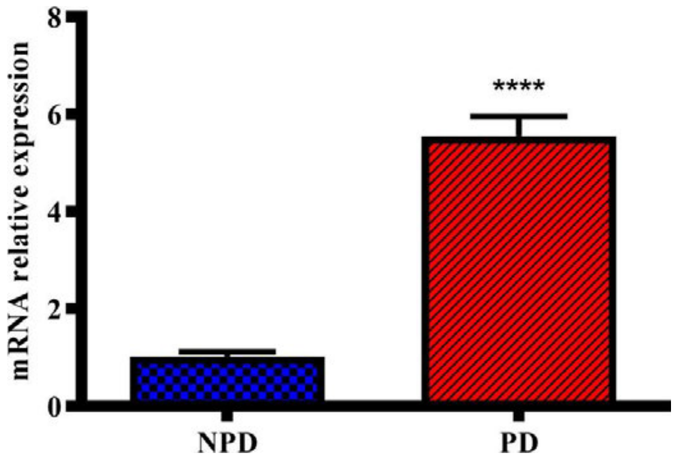

Figure 6: Intestinal VDR gene expression in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group).Values are depicted as mean ± SEM. **** = $p < 0.0001$ when compared to NPD

Intestinal calbindin-D_{9k} mRNA

Intestinal calbindin-D_{9k} gene expression was measured in the non-pre-diabetic (NPD) group (n=6) and pre-diabetic (PD) group (n=6) after the experimental period. It was evident (Fig 7) that the relative expression of intestinal calbindin-D_{9k} expression was significantly (p=<0.0001) increased by 9.13-fold in the PD group relative to the NPD group.

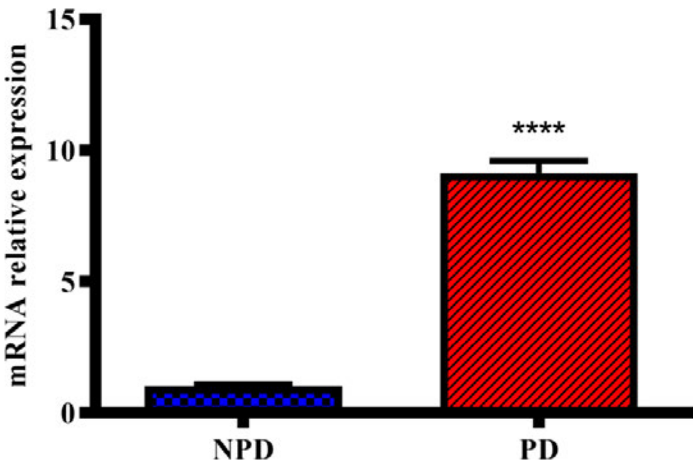

Figure 7: Intestinal calbindin-D_{9k} gene expression in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group).Values are depicted as mean ± SEM. **** = $p < 0.0001$ when compared to NPD

Discussion

Calcium-regulating organs work in association with calciotropic hormones such as parathyroid hormone (PTH), calcitonin and calcitriol to maintain calcium homeostasis [20]. Several studies have shown that the functioning of calcium-regulating organs are disturbed in T2DM [7, 10]. There is increased bone resorption, renal calcium wastage and intestinal malabsorption of calcium during the diabetic state [4, 10]. Furthermore, studies have recognized pre-diabetes as the state that occurs before the onset of T2DM as well as the state where numerous complications associated with T2DM begin [11, 12]. In our laboratory, pre-diabetes was induced in rats through consumption of a HFHC diet for a prolonged period according to a well-established protocol [11, 12]. According to studies, complications of T2DM, such as cardiovascular disease, renal failure and aberrant immunological function begin in pre-diabetes [11, 12]. However, previous studies have not elucidated the changes to the functioning of calcium-regulating organs during the pre-diabetic state. Hence, this study aimed to investigate the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs, namely the kidney, intestine and bone.

Pre-diabetes is characterised as a combination of impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), both of which can be attributed to moderate insulin resistance in insulin-dependent tissues [18]. Blood glucose levels must be constantly maintained within a physiological range including a postprandial glucose concentration less than 7.8 mmol/L while a fasting glucose concentration below 5.6 mmol/L [21]. In the postprandial state of normal glucose tolerant (NGT) individuals, blood glucose concentration increases and insulin is secreted to enhance glycogenesis and inhibit glycogenolysis [1]. As a result, plasma glucose levels are maintained followed by plasma insulin levels returning towards the homeostatic range [22]. However, in the PD state endogenous glucose production is high prior to food consumption and fails to decrease after food consumption in pre-diabetic patients [23]. This is a result of insulin-induced peripheral glucose uptake being impaired in insulin-dependent tissue [11]. This accounts for insulin, postprandial glucose levels, HOMA-IR and fasting plasma glucose being higher in pre-diabetic individuals by comparison to NGT individuals [18, 24]. In this study, the postprandial glucose concentration at 120 min, AUC and HOMA-IR value in PD group was significantly higher by comparison to NPD. These findings corroborated with previous findings that have shown significantly higher plasma glucose, insulin, 2-hour postprandial glucose levels and HOMA-IR in pre-diabetic patients by comparison to NPD [2, 25]. In the PD group, the elevated plasma insulin, impaired fasting glucose and HOMA-IR value in the range of insulin resistance may suggest that there is some insulin resistance from peripheral tissue against the uptake of glucose. High dietary fat promotes an increase in circulating triacylglyceride which breakdown to free fatty acids (FFA) [26]. The increase in FFAs around insulin-dependent tissue results in insulin resistance which decreases glucose uptake resulting in compensatory hyperinsulinemia, as seen in the PD group [27]. Increased concentrations of plasma glucose and the onset of insulin resistance in T2DM have been shown to interfere with the functioning of calcium-regulating organs in the diabetic state [4, 10].

Calcium plays a crucial role in various physiological processes and plasma calcium levels are kept within a narrow range through the interplay of calcium-regulating organs [28]. Calcium-regulating organs maintain plasma calcium levels by regulating renal calcium reabsorption, bone turnover and intestinal calcium absorption [6]. Previous studies have shown decreased plasma calcium concentrations in patients with type 2 diabetes by comparison to normoglycaemic individuals [10, 29]. These observations suggested that renal dysfunction and abnormal metabolism of vitamin D were responsible for inducing a hypocalcaemic state in diabetes [28]. However, other studies have shown no significant change to plasma calcium levels in diabetic individuals in comparison to NGT individuals [30, 31]. These studies have stated that calcium-regulating organs compensate for the reduced plasma calcium levels by inducing an increase in bone resorption, renal calcium reabsorption and intestinal calcium absorption [30, 31]. In this study, the plasma calcium levels of the PD group did not change significantly by comparison to the NPD. These results coincided with prior literature that have demonstrated no significant change to calcium levels in plasma of T2DM patients [30, 31]. The possible reason for no significant change to plasma calcium levels in the PD state may have been due to calcium-regulating organs compensating for the changes to plasma calcium levels.

The kidneys help maintain calcium homeostasis by regulating filtered calcium reabsorption and excretion [6]. Disturbances in renal calcium reabsorption can lead to excessive urinary calcium excretion and formation of kidney stones [9]. TRPV5 is a calcium channel that controls renal calcium reabsorption and is involved in urinary calcium homeostasis [32]. Studies have reported elevated urinary calcium levels along with decreased renal TRPV5 expression in diabetes [33, 34]. Studies have also shown that decreased renal TRPV5 expression was associated with reduced renal calcium reabsorption [35, 36]. These observations suggested that hyperglycaemia-induced renal damage may have downregulated renal TRPV5 expression [35]. The downregulation in renal TRPV5 expression promotes renal calcium wastage and hypocalcaemia in diabetes [37]. In addition, elevated urinary calcium levels have shown to result from intestinal hyperabsorption of calcium and excessive bone resorption in T2DM [38, 39]. Hence, the present study evaluated urine calcium and renal TRPV5 expression to evaluate kidney function. In this study, the PD group had significantly increased urinary calcium concentrations in the range of hypercalciuria by comparison to NPD. Furthermore, renal TRPV5 expression in the PD group was significantly higher in comparison to the NPD group. These results corroborated with previous findings that have shown elevated urine calcium and an upregulation in renal TRPV5 expression in T2DM patients [40, 41]. The increased urine calcium may have occurred as a result of kidney damage in the pre-diabetic state, which may have decreased the ability of the kidneys to reabsorb calcium. There may have been other contributors to the increased urine calcium such as increased intestinal calcium absorption and bone resorption [42]. Hence, the kidneys may try to compensate for the increased plasma calcium by excreting it in urine. However, the simultaneous increase in urinary calcium excretion and renal TRPV5 may suggest a compensatory mechanism against renal calcium wastage. The increased renal TRPV5 expression in the PD group may have promoted increased renal calcium reabsorption from urinary filtrate. Interestingly, renal TRPV5 expression is regulated by vitamin D, which is known to be catalysed to its active form in the kidney [5].

Renal 1-alpha-hydroxylase resides within the proximal convoluted tubules and is the principal enzyme associated in calcitriol synthesis [43]. Disturbances in kidney function and vitamin D metabolism can lead to excessive urinary calcium loss and hyperparathyroidism [28]. Studies have shown that a loss of kidney function in T2DM leads to a decline in circulating plasma calcitriol concentrations [44, 45]. Renal injury and the accumulation of metabolites in the diabetic kidney contribute to 1-alpha hydroxylase inhibition and lower circulating calcitriol levels [44, 45]. In this study, the PD group had significantly increased renal1-alpha hydroxylase expression by comparison to the NPD group. These results corroborated with previous studies that have shown an upregulation in renal 1-alpha hydroxylase expression in diabetic patients [43, 46]. However, these results have contrasted other studies that have shown decreased renal 1-alpha hydroxylase expression in the diabetic state [44, 45]. The upregulation in renal 1-alpha hydroxylase in the PD group may suggest that there is an increased demand to synthesis calcitriol in the PD state. The kidneys may compensate for the hypercalciuria by upregulating the expression of renal 1-alpha hydroxylase, in order to maintain normal calcium levels in plasma. In addition, the regulation of renal 1-alpha hydroxylase is dependent on the calciotropic hormones [47]. It is evident that renal cells still appear to be responsive to calciotropic hormones in the PD state, in attempt to conserve plasma calcium levels.

Intestinal calcium absorption is a pivotal physiological process for the maintenance of calcium homeostasis [48]. The small intestine absorbs dietary calcium and physiologically adapt according to the conditions of the body [49]. The expression of calcium-binding proteins and vitamin D receptors (VDR) are vital for efficient absorption of calcium in the small intestine [49]. Vitamin D metabolites control intestinal calcium absorption through activating the vitamin D receptor (VDR), which causes increased production of calcium transport proteins such as calbindin-D_{9k} [49]. Type 2 diabetes is linked to a significant decline in calcium metabolism, partially from impaired intestinal calcium absorption [6, 50]. Previous studies on diabetic rats reported that the decrease in intestinal calcium absorption occurred simultaneously with a reduction in VDR and calcium-binding protein calbindin-D_{9k} in enterocytes [4, 51]. It was noted that intestinal VDR and calcium-binding proteins were downregulated due to impaired production of calciotropic hormones in T2DM [6]. Subsequently, the ability of the intestine to adapt to disturbances to low plasma calcium levels is compromised during the diabetic state [6]. However, other studies have shown an upregulation in intestinal calcium transporter expression in diabetic rats [39, 49]. The increased intestinal VDR number promoted increased VDR-calcitriol complexes and increased intestinal calcium transport [6]. It was stated that the increased intestinal calcium absorption may have been a compensatory response to renal calcium wastage and hypocalcaemia [39, 49]. Hence, the present study investigated intestinal VDR and calbindin-D_{9k} expression to evaluate intestinal calcium transport. In this study, intestinal VDR and calbindin-D_{9k} expression in the PD group were significantly higher as compared to the NPD. These results contrasted previous findings that have shown a downregulation in intestinal VDR and calbindin-D_{9k} expression in the diabetic state [4, 51]. The elevated intestinal VDR and calbindin-D_{9k} expression in the PD group may suggest an increase in intestinal calcium absorption. The upregulation of calcium transport genes in the intestine of the PD group may have been a compensatory response to renal calcium wastage.

Bone regulates plasma calcium levels by releasing calcium and storing calcium through processes such as bone resorption and bone formation [52]. Bone resorption is coupled with bone formation, where elevated bone resorption precedes elevated bone formation [5]. An imbalance between bone formation and resorption may result in bone diseases including osteoporosis [53, 54]. Bone resorption and formation can be determined indirectly by measurement of plasma concentrations of bone markers [55]. These markers include constituents of the bone matrix released into the bloodstream during bone resorption or formation [56]. Osteocalcin is a marker of bone formation, whereas deoxypyridinoline is a marker of bone resorption [56]. Some studies have shown increased bone turnover in type 2 diabetic patients, where bone resorption exceeds formation [21, 57]. This was evidenced by decreased plasma osteocalcin levels and increased deoxypyridinoline levels [58, 59]. These observations suggested that during the diabetic state there is an increased demand to mobilize calcium from bone to compensate for hypocalcaemia; however the normal bone coupling process becomes compromised [58, 59]. Hyperglycaemia has shown to decrease bone formation by inhibiting osteoblast synthesis and differentiation [42]. However, other studies have reported increased bone formation in type 2 diabetes [21, 60]. It was stated that hyperinsulinemia alters the balance between bone formation and resorption by favouring bone formation [56]. Hence, the current study focused on investigating the levels of plasma osteocalcin and urine deoxypyridinoline in the PD state, to evaluate bone turnover. In this study, the PD group had increased plasma osteocalcin concentration and decreased urinary deoxypyridinoline concentration by comparison to NPD. These results corroborated with previous findings that have shown increased plasma osteocalcin levels and decreased urinary deoxypyridinoline levels [60, 61]. These observations may suggest that there is increased bone formation and decreased resorption in the pre-diabetic state. The increased bone formation and decreased bone resorption may have been induced by calciotropic hormones to compensate for hypercalcaemia [62, 63]. Insulin is an anabolic hormone which has shown to promote bone formation and inhibit bone resorption [60]. In the pre-diabetic state, early insulin resistance leads to a compensatory increase in insulin secretion [36]. The elevated plasma insulin levels in the PD group may have promoted increased bone formation together with decreased bone resorption. Furthermore, previous research have shown that HOMA-IR and plasma osteocalcin levels were positively correlated in diabetic patients [64, 65]. It was observed that osteocalcin can trigger secretion of insulin, by acting directly on secretion and proliferation of pancreatic beta (β)-cells. Interestingly, there was a positive correlation between plasma osteocalcin and HOMA-IR in the PD group. It may be speculated that the elevated plasma osteocalcin concentration in the PD group may have been a compensatory response to cope with the early insulin resistance. This may be an early adaptation mechanism for insulin resistance, which fails with the onset of overt T2DM.

The findings elucidated in this study may have the potential to provide an understanding into the physiological processes that occur in calcium-regulating organs during pre-diabetes. From a clinical perspective, pre-diabetes is asymptomatic and many people progress towards the development of T2DM due to being unaware. The findings of this study will not only add to academic knowledge but may serve as a novel marker in the identification of pre-diabetes. This study targets some of the complications and disrupted processes involved in T2DM. Furthermore, these findings may provide an early insight into the pathogenesis involved in the associated complications of T2DM. A future prospective would be to use these findings as insights to understand the possible changes that may occur to pre-diabetic humans.

It is evident that during the PD state there are changes to the functioning of calcium-regulating organs which compensate for disturbances to plasma calcium levels. This was made evident by increased urinary calcium levels along with increased expressions of renal TRPV5, renal 1-alpha hydroxylase, intestinal VDR and intestinal calbindinD_{9k}. In addition, there was increased plasma osteocalcin and decreased urinary deoxypyridinoline concentrations along with unchanged plasma calcium in the PD state. The normocalcaemia present in the PD state may have been conserved due to increased renal calcium reabsorption, increased renal vitamin D activation, increased intestinal calcium absorption and increased bone formation followed by decreased bone resorption.

Conclusion

Taken together, calcium-regulating organs compensate for renal calcium wastage and are aimed at maintaining normocalcaemia in HFHC diet-induced pre-diabetes. The effects associated with pre-diabetes on calcium-regulating organs are directed towards promoting increased renal calcium reabsorption, increased renal vitamin D activation, increased intestinal calcium absorption and decreased bone resorption followed by increased bone formation. This was evidenced by increased expression of renal calcium transport markers and intestinal calcium transport markers in addition to increased osteocalcin and decreased deoxypyridinoline levels. Collectively, these observations may suggest that calcium-regulating organs compensate for the changes to calcium homeostasis in the PD state.

Future recommendations

In future, a further insight into the mechanisms in which bone turnover by-products participate in glucose homeostasis in the PD state should be investigated.

Competing interests

The authors declare that they have no competing interests.

Ethics approval

This study was approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, Durban, South Africa (AREC/024/018D).

Author contributions

K.N contributed to the study design, conducted the experiments, collected, analysed and interpreted data as well as being involved in writing the manuscript. P.S.N and A.K. was involved in the conceptualization of the study, study design and editing of the manuscript as well as provide funding.

All authors have read the manuscript and approved its submission.

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Supplementary Material

Osteocalcin-HOMA-IR correlation

Supplementary Table 1: Pearson's correlation between plasma osteocalcin and HOMA-IR in the non-pre-diabetic (NPD) group and pre-diabetic group (PD) (n=6, per group). Values are depicted as mean ± SEM. *= $p<0.05$

Metabolic Parameter		**Plasma osteocalcin**
HOMA-IR values	**NPD**	r= 0.80 p= 0.06
	PD	r= 0.87 p= 0.02*

CHAPTER 4: SYNTHESIS AND CONCLUSION

Increased intake of high-caloric diets has correlated with increased prevalence of type 2 diabetes mellitus (T2DM) [1]. Pre-diabetes is a long-term, asymptomatic state of moderate hyperglycemia that precedes T2DM [2]. Several studies reported that often life-threatening complications associated with T2DM begin in the pre-diabetic state [3, 4]. Hyperglycaemia in T2DM has been observed to disrupt calcium homeostasis by disturbing calciotropic hormones and the functioning calcium-regulating organs [5]. Furthermore, altered levels of calciotropic hormones have shown to promote the development of hyperglycaemia and insulin resistance in T2DM [6]. In addition, diabetes has been shown to promote impaired intestinal calcium absorption, kidney calcium reabsorption and bone turnover [5, 7]. However, the changes to calcium homeostasis during the pre-diabetic state have not been elucidated. Hence, this study aimed to investigate the changes to calcium homeostasis by looking at calciotropic hormones and its association with glycated haemoglobin and insulin resistance in the pre-diabetic state. Furthermore, this study also sought to investigate the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs.

Previous studies have shown that calcium homeostasis is disturbed in diabetes evidenced by altered levels of calciotropic hormones [8, 9]. Some studies have shown the presence of either hypocalcaemia or hypercalcaemia in diabetic patients [10, 11]. However, other studies have shown normal plasma calcium levels in type 2 diabetic individuals [12, 13]. These observations reported that plasma calcium levels are conserved within the homeostatic range due to calciotropic hormones compensating for changes [12, 13]. In our laboratory, a pre-diabetic animal model mimicking the human pre-diabetic state has been developed and used to observe the changes that may occur in pre-diabetes [1, 14]. In the first manuscript, the diet-induced pre-diabetic group had no significant change to plasma calcium levels along with elevated plasma glucose, insulin, HbA1c and HOMA-IR by comparison to the NPD group. The elevated plasma glucose, insulin, HbA1c and HOMA-IR index in the PD group suggested that there was insulin resistance from peripheral tissue which promoted glucose accumulation in blood and compensatory hyperinsulinemia. The unchanged calcium concentrations in the plasma of the PD group may be attributed to calciotropic hormones maintaining calcium homeostasis. This led us to determine the levels of calciotropic hormones in the pre-diabetic state, to elucidate whether calciotropic hormones compensate for the changes to plasma calcium. In this study, plasma PTH, calcitonin, vitamin D and calcitriol were measured via ELISA in the PD and NPD groups. The results have shown that the PD group had significantly higher plasma PTH, calcitonin, vitamin D and 1,25-dihydroxyvitamin D3 (calcitriol) concentrations when compared to the NPD. These results concurred with the observations made by previous studies which revealed that in T2DM, due to increased plasma PTH, calcitonin and calcitriol, there are normal plasma calcium concentrations [15, 16]. The elevated plasma PTH and calcitonin levels may be due to the co-ordination between these hormones in order to compensate for changes to plasma calcium levels in the PD group. Plasma PTH levels may have increased to compensate for renal calcium wastage which has shown to be present in the diabetic state. However, increased parathyroid gland stimulation in response to renal calcium wastage may lead to secondary hyperparathyroidism [17]. Therefore, plasma calcitonin concentrations may have increased to protect the body from PTH oversecretion. Moreover, increased dietary content of fats and high fructose may have promoted increased intestinal vitamin D absorption [18, 19]. The increased plasma calcitriol levels in the PD group may have been a compensatory mechanism for low plasma calcium. Calcitriol production is dependent on adequate substrate supply and vitamin D deficiency compromises calcium homeostasis [20]. The increased intestinal vitamin D absorption may be a compensatory mechanism to cope with hypocalcaemia in pre-diabetes. Furthermore, hyperglycaemia is associated with hypercalciuria as this condition results in damage to the glomerular filtration system thus, impairing the kidneys ability to filter and reabsorb calcium [10, 21]. In this study, urine calcium levels were significantly higher in the PD when compared to the NPD group, in the range of hypercalciuria. Renal dysfunction and injury in the pre-diabetic state were observed in studies conducted in our laboratory [3, 22]. These observations confirm that renal dysfunction in pre-diabetes may have impaired the ability of the kidneys to reabsorb calcium [3, 22]. However, researchers may argue that hypercalciuria could have been due to increased plasma calcium levels which may have induced a compensatory increase in calcium excretion [23, 24]. Therefore, taking into account the significantly increased calciotropic hormones such as plasma PTH, calcitonin, calcitriol and vitamin D concentration, the significantly elevated urinary calcium concentration, and the unchanged plasma calcium levels, this study for the first time shows that calciotropic hormones compensate for the disturbances to plasma calcium in pre-diabetes. Furthermore, studies have shown that altered calciotropic hormones levels promote the development of hyperglycaemia and insulin resistance [15, 25]. Elevated plasma PTH and calcitonin levels have shown to positively correlate with insulin resistance and hyperglycaemia in T2DM [15, 25]. However, plasma calcitriol levels have shown to negatively correlate with insulin resistance and hyperglycaemia in T2DM [9, 15]. Therefore, this study also sought to investigate the association of calciotropic hormones with insulin resistance and hyperglycaemia. Accordingly, the correlation of PTH, calcitonin and calcitriol with HbA1c and HOMA-IR were analysed in the current study. The results revealed that plasma PTH and calcitonin levels were positively correlated with glycated haemoglobin but not insulin resistance in the PD group. Furthermore, plasma calcitriol levels were negatively correlated with glycated haemoglobin in the PD group. These results concurred with the observations made by previous studies which revealed that in T2DM, calciotropic hormones were associated with glycated haemoglobin [15, 26]. Elevated plasma PTH and calcitonin promote an increase in blood glucose concentrations by upregulating gluconeogenesis and glycogenolysis [6, 26]. For the first time this study has shown that pre-diabetes induced by the HFHC diet is not only associated with disturbances to calcium homeostasis but that elevated calciotropic hormones in the PD state may further contribute to the progression of T2DM. Furthermore, plasma calcitriol may serve as an early adaptation for intermediate hyperglycaemia, which fails with the onset of T2DM. In conclusion, we may postulate that diet-induced pre-diabetes promotes a disturbance to calcium homeostasis which may in part be the mechanistic pathway that leads to the development of hyperglycaemia in T2DM.

Since, calcium homeostasis is regulated by calciotropic hormones and calcium-regulating organs, it was essential to investigate the functioning of calcium-regulating organs in the PD state. Furthermore, the unchanged plasma calcium concentration and altered levels of calciotropic hormones in the PD group in manuscript 1 prompted us to investigate the changes to calcium-regulating organs in pre-diabetes. The changes to the functioning of calcium-regulating organs in T2DM have been well-characterised, where it was demonstrated that there was impaired calcium absorption in intestine, renal calcium wastage and increased bone resorption [7, 27]. However, there is no scientific evidence on the functioning of calcium-regulating organs during the PD state. Hence, the second study aimed to investigate the effects of diet-induced pre-diabetes on the functioning of calcium-regulating organs in the PD state.

To investigate the functioning of calcium-regulating organs during HFHC diet-induced moderate hyperglycaemia; markers associated with kidney calcium transport, intestinal calcium transport and bone turnover were analysed. Additionally, urine calcium and plasma calcium concentrations were measured in the PD and NPD groups. The diet-induced pre-diabetic animal model was used again in manuscript 2. In this study, the unchanged plasma calcium levels in the PD group in comparison to the NPD group suggested that calcium-regulating organs may have compensated for the changes. Previous studies have shown that renal dysfunction in diabetes impairs renal calcium reabsorption by downregulating TRPV5 expression and compromising the ability of the kidneys to regulate vitamin D metabolism [28, 29]. Therefore, the present study evaluated the expressions of renal TRPV5 and 1-alpha hydroxylase via RT-qPCR in addition to urine calcium concentration. In this study, there was elevated urine calcium along with increased expression of renal TRPV5 and 1-alpha hydroxylase expressions in the PD group by comparison to NPD. The simultaneous upregulation in renal TRPV5, 1-alpha hydroxylase and urine calcium insinuate that the kidneys function to increase calcium reabsorption in the PD state. These results contrasted observations made by previous studies which revealed a decrease in renal TRPV5 and 1-alpha hydroxylase in T2DM [30, 31]. This suggests that during the PD state there is an increased demand to conserve plasma calcium levels and depress renal calcium wastage. Interestingly, the renal calcium wastage in the second manuscript provides an explanation for the elevated plasma PTH and calcitriol levels in the first manuscript. The increased urinary calcium loss in the pre-diabetic state may have induced a state of hypocalcaemia, as a result plasma PTH and calcitriol concentrations may have increased, as seen in manuscript 1. Furthermore, the second manuscript confirmed that the possible elevated plasma calcitriol in the PD group of the first study may have been due to the elevated renal 1-alpha hydroxylase expression. Disturbed calcium homeostasis due to impaired functioning of calcium-regulating organs have been evidenced in the small intestine; hence calcium transport was investigated in the small intestine [32, 33]. Previous studies have shown suppressed intestinal calcium absorption evidenced by decreased expression of VDR and calbindin-D_{9k} in diabetic patients [5, 34]. Hyperglycaemia has shown to downregulate intestinal calcium transport proteins inducing intestinal calcium malabsorption [5, 34]. Furthermore, decreased plasma calcitriol levels have shown to impair the ability of the intestine to adjust its absorption of calcium according to the needs of the body in T2DM [32]. Therefore, the present study evaluated the expression of intestinal VDR and calbindin-D_{9k} via RT-qPCR in the pre-diabetic state. The results showed that intestinal VDR and calbindin-D_{9k} expression was significantly higher within the PD group in comparison to the NPD. These observations implied that intestinal calcium absorption is increased in the PD state. The increased intestinal calcium absorption may have been a compensatory response to renal calcium wastage and hypocalcaemia. It is evident that during the PD state, the ability of the intestine to compensate for the changes to plasma calcium is not compromised and responds to altered calcium homeostasis. The elevated concentrations of plasma PTH and calcitriol in the PD group of the first manuscript may have promoted the increased intestinal calcium absorption. Researchers have argued that increased intestinal calcium absorption in the diabetic state may overcompensate for renal calcium wastage inducing a state of hypercalcaemia [23, 35]. The increased plasma calcium absorbed by the small intestine calcium gets shunted to the bone promoting increased bone formation and decreased bone resorption [23, 35]. However, other studies have shown decreased bone formation and increased bone resorption in diabetic patients [36, 37]. These observations suggested that to compensate for hypocalcaemia, there is excessive mobilization of calcium from bone [36, 37]. Subsequently, the increased bone resorption followed by decreased bone formation leads to conditions such as osteoporosis and bone weakness [38, 39]. Therefore, in this study bone turnover was analysed by measuring bone resorption marker deoxypyridinoline and bone formation marker osteocalcin. In this study, the PD group had decreased urine deoxypyridinoline and increased osteocalcin concentrations by comparison to NPD. These observations suggested that during the PD state there is decreased bone resorption and increased bone formation. The elevated plasma calcitonin concentrations in the first manuscript may have suppressed bone resorption and promoted bone formation in study 2. The depressed bone resorption and increased bone formation may suggest a compensatory response to elevated plasma calcium levels. Previous studies have shown that there is hyperplasia of the parathyroid gland in T2DM which promotes the continuous secretion of PTH and altered calcium sensing by parathyroid glands. The increased PTH concentrations in the PD group of study 1 may have overcompensated for renal calcium wastage by upregulating intestinal absorption and renal reabsorption of calcium inducing a state of hypercalcaemia. Subsequently, in this study the bone may have responded to the elevated plasma calcium by promoting bone formation and inhibiting bone resorption. Taking into account the upregulation of intestinal calcium absorption and renal calcium reabsorption, this study for the first time shows that bone may compensate for excess plasma calcium retention induced by the above-mentioned organs in pre-diabetes. Insulin was evidenced to serve an integral function in formation of bone [40]. In this study there was increased plasma insulin concentration, in addition to increased plasma osteocalcin during diet-induced moderate hyperglycaemia. In T2DM, hyperinsulinemia has shown to have anabolic effects on bone by promoting osteoblast differentiation and supressing bone resorption [40, 41]. These findings are consistent with the elevated osteocalcin and decreased deoxypyridinoline concentrations observed in this study. Interestingly, extensive literature has demonstrated a link between plasma osteocalcin concentrations and insulin resistance [41, 42]. In T2DM, plasma osteocalcin has shown to alleviate insulin resistance and glucose intolerance by promoting glucose uptake in peripheral tissue [43, 44]. In the current study, elevated HOMA-IR in the PD group has been positively correlated with plasma osteocalcin concentration. The results corroborated with previous findings that have shown a positively correlation between osteocalcin and HOMA-IR [41, 42]. These observations may be a result of the β-cell compensatory mechanism, whereby hyperinsulinemia in the pre-diabetes stage induces an increase in osteocalcin levels to mediate insulin resistance. For the first time, our research reveals that calcium-regulating organs maintain normocalcaemia in pre-diabetes by upregulating genes associated with intestinal calcium transport, renal calcium reabsorption and decreasing bone resorption followed by increased bone formation.

Biologically, study 1 and study 2 have shown the presence of disturbances to calcium homeostasis. The altered levels of calciotropic hormones in study 1 and the markers associated with calcium-regulating organ functioning in study 2 may serve as novel markers in the early detection of T2DM and its associated complications. This may change guidelines in the detection of type 2 diabetes mellitus. Medical practitioners and laboratory technicians may use the markers associated with calcium homeostasis in this study to aid in early detection of T2DM and calcium-related complications. The future prospects of this study would be to include calcium homeostasis as a marker in pre-diabetes and T2DM diagnosis. These findings warrant the importance of screening individuals at risk for developing T2DM by evaluating markers of calcium homeostasis.

Calcium homeostasis is disturbed in the pre-diabetic state, which was evidenced by changes to calciotropic hormones and calcium-regulating organs. The effects associated with diet-induced pre-diabetes on calcium homeostasis include increased urine calcium along with unchanged plasma calcium. This was accompanied by elevated plasma PTH, calcitonin, vitamin D and calcitriol. Furthermore, plasma PTH and calcitonin in the PD state were positively correlated with glycated haemoglobin. Furthermore, there was an upregulation in renal calcium reabsorption, intestinal calcium absorption and decrease in bone resorption followed by increased bone formation. Taken together, these observations suggested that calciotropic hormones and calcium-regulating organs compensate for the changes to plasma calcium in the PD state. However, the changes to calciotropic hormones in the pre-diabetic state may promote the progression of hyperglycaemia in T2DM. Therefore, with the accumulative evidence of study 1 and study 2 we accept the hypothesis which states that during the pre-diabetic state there will be changes to calciotropic hormones and the functioning of calcium-regulating organs indicative of disturbed calcium homeostasis.

In summary, as shown in Figure 1, elevated plasma PTH, calcitriol and vitamin D in pre-diabetes may be a compensatory response to hypocalcaemia which promotes an increase in intestinal calcium absorption and renal calcium reabsorption. However, this may overcompensate for renal calcium wastage inducing a state of hypercalcaemia. This disturbance to plasma calcium elicits a compensatory increase in calcitonin secretion which directs calcium to be stored in bone and suppresses bone resorption, returning plasma calcium levels back to its homeostatic range. The elevated calciotropic hormones in study 1 compensates for disturbances to plasma calcium by inducing corresponding changes to calcium-regulating organs in study 2 in favour of normocalcaemia. However, altered levels of calciotropic hormones in the PD state may promote hyperglycaemia in T2DM, and furthermore be responsible for the overcompensation. Therefore, we accept the hypothesis that during the pre-diabetic state there are changes to calciotropic hormones and the functioning of calcium-regulating organs.

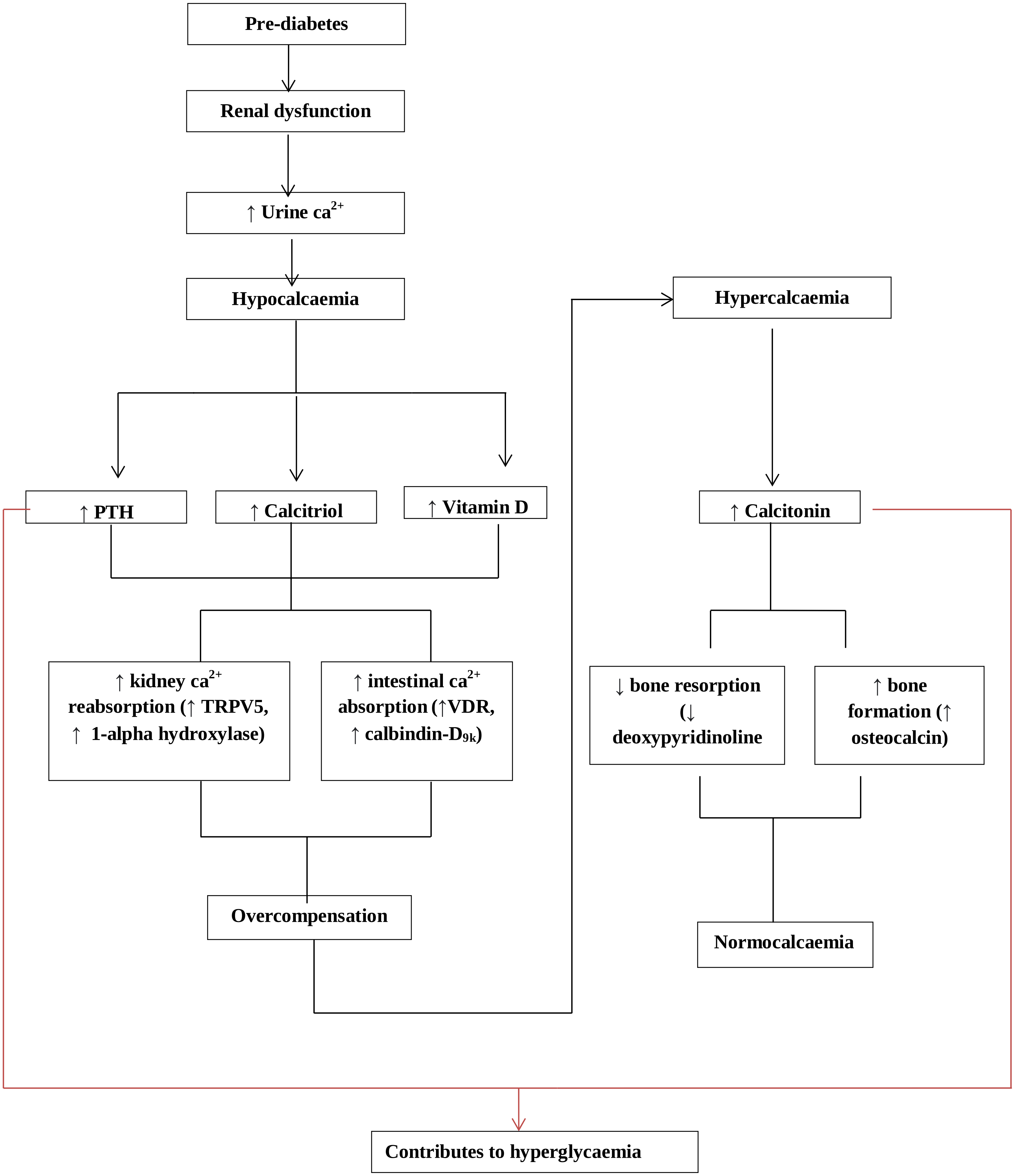

Figure 1: Diagram illustrating the changes to calcium homeostasis in a diet-induced pre-diabetic rat model

Conclusion

In the pre-diabetic state, calcium homeostasis is disturbed however calciotropic hormones and calcium-regulating organs maintain normoglycaemia. The hypothesis of this study is accepted due to the presence of changes to calciotropic hormones and calcium-regulating organs in the pre-diabetic state. The effects of diet-induced pre-diabetes on calcium homeostasis include elevated calciotropic hormones, intestinal calcium transport genes, renal calcium transport genes and decreased bone resorption followed by increased formation in addition to elevated urine calcium and unchanged plasma calcium. Furthermore, altered levels of calciotropic hormones in the PD state were positively correlated with glycated haemoglobin. These observations suggest that despite plasma calcium levels been conserved in the PD state, altered levels of calciotropic hormones may promote the development of type 2 diabetes.

Shortfalls and future studies

In the present study, we investigated calcium homeostasis by looking at the changes to calciotropic hormones and calcium-regulating organs. We established that calcium homeostasis was disturbed, however calciotropic hormones and calcium-regulating organs compensate for the disturbances to plasma calcium. Furthermore, we established that calciotropic hormones may contribute to the development of hyperglycaemia in T2DM. The histology of the parathyroid gland, as well as the expression of calcium-sensing receptors in parathyroid gland should be studied in the future, to determine whether there is any early resistance in the detection of plasma calcium levels.

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APPENDICES

APPENDIX 1: ETHICAL CLEARANCE

UNIVERSITY OF KWAZULU-NATAL™
INYUVESI YAKWAZULU-NATALI

04 May 2018

Mr Akinjide Akinnuga (217081429)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Mr Akinnuga,

Protocol reference number: AREC/024/018D
Project title: Investigating the effects of bredemolic acid on pre-diabetic rats model

Full Approval – Research Application

With regards to your revised application received on 16 April 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

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

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

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

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 04 May 2019.

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

[redacted]

....

Professor Shahidul Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Andile Khathi
Cc Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Anita Engelbrecht
Cc BRU – Dr Linda Bester

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
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Authors are asked to follow the outline set below: Page 1: a) title, b) short running title (limit: 40 char-acters), c) name of the author (no titles or academic grades) and address of the institute(s) where the investigations have been carried out. Should the ad-dress of the author at the time of publication differ from the one stated in the paper, the current address should be stated in a footnote, d) complete mailing address of corresponding author including telephone and telefax numbers and e-mail addresses. Page 2: a) an abstract containing not more than 250 words withno abbreviations, b) keywords (3–6 without repeating words in the title). Page 3 and onwards: a) introduc- tion also indicating the aim of the study, b) materials and methods, c) results: double presentation of data in the form of text, tables or figures should be avoid-ed, d)discussionandconclusions, e) listofreferences, f) legends of tables and figures.

3. References a) *Text*: Citations and references should be numbered consecutively using square brackets in the order in which they are mentioned in the text, followed by any tables or legends. Please do not al- phabetize references and bibliographics. Do not use footnotes and hyperlinks. Ifauthors are mentioned inthe text, onlythe first author should be given followed by "et al." whenever the reference has three or moreauthors. Example: " …protein concentrations were determined according to Lowry et al. [12]. b) List ofReferences: References should be given as plain text. Do not use fields in MS Word, as these are difficult toprocess later. The references should be listed in num-bered order according to the sequence they appear in the text. All authors or groups of authors of each publication should be mentioned. The name of the author(s) should be followed by the full title of the paper, name of the journal in which it has been pub-lished (abbreviaions according to Index Medicus viz. PubMed/Medline), year of publication, volume, firstand last page. Abstracts and supplements have to be clearly marked. Chapters from books have to be citedas follows: author(s), title of chapter, title of book, editor(s), place of publication, publisher, year of pub- lication, first and last page ofthe chapter. Examples:

9 Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin-phenol reagent. J Biol Chem 1951; 193: 265–275

10 Kerner W, Pfeiffer EF. The artificial pancreas. In: Samols E, ed. The endocrine pancreas. New York: Raven Press, 1991: 441–456

Original papers should not exceed 6 printed pages, i.e. 15 typewritten double-spaced manu- script pages of 30 lines of 60 letters each, includ- ing references, tables, figures and legends. Please do not use more than one blank space between words and sentences. A maximum of 4 figures and 3 tables is allowed. Longer manu- scripts will be subject to editing and a page charge of € 180 per printed page (including 19% VAT) starting with the seventh printed page.

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Reviews are normally published by invitation only.Reviews deal with previous research on a certain topic and serve to summarize the current state of the art. Their structure varies from an original paper ac- cording to the nature of the review. Reviews coveringbasic research should take a causal and mechanisticapproach, whereas reviews dealing withclinicaltopicsshould focus on therapeutic relevance. They shouldnot exceed 20 word-processed pages (double-spread, Times New Roman 12 point) and 100 references. All reviews will be peer reviewed. Please refrain from submitting uninvited reviews. If you plan to submit a review, please contact the editors first, explaining in your letter to the editor why this review is unique andsuited to advance the field.

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For formal requirements please refer to the instruc-tions fororiginalarticles above.

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Research Ethics

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Journal of Endocrine Pathology

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- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June2007

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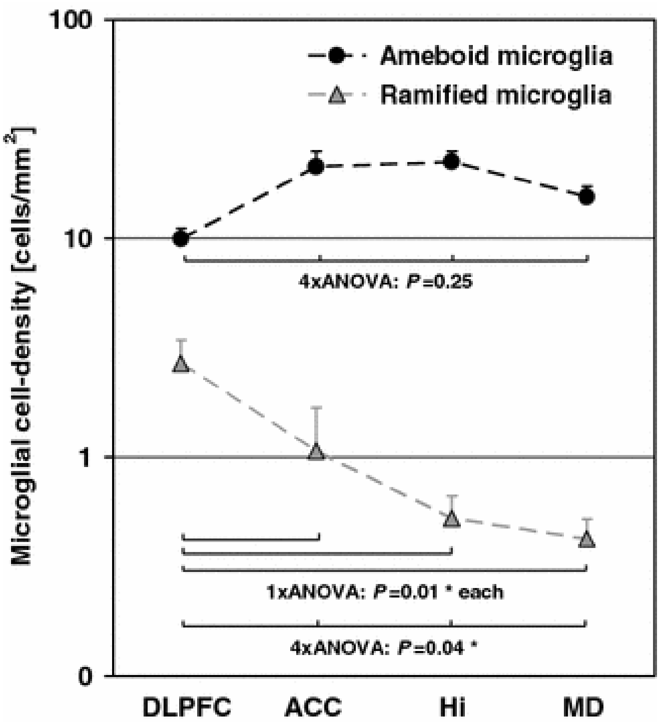

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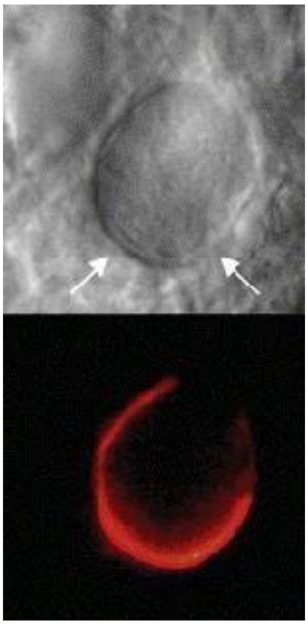

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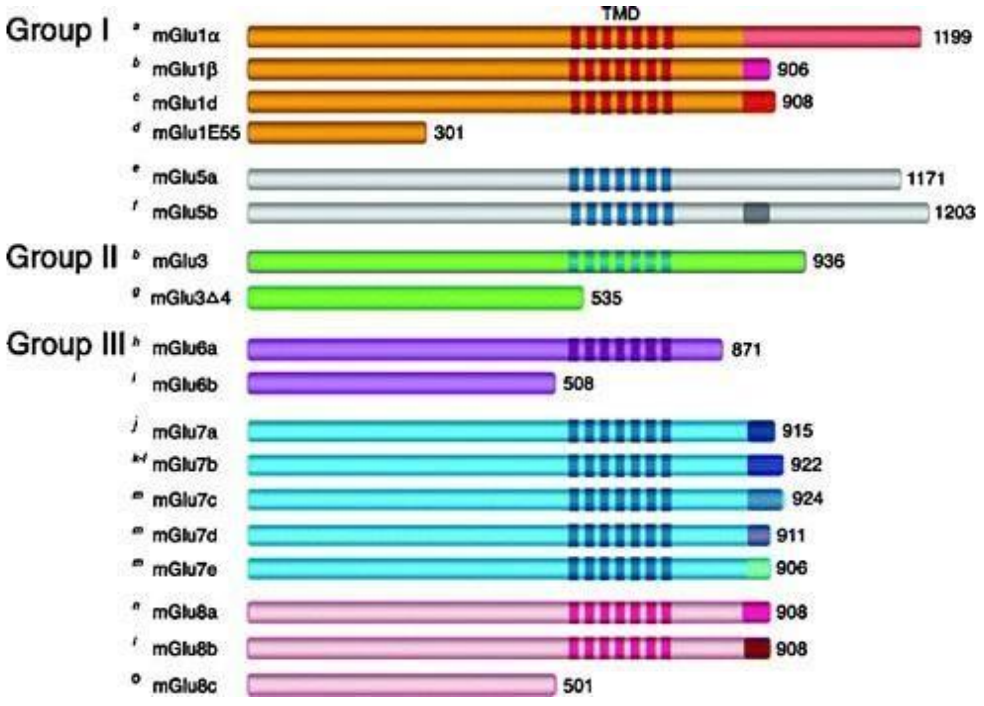

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When reporting a study that involved human participants, their data or biological material, authors should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that an independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. If a study was granted exemption from requiring ethics approval, this should also be detailed in the manuscript (including the reasons for the exemption).

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Although retrospective studies are conducted on already available data or biological material (for which formal consent may not be needed or is difficult to obtain) ethics approval may be required dependent on the law and the national ethical guidelines of a country. Authors should check with their institution to make sure they are complying with the specific requirements of their country.

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Case reports require ethics approval. Most institutions will have specific policies on this subject. Authors should check with their institution to make sure they are complying with the specific requirements of their institution and seek ethics approval where needed. Authors should be aware to secure informed consent from the individual (or parent or guardian if the participant is a minor or incapable) See also section on **Informed Consent**.

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- This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No.................................).

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- The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number ....).

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- Ethical approval was waived by the local Ethics Committee of University A in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

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Summary of requirements

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Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

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APPENDIX 3: DIET COMPOSITIONS

Composition of the high fats high carbohydrates (HFHC) diet

Ingredient	Incl(%)	Mix(kg)
Maize	38.98	390.000
Palm Oil	20.99	210.000
Soya Full Fat	14.99	150.000
Wheat Gluten	6.50	65.000
Flour	6.00	60.000
Monodex	5.00	50.000
Sugar - White	5.00	50.000
Limestone	1.00	10.000
Dicalcium Phosphate	0.50	5.000
Vitamin Premix	0.35	3.500
Salt - Fine	0.30	3.000
Amino Acid - DL Methionine	0.30	3.000
Mineral Premix	0.10	1.000
	100.01	**1000.50**

Nutritional value of the high-fats high-carbohydrate (HFHC) diet

Nutrient	Units	Actual
Dry Matter	g/kg	919.93
Metabolizable Energy	MJ/kg	15.86
Crude Protein	g/kg	151.27
AShreonine	g/kg	4.51
ASIsoluecine	g/kg	5.24
ASLysine	g/kg	6.54
ASMethionine	g/kg	4.86
ASryptophan	g/kg	1.30
ASstidine	g/kg	3.30
ASTSAA	g/kg	6.79
ASValine	g/kg	5.80
Fat	g/kg	250.46
Carbohydrate	g/kg	427.29
Fibre	g/kg	22.08
Ash	g/kg	26.31
Avl Phosphorus	g/kg	1.66
Calcium	g/kg	5.47
Total Phosphorus	g/kg	3.60

Composition of fats, proteins and carbohydrates of the normal diet (Diet intervention)

Fats	15 %
Proteins	25 %
Carbohydrates	65 %

APPENDIX 4: TURNITIN REPORT

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