AN INVESTIGATION INTO PRE-DIABETES-ASSOCIATED CHANGES IN IMMUNE CELLS, RED BLOOD CELL INDICES AND LONG NONCODING RIBONUCLEIC ACIDS IN PATIENTS WITH PRE-DIABETES FROM DURBAN, SOUTH AFRICA

By

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Submitted as the dissertation component in fulfilment for the Doctor of Philosophy inHealth Sciences degree in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal.

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PREFACE

Chronic consumption of unhealthy diets combined with living a sedentary lifestyle has been reported to cause type 2 diabetes (T2D). However, the onset of T2D is often preceded by a long-lasting asymptomatic state of moderate hyperglycaemia known as pre-diabetes. Immune activation, upregulation of inflammatory markers and haematological changes have been reported during T2D in humans. While similar findings have been shown during pre-diabetes, these have only been shown in a diet-induced animal model of pre-diabetes and has not been shown in humans. Additionally, reports have shown that immune cells such as T-cells and dendric cells, express long noncoding ribonucleic acids which contribute to the immune response, inflammation, and exacerbation of cardiovascular diseases. However, no research has reported on long noncoding RNAs during the pre-diabetic state. The city of Durban in South Africa is a rapidly urbanizing area has been reported to have a high prevalence of pre-diabetes with the highest prevalence being in those people aged from 25 years to 45 years. Using this population, this study sought to investigate and characterize changes in selected immune cells, inflammatory markers and red blood cell indices in patients with pre-diabetes aged 25 to 45 years. This study further investigated if there is expression of long noncoding ribonucleic acids in these patients with pre-diabetes.

DECLARATION

I, Nomusa Christina Mzimela hereby declare that the dissertation entitled: **"The state of selected immune cells, inflammatory markers, red blood cells indices and long noncoding ribonucleic acids on multi-ethnic prediabetic males and females from the age of 25 to 45 years in Durban, South Africa."** is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

Student: **N.C Mzimela** **Signature** [redacted] **Date:** **26March 2024**

Supervisor: **Prof A Khathi** **Signature** [redacted] **Date:** **26 March 2024**

Co-Supervisor: **Dr P.S Ngubane** **Signature** [redacted] **Date:** 28 March 2024

PLAGIARISM DECLARATION

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

DOCTOR OF PHILOSOPHY IN HEALTH SCIENCES 2023

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

2. I have used the Vancouver convention for citation and referencing. Each contribution to, and quotation in, this thesis from the works of other people has been attributed and has been cited and referenced.

3. This thesis is my own work.

4. I have not allowed and will not allow anyone to copy my work with the intention of passing it off as his or her own work.

Signature_____  _____

DETAILS OF ALL DISSERTATION MANUSCRIPTS

The manuscripts are divided into a) Publications emanating from this dissertation and b) Manuscripts from this dissertation under review and c) Other manuscripts written during PhD.

a) Publications emanating from this thesis.

1. **Mzimela NC**, Sosibo AM, Ngubane PS, Khathi A. **The Changes That Occur in the Immune System During Immune Activation in Patients with Pre-diabetes from All Ethnicities, Aged 25-45 Years: Protocol for a Systematic Review and Meta-analysis.** *JMIR Research Protocols.* 2022 Nov 14;11(11): e31619. https://doi.org/10.2196/31619

2. **Mzimela NC**, Sosibo AM, Ngubane PS, Khathi A. **The changes that occur in the immune system during immune activation in pre-diabetic patients of all ethnicities, from the age of 25- to 45-years: A systematic review and meta-analysis**. *Medicine (Baltimore).* 2022 Dec 23;101(51): e30903. https://doi.org/10.1097/md.0000000000030903

3. **Mzimela NC,** Sosibo AM, Ngubane PS, Khathi A. **The Changes in Red Blood Cell Indices That Occur in Pre-Diabetic Patients of all Ethnicities from the 25–45 Years of Age: A Protocol for a Systematic Review and Meta-Analysis.** *Methods and Protocols*. 2023 Jan 24;6(1):13. https://doi.org/10.3390/mps6010013

4. **Mzimela NC**, Sosibo AM, Ngubane PS, Khathi A. (2023**). The changes that occur in blood indices that occur on pre-diabetic patients of all ethnicities, from the age of 25 to 45 years: a systematic review and meta-analysis**. *Hematology and Medical Oncology* **(REF: HMO-7-241)**

5. **Mzimela NC**, Sosibo AM, Ngubane PS, Khathi A. **Investigation into changes in inflammatory and immune cell markers in pre-diabetic patients from Durban, South Africa**.*Journal of Immunotoxicology*.2024 Dec 31;21(1):2290282. https://doi.org/10.1080/1547691X.2023.2290282

6. **Mzimela NC**, Sosibo AM, Ngubane PS, Khathi A. (2023). **Evaluating the changes in red blood cells indices in pre-diabetic patients from the age of 25- to 45- years in Durban, South Africa**. *Journal of Blood Medicine* **(REF: 470181).**

b) Manuscripts from this thesis under review

1. **Mzimela NC**, Tata FY, Sosibo AM, Ngubane PS, Khathi A. **The novel role of lncRNAs in prediabetic patients from Durban, South Africa and their potential use as biomarkers in the pathophysiology and prognosis of pre-diabetes** (2023). *Endocrine Journal* **(REF: EJ24-0477).**

c) Other manuscripts written during PhD.

1. Sosibo AM, **Mzimela NC**, Ngubane PS, Khathi A. **Prevalence and correlates of pre-diabetes in adults of mixed ethnicities in the South African population: a systematic review and meta-analysis protocol**. *BMJ Open*. 2021 Oct 18;11(10): e048266.

   https://doi.org/10.1136/bmjopen-2020-048266

2. Sosibo AM, **Mzimela NC**, Ngubane PS, *Khathi A.* **Prevalence and correlates of pre-diabetes in adults of mixed ethnicities in the South African population: A systematic review and meta-analysis.** *PLoS One*. 2022 Nov 29;17(11): e0278347.

   https://doi.org/10.1371/journal.pone.0278347

3. Sosibo AM, **Mzimela NC**, Ngubane PS, Khathi A. **Prevalence of pre-diabetes in adults aged 25–45 years in a Durban-based clinical setting, South Africa: A retrospective study**. *Primary Care Diabetes*. 2023 Dec 1;17(6):650-4.

   https://doi.org/10.1016/j.pcd.2023.10.004

4. Sosibo AM, **Mzimela NC**, Ngubane PS, Khathi A. **Hormone imbalances detected in study participants with pre-diabetes in a Durban-based clinical setting, South Africa**. *Int J Diabetes Dev Countries*. 2024 June 24 .

https://doi.org/10.1007/s13410-024-01363-z

PRESENTATIONS

National Conference Presentation

Mzimela, NC., Sosibo AM., Ngubane, PS., Khathi, A. IMMUNE CELLS AND INFLAMMATORY MARKERS STATUS IN PRE-DIABETIC PATIENTS FROM DURBAN, SOUTH AFRICA. 56th Society for Endocrinology Metabolism and Diabetes of South Africa (SEMDSA) Congress **7-10 September 2023**. Radisson Hotel and Convention Centre, Johannesburg, South Africa.

Symposium Presentation

Mzimela, NC., Sosibo AM., Ngubane, PS., Khathi, A. IMMUNE CELLS AND INFLAMMATORY MARKERS STATUS IN PRE-DIABETIC PATIENTS FROM DURBAN, SOUTH AFRICA. College of Health Sciences Research Symposium **15-16 August 2023**. University of KwaZulu-Natal, Durban, South Africa.

Mzimela, NC., Tata FY., Sosibo AM., Ngubane, PS., Khathi, A. THE NOVEL ROLE OF LNCRNAS AT THE PRE-DIABETES STAGE IN MULTI-ETHNIC PATIENTS AGED FROM 25-45 YEARS AT ETHEKWINI DISTRICT, AS PLAUSIBLE BIOMARKERS IN THE PATHOPHYSIOLOGY AND PROGNOSIS OF PRE-DIABETES. School of Laboratory Medicine and Medical Sciences Research Symposium **04 October 2023**. University of KwaZulu-Natal, Durban, South Africa.

MEDIA ARTICLES PUBLISHED FROM RESEARCH

The research in this thesis titled below produced the articles published at UKZN Ndaba online and newspaper articles (Sunday tribune and Mercury).

Manuscript

Mzimela NC, Sosibo AM, Ngubane PS, Khathi A**. Investigation into changes in inflammatory and immune cell markers in pre-diabetic patients from Durban, South Africa.** *Journal of Immunotoxicology.*2024 Dec 31;21(1):2290282. **https://doi.org/10.1080/1547691X.2023.2290282**

Articles

1. Article on NDABAONLINE UKZN (05 October 2023 Volume :11 Issue :53)

Title of article: **Raising Pre-diabetes Awareness at National Society's Congress**

Link: https://ndabaonline.ukzn.ac.za/UkzndabaStory/Vol11Issue53/Raising%20Pre-diabetes%20Awareness%20at%20National%20Societys%20Congress

2. Article on SUNDAY TRIBUNE and MERCURY (By Mervyn Naidoo| Published Oct 22, 2023)

Title of article: **A study on pre-diabetes in the era of 'diabesity'**

Sunday Tribune Link:

https://www.iol.co.za/sunday-tribune/news/a-study-on-pre-diabetes-in-the-era-of-diabesity-f5942f84-5df2-4a6e-9813-ea36ad288cd0

Mercury Link:

https://www.msn.com/en-za/news/other/a-study-on-pre-diabetes-in-the-era-of-diabesity/arAA1iEXmi?ocid=msedgdhp&pc=HCTS&cvid=100e1ea72d4045a9b73d6f0051af1edd&ei=45

DEDICATION

I would like to dedicate this to my late parents (Mr and Mrs Mzimela), my brothers (ONjingili abahle) and my ninjas (Sphamandla and Thabiso). I am grateful and indeed still blessed that an appointment with God has been accomplished. Nothing can measure how thankful I am for all the support, how you have all contributed to upgrade me to this position of my life journey and for prioritizing for my better life over your personal goals. I am humbled. I would be making a mistake, for not also dedicating this work to my supervisor, Prof Andile Khathi. It has been a very long and a roller coaster journey from my first year of registration of this chapter, and you never gave up on me. You believed in me, even when I could not believe I can do it. I cannot ask for more, I am grateful and humbled.

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To God be the glory. I would like to express my very profound gratitude to my family for believing in me, continuous encouragements, and unfailing support. Ngithi ngiyabonga boNjingili abahle, Mnguni, Lulwandle aluwelwa, Luwelwa zinkonjane ngoba zindizela phezulu, Zimeme, Sheleza, Maconi, Donda kaMlimandlela, Ntaka, Mfeka

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ABBREVIATIONS

ADA - American Diabetes Association

BRU - Biomedical Research Unit

CD40L - cell differentiation 40 ligands

CRP - C-reactive protein

ELISA - Enzyme linked immunosorbent assay.

EPO - erythropoietin

HBA1c - Glycated haemoglobin

HFHC - High fat high carbohydrate

HCT - haematocrit

HGB - haemoglobin

IDF - International Diabetes Federation

IFG - Impaired fasting glucose

IFN-γ - Interferon gamma

IGT - Impaired glucose tolerance

IL-1 - interleukin-1

IL-6 - Interleukin 6

JNK-1 - Jun N-terminal kinases

KZN - KwaZulu Natal

LncRNA - long noncoding ribonucleic acid

LncRNA-DC - noncoding RNA expressed in dendric cells.

LncRNA-NRON - Noncoding repressor of nuclear factor activated T-cells.

LncRNA-NTT - noncoding transcript in T- cells

MCH - mean cell haemoglobin

MCHC - mean cell haemoglobin content

MCV- mean cell volume

NFAT1 - the nuclear factor of activated T cells

NFATc3 - nuclear factor of activated T-cells cytoplasmic-3

NF-Kβ -Nuclear factor kappa B

OGTT- Oral glucose tolerance test

PD - Pre-diabetes

PKC- protein kinase C

RBC - red blood cells

RDW - red cell distribution width

T2D - Type 2 diabetes Mellitus

TLR - toll-like receptors

TNF-α - Tumor necrosis factor-alpha

UKZN - University of Kwazulu-Natal

WHO - World Health Organisation

STUDY OUTLINE

The current dissertation is presented in manuscript format, consisting of 10 sections viz. chapter 1: introduction /literature review, chapter 2: methodology, chapter 3: immunity protocol, immunity systematic review and manuscript 1, chapter 4: hematology protocol, hematology systematic review and manuscript 2, chapter 5: manuscript 3, chapter 6: synthesis.

Chapter 1 covers the introduction of the study.

Chapter 2 covers the foundation of the literature and the information that has been discovered based on immune cells, inflammatory markers, red blood cell indices and long noncoding ribonucleic acids at type 2 diabetes and pre-diabetes stage, in order to link the area of interest of the study and cover a gab that is unclear at pre-diabetes stage.

Chapter 3 covers a summary of the methodology of all experimental work contained in the whole study.

Chapter 4 contains a protocol of immunity systematic review of the research of interest titled; The Changes That Occur in the Immune System During Immune Activation in Patients with Pre-diabetes from All Ethnicities, Aged 25-45 Years: Protocol for a Systematic Review and Meta-analysis. This is the work authored by N.C. Mzimela, A.M. Sosibo, P.S. Ngubane and A. Khathi has been published in **JMIR Research Protocols** according to journal's guidelines to authors (https://doi.org/10.2196/31619). Chapter 4 also contain a systematic review titled; The changes that occur in the immune system during immune activation in pre-diabetic patients of all ethnicities, from the age of 25- to 45-years: A systematic review and meta-analysis. This is the work authored by N.C. Mzimela, A.M. Sosibo, P.S. Ngubane and A. Khathi, has been published in **Medicine** according to journal's guidelines to authors (https://doi.org/10.1097/md.0000000000030903). Lastly, chapter 2 also contains an original research manuscript. This is the work authored by N.C. Mzimela, A.M. Sosibo, P.S. Ngubane

and A. Khathi has been accepted for publication in **Immunotoxicology** and has been formatted according to the journal's guidelines to authors (https://doi.org/10.1080/1547691X.2023.2290282).

Chapter 5 contains a protocol of a systematic review of the research of interest titled; The Changes in Red Blood Cell Indices that Occur in Pre-Diabetic Patients of all Ethnicities from the 25–45 Years of Age: A Protocol for a Systematic Review and Meta-Analysis. This is the work authored by N.C. Mzimela, A.M. Sosibo, P.S. Ngubane and A. Khathi has been published in **Methods and Protocols** according to journal's guidelines to authors (https://doi.org/10.3390/mps6010013). Chapter 3 also contains a systematic review titled; The changes that occur in blood indices that occur on pre-diabetic patients of all ethnicities, from the age of 25 to 45 years: a systematic review and meta-analysis. This is the work authored by N.C. Mzimela, A.M. Sosibo, P.S. Ngubane and A Khathi has been accepted for publication in the journal **Hematology and Medical Oncology** and has been formatted according to journal's guidelines to authors (**REF Number: HMO-7-241**). The last section of chapter 5 is an original research manuscript. This is the work authored by N.C. Mzimela, A.M. Sosibo, P.S. Ngubane and A Khathi has been accepted for publication **Journal of Blood Medicine (ISSN: 1179-2736)** and has been formatted according to journal's guidelines to authors **(REF Number: 470181).**

Chapter 6 contains an original research manuscript 3. This is the work authored by N.C. Mzimela, F.Y. Tata, A.M. Sosibo, P.S. Ngubane and A Khathi is under review for publication in **Endocrine Journal** and has been formatted according to journal's guidelines to authors **(REF Number: EJ24-0477)).**

Chapter 7 discuss the synthesis and conclusion of the study and the appendix consist of the journal's guideline to authors.

ABSTRACT

Background: Pre-diabetes is a metabolic condition that often precedes the onset of type 2 diabetes (T2D). This asymptomatic condition is characterised by moderate hyperglycaemia that is below the threshold for a diagnosis of T2D. Risk factors that are implicated in the development of pre-diabetes includes chronic consumption of unhealthy diets, as well as sedentary lifestyles. The asymptomatic nature of this condition has made it difficult to diagnose and study the condition in humans. Studies using an animal model of pre-diabetes have reported that there are abnormalities such as immune activation, upregulation of inflammatory markers and haematological changes during this condition. The findings indicated changes in immune cells such as neutrophils, lymphocytes, monocytes, basophils and eosinophils. The studies also showed upregulation of inflammatory markers such as CRP, IL-6, TNF-α, fibrinogen, sCD40L and P-selectin during pre-diabetes. Additionally, these studies reported on changes on red blood cell indices such as MCH, MCHC, RBCs, HCT, HGB, MCV and RDW. These findings from animal studies raised the question if these also occur in humans with pre-diabetes. Furthermore, studies have shown that long noncoding ribonucleic acids (lncRNAs) expressed during inflammatory conditions such as T2D contribute to abnormalities such as atherosclerosis. These include lncRNAs such as noncoding RNA expressed in dendric cells (LncRNA-DC), noncoding transcript in T- cells (lncRNA-NTT) and noncoding Repressor of nuclear factor activated T-cells (lncRNA-NRON), however, there have been no studies to investigate if these are expressed during pre-diabetes. Recent studies have reported on increasing prevalence of pre-diabetes among adults in Durban, South Africa with the highest prevalence found in those between 25-45 years of age. This made this area and population ideal to study pre-diabetes to investigate pre-diabetes-associated changes in immune cells, red blood cell indices and long noncoding ribonucleic acids in patients with pre-diabetes.

Methods: Upon ethics approval, the blood samples (n=292) were collected from King Edward Hospital. They were divided into 3 experimental groups; non-diabetic (ND, n = 30 which consist of samples from 20 females and 10 males), pre-diabetic (PD, n = 90 which consists of samples from 56 females and 34 males) and type 2 diabetic (T2D, n = 172 which consists of samples from 113 females and 59 males). This was done using the American Diabetes Association criteria. In each sample, the concentration of immune cells and red blood cell indices were determined using haemocytometer which were analysed and reported on study 1 and study 2 respectively. Additionally, ELISA and Multiplex assay were used to measure concentration of select inflammatory markers for both study 1 and study 2. For study 3, samples

were divided into 3 experimental groups; non-diabetic (ND, n = 9 which consist of samples from 6 females and 3 males), pre-diabetic (PD, n = 15 which consists of samples from 11 females and 4 males) and type 2 diabetic (T2D, n = 22 which consist of samples from 16 females and 6 males). Real time polymerase chain reaction was used to measure the relative expression of lncRNAs.

Results: Results showed abnormal ranges of neutrophils (below normal range = 40-60%) and basophils (above normal range = 0.5-1%) in all 3 groups. Lymphocytes, monocytes and eosinophils were within the normal range. Results showed an increase in basophils, eosinophils, and fibrinogen on PD group by comparison with ND group. There was also a statistically significant ($p < 0.05$) increase in CD40L and TNF-α on PD group by comparison with the ND group. The results showed a decrease in neutrophils, lymphocyte, monocytes, IL-6, CRP and P-selectin on PD group by comparison with ND group. The results also showed a decrease in RBC, HGB, HCT, MCV, MCH, and MCHC for all females per group by comparison to males and an increase in RDW in females by comparison to males per group. Results showed an increase in all RBC indices in the PD group by comparison with ND group. Findings also showed a statistically significant ($p < 0.05$) increase in expression of lncRNA-NRON in the PD group by comparison with the ND group. There was an increase in the expression lncRNA-DC in the PD group by comparison with the ND group while there was a decrease in expression of lncRNA-NTT in the PD group by comparison with the ND group.

Conclusion: The findings of this study indicated that there is immune activation, sub-clinical inflammation, hematological changes, and the expression of various lncRNAs during the pre-diabetic state in humans. While these findings warrant further investigations, they will form a foundation for further investigation of pre-diabetes-associated changes in immune and haematological indices.

CHAPTER 1: INTRODUCTION

1.0 Background

Type 2 diabetes (T2D) is a condition characterized by persistent metabolic disturbances caused by hyperglycemia that is a result of insulin resistance [1]. This condition has been shown to lead to the development of various micro-and macrovascular complications. However, the onset of T2D is often preceded by a state of intermediate insulin resistance and moderate hyperglycemia known as pre-diabetes [2]. Pre-diabetes is an asymptomatic condition that is characterized by glucose levels are above the homeostatic range, but not high enough for a diagnosis of T2D [2]. According to the International Diabetes Federation (IDF), by 2035, there will be a global increase in the number of people with pre-diabetes to approximately 471 million from 316 million data captured in 2013 [3]. Furthermore, it has been shown that the incidence of both pre-diabetes and T2D is increasing in developing countries and this has been associated with the observed rapid urbanization. This urbanization has been linked to sedentary lifestyles, as well as increased consumption of foods rich in refined carbohydrates and saturated fats which have been shown to be leading factors in the development of insulin resistance. Long noncoding ribonucleic acids (lncRNAs) are noncoding RNAs that are not translated to proteins, consisting of larger than 200 nucleotides in length [4, 5]. LncRNAs has been targeted as novel therapeutic strategies since they have been reported to regulate the expression of genes (tissue- and cell-specific expression) in health and diseases such as T2D [4-6].

There are numerous studies that have reported on the effect of insulin resistance and hyperglycaemia in T2D on white blood cells and chronic inflammation [7-10]. However, no research has been done to investigate the changes in white blood cells and inflammation during the prediabetic state. Furthermore, T2D has been reported to result in changes to the red blood cells indices and erythropoietin expression [7, 11-15]. While there have been some studies that have investigated haematological changes during the prediabetic state, there are limited studies that have investigated these changes in humans with pre-diabetes. Additionally, while there are several studies that have reported that there is expression of lncRNAs by immune cells during T2D, none have reported on this phenomenon during pre-diabetes.

The city of Durban in South Africa is a multi-ethnic, rapidly urbanizing area. A recent study by Sosibo and colleagues, reported on the increasing prevalence of pre-diabetes among this population particularly in the age group of 25 years to 45 years [16]. These findings on the prevalence of pre-diabetes in this population provided the opportunity to study the changes in the body that are brought about by pre-diabetes. Therefore, this study sought to investigate changes in immune cells, red blood

cell (RBCs) indices and long noncoding ribonucleic acids (lncRNAs) in patients with pre-diabetes, aged 25-45 years from Durban, South Africa.

1.1 AIM

To investigate pre-diabetes-associated changes in immune cells, red blood cell indices and long noncoding ribonucleic acids in patients with pre-diabetes from aged 25 to 45 years from Durban, South Africa.

1.2 RESEARCH QUESTIONS

1.2.1 What is the effect of pre-diabetes on immune cell and inflammatory marker concentrations?

1.2.2 What is the effect of pre-diabetes on red blood cell indices and erythropoietin concentrations?

1.2.3 What is the effect of pre-diabetes on the expression of long noncoding RNA's such as noncoding transcript in T- cells (lncRNA-NTT), noncoding Repressor of nuclear factor activated T-cells (lncRNA-NRON) and noncoding RNA expressed in dendric cells (lncRNA-DC)?

1.3 OBJECTIVES

The aim and objectives for each of the respective research questions stated in 1.2.1; 1.2.2 and 1.2.3 are stated below. The objectives for research question 1.2.1 are addressed under study 1, while the objectives for research questions 1.2.2 and 1.2.3 are addressed in study 2 and study 3 respectively.

Study 1

- Investigate the changes in concentration of immune cells in patients with pre-diabetes aged from 25 to 45 years in Durban, South Africa. This will include immune cells such as neutrophils, lymphocytes, monocytes, eosinophils and basophils.

- Investigate the changes in concentration of inflammatory markers in patients with pre-diabetes aged from 25 to 45 years in Durban, South Africa. This will include inflammatory markers such as interleukin-6 (IL- 6), Tumor necrosis factor- α (TNF-α), c-reactive protein (CRP), fibrinogen, P-selectin and cell differentiation 40 ligands (CD40L).

Study 2

- Investigate the changes in red blood cell indices in patients with pre-diabetes aged 25 to 45 years in Durban, South Africa. The red blood cells indices will include as mean cell volume (MCV), red cell distribution width (RDW), mean cell haemoglobin (MCH), mean cell haemoglobin content (MCHC), haemoglobin (HGB) and haematocrit (HCT).

- Investigate the changes in erythropoietin concentration in patients with pre-diabetes aged from 25- to 45 years in Durban, South Africa

Study 3

- Investigate the changes in expression of lncRNAs during the pre-diabetic stage on multi-ethnic patients from 25 to 45 years in Durban, South Africa. The lncRNAs are lncRNA-NTT, lncRNA-NRON and lncRNA-DC.

HYPOTHESIS

Pre-diabetes will cause immune activation, sub-clinical inflammation, dysregulate red blood cell indices and dysregulate expression of lncRNA-NTT, lncRNA-NRON and lncRNA-DC.

NULL HYPOTHESIS

Pre-diabetes will not cause immune activation and sub-clinical inflammation. Pre-diabetes will also not dysregulate red blood cell indices and cause dysregulation in the expression of lncRNA-NTT, lncRNA-NRON and lncRNA-DC.

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CHAPTER 2: LITERATURE REVIEW

INTRODUCTION

Type 2 diabetes (T2D) is characterized by metabolic and signalling abnormalities such as an increase in oxidative stress, shunting of the polyol pathway, activation of protein kinase C (PKC) pathway and formation of advanced glycation end products due to chronic hyperglycaemia (1). These metabolic and signalling abnormalities have been shown to trigger immune responses resulting in a dysregulated innate immune system (1, 2). During hyperglycaemic conditions, the production of neutrophils is increased (3). They are the first immune cells to migrate from circulation to the inflamed area where they secrete cytokines such as TNF-α, interleukin-1 (IL-1), and IL-6 (3). There is also increased production of lymphocytes in the bone marrow resulting in T-cells secreting cytokines such as IL-6 A and soluble CD40L (4, 5). The onset of T2D, however, is often preceded by a long-lasting condition called pre-diabetes (6). Pre-diabetes has been reported to be asymptomatic, therefore making it hard to study its progression in humans. Recently, a diet-induced prediabetic rat model was found to mimic the pre-diabetes condition so this model has allowed us to gain insights into the changes in the body during pre-diabetes (7). Studies done using this rat model showed various changes in immune cell concentration and upregulation of immune markers such as interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), c-reactive protein (CRP), fibrinogen, P-selectin and cell differentiation 40 ligands (CD40L) (8). However, none of these findings had been verified in human subjects. Recent studies show that Durban, a city in South Africa has rapidly increasing prevalence of pre-diabetes especially those in the age range of 25-45 years thus making it an ideal area for studying pre-diabetes (9-11). In manuscript 1 of this study, we used this population to investigate changes in immune cells and inflammatory marker status in the pre-diabetic state.

One of the characteristics of T2D is a shortened life span of erythrocytes and low levels of erythropoietin (EPO) (12-14). According to Buys *et al.,* During T2D, red blood cells (RBC) change ultra-structure due to overload of iron and non-enzymatic fibrinogen, which is subsequently associated with inflammation (15). Furthermore, Kim *et al.* mentioned that aged RBC and diabetic-RBC deformability are also affected by increased intracellular Ca^{2+} concentration, reduced ATP concentration and decreased nitric oxide levels (NO) (16). These effects have also been shown to affect the changes in blood cell indices such as mean cell volume (MCV), red cell distribution width (RDW), mean cell haemoglobin (MCH), mean cell haemoglobin content (MCHC), haemoglobin (HGB) and haematocrit (HCT) (17-19). In a

previous study, using a diet-induced pre-diabetic rat model, it was reported that there are changes in red blood cells indices (7, 20). However, these findings have not been verified on human pre-diabetic subjects. Therefore, manuscript 2 is sought to investigate the changes in red blood cells indices using population form age 25 to 45 years in Durban, South Africa.

Noncoding repressor of nuclear factor activated T-cells (lncRNA-NRON) has been reported to inhibit activity of nuclear factor of activated T-cells cytoplasmic-3 (NFATc3) when over-expressed which act as a NFAT trap, resulting in reduction of endothelial cells proliferation, and migration, exacerbating progression of atherosclerosis, which is one of the T2D abnormalities (21, 22). LncRNAs such as noncoding transcript in T- cells (lncRNA-NTT), noncoding Repressor of nuclear factor activated T-cells (lncRNA-NRON), and noncoding RNA expressed in dendric cells (lncRNA-DC) have been reported to contribute to immunity and inflammation (23-25). However, no reports have been explored during the prediabetic state. Therefore, manuscript 3 is sought investigate the state of long noncoding ribonucleic acids (lncRNA-NTT, lncRNA-NRON, and lncRNA-DC) on prediabetic patients aged of 25 to 45- years in Durban, South Africa.

NORMAL PHYSIOLOGY

Immune cells and inflammatory markers

Immune cells are white blood cells that respond to infection from invading particles and secrete cytokines for inflammation (26). These immune cells are categorised as neutrophils, lymphocytes, monocytes, basophils and eosinophils (26). Additionally, inflammatory markers allow cross-communication between the cells (27-29). According to Rogero and Calder, saturated fatty acids modulate the inflammatory response via toll-like receptors (TLR) signalling pathway (30). This is achieved via activation of neutrophil elastase in the gut, which then triggers activation of TLR4 and then resulting in activation of NF-kβ signalling pathways (30). Therefore, translocation of NF-kβ promotes pro-inflammatory signalling, resulting in the recruitment of other immune cells such as lymphocytes and monocytes (30, 31). According to Piwowar and co-workers, human plasma neutrophil elastase is targeted as the neutrophil activation marker, which correlates with inflammation degree (32). Under normal conditions, lymphocytes are produced from bone marrow and they are divided into T-cells and B-cells which then play a role in adaptive immune system (33). B-cells release antibodies such as IgE antibodies and IgA antibodies, that attack the invading particles therefore delivering antigen to

T-cells and these T-cells eliminate foreign articles through the release of cytokines for inflammation(33). Monocytes are immune cells that circulate in blood and later differentiate into macrophages and also undergo phagocytosis to eliminate the invading particles (34). These differentiated macrophages and dendric cells have been reported to present antigen to T-cells and secrete pro-inflammatory cytokines thereby activating the naïve $CD4^+$ T-cells (34). The basophils and eosinophils are produced in the bone marrow upon immune activation and are involved in inflammation since they also secrete cytokines upon activation (34). Additionally, basophils display the IgE antibody receptor on their surface (35, 36). Once they are recruited, they bind to IgE antibodies secreted by B-cells to activate them since a high-carbohydrate diet is associated with increased IgE serum, resulting in the release of cytokines such as IL-6, VCAM-1, and IL-4 for inflammation (35, 37). In manuscript 1 of this study, immune cell, and inflammatory marker levels from non-prediabetic individuals have been used as a control group that will guide on the changes in both the prediabetic and diabetic states.

Red blood cells

Red blood cells (RBCs) are the amplest type of cells in human blood and their function is to transport oxygen (O_2) from the lungs to all tissues and cells as well as to transport carbon dioxide (CO_2) from tissues to the lung for expulsion (38). There are RBCs indices used to detect the chances in RBCs amount, shape and effects caused by disturbances in these cells. These indices are MCV, RDW, MCH, MCHC, HGB and HCT. MCV is a measure of the average volume of the red cells (39). RDW is a measure of the size variability of circulating erythrocytes (18, 19). According to the literature, RDW is an inflammatory marker for cardiovascular mortality and morbidity and has been used as a predictive utility in heart failure patients (17, 18, 40, 41). The normal range of RDW is 11.5 % -14.5 % (18). According to recent literature, MCH measures the average haemoglobin amount in the average red blood cell (42). Kim and co-workers reported that MCHC is the parameter that is used to determine RBCs cytoplasmic viscosity and it also affects deformability of RBCs (16). HCT is defined as the volume percentage of the RBCs in the blood (43). The effect observed on RBCs under normal conditions are also influenced by erythropoietin (EPO). EPO is a hormone produced by kidneys and a small amount by the liver, which plays a pivotal role in the availability of circulating RBCs (13, 44, 45). However, the production of this endogenous hormone is induced by hypoxemia (44, 46). In response to hypoxemia, EPO produced is released to the blood and then carried to the bone marrow, where it has been reported to stimulate RBCs production (44, 46). Additionally, EPO production activity decreases as the increase of oxygen levels increases

to a normal state (44, 46). In manuscript 2 of this study, we investigated changes in RBC indices in prediabetic patients by comparison to non-prediabetic and diabetic individuals.

Long noncoding RNA

Emerging research has introduced long noncoding ribonucleic acids (lncRNAs) that are found in the human genome and are reported to be expressed by immune cells (25, 47-49). Long noncoding RNAs are noncoding RNAs that have been reported to display no evidence of protein-coding capacity and consist of more than 200 nucleotides that are expressed in cells, tissues and in developmental processes (24, 50). They have been reported to function in controlling cellular development processes, lineage specific differentiation and activation (50). LncRNAs have also been reported to be able to regulate the immune response through several different pathways, such as Nf-kβ /MAPK and JAK/STAT (51, 52). LncRNA-NTT is a 17 kb lncRNA which is expressed in the nucleus which was discovered by Liu and colleagues in 1997 (53). LncRNA-NTT is a noncoding transcript in $CD4^+$ T cells that was found to be expressed by $CD4^+$T-cell upon activation but has not been found on resting $CD4^+$T-cell (24, 53). LncRNA-NTT has been reported to be one of the lncRNAs with no open reading frame that is larger than 270 bp (53). Furthermore, this lncRNA has been reported to be a single-copy gene that reside in chromosome 6q23–q24, close to the interferon-γ receptor gene (*IFN-γR*)(53). A recent study by Yang et al., discovered that that lncRNA-NTT is also expressed by human monocytes/macrophages and is highly around 100- to 1000-fold upregulated in normal control (54). However, the function of lncRNA-NTT in regulating macrophage—T cell interactions still remain unclear.

LncRNA- NRON is a noncoding RNA found enriched in muscle which also include cardiac muscle, placenta, lymph nodes, thymus and spleen (55). In 2005, Willingham and colleagues discovered a 2.7 kb in length intronic repressor lncRNA of the nuclear factor of activated T cells (NFAT1) using short hairpin RNA (shRNA) library screen against 512 lncRNAs which is called lncRNA-NRON (56, 57). LncRNA-NRON consists of three exons, which can be alternatively spliced to yield transcripts that range in size from 0.8 to 3.7 kb (21, 58). Therefore, it has been reported that the NFAT1 function in T cells is regulated by this lncRNA-NRON (52). At rest, the NFAT1 of the T-cells is inactive and sequenced at the cytoplasm of the cells (52). Upon activation of T-cells, phosphatase calcineurin dephosphorylate NFAT1 which then trigger the translocation of NFAT1 from cytoplasm to the nucleus (52, 59). Furthermore, in the NFAT1 translocation pathway, lncRNA-NRON serve as the scaffolding of the interaction

of NFAT1 with IQGAP (IQ motif-containing GTPase activating protein) and other three inhibitory NFAT1 kinases which are CKe, GSKb, and DYRK (52, 59). This scaffolding with lncRNA-NRON leads to the retention of NFAT1 in the cytoplasm of the cell (52, 59). Functional synergy between lncRNA-NRON and IQGAP1that is reported to be the blocking interaction of NFAT de-phosphorylation, has been shown to be required for nuclear translocation/activation and induction of IL-2 (24, 52). Furthermore, a lncRNA-NRON-IQGAP axis in regulating NFAT-dependent cytokine such as IFN-γ (an NFAT1 targeting cytokine), production in activated $CD8^{+}$T-cells is still unclear.

LncRNA-DC in a long noncoding RNA that has been reported to regulate the differentiation of human monocytes into dendritic cells (DCs) and activation of profile of DCs in human subjects (52, 59). LncRNA-DC function in binding with STAT3 in the cytoplasm and this binding then further prevent STAT3 dephosphorylation and inactivation by the hormone tyrosine phosphatase SHP1 (25, 52). This then directly promotes the activity of STAT3 and reduce CD86 and CD80, as well as MHC-II expression on DCs (25, 52). LncRNA-DC has been reported to be highly expressed in all populations of the DCs (52). LncRNA-DC also promotes the expression of CD40, CD80, HLA-DR on DCs (52). According to Pi *et al*., lncRNA-DC promotes maturation of DC and inhibits invasion of trophoblast without $CD4^{+}$ T cells involvement (51). Furthermore, lncRNA-DC has been shown to control the immune response by causing the reduction of cytokines such as TNF-a, IL-12, IL-6, and IFN-γ (51). LncRNA-DC has also been reported to contribute to increasing of the concentration of IL-1b that is secreted by the dendritic cells (51). In manuscript 3 of this study, we measured (lncRNA-NTT, lncRNA-NRON and lncRNA-DC) in prediabetic patients and compared these with levels found in non-diabetic and T2D patients.

TYPE 2 DIABETES PHYSIOLOGY

Immune cells and inflammatory markers

T2D a metabolic disorder that arises due to insulin resistance and is associated with obesity, hyperlipidaemia and hypertension, which can all trigger the immune response (60, 61). During the immune response, there is increased production of immune cells such as neutrophils, monocytes, lymphocytes, eosinophils, and basophils in the bone marrow contributing to inflammation and progression of T2D (4, 5, 26, 62). During T2D, neutrophils have been reported to migrate from the bone marrow to inflamed areas such as adipose tissue where they

undergo NETosis and also release pro-inflammatory cytokines such as TNF-α and IL-6 (3, 31, 63). Due to glucotoxicity caused by hyperglycaemia in T2D, there is increased production of lymphocytes in the bone marrow resulting in T-cells secreting cytokines such as IL-6 for inflammation (4, 5). Recruited B-cells mature into plasma cells and then secrete antibodies such as IgE, IgM. B-cells also secrete cytokines such as IL-6 during immune response for inflammation due to chronic hyperglycaemia in T2D subjects (35, 37). The increased production of monocytes during T2D results in the monocytes secreting cytokines upon activation during the immune response (4). Monocytes are recruited into inflamed areas where they differentiate into macrophages (63, 64). Once in the inflamed area, the macrophages secrete cytokines such as monocyte chemoattractant protein-1 (MCP-1) due to inflammation, resulting in the attraction of more monocytes to the inflamed area (36). In T2D, the produced and circulating eosinophils have been shown to migrate to inflamed areas where they secrete cytokines such as IL-4 to sustain alternatively-activated macrophages for inflammation which contribute to the progression of T2D (65). Cytokines such as TNF-α, CRP and IL-6 have been reported to be elevated in T2D (66-71). These three cytokines have also been shown to contribute to inflammation and cardiovascular complications in T2D (69-72). T2D has been shown to have hyperfibrinogenaemia due to elevated fibrinogen levels (73-75). Additionally, due to endothelial damage caused by oxidative stress, T2D has been reported to have increased P-selectin levels and soluble CD40L, contributing to the progression of T2D and the development of cardiovascular diseases such as atherosclerosis (76-81). In manuscript 1 of this study, the concentration of immune cells and inflammatory markers were measured in plasma from non-diabetic and T2D patients to verify the existing literature while of more interest to this dissertation, these markers were investigated in the prediabetic state.

Red blood cells

In adults, aged above 55 years, red blood cells are affected by abnormalities caused by T2D with several factors seeming to play a role in these changes (38, 82, 83). According to the literature, the increase in age from around twenties, has been reported to contribute to a compromised immune system (83-87). Additionally, T2D has also been shown to suppress the immune system (60). Therefore, in T2D, age is also a factor contributing to changes in the immune system and progression of T2D.

Furthermore, it has also been shown that an increase in age, when combined with diabetes, elevates the effects of changes in blood constituents such as red blood cells and immune cells

(88, 89). According to recent literature, RBCs undergo haematological changes to their indices due to the challenges caused by oxidative stress resulting from hyperglycaemia in T2D (18, 42). It has also been reported that an increase in MCH and RDW is associated with T2D and coronary artery disease mortality (18). In a study by Jabeen *et al.* 51 people with diabetes (23 males and 28 females) between 35 and 65 years and discovered that there was a decrease in MCH in T2D patients regardless of gender (42). When the RBCs had been exposed to hyperglycaemic conditions for a long period in T2D, glycation occurred, resulting in changes in rheological properties and increased the glycation of haemoglobin (16). According to Jabeen *et al.*, poor glycaemic control correlates with an increase in HGB (42). The HGB level less than 13 g/dL in men and 12 g/dL in women has been reported to indicate anaemia, another long-term complication of T2D (90). According to Bizjak, MCV is decreased in T2D due to the reduced capacity of the vesicular, limiting the life span of the RBCs (91). In T2D, the RDW has been reported to be higher than normal, indicating anisocytosis, erythrocyte degradation, cell deformability and impairment of erythropoiesis (18, 41). These abnormalities have been reported to be reflecting increased oxidative stress and chronic inflammation (40). It has been reported that in T2D that there is an increase in HCT due to conditions such as hypercholesterolemia (92). Research findings also indicate that as T2D progresses, there are further increases in HCT (93). According to Natali *et al.*, increased HCT is an ischaemic heart disease risk factor through blood viscosity increase and altered blood rheology (92). One of the complications reported in T2D is chronic kidney disease, which also contributes to low EPO levels that are observed in T2D patients (39). In manuscript 2 of this study, the RBC indices and EPO levels were measured in plasma from non-diabetic, prediabetic and T2D patients.

Long noncoding RNA

At present, there are very few studies that have reported on the expression long noncoding RNAs expression during T2D (47, 94, 95). Additionally, most of these human studies report on other lncRNAs such as H19, MALAT1 and ADIPOQ-AS without reporting on the lncRNAs involved on this study. These studies showed that there is expression of lncRNAs in liver, muscles, heart, and adipose tissue on T2D patients (47, 94, 96). However, there is a report based on other abnormalities such as atherosclerosis (22, 23, 47, 97). Atherosclerosis has been reported as one of the inflammatory complications reported in T2D patients (21, 81).According to recent findings by Du and colleagues, lncRNA- NRON promotes the progression of atherosclerosis and contributes to instability of plaques (21). Du and colleagues further reported that lncRNA-NRON cause negative regulation of activity of NFATc3 which then

contributes to VSMC (vascular smooth muscle cells) function impairment and apoptosis (21). Additionally, this results in the increase in production of VEGFA, exacerbating intra-plaque angiogenesis in atherosclerosis (21). Circulating lncRNA-NRON levels has been reported to serve as a possible heart failure biomarker in the cardiac system and reported to cause reduction of fibrosis by inducing fibroblast NFATc3 phosphorylation (22, 55, 57). Furthermore, no study has reported on expression of lncRNA-NTT in the T2D state. In manuscript 3 of this study, we investigated changes in the expression of the selected lncRNAs in both the prediabetic and T2D state.

PRE-DIABETES

Pre-diabetes is the disturbance of glucose metabolism that often precedes the onset of type 2 diabetes (98, 99) In this condition, the blood glucose concentrations are above the homeostatic range but not high enough for a diagnosis of T2D (99). Pre-diabetes has been reported to be diagnosed using the following 2 criteria's which are the American Diabetes Association (ADA) and World Health Organisation (WHO).

WHO criteria: Fasting blood sugar level (FBG) (5.6 -6.9mmol/L), glycated haemoglobin (HbA1c) between 5.7 and 6.4% and glucose tolerance test, 2 hours after ingestion of a standardised 75-gram solution of glucose (7.8 -11.0mmol/L)**.**

ADA criteria : Impaired fasting blood glucose (FBG) (5.6 -7.0 mmol/L), glycated haemoglobin (HbA1c) (5.7-6.4%) and elevated 2h postprandial blood glucose (2hour-OGTT) (7.8 - 11.0mmol/L) (99)**.**

In this study, the ADA criteria was used to diagnose pre-diabetes in all manuscripts.

PREVALENCE OF PRE-DIABETES

Recent literature shows an increase of prevalence of T2D globally, in South Africa as well as in the South African province of KwaZulu Natal (KZN) (9-11). Since the onset of T2D is often preceded by pre-diabetes, it is therefore important to also look at the prevalence of pre-diabetes. According to the International Diabetes Federation, globally, by 2045, the people estimated prevalence of pre-diabetes will increase to 8.6% (548.4 million) from 7.5% (373.9 million), that was reported in 2019 (100). In South Africa, the prevalence of pre-diabetes has been reported to be about 15,56% (over 9 million) based on the findings by Sosibo *et al*.(10). That

study further reported from their findings that the estimate of pre-diabetes prevalence among provinces in South Africa is as follows; Limpopo with 48,0% pre-diabetes prevalence, Gauteng (24,7%), Free State (6,3%), Cape town (15,4%), KwaZulu-Natal (12,96%) and Eastern cape (9,81%) (10). Govender et al., reported that one of the provinces with co-existence of under- and over-nutrition where most females are affected by obesity is KwaZulu Natal (KZN), which include the city of Durban (9). Govender *et al*., further reported that these disturbances are caused by factors such as affordability, seasonal production or availability of certain foods, practice of culture, environment and how the people residing in KZN prefer their food and preparation (9). Sosibo and colleagues further noted that the highest high prevalence of pre-diabetes was among those aged 25-45 years (10, 11). Additionally, in 2018, Therin reported that pre-diabetes has been discovered to be the condition that last between 10-20 years (101). In addition to reports by Therin, other reports show that the average age of diagnosis of T2D is between 55 and 65 which aligns with the study by Sosibo *et al* (10, 11, 101-103).

CHALLENGES ON STUDYING PRE-DIABETES

Exploration of the progression of pre-diabetes stage has been hard, due to pre-diabetes being asymptomatic. Most of the studies that have reported on pre-diabetes progression have relied on the use of animal models (7, 8, 20, 104-108). One of the animal models which has been found to mimic the human condition of pre-diabetes uses chronic ingestion of a high-fat high-carbohydrate (HFHC) diet to induce pre-diabetes (7). The HFHC diet had diet consists of proteins (15 % Kcal/g), carbohydrate (55 % Kcal/g) and fats (30 % Kcal/g) and was derived from the “hamburger, fries and cold drink” diet composition (7). After the induction period, the rat model met the ADA criteria to diagnose pre-diabetes (7). The next section discusses some of the findings on pre-diabetes-associated changes in immune cells, inflammatory markers and RBCs indices (8, 20).

OBSERVED EFFECTS OF PRE-DIABETES IN ANIMAL MODELS

Immune cells and inflammatory markers

Using the HFHC diet-induced animal model, It has been reported that there are changes in immune cell concentration during the progression of pre-diabetes (8). The study found that cytokines such as IL-6 and TNF-α were increased, indicating a contribution to inflammation and progression of the prediabetic stage to the onset of T2D (8). Neutrophils and eosinophils

were shown to decrease from circulation during the progression of pre-diabetes indicating their recruitment in the inflamed areas such as adipose tissues(8). The neutrophils and eosinophils were also observed in adipose tissues embedded in-between the adipocytes at the end of the experimental period, indicating inflammation, which contributes to the progression towards the development of T2D (8). There was a sharp increase in blood percentage of the lymphocytes and basophils indicating that the stage is progressing to T2D (8). Research by Mzimela *et al.,* showed that the blood count of monocytes fluctuates in the diet-induced prediabetic rats as the stage progresses (8). This monocyte fluctuation was shown by an increase in monocyte percentage count at the onset of pre-diabetes, followed by a decrease 4 weeks later and then another increase after another 4 weeks during prediabetic state indicating that some monocytes might have been differentiating into macrophages and dendric cells (8).

Furthermore, this study by Mzimela *et al*., reported that TNF-α, CRP, IL-6, fibrinogen, P-selectin and soluble CD40L are upregulated during the pre-diabetes (8). These findings using animal models prompted further exploration of the effects of pre-diabetes on immune function in human subjects therefore manuscript 1 of this study sought to investigate the immune cells and inflammatory marker status on pre-diabetic patients from 25 years to 45 years in Durban, South Africa.

Red blood cells

Luvuno and colleagues showed that there is impaired renal function while Mzimela and colleagues showed that EPO levels remained normal in diet-induced pre-diabetic rats (20, 104). Other findings showed that that there is an increase in RBC production, which corresponds to the normal functioning of EPO production in kidneys (20). Other results showed that the MCH, MCHC, RDW and HCT decreases during the prediabetic state (20). Mzimela *et al.* also reported an increase in HGB during the progression of pre-diabetes (20). These findings paved the way for this study to investigate changes in human subjects therefore manuscript 2 of this study sought to investigate the changes in RBCs indices and state of EPO on pre-diabetic patients from 25 years to 45 years in Durban, South Africa.

Long noncoding RNA

Long noncoding RNAs has recently been discovered to have unique functions and as much as there are studies on other lncRNAs, but there is less evidence yet available based on the selected lncRNAs explored in this study. Using a rat model, it has been reported that lncRNA-NRON predominantly localizes to the nucleus in cardiomyocytes (57). Hoepfner et al., reported an

anti-hypertrophic role of lncRNA-NRON using neonatal mouse cardiomyocytes demonstrating that hypertrophic stimulation caused downregulation of lncRNA-NRON and expression of lncRNA-NRON upregulated hypertrophic markers (57). However, there is limited evidence on research based on lncRNAs such as lncRNA-NTT and lncRNA-DC. This highlighted a gap in the study of these long noncoding RNAs and their potential use as biomarkers of diseases that involve immune cells and inflammation. Therefore, manuscript 3 of this study sought to investigate the expression of lncRNAs (lncRNA-NRON, lncRNA-NTT and lncRNA-DC) in prediabetic patients from 25-45 years in Durban, South Africa, and their potential use as biomarkers in the pathophysiology and prognosis of pre-diabetes.

JUSTIFICATION OF THE STUDY

Type 2 diabetes is a metabolic disease characterised by impaired glucose regulation which results in chronic hyperglycaemia (1, 2). Chronic hyperglycaemia reported on T2D causes abnormalities such as suppressed immunity, chronic inflammation and altered haematological indices (1, 2, 14). Additionally, recent evidence has shown that lncRNAs contribute to immunity, inflammation, and exacerbation of T2D abnormalities such as atherosclerosis (21, 24, 49). Recent evidence has shown that long noncoding RNAs such as lncRNA-NRON, lncRNA-NTT and lncRNA-DC are expressed by certain immune cells (47-49, 97). Pre-diabetes is a long-lasting state of intermediate hyperglycaemia that often precedes the onset of T2D (109). Due to the asymptomatic nature of this condition, it is often hard to diagnose, and it has consequently been challenging to document the changes associated with immune function during pre-diabetes. A high fat, high carbohydrate (HFHC)-diet induced rat model of pre-diabetes that has been shown to mimic the human condition has been further shown to report on immune activation, upregulation of inflammatory markers and changes in RBCs indices (7, 8, 20). However, the changes seen in the animal model have not been shown in humans with pre-diabetes. Recently, Sosibo and colleagues reported on increased prevalence of pre-diabetes among population from 25 years to 45 years in Durban, South Africa (11). The city of Durban in South Africa has a very culturally diverse population with an evident increase in urbanisation. Additionally, this city has been reported to be affected by different factors such as unhealthy diets and sedentary lifestyles that predispose it's population to developing metabolic conditions such as pre-diabetes. However, there is no research that has investigated changes in immune cells, inflammatory markers, RBC indices and lncRNAs in the pre-diabetic

stat in this population. Therefore, manuscript 1 sought to investigate the changes in immune cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) and inflammatory markers (IL-6, TNF-α, CRP, fibrinogen, P-selectin, and CD40L) status in patients with pre-diabetes aged from 25 years to 45 years in Durban, South Africa. Manuscript 2 sought to investigate the changes in red blood cells indices ((MCV, RDW, MCH, MCHC, HGB and HCT)) and the concentration of EPO in this population. Manuscript 3 sought to investigate the expression of lncRNAs (lncRNA-NRON, lncRNA-NTT and lncRNA-DC) in patients with pre-diabetes from the age of 25 years to 45 years in Durban, South Africa

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CHAPTER 3: METHODOLOGY

Introduction

Chapter 3 gives a general description of how the study was conducted. The sub-sections of this chapter include, study design, sample size calculation of the various manuscripts, the research area, population of the study, the inclusion and exclusion criteria, data collection, diagnosis of pre-diabetes, experimental work, and the statistical analysis.

Chemicals/reagents

All chemicals and reagents of were of analytical grade and sourced from standard suppliers.

Ethical considerations

The ethical clearance of the study was approved by Biomedical Research Ethics Committee (BREC) of the College of Health Sciences, the University of KwaZulu Natal (BREC REF NO: BE266/2019). Ethical clearance was obtained before the collection of samples from King Edward Hospital

Study site, population, and design

Study site.

Blood samples were collected from King Edward hospital upon approval by UKZN Biomedical Research Ethics Committee. The study was then conducted at University of KwaZulu Natal in Durban, South Africa.

Study Design

A quantitative cross-sectional analytical study was conducted using the stored blood sample collected from population of the study. The blood sample of the study population was divided into 3 groups. The 3 groups were blood samples collected from non-diabetic, pre-diabetic and T2D individuals. The blood samples were collected from population from all ethnicities that are from ages 25 years to 45 years from February 2021 to December 2022 upon signed informed consent. The samples selection was according to selection criteria of the study and data provided by hospitals.

Sample size calculation

To calculate the desired sample size necessary for prevalence of pre-diabetes in Durban population targeted in this the study, a reported prevalence of approximately 15 % was used (1). The formular was as follows.

$$n = \frac{z^2 \times P \times (1-P)}{E^2}$$

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.

In this study, 95% confidence level, which is a Z-score of 1.96 was used. The assumed prevalence (p) of approximately 15 % and 5% of margin desired precision (E) was used. Interestingly the results obtained yielded a required sample size of 202 to have 95% confidence level. In study 1 and 2 we then had a sample size of 292 (n=292). In Chapter 5, The Gpower software 3.1.9.4 was used to calculate the sample size, and the sample size obtained for power of 0.95 was 36 (2). However, for manuscript 3 we had sample size of 46 where there were 9 in non-diabetic (ND), 15 pre-diabetic (PD) and 22 type 2 diabetics (T2D). The input and output are displayed as follows.

F tests - ANOVA: Repeated measures, within factors

Analysis: A priori: Compute required sample size

Input:

Effect size f = 0.25

α err prob = 0.05

Power (1-β err prob) = 0.95

Number of groups = 3

Number of measurements = 4

Corr among rep measures = 0.5

Nonsphericity correction ε = 1

Output:

Noncentrality parameter λ = 18.0000000

Critical F = 2.6964690

Numerator df = 3.0000000

Denominator df = 99.0000000

Total sample size = 36

Actual power = 0.9515295

Sample screening and pre-diabetes diagnosis

Inclusion and exclusion criteria

The blood sampling inclusion criteria were as follows.

- Bood sample of the patient that is non-diabetic, pre-diabetic and T2D.
- Blood samples of patients that are between the age of 25 years and 45 years, both genders and all races.

The blood sampling exclusion criteria included the following samples,

- Patients that were below the age of 25 years and above 45 years of age.
- Patients without history of liver disease, thyroid disease, kidney disease, heart disease, depression, and HIV.
- Professional sport athletes
- Blood samples of patients under the influence of alcohol and also pregnant females.

Diagnosis of pre-diabetes

The American Diabetes Association (ADA) criteria was used to confirm whether a study participants was non-diabetic, pre-diabetic or type 2 diabetic (3). In addition to glucose levels provided by hospital, glycated hemoglobin was measured using respective human ELISA kits from Elabscience, as per manufacturer's instructions. From the results obtained from human ELISA, with glucose levels, then the samples that had HbA1c that was below 5.7% were considered to be non-diabetic, HbA1c between 5.7% and 6.4% considered to be pre-diabetic and then HbA1c above 6.4 % considered to be T2D.

Population of the study

Study 1 and 2 had a population of 292 samples. For study 1 and 2, the population was divided into the following groups: The non-diabetic (ND) group with 30 blood samples which were obtained from 20 females and 10 males; The pre-diabetes (PD) group with 90 blood samples which were obtained from 56 females and 34 males; and the type 2 diabetes (T2D) group with

172 blood samples which were obtained from 113 females and 59 males. For study 3, there was population of 46 samples. The population was divided into the following groups: The non-diabetic (ND) group with 9 blood samples which were obtained from 6 females and 3 males; The pre-diabetes (PD) group with 15 blood samples which were obtained from 11 females and 4 males; and the type 2 diabetes (T2D) group with 22 blood samples which were obtained from 16 females and 6 males.

Experimental design of all 3 studies

Study 1

In study 1, the concentration of immune cells (neutrophils, lymphocytes, monocytes, basophils, and eosinophils) of all 3 groups were measured from fresh blood using hemocytometer (Beckman Coulter, Indianapolis, United States) (Beckman Coulter, Indianapolis, United States). The inflammatory markers (TNR- α, IL-6, P-selectin and CD40L) of all 3 groups were measured from plasma using, a customized human Invitrogen "ProcartaPlex", 4-plex multiplex assay kit from Thermofisher Scientific as per manufacturer's protocol. Additionally, other inflammatory markers for study 1(CRP and fibrinogen) of all 3 groups were measured using respective human ELISA kits, as per manufacturer's instructions. More information on the methodology for study 1 is given in manuscript 1 (Chapter 3).

Study 2

In study 2, the red blood cells indices (HGB, HCT, WBC, MCV, MCH, MCH, and RDW) of all 3 groups were measured from fresh blood using hemocytometer (Beckman Coulter, Indianapolis, United States) (Beckman Coulter, Indianapolis, United States). Additionally, in study 2, human EPO ELISA kits form Elabscience was used to measure the concentration of EPO of all 3 groups, as per manufacturer's instructions. More information on the methodology for study 2 is given in manuscript 2 (Chapter 4).

Study 3

In study 3, to accomplish measurement of the expression of lncRNAs of all 3 groups, RNA was first extracted and purified from plasma using Pure Link RNA Mini Kit (Thermofisher scientific, Carlsband, USA) as per manufactures instructions. Purity of extracted RAN was then confirmed using a nano drop (Thermofisher scientific, Carlsband, USA) and an OD range 260/280 ratio greater than 1.8 (>1.8) indicated pure extracted RNA. Furthermore, RNA was then reverse transcribed to synthesize complementary DNA (cDNA) from extracted RNA was

completed using the SuperScript III first-strand synthesis super mix (Thermofisher scientific, Carlsband, USA) following the qRT-PCR protocol as per manufacturer's guidance. Before doing PCR, the obtained cDNA had to be normalized and standardized to a concentration of 400 ng/mL and 10 µL qPCR reaction volume per well was then prepared with TaqMan advanced master mix (2X) (Thermofisher scientific, Carlsband, USA), TaqMan Assay (20X) and nuclease-free water as per manufacturer's protocol. Lastly, the Quantitative Real-Time qPCR (RT-qPCR) was used to measure the expression of all lncRNAs (NRON, DC, and NTT) in all 3 groups of study 3 and all samples were measured in triplicate for all assays performed. More information on the methodology for study 2 is given in manuscript 3 (Chapter 5).

Statistical data analysis

SPSS statistics v28 was used to analysed data for all 3 groups in all studies (Study 1, study 2 and study 3). Comparison of the groups per study was done by application of One-way ANOVA and Tukey's post hoc test using SPSS v28. Data expressed as means ± standard error of means (±SEM) and values of $p < 0.05$ indicated statistical significance.

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BRIDGE 1

Chapter 4 will provide an overview of studies investigating the changes in circulating levels of immune cells as well as the status of selected markers of inflammation in pre-diabetic individuals from age of 25 years to 45 years in Durban, South Africa. Chapter 4 consists of 3 sections: a protocol for a systematic review, a systematic review, and an original research manuscript. All 3 manuscripts have been published, and the details are shared within the chapter.

CHAPTER 4: SYSTEMATIC REVIEW PROTOCOL, SYSTEMATIC REVIEW AND RESEARCH MANUSCRIPT 1

(IMMUNITY)

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled "**The Changes That Occur in the Immune System During Immune Activation in Patients with Pre-diabetes from All Ethnicities, aged 25-45 Years: Protocol for a Systematic Review and Meta-analysis**" and is authored by N.C Mzimela, A.M Sosibo, P.S Ngubane, and A. Khathi. Manuscript is published in **JMIR Research Protocols (ISSN: 1929-0748)** and has been formatted according to journal's guidelines for authors (https://doi.org/10.2196/31619). This journal is accredited by Department of Higher Education and Training South Africa and appears in Scopus accredited list (2021).

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

The Changes That Occur in the Immune System During Immune Activation in Patients with Pre-diabetes from All Ethnicities, From the Age of 25 to 45 Years: Protocol for Systematic Review and Meta-analysis

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Abstract

Background: Pre-diabetes is an asymptomatic, intermediate state between normoglycemia and the onset of type 2 diabetes mellitus. Recent reports indicate that during pre-diabetes, there are subclinical changes to immune cells and inflammatory markers. Therefore, this systematic review will provide a synthesis of the available data on the changes in the concentration of immune cells and selective inflammatory markers. It will also give evidence of a demographic impact on changes or complications in the pre-diabetes state.

Objectives: The objectives of the study are to create a protocol that will be used to analyze the collected data of previously published research based on immune cells such as neutrophils, lymphocytes, monocytes, eosinophils, and basophils, as well as inflammatory markers such as C-reactive protein, tumor necrosis factor-alpha, interleukin-6, P-selectin, cluster of differentiation 40 ligand, and fibrinogen. Additionally, an impact of demographics will be determined using the previously published data collected.

Methods: This protocol was prepared through adhering to the PRISMA (Preferred Reporting Items for Systemic Reviews and Meta-Analysis) 2015 guidelines for reporting protocols. Published clinical studies that involve observational—whether it is cross-sectional, comparative cross-sectional, case-control, or cohort—study designs that include normal or nondiabetic and pre-diabetes reports will be used in this systematic review and meta-analysis. This will be accomplished by using clinical MeSH (Medical Subject Headings) to search on MEDLINE, Cochrane library, and African Journal Online. Reviewers (NCM, AMS, and AK) will screen all the results and select the studies that meet the eligibility criteria. Downs and Black Checklist will be used to check the risk of bias, and then a Review Manager v5.4 forest plot will be used for meta-analysis. Additionally, the forest plot will also be used for sensitivity analysis. The strength of evidence will then be assessed using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) approach.

Results: Since July 5, 2020, there are no participants recruited. Publicly available data will be used in the review and will be collected after this protocol publication. No ethics approval is required as no subjects will be used, and analysis will be based on reported data. Authors will be contacted if there was a misunderstanding related to reading their reported data.

Conclusion: The findings will clarify changes that might be observed in a study of interest based in the eThekwini district in South Africa.

Registration details: This protocol has been registered with the International Prospective Registry of Systematic Reviews (PROSPERO) registration (CRD42020184828) dated 05-07-2020.

Keywords: systematic review; meta-analysis; pre-diabetes; immune cells; inflammatory markers; diabetes; inflammatory response; immunology; demographics; risk factors

Article Summary

- ✓ Pre-diabetes stage is an asymptomatic state where the blood levels of glucose are above normal but below the threshold for diagnosis of type 2 diabetes.
- ✓ Patients who are prediabetic have been reported to have complications on the immune system globally, which will be reported in the systematic review.
- ✓ The protocols that will be used for checking bias will be Downs and Black checklist, while for synthesis, review manager forest plot will be used.

GRADE approach will be applied to assess the strength of evidence.

Introduction

Type 2 diabetes (T2D) is a metabolic disorder characterized by chronic hyperglycemia, which gives rise to metabolic and signaling abnormalities [1,2,8]. According to Kayal and Graves [8], these metabolic and signaling abnormalities have been reported to cause dysregulated innate immunity. Chronic dysregulated immunity includes changes in immune cells such as neutrophils, lymphocytes, monocytes, eosinophils, and basophils [15,20,29,32]. Upon activation, these immune cells play a different role, including secretion of inflammatory markers such as C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), P-selectin, cluster of differentiation 40 ligand (CD40L), and fibrinogen [6,7,15,17,20,23]. The chronic immune activation in T2D results in a suppressed immune system [8,22]. According to Lam and LeRoith [13], a fundamental change in the population with T2D is witnessed by the health care communities. This was confirmed by the International Diabetes Federation statistics, reporting that in 2019, there were 19 million people with diabetes in Africa aged 20-79 years [1]. The International Diabetes Federation also reported that there were 12 million Africans aged 20-79 years living with undiagnosed diabetes in 2019 [1]. South Africa is the highest with 4.6 million adults with diabetes (20-79 years) [1]. In 2017, the Indian population was reported to have the highest prevalence of diabetes in South Africa by 11%-13%, followed by people of color by 8%-10%, then Black people by 5%-8%, and White being the lowest by 4% [3,24]. The Indian population among people with diabetes has been shown to be high due to their strong diabetes genetic predisposition [3,24]. However, the onset of T2D arises from the progression of pre-diabetes [19]. Pre-diabetes has been reported to be an asymptomatic state, creating a research complication in its documentation of the statistics and prevalence. There is less evidence on the changes in immune cells and selective inflammatory markers at the pre-diabetes stage [21,26,28,33]. However, in our laboratory, research has been conducted on animals in addition to the available research reporting the

metabolic and signaling abnormalities, including immune activation during pre-diabetes [14,16,18,25]. This then raised a debatable issue if the same abnormalities occur during pre-diabetes in human individuals owing to limitations in the animal models, even though the research mimicked the human diet. From the search conducted, we found no report or evidence of the systematic review that reports on the changes in immune cell concentration and the level of secretion of selective inflammatory markers that occur during immune activation during pre-diabetes. Therefore, our research presents an opportunity to compile a systematic review that will yield an exhaustive synthesis obtained from the available studies that previously reported on immune cells and selective inflammatory markers of interest in pre-diabetes. Additionally, this systematic review will give reports on the impact of demographics on changes of immune cells and secretion of selective inflammatory markers during pre-diabetes.

The objectives of this study are as follows: (1) to determine the changes in concentration of immune cells, such as neutrophils, lymphocytes, monocytes, eosinophils, and basophils during pre-diabetes; (2) to investigate if there are changes in concentration on selected inflammatory markers, such as CRP, TNF-α, IL-6, P-selectin, CD40L, and fibrinogen, during pre-diabetes; and (3) to assess the variation of pre-diabetes-associated changes in immune function among different demographic groups.

Methods

This protocol was prepared by adhering to the PRISMA (Preferred Reporting Items for Systemic Reviews and Meta-Analysis) 2015 guidelines for reporting protocols [27].

Systematic Review Registration

The protocol has been registered with PROSPERO with registration number "CRD42020184828," dated July- 05-2020.

Ethics Approval and Consent to Participate

The data analyzed will be those that have already been published, and there will be no data collection from individuals. The authors declare that there will be no informed consent required to be signed; therefore, no ethics approval is required for the systematic review and meta-analysis.

Eligibility Criteria for the Study

Studies with a minimum of 100 participants (N=100) and the studies that report community-based clinical cross-sectional study will be eligible. The inclusion and exclusion criteria will be as follows: inclusion—the information reported from nondiabetic adult patients aged 25-45 years from all ethnicities will be used; exclusion—information reported from people with a history of liver disease, kidney disease, heart disease, and depression will not be used. Information from pregnant women will also not be used. Additionally, no samples from professional sports athletes will be allowed in the study. Full-text articles or reports indicating that individuals who were used were free from all the mentioned criteria will then be eligible.

Pre-diabetes Diagnosis Criteria

Diagnostic criteria for pre-diabetes will be as follows (participants should meet 1 of the following diagnoses): fasting blood glucose—5.6 to 7.0 mmol/L; 2 hours postprandial blood glucose (2 hours oral glucose tolerance test)—7.8 to 11.0 mmol/L with glycated hemoglobin (5.7%-6.4%).

Study Design

Participants

Intervention

These will be clinical studies that involve observational studies if they are cross-sectional, comparative cross-sectional, case-control, or cohort study designs that involve normal (nondiabetic) and pre-diabetes reports. The reported information that involves one or more immune cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) in the

prediabetic state will be eligible for this systematic review. Additionally, studies that report information that involves at least one or more inflammatory markers of interest, which are CRP, TNF-α, IL-6, P-selectin, CD40L, and fibrinogen, will also be eligible for this systematic review.

Comparators

In this systematic review, the eligible comparing control groups will be normal (nondiabetic) control and T2D control groups.

Outcomes

This systematic review is expected to show the following results: (1) the changes in concentration of immune cells such as neutrophils, lymphocytes, monocytes, eosinophils, and basophils during pre-diabetes (reported as odds ratio and 95% CI); (2) the changes in concentration of selected markers such as CRP, TNF-α, IL-6, P-selectin, CD40L, and fibrinogen during pre-diabetes (reported as odds ratio and 95% CI); and (3) variations in markers of immune function among different demographic groups based on gender, age, and race (reported as the mean).

Search Strategy

The electronic search strategy will be used as an identification for studies involving cohorts that have been studied that are related to the study of interest [30]. This strategy will be accomplished by search on MEDLINE (from 1963 to 2020), Cochrane library displaying results of trials from PubMed, CT.gov, Embase, ICTRP (from 1963 to 2020), as well as African Journal Online (from 1998 to 2020) [30]. In addition to these search strategies, clinical MeSH (Medical Subject Headings) and text will be applied to filter the available information. For all search conducted, the keywords to be used will be “pre-diabetes and immunity,” “pre-diabetes and immune cells,” “pre-diabetes and leucocytes,” and “pre-diabetes and inflammation.”

Identification of Eligible Studies

The title and abstracts of all the obtained results will be screened by reviewers (NCM, AMS, and AK), and the studies that meet the eligibility criteria will then be selected. Each reviewer will be responsible for screening all the selected study reports before the decision-making of the eligible reports. The PRISMA flowchart for selection of studies will then be provided in the reports from the systematic review.

Patient and Public Involvement

No patient was involved.

Data Management

Study Records and Data Extraction

The data of the study records that are selected as eligible reports will then be extracted and recorded in an Excel (Microsoft Corp) file. The predefined list of variables to be considered in each report will be used as categories in an Excel file. Considering the research of interest, the outcome of interest will mainly be the immune cell response and concentration of selected markers in both genders, at an age parameter of interest, and in all ethnicities. However, the value of the baseline characteristic of the data reported will also be considered. Therefore, the baseline characteristics of the eligible research reports obtained will be author, year of publication, country, and study setting. The methodology of the study reported will also be considered with categories including design, period, sampling strategy, and whether participants are from a normal or prediabetic population. Finally, the outcomes from different genders, ages, ethnicities, and immune cell changes or inflammatory markers will then be extracted.

Data Simplification

Studies that report on the immune cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) will be combined into a single group. Additionally, the studies that report on selected

inflammatory markers (CRP, TNF-α, IL-6, P-selectin, CD40L, and fibrinogen) will also be combined into a single group.

Risk of Bias

The potential risk of bias in individual studies will be obtained using the Downs and Black Checklist [9]. The scores will be rated as follows: excellent (25-26), good (20-24), moderate (14-19), poor (11-13), and very poor (<10). Three reviewers (NCM, AMS, and AK) will be responsible for the independent judgments, which will be based on the 4 domains of the Black and Downs checklist tool, which are reporting bias (10 items), external validity (3 items), internal validity (6 items), and selection bias (7 items). In a situation where there will be a difference of opinions between NCM, AMS, and AK, author PSN will be responsible for adjudication. In situations where the data are not clear, the investigator who reported the data will be contacted 3 times. If no response is obtained, data will be then excluded from the eligible report.

Data Synthesis

For the meta-analysis of reported data, a forest plot will be used from Review Manager software version 5.4 (RevMan) [4,5,11]. Using this forest plot, eligible data from all reported studies will be analyzed depending on their sample size and the mean of the concentration of immune cells or inflammatory markers in prediabetic and control groups. Additionally, an odds ratio and CI will be used to make the forest plot where the solid lines will represent the 95% CI. Each reported study will be represented as a horizontal line on the y-axis to list the primary author and year of study. The forest plot will also include the weight of the study results that will be automatically obtained using the Review Manager software.

Sensitivity Analysis

RevMan forest plot will also test for heterogeneity, where greater homogeneity will be indicated by a greater overlap between the CIs [11]. Using the forest plot, I^2 will then be

calculated where a value between 0% and 100% will be obtained. A value obtained less than 25% will be an indication of a strong homogeneity, and a value obtained greater than 75% will be an indication of a strong heterogeneity. However, a value of 50% will be considered as an average value.

Assessment of Strength of Evidence

NCM, AMS, and AK will then be responsible for the assessment of the strength of evidence. The studies included in the review will then be evaluated using the Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE) [10,11,31]. Furthermore, the summary of findings table will then be created using a GRADE pro (McMaster University and Evidence Prime Inc) tool.

Results

As of July 5, 2020, no participants have been recruited as publicly available data will be used. These data will be collected when this protocol has been published. There will be no ethics approval required as the review is based on published data, and authors will be contacted if there is a misunderstanding from reading their reported data for clarity of their published work.

Discussion

Principal Findings

The synthesis of previous study reports obtained from this systematic review and meta-analysis will clarify the complications on the immune system at pre-diabetes such as the changes that have been reported on immune cells, which are neutrophils, lymphocytes, monocytes, eosinophils, and basophils. This systematic review and meta-analysis will also give an outstanding synthesis of data from previous reports based on selected inflammatory markers of interest.

Conclusion

The synthesis from this systematic review and meta-analysis will create a hallmark of association between demographics and pre-diabetes. This will clarify changes that might be observed in a study of interest based in the eThekwini district in South Africa.

Acknowledgments

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Authors' Contributions

NCM, AMS, and AK were responsible for brainstorming, designing the study, and drafting the protocol. NCM, AMS, PS, and AK were responsible for reviewing the eligible study and final draft of the manuscript. Funders had no role in developing the protocol.

Conflicts of Interest

None declared.

Abbreviations

CD40L: cluster of differentiation 40 ligands

CRP: C-reactive protein

GRADE: Grading of Recommendations Assessment, Development, and Evaluation

IL-6: interleukin-6

MeSH: Medical Subject Headings

PRISMA: Preferred Reporting Items for Systemic Reviews and Meta-AnalysisT2d: type 2 diabetes

TNF-α: tumor necrosis factor-alpha

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Additional file 1

PRISMA-P 2015 Checklist

This checklist has been adapted for use with protocol submissions to *Systematic Reviews* from Table 3 in Moher D et al: Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Systematic Reviews* 2015 **4**:1

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☐	☐	
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☒	☐	
Authors					
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	☒	☐	
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☒	☐	
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☐	☐	
Support					
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
Sponsor	5b	Provide name for the review funder and/or sponsor	☒	☐	
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☒	☐	
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	☒	☐	
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e.,	☒	☐	

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
		screening, eligibility, and inclusion in meta-analysis)			
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☒	☐	
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	☒	☐	
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☒	☐	
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☒	☐	
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)	☒	☐	
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☒	☐	
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☒	☐	

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☒	☐	
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☒	☐	

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled "**The changes that occur in the immune system during immune activation in pre-diabetic patients of all ethnicities, from the age of 25- to 45-years: A systematic review and meta-analysis**" and is authored by N.C Mzimela, A.M Sosibo, P.S Ngubane, and A. Khathi. Manuscript is published in **Medicine (ISSN: 1536-5964)** and has been formatted according to the journal's guidelines for authors.(https://doi.org/10.1097/md.0000000000030903). This journal is accredited by Department of Higher Education and Training South Africa and appears in ISI accredited list (2022).

Author Contribution: NC Mzimela was responsible for study conceptualization, study design, investigation, data analysis, first draft writing, and manuscript editing.

The changes that occur in the immune system during immune activation in pre-diabetic patients of all ethnicities, from the age of 25- to 45- years: a systematic review and meta-analysis (PRISMA)

Short title: Changes in immune cells at pre-diabetes stage

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List of Abbreviations

Type 2 diabetes (T2D)

C-reactive protein (CRP)

Tumour necrosis factor-alpha (TNF-α)

Interleukin-6 (IL-6)

Cluster of differentiation 40 ligands (CD40L)

International Diabetes Federation (IDF)

Reporting items for systemic reviews and meta-analysis (PRISMA)

Fasting blood glucose (FBG)

Medical Subject Heading (MeSH)

Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE)

Summary of findings (SoF)

National Research Foundation (NRF)

ABSTRACT

Background: Pre-diabetes is an intermediate state between normoglycaemia and type 2 diabetes (T2D). This condition has been shown to be asymptomatic thus making it hard to investigate the changes that occur in the body during this state. Recent findings stipulate that in this state, there are changes that are often associated with T2D. These include changes in concentration of immune cells and inflammatory markers. This systematic review will provide a synthesis of the data that is available reporting on the changes in the concentration of immune cells and selected markers during pre-diabetes. It will also give clarity of the variation of the complications of the condition among the various demographic groups.

Methods: The assembly of this systematic review was through strict adherence to the PRISMA 2020 guidelines for reporting systematic reviews. This systematic review has been registered with the International Prospective Registry of Systematic Reviews (PROSPERO), registration number "**CRD42020184828**" dated 05-07-2020). In this systematic review, published clinical studies articles that involve observational reports, whether it is case-control, cross-sectional, and comparative cross-sectional will be used. Cohort study designs that involve normal/non-diabetic and pre-diabetes reports will be used in this systematic review and meta-analysis. Clinical MeSH headings to search on MEDLINE, COCHRANE library, EMBASE, and ICTRP and African Journal Online will be a tool used to achieve the required report. Reviewers (NCM, AMS & AK) will screen all the results and select the studies that will be eligible by guidance according to eligibility criteria. Downs and Black Checklist will be used to check the risk of bias and then for meta-analysis Review Manager v5.4 Forrest plot will be used. Additionally, the Forrest plot will also be used for sensitivity analysis. The strength of evidence will then be assessed using the Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE).

Results: Only 4 reports were eligible, and risk of bias checked. The results indicated the outcomes even though there were only few reports**.**

Discussion and conclusion: This systematic review will give an indication on the available data on this research area and lay a foundation for future studies.

Keywords: Immune cells; Pre-diabetes; Systematic review; Meta-analysis; Inflammatory markers

INTRODUCTION

Pre-diabetes is an intermediate state between normoglycemia and the onset of type 2 diabetes (T2D) stage [1]. This condition is characterized by blood glucose concentrations being higher than normal but below the threshold for diagnosis of T2D [2,3]. According to projections made by the International Diabetes Federation (IDF) in 2019, the number of diabetic adults (20-79 years) in Africa is 19 million, where South Africa has the highest with 4.6 million diabetic adults [3]. In 2017, the Indian population within South Africa had been reported to have the highest prevalence (11-13%) of diabetes in the country, followed by the Coloured population with 8-10%, then Blacks with 5-8% and Whites being lowest with 4% [4,5]. The Indian population has been shown to be high due to their strong genetic predisposition to developing T2DM [4,5]. The statistics from the IDF indicate that South Africa has a high prevalence of T2D and also postulate that there is also a high prevalence of undiagnosed pre-diabetes [5]. The pre-diabetic state is often asymptomatic, thus making it difficult to document the prevalence of the condition. Recently, this condition has been the focus of many studies so as to identify and understand the metabolic and signalling complications occurring in this condition. The eThekwini district is an ethnically and culturally diverse area which is South Africa's third-largest city. Additionally, it offers a convenient lifestyle with a host of amenities that cater to every budget enabling easy access to consumption of high calorie diets and sedentary lifestyles. These living conditions raise the risk of developing metabolic disorders such as pre-diabetes and T2D. Taking into account these statistics, it is then of utmost importance to report on the abnormalities that occur during pre-diabetes. More recently, studies in our laboratory using animal models have shown that complications often associated with T2D begin during the pre-diabetic state [6-11]. In these studies, it was shown that the presence of pre-diabetes could lead to compromised immunity [6,7]. These reports indicated that during the progression from pre-diabetes to overt T2D, there are changes in immune cell concentrations (neutrophils, lymphocytes, monocytes, eosinophils, basophils) and upregulation of inflammatory markers (CRP, TNF-α, IL-6, P-Selectin, CD40L, and Fibrinogen) [7]. This report on animal models lays a platform for research on individuals that are found to be pre-diabetic. Globally, there are reports on innate immunity based on immune cell changes and inflammation during pre-diabetes. One of the earliest research on one of the immune cells was done by Hitchcock and his co-workers on lymphocyte subsets during pre-diabetes in 1986 [12]. Hitchcock and his co-workers only used 65 participants and the focus was only on lymphocytes [12]. However, based on the obtained data, it shows that the researcher's focus was not on pre-diabetes and immunity

until the increase in statistics of T2D around the 1990s and 2000s [13-16]. Research on pre-diabetes in South Africa or specifically in the eThekwini district is very scarce even though the rate of the increase in T2D is very high. Additionally, there is no data obtained based on pre-diabetes and immune activation on different ethnicities at the eThekwini district. The average age of diagnosis for T2D in South Africa is between 55- to 65 years old while pre-diabetes is said to last between 10 to 20 years before the onset of T2D [17]. Even among the age of 25- to 45- years, no data is reporting on immune cells and selective markers on pre-diabetic human subjects in the eThekwini district. This systematic review sought to identify the gaps regarding changes in the immune system during pre-diabetes. Another objective was to highlight differences amongst the different genders and ethnicities of people living in the eThekwini District.

METHODS

Adhering to the preferred reporting items for systemic reviews and meta-analysis (PRISMA) 2015 guidelines for reporting protocols (PRISMA checklist attached in additional file 1), the protocol was registered with the International Prospective Registry of Systematic Reviews (PROSPERO) registration number "**CRD42020184828**" dated 05-07-2020).

Eligibility Criteria for the study

Only studies with a minimum of 100 population size and community-based clinical cross-sectional studies were eligible. This study worked with stored blood samples. The inclusion and exclusion criteria were as follows.

Inclusion: The information reported from non-diabetic adults within the ages of 25-45 of all ethnicities was used.

Exclusion: Information reported from people with a history of liver disease, kidney disease, heart disease and depression were not used. Information from pregnant women was also not used. Additionally, no studies from professional sports athletes were allowed in the study. The full-text article/reports that indicate that the subjects that were used were free from all the mentioned criteria were then eligible.

Ethics Approval and consent to participate.

The data that will be analysed will be the data that is published and there will be no data collection from subjects. The authors declare that there will be no informed consent required to be signed and therefore no ethics approval required for the systematic review and meta-analysis.

Pre-diabetes diagnosis criteria

Diagnostic criteria for pre-diabetes were as follows (participants should meet one of the following diagnoses): fasting blood glucose (FBG): 5.6 -7.0 mmol/L; 2 h postprandial blood glucose (2 h - OGTT): 7.8 -11.0mmol/L with glycated haemoglobin (HbA1c): 5.7-6.4%.

Information Sources

The information sources were any reported clinical study that involved a minimum of 100 participants, either males or females and both genders, aged from 25 to 45 years from all ethnicities. These clinical studies involved observational studies if they were cross-sectional, comparative cross-sectional, case-control or cohort study designs that involve normal and pre-diabetes reports. The reports that contained information that involves specifically one or more immune cells (neutrophils, lymphocytes, monocytes, eosinophils, basophils) in the pre-diabetic state were eligible. Additionally, any study that reports information that involves at least one or more inflammatory markers of interest, which are CRP, TNF-α, IL-6, P-Selectin, CD40L, and fibrinogen was also eligible.

Search Strategy

The electronic search strategy was used for identification of studies involving cohorts that have been done that are related to the study of interest. This strategy was accomplished by search on MEDLINE (from 1963 to 2020), COCHRANE library displaying results of trials from PubMed, CT.gov, EMBASE, and ICTRP (from 1963 to 2020) and African Journal Online (from 1998 to 2020). Added to these search strategies, the use of clinical MeSH headings and text words was applied to filter the available information. For all searches done, the keywords used were “pre-diabetes and immunity,” “pre-diabetes and immune cells,” “pre-diabetes and leucocytes,” and “pre-diabetes and inflammation.”

Identification of eligible studies

The title and abstracts of all the obtained results were screened by reviewers (NCM, AMS & AK) and the studies that met the eligibility criteria were then selected. Basically, each reviewer was responsible for screening all the selected study reports before the decision making of the eligible reports. The PRISMA flow chart for the selection of studies is shown in Additional file 2. The author was contacted twice if data reported was unclear for clarity.

Study Records and data extraction.

The data of study records selected as eligible reports were then extracted and recorded in a Microsoft Excel file. The pre-defined list of variables to be considered in each and every report was used as categories in a Microsoft Excel file. Considering the research of interest, the outcome of interest was mainly the immune cell response and concentration of selective markers in both genders, at an age parameter of interest in all ethnicities. However, the value of the baseline characteristic of the data reported was considered. Therefore, the baseline characteristics of eligible research reports obtained were author, year of publication, country, and study setting. The methodology of the study reported was then considered with the categories (design, time period, sampling strategy and whether participants are normal or pre-diabetic population) considered. Finally, the outcomes from different gender, ages, ethnicity, immune cell changes /inflammatory markers were then extracted.

Data simplification

Studies that report on the immune cells (neutrophils, lymphocytes, monocytes, eosinophils, basophils) were combined into a single group. Additionally, the studies that report on selective inflammatory markers (CRP, TNF-α, IL-6, P-Selectin, CD40L, and Fibrinogen) were also combined into a single group.

Risk of bias

The potential risk of bias in individual studies was obtained by using the Downs and Black Checklist [18]. The scores were rated as follows; excellent (25–26), good (20–24), moderate (14–19), poor (11–13), and very poor (< 10). Three reviewers (NCM, AMS and AK) were responsible for the independent judgments which were based on the four domains of the Black and Downs checklist tool which is reporting bias (10 items), external validity (3 items), internal validity (6 items), and selection bias (7 items). In a situation where there was a difference in opinions between NCM, AMS and AK. PSN was then responsible for adjudication.

Data synthesis

For the meta-analysis of reported data, a Review Manager version 5.4 software (RevMan) Forrest plot was used [19]. Using this Forrest plot, eligible data from all reported studies were analyzed depending on their sample size and the mean of the concentration of immune cells or inflammatory markers in both pre-diabetic and control groups. Additionally, the odds ratio and confidence interval were used to plot the Forrest plot where the solid lines represented the 95% confidence interval. Each reported study was represented as a horizontal line on the y-axis to

list the primary author and year of study. The forest plot also included the weight of the study results at automatically detected by RevMan software.

Sensitivity Analysis

The RevMan forest plot was also used to test for heterogeneity, where a greater overlap indicated greater homogeneity between the confidence intervals [19]. Using the forest plot, I^2 was then calculated where a value between 0 and 100 % obtained. A value obtained less than 25% was considered to be an indication of a strong homogeneity and a value obtained greater than 75 % was then considered to be an indication of a strong heterogeneity. However, a value of 50 % was considered as an average value.

Assessment of Strength of Evidence

NCM, AMS and AK will then be responsible for the assessment of the strength of evidence. The studies included in the review will then be evaluated using the Grading of Recommendations Assessment, Development and Evaluation approach (GRADE) [20-22]. Furthermore, the summary of finding (SoF) table was then created using a GRADE pro tool.

RESULTS

Search report results and eligible reports

Using the six databases mentioned in the search strategy paragraph, there were 4924 reports results captured. However, there were only five reports that were eligible for this systematic review and meta-analysis.

Scope of the reviewed reports

Among these five reports, two articles had information on the overall white blood cell count (WBCs) [23-25]. However, one article by Zhang *et al* was then eliminated due to the fact that it had the hazard ratio calculated not odds ration due to the type of study conducted [24]. Additionally, Grossman *et al.* also had additional reports on lymphocytes and monocytes [23]. Interestingly, 4 of the eligible articles reported on inflammatory markers [23,25-32]. The inflammatory markers reported were CRP, IL-6, fibrinogen and TNF-α. However, no information was obtained from markers P-selectin and soluble CD40L in the pre-diabetic state. A Microsoft Excel table was then created according to the categories mentioned in the extraction of the data paragraph. (Additional file 3). Additionally, from the reports obtained, we could extract the CRP and TNF-α in different races to investigate if there is an impact of

demographic changes on CRP levels at the pre-diabetes stage. However, with the other markers, it was not possible.

Risk of Bias Assessment

All the four eligible reports undergo the risk of bias assessment using a Downs and Black checklist. The Aukour *et al* obtained 11 points and both Grossmann *et al* and Lucas *et al* obtained 18 points. Additionally, Sabanayagam *et al* obtained 19 points. However, we used all the four eligible reports since we already have less reports for the study (see Additional file 4)

Forrest plot report of meta-analysis and predictor of heterogeneity on immune cells and inflammatory markers articles

All the eligible reports were assessed for meta-bias, as shown in Figures 1 and 2. Figure 1 shows the RevMan Forrest plot of eligible evidence obtained from (a) monocytes, (b) lymphocytes and (c) WBC. In figure 1a, heterogeneity could not be calculated because there was only one article reporting on monocytes in pre-diabetes. However, the odds ratio obtained was 1.27 with (1.19,1.36) CI, which favoured the control ($p<0.00001$). Heterogeneity could also not be calculated on figure 1b because there was only one article reporting on lymphocytes in pre-diabetes. However, the odds ratio obtained was 1.10 with (1.05,1.15) CI, which indicated the slight shift from control to experimental shift in pre-diabetes ($p<0.0001$). Heterogeneity was not obtained from the analysis of WBC evidence collected due to one article used. However, the odds ratio of 1.22 and (1.14,1.31) CI ($p<0.00001$) was obtained, as shown in figure 1c.

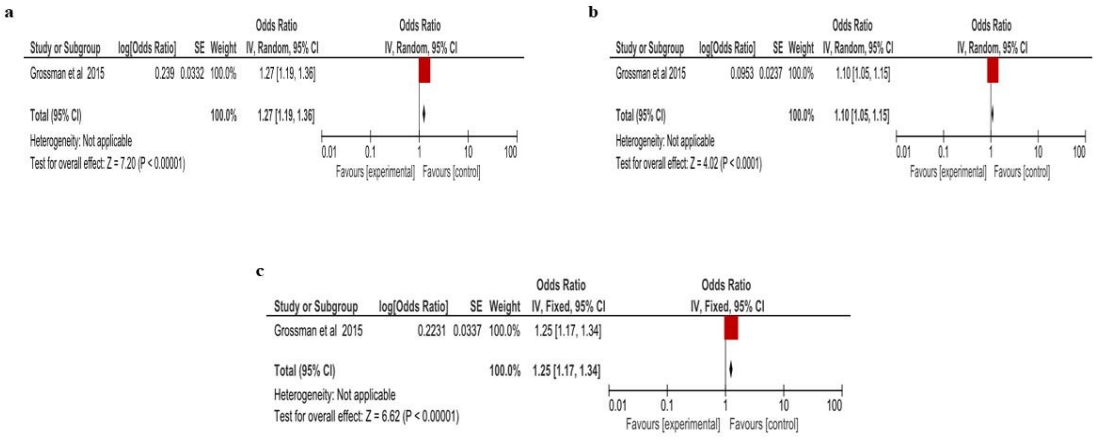

Figure 1: Forest plot of meta-analysis of ND vs PD in different studies where graph a represent Monocytes, b represents Lymphocytes, and c represent WBC.

The Forrest plot on figure 2a shows a meta-analysis of IL-6 evidence of obtained indicating a strong heterogeneity of 89% at the pre-diabetes stage and pooled estimate of 0.94 (OD) and (0.56,1.57) CL which indicates favouring the experimental group. Figure 2b shows the meta-analysis of TNF-α where I^2 indicated a slightly above average heterogeneity of 61% with the pooled estimate of 1.07 (OD) and (1.01,1.13) CI ($p<0.02$) indicating favouring the control. Figure 2c reports on CRP meta-analysis of evidence obtained showing a strong heterogeneity of 86% with a pooled estimate of 1.09 (OD) and (1.00,1.19) CI ($p<0.05$) which favours control. Fibrinogen report obtained is indicated in the Forrest plot on figure 2d, where an odd ratio of 1.33 and (1.25,1.42) CI was reported at $p<0.00001$.

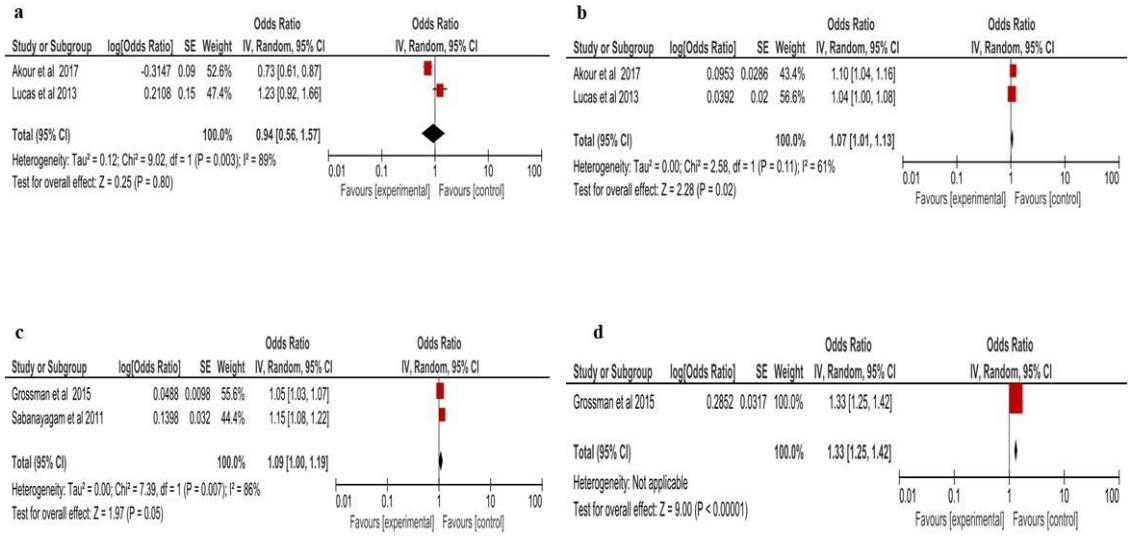

Figure 2: Forest plot of meta-analysis of ND vs PD in different studies where graph a represent IL-6, b represents TNF-α, c represents CRP and d represent fibrinogen.

Quality assessment of the pre-diabetic research reports

Table 1 shows the SoF of assessed reports with the number of studies columns, quality assessment column, effects column, quality column and importance column. These columns

were then created to summarise the assessed eligible reports by the reviewers enabling them to draw conclusion to the available evidence obtained.

Table1: SoF of eligible assessed reports

		Quality assessment				Effect	Heterogeneity %	Quality	Importance
	n	Indirectness	Inconsistency	Imprecision	Risk of Bias	Odds ratio and Confidence intervals			
Immune cells									
WBC	2	high	moderate	moderate	moderate	1.22 (1.14,1.31)	18	high	important
Monocytes	1	high	moderate	moderate	moderate	1.27 (1.19,1.36)	n/a	average	important
Lymphocytes	1	high	moderate	moderate	moderate	1.10 (1.05,1.15)	n/a	average	important
Inflammatory markers									
IL-6	2	moderate	high	moderate	moderate	0.94 (0.96,1.57)	89	high	important
TNF-α	2	high	high	moderate	moderate	1.07 (1.01,1.13)	61	high	important
CRP	2	high	high	moderate	moderate	1.09 (1.00,1.19)	86	high	important
Fibrinogen	1	high	high	moderate	moderate	1.33 (1.25,1.42)	n/a	average	important

DISCUSSION

The search of eligible data or reports in relation to the study based on immune cells concentration and selective inflammatory markers in the pre-diabetic state was very challenging due to a small amount of data available on the research of interest. Additionally, pre-diabetes is generally asymptomatic thus limiting the number of people that get diagnosed and thereby leading to few studies being conducted on the condition. Several studies have shown that a number of abnormalities previously ascribed only to T2DM actually begin during pre-diabetes [2,13,33]. These abnormalities include low-grade inflammation and innate immune system suppression [34-37]. However, 5 of the studies from the search obtained, were eligible for meta-analysis of this review displaying different characteristics from the criteria mentioned above [23,24,27,29,32]. However, in these 5 articles one article required request on information from the authors since the report was on hazard ratio instead of odds ratio [24]. The authors then indicated that the study was prospective research which then required them to use the survival analysis and cox regression analysis to estimate the hazard ratios [24]. This issue then resulted in elimination of the article, as odd ratios are not applicable for the study [24]. The selection of these eligible reports was also due to reviewers prioritizing the diagnostic criteria and eligibility criteria with more challenge being the sample size, age, gender, and race not being in line with the required criteria in some studies for them to be meta-analyzed in this review. As an

example, research by Lucas and his co-workers consists of 41 subjects which were all females and also the age ranged from 18 to 45 years which was less than the sample size indicated in our criteria and the age gap ranged from below 25 years [32]. However, a study by Lucas *et al.* was of value as it enabled us to meta-analyze the reports on CRP and calculate heterogeneity which indicated a strong heterogeneity with I^2 reported to be 86%.

Interestingly, much data was collected from Grossman and his co-workers, as it reported data on WBC, monocytes, and lymphocytes [23]. As much as we could not obtain the results of heterogeneity of WBC, lymphocytes, and monocytes due to these results being the only single data eligible, we could however conclude that the lymphocytes favoured the experimental group. Additionally, WBC and monocytes favoured the control.

As for the basophils, eosinophils and neutrophils, no data was obtained and eligible for meta-analysis which is raising a strong and outstanding value of our study of interest to report on these three immune cells during pre-diabetes and in subjects aged from 25 to 45 years. The Forrest plot on IL-6 indicated the strong heterogeneity where the pooled estimate favoured the experimental group as indicated using two reports obtained [27,32]. This can be hypothesized that it is possible due to the research obtained from T2D, having elevated levels of IL-6 [38]. Additionally, the meta-analysis for TNF-α using the obtained eligible reports indicated a slightly above average heterogeneity without much shift of the pooled estimate. This does not give us much of a conclusion because the sample size of the two eligible studies was not large enough to give accurate results but indicating a slight shift of change in concentration at pre-diabetic stage. CRP reports meta-analysis indicated a strong heterogeneity with an assumption that the accuracy of the pooled estimate is influenced by the large sample size reported by both eligible articles indicating changes during pre-diabetes [23,29]. However, with other inflammatory markers such as CD40L and P-selectin, there was no information obtained, and fibrinogen was only obtained in one article. This then raises the value of the studies that report on the previously mentioned markers to give enough clarity at the pre-diabetic stage.

From the results obtained, the evidence indicated the good quality even though it was not enough and had limitations. Additionally, there was an elimination of articles that indicated a high risk of bias, more errors on sample selection and contacted authors that did not respond. This review raises a strong value of presenting an understandable research publication that is clear and states all information such as diagnostic criteria, indicating if the participants signed

informed consent and indicating the disadvantages such as loss of participants during the study or contact during follow up to avoid bias.

Strengths

Unavailability of enough reports to analyze for this review give strength to the value of the research of interest for us to explore more on the pre-diabetic stage at the area and country of interest. Additionally, it gives a platform to publish enough information on the immune cells and inflammatory markers that are not yet available on published work on the prediabetic state.

Limitations

Few articles obtained based on immune cells and inflammatory markers of interest at the prediabetic stage. Some of the articles contained small sample size; some did not specify gender and race. These characteristics limited the ability to select outstanding work based on what has been done. Additionally, less research has been done in other countries based on research concerning immune cells and inflammation at the pre-diabetic stage. Some articles do not use the ADA criteria for the diagnosis of T2D.

CONCLUSION

The collected evidence gives clarity that there are changes in WBCs concentration and inflammatory markers such as IL-6, TNF-α and CRP. However, not enough evidence reported if there are changes in immune cells (monocytes, lymphocytes, basophils, eosinophils, and neutrophils) individually and markers CD40L and P-selectin at the pre-diabetes stage. The fact that we could only identify one eligible evidence on monocyte, lymphocyte, and fibrinogen, allows evaluating the changes that may be possible to occur during pre-diabetes in our research of interest. Additionally, due to the reason that some articles did not specify race and gender, we could not collect evidence if there are changes during pre-diabetes, which also adds value to our proposed research based on the impact of demographic changes on immunity and pre-diabetes.

Acknowledgements

The authors would like to express gratitude to National Research Foundation (SA) for funding.

Consent for publication.

Not applicable

Availability of supporting data

No extra data available besides the attached additional files since it is a systematic review.

Authors contributions

NCM, AMS, and AK were responsible for brainstorming, designing the study, and then also drafted the systematic review. NCM, AMS, PS, and AK were responsible for reviewing the eligible study, analysing the articles obtained and final draft of the manuscript. Funders had no role in development of the systematic review.

Competing Interests

The authors declare no competing interests.

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PRISMA 2020 Checklist -Additional file 1

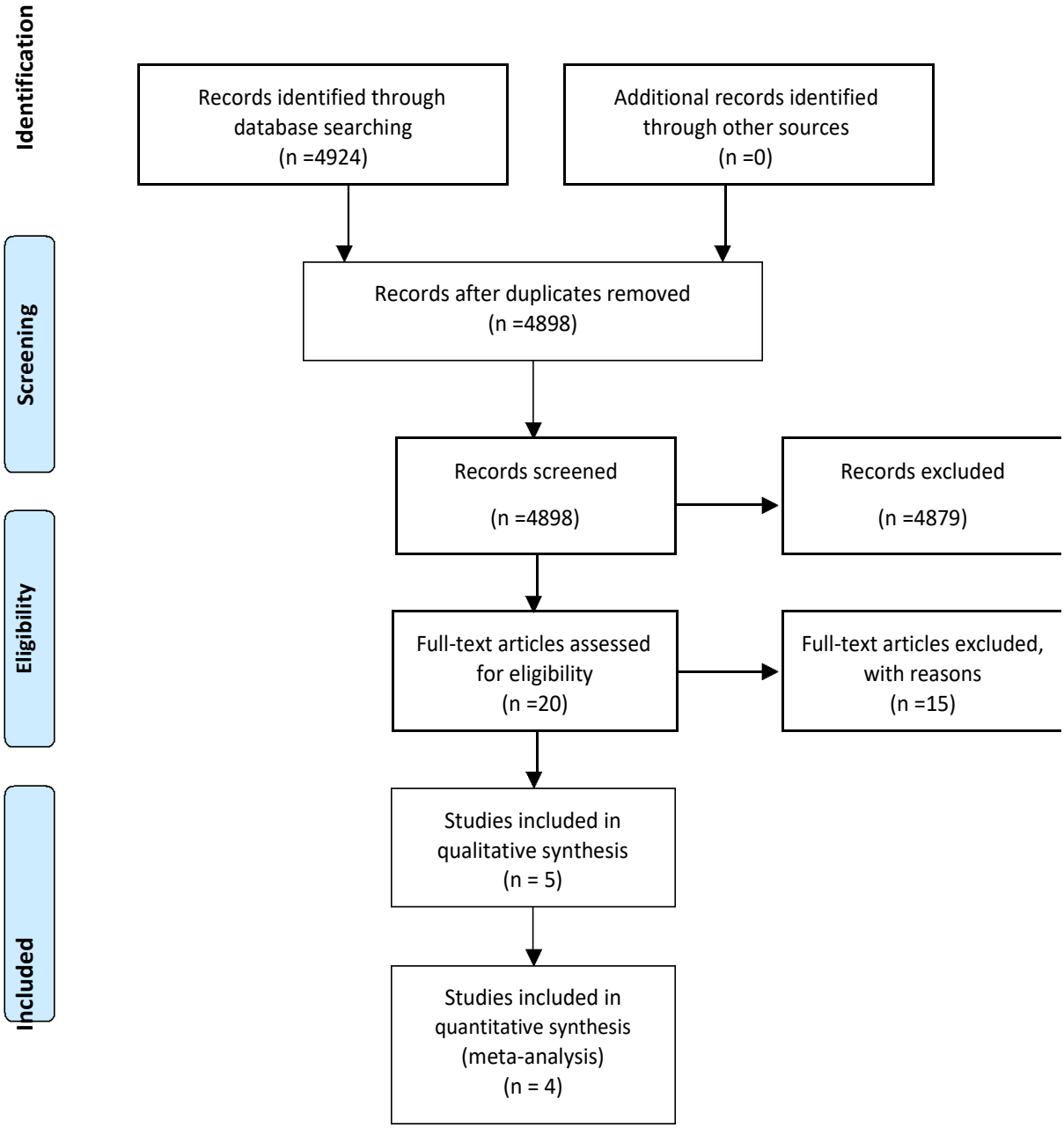
Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Cover page
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Page 2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Page 3
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Page 4
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 5
Search strategy	7	Present the full search strategies for all databases, registers, and websites, inclu6ding any filters and limits used.	Page 5
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Page 5
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Page 5
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Page 6
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Page 5
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 6
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Page 6
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Page 7
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Page 7

Section and Topic	Item #	Checklist item	Location where item is reported
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Page 7
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Page 7
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Page 7
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Page 7
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 7
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Page 7
Study characteristics	17	Cite each included study and present its characteristics.	Page 7
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Page 8 and additional file
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Page 8
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Page 8
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Page 8
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Page 8
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	n/a
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Page 9
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Page 9- Page 11
	23b	Discuss any limitations of the evidence included in the review.	Page 11

Section and Topic	Item #	Checklist item	Location where item is reported
	23c	Discuss any limitations of the review processes used.	Page 11
	23d	Discuss implications of the results for practice, policy, and future research.	Page 11
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Page 4
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Page 4
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	n/a
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Page 12
Competing interests	26	Declare any competing interests of review authors.	Page 12
Availability of data, code, and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Page 12

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71
For more information, visit: http://www.prisma-statement.org/

PRISMA Flow diagram -Additional file 2



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

Eligible reports captured information - Additional file 3

Baseline characteristics				
Author Names	**Year of Publication**	**Country**	**City**	**Study setting**
Akour *et al*	2017	Jordan	Amman	Diabetes,Endocrinology, and Nutrition outpatient clinics at JUH and NCDEG
Grossmann *et al*	2015	Germany	Gutenberg	Population based Health Study (GHS)
Lucas *et al*	2013	Georgia	Augusta	Local communities of Augusta
Sabanayagam *et al*	2011	Republic of Singapore	Singapore	Singapore Prospective Study Programme

Methodology			
Author Names	**Design**	**Time period**	**Sampling strategy**
Akour *et al*	Randomised sample and Experimental	10-hr overnight fast	screening for potential recruitment
Grossmann *et al*	prospective,observational, single-center cohort	04/2007 to 04/2012	clinical data assessment
Lucas *et al*	HbA1c measurement and experimental		recruited from the local communities of Augusta
Sabanayagam *et al*	population-based cross-sectional and experimental		Questionnaire and clinic examinations

Outcomes				
Author Names	**Population**	**Gender**	**Age**	**Ethinicity**
Akour *et al*	235 subjects	79 men and 156 women	>18 year	Caucasians
Grossmann *et al*	15,010 individuals	7,584 men and 7,426 women	35–74 years	
Lucas *et al*	41 subjects	41 women	18–45 years	African American
Sabanayagam *et al*	6,589 subjects	3,054 men and 3,535 women	24–95 years	Chinese, Malay and Indians

Outcomes	
Author Names	**Immune cell /infammatory markers**
Akour *et al*	plasma OXT, high-sensitivity C-reactive protein (hs-CRP), macrophage chemoattractant protein 1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1), matrix metalloproteinase 9 (MMP-9), resistin, adiponectin, leptin, macrophage migration inhibitory factor (MIF), tumor necrosis factor α(TNF-α), thrompospondin 1 (TSP-1), interleukin 10 (IL-10),interleukin 6 (IL-6), and glucagon.
Grossmann *et al*	samples were analyzed for white blood cells (WBCs), granulocytes, lymphocytes, monocytes, platelets, C-reactive protein (CRP), albumin, fibrinogen, and hematocrit. Interleukin-18 (IL-18), IL-1 receptor antagonist (IL-1RA), and neopterin concentrations
Lucas *et al*	interleukin (IL)-5, IL-6, IL-7, tumor necrosis factor-α (TNF-α), granulocyte-monocyte colony-stimulating factor (GM-CSF),interferon-γ (IFN-γ), IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12p70 and IL-13
Sabanayagam *et al*	sensitive CRP

Downs and Black - additional file 4

Downs and Black checklist	Grossmann *et al*	Akour *et al*	Sabanayagam *et al*	Lucas *et al*
Reporting				
1. Is the hypothesis/aim/objective of the study clearly described?	yes	yes	yes	yes
2. Are the main outcomes to be measured clearly described in the Introduction or Methods section?	yes	yes	yes	yes
3. Are the characteristics of the participants included in the study clearly described?	yes	no	yes	yes
4. Are interventions of interest clearly described?	yes	no	yes	yes
5. Are the distributions of principal confounders in each group of subjects to be compared clearly described?	yes	yes	yes	yes
6. Are the main findings of the study clearly described?	yes		yes	yes
7. Does the study provide estimates of the random variability in the data for the main outcomes?	yes	yes	yes	yes
8. Are all adverse events of interventions reported?	yes	n/a	yes	yes
9. Are characteristics of patients lost to follow-up described?	n/a	n/a	yes	n/a
10. Are probability values reported to main outcomes?	yes		yes	yes
External Validity				
11. Were the subjects asked to participate in the study representative of the entire population from which they were recruited?	yes	yes	yes	yes
12. Were those subjects prepared to participate representative of the entire population from which they were recruited?	yes	yes	yes	yes
13. Were the location and delivery of study treatment a represantative of source population?	n/a	n/a	n/a	n/a
External Validity-BIAS and Confounding				
14. Were the participants blinded to treatment?	n/a	n/a	n/a	n/a
15. Was the blinded outcome assessed?	n/a	n/a	n/a	n/a
16. Was there any data dragging clearly described?	n/a	n/a	n/a	n/a
17. Were there any analysis adgustments for differing lengths and follow-ups?	n/a	n/a	n/a	n/a
18. Were the statistical test perfomed appropiate?	yes	yes	yes	yes
19. Was the compliance with interventions reliable?	yes	n/a	yes	yes
20. Were the outcome measure used accurate (valid and reliable)?	yes	yes	yes	yes
21. Were the participants in different intervention groups (trials and cohort studies) or were the cases and controls (case-control studies) recruited from the same population?	yes	yes	yes	yes
22. Were study participants in different intervention groups (trials and cohort studies) or were the cases and controls (case-control studies) recruited over the same period of time?	yes	yes	yes	yes
23. Were the participants randomised to treatment?	n/a	n/a	n/a	n/a
24. Was the allocation of treatment concealed from investigators and participants?	n/a	n/a	n/a	n/a
25. Was there adequate adjustment for confounding in the analyses from which the main findings were drawn?	yes	yes	yes	yes
26. Were the loss to follow-up taken into account?	n/a	n/a	n/a	n/a
Power				
27. Was there a sufficient power to detect treatment effect at significance 1 level of 0.5?	yes	yes	yes	yes
TOTAL	18	11	19	18

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled " **Investigation into Changes in Inflammatory and Immune Cell Markers in Pre-diabetic Patients from Durban, South Africa**" and is authored by N.C Mzimela, A.M Sosibo, P.S Ngubane, and A. Khathi. The manuscript is published in **Immunotoxicology (ISSN: 1547-6901)** and has been formatted according to journal's guidelines for authors (https://doi.org/10.1080/1547691X.2023.2290282). This journal is accredited by Department of Higher Education and Training South Africa and appears in ISI accredited list (2022)

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

Investigation into Changes in Inflammatory and Immune Cell Markers in Pre-diabetic Patients from Durban, South Africa

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Running title: Inflammatory marker/immune cell changes in patients with pre-diabetes

Keywords: Immune cells, inflammatory markers, pre-diabetes, Durban, South Africa

Abstract

The prevalence of pre-diabetes is increasing in rapidly urbanizing cities, especially in individuals aged 25-45 years old. Studies also indicate that this condition is associated with aberrant immune responses that are also influenced by environmental factors. This study sought to investigate changes in the concentration of immune cells and select inflammatory markers in patients with pre-diabetes in Durban, South Africa. Blood samples collected from King Edward Hospital, after obtaining ethics approval, these were divided into non-diabetic (ND), pre-diabetic (PD) and type 2 diabetic (T2D) using ADA criteria. In each sample, the concentration of immune cells and select inflammatory markers were determined. The results showed a significant increase in eosinophil and basophil levels in the PD group as compared to the ND group. Compared to ND, the PD and T2D groups had significant increases in serum TNFα, CD40L and fibrinogen concentrations. Additionally, there were decreases in serum CRP, IL-6, and P-selectin in the PD group while these markers increased in the T2D group. These findings were indicative of immune activation and highlight the impact of pre-diabetes in this population. More studies are recommended with a higher number of samples that are stratified by gender and represent the gender ratio in the city.

Introduction

Chronic consumption of high-calorie diets has been implicated in the development of insulin resistance in humans and rodents (Arner 2002; Zhanguo Gao 2002; Fonseca 2007; Schrauwen 2007; Myles 2014; Ahmad et al. 2017; Luvuno et al. 2018). Consumption of high-fat diets have been reported to result in increased levels of diacylglycerides that ultimately leads to insulin resistance through activation of protein kinase C (PKC) signalling in the liver and in skeletal muscle (Thompson and Cooney 2000; Bruce et al. 2009; Jornayvaz and Shulman 2012). Prolonged consumption of diets high in carbohydrates have been reported to result in hyperglycemia; concurrently, in these hosts, there is increased activation of nuclear factor (NF)-κB translocation in cells - a phenomenon that leads to exacerbation of acute inflammatory events (Daniel et al. 2021).

Long-term consumption of diets high in both carbohydrates (including some with traces of lipopolysaccharides) and fat (specifically, saturated fatty acids) such as a high-fat high-carbohydrate (HFHC)-diet by rodents and humans has been reported to cause activation of NF-κB signalling through activation of toll-like receptor 4 (TLR 4), an effect that exacerbates insulin resistance (Shi et al. 2006; Erridge et al. 2007; Anderson et al. 2010; Erridge 2010; Baker et al. 2011). Insulin resistance itself has been shown to be associated with abnormalities such as hyperglycemia and hyperlipidemia that also can also trigger immune dysfunction/over-activation (Robertson et al. 2004; Kolb and Mandrup-Poulsen 2005; Schrauwen 2007; Nikolajczyk et al. 2011; Richard et al. 2017). In turn, prolonged/repeated states of hyperglycemia are known to be a primary factor underlying dysregulation of the innate immune system noted in patients with type 2 diabetes (T2D) (Graves and Kayal 2008).

According to the International Diabetes Federation (IDF, 2019), T2D accounts for ≈ 90% of all diabetes mellitus cases globally thus making it the most common type of diabetes worldwide. According to IDF statistics, in 2019, there were 19 million diabetics in Africa

between ages 20 and 79 years; surprisingly, at the same time, 12 million Africans were reported as living with undiagnosed diabetes. In these populations, the onset of T2D is usually preceded by a state of pre-diabetes that has been reported to last ≈ 20 years (Therrin 2018). Pre-diabetes is deemed an intermediate state between normoglycemia and T2D where blood glucose levels are higher than normal but not yet high enough for a diagnosis of T2D (Ryden et al. 2007). Due to the asymptomatic nature of the condition of pre-diabetes, this has led to challenges in documentation of the prevalence of this condition as well as in physiological changes that occur during this condition (Fonseca 2007; Grundy 2012).

Using an HFHC diet-induced animal pre-diabetes model, previous studies from our laboratory demonstrated there were increases in glycated hemoglobin (HbA1c) levels that were accompanied by increases in host blood pressure, impaired renal handling, and impaired cardiovascular function (Gamede et al. 2018, 2019; Luvuno Mluleki et al. 2019). These abnormalities were also seen to be associated with an apparent immune activation as demonstrated by changes in host circulating levels of various immune cell types (including those of neutrophils, lymphocytes, basophils, monocytes, and eosinophils) during progression of the pre-diabetes state (Mzimela et al. 2019). In addition, there was also an up-regulation of circulating levels of inflammatory markers, including those of interleukin (IL)-6, tumor necrosis factor (TNF)-α, C-reactive protein (CRP), fibrinogen, P-selectin, and soluble cell differentiation 40 ligands (CD40L). While this data from the animal model clearly shows a potential utility in monitoring various immune cell types/inflammatory markers to track pre-diabetes in a host, these findings have not yet been verified in humans with pre-diabetes.

Together, these markers give us insight into whether there is immune activation as the changes in immune cells concentration may be an indicator of glucotoxicity. Additionally, most of these cytokines have been reported to be involved in the physiology of T2D upon activation of inflammatory signalling pathways due to hyperglycaemia (Alberts et al. 2002; Baker et al.

2011; Chawla et al. 2011). The city of Durban (South Africa) is populated by a wide variety of ethnic groups. According to a study by Sosibo and colleagues, this city shows increasing prevalence of pre-diabetes among individuals specifically those 25-45 years of age. Additionally, in this city, have been reported to be affected by different factors such as unhealthy diets and occupational exposures to immunotoxins suggesting a compromise in immune system and exposure to development of neutropenia (Govender et al. 2021). Building on those findings, the present study was undertaken to investigate if - as in the animal models noted above - there were changes in circulating levels of immune cell types and any dysregulation of a select set of inflammatory markers that could corresponded with a presence of pre-diabetes in this age group.

Materials and methods

Chemicals/reagents

All chemicals and reagents used were of analytical grade. The materials and analytic kits utilized here were as follows: Human HbA1c ELISA kit (Elabscience, Houston, Texas, USA); Human Customized Invitrogen "ProcartaPlex", 4-plex (IL-6, TNFα, sCD40L, and P-selectin) multiplex assay kit (Thermofisher Scientific, Waltham, MA) and, Human CRP and Human Fibrinogen ELISA kits (Elabscience).

Study site, population, and design

The study was carried out at laboratories of the University of KwaZulu Natal (UKZN, Durban, South Africa). A quantitative cross-sectional analytical study was conducted with blood samples (n = 292) collected at King Edward Hospital after UKZN Biomedical Research Ethics Committee (BREC) approval (#BE266/2019). The blood samples were collected from February 2021 to December 2022 from patients of all ethnicities and both genders, who ranged in age from 25-45 years. The selection of samples was done according to selection criteria and data provided by the hospital. The sampling exclusion criteria included: patients < 25 and > 46

yr-of-age; samples from patients displaying other diseases other than T2D/pre-diabetes; patients with no history of liver disease, thyroid disease, kidney disease, heart disease, depression, HIV; no professional sport athletes; patients under the influence of alcohol and pregnant females. All samples were collected only after a signed informed consent was obtained from each individual.

Pre-diabetes confirmation

To confirm whether samples should be categorized as normal, pre-diabetic, or T2D, the criteria of the American Diabetes Association (ADA, 2016) were applied. Additionally, based on glucose level data obtained from the hospital, HbA1c levels in the samples were measured using a human ELISA kit and following manufacturer instructions. Samples that indicated an HbA1c of < 5.7% were considered normal, between 5.7-6.4% pre-diabetic, and > 6.4 % T2D.

Immune cells and inflammatory markers measurements

An automated Beckman Coulter cell counter (Indianapolis, IN) was used to measure the levels of various immune cell types (e.g., neutrophils, lymphocytes, monocytes, eosinophils, and basophils) in each blood sample. The remaining blood was centrifuged at 3000 rpm for 15 min to obtain plasma that was then collected and stored at -80°C until used for biochemical analysis.

To measure IL-6, TNFα, sCD40L, and P-selectin in each plasma sample, a customized human Invitrogen "Procarta Plex" 4-plex multiplex assay kit was used, following manufacturer protocols. All results were processed using a Bio-plex MEGAPIX Multiplex reader (BioRad, Hercules, CA). Levels of CRP and fibrinogen in the samples were measured using their respective human ELISA kits. All measurements from the plate wells were obtained using a Spectro star nanoplate spectrophotometer (BMG Labtech, Ortenburg, Baden-Württernberg, Germany). The level of sensitivity of the kits were: 0.23 ng CRP/ml, 5.63 ng fibrinogen/ml,

52.8 ng IL-6/ml, 25.2 ng TNFα/ml, 10.6 ng sCD40L/ml, and 53.900 ng P-selectin/ml. All samples were evaluated in triplicate, following manufacturer protocols.

Data analysis

All data is expressed as means ± SEM. For the blood levels of the various immune cell types and inflammatory markers (CRP and fibrinogen), data were analyzed using SPSS v.28 software (SPSS, Cary, NC). For these analyses, all groups were compared by applying a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer *post-hoc* test. For the measures of inflammatory markers obtained with the multiplex assay (e.g., IL-6, TNFα, sCD40L, P-selectin), all data was evaluated using Bio-Plex Manager software v.5.0 and outcomes were then compared using Prism software (v.8; GraphPad, San Diego, CA). For these endpoints, all groups were compared by applying a one-way ANOVA and a Tukey-Kramer *post-hoc* test. In all cases, a p-value < 0.05 was considered as statistically significant.

Results

The study here utilized a total of 292 blood samples from various test subjects. The study had about 76 % of African, 17 Indians and 7 whites. Based on established parameters, these samples were sub-categorized into three groups (Figure 1): a non-diabetic group (ND, n = 30) with samples from 20 females and 10 males; a pre-diabetes group (PD, n = 90) with samples from 56 females and 34 males; and a Type 2 diabetes group (T2D, n = 172) with samples from 113 females and 59 males. Our samples were due to sampling criterias and hospitals are populated by more people who need medical assistance which contribute to a small size for non-diabetic group. We can notice that the type 2 diabetes group has a large sample size indicating that there are more people struggling with type 2 diabetes. Additionally, with a very large sample size, we would have got a significance on some of the markers investigates as we noticed the clinical significance on our graphs obtained.

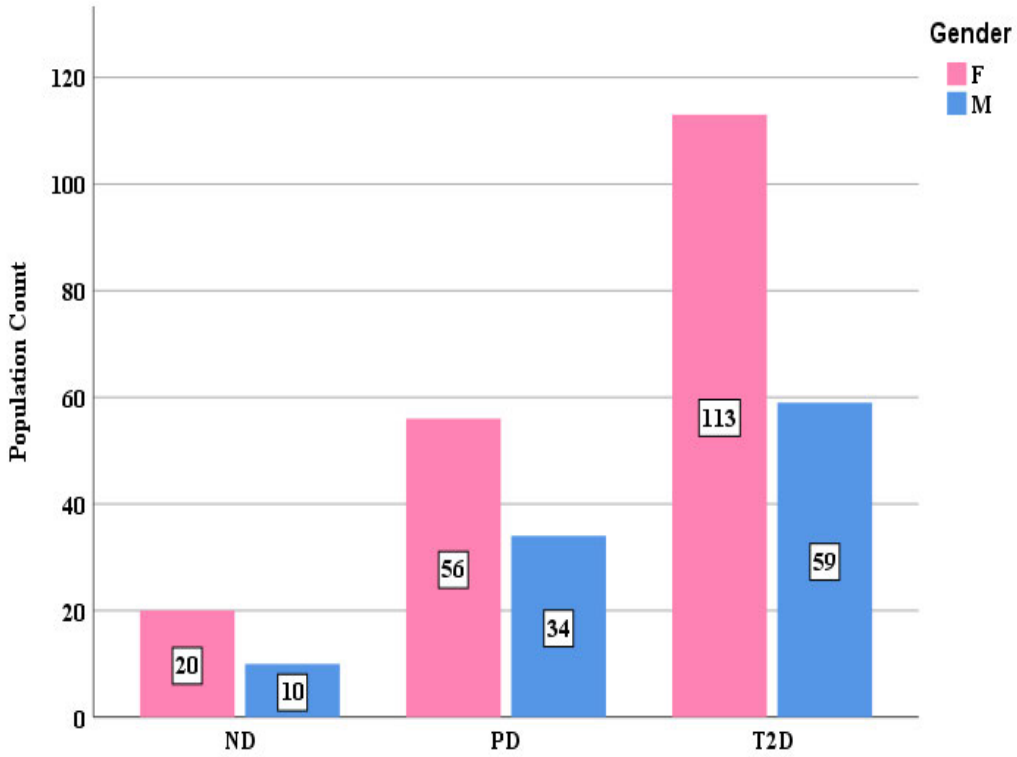


Figure 1. Gender distributions within each group. ND = non-diabetes group, PD = pre-diabetes group, T2D = Type 2 diabetes group.

Blood immune cell (neutrophil, lymphocyte, monocyte, eosinophil, and basophil) levels

Neutrophil (PMN) presence in the fresh blood samples was measured in all experimental groups. Results across all three groups indicated that circulating PMN levels were below the expected normal range (NR; 40-60%). The results showed there was a non-significant decrease in PMN levels in the T2D group in comparison within ND subjects (28.4% ND vs 27.0% T2D), p = 0.84 (Figure 2A). The pre-diabetics (PD) also had non-significantly lower blood PMN levels in comparison to the ND hosts (26.7% PD, p = 0.78. The small decrease (0.3%) between the PD and T2D hosts was deemed to fall within the margin of sampling error, p = 0.97.

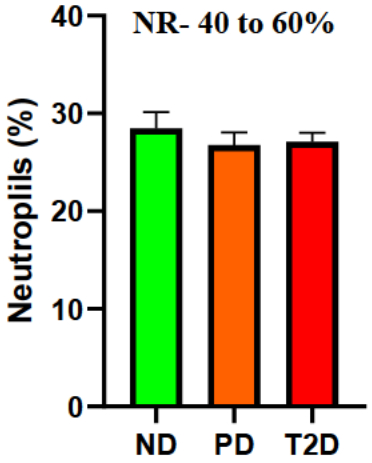
Analysis of blood lymphocyte levels found that all three groups had circulating lymphocyte levels within the normal range (20-40%). The results showed there was a non-significant decrease in lymphocyte levels in the T2D group in comparison to in the ND hosts (61.6% ND vs. 61.1% T2D), p = 0.99 (Figure 2B). The shift seen in the PD hosts was even less notable (61.2% PD), p = 0.99. The small increase (0.1%) between the PD and T2D hosts was deemed to fall within the margin of sampling error, p = 0.99.

Blood monocyte levels analysis showed that all three groups had circulating levels within the normal range (2-8%). The analysis revealed a non-significant increase in monocyte levels in the blood of the T2D group relative to that seen in ND host samples (4.6% ND vs. 5.4% T2D), p = 0.77 (Figure 2C). Interestingly, the shift in levels was not as great with the PD patients and actually was slightly (albeit non-significantly) reduced (4.4% PD), p = 0.99. Comparisons between the PD and T2D hosts revealed that while the net difference was 1.0%, this difference was not significant, p = 0.46.

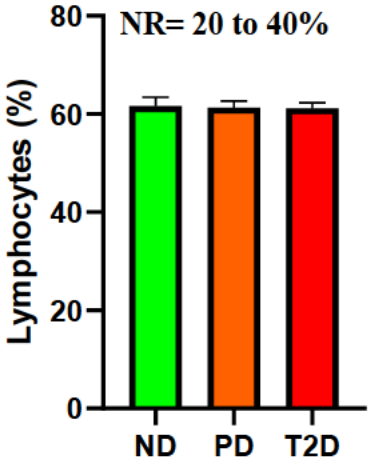
Evaluations of blood eosinophil (EOS) levels showed that all three groups had levels within the normal range (1-4%). The results indicated that there was a non-significant increase in blood EOS levels in the T2D group in comparison to in ND subjects (2.41% ND vs. 2.50% T2D), p = 0.98 (Figure 2D). The non-significant increase seen in the blood of PD hosts was even smaller (2.44% PD), p = 0.99. These low levels indicated there was no significant difference in blood EOS levels between the PD and T2D subjects, p = 0.98.

Measures of basophils in the blood showed all three groups had levels within the normal range (0.5-1%). As with the monocyte outcomes, comparisons among the groups showed that vs. both the ND and T2D hosts, there were small non-significant decreases in circulating basophils in the blood of the PD subjects (2.91% ND, 2.44% T2D, 2.35% PD) (Figure 2E). The p values were 0.95 (NT vs T2D), 0.80 (ND vs PD) and 0.85 (PD vs T2D).

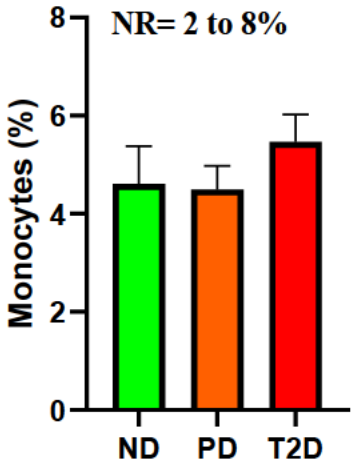
A.


NR- 40 to 60%
Neutroplils (%)
40
30
20
10
0
ND
PD
T2D

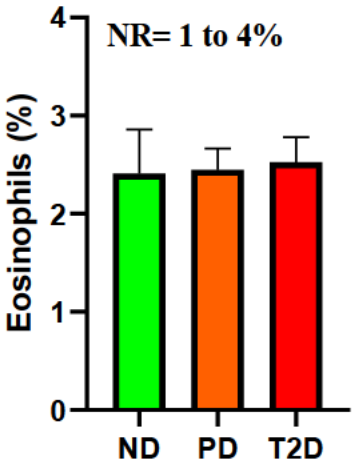
B.


NR= 20 to 40%
Lymphocytes (%)
80
60
40
20
0
ND
PD
T2D

C.


NR= 2 to 8%
Monocytes (%)
8
6
4
2
0
ND
PD
T2D

D.


NR= 1 to 4%
Eosinophils (%)
4
3
2
1
0
ND
PD
T2D

E.

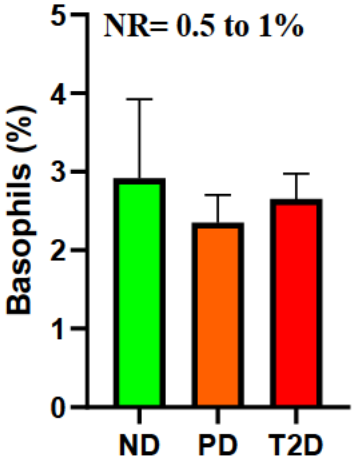

NR= 0.5 to 1%
Basophils (%)
5
4
3
2
1
0
ND
PD
T2D

Figure 2. Comparison of blood immune cells levels in human subjects. **(A)** neutrophils (PMN), **(B)** lymphocytes, **(C)** monocytes, **(D)** eosinophils, **(E)** basophils. ND = non-diabetic group, PD = pre-diabetes group, T2D = Type 2 diabetes group. Values shown are means ± SEM.

Inflammatory markers

Levels of select inflammatory markers (CD40L, P-selectin, IL-6, TNFα) were measured in plasma obtained from hosts in each experimental group. Figure 3A illustrates how there was a non-significant increase in circulating CD40L levels in T2D hosts compared to in the ND group (10.40 pg/ml ND vs. 27.34 pg/ml T2D; p = 0.98). The results showed that while the observed increase in circulating CD40L levels in the blood of the PD group was more substantial relative to levels in the ND group (10.40 pg/ml ND vs. 151.49 pg/ml PD; p = 0.34), this change ultimately was not significant, (nor was the increase relative to the levels seen in the T2D hosts; p = 0.28).

Figure 3B shows that while there was a non-significant increase in plasma P-selectin levels in the T2D group (442.12 μg/ml) in comparison to in ND hosts (279.23 μg/ml; p = 0.06), there was now a non-significant decrease in plasma P-selectin in the PD group (220.33 μg/ml) relative to that in the ND group (p = 0.70). On the other hand, these depressed PD levels were significantly lower than in the T2D group (p = 0.001).

With respect to plasma IL-6, the data in Figure 3C indicates there was a non-significant increase in circulating IL-6 in the T2D hosts in comparison to in the ND subjects (464.74 ng/ml ND vs.703.64 ng/ml T2D; p = 0.17). In contrast again, compared against levels in the ND group, a non-significant decrease in IL-6 levels was noted in in the PD group (355.51 ng/ml; p = 0.70). Unlike for some of the other markers evaluated here, the net difference between the T2D and PD plasma IL-6 values were significant (p = 0.007).

A different overall trend was noted with respect to plasma TNFα levels (Figure 3D). Specifically, the data revealed there was a non-significant increase in circulating TNFα in the T2D group in comparison to in the ND hosts (25.37 ng/ml ND vs. 82.10 ng/ml T2D; $p = 0.49$), and that there was an even much greater significant increase in circulating TNFα (150.73 ng/ml) associated with the PD state ($p = 0.05$). Oddly, the PD levels, albeit almost double that found with the T2D subjects, were not significant different from the T2D levels ($p = 0.24$).

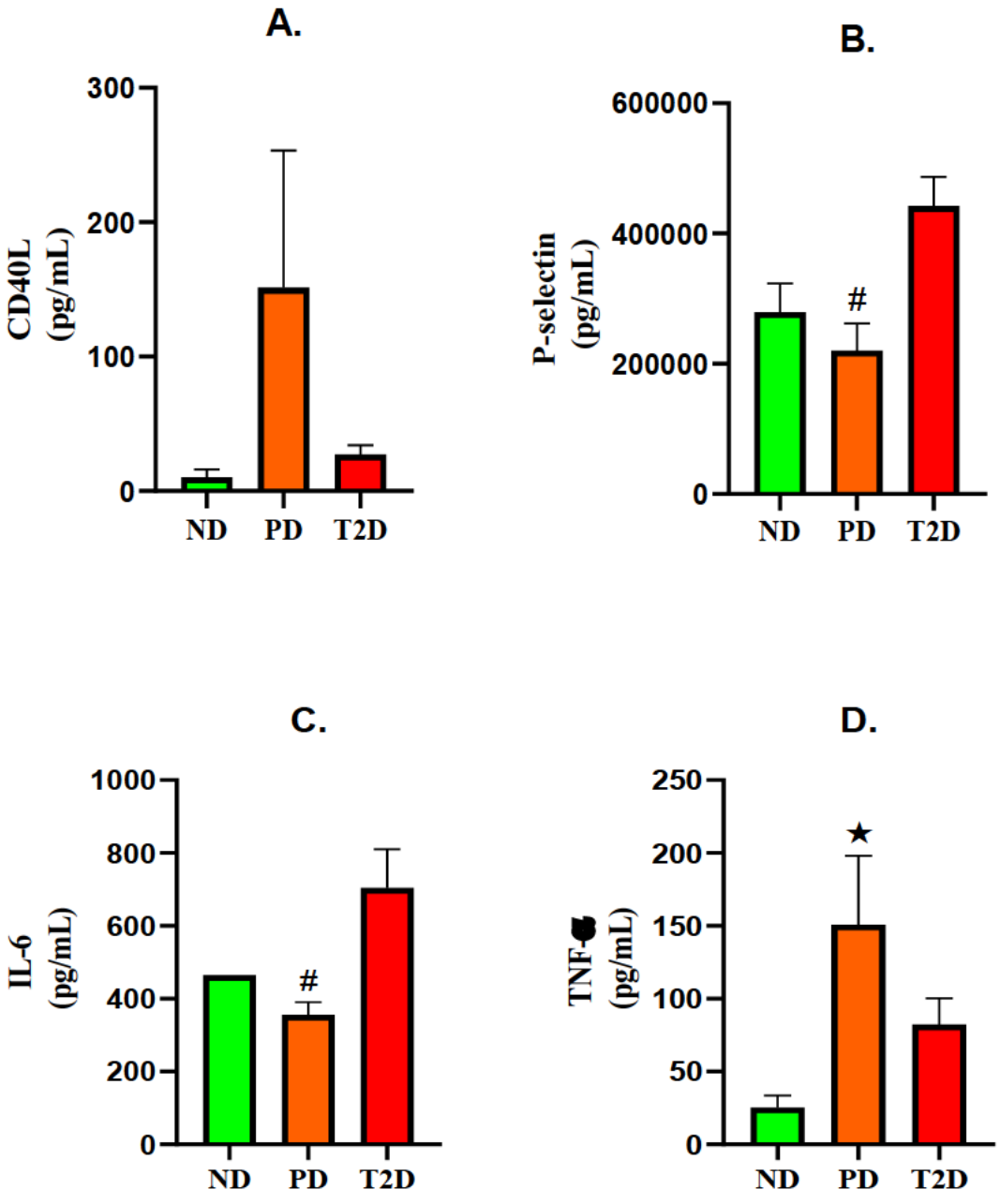


Figure 3. Levels of inflammatory markers in sampled blood. **(A)** CD40L, **(B)** P-selectin, **(C)** IL-6, **(D)** TNFα. Values shown are means ± SEM. *Value significantly different ($p < 0.05$) between ND and PD, #between PD and T2D ($p < 0.05$).

CRP and fibrinogen

The results in Figure 4A indicate there was a non-significant increase in circulating CRP levels in the T2D group when compared to in the ND hosts (4.27 ng/ml ND vs. 4.57 ng/ml T2D; $p = 0.98$). In contrast, there was a non-significant decrease in circulating CRP in the PD group (1.93 ng/ml) when compared to the ND hosts ($p = 0.31$). Oddly again, these PD levels, albeit almost half that in the T2D hosts, were not significant different from the T2D levels ($p = 0.11$).

Analysis of circulating fibrinogen levels (Figure 4B) showed there was a concurrent non-significant increase in the T2D host levels relative to those in the ND subjects (13.40 ng/ml ND vs. 45.63 ng/ml T2D; $p = 0.32$). Unlike with CRP, in this case, there were concurrent non-significant increases in circulating fibrinogen in the PD group (34.52 ng/ml) compared with in the ND subjects ($p = 0.63$). Though fibrinogen levels were increased in both groups relative to in the non-diabetics, these levels were found ultimately to not significantly differ from one another, ($p = 0.82$).

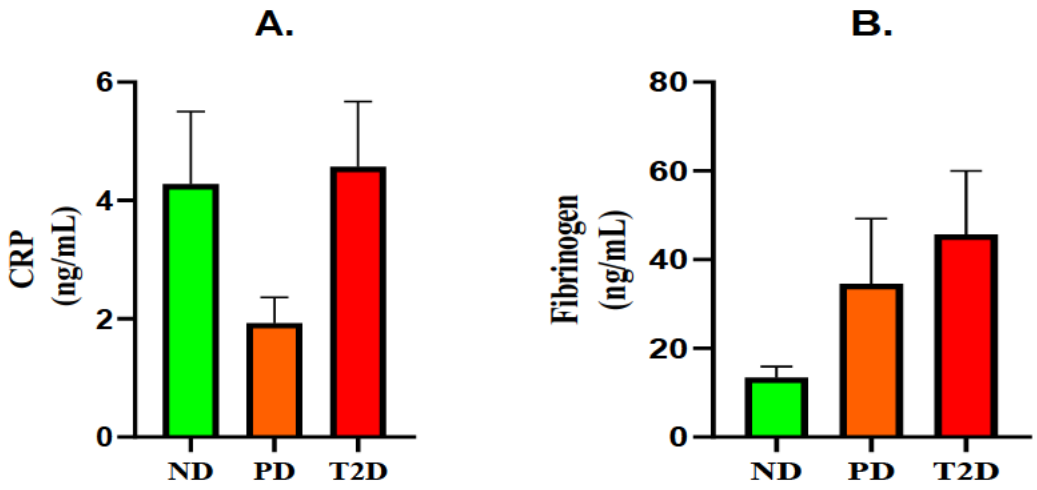


Figure 4. Levels of blood **(A)** CRP and **(B)** fibrinogen in samples. ND = non-diabetic group, PD = pre-diabetes group, T2D = Type 2 diabetes group. Values shown are means ± SEM.

Discussion

One of the complications associated with Type 2 diabetes (T2D) is a dysregulation in the innate immune system (Graves and Kayal 2008). This dysregulation has been reported to be a result of the chronic hyperglycemia observed in T2D subjects (Evans et al. 2002; Monnier et al. Evans et al. 2002; Monnier et al.). However, before the onset of T2D, there is often a long-lasting state of intermediate hyperglycemia known as pre-diabetes (Grundy 2012; Therrin 2018). Several studies conducted in animal models have suggested that the abnormalities observed in T2D begin during pre-diabetes (Luvuno et al. 2018; Gamede et al. 2019; Mabuza et al. 2019; Mzimela et al. 2019).

The city of Durban in South Arica is a culturally diverse urbanized area characterized by increasing levels of consumption of high calorie diets and sedentary lifestyles (Bikombo 2014; Mposula 2019). These trends have coincided with an increasing incidence of pre-diabetes, specifically among individuals in the 25-45 yr-of-age group (Sosibo et al. 2022). Additionally, KZN people, which include Durban, has been reported to be affected by factors

that contribute to immune toxicity such as chronic consumption of high calorie diets, sedentary lifestyles and occupational exposure to immune toxifying agents (Govender et al. 2021). To date, there has been no research done to investigate potential changes in immune cell/inflammatory markers associated with either T2D or pre-diabetes in this population. Such information could prove useful for earlier detection of the onset of diabetes and thus afford an earlier start to treatment or initiation of changes in lifestyle. To gain insight into identification of potential markers of pre-diabetes in this age group, the present study was undertaken to evaluate changes in circulating immune cells as well as in levels of select inflammatory markers in 25-45-yr-old patients with pre-diabetes in the city of Durban, South Africa. The outcomes would then hopefully build upon results of a previous study that investigated the effects of pre-diabetes on immune cells in an animal model of diet-induced pre-diabetes (Mzimela et al. 2019). For this discussion, the outcomes regarding the neutrophils, lymophocytes and monocytes are addressed; the data showing minimal impact on blood eosinophil and basophil levels allows for those cell types to not be discussed further as potential markers of pre-diabetes in this age group.

While neutrophils (PMN) are needed by the immune system to fight invading pathogens and in injury healing (Honda et al. 2016), the current study detected generalized neutropenia in all the different groups evaluated. One could speculate that this could be due to decreased production or differentiation of PMN in the bone marrow, an event potentially related to effects from overall nutritional disparities in these hosts (Govender et al. 2021). The province of KwaZulu-Natal (KZN) wherein Durban is located, is characterized by an odd co-existence of under- *and* over-nutrition (Govender et al. 2021). This suggested to us that even though people in this area could still be categorized as non-diabetics, they ultimately can be affected by different factors arising from the local environment, including dietary habits. Other factors that could be contributing to a state of neutropenia are gastrointestinal disorders (which lead to

repeated inflammatory states) as well as personal/occupational exposures to immunotoxins. Additionally, according to Reich and colleagues, people of African ancestral and Yemenite Jews have a low neutrophil count due to high *FY*-allele frequency which disturb the capacity of mobilization of bone marrow neutrophils reserves, upon response to availability of circulating corticosteroids (Reich et al. 2009). In the Duffy antigen Receptor for Chemokine gene (DARC) gene, the *FY*-allele of an African ancestral and Yemenites Jews also have a noncoding strand that disturb or destroy the gene expression in white blood cells, thereby contributing to low neutrophils count (Reich et al. 2009). The study population consisted mainly of people of African and Indian ancestry (93%) which may provide another explanation of the low neutrophil count observed in the study. The results presented here showed there seemed to be a trend toward a decrease in circulating PMN levels in T2D hosts. Such results would be in keeping with what is known about PMN during T2D as PMN migrate to chronically inflamed areas such as adipose tissue and endothelial cells (Daryabor et al. 2020), and thus are less present in the blood at any given moment. The findings here appear to extend this trend to the PD state. Still, it is interesting that states of hyperglycemia and hyperlipidemia induce a chronic inflammation condition that is expected to stimulate PMN production, thereby *increasing* circulating PMN levels (Soehnlein et al. 2017). Clearly, some-thing is occurring during the development of PD and subsequent progression to T2D that allows for circulating levels of these cells to drop even during ongoing states of hyperglycemia and hyperlipidemia.

Lymphocytes are also implicated in the pathology of diabetes (Hampton and Chtanova 2019). For example, studies have shown that in T2D, there are increased levels of circulating activated T-cells due to chronic hyperglycemia and these T-cells secrete cytokines such as IL-6 and TNFα that contribute to the immune dysregulation associated with diabetes (Butcher et al. 2014; Xia et al. 2017). In addition, CD4 and CD8 T-cells will migrate to adipose tissues upon activation and cause local cells to release inflammatory cytokines that further promote

the pathology (McLaughlin et al. 2014). In the present study, in the PD group, there was a decrease in plasma lymphocyte levels relative to in ND hosts. The PD results suggest that it is likely that during any induced hyperglycemia/inflammation in pre-diabetes, lymphocytes are recruited to inflamed areas where they then secrete additional inflammatory cytokines like TNFα and IL-6. That these PD hosts also displayed relative increases (vs. in ND hosts) in plasma CD40L suggests to us that the immune cells secreting the CD40L and TNFα were more activated.

It may be that lymphocytes were not the only cell source impacted by the pre-diabetes and that contributed to the observed changes in select cytokine/inflammatory protein express noted. For example, monocytes have also been reported to release TNFα and IL-6 due to hyperglycemia (Chomarat et al. 2000; Nikiforov et al. 2017). Monocytes (which can differentiate into either antigen-presenting dendritic cells or macrophages upon reproduction and activation (Chomarat et al. 2000; Shrestha et al. 2014; Mustafa 2022) can play an important role in exacerbating T2D as they secrete IL-6 (via induction of protein kinase C (Ngcobo et al. 2022). In T2D, monocytes are recruited to inflamed areas such as adipose tissue (Xu et al. 2015), with the latter sties being a source of monocyte chemoattractant protein-1 (MCP-1) (Degirmenci et al. 2019). In a self-promoting manner then, the newly released MCP-1 induces monocyte migration to the inflamed area. In addition to MCP-1, monocytes are also a good source of IL-6, IL-8, TNFα, and IL-1β, each of which can then act to exacerbate any ongoing inflammation during hyperglycemia as well as during T2D (Jagannathan-Bogdan et al. 2011; Ngcobo et al. 2022). The results in the present study showing a slight increase in circulating monocytes in the T2D but a nominal decrease in the PD group suggested to us that as a moderate hyperglycemia and chronic sub-clinical states of inflammation eventually gave rise to T2D, more and more monocytes were likely being recruited to inflammatory sites. An

explanation then for why there was an increase in relative levels of blood monocytes in T2D hosts compared to in the normal hosts remains elusive.

The population of the current study contained more women than men per group. Therefore, it is important to consider the potential impact of sex-related hormones on the results of markers and immune cell obtained herein. One protein whose circulating levels are known to be affected by gender is CRP (Gaskins et al. 2012). This is odd in that CRP is routinely used as a clinical predictor of cardiovascular disease in both males and females. CRP is produced in the liver and its release is induced by circulating IL-6 and TNFα (Lee et al. 2009). Stimulation of adipose tissues has been reported to result in elevated CRP levels in T2D patients. Indeed, the results obtained in the present study showed an increase in CRP levels in the T2D hosts. It would be logical to then surmise that during the chronic hyperglycemia/inflammation that led to T2D, one would expect a triggering of CRP release from the liver. Unexpectedly, in the PD group here, plasma CRP levels were decreased in comparison with those in the ND hosts. One potential explanation for this decrease can be gleaned from the findings of Gaskins et al. (2012) where it was seen that among female subjects, decreases in plasma CRP levels were common due to endogenous estradiol. It could very well be here, that because the PD group was populated primarily with women (see Figure 5), these observed decreases in circulating CRP were artefactual and more heavily impacted upon by the hormone than by any hyperglycemic state in the hosts.

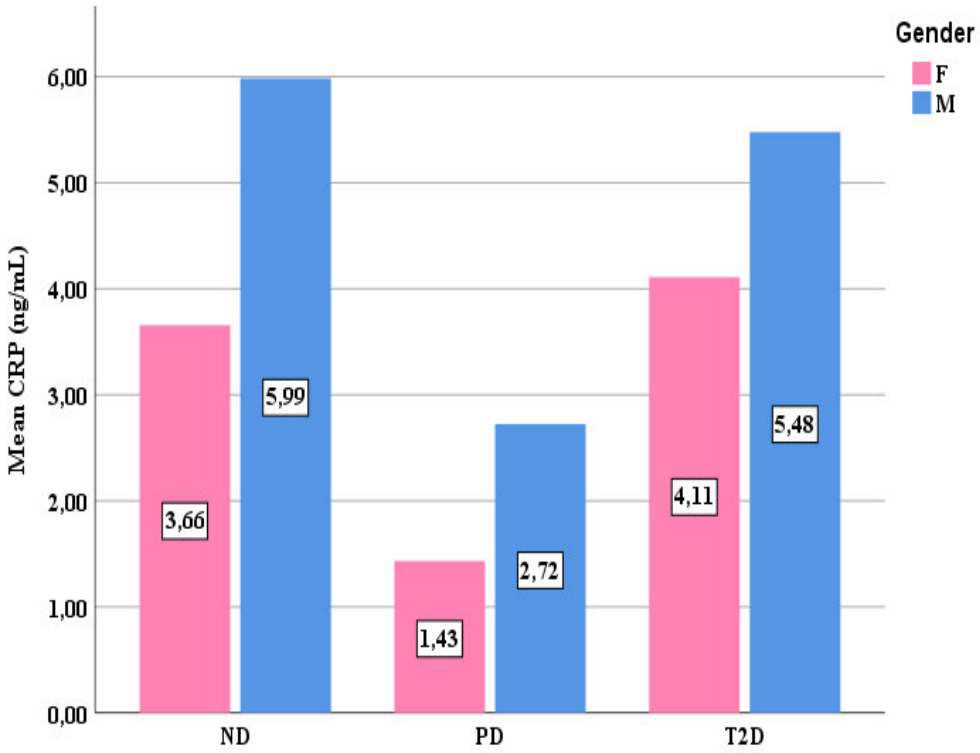


Figure 5. Mean blood concentration of CRP as a function of gender within each group. ND = non-diabetes group, PD = pre-diabetes group, T2D = Type 2 diabetes group.

Conclusions

There remains much to be done to better understand the changes that occur during pre-diabetes that led to the development of T2D. Activation of the immune system and inflammation have each been shown to contribute significantly to this process. The results obtained from the present study suggest that that there are other factors that can contribute to the changes observed such as gender, race, and age. The observed changes in immune cell levels and some of the evaluated inflammatory markers indicate it is increasingly likely that chronic consumption of high-calorie/high-fat diets and living sedentary lifestyles by this Durban population in is having a multiplicity of effects. These seem to include immune system activation and inflammation during the pre-diabetic state that only is amplified as the pathology progresses to T2D.

While these findings provide a basis for more refined marker-defining studies of pre-diabetics, as authors, we wish to acknowledge key limitations in the current study. Of note, a lack of equal numbers of male and female subjects per group was likely a limiting factor in that this imbalance may have skewed some of the results. Further, the current study could not measure all the various inflammatory markers (and hormones) possibly involved in host immune responses due to constraints of sample, time, and funding. Follow-on studies will be better designed to over-come these limitations and expand the scope of endpoints measured in the three groups.

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Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Availability of data and materials

The datasets used/analyzed in the current study are available from the corresponding author upon reasonable request.

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BRIDGE 2

Chapter 4 provided an overview of immune cells and selected markers of inflammation during pre-diabetes. The findings of the research manuscript showed that there is immune activation and sub-clinical inflammation possibly caused by the moderate hyperglycemia observed during pre-diabetes. Chapter 5 will provide an overview of studies related to changes in RBC indices during pre-diabetes. This chapter consists of 3 sections: a protocol for a systematic review, a systematic review, and an original research manuscript.

CHAPTER 5: SYSTEMATIC REVIEW PROTOCOL, SYSTEMATIC REVIEW AND RESEARCH MANUSCRIPT 2
(HEMATOLOGY)

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled "**The Changes in Red Blood Cell Indices that Occur in Pre-Diabetic Patients of all Ethnicities from the 25–45 Years of Age: A Protocol for a Systematic Review and Meta-Analysis "** and is authored by N.C Mzimela, A.M Sosibo, P.S Ngubane, and A. Khathi. The manuscript is published **Methods and Protocols (ISSN: 2409-9279)** and has been formatted according to journal's guidelines for authors (https://doi.org/10.3390/mps6010013). This journal is accredited by Department of Higher Education and Training South Africa DHET and appears in Scopus accredited list (2022).

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

The Changes in Red Blood Cell Indices that Occur in Pre-Diabetic Patients of all Ethnicities from the 25–45 Years of Age: A Protocol for a Systematic Review and Meta-Analysis

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ABSTRACT

Introduction: Pre-diabetes is an intermediate, asymptomatic state between normoglycaemia and the onset of type 2 diabetes mellitus (T2D). Recent reports indicate that there are sub-clinical changes observed in red blood cells during pre-diabetes. This systematic review protocol will provide an outline of all procedures in the synthesis of the available data on the changes in red blood cell indices. **Methods and Analysis:** This protocol was prepared by adhering to the PRISMA 2015 guidelines for reporting protocols. Published clinical studies that involve observation, whether it is cross-sectional, comparative cross-sectional, case-control or cohort study designs that involve normal/non-diabetic and pre-diabetes reports were used. Additionally, this was accomplished by using clinical MeSH headings to search on MEDLINE, COCHRANE library and African Journal Online. Three reviewers (NCM, AMS & AK) screened all the results for eligibility criteria. Then, Downs and Black checklist was used to check the risk of bias. Review Manager v5.4 Forrest plot was used for meta-analysis and sensitivity analysis. Strength of evidence was then assessed using the Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE). **Results and Conclusion:** This protocol will give direction on the exploration of articles that report on changes in red blood cell indices in the pre-diabetic state. The results obtained from this protocol will further give direction on the research to be done at in the eThekwini district of South Africa. **Ethics and Dissemination:** The data that will be analyzed will be data that has already been published thus there will be no data collection from subjects. Therefore, no ethical clearance is required. Registration Details: This protocol has been registered with the International Prospective Registry of Systematic Reviews **(PROSPERO) registration number "CRD42020189080" dated 05-07-2020.**

Keywords: Systematic review; Meta-analysis; Pre-diabetes; Red blood cells; Inflammatory markers, hematologic changes.

Background

Pre-diabetes is an asymptomatic state of intermediate moderate insulin resistance that occurs before onset of type 2 diabetes mellitus T2D [1]. It is characterised by fasting blood glucose (FBG) from 5.6 to 7.0 mmol/L; 2 h postprandial blood glucose (2 h - OGTT) from 7.8 to 11.0mmol/L and glycated haemoglobin (HbA1c) from 5.7 to 6.4% [2,3]. According to the International Diabetes Federation (IDF) atlas reports , around 90% of all diabetes globally is T2D, therefore, indicating that it is the most common diabetes worldwide [4]. However, the onset of T2D is preceded by a pre-diabetes stage which has been reported to last about 20 years in human beings [5]. IDF statistics reported that there were 19 million diabetic people in Africa aged between 20 and 79 years in 2019 [4]. Surprisingly, according to IDF reports, 12 million Africans aged between 20-79 years were reported to live with undiagnosed diabetes in 2019 [4]. The age range with the highest diagnosis of type 2 diabetes in South Africa is 45-65 years while pre-diabetes is said to last anywhere between 10-20 years [6]. It is for this reason that in this study we chose to look at patients in the age range of 25-45 years old. One of the complications of T2D is a reduction in red blood cell (RBC) deformability and changes in concentration contributing to changes in blood indices [7-9]. Reports indicated that T2D patients display changes in RBC indices such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin content (MCHC), haemoglobin (HGB) and haematocrit (HCT)[9-11]. Additionally, Nada reported that people with T2D also have impaired erythropoiesis which is indicated by low levels of erythropoietin (EPO)[10]. These RBCs of T2D also have reduced deformability and a reduced life span[9,12]. Studies further indicate that red blood cell distribution width (RDW) in T2D is increased due to anisocytosis and RBC degradation [10]. Moreover, increased T2D RBCs aggregation is also reported to cause an increase in blood viscosity and the development of high blood pressure [13]. This then contributes to the cardiovascular complication's development due to the clogging of vessels [14-16]. Furthermore, according to Sharif *et al.*, anemia is the key indicator of chronic kidney diseases, cardiovascular factors and retinopathy [17]. It is a debatable issue if these complications occur during pre-diabetes. Research has recently been done based on animals in our laboratory, which is an addition to the available research reporting the metabolic and signalling abnormalities, including changes in red blood cell indices at the pre-diabetes stage [18-22]. This research from our laboratory raised a debatable issue if the same abnormalities occur during the pre-diabetes state in human subjects considering the limitations in the high fat high carbohydrate diet-induced animal model. From the search done, we could

not obtain any report or evidence of the systematic review that reports on the changes in red blood cell indices and the level of secretion of EPO and endothelial nitric oxide synthase(eNOS) in the pre-diabetic state at eThekwini district (South Africa). Therefore, this presents an opportunity to deliver a systematic review that will yield a comprehensive synthesis obtained from the available collected studies that previously reported on the red blood cell indices and concentration of EPO and eNOS during pre-diabetes.

Objectives

1. To determine the changes in RBCs indices (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) concentration in the pre-diabetic state.
2. To investigate if there are changes in concentration with respect to WBC, EPO and eNOS in the pre-diabetes state.
3. To determine the impact of demographics on hematologic changes and secretion of EPO and eNOS in the pre-diabetic state.

Methods

This protocol was prepared by adhering to the preferred reporting items for systemic reviews and meta-analysis (PRISMA) 2015 guidelines for reporting protocols (PRISMA checklist attached in additional file).

Systematic review registration

The protocol has been registered with the International Prospective Registry of Systematic Reviews (PROSPERO registration number "CRD42020189080" dated 05-07-2020).

Eligibility Criteria for the study

A minimum of 100 population studies that report community-based clinical cross-sectional study will be eligible. The inclusion and exclusion criteria will be as follows.

Inclusion: Information that is obtained from non-diabetic adults within the ages of 25-45 of all ethnicities will be eligible.

Exclusion: The study will not use reports from people with a history of liver disease, kidney disease, heart disease, and depression. Additionally, reports from pregnant women will also not be used. Articles from professional sports athletes will not be allowed in the study.

Pre-diabetes diagnosis criteria

The diagnostic criteria that will be used is in line with the criteria used by the American Diabetes Association [3]. Pre-diabetes diagnostic criteria will be as follows (participants used in reports should meet one of the following diagnoses): fasting blood glucose (FBG): 5.6 -7.0 mmol/L; 2 h postprandial blood glucose (2 h - OGTT): 7.8 -11.0mmol/L with Glycated haemoglobin (HbA1c): 5.7-6.4%.

2.4. Study design.

Information Sources

*Participants***:** The target of the information source will be any reported clinical study that involves participants that are more than 100 in minimum, either males or females, and both genders, aged from 25 to 45 years from all ethnicities. The study with a minimum of 100 population size in human studies is recommended to yield accurate results due to involvement of human subjects and accurate results are required. .

*Intervention***:** The clinical studies that involve observational studies if they will be cross-sectional, comparative cross-sectional, case-control, or cohort study designs that involve normal/non-diabetic and pre-diabetes reports. The reported information that involves specifically one or more RBCs indices (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) at the pre-diabetic stage will be eligible for this systematic review. Additionally, a study that reports information that involves WBC, EPO and eNOS will also be eligible for this systematic review.

*Comparators***:** In this systematic review, the eligible comparing control groups will be non-diabetic/normal control.

Outcomes

This systematic review is expected to have the outcome as follows. The primary outcomes

1. The changes in RBCs indices (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) concentration at the pre-diabetes stage (reported as odds ratios and 95 % confidence interval).
2. The changes in concentration of WBC, EPO and eNOS at the pre-diabetes stage (reported as odd ratios and 95 % confidence interval).

The secondary outcome

1. Changes in RBCs indices, EPO and eNOS markers due to demographic impact such as the effect of gender, age, and race (reported as the mean).

Search Strategy

To identify studies involving cohorts, the electronic search strategy will be used that is related to the study of interest. This strategy will be accomplished by search on MEDLINE (from 1963 to 2020), COCHRANE library displaying results of trials from PubMed, CT.gov, EMBASE, and ICTRP (from 1963 to 2020), and African Journal Online (from 1998 to 2020). Additionally, to these search strategies, the use of clinical MeSH headings and text words will be applied to filter the available information. For all searches done, the keywords to be used will be “pre-diabetes and erythrocytes,” “pre-diabetes and red blood cells,” “pre-diabetes and red blood cell indices,” “pre-diabetes and red blood cell parameters,” “pre-diabetes and erythropoietin,” and "pre-diabetes and endothelial nitric oxide synthase."

Identification of eligible studies

NCM, AMS & AK will then screen the title and abstracts of all the obtained results, and the studies that meet the eligibility criteria will then be selected. Basically, each reviewer will be responsible for screening all the selected study reports before the decision making of the eligible reports. The PRISMA flow chart for the selection of studies will then be provided on reports from the systematic review.

Patient and Public Involvement

No patient involved.

Data management

Study Records and data extraction.

A Microsoft Excel file will be used to record the extracted data of study records selected as eligible reports. The pre-defined list of variables to be considered in each report will be used as categories in an Excel file. Considering the research of interest, the outcome of interest will mainly be the RBCs indices response and concentration of EPO and eNOS in both genders, at an age parameter of interest in all ethnicities. Additionally, the value of the baseline characteristic of the data reported will also be considered. Therefore, the baseline characteristics of eligible research reports obtained will be author, year of publication, country,

and study setting. The methodology of the study reported will also be considered with the categories (design, period, sampling strategy, and whether participants are normal or pre-diabetic population) considered. Finally, the outcomes from different gender, ages, ethnicity, RBCs indices changes / markers will then be extracted.

Data simplification

For the simplification of data, the studies that report on the RBCs indices (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) will be grouped into a single group. Additionally, the studies that report on WBC, EPO and eNOS will also be grouped into a single group.

Risk of bias

To measure the potential risk of bias in individual studies, the Downs and Black Checklist will be used [23]. For clarity, the scores will be rated as follows; excellent (25–26), good (20–24), moderate (14–19), poor (11–13), and very poor (< 10).Three reviewers (NCM, AMS, and AK) will be responsible for the independent judgments which will be based on the four domains of the Black and Downs checklist tool which is reporting bias (10 items), external validity (3 items), internal validity (6 items), and selection bias (7 items). In a situation where there will be a difference in opinions between NCM, AMS, and AK. PSN will then be responsible for adjudication.

Data synthesis

For the meta-analysis of reported data, a Review Manager version 5.4 software Forrest plot will be used [24,25]. Using this RevMan Forrest plot, eligible data from all reported studies will be meta-analysed depending on their sample size and the odd ratio of the RBCs indices (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) or markers (EPO and eNOS) and WBC in both pre-diabetic and control groups. Additionally, an odd ratio and confidence interval will be used to plot the forest plot where the solid lines will represent the 95% confidence interval. Each reported study will be represented as a horizontal line on the y-axis to list the primary author and year of study. The forest plot will also include the weight of the study results that will be automatically obtained using RevMan software.

Sensitivity analysis

Heterogeneity will also be automatically calculated using the RevMan software Forrest plot. The greater homogeneity will be indicated by a greater overlap between the confidence intervals[26]. Using the forest plot, I^2 will be calculated where a value between 0 and 100 % will be obtained. Additionally, a value obtained less than 25% will be an indication of a strong

homogeneity, and a value obtained greater than 75 % will then be an indication of a strong heterogeneity. However, a value of 50 % will be considered as an average value.

Assessment of Strength of Evidence

Assessment of the strength of evidence will be done by NCM, AMS, and AK. The studies included in the review will then be evaluated using the Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE) [26-28]. Furthermore, using a GRADE pro tool, the summary of the finding (SoF) table will then be created.

Discussion

The synthesis of previous study reports obtained from this systematic review and meta-analysis will provide clarity of the contribution of RBCs indices (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) on hematologic changes at pre-diabetes, such as the changes that have been reported on each and every RBC index. This systematic review and meta-analysis will also give an outstanding synthesis of data from previous reports based on markers that are EPO and eNOS and the WBC concentration. Additionally, the synthesis from this systematic review and meta-analysis will create a hallmark of an impact of demographics on hematologic changes at the pre-diabetes stage as this will contribute to the clarification of changes that might be observed on a study of interest-based on eThekwini district (South Africa).

Acknowledgements

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Ethics Approval and consent to participate.

The data that will be analysed will be the data that is published and there will be no data collection from subjects. The authors declare that there will be no informed consent required to be signed and therefore no ethics approval required for the systematic review and meta-analysis.

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Disclosure of interest

The authors report no conflict of interest.

Authors contributions

NCM, AMS, PS and AK were responsible for brainstorming, designing the study and then also drafted the protocol. NCM, AMS, PS, and AK were responsible for reviewing the eligible study and final draft of the manuscript. Funders had no role in developing the protocol.

Availability of supporting data

No extra data available besides the attached additional file since it is a protocol for systematic review.

Consent for publication.

Not applicable

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Additional file 1

PRISMA-P 2015 Checklist

This checklist has been adapted for use with protocol submissions to *Systematic Reviews* from Table 3 in Moher D et al: Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. *Systematic Reviews* 2015 **4**:1

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☐	☐	
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☒	☐	
Authors					
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	☒	☐	
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☒	☐	
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☐	☐	
Support					
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	
Sponsor	5b	Provide name for the review funder and/or sponsor	☒	☐	
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☒	☐	
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	☒	☐	
METHODS					

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☒	☐	
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☒	☐	
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	☒	☐	
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☒	☐	
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☒	☐	
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)	☒	☐	
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☒	☐	

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☒	☐	
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☒	☐	
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☒	☐	

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled " **The changes in blood indices that occur in pre-diabetic patients of all ethnicities from the 25-45 years of age: a systematic review and meta-analysis"** and is authored by N.C Mzimela, A.M Sosibo, P.S Ngubane, and A. Khathi. The manuscript is accepted for publication in **Hematology and Medical Oncology (ISSN: 2398-8495)** and has been formatted according to journal's guidelines for authors **(REF Number: HMO-7-241).** This journal is accredited by Department of Higher Education and Training South Africa DHET and appears in the ISI accredited list (2022).

Author Contribution: NC Mzimela was responsible for study conceptualization, study design, investigation, data analysis, first draft writing, and manuscript editing.

The changes in blood indices that occur in pre-diabetic patients of all ethnicities from the 25-45 years of age: a systematic review and meta-analysis

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ABSTRACT

Background: Pre-diabetes is an in-between stage between normoglycemia and onset of type 2 diabetes. This stage has been categorized as an asymptomatic stage before the onset of T2D. However, recent reports have indicated that there are complications at this stage, including the red blood cells changes and changes in inflammatory markers such as endothelial nitric acid synthase and erythropoietin. Therefore, the aim of this systematic review is to provide a synthesis of the available data on the changes in the red blood cells and selective markers. Another aim is also give clarity of a pre-diabetes stage demographic impact on these changes or complications**. Methods:** This systematic review was compiled through strictly adhering to the preferred reporting items for systemic reviews and meta-analysis (PRISMA) 2020 guidelines for reporting systematic reviews and it has been registered with the International Prospective Registry of Systematic Reviews (PROSPERO) registration number "**CRD42020189080**" dated 05-07-2020). In this systematic review published clinical studies articles that involve observational reports, whether it is case-control, cross-sectional, and comparative cross-sectional will be analysed. A cohort study designs that report on normal/non-diabetic group and pre-diabetes group, will be used in this systematic review and meta-analysis. This will be achieved by using clinical MeSH headings to search on MEDLINE, COCHRANE library, EMBASE, and ICTRP and African Journal Online. Reviewers (NCM, AMS & AK) will screen all the results and select the studies that meet the eligibility criteria. Downs and Black Checklist will be used to check the risk of bias, and then for meta-analysis Review Manager v5.4 Forrest plot will be used. Additionally, the Forrest plot will also be used for sensitivity analysis. The strength of evidence will then be assessed using the Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE).

Results: No report was eligible for this systematic review and meta-analysis.

Conclusion: There is insufficient information on human studies, that is based on RBCs indices at pre-diabetes stage and this, therefore creates a huge gap in the research field. This suggest that more research is required where there will be exploration of RBCs parameters, and all mechanisms involved at pre-diabetes stage since findings on animals have indicated that there is change at pre-diabetes stage.

Ethics: There is no ethics approval that will be required because no subjects will be used but, analysis will be based on a publicly available data.

Keywords: Systematic review; meta-analysis; pre-diabetes; red blood cells; inflammatory markers

BACKGROUND

Type 2 diabetes (T2D) is an endocrine disease where chronic hyperglycaemia cause abnormal complications which affect glucose regulation and building up more glucose in blood (Graves and Kayal, 2008). Pre-diabetes is a stage that precedes T2D (Grundy, 2012). International Diabetes Federation (IDF) prediction indicate that, in 2019, in Africa there were 19 million diabetic adults (20-79 years), predicting South Africa with the highest of 4.6 million diabetic adults (20-79 yrs.) (Federation, 2019). In 2017 reports, highest prevalence of diabetes in South Africa by 11-13% were Indian population, followed by coloured population by 8-10%, then blacks by 5-8%, and the lowest were whites by 4% (Motala et al., 2003, 24, 2017). Strong diabetes genetic predisposition has been reported in Indian population which contribute to their high prevalence rate (24, 2017 , Motala et al., 2003). IDF statistics reports indicate that South Africa has a high prevalence of T2D, which then hypothesizes that there is also a high prevalence of undiagnosed pre-diabetes (24, 2017). Pre-diabetes is an intermediate stage between normoglycemia and type 2 diabetes which has been categorised as an asymptomatic stage. Due to pre-diabetes categorised as being asymptomatic, there is shifting the emphasis of research to T2D as the stage where abnormalities or symptoms are discovered and enabling documentation of statistics. However recent studies have now indicated that there are symptoms and abnormalities occurring at pre-diabetes stage even though it is ongoing research aiming for more reports published focusing on this stage. One of the abnormalities discovered in pre-diabetic rats is the changes in red blood cells (RBCs) indices and the state of endothelial nitric acid synthase (eNOS) and erythropoietin (EPO)(Akinnuga et al., 2020, Mzimela et al., 2021). These RBCs indices are mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), haemoglobin (HGB), haematocrit (HCT), and red cell distribution width (RDW). RBCs indices has been reported to contribute to cardiovascular complications and anaemia (Ziaee et al., 2017, Agrawal et al., 2016, Bahlmann et al., 2009, Belonje et al., 2010, Biadgo et al., 2016, Bizjak et al., 2015). According to Ezenwaka and co-workers, it is the presence of anaemia that may exacerbate the cardiovascular complications in subjects with diabetes (Ezenwaka et al., 2008). According to Khoshdel *et al*., the downgraded production and lessen response to EPO has been reported to T2D subjects that have early developed renal damage(Khoshdel et al., 2008). In addition to renal damage, chronic inflammation, autonomic neuropathy and iron deficiency are also reported to be influential to decreased levels of EPO (Khoshdel et al., 2008). However, these complications remain unclear in pre-diabetic subjects. There is a research gap in south Africa

based on RBCs indices and state of EPO and eNOS. To the best of our knowledge, a systematic review that will investigate and pool the prevalence of abnormal RBCs indices, state of EPO and eNOS in people with pre-diabetes is yet to be conducted. This systematic review will explore worldwide reports based on RBCs indices, the state of eNOS and EPO at pre-diabetes stage. This exploration will give clarity if there are changes that occur on RBCs indices, selective markers and if demographics have impact on those changes. Therefore, laying a hall mark of the research of interest based at eThekwini district (South Africa) since it is a district with biodiversity and people living in the area exposed to all the possibilities that contribute to development of pre-diabetes.

METHOD

This systematic review adhered to the preferred reporting items for systemic reviews and meta-analysis (PRISMA) 2020 guidelines for reporting systematic review and PRISMA checklist attached as additional file 1. The protocol was registered with the International Prospective Registry of Systematic Reviews (PROSPERO registration number "**CRD42020189080**" dated 05-07-2020). Additionally, search strategy, data extraction and simplification, checking risk of bias, data synthesis, sensitivity analysis and assessment of strength of evidence was carried out according to published protocol by Mzimela *et.al* as follows (Mzimela et al., 2023)

Search Strategy

The studies involving cohorts were identified using the electronic search strategy that is related to the study of interest. This search strategy was accomplished by search on COCHRANE library displaying results of trials from PubMed, CT.gov, EMBASE, and ICTRP (from 1963 to 2021), MEDLINE (from 1963 to 2021), and African Journal Online (from 1998 to 2021). MeSH headings and text words were applied to filter the available information online. Additionally, the keywords that were used were “pre-diabetes and erythrocytes,” “pre-diabetes and red blood cells,” “pre-diabetes and red blood cell indices,” “pre-diabetes and red blood cell parameters,” “pre-diabetes and erythropoietin,” and "pre-diabetes and endothelial nitric oxide synthase". Endnote X9 software was then used to site and identify the duplicates.

Selection of Eligible Reports

After the capturing the available reports related to the research of interest, three reviewers (NCM, AMS & AK) were responsible for the selection of eligible reports. In cases of a misunderstanding PS was responsible for making a final decision. In cases of

misunderstanding, the corresponding author was contacted for clarity of the published work. The following criteria were used to select eligible reports.

Information Sources

Clinical studies and population: The main target of the information source was any available full text article that reported on clinical study that involved more than 100 participants, in minimum. The participants eligible were either males or females and both genders, aged from 25 to 45 years from all ethnicities. Clinical studies eligible involved observational studies if they were cross-sectional, comparative cross-sectional, case-control, or cohort study designs that involve normal and pre-diabetes reports. Specifically, reported information that involved one or more RBCs indices/parameters (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) at the pre-diabetic stage was eligible. Additionally, a study that reports on EPO and eNOS information at pre-diabetes stage was also eligible.

Inclusion criteria: non-diabetic adults report and pre-diabetic reports within the ages of 25-45 of all ethnicities.

Exclusion criteria: Reports from participants with a history of liver disease, kidney disease, heart disease, and depression were eliminated from this study. Pregnant women reports were also eliminated. Additionally, studies from professional sports athletes were not allowed in the study.

Pre-diabetes diagnosis criteria

Reports using the following diagnostic criteria for pre-diabetes were eligible; (participants should meet one of the following diagnoses): fasting blood glucose (FBG): 5.6 -7.0 mmol/L; 2 h postprandial blood glucose (2 h - OGTT): 7.8 -11.0mmol/L with Glycated haemoglobin (HbA1c): 5.7-6.4%.

StudyExtraction and Simplification

Microsoft excel was used to record extracted data where a pre-defined list of categories was used. The main category was RBCs response and with concentration of EPO and eNOS being the minor focus in both genders, at an age from 25 to 45 years from all ethnicities. In addition to these categories, the baseline characteristics of reported eligible data captured were also in consideration. These baseline characteristics of eligible reports were author, year of publication, country, and study setting. Additionally, reported study methodology was also

considered with the categories the following categories: design, period, sampling strategy, and whether participants are normal or pre-diabetic population. Finally, different gender, ages, ethnicity reports outcomes in based on RBCs indices changes / markers were then be extracted. The reports were simplified by being grouped into 2 groups, the RBCs indices group, and markers group.

Measurement of a Potential Risk of bias

The Downs and Black checklist method were used to measure the risk of bias on individual eligible reports. The three reviewers (NCM, AMS, and AK) were responsible for the independent judgments. These judgements were based on the four domains of the Black and Downs checklist tool which is reporting bias (10 items), external validity (3 items), internal validity (6 items), and selection bias (7 items). Finally, from the judgements obtained results, the scores obtained were then ranked as follows; excellent (25–26), good (20–24), moderate (14–19), poor (11–13), and very poor (< 10). PS was then responsible for final decision where there was difference in opinions between NCM, AMS, and AK.

Eligible data Synthesis and Predictor of Heterogeneity

Following the Downs and Black checklist method was the Forest plot using Review Manager (version 5.4) software (RevMan). The study weight, odds ratio and 95% confidence interval (CI) was the focus areas on plotting the Forest plot. Additionally, in the Forest plot, the solid lines representing 95 % CI and the study weight were automatically calculated upon addition of eligible individual articles of the plot. After all the eligible articles were added on corresponding Forest plot, the RevMan software automatically calculate and generate the heterogeneity of inserted reports in each Forest plot. Ability of RevMan to predict greater heterogeneity is indicated by a greater overlap between the confidence intervals in a Forest plot. The value of I^2 between 0 and 100% is then detected where value less than 25% indicate a strong homogeneity, value greater than 75% indicating strong heterogeneity and value of 50% being considered as an average.

Assessment of quality of evidence

Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE) was used to evaluate the quality of evidence, where a GRADE pro tool summary of findings (SoF) table was created by reviewers (NCM, AMS, and AK).

RESULTS

Information search results and eligible reports

Using the protocol by Mzimela *et al.(Mzimela et al., 2023),* 951 full text articles we obtained by the reviewers. From these 951 results, 21 of the reports were duplicates. This resulted in 930 full text articles that were captured and then screened for eligibility using the criteria mentioned above in methods. However, no report was eligible for this systematic review and meta-analysis, as shown (Additional file 2)**.** Interestingly, there was one article by Ziaee and co-workers that reported on haematological indices but not meeting the criteria for the study (Ziaee et al., 2017). Ziaee *et al* compared haematological indices for pre-diabetes with type 2 diabetic subjects, which lead to elimination of the article (Ziaee et al., 2017).

DISCUSSION

RBCs indices has been one of the hallmarks that enable prediction of diabetes complications such as anaemia. However, insufficient information is available based on RBCs at pre-diabetes stage. Additionally, we can also assume that there is insufficient information based on anaemia and pre-diabetes stage. This gap in research also contributes on the cardiovascular complications that have been reported on pre-diabetic rats which has not been explored in human subjects (Gamede et al., 2019, Khathi et al., 2019, Mabuza et al., 2019). Since pre-diabetes stage is classified by the glucose levels being more than normal state, it is then abnormal (Ziaee et al., 2017). The abnormality of glucose levels therefore raised a challenge to explore the damages that can be done by this abnormality at pre-diabetes. RBCs indices also change due to change in glucose levels (Ziaee et al., 2017). However, the challenge on report by Ziaee *et al* is observed since it does not have reports on a normal state to compare with pre-diabetes and T2D (Ziaee et al., 2017). The population size meets the inclusion criteria and age rage was 20-70 year creating a clash with our inclusion criteria which is 25-45 years (Ziaee et al., 2017). Interestingly, Ziaee *et al*, indicated that most of the pre-diabetics were younger than T2D subjects and also that most pre-diabetic subjects were males and more females being T2D (Ziaee et al., 2017). This was also seconded by a study by Ezenwaka *et al*, that more non-diabetics are younger compared to T2D (Ezenwaka et al., 2008). However, it is a debatable issue if there is a difference on pre-diabetes subjects. Additionally, using Caribbean's subjects, Ezenwaka and co-workers reported that the MCHC was the only RBCs indices that was significantly different in female participants at pre-diabetes stage and T2D (Ziaee et al., 2017). However, these changes are observed and reported based on results on pre-diabetes and T2D,

raising a questionable stage at a normal healthy condition. Ezenwaka *et al* also indicated that non-diabetics and all males had higher RBC count, Hb and HCT compared to diabetic subjects which also raised a gap on the pre-diabetic subjects (Ezenwaka et al., 2008). Their report also indicated that all female subjects had Hb concentration which was similar to each other (Ezenwaka et al., 2008). However, the there is a gap at pre-diabetes stage. This then create a challenge of exploring the pre-diabetic stage to close the gap due to insufficient information reporting at pre-diabetic stage.

CONCLUSION

The insufficient information based on RBCs indices at pre-diabetes stage creates a huge gap in the research field. There is more research, or investigation is required where there will be exploration of RBCs and mechanisms involved at pre-diabetes stage to give clear understanding if abnormalities reported at type 2 diabetes stage begin during pre-diabetes stage. since findings have indicated that there is change at pre-diabetes stage using animal model.

LIMITATIONS

Unavailability of related articles created a limitation of what to expect on our research based on RBCs indices. Unavailability of articles raise a challenge whether the demographics have effect on changes at pre-diabetes stage, if there is any change.

RECOMMENDATIONS

Insufficient information gives an advantage to close a gap available on pre-diabetes research base on RBCs. Gab will be closed by investigating pre-diabetes stage mechanisms that involve RBCs and investigating if there are any abnormalities during progression of pre-diabetes stage.

Abbreviations

Type 2 diabetes (T2D), International Diabetes Federation (IDF), red blood cells (RBCs), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), haemoglobin (HGB), haematocrit (HCT), red cell distribution width (RDW), endothelial nitric acid synthase (eNOS), erythropoietin (EPO), Reporting items for systemic reviews and meta-analysis (PRISMA), Fasting blood glucose (FBG), Medical Subject Heading (MeSH), Grading of Recommendations Assessment, Development, and Evaluation approach (GRADE), summary of findings (SoF), National Research Foundation (NRF)

Authors contributions

NCM, AMS, and AK were responsible for brainstorming, designing the study, and then also drafted the systematic review. NCM, AMS, PS, and AK were responsible for reviewing the eligible study and final draft of the manuscript. Funders had no role in developing the systematic review.

Ethics Approval and consent to participate

The data that will be analysed will be the data that is published, and they will be no data collection from subjects. The authors declare that there will be no informed consent required to be signed and therefore no ethics approval required for the systematic review and meta-analysis.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Consent for publication

Not applicable

Availability of supporting data

No extra data available besides the attached additional file since it is a protocol for systematic review.

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Additional file 1

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Page2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Page 3
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Line 90-116
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Line 80-89
Search strategy	7	Present the full search strategies for all databases, registers, and websites, including any filters and limits used.	Line 80-89
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Line 91-94
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Line 91-129
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Line 118-129
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Line 118-129
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Line 131-139
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Line 141-152
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Line 141-152
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Line 141-152
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Line 141-152
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Line 141-152

Section and Topic	Item #	Checklist item	Location where item is reported
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Line 141-152
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	n/a
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	n/a
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Line 154-157
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Line 160-165
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Line 165-168
Study characteristics	17	Cite each included study and present its characteristics.	165-168
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	n/a
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	n/a
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	n/a
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	n/a
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	n/a
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	n/a
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	n/a
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	n/a
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Line 169-194
	23b	Discuss any limitations of the evidence included in the review.	Line 201-203
	23c	Discuss any limitations of the review processes used.	Line 201-203
	23d	Discuss implications of the results for practice, policy, and future research.	Line 201-203
OTHER INFORMATION			

Section and Topic	Item #	Checklist item	Location where item is reported
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Line 75-77
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Line 77-79
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	n/a
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Line 228-229
Competing interests	26	Declare any competing interests of review authors.	Line 226-227
Availability of data, code, and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Line 223-225

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71
For more information, visit: http://www.prisma-statement.org/

Additional file 2

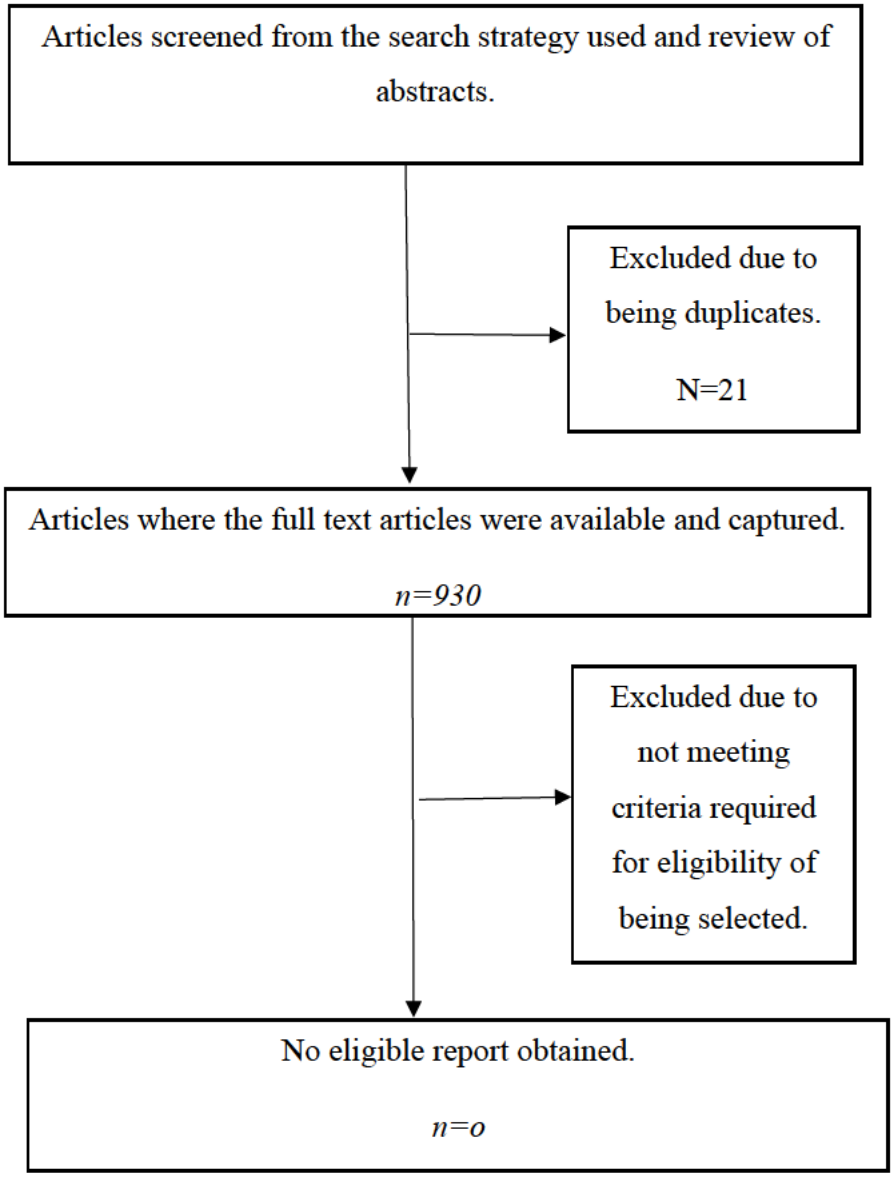

Figure1: Flow diagram of studies in the review.

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled " **Evaluating the changes in red blood cells indices in pre-diabetic patients from the age of 25- to 45- years in Durban, South Africa "** and is authored by N.C Mzimela, A.M Sosibo, P.S Ngubane, and A. Khathi. The manuscript is accepted for publication in **Journal of Blood Medicine (ISSN: 1179-2736)** and has been formatted according to journal's guidelines for authors **(REF Number: 470181).).** This journal is accredited by Department of Higher Education and Training South Africa and appears in Scopus accredited list (2022).

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

Evaluating the changes in red blood cells indices in pre-diabetic patients from the age of 25- to 45- years in Durban, South Africa

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Abstract

Pre-diabetes is an asymptomatic, long-lasting condition that often precedes the onset of T2D. Studies using a diet-induced model of pre-diabetes suggest that this condition results in changes in red blood cells indices. Recently, reports indicated an increase in pre-diabetes prevalence among people of from 25-45 years of age in Durban, South Africa. Using this population, this study is sought to investigate the changes in RBCs indices and the state of erythropoietin in Durban, South Africa. Upon ethical clearance, 292 samples were collected at King Edward hospital and red blood cell indices were measured using a haemocytometer. The samples were then grouped into three experimental group according to American Diabetes Association criteria (30 = non-diabetic (ND), 90 = pre-diabetic (PD) and 172 = T2D). Plasma erythropoietin concentration was also measured. The results showed a non-significant increase in WBC, RBC, HGB, HCT, MCV, MCH and MCHC when comparing T2D with ND. There was also a non-significant decrease in EPO and RDW when comparing T2D with ND. However, by comparing PD with ND, the results showed a non-significant increase in all red blood cell indices and EPO concentration. The change in RBC indices during pre-diabetes stage indicate that while there are insignificant changes in RBC production, there are, however, changes in RBC structure and pathways involved due to the moderate hyperglycaemia observed in pre-diabetes.

Key words: Red blood cells, Pre-diabetes, erythropoietin, Durban, South Africa

Highlights

- ✓ Changes in red blood cells indices in pre-diabetic patients from 25 to 45 years.
- ✓ Male and females have different red blood cells indices due to hormonal changes.
- ✓ Increase in erythropoietin levels in pre-diabetic patients from 25- to 45- years.

Introduction

Red blood Cell (RBC) indices are the individual components of blood tests that measure the characteristic and quantity of red blood cell found in the organism body (1). RBC indices have served as important clinical markers for the diagnosis of cardiovascular diseases (2). Literature has also reported that these red blood cell indices also display changes in type 2 diabetic human subjects (3, 4). In 2019, according to IDF(International Diabetes Federation) statistics, there were 19 million diabetic people in Africa aged between 20 and 79 years (5). According to Statistics South Africa, diabetes has been observed to be the second leading underlying cause of death in South Africa in 2016 and 2017(6). Surprisingly, the IDF further estimated that of the 4.58 million people between 20 and 79 years old with diabetes in South Africa, about 52.4% were undiagnosed in 2019 (7). Pre-diabetes is a state of intermediate hyperglycaemia that often occurs between normoglycaemia and the onset of type 2 diabetes (8). Pre-diabetes has been reported to be asymptomatic contributing to a challenge on its statistical documentation (8). This has also made it difficult to study changes in the body during this state. A systematic review conducted by Sosibo and colleagues estimated that there is an increase in pre-diabetes prevalence in South Africa (9). This study also showed that the high prevalence of pre-diabetes is among population from 25-45 years (9).

Luvuno and colleagues created a diet-induced pre-diabetic rat model using high-fat high-carbohydrate diet (10). This model has been shown to mimic the human condition and thus has been used to study changes during pre-diabetes (10). Other studies using this model have reported that there are metabolic and signal abnormalities during the prediabetic state (10-13). A study by Mzimela and colleagues reported that there is an increase in RBCs, hemoglobin (HGB), hematocrit (HCT) in the prediabetic state by comparison to the non-prediabetic state and a positive correlation of EPO between non-diabetic (ND) and pre-diabetes (PD) group (11). Decreases in white blood cell (WBC) concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW) in the prediabetic state were further observed (14). However, no study has been conducted using human subjects. The city of Durban in South Africa provides a rapidly urbanizing and culturally diverse population which is an advantage for exploration of research based on hematological changes in the pre-diabetic stage. Therefore, this study sought to investigate the haematological changes that occur during pre-diabetic stage in pre-diabetic human subjects aged between 25 and 45 years in a population from Durban, South Africa.

Materials and Methods

The standard pharmaceutical supplier was the source of all chemicals and reagents of analytical grade. The standard pharmaceutical supplier was Elabscience for human HbA1c and EPO (Catalog No: E-EL-H3640) ELISA kits.

Methods

Study site, population, and design

From February 2021 to December 2022, a study was carried out at the University of KwaZulu Natal, Durban (South Africa) laboratories. A quantitative cross-sectional analytical study was conducted with blood sample population (n=292) that was collected from King Edward hospital. Blood samples from patients aged from 25 to 45 years that were without any terminal disease and meeting inclusion criteria, were collected for the study approved by UKZN Biomedical Research Ethics Committee. The samples were collected according to the inclusion and exclusion criteria of the study.

Ethics Approval

Before sample collection from the hospitals mentioned above, study ethics approval was obtained from the College of Health Sciences, Biomedical Research Ethics Committee (BREC), the University of KwaZulu Natal with BREC REF NO: BE266/2019.

Blood sample screening

Selection criteria

The sampling exclusion criteria were any sample of a patient that was below age 25 years and over 45 years of age, the sample of patients displaying other diseases either than T2D and pre-diabetes, blood samples of patients under the influence of alcohol and also pregnant females. The sampling inclusion criteria were any blood sample of the patient that is non-prediabetic, pre-diabetic and T2D without any disease display upon screening, blood samples of patients that are between the age of 25 years and 45 years, both genders and all races.

Pre-diabetes confirmation

The American Diabetes Association (ADA) criteria was applied to confirm whether samples should be categorised as non-prediabetic, pre-diabetic or T2D (15, 16). ADA criteria is: Impaired fasting blood glucose (FBG) (5.6 -7.0 mmol/L), glycated haemoglobin (HbA1c) (5.7-6.4%) and elevated 2h postprandial blood glucose (2hour-OGTT) (7.8 -11.0mmol/L). Additionally, from the data obtained from the hospital for glucose levels, HbA1c was measured

using respective human ELISA kits from Elabscience, as per manufacturer's instructions. The samples that showed HbA1c below 5.7% were considered normal, HbA1c between 5.7% and 6.4% considered pre-diabetic and then HbA1c above 6.4 % considered T2D.

Blood indices measurements

An automated haemocytometer (Beckman Coulter, Indianapolis, United States) was used to measure the concentration of blood cell indices (HGB, HCT, WBC, MCV, MCH, MCH, and RDW) in fresh blood of all three groups. The plasma was collected by centrifuging the whole blood for 15 mins using the centrifuge at 3000 rpm. The plasma was then stored at -80°C until analysed using ELISA.

EPO concentration measurement

To check for the concentration EPO, Human EPO ELISA kits form Elabscience were used, as per manufacturer's instructions. The optical density of each well determined using a Spectro star nanoplate spectrophotometer (BMG Labtech, Ortenburg, Baden-Württernberg, Germany) at 450 nm.

Data analysis

The Statistical Package for the Social Sciences v28 (SPSS Inc., Chicago, USA) software was used to analyse both blood cell indices and EPO in all 3 groups (ND, PD and T2D). One-way ANOVA analysis was used to analyse RBCs parameters and EPO, followed by Tukey-Krammer *Post Hoc* on SPSS. The results are expressed as mean ± SE. A p-value < 0.05 was considered as statistically significant.

Results

The study had a total of 292 participants (30 non-diabetic, 90 pre-diabetic and 172 T2D). The graph shows a percentage of males and females per group. The 3 groups were non-diabetic group (ND) with 20 females and 10 males, pre-diabetes group (PD) with 56 females and 34 males and type 2 diabetes group (T2D) had 113 females and 59 males.

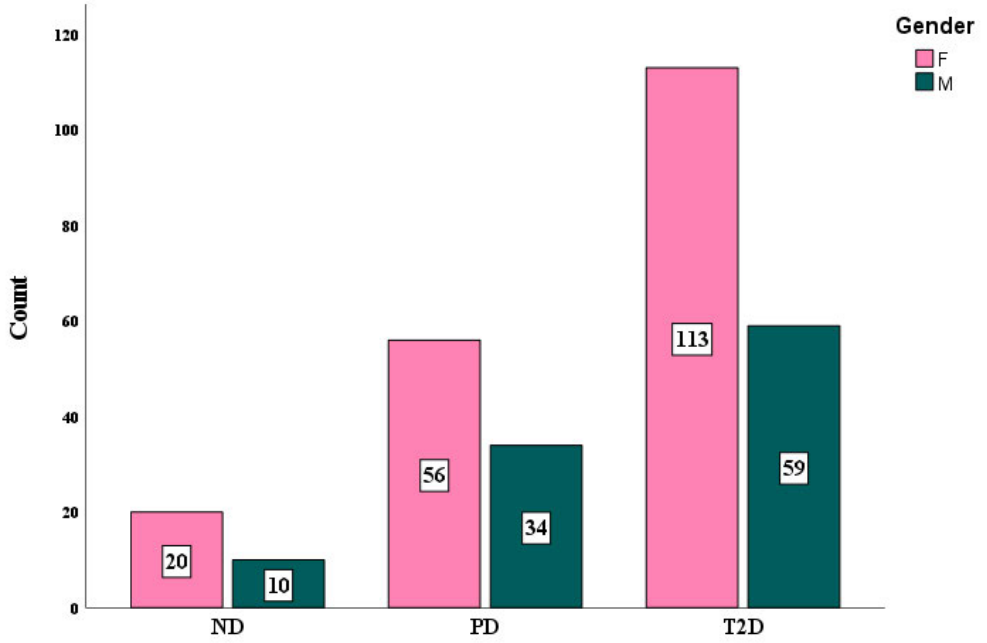


Figure 1: Graph showing the count of males and females per group (ND, PD and T2D)

Blood Glucose and glycated haemoglobin levels

Glucose and glycated haemoglobin levels were measured in the blood that was used in the study in all the three experimental groups (ND, PD and T2D). The results showed an increase in fasting glucose levels in T2D group by comparing it to the ND group (see Table 1A). The results also showed an increase in fasting glucose levels in the PD group by comparison to the fasting glucose levels of the ND group (see table 1A). The results showed an increase in glycated haemoglobin levels in T2D group by comparing it to the ND group (see table 1A). There was also an increase in glycated haemoglobin in the T2D group by comparison to the PD group (see table 1A). All groups showed that they were within the ranges that are outlined by ADA guidelines. Additionally in table 1B, the results showed the changes in glucose levels and glycated haemoglobin levels which still correspond to the ADA guidelines.

Table 1: Table showing the glucose and glycated haemoglobin levels per group where (A) shows glucose and glycated haemoglobin levels per group and (B) shows glucose and glycated haemoglobin levels of gender per group of all 3 groups.

A.

	GROUPS		
	Non-diabetes	**Pre-diabetes**	**Type 2 diabetes**
GLUCOSE(MMOL/L)	5.2	6.7	13.2
HBA1C (%)	4.9	5.8	9.9

B.

	GROUPS					
	Non-diabetes		**Pre-diabetes**		**Type 2 diabetes**	
	Females	**Males**	**Females**	**Males**	**Females**	**Males**
GLUCOSE (MMOL/L)	5.2	5.1	6.7	6.7	13.5	13.0
HBA1C (%)	4.9	4.8	5.8	5.8	9.7	10.3

White blood cells concentration

Circulating WBCs counts were measured in all 3 groups (ND, PD, and T2D). The results from all three groups were within the normal range which is 4.00-11.0 k/μL. Figure 2 shows a non-significant increase ($p = 0.10$) in circulating WBCs counts in the T2D group compared with the ND host group. Additionally, the results showed a non-significant increase ($p = 0.29$) in circulating white blood cells in the PD group compared to the ND group, and there was a non-significant decrease ($p = 0.78$) in circulating WBCs concentration in the PD group compared to the T2D group (see figure 2). Additionally, Multiple linear regression where glycated haemoglobin was a dependant factor for WBC, indicated a statistical significance ($p = 0,05$) (see supplementary data)

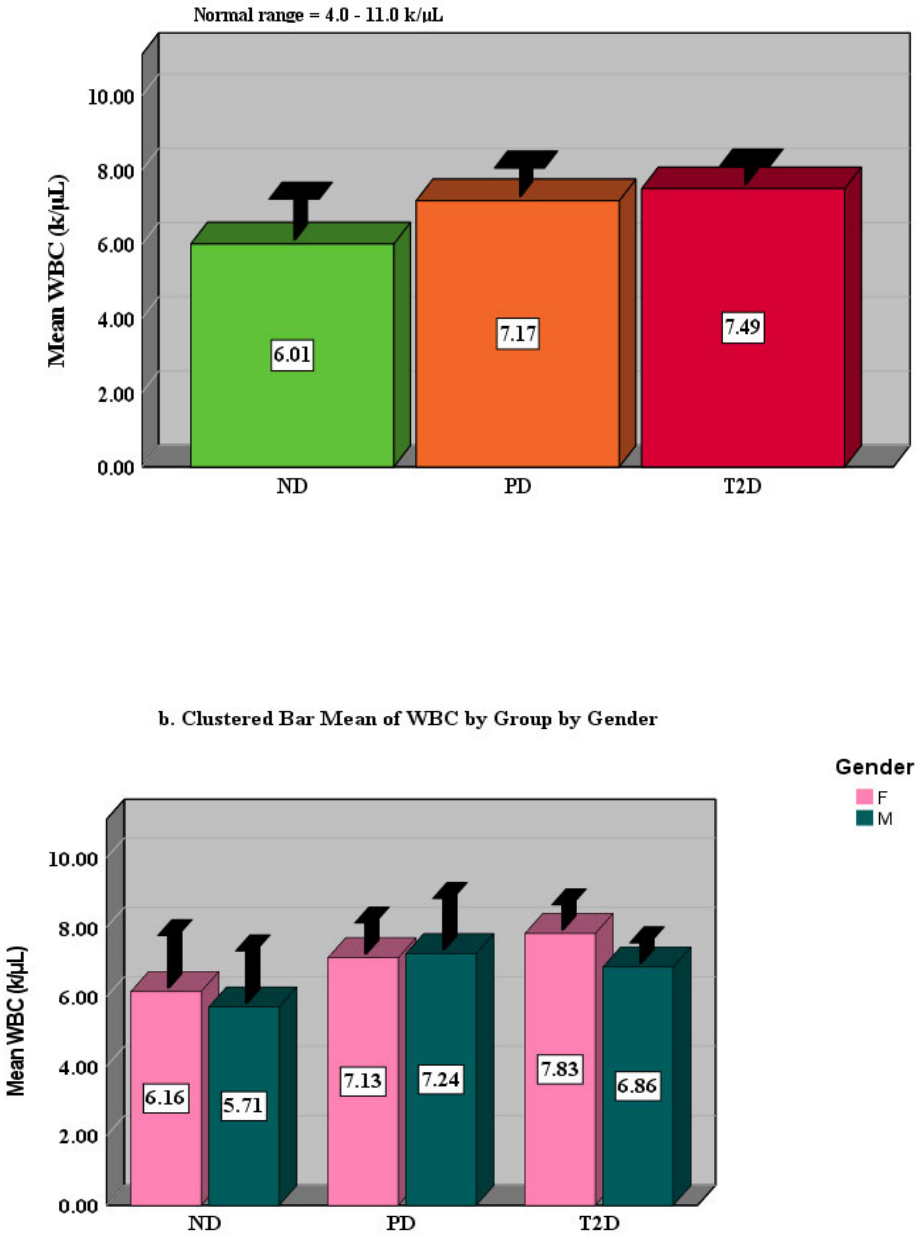


Figure 2: Concentration of white blood cells per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

Red blood cells concentration

The RBC concentration was measured in the ND, PD, and T2D groups. The results of the three groups were within the normal range for RBC which was 4.40-6.60 M/µL. Figure 3 shows a non-significant increase ($p = 0.19$) in circulating RBCs in the T2D group compared with that

in the ND host group. The results showed an insignificant increase (p = 0.24) in RBCs in the PD group compared with that in the ND group. However, when the PD group was compared to the T2D group, no changes were observed (p = 1.00) (see figure 3).

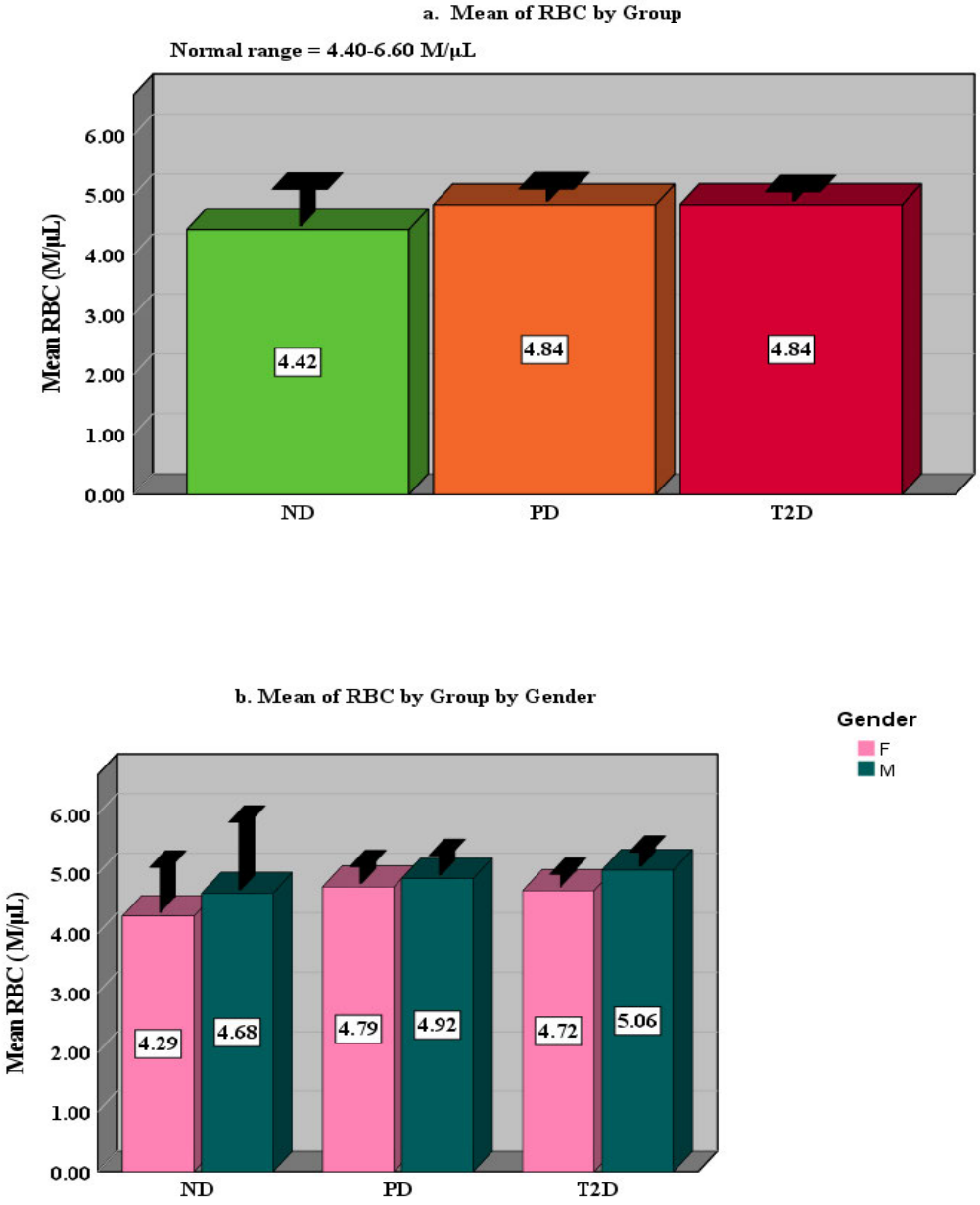
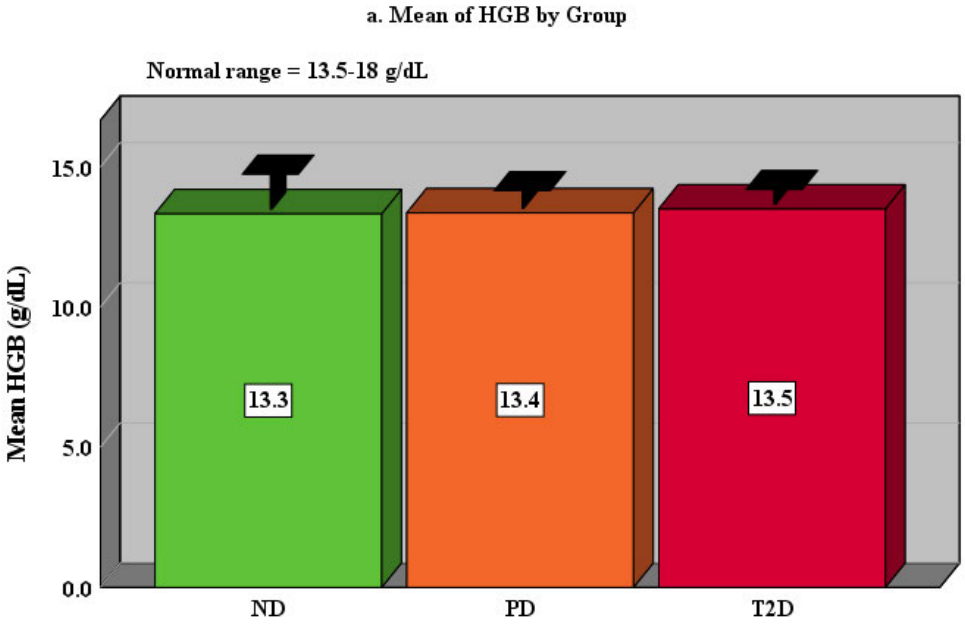


Figure 3: Concentration of red blood cells per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

Haemoglobin concentration

The concentration of HGB was measured in the three experimental groups (ND, PD. And T2D). The three groups measured had HGB concentrations for ND and PD slightly below the normal range, and the T2D group had a range within the normal range which was 13.5-18 g/dL. Figure 4 shows a non-significant increase ($p = 0.97$ in the circulating haemoglobin percentage in the T2D group compared to that in the ND host group. Additionally, there was no significant increase ($p = 0.99$) in HGB levels in the PD group compared to those in the ND group. A further increase ($p = 0.95$) was observed in the T2D group compared to the ND. (see figure 4). The results for males and females per group are within a normal range for both females and males which is 12.1-15 g/dL for females and 13.8 -17.2 g/dL for males (see figure 4b)



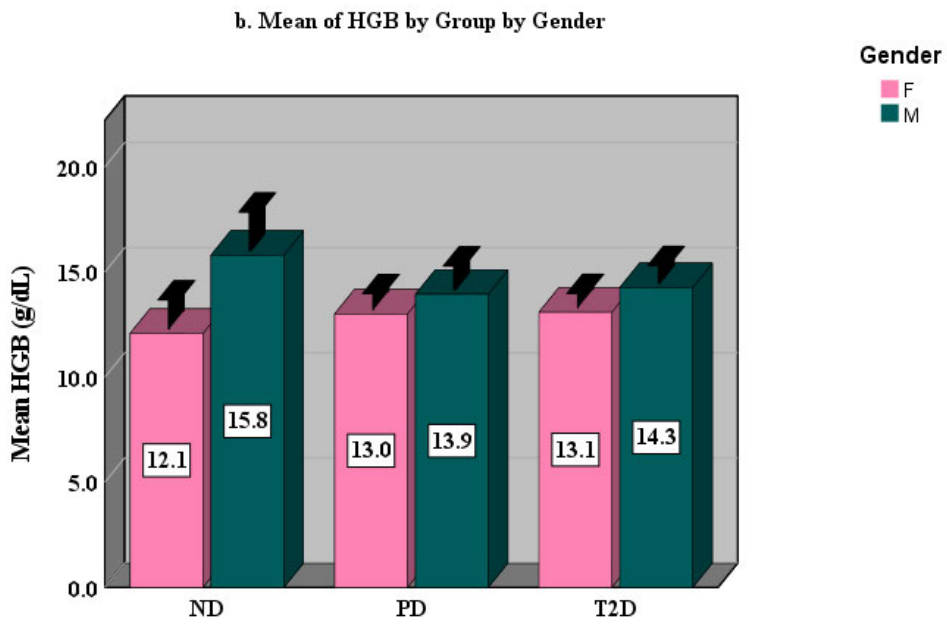
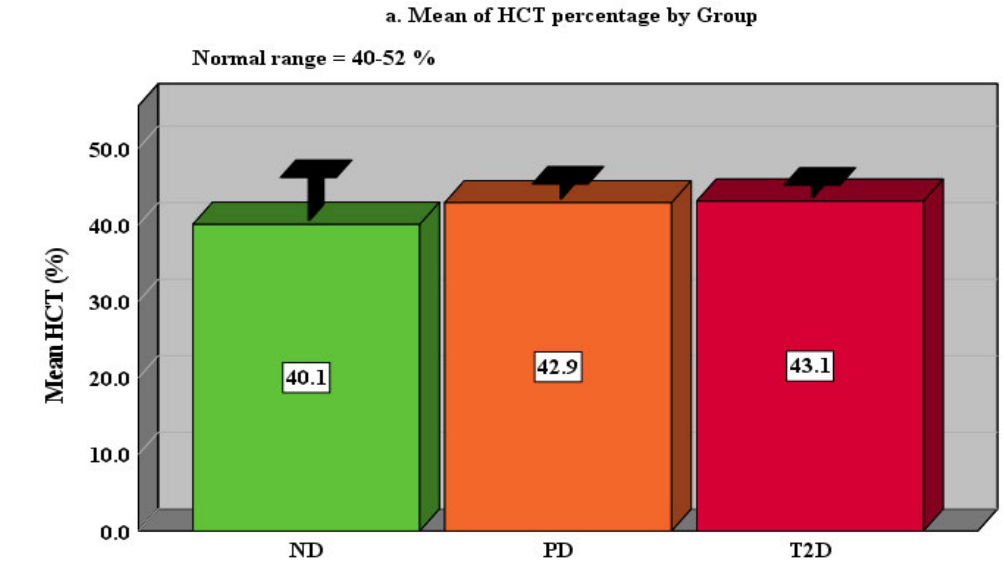


Figure 4: Concentration of hemoglobin per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

Haematocrit percentage

The haematocrit percentage was measured in the ND, PD, and T2D groups. The results obtained from three groups were still within the normal range which is 40-52 %, even though there were changes observed. Figure 5 shows a non-significant increase ($p = 0.40$) in the haematocrit percentage in the T2D group compared to the ND host group. The results also showed a non-significant increase ($p = 0.49$) in the haematocrit percentage in the PD group compared with that in the ND group. There was also a non-significant decrease ($p = 0.99$) in the haematocrit percentage in the PD group compared to that in the T2D group (see figure 5). The results for males and females per group were within the normal range for both females and males which is 38-46% for females and 42-54% for males (see figure 5b).



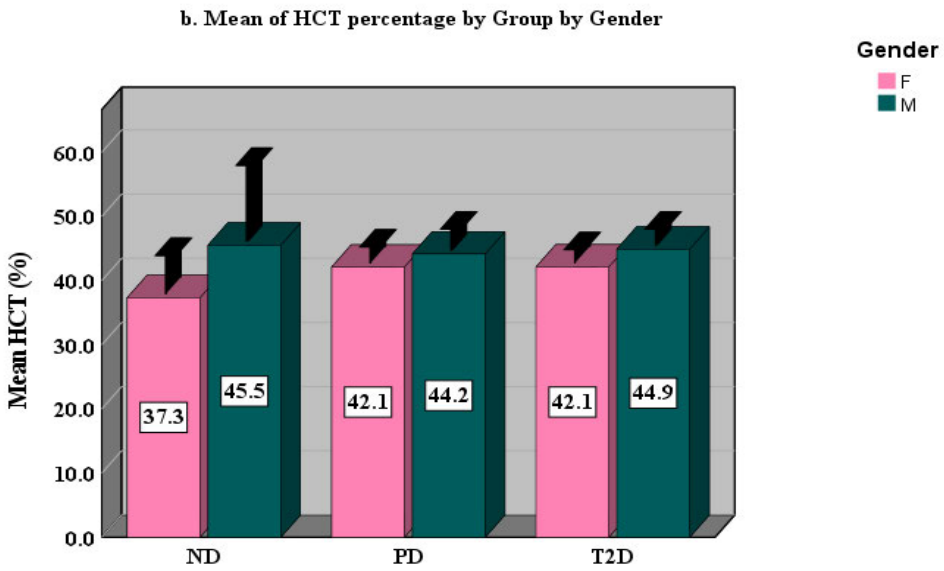
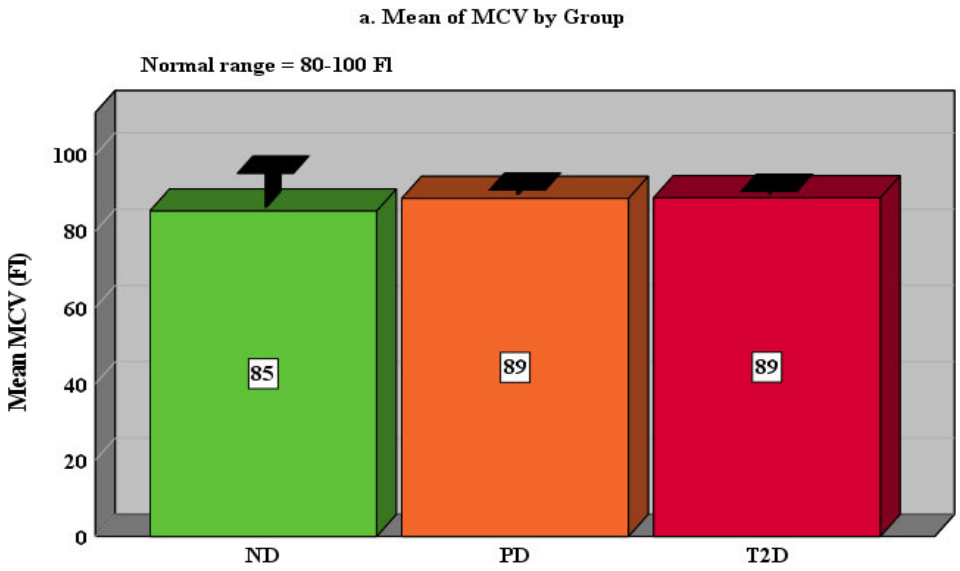


Figure 5: Hematocrit percentage per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

MCV concentration

The MCV concentration was measured in the three groups (ND, PD, and T2D). The results showed changes, but the MCV remained in the normal range of which is 80-100 Fl. Figure 6 shows a non-significant increase (p = 0.22) in MCV concentration in the T2D group compared to that in the ND host group. A non-significant increase (p = 0.30) in MCV concentration was also observed in the PD group compared to that in the ND group. However, no change (p = 0.99) was observed in the comparison between the PD and T2D groups (see figure 6).



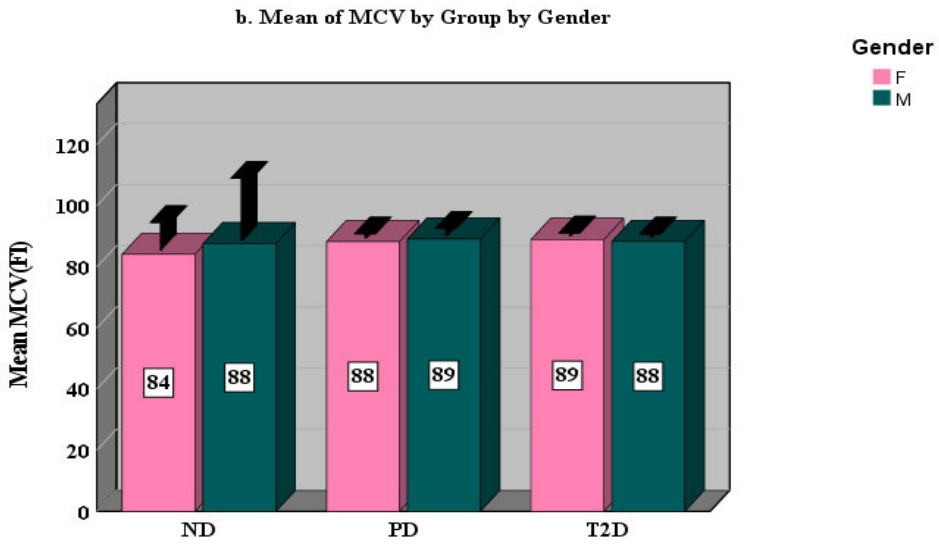


Figure 6: Concentration of MCV per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

MCH concentration

The MCH concentration in RBCs was measured in the ND, PD, and T2D groups. All groups were still within the normal range which was 27.0 -33.0 pg. Figure 7 shows a non-significant increase (p = 0.80) in MCH concentration in the T2D group compared with that in the ND host group. The results also showed a non-significant increase (p = 0.88) in MCH concentration in the PD group compared to the ND group, and a non-significant decrease (p = 0.98) in the PD group compared to the T2D group (see figure 7). Additionally, Multiple linear regression where glycated haemoglobin was a dependant factor for MCH, indicated a statistical significance (p = 0,03) (see supplementary data)

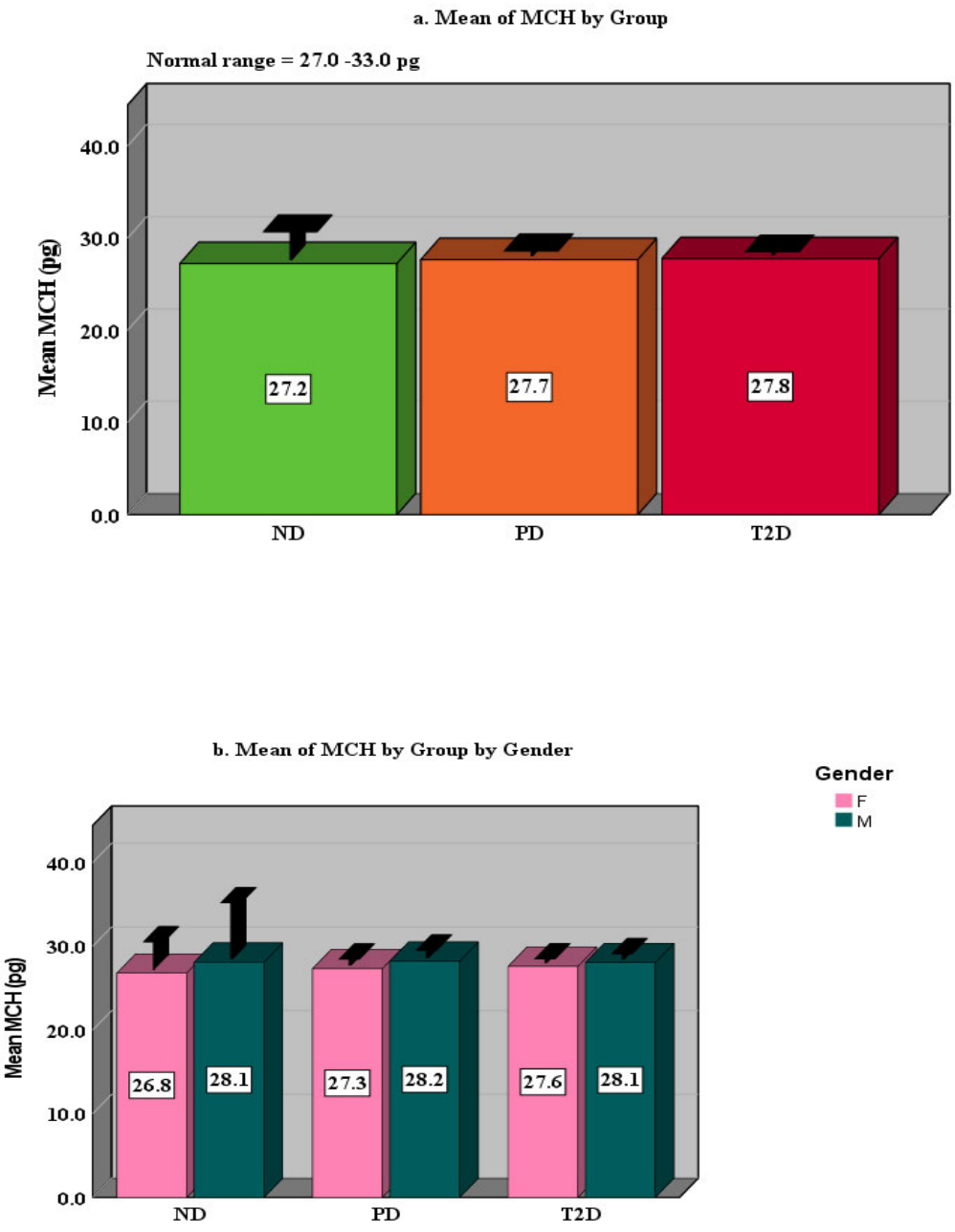
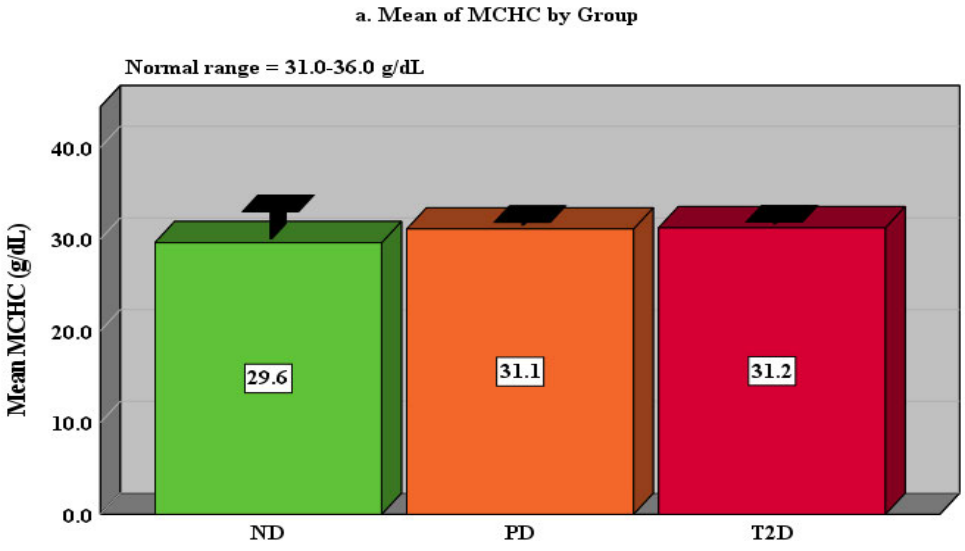


Figure 7: Concentration of MCH per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

MCHC concentration

MCHC concentration was measured in the ND, PD, and T2D groups. The ND group had a value below the normal range of 31.0-36.0 g/dL. However, the PD and T2D groups had a range

within the normal range of MCHC. Figure 8 shows a significant increase (p = 0.05) in MCHC concentration in the T2D group compared with that in the ND host group. Additionally, the results showed a non-significant increase (p = 0.11) in MCHC concentration in the PD group compared to that in the ND group. Furthermore, a non-significant decrease (p = 0.95) in the MCHC concentration was observed in the PD group compared to that in the T2D group (see figure 8). Additionally, Multiple linear regression where glycated haemoglobin was a dependant factor for MCHC, indicated a statistical significance (p = 0,02) (see supplementary data).



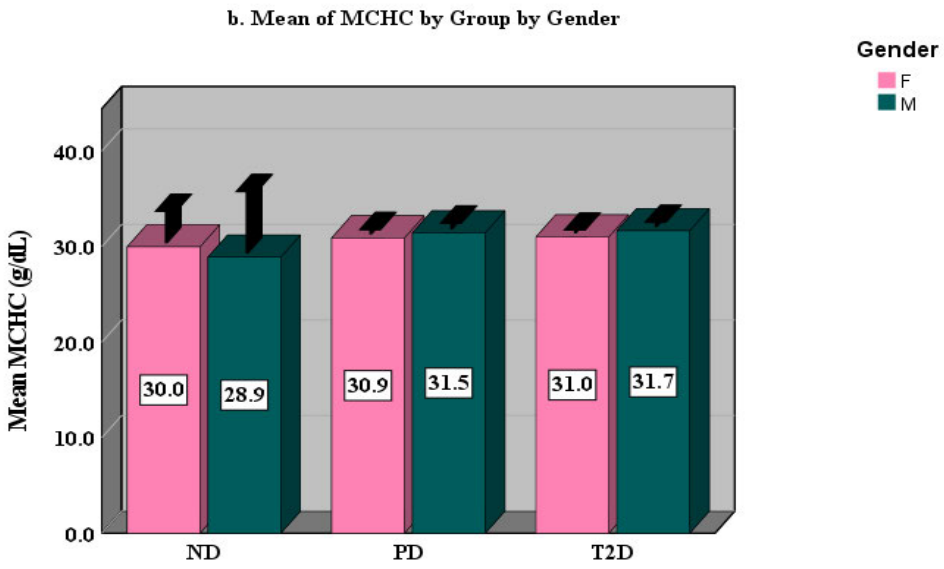


Figure 8: Concentration of MCHC per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

RDW concentration

RDW percentages were measured for ND, PD, and T2D. All groups measured had a range below 16.4%, which is the normal range. Figure 9 shows a non-significant increase ($p = 0.63$) in RDW concentration in the T2D group compared with that in the ND host group. The results also showed a non-significant increase ($p = 0.51$) in the RDW percentage of PD compared with ND, and the PD group had a non-significant increase ($p = 0.92$) in percentage compared to the T2D group (see figure 9).

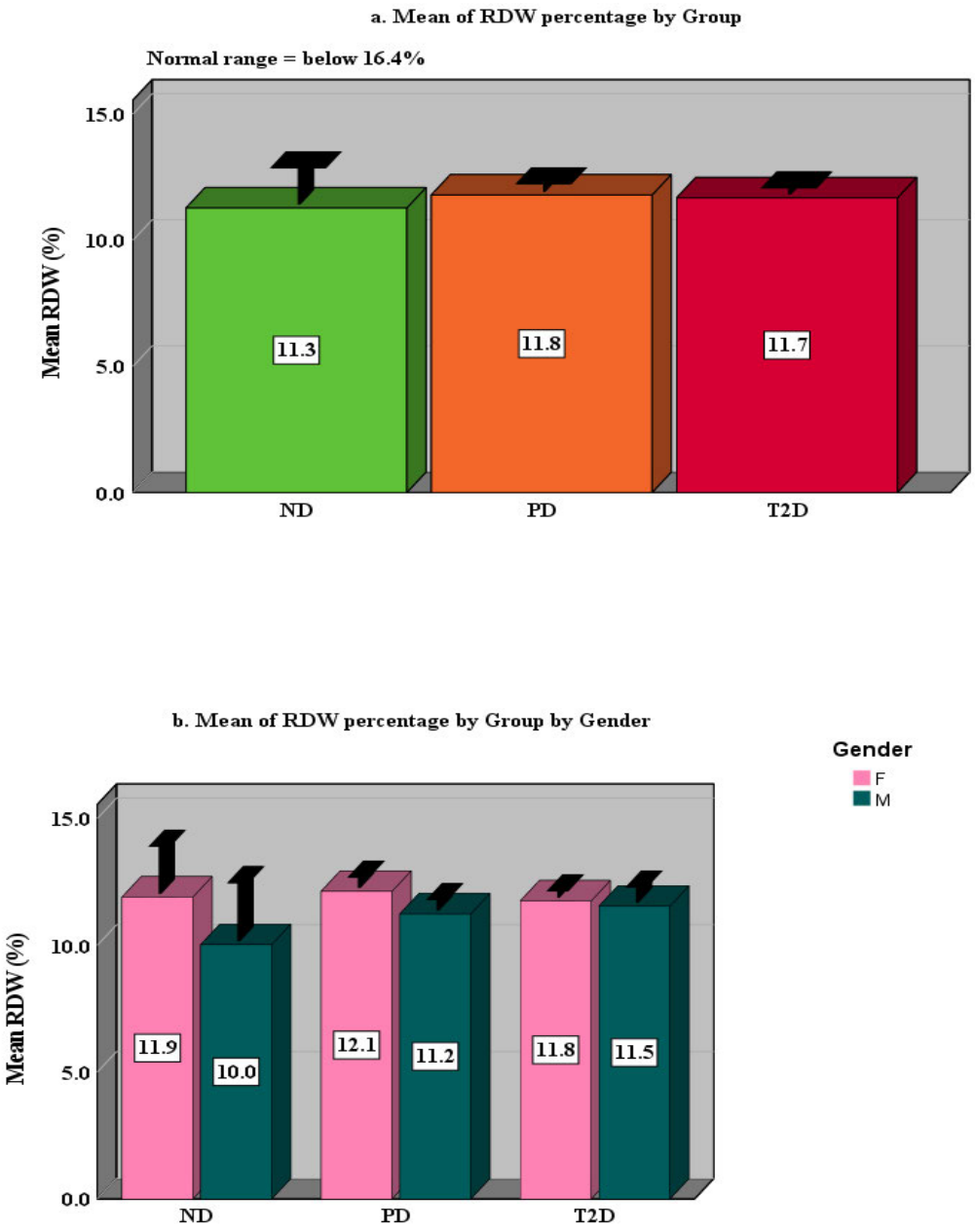


Figure 9: Percentage of RDW per group (a) and gender per group (b)**,** where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group**.** Data presented as mean ± SE.

EPO concentration

Erythropoietin concentrations were measured in the three groups (ND, PD, and T2D). Figure 10 shows a non-significant decrease (p = 0.42) in EPO concentration in the T2D group compared with that in the ND host group. There was also a non-significant increase (p = 0,95) in EPO concentration in the PD group compared with that in the ND group. Additionally, the

results showed a non-significant increase (p = 0.15) in EPO concentration in the PD group compared to that in the T2D group (see figure 10).

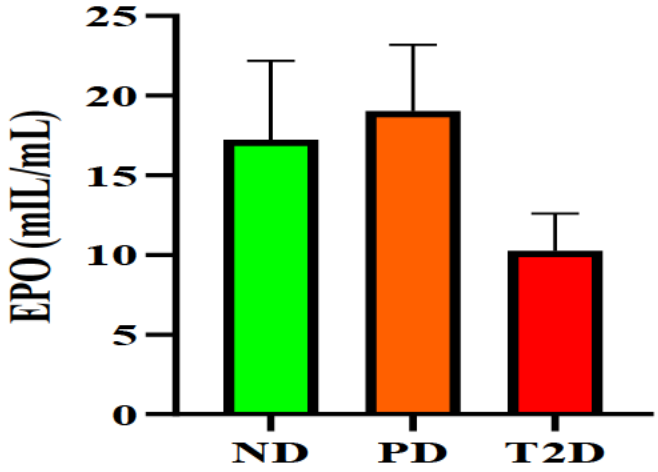


Figure 10: Graphs of erythropoietin, where ND represent a non-diabetic group, PD represent a pre-diabetic group and T2D represent type 2 diabetes group.

Discussion

Blood consists of plasma and different kinds of cells such red blood cells (erythrocytes), white blood cells (leukocytes) and thrombocytes (platelets) (17). We have previously reported on the state of white blood cells (WBCs) and red blood cells (RBCs) during the progression of pre-diabetes using a diet-induced animal model of pre-diabetes (11, 18) . Using this model, we reported on a decrease in WBCs, RDW, MCH, MCHC and MCV during pre-diabetes (11). Additionally, we reported an increase in RBCs, HCT and HGB during pre-diabetes (18). These findings have never been verified in prediabetic human subjects. The prevalence of pre-diabetes has been shown to be increasing in rapidly urbanizing areas such as Durban in South Africa (9, 19). This therefore makes Durban an ideal area to study pre-diabetes. In this study, we looked at RBCs and their parameters in pre-diabetic patients subjects from Durban, South Africa.

According to Vozarova and co-workers, one of the modifications that signal the activation of the immune system and inflammation is the increase in circulating levels of the WBCs (20). WBCs play different roles upon recognition of an invading agent and are divided into agranulocytes which are monocytes and lymphocytes a well as granulocytes which are neutrophils, eosinophils, and basophils (21, 22). During T2D, there is an increase in WBC due to the toxic effect of hyperglycaemia which results in dysregulated immunity and chronic

inflammation (21, 23). Indeed, in this study, this was evidenced by increased concentration of WBCs in the T2D group by comparison with the ND group. The results showed on figure 2 of this study further demonstrated an increase in WBCs in the PD group by comparison with the ND group. These observations may indicate that the moderate insulin resistance during the prediabetic state may cause activation of the immune system, thereby causing the increase in immune cells produced by bone marrow.

According to literature, disturbances in hematopoietic milieu has been reported to affect the RBCs (24, 25). RBCs have been reported to account for about 99.9% of cells on circulation and their main function is oxygen transportation from the lungs to respiring tissues and then transporting carbon dioxide back to the lungs from tissues (21, 22). One of the factors that has been reported to cause disturbances in RBCs is chronic hyperglycaemia, which is observed in T2D and at some stage, it is the very same metabolic elevated glucose levels that contribute to pre-diabetes (14, 26, 27). However, as previously stated, these glucose levels are not yet high enough to be characterised as T2D (28). Under hyperglycaemic states, RBCs have been reported to change in form (reduced cell deformability) which is hypothesised to be due to glycosylation and oxidation of haemoglobin (26, 29, 30). Additionally, it has been reported that chronic insulin resistance and hyperinsulinemia can result in hyperglycaemia which then cause pre-diabetes and onset of T2D (31). Furthermore, insulin has been reported to perturbate various physiological processes by stimulating factors such as receptor substrates thereby causing insulin resistance(31). Insulin has also been reported to cause increase in circulating RBCs count through regulation of erythropoiesis (32). Human RBCs display insulin receptors, which enable the binding of circulating insulin to its receptor which then results in increased circulating RBCs (32-34). This is indeed demonstrated by the results observed in this study where, there is an increase in RBCs on T2D group by comparison with the ND group. Figures 3b and 4b showed a decreased in RBC count and HGB concentration (normal range which is 12.1-15 g/dL for females and 13.8 -17.2 g/dL for males) on all females by comparison to males per group in all groups(35, 36). According to Murphy, under normal physiological conditions females have about 12% lower HGB levels when compared to males (37). This was ascribed to the concentration of hormones such as oestrogens and androgens (37). Estrogen is involved in dilating the vessels in renal microvasculature while androgens are involved in constriction in renal microvasculature there by affecting erythropoiesis and causing changes in haematocrit (35, 37). Interestingly, figure 5b, shows a decrease in HCT (normal range which is 38-46% for females and 42-54% for males) in females by comparison to males per group in all groups,

which correlates with the decrease in RBCs and HGB in females by comparison to males (35). (38). RBC eNOS has been reported to be the central role player in regulating RBC deformability (39, 40). According to Vona *et al*., estrogen recruits the estrogen receptors(ERs) at a membrane level in both male and females, but in males the estrogen receptors are localised more on cytoplasm and membrane localised in females (38). The binding of estrogen with ERs therefore induces eNOS phosphorylation and NO production in females through several intracellular signalling mechanisms (38, 39). However, with the males, different pathways are involved that are not the same as in females (38). In this study, there is an increase in levels of RBC, HGB and HCT in T2D groups by comparison to ND.

We speculate that the insulin resistance in T2D triggered erythropoiesis which caused more production of RBCs. In this study, we observed an increase in RBCs in the PD group by comparison to the ND group. This may suggest that in the prediabetic subjects, insulin resistance triggered erythropoiesis which resulted in the elevated levels of RBCs. However, no change was observed in RBCs concentration of in the PD group by comparison to the T2D group suggesting that insulin resistance still triggers erythropoiesis and that there is no disturbance on erythropoietin-producing cells in kidneys.

HGB levels have been reported to be directly correlated with RBC count (32). HGB has a tetrameric structure formed by α2β2 molecule which is an active oxygen transport regulator in RBCs which also interact with carbon dioxide and nitric oxide (NO) upon its functions (41). Furthermore, as the RBCs reach the relative hypoxia regions, NO is generated resulting in blood flow control via hypoxic vasodilation (41). Additionally, a heme ring of haemoglobin contains reduced iron which makes it the main component of haemoglobin and a prime oxygen carrier (42, 43). Therefore, deficiency in iron can decrease the transport of oxygen and contribute to development of anemia and tissue hypoxia (42, 43) In T2D, low levels of HGB have been reported and the elevated levels of IL-6 reported in T2D also upregulate hepcidin production (44). Hepcidin upregulation causes downregulation of the absorption of intestinal iron and causes impairment of iron transport from the reticuloendothelial system to the bone marrow (44). In addition to EPO deficiency in T2D, there is low transport of iron to the blood, from the macrophages, contributing to iron deficiency (44). This was indeed demonstrated by a low in HGB concentration on all three groups. However, due to the increase in blood RBCs, we indeed observed an increase in HGB concentration of T2D group by comparison with ND group. In this study, there was an also a clinical increase in HGB concentration in the PD group by comparison with the ND group. This suggests that even though the ranges of HGB are below

normal, there is still the effect of hyperglycaemia and inflammation which exacerbate the progression of pre-diabetes to T2D. However, it should also be noted that the level of HGB for all 3 groups were below the normal range, which may be due to poor absorption of iron (42-44). Additionally, it can be hypothesised that the low levels of iron are may be explained by findings by Bourne and colleagues which reported that South African adults display average diet that is low in iron, calcium, folate and vitamin B6, particularly in women and in rural areas. (45). The environmental factors can affect eating patterns since rural areas in south Africa are still socially and economically deprived (46). These factors result in the decrease in the oxygen transport and thus contributing to anaemia development and tissue hypoxia (45, 46).

In addition to insulin, EPO and Angiotensin II have also been reported to team up in maintaining the volume of blood (47). EPO is also a cytokine that is responsible for reproduction of RBC(47, 48). However, EPO activity is altered when there are low levels of oxygen (O_2) in the kidneys (47, 48). According to Donelly, kidneys function as a critmeter, which regulate haematocrit (49). This is because they are able to sense tension of oxygen and trigger production of EPO as well as control the extracellular volume by regulating the excretion of salt and water (49). Therefore, the balance of consumption of oxygen for reabsorption of sodium and the delivery of oxygen to the kidneys proximal tubule is reflected by the oxygen pressure to the tissues and that then determines red blood cells mass to be adjusted to plasma volume (49, 50). However, in conditions such as T2D, the kidneys have been reported to be damaged due to chronic hyperglycaemia and chronic inflammation, which result in hypoxia in the renal interstitium, thereby causing damage in the tubulointerstitial and impaired erythropoietin production (51). Due to the damage of the kidneys, there is a decrease in EPO production, resulting in low levels of EPO. Indeed, this is demonstrated by the results obtained showing a decline in EPO levels in the T2D group by comparison with ND group. This may indicate the beginning of chronic kidney failure which has been reported in T2D subjects. In this study, the EPO levels were increased in the PD group by comparison with the ND group. We can also hypothesise that there is acute kidney damage during pre-diabetes which is due to the moderate inflammation and hyperglycaemia. This acute renal damage might not be severe enough to disturb EPO production and further investigation is required to confirm the state of the kidneys in the pre-diabetes state.

Another marker that has been reported to determine the changes in RBCs is MCHC (52, 53). According to Kim and co-workers, MCHC is the determinant of cytoplasmic viscosity and it

also affects deformability of RBCs (52). An increase in factors such as superoxide ions caused by hyperglycaemia in RBCs have been reported to contribute to changes in structure and function of the RBC (53). Furthermore, The RBCs structural and functional modifications involve the way the RBCs haemoglobin aggregate and attach inside the membrane network of the RBC, which also contribute to an increase its cytosolic viscosity (3, 53). These complications in RBCs exposed to the hyperglycaemic environment cause an increase in MCHC (52, 53). Additionally, the hyperglycaemia-mediated increases in MCHC levels result in the stagnant RBCs causing thrombosis and the abnormalities such as atherosclerosis (4). Indeed, the results of the present study demonstrated an increase in MCHC levels in the T2D group by comparison with ND group, which suggest a change in structure of RBCs due to chronic hyperglycaemia. In this study, we further reported a clinical increase in MCHC in the PD group by comparison to the ND group. We hypothesise that the acute hyperglycaemia that is observed in pre-diabetes causes a disturbance of RBC cytoplasmic viscosity and this correlates with the reported increase in HGB (figure 4).

RDW is a parameter that evaluates the degree of the size and volume differences of the RBCs (54). Elevated levels of RDW are associated with a reduction in anti-oxidants and increased inflammation (24, 55). This is one of the reasons, RDW has been reported to be one of the inflammatory markers of complications such as atherosclerosis in clinical practice (24, 56). An increase in levels of RDW indicate the high degree of anisocytosis (24, 56). Anisocytosis is reported to be associated with the RBC degradation (24).Therefore, increased levels of RDW are associated with the decrease in deformability in RBCs and deformability which then results in blood flow being impaired through microcirculation (24, 55). In T2D, impaired blood flow may result in insufficient blood supply and also cause vascular damage (32, 57). According to Knychala and collegues, increased RDW is associated with elevated inflammatory status (58). Damage at the vascular system may then trigger inflammation and the release of inflammatory cytokines which may cause more insulin resistance and more endothelial system dysfunction (57, 58). Additionally, inflammation may cause changes in RBC maturation, thereby changing the RBC cell membrane which is also observed through increase levels of RDW (24). It is indeed reported in the study, that there is an increase in RDW in the T2D group by comparison with the ND group suggesting that T2D increased inflammation, impaired blood flow and RBC deformability as well as caused insufficient blood supply contributing to vascular damage. Additionally, the observed increase in levels of RDW may also be a result of the complications of T2D called anemia (24). Additionally, in this study there was an increase in RDW of

females per group by comparison with males and also by comparing females in all groups we observed an increase in RDW. This suggest that females are developing erythrocyte degradation or impaired erythropoiesis (25). Factors such as inflammation can influence RBCs half-life and RBCs deformability which therefore exacerbate LDW levels (25). In this study, we observed an increase in RDW in the PD group by comparison with the ND group. From the results obtained, we speculate that there is inflammation that causes disturbances in the RBCs as we have reported on upregulation of inflammatory markers during pre-diabetes (59). In addition to RDW, another factor that can be used in diagnosis of T2D is MCV (60). MCV is a RBCs parameter that ascertains the average size of the RBCs in circulation (61). According to Martina, MCV is the marker that can be used in macro vascular complications prognosis of diabetes (60). According to research done by Hashemi *et al*, hyperglycaemia and hyperlipidaemia cause an increase in MCV and MCH (62). These are both abnormalities that are reported at pre-diabetes stage due to oxidative stress caused by insulin resistance (32, 62). Free radicals observed during oxidative stress can directly damage red blood cell membranes by peroxidation of membrane polyunsaturated fatty acids which then damage the RBCs and contribute to increase in MCV and MCH (32, 63). This is indeed demonstrated by the results obtained for MCV and MCH which report an increase in MCV and MCH of T2D by comparison with the ND group, suggesting that an increase is due to hyperglycaemia and hyperlipidaemia during T2D. In this study, we reported an increase in MCV and MCH in the PD group by comparison with ND group. We hypothesise that hyperglycaemia and hyperlipidaemia contributed to the increase in both MCV and MCH, due to abnormalities on red blood cells membranes.

Haematocrit is a count of the volume percentage of RBCs in the blood (61). Haematocrit has been reported to be the risk factor associated with cardiovascular complications such as ischemic heart disease and atherosclerosis (64). Additionally, T2D patients have been reported to have increased haematocrit that is hypothesised to be due to impaired endothelial function (64). This impairment decreases the bioavailability of nitric oxide (NO)(64, 65). This increase in haematocrit has been shown in the T2D group by comparison with the ND group, suggesting association of T2D with cardiovascular complications and impaired endothelial function. This may suggest that insulin resistance causes disturbances that result in increased blood viscosity thereby limiting the level of delivery of components such as glucose, insulin and oxygen to the tissues thus elevating the haematocrit (66). Additionally as blood viscosity increases, it could further contribute to the development of insulin resistance due to insufficient muscle oxidative

capacity in T2D (66). In this study, there was a clinical increase of haematocrit levels in the PD group by comparison with the ND group. Furthermore, we also noticed a further increase in haematocrit in the T2D group indicating the exacerbation of endothelial damage. We can hypothesise that there is a damage on the endothelial functions during pre-diabetes. However, in this study we did not measure NO concentration.

Conclusion

The results reported in this study indicated that there is a change in RBC indices during pre-diabetes. However, the conclusions from these results are limited as the study had more females than males. Additionally, the results obtained from this study create a hallmark of investigating all the mechanisms that are linked to changes in red blood cells and its production in the bone marrow. These mechanisms include renal system and cardiovascular system, as these changes may have an impact on the development of conditions such as atherosclerosis and renal failure. Further investigation is required to give clarity on the abnormalities at pre-diabetes stage.

Limitations

The study checked the blood parameters on 3 groups, but it will be of interest to look at the results based on the demographics such as gender and race. The second limitation was the number of males and females not being equal. We also believe the results may have been slightly different if we had an equal number of males and females, per group. Due to the nature of the collection of the samples, we could not measure NO or eNOS and this could be implemented in future studies.

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Declaration of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

Availability of data and materials

The datasets used/analyzed in the current study are available from the corresponding author upon reasonable request.

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Supplementary data

Multiple Linear regression table					
Dependent variable	A : Glycated Heme %				
Regression type	Least squares				
Model					
Parameter estimates	**Variable**	**Estimate**	**Standard error**	**95% confidence interval**	
β0	Intercept	1,091	4,031	-6,844 to 9,027	
β1	B : WBC	0,09767	0,04942	0,0003803 to 0,1950	
β2	C : RBC	-0,9326	1,311	-3,513 to 1,648	
β3	D : HGB	0,3536	0,365	-0,3649 to 1,072	
β4	E : HCT	0,005322	0,2019	-0,3921 to 0,4028	
β5	F : MCV	0,1441	0,09539	-0,04366 to 0,3319	
β6	G : MCH	-0,7676	0,3425	-1,442 to -0,09339	
β7	H : MCHC	0,5141	0,2174	0,08614 to 0,9420	
β8	I : RDW	-0,1384	0,1066	-0,3481 to 0,07138	
Sig. diff. than zero?	**Variable**	**|t|**	**P value**	**P value summary**	
β0	Intercept	0,2707	0,7868	ns	
β1	B : WBC	1,976	0,0491	*	
β2	C : RBC	0,7113	0,4775	ns	
β3	D : HGB	0,9687	0,3335	ns	
β4	E : HCT	0,02636	0,979	ns	
β5	F : MCV	1,511	0,132	ns	
β6	G : MCH	2,241	0,0258	*	
β7	H : MCHC	2,365	0,0187	*	
β8	I : RDW	1,299	0,1952	ns	
Normality of Residuals	**Statistics**	**P value**	**rmality test (alp**	**P value summary**	
Anderson-Darling (A2*)	12,66	<0,0001	No	****	
D'Agostino-Pearson omnibu	44,22	<0,0001	No	****	
Shapiro-Wilk (W)	0,878	<0,0001	No	****	
Kolmogorov-Smirnov (dist	0,1671	<0,0001	No	****	
Data summary					
Rows in table	292				
Rows skipped (missing data	1				
Rows analyzed (# cases)	291				
Number of independent vari	9				
#cases/#variables	32,3				
Goodness of Fit					
Degrees of Freedom	282				
R squared	0,04984				
Analysis of Variance	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Regression	137,6	8	17,2	F (8, 282) = 1,849	P=0,0681
Residual	2623	282	9,303		
Total	2761	290			

BRIDGE

Chapter 5 had an overview of RBC indices (RBC, MCV, MCH, MCHC, HGB, HCT, and RDW) and a state of erythropoietin at pre-diabetes stage.

The findings on systematic review showed that there is insufficient information based on RBCs indices at pre-diabetes stage. In Manuscript 2, the results showed change in RBC indices and upregulation of EPO during pre-diabetes caused by moderate hyperglycemia in pre-diabetic multi-ethic population aged from 25 years to 45 years in Durban, South Africa.

Chapter 6 will provide an overview of the expression of LncRNA-DC, lncRNA-NTT and lncRNA-NRON at pre-diabetes stage in multi-ethnic population aged from 25 years to 45 years from Durban, South Africa. Chapter 6 consists of 1 section: an original research manuscript.

CHAPTER 6: RESEARCH MANUSCRIPT 3

(LncRNA's)

DETAILS OF NEXT MANUSCRIPT

The next manuscript is titled " **The novel role of lncRNAs in prediabetic patients from Durban, South Africa and their potential use as biomarkers in the pathophysiology and prognosis of pre-diabetes"** and is authored by N.C Mzimela, F.Y Tata, A.M Sosibo, P.S Ngubane, and A. Khathi. The manuscript is under review in **Endocrine Journal (ISSN: 0918-8959)** and has been formatted according to journal's guidelines for authors **(REF Number: EJ24-0477).** This journal is accredited by Department of Higher Education and Training South Africa and appears in ISI accredited list (2022).

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

The novel role of lncRNAs in prediabetic patients from Durban, South Africa and their potential use as biomarkers in the pathophysiology and prognosis of pre-diabetes

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ABSTRACT

Pre-diabetes is a condition where the body has high blood glucose levels but not high enough for a diagnosis of type 2 diabetes (T2D). This condition often precedes the onset of T2D and is said to arise from sedentary lifestyles and chronic consumption of high calorie diets. LncRNAs have been reported to be expressed in immune cells such as noncoding transcript in T- cells (NTT), Noncoding Repressor of nuclear factor activated T-cells (NRON) and Noncoding RNA expressed in dendric cells (DC). The city of Durban in South Africa has been reported to have high prevalence of pre-diabetes among population aged from 25 to 45 yrs. In this study, we sought to investigate the expression of selected lncRNAs in this population. Upon ethical approval, samples (n=46) were collected from King Edward hospital. Blood glycated haemoglobin and glucose levels were used to group them into 3 groups where non-diabetic (n=9), pre-diabetic (n=15) and T2D (N=22). Relative expression of lncRNAs were measured using qPCR and data analysed using SPSS statistics v28. There results showed a statistically significant ($p<0.05$) increase in lncRNA-NRON expression in the PD group by comparison with ND group. There was a decrease in expression of lncRNA-NTT in PD group by comparison with ND group. There was also an increase in lncRNA-DC expression of PD group in comparison with ND group. Expression of lncRNAs reported during the immune response paves the way for the exploration of immunity at a genetic level using immune cells.

Keywords: long noncoding RNAs, NTT, NRON, DC, pre-diabetes, eThekwini district

INTRODUCTION

The prediabetic state occurs when blood glucose levels are higher than normal but not high enough for a diagnosis of type 2 diabetes mellitus (T2DM). Pre-diabetes is characterised by impaired fasting blood glucose (FBG): 5.6 -7.0 mmol/L, elevated 2 h postprandial blood glucose (2 h - OGTT): 7.8 -11.0mmol/L and increased glycated haemoglobin levels (HbA1c): 5.7-6.4% (1). Pre-diabetes often precedes the onset of T2DM and there have been studies that show that the complications associated with T2DM begin during the prediabetic state (1, 2). Pre-diabetes often precedes the onset of T2DM and there have been studies that show that the complications associated with T2DM begin during the prediabetic state is considered a risk factor for developing type 2 diabetes is on the increase, remains neglected and unnoticeable. Some of the complications reported in T2D are dysregulated immune function and chronic inflammation (3-5). In a diet-induced animal model of pre-diabetes, it has been similarly shown that pre-diabetes is associated with immune activation and chronic inflammation (6, 7). This indicates that early detection of pre-diabetes accompanied by timely interventions could significantly decrease the risk of progression to T2DM and the associated complications.

However, the expression of long noncoding RNAs (lncRNA) that are invariably involved in immune response, immune cell development and inflammatory pathways has never been explored for diagnosis of pre-diabetes and taming the risk of developing type 2 diabetes and other related conditions. The long noncoding RNAs (lncRNA) are RNA molecules that are characterised by more than 200 nucleotides that also lack protein transcription capacity (8, 9). They are present in extracellular fluids such as serum, plasma, and urine. According to Jain and colleagues, in normal endocrine physiology and development, the lncRNAs have been implicated to play important roles such as several lncRNAs are reported to be highly expressed upon addition of glucose on culture of β-cell, lncRNA- HI-LNC25 playing role in insulin regulation and lncRNA ANRIL regulating glucose homeostasis in diabetes (10). Additionally, lncRNAs have been reported to be indispensable during the immune cell's development and immune response regulation (10, 11). Interestingly, lncRNAs have been reported to be involved in the inflammatory signalling pathways that involve the pathways such as NF-$_k$B, p38/MAPK, and JAK/STAT (12-16). Reports have detected that the human genome has about 27, 919 lncRNAs and about 70 % of these lncRNAs are reported to be functional (17). LncRNAs such as Noncoding transcript in T- cells (NTT), Noncoding Repressor of nuclear factor activated T-cells (NRON) and Noncoding RNA expressed in dendric cells (DC) have been reported to play

a role in T2D, immunity and inflammation (10, 18). In T2D, LncRNA-DC has been reported to be one of the lncRNA responsible for the differentiation of human monocytes to dendric cells by promoting STAT3 phosphorylation and activation (12, 16). Furthermore, lncRNA-DC has been reported to block domain-containing phosphatase-1 (SHP1) dephosphorylation during human monocyte differentiation to dendric cells (12, 16). LncRNA-NRON and lncRNA-NTT are the two RNAs that are representing the earliest genes that were identified in immune cells and are expressed in T-cells (19). LncRNA-NTT is mainly expressed by human $CD4^+$ T-cells when they are activated (19). However, lncRNA-NRON has been reported to be a Ca^{2+} - activated transcription factor, a regulator of the nuclear factor of activated T-cells (NFAT) and also responsible for IL-2 expression in activated T-cells (19). While these lncRNAs have been reported to play a role in several mechanisms of T2DM, their role in the prediabetic state is yet to be explored. Therefore, this study will explore the expression of selected lncRNAs (DC, NTT, and NRON) during the prediabetic state. According to Sosibo and colleagues, the eThekwini district in Durban, South Africa is categorised as one of the places with increasing prevalence of pre-diabetes (2, 20). The multi-ethnic population of this area is exposed to multifarious aspects that can lead to the development of pre-diabetes (21, 22). Therefore, using this population, this study sought to explore the selected lncRNAs (DC, NTT and NRON) expression at during the prediabetic state.

METHODS

The study was carried out using laboratory equipment at the University of KwaZulu Natal, in Durban (South Africa) laboratories. A quantitative cross-sectional analytical study has been carried out with using human (n= 46) blood samples that had been collected from King Edward clinic upon approval through UKZN Biomedical Research Ethics Committee. From February 2021 to December 2022, the blood samples were collected from patients from all ethnicities who are of ages from 25 to 45 years. Selection of samples was according to selection criteria and data provided by hospitals mentioned upon signed informed consent.

Ethics Approval

Before the collection of samples from hospitals mentioned above, we obtained the ethics approval from the Biomedical Research Ethics Committee (BREC) of the College of Health Sciences, the University of KwaZulu Natal (BREC REF NO: BE266/2019).

Inclusion and exclusion criteria:

This study worked with stored blood samples for normal, pre-diabetic and T2D patients with the subsequent inclusion and exclusion criteria as follows.

The sampling exclusion criteria

Any samples of patients that were below the age of 25 years and above 45 years. Blood samples of patients displaying other diseases either than T2D and pre-diabetes. Samples of patients with no history of liver disease, thyroid disease, kidney disease, heart disease, depression, and HIV. Blood samples of patients that are not professional sport athletes. Blood samples of patients under the influence of alcohol and also pregnant females.

The sampling inclusion criteria

Any blood sample of the patient that is non-prediabetic, pre-diabetic and type 2 diabetic upon screening. Blood samples of patients that are between the age of 25 years and 45 years, all genders, and all races.

Pre-diabetes diagnosis criteria

To affirm whether or not samples have to be categorized as non-prediabetic, pre-diabetic or T2D, the American Diabetes Association (ADA) standards were applied. Additionally, from the facts received from the health facility for fasting glucose levels, HbA1c was measured using respective human ELISA kits from Elabscience, in line with the manufacturer's instructions. The samples that confirmed HbA1c under 5.7% were considered non-prediabetic, while those between 5.7% and 6.4% were considered pre-diabetic. Samples with HbA1c levels above 6.4 % were considered as type 2 diabetic.

Sample preparation and RNA extraction and purification

The blood samples of patients collected from King Edward Hospital were grouped into ND, PD and T2D groups. They were then centrifuged (Eppendorf centrifuge 5403, Germany) for 15 min at 3000 rpm to separate and collect plasma. The samples were subsequently refrigerated at − 80°C using bio-freezer (Snijders Scientific, Holland) prior to analysis. Pure Link RNA Mini Kit (Thermofisher scientific, Carlsband, USA) was used to extract and purify total RNA from plasma, as per manufactures instructions. To confirm the purity of RNA extracted, a nano drop (Thermofisher scientific, Carlsband, USA) was used and a range of an OD 260/280 ratio greater than 1.8 (>1.8) indicated pure RNA extracted. Extracted and purified RNA was then kept at − 80°C in bio-freezer (Snijders Scientific, Holland), for further analysis.

Reverse Transcription of RNA into cDNA

The complementary DNA (cDNA) was synthesized from extracted RNA using the SuperScript III first-strand synthesis super mix (Thermofisher scientific, Carlsband, USA) for qRT-PCR following the protocol: Incubation of 50°C for 30 mins was set as a general starting point of a thermal cycler (Thermofisher scientific, Carlsband, USA). In a tube on ice to make 20 µL reaction mix, 2X RT Reaction mix, RT Enzyme mix, RNA and DEPC-treated water were used according to the manufacturer's instructions. The tube with the mixture was incubated at 25°C for 10 minutes and was further incubated at 50°C for 30 minutes. The reaction was terminated at 85°C at 15 min and then chilled on ice. While chilled on ice, 1µL of *E. coli* RNAse H was added to a reaction and then incubated at 37°C for 20 minutes. The concentration and purity of the cDNA were measured per sample using nanodrop (Thermofisher scientific, Carlsband, USA) and stored at − 20°C for qPCR.

Quantitative Real-Time Polymerase Chain Reaction(qRT-PCR) per LncRNA

Quantitative Real-Time qPCR (RT-qPCR) was used to assess the expression of selective lncRNAs (NRON, DC, and NTT). The complementary DNA (cDNA) obtained was standardized and normalized to a concentration of 400 ng/mL for the preparation of 10 µL qPCR reaction volume per well with TaqMan advanced master mix (2X) (Thermofisher scientific, Carlsband, USA), TaqMan Assay (20X) and nuclease-free water as per manufacturer's protocol. RT-qPCR amplification and measurement were performed using the TaqMan pre-designed primers obtained from Thermofisher scientific, for NR0N *(Catalogue number: 4426961, Assay ID: Hs04274940),* NTT *(Catalogue number: 4331348, Assay ID: APGZNP3),* and DC *(Catalogue number: 4331348, Assay ID: APZTMK2)* on the BioRad CFX96 thermocycler in the following PCR conditions: hot start activation at 95 °C for 20 seconds (s), followed by 35 cycles of 3 s at 95 °C for denaturation and 30 s at 60 °C for annealing and extension. The expression of the lncRNAs were measured using GAPDH as an internal control for normalization. Gene expression was then analysed and presented as relative fold change compared to the control ($2^{-\Delta\Delta Ct}$)

Statistical analysis

Bio-Rad CFX Maestro 2.2 software version 5.2 to was used to analyse the relative expression of each target in the samples (ND, PD and T2D) and reference gene used in triplicate for all assays performed. Data were then analysed using SPSS statistics v28. The glucose, HbA1c and lncRNAs data of the groups were analysed and compared by applying One way ANOVA and

Tukey's post hoc test using SPSS v28. Data expressed as means ± standard error of means (±SEM). Values of $p < 0.05$ indicate statistical significance.

RESULTS

The study had a total of 46 participants that were divided into 3 groups; 9 in non-diabetic (ND), 15 pre-diabetic (PD) and 22 type 2 diabetics (T2D). This study had ND group with 6 females and 3 males, PD group (PD) with 11 females and 4 males and T2D group had 16 females and 6 males.

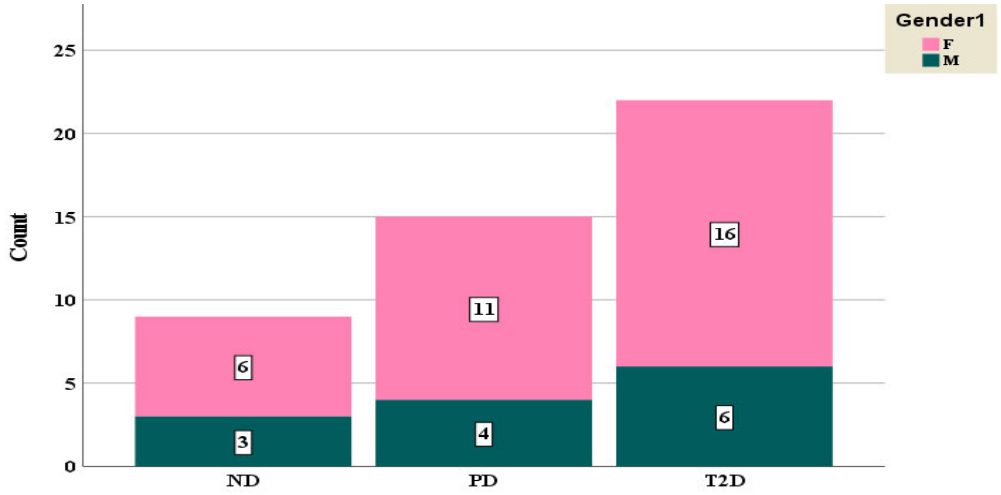

Figure 1: Graph showing the gender count of males and females per group (ND, PD and T2D)

Blood glucose and glycated haemoglobin levels

Levels of glucose and glycated haemoglobin were measured in the blood in all the three experimental groups. The results showed an increase in fasting glucose levels in T2D group by comparing it to the ND group. The results also showed an increase in fasting glucose levels in the PD group by comparison to the fasting glucose levels of the ND group. The results showed an increase in glycated haemoglobin levels in T2D group by comparing it to the ND group. There was also an increase in glycated haemoglobin in the T2D group by comparison to the PD group. All groups were within the ranges outlined by ADA guidelines.

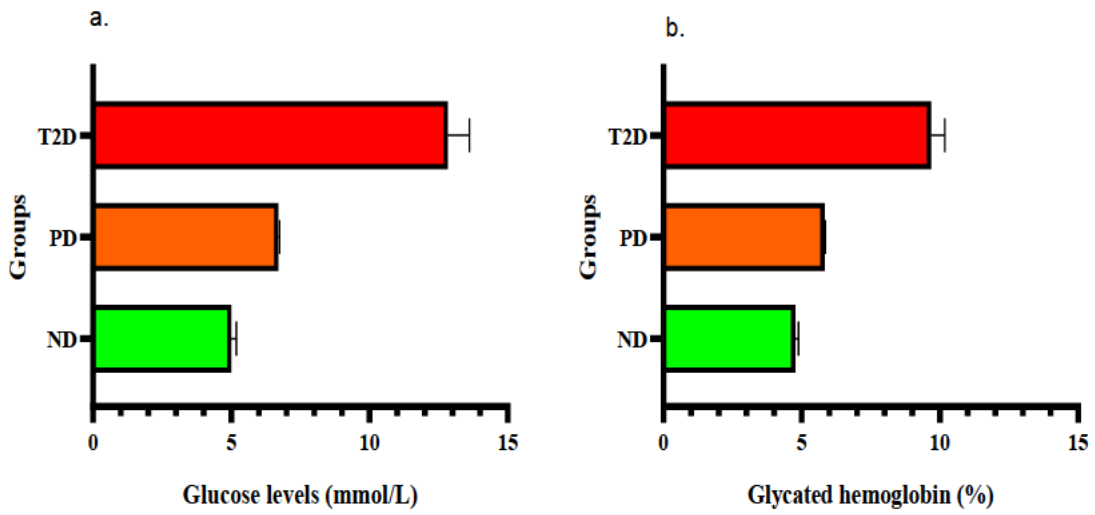

Figure 2: Graph showing the fasting glucose levels and glycated haemoglobin percentage of all 3 groups (ND, PD and T2D). Data presented in ± SEM.

Relative expression of lncRNA-NRON

The relative expression of *lncRNA-NRON* was measured in all experimental groups. The results showed a significant increase in the expression of *lncRNA-NRON* in the PD group by comparison to the expression observed in ND group ($p = 0.03$) (see figure 3). However, the expression of *lncRNA-NRON* observed in PD group was significantly lower by comparison to the T2D group ($p = 0.007$) (see figure 3). When comparing ND group with T2D group, there was a decrease in *lncRNA-NRON* of the T2D Group ($p = 0.83$) (See figure 3).

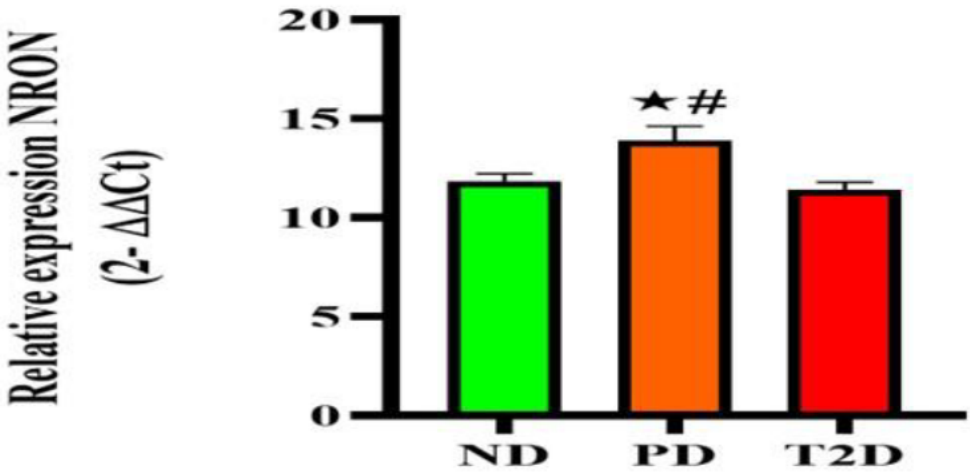

Figure 3: Graph showing relative expression of lncRNA-NRON of 3 groups (ND, PD and T2D), where ★ represent significance between ND and PD ($p < 0.05$) and # represent significance between PD and T2D ($p < 0.05$). Data presented in ± SEM.

Relative expression of lncRNA-NTT

The relative expression of *lncRNA-NTT* was measured in all experimental groups. The results showed a decrease in the expression of *lncRNA-NTT* in the PD group by comparison to the expression observed in the ND group ($p = 0.99$) (see figure 4). However, the expression of *lncRNA-NTT* observed in PD group was higher by comparison to the T2D group ($p = 0.68$) (see figure 4). When comparing ND group with T2D group, there was a decrease in *lncRNA-NTT* of the T2D Group ($p = 0.59$) (See figure 4).

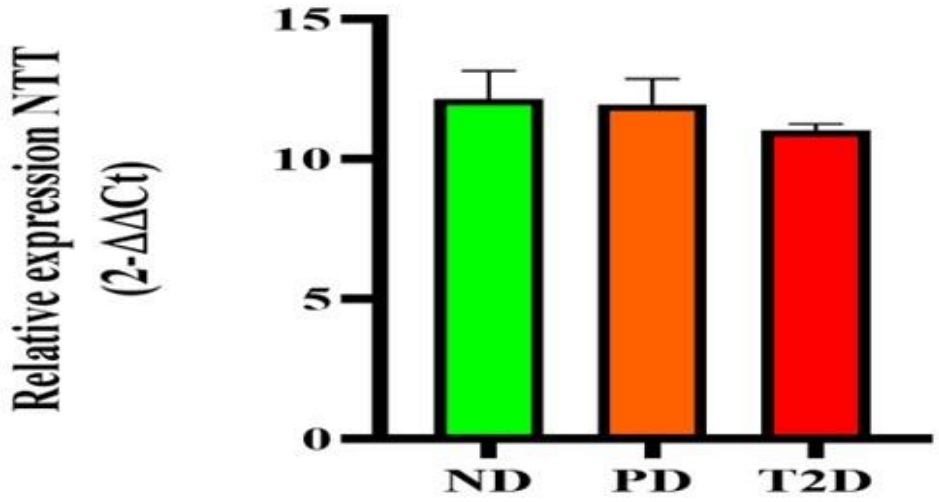

Figure 4: Graph showing relative expression of lncRNA-NTT ND, PD and T2D groups. Data presented in ±SEM.

Relative expression of lncRNA-DC

The relative expression of *LncRNA-DC* expression was measured in all experimental groups. The results showed an increase in the expression of *lncRNA-DC* in PD group by comparison to the expression observed in the ND group ($p = 0.21$) (see figure 5). However, the expression of *lncRNA-DC* expression observed in PD group was lower by comparison to the T2D group ($p = 0.41$) (see figure 5). When comparing ND group with T2D group, there was a significant increase in *lncRNA-DC* of the T2D Group ($p = 0.02$) (See figure 5).

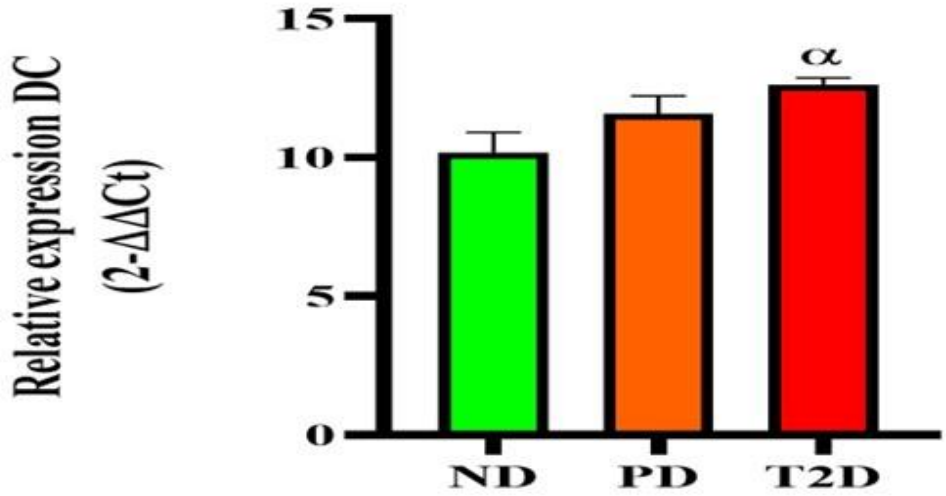

Figure 5: Graph showing relative expression of lncRNA-DC of 3 groups (ND, PD and T2D) where **α** represent significance between PD and T2D ($p < 0.05$).Data presented in ±SEM.

DISCUSSION

Recent evidence shows that lncRNAs play a vital role in directing the improvement of numerous immune cells and controlling the dynamic transcriptional applications which are a hallmark of immune cell activation (13, 23, 24). LncRNA-DC, lncRNA-NTT and lncRNA-NRON have been reported to be involved in T2D, inflammation and immunity(10, 18, 24). However, the onset of T2D is often preceded by pre-diabetes, where the blood glucose levels are above normal homeostatic range but below the level of a T2D diagnosis (25). The state of these three lncRNAs has never been explored even at pre-diabetes stage. The city of Durban in South Africa has a multi-ethic, biodiverse population where the prevalence of pre-diabetes has been shown to be on the rise, specifically in the age range of 25-45 years (21, 22). Using this population, this study discusses the expression of these three lncRNAs in patients that are non-diabetic, prediabetic and type 2 diabetic to assess any their possible role in activation of immune cells during pre-diabetes.

According to Lau and colleagues, T2D patients have an extended quantity of senescent T cells in each of the $CD4^+$ and $CD8^+$ T cell compartments which is causative for the development and progression of T2D (26). Additionally, senescent T-cells have been reported to be a proinflammatory subset that is secreting their precise array of proinflammatory products upon activation (26). A multiplied presence of senescent T cells has an unfavourable effect on immune function throughout T2D stage (26). $CD4^+$ helper T cells also play a critical role in a series of T2D associated complications (27). Upon activation, naïve T cells go through

transcriptional and epigenetic adjustments as they proliferate and differentiate into specific effector subsets which include the production of interleukin-2 (IL-2)(28, 29). This produced IL-2 has been reported to promote naïve T cell proliferation and survival (28, 30). Additionally, IL-2 production requires the NFAT1 transcription, activating protein-1(AP-1) and nuclear factor kappa B (NF-κB) (11, 28, 31). Remarkably, the function of NFAT1 that is required in T cell activation is regulated by the lncRNA-NRON (28). One of the lncRNA discovered on T-cells is phosphorylated in resting T-cells called lncRNA-NRON (23, 32). LncRNA-NRON is a noncoding transcription repressor of NFAT (Nuclear Factor of Activated T cells) (29). This function of lncRNA-NRON is achieved by inhibiting nucleocytoplasmic shuttling of NFAT (29). It is this function of lncRNA-NRON that permits it to be labelled as a transcription regulator for the immune system at some point of immune response (29). According to Mowel and colleagues, lncRNA-NRON facilitates the scaffold of the interaction of NFAT1 with GTPase activating protein (IQGAP) and three inhibitory NFAT1 kinases CKe, GSKb, and DYRK which results in translocation of NFAT1 from the cytoplasm to the nucleus (28). IQGAP1- NRON interaction causes an increase in NFAT dephosphorylation and nuclear translocation (32). This results in inflammation and the release of certain cytokines such as IFN-γ (28).

T2D is characterised by chronic hyperglycaemia which exacerbates chronic inflammation and the release of cytokines for inflammation (33). From the results obtained in this study we observed an increase in fasting glucose levels and glycated haemoglobin levels in PD group by comparison with ND group. Furthermore, there was a further increase in fasting glucose levels and glycated haemoglobin levels in T2D group by comparison with ND group. These observations suggest that there is a hyperglycaemic condition at PD stage, and it exacerbate to contribute to the onset of T2D. Additionally, this chronic hyperglycaemia has been reported to cause chronic inflammation resulting in cytokine storm which is in line with the results obtained.

However, in the prediabetic state, hyperglycaemia is acute (34). Inflammation is also acute as the produced T-cells are still reacting to the toxic effect of hyperglycaemia caused by insulin resistance. It is indeed seconded by the results of the study on PD group in comparison with ND group where there was a significant increase in the expression of lncRNA-NRON in the PD group. By further looking at the results obtained, we also observed that the expression of lncRNA-NRON in PD group was significantly higher in comparison with the T2D group. From

the results we can suggest that there was acute hyperglycaemia in the prediabetic state that caused more production of T-cells from bone marrow. As these T-cell are release to circulation they were exposed to the toxic effect of hyperglycaemia which then activate them, resulting in increased levels of lncRNA-NRON in circulation. However, during T2D, it has been reported that hyperglycaemia is chronic and inflammation which the lead to suggestion that the activation of T-cells is neutral as the abnormalities are now chronic as shown by our T2D group.

Furthermore, the results of *lncRNA-NRON* at prediabetic stage can be used to detect the availability of resting T-cells at pre-diabetes stage. Therefore, *lncRNA-NRON* can be a biomarker to detect the resting T-cells and regulation of NFAT1 function that is required in T cell activation.

Another noncoding transcript in T-cells, specifically $CD4^+$ T cells, is lncRNA-NTT (35). $CD4^+$ T-cells have been reported to be increased in the visceral adipose tissue of obese and T2D subjects, which indicates adipose tissue inflammation (36). Additionally, $CD4^+$ T cells have been reported to function in controlling immune-adipose tissue crosstalk during the progression of T2D (36). LncRNA-NTT is a lncRNA located at chromosome 6q23-q24 and has been reported to have a close relationship to *IFNGR1, TNFAIP2, PBOV1 and MYB* genes that are involved in proliferation, immunity and haematopoiesis (32). Additionally, Liu and co-workers reported that lncRNA-NTT is located adjacent to both the IFNγ (12q15) and IFNγ Receptor (6q23) encoding gene (37). It is a 17-kb long noncoding transcript that is polyadenylated, unspliced and localised in the nucleus (19, 37). LncRNA-NTT transcripts are only found only in activated $CD4^+$T-cells and have not been found on resting $CD4^+$T-cells (37). An experiment by Liu and co-workers indicated that the induction functions of lncRNA-NTT are altered upon activation of T-cells (37). Its' function is to regulate the expression of IFN-γR (37). However, further evidence needs to be collected based on the function of NTT. According to Liu and co-workers, inflammatory profiles produced by $CD4^+$ T cells, define human pre-diabetes as a unique inflammatory state as pre-diabetes produces a unique cytokine profile when compare with non-diabetic and T2D human subjects(38). Additionally, during immune response such as T2D caused by hyperglycaemia, T-cells are produced and the cells such as $CD4^+$ are recruited to the inflamed area where they undergo inflammation by secreting inflammatory cytokines. The results showed a decrease in lncRNA-NTT expression in the T2D group by comparison with the ND group. These results suggest that at T2D stage, the activation of T-

cells become neutral due to chronic hyperglycaemic condition and chronic inflammation. The toxic effect of hyperglycaemia is more chronic to cause more T-cells to trigger the expression of lncRNA-NTT, which is contributing to suppression of immune system reported in T2D.

In our results, we observed a decrease in lncRNA-NTT expression in the PD group by comparison with the ND group. Additionally, when comparing expression of lncRNA-NTT of PD with T2D, there was a higher expression of lncRNA-NTT in the PD group. This indeed suggests that there is inflammation during the progression of the PD to T2D. We postulate that the activated $CD4^+$ T-cells are recruited to an inflamed area therefore causing a decrease in T-cells in circulation. This recruitment also has an effect on the expression of lncRNA-NTT expression in circulating $CD4^+$ T-cells because the recruitment of cells to an inflamed area decreases them from circulation. Further studies are needed to confirm this by measuring the concentration of IFN-γ in these groups.

Furthermore, *lncRNA-NTT* can therefore be used as a biomarker to detect the availability, or the amount of circulation activated T-cells at pre-diabetes stage. This biomarker can thereby show that there is more inflammation or moderate hyperglycemia which activate the T-cells and induce their recruitment to the inflamed area. This will also enable in regulating the expression of IFN-γR to control or manipulate proliferation, immunity and haematopoiesis, during pre-diabetes stage.

Macrophage activation is stricken by T cells and those T cells play a dominant function in maintaining inflammatory methods and insulin resistance by inducing proinflammatory cytokines in metabolic organs (27). However, monocytes have been reported to be the central players in orchestrating complex immune responses (39). Monocytes have been reported to further differentiate into macrophages and dendric cells(DCs) (39). DCs have been reported to be cells that link innate immunity with adaptive immunity (40). They play a role in shaping the immune response by presenting antigens and stimulating naïve T-cells (41-43). Interestingly, conversional DC for skin and blood has been reported to exclusively express lncRNA-DC upon activation (43, 44). Additionally, this lncRNA-DC is required for the differentiation of monocytes to DCs (45). LncRNA-DC is reported to be essential for the activation of T-cells and the uptake antigen (43). LncRNA-DC directly binds with STAT3, which then prevents the dephosphorylation of transcription factor STAT3 by domain-containing phosphatase 1(SHP1)(43, 46). This binding therefore activates STAT3-dependent transcription (43). Upon activation, STAT3 mediates the transcription of genes encoding for DC differentiation (43).

This activation, therefore, promotes the differentiation of monocytes to conventional dendritic cells and the production of certain cytokines, such as IL-12 (30, 45). Furthermore, reports have indicated that JAK/STAT pathway is a crucial pathway for cell growth, development of cells, differentiation of cells and immune cells survival (47). A study by Alikhah and colleagues reported an increase in lncRNA-DC in T2D subjects (16). They further mentioned the effect of hormones in increasing lncRNA-DC on women such as one potential estrogen receptor-binding site in lncRNA-DC promoter (16). However, further investigation is required for the oestrogen-binding site in the lncRNA-DC promoter. Indeed, our results showed an increase in lncRNA-DC expression in the T2D group which is in line with a report by Alikhah and colleagues. From the results obtained, it can be suggested that hyperglycaemia reported in T2D induces the differentiation of monocytes to macrophages and DCs thereby causing expression of lncRNA-DC on activated DCs. The results of our study showed an increase in lncRNA-DC expression in the PD group by comparison to the ND group. This suggests that there is upregulation of inflammatory pathways and that the moderate hyperglycaemia seen in pre-diabetes exacerbates the activation of differentiated DC cells. Additionally, since inflammation involves cytokines such as IL-6 and interferons type I(IFN-I), they have also been reported to activate the JAK/STAT3 signalling pathway which also causes more activation of the DCs and further expression of lncRNA-DC (48). IL-6 and its ligand has been reported to be able to effectively activate STAT and the target gene of NF-κB has been shown to encode IL-6 (48). This then suggests that STAT3 may play a role in the activation of the NF-κB pathway which further exacerbate inflammation resulting in chronic inflammation and more expression of lncRNA-DC (48). This worsening of inflammation could further contribute to progression of pre-diabetes to T2D. Therefore *lncRNA-DC* can be used as the biomarker to trace the level of activation of T-cells and the uptake antigen at pre-diabetes stage and also be a biomarker to indicate the pathophysiology of the JAK/STAT3 signalling pathway activation.

CONCLUSION

The involvement of long noncoding RNAs during the immune response paves the way for the exploration of immunity on a genetic level using immune cells. The results obtained in this study gave pivotal insights into the expression of these lncRNAs during the pre-diabetes state. Further investigation would be very useful as this activation involve certain cells such as macrophages and dendric cells which have not yet been measured at pre-diabetic stage and also the measurement of other inflammatory cytokines such as IL-2 and type I interferons. In addition to these 3 RNAs (NRON, NTT and DC), more lncRNAs can be explored. The

expression of the RNAs during pre-diabetes stage could potentially be used to detect the progression of pre-diabetes towards T2D. Additionally, it would also be of great interest to explore how these lncRNAs can be manipulated to control disease progression at a genetic level.

Recommendations

Further investigation on the expression of the cytokines such as IL-12 and IFNγ in the pre-diabetic state. We also recommend exploring the pathways such as NF-kβ, p38/MAPK, and JAK/STAT. The study indicates that the production of IL-12 requires NFAT1 transcription which then activate inflammatory signalling pathways such as NF-kβ. Additionally, the interaction of IQGAP1- NRON results in dephosphorylation of NFAT thereby inducing release the of cytokines such as IFN-γ. *lncRNA-DC* is involved in JAK/STAT3 signalling. It can also be recommended to explore other lncRNAs in the pre-diabetic state such as NEAT1, NeST, H19 and MALAT1 as they have been shown to be expressed during type 2 diabetes stage.

Limitations

Due to budget constraints, we could not measure all the cytokines involved and pathways mentioned. The concentration of the cells that express these lncRNAs were not measured.

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Declaration of interest statement

Competing interest -The authors declare no competing interests.

Ethics Approval - The ethics approval was obtained from the Biomedical Research Ethics Committee (BREC) of the College of Health Sciences, the University of KwaZulu Natal (BREC REF NO: BE266/2019)

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

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BRIDGE

Chapter 6 had overview of the expression of LncRNA-DC, lncRNA-NTT and lncRNA-NRON at pre-diabetes stage.

Findings on manuscript 3 showed that there was expression of LncRNA-DC, lncRNA-NTT and lncRNA-NRON caused by moderate hyperglycaemia in pre-diabetes stage multi-ethic population aged from 25 years to 45 years in Durban, South Africa.

Chapter 7 will provide a synthesis and conclusions of the study.

CHAPTER 7: SYNTHESIS AND CONCLUSIONS

SYNTHESIS AND CONCLUSION

Type 2 diabetes (T2D) has been reported to emerge from the abnormalities caused by insulin resistance which then cause hyperglycaemia (1). There are various complications associated with T2D and these include suppression of the immune system, chronic inflammation, and changes in red blood cell indices (1-5). Additionally, recent reports have shown that there are long noncoding ribonucleic acids (lncRNAs) that are expressed by immune cells during immune responses, inflammation and in diseases such as atherosclerosis (6-8). The onset of T2D has been shown to be often preceded by a condition known as pre-diabetes which is a condition whereby the blood glucose levels are above the homeostatic range but below the threshold for the diagnosis of T2D (9). This condition is characterized by moderate insulin resistance and intermediate hyperglycaemia however the condition is largely asymptomatic (9). This has not only caused challenges in accurately determining the prevalence of pre-diabetes, but this has led to difficulties in studying the metabolic changes brought about by the condition.

The use of animals in research, including the study of metabolism continues to be very important in medical research. Recently, a diet-induced animal model of pre-diabetes was shown to mimic the human condition(10-13). This model was developed by allowing chronic consumption of a high fat, high carbohydrate diet that consists of refined carbohydrates and saturated fats thus resembling the "unhealthy" diets consumed by humans (12). Using this animal model, the changes in immune cells such as neutrophils, lymphocytes, monocytes, basophils and eosinophils were shown in the prediabetic state (10, 12). This model also showed upregulation of inflammatory markers such as CRP, IL-6, TNF-α, fibrinogen, sCD40L and P-selectin during this condition (10, 12). Furthermore, this model characterized the changes in red blood cell indices during the progression of pre-diabetes (11, 12). However, none of these findings have been verified in humans with pre-diabetes and this, in part, prompted this study.

Other than other communicable and non-communicable diseases, changes in metabolic parameters such as immune and RBC indices have been shown to be also influenced by factors such as exposure to occupational hazards and nutritional status (14, 15). The city of Durban in South Africa is a rapidly urbanizing area with a culturally diverse population. A recent study reported on nutritional challenges facing this population which may predispose them to the development of T2D and the associated complications (16). Coincidentally, another study recently reported on increased prevalence of pre-diabetes in this population, more especially among those in the age range of 25 to 45 years (17). Taken together, these findings made

Durban to be an ideal location to investigate the changes in immune cell concentration, inflammatory marker status and changes in RBCs indices in people with pre-diabetes. Additionally, this made this population ideal to study the expression of lncRNA that are expressed by immune cells, in people who are pre-diabetic.

Manuscript 1 of this study investigated the status of immune cells such as neutrophils, lymphocytes, monocytes, basophils, and eosinophils as well as the status of inflammatory markers such as CRP, IL-6, TNF-α, fibrinogen, sCD40L and P-selectin in patients with pre-diabetes aged from 25 years to 45 years in Durban, South Africa. Previous studies have extensively illustrated the changes in these markers during T2D (18-22). Briefly, the neutrophils in T2D patients are reported to show deficiencies in almost all functions, such as releasing of lytic proteases, phagocytosis, migration to inflammatory sites, production of ROS and apoptosis (18). These reports show that neutrophils and monocytes are produced in bone marrow and then recruited to the inflammatory sites, from circulating blood, for inflammation (18, 19, 21). Neutrophils and monocytes have been reported to exacerbate T2D as they induce mechanisms that trigger the release of cytokines such as IL-6, IL-8, TNF-α, and IL-1β (19, 20, 22, 23). Furthermore, in T2D, monocytes differentiate into macrophages which then contribute to atherosclerosis pathogenesis as they contribute to the uptake of lipids at the intima in a form of foam cells (24). Additionally, an increased concentration of P-selectin in T2D, has been reported to induce monocyte adhesion, activate the NF-kB and AP-1 transcription factors and also increase ROS activity (25). Indeed, the literature is consistent with the results obtained from monocytes and P-selectin in this study. There was an increase in monocytes and P-selectin on T2D group by comparison with the ND group. One of the other functions of IL-6 is to trigger the reproduction and activation of lymphocytes during immune response and also active during maturation of B-lymphocytes (26). According to Obeagu and colleagues, in early stages of on inflammation, there is synthesis of IL-6 in blood stream and this IL-6 is then transported to the liver where it then trigger the induction of other cytokines such as fibrinogen and CRP (26). Indeed, the findings in our T2D group were consistent with the literature based on neutrophils, IL-6, CRP, and fibrinogen as there was a decrease in neutrophils and an increase in IL-6, CRP and fibrinogen in the T2D group by comparison with ND group.

In this study, the findings of neutrophils (normal range = 40-60%) and basophils (normal range = 0.5-1%) percentages were abnormal that they were below the normal range for neutrophils and above the normal range for basophils. According to Govender *et al*., Durban has been

reported as one of the areas that are affected by the factors that compromise the immune system (16). These factors include the consumption of unhealthy diets due to affordability as well as environmental factors such as work exposure to immune toxic agents (16). Such factors compromise the immune system and may therefore explain the neutropenia and abnormal levels of basophils observed in the results obtained (16). Additionally, according to Reich and colleagues, people of African ancestral and Yemenite Jews have a low neutrophil count due to high *FY*-allele frequency which disturb the capacity of mobilization of bone marrow neutrophils reserves, upon response to availability of circulating corticosteroids (77). In the Duffy antigen Receptor for Chemokine gene (DARC) gene, the *FY*-allele of an African ancestral and Yemenites Jews also have a noncoding strand that disturb or destroy the gene expression in white blood cells, thereby contributing to low neutrophils count (77). The study population consisted mainly of people of African and Indian ancestry (93%) which may provide another explanation of the low neutrophil count observed in the study. The findings for lymphocytes, monocytes and eosinophils were within the normal range. The findings on the neutrophils being below normal range may suggest compromised immune function as the neutrophils are the first immune cells reported to be released during an immune response (27). Evidence of immune activation is indicated by a decrease in neutrophils percentage count in the pre-diabetes group by comparison with ND group. Interestingly, the concentration of neutrophils was also lower than that observed in T2D, suggesting that there is more inflammation during the acute hyperglycaemia observed in pre-diabetes. Interestingly, the findings in human subjects are the same as the results reported using a diet-induced rat model of pre-diabetes where the results suggested that neutrophils are recruited to the inflammatory sites from circulating blood for inflammation (18). Additionally, neutrophils have been reported to secrete the inflammatory markers such as IL-6 and TNF-α. In this study, the results showed sub-clinical inflammation where there was an increase in TNF-α concentration in the PD and PD group had the highest increase, even more than that observed in T2D. Additionally, sub-clinical inflammation is indicated by a decrease in IL-6 concentration in the PD group, while the T2D group had elevated levels of IL-6 concentration. The results of TNF-α concentration are indeed the same as results obtained on the diet-induced rat model study (10). Surprisingly, with IL-6 concentration, the results we obtained in the pre-diabetic group were inconsistent with results obtained using diet-induced rat model. Estrogen levels have been reported to decrease IL-6 concentration in females and this study consisted of more females than males (28). This may account for the difference in IL-6 results found in this study. IL-6

has also been reported to induce the synthesis of acute and acute phase proteins such as CRP and fibrinogen in the hepatocytes(26). The findings in manuscript 1 reported an increase in fibrinogen concentration in the PD group, with T2D group showing further increases in fibrinogen concentrations. Additionally, there was sub-clinical inflammation indicated by a decrease in CRP concentration in the PD group, however, the T2D group exhibited an increase in CRP levels. These results may suggest that the circulating IL-6 triggered the increase in plasma during acute phase inflammation in the pre-diabetic state. Once again, this may be due to the study having more female participants (29). Our results also showed a decrease in monocytes percentage count in the PD group, however, the T2D group showed elevated levels of monocytes. These findings were consistent with previous studies conducted in diet-induced prediabetic animals where it was suggested that the moderate hyperglycaemia in the pre-diabetic state triggers the production of monocytes in the bone marrow which are then recruited to the inflamed areas due to the induction of monocyte chemoattractant protein-1 (MCP-1). Our findings also reported on a subclinical inflammation indicated by a decrease in P-selectin concentration in the pre-diabetic state. However, T2D showed elevated levels of P-selectin concentration. This study, for the first time, indicated that there is inflammation in the endothelial cell walls but not high enough to cause atherosclerosis.

Other immune cells that were investigated in manuscript 1 were the lymphocytes, basophils, and eosinophils. Lymphocytes consist of T-cells and B-cells subsets. Sefil and colleagues reported on a decrease in lymphocyte concentration on T2D, indicating that hyperglycaemia in T2D and low expression of IL-2 receptors contribute to a decrease reported (30). In T2D, T-cells are produced in bone marrow upon toxic effect of glucose, and then become activated and synthesize IL-6 and soluble CD40L(31). B-cells produce antibodies such as IgM and IgE antibodies upon activation (32). These antibody IgE then bind to IgE receptor for basophils and activate them, resulting in the secretion of IL-4 and IL-3 (33, 34). IL-3 triggers more production of basophils in bone marrow, thereby increasing them in circulation (34). Additionally, the secreted IL-4 supports eosinophil migration as well as B-cell activation (34). The findings on this study were consistent to the decrease in lymphocytes, increase in CD40L and basophils of T2D by comparison with non-diabetic control. T2D adipose tissues has been reported to secrete IL-5 which induce the production of eosinophils upon chronic hyperglycaemic conditions result in an increase in circulation of eosinophils (34, 35). The T2D group eosinophils showed a similar trend as that seen in existing literature.

Our study also showed that there is an increase in lymphocyte percentage count in the PD state and even more decreases in the T2D group. While this is the first time this has been shown in humans, these results are consistent with findings from studies conducted in a diet-induced pre-diabetes rat model where it was postulated that there is more production of lymphocytes due to glucotoxicity in the pre-diabetic state (10). Upon activation, lymphocytes secrete inflammatory cytokines such as IL-6 and sCD40L (36). CD40 triggering has been reported to induces B-lymphocytes to secrete IL-6, TNF-α, IL-5, IL-2 etc (37). The same trend of results was observed with the CD40L concentration which has also been shown for the first time in human studies. These findings on CD40L concentration may indicate that the activated lymphocytes secreted CD40L which also triggers more secretion of other inflammatory markers under hyperglycaemic conditions. B-cells have been reported to express antibodies upon activation such as IgE antibodies (32, 38). Basophils has been reported to display IgE binding receptors, upon activation caused by glucotoxicity, the basophils bind to IgE antibodies (39, 40). The activated basophils trigger the release of IL-4 and IL-3 (33).

Lastly, the results in manuscript 1 also showed an increase of basophils percentage count in the prediabetic state but this increase was not as high as that seen in the T2D state. Interestingly, showing the same result obtained on the last week of the study on pre-diabetes diet-induced rat model. These results suggest that the acute hyperglycaemia in the pre-diabetic state caused production of basophils (33, 40). The produced basophils were activated due to glucotoxicity and released IL-3 which the caused more production of basophils by the bone marrow during pre-diabetes (41, 42). Additionally, hyperglycaemia triggers the release of eosinophils, and the recruited eosinophils are supported by IL-4 at the inflamed areas. This was also consistent with the other findings of manuscript 1 which showed an increase of eosinophil percentage count in the PD, but the increase was not as high as that seen in T2D. Taken together, manuscript 1 showed that moderate hyperglycaemia seen in pre-diabetes may be associated with of immune activation accompanied with acute sub-clinical inflammation.

After observing the changes in manuscript 1, we sought to investigate if the moderate hyperglycaemia observed in pre-diabetes may have any effects on red blood cell indices. Manuscript 2 of this study therefore investigated the status of red blood cell indices such as MCH, MCHC, RBCs, HCT, HGB, MCV and RDW in the prediabetic state. In T2D, RDW has been reported to be an inflammatory marker that measures the heterogeneity of RBC volume with high levels of RDW indicating anisocytosis (43, 44). Indeed, our results are consistent

with those seen in literature where there was an increase in RDW reported in T2D by comparison with the ND group. The findings of this study showed an increase in RDW during the pre-diabetic state which has only been shown in a diet-induced animal model of pre-diabetes (11). In that study, the increase in RDW was said to indicate reduction of RBC deformability and half-life due to glucotoxicity and oxidative stress (45). The results of the present study suggested this for the first time in pre-diabetic humans.

Other RBCs indices that were investigated in manuscript 2 were mean RBC count, HGB, HCT, MCH, MCV and MCHC. A study by Arkew *et al*., reported a decrease in mean RBC count, HGB, HCT, MCH and MCHC in patients with T2D compared to the ND control group (44). However, there was an increase in MCV in the T2D group (44). In the present study, we also observed an increase in MCV in the T2D group by comparison with ND group. The findings on the T2D parameters being decreased, reported by Arkew *et al.*, suggested that chronic hyperglycemia in T2D induced more ROS formation and glycation of hemoglobin, thereby reducing RBC deformability and more aggregation which then changes blood viscosity (44). Surprisingly, other studies report on an increase in RBCs and HGB which has been reported to suggest that hyperinsulinemia causes a synergistic effect with erythropoietin contributing to increased erythropoiesis (46, 47). Additionally, other studies reported increased levels of MCHC indicating that chronic hyperglycemia contributes to morphological and functional modifications (48-50). In the present study, there was an increase in mean RBC count, HGB, HCT, MCH and MCHC indicating that the effect of hyperglycemia and hyperinsulinemia still affects the morphological changes, functional modifications and erythropoiesis which was reported in the T2D group.

Manuscript 2 showed an increase in RBC and MCV in the PD group while no change was observed with T2D. According to literature, hyperglycemic conditions reduce the cell deformability as a result of oxidation of hemoglobin and glycosylation (51, 52). Circulating insulin bind to RBC's insulin receptors which then trigger more production of RBCs and cause increase of circulating RBCs (53, 54). Additionally, increased RBCs count suggests that insulin still regulate erythropoiesis and that EPO is still released indicating that there is no disturbance in the kidney's erythropoietin-producing cells, even though, there is moderate hyperglycemia in the pre-diabetic state. The HGB levels have been shown to directly correlate with RBC count under hyperglycemic conditions (45). Interestingly, manuscript 2 also reported on a decrease in RBC count and HGB concentration on females by comparison to males per group. This

could be due to sex-related hormones such as androgens and eostrogens which also have been reported to affect erythropoiesis (55). Females has also been reported to have lower HGB at about 12% when compared to males, under normal physiological conditions (55). Estrogen functions in causing dilation of renal microvascular vessels while androgens induce constriction of renal microvascular vessels which then results in changes in hematocrit levels (55). Indeed, the decrease in hematocrit on females per group by comparison with males, was observed in all groups, which correlate with a decrease in HGB concentration and RBC count in females compared to males per group in all groups. Findings of this study also showed an increase in HGB, HCT, MCHC and MCH in the pre-diabetic group when compared to the ND group however the concentrations of these markers were lower when compared to the T2D group. These findings are consistent with those seen in the diet-induced rat model of pre-diabetes (11). The increase in HGB indicate that while there is moderate hyperglycemia during the pre-diabetic state, it does not lead to disturbances in RBCs production. The only concern based on the HGB findings, is their ranges in all experimental groups below normal range [13.5-18.0 g\dL], which suggest that it is due to iron absorption disturbances (56, 57). Additionally, the disturbances in iron absorption can trigger disturbances in oxygen transportation, thereby contributing to development of anemia (57). An increase in MCHC suggest that hyperglycemia cause an increase its cytosolic viscosity, which is due to disturbances in hemoglobin aggregation. These changes in cytosolic viscosity contribute to thrombosis and cardiovascular abnormalities such as atherosclerosis suggesting that more research needs to be done based on these abnormalities during pre-diabetes. Additionally, an increase in HCT suggests endothelial damage in the pre-diabetic state which contributes to CVD such as ischemic heart disease and atherosclerosis. These changes suggest that the changes observed on red blood cells during T2D are due to disturbances that begin during the pre-diabetic state.

EPO has been reported to be decreased in T2D due to nephropathy caused by chronic hyperglycemia, contributing to anemia (58). Findings on this study are consistent with the literature on T2D group, showing a decrease in EPO concentration in the T2D group by comparison with ND group. Interestingly, the findings on normal erythropoiesis were proven by the results of EPO concentration obtained in this study, where the results showed an increase in EPO concentration in the PD group by comparison to the ND group but lower than those observed in the T2D group. This also suggests that circulating levels of EPO trigger the production of RBCs therefore increasing them on circulation. Additionally, the increase in EPO

levels in the pre-diabetic state suggests that acute hyperglycemia has not caused a lot of damage on renal function since EPO is produced from kidneys. However, more research needs to be done on the effects on pre-diabetes on renal function. Taken together, manuscript 2 showed that the moderate hyperglycaemia in the pre-diabetic state causes hematological changes that have been implicated in the development of other blood-related complications.

After observing that there is immune activation during the pre-diabetic state in manuscript 1, we further sought to investigate if there are lncRNAs expressed by immune cells. LncRNAs are defined as non-protein coding RNAs of more than 200 nucleotides, involved in regulation of biological functions such as gene control, differentiation and development (59, 60). They have also been involved in other diseases such as cardiovascular diseases, HIV and cancer (61). These studies therefore provide novel biomarkers and pharmaceutical targets of those diseases (61-63). In diseases, lncRNA play roles such as stabilizing mRNA by recruiting proteins to prevent degradation, bind and sequest proteins to attenuate their action, act as scaffold to link different proteins that are required for concerted action as well as recruit proteins to specific target genome sites (61). For an example, in cancer, lncRNA *FAL1* has been reported to be found in different tumor types, its expression level detect patient survival (61). Furthermore, depletion of certain lncRNAs has been reported to led to reduced malignancy (61). However, there is paucity of research that report on lncRNAs expressed by immune cells during pre-diabetes and T2D. It may be of great interest to consider certain lncRNAs as biomarkers of prognosis and pathogenesis of during pre-diabetes and T2D. Therefore, manuscript 3 investigated the expression of LncRNA-DC, lncRNA-NTT and lncRNA-NRON during the pre-diabetic state.

LncRNA-NTT and lncRNA-NRON are two lncRNAs expressed by T-cells. Manuscript 1 reported on lymphocytes which include T-lymphocytes which are T-cells and $CD4^+$ T-cells, involved in manuscript 3. LncRNA-NRON is a repressor of the nuclear factor of activated T cells (NFAT), long noncoding RNA expressed by the human resting T-cells (64). LncRNA-NRON plays a role in differentiation of T cells by trapping cytoplasmic NFAT protein in T-cells which modulate NFAT signalling and also contribute to the progression of atherosclerosis (7). Zebardast and colleagues recently reported on the upregulation of lncRNA-NRON in T2D patients from the age of 40 to 65 years (48% males and 52% females)(64). LncRNA-NRON facilitates the scaffold of the interaction of NFAT1 with GTPase activating protein (IQGAP) and three inhibitory NFAT1 kinases CKe, GSKb, and DYRK which results in translocation of

NFAT1 from the cytoplasm to the nucleus (60). However, in the present study, there was a clinical decrease in the expression of LncRNA-NRON in the T2D group by comparison to the ND group. The decrease in LncRNA-NRON expression in the T2D group suggested that due to the chronic hyperglycemia, there was less circulating resting T-cells as other factors such as IL-6 activate T-cells and more inflammation. However, the state of NFAT1 was not measured. The study further showed a significant increase in expression of lncRNA-NRON in the PD group by comparison with the ND group but was also significantly less when compared with that observed in the T2D group. These findings on the lncRNA-NRON correlate with the increase in the lymphocytes produced in the PD group on manuscript 1. These findings may also suggest that during the pre-diabetic state, there is increased production of T-cells, but not at a level to cause an increase in lncRNA-NRON expression. This, to our knowledge is the first study reporting on lncRNA-NRON during the pre-diabetic state. These findings may suggest that the expression of lncRNA-NRON may be used as a biomarker to measure the concentration of resting T-cells at during pre-diabetes which can also indicate the level of inflammation.

Manuscript 3 also investigated the expression of lncRNA-NTT, which is a lncRNA expressed by activated $CD4^+$ T-cells (65). An experiment by Liu and co-workers indicated that the induction functions of lncRNA-NTT are altered upon activation of T-cells (66). According to literature, in conditions such as T2D, activated $CD4^+$ T-cells are reported to be accumulated in inflamed areas such as adipose tissue (67). $CD4^+$ T-cells have been reported to sustain or promote inflammatory processes and insulin resistance through the induction of proinflammatory cytokines in metabolic organs such as liver, adipose tissue and skeletal muscle (67). Surprisingly, there were no reports based on the expression of lncRNA-NTT in T2D. In manuscript 3, our study, for the first time, showed that there was a decrease in lncRNA-NTT expression in T2D by comparison with ND. These findings corroborated previous data that showed that a higher amount of activated $CD4^+$ T -cells are recruited to inflamed areas from circulation indicating chronic inflammation (68). The study further showed a decrease in lncRNA-NTT in the PD group by comparison with ND group but was also higher when compared with that observed in the T2D group. These findings suggest that the moderate hyperglycaemia seen in pre-diabetes triggers recruitment of activated $CD4^+$ T-cells to inflamed areas, therefore decreasing the concentration of activated $CD4^+$ T-cells from the circulation. This could possibly explain the decrease in lncRNA-NTT expression that is observed during pre-diabetes.

Lastly, manuscript 3 measured the expression of lncRNA-DC. This is a long noncoding RNA that is expressed by dendric cells. Dendric cells are produced on a bone marrow and have been reported to be one of the immune cells that are a bridge between innate immunity and adaptive immunity (69-71). Additionally, lncRNA-DC is required for monocyte differentiation into DCs (8, 72). Reports by Wang *et al*., show that binding of lncRNA-DC to STAT3 controls the differentiation of human dendric cells (72). LncRNA-DC has been reported to directly bind with STAT3, which then prevents the dephosphorylation of transcription factor STAT3 by domain-containing phosphatase 1(SHP1)(8, 73). This binding of lncRNA-DC therefore activates STAT3-dependent transcription (73). Upon activation, STAT3 mediates the transcription of genes encoding for DC differentiation (73). Alikhah and colleagues reported an increase in lncRNA-DC in T2D subjects and further mentioned that estrogen contributes to increasing lncRNA-DC in women (74). Indeed, the findings in manuscript 3 are consistent with literature as we reported an increase in expression of lncRNA-DC in the T2D group by comparison with the ND group. The findings further indicate that there was an increase in expression of lncRNA-DC in the PD group by comparison with ND group but was also lower when compared with that observed in the T2D group. These novel findings, continue to suggest that pre-diabetes is a highly inflammatory state which contributes to an increase in the lncRNA-DC. This increase in lncRNA-DC expression associated with the moderate hyperglycaemia in pre-diabetes, triggers the differentiation of monocytes to DCs (8, 75, 76). These findings suggest that the expression of lncRNA-DC may potentially be used as biomarkers with measurement of monocytes and dendric cells in prognosis and pathophysiology of genetic complications in both pre-diabetes and T2D.

Taken together, manuscript 3 showed that the moderate hyperglycemia observed during pre-diabetes triggers the expression of the lncRNAs which contribute to immunogenetic changes that trigger immune activation and acute sub-clinical inflammation.

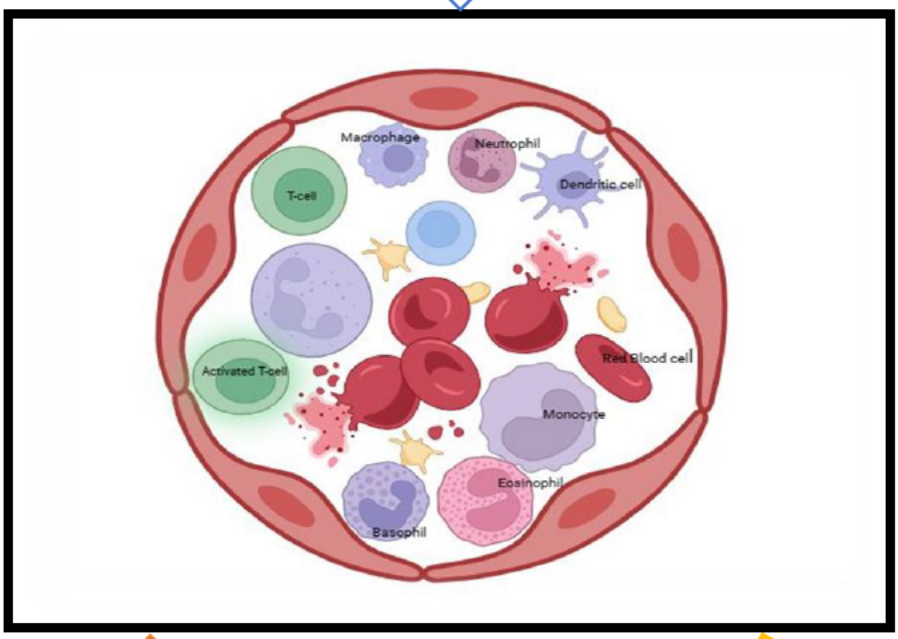
Beginning of Study 1

Diagnosis of Prediabetes

Immune activation: ↑ [basophile, eosinophils], ↓ [neutrophils, lymphocytes, monocytes]

↑ subclinical inflammation: ↑ [TNF–α, CD40L, Fibrinogen], ↓ [IL-6, CRP, P-selectin]

End of Study 1



Beginning of Study 2

Moderate hyperglycaemia

Changes in red blood cells indices: ↑ RBC, HGB, HCT,

Erythropoiesis: ↑ EPO

End of Study 2

Beginning of Study 3

Moderate hyperglycaemia

Expression of LncRNA by immune cells (T-cells & dendric

↑ LncRNA-NRON, LncRNA-DC ↓ LncRNA-NTT.

End of Study 3

Figure 1: Diagram showing a summary of findings in the manuscripts in the dissertation (diagram created using www.biorender.com).

Conclusion

The findings of this study suggest that some of the metabolic abnormalities found in T2D are also present in the pre-diabetic state due to the moderate hyperglycaemia. These include immune activation, sub-clinical inflammation, upregulation of erythropoiesis and expression of lncRNAs by immune cells. While not fully conclusive, these studies pave the way for future research in this area so as to better understand the pathophysiology of pre-diabetes.

Limitations of the study

Apart from the inclusion and exclusion criteria of the study, using the age 25-45 years enabled delivery of accurate results since age has an impact on red blood cells. Additionally, studies have showed that the highest prevalence of prediabetes occurs in the age range of 25-45 which is why we concentrated on this group.

Recommendations for future studies

The findings of manuscript 1 pave a way for more in-depth exploration of the changes in sub-populations of immune cells such as T-cells. More research needs to be done on other immune cells such as macrophages and dendric cells. It has also been observed from manuscript 1 that gender can contribute to immune changes and inflammation involving different mechanism in men and woman which need further investigation. Hormones such as those of the reproductive system need to be explored as they might contribute to changes observed during pre-diabetes. Findings in manuscript 2 highlighted a need to look at the state of red blood cells during pre-diabetes as well as investigate other factors and hormones contributing to changes in red blood cell indices. Findings reported on manuscript 3, lay a foundation to explore pre-diabetes at a genetic level using lncRNAs as biomarkers for pathophysiology and prognosis of pre-diabetes stage.

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APPENDIX

APPENDIX 1

Ethical Clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 May 2021

Dr A Khathi
School of Laboratory Medicine and Medical Sciences
College of Health Sciences

Dear Dr Khathi

Protocol: Investigating the prevalence of pre-diabetes as well as its effects on selected metabolic parameters in KwaZulu-Natal in individuals aged 25-45.
Non-Degree Purposes
BREC REF NO.: BE266/2019

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 04 May 2021. Please ensure that outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 04 May 2021. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 08 June 2021.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

APPENDIX 2

Email of Acceptance of manuscript from journal (Hematology Systematic review)

Re: Article Accepted for Publication

Inbox

editor.hmo <editor.hmo@oatext.com>

Mon, Apr 17, 2023, 3:59 PM

to me

Dear Ms. Nomusa Mzimela,

Greetings!

Thank you for your submission entitled "The changes in blood indices that occur on pre-diabetic patients of all ethnicities, from the age of 25- to 45- years: a systematic review and meta-analysis" towards our journal **Hematology and Medical Oncology**.

We are glad to inform you that your article has been accepted for publication in our journal. We will send you the Galley proof for corrections soon. Meanwhile we request you to make the payment of publication charges, please let us know your mode of payment (Card payment / Bank transfer) so that we can send you Invoice from billing team.

Awaiting your kind Response.

Thanks & Regards

Regina Mathew

Editorial Coordinator

APPENDIX 3

Email of Acceptance of manuscript from journal (hematology manuscript)

Dove Medical Press: Submission accepted for publication

Inbox

Ms Sandi McIver <sandi@dovepress.com> Jul 23, 2024,

5:23 AM

to me

Dear Miss Mzimela,

I am pleased to inform you that your manuscript "Evaluating the changes in red blood cells indices in individuals with pre-diabetes from the age of 25- to 45- years in Durban, South Africa".

Subtitle: Changes in red blood cells indices in pre-diabetic patients" has been accepted for publication in "**Journal of Blood Medicine**".

The article publishing charge of USD 2990 is now payable and an invoice for this amount has been sent to University of KwaZulu Natal.

If you have any questions about your paper, please contact me at any time sandi@dovepress.com

Yours sincerely

Ms Sandi McIver

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APPENDIX 4

Article on NDABAONLINE UKZN (05 October 2023 Volume :11 Issue :53)

Title of article**: Raising Pre-diabetes Awareness at National Society's Congress**

Link:
https://ndabaonline.ukzn.ac.za/UkzndabaStory/Vol11Issue53/Raising%20Prediabetes%20Awareness%20at%20National%20Societys%20Congress

'Raising Pre-diabetes Awareness at National Society's Congress'

Written by Lunga Memela

Pre-diabetes is the intermediate stage between normoglycaemia (when there is normal concentration of sugar in the blood) and type 2 diabetes (the long-term medical condition in which the body does not use insulin properly, resulting in unusual blood sugar levels).'

This was explained by UKZN's Human Physiology PhD candidate, Ms Nomusa Mzimela, who recently presented her research findings at the 56th Society for Endocrinology Metabolism and Diabetes of South Africa (SEMDSA) Congress in Johannesburg.

Mzimela said pre-diabetes generally develops from living an inactive lifestyle and from the chronic consumption of unhealthy foods. She noted that Durban has one of the fastest-growing rates of pre-diabetes prevalence on the continent, especially in people between the ages of 25 and 45, but there is a lack of context-specific investigation into how this condition develops and affects the people in this city. She said the insulin resistance that arises leads to abnormalities such as hyperglycaemia and hyperlipidaemia, which trigger the immune response and inflammation.

Supervised by the Physiology Discipline's Professor Andile Khathi, Mzimela's study investigated the inflammatory status of proteins TNF-a, IL-6, CRP, soluble CD40L, P-selectin and fibrinogen, confirming that there is immune activation and sub-clinical inflammation in prediabetic patients from Durban, with a new unexpected report of the effect of gender on immunity and inflammation.

Mzimela said: ‘Since pre-diabetes is asymptomatic, we recommend improved and increased testing of the condition as it impacts general health and the progression of other diseases.’ Mzimela said it was an honour to present her research to captains of industry at the congress. She also presented her master’s research at the inaugural Conference of Biomedical and Natural Sciences and Therapeutics (CoBNEST) in 2018 in Cape Town.

‘The congress in Johannesburg explored clinical and laboratory research based on diabetes, endocrinology, metabolism, lipid metabolism and atherosclerosis. It was also a space where medical specialists and researchers engaged with each other and explored collaboration to find solutions to challenges faced in healthcare,’ she said.

Mzimela said becoming a researcher has always been at the top of her to-do list. ‘I aspire to become a lecturer or a scientist, as long as it involves research and exploring the interchanging world of medical science.’ She said given the opportunity, she would definitely pursue postdoctoral research - allowing her to interact meaningfully with the world through scientific research and publications.

Mzimela holds a BSc in Physiology and Biochemistry from UNISA, a BSc Honours in the Medical Sciences from UKZN, as well as a Master of Medical Sciences in Human Physiology, also from UKZN.

She enjoys reading books, cooking and baking.

Words: Lunga Memela

Photograph: Supplied

APPENDIX 5

Article on SUNDAY TRIBUNE and MERCURY (By Mervyn Naidoo| Published Oct 22, 2023)

Title of article**: A study on pre-diabetes in the era of 'diabesity'**

Sunday Tribune Link: https://www.iol.co.za/sunday-tribune/news/a-study-on-pre-diabetes-in-the-era-of-diabesity-f5942f84-5df2-4a6e-9813-ea36ad288cd0

Mercury Link: https://www.msn.com/en-za/news/other/a-study-on-pre-diabetes-in-the-era-of-diabesity/ar-AA1iEXmi?ocid=msedgdhp&pc=HCTS&cvid=100e1ea72d4045a9b73d6f0051af1edd&ei=45

'A study on pre-diabetes in the era of 'diabesity'

Written by Mervyn Naidoo

When Nomusa Mzimela picked her path of study she chose to focus on pre-diabetes prevalence because the dreaded disease was "close to home". Outstanding for Mzimela, who is pursuing the qualification with the University of KwaZulu-Natal, was learning that the pre-diabetes stage could be reversed with good diet and exercise.

Mzimela, 38, was also amazed at the rate of pre-diabetes prevalence in people between the ages of 25 and 45, and living in Durban, which ranked the highest in the province. Mzimela said her colleague was doing a pre-diabetes prevalence scan and made the find. "I understand the findings. That is because of the food, lifestyle choices and the environment we live in. Durban lifestyles meant that due to the number of franchise restaurant options available, you end up eating out often, without realising the food might be more unhealthy than meals cooked at home. You then place yourself at risk of contracting the disease. Also, in Durban, I noticed that at tuck-shops, children and students were eating food like 'vetkoeks', which were not good for the body. "It fills stomachs. People can't help themselves because they can't afford healthy alternatives."

Mzimela, who was born and raised in Empangeni, said that unhealthy living trends had also extended to rural areas. “Another researcher said that there is an increase of pre-diabetes in rural areas because of food choices and affordability. “She said the environment you lived in was important because if your family could not afford healthy food, it could become a factor. “I also had family members who had diabetes. Therefore, this study is close to my heart.”

Mzimela’s studies showed her that the disease could be beaten through lifestyle changes and eating choices, instead of dying oblivious to it. “That sparked my interest”.

“I focused more on immunity and haematology. At the back of mind, I have an idea that the immune system is like a bridge to diabetes, cancer, HIV ,TB and other diseases that people get infected with. Therefore, the immune system is something you need to care for. It can easily be suppressed because of the food you eat, the medication you take in. She said a suppressed immune system could lead to infection.

“I’m really keen to explore more how the immune system works in a pre-diabetic person. I strongly believe there will be a way forward based on that.”

Mzimela also got to make her findings known recently at the 56th Society for Endocrinology Metabolism and Diabetes of South Africa (SEMDSA) Congress in Johannesburg, where researchers and doctors collaborated to find solutions to diseases affecting people.

Mzimela used the opportunity to raise awareness on pre-diabetic conditions and gather knowledge from other presenters. “I think the pre-diabetic phase is of critical importance because at the laboratory we found that most abnormalities were traced to this stage in rat specimens we used. “She was now applying the finishing touches to her PhD studies and would be ready for submission next week.

“I’m nervous, but I’m looking forward to the response from the examiners.

“My brothers are also excited about me completing my PhD since my parents are late. They were the ones who motivated me to do this and have been my support structure,” said Mzimela, who holds a BSc in physiology and biochemistry from Unisa, a BSc Honours in the medical sciences and Master of medical sciences in human physiology from UKZN.

She was grateful to her supervisor, Professor Andile Khathi, for her progress thus far and the confidence to explore and learn. “He also gave us opportunities to communicate with him about our research struggles,” she said.

Professor Khathi said Mzimela started with her study before the Covid-19 outbreak. This greatly impacted the collection of samples from the hospitals, but she continued to produce work of good quality, which is evidenced by it being published even before she submitted her dissertation. “I think it helped that she had a really good idea of the type of work that she wanted to do even before she began the study, which is relatively new. I helped her with the best way to achieve her study goals.”

Khathi said he was also initially shocked with her study results but over time it made sense. “Durban is urbanising quite rapidly which gives access to high-calorie, highly-processed foods often in the form of takeaways. It also explains why so many people develop type 2 diabetes mellitus later on in life,” he said.

Dietician Radha Joshi said much has changed since the Covid-19 pandemic for most people globally. “This is not only due to physical implications but mental health has also been compromised. The rate of depression and other mental health illnesses has skyrocketed, resulting in younger people facing type 2 diabetes mellitus as a post-Covid complication, and a sedentary lifestyle culture of caloric surplus and digitalisation. “We are now living in an obesogenic environment and dealing with another pandemic coined as ‘diabesity’, leading to other chronic diseases and a downward spiral,” she said.

SUNDAY TRIBUNE

Related Topics:

education2023ukzndurbanhealth welfare

APPENDIX 6

Immunity protocol in chapter 3 (Journal Guide): JMIR Research Protocol

Our requirements for submitted manuscripts are in accordance with the recommendations drawn up by the International Committee of Medical Journal Editors. For general information about the structure and content of a biomedical manuscript, authors should become familiar with the ICMJE Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals before reading our specific instructions for authors below.

The following instructions for authors are valid for all JMIR journals. Additional journal-specific instructions may also apply so please also be sure to look for those below. Author instructions are subject to revision so please refer to them frequently before submitting your manuscript.

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The following format ("**IMRAD Format**") must be used for the paper:

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Results (e.g. user statistics, evaluation outcomes). If your study consists of different stages/parts, subheadings in this section should mirror subheadings in the methods section to describe these parts.

Discussion (e.g. with the subheadings "Principal Results", "Limitations", "Comparison with Prior Work", "Conclusions")

Acknowledgements

Conflicts of Interest

[optional] Multimedia Appendix of supplementary files (e.g. a PowerPoint presentation of a conference talk about the study, additional screenshots of a website, mpeg/ Quicktime video or audio files, or Excel, Access, SAS, or SPSS files containing original data)

References

Abbreviations

Please use further subheadings within the main "Introduction," "Methods," "Results," and "Discussion" sections. For example, if you describe three different methods, use three subheadings within the "Methods" section. Also, use matching subheadings in the "Results" section if you report the results from each of the described methods.

APPENDIX 7

Immunity Systematic review in chapter 3 (Journal Guide): Medicine

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Role	Definition
Conceptualization	Ideas; formulation or evolution of overarching research goals and aims.
Data curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later re-use.
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyse or synthesize study data.
Funding acquisition	Acquisition of the financial support for the project leading to this publication.
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.

Methodology	Development or design of methodology; creation of models.
Project administration	Management and coordination responsibility for the research activity planning and execution.
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools.
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components.
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.
Validation	Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs.
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.
Writing – original draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation).
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Typeface should be Times/Times New Roman or similar serif typeface.

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Title

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The full title should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).

Authors and Affiliation

All author names should be listed in the following order:

First names (or initials, if used),

Middle names (or initials, if used), and

Last names (surname, family name)

Medical and/or highest academic degrees (eg, MD, PhD)

Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country.

When a large group or center has conducted the work, the author list should include the individuals whose contributions meet the authorship criteria defined above, as well as the group name. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article in the Acknowledgements section.

One author should be designated as the corresponding author, and his or her email address should be included on the manuscript cover page. This information will be published with the article if accepted.

For questions regarding authorship requirements, please consult the ICMJE Uniform Requirements for Manuscripts Submitted to Biomedical web page at http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html.

Abbreviations, Nomenclature and Symbols

Abbreviations, nomenclature and symbols should conform to those found in the *AMA Manual of Style*. The use of standard international units is encouraged. Abbreviations should be used sparingly and should be spelled out the first time they are used. A list of abbreviations should be included as part of the manuscript following the title page.

Abstract

A structured abstract should be **no more than 350 words**, summarizing the problem being considered, how the study was performed, the salient results, and the principal conclusions. Specific instructions regarding abstract structure are often included in the relevant reporting guidelines checklist.

Introduction/Materials and Methods/Results/Discussion and Conclusions

The overall structure of your manuscript text should follow the corresponding reporting guideline. For example, a CONSORT compliant manuscript should include the following sections, as defined by the CONSORT checklist:

Introduction

Methods

Results

Discussion

Other Information

Acknowledgements

All contributors who do not meet the criteria for authorship should be listed in an 'Acknowledgements' section. Additionally, if the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article in the Acknowledgements section. Authors should also disclose whether they had any writing assistance.

References

The style of references conforms to the guidelines set forth by the *AMA Manual of Style*. For Specific examples and information regarding references, see the manual or visit online: http://www.amamanualofstyle.com. EndNote users can access a direct download of the JAMA style at Medicine's Editorial Manager site. Authors using other forms of reference management software should use JAMA style.

All references cited in the text must be both listed and cited by the reference number (footnotes are not accepted).

Each reference should be cited in the text, tables, or figures in consecutive numerical order by means of superscript arabic numerals. Use superscript numerals outside periods and commas, inside colons and semicolons. When more than 2 references are cited at a given place in the manuscript, use hyphens to join the first and last numbers of a closed series; use commas without space to separate other parts of a multiple citation (eg, As reported previously,1,3-8,19...The derived data were as follows3,4,12:)

References should be numbered consecutively in the order in which they are cited in the text.

References in tables and in figure legends must appear in the reference page(s).

In listed references, use the author's surname followed by initials without periods. (eg, Doe JF)

For references with 6 or fewer authors, list all authors. For references with more than 6 authors, list the first 3 authors followed by "et al."

1 author Doe JF.

2 authors Doe JF, Roe JP III.

6 authors Doe JF, Roe JP III, Coe RT Jr, Loe JT Sr, Poe EA, van Voe AE.

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Full-page ranges should be given in expanded form (eg, 426–429, not 426–9).

If non-English-language titles are translated into English, bracketed indication of the original language should follow the title.

Abbreviate and italicize names of journals according to the style in PubMed; refer to the National Library of Medicine (NLM) Journals Database (http://www.ncbi.nlm.nih.gov/nlmcatalog/journals) if needed.

In references to journals that have no volume or issue numbers, use the issue date, as shown in example 1 below. If there is an issue number but no volume number, use the style shown in example 2. Conversely, if there is a volume number but no issue number, follow example 3.

Author(s). Article Title. Journal Name. Month Year:inclusive pages.

Author(s). Article Title. Journal Name. Year;(Issue No.):inclusive pages.

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Papers "submitted for publication" but not yet accepted and citations such as "personal communication" or "unpublished data" are not acceptable as listed references and instead should be included parenthetically in the text. This material, with its date, should be noted in the text as "unpublished data" as follows: (J. F. Doe, MD, unpublished data, January 2010).

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Authors are responsible for the accuracy and completeness of the references.

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Create tables using the table formatting and editing feature of your word processing software. Do not use Excel or comparable spreadsheet programs. Tables should be self-explanatory and should supplement, rather than duplicate, the material in the text.

Tables are text-only items. Do not embed images within the table file.

Each table file should include the table title, appropriate column heads, and any legends.

Save each table in a separate word processing document file and upload individually.

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Tables are numbered with arabic numerals (1, 2, 3, etc.) when there is more than one. Do not use roman numerals.

Cite tables consecutively in the manuscript, and number them in the order in which they are discussed.

Abbreviations are not permitted in table titles. Any abbreviation(s) used in the body of the table, including dashes, must be defined in a footnote to the table, listed in reading order.

Many tables include information from other articles and series of patients. In these tables, include the name of the first author of the previous series, and include the reference number and year alongside the author's name. Each series mentioned in a table must be listed in the Reference section.

For further information on table formatting, please see the *AMA Manual of Style*.

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To ensure the highest-quality reproduction of figures, please follow these guidelines carefully. For further information, please see the "Creating Digital Artwork" file available on Medicine's Editorial Manager site in the "Files & Resources" section of the home page.

Medicine® is not responsible for the quality of images; it is the responsibility of the authors to submit publication-quality, high-resolution images. If you have questions, consult a graphics specialist. The term "Figures" refers to both photographic and computer-generated graphs and charts.

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Composite figures may be either submitted as one single print-quality image that is neatly labeled with uppercase letters using Arial/Helvetica bold font or submitted as separate panels (without labels), eg, Figure 1A.tif, Figure 1B.tif, to be combined during production if accepted for publication.

APPENDIX 8

Immunity research manuscript in chapter 3 (Journal Guide): Journal of immunotoxicology

Article Types

Research Article

Should be written with the following elements in the following order: title page; abstract; keywords; main text introduction, materials and methods, results, discussion; acknowledgments; declaration of interest statement; references; appendices (as appropriate); table(s) with caption(s) (on individual pages); figures; figure captions (as a list)

Should be no more than 8,000 words, inclusive of:

Tables

References

Figure or table captions

Should contain an unstructured abstract of 200 words.

Should contain between 3 and 7 **keywords**. Read making your article more discoverable, including information on choosing a title and search engine optimization.

Methods

Should be written with the following elements in the following order: title page; abstract; keywords; main text introduction, materials and methods, results, discussion; acknowledgments; declaration of interest statement; references; appendices (as appropriate); table(s) with caption(s) (on individual pages); figures; figure captions (as a list)

Should be between 2,500 and 4,000 words

Should contain a structured abstract of 200 words.

Introduction, Methods, Results, Discussion.

Should contain between 3 and 7 **keywords**. Read making your article more discoverable, including information on choosing a title and search engine optimization.

Author Contributions Statement: Please provide an author contributions statement at the end of your article, before the references, that outlines which author(s) were involved in the conception and design, or analysis and interpretation of the data; the drafting of the paper, revising it critically for intellectual content; and the final approval of the version to be published; and that all authors agree to be accountable for all aspects of the work.

Further information about this article type: Methods articles are a medium-length, peer-reviewed article type that describes an advancement or development of current methods and research procedures. These should include adequate and appropriate validation to be considered. Any datasets associated with the paper must publish all experimental controls and make full datasets available where possible. If there are concerns about identifying factors in datasets, these should be discussed with the Editor-in-Chief prior to submission.

Please note, that authors submitting protocol and methodology articles have the option to share their methods on protocols.io. Please note, this is not required for submission but is encouraged.

Further to the data sharing policy for the journal, Data Note submissions must include a data availability statement and describe data available via a repository.

Checklist: What to Include

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Data availability statement. If there is a data set associated with the paper, please provide information about where the data supporting the results or analyses presented in the paper can be found. Where applicable, this should include the hyperlink, DOI or other persistent identifier associated with the data set(s). Templates are also available to support authors.

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In order to be published in a Taylor & Francis journal, all clinical trials must have been registered in a public repository, ideally at the beginning of the research process (prior to participant recruitment). Trial registration numbers should be included in the abstract, with full details in the methods section. Clinical trials should be registered prospectively – i.e. before participant recruitment. However, for clinical trials that have not been registered prospectively, Taylor & Francis journals requires retrospective registration to ensure the transparent and

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Please ensure that all research reported in submitted papers has been conducted in an ethical and responsible manner, and is in full compliance with all relevant codes of experimentation and legislation. All original research papers involving humans, animals, plants, biological material, protected or non-public datasets, collections or sites, must include a written statement in the Methods section, confirming ethical approval has been obtained from the appropriate local ethics committee or Institutional Review Board and that where relevant, informed consent has been obtained. For animal studies, approval must have been obtained from the local or institutional animal use and care committee. All research studies on humans (individuals, samples, or data) must have been performed in accordance with the principles stated in the Declaration of Helsinki. In settings where ethics approval for non-interventional studies (e.g. surveys) is not required, authors must include a statement to explain this. In settings where there are no ethics committees in place to provide ethical approval, authors are advised to contact the Editor to discuss further. Detailed guidance on ethics considerations and mandatory declarations can be found in our Editorial Policies section on Research Ethics.

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APPENDIX 9

Hematology protocol in chapter 4 (Journal Guide): Methods and Protocols

General Considerations

Research manuscripts should comprise:

Front matter: Title, Author list, Affiliations, Abstract, Keywords.

Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).

Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.

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Front matter: Title, Author list, Affiliations, Abstract, Keywords.

Review sections: a literature review organized logically within specific sections and subsections (optional).

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Graphical Abstract:

A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

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Acronyms/Abbreviations/Initialisms should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.

SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.

Accession numbers of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and Expression Data.

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Front Matter

These sections should appear in all manuscript types.

Title: The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than

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Results: Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

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Author Contributions: Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author’s contribution to the study); AND agrees to be personally accountable for the author’s own contributions and for ensuring that questions related to the accuracy or

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The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote and Zotero are available.

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All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme 1, Figure 2, Scheme 2, Table 1, etc.).

All Figures, Schemes and Tables should have a short explanatory title and caption.

All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.

Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

Supplementary Materials, Data Deposit and Software Source Code

MDPI Research Data Policies

MDPI is committed to supporting open scientific exchange and enabling our authors to achieve best practices in sharing and archiving research data. We encourage all authors of articles published in MDPI journals to share their research data including, but not limited to protocols,

analytic methods, raw data, processed data, code, software, algorithms, and study material. The data should be FAIR – findable, accessible, interoperable, and reusable – so that other researchers can locate and use the data.

We recommend that data and code should be deposited in a trusted repository that will allow for maximum reuse (see the Data Preservation section below). If this is not possible, authors are encouraged to share the specific reason in the Data Availability Statement and make this material available upon request to interested researchers. In addition, research materials necessary to enable the reproduction of an experiment should be indicated in the Materials and Methods section. Individual journal guidelines can be found at the journal 'Instructions for Authors' page. Data sharing policies concern the minimal dataset that supports the central findings of a published study. Generated data should be publicly available and cited in accordance with journal guidelines.

MDPI data policies are informed by TOP Guidelines.

Where ethical, legal, or privacy issues are present, data should not be shared. The authors should clarify the availability status of the data upon submission and make any limitations or exceptions clear in the Data Availability Statement. Authors should ensure that the data shared is in accordance with consent provided by participants on the use of confidential data. Authors should ensure that the publication of such data does not compromise the anonymity of the participants or breach local data protection laws.

In situations where access is restricted to protect confidential or proprietary information, authors will be requested to clearly explain the restrictions on the dataset and make the data available upon request, with permission for the purposes of peer review.

MDPI recognizes that some institutions and funding agencies only require the retention of research data for a finite period after a project's completion or publication. However, there are no such limits specified within the MDPI Data Availability Policy and, therefore, we encourage the authors to archive their research data through appropriate data repositories or provide us with minimal datasets within Supplementary Material.

Data availability statements

Data availability statements are required for all articles published with MDPI. During the peer review and editorial decision process, authors can be asked to share existing datasets or raw data that have been analyzed in the manuscript, and whether they will be made available to

other researchers following publication. Authors will also be asked for the details of any existing datasets that have been analyzed in the manuscript.

Below are the recommended Data Availability Statements:

Data availability status	Recommended Data Availability Statement
Data available in a publicly accessible repository	The data presented in this study are openly available in [repository name, e.g., FigShare] at [doi], reference number [reference number].
Data available in a publicly accessible repository that does not issue DOIs	Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number].
Data available on request due to restrictions (e.g., privacy, legal or ethical reasons)	The data presented in this study are available on request from the corresponding author (accurately indicate status).
3rd Party Data	Restrictions apply to the availability of these data. Data were obtained from [third party] and are available [from the authors/at URL] with the permission of [third party].
Embargo on data due to commercial restrictions	The data that support the findings will be available in [repository name] at [URL / DOI link] following an embargo from the date of publication to allow for commercialization of research findings.
Restrictions apply to the datasets:	The datasets presented in this article are not readily available because [include reason, e.g., the data are part of an ongoing study or due to technical/ time limitations]. Requests to access the datasets should be directed to [text input].
Data derived from public domain resources:	The data presented in this study are available in [repository name] at [URL/DOI], reference number [reference number]. These data

	were derived from the following resources available in the public domain: [list resources and URLs]
Data sharing is not applicable (only appropriate if no new data is generated or the article describes entirely theoretical research.	No new data were created or analyzed in this study. Data sharing is not applicable to this article
Data is contained within the article or supplementary material:	The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.
Dataset available on request from the authors.	The raw data supporting the conclusions of this article will be made available by the authors on request.

APPENDIX 10

Hematology systematic review in chapter 4 (Journal Guide): Hematology and Medical oncology

Abstract

Approximately 100-300 words, you will need to summarize your findings and what the implications of those findings are.The abstract must be accurate as a reflection of what is in your article. Because abstracts are the only substantive portion of the article indexed in many electronic databases, and the only portion many readers read, authors need to be careful that abstracts reflect the content of the article accurately. It should emphasize new and important aspects of the study or observations. Please do not include citations in your abstract and avoid the use of abbreviations, if possible and must be self-contained. It is a good idea to include keywords in your abstract, as this will help readers to find it. Key phrases need to make sense within the abstract. Try to keep to a maximum of three or four different keyword phrases and avoid over-repetition of such phrases as this can look like an attempt to trick a search engine, which may result in a page being rejected. Check that the abstract reads well.

Key words

Key words can be up to 6 which may include the species, variables tested, and the major response criteria. The first letter of each key word is lowercase (unless a proper noun); key words are separated by commas and presented in alphabetical order; and no abbreviations should be used. Key words will assist indexers in cross-indexing the article and may be published with the abstract.

Introduction

The introduction must not exceed 2,000 keystrokes (characters plus spaces) and briefly justifies the research, specifies the hypotheses to be tested, and gives the objective(s).

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study.
- Define the problem addressed and why it is important.
- Include a brief review of the key literature.

- Reports of clinical research should, where appropriate, include a summary of a search of the literature to indicate why this study was necessary and what it aimed to contribute to the field.
- Note any relevant controversies or disagreements in the field.
- The section should end with a very brief statement of what is being reported in the article.
- The main and secondary objectives should be made clear, and any pre-specified subgroup analysis should be described.
- Give only strictly pertinent references and do not include data or conclusions from the work being reported.
- Extensive discussion of relevant literature should be included in the discussion.

Materials and methods

Sufficient information should be given to permit repetition of the experimental work. This should include the design of the study, the setting, the type of participants or materials involved, a clear description of all interventions and comparisons, and the type of analysis used, including a power calculation if appropriate. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references. We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information. Methods sections of papers with data that should be deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.

Results

Present your results in logical sequence in the text or in the form of tables or figures/illustrations when feasible, giving the main or most important findings first. The text should explain or elaborate on the tabular data, but numbers should not be repeated within text. Extra or supplementary data and technical details can be placed in an appendix where it will be accessible but will not interrupt the flow of the text.

When data are summarized in the results section, sufficient data, all with some index of variation attached should be presented to allow the readers to interpret the results of the experiment. Give numeric results not only as derivatives (For example %) but also as the absolute numbers from which the derivatives were calculated and specify the statistical methods used to analyse them.

Restrict tables and figures to those needed to explain the argument of the paper and to assess its support. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid non –technical uses of technical terms in statistics, such as “random” (which implies a randomizing device), “normal”, “significant”, “correlations”, and “sample”.

Results section may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Discussion

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion").

Emphasize the new and important aspects of the study and the conclusions that follow from them. Do not repeat in detail data or other material given in the Introduction or the Results section. For experimental studies it is useful to begin the discussion by summarizing briefly the main findings, then explore possible mechanisms or explanations for these findings, compare and contrast how your research is different from previous reported and how your observations will significantly advance the current knowledge of the subject, state the limitations of the study. Do not repeat the details given in the introduction. Link the conclusions with the goals of the study but avoid unqualified statements and conclusions not adequately

supported by the data. Avoid claiming priority and alluding to work that has not been completed. State new hypotheses when warranted, but clearly label them as such.

References

Published works, works accepted for publication, and citable datasets should appear in the reference list. Mentions of unpublished work should be cited parenthetically within the main text of the article as personal communications.

OA Text employees the name-year (or "Harvard") system of in-text references, in which the author's surname and year of publication are cited in the text of your work, enclosed in parentheses. References should be numbered consecutively in the order in which they are first mentioned in the text. Identify references in text, tables, and legends by Arabic numerals in parentheses. References cited only in tables or figure legends should be numbered in accordance with the sequence established by the first identification in the text of the particular table or figure. Journals' titles should be abbreviated following theISI Journal Title Abbreviation List, but set in title case and italicized. The list can also be obtained through theLibrary's website.

Detailed information on formatting references can be found in ourReference Style Guide. We use Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers (7th Edition, 2006) as our primary style guide and highly recommend that authors consult it.

Tables

Tables capture information concisely, and display it efficiently; they also provide information at any desired level of detail and precision. Including data in tables rather than text frequently makes it possible to reduce the length of the text.

Type or print each table with double spacing on a separate sheet of paper. Number tables consecutively in the order of their first citation in the text and supply a brief title for each. Do not use internal horizontal or vertical lines. Give each column a short or abbreviated heading. Authors should place explanatory matter in footnotes, not in the heading. Explain in footnotes all nonstandard abbreviations. For footnotes use the following symbols, in sequence:

*,†,‡,§,¶,**,††,‡‡,§§,¶¶

Identify statistical measures of variations, such as standard deviation and standard error of the mean.

Be sure that each table is cited in the text.

If you use data from another published or unpublished source, obtain permission and acknowledge them fully.

Additional tables containing backup data too extensive to publish in print may be appropriate for publication in the electronic version of the journal, deposited with an archival service, or made available to readers directly by the authors. In that event an appropriate statement will be added to the text. Submit such tables for consideration with the paper so that they will be available to the peer reviewers. Please consult Table Preparation for detailed guidelines on formatting tables.

Figures

Figures should be either professionally drawn and photographed or submitted as photographic quality digital prints. In addition to requiring a version of the figures suitable for printing, some journals now ask authors for electronic files of figures in a format (e.g., JPEG or GIF) that will produce high quality images in the web version of the journal; authors should review the images of such files on a computer screen before submitting them, to be sure they meet their own quality standard.

For x-ray films, scans, and other diagnostic images, as well as pictures of pathology specimens or photomicrographs, send sharp, glossy, black-and-white or color photographic prints, usually 127 x 173 mm (5 x 7 inches). Although some journals redraw figures, many do not. Letters, numbers, and symbols on Figures should therefore be clear and even throughout, and of sufficient size that when reduced for publication each item will still be legible. Figures should be made as self-explanatory as possible, since many will be used directly in slide presentations. Titles and detailed explanations belong in the legends, however, not on the illustrations themselves.

Photomicrographs should have internal scale markers. Symbols, arrows, or letters used in photomicrographs should contrast with the background.

If photographs of people are used, either the subjects must not be identifiable, or their pictures must be accompanied by written permission to use the photograph. Whenever possible permission for publication should be obtained.

Figures should be numbered consecutively according to the order in which they have been first cited in the text. If a figure has been published, acknowledge the original source and submit written permission from the copyright holder to reproduce the material. Permission is required irrespective of authorship or publisher except for documents in the public domain.

For illustrations in color, ascertain whether the journal requires color negatives, positive transparencies, or color prints. Accompanying drawings marked to indicate the region to be reproduced might be useful to the editor.

Legends of figures

Type or print out legends for illustrations using double spacing, starting on a separate page, with Arabic numerals corresponding to the illustrations. When symbols, arrows, numbers, or letters are used to identify parts of the illustrations, identify and explain each one clearly in the legend. Explain the internal scale and identify the method of staining in photomicrographs.

Supplementary materials

Although we do not limit the number or type of Supplemental Material items authors may include, we do require that they provide a relevant and useful expansion of the article, and that they be as well described as are figures and tables included within the body of the article. Good metadata of this material are key to discoverability and usefulness. All Supplemental Material should include the following:

Type and number: Supplemental material can be named in almost any way, provided that the files are consistently named, and numbers are preceded by “S” and closed with a period. Examples:

Figure S1.

- Table S1.
- Text S1.

- Video S1.
- Animation S1.
- Alternative Language Abstract S1.

Figures, Tables, Videos, Animations should be provided with titles should be no more than 15 words and set in bold type, using sentence case.

Supplemental material figures and tables should follow the requirements for main-text figures and tables (see Figure Preparation and Table Preparation).

Other types of supplementary material files should include a caption of no more than 300 words, should be describing the key message of the figure/video/animation in such a way that readers can interpret the file without referring to the text.

APPENDIX 11

Hematology research manuscript in chapter 4 (Journal Guide): Journal of Blood Medicine

Article structure

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address.

All necessary files have been uploaded:

Manuscript:

• Include keywords

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

Graphical Abstracts / Highlights files (where applicable)

Supplemental files (where applicable)

Further considerations

• Manuscript has been 'spell checked' and 'grammar checked'

• All references mentioned in the Reference List are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Internet)

• A competing interests statement is provided, even if the authors have no competing interests to declare

• Journal policies detailed in this guide have been reviewed

• Referee suggestions and contact details provided, based on journal requirements

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

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

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results

Results should be clear and concise.

Discussion

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

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first

page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proofreading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, it is recommended to include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
• Ensure that color images are accessible to all, including those with impaired color vision.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.

Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.

TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.

TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures these will be reproduced in color at no cost. For further information on the preparation of electronic artwork, please

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the

figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list but may be mentioned in the text. If these references are included in the reference list, they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired or can be included in the reference list.

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset]

immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Preprint references

Where a preprint has subsequently become available as a peer-reviewed publication, the formal publication should be used as the reference. If there are preprints that are central to your work or that cover crucial developments in the topic, but are not yet formally published, these may be referenced. Preprints should be clearly marked as such, for example by including the word preprint, or the name of the preprint server, as part of the reference. The preprint DOI should also be provided.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript.

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references can be listed either first alphabetically, then chronologically, or vice versa.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999)…. Or, as demonstrated (Jones, 1999; Allan, 2000)… Kramer et al. (2010) have recently shown …'

List: References should be arranged first alphabetically and then further sorted

chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Reference to a website:

Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).

Reference to a dataset:

[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T., 2015. Mortality data for Japanese oak wilt disease and surrounding forest compositions. Mendeley Data, v1. https://doi.org/10.17632/xwj98nb39r.1.

Reference to software:

Coon, E., Berndt, M., Jan, A., Svyatsky, D., Atchley, A., Kikinzon, E., Harp, D., Manzini, G., Shelef, E., Lipnikov, K., Garimella, R., Xu, C., Moulton, D., Karra, S., Painter, S., Jafarov, E., & Molins, S., 2020. Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88). Zenodo. https://doi.org/10.5281/zenodo.3727209.

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material

together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

APPENDIX 12

Genetics research manuscript in chapter 5 (Journal Guide): Endocrine Journal

Research papers

A full-length report of original, hypothesis-driven basic or clinical research, with new data, investigated using the scientific method, may be submitted as a research paper.

Limit- 4000 words excluding references, tables, and figures

Abstract- 200 words maximum

References- up to 75.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided,

but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 5 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

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Reference to a journal publication with an article number:

[2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.

Reference to a book:

[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).

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[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/xwj98nb39r.1.

Reference to software:

[7] E. Coon, M. Berndt, A. Jan, D. Svyatsky, A. Atchley, E. Kikinzon, D. Harp, G. Manzini, E. Shelef, K. Lipnikov, R. Garimella, C. Xu, D. Moulton, S. Karra, S. Painter, E. Jafarov, S. Molins, Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88), Zenodo, March 25, 2020. https://doi.org/10.5281/zenodo.3727209.

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