An Investigation into the Prevalence of Prediabetes in Durban, South Africa, as well as the associated Hormonal Imbalances and MicroRNA Expression Patterns in Patients with Prediabetes.

By

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PREFACE

The thesis was conducted under the supervision of Professor Andile Khathi and Dr Phikelelani Ngubane at the University of KwaZulu-Natal, Discipline of Physiology, in fulfilment of the requirements for a Doctor of Philosophy degree in Health Sciences. This research project begins with an introduction and stated objectives (Chapter 1), followed by a comprehensive literature review (Chapter 2) and methodology overview (Chapter 3). Chapter 4 presents a systematic review with meta-analysis of pre- diabetes prevalence in South Africa and a clinical study at King Edward Hospital, Durban. Subsequent chapters investigate pathophysiological mechanisms: hormonal imbalances in pre- diabetic individuals (Chapter 5) and differential microRNA expression between pre-diabetic and normoglycemic patients (Chapter 6). The final chapter (7) synthesises all findings, discussing implications, limitations, and conclusions.

This work has not been submitted in any form for any other degree or diploma at another institution. The use of other people's work has been acknowledged accordingly in-text.

A.M. Sosib **.....** **Date:** 18 April 2024

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P.S. Ngubane **Date:** 18 April 2024

DECLARATIONS

I, Aubrey Mbulelo Sosibo, declare that:

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(ii) This dissertation has not been submitted for any degree or examination at any other university.

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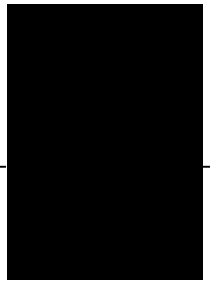
Signed: 

Date: 18 April 2024

DEDICATION

To my loving mother, Mrs H.C. Sosibo and the Sosibo family (Sthembile, Thulile and Peatra).

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I want to acknowledge the Lord God Almighty for the gift of life. My faith in Him has been my pillar through this research project.

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CONTENTS

LIST OF ABBREVIATIONS

ADA	American Diabetes Association
cDNA	Complementary DNA
CI	Confidence Interval
C-peptide	Connecting peptide
GH	Growth hormone
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-Like Peptide 1
GLUT-4	Glucose transporter type 4
HbAc1	Glycated haemoglobin
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
IFG	Impaired fasting glucose
IR	Insulin resistance
KEH	King Edward Hospital
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
NPD	Non-pre-diabetes
PRISMA	Preferred Reporting Items for Systematic Review and Meta-Analysis
PD	Pre-diabetes
pg/mL	Picograms per millilitre
T2DM	Type 2 diabetes Mellitus.
T3	Triiodothyronine
T4	Thyroxine
UKZN	University of KwaZulu-Natal.
WHO	World Health Organization

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ABSTRACT

Background

Prediabetes constitutes a metabolic state linked with an elevated risk of all-cause mortality. The International diabetes federation has projected a substantial increase in the prevalence of pre-diabetes, particularly in middle-income countries. Despite this, there is a lack of research looking into pre-diabetes in middle-income countries, leading to a significant number of undiagnosed cases. Additionally, there exists a limited understanding of hormonal imbalances and their correlation with pre-diabetes. The exploration of the link between epigenetic factors and pre-diabetes is also insufficient, particularly in developing countries such as South Africa. There is a compelling need for more pre-diabetes-related research that will represent middle-income populations in order to assist in mitigating the anticipated surge in prediabetes and type 2 diabetes mellitus (T2DM). Therefore, this research project aimed to assess the systematic prevalence of pre-diabetes and its correlates in South Africa and the specific prevalence of pre-diabetes in King Edward Hospital, the second-largest tertiary hospital in Southern Africa, based in Durban, South Africa. The paucity of pre-diabetes-related research on hormonal imbalances relative to T2DM led us to quantify the difference in hormone concentration levels between persons with pre-diabetes and those without pre-diabetes. Lastly, the miRNA expression changes in pre-diabetes participants and the association between microRNAs and HbA1c-determined dysglycemia were examined for the first time in a population based in Durban.

Methods

Per the PRISMA 2020 guidelines, a first-of-its-kind systematic and meta-analysis review was conducted to estimate the overall prevalence of pre-diabetes and its correlates in South Africa. Then, a retrospective study design was adopted, with a sample size of 364, to investigate the prevalence of pre-diabetes in a clinical setting based in Durban. After that, hormone concentrations were measured from stored plasma samples through multiplexing using the Luminex MAGPIX System to determine the occurrence of hormonal imbalances in subjects with pre-diabetes. Lastly, microRNAs were extracted from plasma samples and converted to cDNA in order to perform RT-qPCR. The RT-qPCR quantified the expression of microRNA targets previously unexplored in the study population (miRNA-34a-5p, miRNA-145-5p, and let-7f-5p) between persons with pre-diabetes and normoglycemia.

Results

The meta-analysis generated a pre-diabetes pooled prevalence of 15,56 % in the South African population. The prevalence differed across the ethnic groups, mainly affecting the Indian, Coloured, African and then white ethnic groups, respectively. The common correlates of pre-diabetes were hypertension, obesity and a sedentary lifestyle. The retrospective prevalence study showed a mammoth 54 % and 67 % prevalence of pre-diabetes when using the respective WHO and ADA diagnostic criteria

for pre-diabetes. The following hormones, c-peptide, cortisol, progesterone, estradiol, T3, T4, and adiponectin, were dysregulated in persons with pre-diabetes and significantly correlated with HbA1c. Of the three candidate miRNAs, only miRNA-34a-5p showed a significant relative fold expression difference between pre-diabetes and individuals with normoglycaemia. Moreover, the receiver operator characteristic curve analysis for miR-34a-5p produced an AUC of 0.806,

Conclusion

The meta-analysis study showed a substantial prevalence of pre-diabetes across the country, and the study's prevalence analysis in King Edward Hospital indicates that it is burgeoning. The growing pre-diabetes prevalence suggests a looming incidence of overt T2DM if left unattended. Hence, the outcomes highlight the need to strengthen pre-diabetes surveillance in order to implement diabetes prevention policies and interventions effectively. Moreover, the management of pre-diabetes care may need to consider a more holistic approach by monitoring the hormonal imbalances that were found to correlate with HbA1c. Future research should explore the therapeutic potential of targeting these hormonal pathways, including the possibility of hormone replacement interventions. Notably, miR-34a-5p was found to be associated with dysglycemia and a potential future risk marker or diagnostic marker for pre-diabetes.

Keywords: pre-diabetes, microRNA, hormonal imbalances, prevalence rate

CHAPTER 1: INTRODUCTION

1. Introduction

According to projections by the International Diabetes Federation (IDF), the global prevalence of pre-diabetes is expected to rise significantly in the coming years (1). This increase is particularly pronounced in middle-income countries like South Africa, which, over the years, experienced rapid economic growth, urbanisation and lifestyle changes that contribute to an uptick in metabolic disorders like pre-diabetes (1). Pre-diabetes is a metabolic state characterised by elevated blood glucose levels that are higher than normal but not yet high enough for a diagnosis of type 2 diabetes mellitus (2). A diagnosis of pre-diabetes can be confirmed by the presence of impaired fasting glucose (IFG), impaired glucose tolerance (IGT) as well as elevated concentrations of glycated haemoglobin (HbA1c) using either the World Health Organisation (WHO) or American Diabetes Association (ADA) diagnosis criteria (2-4). Pre-diabetes has been linked with an increased risk for several adverse health outcomes, particularly the progression to overt type 2 diabetes (5-7). Moreover, pre-diabetes is strongly associated with an elevated risk of all-cause mortality, meaning that individuals with this condition face a higher probability of death from any cause compared to those without pre-diabetes (8). The elevated risk of mortality and morbidity highlights the seriousness of pre-diabetes as a public health concern, emphasising the need for early detection and intervention.

Additionally, hormonal imbalances of C-peptide, cortisol, adipokines, thyroids, incretins and sex steroids have been reported in persons diagnosed with T2DM (9-12). Hormones play a pivotal role in glucose regulation and metabolic processes; thus, dysregulation of hormones is also likely to contribute significantly to the development of pre-diabetes. However, the understanding of these hormonal imbalances and their correlation with pre-diabetes remains underexplored. Furthermore, there is limited exploration of epigenetic factors in the context of pre-diabetes. Epigenetic modifications, such as circulating microRNA (miRNA) expression, have been shown to influence gene expression without altering the underlying genetic code, thereby playing a role in metabolic disorders such as diabetes mellitus (13-15). Despite increasing evidence of epigenetic regulation in metabolic diseases, studies investigating the relationship between miRNA patterns of expression changes and pre-diabetes, particularly in diverse populations, are scarce.

Therefore, given the predicted surge in T2DM and pre-diabetes cases, particularly in middle-income countries like South Africa, there is an urgent need for more research that accurately represents these populations and contributes to a more comprehensive understanding of pre-diabetes in diverse socioeconomic and geographic settings. The city of Durban in South Africa is a rapidly urbanising area that has been shown to have an increasing prevalence of type 2 diabetes (16, 17). However, there is limited data on the prevalence of pre-diabetes in this city and the possible physiological changes that

may be brought about by this condition. Therefore, this research project sought to appraise, for the first time, the systematic prevalence of pre-diabetes in South Africa over the past two decades and then investigate the current prevalence of pre-diabetes, aged 25-45, in Durban, South Africa. Thereafter, the study explored any associated changes in hormonal concentrations and miRNA expression changes during the pre-diabetic state.

1.2 Aim

The aim of the study was to investigate the overall and population-specific prevalence of pre-diabetes in Durban, South Africa, as well as the various hormonal and expression of specific miRNA changes that exist in individuals diagnosed with pre-diabetes.

1.3 Research questions

1.3.1 What is the overall prevalence of pre-diabetes and its correlates in existing literature evidence from 2000 to 2020 in South Africa?

1.3.2 What is the present prevalence of pre-diabetes in a Durban-based clinical setting?

1.3.3 Are incretins, thyroid, steroid, and c-peptide hormones altered in pre-diabetes?

1.3.4 Does the expression of miRNA correlate with HbA1c to determine pre-diabetes?

1.4 Objectives:

The aim and objectives for each of the respective research questions stated in 1.3.1, 1.3.2, 1.3.3, and 1.3.4 are stated below. The objectives for research questions 1.3.1 and 1.3.2 are addressed under study 1, while the objectives for research questions 1.3.3 and 1.3.4 are addressed in study 2 and study 3, respectively.

Study 1

- To conduct a systematic review and meta-analysis to determine the summarised prevalence of pre-diabetes in South Africa
- To identify the most common correlates of pre-diabetes in the South African population.
- To measure HbA1c and IFG to determine pre-diabetes prevalence in study participants aged 25 – 45 in a Durban-based clinical setting.

Study 2

- To measure the incretins (GLP-1 and GIP) hormones in study participants with pre-diabetes and non-pre-diabetes to determine if incretin imbalance exists during pre-diabetes.
- To measure thyroid hormones (T3 and T4) in study samples with pre-diabetes and control group to determine whether pre-diabetes affects thyroid regulation,
- To measure adipokines (adiponectin and adipsin) in persons with pre-diabetes and without.
- To measure steroid hormones (cortisol, estradiol, progesterone and testosterone) in pre-diabetes samples and non-prediabetes to determine if the imbalance of these hormones exists in pre-diabetes.

Study 3

- To appraise the expression of miRNAs in HbA1c-determined pre-diabetes participants and control participants to determine if there are any changes in expression between them.
- To appraise the correlation of the miRNAs and pre-diabetes to determine new miRNAs linked with increased risk of pre-diabetes onset.

1.5 Hypothesis

There is a high prevalence of pre-diabetes in Durban South Africa, and individuals diagnosed with pre-diabetes exhibit a significant difference in hormonal changes and miRNA expression patterns compared to persons without pre-diabetes.

1.6 Null-hypothesis

There is a low prevalence of pre-diabetes, and individuals diagnosed with pre-diabetes exhibit no statistically significant changes in miRNA expression patterns compared to persons without pre-diabetes.

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CHAPTER 2: LITERATURE REVIEW

1. Background

The overwhelming global prevalence rate of type 2 diabetes mellitus (T2DM) has resulted in an exponential increase in recognition of pre-diabetes, a condition that often precedes the onset of T2DM (1, 2). Pre-diabetes is established when blood glucose concentrations are above normal and below the Type 2 Diabetes Mellitus (T2DM) threshold (3). Persons diagnosed with pre-diabetes are more at risk of developing T2DM and other metabolic complications (4, 5). Health organisations across the globe call attention to the necessity of addressing pre-diabetes to alleviate the rising prevalence of T2DM and other metabolic complications. Around the middle of the 20th century, pre-diabetes was initially recognised as a phase of elevated blood glucose levels below the T2DM threshold (6). This stage was termed borderline diabetes or impaired glucose tolerance. As pre-diabetes awareness increased, diagnostic measurements and criteria were developed to determine prevalence rates. The American Diabetes Association (ADA) and the World Health Organisation (WHO) have since developed diagnostic criteria that are used for the diagnosis of pre-diabetes (7, 8).

The global prevalence of pre-diabetes is increasing exponentially, with 464 million cases confirmed in 2021 (9). The projections for the year 2045 indicate that middle-income countries will experience the most significant increase in the prevalence of pre-diabetes compared to high-income and low-income countries (9, 10). Over and above these estimates, it is further estimated that there is an additional 80% of people with undiagnosed pre-diabetes in developing and middle-income countries (11, 12). Hence, these countries must improve the surveillance of pre-diabetes in order to roll out strategies for effective treatments and pre-diabetes prevention policies. South Africa is a middle-income country, and there has been a paucity of recent studies reporting the prevalence of prediabetes. Therefore, more prevalence studies are warranted to obtain an updated estimate of the burden of pre-diabetes using the latest criteria, which led to the formation of the objectives for Chapter Three.

The pathophysiology of pre-diabetes is linked with moderate vascular complications and sub-clinical chronic inflammation (13-15). This condition is reportedly asymptomatic, thus making it difficult to diagnose. This often results in clinical inertia in patients with pre-diabetes, often leading to the development of T2DM (2). The asymptomatic nature of pre-diabetes has also made it difficult to study the progression of this condition, which has often led to it being studied in animal models. The study of pre-diabetes using animal models has shown a variety of metabolic derangements during this condition. These studies have also revealed the pre-diabetic state to be the point where the metabolic complications associated with T2DM begin. Thus, early detection of pre-diabetes can play a pivotal role in mitigating future metabolic complications and improving health outcomes in T2DM associated comorbidities. However, the physiological changes that occur during this condition in humans remain underexplored due to a limited understanding of the pathology of pre-diabetes.

The investigation of the hormonal regulation of various hormones in persons with pre-diabetes has the potential to reveal new therapeutic targets that can improve pre-diabetes care and contribute to precision medicine. Relative to T2DM, there is limited literature exploring the possible hormone imbalances in pre-diabetes. Another possible factor contributing to the development of pre-diabetes is genetic predisposition, mainly through circulating microRNAs (miRNAs). They are indicated as one of the reasons that explain why the proportion of people diagnosed with diabetes over different racial groupings varies significantly internationally (16). A study focusing on European and East Asian racial groups showed that the expressions of miRNAs in study participants with T2DM are linked with ethnic factors (17). However, limited research has been done on South African ethnic groups, leading to the formation of the objectives in chapter five of the dissertation.

Durban is a metropolitan city in the province of KwaZulu-Natal in South Africa. The United Nations World Urbanisation Prospects estimated findings using the urban agglomeration statistical concept and showed that the population size of Durban grew from 2.6 to 3.1 million between 2000 and 2018 (18). The growth in urban population size will often mirror the increasing incidence of metabolic disorders (19). Given the lack of prior research on the prevalence of pre-diabetes in Durban, it was necessary to conduct a study in order to comprehensively grasp the current extent of the pre-diabetes burden within the population. This population is also ideal for appraising metabolic complications such as hormonal imbalances, which may be linked with pre-diabetes. The city comprises individuals from various ethnic backgrounds, which makes it suitable for exploring genetic predispositions that may be induced by genetic markers like miRNAs. Therefore, we sought to investigate the prevalence of pre-diabetes, the hormonal dysregulation in pre-diabetes and the association between miRNA expression and pre-diabetes in patients from Durban, South Africa.

2. History of pre-diabetes

It was in the late 1970s that the concept of pre-diabetes began to surface due to an improved understanding of the development of type 2 diabetes (6, 20). Pre-diabetes was signalled as the initial marker of a disturbance in glucose homeostasis. In 1997, impaired fasting glucose (IFG) was recognised by the American Diabetes Association (ADA) as pre-diabetes (20). The World Health Organisation (WHO) concurred with the definition of IFG. However, they utilised the term intermediate hyperglycaemia to describe pre-diabetes (21). In 2003, the ADA adjusted the IFG criterion for pre-diabetes to a concentration range of 5.7-6.9 mmol/L. The rationale for lowering the criterion was founded on the examination of fewer IFG-diagnosed individuals progressing to overt diabetes than those whose pre-diabetes was diagnosed as impaired glucose tolerance (IGT) (22). The adjustment proved to correct the differences in diabetes progression between the criteria. However, the WHO did not incorporate the ADA-lowered IFG range; thus, it remains in the 6.0-6.9 mmol/L range.

Then, in 2008, glycated haemoglobin (HbA1c) was introduced to the diabetes diagnosis criteria following a convocation of experts in diabetes research (23). The panel of experts included representatives from ADA, the European Association for the Study of Diabetes and the International Diabetes Federation (IDF) (23). The outcome of the gathering was that an HbA1c value of more than 6.5% would identify a person with overt diabetes. A value greater than 6.0% would define someone at risk of developing diabetes, and that individual should be monitored closely so they do not develop diabetes. After that, the ADA expanded its pre-diabetes criteria by including HbA1c. They defined pre-diabetes with an HbA1c criterion of 5.7-6.4% (24). The criterion was entrenched in findings from a large survey population fed into models that estimated the risk of developing diabetes and cardiovascular diseases. The calculated risk generated by the models showed a significant risk for individuals within the HbA1c criterion to develop diabetes. Scientific institutes may have different descriptions of pre-diabetes; however, the general idea is that pre-diabetes is a condition between normoglycaemia and T2DM. The prevalence rate of pre-diabetes depends on the type of criterion utilised, the test chosen, and the population being investigated.

3. Prevalence of Pre-diabetes

The global prevalence rates of pre-diabetes determined by IGT and IFG were estimated at approximately 9% and 6% in adults above 20 years old using the WHO criteria (9). There is limited epidemiological data to give a global scale HbA1c-defined prevalence of pre-diabetes, which may be explained by the fact that the criterion is relatively new. Hence, countries, particularly those with low and middle incomes, are encouraged to conduct HbA1c measurements. The 8th edition of the International Diabetes Federation (IDF) Atlas revealed that the prevalence of pre-diabetes differs by population studied (25). The variation in prevalence across different populations demonstrates the necessity for each continent and country to conduct its prevalence studies (25, 26). Of all the seven regions represented, Africa had the third highest IGT-determined pre-diabetes prevalence, as shown in Figure 2 (25).

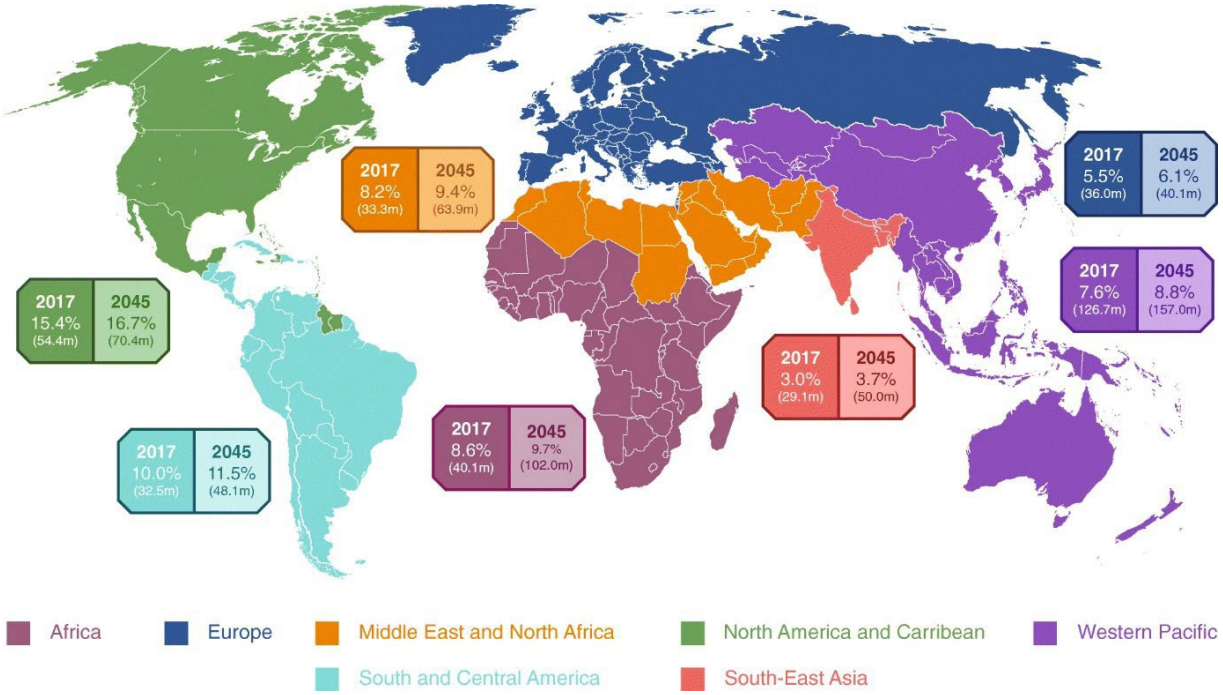

Figure 1. Demonstrates the global scale of prediabetes prevalence (adapted from Ulrike Hostalek, 2019)**.**

The IGT and IFG estimated prevalence of pre-diabetes in Africa was reported to be 12.6% and 8%, respectively, in the latest IDF global report (27). The worldwide prevalence of undiagnosed diabetes is around 240 million, and more than half of them are estimated to be from the African region (12). This suggests that there is poor screening of pre-diabetes in the African population (12). A bibliometric analysis study highlighted that low-middle-income countries are less attentive to pre-diabetes, which makes them more susceptible to increasing pre-diabetes and T2DM prevalence (28). Additionally, the escalation of urbanisation correlates with an augmented prevalence of T2DM.

The impact of rising urbanisation on T2DM prevalence manifests greater significance in middle-income countries than in high-income nations (29). Therefore, in middle-income nations, the urbanisation rate emerges as a more potent risk indicator for the incidence of T2DM (29). South Africa is a middle-income country, and from 2011 to 2021, the prevalence of diabetes increased from approximately 1.9 to 4.2 million (11). Records from the United Nations show that the South African percentage of the population residing in urban areas from 2000 to 2018 increased by 10,9 % (18). The high rate of urbanisation in South Africa is attributed mainly to its metropolitan cities, such as Durban, in the province of KwaZulu-Natal. According to the Statistics of South Africa census, the urban population of eThekwini has grown from 3,476,686 in 2000 to 4,239,901 in 2022 (30). The rising urbanisation in Durban designates it as a region highly susceptible to an elevated prevalence of T2DM (31). In a study conducted by Hird et al., the prevalence of T2DM was 11.9% based on fasting blood glucose and 13.1% based on HbA1c (32). However, the onset of T2DM is often preceded by pre-diabetes. Therefore, the paucity of research on the prevalence of pre-diabetes in South Africa, especially in urban areas like Durban, needs to be

addressed.

This led to one of the purposes of the current study: to understand the current pre-diabetes burden in the South African and Durban-based population. A systematic review and meta-analysis were conducted and reported in chapters 3.1 and 3.2 to get the overall prevalence of pre-diabetes and its covariates in South Africa. In an attempt to halt the rising prevalence and identify novel therapeutic targets, there is growing interest in better understanding the transition from normal glucose homeostasis physiology to pre-diabetes onset.

4. Normal glucose homeostasis and its regulatory hormones.

In normal physiological conditions, blood glucose concentrations are kept within 4.0 – 5.4 mmol/L (33). Various hormones and genetic markers ensure that glucose levels in the blood are kept within a range. In particular, the endocrine cells of the pancreas, termed islets of Langerhans, are responsible for releasing hormones that regulate blood sugar levels into the blood circulation (34). The α-cells secrete the hormone glucagon, and β-cells secrete and synthesise insulin and C-peptide (34). Glucagon and insulin are the two well-known pancreatic hormones that play a significant role in ensuring glucose homeostasis. Insulin is responsible for lowering elevated blood sugar levels (35). Glucagon exerts an opposing effect to insulin by stimulating an increase in blood glucose concentrations when they fall below the normal physiological range (35). Moreover, the functions of these two hormones are influenced by other endocrine hormones that contribute to regulating the glucose homeostatic range.

Reproductive, thyroid, adipokine and incretins are hormones that contribute to glucose homeostasis control. The thyroid gland generates thyroid hormones thyroxine (T4) and triiodothyronine (T3). They can control glucose production by stimulating hepatic gluconeogenesis and glycogenolysis (36, 37). The thyroid hormones also positively influence insulin sensitivity, which allows more glucose uptake in insulin-dependent tissues (38, 39). Reproductive hormones such as estrogen and testosterone impact the glucose homeostatic range. For example, estrogen is the main hormone produced in females, and it exerts protective effects towards insulin sensitivity and pancreatic beta-cell function (40, 41). Therefore, estrogen promotes glucose homeostasis. Testosterone is primarily produced in males. The levels of testosterone appear to affect males differently from females. High testosterone concentrations in males are associated with better sensitivity to insulin (42). On the contrary, elevated testosterone levels in females are linked to insulin resistance (43). Incretins, mainly glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are a gut-derived group of hormones secreted after a meal. They play a role in glucose metabolism through their insulinotropic effect, which leads to decreased blood glucose levels (44, 45). Adipokines such as adipsin and adiponectin are also known to contribute to glucose metabolism control by inducing glucose-lowering effects (46). Therefore,

hormonal balance plays an integral role in ensuring glucose homeostasis and any hormonal imbalances will negatively affect glucose metabolism and its homeostatic range. Additionally, there is growing interest in the association of epigenetics and glucose control.

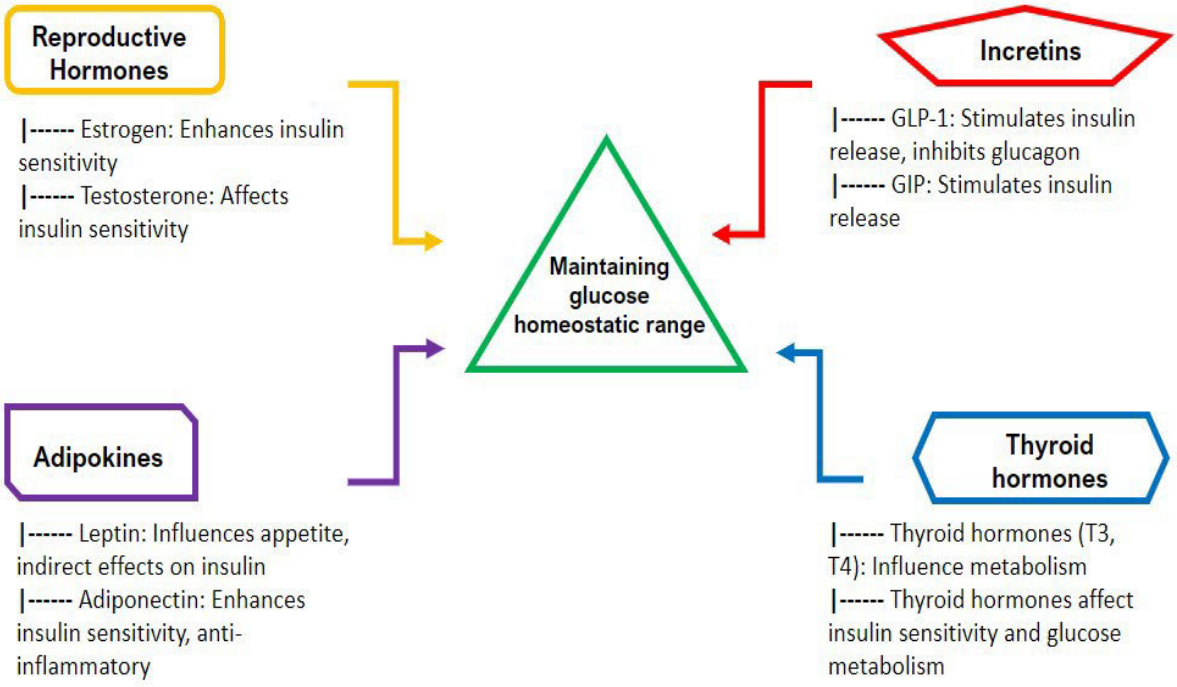

***Figure 2*:** Illustrates the contributions of reproductive, adipokines, thyroid and incretin hormones in maintaining glucose homeostasis.[1]

MicroRNA and their contributions to the control of blood glucose.

Epigenetic changes occur when a particular gene function is altered due to modifications in the gene expression levels rather than a nucleotide sequence change (47, 48). One of the epigenetic mechanisms involved in modulating gene expression is microRNA (miRNA) (48). These miRNAs are characterised as a class of single-stranded, non-coding RNA with a short sequence of 19 – 25 nucleotides in length. miRNA can regulate gene expression post-transcriptionally, achieved through repression of protein-coding mRNA during the translational stage or mRNA degradation (49). Some literature suggests that translational repression and miRNA degradation do not occur independently. Translational control is the initial event followed by mRNA degradation (50). Therefore, these genes can potentially influence pathways that regulate glucose homeostasis. For example, microRNA-181b positively influenced maintaining the glucose homeostatic range (51). In another study, miRNA let-7 was demonstrated to disturb glucose homeostasis (52). This proves that gene expression regulation by miRNA can affect

[1] | ------indicates how respective hormones influence the regulation of insulin, glucagon, and glucose to keep blood glucose levels within a normal range

how glucose levels are kept within the desired range. A disturbance in glucose homeostasis will result in metabolic complications such as T2DM.

5. The contributions of hormonal imbalances in T2DM and pre-diabetes pathophysiology.

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder induced by the body's diminished insulin sensitivity, resulting in excessive blood glucose levels (53, 54). Reduced insulin sensitivity and impaired beta cell function are the hallmarks of T2DM (53). The reduction in insulin sensitivity diminishes the body's ability to maintain the glucose homeostatic range (53). To compensate for the insulin resistance state experienced by the body, the secretion of insulin will be further stimulated in an attempt to bring glucose levels back to normal (55, 56). Hence, T2DM is characterised by hyperinsulinemia. Additionally, persons with T2DM often present pancreatic beta-cell dysfunction, which causes relative insulin deficiency that perpetuates hyperglycaemia (57, 58). The chronically high glucose levels in T2DM are associated with metabolic complications and increased mortality risk (59, 60). T2DM is one of the frontal causes of death and disability (11). Therefore, there is a growing concern with the rising T2DM prevalence.

Globally, over half a billion (536.6 million) people were reported to be living with diabetes in 2021, and 96% of the cases are attributed to T2DM (26, 61). The T2DM prevalence is estimated to rise to close to one billion people (783.2 million) by 2045 (26). Notably, middle-income countries such as South Africa are expected to experience more significant increases in T2DM prevalence compared to low and high-income countries (26). IFD further estimates that about 4.6 million South Africans suffer from T2DM and that many remain undiagnosed (11, 26). In another recent study, the South African prevalence of T2DM was found to be 22% (62). The prevalence statistics highlight the monumental burden T2DM exerts on public health worldwide. Hence, the current global challenge is finding effective measures to prevent T2DM incidence. Consequently, this research study centres its investigation on the pre-diabetes phase, which is imperative to understand in order to attenuate rising T2DM prevalence.

How hormone changes influence the pathophysiology of T2DM and pre-diabetes.

The endocrine system achieves the production and circulation of hormones throughout the human body. An endocrine disorder is characterised by failure to maintain the homeostatic range of a particular hormone. The dysregulated hormone concentration is often termed hormonal imbalance, which is one of the mechanisms attributed to the induction of pre-diabetes and T2DM. However, it remains elusive whether some of the hormonal imbalances linked with T2DM would be seen in the pre-diabetes phase.

Therefore, we pooled the available evidence about the following hormones and their possible role in the onset of T2DM.

Insulin

In response to rising glucose levels, the hormone insulin is secreted by pancreatic β cells (63). Insulin consists of two chains of amino acids linked by disulphide bridges (64). These two chains of amino acids are differentiated as the A chains and B chains, with 21 and 30 amino acids, respectively (65). Consequently, insulin is composed of 51 amino acids, constituting its molecular weight of 5808 Da (65, 66). Functionally, insulin exerts an anabolic effect (63).

As demonstrated in Figure 4, for insulin to function, it binds to insulin receptors on the insulin-sensitive cell and generates signal transduction (67). As a result, insulin promotes glucose uptake into the cells, which causes a decrease in blood glucose levels (68). Insulin has an anabolic function, increasing the storage of macromolecules such as amino acids, glucose, and fatty acids (69). The build-up of these macromolecules stimulates body gain (69). The fundamental function of insulin is to regulate the storage and oxidation of energy-carrying substances. Therefore, insulin can inhibit gluconeogenesis and glycogenolysis (63).

Furthermore, insulin induces glucose breakdown and glycogenesis (70). The effects of insulin also include promoting the utilisation of amino acids into protein and preventing protein degradation (70). Regarding fat metabolism, insulin induces lipogenesis and suppresses lipolysis (63). The insulin blood levels are linked to the concentration of C-peptide, which also contributes to glucose metabolism.

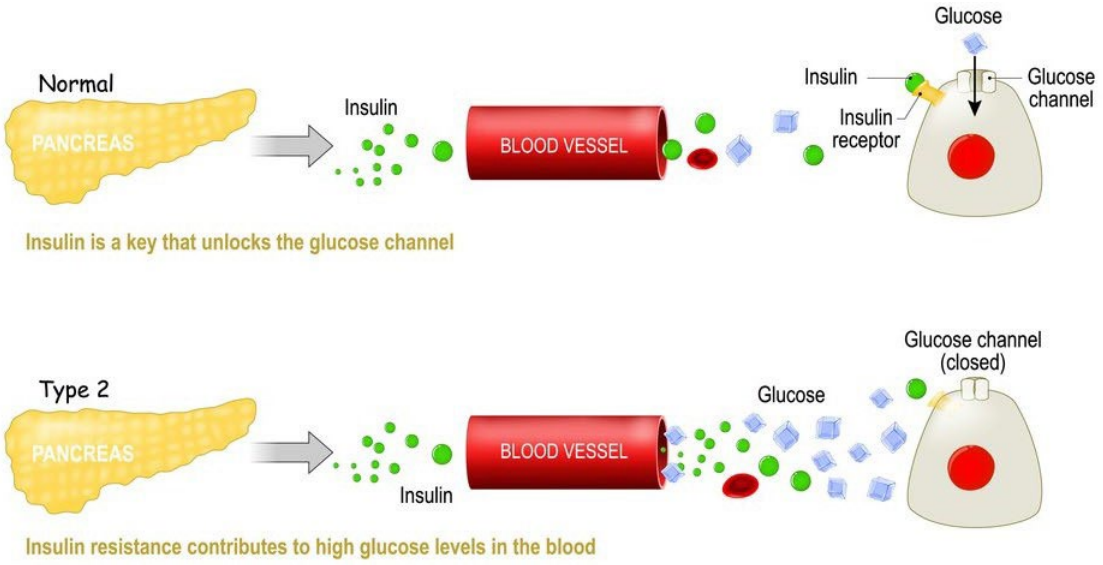

Figure 3. Illustrates how insulin regulates glucose homeostasis.[2]

Connecting peptide

The connecting peptide (C-peptide) comprises 31 amino acids and is produced as a by-product whenever proinsulin is cleaved to generate insulin inside the pancreatic beta-cells (71, 72). C-peptide was recognised as an inactive by-product of insulin processing (71). However, growing evidence suggests that c-peptide is a bioactive compound as it can cause significant effects on different cells and tissues (71, 73, 74). Compared to insulin, c-peptide has a lower degradation rate (74). Thus, it has a relatively long shelf life of 30 minutes and is a valuable beta cell function marker (74, 75). Notably, the concentrations of C-peptide have been shown to correlate with increased insulin secretion (71, 76). Therefore, changes in C-peptide plasma levels could be used to evaluate the state of pancreatic beta cell function.

The plasma concentration of c-peptide in healthy persons ranges from 0.3 to 0.6 nmol/l in the fasting state to 1-3 nmol/l after meals (77). A systematic review found that c-peptides were endorsed as the primary measure for insulin deficiency. They may be used for diagnostic purposes, mainly when there is a need to distinguish between Type 1 and Type 2 diabetes (78, 79). Therefore, c-peptide is considered an appropriate measure for beta-cell safeguarding (76). In patients newly diagnosed with T2DM,

[2] When blood glucose levels rise, the pancreatic beta-cells are stimulated to secrete the hormone insulin. Insulin will bind to the insulin receptor in insulin-dependent cells, causing the glucose channels to open. Then, there is an influx of glucose into the insulin-dependent cells, which results in lower blood glucose concentrations. However, the presence of insulin resistance in type 2 diabetes impairs insulin's ability to reduce blood glucose levels, causing hyperglycaemia and hyperinsulinaemia.

elevated c-peptide levels strongly predict all-cause and cardiovascular deaths (80). It is plausible, therefore, that the increase in c-peptide levels may begin during pre-diabetes. Hence, based on this presumption, an investigation of c-peptide levels in pre-diabetes concentrations is warranted. The outcome of this measurement may assist in managing pre-diabetes and the early identification of individuals at risk of macrovascular complications and death.

Cortisol

The hormone cortisol is a product of the hypothalamic pituitary adrenal axis (81, 82). The literature indicates that cortisol contributes to the events that lead to T2DM (83, 84). There is evidence that individuals with T2DM have a more impaired response to acute laboratory stress than matched healthy controls (85- 87). In normal physiological conditions, there is a specific diurnal pattern of cortisol, which is characterised by high concentrations upon waking, a peak 30-45 minutes after waking, and then a decline throughout the day (88). However, one study showed that individuals with T2DM had higher evening cortisol levels and a flatter diurnal drop in cortisol than those without T2DM (83). Hypercortisolism encourages disturbance in glucose homeostasis by increasing visceral adipose tissue, impairing insulin signalling in skeletal muscle, and activating lipolysis, which releases free fatty acids (86).

Ghrelin

The hormone ghrelin is secreted by the stomach, mainly when the stomach is empty (89, 90). Therefore, immediately before ingestion, ghrelin concentrations are highest (91, 92). Then, after food consumption, ghrelin will drop to its lowest concentration for approximately three hours (89, 91, 92). Interestingly, it has been shown that specific individuals can be genetically inclined to secrete more ghrelin, which stimulates appetite faster than usual (89, 93, 94). Ghrelin is multifunctional; however, one of its primary functions is elevating appetite and nutrient uptake (92). Therefore, ghrelin contributes to maintaining glucose homeostasis by stimulating appetite when blood glucose levels are low(89). The appetite-stimulating function is triggered by its signal to the hypothalamic area of the brain (91, 95).

Incretins (GIP & GLP-1)

When the gastric inhibitory polypeptide was discovered, its role in inhibiting gastric acid secretion was established (96). However, it was later revealed that GIP functions more potently as an insulinotropic polypeptide (44, 97). Therefore, it was then termed a glucose-dependent insulinotropic polypeptide (GIP) instead of gastric inhibitory polypeptide (44). The GIP hormone exhibits an incretin effect that assists pancreatic secretions (97). The incretin effect is realised by GIP's ability to improve insulin secretion in the first phase of glucose-induced secretion in people without diabetes (98). In addition, GIP has been shown to foster the growth of insulin-secreting cell lines and islet beta cells in rodent

models (99-101). Therefore, decreased levels of GIP may reduce the amount of insulin secreted, leading to relative insulin deficiency.

Similar to GIP, the established functions of glucagon-like peptide-1 (GLP-1) are linked to glucose metabolism (96). The GLP-1 hormone increases pancreatic ß-cell insulin secretion after administering glucose orally (102). GLP-1 also inhibits hepatic glucose production via reduced α-cell glucagon secretion (103). Therefore, GLP-1 is pivotal in maintaining glucose homeostasis (96). Hence, metabolic complications such as T2DM exhibit lower GLP-1 levels and have been shown to diminish the incretin effect (104). However, the pancreas remains responsive to GLP-1 but is no longer responsive to GIP. This explains how pharmacological levels of GLP-1 can revive insulin secretion (105).

The role of adipokines in diabetes (adiponectin, adipsin)

Adiponectin is an endocrine hormone discovered in 1994 as a novel protein released by the adipose tissue into the blood circulation (106). The hormone is capable of elevating the utilisation of glucose and causing lipid oxidation (107). Additionally, it has been shown to impair gluconeogenesis in the liver (107). These functions are achieved through the stimulation of the AMP-activated protein kinase pathway (108). The functions of the hormone show that it is a significant contributor to maintaining the glucose homeostatic range. There is growing evidence linking T2DM with dysregulated plasma levels of adiponectin. Some study findings indicate that the concentration of adiponectin in the blood is increased in participants with T2DM (109). However, some reports found that circulating adiponectin levels are reduced in persons with diabetes (110-112). Furthermore, limited evidence exists about the association between plasma adiponectin and pre-diabetes.

The adipokine adipsin, later termed the complement factor D, constitutes one of the critical cells of the adipose tissue (113). It was named complement factor D because of its role in the alternative complement activation (114, 115). In turn, adipsin induces the generation of anaphylatoxins C3a and C5a that act as signalling molecules (116, 117). In addition, there is growing evidence of the role of the adipsin/C3a pathway and beta-cell physiology (118). The peptide C3a was identified as a noteworthy insulin secretagogue. Studies conducted in mice deficient in adipsin displayed poor glycaemic control (118). It was found that there is an association between adipose cells and the function of the beta-cells (118).

Triiodothyronine and thyroxine

The hypothalamus and pituitary gland regulate the thyroid gland by secretion of thyrotropin-releasing and thyroid-stimulating hormones (119). The thyroid gland releases triiodothyronine (T3) and thyroxine (T4) upon stimulation (120). The thyroid hormones are maintained within the homeostatic range through the hypothalamic-pituitary-thyroid axis, which is self-regulated by the thyroid hormone's negative and positive feedback mechanisms (120, 121). Functionally, thyroid hormones are a crucial

component of the endocrine system that help manage metabolism, weight and energy levels (122). T3 and T4 hormones are released upon the stimulation of the thyroid follicular cells by the thyroid stimulating hormone (TSH), which is released by the anterior pituitary (120). The active form of thyroid hormone is called T3 (123). The amount of T3 secreted by the anterior pituitary accounts for 20% of the released hormone. The remaining 80% is derived from the peripheral conversion of T4 to T3 through the de-iodination process.

T3 can interact with most of the cells in the body to raise the resting metabolic rate. Specifically, T3 stimulates the activity of the enzyme Na/k-ATPase, which elevates heat output and oxygen utilisation (124, 125). Additionally, thyroid hormones stimulate metabolism by increasing glucose uptake, lipolysis, gluconeogenesis, glycogenolysis, and protein synthesis and breakdown (126). Therefore, the thyroid hormone plays a role in maintaining glucose homeostasis and any impairment can result in glycaemic dysregulation (127).

Thyroid disorders can be divided into two: hyperthyroidism and hypothyroidism (128, 129). Hyperthyroidism, defined by elevated levels of thyroid hormones, is shown to decrease blood glucose levels by enhancing insulin sensitivity (130, 131). Hyperthyroidism may be one of the compensatory mechanisms that the body uses to combat insulin resistance and delay overt diabetes onset (128). Paradoxically, hyperthyroidism can also influence glucose production by stimulating hepatic gluconeogenesis. Hypothyroidism occurs when there is an insufficient generation of thyroid hormones, which has been associated with reduced sensitivity to insulin and diabetes onset (129). If thyroid disorders are present in individuals with pre-diabetes, then thyroid disorders can be targeted therapeutically to help regulate glucose homeostasis in pre-diabetes.

Sex hormones (estradiol, progesterone and testosterone)

The human body's reproductive physiology relies on endocrine glands, like the adrenal cortex and gonads, to produce sex hormones such as testosterone, estradiol and progesterone (132, 133). They are all synthesised from cholesterol and are termed steroid hormones (134, 135). The primary role of these hormones is to promote the development of the reproductive system, puberty and sexual differentiation. In addition, sex hormones also play a role in regulating glucose homeostasis (136, 137). Hence, disturbances in the homeostatic range of sex hormone concentrations have been linked with diabetes.

Testosterone is an important hormone produced mainly from the Leydig cells located in the testicles of men (138, 139). In females, testosterone is synthesised in the adrenal glands and ovaries (139). The hormone's primary function is to promote the growth of male secondary features such as pubic hair, deep voice, and muscle growth (140). Nevertheless, testosterone is also known to play a significant role in metabolism. Low blood testosterone concentrations in males are associated with a greater risk of developing Type 2 Diabetes Mellitus (T2DM) (136, 141). In females, contrary results were reported.

The low testosterone levels in the blood were not associated with T2DM. However, the plausible link between pre-diabetes and the imbalance of testosterone remains underexplored.

Estradiol and progesterone are present in both males and females but predominantly in females (142). They are mainly known for promoting the growth and maintenance of the reproductive organs in women and menstrual cycle control (143, 144). Like testosterone, estradiol also contributes to the regulation of glucose homeostasis (40). Increased estradiol levels in females provide protective effects against the onset of diabetes (40, 145). The steroid is believed to exert protective action through anti-inflammatory properties and enhancing insulin sensitivity (145, 146). During menopause, estradiol concentrations drop significantly, and women lose the protection (40, 145). Then, they become more likely to develop T2DM. This is reflected in the differences in T2DM prevalence between adolescent, middle-aged, and post-menopausal women (40, 137). Adolescent and post-menopausal females have a higher prevalence. Interestingly, high estradiol levels in the circulation are reported to predict T2DM independent of obesity in men (42). Therefore, research to fully understand the relationship between estradiol and diabetes is ongoing. Also, progesterone contributes to blood glucose control but is less significant than estradiol (147, 148). So far, research has linked progesterone with insulin resistance. It is reported that progesterone can increase blood glucose concentrations by disturbing insulin action (148). Establishing the link between pre-diabetes and sex hormones is imperative as it could contribute to new knowledge to improve pre-diabetes care and prevent T2DM.

MicroRNAs (miRNA)

MicroRNA (miRNA) forms part of the short, non-coding RNAs with approximately 22 nucleotides (149). These miRNAs can regulate gene expression post-transcriptionally, achieved through repression of protein-coding mRNA during the translational stage or mRNA degradation (47, 48, 150). Some literature evidence suggests that translational repression and miRNA degradation do not occur independently (50). The translational control is said to be the initial event that is followed by mRNA degradation (50). Consequently, miRNAs play a role in most physiological processes like regulating cell division, differentiation, growth and apoptosis (150). There is accumulating research showing the significant involvement of miRNA in regulatory mechanisms that contribute to many pathological conditions such as cancer, inflammation, cardiovascular disorders and diabetes (150, 151). There is growing interest in finding altered expression of various miRNAs linked to diabetes that can be used as biomarkers and therapeutic targets (152).

The features of miRNAs that have attracted much interest are their ability to capture underlying genetic risk and responses to environmental, social, and lifestyle factors. Therefore, miRNA findings can assist in detecting individuals who are at risk of developing pre-diabetes and diabetes. In addition, miRNA outcomes can provide knowledge about the interactions between underlying genetic predisposition and

environmental and lifestyle factors by analysing specific gene expression patterns in persons with genetic (specific miRNA biomarkers) predisposition.

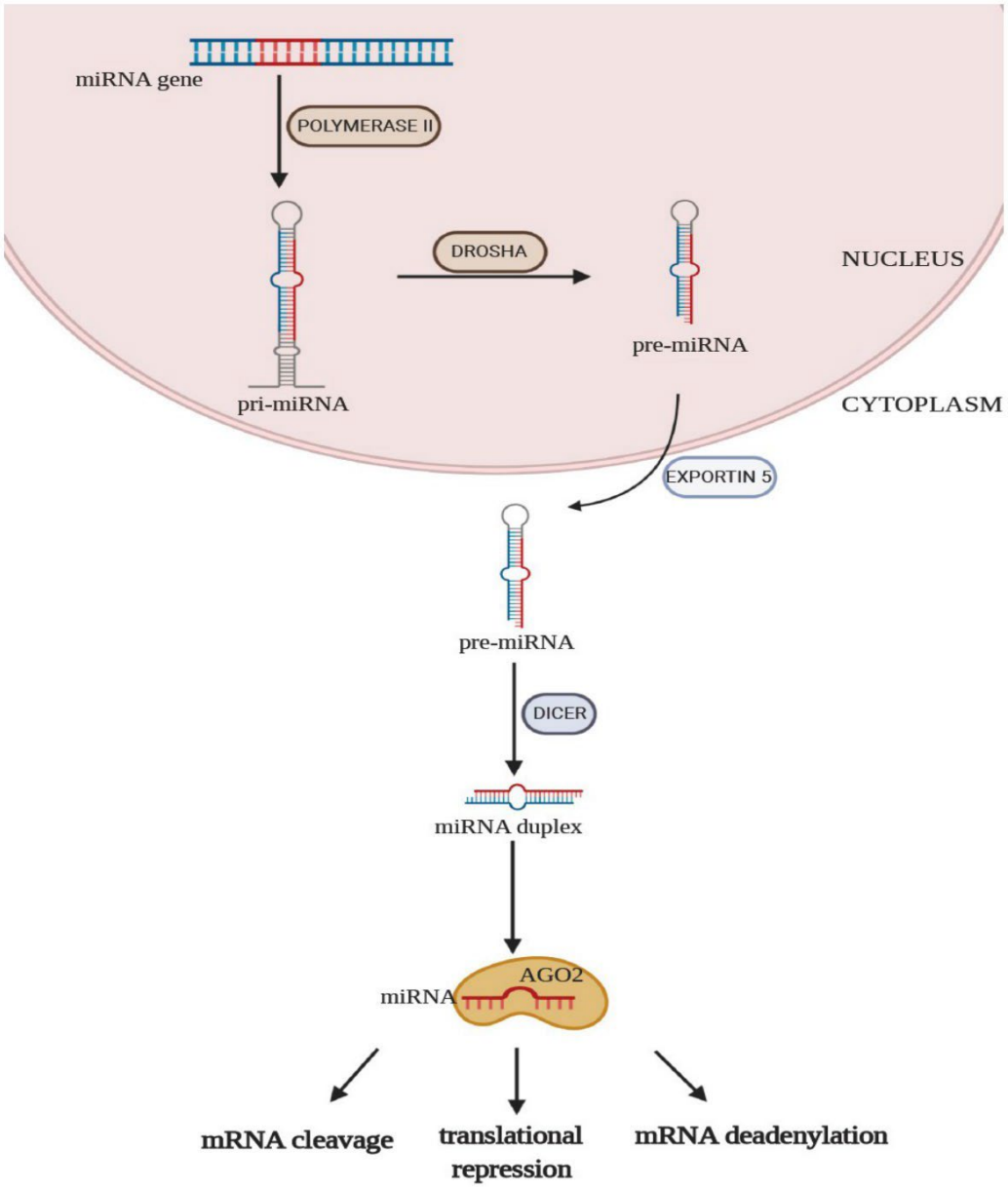

Figure 4. Displays the biogenesis and functions of miRNA. The figure was adapted from Bielska *et al*., (2021) (153).

The nucleus is the site where miRNA synthesis begins. Inside the nucleus, polymerase II will cause transcription of the miRNA genes to primary RNA (pri-miRNA), which is converted to pre-miRNA by the ribonuclease Drosha. After that, the pre-miRNA is exported out of the cell's nucleus to the cytoplasm by Exportin 5. In the cell's cytoplasm, the pre-miRNA is cleaved into short miRNA duplexes

by an endonuclease called the dicer. Finally, the mature miRNA strand binds to an Argonaute (Ago) protein, forming a complex.

MicroRNAs are also shown to be involved in glucose homeostasis and diabetes (154). One of the miRNAs specific to pancreatic islets is miR-375, and the overexpression of this miRNA is found to reduce glucose-stimulated insulin release, while its inhibition increases insulin secretion (155). In previous literature articles, miRNA-145-5p was demonstrated to be enriched significantly in persons with T2DM (156-158). An inverse correlation between miR-192, miR-34a-5p, miR-9 and insulin secretion was also reported, indicating that miRNAs may play a role in diabetes. Prevention of T2DM is one of the critical strategies in T2DM management. T2DM is often preceded by another metabolic condition, pre-diabetes (9, 26, 159). Since miRNA expression can be influenced by ethnicity, research conducted in other countries cannot be generalised to other countries. To the best of our knowledge, no study has assessed the miRNA expression and its relationship with pre-diabetes in study participants based in Durban, KwaZulu-Natal province, South Africa.

6. Study rationale

Based on the reviewed literature, developing and middle-income countries are expected to play a significant role in the future increase of both type 2 diabetes mellitus (T2DM) and pre-diabetes cases worldwide. South Africa is classified as a middle-income country experiencing rapid urbanization. Moreover, due to limited screening for pre-diabetes, a large proportion of individuals (80%) with pre-diabetes remain unaware of their condition. However, to fully understand the extent of pre-diabetes within the South African population, a systematic review and meta-analysis study was necessary to assess its prevalence and associated factors. Based on the systematic review findings, a prevalence study was conducted at King Edward Hospital in Durban using the latest criteria for diagnosing pre-diabetes. These studies, outlined in Chapter 3, shed light on the burden of pre-diabetes in the population.

Previous studies have shown that both T2DM and pre-diabetes pose a risk for the development of vascular complications, chronic inflammation and adverse health outcomes linked to other conditions. While hormonal changes have been extensively studied in T2DM, their association with pre-diabetes remains underexplored. In Chapter 4, we investigate the relationship between hormonal imbalances and pre-diabetes. Furthermore, microRNAs (miRNAs) have emerged as potential biomarkers and therapeutic targets for diseases like T2DM. While positive findings regarding miRNA and T2DM have been reported, their role in pre-diabetes remains under-explored. Chapter 5 explores the expression of miRNA-34a-5p, miRNA-145-5p, and let-7f-5p miRNAs in pre-diabetic and non-pre-diabetic groups. Recognizing and managing the hormonal imbalances that exist in pre-diabetes, along with

understanding the genetic effects of miRNAs, are crucial steps in the pursuit of effective clinical management in individuals with pre-diabetes.

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CHAPTER 3: METHODOLOGY

1. Chemicals and Reagents

All kits and reagents used were of analytical grade and purchased from standard commercial suppliers. The supplier details and protocols are described in the manuscripts.

2. Ethical clearance

The study obtained ethical clearance from the Biomedical Research Ethics Committee (BREC) of the College of Health Sciences, University of KwaZulu-Natal (BREC REF NO: BE266/2019). Ethical approval was secured prior to sample collection at King Edward Hospital.

3. Study site, population and design

Study setting and population

The study was conducted in collaboration with the King Edward Hospital, a tertiary clinical setting based in the metropolitan city of Durban, South Africa. Durban is part of the eThekwini municipality in the province of KwaZulu-Natal. The population characteristics are described in detail in the respective manuscript in Chapter 3.3.

Study design

The dissertation utilised a retrospective study design involving the analysis of blood samples obtained from both healthy participants and individuals diagnosed with prediabetes. The experimental design is described fully in the respective manuscript.

4. Sample screening (inclusion and exclusion criteria) and diagnosis

Inclusion:

- Therefore, the fasting blood glucose concentrations must be less than 6.9 mmol/L and the glycated haemoglobin (HbA1c) of less than 6.5 %.
- No previous history of diabetes diagnosis.
- Between the ages of 25 – 45.
- A documented citizen of the Republic of South Africa residing in the eThekwini region of KwaZulu-Natal.

Exclusion:

- Study subjects with pre-existing conditions such as anaemia, kidney failure, pregnancy, liver disease, hypertension and haemoglobin variants were excluded. These conditions could adversely affect glycated haemoglobin results.
- Participants diagnosed with depression were omitted, as it may impact cortisol concentrations.
- HIV-positive patients and those undergoing HIV treatments were excluded because of the heightened risk of developing T2DM in these individuals.

5. Diagnosis

The study employed the glycated haemoglobin (HbA1c) and Impaired fasting glucose (IFG) criteria to determine the pre-diabetes diagnosis as per the American Diabetes Association (ADA) and World Health Organisation (WHO). The ADA defines pre-diabetes as IFG of 5,6 – 6,9 mmol/L and HbA1c of 5,7 – 6,4 % . The WHO IFG of 6,1 – 6,9 mmol/L was used to diagnose pre-diabetes (1). Therefore, participants with an IFG less than 5,6 mmol/L and HbA1c less than 5,7 were grouped as the non-pre-diabetes or normoglycaemic group.

6. Data and sample collection

The data and sample collection period occurred from March 2022 to November 2022. By November, 364 blood samples and respective patient data files were obtained. Further details about Data and sample collection are alluded to in Chapter 3.3.

7. Biochemical analysis

The Tosoh G8 HPLC Analyzer, Luminex Magpix Multiplexing System, and QuantStudio 5 instruments were employed to measure HbA1c, hormonal levels, and microRNA, respectively. The protocols

utilised in each measurement are detailed extensively in the related manuscripts in chapters 3 – 5. However, some of the research papers did not describe how the sample size was determined.

8. Sample size calculation

The sample size calculation was conducted to obtain the desired sample size necessary to estimate the prevalence of pre-diabetes in the Durban population. This was done to ensure that the research study is adequately powered to provide reliable and valid outcomes. The formula ($n = zz^{2} \times PP \times (1-PP) / EE^{2}$) was used (2).

Where:

- n is the required sample size.
- Z is the Z-score corresponding to the desired confidence level.
- p is the assumed prevalence.
- E is the desired precision.

The study used a 95% confidence level, which is a Z-score of 1.96. The assumed prevalence (p) was based on a systematic review that reported a prevalence of approximately 15 % (3). The margin desired precision (E) was 5%. The calculation result revealed that a sample size of 202 will be necessary to have a confidence level of 95%. Therefore, it was ensured that the manuscript in Chapter 2 used a sample size above 202.

In Chapter 4, the sample size calculation was performed from previously published fasting insulin data using the Gpower software (4). The calculations showed that a total sample size of 10 or more will provide sufficient power for the hormone analysis. The input and output are displayed as follows.

t tests - Means: Difference between two independent means (two groups)

Analysis:	A priori: Compute required sample size		
Input:	Tail(s)	=	Two
	Effect size d	=	2.94
	α err prob	=	0.05
	Power (1-β err prob)	=	0.95
	Allocation ratio N2/N1	=	1,1
Output:	Noncentrality parameter δ	=	4.65
	Critical t	=	2.31
	Df	=	8
	Sample size group 1	=	5
	Sample size group 2	=	5
	Total sample size	=	10
	Actual power	=	0.98

In Chapter 5, a published study evaluating miRNAs in diabetes with a sample size of 55 subjects was sufficiently powered (5). The study was used to calculate the minimum samples required to obtain an adequate sample size. The Gpower software was used, and the calculations suggest that a minimum sample size of six is required. However, a minimum of 25 samples will be required for regression analysis (6). The input and output are displayed as follows.

t tests - Means: Difference between two independent means (two groups)

Analysis:	A priori: Compute required sample size		
Input:	Tail(s)	=	Two
	Effect size d	=	5.1693049
	α err prob	=	0.05
	Power (1-β err prob)	=	0.95
	Allocation ratio N2/N1	=	1
Output:	Noncentrality parameter δ	=	6.3310797
	Critical t	=	2.7764451
	Df	=	4
	Sample size group 1	=	3
	Sample size group 2	=	3
	Total sample size	=	6
	Actual power	=	0.9951022

9. Statistical analysis

All statistical analysis methods are mentioned in detail in each research article.

10. Conclusion

The full details of the methodologies employed to accomplish the objectives outlined in the thesis have been comprehensively elucidated within each manuscript. Each methodological approach has been meticulously described, providing a thorough understanding of the research procedures and ensuring transparency and reproducibility in how the research project was conducted.

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CHAPTER 4

4.1 SYSTEMATIC REVIEW AND META-ANALYSIS PROTOCOL

DETAILS OF NEXT MANUSCRIPT

The following manuscript is titled "Prevalence and correlates of pre-diabetes in adults of mixed ethnicities in the South African population: A Systematic Review and Meta-analysis Protocol" is authored by A.M. Sosibo, N.C Mzimela, P.S Ngubane, and A. Khathi. The manuscript is published in the BMJ Open journal and has been formatted according to the journal's guidelines for authors (10.1136/bmjopen-2020-048266). This journal is accredited by the Department of Higher Education and Training South Africa (2021) and appears in the Scopus accredited list (2021)

Author Contribution: AM Sosibo was responsible for study conceptualization, study design, first draft writing, and manuscript editing.

Prevalence and correlates of pre-diabetes in adults of mixed ethnicities in the South African population: A Systematic Review and Meta-analysis Protocol

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Abstract

Introduction

Pre-diabetes is a metabolic condition characterised by moderate glycaemic dysregulation and is a frontline risk factor for multiple metabolic complications such as overt diabetes. To the best of our knowledge, this will be the first systematic review and meta-analysis focusing on generating a comprehensive pool of studies reporting pre-diabetes prevalence in South Africa. Therefore, the purpose of the review will be to screen and select reports that can be used to synthesise and provide the best estimate of the prevalence and correlates of pre-diabetes in the South African population.

Methods and analysis

To determine the prevalence and correlates of pre-diabetes in South Africa, we will search PubMed, Embase, and African Journal online for published or unpublished studies reporting the prevalence of pre-diabetes in South Africa from 2000 to 2020. Studies will be assessed for eligibility by checking if they meet the inclusion criteria. Eligible studies will undergo data extraction and risk of bias assessment. We will perform a subgroup analysis to detect probable causes of heterogeneity.

Ethics and dissemination

The review will not require ethics clearance because non-identifiable data will be used. The review outcomes will give more insight into the current burden that pre-diabetes has in South Africa.

PROSPERO registration number: CRD42020182430

1. Introduction

Pre-diabetes (or intermediate hyperglycaemia) is a severe health condition characterised by blood glucose concentration that is higher than normal but not high enough to diagnose overt type 2 diabetes mellitus (T2DM) (1,2). The onset of pre-diabetes is preceded by insulin resistance (IR) or pancreatic β cell dysfunction, which, in turn, causes a gradual increase in glucose levels(1,3). The diagnosis of pre-diabetes is confirmed in individuals with impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or elevated glycosylated haemoglobin (HbA1c) using either the World Health Organisation (WHO) or the American Diabetes Association (ADA) (4). Therefore, the WHO and ADA criteria are generally utilised when measuring the prevalence of pre-diabetes.

The International Diabetes Federation (IDF) estimated that the 2019 pre-diabetes global prevalence was 7.5% (373.9 million people). By 2045, the projected prevalence will increase to 8.6% (548.4 million) (5). In all the seven regions of the International Diabetes Federation (IDF), predictions state that the African region will have the most significant increase in pre-diabetes prevalence. The projection is that the 2019 prevalence of 45.3 million will rise by 143.3% to 110.2 million (5). However, studies conducted in populations with various ethnic groups reported that pre-diabetes prevalence in certain ethnic groups is disproportionally high. (6). In the United Kingdom, the observations showed that minority ethnic groups have a higher prevalence of pre-diabetes (7)**.** Furthermore, Another report revealed that 15.3% of adults of the 88 million estimated to have pre-diabetes did not know, which indicates that most people remain with this condition undiagnosed (5). This is concerning because the pre-diabetes condition can be very harmful.

Researchers have shown that pre-diabetes can be a toxic environment that instigates multiple metabolic complications, such as T2DM (1). The literature indicates that pre-diabetes promotes severe chronic microvascular and macrovascular complications, blood pressure changes, fatty liver disease conditions, and T2DM (3,4). A prevention study spanning over 20 years displayed a greater than 90% cumulative incidence of progression to T2DM from IGT (8). This development rate is a concern because we know that diabetes causes the death of approximately 1.3 million people annually (9). In addition, the literature reveals that there is an increased risk of heart failure and all-cause mortality in people with pre-diabetes (10,11). Therefore, the synthesis of available prevalence data for pre-diabetes in a population may prove crucial in many ways.

The pooling of studies with pre-diabetes prevalence data will help determine the current burden that pre-diabetes has on a specific community and assist in mitigating the future prevalence of pre-diabetes and its associated metabolic complications. Notably, the prevalence of pre-diabetes is shown to vary amongst different populations (5). Therefore, populations must have their own summarised prevalence data so that relevant and empirical evidence can be at the policymakers' disposal, which will result in better-informed decisions.

Hence, the review will focus on the population in South Africa, which could also be a representative of Southern Africa. Various studies have reported on the prevalence of pre-diabetes in South Africa (12–14). However, none of the studies can independently give us an overall prevalence of pre-diabetes and account for all the ethnic groups. Moreover, no systematic review focuses on the primary outcome of determining the total prevalence of pre-diabetes in South Africa. Therefore, this led us to generate the following research questions and objectives.

Research questions

1. What is the overall prevalence of pre-diabetes, and is there a fair representation of all the ethnic groups living in South Africa?
2. What are the common correlates of pre-diabetes?

Objectives

1. To determine the prevalence of pre-diabetes in the adult population of mixed ethnicities.
2. To determine the common correlates of pre-diabetes.

2. Methods and Analysis

Study Design

This systematic review protocol has been prepared following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Protocols 2015 guidelines. The protocol was registered with PROSPERO (CRD42020182430).

Search strategy

With a librarian's aid, two independent reviewers will conduct a comprehensive search of databases to find all related articles published on diabetes mellitus and pre-diabetes in South Africa from January 2000 to May 2020, regardless of the language of publication. The databases that will be screened will include MEDLINE through PubMed, Google Scholar, Embase and African Journals Online. They will utilise some of the following Medical Subject Heading (MeSH) in our search strategy: "Pre-diabetes," "South Africa," "Prevalence," "Type 2 diabetes mellitus," "Impaired fasting glucose," and "Impaired glucose tolerance." A detailed method in Table 1 (supplementary file) will be used to search PubMed. We will use the Mendeley referencing manager (V1.19.10) to remove duplicates. Moreover, they will perform a hand search to identify other eligible studies not indexed in the databases, especially in the included studies' bibliography and relevant literature reviews. We will also request and screen unpublished manuscripts and thesis from the University of KwaZulu-Natal registry and contact researchers.

Types of study eligible

The studies that we will consider for this review consist of prospective or retrospective cross-sectional population-based and cohort studies reporting pre-diabetes prevalence in South Africa. The study must have a minimum of 100 participants to pass eligibility. There will be no language restriction for eligibility for studies. Furthermore, the most up-to-date and comprehensive version will be selected for studies reporting the same results in multiple articles.

Types of participants

The participants in the studies included will need to be adults (≥18) located in South Africa and registered citizens who are black, coloured, Indian/Asian, or white. The participants used in the studies will need to be clinically diagnosed with pre-diabetes using the ADA or WHO diagnosis criteria. The diagnosis criteria defined by the American diabetes association (ADA) and the World Health Organization (WHO) will be considered. Accordingly, the diagnosis is determined by observing impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and elevated glycosylated haemoglobin (HbA1c) (15). IFG is defined as fasting plasma glucose (FPG) of 5.6-6.9 mmol/L. IGT is defined as the two-hour plasma glucose of 7.8-11.0 mmol/L after a two-hour interval following the ingestion of 75 g of an oral glucose load or a combination of both the IGT and the IFG recorded during the oral glucose tolerance test (OGTT). Also, pre-diabetes diagnosis with HbA1c will be accepted for any value between 5.7-6.5% (15,16). Any study lacking a clear diagnosis criteria description will be excluded if, after contacting authors twice, the information is not provided.

Inclusion and Exclusion criteria

The two independent reviewers will be responsible for choosing eligible studies that meet the type of study and participant requirements. The articles or thesis included will have to be studies done between 2000 and May 2020. Therefore, all studies that do not adhere to the eligible type of study and participant requirements and that fall outside the 20-year range between 2000 and May 2020 will be excluded. Studies with insufficient data to calculate the primary outcome will face exclusion if the requested data is not provided after contacting the corresponding author twice.

Primary outcomes

- Prevalence of pre-diabetes
- Prevalence of pre-diabetes across different ethnic groups

Secondary outcome

- Determine the most conventional risk factors associated with pre-diabetes.

Data Extraction

Using a pre-designed Excel form, one author will extract the applicable data. To ensure the quality of extracted data, another author will independently check all data. If there are any disagreements, they will be deliberated and solved with the assistance of a third reviewer. The data to be extracted will

include the population sampled, crude pre-diabetes prevalence estimates, and any prevalence estimates reported stratified by age, sex, or location (within South Africa). Data on parameters such as weight, hypertension and family history of diabetes will be pulled to appraise the most conventional risk factors associated with pre-diabetes. Prevalence figures and 95%confidence intervals (CIs) will be extracted or calculated, provided all necessary data is available. Where data is insufficient or presented graphically, we will contact the article's first author to request more data or calculate from the available data using Wilson's method. Given that all essential data will be provided, we will pull all correlates for pre-diabetes present in the clinically diagnosed participants.

Assessment of the quality of included studies

Two investigators (AMS & AK) will independently assess the methodological quality of comprised studies using the risk of bias tool for prevalence studies developed by Hoy and colleagues (17). A score of 0–4, 5–7, or 8–10 rated the risk of bias as high, moderate, or low, respectively. A third review author (NCM) will resolve disagreements between the two investigators by consensus or arbitration.

Data synthesis and analysis

The MetaXL (www.epigear.com) add-in for Microsoft Excel will be utilised for the synthesis and analysis. The point estimate for each study will be transformed with the Freeman-Tukey double arcsine method to stabilise the variance. The normal distribution of data will be validated using the D'Agostino & Pearson omnibus normality test. The study-specific estimates with 95% CI (Confidence Interval) will be pooled to generate an overall summary prevalence figure across the selected studies. After that, the heterogeneity between estimates will be assessed using the I^2 statistics.

The I^2 value describes the percentage of variation not because of chance or sampling error across studies (18). Suppose the I2 value is higher than 75%. In that case, the heterogeneity between studies will be deemed high (19). Any possible influences on prevalence estimates will be investigated using subgroup analyses and meta-regression. Where studies allow, we will descriptively compare prevalence estimates by the following subgroups: sex, race, age, Body Mass Index (BMI), lipid profile, family history, exercise, and education. After that, we will calculate the regression coefficient to ascertain a linear relationship between the effect estimate (i.e.outcome variable) and the explanatory variable or subgroup. We will then assess the influence on estimates of the following study-level variables identified a priori as potential sources of variation in the estimates of prevalence: (1) risk of bias, (2) geographical location, and (3) data collection method. Finally, where fitting, the outcomes will be displayed in tables or forest plots.

Confidence in cumulative evidence

The Grading of Recommendations Assessment, Development, and Evaluation (GRADE) method will assess the strength of evidence (20). The GRADE method will provide a score of the quality of the

studies and the strength of the evidence depending on methodological flaws within the included studies, consistency of results across diverse studies, precision estimates, and publication bias.

Ethics and Dissemination

The systematic review and meta-analysis do not require ethics clearance since studies with non-identifiable data will be used. The review will give insight into the current burden that pre-diabetes has on specific areas in the country and may assist in predicting and mitigating the future prevalence of other associated conditions like T2DM.

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Authors contributions

AMS, NCM, and AK were responsible for brainstorming, designing the study, and then drafting the protocol. AMS, AK, and PS were responsible for reviewing and approving the final draft of the manuscript.

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Competing Interest

The authors declare no competing interest

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4.2 SYSTEMATIC REVIEW AND META-ANALYSIS

DETAILS OF NEXT MANUSCRIPT

The following manuscript is titled "Prevalence and correlates of pre-diabetes in adults of mixed ethnicities in the South African population: A Systematic Review and Meta-analysis" is authored by A.M. Sosibo, N.C Mzimela, P.S Ngubane, and A. Khathi. The manuscript has been published in the PLOS ONE journal and has been formatted according to the journal's guidelines for authors (https://doi.org/10.1371/journal.pone.0278347). This journal is accredited by the Department of Higher Education and Training South Africa (2022) and appears in the Scopus accredited list (2022).

Author Contribution: AM Sosibo was responsible for study conceptualization, study design, data collection, data analysis, first draft writing, and manuscript editing.

Prevalence and correlates of pre-diabetes in adults of mixed ethnicities in the South African population: A Systematic Review and Meta-analysis

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Abstract

Introduction

Pre-diabetes is a metabolic condition characterised by moderate glycaemic dysregulation and is a frontline risk factor for multiple metabolic complications such as overt diabetes. To the best of our knowledge, this will be the first systematic review and meta-analysis focusing on generating a comprehensive pool of studies reporting on pre-diabetes prevalence in South Africa. Therefore, the purpose of the review will be to screen and elect reports that can be used to synthesise and provide the best estimate of the prevalence of pre-diabetes and its associated correlates in the South African population.

Methods and analysis

To determine the prevalence and correlates of pre-diabetes in South Africa, we searched PubMed, Web of Science, Google Scholar and African Journal online for published or unpublished studies reporting the prevalence of pre-diabetes in South Africa starting from the year 2000 to 2020. Studies were assessed for eligibility by checking if they met the inclusion criteria.

Results & conclusion

The total number of studies deemed eligible is 13, and from these studies, an overall prevalence of pre-diabetes was reported to be 15,56% in the South African population. Hypertension, obesity and sedentary lifestyle were the common correlates recorded for the population of interest. Therefore, the review highlights the disturbingly high prevalence of pre-diabetes in South Africa and necessitates further investigations into the possible genetics, biochemical and hormonal changes in pre-diabetes.

Ethics and dissemination

The review will not require ethics clearance because non-identifiable data will be used. The review outcomes will give insight into the current burden that pre-diabetes has in South Africa.PROSPERO registration number: CRD42020182430

Keywords: Pre-diabetes, Prevalence, South Africa

1. Introduction

Pre-diabetes is a condition that exists when the blood glucose concentration is higher than normal but not high enough to diagnose type 2 diabetes mellitus (T2DM) (1, 2). The diagnosis of pre-diabetes can be confirmed by impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or glycated haemoglobin (HbA1c) (3). The co-existence of moderate insulin resistance and beta-cell dysfunction characterises the pre-diabetic state (2, 3). Pre-diabetes is associated with metabolic complications often observed in T2DM, such as microvascular and macrovascular complications (4-7). Presently, lifestyle modifications and metformin administration are the recommended treatments for pre-diabetes (1, 8, 9).

Interestingly, the pathophysiology of pre-diabetes in subjects with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) displays different pathophysiological mechanisms (10, 11). In people with IFG, hepatic insulin resistance is observed; in those with IGT, muscular insulin resistance is seen (10). In the liver cells, insulin will stimulate glycogen synthesis by activating the enzyme hexokinase, inducing the phosphorylation of glucose to glucose-6-phosphate. Simultaneously, insulin inhibits the gluconeogenesis process in the hepatic cells. However, in the IFG state, diminished insulin sensitivity in the hepatocytes will stimulate gluconeogenesis and glycogenolysis processes (11, 12). Glucose concentration in the plasma will consequently rise.

The skeletal muscle insulin signalling pathway is initiated upon insulin binding to the α-subunit of the insulin receptor, which then causes the tyrosine residues in the intracellular β-domain of the insulin receptor to undergo autophosphorylation (13, 14). After that, a cascade of events leads to the activation of Akt, which causes the translocation of the glucose transporter-4 (GLUT-4) to the plasma membrane, thereby allowing glucose uptake into the skeletal muscle tissue (14). However, the insulin signal pathway is impaired in the IGT state, resulting in glucose homeostasis dysregulation(11). The consequences of leaving pre-diabetes unattended are significant.

Pre-diabetes is the frontline risk factor that can lead to the development of T2DM (3). This was amplified further by the American Diabetes Association (ADA) findings, which estimate that up to 70% of people with pre-diabetes will, in the long run, have T2DM (15, 16). In 2019, the prevalence of diabetes stood at 463 million, and a further estimate of 578 million people are predicted to have T2DM by 2035 (17, 18). T2DM is responsible for the deaths of approximately 1.5 million people annually (19). In addition, other causes of fatalities, such as those related to cardiovascular disease and the more recent COVID-19 virus, occur in individuals in which diabetes exists as a comorbidity (20). Therefore, understanding pre-diabetes can be pivotal in mitigating the incidence of T2DM and the mortality rate. Consequently, this calls for further research to be conducted in investigating pre-diabetes.

The global prevalence of pre-diabetes or impaired glucose tolerance was estimated to be 7.5% (374 million) in 2019 and is projected to reach 8.0% (454 million) by 2030 (18). Interestingly, some literature has documented the prevalence of diagnosed and undiagnosed pre-diabetes and how it affects different

ethnic groups. A study by Geert Roeyen *et al.* showed that 77.7% of patients referred for pancreatic surgery had some degree of pre-diabetes (21). Another report revealed that 15.3% of adults of the 88 million estimated to have pre-diabetes did not know, which indicates that most people remain with this condition undiagnosed (22). This high number of undiagnosed pre-diabetes cases in people is a worrying matter because if it is left unchecked, the incidence of T2DM will continue to rise (10). Additionally, a published study conducted in Canada, a country with diverse ethnicities, has shown that different ethnic groups are disproportionately affected by T2DM in adolescents (23). This disproportion was also evident in a study with adults conducted in England, whereby the minority ethnic groups displayed a higher prevalence of pre-diabetes (24)**.**

Interestingly, South Africa, also known as the "rainbow nation", has a significant mixture of ethnicities, but whether certain ethnic groups in the country are disproportionally diagnosed with pre-diabetes remains unknown. Furthermore, there seems to be a paucity of pre-diabetes-related research work done within the southern African scale. Therefore, the overall prevalence of pre-diabetes in a South African population of mixed ethnicities remains uncertain or minimal.

Research question

1. What is the prevalence of pre-diabetes, and are any ethnic groups disproportionally affected by pre-diabetes in South Africa?
2. What are the common correlates of pre-diabetes?

Objectives

1. To determine the prevalence of pre-diabetes in the adult population of mixed ethnicities.
2. To determine the common correlates of pre-diabetes.

2. Methods

Design

This systematic review protocol has been prepared following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Protocols 2015 guidelines. This review was registered in the PROSPERO International Prospective Register of Systematic Reviews, registration number CRD42020182430.

Setting

South Africa is a country on the southernmost tip of the African continent. South Africa is the largest country in Southern Africa, with over 58 million people.

Criteria for considering studies for the review

Category of study*:*

The studies considered for this review consisted of cross-sectional population-based studies, prospective or retrospective cohort studies and population-based surveys that report on the prevalence of pre-diabetes and how different ethnicities are affected. Also, a minimum of 100 participants had to be included in these studies. Furthermore, the most up-to-date and comprehensive version was selected for studies reported in more than one report.

Types of participants:

The study participants included adults (≥18) located in South Africa who are registered citizens who are black, coloured, Indian/Asian, or white. The participants used in the studies had to be clinically diagnosed with pre-diabetes using the ADA or WHO diagnosis criteria.

The outcome of interest:

Studies included in this review had to report the prevalence of pre-diabetes with sufficient data to calculate this estimate. Studies lacking primary data were excluded if the information was not provided after contacting authors at least twice.

Diagnosis criteria:

The diagnosis criteria defined by the American diabetes association (ADA) and the World Health Organization (WHO) were considered. Accordingly, the diagnosis is determined by observing impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and elevated glycosylated haemoglobin (HbA1c). IFG is defined as the fasting plasma glucose (FPG) of 6.1-6.9 mmol/L. IGT is defined as the two-hour plasma glucose of 7.8-11.0 mmol/L after the ingestion of 75 g of oral glucose load or a combination of both the IGT and the IFG recorded during the oral glucose tolerance test (OGTT). Any study that lacked a clear method description had to be excluded if, after contacting authors at least twice, the information was not provided.

Search strategy

Two independent (AMS & AK) reviewers conducted a comprehensive search of databases to find all related articles published on diabetes mellitus and pre-diabetes in South Africa from 2000 to 2020, regardless of the language of publication. The databases screened included MEDLINE through PubMed, Google Scholar, Web of Science and African Journals Online. The following Medical Subject Headings in our search strategy: "Pre-diabetes," "South Africa," "Prevalence," "Type 2 diabetes mellitus," "Glycated Hemoglobin," "Impaired fasting glucose," and "Impaired glucose tolerance." A detailed method is shown in the search strategy in S1 Table. The Mendeley referencing manager (V.1.19.10) was used to remove duplicates. Moreover, hand searching was done to identify other eligible studies not indexed in the databases, especially in the included studies' bibliography and relevant literature reviews.

Selection of included study

Two independent reviewers (AMS & AK) selected applicable studies. Each reviewer independently screened the titles, abstracts, and full texts to acquire studies they consider relevant.

Appraisal of the quality of included studies

The methodological quality of included studies for prevalence estimates was assessed using an adapted version of the risk of bias tool for prevalence studies developed by Hoy and colleagues. A score of 0–4, 5–7, or 8–10 rated the risk of bias as high, moderate, or low, respectively. Two investigators (AMS & AK) independently assessed the study quality, with disagreements resolved by consensus or arbitration by a third review author (NM).

Analysis

A descriptive analysis of the eligible studies was first undertaken in the review investigation. The pooled proportion and 95% confidence interval (CI) were taken as the effect size. The normal distribution of data was validated using the D'Agostino & Pearson omnibus normality test. After that, a meta-analysis was undertaken using a random-effects model (to account for heterogeneity) using the MetaXL (www.epigear.com) add-in for Microsoft Excel. Using MetaXL, we calculated the I2 statistics to assess heterogeneity between estimates. The I2 value describes the percentage of variation not because of

chance or sampling error across studies. If the I2 value is higher than 75%, then the heterogeneity between studies is deemed high. We used the 95% CI to calculate a pooled prevalence figure.

Any possible influences on prevalence estimates were investigated using subgroup analyses. We assessed the impact on estimates of the following study-level variables identified a priori as potential sources of variation in the calculations of prevalence: (1) risk of bias, (2) geographical location, and (3) data collection method. We classified studies as being either at low risk of bias (low risk of both participation and outcome measurement bias) or moderate-to-high risk of bias (moderate or high risk of either participation or outcome measurement bias).

3. Results

Study selection

The initial search resulted in a total of 995 articles detected. The last search date was on the 7th of September 2021. After that, the titles and abstracts had to be screened for eligibility. Consequently, 950 articles had to be extracted due to duplication and being unrelated. Then, the full-text assessment of the remaining 45 articles was conducted, which resulted in 13 articles that met the eligibility criteria.

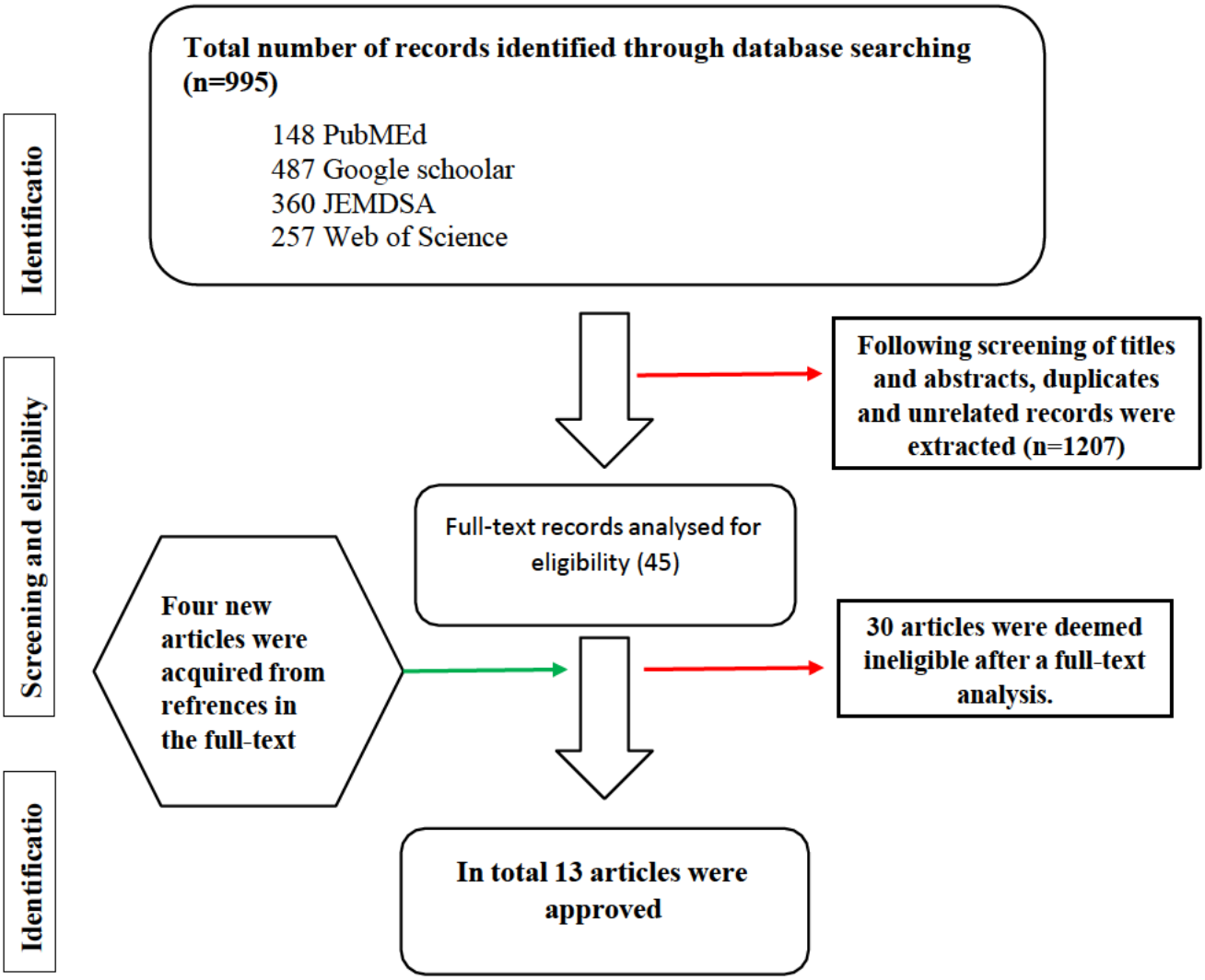


Figure 1. Presents the procedure followed for identifying and selecting studies included in the review.

Appraisal of the quality of included studies

Hoy and colleagues' risk of bias assessment for prevalence studies was used. As shown in Table 1, seven out of the 13 articles had a low risk of bias, six had a moderate risk of bias, and one had a high risk of bias. All the studies with a moderate to high risk of bias were not randomised.

Pre-diabetes prevalence

Figure 1: In a pooled sample of 5257 participants, the combined effect size for the prevalence of pre-diabetes is 15,56% in the South African population. The heterogeneity observed in the forest plot is 98,86%.

Prevalence of pre-diabetes stratified by sex and age

The results from Table 1 show that the female-to-male ratio is 1,18. Therefore, the probability of developing pre-diabetes in females is higher by 0,18 (or 18%) than in males. According to Figure 2, the results for groups A and B show that, generally, pre-diabetes will increase with age.

Summary of Sub-group results

The following results are seen in Table 1. The findings show that according to the geographical location, the province of Limpopo, represented by a single study, has an alarming 48,0% prevalence of pre-diabetes, which is the highest. Other provinces represented by single research include Gauteng (24,7%) and Free State (6,3%). Of the provinces that had more than one study conducted in it, the Western Cape province, represented by Cape Town, had the highest prevalence of pre-diabetes (15,4%), followed by KwaZulu-Natal (12,96%) and Eastern Cape (9,81%). The prevalence of pre-diabetes according to race reveals that the Indian population has a proportion of 29,01%. The Indian population was followed by the Coloured at 17,38% and then the Black population at 13,8%. The risk of bias outcome shows that the pooled data for the group of studies with low risk is 16,7%, 15,6% for the moderate risk group, and 6,1% for the high-risk group, represented by only one study. According to the diagnosis method, the prevalence of pre-diabetes was recorded to be 18,2% in the studies that used the impaired fasting glucose test and 13,9% using the impaired glucose tolerance.

Correlates of Pre-diabetes

From the identified correlates, hypertension has the highest total proportion of 46,8% in the pool of studies elected. People who live a sedentary lifestyle have the second highest total proportion of 43,4% in the pooled studies. In addition, the proportion of obesity (39,1%) and overweight (36,5%) is high in the population. A high number of smokers (26,3%) and people who drink alcohol (30,6%) are represented in the review. Family history of diabetes was represented by a single study that reported that 32,8% of the participants had a history of diabetes. Triglyceride levels had a mean value of 1,43 mmol/L, whilst total cholesterol and LDL had mean values of 4,47 and 2,75 mmol/L.

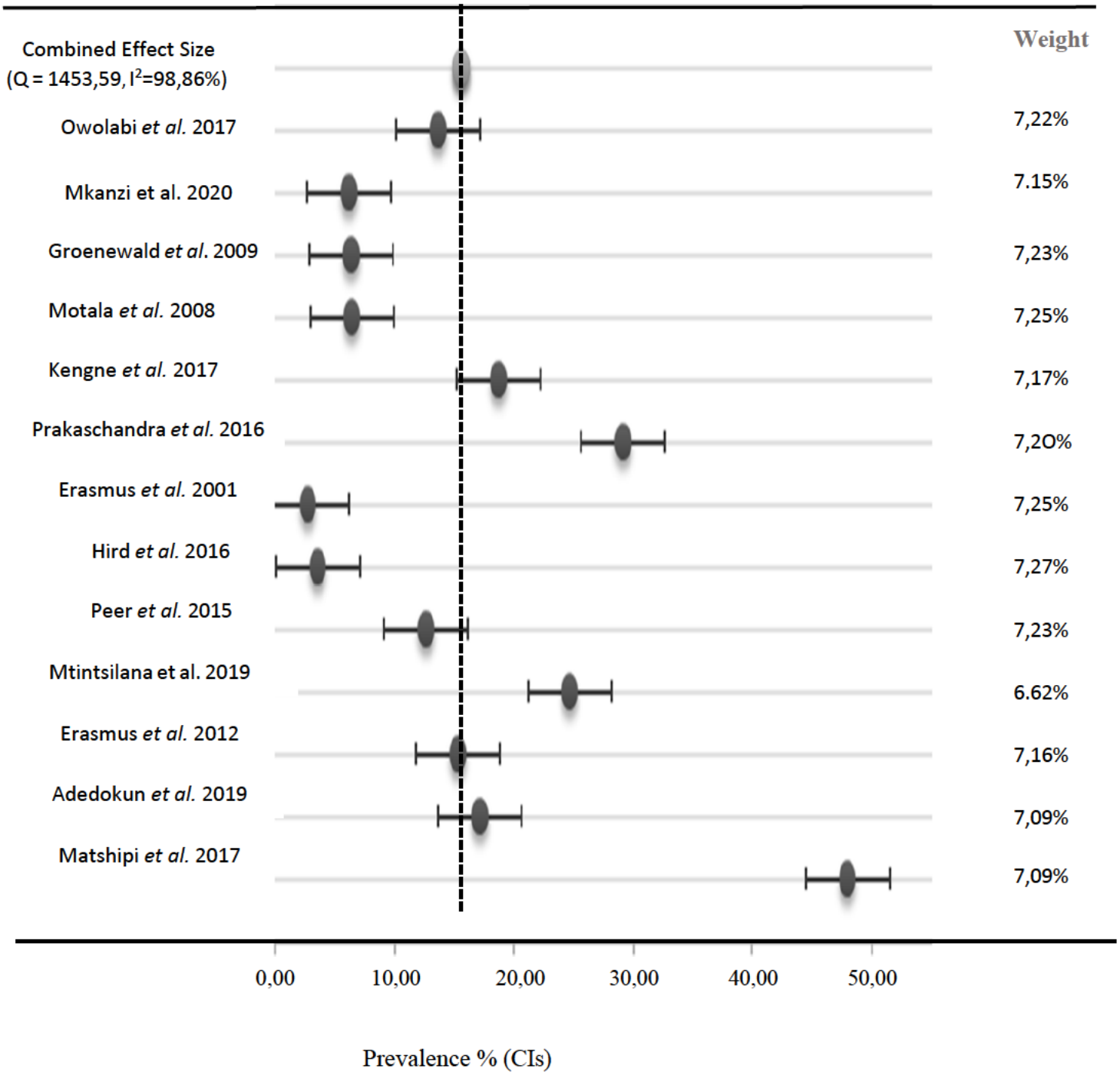


Figure 2. Presents a forest plot of the summary of the analysis of prediabetes prevalence in South Africa.[3]

[3] The diagnosis was determined by either IFG or IGT test. In a pooled sample of 9339 participants, the prevalence of prediabetes was 15,56%, with a heterogeneity of 98,86%.

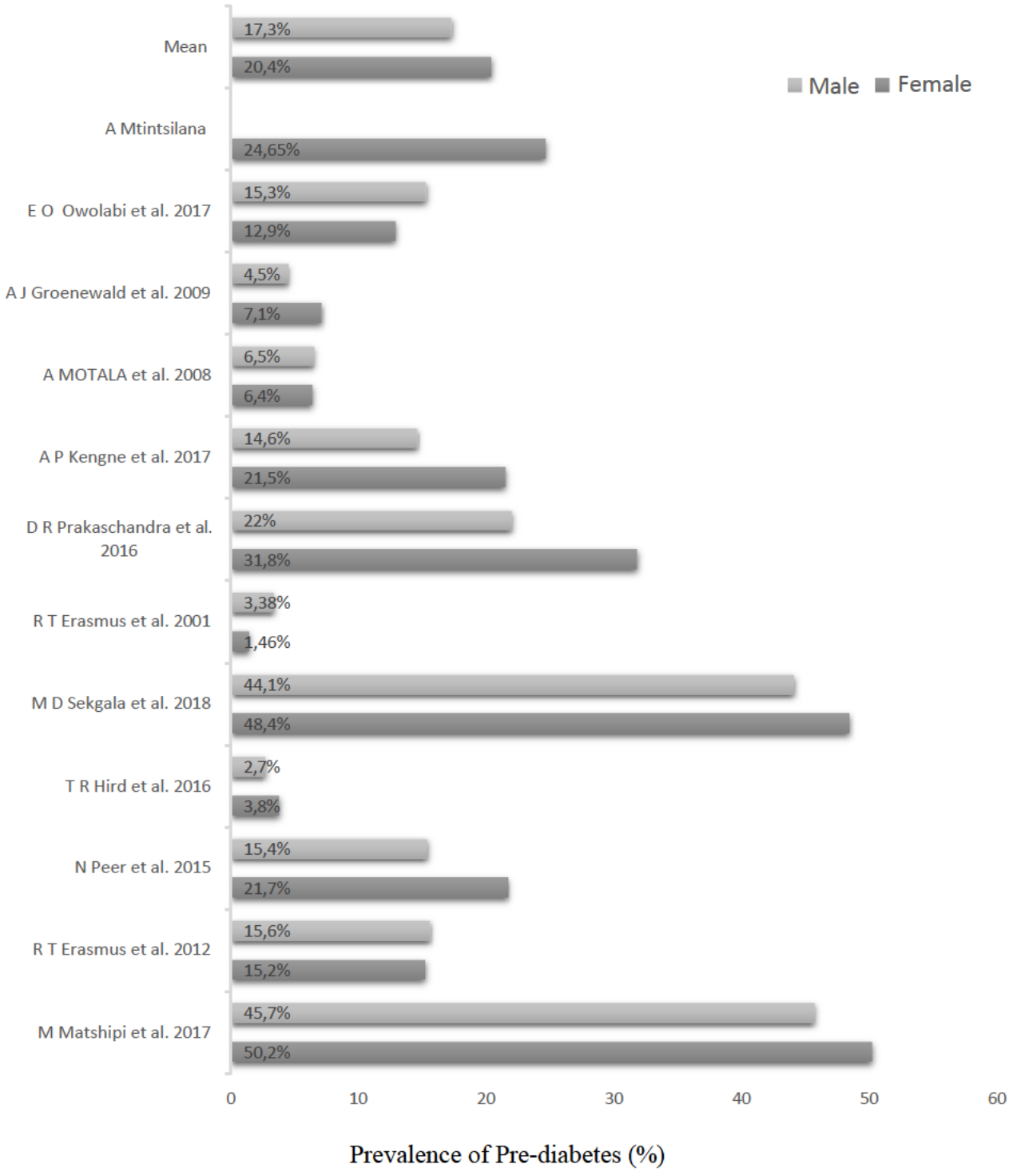
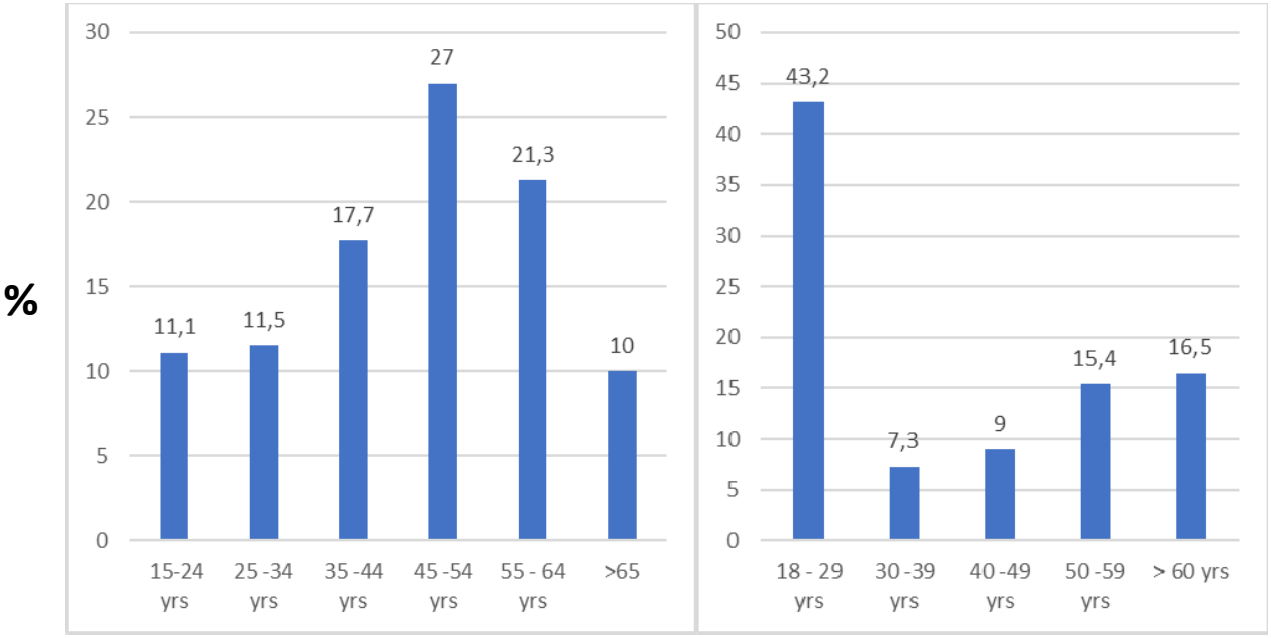


Figure 3. Presents the prevalence of pre-diabetes stratified by sex in the South African population.[4]

[4] The study conducted by A Mtintsilana only had female participants. The overall female-to-male ratio is 1,18



Group A: Age starting from 15

Group B: Age starting from 18

Figure 4. Presents the prevalence of pre-diabetes stratified by age.[5]

[5] Except for the 18 – 29 age range in group B, the proportion of individuals with prediabetes tends to increase with increasing age.

Table 1. Presents the summary of pre-diabetes prevalence in all the considered sub-groups.[6]

Group	Studies	Participants	Cases	Prevalence % (95%CI)	I2 %	p-Egger test
By province						0,009
KZN	3	3567	462	12,96 (-21,8 - 47,7)	99	
EC	4	2003	196	9,81 (-0,8 - 20,4)	97	
CPT	3	2362	364	15,42 (7,8 - 23,1)	84	
Free state	1	552	35	6,3	-	
Gauteng	1	142	35	24,7	-	
Limpopo	1	713	342	48,0	-	
By race						0,009
Black	10	6670	924	13,85 (4, - 23,6)	99	
Coloured	2	1291	230	17,8	64	
Indian	1	1378	401	29,10 (26,6 - 31,5)	-	
By risk of bias						
Low	7	6288	1049	16,69 (1,5 - 31,9)	99	
Moderate	5	2823	441	15,62 (7,4 - 23,8)	95	
High	1	228	14	6,14 (3,1 - 9,2)	-	
By gender						0,000
Male	11	3066	586	19,10 (3,5 -11,1)	98,13	
Female	12	6472	1294	19,99 (8,8 -31,2)	98,95	
By diagnosis						0,009
IFG	5	2894	526	18,19 (-3,2 - 39,6)	427,94	
IGT	8	6445	895	13,89 (5,8 - 22,0)	553,10	

[6] Except for Limpopo, the more industrialised provinces, such as KwaZulu-Natal (KZN), Gauteng and Cape Town/Western Cape, tend to have a higher pre-diabetes prevalence. The coloured and Indian populations have the highest prevalence of pre-diabetes. The risk of bias in most studies was low or moderate. The male and female participants have a similar prevalence of pre-diabetes. The IFG detected more pre-diabetic cases than the IGT.

Table 2. Shows the proportion of risk factors associated with pre-diabetes.

Studies	obesity	overweight	hypertension	tobacco	alcohol	Triglycerides	Chol (mmol/l): Total & LDL	Family history	Sedentary lifestyle
Matshipi *et al.*, 2017	-	-	-	-	-	-	-	-	-
Adedokun *et al.*, 2019	-	-	-		49.4	-	-	-	-
Erasmus *et al.*, 2012	-	-	-	44.4	28.4	-	-	-	-
Mtintsilana *et al.* 2019	70.4	21.1	-	-	-	-	-	-	-
Peer *et al.*, 2015	63	58	49	28	33	-	4.4 & 3.0	-	-
Hird *et al.*, 2016	37.7	60.8	28.9	19.2	14.3	1.4	4.3 & 2.3	32.8	46.5%
Erasmus *et al.*, 2001	10	10	-	-	-	-	-	-	-
Prakaschandra *et al.*, 2016	10,4	-	79	24,9	-	1.8	3.35	-	-
Kengne *et al.*, 2017	-	-	-	-	-	-	3.3 & 5.2	-	-
Motala *et al.*, 2008	-	64	64	-	-	1.1	4.1 & 2.4	-	-
Groenewald *et al.*, 2009	32.1	20.8	-	-	-	-	-	-	9.1%
Mkanzi *et al.*, 2020	44	33	11	-	-	-	-	-	-
Owolabi *et al.*, 2017	45.2	24	49	15	28	-	-	-	74.7%
Mean	39,1	36,5	46,8	26,3	30,62	1,43	4,27 & 2,75	32,8	43.4

4. Discussion

According to the Statistics of South Africa department, the Republic of South Africa has a population of slightly over 60 million (25). Therefore, the present meta-analysis showed that the overall prevalence of pre-diabetes was 15,56% in the South African population, which translates to over 9 million people with pre-diabetes. Interestingly, pre-diabetes diagnosis using the impaired fasting glucose criteria (IFG) had a higher prevalence of 18,2% compared to studies that used the impaired glucose tolerance test (IGT), which recorded 13,9%. Consequently, the heterogeneity recorded in the forest plot was 98,86%, indicating a high variability between the studies. We speculate that one reason for the high heterogeneity is the differences in the diagnosis methods utilised across the selected studies.

Differences in prevalence between IFG and IGT confirm the different pathophysiological mechanisms that contribute to these glucose homeostasis disorders. Some literature suggests that IFG is seen after impaired glucose tolerance IGT is observed (11, 26, 27). Relative to IGT, the IFG is associated with a more significant increase in future T2DM risk (11). Hence, IFG will generally be seen in a slightly more advanced stage of pre-diabetes prognosis relative to IGT (11). However, compensatory mechanisms such as increased insulin secretion by the pancreatic beta-cells can delay the development of pre-diabetes (28). Therefore, these compensatory mechanisms should be considered, as they may be more robust in specific individuals, allowing them to bypass the early phase of IGT. Hence, it is plausible that at this moment, a speculate that a more significant number of people with pre-diabetes remain undiagnosed either because they do not go for health screening or because the clinicians may prefer to use only IFG to determine pre-diabetes. By doing so, they miss many pre-diabetic individuals according to the IGT criteria.

The sex of the participant seems to play a slight role in developing pre-diabetes. The results in Figure 2 indicate that females have an 18% greater chance of developing pre-diabetes when compared to males. This contrasts with what some literature suggests: that males are more susceptible to diabetes than females (29-31). Their justification is that men tend to store fat in their organs, like the liver, compared to women, who store fat mainly on the surface layer, such as the hip and thighs (31). However, literature has also shown that females are more prone to developing IFG than males, independent of age (32, 33).

This suggests that females may have more robust protective mechanisms that delay the onset of T2DM. A European study corroborated that men are often diagnosed with T2DM earlier than women (32, 34). Therefore, the higher prevalence of pre-diabetes observed in women can be explained by their greater susceptibility to developing pre-diabetes.

Another interesting factor that the review looked at is age. The findings in this review show that the association between age and the prevalence of pre-diabetes are directly proportional. The results are consistent with the literature as the prevalence of pre-diabetes rises with increasing age (24, 35, 36). The plausible explanation for the correlation between age and prevalence is that people tend to live a more sedentary lifestyle as they grow older. A study by Zoran Milanović *et al*. revealed that relative to the young elderly, there is a reduction in physical activity amongst the old elderly (37). The decline in physical activity was attributed to decreased muscle strength in both upper and lower limbs and changes in body fat percentage (37). However, the high prevalence observed in the 18-29 age group in Table 3 can be attributed to the study by Sekgala et al., which was reported on the 18-29 age group only (38). Therefore, it is plausible to expect a similar trend had the study by Sekgala included the older age groups.

The prevalence of pre-diabetes according to geographical location shows that the Limpopo and Gauteng provinces have the highest prevalence of 48% and 24,8%, respectively. However, this cannot be taken as conclusive data because these provinces have a small sample size and are represented by a single study. Therefore, more prevalence studies are required in these provinces. The outcomes of the prevalence of pre-diabetes according to the geographical location are more credible in the provinces with multiple studies. These provinces include the province of the Western Cape, represented by Cape Town, which had the highest prevalence of 15,42%, followed by KwaZulu-Natal with 12,96% and Eastern Cape with 9,81%. However, ethnicity is known to play a role in the prevalence of diabetes.

The prevalence of pre-diabetes across different ethnic groups in South Africa suggests a genetic predisposition for developing pre-diabetes. The Indian population have the highest prevalence of 29,01%, followed by the coloured community with 17,38% and the black population with 13,8%. Therefore, ethnicity plays a significant role in predisposition to developing pre-diabetes. There is

increasing evidence in the literature on how epigenetic factors play an essential role in the development of T2DM (39, 40). Epigenetics can be described as transmissible alterations in gene function that arise with no changes in their nucleotide sequence (41). Epigenetic markers like miRNAs and methylation are shown to be involved in the pathogenesis of diabetes and may explain why some ethnic groups are disproportionally affected by pre-diabetes (23, 40, 42). Hence, further studies appraising the role of epigenetics in developing pre-diabetes are needed for the population of South Africa. Furthermore, pre-diabetes is associated with numerous other medical conditions.

Unsurprisingly, the prevalence of pre-diabetes correlates with high occurrences of hypertension, obesity, overweight, and a sedentary lifestyle. These risk factors are well documented in the literature to be associated with the development of diabetes (43-45). Hence, there is expected to be a high pre-diabetes prevalence whenever these correlates are high in a population. Therefore, we must not look at pre-diabetes as an isolated condition; instead, we need to monitor its risk factors, which play a significant role in the onset and prognosis of pre-diabetes. Derek Weycker *et al.* have shown that regardless of Body Mass Index (BMI), age, and sex, all people with hypertension are at a considerable risk of becoming diabetic(46). Also, the sedentary lifestyle over the years has risen with the increasing urbanisation and consumption of Western fast foods, which, according to the literature, is directly proportional to the rise in pre-diabetes prevalence and all-course mortality (47, 48). A sedentary lifestyle causes an imbalance in calorie intake, resulting in a greater intake than output. This imbalance causes the body to store the extra energy source as fats, which accumulate and cause insulin resistance that leads to pre-diabetes and type 2 diabetes(49, 50). Likewise, the obesity and overweight association with diabetes is well established(51). Both conditions of obesity indicate early signs of insulin resistance and can result in the development of diabetes if unattended. Therefore, when assessing the prevalence of pre-diabetes in a particular location, these associated risk factors must also be monitored to identify those at risk.

5. Limitations

One of the limitations of this study is that only seven of the 13 eligible studies had a low level of risk. This suggests that more credible studies are needed to have a pool of studies that will generate a more reliable overall prevalence in the country. Furthermore, some utilised the outdated version of the WHO diagnostic criteria for impaired glucose tolerance and impaired fasting glucose. The lack of a universal definition for determining pre-diabetes is also a limitation for this review because different studies utilised different criteria for deciding pre-diabetes.

6. Conclusion

The selected studies applied different criteria for diagnosing pre-diabetes, and some used outdated measures. Consequently, there is a need for a universal diagnostic marker for pre-diabetes that will improve accuracy when comparing and pooling epidemiological studies. Furthermore, the present review findings are concerning because a high prevalence of pre-diabetes poses a significant healthcare burden. Pre-diabetes is associated with metabolic disorders and is the frontal risk factor for developing the more harmful type 2 diabetes. Thus, there is a gap for more prevalence studies on prediabetes that will use current diagnostic criteria for pre-diabetes and reveal updated data on the prevalence of pre-diabetes.

Additionally, as seen in T2DM, there appears to be a correlation between a high prevalence of pre-diabetes and hypertension, obesity and a sedentary lifestyle. Screenings done in the hospitals should, therefore, be holistic by including markers for pre-diabetes and associated correlates. Finally, the findings suggest that clinicians should put in place preventative measures for preventing the progression from pre-diabetes to type 2 diabetes and have more clinical outreaches to identify early those who have pre-diabetes.

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4.3 RESEARCH ARTICLE 1

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled "Prevalence of pre-diabetes in adults aged 25 – 45 years in a Durban-based clinical setting, South Africa: A retrospective study" is authored by A.M. Sosibo, N.C Mzimela, P.S Ngubane, and A. Khathi. The manuscript has been published in the Primary Care Diabetes journal and has been formatted according to the journal's guidelines for authors (DOI: 10.1016/j.pcd.2023.10.004). This journal is accredited by the Department of Higher Education and Training South Africa (2023) and appears in the Scopus accredited list (2023)

Author Contribution: AM Sosibo was responsible for study conceptualization, study design, sample collection, data analysis, first draft writing, and manuscript editing.

Prevalence of pre-diabetes in adults aged 25 – 45 years in a Durban-based clinical setting, South Africa: A retrospective study

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Abstract

Aim

Due to pre-diabetes being underexplored, its prevalence was investigated in study participants aged 25 to 45 years in a Durban-based tertiary-level clinical setting in South Africa.

Methods

The study was done using a retrospective study design. Fasting blood samples from consented patients with no previous diagnosis of diabetes and within the specified age range were collected from King Edward Hospital in Durban. The pre-diabetes diagnosis was confirmed in participants with fasting glucose concentrations between 5.6 – 6.9 mmol/L and glycated haemoglobin (HbA1c) levels between 5.7 to 6.4% using the American Diabetes Association (ADA) and World Health Organisation (WHO) diagnosis criteria. The study participants' characterisation was stratified according to the diagnosis criterion, age, gender and ethnicity.

Results

An alarming 68% average pre-diabetes prevalence across ADA and WHO criteria in the Durban, eThekwini district sample population. Between the White, Black and Indian ethnic groups, the Indian group were more predisposed to pre-diabetes onset, with a prevalence of 62.7%.

Conclusion

If pre-diabetes management is unattended, an unprecedented increase in metabolic disorders such as T2DM and all-cause mortality incidence can be expected. Therefore, the study reveals a window of opportunity to intensify preventative measures and mitigate the incidence of T2DM.

1. Introduction

Pre-diabetes is characterised by moderate hyperglycaemia, whereby blood glucose concentrations are above the homeostatic range but below the threshold for type 2 diabetes mellitus (T2DM) (1-5). Pre-diabetes is a significant risk factor for developing T2DM. T2DM is one of the leading contributors to global deaths (7). Additionally, the coronavirus disease (COVID-19) highlighted the dangers associated with pre-diabetes, as pre-diabetic patients infected with COVID-19 were significantly associated with adverse outcomes of COVID-19 (6). A diabetes prevention trial conducted in China suggests that in 20 years, more than 90% of individuals with pre-diabetes will progress to overt diabetes (7). Therefore, early detection of pre-diabetes is imperative to prevent the rising incidence of T2DM.

Epidemiological studies have reported that the incidence and prevalence of pre-diabetes are escalating in both developed and developing countries (8, 9). In 2021, 541 million adults were estimated to have pre-diabetes, accounting for 10.6% of the global population. Furthermore, it is anticipated that by 2045, the prevalence of pre-diabetes will increase to 730 million adults or 11.4% of the total population (10). Africa is predicted to experience one of the most significant increases in pre-diabetes prevalence (7). The projections state that relative to the 2019 prevalence estimate, an additional 4.9 million people with pre-diabetes will be probable in Africa by 2045 (7). One of the reasons for the projections made about Africa can be attributed to its swift changes in urbanisation and diet (8, 11). Therefore, preventing or slowing down the anticipated increases in pre-diabetes prevalence in developing countries like South Africa is imperative.

A recently published systematic review reported on the pre-diabetes prevalence (15.56%) in South Africa (12). The study highlighted that the age group of 25 to 45 is at a higher risk of developing pre-diabetes. The study further revealed that some of the prevalence studies conducted in the country used outdated criteria for diagnosing pre-diabetes. Therefore, the present estimate of pre-diabetes prevalence may be underestimated, and research on pre-diabetes prevalence in the eThekwini municipality has been underexplored. Therefore, this study was conducted to understand the current pre-diabetes burden on participants between 25 – 45 years in a clinical setting in eThekwini.

2. Methods and materials

Study design and setting

The study used a retrospective study design, and ethical clearance was obtained from the ethics committee of the University of KwaZulu-Natal (UKZN) (Study ref: BE266/19). The study was conducted in a developing country, South Africa, in a Durban-based clinical setting of eThekwini metropolitan. The metropolitan municipality comprises the city of Durban and its surrounding towns. The eThekwini district is one of eleven municipalities in the province of KwaZulu-Natal. A recent

census estimates that the metro area's population is 3,199,000. The study was conducted in collaboration with the King Edward Hospital (KEH). It is a tertiary-level hospital providing tertiary services to the entire KZN province. The ethnic groups represented in the Metro include black Africans (74%), Indians/Asians (17%), whites (7%), and Coloureds (2%). For sample size calculation, data from a published systematic review was used in which the overall prevalence of pre-diabetes is reported to be 15.56% in the South African population (13). Hence, for the calculation, we used a confidence interval of 95%, a margin of error of 5%, and a population proportion of 15.56%. The calculation result revealed that a sample size of 202 will be necessary to have a confidence level of 95%.

Prediabetes diagnosis and eligibility criteria

The pre-diabetes diagnosis criteria utilised for the study were adopted from the World Health Organisation (WHO) and the American Diabetes Association (ADA). Following the ADA criterion, pre-diabetes was determined by HbA1c levels between 5.7%-6.4% and IFG concentrations between 5.6-6.9 mmol/L. The WHO criterion defines IFG as fasting glucose concentrations between 6.0-6.9 mmol/L. The participants had to fast overnight before the blood glucose concentrations were measured. Studies have shown that the HbA1c and IFG values are reliable and effective risk measures to project progression to overt diabetes (13-15). For eligibility, the participants had to be based within the eThekwini municipality and between the ages of 25 and 45 years. The age limit was chosen after completing a systematic review showing that this age group is more predisposed to developing pre-diabetes. Therefore, we will likely include persons in the early stages of pre-diabetes. They must have an HbA1c of less than 6.5% and an IFG of less than 6.9 mmol/l.

Blood sample collection and analysis

With the assistance of professional nurses, serum and EDTA tubes were used to collect blood samples from participants aged 25 to 45 with undiagnosed diabetes at the Durban-located King Edward Hospital and linked clinics in the eThekwini metropolitan area of South Africa. The participants recorded data, and sample collection for the study began in March 2022 and continued to November 2022, and a total of 364 samples were collected.

Inclusion and exclusion criteria

The samples were collected from patients who were going to the hospital for check-ups on conditions unrelated to diabetes. The inclusion criteria included samples obtained from non-diabetic adults between the ages of (25-45) years of all ethnicities. The study excluded samples from patients who had a previous diagnosis of diabetes mellitus and were on treatment. The study further excluded samples from patients with a history of liver disease, kidney disease, heart disease, depression and those who were pregnant at the time of the study.

Sample processing and analysis

Blood samples were obtained from participants who confirmed overnight fasting and met the eligibility. Blood sample quality control was ensured following the guidelines reported by Lippi and colleagues (16). Then, the tubes were centrifuged (Eppendorf centrifuge 5403, Germany) to collect serum stored in a -80ºC freezer (Snijders Scientific, Holland). The fasting plasma glucose was measured using a blood glucose meter. The glycated haemoglobin (HbA1c) was measured using a clinically validated NGSP and IFCC-certified, laboratory-based Tosoh G8 HPLC Analyzer (17). The participants were divided into persons with pre-diabetes (PD) and those without groups.

Statistical analysis

The GraphPad Prism 8 software was used for statistical analysis. A normality test assessed whether the data was parametric or non-parametric. Non-parametric data was presented as Median + Interquartile range. The prevalence data was presented as a percentage ± 95% Confidence Interval (CI).

3. Results

We investigated the prevalence of pre-diabetes in a Durban-based clinical setting in South Africa. To determine the proportion, we measured the Hb1A1c and fasting glucose levels in study participants with no previous history of diabetes at King Edward Hospital (KEH). Our findings revealed an alarming pre-diabetes prevalence and confirmed the International Diabetes Federation (IDF) prediction of an exponential increase in pre-diabetes prevalence in developing countries.

Table 1. The characteristics of the participants grouped according to their diabetic status.[7]

	Non-pre-diabetes(NPD) *n =168*	*Pre-diabetes(PD)* *n =196*	*Total* *364*
Age			
Mean	36	39	
25-29, n	32	18	50
30-34, n	28	26	54
35-39, n	43	51	94
40-45, n	65	101	166
Gender			
Men, n (%)	65 (42,2)	89 (57,8)	154
Women, n (%)	103 (49,0)	107 (51,0)	210
Ethnicity			
African, n (%)	124 (47,0)	140 (53,0)	264 (72,5)
Indian, n (%)	25 (37,3)	42 (62,7)	67 (18,4)
White, n (%)	18 (64,3)	10 (35,7)	28 (7,7)
Coloured, n (%)	1 (20)	4 (80)	5 (1,4)
Diagnosis criteria			
%HbA1c median (95% CI)	5,3 (5,2-5,3)	5,9 (5,9-6,0)	
WHO IFG median (95% CI)	5,7(5,5-5,7)	6,5 (6,3-6,5)	
ADA IFG median (95% CI)	5,2 (5,2-5,4)	6,3 (6,3-6,5)	

[7] The non-parametric data is presented as a median with 95% Confidence and percentage.

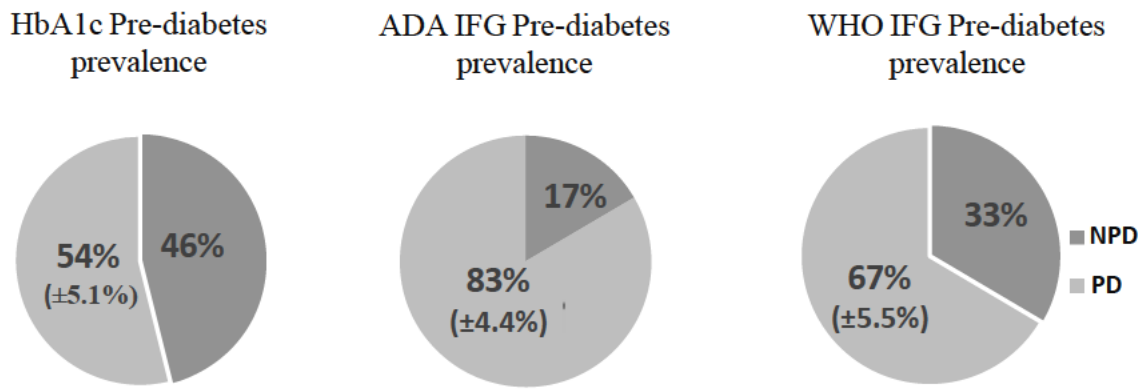

Figure 1. The pie charts display the prevalence of pre-diabetes (PD) according to the criteria utilised.[8]

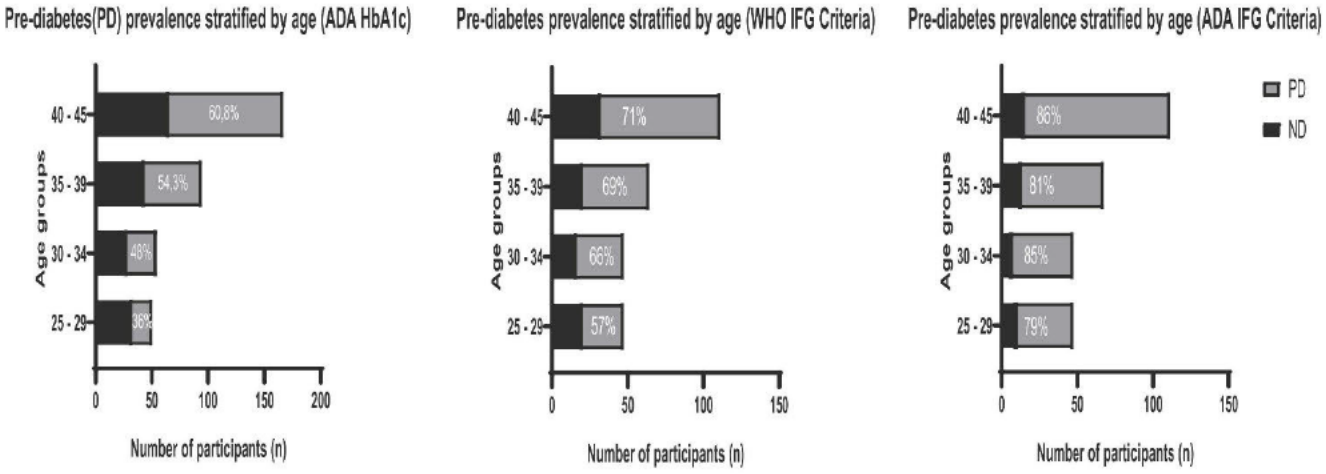

Figure 2. The criterion-specific pre-diabetes prevalence stratified by age in the urban population of eThekwini.[9]

[8] The HbA1c and IFG total number of participants were 364 and 272, respectively. A 54% (±5.1%), 83% (±4.4%), and 67% (±5.5%) prevalence were recorded using the HbA1c, ADA and WHO criteria in the population of adults aged between 25 – 45 years in the eThekwini region.

[9] The highest prevalence is observed in the uppermost age range of 40 – 45, and the prevalence is at its lowest in the youngest age group of 25 – 29.

4. Discussion

The pre-diabetes condition is characterised by moderate hyperglycaemia. If not managed timely, persons with pre-diabetes will often progress to type 2 diabetes mellitus (T2DM). This study aimed to determine the current prevalence of pre-diabetes in a clinical setting in Durban. The findings will provide empirical evidence on the current burden of the condition and help determine the measures required in primary care settings to improve pre-diabetes management in clinical settings and help alleviate progression to T2DM.

In Figure 1, the WHO and ADA IFG diagnosis criteria detected more individuals with pre-diabetes than the HbA1c criteria. The literature has shown lower HbA1c sensitivity in detecting pre-diabetes compared to using IFG (18). This may be due to other factors that affect haemoglobin glycation independently of blood glucose concentrations, such as anaemia, pregnancy, HIV treatment, race and haemodialysis (18, 19). However, the pre-diabetes prevalence remained extremely high, above 50% in both the IFG and HbA1c diagnosis. The average pre-diabetes prevalence across ADA and WHO criteria is a staggering 68%, which indicates an excessive pre-diabetes burden in this study population of adult patients aged between 25 and 45 years in urban clinical settings under the tertiary-level King Edward Hospital (KEH) located in the eThekwini metropolitan. Therefore, more than half the population is at risk of developing Type 2 diabetes. Furthermore, pre-diabetes is shown to be associated with macrovascular and microvascular complications often seen in T2DM individuals (4, 20-22). Therefore, based on these findings, participants with pre-diabetes may be at risk of developing diabetic complications and poor health outcomes in persons with pre-diabetes-associated comorbidities. Monitoring of pre-diabetes to prevent metabolic complications, progression to T2DM and poor health outcomes is imperative.

The high pre-diabetes prevalence in the KEH findings supports the results reported by the International Diabetes Federation. IDF published a study that predicted an increase in the incidence of diabetes and pre-diabetes in the future. In addition, the study indicated that out of the seven regions investigated, the African region is expected to experience one of the most significant increases in pre-diabetes prevalence (8, 9). The increase in pre-diabetes means there is a greater probability of T2DM incidence increasing. A comprehensive systematic review showed that the risk of diabetes increased dramatically between A1c values of 5 and 6.5% (14). For individuals with A1C values between 5.5 to 6.0% and 6.0 to 6.5%, the predicted risk of diabetes over five years was 9 to 25% and 25 to 50%, respectively (14). In this study population, over half of the participants had A1c values greater than 5.5%. This high prevalence also suggests that there may be high prediabetes prevalence in the general population of Durban. Hence, studies conducted outside clinical settings are warranted, especially because prediabetes is asymptomatic. Therefore, measures that prevent the progression of pre-diabetes to overt T2DM should be implemented. If nothing is done, the healthcare system should expect an exponential increase in the admission of persons with the fatal T2DM. A diabetes prevention program trial has shown that intensive

lifestyle interventions can reduce the risk of T2DM by 58% over three years (23). Therefore, preventative measures that mitigate the incidence of T2DM should be amplified in the population of the eThekwini metropolis.

The study was undertaken in a metropolitan population, which could have been one of the factors that contributed to the high pre-diabetes prevalence. Individuals in cities or urban areas will be at a greater risk of developing pre-diabetes than those in rural areas. The 2021 International Diabetes Federation (IDF) findings show that diabetes prevalence in urban areas is 12.1% and in rural areas 8.3% (10). In urban areas, people are more exposed and prompted to adapt to risk factors linked to pre-diabetes, such as consuming unhealthy high-caloric diets, sedentary lifestyles and being overweight or obese (24). The study population may consist primarily of professionals who live a sedentary lifestyle and consume fast foods because of work demands. This leads to excess calories, often leading to metabolic complications such as pre-diabetes. According to a study conducted on an urban population in India, the prevalence of diabetes in people engaged in light physical activity at work was three times higher than in people involved in heavy physical activity at work (25).

The relationship between age and the prevalence of pre-diabetes is directly proportional. This is supported by the apparent trend in Figure 2, showing how an increase in age results in an increase in pre-diabetes onset. This may be because people become less physically active as they age. In addition, this age group mainly consists of working professionals and most working environments promote sedentary lifestyles and the consumption of fast foods (26). The trend is consistent with other findings in the literature (12, 27, 28).

In this sample population, percentage-wise, more men (57.8%) develop pre-diabetes than women (51.0%). The finding in this study supports other literature outcomes that suggest men are more predisposed to diabetes (29). Anna Nordström *et al.* reported that compared to females, males store more visceral fat, making them more susceptible to T2DM onset (30). However, other studies found impaired glucose tolerance (IGT) more prevalent in women than men (31-33). Nevertheless, it is argued that higher IGT prevalence in women may be associated with lower height often observed in women and using a fixed glucose load in OGTT, regardless of body size (32). This suggests that pre-diabetes prevalence in men and women may depend on the diagnosis used to confirm pre-diabetes. Other literature indicates that the differences in prevalence may perhaps involve the effect of gonadal hormones. Nevertheless, the reason for these differences in pre-diabetes prevalence between men and women is yet to be fully elucidated.

The prevalence of pre-diabetes stratified by race results suggests that race plays a pivotal role in pre-diabetes development. The Indian race (63%) appears to be the most predisposed to pre-diabetes onset, followed by the African (53%) and the white race (28%). Genetic predisposition may be a plausible cause for the observed variations in prevalence across the different ethnic groups. Diabetes is reported

to be influenced by genetic determinants and heritability (34-36). Growing evidence implicates genetic alterations caused by epigenetic changes or SNPs as the cause of diabetes induction (34, 36-38). Therefore, research is needed to conduct genomic sequencing and investigate the association between genetics and pre-diabetes onset. This research can potentially improve diabetes management in the studied population by detecting novel genetic biomarkers that may be used to diagnose pre-diabetes early or identify a therapeutic target. This will assist clinicians in timeously recommending lifestyle interventions or treatments. In turn, this can attenuate the incidence of T2DM in the future.

In a study by Phillip Tuso, it was estimated that in four years, 37% of persons with pre-diabetes will progress to overt diabetes if unattended (39). The observed prevalence of pre-diabetes is alarming and suggests that the future incidence of T2DM will rise exponentially. In 2021, the IDF reported that 6.7 million people died from diabetes (10). The high prevalence observed in the study's clinical setting shows the importance of having preventative measures. Preventing the progression from pre-diabetes to overt diabetes is imperative to ease the burden in clinical settings. This may also improve population health in the eThekwini region of Durban. In addition, the results emphasise the necessity to promote pre-diabetes screening in hospital settings because persons with pre-diabetes linked comorbid will often experience poor health outcomes.

5. Limitations and future plans

One of the study's limitations is that we could not gather data on the common correlates associated with pre-diabetes, such as data about the participants' lifestyle habits, exercise, BMI and diets. Consequently, we could not determine the critical contributors to the onset of pre-diabetes. Therefore, a follow-up study is warranted to determine the leading causes of pre-diabetes onset. The HbA1c measures are not recommended in persons with acute and chronic blood loss, hemolytic anaemia, splenomegaly and renal diseases. These conditions are known to negatively impact haemoglobin glycation results. However, we incorporated the IFG results to validate pre-diabetes diagnosis. The study did not evaluate the prevalence of diabetic complications associated with pre-diabetes.

Further investigations are warranted to establish the association between pre-diabetes and the onset of macro/microvascular complications in the eThekwini population. The study showed a disproportion of pre-diabetes prevalence across the different ethnic groups in the targeted population. Therefore, as a future study, it will be essential to appraise the genetic inheritance that may be at play in the population. Also, the effect of hormonal changes in both men and women who develop pre-diabetes is yet to be fully understood and thus could be another future investigation.

6. Conflict of interest

None.

7. Author contributions

AMS and AK brainstormed, designed the study, and drafted the manuscript. AMS, NCM, AK and PSN were responsible for reviewing and approving the final draft of the manuscript

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Chapter 3 exhibited the present burden of pre-diabetes in South Africa from the sample population in Durban. This burgeoning prevalence of pre-diabetes in the targeted population warrants further research to appraise and understand endocrine changes that characterise pre-diabetes. Overt type 2 diabetes has been linked with multiple hormone imbalances, yet it remains underexplored in the pre-diabetic stage. Therefore, Chapter 4 includes an original research paper evaluating the occurrence of hormonal imbalances during the pre-diabetes phase and assessing its correlation with glycaemic control.

CHAPTER 5: RESEARCH ARTICLE 2

DETAILS OF THE NEXT MANUSCRIPT

The subsequent manuscript is titled "Hormone Imbalances Detected in Participants with Pre-Diabetes in a Durban-based Clinical Setting in South Africa" is authored by A.M. Sosibo, N.C Mzimela, P.S Ngubane, and A. Khathi. The manuscript is currently under peer review in the International Journal of Diabetes in Developing Countries and has been formatted according to the journal's guidelines for authors. This journal is accredited by the Department of Higher Education and Training South Africa (2024) and appears in the Scopus accredited list (2024)

Author Contribution: AM Sosibo was responsible for study conceptualization, study design, sample collection, carrying out experiments, data analysis, first draft writing, and manuscript editing.

Hormone Imbalances Detected in Participants with Pre-Diabetes in a Durban-based Clinical Setting in South Africa.

Abstract

Background: Type 2 diabetes mellitus onset is linked with hormonal imbalances. However, the knowledge about hormonal alterations in pre-diabetes is limited. The study aimed to examine type 2 diabetes mellitus-associated hormone levels during the pre-diabetes phase in participants aged 25-45 in a Durban-based clinical setting in South Africa.

Methods: Stored plasma samples from a retrospective study collected 364 samples divided into pre-diabetes and non-pre-diabetes groups. From the 364, 38 samples from persons without pre-diabetes and 38 from persons with glycated haemoglobin determined pre-diabetes were blindly selected. The hormone concentrations (C-peptide, cortisol, adipokines, thyroids, incretins, and sex steroids) of the study participants were measured using the BIO-RAD Bio-Plex MAGPIX instrument.

Results: Hormone imbalances in several hormones were detected in study participants with pre-diabetes. Most of the hormone dysregulation associated with T2DM begins in pre-diabetes but at a moderate level.

Conclusion: The findings reveal new possible hormone therapy targets for pre-diabetes and contribute to the growing support for targeting pre-diabetes as a preventative measure for T2DM prevention.

Keywords: Pre-diabetes; intermediate hyperglycaemia; glycated haemoglobin; incretins; adipokines; sex steroids; thyroid hormones.

1. Introduction

The African region has the highest proportion (73%) of deaths linked to diabetes in persons aged below 60 years (1, 2). The prevalence of Type II Diabetes Mellitus (T2DM) continues to rise, especially in developing countries (2, 3). This adverse metabolic disease is characterised by hyperglycaemia caused by insulin resistance, insufficient insulin production, or both. The elevated blood glucose levels seen in T2DM are linked with hormonal imbalances. Nevertheless, T2DM will often be preceded by pre-diabetes (4, 5). The pre-diabetic state is defined as an intermediate state of hyperglycemia, whereby the glucose concentration in the blood is higher than usual but below the T2DM threshold (6-9). Pre-diabetes is considered a viable target for preventative measures to attenuate the rising prevalence of T2DM (10). However, in hormonal imbalances, pre-diabetes remains underexplored when compared to T2DM.

In T2DM, studies have shown that hormonal differences between the sexes influence the prevalence of diabetes (11). The imbalance of sex hormones such as progesterone, estradiol and testosterone was linked with the development of T2DM (12-14). The stress hormone cortisol was reported to alter insulin sensitivity in fat and muscle cells, thus rendering them resistant (15-18). Incretins such as GLP-1 and GIP are another group of hormones that regulate glucose metabolism by promoting pancreatic beta-cells to secrete the hormone insulin. In T2DM, the incretin hormone levels are dysregulated, resulting in hyperglycaemia (19, 20). In addition, thyroid and adipokine hormone changes in the blood are associated with the development of T2DM. These investigations into hormonal imbalances in T2DM have assisted immensely in T2DM care and creating antidiabetic drugs like GLP-1 agonists and Tirzepatide. Whether or not the dysregulation of hormones observed in T2DM will reflect in pre-diabetes remains elusive.

Pre-diabetes shares similar metabolic characteristics to T2DM. However, the relationship between pre-diabetes and hormonal imbalances is not well studied. The outcomes have the potential to reveal specific hormone imbalances in pre-diabetes that can be considered therapeutic targets to improve pre-diabetes care and prevent T2DM onset. Therefore, the objective of the study was to appraise whether or not these hormone imbalances exist in individuals diagnosed with pre-diabetes aged between 25 and 45 in a Durban-based clinical setting.

2. Materials and Methods

Study population

Ethical approval was obtained from the Ethics Committee of the University of KwaZulu Natal (Study ref: BE266/19). The study was part of a retrospective study. The initial data and sample collection were conducted, from March 2022 to November 2022 at the King Edward Hospital located in an urban area of Durban, South Africa, a developing country. Three hundred and sixty-four (364) persons aged 25-45 participated, of which 76 samples were selected in a blind study where only their diabetes status was known. The 76 samples included 38 samples selected from the group without pre-diabetes, and the other 38 were selected from the group with pre-diabetes.

The inclusion and exclusion criteria for the study are described as follows: The included samples had to be from participants of any ethnicity who fell within the age group of 25 – 45 years. The 25-45 age limit was chosen to eliminate the influence of the ageing factor on hormone concentration changes (21). The American Diabetes Association (ADA) criteria were used to diagnose pre-diabetes by measuring the glycated haemoglobin (HbA1c). The ADA criterion confirms pre-diabetes with an HbA1c value between 5.7-6.4%. Therefore, for eligibility, the patients had to be within the age range of 25-45 have an HbA1c of less than 6.5%. Haemoglobin variants, kidney failure, anaemia, pregnancy, and medications such as opioids are known to affect the reliabilities of HbA1c readings (1). To circumnavigate these conditions, study participants with hypertension, anaemia, kidney disease, liver disease, a previous history of diabetes and who were pregnant were excluded. Furthermore, the impaired fasting glucose results were also used to validate HbA1c outcomes.

Clinical data collection and biochemical analysis

All participants were asked to fast overnight and arrive in the morning for blood collection. Therefore, the blood samples of all participants were drawn in the morning. In turn, the diurnal variation in any hormonal concentrations was minimised. Fasting blood glucose was measured by study participants who fasted overnight. Before the whole blood was separated, the HbA1c was measured using a clinically validated NGSP and IFCC-certified, laboratory-based Tosoh G8 HPLC Analyzer (22). The analyser is an ion-exchange HPLC instrument for separating and quantifying HbA1c in whole blood. The whole blood was centrifuged (Eppendorf centrifuge 5403, Germany) to collect plasma that was stored in a -80^{o}C freezer (Snijders Scientific, Holland). The Luminex MAGPIX System was utilised to measure the hormone concentrations in plasma samples using Multiplex immunoassays. The manufacturer's instructions were followed for each immunoassay kit (MilliPlex, Merck, USA and Procarta Plex, ThermoFisher, USA).

Statistical Analysis

A normality test assessed whether or not the data was parametric or non-parametric. Parametric data were presented as Mean ± 95% Confidence Interval (CI) and Median + Interquartile range for non-parametric data or in proportion (percentage) where appropriate. To compare the two groups, the Independent Student's t-test or Mann-Whitney U test was utilised for parametric and non-parametric data to detect the difference between them. Spearman correlation analysis was conducted to determine hormones that are associated with HbA1c. Given that gender is a possible confounder for hormone concentrations, the data was therefore stratified by gender in Table 3.

3. Results

Various T2DM-linked hormonal imbalances in persons with pre-diabetes compared to persons without were investigated. This was achieved by conducting multiplex analyses containing the hormones of interest. The study outcomes showed that specific hormone concentrations are dysregulated significantly in the Pre-Diabetes (PD) group compared to the Non-Diabetes (ND) group in the pre-diabetes condition.

The characterisation of study participants is recorded in Table 1. The multiplexed assay results for hormone concentrations are recorded in the Figure 1 layout. The adipokines (adiponectin and adipsin), thyroid (T3 and T4), GIP, C-peptide, sex steroids (testosterone, estradiol and progesterone), and cortisol hormones were elevated in the pre-diabetes (PD) group when compared to the non-prediabetes (NPD) group ($p < 0.05$). In Table 2, the Spearman correlation analysis indicates that c-peptide, cortisol, progesterone, estradiol, T3, T4, and adiponectin are correlated with the HbA1c significantly.

Table 1. Characteristics of participants in the NPD and PD groups involved in analysing hormonal changes[10]

	Non-Pre-Diabetes (NPD) 38	*Pre-diabetes (PD) 38*
Age; mean (95% CI)	37,45 (35,47- 39,42)	37,82 (35,77 - 39,86)
Race		
African	25 (66%)	24 (63%)
White	6 (16%)	2 (5%)
Indian	7 (18%)	10 (26%)
Coloured	0 (0%)	2 (5%)
Gender		
Male	13	18
Female	25	20
IFG, median (IQR), mmol/l	5,7 (0,7)	6,5 (0,5)
HbA1c, mean % (95% CI)	5.3% (5,2-5,4)	6.1% (6,0 - 6,2)
Insulin, median (IQR) pg/ml	12232 (11341 - 13123)	12413 (11789 - 13037)

[10] The data are presented as mean (95% CI) or median (IQR) depending on the normality and proportion.

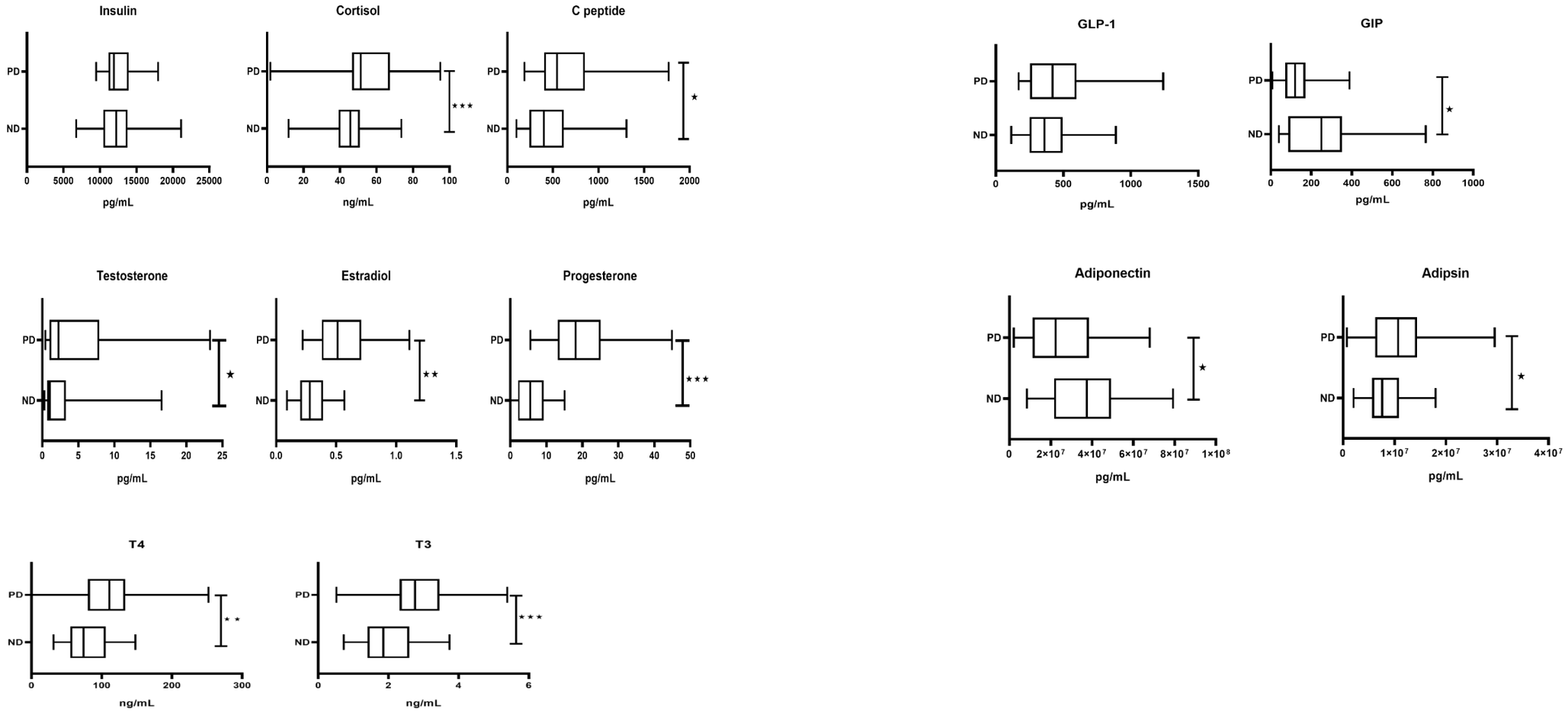

Figure 1. *Hormone concentration changes observed between the PD and ND groups.*[11]

[11] Values are presented as mean ± 95% CI for parametric data or median ± IQR for non-parametric. ★ (ND vs PD, $P < 0.05$); ★★ (ND vs PD, $P < 0.01$); ★★★ (ND vs PD, $P < 0.001$)

Table 2. Hormones that are correlated with changes in HbA1c [12]

Correlation	HbA1c, Spearman r value	95% CI	P value
HbA1c	1	1,000 to 1,000	0,000
Male	-0,141	-0,37 - 0,07	0,22
C-peptide	0,343	0,02 - 0,49	0,006 **
Cortisol	0,314	-0,08 - 0,43	0,018 *
estradiol	0,501	-0,03 - 0,42	< 0,001 ***
Progesterone	0,626	0,37- 0,70	< 0,001 ***
Testosterone	0,216	-0,09 - 0,36	0,064
T3	0,384	0,12 - 0,53	< 0,001 ***
T4	0,355	0,10 - 0,52	0,002 **
Adiponectin	-0,314	-0,53 - -0,12	0,006 **
Adipsin	0,195	0,02 - 0,45	0,099
GIP	-0,237	-0,50 - 0,05	0,054
GLP-1	0,119	-0,10 - 0,38	0,341

[12] The non-parametric data is presented as Spearman r and 95% CI. A p-value < 0,05 is considered statistically significant. * represents a p-value < 0,05; ** represents a p-value < 0,01; p-value < 0,001.

Table 3. Hormone changes stratified by gender.[13]

Hormone (units)	NPD	PD	P value
GIP (pg/mL)			
Male, median (IQR)	125,0 (80,3 - 296,2)	123,8 (100,1-290,8)	*P* = 0,7438
Female, mean (95% CI)	355,7 (235,7-475,7)	126,6 (78,97-174,2)	***P* = 0,0055**
C-peptide (pg/mL)			
Male, mean (95% CI)	675,8 (208,1-1144)	502,0 (372,7-631,3)	*P* = 0,3742
Female, mean (95% CI)	476,7 (339,6-613,8)	886,3 (623,8-1149)	***P* = 0,0051**
Insulin (pg/mL)			
Male	12801 (10492-15110)	12451 (11436-13466)	*P* = 0,7406
Female	11969 (11081-12858)	12382 (11525-13239)	*P* = 0,4997
GLP-1 (pg/mL)			
Male	415,6 (265,9-565,4)	469,0 (279,8-658,1)	*P* = 0,6559
Female	369,1 (287,1-470,0)	466,4 (310,9-635,4)	*P* = 0,1892
Adiponectin (ng/mL)			
Male, mean (95% CI)	32835,6 (22953-42718,2)	26818,3 (18318-35318,5)	*P* = 0,3316
Female, mean (95% CI)	38926,4 (30462,3-47390,4)	26224,5 (18018,4-34430,6)	***P* = 0,0324**
Adipsin (ng/mL)			
Male, mean (95% CI)	7272,7 (6014,5-8530,9)	17284,5 (10925,3-23643,7)	***P* = 0,0183**
Female, mean (95% CI)	9266,8 (7206,7-11326,9)	11602,1 (7860,3-15344,8)	*P* = 0,2370
Cortisol (ng/mL)			
Male, mean (95% CI)	50,4 (44-56,9)	61,9 (53,8-70,1)	***P* = 0,0455**
Female, median (IQR)	42,9 (37,3- 47,9)	49,6 (41,4- 63,7)	***P* = 0,0232**
Estradiol (ng/mL)			
Male, median (IQR)	0,39 (0,25-0,44)	0,570(0,42-0,83)	***P* = 0,0007**
Female, mean (95% CI)	0,27 (0,2-0,3)	0,5(0,4-0,6)	***P* = 0,0001**
Progesterone (ng/mL)			
Male	7,6 (4,5-10,7)	18,9 (13,9-23,9)	**P = 0,0030**
Female	5,5 (3,5-7,5)	20,6 (16,2-24,9)	**P = <0,0001**
Testosterone (ng/mL)			
Male, mean (95% CI)	7,8 (4,62-10,96)	8,6 (6,2-11,1)	P = 0,6518
Female, mean (95% CI)	0,9 (0,7-1,1)	1,2(0,9-1,5)	**P = 0,0344**
T3 (ng/mL)			
Male, mean (95% CI)	1,8 (1,4-2,2)	2,7 (2,3-3,1)	**P = 0,0026**
Female, median (IQR)	2,1 (1,5- 2,7)	2,8 (2,5- 3,5)	**P = 0,0036**
T4 (ng/mL)			
Male	70 (47,2-92,7)	106,4 (87,9-124,9)	**P = 0,0133**
Female	86,9 (75,5-98,4)	120,9 (92,9-149,0)	**P = 0,0116**

[13] Parametric and non-parametric data are presented as mean ± 95% CI and median ± IQR. A $P < 0.05$ is regarded as statistically significant.

4. Discussion

Overt T2DM is a complex metabolic condition involving various endocrine hormone interactions. Since hormones tend to fluctuate with ageing, the findings for this study are adjusted for ageing by only including participants between 25-45 years of age. It is unclear if the hormone dysregulations observed in T2DM will be seen in individuals with pre-diabetes between 25 – 45 years. Hence, this study highlights the various hormones linked with T2DM in persons with pre-diabetes.

C-peptide is a parameter for insulin sensitivity and insulin production. Therefore, in Figure 1, it is unsurprising that high c-peptide levels were detected in the pre-diabetes participants. Another study showed increased concentrations of C-peptide detected in insulin-resistant individuals recently diagnosed with T2DM, which supports the study's C-peptide outcome in subjects with pre-diabetes (23). In Table 2, c-peptide positively correlates with HbA1c, suggesting that c-peptide contributes to glycemic control. We speculate that C-peptides remain elevated chronically during pre-diabetes to compensate for insulin resistance. If insulin resistance is unattended during pre-diabetes, then the C-peptide levels will continue to rise and reach the levels observed in T2DM. Interestingly, in Table 1, the hormone insulin levels are higher but not statistically significant in pre-diabetes participants. This may indicate that the level of insulin resistance is still moderate in persons with pre-diabetes.

The incretin, glucose-dependent insulinotropic polypeptide (GIP) levels shown in Figure 1 are significantly lower in the pre-diabetes group compared to the non-pre-diabetes group. The GIP hormone is critical in maintaining the glucose homeostatic range by stimulating beta-cell proliferation and insulin secretion (24). Comparable results have been observed in studies using participants with T2DM, where the effectiveness of GIP is almost completely lost (25, 26). A meta-analysis study on individuals with T2DM showed that higher glycated haemoglobin (HbA1c) concentrations and ageing were linked with reduced GIP responses (27). However, the results of this study reveal that the decrease in GIP levels begins at the pre-diabetic stage. We speculate that sustained low levels of GIP may be necessary to trigger the onset of hyperglycaemia and, in turn, T2DM. During the pre-diabetes phase, the low levels of GIP may be compensated by another incretin hormone known as GLP-1 to keep glucose levels below the T2DM diagnosis threshold.

The concentrations of GLP-1 in Figure 1 remained relatively the same between the two groups, as no statistical significance was observed. A prospective study by Hoffmann *et al.* (2023) also detected no changes in GLP-1 levels at the pre-diabetes state, but a decline in GLP-1 concentrations was seen in patients progressing to diabetes (28). Therefore, it is plausible that the imbalance of GLP-1 may be a triggering point for the development of T2DM. Therefore, a longitudinal study is warranted to investigate the role of both GIP and GLP-1 in the progression from pre-diabetes to T2DM. A clear understanding of their roles in developing pre-diabetes and its passage to T2DM can play a pivotal role in introducing new therapeutic medications for pre-diabetes patients.

The cortisol concentration in Figure 1 is significantly higher in individuals with pre-diabetes when compared to those in the non-pre-diabetes group. In Table 2, a positive correlation between cortisol and HbA1c was determined. Elevated cortisol levels have been shown to promote disturbance in glucose homeostasis by inducing visceral fat accumulation, reducing insulin sensitivity in skeletal muscles, and causing lipolysis. The reduction in insulin sensitivity may be exacerbated by the significantly lower levels of the hormone adiponectin. Studies have linked the decrease of adiponectin with insulin resistance and T2DM (29, 30). Moreover, dysregulation of adiponectin has been observed in non-obese individuals, indicating its potential utility in studies where BMI data is unavailable. (31). Therefore, adiponectin may be one of the critical causes and markers of interest that promote glycaemic dysregulation, resulting in pre-diabetes and T2DM if left unattended. However, the adverse complications of high cortisol and low adiponectin levels seen in pre-diabetes may be attenuated by the significantly elevated adipsin levels. The hormone adipsin is responsible for sustaining adipose tissue homeostasis and boosts insulin secretion in response to glucose (32). Hence, adipsin is associated with protection from T2DM and may be one of the key compensatory mechanisms that delay pre-diabetes progression to overt diabetes. However, a longitudinal study that will adjust for the confounding variable, body weight, and trace adipsin levels from pre-diabetes to T2DM is necessary to have a conclusive statement.

The role of sex hormones in T2DM is well-documented (33-35). Their role in pre-diabetes remains limited. The risk of developing diabetes is more significant in men with low testosterone (36). In females, however, high testosterone concentrations were associated with an elevated risk of diabetes onset (37). In Table 2, this study observed no significant changes in testosterone levels in the male groups. This suggests that in males aged 25-45 with pre-diabetes, the testosterone levels are not yet dysregulated and may decrease upon the onset of overt diabetes. This also suggests that the leading cause of testosterone drop observed in other studies may be ageing since testosterone levels are known to decrease with age (38). In females, Table 2 results show that testosterone was higher in participants with pre-diabetes, which supports other literature findings that indicate a higher risk of diabetes in females with high testosterone levels. The other sex hormone investigated is estradiol. Estradiol has a protective impact on the insulin β cells. Compared to the control group, females with pre-diabetes have higher estradiol levels. This may explain why the proportion of females with pre-diabetes is less when compared to males. There is literature evidence showing an increased incidence of diabetes in females who have reached menopause. Estradiol deficiency occurs after menopause, increasing the risk of T2DM onset (39). Therefore, maintaining the homeostatic range of estradiol may be part of the compensatory mechanisms responsible for preventing T2DM and opens the door for designing new therapeutic targets (40).

Triiodothyronine (T3) and tetraiodothyronine (T4) levels were significantly elevated in the pre-diabetes group and correlated with HbA1c levels, indicating a proportional relationship between thyroid

hormones and pre-diabetes onset. In literature, both hypothyroidism and hyperthyroidism have been shown to be associated with T2DM (41). Hyperthyroidism has been reported to worsen glycaemic control in patients with T2DM. Therefore, it can be one of the essential hormones responsible for the progression from pre-diabetes to T2DM over time (42). Females are more susceptible to developing thyroid dysfunction than males (43). The study observed the same trend for women with pre-diabetes in Table 2. However, the understanding of the association remains unclear and necessitates further investigation into the pre-diabetes condition. An intervention study targeting hyperthyroidism in women can be done on participants with diabetes and pre-diabetes to provide empirical evidence that will improve individualised care and management of diabetes.

The findings, although not yet conclusive, raise a question about possible alternative treatments to metformin, such as hormone replacement medications and GIP/GLP-1 agonists, which could be administered in persons with pre-diabetes. For example, a study by Iskra Bitoska *et al.* (2016) showed that hormone replacement therapy improves insulin sensitivity in women with T2DM (44). A dual GIP/GLP-1 receptor co-agonist, Tirzepatide, has been sanctioned for managing T2DM. It is a modified acylated peptide that stimulates GIP and GLP-1 receptors (45). These receptors play pivotal roles in insulin secretion and are also present in areas of the brain responsible for regulating food intake. These interventions may prove beneficial in the management of pre-diabetes; thus, preclinical trials are warranted.

5. Conclusion and limitations of the study

The irregular hormonal changes of adipsin, thyroid, GIP, C-peptide, sex steroids, and cortisol hormones detected in pre-diabetes contribute to the growing body of research suggesting that pre-diabetes is an unfavourable metabolic condition. The study could not determine conclusively the causes of these imbalances due to the retrospective nature of the study as it lacked some confounding variable data, such as the subjects' weight. Adiponectin, C-peptide, progesterone, estradiol, T3, T4, and cortisol hormones exhibit correlations with HbA1c levels, indicating that persistent dysregulation of these hormones may disrupt glucose homeostasis and predispose individuals to the development of overt diabetes if left unaddressed. Hence, our study supports Bailey (2023) in that intervening for pre-diabetes is never too early (10). Subsequently, it opens a window for clinical trials investigating pharmacological interventions that would restore hormonal homeostasis, which may attenuate intermediate hyperglycaemia. However, prior to the intervention trials, future studies evaluating the mechanism behind the observed impairment in the hormonal control of adipokines, sex, and thyroid hormones are needed. In addition, a longitudinal study that tracks these hormone changes from the pre-diabetes phase to diabetes is necessary. Although surrogate markers for obesity were reported, the unavailability of data for other possible confounders was a limitation in the study, as it did not allow adjustment for

confounding variables like BMI (Body Mass Index), diet, and physical activity. A future study with a larger sample size is recommended to generate results that can be generalised for the entire country and, in turn, validate our suggestion to consider incorporating these hormones in the screening processes to improve pre-diabetes care.

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7. Statements and Declarations

Ethical approval

The study was approved by the Ethics Committee of the University of KwaZulu Natal (Study ref: BE266/19) and was performed in line with the principles of the Declaration of Helsinki.

Informed consent

Informed consent was obtained from all individual study participants.

8. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

9. Funding sources

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10. Author contributions

AMS and AK conceptualised the research, designed the study, and drafted the manuscript. AMS, NCM, AK and PSN were responsible for reviewing and approving the final draft of the manuscript.

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Chapter 4 demonstrated multiple hormonal concentration changes during pre-diabetes and how they are associated with the glycaemic control marker, HbA1c. However, the onset of T2DM is also linked to genetic predisposition. Research findings have established microRNAs as a crucial player in the development of overt T2DM. Yet, there is limited research conducted on persons diagnosed with pre-diabetes and on the South African population. Hence, Chapter 5 consists of an original research manuscript that investigated the association between miRNAs (miRNA-34a-5p, miRNA-145-5p, and let-7f-5p) and pre-diabetes and evaluated the diagnostic ability of the miRNA.

CHAPTER 6: RESEARCH ARTICLE 3

DETAILS OF THE NEXT MANUSCRIPT

The following manuscript is titled "MicroRNA-34a-5p Correlated with Pre-Diabetes Onset: Implications for Early Detection and Therapeutic Targets" is authored by A.M. Sosibo, N.C Mzimela, P.S Ngubane, and A. Khathi. The manuscript is under review in the International Journal of Molecular Sciences, a peer-reviewed journal.

Author Contribution: AM Sosibo was responsible for study conceptualization, study design, sample collection, carrying out experiments, data analysis, first draft writing, and manuscript editing.

MicroRNA-34a-5p Correlated with Pre-Diabetes Onset: A Pilot Study Indicating Implications for Early Detection and Therapeutic Targets

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Abstract

Background: MicroRNAs are linked to Type 2 Diabetes Mellitus (T2DM) pathogenesis. However, their role in pre-diabetes remains underexplored. Therefore, miRNA-34a-5p, miRNA-145-5p, and let-7f-5p expression in pre-diabetes were examined to understand their possible association with the onset of pre-diabetes and determine their diagnostic ability.

Methods: The study included 48 plasma samples, equally distributed into two groups of non-pre-diabetes (24) and pre-diabetes (24), which were obtained from a retrospective study. MicroRNA isolation from plasma samples was conducted, followed by cDNA synthesis, which was used to measure the relative amplification of the miRNA genes. Spearman's r was used to assess the correlation between miRNAs and glycated haemoglobin, while the receiver operating characteristic curve evaluated the diagnostic ability of the gene.

Results/Discussion: The relative expression of miRNA-34a-5p was upregulated (7,25 fold increase) significantly in the pre-diabetes group compared to the non-pre-diabetes. The miRNA also displaced a strong positive correlation with glycaemic control. An area under the receiver operating characteristic curve of 0,806 was reported for miRNA-34a-5p.

Conclusion: In the population representing Durban, this novel exploration of miRNA-34a-5p showed promising results as a potential biomarker that can be employed for pre-diabetes diagnosis and targeted therapeutically. The mechanics behind the observed association of miRNA-34a-5p and pre-diabetes onset require further research.

Keywords: miRNA-34a-5p; intermediate hyperglycaemia; epigenetics; non-coding RNA

1. Introduction

Pre-diabetes presents a substantial and escalating global health challenge (1-4). Recent epidemiological evidence indicates its prevalence will rise from 464 million in 2021 to 638 million in 2045 (3). The pre-diabetic state is characterised by intermediate hyperglycaemia, where glucose levels surpass normal but fall below the diabetic threshold (5, 6). Depending on the criterion utilised, pre-diabetes can be diagnosed by Impaired Fasting Glucose (IFG) levels between the concentration range of 5.7-6.9 mmol/L, Impaired Glucose Tolerance (IGT) confirmed by glucose concentrations between 7.8 and 11 mmol/L after an oral glucose tolerance test, or 5.7% - 6.4% glycated haemoglobin levels (HbA1c) (3). In most cases, the latter diagnosis is considered superior and more practical due to the ability to provide an average blood glucose value over the past 2-3 months (7). The observed increase in glucose concentrations is often linked to moderate insulin resistance or the moderate functional failure of pancreatic beta-cells (8). If left untreated, pre-diabetes often progresses to overt type 2 diabetes mellitus (T2DM), which has a more significant impact on healthcare (9). Moreover, the onset of T2DM is complex, involving multiple factors. For example, gene expression alterations involving microRNAs (miRNA) have been linked to the pathogenesis of overt diabetes (10, 11). A study conducted by Ghai et al. identified 57 miRNAs linked to the onset of type 2 diabetes mellitus (T2DM) (10). In another study, miRNA-143 was recognised as a key regulator of T2DM because it disrupted the insulin-AKT signalling pathway, leading to insulin resistance and hyperglycemia (12). Consequently, there is an increasing interest towards microRNAs (miRNAs), emerging epigenetic regulators that hold promise as early biomarkers for metabolic complications (13-15).

MicroRNAs (miRNAs) are encoded within the human genome, where their biogenesis occurs through transcription into primary miRNAs (pri-miRNA) by the enzyme RNA polymerase II. In turn, pri-miRNA becomes cleaved by the DROSHA-DGCR8 complex, resulting in the formation of pre-miRNA (16, 17). The pre-miRNAs are transported from the nucleus to the cytoplasm via a process facilitated by exportin 5. Once in the cytoplasm, the stem-loop structure of the pre-miRNA is cleaved by the RNase III-like enzyme DICER, generating a double-stranded miRNA that is loaded onto the RNA-induced silencing complex (RISC) through its association with an Argonaute family protein (18). Within the RISC complex, one strand of the double-stranded miRNA, known as the guide strand, is retained, while the other strand, known as the passenger strand, is degraded, leading to the formation of the mature RISC complex (18). The guide strand then guides the RISC complex to its target messenger RNA (mRNA), where it mediates post-transcriptional gene silencing by inhibiting mRNA translation or promoting mRNA degradation (16, 18).

Consequently, these mature, single-stranded microRNA guide strands can negatively modulate gene expression at the post-transcriptional level through two distinct mechanisms: repressing translation of the target mRNA or inducing degradation of the target mRNA transcripts (18, 19). These miRNAs are

capable of being either stimulants or suppressants of various pathological states (18, 20). Furthermore, their stability in blood circulation has been established, and as a result, they are increasingly recognised as promising therapeutic targets and diagnostic markers for various diseases (21, 22). Notably, mounting literature evidence links miRNAs to the onset of T2DM (14, 15, 23, 24). For example, the deregulation of let-7f-5p miRNA has been implicated in the development of T2DM (25). The study showed that the downregulation of let-7f-5p correlated with the development of overt diabetes and metabolic syndrome, suggesting it could play a considerable role in the initial disturbance of glucose homeostasis (25, 26). Additionally, the miRNA-145p-5p has been reported to exhibit ameliorative effects on high glucose-induced oxidative stress and is proposed to be a biomarker for patients with diabetes (27, 28). The impaired insulin signalling pathway and secretion are implicated with abnormal expression of miRNA-143 and miRNA-34a-5p in persons with T2DM (12, 13, 29).

However, research exploring miRNAs in pre-diabetes is minimal when compared to T2DM. Therefore, whether the miRNA changes observed in T2DM will be detected in pre-diabetic states remains unknown. In addition, miRNA expression can be influenced by ethnicity, social, environmental, and lifestyle factors, as well as the underlying genetic risk for a specific disease (30, 31). Subsequently, there is a concern about the lack of genomic data representing the African population, which leaves Africans behind in the potential of future genetic therapy, especially in precision medicine.

To the best of our knowledge, this study is the first to evaluate the expression of the three candidate miRNAs (miRNA-34a-5p, miRNA-145-5p, and let-7f-5p) and their potential association with pre-diabetes induction in the diverse population of Durban, South Africa. The outcomes have the potential to provide insight into the role of miRNA in the pathophysiology of pre-diabetes. Since miRNAs are quantifiable in circulating serum and plasma, they serve as potential biomarkers to determine pre-diabetes risk (32).

2. Materials and Methods

Prior to the study being conducted, ethical approval was obtained from the ethics committee of the University of KwaZulu Natal (Study ref: BE266/19).

Eligibility criteria

The participants were selected based on the following inclusion criteria: ages between 25–45, no previous diagnosis of diabetes, glycated haemoglobin less than 5.7%, fasting glucose less than 5.7 mmol/L, and no hypertension. Exclusion criteria comprised individuals with a prior diagnosis of diabetes mellitus undergoing treatment, persons who did not fast overnight, as well as those with a history of liver, kidney, or heart disease, depression, or pregnancy during the study period. Then, the tubes of the eligible samples

were centrifuged (Eppendorf centrifuge 5403, Germany) to collect serum that was stored in a -80 degrees Celsius freezer (Snijders Scientific, Holland).

Data collection and biochemical analysis

The data and sample collection for the study were conducted from March 2022 to November 2022, with 364 participants involved in a previous retrospective study (33). A total of 48 samples were blindly selected from the retrospective study and equally distributed to persons with pre-diabetes (n=24) and without pre-diabetes (n=24). The American Diabetes Association (ADA) criterion was used to diagnose pre-diabetes (34). A professional nurse measured fasting plasma glucose using a blood glucose meter. The HbA1c was measured using a validated NGSP and IFCC-certified, laboratory-based Tosoh G8 HPLC Analyse (35).

Molecular analysis

Three steps are necessary to perform the molecular investigation: miRNA extraction, cDNA synthesis, and miRNA amplification. For the extraction step, the automated Maxwell RSC instrument was utilised to extract and purify microRNA from plasma samples using the Maxwell® RSC miRNA Plasma and Serum kit (Promega, USA) (36). This step involved the following steps: 200 µl of plasma samples was used from the stock sample. Then, 80 µl of Proteinase K and 230 µl of Lysis Buffer C were added to the sample, followed by vortexing for 5 seconds. The mixture was then incubated at 37°C for 15 minutes. After incubation, the lysate is transferred to well number one of the cartridge. Additionally, 10 µl of blue DNase I Solution is added to well number four, causing the reagent in well number four to turn green. Finally, the deck tray was loaded onto the instrument, and the automated purification run was initiated and completed.

The TaqMan Advanced miRNA cDNA Synthesis kit was used per manufacturer guidelines to achieve the second step of cDNA synthesis (Applied Biosystems; Thermofisher Scientific, USA). The pure and mature isolated miRNA was modified by adding a poly(A) tail (3') and an adaptor (5'). Then, to generate cDNA, a universal reverse transcription (RT) primer bound to the 3' poly(A) tail, and the miRNA was reverse transcribed. After that, the cDNA had to undergo a miRNA amplification reaction to generate the amplified microRNA reaction product. The amplification step was conducted to increase the number of cDNA molecules before qPCR analysis.

The amplified cDNA template (5 µL) was combined with a total PCR reaction mix (15 µL) consisting of the TaqMan fast advanced master mix, TaqMan advanced miRNA assay (hsa-miRNA-34a-5p, hsa-let-7f-5p and hsa-miRNA-145-5p) and RNase-free water. The analysis of miRNA profiles by qRT-PCR was achieved through the cDNA product and TaqMan Advanced miRNA assays. The QuantStudio 5 system was used to perform the PCR run, which generated Ct values. The resulting Ct values were assessed using

the ΔΔCt method. The endogenous miRNA-361-5p was used as a reference gene to normalise data as ΔCt in order to obtain the relative expression. The final expression results are presented as the $2^{-\Delta\Delta CT}$.

Statistical Analysis

Parametric data were presented as Mean ± 95% Confidence Interval (CI) and Median + Interquartile range for non-parametric data. To compare the two groups, the Independent Student's t-test or Mann-Whitney U test was utilised for parametric and non-parametric data to detect the difference between them. Categorical variables were expressed as numbers (percentages). The Spearman r correlation test was employed to measure the association between the miRNA expression and % HbA1c. A Spearman rank correlation characterises the monotonic association between two variables. A value of +1 means a perfect association of rank, and A value of -1 means a perfect negative association of rank. In addition, a multivariable regression was conducted to understand the relationship between a miRNA-34a-5p expression as the dependent variable and gender, age and HbA1c as independent variables.

The Receiver Operating Characteristic Curve (ROC Curve) analysis was used to measure the diagnostic ability of the miRNA. From the generated curve, an Area Under the Curve (AUC) was calculated, which measures the overall performance of the diagnostic test. The AUC ranges from 0 to 1, where a value closer to 1 indicates better discrimination between true positive and false positive cases. An AUC of 0.5 suggests random chance, while an AUC of 1 indicates perfect discrimination. A p-value < 0.05 will be considered statistically significant.

3. Results

The study received a total of 48 blood samples from enrolled participants, with females constituting 54% of the samples. Participants were stratified into non-pre-diabetes (NPD) and pre-diabetes (PD) groups based on the American Diabetes Association (ADA) criterion for HbA1c levels. This study comprised individuals from three distinct ethnic groups. In the NPD group, there were 20 African (or Black) individuals, two of Indian ethnicity and two of White ethnicity. Conversely, the PD group consisted of 18 African individuals, five of Indian ethnicity and one of White ethnicity. The study specifically targeted individuals aged 25 to 45 years.

The relative expressions of four miRNAs (miRNA-34a-5p, miRNA-193b-5p, miRNA-145-5p, and miRNA-7f) were assessed in both the NPD and PD groups. Notably, only miRNA-34a-5p demonstrated significant results with a relative expression fold increase of 7.25 in the PD group compared to 0.90 in the NPD group (p-value = 0.0049), as illustrated in Figure 1. The role of gender in expression levels on the investigated miRNAs was limited, as shown in Figure 2. A statistically significant difference was observed only in the expression of miRNA-34a-5p in males. Furthermore, Table 2 displays the Spearman

correlation analysis results, revealing a notable association between miRNA-34a-5p expression and HbA1c levels (p = 0.0022). Multivariate regression analysis in Table 3 indicates that race and gender are not independent influencers of the expression of miRNA-34a-5p. Figure 3 shows that the ROC curve analysis presented a significant AUC for miRNA-34a-5p of 0.806 (0.7003 – 0.9123).

Table 4. This presents a general characterisation of the study participants who were grouped into Non-Pre-diabetes (NPD) and Pre-diabetes (PD), respectively.

The total sample size is 48, with each group comprising a sample size of 24. The student t-test (Mann-Whitney test) and Chi-square were conducted to measure the statistical difference of the continuous and categorical data between the two groups. ***** (NPD vs PD, P < 0.01).

	Non-Pre-diabetes (NPD)	**Pre-diabetes (PD)**	**P value**
Sample size (n)	24	24	
Age median (IQR)	37.0 (4)	40.5 (5)	0.0430*
Gender			0.247
Male (%)	9 (38)	13 (54)	
Female (%)	15 (62)	11 (46)	
Ethnicity			0,422
African	20	18	
Indian	2	5	
White	2	1	
IFG median (95% CI)	6.2 (5.9 – 6.3)	7.0 (6.7 – 7.1)	<0.0001*
%HbA1c median (95% CI)	5.5 (5.3 – 5.6)	6.1 (5.8 – 6.2)	<0.0001*

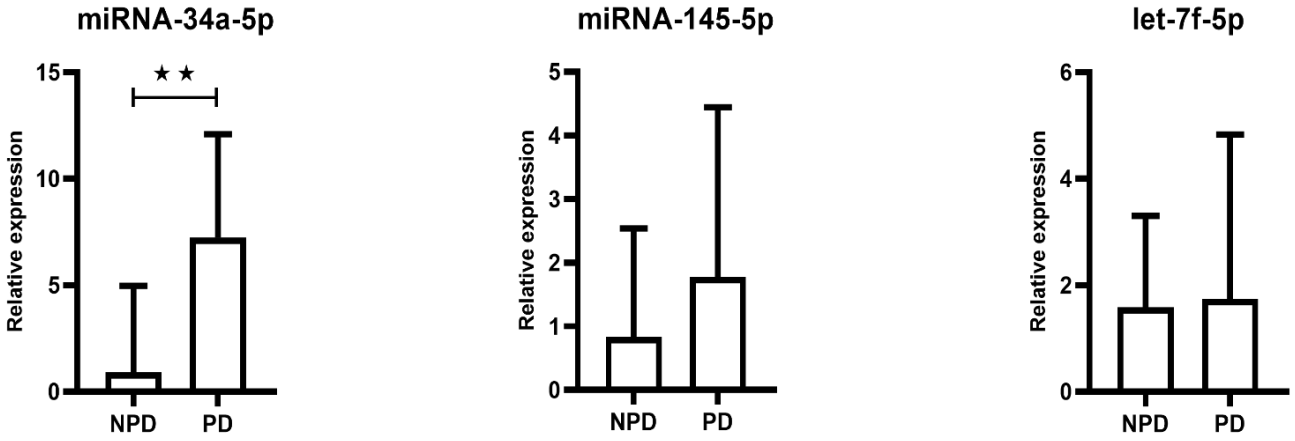

Figure 5. Shows the relative expression of circulatory microRNAs in plasma in the NPD vs PD groups, respectively.

Both the NPD and PD groups consisted of a sample size of 24. The endogenous miRNA-361-5p was employed as the reference gene for normalisation, and the relative fold expression for each group was calculated using the $2^{-\Delta\Delta CT}$ method. The Mann–Whitney U test was used to evaluate differences in relative expressions between the NPD and PD study groups. ★ ★ (NPD vs PD, $P < 0.01$).

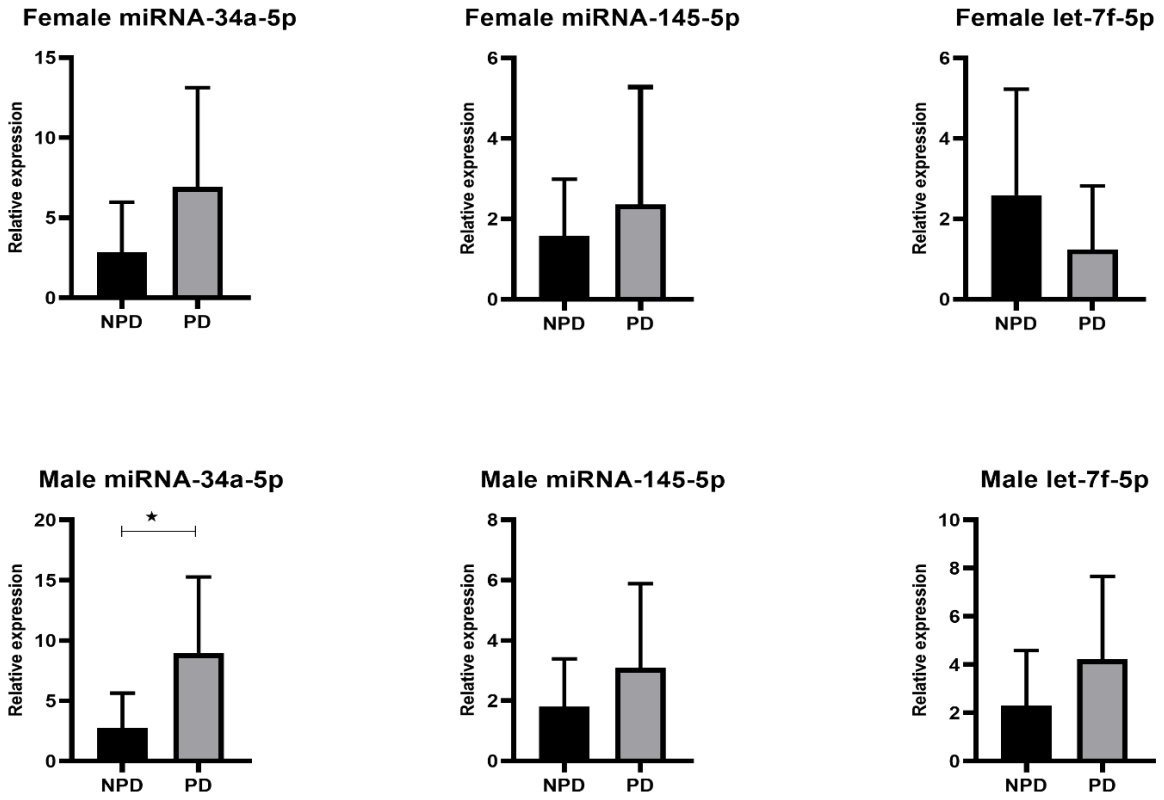

Figure 6. Displays the gender-stratified miRNA relative expression in the NPD vs PD groups.

The NPD group had 9 males and 15 females represented in its total sample size of 24. The PD group had 13 males and 11 females represented in its total sample size of 24. The student's t-test or Mann-Whitney U test was adopted to measure the differences between the groups depending on whether the data was parametric or non-parametric. ★ (NPD vs PD, $P < 0.05$).

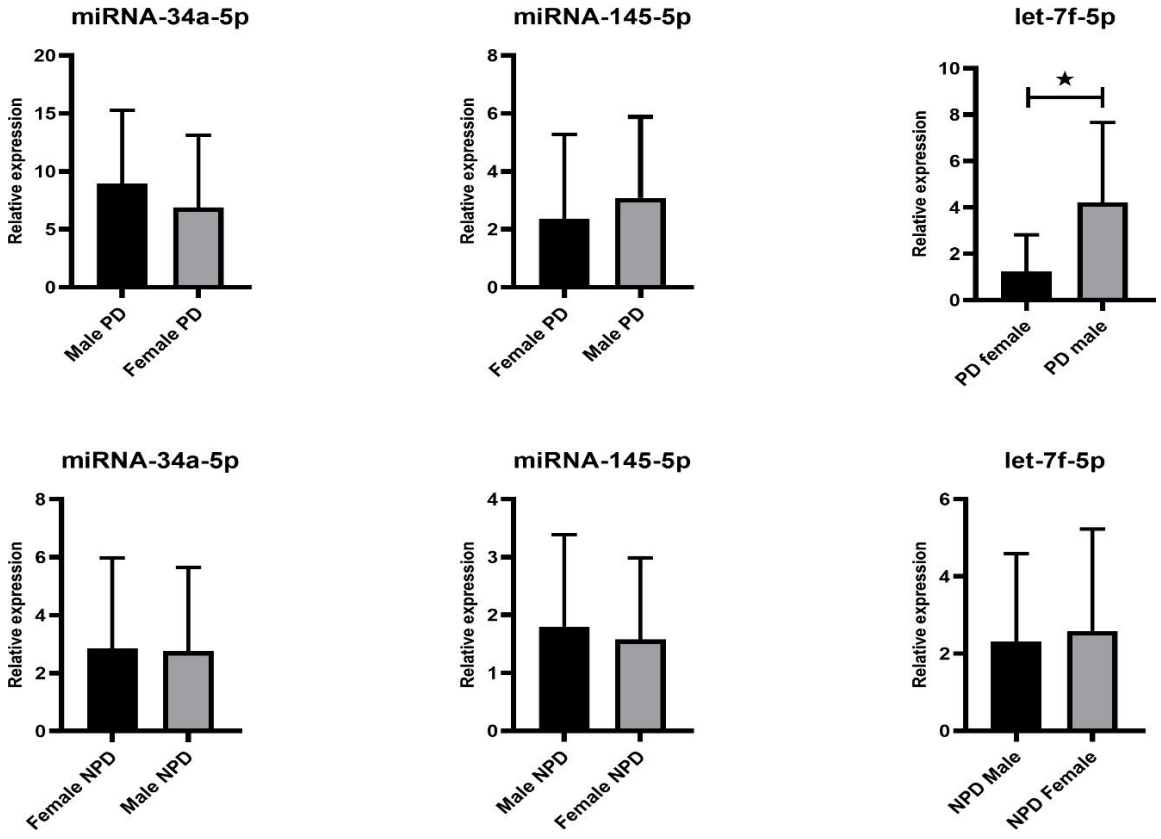

Figure 7. Shows the outcomes comparing the miRNA relative expression difference between men and women in the same status group.

The student's t-test or Mann-Whitney U test was adopted to measure the differences between males and females depending on whether the data was parametric or non-parametric. The let-7f-5p miRNA result displayed an elevated expression in PD males compared to PD females. ★ ($P < 0.05$).

Table 5. The Spearman r correlation analysis was conducted to compare the association between HbA1c and miRNA-34a-5p gene expression.

A strong correlation (r = 0.5454) was observed between the HbA1c and miRNA-34a-5p (p = 0.0022).

Spearman r	**A1c (%) vs miRNA-34a-5p**
r	0.5454
95% CI	0.2128 to 0.7648
P value (two-tailed)	**0.0022 ****

Table 6. A multivariate regression analysis. β1 is unadjusted; β2 is adjusted for race and β3 is adjusted for gender. ***** = $p < 0.01$.

Parameter estimate	Variable	Estimate	Standard error	95% confidence interval	P value
β1	HbA1c	4.499	2.011	0.3476 to 8.650	0.0349 *
β2	Race	0.4949	1.620	-2.849 to 3.839	0.7627
β3	Gender	-0.7320	1.860	-4.570 to 3.106	0.6973

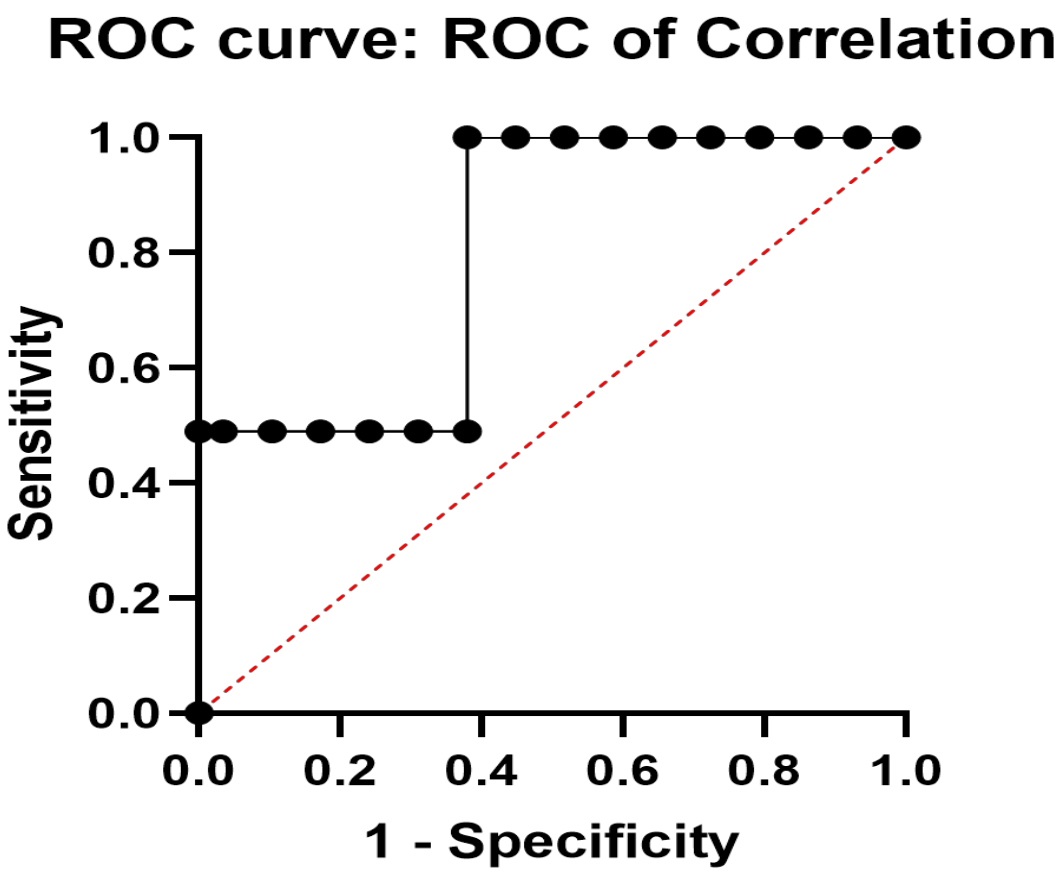

Figure 8. Displays the receiver operator characteristic curve for miRNA-34a-5p in the prediction of HbA1c-determined pre-diabetes.

The AUC for miRNA-34a-5p was 0.806 (0.7003 – 0.9123) and had a p-value of <0.0001. To determine the optimal cutoff, we choose the method that uses the point of the curve where the product of the Sensitivity and specificity indices is maximum (37). Thus, the optimal sensitivity was 85.71%, and the specificity was 93.75 for discriminating pre-diabetes individuals from healthy controls.

4. Discussion

T2DM is a chronic metabolic disorder characterised by hyperglycaemia, often resulting from unhealthy dietary habits, sedentary lifestyle and genetic predisposition (38, 39). Notably, miRNAs have emerged as crucial regulatory molecules implicated in the onset of T2DM (11). The association between certain miRNAs and the pathogenesis of T2DM is well-established (11, 40). T2DM is often preceded by pre-diabetes, an intermediate hyperglycemic state, which offers an opportunity for early intervention and prevention strategies (41). However, the relationship between miRNA expression and pre-diabetes remains relatively unexplored. Therefore, this study aims to investigate the expression levels of T2DM-

associated miRNAs, specifically miRNA-34a-5p, let-7f-5p, and miRNA-145-5p, in individuals with pre-diabetes.

The let-7f-5p miRNA did not show a significant difference in relative expression between the NPD and PD groups. This suggests that these miRNAs may not play a role in the development of pre-diabetes. However, untreated or poorly managed pre-diabetes could potentially lead to dysregulation of these miRNAs, contributing to the onset of T2DM. Our speculation is based on previous research indicating that elevated levels of let-7f-5p are associated with T2DM (25). Some literature indicates the influence of gender on miRNA expression patterns (42, 43). Hence, we also explored the let-7f-5p expression stratified by gender, and gender did not influence the let-7f-5p expression, as seen in Figure 2. However, supported by the Figure 3 result, females diagnosed with pre-diabetes appear to exhibit a lower expression of let-7f-5p compared to males diagnosed with pre-diabetes. We speculate that the compensatory mechanisms involved in maintaining glucose levels below the threshold for T2DM during the pre-diabetic stage may impact the expression of let-7f-5p, resulting in levels comparable to those observed in healthy individuals. Studies indicate that in ages 25 – 45, as seen in Table 1, females are less likely to develop diabetes than males, with the estrogen protective effect on glucose regulation considered as the key reason (44, 45). It is plausible that the estrogen protective effect could also influence the expression of let-7f-5p expression, explaining its reduced expression in females. Therefore, a longitudinal study that investigates the expression of let-7f-5p during the progression of pre-diabetes to T2DM is necessary to understand the miRNA role further.

The expression of miRNA-145-5p did not exhibit a statistically significant difference between individuals diagnosed with pre-diabetes and those with normoglycemia. Moreover, gender-stratified analysis of miRNA-145-5p expression revealed no disparity between males and females, indicating that gender does not exert an influence on this miRNA. Contrary to our results, a study conducted in the Middle East reported deregulated levels of plasma miRNA-145-5p in persons with pre-diabetes and T2DM (28). Studies have shown racial differences in miRNA expression (31, 46). Hence, the influence of ethnicity on miRNA expression may be the reason for the disparity. This study lacked sufficient sample size to ascertain statistically significant differences in the expression of the investigated miRNAs within the White and Indian populations. Nevertheless, the findings available suggest a trend where the Indian ethnic group may exhibit greater susceptibility to alterations in miRNA expression levels. Therefore, further research with larger sample sizes within the Durban Indian population is warranted. This would allow a more comprehensive investigation into the genetic factors contributing to these differences across ethnic groups and their potential implications for pre-diabetes susceptibility and management strategies.

Additionally, a study by Aladel et al. found increased expression of miRNA-145p-5p in both non-obese and obese individuals with T2DM, with a more significant increase observed in the obese T2DM group

(47). This suggests a potential influence of obesity on miRNA-145p-5p expression. It is possible that the control group in our study had more obese participants compared to the pre-diabetes group, leading to the non-significant difference observed. Future studies are recommended to validate this by including data on obesity status in both obese and non-obese pre-diabetes groups.

The miRNA-34a-5p displayed a significantly upregulated expression in persons with pre-diabetes compared to those with non-pre-diabetes. These results correlate with most literature outcomes in animal models and clinical studies showing deregulation in the diabetes group compared to the non-diabetic group (48-51). For example, a study by Elodie Roggli et al., 2010 reported elevated levels of miRNA-34a-5p expression and linked the miRNA-34a-5p with pancreatic β-cell failure and proinflammatory cytokines (48). Its' negative impact on the pancreatic β-cell is said to cause impaired glucose tolerance and diminished insulin secretion (48, 49). In another study, the miRNA-34a-5p has been reported to influence how hepatic gluconeogenesis is controlled and how that contributes to dysglycaemia (50). Furthermore, a study by Su *et al*. 2021, demonstrated a notable association between the enhanced expression of miRNA-34a-5p and pancreatic insulin function. This association was elucidated through the modulation of the insulin signalling pathway, whereby miRNA-34a-5p exerts regulatory control by targeting multiple pivotal genes, including MAPK10, PPP1CC, CRKL, PRKAG1, LIPE, HK1, PCK1, and MKNK1 (49).

Roggli *et al*., 2010 linked increased miRNA-34a-5p expression with pancreatic β-cell failure and proinflammatory cytokines (48). The gender-stratified expression of miRNA-34a-5p showed that the expression of miRNA-34a-5p in PD males was significantly higher than in NPD, which signifies a possible gender impact on the miRNA expression. However, most literature evidence suggests that the upregulation of this gene is the cause of metabolic complications through its negative impact on the pancreatic β-cell, which is likely the cause of impaired glucose tolerance and diminished insulin secretion (48, 49). Additionally, the miRNA-34a-5p has been reported to influence how hepatic gluconeogenesis is controlled and how that contributes to dysglycaemia (50). This further suggests a multifactorial function of the miRNA-34a-5p that requires more research to understand its mechanics fully.

The impact of ethnicity on miRNA expression patterns could not be confirmed in this study. Previous research indicated that ethnicity affects the overall prevalence of pre-diabetes (33, 52, 53). A similar trend is likely to be observed in this population. However, the statistical analysis of miRNA expression between the ethnic groups was constrained by the relatively small sample size, as shown in Table 1. Therefore, further research with a larger sample size is essential to understand better miRNA expression levels across different ethnic groups in Durban, South Africa.

The study then investigated the association between HbA1c and miRNA-34a-5p to determine whether this gene may influence glycemic control. The study findings revealed a strong correlation between

miRNA-34a-5p expression and HbA1c levels, suggesting a pivotal role in maintaining glucose homeostasis. Thus, miRNA-34a-5p expression appears directly proportional to % HbA1c and could serve as a potential biomarker for assessing glycemic control or diagnosing pre-diabetes. Moreover, in Table 3, the multivariate regression analysis indicated that miRNA-34a-5p is associated with HbA1c levels after adjusting for race and gender. To further evaluate its diagnostic potential, we conducted an ROC curve analysis to determine if miRNA-34a-5p expression could discriminate between individuals with pre-diabetes and healthy individuals. The AUC value of 0.806 (95% CI: 0.7003 - 0.9123) indicates a great discriminatory ability of miRNA-34a-5p. While these results are promising, validation through larger clinical studies involving diverse populations across South Africa is necessary to establish its diagnostic utility in clinical practice.

5. Limitations and recommendations

The study findings suggest a potential role for miRNA-34a-5p as a biomarker for pre-diabetes onset and warrant further investigation into its mechanistic involvement in dysglycemia. However, the study was not able to adjust for possible confounding variables such as obesity, indicating that future studies are necessary to validate the outcomes of this research. The study is also restricted by a small sample size that limits the generalizability of the outcomes to the general population of South Africa. The dysregulated microRNAs can be further investigated as therapeutic targets (54). For example, dysregulated microRNAs in persons with cancer have been targeted in a novel microRNA replacement therapy. This new treatment strategy has the potential to be incorporated into diabetes. Therefore, future pre-clinical studies can look into miRNA-34a-5p as a replacement therapy. Additionally, we recommend exploring large-scale miRNA sequencing to elucidate the possible mechanics behind the induction of pre-diabetes. The dysregulated microRNAs can be further investigated as potential targets in microRNA replacement therapy (54). Future studies should measure the expression of the miRNA along with the targeted genes to confirm the association.

6. Conclusions

The study sheds light on the evolving significance of microRNAs as biomarkers in unravelling the mechanisms underlying pre-diabetes development. The findings presented herein contribute to the growing body of knowledge on the involvement of microRNAs in the progression from normoglycaemia to pre-diabetes, providing valuable insights for further research and potential therapeutic interventions. Notably, miRNA-34a-5p showed promise as a biomarker for determining the risk of pre-diabetes onset and as a diagnostic marker. Furthermore, miRNA-34a-5p remained significant after adjusting for race and gender. Due to the low number of White and Indian ethnic groups, the results were not statistically

analysed. Hence, larger clinical studies, including all possible confounders, are imperative to validate study findings. In future studies, the increased sample size will enhance the statistical power and generalizability of the results. We expect the association between miRNA-34a-5p and pre-diabetes to remain significant, further validating miRNA-34a-5p as a potential biomarker for early detection and management of pre-diabetes across diverse populations.

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8. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 7

The increasing prevalence of diabetes mellitus presents a significant global healthcare challenge (1). Diabetes mellitus is considered one of the leading causes of lower life expectancy and mortality worldwide (2). In 2021, approximately 10,5% of the global adult population was diagnosed with diabetes mellitus, and the prevalence is expected to increase to 12,2% by the year 2045 (3). Notably, over 90 % of the cases are attributed to type 2 diabetes mellitus (T2DM), a multi-organ metabolic disorder characterised by deficient insulin secretion by pancreatic beta cells and insulin resistance in insulin-sensitive tissues (3, 4). The diminished insulin availability and action result in elevated blood glucose concentrations, which contribute to other metabolic complications such as microvascular and macrovascular disorders (4, 5).

Since T2DM is a multi-organ metabolic disorder, its pathogenesis involves a loss in the inter-organ crosstalk demonstrated by the endocrine system, which, under normal physiological conditions, releases hormones into the circulation that allows communication between distant organs and maintains glucose homeostasis (6). Hence, multiple hormonal imbalances have been implicated in the onset of T2DM. For example, hormones secreted by the thyroid, adipose tissue, gonads, pancreas and intestine are known to be imbalanced in individuals diagnosed with T2DM (7-10). However, a paucity of studies explored all these hormones collectively in a single study.

Moreover, accumulating genome-wide association studies show that genetic factors contribute significantly to the pathogenesis and epidemiology of T2DM (11). Increasing attention has been placed on regulatory microRNAs, which led to the establishment of the association between T2DM and miRNAs (12, 13). They are described as small, non-coding RNA molecules regulating gene expression, typically about 21 to 25 nucleotides in length (14, 15). They control gene expression by binding to specific messenger RNA (mRNA) molecules, thereby regulating their translation into proteins or marking them for degradation (14). The following miRNA-34a-5p, miRNA-145-5p, and let-7f-5p have been linked with the development of T2DM by interfering with genes that influence glucose homeostasis (16).

However, the onset of T2DM is often preceded by a state of intermediate hyperglycaemia known as pre-diabetes (17). People with pre-diabetes present with moderate insulin resistance, resulting in blood glucose levels above the homeostatic range but below the overt T2DM threshold (17). According to the American Diabetes Association, pre-diabetes can be diagnosed by glycated haemoglobin (HbA1c) levels of 5,7 - 6,4% or fasting blood glucose concentrations of 5,7 – 6,9 mmol/L. The global prevalence of pre-diabetes was estimated at 298 million individuals in 2021 and is expected to increase to 414 million people by 2045 (3). The annual conversion rate from pre-diabetes to T2DM typically ranges

from 5% to 15%, emphasising its significance as a critical focus in preventive strategies against T2DM (17, 18). In addition, pre-diabetes has been deemed as an unfavourable state that exacerbates metabolic complications and correlates with increased mortality rates (19). Therefore, understanding the pathogenesis and burden of pre-diabetes is pivotal in determining the measures necessary to treat pre- diabetes and prevent the onset of T2DM. The prevalence and conversion rates also depend on the population's ethnicity, highlighting the importance of pre-diabetes prevalence studies across various populations, such as South Africa (18). Upon thoroughly examining the existing literature outlined in Chapter 1, it was observed that studies exploring the involvement of hormonal imbalances and microRNAs in the progression of pre-diabetes are comparatively less explored in contrast to those focusing on T2DM.

The first research objective was to determine the overall prevalence and correlates of pre-diabetes in South Africa from previously published literature and to estimate the current prevalence in the population of Durban, South Africa. The King Edward Hospital in Durban was deemed appropriate for this study as it was central and dealt with patients throughout the city. The second research objective was to determine the level of change in hormones associated with glucose homeostasis in people with pre-diabetes by comparing them with individuals who do not have pre-diabetes. The third and final objective was to evaluate the relative expression of miRNA-34a-5p, miRNA-145-5p, and let-7f-5p miRNAs between persons with pre-diabetes and those without pre-diabetes.

Prior to this research project, no study had conducted a systematic review and meta-analysis evaluating the prevalence and correlates of pre-diabetes in South Africa. It was imperative first to assess the burden of pre-diabetes in the country and provide insights and research gaps in the available empirical evidence. Therefore, the systematic review and meta-analysis process yielded a synthesised prevalence rate of 15,6% for pre-diabetes across South Africa. It became evident that many existing studies relied on outdated diagnostic criteria to determine the prevalence of pre-diabetes, which may have led to an underestimation of the prevalence of pre-diabetes in the country. In response to the review findings, we incorporated the latest criteria for the diagnosis of pre-diabetes and evaluated the prevalence of pre- diabetes in a clinical setting in Durban, South Africa. The study employed both the latest ADA HbA1c and WHO IFG criteria and reported an alarming 54 % and 67 % prevalence rate of pre-diabetes in King Edward Hospital, serving the Durban population. These findings align with predictive studies suggesting that the most notable increase in diabetes prevalence will occur in developing nations like South Africa (3, 20). This poses a substantial concern for public health, particularly to the King Edward Hospital management, as untreated pre-diabetes often progresses to overt T2DM, which will be more challenging and costlier to manage (21-23). Hence, this high prevalence cannot be left unattended. The combined findings from the systematic review and prevalence research article will provide valuable insights into the current burden of pre-diabetes, aiding policymakers in

their planning and intervention strategies moving forward.

The alarming pre-diabetes prevalence motivates the necessity of increasing the knowledge and understanding of the metabolic state of pre-diabetes. For instance, hormonal imbalances in persons with T2DM are well documented (24-26). However, these hormonal changes in the prediabetic phase are underexplored. Findings in Chapter Three showed that an imbalance of hormone concentrations such as T3, T4, cortisol, testosterone, estrogen, progesterone and GLP was present during pre-diabetes. These hormones could be used as markers to monitor the management of pre-diabetes and identify individuals who are at the most risk of developing pre-diabetes and possibly progressing to overt diabetes. They can be used as therapeutic targets to restore hormonal balance and reduce the incidence of T2DM. Presently, it remains unclear whether restoring the altered hormone levels during pre-diabetes can alleviate complications linked with pre-diabetes and re-establish normoglycaemia. Moreover, the association of the pre-diabetic state and hormonal imbalances suggests that pre-diabetes is a complex multi-organ disease, and future studies should, therefore, investigate the pre-diabetic state as a multi-organ condition.

Furthermore, genetic predisposition is a significant factor contributing to the onset of T2DM (27). MicroRNAs have emerged as biomarkers linked with the pathogenesis of T2DM (13). However, there is limited research on their role in the development of pre-diabetes. The miRNA-34a-5p, miRNA-145- 5p, and let-7f-5p miRNAs associated with T2DM were explored in participants with pre-diabetes to ascertain their involvement in the onset of pre-diabetes. The relative fold expression of miRNA-34a-5p was elevated in persons with pre-diabetes than in non-prediabetic individuals. The Spearman's correlation test showed a strong association between HbA1c and miR-34a-5p expression, and the miRNA-34a-5p was able to discriminate between the two groups, with a ROC curve AUC of 0,081. This study, for the first time, suggested that it can be employed as a pre-diabetes diagnostic marker.

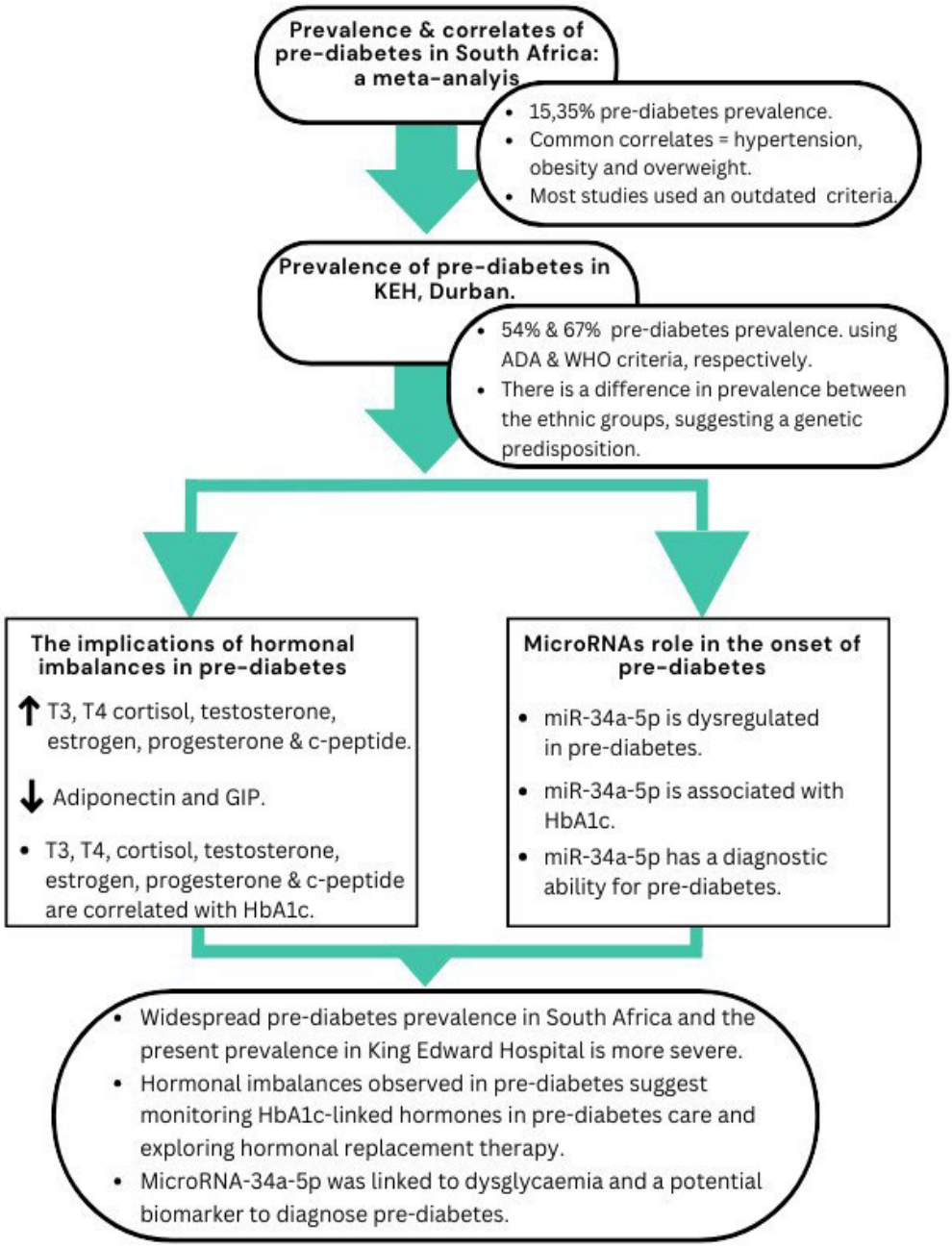

Figure 1. *Diagram illustrating the main findings from this dissertation on pre-diabetes prevalence, as well as hormonal and* microRNA changes in individuals with pre-diabetes.

Conclusions, shortfalls & recommendations for future studies

There is an indication of a country-wide pre-diabetes burden, and this study shows that the present analysis of the prevalence of pre-diabetes is on the rise. If left unattended, a massive T2DM incidence looms in the population serviced by the King Edward Hospital, and this could be a microcosm of the entire country of South Africa. This could be an indicator of the looming burden of T2DM in the country. Hormonal imbalances in multiple hormones secreted by different organs were also observed in people with pre-diabetes, suggesting a more holistic approach should be taken when managing pre-diabetes, as it appears to be a multi-organ disease. Also, the possibility of introducing hormonal replacements as a treatment for pre-diabetes should be considered in future studies. The miR-34a-5p gene proved to be a promising biomarker for determining those at risk of developing pre-diabetes, and that may be used as a diagnostic marker. The results further identify miR-34a-5p as a possible therapeutic target in treating pre-diabetes. Hence, future genetic therapy studies are recommended to evaluate treatments targeting the miR-34a-5p. However, these findings are not conclusive due to study limitations, including a small sample size that restricts generalisability. Potential confounders such as BMI and lifestyle factors were not adjusted for, which may have affected the results. Further research with larger cohorts and robust adjustment for confounding variables is needed to validate the findings for clinical relevance.

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APPENDICES

Appendix 1: Ethical clearance

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04 May 20'21

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A sub-commftt.ee of the Biomedical Researcih EU.ics. Ciommittee h,as ca,ns.fdered and noted your apptfca,tfon.

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Yours sincerely,

[redacted]

Prof D Wassena.arr
Chair: Bio:medica.l Researcih Ethics Comnrittece

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Founding Campuses: Edgewood Howard College -b,I Scl!iooi Pietermaritzburg

INSPIRING GREATNESS

Appendix 2: Chapter 3.2 Supplementary file

Table 1: Search strategy (PubMed)	
Search	**Search terms**
4	Search (#3 NOT animal[mh]) AND ("2000/01/01"[Date-Publication] : "2021/09/7"[Date-Publication])
3	Search (#1 AND #2 AND #3)
2	Search (South Africa[mh] OR "South Africa*"[tiab] OR RSA[tiab] OR Southern Africa[tiab])
1	Search (Diabetes OR Type 2 diabetes mellitus OR Hemoglobin A, glycosylated OR Pre-diabetes OR Glycosylated haemoglobin OR Impaired glucose tolerance OR Impaired fasting glucose