ASSOCIATION BETWEEN HEPCIDIN, INSULIN RESISTANCE AND CRP IN TYPE 2 DIABETES PATIENTS AT THE KORLE- BU TEACHING HOSPITAL

BY

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DECLARATION

I, Linda Adu-Gyamfi Boadi of the department of Medical Biochemistry, University of Ghana Medical School, do hereby declare that, with the exception of quoted articles and references to other works which have dully been acknowledge, this thesis is the result of my own research under the supervision of Dr. Kwame Yeboah of the department of Physiology, University of Ghana. Medical School and Dr. Nii Ayitey Aryee of the department of Medical Biochemistry, University of Ghana. School of biomedical and Allied Health Sciences.

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ABSTRACT

Background: Diabetes Mellitus (DM) is a significant cause of morbidity and mortality and remains a global health challenge. Type 2 diabetes (T2DM) is a leading root of cardiovascular diseases worldwide. Growing evidence indicates a strong association between T2DM and high level of hepcidin; a major regulator of iron homeostasis. Iron overload is an important risk factor that plays a major role in pathogenesis of diabetes and its complications. Insulin resistance and elevated c-reactive proteins have been shown to predict the occurrence of target organ damage in diabetes and in the general population, possibly across all age ranges. The study of the relationship between the major regulator of iron metabolism; hepcidin, C- reactive protein (CRP) and insulin resistance (IR) in type 2 diabetes patients may shed more light on the mechanisms of diabetes complications which may ultimately improve prevention and management strategies of this debilitating disease.

General Aim: this study sought to investigate the association between hepcidin, insulin resistance and C-reactive protein levels with T2DM and their connected variables in samples of diabetes patients at Korle-Bu teaching Hospital.

Method: In the case-control study design, 111 T2DM patients were recruited from the National Diabetes Management and Research Centre (NDMRC), Korle-Bu, and 29 non-diabetic adults (controls) were recruited from surrounding communities. Body mass index, triglycerides, glucose, insulin, hepcidin and C- reactive protein levels were determined for each participant. HOMA2-IR was calculated. T2DM was defined as a fasting plasma glucose (FPG) level $\geq$ 7.0 mmol/L, and the use of anti-diabetic drugs. Statistical analyses were performed using SPSS version 20.

Anthropometric measurements such as body fat, weight (kg), height (m), waist circumference (m) and hip circumference (m) were also measured.

Results: in unadjusted analysis, a significant difference in mean values of FPG, Total cholesterol, HDL cholesterol, Low density Lipoprotein cholesterol, Triglyceride, insulin, and HOMA-IR was observed between subjects with and without T2DM. CRP level was significantly higher in both T2DM and non T2DM subjects ($p = 0.02$). In both cases and controls, there was a significant association and positive correlation between CRP and hepcidin ($p< 0.01$), log insulin ($p= 0.02$), and HOMA-IR ($p= 0.05$). Subjects with higher CRP levels (> 3.0 mg/L) are at increased risk of T2DM compared to those with low CRP levels. Hepcidin correlated negatively with IR ($p= 0.05$) and Insulin ($p= 0.02$) and correlated negatively with β cell function ($p= 0.04$) and insulin sensitivity ($p= 0.04$).

Conclusions: These results suggest that elevated CRP, increased IR and reduced hepcidin levels are associated with T2DM.

Recommendations: Further work with larger sample size as well as other confounding protein analysis should be carried out in the future. Further longitudinal studies should be carried out to identify the causative link which might be unique to the Ghanaian.

DEDICATION

I dedicate this work to my Uncle, Dr. Douglas Adu- Gyamfi for his immense contribution to my academic life. I also dedicate this work to my parents, Mr. Francis Fofie and Ms. Regina Adu-Gyamfi for their understanding and support. I also dedicate this work to my better half Mr. Yaw Kankam Frimpong- Mansoh for his love and understanding and finally to my lovely siblings.

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TABLE OF CONTENT

LSIT OF FIGURES

LSIT OF TABLES

ABBREVIATIONS

AGE	Advanced glycation end products
BMI	Body mass index
BIA	Body adiposity Index
CDC	Centre for Disease Control
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardio vascular disease
DA	Diacyl glycerol
DM	Diabetes mellitus
DBP	Diastolic blood pressure
ELISA	Enzyme-linked immunoabsorbent assay
FPN	ferroportin
HDL	High density lipoprotein cholesterol
HR	Heart Rate
LDL	Low density lipoprotein cholesterol
MAP	Mean Arterial Pressure
NDMRC	National Diabetes Management and Research Centre
NIDDM	Noninsulin dependent diabetes mellitus
NTBI	Non-transferrin bound iron
OGTT	Oral glucose tolerance test

PBS	Phosphate Buffer Saline
PI	Ponderal index
PKC	Protein kinase C
PP	Pulse pressure
SBP	Systolic blood pressure
STEAP	Six-transmembrane epithelial antigen of prostate
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 Diabetes Mellitus
TChol	Total cholesterol
TG	Triglycerides
THR	Thigh circumference/ head circumference ratio
WHO	World Health Organization
WHR	Waist-to-hip ratio
WSR	Waist-to-stature ratio

CHAPTER ONE

INTRODUCTION

1.1 Background

Diabetes Mellitus (DM) is a metabolic disorder categorized by the rise in plasma glucose level resulting from either insufficient secretion of insulin or inefficient stimulation of the target cells (WHO, 2010 ; Himsworth., 1936). Two major forms of DM exist; type 1 diabetes mellitus (T1DM), constituting 10% of DM cases, results from an auto immune destruction of insulin secreting pancreatic β cells. Type 2 diabetes mellitus (T2DM), which is the other type constitutes 90% of DM cases and is considered as resistance in the action of glucose (WHO 2015). T2DM is either triggered by reduction in the level of insulin receptors or weakening of the binding affinity of insulin (American Diabetes Association. 2013). T2DM remains a major health challenge worldwide, predominantly in low and middle-income countries where it comprises a serious risk factor of cardiovascular diseases (CVD) and kidney failure (Mazzone et al., 2008). Other clinical conditions such as centralized obesity, high plasma iron concentration, vascular disease, neuropathy and increased predisposition to microbial infections are also associated with T2DM (Alberti et al, 2006 ; Zimmet et al, 2004).

Basically, the insulin resistant (IR) state in T2DM is the inability of the muscle to take up glucose and also rise in glucose production by the liver which results in hyperglycemia (Catalano et al., 2014). Also, the pathophysiological occurrence of T2DM in addition to β cell failure is the inability of target tissues to take up insulin, thus resulting in irregular insulin secretion (Muoio and Newgard., 2008). Although, the exact sequence of events leading to insulin resistance are yet to

be known, current studies have however added to the knowledge of the fundamental molecular mechanisms (Higgins et al., 1996).

T2DM has been the sequeale of iron overload, associated with excess plasma iron. Elevated serum hepcidin level gives an indication of reduced iron absorption, and low level of hepcidin results in enhanced activity of ferriportin and excessive availability of plasma iron (Jiang et al. 2011). Slightly elevated body iron is known to be a contributing factor of T2DM (Aregbesola et al. 2015). Also, according to Fernandez- Real et al (2013) "serum glucose concentration is known to normalize serum hepcidin" (Fernández-Real et al., 2015; Aigner et al., 2013). However, the exact mechanism is not well understood, with some recent epidemiologic studies that reported association between serum hepcidin and T2DM with inconsistent results (Jiang et al., 2011; Simcox and McClain.., 2013)). Hepcidin level as indicated by various studies is controlled by iron stores, low oxygen level as well as inflammation (Simcox and McClain., 2013).

The idea of diabetes as inflammatory disease is now established. C-reactive protein (CRP); an inflammatory marker has gradually evolved as a risk factor for CVD (Hansson. 2005; Libby et al. 2002; Pepys et al. 2003). Rise in the level of this marker is known to be linked with increase in risk of T2DM progress. (Belfki et al. 2012; Barzilay et al. 2001; Doi et al. 2005). In 2003, Pepys conducted a study which established a higher CRP levels in diabetes as compared to non-diabetes (Pepys et al., 2003). Recently, a cross-sectional study by Belfki et al (2012) demonstrated an association of elevated CRP levels with insulin resistance and obesity. Glucose intolerance is also associated with elevated CRP level indicating the involvement of inflammation in the aetiology of T2DM (Dehghan et al., 2007; Pickup and Crook, 1998).

Epidemiologic observations, as well as investigational studies in mammals, conducted by Fernandez and colleagues showed a strong link between the tissue iron stores, which is primarily regulated by hepcidin, and diabetes risk (Fernández-Real et al., 2015). It is evident that plasma iron level at a particular time has an array of effects on numerous tissues which is either pro- or anti-diabetic, depending on the concentration (Swaminathan et al, 2007; Catalano et al, 1997). A section of such studies has proposed a causative relation. Thus, increased plasma iron level, indicating low hepcidin is enough to cause diabetes and excess iron in the pancreatic β cell which can be deadly (Pechlaner et al., 2016; Kulaksiz et al., 2008). As a result, minimal iron concentration is required for complete methylation of the proteins necessary for oxidation and sensing of glucose (Simcox and McClain., 2013 ; Finch. 1994). Findings of various studies have therefore proven that, increased plasma iron associated with diabetes risk as iron deficiency also poses a fundamental risk for diabetes and obesity. Hence, normal iron level is essential for physiological balance (Swaminathan et al., 2007; Simcox and McClain., 2013).

Although some studies have reported on T2DM in relation to various clinical conditions, few studies have assessed its association with iron metabolic regulator, hepcidin, in T2DM patients. However, the levels of serum hepcidin, insulin resistance and CRP in Ghanaians with T2DM has not been investigated, despite the essential role of iron metabolism in diabetes

1.2. Problem statement

The prevalence of T2DM, which is 3.5% in Ghana, implies that there will be drastic increase in burden and mortality of CVDs complications in the future (Mazzone et al. 2008). T2DM is linked with hyperinsulinemia and insulin resistance, both conditions poses major insults to the walls of

the blood vessels, resulting in arteriosclerosis. Cardiovascular complications of diabetes such as peripheral arterial disease (Yeboah et al. 2016) coronary arterial disease (Amoah et al., 2003), stroke (Agyemang et al, 2012) and kidney failure (Osafo et al, 2011) are reported to be on the rise in Ghana. This will put a lot of constraints on the already deprived health care system in Ghana.

T2DM patients are reported to have anomalies in their iron regulation and this may be suspected to surge the risk of CV mortality (Jiang et al. 2011; Liu et al. 2009). Hepcidin is the major hormone regulating iron bioavailability and hence, abnormalities in hepcidin levels affects iron metabolism (Fernández-Real et al., 2009; Ganz, 2006). Because hepcidin prevent cellular uptake of iron, abnormal increase in plasma hepcidin is associated with elevated plasma iron level in T2DM patients (Simcox and McClain, 2013; Aregbesola et al., 2015), whereas low level of plasma hepcidin could as well result in iron accumulation in the vessels wall, contributing to vascular damage, and hence the utility of plasma hepcidin levels as a predictive biomarker for CVDs (Liu et al., 2009; Ganz, 2006)). Since inflammation is considered as the significant regulator of T2DM pathogenesis, there is the need to investigate the link between C-reactive protein and T2DM as well.

1.3 Rational/Justification

The relation between diabetes mellitus and its complications remain an area of public health concern. Until recently, metabolic diseases were not given much attention as the disease was noted to be confined to the wealthy people due to the kind of lifestyle lived. This ever changing lifestyle in low income countries has led to the rapid upsurge in the burden of T2DM, of which epidemiological trend has caught up with Ghana ((Eckel et al., 2004; Amoah et al. 2003; (Amoah

et al., 2002a). With the current increase in the trend of diabetes among all the different social categories of people, much research has to focus on the pathogenesis and possible targets of the pathophysiological pathway that could be reversed to obstruct the commencement of diabetic complications. In the sub-Saharan region of Africa, diabetes is linked to increased risk of failure and mortality during CVDs treatment.

Dysregulation of hepcidin, resulting in excess iron level, is established as an autonomous factor that contributes to DM pathogenesis and cardiovascular problems. This eventually causes hepatic injury as a result of oxidative stress (Aregbesola et al., 2015; Pechlaner et al., 2016). This may eventually result in resistance in insulin activity and reduction in insulin level as well as secretion (Simcox and McClain. 2013). Prospective clinical studies conducted by Fernández-Real et al. (2002) have proven that "body iron load is positively correlated with the prevalence of T2DM". Insulin resistance, the hallmark of T2DM is associated with decreased serum hepcidin level which is an indication of iron overload in T2DM (Jiang et al., 2011). However, a direct relationship between the two remains unclear. But, molecular and genetic studies have indicated that, hepcidin expression is stimulated by insulin via STAT3, a novel transcription modulator of hepcidin (Campos et al., 1996; Kartikasari et al., 2008). These regulatory signal pathway links glucose and iron metabolism in the liver and identifies hepcidin, the iron hormone, as a gluconeogenic sensor. Inflammations involved in the pathogenesis of T2DM stimulate hepcidin synthesis (Fleming, 2008). Studies have shown that, C-reactive proteins and other pro-inflammatory cytokines responsible for this inflammation has a positive correlation with T2DM (Barzilay et al., 2001; Pradhan et al., 2001). Understanding manifestation as well as related risk of this link however, is necessary to guide diagnosis, management and prevention in both cases.

1.4. Hypothesis

- Compared to non-diabetes controls, T2DM patients have low levels of plasma hepcidin.
- Elevated plasma hepcidin and insulin levels are linked to plasma C-reactive protein elevation in T2DM patients.
- Insulin resistance affect iron metabolism with increased levels of plasma hepcidin.

1.5 Aim

This study purpose to examine the association between hepcidin, insulin resistance and C-reactive protein in T2DM patients.

1.6 Specific Objectives

The specific objectives of this study are to;

- Determine and compare the levels of insulin resistance, plasma C-reactive protein and plasma hepcidin in T2DM and non-T2DM patients
- Study the association of insulin resistance with hepcidin in T2DM and non-T2DM patients.
- To study the association of hepcidin and C-reactive protein in T2DM and non-T2DM patients.
- Study the association of insulin resistance with C-reactive proteins in T2DM and non-T2DM patients

CHAPTER TWO

LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder which is categorized by prolonged hyper glycaemia with alterations in carbohydrate, fat and protein metabolism. This disorder occurs as a result of abnormalities in the secretion of insulin, its action or both and can happen when abnormal chemical reactions in the body alters the normal metabolic process(Alberti and Zimmet., 1998; American Diabetes Association, 2004).

DM presents with distinctive signs such as polydipsea, polyuria, blurred vision and loss in weight (Alberti and Zimmet., 1998). In severe case, ketoacidosis may develop which can lead to stupor and/or coma. There is also the likelihood of occurrence of death if not effectively treated (Brown & Löe., 1993). Symptoms of DM are often mild and therefore hyperglycemia appropriate to cause disease and metabolic changes may be not be visible before diagnosis (Hanson et al., 2002). DM effects varies among individuals, thus from mild to deleterious effects. This includes progressive development of complications of vision impairment resulting in blindness, nephropathy; can result in renal failure and neuropathy resulting in foot ulcers, amputation and characters of autonomic dysfunction, which includes infertility in males (Hanson et al., 2002). The risk of cerebrovascular disease, CVD and peripheral disease is on the rise in diabetes patients as compared with non-diabetes (Mazzone et al., 2008; Gleissner et al ., 2007).

2.1.2 Classification and Diagnosis of DM

The first accepted classification was published in 1980 which was later revised in 1985 (WHO. 1980; WHO. 1985). In 1980, the expert committee came up with proposal of 2 major classes of DM, these were type 1 or IDDM, type 2 or NIDDM. During the 1985 publication, Type 1 and 2 were absent leaving IDDM and NIDDM. A new class; Malnutrition-related diabetes mellitus (MRDM) was then introduced (Alberti & Zimmet, 1998). Other types including IGT and Gestational diabetes among others were included in both 1980 and 1983 classifications. these were reviewed in 1991 and 1992 in International Nomenclature of Disease (IND) and the 10th revision of the International classification of disease (ICD-10) (Alberti & Zimmet, 1998). The 1985 classification is internationally accepted and currently in use.

The diagnostic requirements for a person with severe signs and hyperglycemia differs from persons without DM symptoms but have blood glucose values are a little above the diagnostic cut-off value. DM diagnosis in an asymptomatic person requires further plasma blood glucose test (Himsworth., 1936) result, as recommended by WHO guidelines, either FBS or OGTT (American Diabetes Association, 2004). Failure of these samples to confirm the diagnosis usually requires surveillance with periodic follow-ups until the confirmatory result is attained (Wiener and Roberts., 1998). In this case, further investigations into the family background, ethnicity, time of life, TG, and associated disorders are considered before any decision on drug administration. WHO now recommends the use of glycated haemoglobin (HbA1c) as a means of diagnosis regarding the inconvenience in measuring fasting plasma glucose levels or OGTT (Drouin et al., 2009).

2.2 Global Prevalence and Impact of Diabetes Mellitus on Health.

Globally, DM is a disorder of major public health concern with serious economic challenges (Mbanya et al., 2010). DM associated mortality is recorded in every 6 seconds. Currently, 382 million people are estimated to live with DM and the situation is projected to aggravate to by 2035 (International Diabetes Federation 2013 as cited by (Harries et al., 2013). Almost 5.1 million mortalities were recorded in 2013 as a result of T2DM complications. The Global health expenditure on DM in 2013 was approximately 548 billion US dollars. Recently, a global increase in the T2DM incidence has been reported and is attributed to behavior as well as lifestyle modifications. These ranges from sedentary lifestyles, poor nutrition, physical inactivity, and excessive fat deposition (Zimmet et al., 2001; Wild et al., 2004; Mbanya et al., 2010)

2.2.1 Aetiological Types

The types comprise disorders, defects and processes that usually result in DM. These specifically are T1DM and T2DM ; insulin dependent and non-insulin dependent diabetes mellitus (National Diabetes Data Group, 1979).

2.2.1.1 Type 1 Diabetes Mellitus (T1DM)

T1DM is a disorder of glucose metabolism characterized by autoimmune destruction of insulin that produces pancreatic β-cell. This disorder gradually leads to insulin deficiency and finally results in hyperglycemia (Flier et al., 1986). The predisposition of T1DM begins at birth giving the name juvenile onset diabetes mellitus. T1DM presents with classic outcomes of polyuria, polydipsia polyphagia, and loss of weight. (Cudworth, 1978; Atkinson et al., 2001). The actual cause is not known but ranges from genetic susceptibility to exposure to a diabetogenic trigger or

antigen. Treatment of Type 1 diabetes require insulin injection (Atkinson et al., 2001; Nathan et al., 2005).

2.2.1.2 Type 2 Diabetes Mellitus (T2DM)

T2DM is a disorder of insulin production where there is inadequate production of insulin by the pancreas to transport the glucose produced by the consumption of carbohydrate (Edelman, 1998). T2DM has become pandemic in all societies (Chen et al., 2011). Its effect ranges from impaired function of the renal, eye, brain, vascular tissues, and cardiac tissues to their destruction. T2DM also complicates a massive range of other tertiary diseases which consumes almost 10-12% of the total health care costs worldwide (Chen et al., 2011) . Mostly, T2DM is a disease of life style headed by metabolic syndrome. The effect of this misfortune is an excessive rise in plasma glucose level. The excess glucose stimulates many reactions causing circulatory, renal, optic, and neurological injury.

2.2.2 Diabetes Mellitus in Sub-Saharan Africa (SSA) and Ghana

DM was once considered a rare disease in SSA until 2010, when more than 12 million people in SSA were projected to have the disease with 330,000 expected mortality from its complications such as CVD, stoke and kidney failure (WHO. 2010). Studies predict that in the next 15 years, SSA will be the leading region with the highest growth in DM prevalence in the world with a doubling of the 2010 estimated number reaching 23.9 million by 2030 (Patel and Burke, 2009). The consistent increase in T2DM prevalence in SSA is principally attributable to rural–urban

relocation (Patel and Burke, 2009), where a healthy traditional way of life is shifted to lack of physical activity and associated unhealthy diet smoking, alcoholism and epidemiological transition (Hu et al., 2003). Middle income countries like Ghana are at highest risk for T2DM with 80% DM patients living in low- and middle- income countries (IDF. 2014).

In Ghana, DM is evolving as a prevalent of vast public health concern (Mbanya et al., 2010). It is the commonest endocrine-(carbohydrate, protein and fat) metabolism disorder resulting in obesity in 23% of Ghanaian adults (Amoah et al., 2002b). Statistics suggest the relations between environmental and genetic predisposition being involved in T2DM pathogenesis among Ghanaians. In SSA, DM risk increases about 3 times after age 45, with its prevalence peaking between the ages 45 and 50 (Ekpenyong et al., 2011). T2DM incidence rises with age as insulin resistance deteriorates with age and reduced physical activity (Ekpenyong et al., 2011).

2.3 Type 2 diabetes and cardiovascular risk

T2DM is significant for increased rate of its associated CVD (Grundy et al., 1999). The manifestation of this CVD ranges from coronary artery disease to carotid artery disease. Most T2D patients have equal risk of a cardiovascular event as non-diabetes with a predisposition of heart attack (Mazzone and Chait., 2008). Consequently, management of cardiovascular risk factors plays a great role in T2DM care, and is mostly cost effective (Mazzone and Chait., 2008). Due to the problem associated with maintaining normo-glycaemia, which is linked to insulin deficiency, the degree of hyperglycemia in most individuals is enough results in diabetes complications (Barzilay et al., 2001). Due to premature mortality caused by CVD, the complications are less common in T1DM patents (Gleissner et al., 2007).

2.3.2 Inflammation and role in T2DM pathogenesis

Inflammation is principally involved in T2DM pathogenesis (Pickup and Crook, 1998). Many pro-inflammatory cytokines play a significant role in inflammatory reaction. and are known to upsurge the risk of T2DM (Tutuncu et al., 2016). The pro-inflammatory cytokines also boost insulin resistance in adipose tissues, hepatocytes. This can lead to complete distraction of insulin sensitivity and impaired glucose homeostasis. Rise in the levels of proinflammatory cytokines result in production and secretion of CRP by the liver. CRP appear in the early stage of T2DM with increase in their circulating concentrations as the condition deteriorates. Prospective epidemiological studies have indicated that elevated CRP levels predict T2DM development (Dehghan et al., 2007; Doi et al., 2005)

Fig. 1 Inflammation in diabetes atherosclerosis

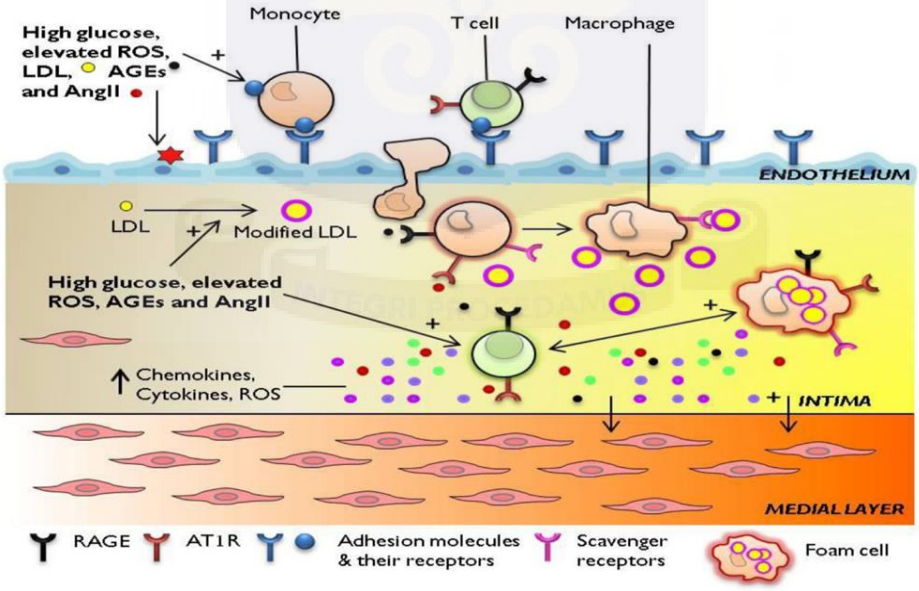

Fig.1 Involvement of inflammation in diabetes atherosclerosis. (Dehghan et al., 2007

2.3.4 Role of Inflammation in Diabetes atherosclerosis

Inflammation plays a significant role in CVD. It normally initiates with endothelial changes, which then expresses the adhesion molecule VCAM-1 (Gimbrone, Topper, Nagel, Anderson, & Garcia-Cardeña, 2000).,2000). VCAM-1 traps monocytes and migrate through the endothelial layer by the guidance of other proinflammatory cytokines. These monocytes transform into macrophages, which engulf lipids and finally form foam cells (Williams and Nadler, 2007). Migration of T lymphocytes also follows where there is a release of proinflammatory cytokines that intensify the inflammatory activity. These inflammatory processes lead to the formation of a fatty streak; signifying atherosclerosis lesion (Natarajan and Nadler, 2004).

Inflammation aids in fatty streak advancement from fatty streak to multifaceted plaque. During the evolution of plaques, macrophages are activated by T cells either by cyto-signaling or direct contact through CD40 ligation. Activated macrophages secretes collection of molecules that includes cytokines and matrix metalloproteinase. This comprises of collagen that forms the fibrous cap, which protects the plaque. The fibrous cap thin out and may rupture. This forms a thrombus that can result in further complications (Williams and Nadler, 2007).

2.4 Mechanisms related to insulin resistance and hyperinsulinemia

Several mechanisms that leads to insulin resistance and hyperinsulinemia have been proposed. These anomalies are both features of T2DM (Catalano et al., 2014). According to (Chiu et al., 2007)Chiu et al (2007) Impaired “mitochondrial activity results in dysregulation of intracellular fatty acid metabolism. Increased intracellular fatty acids may activate protein Kinase C and δ via DAG and thereby lead to serine phosphorylation of the insulin receptor substrate (IRS)-1, resulting

in reduced glucose transport". According to Natarajan and Nadler (2004) these "deficiencies appear to be closely associated with increased body fat". Also, "visceral abdominal fat has important endocrine functions that leads to increased serum levels of TNF-α, interleukin-6, plasminogen activator inhibitor (PAI-) 1, and reduced expression of adiponectin". (Natarajan and Nadler, 2004). Such changes in adipose tissues result in impaired insulin signaling and cytokine activation as well as suppression of transcriptional factors which will lead to resistance in insulin action.

2.5 Diabetes-associated hyperlipidemia

T2DM is linked to high lipid level which constitutes; "elevated TG, reduced high density lipoprotein (HDL) and increased low density lipoprotein (LDL) levels" (Grundy et al., 2004; O'Brien et al, 1998). These abnormalities in lipid composition occurs as a result of overproduction of triglyceride-rich lipoproteins in the liver. In addition to lipoproteins oxidation, hyperglycemia could also lead to glycation that could result in the formation of pro-atherogenic glycoxidation products (Virella and Lopes-Virella, 2012). This might also initiate the synthesis of advanced glycation end products (AGE) (Virella and Lopes-Virella, 2012). Instances "where LDL levels are normal, the composition of LDL particles could still be altered which is more atherogenic as compared to healthy individuals" (American Diabetes Association, 2004; Virella and Lopes-Virella, 2012).

2.6 Glucose and cellular inflammation

Numerous pathways have been proposed on glucose contribution to cellular injury and subsequent inflammation. (Peiró et al., 2016). Cells that are unable to withstand and adapt to the stress accompanied with higher levels of intracellular glucose might activate pathways of cellular

inflammation. Persistence of excess glucose could lead to cell injury (Barzilay et al., 2001). The processes involved in cellular inflammation ranges from protein kinase C (PKC) activation, polyols formation, to activation of hexosamine with later rise in the level of reactive oxidant species and mitochondrial stress (Peiró et al., 2016). Although this evidence has been associated with diabetes micro vascular disease, fluctuations of free fatty acids in the endothelium could cause macro vascular diseases through related pathways, hence, induce inflammation (Peiró et al., 2016).

2.6.1 C-reactive protein (CRP)

CRP is an acute-phase protein that acts as early inflammatory marker (Black et al. 2004). This protein is synthesized in the liver at concentrations less than 10 mg/L. During infections, the level of CRP rise rapidly within the first 6 to 8 hours and peak up to 350–400 mg/L after 48 hours of infection (Clyne et al., 1930). CRP binds to phosphocholine and stimulates the classical complement cascade of the immune system. (Volanakis & Wirtz, 1979). The level of CRP falls after inflammation is resolved. Hence, makes it a useful marker for diagnosis (Pepys et al., 2003).

2.7 Insulin and its action on Glucose

Insulin, as defined by Drury (1940) is a peptide hormone produced by beta cells of the pancreatic islets. It is synthesized as pre-proinsulin. Within a minute after synthesis it is discharged into cisternae space of rough endoplasmic reticulum where it is cleaved into proinsulin A and B by proteolytic enzymes. Insulin has significant effects on carbohydrate metabolism, proteins and fats. Insulin contributes to the metabolism by stimulating the absorption of circulating glucose into the liver, fats and skeletal muscle cells (Drury, 1940).

Glucose is transported into the b cellvia GLUT-2. The transported glucose is then oxidized by glucokinase. Glucokinase therefore acts as a glucose sensor (Aronoff et al., 2004). Oxidized glucose is transformed into Triglycerides. In the liver, glucose production and release into the blood is strongly repressed by elevated level of plasma insulin (Drury, 1940). Circulating insulin also affects the synthesis of proteins in a wide variety of tissues. In high concentrations, it functions as an anabolic hormone promoting the conversion of small molecules in the blood into large molecules inside the cells. Low insulin levels in the blood have the opposite effect by promoting widespread catabolism (Aronoff et al., 2004; Drury, 1940).

2.7.1 Hyperinsulinemia and insulin resistance in T2DM

T2DM patients might not necessarily have significantly higher concentrations of insulin compared to corresponding non-diabetes, but for any plasma insulin concentration, diabetics generally have a reduced concentration due to the impaired function of pancreatic B-cell in its secretion (Ferrannini et al., 2004). Moreover, hyperinsulinemia which results in its resistance is said to be the hallmark of T2DM (Shanik et al., 2008). Hyperinsulinemia is a persistent elevation of plasma insulin in the fasting state. Hyperinsulinemia as widely acknowledged that it results from insulin resistance (IR). Shanik et al (2008) stated that "high level of insulin may lead to insulin resistance (Shanik et al., 2008), especially in the presence of fatty acid" (Lim et al., 2011). IR is associated with hyperinsulinemia among T2DM (Shanik et al., 2008).

2.7.2 Obesity, Insulin Resistance and Diabetes mellitus

Obesity is related to insulin resistance and diabetes mellitus as a result contributes to hypertension, hypercholesterolemia, low level of High Density Lipo-protein (HDL) and is independently associated with higher risk of CVD (Eckel et al., 2004; Anderson et al., 2001). the menace of severe health implications in the form of T2DM, coronary heart disease (CHD) and some forms of cancer is known to increase with a rise in body mass index (BMI) and Body adiposity Index (BAI) (Rexrode et al., 1998; Ferreira et al., 2004). But an excessive abdominal fat is more suggestive of the metabolic syndrome profile than BMI (Pouliot et al., 1994)

2.8. Mechanisms linking hepcidin and diabetes mellitus

A major feature of DM is insulin resistance which has a bidirectional relation with elevated plasma iron concentration. A direct relationship between IR and hepcidin however remains unclear. As stated in many molecular studies, hepcidin expression is stimulated by insulin via STAT3; a novel transcription modulator of hepcidin (Campos et al., 1996; Aregbesola et al., 2015). Study conducted by Fernandez and colleagues in 2015 indicated a reduction in hepcidin and prohepcidin concentrations in T2DM subjects. This suggests loss of insulin signal in diabetes subjects with iron overload (Fernández-Real et al., 2015). Another work conducted by Aigner (2013) established an association among hepcidin and insulin through glucose stimulation (Aigner et al., 2013). Hepcidin and insulin secretion is being confined in β-cell granules, where hepcidin induces its paracrine function (Kulaksiz et al., 2008). A correlated release of hepcidin is observed as glucose fuel insulin release (Aigner et al., 2013).

2.8.1. Hepcidin

Hepcidin; as defined in the work of Kartikassari et al (2008) is" a 25- amino acid peptide hormone is secreted by the liver as a pre-prohormone, an 84 amino acid encoded by the human hepcidin gene (HAMP; OMM 606464), containing three exons and its located on chromosome 19q13". The pre-prohormone is then converted into a 60-64 amino acid prohepcidin and finally converted into hepcidin 25 through posttranslational modification in the cytoplasm (Fernández-Real et al., 2009). Hepcidin was originally named as liver- expressed antimicrobial peptide (LEAP-1) in 2001 upon its discovery by three independent research groups (Park et al., 2001). It is primarily considered to be involved in Iron metabolism through negative feedback mechanism which is not clearly understood (Kartikasari et al., 2008; Ganz T, 2006). It also plays a role as antimicrobial peptide during infections (Park et al., 2001). During infections and inflammatory conditions, hepcidin is expressed forming part of the acute phase inflammatory response, principally through the IL-6/STAT 3 pathway (Wrighting and Andrews, 2006).

2.8.2 Iron Homeostasis in type 2 diabetes mellitus

Iron acts as a cofactor in the oxidation of fuel and electron transport. excess plasma iron could be involved in oxidative damage when carefully unregulated, conducted, and sequestered (Swaminathan et al., 2007). Widespread mechanisms to regulate iron uptake and fate have developed. Iron entry into cells surges during the oxidation of fuel. Adversely, the fate of ethanol as well as glucose metabolism are dependent on iron availability (Finch, 1994). During absorption, dietary ferric iron (Fe3+) is reduced to ferrous iron (Fe2+) by ferrireductase "duodenal cytochrome B". Absorption of iron occurs through divalent metal transporter 1 "Nramp2 (natural-resistance-

associated macrophage protein 2). Divalent metal transporter 1 is found in apical intestinal epithelium (Mackenzie et al., 2006). Divalent metal transporter 1 catalyzes passage of non-transferrin bound iron (NTBI) with other divalent metals (Zn2+ and Mn2+). According to Liuzzi et al (2006) "non-transferritin bound iron can also enter cells through DMT1 and other transporters such as Zip14" (Liuzzi et al., 2006; Mackenzie et al., 2006).

Iron in enterocytes is either deposited in ferritin or transported by an iron exporter (ferroportin) to the basolateral surface of the cell. Transported iron is deoxidized by hephaestin and incorporated into transferrin. Transferrin-bound iron is then distributed through various cells and tissues (Rajpathak et al., 2009). Uptake of iron by various cells is mediated by transferritin receptors (TfRs). Upon endocytosis, transferrin releases ferric iron from as the endosome becomes acidified, transported Iron is then reduced by the STEAP family (Six-transmembrane epithelial antigen of prostate) of ferrireductases, and finally enters cytosol through DMT1 (Simcox and McClain, 2013).

2.9 Anthropometric indices and T2DM

According to Dhakail et al (2013), "Anthropometric measurement is the art of assessing human body parts to study and compare the relative proportions under normal and abnormal conditions" (Dhakaì1 et al., 2013). Anthropometric indices includes weight (Wt), height (Ht), ponderal index (PI), body mass index (BMI), among others. Anthropometric measurements are amongst the commonly applied systems for evaluating dietary status in to ensure effective management in DM patients (Hadaegh et al., 2009) . Several studies have shown the existing link between anthropometric indicators and DM incidence (Sharp et al., 2003; Dhakaì1 et al., 2013; Wang et al, 2007)

2.9.1 Body Mass Index (BMI)

As invented by a Belgian, Adolphe Quetelet (1796- 1874), BMI is a homogeneous estimate of an individual's virtual body fat calculated from height and weight of an individual. BMI is expressed as kilogram per meter square; ratio of weight to the square of height (Centers of disease control, 2011; Jerant et al., 2015). On the basis of BMI, an individual is said to be underweight if BMI is less than 18.5 kg/m^2, Normal weight ranges from 18.5kg/m^2 to 24.9 kg/m^2, when BMI is between 25 and 29.9 kg/m^2 one is said to be overweight, and Obese when BMI is 30 kg/m or greater (Centers of disease control, 2011). There is a strong correlation link between BMI and obesity (Bays et al., 2007; M. L. Ganz et al., 2014). Higher BMI is correlated with a greater DM risk, hypertension and additional CV risk factors (Field et al., 2001; Narayan et al., 2007). Obesity as categorized as excess body fat is defined conventionally as body fat of 25 % in males and 35 % in females (Brancati et al., 1999; Everhart et al. 1992)

2.9.2 Waist-To-Hip Ratio (WHR)

WHR can be determined as, the waist circumference / hip circumference. It measures the proportion of fat distribution in the upper body (Schmidt et al., 1992). So far, the most extensively used index of central fat distribution is WHR due to its benefits in routine monitoring and assessment in patients (Czernichow et al., 2011). Studies concerning WHR in DM patients have focused largely on its value as a biomarker for health and attractiveness. According to Schmidt et al (1992) "A WHR of 0.7 for females and 0.9 for males is said to correlate strongly with general health" (Schmidt et al., 1992). Women with high WHR exhibit cardiovascular disease risk factors (Czernichow et al., 2011). A correlation between WHR and hypertensive diabetes has being

established (Schmidt et al. 1992; Czernichow et al., 2011). As established by Yamamoto et al., (2001) "higher WHR is an important predictor of T2DM pathogenesis and its associated CVD regardless of total adiposity; BMI and BW.

2.9.3 Waist Circumference (WC)

Waist circumference is the simplest anthropometric index of abdominal visceral adiposity, (Pouliot et al., 1994). Measurement of WC in determining abdominal adiposity correlates better with CV risk factors as equated to BMI (Lee et al., 2008; Janssen et al., 2004) The correction of waist circumference for stature or hip circumference improves its performance in the prediction of the incidence of hypertension (Bozorgmanesh et al., 2011). According to Okusun et al (2000), "Differing values of WC are associated with increased risk of hypertension in different populations". The study revealed that, "WC cut-off points of 76, 81, 80, 83 and 87 cm provides the highest sensitivity for identifying hypertensive in some countries in West Africa. The equivalent cut-off points in women are 72, 82, 85, 86 and 88 cm" (Okosun et al., 2000)

CHAPTER THREE

3.0 METHOD

3.1. Study Design

The study is a case control design. The study subjects were male and female T2DM patients, compared to age and gender matched non-DM individuals. The design and protocol of the study was approved by the College of Health Sciences Ethics and Protocol Review committee (Protocol ID number: CHS-Et/M.9 – P 4.5/2015-2016).

3.1.1 Study site

The cases were recruited from the National Diabetes Management and Research Centre (NDMRC). NDMRC is an excellent referral center for diabetes care, training and research. It provides medical care and consultation, involving eye services for diabetes management. Non-T2DM controls were recruited from the surrounding communities of comparable age and gender as the T2DM cases.

3.2 Target Population

The study targeted T2DM patients between the ages of 30 – 70 years reporting at the NDMRC clinic. T2DM status was essentially clinical and based on non-requirement of insulin administration at the time of T2DM diagnosis as well as older age of presentation ($\geq$ 30 years).

The controls were gender and age matched individuals with normal glucose tolerant status (ADA, 2012).

3.3. Definition of cases

1. Type 2 diabetes subjects: Individuals diagnosed of diabetes in adulthood (between 30 and 70 years), initially treated with lifestyle modification and/or oral hypoglycemic medication.

2. Non-diabetes subjects: Individuals with no T2DM history. Thus, FPG < 7.0 and plasma glucose after 2 hours post load plasma glucose < 11.2 mmol/L

3.4 Eligibility criteria

Inclusion criteria	Exclusion criteria
• T2DM patients (cases) and non-diabetes individuals (controls) between the ages 30 and 70 years	• Type 1 diabetes patients, newly diagnosed, pregnant women with diabetes and type 2 diabetes patients who fall outside the age range of 30 – 70 years.
• Subjects capable and willing to provide written informed consent.	• Subjects with diabetic foot ulcers, amputation.

3.5 Sample Size and Power Determination

Sample size and power were computed based on the expected standardized effect size [ratio of the planned mean difference (δ) to combine standard deviations (σ) between T2DM subjects and controls] of the various parameters being measured in the study. With the level of significance, α=5%, and power of 80% (β = 20%), the results are shown in the Table 3.1 below.

Parameter	Hypothesized effect	A	Power	N
Hepcidin	0.70	0.05	80%	64
Insulin	0.70	0.05	80%	64
CRP	0.70	0.05	80%	64

Table 3.0.1 Postulation for sample calculation Parameter Hypothesized effect α Power N

3.6 Subject Recruitment and Data collection

The T2DM patients arrive at the NDMRC early in the morning and register at the sample collection point with their Diabetes record book, popularly known as 'diabetes traffic light book'. It was based on the traffic light book that systematic sampling was performed. IBM SPSS version 20 software was used to select the participants for the study based on the age and gender status. Other factors such as BMI, duration of diabetes and forms of diabetes treatment were not considered in the selection of subjects. The selected patients were gathered in a separate room and we explained to them as a group the nature, rationale, procedure, effects and welfares of the study. They were allowed to ask questions and we provided answers to their concerns. Afterwards, we met privately

with the invited individuals and addressed any personal concerns of the invitee, clarifying any doubts before recruiting him/her into the study by registration and signing or thumb-printing the informed consent form. Special identification numbers were given to the study subjects. Those who were unwilling to join the study or had any reservations were allowed to go for their normal clinical routine care.

The controls were recruited from the general public who responded to the invitation to partake in the research. An invitation was sent out for research participants. Those willing to participate were contacted, mainly on the phone. A brief explanation of the nature of the research, detailed instruction of the fasting procedure was given to them. Afterwards, they were scheduled to come to the NDMRC to participate in the research. The evening prior to their appointment date, the researcher contacted them on phone to remind the volunteers of the fasting procedure. The study volunteers arrived at the NDMRC in the morning, between 6:30 – 8:30 am. Upon their arrival at the NDMRC, the non-diabetes invitees were taken through a process similar to the diabetes invitees, as stated in the preceding paragraph.

3.6.1 Fasting instructions

Prior to the participation in the research, subjects were asked to fast overnight between 8 – 12 hours. To prevent over fasting, subjects were asked to take light food like fruits, biscuits or slice of bread around 9 pm, before going to bed, and report to the NDMRC at 7:00 am. At the NDMRC, subjects were asked the time of their last food intake to estimate the duration of fasting. Subjects who did not meet the fasting requirement had their appointment rescheduled.

3.6.2 Questionnaire administration

Structured questionnaire was used to obtain the following data from study participants: socio-demographic characteristics [age, gender, highest education level, lifestyle (level of tobacco use, alcohol use, and medical history (personal, family background of T2DM, heart failure, renal failure, amputation and stroke)].

3.6.3 Anthropometric assessment

Weight was measured in kilograms (kg) and reported in 0.1 kg, using a floor scale from Secca, Hamburg, Germany, with the subject standing upright on the platform, in light clothing with footwear and any heavy jewellery removed from the body; both feet directed forward and arm by the side of the body. Body height was measured in meters (m) and reported in 0.01 m, with a clinical measuring rule in a similar faction. BMI was calculated by the ratio of the weight (kg) to height (m) square. As adapted from Cecilia Ramlaus work, BMI was categorized as "underweight (BMI < 18.50 kg/m2), normal weight (BMI: 18.50 – 24.99 kg/m2), overweight (BMI: 25.00 – 29.99 kg/m2) and obese (BMI ≥ 30 kg/m2)".

waist and hip circumferences measurements were done in centimeters (cm) and reported in 0.1 cm using stretch-resistant tape measure. WC measurement was made at the estimated line between the lower border of the last physical rib and the uppermost of the iliac crest. Measurement of hip circumference was taken around the broadest part of the bottoms. Waist and hip measurements were made with the tape held tightly but not squeezing parallel to the floor. WHR was computed by waist girth/hip girth. The end point for abnormal waist girth was set at 92 cm for men and 90 cm for women.

3.7 Sample collection, processing and storage

An amount of 10 ml of intravenous blood sample was collected from the antecubital area into vacutainer tubes, using single-use disposable sterile syringe under aseptic technique. The blood samples were collected into three vacutainer collection tubes: 4 ml into plain tube with clotting activator (red-top), 4 ml into sodium ethylene diaminetetraacetic acid (Na+-EDTA) tubes (purple-top) and 2 ml into fluoride oxalate (ash-top) tubes. The collection tubes, containing the blood sample, were immediately chilled on ice prior to centrifugation. Within 15 minutes of sample collection, the collection tubes were centrifuged at 4000g: 5 minutes for fluoride oxalate collection tubes and 15 minutes for plain and Na+-EDTA collection tubes. Plasma and serum samples were collected into Eppendorf tubes. Blood lipids and glucose were analyzed immediately before storage. The plasma and serum samples were than stored at -80ºC.

3.8 Preparation and administration of glucose drink for OGTT

An amount of 75g of anhydrous glucose was dissolved in 400 mL of water, sterilized by boiling, and refrigerated overnight at 8ºC in a well labelled bottle. Dye and flavor was added to make it easier for the subjects to ingest. The subjects drank all the glucose solution within 5 minutes and sat on a bench in a waiting room for 2 hours, after which 2 mL of blood was drawn from the antecubital area into fluoridated tubes for post glucose-load plasma glucose assay.

3.9 Biochemical analysis

3.9.1 Plasma glucose analysis

The level FBS and O.G.T.T. were measured using BS 120 chemical autoanalyser (Mindray, China), using Randox Gluc-Pap reagent (Randox Laboratory Reagents, UK), following the manufacturer's guidelines.

3.9.2 Plasma Lipid Profile assay

Plasma Lipid profile was analyzed using BS 120 chemical autoanalyser with Randox reagents, following the manufacturer's protocol.

Total Cholesterol (TChol)

The total amount of cholesterol (TChol) in the plasma was assayed after enzymatic hydrolysis and oxidation.

Triglycerides (TG)

Plasma triglycerides (TG) were assayed after enzymatic hydrolysis with lipases. The concentration was determined by the equipment after reading the absorbance of the indicator at a wavelength of 500 nm.

HDL cholesterol

HDL cholesterol was assayed by the precipitation method. 500 µL of diluted precipitant solution, containing phosphotungstic acid in the presence of magnesium, was added to 200 µL of the plasma sample. The sample was allowed to sit for 10 mins at room temperature and centrifuged afterwards at 4000g for 10 mins to precipitate low density lipoproteins and chylomicrons. The HDL cholesterol was assayed from the supernatant solution at an absorbance of 500nm.

LDL cholesterol

The levels of LDL cholesterol were calculated from Frieldwald's equation,

LDL = TChol – (HDL+TG/2.2).

3.10 Hepcidin Enzyme Immunoassay

The serum levels of hepcidin were assayed using enzyme linked immunoassay (ELISA) method.

Sample Dilution

An amount of 5 µL of the serum samples was added to 95 µL of regent diluent to achieve 1:100 dilution. The samples were then stored in a refrigerator at a temperature of 2ºC and used for the assay within one week.

Plate Preparation

Plate Coating with Capture Antibody: Before the assay of each human serum hepcidin, capture antibody was used to coat the wells in ELISA micro plate overnight. Rabbit anti-human hepcidin was reconstituted with 0.5 mL of PBS to obtain a concentration of 333µg/mL, which was further diluted in phosphate buffer saline to achieve the working concentration of 4 µg/mL. All these dilutions were performed without a carrier antibody. The 96-well microplate was coated with 100 µL of the diluted capture antibody solution and left overnight at room temperature.

Washing: After incubating overnight, each well was aspirated and washing was done by filing each well with 400 µL of wash buffer. This was repeated for three times, using an auto washer. The micro plate was blotted against paper towel to remove the remaining wash buffer by aspiration.

200 µL of reagent diluent was added to each well to enhance blocking and incubated for one hour.

Washing repeated as in step '2'.

Assay technique

Sample and standard addition: addition of 100 µL of sample was pipetted in respective well, followed by covering with adhesive strip and finally, 2-hour incubation.

Washing: Washing process repeated as in step '2'.

Detection antibody addition: a volume of 100 µL of detection antibody was diluted in reagent diluent with normal goat serum. It was added to each well, covered with a new adhesive strip and incubated for 2 hours at room temperature.

Washing: The washing process was repeated as in step '2'.

a. A volume of 100 µL of diluted Streptavidin-HRP was added to each well, covered and incubated for 20 minutes at room temperature.

The washing process was repeated as in step '3'.

A volume of 100 µL of substrate solution was added to each well, incubated for 20 minutes at room temperature in darkness.

A volume of 50 µL of stop solution was added to each well.

The optical density was read at 450 nm using a microplate reader.

The optical densities were converted into concentrations by using four-parameter logistic curve fit programme, Auditable Data Analysis and Management System for ELISA (ADAMSEL v1.1).

3.10.1 Insulin Enzyme Immunoassay

The serum levels of insulin were assayed using enzyme linked immunoassay (ELISA) method.

Sample Dilution

An amount of 50 µL of the serum samples was added to 50 µL of regent diluent to achieve 2x dilution. The samples were then stored in a refrigerator at a temperature of 2ºC and used for the assay within one week.

Plate Preparation

Plate Coating with Capture Antibody: Before the assay of each human serum insulin, capture antibody was used to coat the wells in ELISA micro plate overnight. mouse anti-Human/carnine/porcine insulin was reconstituted with 0.5 mL of PBS to obtain a concentration

of 180µg/mL, which was further diluted in PBS to achieve the working concentration of 4 µg/mL. All these dilutions were performed without a carrier antibody. the 96-well microplate was coated with 100 µL of the diluted capture antibody solution and left overnight at room temperature.

Washing: After incubating overnight, each well was aspirated and washing was done by filing each well with 400 µL of wash buffer. This was repeated for three times, using an auto washer. The micro plate was blotted against paper towel to remove the remaining wash buffer by aspiration.

300 µL of reagent diluent was added to each well to enhance blocking and left for an hour incubation.

Washing: this process was repetitive as in step '2'.

Assay technique

Sample and standard addition: addition of 100 µL of sample were pipetted into their respective wells, covered with adhesive strip and incubated for 2-hours.

Washing: Washing process repeated as in step '2' .

Detection antibody addition: a volume of 100 µL of detection antibody was diluted in reagent diluent with normal goat serum. it was then added to each well, covered with a new adhesive strip and incubated for 2 hours at room temperature.

Washing: The washing process was repeated as in step '2'.

A volume of 100 µL of diluted Streptavidin-HRP was added to each well, covered and incubated for 20 minutes at room temperature.

The washing process was repeated as in step '3'.

A volume of 100 µL of substrate solution was added to each well, incubated for 20 minutes at room temperature in darkness.

A volume of 50 µL of stop solution was added to each well.

The optical density was read at 450 nm using a microplate reader.

The optical densities were converted into concentrations by using four-parameter logistic curve fit programme, Auditable Data Analysis and Management System for ELISA (ADAMSEL v1.1).

3.10.2 C-reactive protein Enzyme Immunoassay

The serum levels of CRP were assayed using enzyme linked immunoassay (ELISA) method.

Sample Dilution

An amount of 60 µL of the serum samples was added to 240 µL of regent diluent to achieve 1:50 dilution. The samples were then stored in a refrigerator at a temperature of 2ºC and used for the assay within one week.

Plate Preparation

Plate Coating with Capture Antibody: Before the assay of each CRP capture antibody was used to coat the wells in ELISA micro plate overnight. Mouse anti-human CRP was reconstituted with 1.0 mL of PBS to obtain a concentration of 720µg/mL, which was further diluted in PBS to achieve the working concentration of 4 µg/mL. All these dilutions were performed without a carrier antibody. The 96-well microplate was coated with 100 µL of the diluted capture antibody solution and left overnight at room temperature.

Washing: After incubating overnight, each well was aspirated and washing was done by filing each well with 400 µL of wash buffer. This was repeated for three times, using an auto washer. The micro plate was blotted against paper towel to remove the remaining wash buffer by aspiration.

300 µL of reagent diluent was added to each well to enhance blocking and left for an hour incubation.

Washing: this process was repetitive as in step '2'.

Assay technique

Sample and standard addition: addition of 100 µL of sample was pipetted in respective well, followed by covering with adhesive strip and finally, 2-hour incubation.

Washing: Washing process repeated as in step '2'.

Detection antibody addition: 100 µL of detection antibody was diluted in reagent diluent with normal goat serum and added to each well, covered with a new adhesive strip and incubated for 2 hours at room temperature.

Washing: The washing process was repeated as in step '2'.

A volume of 100 µL of diluted Streptavidin-HRP was added to each well, covered and incubated for 20 minutes at room temperature.

The washing process was repeated as in step '3'.

A volume of 100 µL of substrate solution was added to each well, incubated for 20 minutes at room temperature in darkness.

A volume of 50 µL of stop solution was added to each well.

The optical density was read at 450 nm using a microplate reader. The optical densities were converted into concentrations by using four-parameter logistic curve fit programme, Auditable Data Analysis and Management System for ELISA (ADAMSEL v1.1).

\3.11 HOMA- IR CALCULATION

Insulin resistance was then calculated using insulin level in the Homeostatic Model Assessment (HOMA-1R). Using the formulae

$$\text{HOMA-IR} = \frac{\text{fasting insulin (Umol/L)} * \text{fasting glucose (mmol/L)}}{22.5}$$

CHAPTER FOUR

RESULTS

4.0 Description of the Study Population

4.1 General Description

The description of study participants; gender, age distribution, alcohol and smoking status are presented in Table 4. 1. A total of 140 volunteers, with equal gender distribution participated in the study. Majority of the participants were diabetic (79.3% vs 20.7%). In diabetic patients, the males were more than females (53.1% vs 46.8%). Diabetes patients were insignificantly younger than non-diabetes participant (53.9±11.9 vs. 56.8±11.5, p= 0.39). Alcohol intake was significantly associated with diabetes status whereas gender, age decade and cigarette smoking were not associated with diabetes status. (Table 4.1)

Table 4.1. General description of the study subjects

Parameter	**Subjects category n (%)**			***p***
	T2DM (n=111)	**NDM(n=29)**	**Total**	
Gender				
Male	59 (84.3)	11 (15.7)	70 (100)	0.14
Female	52 (74.3)	18 (25.7)	70 (100)	
Age decade in years				
<50	39 (84.8)	7 (15.2)	46 (100)	
50-59	33 (78.6)	9 (21.4)	42 (100)	0.39
60-69	34 (77.3)	10 (22.7)	44 (100)	
70+	4 (57.1)	3 (42.9)	7 (100)	
Alcohol intake	**37 (67.3)**	**18 (32.7)**	55 (100)	< 0.01
Previous smokers	21 (77.8)	6 (22.2)	27 (100)	0.83

Data presented in frequency (percentages). T2DM: type 2 diabetes subjects; NDM; non type 2 diabetes subjects

4.2 Anthropometric characteristics of the study subjects

The mean levels of anthropometric features of study participants were compared and presented in Table 4. 2. There was no significant difference in the mean values of anthropometric indices between diabetes patients and non-diabetes controls.

Table 4. 2. Mean levels Anthropometric Indices of Study Subjects

Anthropometric Parameter	**Subjects category (̄x± SD)**		***P***
	T2DM	NDM	
BMI (kg/m^2)	29.0±5.6	29.1±5.5	0.98
Body fat (%)	34.2±12.1	37.3±12.2	0.22
Visceral fat	11.8±4.7	10.4±3.4	0.13
Waist circumference (cm)	99±12	95.±19	0.16
WHR	0.9±.1	0.9±.2	0.41
WSR	0.6±.1	0.6±.11	0.41

Data presented as mean ± standard deviations. BMI: body mass index; WHR: waist-to-hip ratio; WSR: waist to height ratio.

Mean Biochemical characteristics of study subjects

Mean levels of biochemical parameters of study participants were compared by diabetes status and presented in Table 4.3. With the exception HDL cholesterol, a common trend seems to run through all the biochemical characteristics measured. Subjects with diabetes had higher mean biochemical characteristics than non-diabetes except HDL cholesterol (0.73±0.46 vs 1.28±0.23, p= 0.06).

Table 4.3. Mean Biochemical characteristics of study subjects

Biochemical characteristics	Subjects category (ẋ± SD)		*P*
	T2DM	**NDM**	
Fasting plasma glucose	8.61±3.03	4.703±2.18	< 0.01
Fasting triglycerides	1.61±0.45	1.04±0.42	0.02
Total cholesterol	5.28±1.29	3.08±1.50	0.01
HDL chol	0.73±0.46	1.28±0.23	0.06
LDL chol	3.93±1.19	2.65±1.61	0.01
TChol/HDL	7.53±2.63	5.12±3.08	0.04
LDL/HDL	5.45±2.17	4.19±2.92	0.01
TG/HDL	1.63±1.12	1.31±0.88	0.2
non HDL cholesterol	4.54±1.32	3.30±1.53	0.01

Data presented as mean ± standard deviations. HDL cholesterol; high density lipoprotein cholesterol, LDL cholesterol; low density lipoprotein cholesterol, Tchol/HDL; ratio of total cholesterol to high density lipoprotein, LDL/HDL; ratio of low density lipoprotein to high density lipoprotein.

4.3 Mean BP levels among study subjects

As shown in Table 4.4, diabetes patients had non-significantly higher heart rate than non-diabetes controls.

Table 4.4 Brachial BP of study subjects

Blood Pressure Indices (mm Hg)	**Diabetes status (x± SD)**		***P***
	T2DM	NDM	
Systolic BP	140±35	141±18	0.8
Diastolic BP	84±13	85±13	0.6
PP	61±14	56±11	0.1
HR	**74±18**	**66±12**	0.03
MAP	104±16	104±14	0.9

Data presented as mean ± standard deviation. PP: pulse pressure; MAP: mean arterial pressure; HR: heart rate

4.4 Median levels of Insulin function among Study Subjects

T2DM subjects had higher levels of C- reactive protein and insulin resistance compared to non-diabetes controls. Non-diabetes on the other hand had higher levels of β cell function, hepcidin and insulin sensitivity (Table 4. 5).

Table 4.5. Median levels of Insulin function among study subjects

Biochemical parameter	Diabetes status		*P*
F	T2DM	non T2DM	
Hepcidin	**23.5 (8.8-31.5)**	**25.6 (13.4-30.4)**	0.03
CRP	107.7 (22.6-142.5)	77.8 (95.5-147.9)	0.49
Insulin	15.8 (8.6-30.5)	24.5 (18.4-38.8)	0.17
B cell function	**17.7 (9.2-33.9)**	**40.6 (20.5-58.0)**	<0.001
Insulin sensitivity	**24.1 (16.5-33.3)**	**45.7 (35.1-69.0)**	0.04
Insulin resistance	**1.8 (1.2-2.9)**	**0.9 (0.4-1.1)**	0.005

Data presented as marginal median ± interquartile range are provided. Non- T2DM- non diabetes subjects.

These results show that, insulin function which is reduced in diabetes is associated with reduced iron homeostasis which is controlled by plasma hepcidin.

4.6 Association between Insulin function with Hepcidin and CRP

The correlation between indices of diabetes vs hepcidin and CRP were performed and presented as in Table 6. Hepcidin and CRP correlated negatively with insulin sensitivity whilst β cell function correlated negatively with Hepcidin and positively with CRP (Table 5).

Table 4.6. Correlation of Insulin function with hepcidin and CRP.

Biochemical index	Hepcidin		CRP	
	R	*P*	r	*p*
Hepcidin	--	--	0.4	<0.01
Insulin	0.3	0.02	0.03	0.04
B cell function	**-0.26**	0.04	0.07	0.05
Insulin sensitivity	**-0.34**	0.04	**-0.18**	0.04
Insulin resistance	0.3	0.05	0.20	0.05

Data presented as Pearson's correlation coefficient, r. CRP: c reactive protein.

These results show

4.7. Association between HOMA and Fasting plasma glucose

As shown in Fig.2 A positive linear correlation was observed when Insulin resistance levels were plotted against Fasting plasma glucose

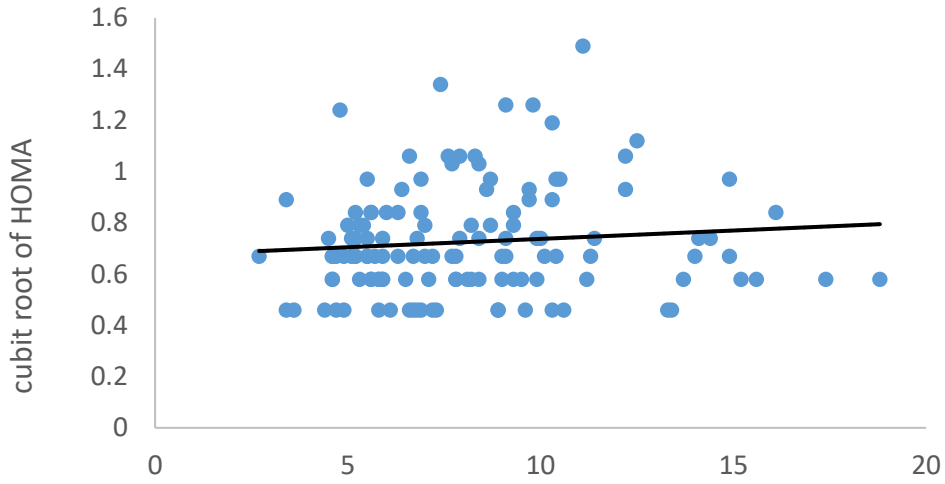

Fig 2. Fasting plasma glucose (mmol/L)

4.7.1. Association between HOMA and Total cholesterol

As shown in Fig.3 A weak positive correlation was observed when Insulin resistance levels were plotted against Total Cholesterol

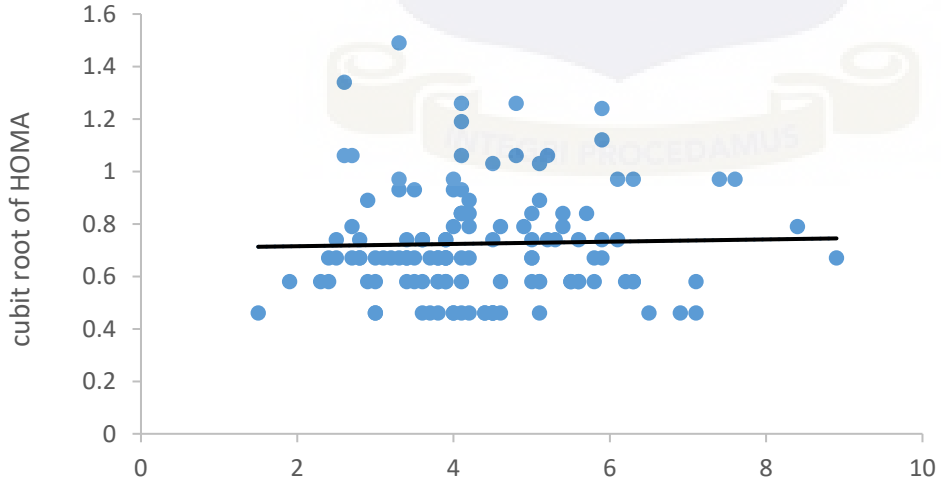

Fig. 3 Total cholesterol (mmol/L)

4.7. 2 Association between HOMA and Triglyceride/ HDL cholesterol

As shown in Fig.4. A positive linear correlation was observed when Insulin resistance levels were plotted against Tryglyceride/HDL cholesterol.

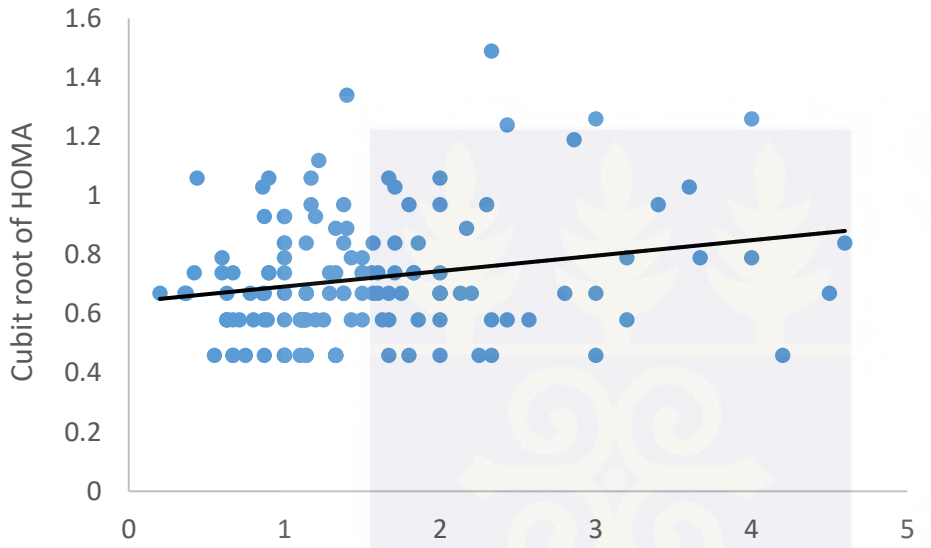

Fig. 4 Triglyceride/HDL cholesterol ratio

4.8. Association between Plasma Hepcidin and Fasting plasma glucose

As shown in Fig.5. A positive linear correlation was observed when plasma hepcidin levels were plotted against Fasting Plasma glucose

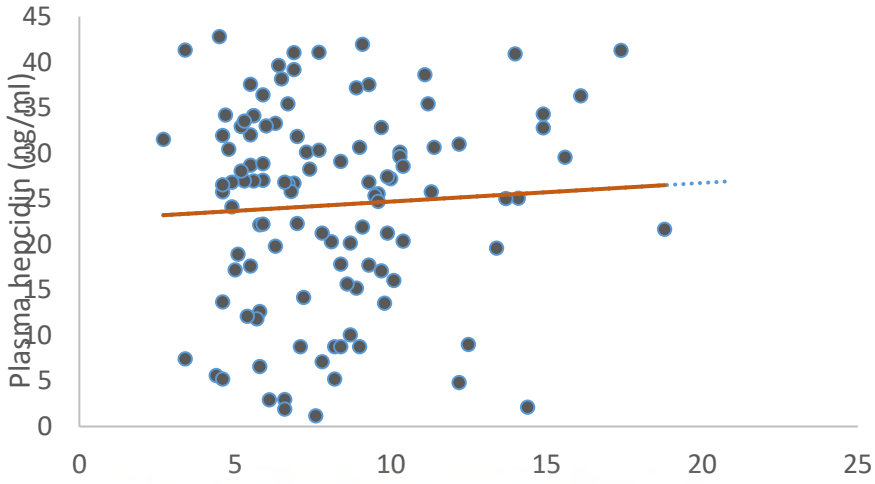

Fig. 5 Fasting plasma glucose (mmol/L)

4.8.1. Association between Plasma hepcidin and Total cholesterol

As shown in Fig.6. a negative correlation was detected when plasma hepcidin levels were plotted against Total cholesterol

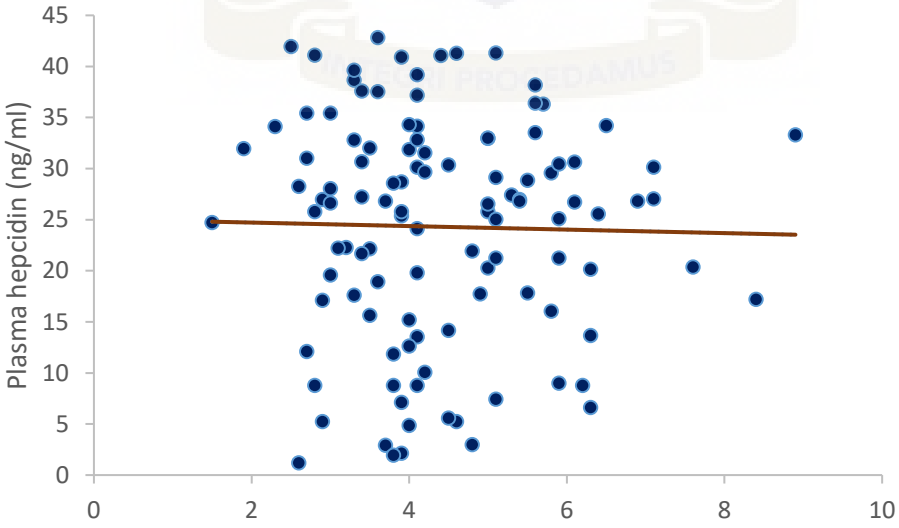

Fig. 6 Total cholesterol (mmol/L)

4.8.2. Association between plasma Hepcidin and Triglyceride/HDL cholesterol

As shown in Fig.7 a positive linear correlation was observed when plasma hepcidin levels were plotted against Triglyceride/ HDL cholesterol

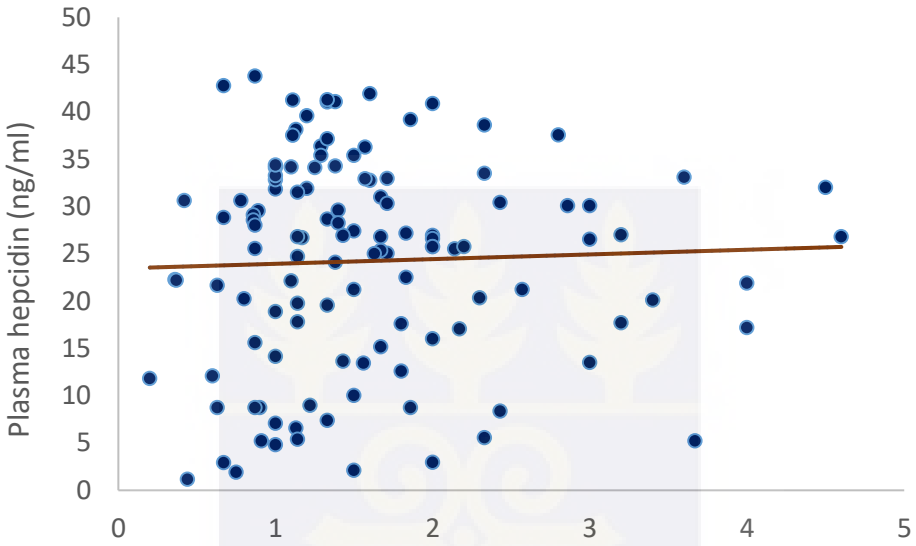

Fig. 7 Triglyceride/HDL cholesterol ratio

4.9. C- reactive protein and Fasting plasma glucose

As shown in Fig.8 a negative correlation was observed when CRP levels were plotted against Fasting plasma glucose.

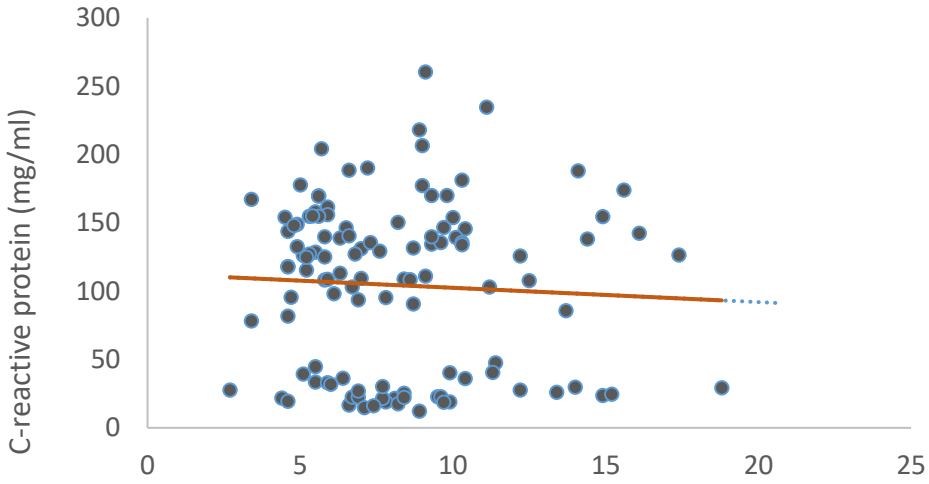

Fig. 8 Fasting plasma glucose (mmol/L)

4.9.1. Total cholesterol and C- reactive protein

As shown in Fig.9. A strong correlation was observed when CRP levels were plotted against Total cholesterol

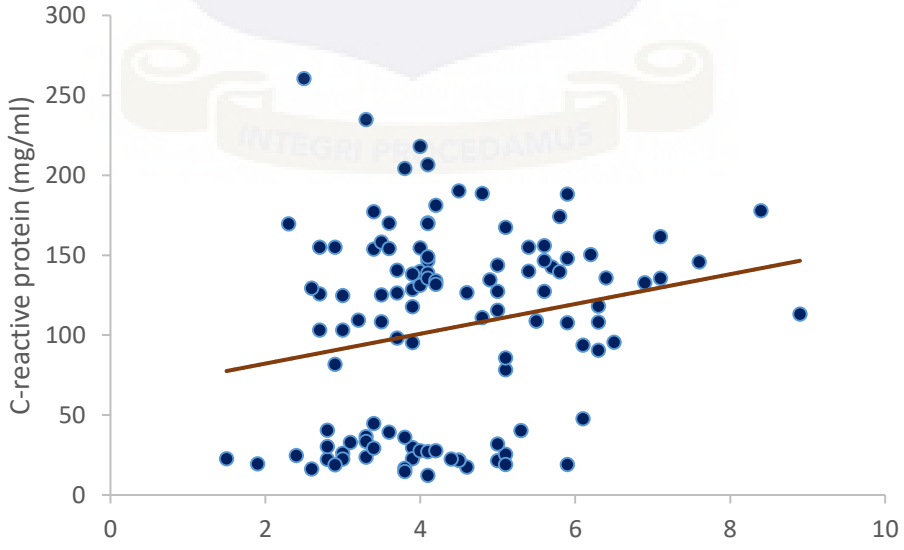

Fig. 9 Total cholesterol (mmol/L)

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussions

The study examined association between hepcidin and CRP in conjunction with insulin resistance in a case-control design involving Ghanaian T2DM patients. The study outcome indicated no correlation between insulin resistance and CRP, as well as hepcidin. This can be best explained by the contribution of inflammation in T2DM pathogenesis. Few studies in Africa had reported these parameters in healthy individuals, and there is paucity of data on this in SSA (Aregbesola et al., 2015; Bansal et al., 2010; Busbridge et al., 2009; Doi et al., 2005; Ganz et al., 2008).

In Ghana, no study was found in literature that has reported the levels of Hepcidin in T2DM patients. This investigation, therefore, is foremost to account for the levels of hepcidin, a regulator of iron homeostasis, in T2DM patients in Ghana. Also, variations in data on the link existing between hepcidin and inflammatory cytokines in diabetes patients as well as healthy individuals have been widely investigated and observed. Whilst most studies have reported associations between diabetes and hepcidin levels in diabetes (Aregbesola et al., 2015; Derbent et al., 2013; Aigner et al., 2013; Jiang et al., 2011; Pechlaner et al., 2016; Fernández-Real et al., 2009; Fernández-Real et al., 2015), others did not report on associations between them (Bekri et al., 2006). This study provides the baseline information on the association between these clinical parameters among T2DM for the first time in Ghana. A total of 140 subjects with 111 T2DM and 29 non-T2DM were analyzed.

5.2 CRP and diabetes

This study demonstrated a higher level of CRP in T2DM subjects matched with non-DM individuals. According to Pradhan et al (2001) "inflammatory markers such as CRP, have been related to the development of insulin resistance and type 2 diabetes" (Pradhan et al., 2001; Barzilay et al., 2001; King et al.,2003; Schmidt et al., 1999; Festa et al., 2000). higher levels of CRP is observed in T2DM and CVD patients and also associated with glucose level in people without diabetes (Wu et al., 2002). Cause and effect relation could not be inferred from the study because of the case control design of the study. Thus, whether poor glycaemic control leads to inflammation or vice versa. Or whether confounding factors influences them. Prospective studies are however, required to evaluate this query. Nevertheless, either direction of causality will have significant implication (Dehghan et al., 2007; Belfki et al., 2012). Thus, if uncontrolled glycaemia results in inflammation; increased CRP levels, then improved normoglycemia should lessen the level of inflammation and also lower the risk of CV complications (Doi et al., 2005; Belfki et al., 2012).

According to Ford (1999) "CRP is known to be higher in people with impaired glucose tolerance and frank diabetes" (Ford, 1999; Wu et al., 2002; Dehghan et al., 2007). Also, high CRP is found to be a possible factor for T2DM pathogenesis (Pradhan et al., 2001; Schmidt et al., 1999; Festa et al., 2000). Festa et al (2000) found the existing link between CRP and insulin resistance (Festa et al., 2000). In this study, a positive correlation was detected between CRP and IR. We also observed from table 4. That as CRP levels increased, insulin resistance levels also increased in the same population of T2DM. this can be inferred from the involvement of inflammation in T2DM pathogenesis.

Other investigations have linked hyperglycaemia and inflammation by demonstrating concurrent inflammation and IR at the physiologic level (Yudkin et al., 1999; Fichtlscherer et al., 2000). One of the various mechanisms projected is oxidative stress on the endothelium, thus promotes inflammation and enhanced by hyperglycaemia (Mohanty et al., 2000; Dandona and Aljada, 2002) and these findings is in line with this study which establish an association between hyperglycaemia and inflammation using CRP as an inflammatory indicator in adults with diabetes.

5.3 Relationship between Hepcidin and diabetes

The present study showed that insulin resistance interferes with iron balance in diabetes. Reduced levels of hepcidin was observed in the T2DM patients with negative correlation with IR. The mean levels were compared among the categories of study subjects as shown in Table 5, in an unadjusted analysis.

According to Rajpathak et al (2009), in type 2 diabetes individuals, "reduced hepcidin concentrations may contribute to iron overload by increasing the intestinal absorption of iron". gradual elevation of iron levels, are associated with elevated risk of Type 2 diabetes (Rajpathak et al., 2009).

It is established that insulin resistance can suppress hepcidin expression in mammals on a high fat diet (Le Guenno, Chanséaume, Ruivard, Morio, & Mazur, 2007). Whether the decrease in hepcidin level in T2DM individuals is key or minor event to IR is yet to be resolved. Nonetheless, the link between iron homeostasis (hepcidin) and insulin resistance is bidirectional. According to Fernandez-real et al (2002) "iron accumulation favors insulin resistance and insulin resistance may in turn result in increased body iron stores" (Fernández-Real et al, 2002b). Hence, decrease in the

concentration of hepcidin that results in improved absorption of intestinal iron can worsen insulin resistance (Pechlaner et al., 2016).

5.4 Relation between Hepcidin and CRP in diabetes

The study involved the link between hepcidin; iron regulation and CRP; inflammation in T2DM and non-T2DM individuals. Reduced hepcidin concentration was observed in the diabetes patients than that of the control arm. CRP concentration in T2DM patients was higher (Table 4).

Studies have proven that, serum iron and inflammation are the major stimulants for hepcidin expression and secretion (Theurl et al., 2008; Fleming, 2008). we found out that non diabetes had higher levels of serum hepcidin than in T2DMs (Table 4). Increase visceral adipose tissues also contributes to increase in hepcidin levels to some extent (Coimbra et al., 2013). In this investigation, we couldn't determine whether the decreased concentrations of serum hepcidin occurred as a result of excess plasma iron or as a result of inflammation as iron was not measured. Conversely, comparable observations were found in most studies where low hepcidin concentrations was suggested as one of many constituents of the inflammatory insulin resistance syndrome (Amir et al., 2012). low hepcidin level is also known to be associated with diabetes onset (Rolo and Palmeira, 2006). However, few studies have correlated increased hepcidin levels with insulin resistance in diabetes (Al-Hakeim et al., 2012).

Earlier postulation indicate how elevated hepcidin level reflects increase in plasma iron concentrations. The increase hepcidin level can change hepatic insulin clearance and result in hyperinsulinemia as a result impairs insulin secretion in the pancreas (Lipinski et al., 2011); and induce β- cell apoptosis (Martyn et al., 2008).

Literature has shown that monocytes which are inflammatory cells express and secrete hepcidin. Though the exact mechanism is yet to be determined. In a study of 30 gestational diabetes women, hepcidin was found to be linked with inflammation, compared with controls and impaired glucose tolerance (Derbent et al., 2013). These women also had increased CRP levels but did not correlate with other inflammatory markers. outcomes suggest the role of hepcidin in iron regulation under an inflammatory milieu (Fleming, 2008)

Findings of the current study revealed that, inflammation in T2DM patients correlated positively with hepcidin levels. Also we observed that the highest hepcidin levels were found in T2DM individuals, β cell function and insulin sensitivity were also reduced in T2DM subjects at significant levels and these were linked with reduced insulin levels compared with non-diabetes controls.

CONCLUSION AND RECOMMENDATIONS

5.5. Limitations

1. Cross sectional data was collected in this study. As a result, casualty cannot be inferred from the findings of the study. A longitudinal study can be used to assess the causes of these relations in Ghanaian population.

2. Older participants were recruited for this study. This confounds the results with natural ageing process of diabetes complications. Also the older population had been exposed to many stimuli such as acute inflammatory diseases which might influencing CRP levels.

3. Also the controls recruited was far less than the cases, hence affecting the true reflection of the findings.

5.6 Conclusions

The focus of this study was to investigate and evaluate the association of hepcidin- being a major regulator of plasma iron, insulin resistance and CRP- an inflammatory protein among type 2 diabetes patients at the NDMRC of the Korle-Bu teaching hospital, Accra compared to non-diabetes controls. From the results and discussions, the following conclusions were drawn.

- A higher level of CRP in T2DM subjects compared with non-DM subjects
- Reduced level of hepcidin concentrations was observed in T2DM patients as compared to non-diabetes subjects
- Strong association was observed between iron regulation and inflammation in type 2 diabetes cases as hepcidin concentration decreased with increasing CRP levels.

- Insulin resistance could result in down regulation of hepcidin and hence can be said to interfere in hepcidin expression in diabetes hence reduction in hepcidin concentration as insulin resistance increased.

5.7 Recommendation

1. A study involving treatment of naïve diabetes with cardiovascular complications subjects might be able to determine the actual burden of attributable to hypertension and diabetes. Also, this design could be used to test the association between hepcidin, CRP and biochemical parameters.

2. Longitudinal studies could be designed to assess the impact of diabetes with time. The study could be designed to assess the ability of various indices of cardiovascular complications of diabetes to predict end-organ damage. The study could involve younger subjects who might be free from most pro-inflammatory agents.

3. The validity of the various tools used to assess the levels of hepcidin, CRP as well as insulin resistance in diabetes need to be determined in a larger Ghanaian population, taking into account age, gender, disease state and various risk factors.

4. As performed in other studies, a community-based study could be designed to assess the levels of iron, its regulatory hormones as well as other proinflammatory cytokines in Ghanaian population.

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APPENDICES

CONSENT FORM

Appendix A:

INFORMED CONSENT FORM

Participant ID Number: _____ Participant Name: _____

Study Title: Association between hepcidin, insulin resistance and CRP among T2DM patients at the korle-bu teaching hospital

Diabetes mellitus is a sugar-related disease, which may lead to diseases of the heart and blood vessels. Subjects with Diabetes also tend to develop problems with a metal called iron that is found in small quantity in our blood. High levels of iron in blood can destroy the tubes that carry blood called arteries, leading to the formation of fatty blockages (atherosclerosis). How important problems with iron levels, inflammation and resistance in insulin levels are in Ghanaians with diabetes is not known. Iron levels in the blood is determined by the levels of a certain protein in the blood called ferritin and regulated by another protein called hepcidin. Researchers from the School of Biomedical and Allied Health Sciences, Korle Bu, Accra are conducting a research to find out how many persons with diabetes compared to those without diabetes have high levels of iron, high rates of inflammation as well in increased resistance in insulin level measured as plasma levels of hepcidin, CRP and insulin.

You are to understand that taking part in the research is entirely voluntary. You are further to note that you may refuse to take part or withdraw from the study at any time without anyone objecting.

You are likely to spend the best part of the morning at the Diabetes Centre. For you to qualify to be part of this research, you should be between the ages of 30 and 70years. If you want to take part in the research, you would be asked to come to the hospital after you have fasted overnight for 8-12 hours and report to the Diabetes Clinic at 7:00 in the morning. We will ask you to provide information about yourself, your health and that of your family. You may feel uncomfortable providing such information. In addition, your blood pressure, height, weight and amount of fat in you will be measured. In addition, some special medical equipment that measure blood pressure

will be applied to your arms. These procedures are painless and might give slight tingling sensations for few seconds when the cuffs inflate. A gel will be applied to the site of measurement to allow us obtain the measurement. An amount of blood which is not very different from what you are normally asked to provide will be drawn for this analyses when you first come to our center. You are assured that this amount will not affect your health. You may be asked to take a glucose drink if you do not already have diabetes to find out if you have diabetes or not. All the tests we will do for you in connection with this research will be free of charge.

Information we collect on you in this study will be kept confidential and secure. The information will only be available to the doctors and scientists conducting this study. You are further assured that if a report of this study is prepared for the scientific and medical community you will not be identified by name.

You may experience a minor bruise and/or temporary discomfort at the site of the blood draw and this risk is no more than you will normally be exposed to for having a blood draw routinely at our hospital. We will reduce this happening by asking experienced staff to take the blood. There may not be any direct benefit to you for taking part in the study. For those who do not have diabetes you will be informed of your diabetes status; if you should have diabetes you will be asked to see one of our diabetes doctors for care. The study will, help us appreciate the importance of the above conditions in Ghanaians with diabetes. The research may also help us put in place strategies to screen for these conditions to allow early treatment.

All your test results will be explained to you. You may through this study discover that you have fatty blockages in your arteries, stiff arteries or diabetes if you do not already have diabetes. You will be advised to attend our Centre if you should have any of these conditions on testing. Is there something you do not understand or do you have any questions or concerns about this Research? Should you later wish to have any matter or question relating to this study clarified do not hesitate to contact Miss. Linda Adu-Gyamfi Boadi, Department of Medical Biochemistry, School of Biomedical and Allied Health Sciences, (Tel number, 024 07 856 79),

This study is conducted under the supervision of Dr. Kwame Yeboah. Department of Physiology. Korle-bu. And Dr. Nii Aryee Aryitey. If you have any question about your rights as a research participant, please do feel free to contact Linda Adu- Gyamfi Boadi on 0240785679.

CONSENT

I have fully explained to _____ the nature and purpose of the above described research, its procedures, risks and benefits. I have allowed the subject to ask questions and have answered and will answer to the best of my ability, all questions relating to the study.

_____ _____ _____

Signature Full Name of Staff Member Date

I _____, have read (or have had read to me in a language that I fully understand) the proposed study and that I have understood what is going to be done. Also, any concerns I have, have fully been addressed. My signature or thumbprint below indicates that I have understood what is going to be done and that I agree to take part in the study.

_____ Date:_____

(Signature/thumbprint of Subject)

_____ Date:_____

(Signature: Witness)

Appendix B: Questionnaire Form

Association between hepcidin, insulin resistance and CRP among T2DM patients

Date of Evaluation: _____ Hosp. #._____

SECTION A: DEMOGRAPHIC DATA

(1) (a) Name of patient_____ (b) Age_____

(c)Gender_____ (1 =M; 2=F)

(2) Level of highest education attained? _____

(0=none, 1 =elementary (primary/middle); 2= secondary/ vocational/ commercial; 3=tertiary/university 4= other (specify) _____

(3) Current occupation? _____ (please circle where appropriate) 1=executive/professional/Managerial/Administration; 2=Paraprofessional teachers, nurses, technologist, technicians) 3=Business person; 4=Trades person 5= Hawker/Trader; 6= clerical staff /accounts clerk/messengers 7=skilled manual worker/ laborer; 8= unskilled laborer; 9= housewife; 1) = student; 11 = other (specify) _____

(4) Employment status_____ (1 = unemployed; 2= part-time employment; 3= full employment)

(5) Retirement status_____ (1 = yet to retire; 2= retired and not working; 3= retired but working)

(6) How long have you had diabetes _____years _____months (if less than a year).

(7) (a) what treatment do you currently take for your diabetes____(1 = diet only

2=daonil /glibenclamide (other sulplonylurea); 3=metformin/glucophage;

4= daonil + metformin; 5= insulin; 6= thiazolidindione

7. Other? (combination), specify_____

(b) Apart from your current treatment, have you had other treatment for diabetes? _____ (yes=1; No=2)

(c) If yes, which treatment? _____ (1=diet only; 2=daonil/glibenclamide (other sulphonylurea); 3= metphormin/glucophage; 4= daonil+metformin: 5= insulin; 6=thiazolidindione

7. other (combination), specify_____

(d) How long were you on that treatment? _____ yrs. ____months (if less than a year)

SECTION B: RISK FACTOR DATA

{For questions 8-11 Use 1 =Yes, and 2=No}

(8) Do you currently smoke cigarette? _____

a) If yes, how many sticks on average do you smoke a day? _____

b) At what age did you start smoking? _____

(9) Have you ever smoked cigarettes? _____

a) if yes, for how long did you smoke? _____ yrs _____ months

b) if yes, how many sticks did you smoke a day? _____

(10) Are you in a close relation with a smoker? _____

a) if yes, who? _____ (eg. wife, sister, father, son, grandfather, etc)

(b) Did you live with that person(s)? _____ (Yes=1; No=2)

(11.) Do you currently drink alcohol? _____

(a) If yes, how many bottles or glass of drinks on average do you consume in a week? _____

(b)If not that frequent, how many drinks in a month? _____

(Please note that 1 unit of alcohol = half a pint of beer, a tot of gin / whisky, a glass of wine)

Anthropometry/Physical Measurements

(a) Height in (m) _____ Weight (kg) _____

(b) Waist girth 1 (cm) _____ Waist girth 2 (cm) _____

(c) Hip " 1 (cm)_____ Hip " 2(cm)_____

(f)SBP1_____ DBP1_____ Heart rate_____/min

(After 10 minutes)

(h) SBP2_____ DBP2 _____ Heart rate_____/min

(After 10minutes)

(i) SBP3_____ DBP3_____ Heart rate_____/min