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Iron metabolism and its association with chronic inflammation and cardiovascular risk in Type 2 diabetes mellitus.

By

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Declaration

I, Fransina Ndevahoma hereby declare that the work contained in the thesis entitled" iron metabolism and its association with chronic inflammation and cardiovascular risk in type 2 diabetes patients in Windhoek, Namibia"is my own original work and that I have not previously, in its entirety or in part, submitted it at any university or other higher education institution for the award of a degree.

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Dedication

To my parents, Mr & Mrs Ndevahoma:

I love you!

Abstract

Background

Type 2 diabetes mellitus (T2D) is a low-grade systemic inflammatory condition that is characterised by hyperglycaemia driven by defects in insulin resistance, insulin secretion, or both. Hyperglycaemia, obesity-induced inflammation, and dyslipidaemia have been implicated in increasing cardiovascular risk in patients with T2D. Interestingly, a growing body of evidence has also linked these abnormalities to altered iron metabolism in patients with T2D. However, the exact mechanisms behind this dysregulation are not well understood. Therefore, understanding the iron profiles in poor glucose control may pave ways to the identification of pathways involved in iron dysmetabolism and the development of therapeutic interventions. Therefore, the primary aim of this study was to investigate iron profiles in patients with T2D and to further assess the impact of inflammation on these profiles. In addition, it aimed to assess the cardiovascular risk in patients with T2D and to determine whether there are any associations between iron lipids and inflammation profiles in these patients.

Methods

This descriptive observational study involved a cohort of clinically known outpatients with T2D who visited an urban healthcare center from September 2020 to December 2020. The participants were recruited from Katutura Community Health Centre, Windhoek, Namibia. The study randomly recruited a total of one hundred and fifteen adult patients (n=115) of both genders. The diagnoses of T2D were diagnosed by a registered and qualified clinician following the American Diabetes Association guidelines. Analysis of the measured parameters was done in two parts, which is based on the patients' inflammatory status and the degree of obesity. Standard laboratory instruments and validated assays were used to measure and determine the glucose, iron, inflammatory, and lipid profiles, as well as haematological indices.

Results

Patients presenting with underlying inflammation had significantly elevated levels of fasting plasma glucose (FPG) (11.12 ± 3.95) when compared to those without underlying inflammation (8.86 ± 3.68), p=0.0413. However, the glycated haemoglobin (Hb1Ac) levels were comparable between the two groups (p>0.05). Interestingly, patients with underlying inflammation had significantly lower levels of total serum iron (12.78 ± 3.50) and red cell mean volume (87.66 ± 3.62) in comparison to patients without underlying inflammation (15.26 ± 4.64), p=0.0381 and (90.79 ± 4.52), p=

0.0096, respectively. However, the levels of ferritin, transferrin, and other red cell indices were comparable between the groups (p>0.05). Similarly, there were no differences in the lipid profiles between these patients (p>0.05). The correlation analysis showed a medium association between the levels of C-reactive protein (CRP) and total serum iron levels (Spearman r=-0.38, p=0.006). However, no associations were found with lipids profiles. Assessment of iron metabolism based on the class of body mass index (BMI) showed comparable glucose, iron, and haematological profiles amongst the three groups assessed, that is lean, overweight, and obese (p>0.05). However, systolic (139.3 ± 19.12) and diastolic (90.49 ± 13.35) blood pressures were significantly higher in patients with obesity compared to lean patients (126.1 ± 17.03), p=0.0163 and (82.71 ± 10.83), p=0.0289, respectively. Notably, patients with obesity had higher levels of triglycerides (1.76 [1.29 – 2.64]) in comparison to lean patients (1.15 [0.75 – 1.87]), p=0.0029. All other lipid profiles were comparable across the three groups. The BMI only positively correlated with triglycerides (Spearman r=0.27, p=0.005) systolic (Spearman r=0.23, p=0.019) and diastolic (Spearman r=0.23, p=0.022) blood pressure.

Conclusion

In conclusion, Iron metabolism in individuals with T2D is dysregulated. Apart from poor glucose control, the presence of underlying inflammation also influences iron metabolism in patients with T2D by decreasing the total serum iron levels and MCV. These are classical features of an early manifestation of iron deficiency anaemia. Therefore, intervention measures such as the use of low-dose aspirin as an anti-inflammation drug might help alleviate the development of iron deficiency anaemia in these patients. In addition, the use of triglycerides lowering drugs might be helpful in preventing future cases of cardiovascular disease in T2D presenting with obesity.

Keywords: type 2 diabetes; inflammation; iron metabolism; cardiovascular risk, obesity

List of Abbreviations

ADA	American Diabetes Association
BMI	Body Mass Index
CRP	C-reactive protein
CVD	Cardiovascular disease
ESR	Erythrocytes Sedimentation rate
HDL-c	High - density lipoprotein cholesterol
HCT	Haematocrit
IL	Interleukin
IDF	International Diabetic Federation
LDL-c	Low - density lipoprotein cholesterol
MCV	Mean cell volume
MCHC	Mean Corpuscular Haemoglobin Concentration
MCH	Mean Corpuscular Haemoglobin
TNF	Tumour necrosis factor
T2D	Type 2 Diabetes Mellitus
WCC	White cells count
WHO	World Health Organisation

Table of Contents

Table of Contents

Declaration ii

Retention and use of thesis iii

Acknowledgments iv

Dedication v

Abstract vi

List of abbreviations viii

Table of contents ix

List of figures xii

List of tables xiii

Chapter One: Introduction 1

1.1 Hypothesis 2

1.2 Research questions 2

1.3 Overall aim 2

1.4 Objectives 2

Chapter Two: Literature review 3

2.1 Introduction 3

2.2 Definition and classification of diabetes mellitus 4

2.2.1 Pre diabetes 4

2.2.2 Gestational diabetes 4

2.2.3 Type 1 diabetes mellitus 5

2.2.4 Type 2 diabetes 5

2.3 Pathogenesis of T2D 6

2.4 Immune response in T2D 9

2.5 Iron metabolism 11

2.51 The regulation of iron metabolism 12
2.52 The impact of inflammation on iron metabolism 14
2.53 Iron metabolism in T2D 16
2.6 Dyslipidaemia and cardiovascular risk in T2D 17
Chapter Three: Materials and Methods 20
3.1 Study design, setting, population and sampling 20
3.1.1 Inclusion criteria 20
3.1.2 Exclusion criteria 20
3.1.3 Sample size 20
3.1.4 Sample collections 21
3.2 Clinical measurements 21
3.2.1 Laboratory measurements 22
3.3 Statistical analysis 22
Chapter Four: Results 24
4.1 Characteristics of included study 24
4.2 The effect of obesity on iron metabolism and cardiovascular risk 24
4.2.1 The impact of body weights on glucose and inflammatory profiles in T2D 25
4.2.2 The effects of obesity on iron profiles 27
4.2.3 Obesity in T2D elevates the cardiovascular risk in patients with T2D 27
4.3 Correlation analysis between obesity, inflammation, iron metabolism and cardiovascular risk 28
4.4 The impact of inflammation on cardiovascular risk, iron and glucose metabolism in T2D 30
4.4.1 Clinical parameters and glucose parameters 30
4.4.2 Inflammatory profiles 32
4.4.3 Iron profiles levels and red cell indices 32
4.4.4 Lipid profiles levels 33
4.5 Correlation and regression analysis of glucose levels, CRP and iron profiles 33
Chapter Five: Discussion 35
5.1 Limitations 37
5.2 Recommendations 37

5.3 Conclusion remarks 38

References 39

Appendices 53

Appendix 1: Ethics clearance Namibia University of Sciences and Technology 53

Appendix 2: Ethics clearance Ministry of Health and Social Services 54

Appendix 3: Ethics clearance Namibia Institute of Pathology 56

Addedumns 57

Addendum 1: Systematic review and meta-analysis on the influence of body weights on hepcidin levels in T2D (Accepted for publication in Heliyon) 57

Addendum 2: Experimental paper 1 (Submitted) 85

List of Figures

Chapter Two: literature review

Figure 2.1. Etiology and pathogenesis of type 2 diabetes ……..7

Figure 2.2. The association between obesity and type 2 diabetes……8

Figure 2.3. Effect of hyperglycaemia on oxidative stress ……10

Figure 2.4. An overview of iron metabolism in the body ……12

Figure 2.5 An overview of potential mechanisms involved in erythroid suppression of hepcidin……14

Figure.2.6 An Illustration of how inflammation affects iron metabolism……15

Figure 2.7. Dyslipidaemia in type 2 diabetes……18

Chapter Four: Results

Figure. 4.1 Iron profiles and haematological indices in T2D.……27

Figure. 4.2 Assessed cardiovascular risks parameter amongst individuals with T2D……28

Figure. 4.3 Inflammatory parameters inT2D with normal and high CRP levels ……32

Figure. 4.4 Iron profiles and red cells indices in T2D with normal and high CRP ……33

Figure. 4.5 Interpretation of predicted and actual values of serum iron levels and the residual ……34

List of Tables

Chapter Two: Literature review

Table 2.1. Diagnosis criteria of pre-diabetes……4

Table 2.2. Diagnosis criteria of type 2 diabetes ……5

Chapter Three. Methods

Table 3.1 Biochemical and haematological measured parameters ……22

Chapter Four. Results

Table 4.1 Characteristic of included participants ……..24

Table 4.2 Characteristic of included participants based on degree of obesity …….. 26

Table 4.3 Correlations analysis between obesity, inflammation, iron and cardiovascular risk ……..29

Table 4.4 Clinical characteristics and laboratory profiles of included participants ……..31

Table 4.5. Multivariate linear regression analysis of independent variables of serum iron……..34

Chapter One: Introduction

Diabetes mellitus is a metabolic disorder that is characterised by hyperglycaemia as a result of either insulin deficiency, resistance, or both (Atyia, Gawaly, Enaam, EL-BAR, et al., 2018; Petersmann et al., 2019). Type 2 diabetes (T2D), is the common form of diabetes that constitutes more than 90% of global cases (Lascar et al., 2018). This disorder is characterised by poor glucose control and insulin resistance which are both closely associated with obesity, dyslipidaemia, and low-grade chronic inflammation (Hameed et al., 2015; Nicholas et al., 2019). Although the exact mediators of inflammation in T2D are still not well understood, obesity and hyperglycaemia are some of the factors implicated in this process (Al-Shukaili et al., 2013; De Rekeneire et al., 2006).

Obesity is associated with the exacerbated release of pro-inflammatory cytokines that modulate chronic inflammation in T2D (Wellen & Hotamisligil, 2005). Notably, the resulting pro-inflammatory state activates various signaling pathways that are involved in cell differentiation and immune activation such as Bone Morphogenic Protein-SMAD pathway (BMP-SMAD), mitogen-activated protein kinase pathway, and (Janus-kinase) JAK/Signal transducer and activator of transcription 3 (STAT 3) signaling pathways (Seif et al., 2017). Notably, these pathways, particularly the JAK/STAT signaling pathway modulate proteins that regulate iron regulation such as hepcidin (Suárez-Ortegón et al., 2015; Vela, 2018). Iron metabolism in T2D is dysregulated, with both anaemia of chronic disease (ACD) and iron overload being described (Pagani et al., 2019; X. Zheng et al., 2011). The difference in the relationship between glucose control and iron metabolism is dependent on various underlining factors such as inflammation (Fernández-Real et al., 2002). Iron is essential for optimal metabolism in most tissues and its dysregulation contributes to different pathogenesis that are associated with iron disorders such as iron overload and iron deficiencies (Kali et al., 2015). Although iron metabolism is well studied, there are reported differences on how iron metabolism is regulated in T2D, and how its levels together with its surrogates markers such as ferritin and transferrin varies (Altamura et al., 2017; Andrews et al., 2015; Atyia, Gawaly, Enaam, EL-BAR, et al., 2018; X. Guo et al., 2013; Jiang et al., 2011; X. Zheng et al., 2011). For instance, reduced (Altamura et al., 2017; X. Zheng et al., 2011), comparable (Andrews et al., 2015; X. Guo et al., 2013) and increased (Atyia, Gawaly, Enaam, EL-BAR, et al., 2018; Jiang et al., 2011) iron levels among T2D has been reported. Thus, these different relationships suggest the presence of other underlying conditions besides inflammation to be involved in iron metabolism. One of which being obesity.

Obesity a major risk factor for T2D is associated with systemic inflammation and insulin resistance (Esser et al., 2014; Nyambuya et al., 2019). Notably, obesity is also associated with dyslipidaemia and

an increased risk of cardiovascular disease (CVD) in patients with T2D (Steinberger et al., 1995). The accumulation of the visceral fat in the adipose tissue of obese individuals promotes insulin resistance and secretions of pro-inflammatory cytokines such as interleukin (IL)-6, Tumor necrosis factor -α (TNF-α), and interferon-γ (IFN-γ) which drives atherogenesis (Fuster et al., 2016). This IL-6 is involved in the up-regulation and overexpression of hepcidin via the JAK/STAT signaling pathway (Diedra M. Wrighting & Andrews, 2006). This causes degradation of ferroportin and a reduction in iron release and absorption from the macrophages, thus decreasing iron levels and eventually resulting in iron deficiency (Bobby J. Cherayil, 2016). Therefore, proper regulation of hepcidin is important, as is involved in the regulation of iron metabolism. Although, the variations of iron profiles in people with T2D is well established (Altamura et al., 2017; Andrews et al., 2015; Atyia, Gawaly, Enaam, EL-BAR, et al., 2018; X. Guo et al., 2013; Suárez-Ortegón et al., 2015), the exact underlining mechanisms that influence these variations are not well-understood. Understanding these factors is important as it will pave way for the development of therapeutic interventions that target iron metabolism to alleviate chronic inflammation in T2D. Therefore, this study aimed to investigate iron profiles in patients with T2D and the impact of inflammation on these profiles. Furthermore, the study aimed to assess cardiovascular risk in T2D.

1.1 Hypothesis

Patients with T2D have altered iron metabolism and increased cardiovascular risk.

1.2 Research questions

This study was conducted to answer the following research questions:

1. Are the iron profiles altered in patients with T2D?
2. Does the inflammatory status of patients with T2D have an impact on the iron profiles?
3. Are there any associations between iron profiles, inflammation, and lipids profiles in T2D?

1.3 Overall aim

To investigate iron metabolism and cardiovascular risk in patients with T2D

1.4 Objectives

- To measure iron profiles (iron, transferrin, and ferritin) in patients with T2D.
- To determine the impact of inflammation and obesity on iron profiles in patients with T2D.
- To measure the lipid profiles and cardiovascular risk in patients with T2D.

Chapter two: Literature review

2.1 Introduction

The global prevalence of diabetes has been rising over the years, and it is estimated to rise to 10.2% by 2030 from 9.3% in 2019 (IDF, 2019). In fact, the Word health organisation (WHO) has reported diabetes mellitus to be the ninth major cause of global death (WHO, 2016). In the low to middle-income countries, the prevalence of diabetes mellitus is approximately at 7.2% (Saeedi et al., 2019) and 6.0% in sub-Saharan Africa (Zimmermann et al., 2018). This high prevalence of T2D in this region is attributed to changes in lifestyle factors such as decreased physical activity which promotes obesity and increased intake of unhealthy foods, particularly those rich in carbohydrates (Li et al., 2012). These changes are associated with the rapid modernisation and urbanisation in these regions (Li et al., 2012). Notably, this high incidence of diabetes mellitus may be alleviated through lifestyle changes including eating healthy food and exercising regularly (Y. Zheng et al., 2018). Astoundingly, over 90% of diabetic cases in sub-Saharan Africa are type 2 diabetes (T2D) (Tripathi B & Srivastava A, 2006), and the remaining cases are type 1 diabetes (Pastakia et al., 2017). In Namibia, the prevalence of T2D is estimated to be at 5.1% and is amongst the top eight causes of death in the country (Adekanmbi et al., 2019).

Type 2 diabetes (T2D) is a systemic-low grade inflammatory condition that is associated with an increase in blood glucose levels due to defects in insulin secretion, resistance, or both (Shersten et al., 2007). The hyperglycaemia observed in T2D is partially attributed to the increased intake of carbohydrates rich food, which leads to an increase in glucose production from the liver and a decrease in its uptake from the muscles (Sheard et al., 2004). This type is most common in people of middle adulthood, hence it is also referred to as an adult-onset condition (Kalin et al., 2017). Patients with this disorder display symptoms such as insulin resistance and increased abdominal adiposity, which worsens their glucose control and puts them at high risk of developing T2D-associated complications such as cardiovascular disease (CVD) and chronic immune activation ref (Guthrie & Guthrie, 2004). The resulting pro-inflammatory state may alter the synthesis and action of important regulatory proteins involved in iron haemostasis. This thesis aimed at assessing iron metabolism and cardiovascular risk in patients with T2D.

2.2 Definition and classification of Diabetes mellitus

Diabetes mellitus is a cluster of disorders that are associated with hyperglycaemia caused by the inability or failure of the pancreas to produce insulin due to defects of insulin secretion, action, or both (Shouhip, 2005). Insulin is a hormone that is produced by the beta cells of Langerhans in the pancreas and regulates blood glucose levels (Patil et al., 2017). There are four main types of diabetes, namely pre-diabetes, type 1 diabetes, gestational diabetes, and type 2 diabetes (N. Holman et al., 2015).

2.2.1 Pre diabetes

Pre-diabetes or intermediate diabetes refers to individuals whose blood glucose levels are higher than the reference ranges, yet not high enough to be considered as clinical diabetes (Beulens et al., 2019). Pre diabetes is diagnosed based on the following criteria, glycated haemoglobin (Hb1Ac) of 5.7 to 6.4%, or impaired fasting blood glucose levels that falls within the range of 5.6 mmol/L (100 mg/dL) to 6.9 mmol/L (125 mg/dL) or a 2 hours plasma glucose level that is between 7.8 mmol/L (140 mg/dL) and 11.0 mmol/L (199 mg/dL) (Perreault & Færch, 2014) (ADA, 2010) (Table 2.1). Notably, approximately 5-10% of pre-diabetes patients progress to T2D annually (Tabák et al., 2012).

Table 2.1 Diagnosis criteria of pre-diabetes (compiled by the author based on work by the American Diabetes Association ADA, 2010).

1. Glycated haemoglobin (HbA1c) of 5.7 to 6.4%.
or
2. Impaired fasting glucose (IFG) of 5.6 – 6.9 mmol/L (100 – 125mg/dl)
or
3. 2-hours plasma glucose of 7.8 to 11.0 mmol/L (140 - 199 mg/dL) (impaired glucose tolerance)

2.2.2 Gestational diabetes

This is a less threatening diabetes that is usually diagnosed and present during pregnancy (Plows et al., 2018). However, in most cases affected mothers and their children might be at higher risk of developing T2D in the future (Dunne, 2004). In fact, this type of diabetes affects the new born compared to their mothers by increasing their weight and putting them at high risk of developing obesity and insulin resistance in the near future (Szmuilowicz et al., 2019).

2.2.3 Type 1 diabetes mellitus

Type 1 diabetes is an insulin-dependent autoimmune disorder that is caused by auto antibodies that attacks the beta cells in the pancreases. The destruction of beta cells results in insulin deficiency and poor glucose control (Acheson, 2012). This condition is most common in children although it can develop at any age. This type of diabetes requires regular lifetime insulin injections in order to lower the blood glucose levels and promote its absorption into cells hyperglycaemia (Linda A et al., 2019). Although the above-mentioned different types of diabetes are also important, this review will primarily focus on the most common type of diabetes (T2D).

2.2.4 Type 2 diabetes

Type 2 diabetes (T2D) accounts for approximately 90% cases of all diabetes (IDF, 2019). Briefly, this disorder is characterised by hyperglycaemia, insulin resistance and low-grade inflammation (ADA, 2010; Kalin et al., 2017) Patients with T2D presents with symptoms such as polyphagia, polyuria, polydipsia, and sometimes blurred vision (Kazi & Blonde, 2001). The diagnosis of T2D is based on apparent poor glucose control and the ADA diagnosis criteria for T2D is shown in Table 2.2 (ADA, 2010). Briefly, a definitive diagnosis is made when the patient present with glycated haemoglobin (Hb1AC) of ≥ 6.5%, fasting plasma glucose (FPG) of ≥7.0 mmol/l (126 mg dl/1), or an oral glucose tolerance test of ≥ 11.1 mmol l/1 (200 mg/dl) based on 2-hour plasma glucose or random glucose of >11.1 mmol/l (200 mg/dl) or higher in patients with classical symptoms of hyperglycaemia (ADA, 2010).

Table 2.2 Diagnosis criteria of type 2 diabetes (compiled by the author based on work by the American Diabetes Association ADA, 2010).

1. Glycated haemoglobin (HbA1c) ≥ 6.5%.
or
2. Fasting plasma glucose (FPG) ≥ 7.0 mmol/l (126 mg/dl). Fasting is defined as no caloric intake for at least 8 hours.
or
3. 2-hours plasma glucose of ≥ 11.1 mmol/l (200 mg/dl) during an oral glucose tolerance test (OGTT).
or
4. A random glucose of 11.1 mmol/l %200 mg/dl) or higher in patients with classical symptoms of hyperglycaemia

2.3 Pathogenesis of T2D

It is now established that T2D is a low-grade chronic inflammatory condition characterised with either insulin resistance, insulin deficiency, or both (Hameed et al., 2015; Petersmann et al., 2019). Glucose is the main source of energy supply in the body (Fernandez-Real et al., 2002). In normal glucose metabolism, plasma glucose levels remain constant, despite changes in demand and supply. For instance, when the blood glucose level rises the pancreas secrets insulin into the bloodstream, which will then lower the blood glucose levels (Villines & Carter, 2019). Conversely, the decrease in plasma glucose levels stimulates the pancreas to release glucagon, in order for the liver to convert glycogen into glucose for usage. However, in T2D this mechanism is altered, leading to an increase in fasting plasma insulin levels, and glucose and thereafter, resulting in insulin resistance and a failure in insulin secretion to compensate for insulin deficiency resulting in hyperglycemia (Kahn et al., 2005; Kalin et al., 2017).

Type 2 diabetes is a multifactorial disease that is caused by various factors, including genetic and environmental. Obesity driven by reduced physical activity coupled with excess calorie intake has a huge impact on the function of the beta cells as well as its associated tissues (Kolb & Martin, 2017). In addition, obesity is closely associated with insulin resistance mediated by the increased production of non-esterified fatty acids (NEFA) and exacerbated secretion of pro-inflammatory cytokines. Collectively, these eventualities lead to impaired glucose uptake in the liver and muscles (Scheen, 2003) In addition, insulin resistance as a result of hyperglycaemia can cause both structural and functional damage of the beta cells in the pancreas, leading to, excess secretion of free fatty acids and glucose in the liver, pancreas, and muscles, which is implicated in the pathogenesis of T2D (Sivitz, 2001), Figure 2.1.

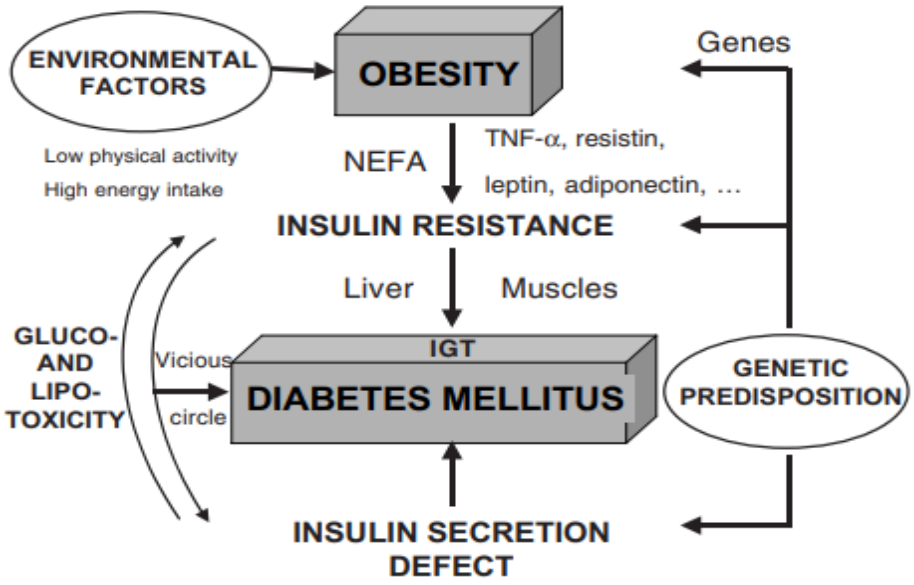

Figure 2.1 Etiology and pathogenesis of type 2 diabetes. (Adapted from Scheen, 2003). Type 2 diabetes is a multifactorial condition that is caused by both genetic and environmental factors, that leads to defects of both insulin secretion and resistance resulting in hyperglycemia. Obesity, the major cause of insulin resistance is associated with increase secretion of excess non-esterified free fatty acids and pro-inflammatory makers, such as tumor necrosis factor. This leads to the decrease in glucose uptake in the liver and muscles resulting in impaired insulin resistance and excess glucose that developed into diabetes mellitus due to defects of insulin secretion.

As previously described, T2D is a chronic inflammatory condition that is closely associated with obesity a major risk factor of insulin resistance in T2D due to altered carbohydrates and fat metabolism (Al-Goblan et al., 2014). In fact, previous studies have found a link between obesity, adipose tissue, and T2D. Whereby, obesity as a result of excess intake of carbohydrates rich food is associated with increased production of fatty acids due to lipid oxidation, which causes excessive growth of adipose tissue as a compensatory mechanism for excess fatty acids production in the muscles (Arya M. Sharma & Lau, 2013). This promotes hyperglycaemia as excess fatty acids will be used for energy production instead of glucose (Greenberg & McDaniel, 2002).

Type 2 diabetes as a chronic inflammatory condition is associated with insulin resistance and an increase in the production of pro-inflammatory cytokines such as C-reactive protein (CRP), interleukin 6, and tumor necrosis factor (TNF-α) (Giorgino et al., 2005). In addition, excess nutrients intake results in hypertrophy of the adipose tissue, which displays both pro-inflammatory (M1) and anti-inflammatory properties (M2) (Calle & Fernandez, 2012). The relationship between inflammation, obesity and insulin resistance have been demonstrated previously in both human and animal models

(Dandona et al., 2004), for instance, a study done in obese mouse found increased levels of TNF-α in their muscles, however, attempts to reduce these levels resulted in insulin resistance (Hotamisligil et al., 1993). In fact, human studies done in obese patients with T2D showed that the increase in adipose tissue is responsible for the excess secretion of TNF-α in the plasma (Tilg & Moschen, 2008), and weight loss was associated with decreased levels of TNF-α (Dandona et al., 1998). Long-term macrovascular complications of T2D are associated with atherosclerosis. Atherosclerosis is caused by the dysregulation of lipids metabolism, which results in endothelial dysfunction due to deposition of fat and narrowing of arteries, a major cause of CVD and hypertension in T2D (Peter Libby, 2002), figure 2.2.

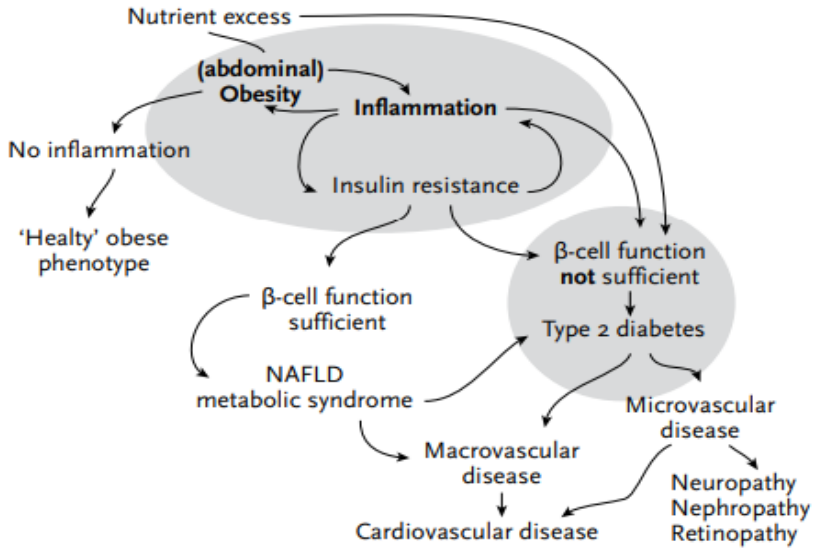

Fig 2.2 The association between obesity and type 2 diabetes. (Adapted from Greevenbroek et al., 2013). Excess intake of nutrients leads to fat accumulation that is associated with inflammation and insulin resistance due to secretion of pro-inflammatory cytokines, both insulin resistance and insufficient secretion of insulin by beta cells causes type 2 diabetes, which on a long term progression is associated with both microvascular and macrovascular complications (van Greevenbroek et al., 2013).

In addition, alteration of glucose metabolism in T2D is also partially attributed to the dysregulation of insulin signaling via the protein kinase B (PKB) pathway and Ras-mitogen-activated protein kinase (MAPK) pathway (Taniguchi et al., 2006). Insulin also plays a role in the muscles and liver, where it promotes the uptake and expression of glucose, respectively (Singla, 2010; Taniguchi et al., 2006). Dysregulation of these pathways can results in insulin resistance as a consequence of hyperglycemia, which in turn may lead to inflammation and eventually activation of the immune response (Berbudi et al., 2019).

2.4 Immune response in T2D

An immune response is the ability of the body to protect itself by recognising foreign antigens (Chaplin, 2010). The hyperglycaemia present in patients with T2D modulate chronic inflammation and activation of the immune response which is associated with increased acute phase reactants such as C-reactive protein (CRP), and white cell counts (WCC) (Dokken, 2008; Farhangi et al., 2013; Mohit. et al., 2017). Even though previous studies have demonstrated a link between increased CRP and WCC in T2D (R. R. Holman et al., 2008), contradictory findings have been reported by others (Barzilay et al., 2001; Chun et al., 2010; Dehghan et al., 2007; Freeman et al., 2002). Whereby, increased levels of WCC and CRP were associated with T2D irrespective of any underlying conditions (Barzilay et al., 2001; Dehghan et al., 2007), whilst others have described their levels to be only associated with obesity (Chun et al., 2010; Freeman et al., 2002). In addition to WCC and CRP, globulins are also acute phase proteins that are synthesised in an inflammatory response (Muhammad et al., 2016; Sproston & Ashworth, 2018). Their increased synthesis is associated with elevated ESR levels as they sit on RBC and increase the cell density (Alende-Castro et al., 2019; S. Guo et al., 2020). As expected, elevated globulins and ESR has been reported in patients with T2D. Hence both are widely accepted as general markers of inflammation. Therefore, these acute phase reactants and associated tests such as ESR may be used as reliable markers to stratify the degree of inflammation in patients with T2D and monitor disease progression and prognosis.

Obesity is a major risk factor for T2D that is associated with hyperinsulinemia and insulin resistance (Tataranni, 2003). Obesity induces an exacerbated release of pro-inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor (TNF-α), and active reactants such as (CRP) and ferritin (Roytblat et al., 2000; W. Wang et al., 2011). The elevated release of IL-6 in obesity modulates iron metabolism by promoting the synthesis and release of hepcidin, an important regulator of iron metabolism (Zhu et al., 2019). Thus, suggesting that inflammation and obesity are some of the underlying factors that influence iron metabolism. However, the exact mechanisms behind this are not fully understood.

Oxidative stress is involved in vascular injuries as well as the pathogenesis of T2D (Folli et al., 2012). Oxidative stress which is caused by increased production of reactive oxygen species (ROS) is triggered by hyperglycemia via several pathways in T2D. Some of these pathways include glucose oxidation, hexosamine, protein kinase C, polyol, and advanced glycation end-product (AGE) pathways (Bikkad et al., 2014). In T2D, poor glycaemic control and hyperglycemia in general cause an increase in ROS as well as the production of glucose from the polyol pathways (Ighodaro, 2018). Figure 2.3

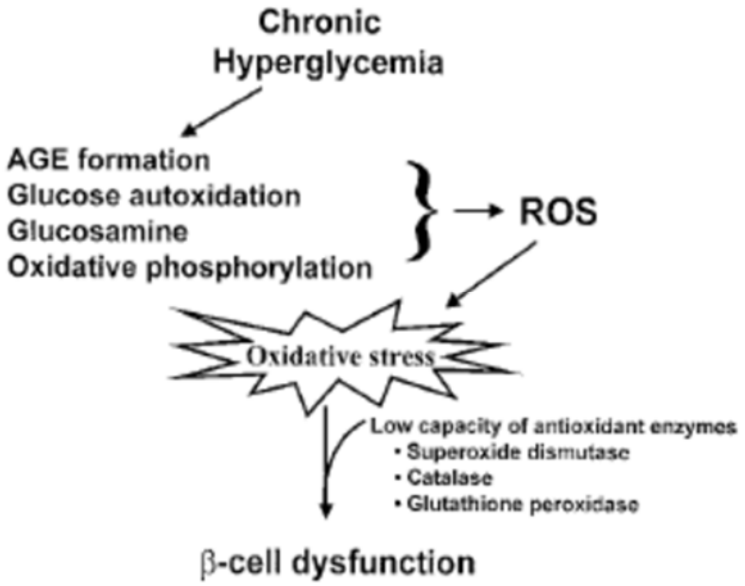

Figure 2.3. Effects of hyperglycemia on oxidative stress (Adapted from Jebur et al., 2016). Poor glycaemic control leads to the activation of AGE and its associate pathways, which results in the accumulation of reactive oxygen species and eventually oxidative stress. This continuous release of oxidative stress causes damage to the beta cells in the pancreas which eventually leads to its dysfunction (Jebur et al., 2016).

Management of T2D requires proper treatment in order to reduce the risk of CVD and achieve proper glycaemic control (Sanchez-Rangel & Inzucchi, 2017). Metformin is one of the most commonly prescribed anti-diabetic drugs for T2D. This drug lowers blood glucose levels by increasing the uptake of glucose from the muscles and decreasing glucose release from the liver (Correia et al., 2008). In addition, metformin controls blood glucose levels by inhibiting lipolysis in the adipose tissue as well as gluconeogenesis in the liver (Rojas L & Gomes M, 2013). Metformin also reduces insulin resistance and is not associated with hypoglycaemia events. This drug is suitable for usage in both lean and obese patients as it controls weight loss (Hollander, 2007). Importantly, metformin protects against CVD (Nathan et al., 2006; Papanas et al., 2009), however, its long-term usage is known to cause an increase in hepcidin levels coupled with a decrease in total serum iron levels (Ahmed et al., 2015).

Insulin therapy is another treatment that is used in elderly patients with T2D, however, this drug is associated with hypoglycaemic events as well as weight gain (Petznick et al., 2011). In addition, insulin therapy is directly associated with the expression of hepcidin (H. Wang et al., 2014). Interestingly, insulin treatments do not offer cardio protection in patients with T2D (Muis et al., 2005). Therefore, treatment strategies in T2D may contribute to the dysregulation of iron metabolism. In this chapter,

the physiology of iron metabolism and how its dysregulation contributes to the pathogenesis and manifestation of iron-related syndromes in T2D will be discussed. In addition, this chapter will discuss the relationships between obesity, inflammation, and abnormal lipid metabolism in T2D.

2.5 Iron metabolism

Iron plays a pivotal role in immunity, erythropoiesis, and glucose control (Fernandez-Real et al., 2002). Approximately 2mg of iron is absorbed daily from the diet by the duodenal enterocytes in the small intestine via the divalent metal transporter 1 (DMT1) (Winter et al., 2014) About 1-2 mg of iron is lost every day through minor bleedings and the skin. However, this loss is compensated by intestinal absorption (Ganz, 2012).

Iron is transported in the body bound to transferrin, by transferrin-receptors before it is distributed to the bone marrow for erythropoiesis, and to the cytoplasm for absorption via intestinal divalent metal transporter 1 (Hentze et al., 2010). Iron is also found in the red cells bound to haeme and is salvaged from the red cells after they reached their lifespan or get damaged, by macrophages in the spleen and liver through phagocytosis. Thereafter, the iron is recycled back to the plasma by transferrin when the need arises and when in excess, the iron is stored by ferritin (Beaumont & Delaby, 2009) (Figure2.1). The regulation of iron is important in preventing different anaemias that are associated with iron disorders such as iron overload and iron deficiencies (Kali et al., 2015).

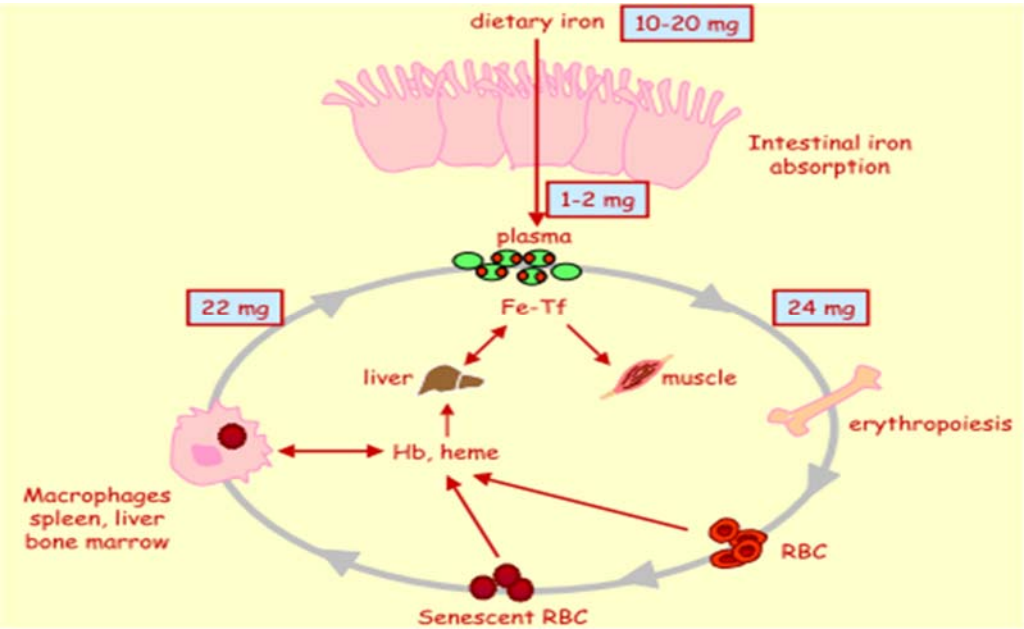

Figure 2.4. An overview of iron metabolism in the body. After dietary intake of iron, iron is absorbed from the small intestines via the enterocytes and transported to plasma bound to transferrin, by transferrin-receptors. These transferrin receptors then distribute the iron to the liver and muscles for usage, some of the iron will get stored in the macrophages after getting recycled through the process of degradation of old and senescent red blood cells that release haeme. The free iron is re-used for the production of new red blood cells in the bone marrow through a process called erythropoiesis. Tf (transferrin) and RBC (red blood cells) (Adapted from Beaumont et al., 2009).

Iron metabolism is mainly regulated by hepcidin, a peptide hormone coded by the gene HAMP that is synthesised in the liver, (Atyia, Gawaly, Enaam, EL-BAR, et al., 2018; Suárez-Ortegón et al., 2015). Notably, the hormone is secreted in response to inflammation, iron status, and erythropoiesis (D'Angelo, 2013; Vela et al., 2018a). Excess iron and inflammation stimulate hepcidin production, which in turn causes internalisation and degradation of ferroportin in the enterocytes and macrophages, thus reducing plasma iron levels. Conversely, hepcidin is suppressed in iron deficiency, to allow more iron to be absorbed from the intestines and increase its uptake from the macrophages (Bobby J. Cherayil, 2016; Nemeth & Ganz, 2009).

2.5.1 The regulation of iron metabolism

Hepcidin is the major regulator of iron metabolism that is synthesised in the liver (Atyia, Gawaly, Enaam, EL-BAR, et al., 2018). It regulates plasma iron through its action on ferroportin, an iron exporter protein that is located in the basolateral membrane of duodenum enterocytes in the small intestines (Sara et al., 2010). Elevated plasma iron levels induce the synthesis of hepcidin through the

signaling of bone morphogenetic protein 6 (BMP-6) and IL-6 activate the Janus kinase/signal transducer the activator of transcription 3 (JAK/STAT3) pathways (Nemeth, Rivera, et al., 2004; Vela et al., 2018). Once synthesised, hepcidin binds to the hemojuvelin (HJV) and activates the mothers against decapentaplegic homolog (SMAD)-signaling pathway that regulated immune responses and iron haemostasis (Ganz & Nemeth, 2012).

In inflammatory conditions such as T2D, the transcription and expression of hepcidin is increased, thus resulting in decreased plasma iron levels (Babitt & Herbert Y. Lin, 2010). This is due to the degradation of ferroportin and reduction in iron exportation and deliveries to the plasma as well as its absorption from the macrophages (Aschemeyer et al., 2018). Interestingly, the dysregulation of hepcidin levels in T2D can result in both iron overload or iron deficiency anaemia (Jiang et al., 2011; Suárez-Ortegón et al., 2015). This altered metabolism impacts the immune system in conditions of poor glucose since iron is required for immune responses (Prentice, 2017). In that context, iron deficiency can cause alteration of the immune response since iron is involved in the proliferation and differentiation of immune cells and also in the differentiation of monocytes to macrophages where it plays a role in the process of phagocytosis (Cherayil, 2010), therefore iron deficiency can result in inadequate immune response (Hassan et al., 2016).

Conversely, during iron overload, there is excess production of free iron radicals through electron transport that leads to catalysation and formation of Reactive Oxygen Species (ROS) which causes oxidative stress (Cherayil, 2010). Oxidative stress causes chronic inflammation by keeping on producing more free radicals that trigger the immune response even when is not needed, thus the association between oxidative stress and chronic inflammation causes damage and insulin resistance that can alter the immune response (Eske, 2020).

Erythropoietin is another important hormone that tightly regulates iron metabolism through the modulation of erythropoiesis (Sara et al., 2010). The hormone promotes the transportation of iron to the bone marrow for red cell synthesis in anaemia and hypoxic conditions (Haase, 2010). The increased release of erythropoietin promotes erythropoiesis which in turn reduces hepatic hepcidin synthesis and increases the release of iron from the macrophages as well as its absorption from the intestines (Pasricha et al., 2016) (Figure 2.2). Therefore, hepcidin and erythropoietin regulate each other under normal physiology but in inflammatory conditions such as T2D, their expression is dysregulated and leads to abnormal iron profiles and related indices.

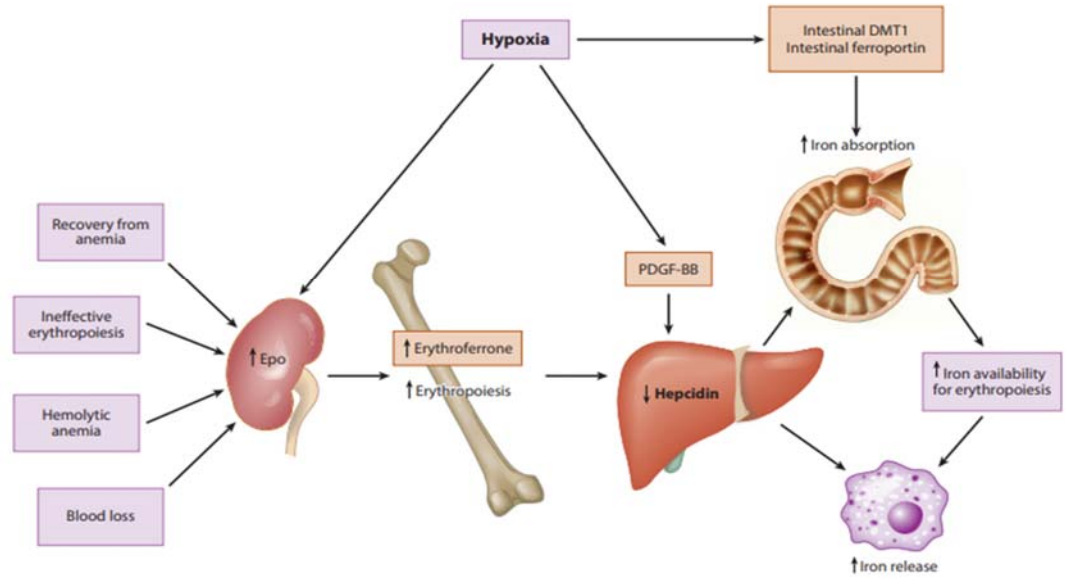

Figure 2.5 An overview of potential mechanisms involved in erythroid suppression of hepcidin (Adapted from D' Angelo., 2013). Certain conditions such as anaemia, blood loss, and hypoxia increase the secretion of erythropoietin hormone in the kidney. This results in increased production of red cells and decreases hepcidin synthesise in the liver, which results in increased release of iron from the macrophage as well as its absorption from the enterocytes by the ferroportin in the small intestine via the divalent metal transporter 1(DMT1).

2.5.2 The impact of inflammation on iron metabolism

It is apparent that inflammation increases hepcidin synthesis (D'Angelo, 2013). Inflammation causes the stimulation and activation of macrophages which release pro-inflammatory cytokines such as IL-6 and TNF-α which promotes hepcidin synthesis (Tomas, 2016). Hepcidin then inhibits the expression of ferroportin and prevents the absorption of iron from the intestines and its release from the macrophages, as a result, the levels of plasma iron reduces and hypoferremia is observed (Kemna et al., 2005).

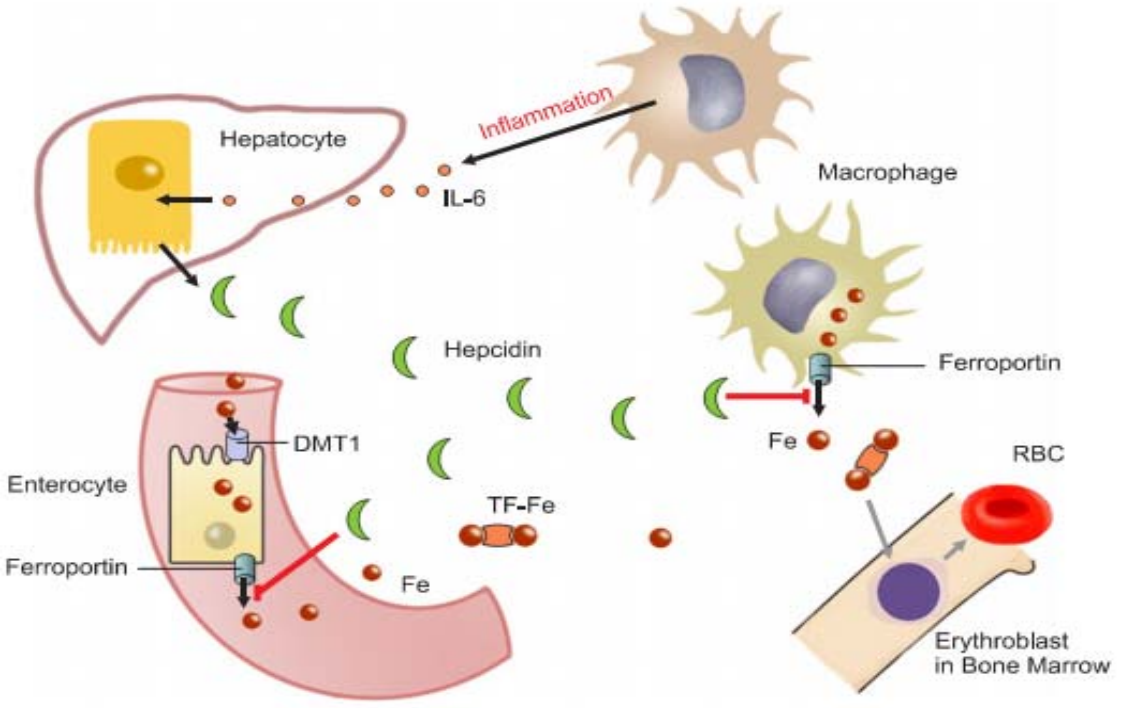

Figure 2.6 An Illustrations of how inflammation affects iron metabolism. The increase in the levels of pro-inflammatory cytokines such as interleukin (IL)-6 increases hepcidin production and expression. Hepcidin inhibits the expression of ferroportin which prevents iron absorption from the intestines and its release from the macrophages, thus, resulting in decreased iron levels. TF -transferrin; Fe- iron; DMT1 - divalent metal transporter (Adapted from D' Angelo., 2013)

Given the essential functions of iron and the importance of it's tight regulation. Failure to maintain iron homeostasis can lead to several iron-associated conditions, such as iron deficiencies and overload. Iron deficiency anaemia usually presents with microcytic and hypochromic red blood cells due to a reduction in iron production that hampers the production of red blood cells in the bone marrow (Dev & Babitt, 2017). As previously described, inflammation and secretion of IL-6 influence iron levels and cause iron deficiency by increasing hepcidin levels. Conversely, a decrease in hepcidin levels increases iron levels. Other abnormalities of iron overload include primary and secondary hereditary hemochromatosis (Barton & Acton, 2017; Dev & Babitt, 2017). These disorders are associated with the accumulation of iron in the pancreatic beta cells, which eventually leads to apoptosis and insulin resistance, a hallmark of T2D (Wallace, 2019). Therefore, proper regulation of iron is crucial, as it prevents different diseases associated with dysregulated iron metabolism.

2.5.3 Iron metabolism in T2D

Altered iron metabolism in T2D is associated with different types of anaemias, particularly iron deficiency (AlDallal & Jena, 2018; Barbieri et al., 2015) and iron overload (Altamura et al., 2017; Zheng et al., 2011) anaemia.. However, the mechanisms behind this are still not well understood. Iron deficiency anaemia usually present with microcytic and hypochromic red blood cells (RBCs) that is associated with decreased total serum iron levels, red blood cell count, haemoglobin, and the mean corpuscular volume (MCV) (AlDallal & Jena, 2018; Soliman et al., 2017). This anaemia is diagnosed based on a haemoglobin level of < 12g/dl in women or <13g/dl in men, coupled with an MCV of <87fl in both genders (Adejumo et al., 2012).

On the contrary, iron overload present with macrocytic and hyperchromic RBCs is associated with excessive iron stores, increased transferrin saturation, and red cell indices (Barton et al., 2000). Failure of the body to get rid of excess iron may cause its deposition in different organs such as the pancreas and eyes as seen in hereditary hemochromatosis, thalassemia, or in patients who receive multiple blood transfusions (Fiorelli, 2007).

Interestingly, patients with T2D may also present with anaemia of chronic disease (ACD) which is usually characterised by normocytic RBCs and an underlying inflammation (Elizabeta & Tomas, 2014). This is notably the second commonest anaemia worldwide after iron deficiency anaemia (Camaschella & Girelli, 2020; Elizabeta & Tomas, 2014). This anaemia is associated with reduced haemoglobin, RBCs, and haematocrit levels (Camaschella & Girelli, 2020). Increased levels of the pro-inflammatory cytokines IL6, hepcidin, and chronic diseases such as T2D block the synthesis of erythropoietin and shortens the life span of immature red cells through iron restricted erythropoiesis (Elizabeta & Tomas, 2014; Fraenkel, 2017).

Previous studies have found an association between poor glucose control and iron profiles (iron, ferritin, transferrin) (Andrews et al., 2015; Atyia, Gawaly, Enaam, EL-BAR, et al., 2018; Fernandez-Real et al., 2002; Vela et al., 2018). Ferritin is the main storage of iron that reflects the body's iron store (Saha & Murgod, 2019). Previous studies have indicated that low ferritin levels are associated with iron deficiency anaemia (Suárez-Ortegón et al., 2015), whilst its increase with iron overload (Atyia, Gawaly, Enaam, EL-BAR, et al., 2018; Saha & Murgod, 2019). However, both decreased (Suárez-Ortegón et al., 2015) and increased (Altamura et al., 2017; Andrews et al., 2015; Vela et al., 2017) ferritin levels were reported in patients with T2D.

Plasma iron levels are used to determine the amount of circulation iron in the body (Nagarajrao & Alharbi, 2015; S., 2015). Previous studies have found serum iron to be decreased in iron deficiency anaemia and increased in iron overload (Goodnough et al., 2010; Miller & Tanno, 2010). However, in T2D both decreased in iron levels (Manikandan et al., 2015) and increased in the levels of iron (Altamura et al., 2017; Nagarajrao & Alharbi, 2015) have been reported. Transferrin, the main iron transporter in the body, and its receptor (transferrin receptor) are influenced by the availability of iron in the body (Gkouvatsos et al., 2012). Thus in iron deficiency anaemia, their levels are expected to be elevated due to lack of available iron and vice versa in iron overload (Saha & Murgod, 2019). However, in patients with T2D, both decrease (Jiang et al., 2011; Shalitin et al., 2018) and increase (Suárez-Ortegón et al., 2015) levels of transferrin and transferrin receptor have been reported.

Therefore, these variations need to be assessed further since these different relationships suggest a dysregulation of iron metabolism in T2D. However, it remains to be investigated whether the underlying factors such as obesity, inflammation, and dyslipidaemia in T2D play a huge role in iron metabolism in T2D.

2.6 Dyslipidaemia and cardiovascular risk in T2D

Obesity is associated with an increased risk of developing cardiovascular disease (CVD) (Klop et al., 2013). Notably, this risk is closely linked with altered lipid metabolism, particularly in obesity and T2D (Feingold KR & Grunfeld C, 2018; Klop et al., 2013). In obesity, the increased visceral adipose tissue secretes increased levels of free fatty acids (FFA) and pro-inflammatory cytokines which promotes insulin resistance (Klop et al., 2013).

Insulin resistance coupled with hyperglycemia, dyslipidaemia , and CVD is closely associated with Non-alcoholic fatty liver disease (NAFLD) and T2D (Hazlehurst et al., 2016), and approximately 70% of T2D presents with NAFLD (Targher et al., 2007) Figure 2.6. In normal insulin metabolism, insulin signaling regulates glucose and lipid metabolisms uptake in the skeletal muscles and adipocytes, insulin is also involved in the conversion of glycogen to glucose, and in the suppression of lipolysis, when adequate levels of glucose are present (Ormazabal et al., 2018). However, in T2D, these functions are altered due to the dysregulation of insulin signaling via the protein kinase B (PKB) pathway and Ras-mitogen-activated protein kinase (MAPK) pathway (Taniguchi et al., 2006), which lead to excess secretion of insulin and impair lipolysis in the adipose tissue, thus increasing the release of free fatty acids, which are transported into the liver, where they stimulate the secretion of very low-density lipoprotein

(VLDL) and cause an increased in the levels of triglycerides which is closely associated with dyslipidaemia in T2D (Wilcox, 2005).

Dyslipidaemia, characterised by decreased High-density lipoprotein cholesterol (HDL-C) coupled with increased levels of triglycerides, total cholesterol, and Low-density lipoprotein cholesterol (LDL-C) promotes endothelial dysfunction and atherosclerosis, which are among the major risk factors of CVD such as myocardia infraction (Matheus et al., 2013). In addition to dyslipidaemia, CVD is also associated with obesity, a key risk factor in insulin resistance (Hedayatnia et al., 2020; Sadeghi et al., 2017). The increased deposit of LDL-C in the arterial wall of individuals with obesity can activate the process of atherosclerosis in cases of macrophages failure to clear chylomicrons remnants from the subendothelial space (Khatana et al., 2020). Atherosclerosis is a chronic inflammatory condition that is caused by an increase in the accumulation of lipids in the arterial wall, due to dysfunctional endothelial which causes an increase in the formation of plagues, that can activate the thrombotic cascade once ruptured and is associated with altered lipids metabolism (Katakami, 2018; Poznyak et al., 2020).

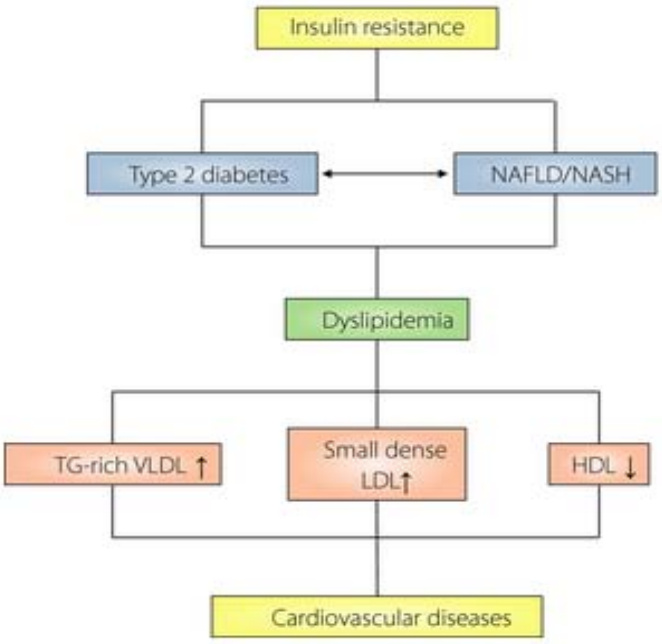

Figure 2.7 Dyslipidaemia in type 2 diabetes (Adapted from Matsuzaka & Simano., 2020). Insulin resistance which is associated with type 2 diabetes and non-alcoholic fatty liver disease, stimulates excess production of triglycerides in the liver as well as the development of non-alcoholic fatty liver disease. Dyslipidaemia is associated with increased levels of triglycerides and low-density lipoprotein coupled with low levels of high-density lipoprotein cholesterol. In addition, the increase in the levels of low-density lipoprotein increases the risk of cardiovascular disease in type 2 diabetes(Matsuzaka & Shimano, 2020)

Altered lipids metabolism is associated with the increase in serum triglycerides caused by, an increased in the hepatic production of VLDL levels as well as a decreased in its clearance from the liver by lipoprotein (Bays et al., 2013; Feingold KR & Grunfeld C, 2018). In this condition, the levels of HDL-C are usually decreased whilst the HDL/cholesterol ratio is increased (Bays et al., 2013; Grundy, 2004; Klop et al., 2013). Therefore, the increase in the levels of LDL-C and HDL/cholesterol ratio is associated with increased events of CVD, thus they can be used to predict CVD events in T2D (Gimeno-Orna et al., 2005). Cholesterol ester transfer protein (CETP) plays an important role in dyslipidaemia, by promoting the transfer of HDL-C to VLDL and LDL-C, and its deficiency is associated with increased levels of HDL-C and decreased levels of LDL-C (Barter et al., 2003).

The relationship between lipids and iron metabolism has been described by others, whereby iron deficiency anaemia was reported to be associated with altered lipid metabolism (Antappanavar et al., 2014; Shirvani et al., 2017). However, studies have found inconsistent results, for instance, a study done in girls with severe iron deficiency anaemia, reported decreased levels of triglycerides and cholesterol which were corrected with iron therapy, and this study further found associations between MCV and cholesterol (Jong Weon Choi et al., 2001), which was in agreement with the correlation study done in adults between iron deficiency and lipid profiles (Antappanavar et al., 2014). Also, another study done in anaemia patients demonstrated a decrease in lipids profile (cholesterol, triglycerides, HDL-C, and LDL-C) among anaemic patients in comparison to non-anaemic individuals, with MCV showing an association between cholesterol and LDL-C (Shirvani et al., 2017). Moreover, others found positive associations between serum ferritin levels and lipids in patients with T2D (Yu et al., 2020).

In conclusion, inflammation and altered lipid metabolism are persistent in patients with T2D. Most importantly, the inflammatory state seems to modulate the altered iron metabolism in patients with T2D. Thus, a deep understanding of the mechanisms and factors that modulate the dysregulated iron metabolism in poor glucose control may pave way for therapeutic interventions to control this anomaly in these patients. Moreover, cardiovascular risk stratification in T2D will be important in reducing the incidences of thrombotic events in these patients. Therefore, this study aimed at assessing iron and lipid profiles in patients with T2D.

Chapter Three: Methods

3.1. Study design, setting, populating, and sampling

This was a descriptive observational study involving clinically known outpatients with type 2 diabetes (T2D) who visited an urban health center clinic from September 2020 to December 2020. The participants were recruited from Katutura Health Centre clinic, Windhoek, Namibia. The study setting was chosen based on Namibia's mostly populated urban city. The study randomly recruited a total of one hundred and fifteen adult patients (n=115) of both genders. All of the patients were confirmed cases of T2D diagnosed by clinicians based on the American Diabetes Association guidelines (ADA, 2020). The ethical clearance for this study was sought from the Namibia University of Science and Technology (NUST) Research Ethics committee, reference number (FHAS 1/2020) (see Appendix 1), the Ministry of Health and Social Services (17/3/3 MN) (see Appendix 2) and from Namibia Institute of Pathology (NIP) ethics committee (see Appendix 3). The study was conducted based on the Code of Ethics for the World Medical Association (Declaration of Helsinki 2013). Participation was voluntary and informed consent was sought from all participants prior to taking part in the study. Patients' confidentiality was maintained by using a computer-generated number instead of their names for identification.

3.1.1 Inclusion criteria

The study included adult patients (>18 years) from both genders, who have agreed to partake in the study with clinically known and diagnosed cases of T2D based on ADA diagnosis criteria that were previously confirmed by registered medical doctors. All participants fasted overnight prior to the collection of blood samples.

3.1.2 Exclusion criteria

This study excluded adult individuals without diagnosed cases of T2D or patients under the age of 18 years old. Patients with known conditions that alter iron metabolisms such as pregnancy, hemochromatosis, and thalassaemia were also excluded.

3.1.3 Sample size

The sample size was calculated based on the prevalence rate of T2D in Namibia, which was 5.1% (Adekanmbi et al., 2019) and the formula was adopted from (Alemseged et al., 2015).

$$n = \left(\frac{(Z)^2 \times p(1-p)}{d^2}\right)$$

$$n = \left(\frac{(1.96)^2 \times 0.051(1-0.051)}{0.05^2}\right)$$

$$n = \left(\frac{3.8416 \times 0.051(0.949)}{0.0025}\right)$$

n = 74.371

Where: n= Number of patients to be recruited.

Z= Value of 95% confidence interval (1.96)

P= Proportion of patients with T2D (5.1%) = 0.051

d= Absolute sampling error (margin of error) that can be tolerated (5%) = 0.05

Therefore, the overall minimum sample size required was **74 patients**. In order to carter for any unforeseen patient loss or voluntary withdrawal from the study, an additional 41 patients were recruited, bringing the sample size to a total of one hundred and fifteen (n=115).

3.1.4 Sample collection

Fasting blood samples for haematological and biochemical tests were collected in three different types of tubes, namely, ethylenediamine tetra acetic acid (EDTA), sodium fluoride, and serum separator tube (SST) by qualified registered/ enrolled nurses. Blood drawn in the EDTA tube (4ml) was used for haematological parameters, erythrocytes sedimentation rate (ESR), and glycated haemoglobin (HbA1C%) analysis, whilst in the sodium fluoride tube (4ml) was used for fasting plasma glucose (FPG) and the SST tube (5ml) was used for C- reactive protein (CRP), iron profiles (iron, transferrin, and ferritin) and lipids profiles (cholesterol, triglycerides, high-density lipo-protein cholesterol (HDL)-C, low-density lipoprotein cholesterol (LDL)-C and high-density lipoprotein/ cholesterol ratio (HDL/Chol ratio). Samples were transported to the laboratory within the time frame of 2-3 hrs. A temperature of 2-8 °C was used and analysis was performed within 3hrs of collection. The SST tubes and sodium fluoride tubes were spun at 3000rpm for 10 minutes before analysis in order to separate serum from the red blood cells.

3.2 Clinical Measurement

Clinical parameters were measured by professional nurses and included blood pressure, height, and weight. The other parameters such as age and disease duration were extracted from the patients' clinical report cards whilst the body mass index (BMI) was calculated as follows:

BMI = $\frac{Body\ mass\ (Kg)}{height\ m2}$(Murguía-Romero et al., 2012)

3.2.1 Laboratory measurements

All laboratory measurements including glucose, inflammatory, haematological, iron, and lipid profiles were analysed and measured by the researcher at an ISO 15189 of 2012 accredited medical laboratory (Namibia Institute of Pathology, Windhoek, Namibia). The measured parameters and the methods of testing used are indicated in Table 3.1. Calibrations and quality control were performed on all instruments before sample analysis in accordance with the laboratory's standard operating procedures and manufacture specifications.

Table 3.1 Biochemical and haematological measured parameters

Parameter	Testing method used	Instrument used
Plasma glucose (mmol/L)	Photometric	Cobas c501 analyser (Roche, Basel, Switzerland)
Glycated haemoglobin (%)	Latex agglutination inhibition reaction	Cobas c501 analyser (Roche, Basel, Switzerland)
Iron (µmol/L)	Ferene method	Alinity c analyser (Abbot, Illinois, USA)
Transferrin (g/L)	Immunoturbidimetric	Alinity c analyser (Abbot, Illinois, USA)
Ferritin (ng/mL)	Chemiluminescent microparticle immunoassay (CMIA)	Alinity i analyser (Abbot, Illinois, USA)
Cholesterol (mmol/L)	Enzymatic	Alinity c analyser (Abbot, Illinois, USA)
Triglycerides (mmol/L)	Glycerol Phosphate Oxidase	Alinity c analyser (Abbot, Illinois, USA)
High density-lipoprotein (mmol/L)	Accelerator Selective Detergent with cholesterol oxidase (CO) with non-HDL unesterified cholesterol	Alinity c analyser (Abbot, Illinois, USA)
Low density-lipoprotein (mmol/L)	chromogenic coupler	Alinity c analyser (Abbot, Illinois, USA)
C-reactive protein (mg/L)	Turbidimetric/Immunoturbidimetric	Alinity c analyser (Abbot, Illinois, USA)
Complete blood count	Impedence	Sysmex 1000 XN automated hematology analyzer (Sysmex Corporation, Kobe, Japan).
Erythrocytes sedimentation rate mm/hr	Photometric capillary stopped flow kinetic analysis	Test 1 THL Alifax S.p.A (Alifax, Udine, Italy),

3.3 Statistical Analysis

Normality testing for data distribution was performed using D'Agostino & Pearson test and all data were expressed as either mean ± SD or median and interquartile range [IQR] based on their distributions. The two-tailed independent student's t-test was used to perform analysis of parametric data and in cases of unequal variance, a Welch's correction test was performed. The non-parametric

data were analysed using the Mann-Whitney *U* test and the associations between two non-parametric variables (correlation) was performed using the spearman coefficient test. Comparison amongst body weights were assessed using the analysis of variance (ANOVA) test. The Brown's Forsythe and Welch's correction test were used for parametric data and followed by the Games-Howell's multiple comparison post hoc test for all significant p values. The Kruskal-Wallis was used for non-parametric data followed by the Dunnes test post hoc analysis, to determine the significant difference between the three groups. In addition, all non parametric data were log transformed. A p-value of < 0.05 was considered statistically significant. Graph Pad Prism 8 version 8.0.2 Software, (Graph Pad Software Inc, San Diego, CA, USA) was used to perform all statistical analysis.

Chapter Four: Results

4.1 Characteristics of included studies

A total of 115 participants with type 2 diabetes (T2D) with a mean age range of 49.72 ± 12.64 years and a male to female ratio of 0.49 were included in the study. Seventy-two patients 63% had T2D-associated complications, which included hypertension (47%), retinopathy (3%), cardiovascular disease (1%), arthritis (3%), late-onset deafness (1%), swollen feet (1%), and asthma (1%). Thirty-five of 1the included patients (30%) had no complications, whilst for eight patients (7%) it was not known if they had complications. The characteristics of included patients are summarised in Table 4.1. The majority of patients were on metformin treatment only (n=70, 61 %), while others were on a combination of metformin and other glucose-lowering drugs (n= 36, 31%). A total of nine patients (8%) were on insulin treatment (Table 4.1).

Table 4.1 Characteristics of included participants (n=115)

Type of complication	Number of patients (n)	Percentage (%)
Hypertension	64	47
Retinopathy	7	3
Cardiovascular disease	1	1
Arthritis	4	3
Late onset deafness	1	1
Swollen feet	1	1
Asthma	1	1
None	35	30
Unknown	8	7
Type of treatment		
Metformin	70	61
Insulin	9	8
Metformin and insulin	17	15
Metformin and sulfonyurea	7	6
Metformin and moduretic	5	4
Metformin, insulin and sulfonyurea	2	2
Metformin and octraphane	1	1
Metformin and warfarin	1	1
Metformin and glindemycin	1	1
Glindermycin and moduretic	1	1

4.2 The effect of body weights on iron metabolism and cardiovascular risk

An analysis based on the degree of obesity was performed to investigate the effect of body weights on iron profiles, the status of inflammation, and lipid profiles. The participants were stratified into weight categories based on their body mass index (BMI) using the WHO guidelines (Sharma & Kushner, 2009). Briefly, a BMI of 18.5-24.9 was considered lean, whilst that of 25.0-29.9 as overweight and ≥

30 kg/m^2 as obese, as a result, the study included a total of twenty-eight lean patients (n=28), forty patients who were overweight (n=40) and thirty-seven patients with obesity (n=37). The study excluded 10 patients because their BMIs were not recorded, hence the total number is 105 but not 115.

4.2.1 The impact of body weights on glucose and inflammatory profiles in patients with T2D

The levels of fasting plasma glucose (FPG) and glycated haemoglobin (HbA1C%) for the included patients were measured based on body weights. Although the levels were comparable across all groups ($p>0.05$), the patients' FPG and HbA1C% levels were not within the normal reference ranges, thus indicating poor glucose control (Table 4.2). In order to assess the influence of inflammation on body weights the levels of C-reactive protein (CRP), erythrocytes sedimentation rate (ESR), and white cells count (WCC) were measured among the groups. There were no significant differences on these inflammatory profiles amongst these groups ($p>0.05$) (Table 4.2).

Table 4.2 Characteristics of included patients based on the degree of obesity

Parameter	Lean (n=28)	Overweight (n=40)	Obese (n=37)	p-value
Clinical characteristics				
SBP (mm/Hg)	126.1 ± 17.03	135.5 ± 19.51	139.3 ± 19.12	**0.0163**
DBP (mm/Hg)	82.71 ± 10.83	83.63 ± 12.80	90.49 ± 13.35	**0.0289**
Age (Yrs.)	46.93 ± 11.30	52.15 ± 13.57	49.81 ± 10.31	0.2353
BMI (kg/m2)	22.87 [21.24 – 24.42]	27.21 [26.23 – 28.33]	33.64 [31.03 – 36.20]	**<0.0001**
Duration ((Yrs.)	3.00 [1.2500 – 8.500]	5.500 [2.00 – 12.75]	3.00 [0.00 – 10.00]	0.2778
Glucose profiles				
Fasting glucose (mmol/l)	9.60 [6.58 – 14.15]	10.55 [8.03 - 13.20]	10.70 [6.80 - 13.80]	0.8023
HbA1c (%)	7.90 [6.10 – 9.90]	9.400 [7.75 – 10.50]	8.50 [7.15 – 9.70]	0.2117
Inflammatory profiles				
ESR (mm/hr)	24.00 [14.75 – 41.5]	21.50 [9.250 – 50.75]	26.00 [9.00 – 47.00]	0.9684
CRP (mg/l)	6.00 [2.750 – 10.20]	3.700 [1.875 – 8.200]	7.200 [3.800 – 11.600]	0.0627
Haematological indices				
White cell count (10^9/L)	6.18 [5.03 – 7.36]	6.36 [5.26 – 8.59]	6.45 [5.51 – 7.39]	0.5645
Red cell count (10^12/L)	4.927 ± 0.67	4.81 ± 0.49	4.92 ± 0.45	0.9995
Haemoglobin (g/dl)	14.03 ± 1.19	14.03 ± 1.65	14.24 ± 1.56	0.9762
Haematocrit (%)	43.11 ± 4.13	42.93 ± 4.87	44.25 ± 5.50	0.5063
MCV (fl)	88.90 [83.28 – 91.98]	88.60 [86.10 – 92.10]	89.00 [83.30 – 93.35]	0.9999
MCH (pg)	28.95 [27.40 – 30.73]	29.20 [28.10 – 30.40]	28.80 [27.70 – 30.65]	0.6991
Iron profiles				
Iron (umol/l)	13.20 [8.80 – 16.69]	12.60 (10.10 – 19.90)	14.70 [11.30 – 17.70]	0.3347
Ferritin (ng/ml)	191.5 ± 108.1	190.8 ± 139.0	204.9 ± 140.1	0.9080
Transferrin (g/L)	2.48 [2.30 – 2.81]	2.63 [2.29 – 2.99]	2.65 [2.32 – 3.01]	0.9762
Lipid profiles (mmol/l))				
Total cholesterol	4.45 [3.81 -5.02]	4.70 [3.80 - 5.42]	4.80[4.32 -5.31]	0.3490
Triglycerides	1.15[0.75 – 1.87]	1.445 [1.14 – 1.73]	1.76 [1.29 – 2.64]	**0.0029**
LDL-cholesterol	2.93 [2.26 – 3.23]	3.00 (2.08 – 3.65)	2.72 [2.33 – 3.61]	0.7575
HDL-cholesterol	1.05 ± 0.29	1.04 ± 0.27	1.03 ± 0.289	0.9742
HDL/Chol ratio	0.22 [0.19 – 0.30]	0.22 [0.18 - 0.27]	0.23 [0.18 – 0.25]	0.7244

T2D: type 2 diabetes; **SPB**: systolic blood pressure; **DPB**: diastolic blood pressure; BMI: body mass index; **ESR:** erythrocyte sedimentation rate; **MCH:** mean corpuscular haemoglobin; **MCHC:** mean corpuscular haemoglobin concentration; **LDL:** low-density lipoprotein; **HDL**: High-density lipoprotein. Results expressed as mean ± standard deviation and median interquartile range. **The bolded p values indicate significant p-values**.

4.2.2 The impact of obesity on iron profiles

Obesity has been reported to have an impact on iron profiles in patients with T2D (Collins et al., 2008). Therefore the levels of iron profiles based on the patients' BMIs were measured and compared. The levels of total serum iron ($K_{(2)}$ = 2.19, p = 0.3347), ferritin ($F_{(2, 94)}$ = 0.13, p=0.8802) and transferrin ($K_{(2)}$ = 0.99, p = 0.60917) were comparable across the groups (Table 4.2, Figure 4.1A-C). A further assessment of haematological indices influenced by iron metabolism also showed no significant differences amongst the groups (p>0.05) (Table 4.2, Figure 4.1D-F).

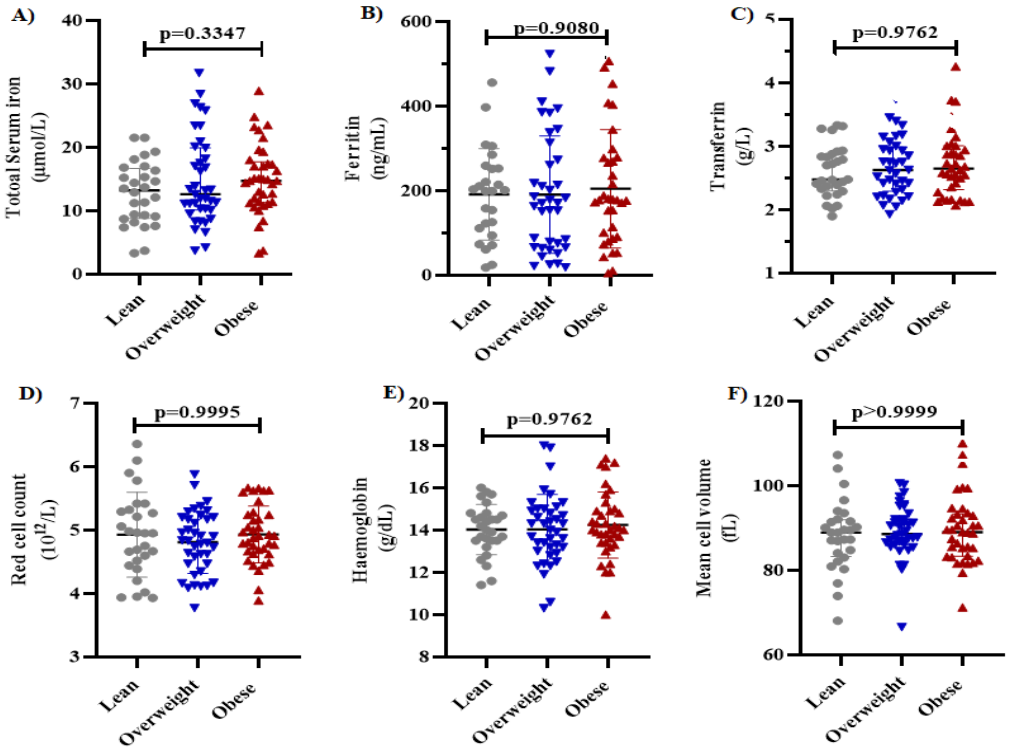

Figure 4.1 Iron profiles and haematological indices in T2D. Figure4.1 **(A-C)** shows comparable iron profiles in T2D and red cell indices influenced by iron metabolism (**D-F**) among individuals with T2D across the groups. Results are reported as mean ± SD or median and interquartile range [IQR] depending on the data distribution

4.2.3 Obesity in T2D elevates the cardiovascular risk in patients with T2D

Patients with T2D are known to be predisposed to developing cardiovascular diseases (Daniel, 2011). In order to assess this risk, blood pressures and lipograms were measured and compared amongst the different classes of body weights. The levels of triglycerides differed across the groups ($K_{(2)}$ = 10.9, p = 0.0029) (Table 4.2). The Dunn's post hoc analysis showed elevated triglyceride levels in the obese group (1.76 [1.29 – 2.64]) when compared to the lean group (1.15 [0.75-1.87]), p=0.0029 (Figure 4.2a). However, the levels were comparable between the lean and obese group as well as the overweight

and obese group (p>0.05). The levels of total cholesterol were comparable amongst the groups ($K_{(2)}$ = 2.11, p = 0.3490) (Table 4.2). However, the systolic blood pressure differed across the groups ($F_{(2, 99)}$ = 4.087, p=0.0197). The post hoc analysis based on the Games-Howells's multiple comparisons indicated an elevated systolic blood pressure in the obese group (139.3 ± 19.12) compared to the lean group (126.1 ± 17.03), p=0.0147 (Figure 4.2c). Similarly, the levels of diastolic blood pressure were different amongst the groups ($F_{(2, 98)}$ = 4.036, p=0.027). The post hoc analysis based on the Games-Howells's multiple comparisons indicated an elevated diastolic blood pressure in the obese group (90.49 ± 13.35) compared to the lean group (82.71 ± 10.83), p=0.0349 (Figure 4.2d).

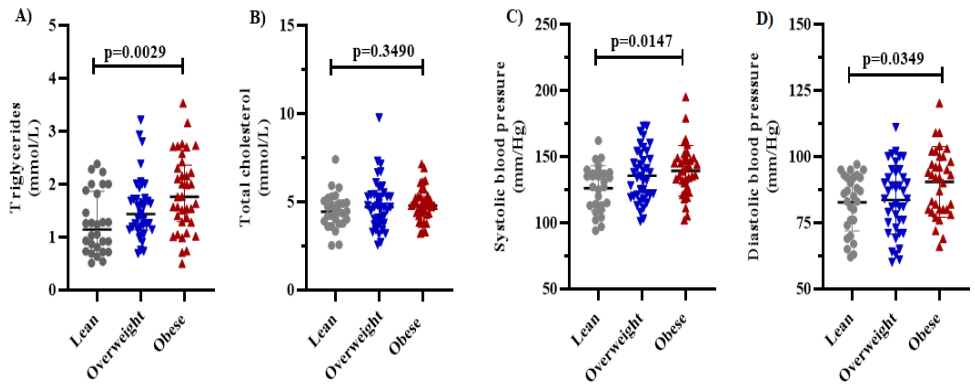

Figure 4.2: Assessed cardiovascular risks parameter amongst individuals with T2D. Figure 4.2 (**A, C** and **D**) shows significant differences in triglycerides levels, the systolic blood pressure and diastolic blood pressure amongst T2D with different body mass indexes across all the groups respectively, whilst Figure (**B**) shows comparable levels of total cholesterol across the group. Results are reported as mean ± SD or median and interquartile range [IQR] depending on the data distribution.

4.3 Correlation analysis between obesity, inflammation, iron metabolism, and cardiovascular risk

A multiple bivariate analysis was performed to determine whether there are any associations between the measured parameters. The spearman coefficients are reported in the correlation matrix below (Table 4.3). The BMI positively correlated with triglycerides (Spearman r=0.27, p=0.005) systolic (Spearman r=0.23, p=0.019) and diastolic (Spearman r=0.23, p=0.022) blood pressure.

Table 4.3 Correlation analysis between obesity, inflammation, iron metabolism, and cardiovascular risk

	BMI	Hb1Ac	FPG	SBP	DBP	Age	Duration	Fe	Ferritin	Transferrin	RBC	Hb	HCT	MCV	MCH	WBC	Plt	Chol	Trigs	HDL-c	LDL-c	Chol:HDL
BMI	-																					
Hb1Ac	0.02	-																				
FPG	0.05	0.02	-																			
SBP	**0.23**	0.07	0.13	-																		
DBP	**0.23**	0.00	0.19	**0.74**	-																	
Age	0.11	0.01	-0.02	**0.22**	0.03	-																
Duration	-0.03	-0.02	0.13	0.10	-0.05	0.17	-															
Fe	0.15	0.08	**0.25**	**0.31**	**0.24**	0.06	0.02	-														
Ferritin	0.02	-0.07	-0.02	-0.07	-0.07	-0.11	0.16	0.02	-													
Transferrin	0.12	0.15	0.11	0.13	0.03	-0.08	0.16	0.12	-0.19	-												
RBC	0.06	**0.26**	0.10	0.07	0.07	-0.15	-0.16	0.06	-0.06	-0.05	-											
Hb	0.04	**0.24**	0.11	**0.24**	**0.29**	-0.07	-0.03	**0.24**	-0.03	0.02	**0.73**	-										
HCT	0.06	**0.28**	0.01	0.15	0.19	-0.13	-0.15	0.15	0.01	-0.01	**0.75**	**0.88**	-									
MCV	0.00	-0.01	-0.10	0.09	0.16	0.06	0.02	0.15	0.19	0.03	**-0.36**	0.16	**0.27**	-								
MCH	-0.04	-0.07	0.08	**0.21**	**0.29**	0.06	0.17	**0.23**	0.07	0.09	**-0.38**	**0.27**	0.11	**0.75**	-							
WBC	0.11	0.06	-0.06	-0.07	-0.20	**0.26**	0.11	0.05	-0.10	-0.03	0.04	0.00	-0.08	-0.18	-0.11	-						
Plt	0.06	0.03	-0.09	0.04	-0.02	0.15	0.09	0.13	0.22	0.00	-0.09	-0.09	-0.14	-0.07	-0.06	0.34	-					
Chol	0.09	-0.03	0.10	-0.02	-0.03	0.13	0.08	0.03	0.10	0.03	0.03	0.08	0.08	0.05	0.03	0.11	0.17	-				
Trigs	**0.27**	0.00	0.16	0.17	0.15	0.18	0.04	0.07	-0.07	0.18	-0.06	0.03	0.04	0.12	0.12	-0.01	0.10	0.18	-			
HDL-c	-0.05	0.08	**-0.23**	-0.14	-0.18	-0.04	0.15	0.11	0.12	-0.12	**-0.23**	-0.13	-0.14	0.08	0.09	-0.04	0.12	**0.35**	**-0.32**	-		
LDL-c	-0.02	-0.15	-0.06	0.01	-0.05	-0.12	0.00	-0.10	0.01	0.04	-0.10	-0.11	-0.12	-0.04	-0.01	-0.01	-0.01	0.14	-0.14	0.22	-	
Chol:HDL	-0.10	0.10	**-0.34**	-0.19	-0.22	-0.18	0.10	0.00	0.05	-0.09	-0.23	-0.21	-0.21	0.00	-0.02	-0.12	-0.03	**-0.43**	**-0.40**	**0.63**	0.13	-

BMI: Body mass index; **HbA1c: glycated haemoglobin; FPG; Fasting blood glucose; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Fe: Serum iron; RBC: Red blood cells; Hb: Haemoglobin; HCT: Haematocrit; MCV: Mean cell volume; MCHC:** mean corpuscular haemoglobin concentration; WBC: white blood cells; Plt: Platelets; **HDL**: High- density lipoprotein; **LDL:** low-density lipoprotein and Chol: HDL: cholesterol /HDL ratio

4.4 The impact of inflammation on cardiovascular risk, iron and glucose metabolism in T2D

In order to investigate the effect of underlying inflammation in patients with T2D on iron metabolism and cardiovascular risk, an analysis based on the degree of inflammation was performed. The patients were grouped based on their levels of CRP. The normal CRP group consisted of patients with CRP levels ≤10 mg/L, whilst the high CRP group had patients with CRP levels of >10 mg/L. As a result, a total of 50 adult patients with T2D were included in this study (25/group). The demographic and characteristics of the included participants are shown in Table 4.4. The groups had similar age and gender distribution, and the patients were from a similar socio-economic and ethnic background as they were recruited from the same community. Overall, the included patients had a mean age of 50.16 ± 12.72 years and a male to female ratio of 0.43.

4.4.1 Clinical parameters and glucose parameters

There were no significant differences in the body mass index (BMI), systolic blood pressure, diastolic blood pressure, and disease duration between the two groups ($p>0.05$) (Table 4.4). The odds of hypertension were higher in patients with underlying inflammation when compared to those without (OR =1.64, 95% CI [0.53; 5.09]). The levels of HbA1C% were comparable between the two groups ($p=0.6125$), however, fasting plasma glucose levels were higher in T2D with high CRP levels (11.12 ± 3.95) when compared to T2D with normal CRP group (8.86 ± 3.68), $p=0.0413$ (Table 4.4).

Table 4.4 Clinical characteristics and laboratory profiles of included patients (n=50)

Parameter	T2D with normal CRP (n=25)	T2D with high CRP (n=25)	p-value
Clinical characteristics			
Age (years)	50.64 ± 13.63	49.68 ± 12.01	0.7927
Male n (%)	7 (28)	8 (32)	-
Body mass index ((kg/m^2)	28.13 ± 4.94	30.43 ± 5.62	0.1394
Systolic blood pressure (mm/Hg)	137.5 ± 21.83	137.4 ± 20.18	0.9833
Diastolic blood pressure (mm/Hg)	84.13 ± 11.38	86.39 ± 13.00	0.5334
Hypertension n (%)	13 (52)	16 (64)	-
Duration of T2D (years)	5.00 [2.00 – 12.00]	4.00 [1.00 – 13.00]	0.9807
Glucose profiles			
Glycated haemoglobin (%)	8.500 [6.20 – 9.90]	8.700 [7.20 – 9.40]	0.6125
Fasting plasma glucose (mmol/L)	8.86 ± 3.68	11.12 ± 3.95	**0.0413**
Inflammatory profiles			
C-reactive protein (mg\L)	3.87 ± 2.56	17.06 ± 7.88	**<0.0001**
ESR (mm/hr)	22.60 ± 19.45	44.57 ± 32.13	**0.0114**
Haematological profiles			
White cell count (10^9/L)	6.77 ± 1.49	7.11 ± 1.99	0.4922
Platelet count (10^9/L)	309.2 ± 81.06	327.9 ± 87.21	0.4357
Red cell count (10^12/L)	4.82 ± 0.53	4.85 ± 0.48	0.8332
Haemoglobin (g\dl)	14.28 ± 1.19	14.08 ± 1.37	0.5841
Haematocrit (%)	42.74 ± 5.17	42.48 ± 4.19	0.8482
Mean cell volume (fL)	90.79 ± 4.52	87.66 ± 3.62	**0.0096**
MCH (pg)	29.32 ± 1.49	28.64 ± 1.44	0.1057
MCHC (g/dL)	32.90 ± 1.08	32.94 ± 0.91	0.8878
Red cells distribution width %	13.33 ± 1.12	13.40 ± 0.86	0.8108
Iron profiles			
Serum iron (µmol/L)	15.26 ± 4.64	12.78 ± 3.50	**0.0381**
Ferritin (ng\mL)	180.9 ± 102.1	175.3 ± 113.4	0.8559
Transferrin (g\L)	2.59 ± 0.35	2.76 ± 0.44	0.1502
Lipid profiles (mmol\l))			
Triglycerides (mmol\L)	1.67 ± 0.68	1.62 ± 0.69	0.8042
Total cholesterol (mmol\L)	4.91 ± 1.13	4.59 ± 0.77	0.2481
LDL-cholesterol (mmol\L)	3.10 ± 1.04	2.80 ± 0.87	0.2617
HDL-cholesterol (mmol\L)	1.02 ± 0.27	1.06 ± 0.32	0.6419
HDL/Cholesterol ratio	0.22 ± 0.06	0.23 ± 0.07	0.5313

T2D: type 2 diabetes; **SPB**: systolic blood pressure; **DPB**: diastolic blood pressure; **BMI**: body mass index; **ESR:** erythrocyte sedimentation rate; **MCH:** mean corpuscular haemoglobin; **MCHC:** mean corpuscular haemoglobin concentration; **LDL:** low-density lipoprotein; **HDL**: low-density lipoprotein. Results expressed as mean ± standard deviation and median interquartile range.

4.4.2 Inflammatory profiles

The levels of CRP were used as a dependent factor to group the patients. As expected, the levels of CRP significantly differed between the groups of T2D with normal and high CRP levels ($p<0.0001$) (Table 4.4). Similarly, the ESR levels were elevated in the T2D with high CRP group (44.57 ± 32.13) when compared to the T2D with normal CRP levels (22.60 ± 19.45), $p=0.0114$) (Figure 4.3a, Table 4.4). However, there were no significant differences in the WCC, and platelet counts between the two groups ($p>0.005$) (Figure 4.3b-c, Table 4.4).

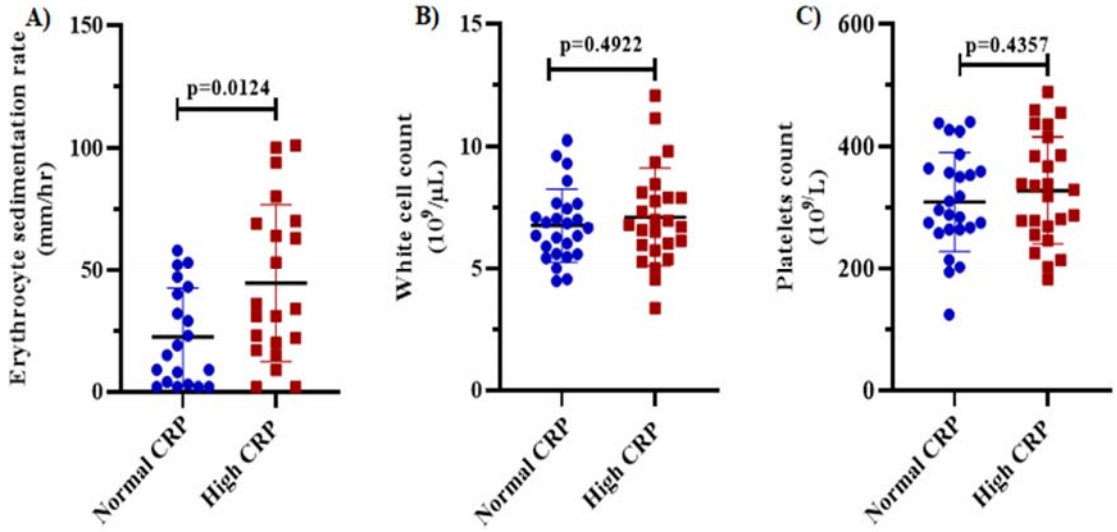

Figure 4.3. Inflammatory parameters in T2D with normal and high CRP. Figure **(A)** shows a significant difference in ESR levels between T2D with normal CRP and high CRP levels. Figure **(B)** and **(C)** illustrate the insignificant levels of white cell count and platelets between T2D with normal and high CRP. All results were expressed as mean ± standard deviation.

4.4.3 Iron profiles levels and red cells indices

In order to assess the impact of underlining inflammation on iron metabolism, the levels of iron profiles in patients with T2D were measured. The T2D with high CRP levels had lower levels of serum iron (12.78 ± 3.50) in comparison to T2D with normal CRP levels (15.26 ± 4.64), ($p=0.0381$) (Figure 4.4a). However, there were no significant differences in the levels of ferritin and transferrin groups ($p>0.005$) (Figure 4.4e-f, Table 4.4). Furthermore, measurements of haematological indices that are closely associated with iron metabolism were determined. Notably, although RBC count, haematocrit, and haemoglobin levels were comparable between the two groups ($p>0.05$) (Figure 4.4a-b), the MCV in T2D with high CRP was lower (87.66 ± 3.62) than in T2D with normal CRP levels (90.79 ± 4.52), $p=0.0096$ (Figure 4.4c).

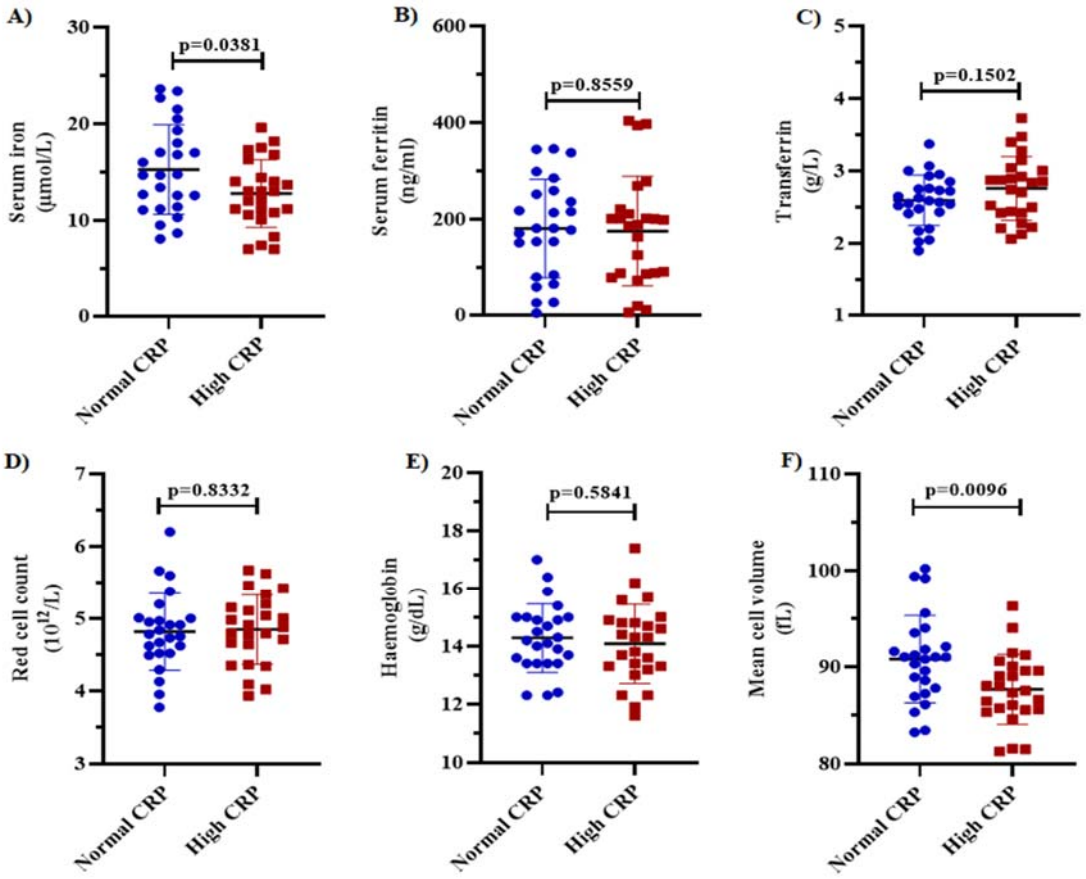

Figure 4.4: Iron profiles and red cell indices in T2D with normal and high CRP. Figure **(A)** illustrates a significant difference in serum iron levels between T2D with normal CRP and high CRP levels. Figure **(B-D)** shows comparable levels of ferritin, transferrin, red cells count and haemoglobin between T2D with normal and high CRP, whilst Figure **(F)** illustrates significant levels of mean cell volume among T2D with normal and high CRP. All results were expressed as mean ± standard deviation.

4.4.4 Lipid profiles levels

Dyslipidaemia is closely associated with increased cardiovascular risk in patients with T2D (Mooradian, 2011). Therefore, lipograms in patients with T2D were measured. The levels of triglycerides (p=0.8042), total cholesterol (Tc) (p=0.2481), low-density lipoprotein (LDL)-c (p=0.2617), high-density lipoprotein (HDL)-c (p=0.6419) and HDL/cholesterol ratio (p=0.5313) were comparable between the two groups (Table 4.4)

4.5 Correlation and regression analysis of glucose levels, CRP, and iron profiles

Correlation analysis was done to determine whether there are any associations between glucose, inflammation, and iron profiles in patients with T2D. Notably, the CRP levels were moderately associated with serum iron levels (Spearman r=-0.38, p=0.006) and the MCV (Spearman r=-0.41, p=0.003), whilst the levels of ESR positively correlated with those of CRP (Spearman r=-0.54, p<0.0001)

and was also associated with the MCV (Spearman r=-0.37, p=0.0008). However, there was no correlation between FPG and iron or inflammation profiles (p>0.05). A multivariate regression analysis was performed to further investigate whether CRP and FPG levels could predict serum iron levels in T2D. The results showed that the model was a significant predictor of iron levels in patients with T2D ($F_{2,47}$) = 5.43, p=0.0075, and could explain a total of only 18.87% of the variance in the regression model. Notably, CRP levels contributed significantly to the prediction of iron levels (β =-0.20, p=0.0032), whilst FPG did not (β =0.18, p=0.2129) (Table 4.5). The relationship of both the actual and predicted values, and the residual is shown in Figure 4.5.

Table 4.5: Multivariate linear regression analysis of independent variables of serum iron levels in T2D

Variable	Beta	Standard error	95% Confidence interval	|t|	p-value
Intercept	14.30	1.63	11.03 to 17.58	8.79	**<0.0001**
C-reactive protein	-0.20	0.06	-0.33 to -0.07	3.11	**0.0032**
Fasting plasma glucose	0.18	0.14	-0.11 to 0.46	1.26	0.2129

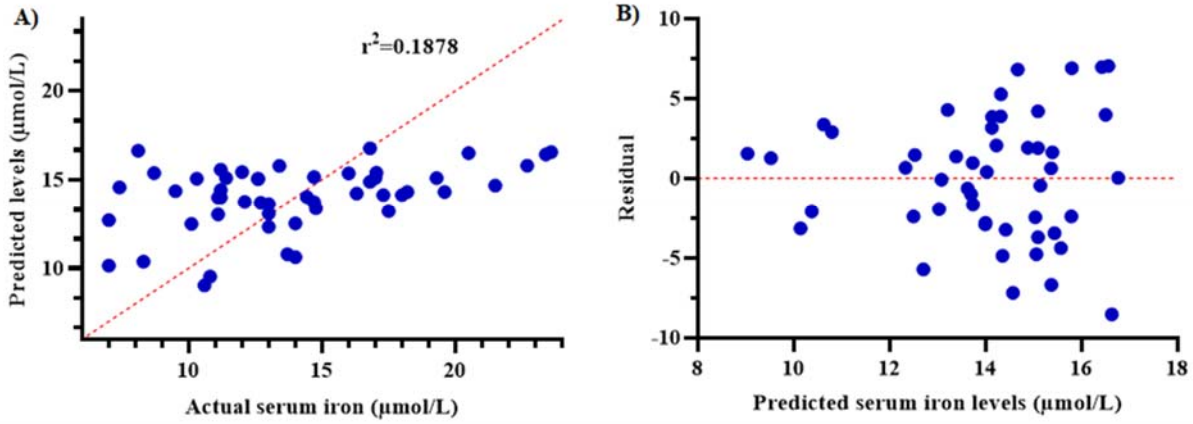

Figure 4.5: The graph shows the interpretation of the predicted and the actual values of total serum iron levels and the residual.

Chapter Five: Discussion

Chronic inflammation is associated with poor glucose control and increased cardiovascular risk in patients with type 2 diabetes (T2D) (Olokoba et al., 2012; Pagani et al., 2019). However, iron metabolism in T2D is poorly understood. Therefore, the primary aim of this study was to investigate iron profiles in patients with T2D. In addition, it aimed to assess the impact of obesity and underlining inflammation on iron metabolism, glucose control, and cardiovascular risk in T2D. Lastly, it investigated whether there are any associations between iron profiles, lipids profiles, and inflammation in these patients. Notably, the results demonstrated abnormal iron profiles in patients with T2D, whereby underlying inflammation was associated with decreased total serum iron levels and red cell mean volume (MCV). Although the levels of haematological profiles and iron profiles were within the reference ranges, they are indicative of an early development of microcytic iron deficiency anaemia (Shersten et al., 2007). However, since this is an early manifestation of iron deficiency anaemia the body is still capable of compensating for the changes in the profiles of iron and red cells indices, hence no changes were observed in these profiles. Moreover, although HbA1c is a good maker of long-term glycaemic control in patients with T2D (Sherwani et al., 2016), our results found no significant difference in its levels between T2D with inflammation and T2D without inflammation. This can be attributed to insignificant haemoglobin levels between the two groups since HbA1c levels are depended on haemoglobin levels (Ye et al., 2016). However, the presence of underlining inflammation in T2D was also associated with significantly elevated levels of fasting plasma glucose which is indicative of worsening glucose impairment.

The correlation results based on the measured inflammatory makers' C-reactive protein (CRP) and erythrocytes sedimentation rate (ESR) showed that the levels of CRP and ESR were moderately associated with low serum iron levels and MCV. This was due to the increase in our measured inflammatory markers (CRP and ESR) that are associated with inflammation. Even though the two hormones, hepcidin, and erythropoietin were not measured in this study, they are known to be synthesis and modulated by inflammation (D'Angelo, 2013). This leads to the blockage of iron release from the macrophages and its absorption from the intestines, causing low serum iron, thus less iron being transported to the bone marrow for erythropoiesis. The subgroup analysis based on the degree of obesity in patients with T2D showed comparable levels in iron, glucose, and inflammatory profiles. Even though low-density lipoprotein (LDL-C) is a better maker of cardiovascular disease (CVD) (Hsu et al., 2019), our results showed

no significant differences. However, the degree of obesity was associated with significantly elevated levels of triglycerides and blood pressures (systolic and diastolic blood pressures). Thus, highlighting the role of obesity in dysfunctional lipid metabolism and increase the risk of CVD in T2D.

Obesity, a major risk factor risk factor for CVD is associated with dyslipidaemia and poor glycaemic control in patients with T2D (Piché et al., 2020; Scherer & Hill, 2016). Dyslipidaemia is a condition that is characterised with increased levels of triglycerides, total cholesterol (Tc), and low-density lipoprotein LDL-C) coupled with reduced levels of high-density lipoprotein cholesterol (HDLC), and is closely associated with the pathogenesis of atherosclerosis (Chehade et al., 2013; Mooradian, 2011). Similarly, our results showed elevated levels of triglycerides in patients with obesity. Notably, hypertriglyceridemia is closely associated with atherosclerosis and thrombosis (Lascar et al., 2018). These abnormalities promote the narrowing of blood vessels and lead to the eventual blockage of the endothelial lumen (Petrie et al., 2018). These changes result in the development of hypertension due to increased blood pressure (Martín-Timón, 2014; Pretorius et al., 2018). Notably, in this current study patients with obesity had elevated blood pressure and over half of the participants had hypertension. Furthermore, an increase in LDL-C and (high-density lipoprotein/cholesterol ratio (HDL/CHOL) coupled with a decrease in HDL-C is associated with increased risk of CVD in T2D (Lemieux et al., 2001). In fact, hypertriglyceridemia in patients with T2D predisposes them to the development of cardiovascular disease. As a result, patients with T2D who have dysfunctional lipids metabolism accompanied by high triglycerides levels should be put on triglycerides lowering drugs that are recommended for T2D such as pioglitazone to prevent their chances of developing CVD (Rosenblit, 2016).

The no clinically significant results of total serum iron in this study might be due to the fact that, all (lean, overweight and obese) participants were having T2D and therefore, is possible for them to have dysregulated iron metabolism. However, the systematic-review and meta-analysis that we conducted on the levels of hepcidin in T2D revealed that serum iron levels positively differ based on body mass index in patients with T2D (Addendum 1) and these differences were associated with varying levels of hepcidin.
Metformin is a commonly used drug in obese T2D that is used to reduce blood glucose levels by inhibiting glucogenesis in patients with T2D (Horakova et al., 2019). Its long-term usage has been found to cause an increase in hepcidin levels and a decrease in serum iron levels (Ahmed et al., 2015). Therefore, its usage in the clinical setting should be monitored, especially in T2D with early manifestation of iron deficiency anaemia. This is because metformin causes an early reduction in the levels of haemoglobin by causing

ineffective erythropoiesis through increased levels of hepcidin, as a result less iron is available for the production of red cells in the bone marrow and this results in incidences of developing moderate anaemia. In fact, anaemia is a common finding in patients with T2D (Donnelly et al., 2020). In addition, Insulin is another commonly used anti-diabetic drug which is given to T2D who have beta cell failure in order to compensate for their low insulin levels, in addition, this drug is also known to correct for hepcidin levels in T2D and prevents incidents of anaemia (Vela et al., 2017) Moreover, the subgroup analysis based on the treatment could not be done as hepcidin levels were not measured in this study, and therefore the findings could not be concluded. However, patients on metformin would be expected to have moderate anaemia in comparison to T2D on other treatments.

Inflammation in conditions of poor glucose control is associated with dysregulated iron metabolism (D'Angelo, 2013). However, its exact role is not well understood as different and conflicting theories have been proposed (Adejumo et al., 2012; Elizabeta & Tomas, 2014). Inflammation modulates the synthesis and action of important regulators of iron metabolism such as hepcidin and erythropoietin (Collins et al., 2008; Vela, 2018). Exacerbated inflammation vial interleukin (IL)-6 signaling promotes the synthesis of hepcidin, which leads to the blockage of iron release from the macrophages and its absorption from the small intestines (D'Angelo, 2013; Wessling-Resnick, 2010). Thus, resulting in reduced iron transportation to the bone marrow for erythropoiesis. Conversely, inflammation inhibits the synthesis of erythropoietin, an important hormone that promotes red cell synthesis (Pagani et al., 2019; Pasricha et al., 2016), based on our experimental paper that we conducted (Addendum 2), we, therefore hypothesise that, the decrease in total serum iron levels in patients with poor glucose control presenting with an underlying inflammation may be attributed to increased hepcidin synthesis, that causes a decrease in iron absorption and uptake from the macrophages (Pasricha et al., 2016). Whilst the decreased in MCV levels may be due to the inhibition of erythropoietin synthesis and its action which is associated with a decrease in red cells production and its indices (Hb, MCV, MCH, and MCH) (D'Angelo, 2013; Pagani et al., 2019). Therefore the use of anti-inflammatory drugs such as aspirin may help in alleviation inflammation and the development of iron deficiency anaemia in these patients (Abi Khalil et al., 2018; Pignone et al., 2010).

5.1 Limitations

This study goes not without limitations. The major limitation of our study was that we did not assess some of the important regulators of iron metabolisms such as hepcidin and erythropoietin, which also play a

role in iron metabolism. In addition, even though the majority of our patients were on metformin, we did not measure the levels of metformin and hepcidin in these patients to determine its effects on hepcidin and iron levels.

5.2 Recommendations

Future longitudinal studies should consider measuring the levels of some of the important iron regulators such as erythropoietin and hepcidin in order to be able to make concrete conclusions on how they are involved in dysregulation of iron metabolism in T2D. In addition, metformin levels should also be measured to assess its impact on hepcidin levels.

5.3 Conclusion remarks

Iron profiles in individuals with T2D are dysregulated and this is coupled with increased cardiovascular risk mediated by altered lipid profiles.

Apart from poor glucose control, the presence of underlying inflammation in patients with T2D also influences iron metabolism by decreasing the total serum iron levels and MCV. These are classical features of an early manifestation of iron deficiency anaemia. Therefore, intervention measures such as the use of anti-inflammation drugs, for example low-dose aspirin might help alleviate the development of iron deficiency anaemia in these patients. In addition, the use of triglycerides lowering drugs might be helpful in preventing future cases of cardiovascular disease in T2D presenting with obesity.

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Appendices

Appendix 1: Ethics clearance Namibia University of Science and Technology Research Ethics committee (NUST).

NAMIBIA UNIVERSITY OF SCIENCE AND TECHNOLOGY
13 Storch Street
Private Bag 13388
Windhoek
NAMIBIA
T: +264 61 207 9111
F: +264 61 207 2444
W: www.nust.na

FACULTY OF HEALTH AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE

(FHAS-REC)

DECISION/FEEDBACK ON RESEARCH PROPOSAL ETHICAL CLEARANCE

Dear: Prof/Dr/Mr/Ms/Other	Mr. Maurice Nyambuya NUST Staff Number: 1006785
Research Topic:	Iron Metabolism and its association with Chronic Inflammation and Cardiovascular Risk in Type 2 Diabetes Mellitus
Supervisor (if applicable):	Nil
Co-supervisor(s): if applicable	Nil
Qualification registered for (if applicable):	ND Biomedical Technology, B Tech Biomedical Technology, MSc Biomedical Technology.

Re: Ethical Screening Application No: FHAS 1/2020

The Faculty of Health and Applied Sciences Research Ethics Screening Committee has reviewed your application for the above-mentioned research project. Based on the recommendation of the expert reviewer, the research as set out in the application is hereby:

(Indicate with an X)

Approved: i.e. may proceed with the project, subject to Ministry of Health and Social Service Clearance.	X
Approved provisionally: i.e. may proceed but subject to compliance with recommendation(s) listed below	
Not approved: Not to proceed with the project until compliance with recommendation listed below and resubmit ethics application for consideration	

It is important to note that as a researcher, you are expected to maintain ethical integrity of your research. You are encouraged to strictly adhere to the research ethics policy of NUST. You should remain within the scope of your research proposal and support evidence as submitted to the FHAS-REC. Should any aspect of your research change from the information as presented, which could have an impact or effect on any research participants/subjects/environment, you are to report this immediately to your supervisor and to the FHAS-REC as applicable in writing. Failure to do so may result in withdrawal of approval.

Kindly consult the committee if you need further clarification in this regard. We wish you success in your research endeavour and are of the belief that it will have positive impact on your career as well as the development of NUST and the society in general.

Ethical issues that require compliance/ must be addressed : None		
No.	**Ethical issues**	**Comment/recommendation**

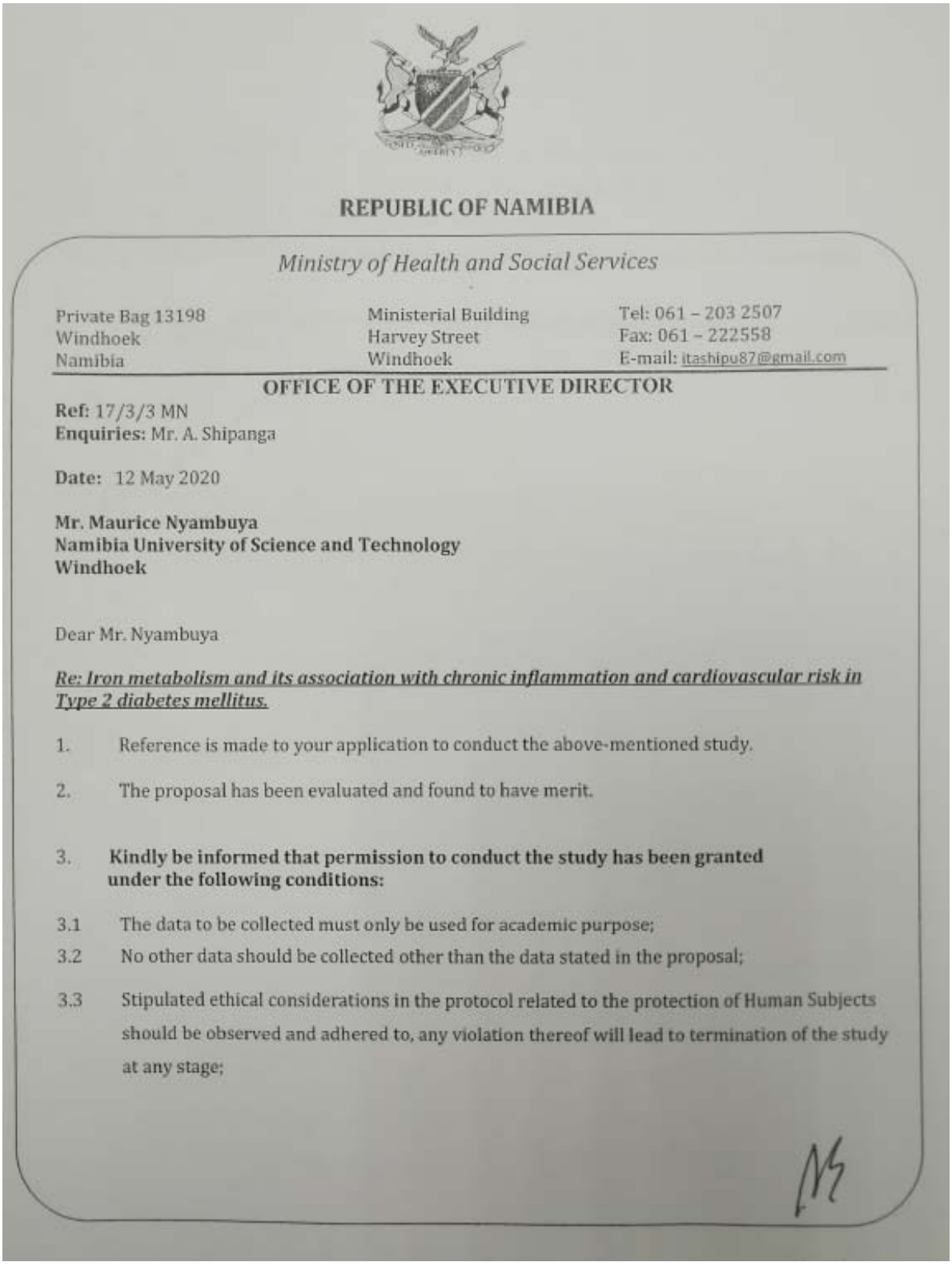
NB: May attach additional page as required

Sincerely Yours,

Name: Prof Sylvester R Moyo Signature: Date: 4th March 2020

Chairperson: FHAS Ethics Screening Committee.

Appendix 2. Ethics clearance the Ministry of Health and Social Services



REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198
Windhoek
Namibia

Ministerial Building
Harvey Street
Windhoek

Tel: 061 – 203 2507
Fax: 061 – 222558
E-mail: itashipu87@gmail.com

OFFICE OF THE EXECUTIVE DIRECTOR

Ref: 17/3/3 MN
Enquiries: Mr. A. Shipanga

Date: 12 May 2020

Mr. Maurice Nyambuya
Namibia University of Science and Technology
Windhoek

Dear Mr. Nyambuya

Re: Iron metabolism and its association with chronic inflammation and cardiovascular risk in Type 2 diabetes mellitus.

1. Reference is made to your application to conduct the above-mentioned study.

2. The proposal has been evaluated and found to have merit.

3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**

3.1 The data to be collected must only be used for academic purpose;

3.2 No other data should be collected other than the data stated in the proposal;

3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;

3.4 A quarterly report to be submitted to the Ministry's Research Unit;

3.5 Preliminary findings to be submitted upon completion of the study;

3.6 Final report to be submitted upon completion of the study;

3.7 Separate permission should be sought from the Ministry for the publication of the findings.

4. All the cost implications that will result from this study will be the responsibility of the applicant and **not** of the MoHSS.

Yours sincerely,

BEN NANGOMBE
EXECUTIVE DIRECTOR

"Health for All"

Appendix 3. Ethics clearance Namibia Institute of Pathology

NAMIBIA INSTITUTE OF PATHOLOGY LIMITED
Reg. No. 2002/[illegible]
Tel: +264-61-295 4300 • Fax: +264-61 255 566 • P.O. Box 277 • Windhoek • Namibia • Info@nip.com.na, www.nip.com.na

OFFICE OF THE CHIEF OPERATIONS OFFICER

Enquiries: Mr Boniface Makumbi; Tel.: 061-295 4210

Date: 01st October 2020

Ms Fransina Ndevahoma
Namibia University of Science and Technology
Windhoek
Namibia

Dear Ms Ndevahoma

RE: IRON PROFILES AND INFLAMMATORY PARAMETERS AMONG INDIVIDUALS WITH TYPE 2 DIABETES MELLITUS, IN WINDHOEK, NAMIBIA

1. The above-mentioned research proposal has been perused and found beneficial to the country.
2. After the review, it is a pleasure to inform you that approval was granted for you to proceed with the research on condition that the following be complied with:
3. Consultation with the relevant Heads of the Business Units upon starting with your research.
4. For the required data, please submit your request to the ICT department two week prior to the commencement of your research.
5. Observe and adhere to all ethical considerations and confidentiality to protect your clientele information.
6. Report to the office of the Senior Manager: Specialised Services upon starting your research.
7. Final report to be shared with the Namibia Institute of Pathology Limited.

NAMIBIA INSTITUTE OF PATHOLOGY (NIP) LTD
HEAD OFFICE
2020 -10- 01
WINDHOEK
NAMIBIA

Yours Sincerely

.....

Boniface Makumbi
Acting Chief Operations Officer

Addendums

Addendum 1: Systematic review and meta-analysis on the influence of body weights on hepcidin levels [Accepted for Publication in Heliyon]

Body weight and its Influence on Hepcidin Levels in Patients with Type 2 Diabetes: A Systematic Review and Meta-analysis of Clinical Studies

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Department of Health Sciences, Faculty of Health and Applied Sciences, Namibia University of Science and Technology, Windhoek 9000, Namibia. Email: mnyambuya@nust.na; Tel.: +26461 2072914.

Abstract

Introduction: Iron profiles in patients with type 2 diabetes (T2D) are inconsistent. In this study, we assessed the levels of hepcidin, a regulatory protein involved in iron homoeostasis, in patients with T2D. We further evaluated the surrogate markers of hepcidin action, particularly those associated with erythropoiesis.

Methods: This systematic review and meta-analysis was reported following the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines. We searched for relevant studies in electronic databases from inception until 31 October 2020 without any language restriction. The random effects model was used to calculate effect estimates, and outcomes we reported as either standardised mean difference (SMD) or mean differences, 95 percent confidence interval (95% CI).

Results: Eleven studies involving 2 620 participants were included in this study. Patients with T2D had a slight increase in hepcidin levels when compared to controls SMD: 0.07 [95% CI: -0.30, 0.44]. The subgroup analysis showed that studies involving T2D patients who were overweight reported elevated hepcidin levels SMD: 0.35 [95% CI: 0.07, 0.62] whilst those with grade I obesity described reduced levels SMD: -0.42 [95% CI: -1.21, 0.38]. All T2D patients had low levels of haemoglobin MD: -0.23g/dl [95% CI: -0.46, -0.01] irrespective of body weight.

Conclusion: The levels of hepcidin are altered in patients with T2D and are disproportionately influenced by weight. Moreover, T2D patients presents with subclinical anaemia despite elevated iron stores. The regulation of hepcidin in patients with T2D is dependent on several factors and vary greatly, thus its sole use in clinical settings may be less beneficial.

Keywords: Hepcidin; haemoglobin, anaemia; iron profiles; obesity; type 2 diabetes

1. Introduction

Obesity is an independent risk-factor for several non-communicable disease (NCD) which includes type 2 diabetes (T2D).[1] An obese state in patients with T2D is associated with exacerbated systemic inflammation and an increased degree of insulin resistance.[2,3] T2D is also considered as a chronic inflammatory condition that is characterised by insulin resistance and hyperglycaemia.[4] A growing body of evidence linking dysregulated iron metabolism and T2D has been described over the years.[5–7] Whereby, both conditions iron deficiency anaemia[8,9] and in some instances iron overload [6,10,11] has been widely reported in patients with T2D. The reported different relationships between poor glucose control and iron metabolism sparked interest in investigating iron metabolism in T2D, particularity on the modulators of iron intake, release, and transportation. One of these regulators is hepcidin, an acute phase protein synthesised in the liver and encoded by the *HAMP* gene, that inhibits iron release from macrophages and uptake from intestinal cells through its interaction with ferroportin, an iron importer protein.[12,13]. The synthesis of hepcidin is induced by the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and bone morphogenetic protein/s-mothers against decapentaplegic (BMP/SMAD) signalling pathways mediated by interleukin (IL)-6 and BMP-6 in response to inflammation and iron status, respectively.[14,15]

Hepcidin plays a pivotal role in regulating iron homeostasis and indirectly modulates red cell production.[16] In that context, the release of erythropoietin, a major regulator of erythropoiesis, blocks hepcidin secretion via erythroferrone action leading to increased iron supply to the bone marrow for erythropoiesis.[16,17] Consequently, aberrant hepcidin levels are associated with anaemia of chronic disease (ACD), a common feature of chronic inflammatory diseases such as T2D.[17] Interestingly, this mild to moderate ACD is attributed to increased release of pro-inflammatory IL-6 which in addition to promoting hepcidin synthesis, has anti-erythropoietic effects mediated by inhibiting the proliferation of erythroid precursor cells and erythropoietin action.[9,14] The reduction of hepcidin expression may also cause ineffective erythropoiesis leading to iron-loading anaemia deficiency as previously reported elsewhere.[18] The dysregulation of hepcidin levels is also associated with non-alcoholic fatty liver disease (NAFLD), a metabolic disorder that is closely associated with hyperglycaemia and insulin resistance.[19,20] The elevated levels of hepcidin in NALFD leads to the excessive accumulation of hepatic iron stores coupled with a deficiency in serum levels.[21] Notably, this increase in hepcidin levels is attributed to obesity[22] and consequently, NALFD has become a growing complication of diabetes with a prevalence of 54% in T2D cases.[23] The resulting dysmetabolic iron overload syndrome (DIOS) in NAFLD and T2D is driven by insulin resistance and low-grade inflammation.[21,22,24]

Although it is apparent that hepcidin is an important regulator of iron metabolism, its levels together with its surrogate in patients with T2D remain elusive. For instance, elevated[5–7,25] and comparable[10,26–28] levels of hepcidin between patients with T2D and healthy controls have been previously shown.[10,11,27,29,30] Whilst others have reported a marked reduction in the levels of hepcidin in patients with T2D.[11,29,30] Therefore, these findings suggest the presence of other underlying factors besides poor glucose control in these patients that may influence hepcidin levels. These include the severity of obesity (which is associated with increased insulin resistance and IL-6 levels[27,31]) and DIOS, but also the presence of hemochromatosis and beta-thalassemia major (which is associated with increased beta-cell damage and impaired insulin synthesis and action).[22,32,33] Therefore, inferences on the levels of hepcidin in poor glucose control will be important in the risk stratification of T2D and the development of its associated complications that are mediated by altered iron metabolism. In this systematic review and meta-analysis, we comprehensively assessed available literature reporting on the expression of hepcidin in patients with T2D and further explored how obesity impacts these levels. Lastly, we assessed the levels of surrogate markers of iron metabolism influenced by hepcidin action.

2. Methods

This systematic review and meta-analysis was reported following the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines.[34] A detailed MOOSE checklist is provided in Table 1S. A protocol was designed for this study and was agreed upon by all authors before conducting the qualitative and quantitative synthesis. However, it was not registered and therefore the protocol does not have a registration number.

2.1 Information sources and search strategy

A comprehensive search strategy was designed by two independent reviewers (FN and PVD) with the help of an experienced librarian. The MEDLINE electronic database, and Google Scholar as well as grey literature including preprints, were searched from inception until 31 October 2020. The reviewers used the following Medical Subject-Heading (MeSH) terms and text words to retrieve relevant studies; “hepcidin” AND ‘type 2 diabetes mellitus’. The reference lists of included studies were further scanned for additional relevant studies. No language restrictions were applied, and a detailed MEDLINE search strategy using PubMed search engine is shown in Table 2S. We retrieved studies to address the following research questions;

1. Are there differences in the levels of regulatory proteins involved in iron homoeostasis in patients with T2D?
2. Does the degree of obesity in these patients influence the levels of iron regulatory proteins?

2.2 Eligibility criteria and study selection

Studies were independently screened and selected by two reviewers (FN and BBN) using a pre-defined inclusion and exclusion criteria. In cases of disagreements, third reviewer (TMN) was consulted for arbitration. We included experimental and observational studies that reported on hepcidin levels in patients with T2D irrespective of the age. We excluded reviews, case studies, letters to the editor and animal studies.

Participants

Patients living with T2D and individuals with normal glucose control (normoglycaemics).

Intervention and comparator

No intervention was considered in this study and the comparators included normoglycaemics (controls).

Outcomes

The primary outcome of this study was to determine the level of regulatory proteins involved in iron homoeostasis. The secondary outcome involved iron profiles modulated by iron regulatory proteins.

2.3 Data extraction and management

Two independent reviewers (FN and TMN) extracted detailed study information and characteristics using a predefined data extraction form adapted from the Cochrane Consumers and Communication Review Group data extraction for included studies template.[35] The following data were extracted from each study, author's name and year of publication, country, number of participants, number of males, age and BMI as well as effect measures (levels of hepcidin, iron, haemoglobin, ferritin and hepcidin:ferritin ratio) and main findings. Discrepancies in the extracted data items were resolved through discussions or consultation of a third reviewer (BBN).

2.4 Risk of bias and confidence in the cumulative evidence

The risk of bias in the included studies was assessed by two independent reviewers (FN and EPN) using the modified Newcastle-Ottawa scale, adapted for observational studies.[36] Briefly, a star system was used to appraise studies based on three domains (selection, comparability, and outcome ascertainment). A study is rated unsatisfactory if score is between 0-4, satisfactory if 5-6, good if 7-8 and very good if 9-10. Inconsistencies in the scores were resolved by consulting the third reviewer (TMN). The quality of the included studies was assessed used the Grading of Recommendations Assessment, development, and Evaluation (GRADE) approach to evaluate cumulative evidence quality.[37]

2.5 Statistical analysis

The random-effects model was used to estimate the effect size and the I^2 was used to test for statistical heterogeneity. The effect estimates were reported as the standardised mean difference (SMD) or mean differences (MD), 95 percent confidence interval (95% CI) depending on the reported effect measure and units of measurement, and the Cohen's d method was used to interpret the calculated pooled estimates.[38] We performed a subgroup-analysis based on the reported BMI (Overweight vs. Obesity), which may have an influence on hepcidin levels.[25] We further conducted a sensitivity analysis to explore the sources of unexplained statistical heterogeneity amongst the included studies and to evaluate the robustness of the reported overall effect estimates. The Cohen's Kappa (κ) was used to assess inter-rate reliability on study selection and risk of bias assessment.[39] A p-value<0.05 was considered statistically significant whilst a p-value of less than 0.1 was considered statistically significant among subgroup analysis.[40] Publication bias was assessed using visual inspection of funnel plots. All statistical analysis was performed using Review Manager (RevMan V.5.3) software.

3. Results

3.1 Selected studies

We identified a total of 37 citations, of which thirty-five were retrieved from PubMed (n=35) and two from other sources (n=2). After the abstract screening phase, we excluded twenty studies (n=20) as these were not relevant to the topic of interest. Furthermore, six studies (n=6) were excluded upon the full-text screening phase, with studies not reporting on suitable control group or hepcidin levels (n=3) and studies not reporting adequate study-level data (n=3). Therefore only 11 studies met the inclusion criteria and had enough data for the primary outcome and quantitative meta- analysis (Figure 1).

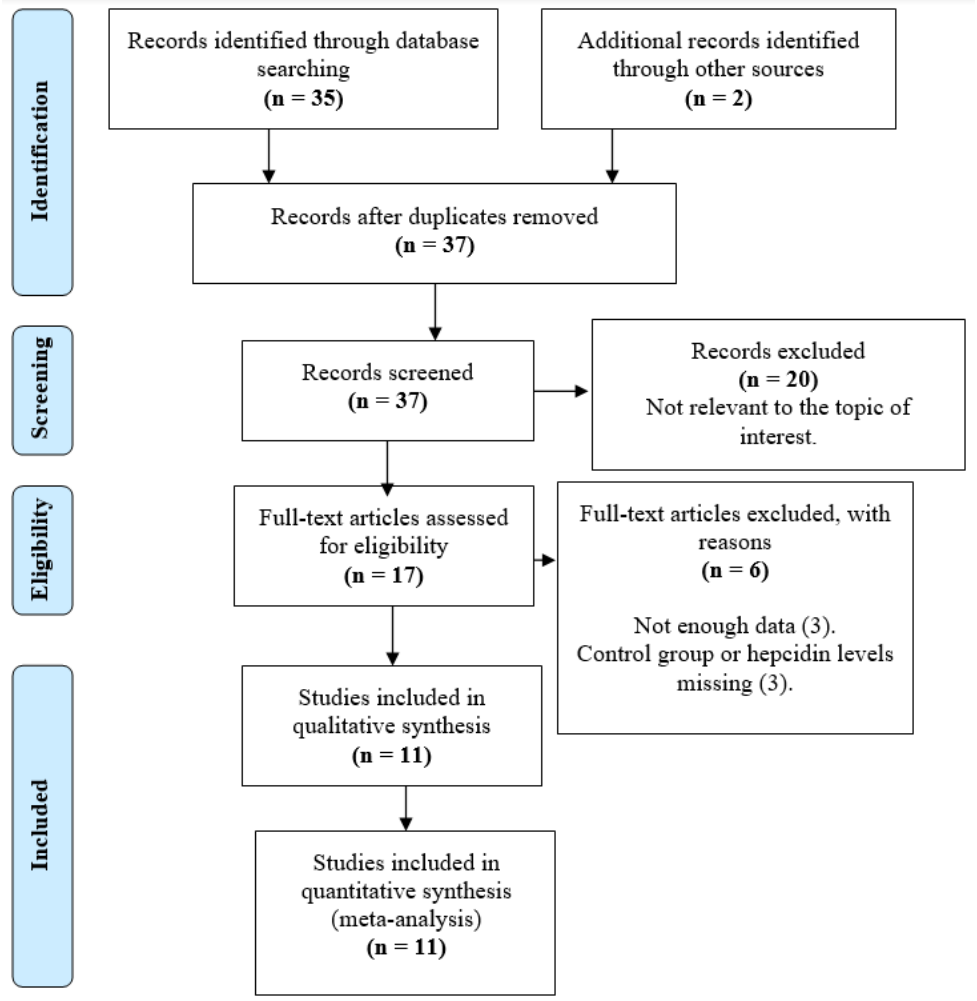

Figure 1: PRISMA flow diagram showing the study selection process

3.2 Characteristics of included studies

All included studies [5-7,10-11,25-30] were observational studies published between 2011 and 2018, and were from Egypt[5], Colombia[30], Germany[11], Chile[25], Finland[29], Israel[28] and the rest from China (n=4).[6,7,10,26] However, one study by Vela and colleagues[27] did not indicate the country where the study was conducted. This systematic review and meta-analysis included a total of 2 620 participants, of which 1276 (49%) were T2D patients with a mean age of 60.49 ± 10.93 years and 1344 (51%) were healthy individuals, with a mean age of 54.03 ± 12.24 years, with a male to female ratio of 0.98. Amongst the T2D group, 899 individuals were overweight (BMI>25 but less than 30 kg/m^2) and were reported in 5 of the included studies[6,7,10,26,27] whilst 337 individuals were grade I obese (BMI>30 but less than 35 kg/m^2) and were reported in 4 of the included studies[11,28–30]. One study reported both overweight and obese T2D patients[25] while the other did not specify the degree of obesity[5] (Table 1).

3.3 Study quality and publication bias

The quality assessment for included studies was assessed using the Newcastle-Ottawa scale (Table 3S). The median score range of included studies was 7 (5-9) out of total 10 scores. Four of the studies were rated as satisfactory[5,25,27,29] and 6 as good[6,7,11,26,28,30] with only one study as very good.[10] Briefly, the selection domain for included studies had a median of 3 (2-4) out of 5 overall score (overall agreement 86.36%, kappa = 0.73), comparability median of 1(1-2) out of 2 maximum stars (overall agreement 54.55%, kappa = 0.09) and outcome median of 2 (2-3) out of 3 possible stars (overall agreement 95.46%, kappa = 0.91) Table 2S. Visual assessment of funnel plots indicated no potential publication bias (Figure 1S).

3.4 Data synthesis

Reported metabolic parameters in included studies

Fasting blood glucose levels were reported in 9 (82%) of the included studies.[5–7,10,11,26–28,30] Overall, the pooled effect estimates of fasting blood glucose levels, showed a large effect size between patients with T2D and controls (SMD: 2.19 [95% CI: 1.41, 2.98]; I^2= 97%, p^H<0.00001) (Figure 2aS). A total of 4 (36%) studies reported on insulin levels[10,27,28,30] and pooled effect showed increased insulin levels in patients with T2D when compared to controls (SMD: 1.34 [95% CI: 0.07, 2.60]; I^2= 97%, p^H<0.00001) (Figure 2bS). Glycated haemoglobin levels were reported in 8 (73%) of the included studies [5,6,10,11,26–29] and were increased in patients with T2D when compared to controls (MD: 2.30% [95% CI: 1.82, 2.77]; I^2= 97%, p^H<0.00001) (Figure 2cS.

Primary findings on hepcidin levels in patients with T2D

A total of 11 (100%) studies reported on hepcidin levels in T2D patients. The qualitative synthesis of 4 included studies (36%)[5–7,25] showed elevated levels of hepcidin in patients with T2D when compared to controls, whilst 3 studies (27%)[11,29,30] described decreased hepcidin levels in T2D patients in comparison to controls. In contrast, the other 4 studies (36%)[10,26–28] reported on comparable hepcidin levels between T2D and control (Table 1**)**. Nonetheless, the pooled effect estimates showed a slight increase in hepcidin levels between patients with T2D and controls (SMD: 0.07 [95% CI: -0.30, 0.44]; I^2= 93%, p^H<0.00001) (Figure 3S).

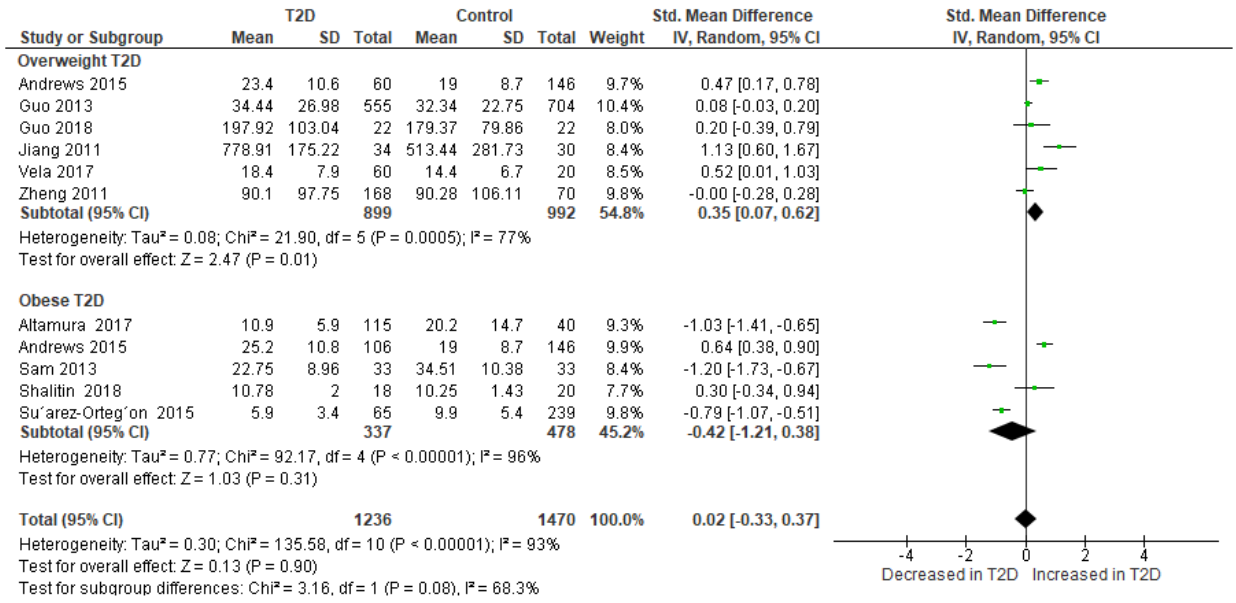

Figure 2: A subgroup analysis of hepcidin levels in type 2 diabetes (T2D) patients based on degree of obesity, between overweight T2D (BMI ≥ 25 but less than 30 kg/m^2) and grade I obese T2D (BMI>30 but less than 35 kg/m^2). Hepcidin levels were significantly increased in overweight T2D when compared to patients with grade 1 obesity.

To investigate sources of heterogeneity amongst the 11 studies, we performed a subgroup analysis based on the classification of weight status by body mass index (BMI). Normal weight is considered as BMI<25 kg/m^2, overweight as BMI ≥ 25 but less than 30 kg/m^2 whilst class I, II and III obesity as BMI >30 but less than 35 kg/m^2, BMI ≥ 35 but less than 40 kg/m^2 and BMI ≥40 kg/m^2, respectively. In this subgroup analysis we included 10 studies[6,7,10,11,25–30] and we omitted the study by Atyia and colleagues[5] as they did not specify the degree of obesity amongst the included participants. Subsequently, the test for subgroup differences showed a significant subgroup effect (p = 0.08).[40] Thus, the overall classification of body weights based on the reported BMI modified the overall effect of hepcidin levels. Studies that reported on T2D patients who were overweight showed significantly elevated hepcidin levels in comparison to controls (SMD: 0.35 [95% CI: 0.07, 0.62]; I^2= 77%, p^H = 0.0005) (Figure 2). In contrast, studies that involved T2D patients with grade I obesity described reduced levels of hepcidin (SMD: -0.42 [95% CI: -1.21, 0.38]; I^2= 96%, p^H<0.00001) (Figure 2). Notably, the sources of heterogeneity remained high in the subgroup analysis. To further investigate sources of heterogeneity and the robustness of our results, we performed a sensitivity analysis based on treatment, age and CRP levels which are known to influence hepcidin levels. The SMDs did not change direction nor the magnitude of our effect size and the levels of heterogeneity remained substantial (Table 4S).

Surrogate markers of hepcidin

Serum iron levels

Overall, 45% of the included studies (n=5) reported on iron levels[5,10,11,25,27] Of these, 3 studies[5,10,42] reported comparable iron levels between T2D and controls. The other 2 studies[11,25] described elevated levels in T2D when compared to controls (Table 1). However, pooled effect estimates showed a slight increase in iron levels in patients with T2D when compared to controls (SMD: 0.06 [95% CI: -0.26, 0.39]; I^2= 80%, p^H = 0.0005) (Table 2). Although the test for subgroup differences was not significant (p=0.29), it is important to note that in the overweight T2D, the iron levels were lower than healthy controls.

Haemoglobin levels

A total of 8 of the included studies reported on haemoglobin levels. Whereby 2 citations[10,26] reported increased haemoglobin levels in T2D in comparison to controls, whilst the other 5 studies described decreased[6,25,27,29] and comparable[11] haemoglobin levels (Table 1). The pooled estimates showed, significantly decreased levels of haemoglobin in patients with T2D when compared to healthy controls (MD: -0.23g/dl [95% CI: -0.46, -0.01]; I^2= 85%, p^H<0.00001) (Table 2). Although the test for subgroup differences was nonsignificant (p=0.28), notably reduced haemoglobin levels suggestive of subclinical anaemia were observed in the obese T2D subgroup (p=0.01).

Ferritin levels

Approximately 91% of the included studies (n=10) reported on ferritin levels, with 9 of these[5–7,10,11,25–27,29] showing elevated levels of ferritin in T2D in comparison to controls, whilst one study described comparable levels.[30] The pooled effect estimates showed significantly increased ferritin levels in T2D patients when compared to healthy controls (SMD: 0.60 [95% CI: 0.32, 0.88]; I^2= 88%, p^H<0.00001) (Table 2). The test for subgroup analysis was insignificant (p=0.85), thus iron stores were not influenced by weight.

Hepcidin: ferritin ratio

A total of 5 of the included studies reported on the hepcidin: ferritin ratio. The hepcidin: ferritin ratio was increased in patients with T2D compared to controls.[5,7] Whilst the other 3 studies described decreased[29,30] and comparable[27] ratios (Table 1). The meta-analysis revealed a slight decrease in the hepcidin: ferritin ratio in patients with T2D when compared to healthy controls (SMD: -0.19 [95% CI: -0.46, 0.88]; I^2= 96%, p^H<0.00001) (Table 2). The test for subgroup differences was significant (0.03) and we therefore performed a subgroup analysis based on weights. The

overweight T2D subgroup had comparable hepcidin: ferritin ratios whilst it was significantly decreased in obese T2D group (p=0.03).

4. Discussion

Patients with T2D are known to have altered liver function, which impacts on the synthesis of regulator proteins involved in iron metabolism. As a consequence, dysregulated iron metabolism may lead to ACD or iron-loading anaemia deficiency.[43,44] In this study, we aimed at evaluating the levels of hepcidin in patients with T2D as well as the surrogate markers of iron synthesis that are influenced by hepcidin action, particularly those that are associated with erythropoiesis. Overall, hepcidin levels were slightly elevated in T2D patients and this was concomitant with a modest reduction in haemoglobin levels and a slight increase in iron and ferritin levels. Interestingly, the levels of hepcidin were dependent on weight, whereby overweight was associated with elevated levels whilst class I obesity was characterised by a reduction in its concentration. The decrease in haemoglobin levels were independent of hepcidin levels, thus T2D patients present with subclinical anaemia. A summary of findings is provided in table 3.

The accumulation of visceral fat in obesity promotes insulin resistance and the secretion of pro-inflammatory cytokines from the adipose tissues, such as IL-6 and TNF-α.[45,46] In addition to inhibiting the proliferation of erythroid precursor cells, these cytokines can inhibit renal erythropoietin synthesis, which is essential for erythropoiesis.[9,14,47] On the other hand, IL-6 via JAK/STAT signalling pathway can induce hepatic hepcidin synthesis[42,48] which promotes iron supply to the bone marrow for erythropoiesis. Therefore, a tight regulation is essential in maintaining a fine balance between hepcidin synthesis and erythropoiesis. However, due to alteration in the cytokine milieu in T2D, this balance is dysregulated. The decreased levels of hepcidin in the obese group may be attributed to modulatory effects of insulin on STAT signalling and hepcidin synthesis.[42,49] Since the relationship between circulating iron levels and hepcidin levels is directly proportional, an elevation in iron levels is expected to induce hepcidin synthesis via the BMP/SMAD signalling pathway. However, this mechanism seems to be altered in patients with T2D. This may be ascribed to increased PI3K/AKT activation, a regulator of glucose metabolism, which outweighs BMP/SMAD signalling as previously reported.[11] Moreover, the decreased levels of hepcidin may also be attributed to the suppression of hepcidin synthesis through nutrient-dependent mechanistic target of rapamycin (mTOR) signalling, which is well-established to be elevated in both obesity and T2D.[50,51] The suppression of hepatic glucose production through the activation of adenosine-monophosphate-activated protein kinase (AMPK) renders metformin its anti-

hyperglycaemic effects.[52] Notably, the phosphorylation of AMPK mediates the suppression of STAT3 signalling and hepcidin synthesis.[53] On the other hand, the use of insulin therapy is an effective strategy to improve glucose control in older patients with T2D, who usually present with poor insulin secretion capacity and are lean.[54] Interestingly, this therapy is closely associated with hypoglycaemia and hyperinsulinemia[54,55], with the latter known to directly promote and induce hepcidin synthesis.[27,49] Therefore, different treatment strategies may be a contributing factor to the variation in hepcidin levels in patients with T2D.

Anaemia, is common in T2D patients and is directly associated with the development of microvascular complications.[56] Normocytic-normochromic ACD, which is characterised by decreased levels of haemoglobin and haematocrit has been reported in patients with T2D.[9] Our study showed a reduction in haemoglobin levels in T2D patients in comparison to healthy individuals. Although this reduction did not constitute overt anaemia, it was suggestive of subclinical anaemia. Notably, the reduction in haemoglobin levels was more pronounced in the obese subgroup. In patients with ACD, elevated ferritin levels are expected due to increased iron retention in the reticuloendothelial cells and inflammation-induced production.[43] Our study showed that included T2D patients presenting with subclinical anaemia had elevated ferritin levels, irrespective of body weights. This increment is responsible for a lower hepcidin: ferritin ratio in patients with T2D.

Our systematic review and meta-analysis had a few limitations. For instance, there were substantial levels of unexplained statistical heterogeneity in the included studies. Moreover, most studies did not control for confounders such as inflammation and obesity which have an impact on hepcidin levels. Most of the included studies did not report on the treatment that these patients were on which could have modified the hepcidin levels. For instance, metformin, the standard drug used in obese T2D patients is known to cause anaemia due to vitamin B12 deficiency.[57] Lastly, the quality of cumulative evidence in this study was low due to the observational nature of the included studies which are associated with a high risk of selection bias due to lack of randomisation. Therefore, caution should be taken when extrapolating these findings. Nonetheless, our current study has significant strengths. A previous systematic review and meta-analysis by Karamzad and colleagues demonstrated a slight increase in hepcidin levels in patients with T2D.[58] Our current meta-analysis further explored the impact body weight on hepcidin levels. Other strengths included the robustness of our results and the methods used as indicated by the sensitivity analysis and high inter-rate reliability, respectively. Lastly, this study showed that hepcidin regulation in poor glucose control is a complex phenomenon that

is influenced by several factors and therefore its sole diagnostic and clinical utility in overweight or obese patients with T2D may lead to variable patient outcomes. However, hepcidin: ferritin ratio was recently proven to be a reliable and better maker for T2D risk stratification.[58]

5. Conclusion

Patients with T2D present with iron overload which is congruent with impaired metabolic function and elevated levels of hepcidin. While hepcidin levels were elevated in overweight patients, these were decreased in patients with grade I obesity, suggesting that the degree of obesity can greatly impact iron metabolism in T2D patients. Despite the body weights, patients with T2D generally present with subclinical anaemia marked by a reduction in haemoglobin levels despite an increase in iron stores. Overall, the findings highlight that the synthesis of hepcidin and its impact on iron metabolism in patients with T2D is a quite complex relationship that is depended on multifactorial factors.

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Table 1: Characteristics of included studies reporting on hepcidin levels and iron profiles in patients with type 2 diabetes (T2D) (n=11)

Study	Country	Study size	Male, n (%)	Age (years) BMI [kg/m^2]	Reported effect measures of iron metabolism in serum	Main findings
Jiang et al., 2011 [6]	China	64 participants (34 T2D and 30 controls)	35 (55%)	T2D (60.88 ± 10.99) [25.72 ± 3.28] Control (60.19 ± 6.74) [22.52 ± 2.44]	Hepcidin, haemoglobin, ferritin and erythropoietin	The levels of hepcidin, ferritin and erythropoietin were elevated in T2D patients, whilst haemoglobin levels were lower than in healthy controls. The levels of hepcidin positively correlated with ferritin levels among T2D individuals and the controls.
Zheng et al., 2011 [10]	China	238 participants (168 T2D and 70 controls)	77 (32%)	T2D (62.7 ± 8.7) [25.4 ± 3.65] Control (52.94 ± 8.03) [24.28 ± 1.25]	Hepcidin, iron, haemoglobin, and ferritin	Levels of hepcidin and iron were comparable between T2D patients and controls. However, the levels of ferritin and haemoglobin were elevated in patients with T2D.
Guo et al., 2013 [26]	China	1259 participants (555 T2D and 704 controls)	422 (34%)	T2D (64.45 ± 9.16) [25.3 ± 3.49] Control (58.55 ± 9.56) [24.46 ± 3.33]	Hepcidin, ferritin and haemoglobin	The levels of hepcidin were comparable between patients with T2D versus controls. However, the levels of ferritin and haemoglobin were elevated in the T2D group. A significant positive correlation was found between hepcidin and haemoglobin levels in patients with T2D.
Sam et al., 2013 [29]	Finland	66 participants (33T2D and 33 controls)	46 (70%)	T2D (53.34 ± 3.69) [32.23 ± 1.09] Control (49.63 ± 6.50) [31.03 ± 1.82]	Hepcidin, ferritin, hepcidin: ferritin ratio, haemoglobin	Patients with T2D had lower levels of hepcidin, hepcidin: ferritin ratio and haemoglobin levels when compared to controls. On the other hand, ferritin levels were increased in T2D patients when compared to controls.
Andrews et al., 2015 [25]	Chile	312 participants (166 T2D and 146 controls)	312 (100%)	T2D[#] (59.38 ± 9.04) [29.23 ± 3.73] Control (51.4 ± 15.7) [24.6 ± 2.2]	Hepcidin, iron, ferritin and haemoglobin	Increased levels of hepcidin, iron and ferritin levels were reported in T2D patients in comparison to controls. These increments were further exacerbated by the presence of obesity in T2D patients. Haemoglobin levels were decreased in T2D patients compared to controls. Notably, increased hepcidin levels were associated with iron and increased risk of developing T2D independent of weight.

Suarez-Ortegon et al., 2015[30]	Colombia	304 participants (65 T2D and 239 controls)	148 (49%)	T2D (53.1 ± 8.3) [30.4 ± 5.4] Control (45.5 ± 7.7) [26.0 ± 3.6]	Hepcidin, hepcidin: ferritin ratio, ferritin	Hepcidin levels and hepcidin: ferritin ratio were significantly decreased in patients with T2D in comparison to controls. However, ferritin levels were comparable between T2D and control.
Vela et al., 2017 [27]	Not indicated	80 participants (60 T2D and 20 controls)	57 (71%)	T2D (55.6 ± 6.1) [28.4 ± 3.7] Control (58.1 ± 9.3) [27.2 ± 3.4]	Hepcidin, hepcidin: ferritin ratio, ferritin, iron, haemoglobin, haematocrit, and red blood cell count	Hepcidin and hepcidin: ferritin ratios were comparable between T2D patients and control group. However, ferritin and iron levels were increased in T2D patients whilst the levels of haemoglobin, haematocrit and red blood cells count were decreased. There was no significant association between hepcidin levels and glucose or haematological parameters.
Altamura et al., 2017[11]	Germany	155 Participants (115 T2D and40 Controls)	113 (73%)	T2D (60.2 ± 6.9) [33.1 ± 5.5] Control (57.9 ± 11.7) [28.0 ± 4.4]	Hepcidin, iron, ferritin and haemoglobin and haematocrit.	Patients with T2D had reduced hepcidin levels when compared to control group. However, the levels of iron and ferritin were increased in T2D patients whilst haemoglobin and haematocrit levels were comparable. Notably, a significant positive association was found between iron and ferritin levels among patients with T2D.
Atyia et al., 2018 [5]	Egypt	60 participants (40 T2D and 20 controls)	Not reported	T2D (49.23 ± 5.62) [Not reported] Control (48.55 ± 7.76) [Not reported]	Hepcidin, ferritin, iron and hepcidin: ferritin ratio.	Hepcidin and ferritin levels were elevated in patients with T2D when compared to controls, whilst iron levels were comparable between T2D and control group with T2D patients exhibiting lower hepcidin: ferritin ratio. Elevated iron levels were associated with high risk of T2D.
Guo et al., 2018 [7]	China	44 participants (22 T2D and 22 Controls)	29 (73%)	T2D (56.45 ± 11.80) [25.48 ± 3.45] Control (52.45 ± 10.01) [24.43 ± 2.33]	Hepcidin, ferritin and hepcidin: ferritin ratio	Hepcidin and ferritin levels were elevated whilst hepcidin: ferritin ratio was decreased in T2D patients in comparison to the controls.

Shalitin et al., 2018 [28]	Israel	38 participants (18 T2D and 20 controls)	25 (66%)	T2D (15.1 ± 3.1) [2.45 ± 0.43] * Control (13.1 ± 3.0) [2.39 ± 0.36]	Hepcidin.	There were no differences in hepcidin levels between T2D the control group.

[#]: T2D group comprised of lean [Age: 61.3 ± 10.0; BMI: 25.4 ± 2.1] and obese [Age: 58.3 ± 8.3; BMI: 31.4 ± 2.5] groups ***:** BMIZ scores

Table 2: Pooled effect estimates of surrogate markers of hepcidin in patients with type 2 diabetes (T2D).

Effect Measure		Number of Studies	Number of participants	Effect Estimate					
				Model	MD	SMD	95% CI	I^2, p-value	Z, p-value
Iron levels	**Overall**	**5** [10,11,25,27]	**931**	**RE**	-	**0.06**	**-0.26 to 0.39**	**80%, p^H =0.0005**	**0.38, p=0.70**
	Overweight T2D	3 [10,25,27]	524		-	-0.11	-0.57 to 0.36	81%, p^H =0.005	0.46, p=0.65
	Obese T2D	2 [11,25]	407		-	0.30	-0.30 to 0.90	86%, p^H =0.007	0.98, p=0.33
Haemoglobin levels	**Overall**	**8** [6,10,11,25–27,29]	**2320**	**RE**	**-0.23**	**-**	**-0.46 to -0.01**	**85%, p^H <0.00001**	**2.02, p=0.04**
	Overweight T2D	5 [6,10,25–27]	1847		-0.43	-	-0.94 to 0.07	87%, p^H <0.00001	1.68, p=0.09
	Obese T2D	3 [11,25,29]	473		-0.15	-	-0.27 to -0.03	0%, p^H=0.37	2.44, p=0.01
Ferritin levels	**Overall**	**10** [6,7,10,11,25–27,29,30]	**2668**	**RE**	-	**0.60**	**0.32 to 0.88**	**88%, p^H <0.00001**	**4.15, p<0.00001**
	Overweight T2D	6 [6,7,10,25–27]	1891		-	0.57	0.31 to 0.83	74%, p^H=0.02	4.28, p<0.0001
	Obese T2D	4 [11,25,29,30]	777		-	0.65	-0.08 to 1.37	95%, p^H <0.00001	1.75, p=0.08
Hepcidin: ferritin ratio	**Overall**	**4** [7,27,29,30]	**494**	**RE**	**-0.19**	**-**	**-0.46 to 0.08**	**96%, p^H<0.00001**	**1.35, p=0.18**
	Overweight T2D	2 [7,27]	124		0.01	-	-0.08 to 0.09	0%, p^H =0.91	0.16, p=0.87
	Obese T2D	2 [29,30])	370		-0.37	-	-0.70 to -0.05	96%, p^H <0.00001	2.23. p=0.03

T2D groups were compared to healthy controls

Table 3: Summary of findings table

Type 2 diabetes compared to healthy controls

Patient or population and Exposure: Individuals with T2D

Comparison: Healthy controls (normoglycaemics)

Outcome: Hepcidin levels and iron overload

Outcomes	Absolute effects* (95% CI)		Relative effect (95% CI)	№ of participants (studies)	Certainty of the evidence (GRADE)	Comments
	Risk with control	Risk in T2D patients				
Iron regulator proteins Measured by the levels of hepcidin	-	The standardised MD in the exposure group was 0.07 higher (-0.30 to 0.44)	-	2 620 (11 observational studies)	⊕⊕OO LOW	
Iron profiles Measured by haemoglobin levels	-	The mean level in the exposure group was -0.23 mg/dl lower (-0.46 to -0.01)	-	2 320 (8 observational studies)	⊕⊕OO LOW	

***The risk in the intervention group** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).

CI: Confidence interval; **MD:** Mean difference; **OR:** Odds ratio; **NE**: Not estimable

GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

Abbreviations: T2D: Type 2 diabetes mellitus; **HAMP**: Hepcidin Antimicrobial Peptide; JAK/**STAT:** Janus kinase/signal transducer and activator of transcription; **BMP/SMAD:** Bone Morphogenetic Protein/s-Mothers Against Decapentaplegic; **TNF-α:** Tumour necrosis factor-α; **IL-6:** Interleukin-6; **PRISMA:** Preferred Reporting Items for Systematic Review and Meta-Analysis; **ACD:** Anaemia of chronic disease; **BMI**: Body mass index; **NAFLD:** Non-Alcoholic Fatty Liver Disease; **DIOS**: Dysmetabolic Iron Overload Syndrome; **mTOR:** Mechanistic Target Of Rapamycin; **AMPK:** Adenosine-Monophosphate-Activated Protein Kinase

Online Supplementary files

Table 1S: MOOSE Checklist for Meta-analyses of Observational Studies

Item No	Recommendation	Reported on Page No
Reporting of background should include		
1	Problem definition	3
2	Hypothesis statement	N/A
3	Description of study outcome(s)	3
4	Type of exposure or intervention used	3
5	Type of study designs used	4
6	Study population	4
Reporting of search strategy should include		
7	Qualifications of searchers (e.g., librarians and investigators)	4
8	Search strategy, including time period included in the synthesis and key words	4
9	Effort to include all available studies, including contact with authors	4
10	Databases and registries searched	4
11	Search software used, name and version, including special features used (e.g., explosion)	4
12	Use of hand searching (e.g., reference lists of obtained articles)	5
13	List of citations located and those excluded, including justification	6
14	Method of addressing articles published in languages other than English	N/A
15	Method of handling abstracts and unpublished studies	N/A
16	Description of any contact with authors	N/A
Reporting of methods should include		
17	Description of relevance or appropriateness of studies assembled for assessing the hypothesis to be tested	5
18	Rationale for the selection and coding of data (e.g., sound clinical principles or convenience)	N/A
19	Documentation of how data were classified and coded (e.g., multiple raters, blinding and interrater reliability)	6
20	Assessment of confounding (e.g., comparability of cases and controls in studies where appropriate)	N/A
21	Assessment of study quality, including blinding of quality assessors, stratification or regression on possible predictors of study results	5-6
22	Assessment of heterogeneity	6
23	Description of statistical methods (e.g., complete description of fixed or random effects models, justification of whether the chosen models account for predictors of study results, dose-response models, or cumulative meta-analysis) in sufficient detail to be replicated	6
24	Provision of appropriate tables and graphics	4
Reporting of results should include		
25	Graphic summarizing individual study estimates and overall estimate	9
26	Table giving descriptive information for each study included	7
27	Results of sensitivity testing (e.g., subgroup analysis)	9

28	Indication of statistical uncertainty of findings	9-10

Table 2S: PubMed search strategy

Terms	Search terms
#1	Hepcidin[MeSH Terms] = **2985 hits**
#2	Type 2 diabetes mellitus[MeSH Terms] = **132,238 hits**
Combined #1 and #2	(type 2 diabetes mellitus[MeSH Terms]) AND (hepcidin[MeSH Terms]) = **29 hits**
Combined #1and type 2 diabetes text	(hepdcidin[MeSH Terms]) AND type 2 diabetes mellitus = **35 hits**

*No restrictions were made on language, study type and publication date.

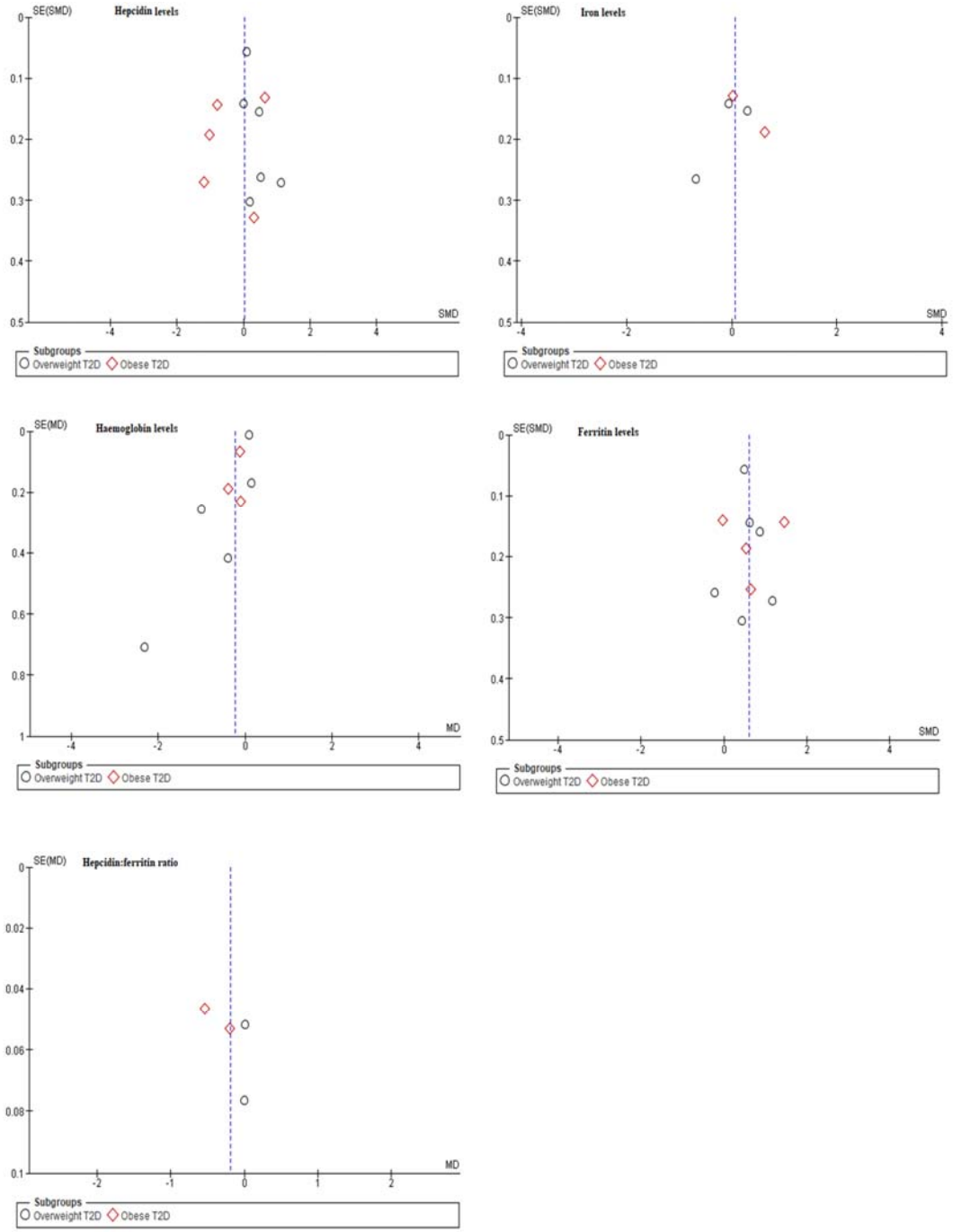

Figure 1S: Funnel plots used to assess publication bias

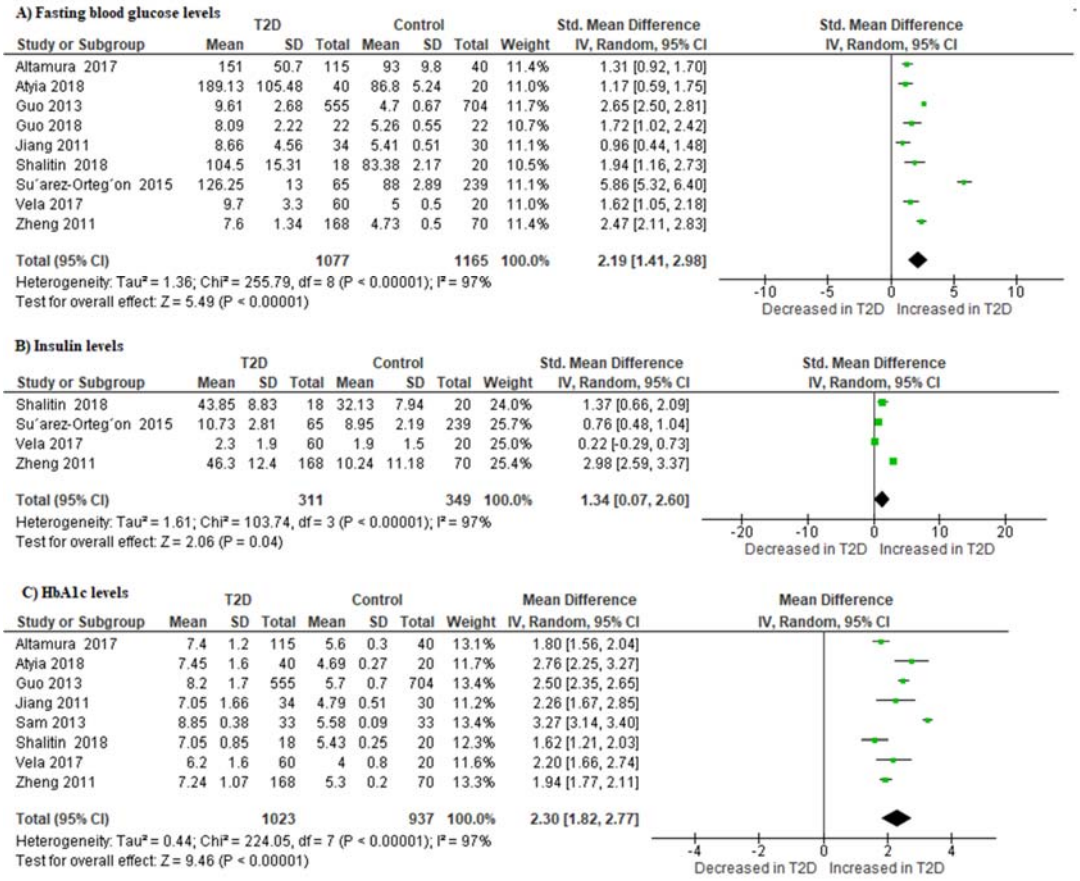

Figure 2S: A comparison of fasting glucose levels (a), insulin levels (b) and HbA1c levels (c) between individuals with type 2 diabetes (T2D) and healthy controls.

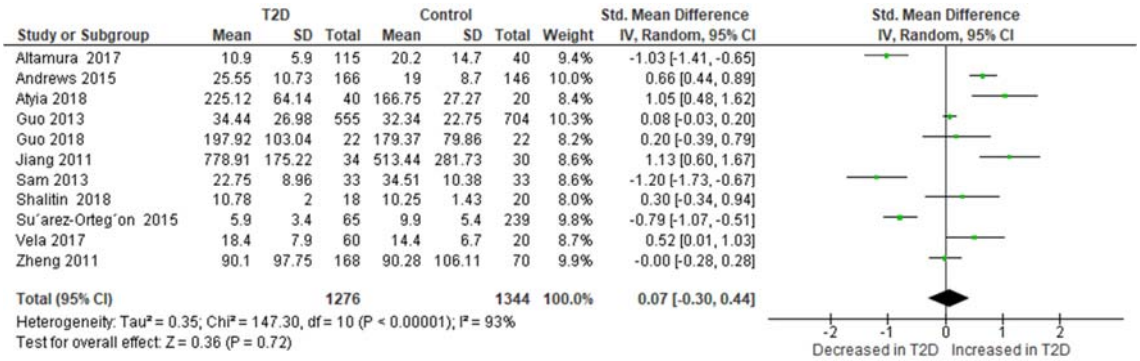

Figure 3S: Hepcidin levels in individuals with type 2 diabetes (T2D) compared to healthy controls

Table 3S: Quality assessment for included studies (Observational studies) using modified Newcastle-Ottawa scale (n=11)

Study and year	Selection				Average	Comparability		Outcome			Total quality score	Rating
	Representativeness of the sample	Selected group of users	Sample size	Diagnose		Age and sex	Score	Assessment of methods	Statistical test	Average		
Jiang 2011	0	*	0	***	**4**	*	**1**	*	*	**2**	**7**	**Good**
Zheng 2011	0	*	0	***	**4**	**	**2**	**	*	**3**	**9**	**Very good**
Guo 2013	0	*	0	***	**4**	*	**1**	*	*	**2**	**7**	**Good**
Sam 2013	0	*	0	*	**2**	**	**2**	*	*	**2**	**6**	**Satisfactory**
Andrews 2015	0	*	0	*	**2**	**	**2**	*	*	**2**	**6**	**Satisfactory**
Suarez-Ortegon 2015	0	*	*	*	**3**	*	**1**	**	*	**3**	**7**	**Good**
Vela 2017	0	*	0	*	**2**	*	**1**	*	*	**2**	**5**	**Satisfactory**
Altamura 2017	0	*	0	***	**4**	*	**1**	*	*	**2**	**7**	**Good**
Atyia 2018	0	*	0	*	**2**	**	**2**	*	*	**2**	**6**	**Satisfactory**
Guo 2018	0	*	0	***	**4**	*	**1**	*	*	**2**	**7**	**Good**
Shalitin 2018	0	*	0	*	**2**	**	**2**	**	*	**3**	**7**	**Good**

Table 4S. Sensitivity analysis of hepcidin levels based on treatment, gender and C-reactive protein levels.

T2D subgroup	Parameter	Number of studies	Omitted studies	SMD [95% CI]	I^2 (%), p^H value	Overall effect Z, p - value
Overweight	**All**	**6** (13,16–18,92,147)	**0** 4 (16–18,147)	**0.35 [0.07, 0.62]** 0.21 [-0.29, 0.72]	**77%, (p^H = 0.0005)** 67%, (p^H = 0.08)	**2.47 (p= 0.01)** 0.83 (p= 0.41)
	Insulin treatment	2 (13,92) 4 (16–18,147)	2 (13,92) 5 (13,16–18,147)	0.44 [0.02, 0.85] 0.52 [0.01, 1.03]	84%, (p^H = 0.0003) Not applicable	2.07 (p= 0.04) 1.99 (p= 0.05)
	Other treatments	1 (92) 5 (13,16–18,147)	1 (92)	0.32 [0.02, 0.62]	80%, (p^H = 0.0005)	2.10 (p= 0.04)
	Controlled CRP					
	Uncontrolled CRP					
Obese	**All**	**5** (11,16,19,99,158)	**0** 4 (11,16,99,158)	**-0.42 [-1.21, 0.38]** -0.13 [-1.41, -0.65]	**96%, (p^H <0.00001)** Not applicable	**1.03 (p= 0.31)** 5.33 (p<0.00001)
	Insulin treatment	1 (19) 4 (11,16,99,158)	1 (19) 4 (11,16,19,158)	-0.26 [-1.19, 0.67] 0.30 [-0.34, 0.94]	96%, (p^H <0.00001) Not applicable	0.55 (p= 0.58) 0.92 (p= 0.36)
	Other treatments	1 (99) 4 (11,16,19,158)	1 (99) 4 (11,16,99,158)	-0.58 [-1.50, 0.33] -1.03 [-1.41, 0.65]	97%, (p^H <0.00001) Not applicable	1.24 (p= 0.21) 5.33 (p< 0.00001)
	Children	1 (19) 4 (11,16,99,158)	1 (19)	-0.26 [-1.19, 0.67]	96%, (p^H <0.00001)	0.55 (p=0.58)
	Adults					
	Controlled CRP					
	Uncontrolled CRP					

Addendum 2 -Experiment paper 1 [Submitted for publication]

Reduced Serum Iron Levels and Red Cell Mean Volume are Apparent Features of Type 2 Diabetes with Underlying Inflammation

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Abstract

Aim: To investigating the impact of underlying inflammation on iron and lipid profiles of patients with type 2 diabetes (T2D).

Material and methods: A total of 50 participants with T2D were included in this study and were grouped based on their levels of c-reactive protein (CRP), *viz* normal (1-10 mg/L) and high (>10 mg/L) levels (n=25/group). All laboratory profiles were measured using standardised methods and conditions.

Results: Fasting plasma glucose (FPG) levels were significantly elevated in T2D patients with high CRP levels (11.12 ± 3.95) in comparison to those with normal CRP levels (8.86 ± 3.68), p=0.0413. However, the glycated haemoglobin levels were comparable between the two groups (p>0.05). The total serum iron levels were significantly reduced in patients with high CRP levels (12.78 ± 3.50) when compared to those with normal CRP levels (15.26 ± 4.64), (p=0.0381). There were no differences in ferritin and transferrin levels between the groups (p>0.005). An assessment of haematological indices showed comparable differences in red blood cell count, haematocrit, and haemoglobin levels between the two groups (p>0.05). However, the MCV in patients with high CRP was lower (87.66 ± 3.62) than in those with normal CRP levels (90.79 ± 4.52), p=0.0096. Triglycerides and cholesterol levels were comparable between the two groups (p>0.05). Correlational analysis only showed moderate association between CRP levels and total serum iron levels (Spearman r=-0.38, p=0.006) as well as the MCV (Spearman r=-0.41, p=0.003). The multivariate regression analysis showed that CRP levels contributed significantly to the prediction of serum iron levels (β =-0.20, p=0.0032), while FPG did not (β =0.18, p=0.2129).

Conclusion: Exacerbated inflammation in patients with T2D is compatible with altered iron metabolism and aggravated poor glucose control. An underlying inflammatory state in these patients is associated with reduced levels of total serum iron and red cell mean volume. Therefore, mitigating inflammation may be of notable therapeutic benefit in circumventing the ultimate development of iron deficiency anaemia in these patients.

Keywords: Anaemia; C-reactive protein; cardiovascular risk, inflammation; iron profiles; type 2 diabetes

1. Introduction

Type 2 diabetes (T2D) is amongst the top four noncommunicable diseases that are currently exerting the greatest burden on the global healthcare sector (WHO, 2016). A recent report estimated the global prevalence of T2D to be at a staggering 9.3% in 2019 (IDF, 2019) (Saeedi et al., 2019) , with most of the cases being from low to middle income countries, including those in sub-Saharan Africa. The high incident of diabetes in these regions is ascribed to increased sedentary lifestyles and unhealthy diets which promotes the manifestation of obesity and insulin resistance (Li et al., 2012). Notably, these risk factors for T2D promote the development of a low-grade inflammatory milieu (Dandona et al., 2004; Shimobayashi et al., 2018) that is closely linked with the pathogenesis of T2D-associated complications such as iron and lipid dysregulation (Chrobak et al., 2017; Tahir et al., 2021; Van Der Wal et al., 2019).

It is now well-acknowledged that T2D is a chronic inflammatory disorder that is characterised by poor glucose control (ADA, 2015; Gonzalez et al., 2018; Hameed et al., 2015). A collection of increasing evidence suggests a strong association between dysregulated iron metabolism and T2D (Andrews et al., 2015; Jiang et al., 2011; Vela et al., 2018b). However, the exact mechanisms involved in the alteration of iron metabolism in T2D are a complex phenomenon that is poorly understood. In that context, both anaemia of iron deficiency (Aregbesola et al., 2013) and overload (Jiang et al., 2011) have been reported in patients with T2D. The apparent differences in the type of anaemia suggest the presence of underlying conditions beyond hyperglycaemia that may mediate the dysmetabolic iron syndromes in these patients (Fernandez-Real et al., 2002). Notably, obesity and the degree of inflammation have been identified as some of the major modulators of iron metabolism in patients with T2D (Vela et al., 2018b). The persistent low-grade inflammation primarily modulates the synthesis and action of important regulators of iron metabolism, namely hepcidin and erythropoietin (Pagani et al., 2019). Briefly, hepcidin is the principal regulator of iron absorption and transport that is coded by the *HAMP* gene, whilst erythropoietin regulates erythropoiesis in hypoxic and anaemic conditions (Jorge, P. Pinto , Sara, Ribeiro, Helena et al., 2008). Thus, these hormones regulate each other's function through a negative feedback mechanism that ultimately influences the iron profiles.

Patients with T2D have been reported to have reduced levels of total serum iron and iron stores (ferritin) (Goodnough et al., 2010; Miller & Tanno, 2010; Suárez-Ortegón et al., 2015), albeit others have described increased levels (Altamura et al., 2017; Andrews et al., 2015; Sam et al., 2013). Similarly, contradictory levels of transferrin, a transporter of iron have been reported in these patients (Jiang et al., 2011; Shalitin et al., 2018; Suárez-Ortegón et al., 2015). Thus, these findings support both hypotheses of iron deficiency and overload in

T2D, and the existence of other underlying factors that influences iron metabolism besides poor glucose control. It is well-acknowledged that patients with T2D have an increased cardiovascular risk that is mainly driven by inflammation and altered lipid metabolism (Battisti et al., 2003; Bitzur et al., 2009; Martín-Timón, 2014). Interestingly, anaemia is closely associated with dyslipidaemia (Shirvani et al., 2017; Zhu et al., 2019). Therefore, this study primarily aimed to investigate the effect of underlying inflammation in patients with T2D on iron profiles and associated haematological indices. In addition, it aimed to assess cardiovascular risk and determine whether there are any correlations between iron, inflammation, and lipid profiles in patients with T2D.

2. Materials and methods

2.1 Study population

Included participants were randomly selected from clinically known outpatients with T2D from Khomas urban areas that visited the Katutura Community Health Centre, Windhoek, Namibia between September 2020 and December 2020. A total of one hundred and sixty-one (n=161) adult patients were enrolled, and only fifty (n=50) were included in this study. The sample size was calculated using G*Power software (Version 3.1.9.2) based on a previous study (Praveen et al., 2020). The following assumptions were used in determining the minimum number of required participants; a two tailed student t test; effect size d = 0.898071; α err prob = 0.05; power (1-β err prob) = 0.80 and an allocation ratio of 1:1. As a result, a minimum of 21 patients per group were required and considering a 5% arbitration rate, we increased the sample size by 4 per group and ultimately included 25 patients per group. The cases of T2D were diagnosed by clinicians using the American Diabetes Association guidelines (ADA, 2020). We excluded pregnant patients, patients under the age of 18 years old and patients without confirmed cases of T2D. The included patients were divided into two groups based on their inflammatory state using the levels of CRP, an acute phase reactant (n=25/group). The normal CRP group consisted of patients with CRP levels $\leq$10 mg/L, whilst the high CRP group had patients with CRP levels of >10 mg/L. Informed consent was sought from all participants and the research was conducted in accordance with the Declaration of Helsinki (2008) of the World Medical Association. The study was approved by the Namibia University of Science and Technology Research Ethics committee (FHAS 1/2020) and the Ministry of Health and Social Services (17/3/3 MN).

2.2 Laboratory measurements

Blood samples were collected into EDTA and SST vacutainers tubes for analysis at an ISO 15189 of 2012 accredited laboratory (Namibia Institute of Pathology, Windhoek, Namibia). The fasting plasma glucose and Hb1Ac levels were measured using the Cobas c501 analyser (Roche, Basel, Switzerland). The CRP levels and

ESR were determined using the Alinity c analyser (Abbot, Illinois, USA) and Test 1 THL Alifax S.p.A (Alifax, Udine, Italy), respectively. Complete blood counts were measured using a Sysmex 1000 XN automated haematology analyzer (Sysmex Corporation, Kobe, Japan). Lipograms (cholesterol levels and triglycerides) and iron parameters (serum iron, transferrin, and ferritin) were determined by the Alinity c and i analysers (Abbot, Illinois, USA).

2.3 Statistical methods and data analysis

The D'Agostino & Pearson test was performed for normality testing. All data were reported as mean ± SD or median and interquartile range [IQR] depending on the data distribution. For parametric data, the unpaired two-tailed student's t-test was used and in cases of unequal variance, a Welch's correction was performed. The Mann-Whitney *U* test was used to compare non-parametric data. Correlations were performed using the Pearson coefficient. A p-value < 0.05 represented statistical significance. All statistical analysis was performed using Graph Pad Prism 8 version 8.0.2 Software (Graph Pad Software Inc, San Diego, CA, USA).

3. Results

A total of 50 adult patients with T2D were included in this study, 25 with normal and 25 with high CRP levels. The demographic and characteristics of the included participants are shown in Table 1. The groups had a similar age and gender distribution, and the patients were from a similar socio-economic and ethnic backgrounds as they were recruited from the same community. Overall, the included patients had a mean age of 50.16 ± 12.72 years and a male to female ratio of 0.43.

3.1 Clinical parameters and glucose parameters

There were no significant differences in the body mass index (BMI), systolic blood pressure, diastolic blood pressure and disease duration between the two groups ($p>0.05$) (Table 1). The odds of hypertension were higher in patients with underlying inflammation when compared to those without (OR =1.64, 95% CI [0.53; 5.09]). The levels of Hb1Ac were comparable between the two groups ($p=0.6125$), however, fasting plasma glucose levels were higher in T2D with high CRP levels (11.12 ± 3.95) when compared to T2D with normal CRP group (8.86 ± 3.68), $p=0.0413$ (Table 1).

Table 1: Clinical characteristics and laboratory profiles of included patients (n=50)

Parameter	T2D with normal CRP (n=25)	T2D with high CRP (n=25)	p-value
Clinical characteristics			
Age (years)	50.64 ± 13.63	49.68 ± 12.01	0.7927
Male n (%)	7 (28)	8 (32)	-
Body mass index ((kg/m^2)	28.13 ± 4.94	30.43 ± 5.62	0.1394
Systolic blood pressure (mm/Hg)	137.5 ± 21.83	137.4 ± 20.18	0.9833
Diastolic blood pressure (mm/Hg)	84.13 ± 11.38	86.39 ± 13.00	0.5334
Hypertension n (%)	13 (52)	16 (64)	-
Duration of T2D (years)	5.00 [2.00 – 12.00]	4.00 [1.00 – 13.00]	0.9807
Glucose profiles			
Glycated haemoglobin (%)	8.500 [6.20 – 9.90]	8.700 [7.20 – 9.40]	0.6125
Fasting plasma glucose (mmol/L)	8.86 ± 3.68	11.12 ± 3.95	**0.0413**
Inflammatory profiles			
C-reactive protein (mg\L)	3.87 ± 2.56	17.06 ± 7.88	**<0.0001**
ESR (mm/hr)	22.60 ± 19.45	44.57 ± 32.13	**0.0114**
Haematological profiles			
White cell count (10^9/L)	6.77 ± 1.49	7.11 ± 1.99	0.4922
Platelet count (10^9/L)	309.2 ± 81.06	327.9 ± 87.21	0.4357
Red cell count (10^12/L)	4.82 ± 0.53	4.85 ± 0.48	0.8332
Haemoglobin (g\dl)	14.28 ± 1.19	14.08 ± 1.37	0.5841
Haematocrit (%)	42.74 ± 5.17	42.48 ± 4.19	0.8482
Mean cell volume (fL)	90.79 ± 4.52	87.66 ± 3.62	**0.0096**
MCH (pg)	29.32 ± 1.49	28.64 ± 1.44	0.1057
MCHC (g/dL)	32.90 ± 1.08	32.94 ± 0.91	0.8878
Red cells distribution width %	13.33 ± 1.12	13.40 ± 0.86	0.8108
Iron profiles			
Serum iron (µmol/L)	15.26 ± 4.64	12.78 ± 3.50	**0.0381**
Ferritin (ng\mL)	180.9 ± 102.1	175.3 ± 113.4	0.8559
Transferrin (g\L)	2.59 ± 0.35	2.76 ± 0.44	0.1502
Lipid profiles (mmol\l))			
Triglycerides (mmol\L)	1.67 ± 0.68	1.62 ± 0.69	0.8042
Total cholesterol (mmol\L)	4.91 ± 1.13	4.59 ± 0.77	0.2481
LDL-cholesterol (mmol\L)	3.10 ± 1.04	2.80 ± 0.87	0.2617
HDL-cholesterol (mmol\L)	1.02 ± 0.27	1.06 ± 0.32	0.6419
HDL/Cholesterol ratio	0.22 ± 0.06	0.23 ± 0.07	0.5313

T2D: type 2 diabetes; **ESR:** erythrocyte sedimentation rate; **MCH:** mean corpuscular haemoglobin; **MCHC:** mean corpuscular haemoglobin concentration; **LDL:** low-density lipoprotein; **HDL**: low-density lipoprotein. Results expressed as mean ± standard deviation and median interquartile range.

3.2 Inflammatory profiles

The levels of CRP were used as a dependent factor to group the patients. As expected, the levels of CRP significantly differed between the groups ($p<0.0001$) (Table 1). Similarly, the ESR levels were elevated in the

T2D with high CRP group (44.57 ± 32.13) when compared to the T2D with normal CRP levels (22.60 ± 19.45), p=0.0114) (Figure 1a, Table 1). However, there were no significant differences in the WCC, and platelet counts between the two groups (p>0.005) (Figure 1b-c, Table 1).

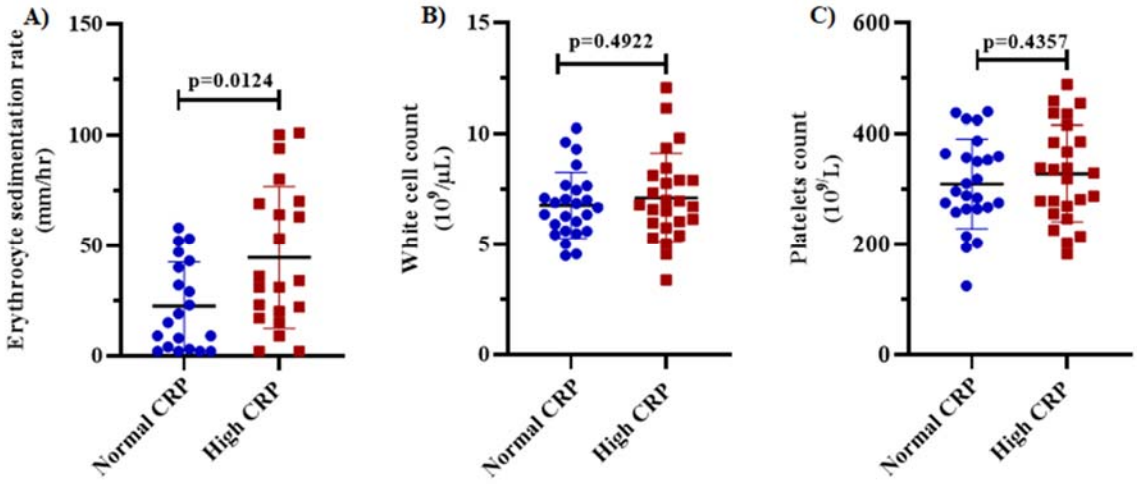

Figure 1. Inflammatory parameters in type 2 diabetes with normal and high C-reactive protein (CRP). Figure **(A)** shows a significantly increased erythrocyte sedimentation rate in patients with high CRP when compared to those with normal levels. Figure **(B)** and **(C)** demonstrates comparable levels of white cell and platelet counts between the two groups. All results were expressed as mean ± standard deviation.

3.3 Iron profiles levels and red cells indices

In order to assess the impact of underlining inflammation on iron metabolism, we measured iron profiles in patients with T2D. The T2D with high CRP levels had lower levels of serum iron (12.78 ± 3.50) in comparison to T2D with normal CRP levels (15.26 ± 4.64), (p=0.0381) (Figure 2a). However, there were no differences in the levels of ferritin and transferrin groups (p>0.005) (Figure 2e-f, Table 1). We further measured haematological indices that are closely associated with iron metabolism. Notably, although RBC count, haematocrit and haemoglobin levels were comparable between the two groups (p>0.05) (Figure 1a-b), the MCV in T2D with high CRP was lower (87.66 ± 3.62) than in T2D with normal CRP levels (90.79 ± 4.52), p=0.0096 (Figure 2c).

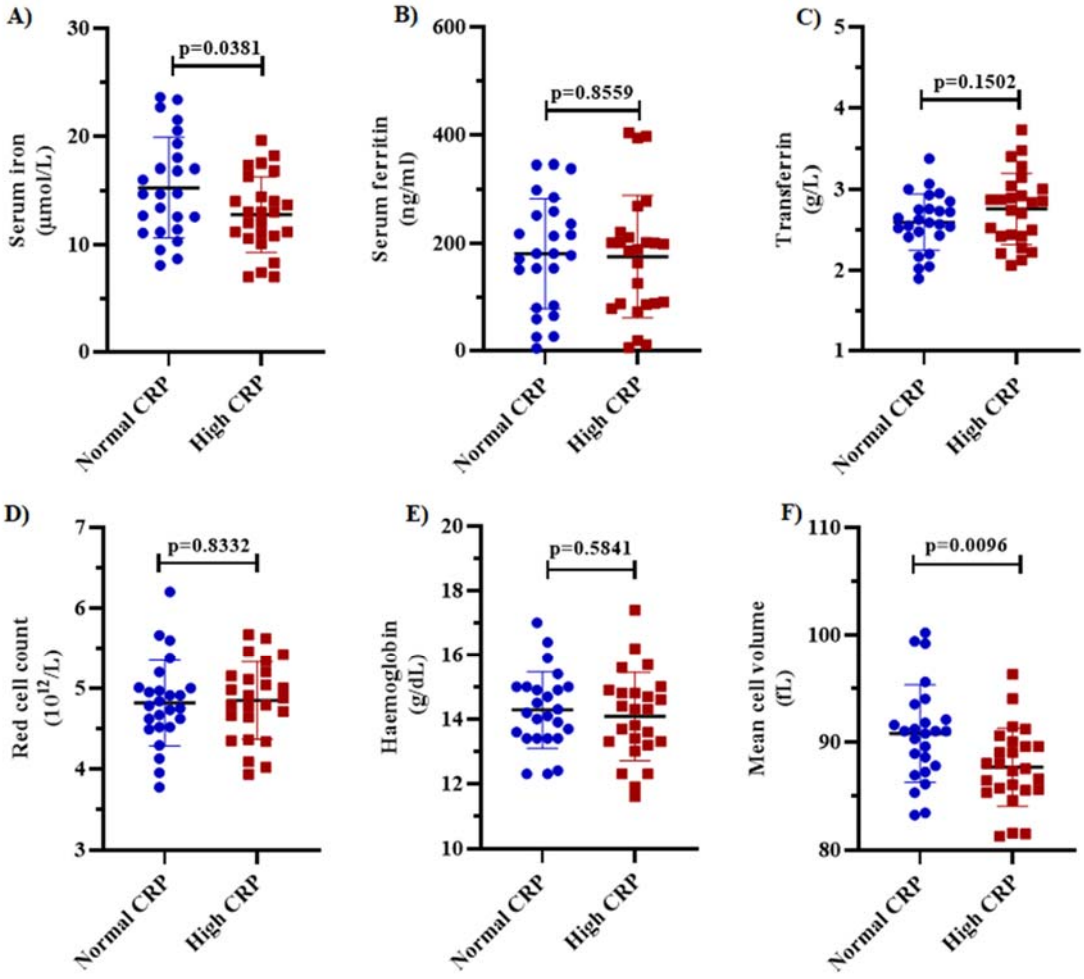

Figure 2: A comparison of iron profiles between patients with normal and high C-reactive protein (CRP) levels. The levels of total serum iron (Figure **A**) and red cell mean volume (Figure **F**) were significantly lower in patients with underlying inflammation when compared to those without. However, comparable levels of ferritin (Figure **B**) and transferrin (Figure **C**) as well as red cell count (Figure **D**) and haemoglobin (Figure **E**) were observed between the two groups. All results were expressed as mean ± standard deviation.

3.4 Lipid profiles levels

Dyslipidaemia is closely associated with increased cardiovascular risk in patients with T2D (Mooradian, 2011).We therefore, measured lipograms in patients with T2D. The levels of triglycerides (p=0.8042), total cholesterol (Tc) (p=0.2481), low-density lipoprotein (LDL)-c (p=0.2617), high-density lipoprotein (HDL)-c (p=0.6419) and HDL/cholesterol ratio (p=0.5313) were comparable between the two groups (Table 1).

3.5 Correlation and regression analysis of glucose levels, CRP and iron profiles

We performed a correlation analysis to determine whether there are any associations between glucose, inflammation, and iron profiles in patients with T2D. Notably, the CRP levels were moderately associated with serum iron levels (Spearman r=-0.38, p=0.006) and the MCV (Spearman r=-0.41, p=0.003). As expected, the levels of ESR positively correlated with those of CRP (Spearman r=-0.54, p<0.0001) and was also associated with the MCV (Spearman r=-0.37, p=0.0008). However, there was no correlation between FPG and iron or inflammation profiles (p>0.05). A multivariate regression analysis was performed to further investigate whether CRP and FPG levels could predict serum iron levels in T2D. The results showed that the model was a significant predictor of iron levels in patients with T2D ($F_{(2,47)}$ = 5.43, p=0.0075), and could only explain a total of 18.87% of the variance in the regression model. Notably, CRP levels contributed significantly to the prediction of iron levels (β =-0.20, p=0.0032), whilst FPG did not (β =0.18, p=0.2129) (Table 2). The relationship of both the actual and predicted values, and the residual is shown in Figure 3.

Table 2: Multivariate linear regression analysis of independent variables of serum iron levels in T2D

Variable	Beta	Standard error	95% Confidence interval	|t|	p-value
Intercept	14.30	1.63	11.03 to 17.58	8.79	**<0.0001**
C-reactive protein	-0.20	0.06	-0.33 to -0.07	3.11	**0.0032**
Fasting plasma glucose	0.18	0.14	-0.11 to 0.46	1.26	0.2129

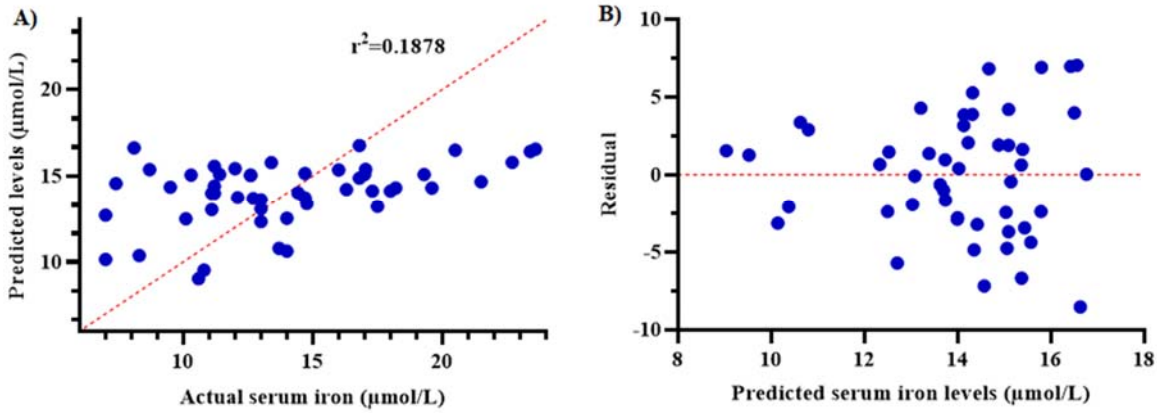

Figure 3: The graph shows the interpretation of the predicted and the actual values of total serum iron levels and the residual.

4. Discussion

This study aimed at investigating the impact of underlying inflammation on iron profiles in patients with T2D and their association with lipid parameters. Our results showed reduced total serum iron levels and red cell mean volume in patients with significant underlying inflammation when compared to those without underlying inflammation. Moreover, the FPG levels in patients with underlying inflammation was significantly higher than

those without, thus suggesting exacerbated impairment of glucose control in a pro-inflammatory state. Interestingly, the levels of CRP in patients with T2D inversely correlated with total serum iron levels and the MCV, whilst ESR was only associated with the latter. Our linear regression model showed that only the levels of CRP were negatively associated with total serum iron levels in patients with T2D. Thus, highlighting the influence of underling inflammation on iron metabolism. Lastly, although a negative association between iron levels and lipid profiles (triglycerides, Tc and LDL-c) has been previously described (Wolide et al., 2017), our current study found no associations.

Hepcidin is one of the hormones that maintains iron haemostasis through the inhibition of iron absorption and release from the intestines and macrophages as well as its subsequent transportation to the bone marrow for erythropoiesis (Collins et al., 2008; Vela, 2018). The synthesis of iron is dependent on the inflammatory status and the oxygen carrying capacity in the body (D'Angelo, 2013; Vela et al., 2018b). In that context, the increased release of IL-6 during inflammation induces the activation of JAK/STAT3 signalling which activates the transcription of the *HAMP* gene (Tomas, 2008). Whereas, an increase in body iron stores activate the bone morphogenetic protein/s-mothers against decapentaplegic (BMP/SMAD) transduction pathway which induces the downstream activation of *HAMP* gene and the translation of hepcidin thereof (Parrow & Fleming, 2014; Silvestri et al., 2019). The synthesis and action of hepcidin is also modulated by erythropoietin, a hormone that is released in anaemic hypoxia to initiate red cell synthesis (Pagani et al., 2019; Pasricha et al., 2016). The dysregulation of these important hormones alters iron metabolism leading to the manifestation of anaemia in T2D (Pagani et al., 2019). Although the patients included in this cohort were not anaemic, the reduced levels of total serum iron levels and MCV suggests the early onset of microcytic iron deficiency anaemia in patients with T2D coupled with underlying inflammation. Therefore, alleviating inflammation in T2D could aid in improving iron metabolism in patients with a significant underlying inflammation since CRP and ESR levels were negatively associated with decreased total serum iron and MCV.

Obesity-induced inflammation is closely associated with insulin resistance and poor glucose control (Zeyda & Stulnig, 2009). Here we report elevated FPG levels in T2D patients with underlying inflammation. Notably, the group of patients with underlying inflammation had class I obesity as denoted by the BMI >30kg/m^2. These findings further highlight the effect of inflammation in aggravating poor glucose control in T2D via the activation of various pathways and the impairment of insulin signalling as previously reviewed (Kuryłowicz & Kózniewski, 2020; Tsalamandris et al., 2019). Obesity predisposes patients with T2D to develop CVD (Piché et al., 2020;

Scherer & Hill, 2016). In fact, about a third of patients with T2D have CVD (Einarson et al., 2018). This association has been ascribed to exacerbated inflammation and altered lipid metabolism which causes atherosclerosis and hypertension (Petrie et al., 2018). With regards to the latter, although the blood pressures were comparable between the two groups, it is evident that the patients were hypertensive, and the odds were higher in individuals with underlying inflammation. Therefore, the use of anti-inflammatory drugs such as low-dose aspirin in patients with T2D is important in reducing cardiovascular risk in these patients as previously described (Abi Khalil et al., 2018; Pignone et al., 2010).

Dyslipidaemia promotes the initiation of atherosclerosis and arterial thrombosis, some of the major risk factors for the pathogenesis of CVD in patients with T2D (Katakami, 2018; Ray & Rosendaal, 2001; Watson et al., 2015). The aberrant cholesterol and triglycerides levels are classical features of dyslipidaemia (Chehade et al., 2013; Mooradian, 2011). Although triglycerides and cholesterol levels were comparable between the groups, elevated triglycerides, Tc, and LDL-c coupled with reduced HDL-c levels were reported in patients with T2D and was closely associated with increased cardiovascular risk (Chahil & Ginsberg, 2006). Therefore, cholesterol lowering drugs such as statins are recommended in patients with T2D in order prevent cardiovascular events (Bitzur, 2011; Sillars & Sattar, 2019). In all, this current study had one major weakness, that is, we did not assess the levels of important regulators of iron metabolism such as hepcidin and erythropoietin. This could have helped in making an inference in the differences in iron levels between the two groups that we investigated. We recommend for future studies to investigate these regulators in T2D with and without underlying inflammation.

5. Conclusion

Apart from the impaired glucose control, iron metabolism is significantly influenced by the inflammatory status in patients with T2D. As such, reduced total serum iron levels and red cell mean volume are apparent features of underlying inflammation in T2D. The exacerbated inflammation in these patients is closely associated with increased incidence of hypertension. Therefore, the amelioration of inflammation in patients with T2D may be an effective intervention to circumvent the eventual development of iron deficiency anaemia.

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Authors' contributions

FN, BBN and TMN conceptualised, designed the study and drafted the manuscript. FN, BBN and TMN performed formal analysis, methodology and validation as well as visualisation. All authors including PVD, MM and KNN wrote, reviewed, edited and approved the final manuscript.

Declaration of Interest

None.

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