EVALUATION OF HEPCIDIN AS A POTENTIAL BIOMARKER FOR THE DIFFERENTIAL DIAGNOSIS OF IRON DEFICIENCY ANAEMIA AND ANAEMIA OF CHRONIC DISEASE AT NDOLA CENTRAL HOSPITAL, ZAMBIA

By;

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A dissertation submitted to the University of Zambia in partial fulfillment of the requirements of the award of Master of Science Degree in Pathology (Haematology)

UNIVERSITY OF ZAMBIA
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DECLARATION

I, Ephraim Chikwanda, do hereby declare that the work presented in this study for the Master of Science degree in Pathology (Haematology) has not been presented whether wholly in part for any other study programme at the University of Zambia or any other university and is not being submitted for any other Masters programme. I further declare that this work is entirely the result of my own independent investigations. All the sources I have quoted and the various persons to which I am indebted are acknowledged both as text and final references.

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CERTIFICATE OF APPROVAL

This dissertation of Ephraim Chikwanda is approved as a partial fulfillment of the requirement for the award of the degree of Master of Science in Pathology (Haematology) by the University of Zambia.

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ABSTRACT

Anaemia affects approximately 1.62 billion people globally corresponding to 24.8% of the world's population. Iron deficiency anaemia (IDA) and anaemia of chronic disease (ACD) are the most common forms of anaemia. A hormone produced by the liver, hepcidin, is the primary regulator of iron homeostasis and its production increases in ACD and decreases in IDA. Usually, ACD and IDA coexist and sometimes look identical on peripheral blood smears.

The current study aims to evaluate the diagnostic value of hepcidin to predict ACD from IDA as well as the diagnostic value of hepcidin to predict ACD from a combination of IDA and ACD.

Specimens presenting with haematological indices suggestive of IDA and/or ACD following World Health Organisation (WHO) standard case definitions were identified among samples coming to the Haematology laboratory for routine investigations. Serum hepcidin, serum ferritin, serum iron and total iron binding capacity (TIBC) were assessed. Demographic data was obtained from specimen requisition forms.

Of the 66 participants, 62.1% (n = 41) were females and IDA was more common among females (36.4%) than males (6.1%) while ACD was more common in males (19.7%) than females (12.1%). Anaemia of chronic disease participants had significantly higher hepcidin levels than IDA and IDA/ACD ($p<0.001$). There was a significant positive correlation between serum hepcidin and serum ferritin levels ($p < 0.001$).

We found that IDA participants had significantly lower hepcidin levels than ACD and IDA/ACD combined. Therefore, serum hepcidin could be considered in diagnosing and distinguishing ACD from IDA or IDA/ACD as it also had high diagnostic sensitivity and specificity compared to other markers.

Key words: *Hepcidin; Iron Deficiency Anaemia; Anaemia of chronic Disease.*

DEDICATION

I dedicate my work to my family for the overwhelming support and encouragement I got throughout my studies. To my loving wife, Shebbah your words of encouragement and push for tenacity gave me the impetus to forge ahead even when things seemed impossible. To my two sons, Ephraim and Stephen, you gave me that smile and motivation I needed most especially in difficult times. I love you.

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ABBREVIATIONS AND ACRONYMS

ACD	Anaemia of Chronic Disease
AUC	Area under the Curve
BMP	Bone Morphogenetic Proteins
CBC	Complete Blood Count
CHr	Reticulocyte Haemoglobin Content
CKD	Chronic Kidney Disease
CRP	C-reactive protein
CV	Coefficient of Variation
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ESA	Erythropoiesis Stimulating Agents
HAMP	Human Antimicrobial Peptide
Hb	Haemoglobin
HCT	Haematocrit concentration
HH	Hereditary hemochromatosis
HRP	Horseradish peroxide
ID	Iron deficiency
IL-6	Interleukin-6
IRIDA	Iron refractory iron deficiency anaemia
IV	Intravenous
LEAP-1	Liver-Expressed Antimicrobial Protein
MCH	Mean Cell Haemoglobin

MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Cell Volume
NCH	Ndola Central Hospital
PBS	Peripheral Blood Smear
RBC	Red blood cell
ROC	Receiver Operating Characteristics Curve
RDW	Red Cell Distribution Width
RES	Reticulo-endothelial system
SOP	Standard Operating Procedures
SPSS	Statistical Package for Social Science
STfR	Serum transferrin receptor
TIBC	Total Iron Binding Capacity
TSAT	Transferrin saturation
UIBC	Unsaturated Iron Binding Capacity
UNZA	University of Zambia
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

Anaemia is a condition in which the number of red blood cells (RBCs) or their oxygen carrying capacity is insufficient to meet physiological needs, and this varies by age, sex, altitude, smoking and pregnancy status (WHO, 2008). World Health Organisation (WHO) further defines anaemia as blood haemoglobin (Hb) values less than 7.7mmol/L (130g/l) in men and less than 7.4mmol/L (120g/l) in women (WHO, 2008). As well as reduced Hb, anaemia is usually accompanied by a reduction in the number of RBC count and packed cell volume (PCV) or haematocrit (HCT) (Rodak, 2007). Broadly, causes of anaemia may be classified as impaired RBC production, increased RBC destruction (haemolytic anaemias), blood loss and fluid overload (Rodak, 2007; Hoffbrand *et al*., 2005). Aetiology of anaemia is frequently difficult to determine even after extensive investigations including the bone marrow (BM) examination. It is reported as nutritional (34%), renal insufficiency (12%), chronic diseases (20%) and unexplained (24%). The anaemia of chronic disease (ACD) and iron deficiency anaemia (IDA) are the two most prevalent causes of anaemia (Karuna *et al*., 2011; Rasheed *et al*., 2013; Joosten *et al*., 1992; Geerts *et al*., 2012). An inflammatory process plays a central role in the pathogenesis of the ACD with low serum iron and normal to increased iron stores, while the absence of iron stores is the hallmark of the IDA (Weiss *et al*., 2005; Geerts *et al*., 2012). However the differential diagnosis between these two causes of anaemia is often difficult in clinical practice. The diagnosis is even a greater challenge in case of the combination of ACD and IDA in the same patient (Ganz, 2006).

The discovery of hepcidin in 2000 by Krause *et al*., and 2001 by Park *et al.*, working independently has drastically increased the understanding of the control of iron metabolism.

Hepcidin hormone is the primary regulator of iron homeostasis and mediator of ACD. Hepcidin is a 25-amino acid (cysteine-rich) peptide hormone produced in the liver. In humans, it is encoded by Hepcidin Antimicrobial Peptide (*HAMP*) gene (Ganz, 2003; Krause *et al*., 2000; Pigeon *et al*., 2001; Park *et al*., 2001). The mechanism of hepcidin activity depends on hepcidin interaction with ferroportin. Hepcidin binds to ferroportin and causes its internalization and degradation in endolysosomes, which in turn blocks the iron transport via ferroportin (Justyna *et al*., 2014). When iron stores are adequate or high, increased hepcidin expression inhibits intestinal iron absorption, release of recycled iron from macrophages and its transport across the placenta. On the other hand when iron stores are low, hepcidin production is suppressed. By modulating hepcidin expression, organisms can control plasma iron level and maintain iron metabolism homeostasis (Kemna *et al*., 2008; Ganz 2006; Atanasiu *et al*., 2007; Knutson *et al*., 2005; Nemeth *et al*., 2004). There is enormous interest in quantifying circulating hepcidin levels in clinical samples. Hepcidin measurement can be a helpful test distinguishing ACD from IDA, as it is known that hepcidin production is induced by inflammation (ACD) and reduced in iron deficiency states (IDA) (Brugnara, 2008; Nemeth *et al*., 2004). In principle, hepcidin measurements could complement the most frequently used indicators of total body iron stores, such as serum iron and ferritin (Ganz 2006; Roe *et al*., 2009), in addition to others such as transferrin receptor, transferrin saturation, and Zinc protoporphyrin (Bain *et al*., 2012; Paiva *et al*., 2000).

The possible diagnostic value of hepcidin determination depends on the limitations of current iron markers. Serum iron has diurnal variation, elevated premenstrually, variability according to methodology and haemolysis, high levels in oral contraceptives, high levels in hepatitis and low to normal in inflammation.

Transferrin saturation (TSAT) increases up to 100% in oral iron intake, and rises to 75% in oral contraceptives. Transferrin is high in pregnancy and estrogen therapy, with decreased synthesis in chronic liver disease or malnutrition and low levels due to loss in nephrotic syndrome or protein losing enteropathy. Measurement of serum ferritin is frequently of little value, as ferritin is an acute phase protein as well as an indicator of iron stores, and levels will be increased in the presence of inflammation (Brugnara, 2003; Wish, 2006). The measurement of serum transferrin receptor (sTFR) has been suggested as a possible tool for differentiating between ACD and IDA. However, in practice interpretation of this assay in differentiating IDA from ACD has proved more difficult (Mast *et al*., 1998), and the assay has not been standardized. The gold standard for assessment of iron stores remains a Perl's stained bone marrow aspirate, but a bone marrow biopsy/aspirate is of limited value in the diagnosis of ACD, so other non-invasive tools for measurement of iron supply are needed.

1.2 Statement of the Problem

Anaemia is a global public health problem affecting both developing and developed countries with major consequences for human health as well as social and economic development. It is the most common disorder of the blood (Janz *et al*., 2013). Globally, anaemia affects 1.62 billion people (95% CI: 1.50–1.74 billion), which corresponds to 24.8% of the population (95% CI: 22.9–26.7%). The highest prevalence is in preschool-age children (47.4%, 95% CI: 45.7–49.1), and the lowest prevalence is in men (12.7%, 95% CI: 8.6–16.9%) (WHO, 2008).

IDA is the most common form of anaemia and affects nearly 1 billion people (Vos *et al*., 2012). It is generally assumed that 50% of the cases of anaemia are due to iron deficiency (WHO, 2005). ACD is the second most common form of anaemia and is the most common form of anaemia among hospitalized patients (Cullis, 2013; Ganz, 2011; Rodak, 2007).

Anaemia of chronic disease and IDA usually coexist and may be difficult to diagnose. The diagnosis of IDA and ACD is typically performed by assessment of values for traditional quantitative laboratory tests: serum iron, total iron binding capacity (TIBC), TSAT, serum ferritin and RBC indices (mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), mean cell volume (MCV) and red cell distribution width (RDW)). Several studies have shown that these biochemical parameters have some limitations in distinguishing IDA from ACD especially when they are used individually (Brugnara, 2003; Wish, 2008; Bain *et al*., 2012). For instance, serum iron has diurnal variation, elevated premenstrually, variability according to methodology and haemolysis, and low to normal levels are seen in inflammation. Measurement of serum ferritin is frequently of little value and the main drawback is that ferritin is an acute phase protein as well as an indicator of iron stores, and levels will be increased in the presence of inflammation (Brugnara, 2003; Wish, 2006). Therefore, normal values may be seen despite iron deficiency in many clinical disorders (e.g., chronic infection or inflammation, liver disease and malignant neoplasms) that have a higher prevalence in hospitalized patients. In Zambia, the diagnosis of anaemia is mostly based on RBC indices which have little value in differentiating IDA from ACD as these conditions show similar changes in RBC indices. Therefore, carrying out this study to evaluate hepcidin as a potential biomarker for the differential diagnosis of IDA and ACD is necessary.

1.3 Study Justification

While no single test is reliable to distinguish IDA from ACD, hepcidin, a key regulator of iron metabolism, which is reduced in IDA and increased in ACD is hoped to be useful as a diagnostic test for differentiating IDA from ACD.

Since the discovery of hepcidin in 2000, several studies have been conducted to evaluate the role of hepcidin in many diseases and whether it can be used as a diagnostic test in distinguishing between IDA and ACD. For instance, the study done by Röhrig *et al*., showed that hepcidin would be used as a diagnostic parameter to differentiate between IDA and ACD in the future. In 2008, Ganz *et al*., performed successful validation of a competitive enzyme-linked immunosorbent assay (C-ELISA) for human hepcidin and noted that this assay could be used in detecting physiological and pathological changes in serum or urine hepcidin (Ganz *et al*., 2008).

This study provided information on the utilization of hepcidin as diagnostic test to differentiate IDA from ACD. The study confirmed the distinct difference between ACD and ACD+IDA or IDA. It is important to distinguish between ACD and ACD+IDA or a pure case of IDA because this difference implicates treatment, making iron supplement necessary in ACD/IDA or pure IDA and contraindicated in ACD. Hepcidin levels may be more useful in distinguishing patients with pure ACD from those with IDA or ACD+IDA as these conditions are believed to contain different levels of hepcidin. Besides, the finding of this study might draw the attention of physicians to consider the analysis of hepcidin hormone level for protocol of anaemia.

1.4 Research Question

Do serum hepcidin levels better predict ACD from IDA than serum ferritin?

1.5.0 Objectives

1.5.1 General Objective

To evaluate hepcidin as a diagnostic test for the differential diagnosis of IDA and ACD at Ndola Central Hospital, Zambia

1.5.2 Specific Objectives

1. To evaluate the diagnostic value of hepcidin to predict ACD from IDA
2. To evaluate the diagnostic value of hepcidin to predict ACD from IDA/ACD

2.4.1 Biology of Hepcidin

Hepcidin, a 25-amino acid peptide hormone produced in the liver and encoded by *HAMP* gene, is a central regulator of iron homeostasis (Ganz, 2003; Fleming, 2008; Nemeth *et al*., 2004). Krause *et al*., in the year 2000 isolated a peptide hormone from human blood and named it liver-expressed antimicrobial peptide (LEAP-1), whereas Park et al. after one year isolated the same peptide from human urine and named it hepcidin (hepatic bactericidal protein) (Krause *et al*., 2000; Park *et al*., 2001; Politou *et al*., 2004).

The gene encoding hepcidin (*HAMP*, 19q13) is expressed in the liver, heart, lungs, brain, spinal cord, intestine, stomach, pancreas, adipocyte, skeletal muscles, testis and macrophages. In humans, expression of hepcidin mRNA is mostly confined to the liver with low levels being found in the muscle, intestine, stomach, colon, lungs, and heart (Justyna *et al*., 2014; Kemna *et al*., 2008). Hepcidin is synthesized as preprohepcidin with 84 amino acids which is then cleaved to prohepcidin having 60 amino acids. Prohepcidin then gives rise to 25 amino acids containing peptide hormone hepcidin. Structural studies have revealed that human hepcidin is a β-sheet-cysteine-rich peptide having a hairpin configuration (Ganz, 2003; Kemna *et al*., 2008; Ganz, 2006; Leong and Leonnerdal, 2004).

2.4.2 Function of Hepcidin

Hepcidin is known to be the principal iron-regulatory hormone maintaining iron balance both in iron deficient states and iron loaded conditions. It down-regulates intestinal iron absorption, blocks iron transport across the placenta and induces iron sequestration in the macrophages, as it has a potent inhibitory role against egress of iron from macrophages (Ganz, 2006).

When body iron requirements are high as a result of decreased iron stores or in cases of stimulated erythropoiesis, expression of hepcidin is decreased. A decline in the iron requirements leads to increased expression of hepcidin (Anderson *et al*., 2007; Munoz *et al*., 2009; De Domenico *et al*., 2007). In all species, the concentration of iron in biological fluids is tightly regulated to provide iron as needed, and also to avoid toxicity, since excess iron can lead to the generation of reactive oxygen species (ROS), and decreased iron levels can lead to anaemia (De Domenico *et al*., 2007; Zhang and Enns, 2008). Thus, maintenance of body iron stores is essential, because many human diets contain sufficient iron only to replace the small iron losses.

2.4.3 Molecular Biology / Mechanism of Action of Hepcidin

Hepcidin accomplishes it activity by reducing the transmembrane iron transporter, ferroportin. Ferroportin is the only mammalian cellular iron transporter and is expressed on the surface of reticulo-endothelial macrophages, hepatocytes, duodenal enterocytes and placenta cells (Atanasiu *et al*., 2007; Ganz 2006; Kemna *et al*., 2008). Hepcidin regulates posttranscriptionally ferroportin expression. Hepcidin, therefore lowers dietary iron absorption as a result of reduced iron transport across mucosal cells, reduces iron exit from macrophages (the main iron storage site), and reduces iron exit from the liver. Abnormally high levels of serum hepcidin in states such as inflammation, serum iron falls due to iron trapping in macrophages and liver cells and decreased gut iron absorption. This typically leads to anaemia due to an inadequate amount of serum iron being available for developing red cells. When hepcidin levels are abnormally low such as in haemachromatosis, iron overload occurs due to increased ferroportin mediated iron efflux from storage and increased gut iron absorption (Kemna *et al*., 2008; Ganz 2006; Atanasiu *et al*., 2007; Nemeth *et al*., 2004; Knutson *et al*., 2005).

Hepcidin synthesis and secretion by the liver is controlled by iron stores within macrophages, inflammation, hypoxia and erythropoiesis. Macrophages communicate with hepatocytes to regulate hepcidin release into the circulation via eight different proteins [hemojuvelin (HJV), hereditary hemochromatosis protein (HFE), transferrin receptor-2 (TfR-2), Bone Morphogenetic protein-6 (BMP-6), Matriptase-2, neogenin, BMP receptors and transferrin] (Fleming, 2008; Piperno *et al*., 2009). Inflammation and increase in iron stores are positive regulators of hepcidin production. Hepcidin is not the only regulatory hormone but also a type II acute phase reactant, meaning that its synthesis can be induced by interleukin-6 (IL-6). Interleukin-6 acts via its receptor and causes phosphorylation of signal transducer and activator of transcription 3 (STAT 3), dimerization of phosphor-STAT 3 and its translocation to the nucleus, where it interacts with hepcidin promoter (Fleming, 2008; Nemeth *et al*., 2004; Nemeth *et al*., 2003; Piperno *et al*., 2009). Hypoxia, anaemia and increased erythropoiesis are negative regulators of hepcidin production. Anaemia and hypoxia regulate the erythrocyte production through erythropoietin (Epo) synthesis. In addition, decrease in iron stores negatively regulates hepcidin production (Fleming, 2008; Piperno *et al*., 2009).

2.4.4 Hepcidin as a Potential Diagnostic and Therapeutic Tool

The discovery of hepcidin not only opened the way to understanding the iron metabolism but also helped to elucidate the pathomechanisms of many diseases such as hemochromatosis (HH) and ACD. Several studies on hepcidin have provided information on possible use of hepcidin as a diagnostic and therapeutic tool in many diseases. Hepcidin measurement can be a helpful test for distinguishing ACD from IDA, as it is known that hepcidin production is induced by inflammation (ACD), and reduced in iron deficiency status (IDA) (Nemeth *et al*., 2004; Brugnara, 2008; Cullis, 2013).

One of the greatest promises for the practical application of the hepcidin assay is the utilization of hepcidin in the diagnosis and monitoring of hemochromatosis. The development of synthetic hepcidin should be useful in the treatment of hemochromatosis and other iron-loading conditions (Gardenghi et al., 2010; Laftah et al., 2004).

In 2008, Ganz *et al*., performed successful validation of a competitive enzyme-linked immunosorbent assay (C-ELISA) for human hepcidin. This simple and robust assay can be used in detecting physiological and pathological changes in serum or urine hepcidin (Ganz *et al*., 2008). Being able to accurately measure hepcidin levels, researchers are exploring the benefits of knowing a patient's hepcidin level, including the possibilities to detect iron deficiency in infants, identify patients with anaemia of chronic disease who are non- responsive to erythropoietin stimulating agents (ESAs), and discover patients who will require intravenous iron supplements before oral iron proves ineffective (Zaritsky *et al*., 2009; Young and Zaritsky, 2009).

Röhrig *et al*., conducted a study on iron deficiency anaemia (IDA) and anaemia of chronic diseases (ACD) in the geriatric population. They aimed at studying whether serum hepcidin levels might serve as a diagnostic parameter to differentiate between IDA and ACD among the elderly. A total of 37 patients (age 69-97 years) were divided into 4 groups: group I (IDA), group II (ACD), group III (controls), and group IV (IDA/ACD). Serum hepcidin levels were analyzed using a commercially available ELISA kit (DRG Instruments, Marburg, Germany). They showed a strong positive correlation between serum hepcidin and ferritin (Spearman rho 0.747) and a statistically significant difference of hepcidin levels among all groups ($p = 0.034$). They concluded that hepcidin would be used as a diagnostic parameter to differentiate between IDA and ACD in the future (Röhrig *et al*., 2014).

CHAPTER THREE
METHODOLOGY

3.1 Study Site and Design

A cross sectional study was undertaken from November, 2015 to February, 2016 at Ndola Central Hospital in Ndola, Zambia.

3.2 Target Population

Specimens from anaemic individuals aged 18 to 60 years presenting with haematology indices suggestive of IDA and/or ACD as defined by WHO standard case definitions were identified among samples coming to Haematology laboratory for routine investigations.

3.3.0 Study Population

Anaemia was defined according to WHO criteria, as a haemoglobin concentration of <7.7mmol/L (13g/dl) for men and <7.4mmol/L (12g/dL) for women. Patients were classified as having IDA if they had serum ferritin levels of <30 ng/mL, TSAT <16% and active inflammation (defined as a level of CRP level of ≥10mg/L) was absent. Patients were classified as having ACD if they had serum ferritin levels of ≥ 100ng/mL, TSAT<16% and active inflammation. Patients were classified as having ACD+IDA if they had serum ferritin levels of < 100 ng/mL, TSAT<16% and active inflammation.

3.3.1 Inclusion Criteria

- IDA and ACD adult individuals aged 18 to 60 years were included in the study.
- Persons who had read and understood the rationale for participating in the study and given written consent without being forced to were included in the study.

3.3.2 Exclusion Criteria

- Individuals with IDA and ACD below the age of 18 and above the age of 60 were excluded from the study because haematological and biochemical parameters are usually affected by age. For instance, in healthy men and women, HB, RBC, HCT and other red cell indices remain remarkably constant until the 6^{th} decade (Bain, 2012). The reference values for children usually differ from adults.
- Pregnant women and individuals on estrogen, erythropoietin stimulating agent (ESA) or iron therapy.
- Patients with thalassemia, sideroblastic anaemia or any other anaemia apart from the ones stated in the inclusion criteria.

3.4.0 Sampling and Sample Size

3.4.1 Sampling Method

Convenience sampling of routine blood samples received in the Haematology Laboratory at Ndola Central Hospital.

3.4.2 Sample Size

To compare mean levels of serum hepcidin between IDA and ACD participants, the following equation was used to calculate the sample size.

$$\mathbf{N = [(Z_{\alpha/2} + Z_{\beta})^2 \times 2\sigma^2] / \delta^2}$$

Where n = the sample size required, σ = estimated standard deviation (7ng/mL) and δ = estimated minimum size difference of diagnostic significance (5ng/mL). The study had an 80% power at a significance level (alpha or p value) of 5%. Therefore, Z_{β} =0.84 and $Z_{\alpha/2}$ = 1.96.

Using the values above, the sample size was calculated as follows:

$$N= [(0.84+1.96)^2 \times 2 \times 7^2] / 5^2$$

$$\underline{\mathbf{N \approx 30}}$$

To account for any losses, a 10% sampling adjustment was factored in. Therefore, the total sample size required for each treatment arm was:

$$\mathbf{(30 + (10 / 100 \times 30)) = 33}$$

This meant that the study composed of a **total of 66 participants** and that a sample size of 33 in each treatment arm was sufficient to detect a minimum diagnostically important difference of 5ng/mL of serum hepcidin, assuming a standard deviation of 7.0ng/mL, using a two-tailed t-test of the difference between means, a power of 80%, and a significance level of 5%.

3.4.0 Data Collection

3.4.1 Clinical and Demographical Data Collection

Participants were recruited after they had been diagnosed with anaemia as described above. As the participants were being seen by the clinician, they were informed and enlightened about the study by the clinician who also provided the participants with the study information sheet.

If a patient autonomously agreed to be part of the study, they were required to sign the consent form, and assigned a serial number. Thereafter information on the patient's demographic and clinical data was collected.

3.4.2.0 Specimen Collection, Storage and Preparation

3.4.2.1 Specimen Collection

Blood samples were collected from 66 anaemic individuals. A total of 8 ml of blood was drawn into two plain containers from each participant.

Blood samples were collected from research participants via venipuncture from an antecubital vein or other visible veins in the forearm using the Evacuated Tube System (ETS). Blood collected in serum tubes (plain container) was used for iron parameters/ELISA. EDTA blood samples received in the Haematology laboratory were used for CBC analysis. These EDTA blood samples were sent to the laboratory for routine investigations. Specimens were collected according to the Clinical Laboratory Standards Institute (CLSI) procedures for collection of diagnostic blood specimens by venipuncture (Dennis et al., 2007).

3.4.2.2.0 Specimen Preparation and Storage

3.4.2.2.1 Preparation and Storage of Blood Specimens

Specimens were processed within 2 hours after collection. Complete blood count was performed using automated analyzers, Sysmex XT 2000i.

The blood in a serum container was left for a minimum of 15 minutes to allow it to clot and then centrifuged at 3000 rpm/10 minutes. Serum specimens were kept at -20 degrees Celsius until analysis.

3.4.2.2.2 Preparation of PBS

Blood films were prepared and fixed within two hours after collection. The blood films were prepared from EDTA-anticoagulated blood using Wedge Slide (push slide) technique developed by Maxwell Wintrobe. Films were made using frosted end clean slides measuring 75 × 25mm and approximately 1mm thick. A 2-3mm drop of blood was placed approximately 1 cm from frosted end of the slide using a wooden applicator stick or glass capillary tube. Then, without delay, a spreader was placed in front of a drop at an angle of about 30˚ to the slide and moved back to make contact with the drop. The drop was spread quickly along the line of contact.

With a steady movement of the hand, the drop of blood was spread along the slide at a rapid, even motion, and letting the weight of the slide (spreader) do the work. The slide was then allowed to air dry for about 10 minutes and then immediately fixed by immersing in a jar of methanol or absolute alcohol for 5-10minutes.

3.5 Quality Control

To ensure reliable results, quality control was performed on all the reagents, procedures and analytical instruments and analyzers to be used for specimen analysis according to the established quality control guidelines. Quality control included equipment calibrations and analytical control runs on all analyzers before each test analysis.

3.6.0 Specimen Analysis

3.6.1 Complete Blood Count (CBC) Analysis

Complete blood count was analyzed using the Sysmex XT Automated Haematology Analyzer. Before the specimen was run, start-up and quality control were performed according to the Sysmex XT 2000i haematology analyzer operator manual. For Patient Sampling-Sampler (Auto) Mode, a minimum of 1.0mL of blood was required.

The specimens were placed in the rack with barcodes facing the front of the rack. On the computer, the sampler icon was clicked, then [sampler start] and [OK] were placed. The specimen was automatically mixed ten times, then aspirated and analyzed according to the tests ordered. For patient sampling manual mode, a minimum of 85uL of sample was required. First manual icon was clicked, and then the specimen number was entered. Then the patient sample was mixed thoroughly and placed under the aspiration pipette. The manual button was then placed. The sample was then removed when 2 beeps sounded or when the green LED stopped blinking.

After running the specimen, a shutdown procedure was performed for every 24 hours or 500 cycles (Sysmex XT 2000 operators Manual).

3.6.2.0 Staining and Examination of Peripheral Blood Films

3.6.2.1 May-Grunwald-Giemsa Staining

Peripheral blood films were stained using May-Grunwald-Giemsa staining technique. The fixed films were transferred into a staining jar containing May-Grunwald stain freshly diluted with equal volume of buffered water. After the films had been allowed to stain for about 15 minutes, they were transferred without washing to a jar containing Giemsa stain freshly diluted with 9 volumes of buffered water, pH 6.8.

After staining for 10-15 minutes , the slides were transferred to a jar containing buffered water, pH 6.8, rapidly washing in three to four changes of water and finally allowed to stand undisturbed in water for a short time (usually 2-5 minutes) for differentiation to take place (Bain *et al.,* 2012).

3.6.2.2 Examination of Blood Films

The stained smears were examined in a systematic approach in order to gather all possible information. The low power (10X ocular, 10X objectives) was used to evaluate the quality of the smear, ascertain the approximate number of WBCs and platelets, and detect rouleaux formation, platelet clumps, and leukocyte clumps and other abnormalities visible at low magnification. Following low power examination of a peripheral smear, 40X objective (total magnification=400X) was selected. At this magnification, it was easy to select the correct area of the smear in which to begin the differential count and to evaluate cellular morphology. Then 100 X objective oil-immersion was selected for examination for any abnormal morphology of RBCs, WBCs and platelets (Bain *et al.,* 2012).

3.6.2.3.0 Determination of Serum Iron and Total Iron Binding Capacity

3.6.2.3.1 Determination of Serum Iron (Iron Chromazurol)

The materials used for determination of serum iron were: Reagent R (Acetate buffer pH (5.0) - 45mmol/L, chromazurol B- 0.2mmol/L, cetyltrimethyl ammonium bromide 2mmol/L, guanidine hydroxide 3mmol/L) and standard: STD (Iron 100µg/dL, 1 mg/dL, and 17.9µmol/L). The reagents were mixed as shown in Table 1 below and incubated for 5 minutes at 37°C. The absorbance of the standard and sample were read against reagent blank.

Serum iron reacted with chromazurol B and cetyltrimethyl ammonium bromide (CTMA) to form a coloured complex with an absorbance measured at 623nm. Serum iron reference range used was 60-170µg/dL (10-30µmol/L.

Table 1: Preparation of the Blank, Calibration and Sample

	Blank	**Calibration**	**Test**
Reagent R	1.0mL	1.0mL	1.0mL
Distilled water	40µL	-	-
Standard	-	40µL	-
Sample	-	-	40µL

3.6.2.3.2 Determination of Total Iron Binding Capacity (TIBC)

The reagents composition was: Reagent 1 (Iron saturating solution 520µg/dL, 5.2 mg/L, and 93µmol/L) and Reagent 2 (Magnesium carbonate). 1mL of reagent R1 and 0.5mL of sample were put in a centrifugation tube. Then they were mixed and incubated for 5 minutes to allow saturation of iron binding sites. After incubation, 1 level measuring spoonful of Reagent 2 was added and then the mixture was incubated for 20 minutes and shaken several times during this period.

The sample mixture was centrifuged at 3000 r.p.m. for 10 minutes and then the supernatant was collected. The Iron content of the supernatant was measured colorimetrically with the Iron Chromazurol method.

3.6.2.3.3 Calculation of Transferrin Saturation (TSAT)

TSAT is the ratio of serum iron and TIBC, multiplied by 100. It was calculated as shown below:

[TSAT = (Serum Iron / Total Iron Binding Capacity) x 100%]

3.6.2.4 Ferritin and C - Reactive Protein (CRP) Analysis

The C-reactive protein and ferritin were determined by turbidimetric immunoassay method on Olympus AU400 analyzer using Olympus diagnostics reagents, according to CRP and ferritin application protocol for Olympus instrument. Immune complexes formed in solutions scatter light in proportion to their size, shape, and concentration. Turbidimeters measured the reduction of the incidence light due to reflection, absorption, or scatter. In the Beckman Coulter AU system procedure, the measurement of the rate of decrease in light intensity transmitted (increased in absorbance) through particles suspended in the solution was the result of complexes formed during the antigen-antibody reaction between patient serum and antibodies coated on latex particles. The specimens (serum or plasma) were analyzed by automated Olympus AU400 chemistry analyzer (www.beckmancoulter.com).

3.6.2.5 Determination of Serum Hepcidin Hormone

Hepcidin levels were measured by the Hepcidin ELISA Kit (DRG-Kit Germany). All patients' samples were assayed in duplicate and were compared with those of the standard curve developed from the calibrator. All kit components and samples were brought to room temperature.

All reagents, working standards, and samples were prepared according to the manufacturer's protocol. The 96-well plates were coated with anti-Hepcidin antibody (monoclonal).

100µL of the sample buffer were added to each of these wells. 20µL of each standard, control and samples with new disposable pipette tips were dispensed into appropriate wells with care to avoid any contamination. The reaction mixture was incubated for 30minutes at room temperature on the plate shaker at 500rpm. A volume of 150µL Assay Buffer and 100µL Enzyme conjugate were added to each of these wells.

The reaction mixture was incubated for 180 minutes at room temperature on a plate shaker at 500rpm, and shaken briskly. The wells were rinsed 5 times with distilled water (400µL per well). The wells were struck sharply on absorbent paper to remove residual droplets. A volume of 100µL of Enzyme complex was dispensed into each well. The reaction mixture was incubated for 45 minutes at room temperature. The contents of the well were briskly shaken out. The wells were rinsed 5 times with distilled water (400µL per well). The wells were struck sharply on the adsorbent paper to remove residual droplets. A volume of 100µL of Substrate solution was added to each well and the reaction mixture was incubated for 30 minutes at room temperature. The enzymatic reaction was stopped by adding 100µL of stop solution to each well. The absorbance (OD) of each well was determined at 450 ± 10 nm with a microtiter plate reader.

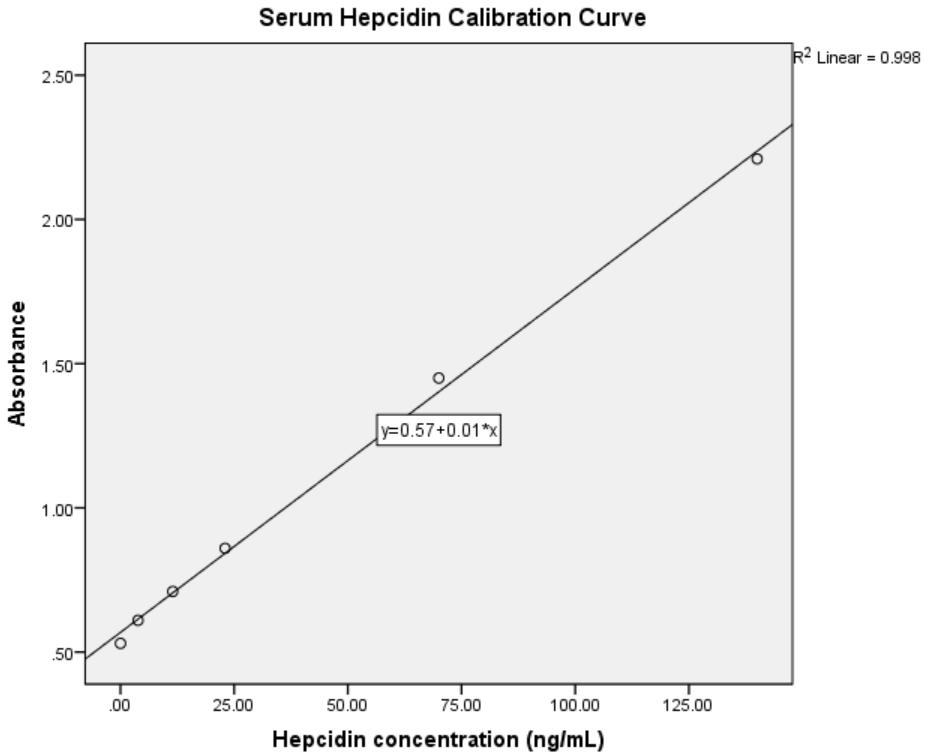


Figure 1: Serum Hepcidin Calibration Curve

Figure 1 shows the serum hepcidin calibration curve plotted from standard absorbances (O.Ds) against concentrations. The regression equation was used to calculate sample concentration from their respective O.Ds. The average absorbance values for each set of standards, controls and specimen samples were calculated. The mean absorbance for each sample was used to determine the corresponding concentration from the standard curve. The concentration of the samples was read directly from this standard curve. The range of this assay was 0.9-140ng/mL.

3.6.2.6 Statistical Analysis

Data was analyzed using IBM SPSS Statistical package version 21. The differences between the means of quantitative variables of study groups were tested by the independent sample t-test. The correlations between variables were calculated using Pearson's correlation analysis of numerical data. The diagnostic utility of serum hepcidin level as a test for differential diagnosis of IDA and ACD was evaluated according to the area under the ROC curve (AUC). The results in all the above mentioned procedures were accepted as statistically significant when the 2-tailed p-value was less than 5% ($p<0.05$)**.** Data was expressed as mean ± SD.

3.6.2.7 Ethical Considerations and Permissions

Ethical clearance was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC) before the commencement of the study. Patient information and results were kept confidential and access to this information was restricted to the researcher, supervisors and attending clinicians only. The specimen containers, request form or any other material for the patients were assigned a serial number hence they were identified by a unique study identifier. In no way was the participant's name or file number linked to the specimen or research results. The study participants were provided with an information sheet and given a thorough explanation of intent and rationale of the research after which the patient was given written informed consent without being forced. This ensured a true meeting of minds between the researcher and the patient. All this was done in private on a one to one basis to avoid undue influence that may affect or substitute the participant's will for that of any other persons. The study participants were free to withdraw from the study when they felt like without any prejudice. Permission to use equipment and facilities in the Department of Pathology at Ndola Central Hospital (NCH) was obtained in writing from the NCH Senior Medical Superintendent.

CHAPTER FOUR
RESULTS

4.1 Demographic Characteristics of the Study Population

Table 2 below shows the demographic characteristics of the study population. The mean age (SD) for the study population was 35.4 (±11.9) years. IDA patients were younger with mean age (SD) of 26.5 (±5.9) compared to 41.9(±10.0) and 42.3 (±12.7) years in ACD and IDA/ACD patients respectively. There were more females 41(62.1%) with anaemia than males 25 (37.9%). Twenty eight (28) (42.4%) study participants had IDA whereas twenty-one (21) (31.8%) and seventeen (17) (25.5%) had ACD and IDA/ACD respectively. IDA was found to be more common among females (36.4%) than males (6.1%) while ACD was more common in males (19.7%) than females (12.1%). Eight (8) (12.1%) males and nine (9) (13.6%) females had a combination of IDA and ACD.

Table 2: Demographic Characteristics of Study Participants by Sex and Age

	IDA	ACD	IDA/ACD	Total
Number cases	28 (42.4%)	21 (31.8%)	17 (25.8%)	66
Male	4 (6.1%)	13 (19.7 %)	8 (12.1%)	25 (37.9%)
Female	24 (36.4%)	8 (12.1%)	9 (13.6%)	41 (62.1%)
Mean age (SD)	26.5 (5.9)	41.9 (10.0)	42.3 (12.7)	35.4 (11.9)

IDA = iron deficiency anaemia; ACD = anaemia of chronic disease; SD = standard deviation

4.2 Haematological and Biochemical Markers of Participants

Table 3 shows the mean concentration of haematological and biochemical parameters of anaemia. Table 4 shows a comparison of laboratory parameters between IDA and ACD; IDA and IDA/ACD; and ACD and IDA/ACD.

The mean ferritin concentrations were 10.87 ±7.76ng/mL , 118.7 ± 43.3ng/mL and 63.60 ± 26.98; the mean CRP concentrations were 5.03 ± 6.69 mg/L, 30.60 ± 8.28 mg/L and 22.14 ± 9.91mg/L ; and the mean hepcidin concentrations were 6.41 ± 2.12 ng/mL, 15.39 ± 4.70 ng/mL and 7.31 ± 2.01 ng/ml for IDA, ACD and IDA/ACD respectively. The study found that IDA participants had significantly lower hepcidin levels than ACD ($p<0.001$). There was also a significant difference in serum hepcidin between IDA/ACD and ACD ($p < 0.001$) (Table 5). Serum ferritin levels were significantly higher in ACD than in IDA ($p < 0.001$). However, there was no statistically significant difference in mean serum ferritin concentration between IDA/ACD and ACD ($p= 0.072$) (Table 4). There was a significant difference in CRP levels between IDA and ACD ($p < 0.001$); IDA and IDA/ACD ($p < 0.001$); and ACD and IDA/ACD ($p =0.007$) as shown in table 5.

Table 3: Levels haematological and biochemical Indices in IDA, ACD and IDA/ACD

	IDA n=28	**ACD** n=21	**IDA/ACD** n=17
RBC (10^12/L)	3.10 ± 1.02	4.05 ± 5.93	3.37 ± 0.69
Hb (g/dL)	6.5 ± 2.5	8.1 ± 1.9	7.4 ± 1.8
HCT (%)	21.1 ± 7.4	25.3 ± 5.1	25.8 ± 7.1
MCV (fL)	65.1 ± 6.9	73.8 ± 3.2	69.1 ± 5.6
MCHC (g/dL)	30.8 ± 2.8	31.8 ± 2.52	31.2 ± 2.7
RDW (%)	21.8 ± 5.1	17.3 ± 1.8	20.7 ± 3.6
Serum iron (µg/dL)	36.85 ± 12.01	42.14 ± 12.61	41.88 ± 13.48
TIBC (µg/dL)	430 ± 69.26	540.2 ± 209.5	438.25 ± 92.56
TSAT (%)	8.02 ± 3.03	7.73 ± 2.87	9.68 ± 2.47
CRP (mg/L)	5.03 ± 6.69	30.60 ± 8.28	22.14 ± 9.91
Ferritin (ng/mL)	10.87 ± 7.76	118.76 ± 42.28	63.60 ± 22.9
Hepcidin (ng/mL)	6.41 ± 2.21	15.39 ± 4.70	7.31 ± 2.01

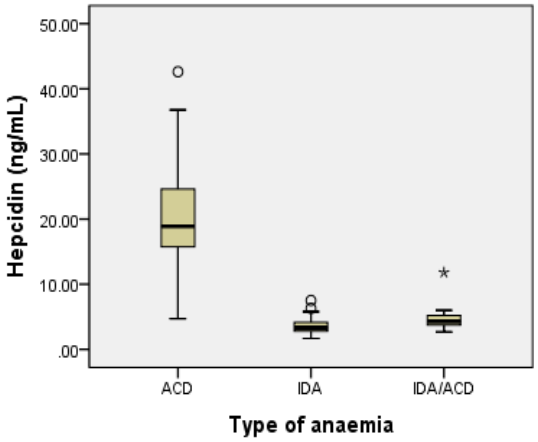
Values are expressed in mean ± SD

Table 4: Comparison of Laboratory Parameters between IDA and ACD; IDA and IDA/ACD; and ACD and IDA/ACD.

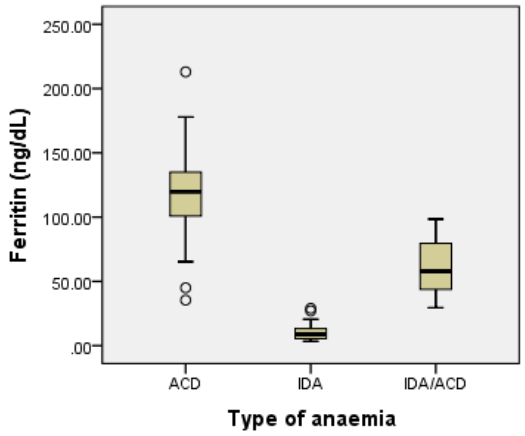
	IDA vs ACD		IDA vs IDA/ACD		ACD vs IDA/ACD	
	t-test	p-value	t-test	p-value	t-test	p-value
Age(years)	-6.768	<0.001	-5.868	<0.001	-0.111	0.912
RBC (10^12/L)	2.645	0.011	2.797	0.008	0.844	0.404
Hb (g/dL)	-1.171	0.247	1.404	0.168	2.16	0.038
HCT (%)	0.264	0.793	2.139	0.038	2.087	0.044
MCV (fL)	-5.398	<0.001	-2.005	0.051	3.294	0.002
MCHC (g/dL)	-1.328	0.191	-0.499	0.62	0.709	0.483
RDW (%)	3.928	<0.001	-0.88	0.384	-3.731	0.001
Serum Fe (µg/dL)	-1.495	0.142	-1.302	0.2	0.61	0.952
TIBC(µg/dL)	-0.379	0.706	-0.327	0.746	0.053	0.958
TSAT (%)	-1.997	0.052	-1.897	0.065	0.063	0.95
CRP (mg/L)	-11.954	<0.001	-6.918	<0.001	2.866	0.007
Ferritin (ng/mL)	-8.799	<0.001	-8.999	<0.001	1.782	0.072
Hepcidin (ng/mL)	-6.27	<0.001	-2.307	0.026	4.356	<0.001

The mean difference is statistically significant if $p < 0.05$

Serum hepcidin, ferritin and CRP distribution in IDA, ACD and IDA/ACD were also presented as box plots as shown in Figure 2. Anaemia of Chronic Disease patients exhibited a wide range of hepcidin distribution from 4.70 to 42.62 ng/dL compared to IDA and IDA/ACD patients which exhibited a narrow distribution of hepcidin from 1.67 to 7.50ng/dL and 2.67 to 11.80ng/dL respectively. The differentiation of median hepcidin levels was negligible in IDA and IDA/ACD. Ferritin levels were widely distributed (from 35.40 to 213.0ng/dL) in ACD compared to IDA (3.40 to 29.0ng/dL). Anaemia of Chronic Disease and IDA patients exhibit significantly distinct levels of hepcidin, ferritin and CRP.



A.



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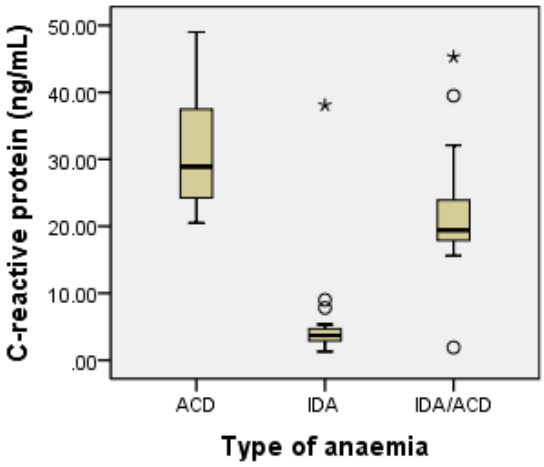


C.

Figure 2: Distribution of serum hepcidin (A), serum ferritin (B) and CRP (C) in IDA, ACD and IDA/ACD. Data are presented as box plots, where boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the minimal and maximal values.

4.3 Correlation between Hepcidin and Haematological and Biochemical Parameters of Study Population

Table 6 shows the relationship between hepcidin and haematological and biochemical markers of anaemia. Serum hepcidin levels correlated slightly better with serum ferritin levels and MCV (pearson's r = 0.624, p < 0.001; r = 0.508, p < 0.001, respectively) than CRP (pearson's r = 0.422, p < 0.001).

There was a weak negative correlation between serum hepcidin and RDW (Pearson's r = -0.279, p = 0.024). However, there was a statistically insignificant correlation between hepcidin and TIBC, serum iron, MCHC, HCT, Hb and RBC.

Table 5: Correlation between hepcidin and biochemical and haematological markers of anaemia

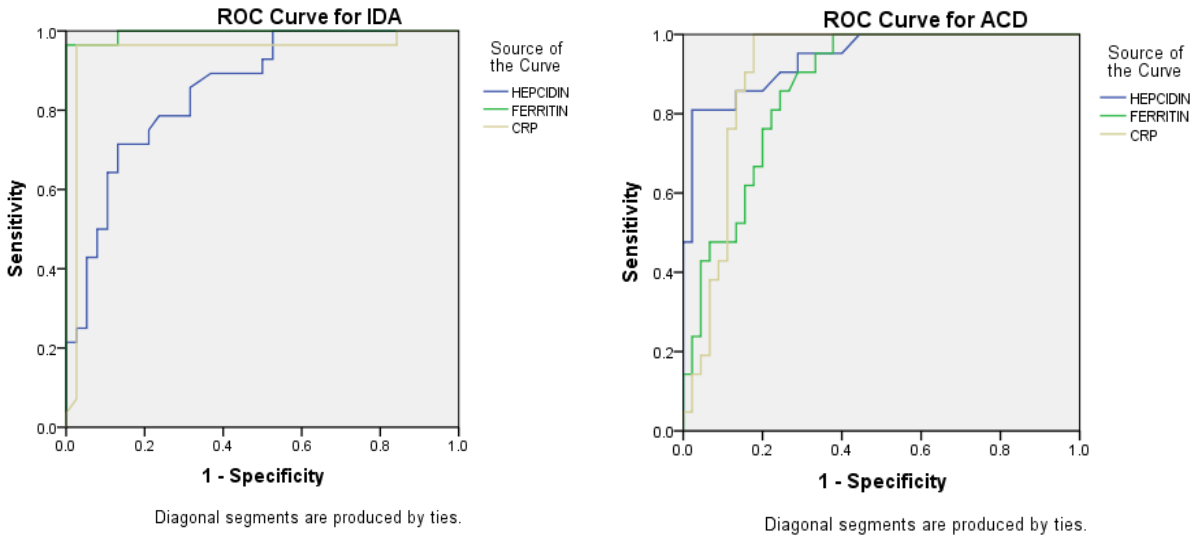
Biomarker	Pearson correlation (r)	P-value
Ferritin	0.624	<0.001
CRP	0.422	<0.001
TSAT	0.163	0.191
TIBC	-0.097	0.439
Serum iron	0.059	0.639
RDW	-0.279	0.024
MCHC	-0.034	0.789
MCV	0.508	<0.001
HCT	0.065	0.602
Hb	0.12	0.337
RBC	-0.15	0.231

The relationship is significant at p-value < 0.05.

4.4 Diagnostic Characteristics for the Detection of Iron Deficiency Anaemia and Anaemia of Chronic Disease

Figure 3 shows the receiver operating characteristics (ROC) curves for markers used to test for IDA and ACD diagnostic performance. Table 6 shows the calculated values of the area under the receiver operating characteristic curve (ROC-AUC) and Table 7 shows the sensitivity and specificity of serum hepcidin, serum ferritin and CRP in the diagnosis IDA and ACD. In IDA, ferritin exhibited the best performance with 99.5% area under the curve (AUC) (95% CI: 98.5 to 100), 96.4 % sensitivity and 73.8% specificity at ≤30ng/mL cutoff value. Hepcidin showed the weakest performance with 85.4% AUC (95% CI: 76.4 to 94.3) and 89.3% sensitivity and 34.2% specificity at ≤5ng/mL cutoff value.

In ACD, however, hepcidin showed the best performance with 94.2% AUC (95% CI: 88.5 to 99.9), 95.2% sensitivity and 63.8% specificity compared to ferritin with 86.8% AUC (95% CI: 78.5 to 95.1), 94.1% sensitivity and 42.9% specificity, and CRP with 90.5% AUC (95% CI: 83.0 to 98.0), 90.2% sensitivity and 35.2% specificity. For all the markers, the AUC is significantly different from 0.5 since p-value < 0.001.



A. B.

Figure 3: Receiver operating characteristic curves for iron deficiency anaemia (A) and anaemia of chronic disease (B).

Table 6: Area under the receiver operating characteristics curves for IDA and ACD

	IDA			ACD		
Test result variable	**Area**	**95% CI**	**P-value**	**Area**	**95% CI**	**P-value**
Hepcidin	0.854	0.764-0.943	<0.001	0.942	0.885-0.999	<0.001
Ferritin	0.995	0.985-1.000	<0.001	0.868	0.785-0.951	<0.001
C-reactive protein	0.946	0.873-1.000	<0.001	0.905	0.830-0.980	<0.001

True area=0.50

Table 7: Sensitivity and specificity of serum hepcidin and ferritin at Selected Cutoff values

Selected Cutoff Values		IDA	ACD
Serum hepcidin at 5ng/mL	Sensitivity	89.3%	95.2%
	Specificity	34.2%	63.8%
Serum ferritin at 30ng/mL	Sensitivity	96.4%	94.1%
	Specificity	73.8%	42.9%
C-reactive protein at 10mg/L	Sensitivity	96.4%	90.2%
	Specificity	55.8%	35.20%

The smallest cutoff value is the minimum observed test value minus 1

CHAPTER FIVE
DISCUSSION

5.1 The Prevalence of IDA, IDA/ACD, and ACD in the Study Participant

We found more females 41(62.1%) with anaemia than males 25 (37.9%) and IDA was the most prevalent form of anaemia accounting for 28 (42.4%) cases of whom 24 (36.4%) were females. Iron deficiency anaemia causes approximately half of all anaemia cases worldwide, and affects females of reproductive age more often than males. Many studies have found that anaemia is a common problem in women of reproductive age resulting from prolonged negative iron balance, caused by inadequate iron intake, or absorption, increased iron demand during pregnancy, and increased iron losses due to menstruation (Global Burden of Disease Study 2013 Collaborators, 2015). Anaemia of chronic disease accounted for 31.8% of the study population and was more common among males (19.7%) than females (12.1%). There was a significant difference between mean age of IDA patients (26.5 years) and ACD (41.9 years) or IDA/ACD (42.3 years), $p < 0.001$. ACD is more common in elderly, primarily from presence of more chronic diseases, rather than as a phenomenon of normal aging (Weiss & Goodnough, 2005). On the contrary, IDA is more common among women of childbearing age and school going children (Global Burden of Disease Study 2013 Collaborators, 2015).

5.2 The Relationships between Hepcidin Hormone and IDA, IDA/ACD, and ACD

The study found that IDA participants had significantly lower hepcidin levels than ACD ($p<0.001$). There was also a significant difference in serum hepcidin between IDA/ACD and ACD ($p < 0.001$) (Table 5). ACD patients exhibited a wide range of distribution of hepcidin levels from 4.70 to 42.62ng/mL compared to IDA and IDA/ACD patients which exhibited a narrow distribution from 1.67 to 7.50ng/mL and 2.67 to 11.80ng/dL respectively. The differentiation of median hepcidin levels was negligible in IDA and IDA/ACD.

This was in agreement with the study done by (Van Santen *et al.*, 2011) which found that hepcidin content in serum from patients in the IDA group and as well as that from patients combined IDA/ACD group differed significantly from that in the ACD group ($p < 0.001$). Another study done by (Röhrig *et al*., 2014) showed a statistical significant difference of hepcidin levels among IDA, ACD and control groups ($p=0.034$).

5.3 The Relationship between Hepcidin Hormone and Biochemical Markers (Ferritin and CRP)

There was a strong positive correlation between serum hepcidin levels and serum ferritin levels (Pearson's $r = 0.624$, $p < 0.001$). A study done by Röhrig *et al*., 2014 showed a strong positive correlation between serum hepcidin and ferritin (spearman $r=0.747$). Serum hepcidin levels also significantly correlated with CRP (Pearson's $r = 0.508$, $p<0.0001$). Serum hepcidin, serum ferritin and CRP are induced by inflammation and therefore high levels of these markers are seen in ACD compared to IDA (Fleming, 2008; Nemeth *eta al*., 2004; Piperno *et al*., 2009; Brugnara, 2008).

The receiver operating characteristic analysis was used to assess the characteristic of hepcidin, ferritin and CRP in IDA and ACD. In IDA, ferritin exhibited the best performance with 99.5% area under the curve (AUC) (95% CI: 98.5 to 100), 96.4 % sensitivity and 73.8% specificity at ≤30ng/mL cutoff value whereas hepcidin showed the weakest performance with 85.4% AUC (95% CI: 76.4 to 94.3) and 89.3% sensitivity and 34.2% specificity at ≤5ng/mL cutoff value.

In ACD, however, hepcidin showed the best performance with 94.2% AUC (95% CI: 88.5 to 99.9), 95.2% sensitivity and 63.8% specificity compared to ferritin with 86.8% AUC (95% CI: 78.5 to 95.1), 94.1% sensitivity and 42.9% specificity, and CRP with 90.5% AUC (95% CI: 83.0 to 98.0), 90.2% sensitivity and 35.2% specificity.

A study done by Choi *et al*., 2012, the AUC for serum hepcidin as predictor of iron deficiency (ID) was 85.2% (95% CI: 75.5 to 95.0) and hepcidin ≤6.895ng/mL had a sensitivity of 79.2% and specificity of 82.8% for the diagnosis of ID whereas hepcidin ≤2.735ng/mL had a sensitivity of 88.1% and specificity of 88.2% for diagnosis IDA (Choi *et al*., 2012). Another study done by Parischa *et al*., 2010, showed that an undetectable hepcidin (<5.4ng/mL) had a sensitivity and specificity of 41.5% and 97.5% respectively, and hepcidin <20ng/mL had a sensitivity and specificity of 74.6% and 83.2% respectively (Parischa *et al*., 2010). In a study done by Svenson and colleagues in 2015, hepcidin cutoff point value of 8ng/mL had a sensitivity of 73% and specificity of 72% in identifying iron deficiency anaemia (Svenson *et al*., 2015).

5.4 The Relationship between Hepcidin Hormone and Haematological Markers of Anaemia

There was a statistically significant correlation between hepcidin and MCV and RDW ($r = 0.508$, $p < 0.001$ and $r = -0.279$, $p = 0.024$, respectively), and a statistically insignificant correlation between hepcidin and Hb and RBC ($r=0.12$, $p=0.337$ and $r=-0.150$, $p=0.231$, respectively).

The study done by Mohammad *et al*., 2013, however, showed an insignificant correlation between hepcidin and MCV and RDW ($r=0.030$, $p=0.706$ and $r=0.106$, $p=184$, respectively) and a statistically significant positive correlation between hepcidin and RBCs and Hb ($r=0.160$, $P=0.043$ and $r=0.165$, $p=0.037$, respectively).

Decreased values of Hb, HCT, MCHC, and MCV were seen in both IDA and ACD, whereas serum hepcidin levels were lower in IDA than ACD. Therefore, complete blood count indices may not have a linear relationship with hepcidin.

5.5 Hepcidin as Biomarker for the Differential Diagnosis of Iron Deficiency Anaemia and Anaemia of Chronic Disease

The study showed that patients with IDA could be differentiated from those with ACD since there was a significant difference in the mean serum hepcidin levels ($p<0.001$). In addition, serum hepcidin performed well as a diagnostic test of deficiency of iron, even in the presence of inflammation and differentiated groups with IDA and IDA/ACD ($p = 0.026$). Both IDA and IDA/ACD had lower hepcidin levels than a pure case of ACD. The findings from this study are consistent with the studies done by Röhrig *et al*., 2011; Van Santen et al., 2011; Manolov *et al*., 2015; Svenson *et al*., 2015; Parischa *et al*., 2011; Choi *et al*., 2012. Hepcidin measurement can be a helpful test for distinguishing ACD from IDA, as it is known that hepcidin production is induced by inflammation (ACD), and reduced in iron deficiency status (IDA) (Nemeth *et al*., 2004; Brugnara, 2008; Cullis, 2013).

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.0 Conclusion

The study found that IDA participants had significantly lower hepcidin levels than ACD and IDA/ACD participants. It was further discovered that serum hepcidin could be used as one of the iron indices especially in the differentiation of ACD from IDA and IDA/ACD as these forms of anaemia require different approaches to treatment. Therefore, serum hepcidin could be considered in diagnosing and distinguishing ACD from IDA or IDA/ACD as it had high diagnostic sensitivity and specificity compared to others.

6.1.0 Recommendations/Limitations/Weaknesses/Future Directions

6.1.1 Recommendations

Validation of different methods for determining blood hepcidin concentration have not all been validated in accordance with specified procedures. Some studies report limits of detection, whereas others report limits of quantification (Iain *et al*., 2010). The absolute hepcidin values differ depending on the method used and cannot easily be related to one another, limiting comparison across studies. Therefore, validation and harmonization of different hepcidin methods should be undertaken to allow establishment of consistent reference values across different populations.

6.1.2 Limitations / Weaknesses

The study could not include all markers of iron status to better classify anaemia. Furthermore, serum hepcidin levels were not done in the normal population to determine the reference values.

6.1.3 Future Direction

Further studies are needed to define precisely the role of hepcidin in iron metabolism, homeostasis and its utility in the diagnosis and treatment of iron disorders. The development of synthetic hepcidin would be useful in the treatment of hemochromatosis and other iron-loading conditions.

7.0 REFERENCES

Ahonkhai , A A, K A Gebo , and M B Streiff . 2008. "Venous thromboembolism in patients with HIV/AIDS: a case-control study." *J. Acquir Immune Defic Syndr* 48 (3): 310-14.

Álvaro , Borges H, Michael J Silverberg, Deborah Wentworth, Andrew E Grulichd, Gerd Fätkenheuer, Ronald Mitsuyasuf, Giuseppe Tambussi, Caroline A Sabin, James D Neaton, and Jens D Lundgren. 2013. "Predicting risk of cancer during HIV infection: the role of inflammatory and coagulation biomarkers." *AIDS* 27 (9): 1433–1441. doi:10.1097/QAD.0b013e32835f6b0c.

Anderson, F A Jr, H B Wheeler , R J Goldberg , and et al. 1991. "A population-based perspective of the hospital incidence and case-fatality rates of deep vein thrombosis and pulmonary embolism." *Arch Intern Med* 151: 933-938.

Anderson, G,J, D Darshan, S,J Wilkins, and D,M Frazer. 2007. "Regulation of systemic iron homeostasis: how the body responds to changes in iron demand." *Biometals* 20: 665-674.

Atanasiu, V, B Manolescu, and Stoian I. 2007. " Hepcidin-central regulator of iron metabolism." *European Journal of Haematology* (European Journal of Haematology) 78: 1-10.

Baars , J W, J P de Boer , J Wagstaff , D Roem , A J Eerenberg-Belmer , J Nauta , and et al. 1992. "Interleukin-2 induces activation of coagulation and fibrinolysis: resemblance to the changes seen during experimental endotoxaemia." *Br J Haematol* 82: 295–301.

Bain, J, Barbara, Bates Imelda, A, Laffan Mike, and Lewis Mitchell. 2012. *Dacie and Lewis Practicle Haematology.* Edited by 11th Edition. Churchill Livingstone: Elsevier.

Baker, J, H Quick, K H Hullsiek, R Tracy, D Duprez, and et a. 2010. "IL-6 and D-dimer levels are associated with vascular dysfunction in patients with untreated HIV infection." *HIV Med* 11: 608-609.

Baker, W F Jr. 1998. "Current Concepts of Thrombosis: Prevalent Trends for Diagnosis and Management." *Med Clin NA* 82 (3): 460-476.

Boos , C J, PKY Goon, and GYH Lip. 2006. "The endothelium, inflammation and coagulation in sepsis." *Clin Pharmacol Ther* 79: 20-22.

Boulware, David R, Katherine Huppler Hullsiek, Camille E Puronen, Adam Rupert, Jason V Baker, Martyn A French, R Paul Bohjanen, Richard M Novak, James D Neaton, and Irini Sereti. 2011. "Higher Levels of CRP, D-dimer, IL-6, and Hyaluronic Acid Before Initiation of Antiretroviral Therapy (ART) Are Associated With Increased Risk of AIDS or Death." *The Journal of Infectious Diseases* (Oxford University Press) 1637-1646. doi:10.1093/infdis/jir134.

Boulware, David R, Katherine Huppler Hullsiek, Camille E Puronen, Adam Rupert, Jason V Baker, Martyn A French, Paul R Bohjanen, Richard M Novak, James D Neaton, and Irini Sereti. 2011. "Higher Levels of CRP, D-dimer, IL-6, and Hyaluronic Acid Before Initiation of Antiretroviral Therapy (ART) Are Associated With Increased Risk of AIDS or Death." *Journal of Infectious Diseases* (203): 1637-1646.

Breen, E C, A R Rezai, K Nakajima, G N Beall, R T Mitsuyasu, T Hirano, T Kishimoto, and O Martinez-Maza. 1990. "Infection with HIV is associated with elevated IL6 levels." *J. Immunol* 144:480.

Bregman, D,B, D Morris, T,A Koch, and A, Goodnough, L,T He. 2013. "Hepcidin levels predict nonresponsiveness to oral iron therapy in patients with iron deficiency anaemia." *American Journal of Hematology* (American Journal of Hematology. Luitpold Pharmaceuticals, Inc, Valley Forge) 88 (2): 97-101.

Brown, T T, S R Cole, X Li, and et al. 2005. "Antiretroviral Therapy and the Prevalence and Incidence of Diabetes Mellitus in the Multicenter AIDS Cohort Study." *Arch Intern Med* 1179–1184.

Brugnara, C. 2008. "An Immunoassay for human serum hepcidin at last:Ganz klar." *Blood* (Blood) 112: 3922-3923.

Brugnara, C. 2003. "Iron deficiency and erythropoiesis: new diagnostic approaches. Clinical Chemistry." *Clinical Journal of the American Society of Nephrology* (Clinical Journal of the American Society of Nephrology) 10: 1573-1578.

Calis, J,C, K,S Phiri, and E,B Faragher. 2008. "Severe anemia in Malawian children." (The New England Journal of Medicine) 358 (9): 888-899.

Calmy A, Gayet-Ageron A, Montecucco F, Nguyen A, Mach F, et al. 2009. "HIV Increases Markers of Cardiovascular Risk: Results From a Randomized, Treatment Interruption Trial." *AIDS* 929-939.

Calmy, A, A Gayet-Ageron, F Montecusso, A Nguyen, and Mach F. 2009. "HIV Increases Markers of Cardiovascular Risk: Results from a Randomized, Treatment Interruption Trial." *AIDS* 929-939.

Camaschella, C, and L Silvestri. 2008. "New and old players in hepcidin pathways." (Haematologica) 93: 1441-1444.

Carter, C J. 1994. "The Natural History and Epidemiology of Venous Thrombosis." *Prog Cardiovasc Dis* 36 (6): 423-438.

Castelnuovo , B, Y C Manabe, A Kiragga , M Kamya , P Easterbrook , and A Kambugu . 2009. "Cause-specific mortality and the contribution of immune reconstitution inflammatory syndrome in the first 3 years after antiretroviral therapy initiation in an urban African cohort." *Clin Infect Dis* 49: 965–72.

Central Intelligence Agecy. 2011. *The world Factbook.* January Friday. Accessed November 15th, 2013. https://www.cia.gov/library/publications/the-world-factbook/.

Choi, Soo, Hyoungi, Hoon, Song Sang, Hee, Lee Jae, Kim Heep-Jin, and Ram, Yang Hye. 2012. "Serum hepcidin levels and iron parameters in children with iron deficiency." *Korean Journey of Hematology* (Korean Journey of Hematology) 47 (4): 286-292.

Collaborators, GBD 2013 Mortality and Causes of Death. 2014.

Collaborators, GBD 2013 Mortality and Causes of Death. 2014. "GBD 2013 Mortality and Causes of Death Collaborators."

Collaborators, GBD 2013 Mortality and Causes of Death. 2014. "Global, regional and national age-sex specific all-cause and cause-specific mortality for 240 causes of Death, 1990-2013." *Lancet* (Lancet) 285 (9963): 117-171.

Correll, P K, M G Law, A M McDonald, D A Cooper, and J M Kaldor. 1998. "HIV Disease Progression in Australia in the time of Combination Antiretroviral Therapies." *Med J Aust* v: 469–472.

Cullis, Jonathan. 2013. "Anaemia of chronic disease." (Royal College of Physicians) 13 (2): 193-6.

Cullis, O, Jonathan. 2011. "Diagnosis and management of anaemia of chronic disease: current status." *British Journal of Haematology* (Blackwell Publishing Ltd) 154: 289-300.

De Domenico, I, D,M Ward, C Langelier, M,B Vaughn, E Nemeth, and W,I Sundquist. 2007. " The molecular mechanism of hepcidin-mediated ferroportin down-regulation. Mol Biol Cell." *Molecular Biology of the Cell* (Molecular Biology of the Cell) 18: 2569-2578.

De, W S, C A Sabin, Weber R, and et al. 2008. "Incidence and Risk Factors for New-onset Diabetes in HIV-Infected Patients: the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) study." *Diabetes Care* 31: 1224–1229.

Deehan , D J, S D Heys , W Simpson , R Herriot , J Broom , and O Eremin. 1994. "Correlation of serum cytokine and acute phase reactant levels with alterations in weight and serum albumin in patients receiving immunotherapy with recombinant IL-2." *Clin Exp Immunol* 95: 366–372.

Dempfle, C. 2000. "Use of D Dimer Assays in the Diagnosis of Venous Thrombosis." *Sem Thromb Hem* 26 (6): 631-641.

Dennis, J, Ernst, O, Ballance Lisa, R, Calam Roger, McCall Ruth, S, Smith Susan, I, Szamosi Diane, and J, wARUNEK David. 2007. *CLSI. Procedures for the collection of diagnostic blood specimens by venepuncture. Approved standard. 6th Ed. Document H3-A6. Wayne PA.* 6th. Vol. 27. 26 vols. Wayne, Pennsylvania: Clinical and Laboratory standard Institute (CLSI).

Dienz , O, and M Rincon . 2009. "The effects of IL-6 on CD4 T cell responses." *Clin Immunol* 130: 27–33.

Doğanavşargil , E. 2003. "Romatolojik hastalıkların tanısında labaratuar yöntemleri." *Klinik Romatoloji El Kitabı* 117-118.

Dougan , M, D Li, D Neuberg , M Mihm , P Googe , and K K Wong . 2011. "A dual role for the immune response in a mouse model of inflammation-associated lung cancer." *J Clin Invest.* 121: 2436–2446.

Drake , Thomas A, Wolfram Ruf, James H morrissey, and Thomas S Edgington. 1989. "Functional Tissue Factor is Entirely Cell Surface Expressed on Lipopolysaccharide-Stimulated Human Blood Monocytes and a Constitutively Tissue Factor- Producing Neoplastic Cell Line." *The Journal of Cell Biology* 109: 389-395.

Esmon, C T, K Fukudome, and T Mather. 1999. "Inflammation, Sepsis and Coagulation." *Haematologica* 84: 254-259.

Ferguson, Smith AC, YF Chen , MS Newman , LT May , PB Sehgal, and FH Ruddle. 1988. "Regional localization of the interferon-beta 2/B-cell stimulatory factor 2/hepatocyte stimulating factor gene to human chromosome." *Genomics* 2 (3): 203–208.

Fisher , S D, T L Miller , and S E Lipshultz . 2006. "Impact of HIV and highly active antiretroviral therapy on leukocyte adhesion molecules, arterial infl ammation, dyslipidemia, and atherosclerosis." *Atherosclerosis* 185: 1-11.

Fleming, R, E. 2008. "Iron and Inflammation: Cross-talk between pathways regulation hepcidin." (Journal of Molecular Medicine) 86: 491-494.

Fortis, C, L Soldini , S Ghezzi , S Colombo , G Tambussi , E Vicenzi , and et al. 2002. "Tumor necrosis factor alpha, interleukin 2, and soluble interleukin 2 receptor levels in human immunodeficiency virus type 1-infected individuals receiving intermittent cycles of interleukin 2." *AIDS Res Hum Retroviruses* 18: 491–499.

Funderburg, Nicholas T, Elizabeth Mayne, Scott F Sieg, Robert Asaad, Wei Jiang, Magdalena Kalinowska, Angel A Luciano, et al. 2010. "Increased Tissue Factor Expression on Circulating Monocytes in Chronic HIV Infection: Relationship to in vivo Coagulation and Immune Activation." *Blood* 161-167.

Furie , B, and B C Furie . 2009. "Mechanisms of thrombus formation." *N Engl J Med* 359 (9): 938-949.

Galesloot, E,T, H, S Vermeulen, J,A Geurts-Moespot, M,S Klaver, and J,J Kroot. 2011. "serum hepcidin: reference ranges and biochemical correlates in the general population." *Blood* (American Society of Hematology) 117 (25).

Gallo, P, K Frei, C Rordorf, J Lazdins, B Tavolato, and A Fontana. 1989. "Human Immunodeficiency Virus Type 1 (HIV 1) Infection of the Central Nervous System: An Evaluation of Cytokines in Cerebrospinal Fluid." *J. Immunol* 23 (109).

Ganz, T. 2003. " Hepcidin, a key regulator of iron metabolism and mediator of anaemia of inflammation." *Blood* (Blood) 102: 783-788.

Ganz, T. 2011. "Hepcidin and iron regulation, 10 years later." (Blood) 117: 4425-4433.

Ganz, T. 2006. "hepcidin and its role in regulating systemic iron metabolism." (American Journal of Hematology) 1: 29-35.

Ganz, T, G Olbina, D Girelli, E Meneth, and M Westerman. 2008. "Immunoassay for human serum hepcidin." *Blood* (blood) 112 (10): 4292-7.

Gardenghi, S, P Ramos, M,F Marongiu, L Melchiori, L Breda, E Guy, and et al. 2010. "Hepcidin as a therapeutic tool to limit iron overload and improve anaemia in B-thassemic mice." *Journal of Clinical Investigation* (Journal of Clinical Investigation) 120 (12): 4466-77.

n.d. "GBD 2013 Mortality and Causes of Death Collaborators, 2014."

Geerts, inge, Vermeersch Pieter, and Etienne Joosten. 2012. "Evaluation of the First Commercial Hepcidin ELISA for the Differential Diagnosis of Anaemia of Chronic Disease and Iron Deficiency Anaemia in Hospitalized Geriatric Patients." *International Scholarly Research Network* (International Scholarly Research Network) 2012, Article ID 567491, 3 pages.

Grunfeld , C, M Pang, W Doerrler, J K Shigenaga, P Jensen, and K R Feingold. 1992. "Lipids, Lipoproteins, Triglyceride Clearance, and Cytokines in Human Immunodeficiency Virus Infection and the Acquired Immunodeficiency Syndrome." *J Clin Endocrinol Metab* 1045–1052.

Guardiola , M, R Ferre , and J Salazar. 2006. "Protease inhibitor-associated dyslipidemia in HIV-infected patients is strongly infl uenced by the APOA5-1131T->C gene variation." *Clin Chem* 52: 1914-19.

Hamlyn, Elizabeth, Wolfgang Stöhr, David A Cooper, Martin Fisher, Giuseppe Tambussi, Mauro Schechter, Jose M Miro, et al. 2015. "The effect of short-course antiretroviral therapy initiated in primary HIV-1 infection on interleukin-6 and D-dimer levels." *AIDS.* doi:10.1097/QAD.0000000000000675.

Hirsh , J, R D Hull , and G E Raskob . 1986. "Clinical features and diagnosis of venous thrombosis." *J Am Coll Cardiol* 8 ((6 Suppl B)): 114B-127B.

Hoffbrand, A, Victor, and Catovsky, Edward, G, Tuddenham Daniel. 2005. *Postgraduate Haematology*. Edited by 5th Edition. Blackwell Publishing Ltd.

Hyers , T M. 1999. "Venous Thromboembolism." *Am J Respir Crit Care Med* 159 (1): 1-14.

Iain, Macdougall C, Jolanta Malyszko, Hider C Robert, and bansal S Sukhvinder. 2010. "Current Status of Measurement of Blood Hepcidin Levels in Chronic kidney Disease." *Clin J Am Soc Nephrol* 5: 1681-1689.

Iffen, T S, H Efobi, C A O Usoro, and N E Udonwa. 2010. "Lipid Profile of HIV-Positive Patients Attending University of Calabar Teaching Hospital, Calabar - Nigeria." *World J. Med. Sci.* 5 (4): 89-93.

Janz, T, G, R, L Johnson, and S, D Rubenstein. 2013. "Anaemia in emergency department: evaluation and treatment." (Emergency medicine practice) 15 (11): 1-15.

Joosten, E, W Pelemans, M Hiele, J Noyen, R Verhaeghe, and M,A Boogaerts. 1992. "Prevalence and causes of anaemia in a geriatric hospitalized population." (Gerontology) 38 (1-2): 111-117.

Justyna, Kwapisz, Slomka Artur, and Zekanowska Ewa. 2014. "Hepcidin and its Role in Iron Homeostasis." *The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine* (IFCC).

Karuna, R, R Brian, and M Peter. 2011. "Prevalence of Anaemia and Correlation with Expression of Hepcidin in Adults." (Journal of Indian Academy of Geriatrics) 7: 25-29.

Kemna, E, H, H Tjalsma, H, L Williems, and D,W Swinkels. 2008. "Hepcidin: From discovery to differential diagnosis." *Haematologica* 93: 90-97.

Klein, Nigel, Chipo Berejena, Godfrey Pimundu, Pietro Pala, Tichaona Vhembo, Victor Musiime, Philip Kasirye, Hannah Poulsom, Sarah Ann Walker, and Andrew J Prendergast. 2012. "Complex Pattern of Inflammatory Biomarkers After ART Initiation in HIV-Infected African Children." *Journal of AIDS* 910.

Knutson, M, D, M Oukka, L,M Koss, F Aydemir, and M Wessling-Resnick. 2005. "Knutson MD, Oukka Iron release from macrophages after erythrophagocytosis is upregulated by ferroportin 1 overexpression and down-regulated by hepcidin." *Proceedings of the National Academy of Sciences, USA* 102: 1324-1328.

Koliaraki, V, M Marinou, T,P Vassilakopoulos, E Vavourakis, E Tsochatzis, and et al. 2009. "A Novel Immunological Assay for Hepcidin Quantification in Human Serum." *PloS ONE* (PloS ONE) 4 (2): e4581.

Krause, A, S Neitz, H,J Magert, A Schulz, and W,G Forssmann. 2000. "LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity." (FEB Letter) 480: 147-150.

Kroot, J,J, H Tjalsma, R,E Fleming, and D,W Swinkels. 2011. "Hepcidin in human iron disorders: diagnostic implications." (Clinical Chemistry) 57: 1650-1669.

Kuller, H Lewis, Russell tracy, Waldo Belloso, Stephane De Wit, Fraser Drummond, H Clifford Lane, Bruno Ledergerber, et al. 2008. "Inflammatory and Coagulation Biomarkers and Mortality in Patients with HIV Infection." *PLos Med 5(10)* 1496-1508.

Kumar, Vinay, Abul K Abbas, and John C Aster. 2013. "Hemodynamic Disorders, Thromboembolism, and Shock." Chap. 3 in *Robbins Basic Pathology*, 79-80. Philadelphia: ELSEVIER.

Laftah, A, H, B Ramesh, R,J Simpson, N Solanky, S Bahram, K Schumann, and et al. 2004. "Effects of hepcidin on intestinal iron absorption in mice." *Blood* (Blood) 103: 3940-3944.

Ledwaba, Lotty, Jorge A Tavel, Paul Khabo, Patrick Maja, Jing Qin, Phumele Sangweni, Xiao Liu, et al. 2012. "Pre-ART Levels of Inflammation and Coagulation Markers Are Strong Predictors of Death in a South African Cohort with Advanced HIV Disease." *PLoS One* 7 (3): 1-9.

Lenos, A, Andrea Dos, L, A, S, Ismael, C,C,M Boato, M,T,F Borges, and P,H,C Rondo. 2010. "Hepcidin as a Biochemical Parameter for the Assessment of iron Deficiency Anemia." *Rev Assoc Med Bras* (Rev Assoc Med Bras) 56 (5): 596-9.

Leong, W, I, and B Lonnerdal. 2004. "Hepcidin, the recently identified peptide that appears to regulate iron absorption." (Journey of Nutrition) 134: 1-4.

Levi, M, TT Keller, and E vanGorp. 2003. "Infection and inflammation and the coagulation system." *Cardiovasc Resp* 60: 26-39.

Lin, Zi-Qing, Toshikazu Kondo, Yugo Ishida, Tatsunori Takayasu, and Naofumi Mukaida. 2003. "Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice." *Journal of Leukocyte Biology* 73: 713 - 721.

Lotz, M, J H Vaughan, and D A Carson. 1988. "Effect of neuropeptides on production of inflammatory cytokines by human monocytes." 1218. Wash. DC.

Lowe , G D. 2005. "Circulating inflammatory markers and risks of cardiovascular and non-cardiovascular disease." *J Thromb Haemost* 3: 1618–1627.

Manolov, V., Paskaleva-Peycheva, V., Bogov, B., Yonova, D., et al.,. 2015. "Serum hepcidin quantification in differentiation of anemia." *International Journal of development research vol. 5 issue, 01* 2918-2920.

Marchetti , G, Giusi M Bellistri, Elisa Borghi, Camilla Tincati, Stefania Ferramosca, Maria LaFrancesca, Giulia Morace, Andrea Gori, and Antonella d'Arminio Monforte. 2008. "Microbial Translocation is Associated with Sustained Failure in CD4 T-Cell Reconstitution in HIV Infected Patients on Long-Term Highly Active Antiretroviral Therapy." *AIDS* 22 (15): 2035-2044.

Martin, D M, C W Boys , and W Ruf . 1995. "Tissue factor: molecular recognition and cofactor function." *FASEB J* 9 (10): 852-859.

Mast, A, E, M, A Blinder, A, M Gronowski, C Chumley, and M, G Scott. 1998. "Clinical utility of the soluble transferrin receptor and comparison with serum ferritin in several populations." *Clinical Chemistry* 44: 45-51.

Matta , F, A Y Yaekoub, and P D Stein . 2008. "Human immunodeficiency virus infection and risk of venous thromboembolism." *Am J Med Sci* 336 (5): 402-406.

McDonald, Bethan, Sikhulile Moyo, Lesego Gabaitiri, Simani Gaseitsiwe, Hermann Bussmann, John R Koethe, Rosemary Musonda, et al. 2013. "Persistently Elevated Serum Interleukin-6 Predicts Mortality Among Adults Receiving Combination Antiretroviral Therapy in Botswana: Results from a Clinical Trial." *AIDS RESEARCH AND HUMAN RETROVIRUSES* 29: 993-999.

Meneth, E, S Rivera, V Gabayan, C Keller, S Taudorf, B,K Pedersen, and T Ganz. 2004. "IL-6 mediates hypoforremia of inflammation by inducing the synthesis of iron regulatory hormone hepcidin." (The Journal of Clinical Investigation) 113: 1271-1276.

Mocroft , A, J Gatell , and P Reiss . 2004. "Causes of death in HIV infection: the key determinant to define the clinical response to anti-HIV therapy." *AIDS* 18: 2333–7.

Mohammad, B, Abosakran, E, Shubair Mohammad, and M, Zaida Tarek. 2014. "Hepcidin Status Correlated with Biochemical Parameters and Hematological Indices among Iron Deficiency Anemic Children aged (6-12) years in Gaza City: A Case Control Study." *Journal of Natural and Engineering Studies* (Journal of Natural and Engineering Studies) 22 (2): 1-13.

Munoz, M, I Villar, and J,A Garcia-Erce. 2009. " An update on iron physiology." *World Journal of Gastroenterology* (World Journal of Gastroenterology) 15: 4617-4626.

Muraguchi, A, T Kishimoto, Y Miki, T Kuritani, T Kaieda, K Yoshizaki, and Y Yamamura. 1981. "Tcell-replacing factor- (TRF) induced IgG secretion in a human B blastoid cell line and demonstration ofacceptors for TRF." *J Immunol* 127: 412.

Nakajima, K, O Martinez-Maza, T Hirano, E C Breen, P G Nishanian, J F Salazar-Gonzalez, J L Fahey, and T Kishimoto. 1989. "Induction of IL6 (B cell stimulatory factor- 2/IFN-Sz) production by HIV." *J. Immunol* 142:531.

Nemeth, E, S Rivera, V Gabayan, C Keller, S Taudorf, B,K Pedersen, and T Ganz. 2004. "IL-6 mediates hypoforremia of inflammation by inducing the synthesis of iron regulatory hormone hepcidin." (The Journal of Clinical Investigation) 113: 1271-1276.

Neuhaus, J, DR Jr Jacobs, JV Baker, A Calmy, and D Duprez. 2010. "Markers of Inflammation, Coagulation and Renal Function are Elevated in Adults with HIV Infection." *J Infect Dis 201* 1788-1795.

Nicholas, T. 2010. "Increased Tissue Factor Expression on Monocytes in Chronic HIV Infection: Relationship to In Vivo Coagulation and Immune Activation." *Blood* 115 (2).

Nordan, R P, and M Potter. 1986. *A macrophage-derived factor required by plasmacytomas for survival and proliferation in vitro. Science.* Vol. 233:566. Wash. DC.

Paiva, A,A, P,H Rondo, and E Guerra-Shinohara. 2000. "Parameter for the Assessment of Iron Status." *Rev Saude Publica* (Rev Saude Publica) 34: 421-426.

Parischa, S,R, Z McQuilten, and M Westerman. 2011. "Serum hepcidin as a diagnostic test of iron deficiency in premenopausal female blood donors." (Haematologica) 96: 1099-105.

Parischa, S,R, Z McQuilten, M Westerman, and et al. 2011. "Serum hepcidin as a diagnostic test of iron deficiency in premenopausal female blood donors." (Haematologica) 96: 1099-105.

Park, C,H, E,V Valore, A,J Waring, and T Ganz. 2001. "Hepcidin, a urinary antimicrobial peptide synthesized in the liver." (The Journal of Biological Chemistry) 276: 7806-7810.

Peng, J, P Friese, and R F Wolf. 1996. "Relative reactivity of platelets from thrombopoietin and IL-6 treated dogs ." *BLOOD* 87: 4158-4163.

Phillips, A N, A Carr, J Neuhaus, and et al. 2008. "Interruption of Antiretroviral Therapy and Risk of Cardiovascular Disease in Persons with HIV-1 Infection: Exploratory Analyses from the SMART Trial. ." *Antivir Ther* 177–187.

Pigeon, C, G Ilyin, B Courselaud, P Leroyer, B Turlin, P Brissot, and O Loreal. 2001. "A new mouth liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is expressed during iron overload." *Journal of Biological Chemistry* (Journal of Biological Chemistry) 276: 7811-7819.

Piperno, A, R Mariani, P Trombini, and D Girelli. 2009. "Hepcidin modulation in human diseases: from research to human disease." (Journal of Gastroenterology) 15: 538-551.

Poli , Guido, Peter Bressler, Audrey Kinter, Elia Duh, William C Timmer, Arnold Rabson, Shawn J Justement, Sharylin Stanley, and Anthony S Fauci. 1990. "Interleukin 6 Induces Human Immunodeficiency Virus Exopression in Infected Monocytic Cells Alone and in Synergy with Tumor Necrosis Factor α by Transcriptional and Post-transcriptional Mechanisms." *The Journal of Experimental Medicine* 172: 151-158.

Politou, M, and G Papanikolaou. 2004. "Hepcidin: A key iron regulator involved in the pathogenesis of chronic disease." *Haematologica* 7: 165-174.

Rasheed, Noor, H, Shatta Ali, Z, M Mazin, and M, Al Shami Adeeb. 2013. "Serum Hepcidin Levels in Anaemia of Chronic Kidney Diseases Compared to Iron Deficiency Anaemia and its Correlation with Serum Levels of HS-C reactive Protein, Interleukin-6 and Ferritin." (Global Journal of Bioscience and Biotechnology) 2 (1): 43-50.

Reber, G. 2000. "D Dimer Assays for the Exclusion of Venous Thromboembolism." *Sem Thromb Hem* 619-624.

Rodak, F, Bernadette. 2007. "Haematology: Principles and applications." In *Haematology: Principles and applications,* by F, Bernadette Rodak, p.220 ISBN 9781416030065. philadelphia: Saunders.

Roe, M,A, R Collings, J,R Dainty, D,W Swinkels, and S,J Fairweather-Trait. 2009. "Plasma hepcidin concentrations significantly predict inerindividual variation in iron absorption in healthy men." *American Journal of Clinical Nutrition* (American Journal of Clinical Nutrition) 89: 1088-1091.

Röhrig, G, G Rappl, B Vahldick, I Kaul, and R, J Schulz. 2014. "Serum hepcidin levels in geriatric patients with iron deficiency anemia or anemia of chronic disease." *Zeitschrift fur Gerontologie und Geriatrie* (Zeitschrift fur Gerontologie und Geriatrie) 47 (1): 51-56.

Roumen-Klappe , E M, M den Heijer , S H van Uum , J van der Ven-Jongekrijg , F van der Graaf , and H Wollersheim . 2002. "Inflammatory response in the acute phase of deep vein thrombosis." *J Vasc Surg* 35: 701–706.

Ruivard, M, F Laine, T Ganz, G, Westerman, M Olbina, E Meneth, R, and et al. 2009. "Iron absorption in dymetabolic iron overload syndromeis decreased and correlates with increased plasma hepcidin." *Journal of Hepatology* (Journal of Hepatology) 50: 1919-1225.

Sabin, C A, S W Worm, R Weber, and et al. 2008. "Use of Nucleoside Reverse Transcriptase Inhibitors and Risk of Myocardial Infarction in HIV-Infected Patients Enrolled in the D:A:D study: a Multicohort Collaboration." *Lancet* 371: 1417–1426.

Saulius. 2009. "Arterisclerosis, Thrombosis and Vascular Biology." *Aha Journals.org* 29: 1989-1996.

Schulze, K,J, P Christian, I Ruczinzki, and et al. 2008. "Hepcidin and iron status among pregnant women in Bangladesh." (Asia Pacific Journal of Clinical Nutrition) 17: 451-6.

Sereti , I, B Herpin , J A Metcalf , R Stevens , M W Baseler, and C W Hallahan . 2001. "CD4 T Cell Expansions are associated with increased apoptosis rates of T lymphocytes during IL-2 cycles in HIV infected patients." *Aids* 15: 1765–1775.

Shafran, S D, L D Mashinter, and S E Roberts. 2005. "The effect of low-Dose Ritonavir Monotherapy on Fasting Serum Lipid Concentrations." *HIV Med* 6: 421–425.

Spira, R, C Marimoutou, C Binquet, D Lacoste, and F Dabis. 1998. "Rapid Change in the use of Antiretroviral Agents and Improvement in a Population of HIV Infected Patients." *J Acquir Immune Defic Syndr Hum Retrovirol* 18: 358–64.

Stec, James J, Halit Silbershatz, Geoffrey H Tofler, Travis H Matheney, Patrice Sutherland, Izabela Lipinska, Joseph M Massaro, Peter F. W Willson, James E Muller, and Ralph B Sr D'Agostino. 2000. "Association of Fibrinogen with Cardiovascular RiskFactors and Cardiovascular Disease in the Framingham Offspring Population." *Journal of the American Heart Association* 1634-1638.

Stein, J H, M A Merwood, J B Bellehumeur, P E McBride , D A Wiebe, and J M Sosman. 2005. "Postprandial Lipoprotein Changes in Patients Taking Antiretroviral Therapy for HIV Infection." *Arterioscler Thromb Vasc Biol* 25: 399–405.

Stone, R L, A M Nick , I A McNeish , F Balkwill , H D Han , and J Bottsford-Miller . 2012. "Paraneoplastic thrombocytosis in ovarian cancer." *N Engl J Med* 366: 610–618.

Svenson, N., Patmore, R., Cox, H., Bailey, J., Holding, S.,. 2015. "Iron Age or New Age: Ironing out the Diagnosis of Anaemia of Inflammation from Iron Deficiency Anaemia." *Hull and East Yorkshire Hospitals NHS Trust, Hull UK.*

Tien , P C, A I Choi , A R Zolopa , C Benson , R Tracy , R Scherzer, P Bacchetti , M Shlipak , and C Grunfeld . 2010. "Inflammation and mortality in HIV-infected adults: Analysis of the FRAM study cohort." *J Acquir Immune Defic Syndr* 55: 316-322.

Torriani , N, B J Thomas , R B Barlow, J Librizzi, S Dolan, and S Grinspoon. 2006. "Increased intramyocellular lipid accumulation in HIV-infected women with fat redistribution." *J Appl Physiol* 100: 609–14.

Van der Poll , T, CV Keogh , X Guirao , WA Buurman , M Kopf , and SF Lowry . 1997. "Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia." *J Infect Dis* 176 (2): 439–444.

van der Poll, T, M Levi, S J van Deventer, H Ten Cate, B L Haagmans, and B J Biemond. 1994. "Differental Effects of anti-tumor necrosis factor Monoclonal Antibodies on Systemic Inflammatory responses in Experimental Endotoxemia in Chimpanzees." *Blood* 446-451.

Van Der Poll, Tom, Harry R Buller, Hugo Ten Cate, Cornelis H Wortel, Kenneth A Bauer, Sander J. H Van Deventer, Erick C Hack, Hans P Sauerwein, Robert D Rosenberg, and Jan W Ten Cate. 1990. "Activation of Coagulation after Administration of Tumor Necrosis Factor to Normal Subjects." *The New England Journal of Medicine* 1622-1627.

Van Santen, S, and E.C de Vegt, F., et al., Van Dongen-Lases. 2011. "hepcidin and hemoglobin Content parameters in the diagnosis of iron deficiency in rheumatoid arthritis patients with anaemia." *Arthritis Rheum* 120: 2395-405.

Van, L F, P Phanuphak, E Stroes, and et al. 2004. "Nevirapine and Efavirenz Elicit Different Changes in Lipid Profiles in Antiretroviral-Therapy- Naive Patients Infected with HIV-1." *PLoS Med* e19.

Vos, T, A,D Flaxman, M Naghavi, R Lozano, C Michaud, M Ezzati, M Shibuya, and K Salomon. 2012. "Years lived with disbility (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for Global Burden of Disease Study 2010." *Lancet* (Lancet) 380 (9859): 2163-2196.

Weber , C, A Zernecke , and P Libby . 2008. "The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models." *Nat Rev Immunol* 8 (10): 802-815.

Weiss, G, and L, T Goodnough. 2005. "Anaemia of Chronic Disease." (The New England Journal of Medicine) 352 (10): 1011-1023.

WHO. 2006. "BMI Classification. Global Database on Body Mass Index." (World Health Organisation).

WHO, World health Organisation. 2008. "Worldwide prevalence of anaemia 1993-2005." *World Health organization.ISBN 978-92-4-1596656-7.*

Wish, B. 2006. "Assessing iron status: beyond serum ferritin and transferritin saturation." *Clinical Journal of the American Society of Nephrology* (Clinical Journal of the American Society of Nephrology) 1: S4-S8.

Worm , S W, and P Hsue . 2010. "Role of biomarkers in predicting CVD risk in the setting of HIV infection." *HIV AIDS* 5 (6): 467–72.

Worm, S W, C Sabin, R Weber, and et al. 2010. "Risk of Myocardial Infarction in Patients with HIV Infection Exposed to Specific Individual Antiretroviral Drugs from the 3 Major Drug Classes: The Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) Study." *J Infect Dis* 318–330.

Young, B, and J Zaritsky. 2009. "Hepcidin for Clinicians." *Clinical Journal of American Society of Nephrology* (Clinical Journal of American Society of Nephrology) 4 (8): 1384-1387.

Zaritsky, J, B Young, H,J Wang, and et al. 2009. "Hepcidin-a potential novel biomarker for iron status in chronic kidney disease4." *Clinical Journal of American Society of Nephrology* (Clinical Journal of American Society of Nephrology) 4: 1051-56.

Zhang, A,S, and C,A Enns. 2008. "Iron homeostasis: recently identified proteins provide insight into novel control mechanism." (Journal of Biological Chemistry) 284: 711-715.

APPENDIX I: INFORMATION SHEET: PARTICIPANT

This form gives you information on the study in which you are being requested to participate in. To make sure that you have all the facts about this study you must read this form or have someone read it for you. If you agree to participate in this study, you must sign the consent form or put your thumbprint in the space provided, if you cannot write. If you feel that you cannot take part in this study, you are free not to participate in it and your refusal will not in any way jeopardize the care you will receive from the health providers.

Purpose of the Research

My name is Ephraim Chikwanda studying for a Master of Science degree (MSc.) in Pathology (Haematology) at the University of Zambia- Ridgeway Campus. I am carrying out a research as a requirement for fulfillment of Master of Science (MSc) degree in Pathology (Haematology). You have been invited to participate in this study which seeks to determine the levels of hepcidin hormone in iron deficiency anaemia (IDA) and anaemia of chronic disease (ACD) to help differentiate these two conditions. You have been requested to participate in this study because you have been diagnosed with anaemia and also based on the clinician's assessment with regard to the study inclusion criteria. If you decide to take part in the study, you will be requested to give 8mL (1 EDTA container and 1 Plain container) of blood. The study requires at least 66 people to participate.

Risks, Discomforts and Benefits of this Study

There are no risks associated with being a participant in this study. However, you will experience some pain when blood is being drawn from the vein. Every effort will be made to reduce the pain that you feel as the blood is being collected.

There are no costs to you for being in this study and the study may not benefit you directly. However, the study results will provide valuable information which can be used to improve the health of individuals in future. The results from the study may identify patients who may benefit from iron supplement or anti-hepcidin therapy (in future).

Confidentiality

The information you will give in this study will remain confidential and will not be made available to anyone who is not connected with the study. Furthermore, your name will not be written on any data collection tool for confidentiality purposes.

Contact Details

Please if you have any queries do not hesistate to contact the following: Mr. Ephraim Chikwanda, P.O. Box 50110, Lusaka, Cell: 0977965425; the Head of Department of Pathology and Microbiology, School of Medicine, The University of Zambia, P.O. Box 50110, Lusaka or the Chairperson, Research Ethics Committee, Ridgeway Campus, P.O. Box 50110, Lusaka, Zambia, telephone: 256067.

NOTE: THE ABOVE SECTION HAD BEEN GIVEN TO THE PARTICIPANT

APPENDIX II: INFORMED CONSENT FORM

Study Title**: Evaluation of Hepcidin as a Potential Biomarker for the Differential Diagnosis of Iron Deficiency Anaemia and Anaemia of Chronic Disease at Ndola Central Hospital, Zambia**

By signing my name below, I ……..…….. confirm the following:

- I have read (or had read to me) this entire consent document and all of my questions have been answered adequately.
- The study's purpose, procedures, risks and possible benefits have been explained to me.
- I freely and voluntarily choose to participate.
- I understand that participating or not will not affect my health care or that of my family members.
- I understand that my rights and privacy will be maintained.

Participant signature …… Date……..

Thumb print if participant can't sign…….

……. …….
Witness (Name and Signature) Date

Please if you have any queries do not hesistate to contact the Chairperson, Research Ethics Committee, Ridgeway Campus, P.O. Box 50110, Lusaka, Zambia, telephone: 256067.

NOTE: **The participant were provided with a signed and dated copy of this consent form. It helped him/her remember what we discussed.**

Appendix III: CLINICAL AND DEMOGRAPHICAL DATA FORM

The University of Zambia
School of Medicine
Department of Pathology and Microbiology

Study Title: Evaluation of Hepcidin as a Potential Biomarker for the Differential Diagnosis of Iron Deficiency Anaemia and Anaemia of Chronic Disease at

Ndola Central Hospital, Zambia

Personal Data

Participant ID number….....

Age……. Gender…….

Clinical Data

Diagnosis: ……

Previous History……

Other Underlying Diseases/Conditions……..

…… …….. ……

Clinician's name **Signature** **Date**