FUNCTIONAL IRON DEFICIENCY ANAEMIA AND ITS GENETICS IN A BLACK SOUTH AFRICAN POPULATION WITH CHRONIC KIDNEY DISEASE

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DECLARATION

I, AISHATU MUHAMMAD NALADO, hereby declare that the work in this thesis, unless otherwise acknowledged, is mine. This work is submitted, in fulfilment for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.

Signed at the University of the Witwatersrand, Johannesburg

15th of October 2019

DEDICATION

To God Almighty

To my father, Alhaji Muhammad Nalado

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS RESEARCH

Publications

1. **Nalado AM,** Mahlangu J, Waziri B, Duarte R, Paget G, Olorunfemi G, Naicker S. Ethnic prevalence of anaemia, and predictors of anaemia among chronic kidney disease patients at a tertiary hospital in Johannesburg, South Africa. International Journal of Nephrology and Renovascular Disease 2019:12. Pages 19-32.
2. **Nalado AM**, Mahlangu JN, Duarte R, Paget G, Olorunfemi Barry F, Naicker S. Utility of reticulocyte haemoglobin content and percentage hypochromic red cells as markers of iron deficiency anaemia among black CKD patients in South Africa. Plos One. 2018 13 (10).
3. **Nalado AM,** Dix-PeekT, Olorunfemi G, Dickens C, Khambule L, Snyman T, Paget G, Mahlangu JN, Duarte R, George JA, Naicker S. Hepcidin and GDF-15 are potential biomarkers of Iron Deficiency Anaemia in Chronic Kidney Disease Patients in South Africa. Under Review (Haematologica).
4. **Nalado AM**, JN Mahlangu, G Paget, MK Muteba, B Waziri, R Duarte, Naicker S. GDF-15, Hepcidin, and Iron deficiency Anaemia as Predictors of Disease Progression and Mortality in CKD Patients. Under Review (KI).
5. **Nalado AM**, Dickens C, Dix-Peek T, Mahlangu JN, Olorunfemi G, Paget G, Duarte R, Naicker S. TMPRSS6 rs 855791 Polymorphism and Susceptibility to Iron Deficiency Anaemia in Non-Dialysis Chronic Kidney Disease Patients in South Africa. In press (International Journal of Molecular Epidemiology and Genetics).

Presentations

1. **Nalado AM,** Naicker S. Utility of Reticulocyte Haemoglobin Content as a Marker of Iron Deficiency Anaemia in Black Chronic Kidney Disease patients in South Africa. Journal of the American Society of Nephrology 2017 28, Abstract supplement; page1003.
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4. **Nalado AM**, Mahlangu JN, Waziri B, Paget G, Olorunfemi G, Duarte R, Naicker S. Anaemia, Mean Corpuscular Volume and Mortality among a Black CKD Population in South Africa. Oral Presentation: South African Renal Congress, October 2018.
5. **Nalado AM**, JN Mahlangu, G Olorunfemi, T Dix-Peek, C Dickens L Khambule, T Snyman, G Paget, R Duarte, Jaya G, S Naicker. GDF-15, Hepcidin, and Iron Deficiency Anaemia as Predictors of Disease Progression and Mortality in CKD Patients. Poster Presentation Accepted for World Congress of Nephrology, April 2019.
6. **Nalado AM**, Mahlangu JN, Waziri B, Paget G, Olorunfemi G, Duarte R, Naicker S. Anaemia, Mean Corpuscular Volume and Mortality among a Black CKD Population in South Africa**.** Poster Presentation Accepted for World Congress of Nephrology, April 2019.

ABSTRACT

Background: Anaemia of chronic kidney disease (CKD) and iron deficiency anaemia (IDA) has been associated with morbidity and mortality in patients with CKD. Consequences of these changes differ across different races. Diagnosis of IDA is usually straightforward, except when it occurs in the context of inflammatory disorders such as CKD, where the conventional parameters used to diagnose IDA are not definitive. Hence, there is a need for newer and more accurate biomarkers in the diagnosis of IDA in CKD patients. Studies in developed countries have reported the usefulness of newer parameters in IDA diagnosis; however, the role of these newer biomarkers in IDA diagnosis in Africa is unknown. It remains unclear whether genetic factors may increase susceptibility to the development of IDA in CKD patients. Therefore, this study has been conducted to determine the prevalence and racial prevalence of IDA; to determine the utility of newer biomarkers in the diagnosis of IDA and the effect of these biomarkers on disease outcomes; and to provide important insights into the prevalence and genetic susceptibility of IDA in CKD patients in sub-Saharan Africa.

Methods: This was a cross-sectional study carried out from May 2016 to December 2018, involving 365 CKD patients from the Renal Outpatient Clinic of the Charlotte Maxeke Johannesburg Academic Hospital, South Africa, and 144 age- and gender-matched healthy controls comprising hospital staff and the patients' relatives.

The study was approved from the Human Research Ethics Committee of the University of the Witwatersrand (clearance certificate number, 150929). The first part of the study described the prevalence of anaemia and IDA in a multi-ethnic CKD population. The second part of the study determined the utility of reticulocyte haemoglobin content (CHr), and % hypochromic red cells (%HYPO), in the diagnosis of IDA, followed by role of novel biomarkers, GDF-15 and hepcidin, as diagnostic markers of IDA. Hepcidin and GDF-15 were measured using mass

spectrometry and ELISA, respectively and was followed by the genetic aspect of the study that determined the susceptibility of a genetic variant to IDA. The last part of the study looked at role of GDF-15 and hepcidin in disease progression and mortality using Spearman's rank correlation and linear and logistic regression analysis. Receiver operator curves (ROCs) were used to evaluate the diagnostic utility of biomarkers. Using Cox proportional hazard models, a composite endpoint of CKD progression was also investigated**.**

Results: The prevalence of anaemia (Hb < 13 g/dl in males and < 12 g/dl in females) among black CKD patients was 46.9 %, with 26.1% of the sample population suffering from IDA, which proved to be three times more frequent among the CKD patients than among the controls. The proportions of functional iron deficiency anaemia (18.6 % vs 1.4 %) and absolute iron deficiency anaemia (16.7 % vs 7.8 %) were higher among the CKD patients than among the controls. However, 32.3 % (95% CI: 27.90 %– 37.10 %) of the study population presented with iron deficiency without anaemia.

The mean age of the CKD patients was higher than that of the controls (52.7 ± 14.3 *vs* 40.4 ± 12.6 years, P < 0.001). The sensitivity and specificity values for diagnosing IDA among CKD participants were 62.6 % and 80.2 % respectively for CHr at a cut-off value lower than 28 pg, and 63.3 % and 79.8 % for the proportion of hypochromic red cells (%HYPO), respectively. The (define here) AUC of CHr (0.81, 95 % CI: 0.76-0.87) was higher than the AUC of %HYPO (0.76, 95 % CI: 0.76-0.87). The predictive value of diagnosing IDA among CKD patients using GDF-15 and hepcidin was high (AUC= 0.723 and 0.714, respectively). The prevalence of rs 855791 C homozygosity was similar for the iron-deficient and non-iron-deficient anaemia groups (86.1 % vs 84.2 %, p= 0.723). When the analysis was confined to participants with or

without functional IDA, however, the C homozygote (88.3 % *vs* 84.4 %, p= 0.425) value was similar for both groups.

Patients with high levels of hepcidin were up to six times more likely to be at risk of dying as opposed to patients with normal levels of hepcidin (HR: 6.14; P< 0.001). On the other hand, it was determined that the levels of GDF-15 and IDA were not associated with mortality in CKD patients. Higher plasma levels of hepcidin were strongly associated with CKD progression when compared to normal hepcidin levels (HR: 3.09; P= 0.002). The risk of CKD progression was two times higher in the presence of hypocalcaemia as opposed to normocalcaemia (HR: 2.24; P= 0.021).

The death rate increased with declining haemoglobin levels from 0.96 among patients with mild anaemia, to 4.29 per 100-person years, among patients with severe anaemia. Anaemic patients with a glomerular filtration rate (GFR) of less than 30 mls/min were susceptible to a significantly greater risk of death, due mainly to end stage kidney disease (ESKD) and failure to commence haemodialysis because of a lack of funding (HR 11.51: 95% CI 1.62–78.32, P < 0.001). Participants with hyperphosphatemia had about a 2.6-fold increased risk of death (HR, 2.60; 95% CI, 1.09-6.20, P= 0.02). Although the odds of anaemia were 3.8-fold higher (odds ratio = 3.8, P = 0.059) among CKD stage 5 participants as opposed to those with CKD stage I, the relationship between anaemia and the stages of CKD proved to be non-linear**.**

Conclusions: Anaemia and IDA were common in our pre-dialysis CKD patients. Anaemia, elevated hepcidin levels, hypocalcaemia and low estimated glomerular filtration rates (eGFRs), were found to be associated with an increased risk of death among pre-dialysis CKD patients. Our study also highlighted that newer biomarkers (novel) can be useful in the diagnosis of IDA

and can perform more effectively than the conventional parameters (transferrin saturation (TSAT) and ferritin) in an African CKD population. In addition, our study showed that newer biomarkers can be used to predict disease progression and mortality in a CKD population. This suggests that health care services for chronic diseases should be prioritise pre-dialysis CKD patients.

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LIST OF ABBREVIATIONS

AUC: Area under the Curve

BMI: Body mass index

ELISA: Enzyme- linked immunosorbent assay

CKD: Chronic Kidney Disease

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration

CHr: Reticulocyte Haemoglobin Content

CRP: C reactive protein

DM: Diabetes mellitus

DBP: Diastolic blood pressure

DNA: Deoxyribonucleic acid

DMTI: Divalent metal transporter 1

EDTA: Ethylene diamine tetra acetic

eGFR: Estimated glomerular flirtation

ESA: Erythropoiesis-stimulating agents

EPO: Erythropoietin

ESKD: End stage kidney disease

FIND-CKD: Ferinject assessment in patients with iron deficiency anaemia and non-dialysis-dependent CKD

GDF-15: Growth Differentiation Factor-15

HAMP: Hepcidin Antimicrobial Peptide

HTN: Hypertension

% HYPO: Percentage hypochromic red cells

IDA: Iron Deficiency Anaemia

FID: Functional Iron Deficiency Anaemia

KDIGO: Kidney Disease Improving Global Outcomes

MCV: Mean Corpuscular Volume

MCH: Mean Corpuscular Haemoglobin

MCHC: Mean Corpuscular Haemoglobin Concentration

NPV: Negative Predictive value

PPV: Positive predictive value

RCT: Randomized Controlled Trial

ROC: Receiver operative characteristic

RRT: Renal replacement therapy

SCr: Serum creatinine

SD: Standard deviation

SNP: Single nucleotide polymorphism

sTfR: Soluble Transferrin Receptor

SBP: Systolic blood pressure

TIBC: Total Iron Binding Capacity

TMPRSS6: Transmembrane Serine Protease 6

TSAT: Transferrin Saturation

1. CHAPTER ONE: INTRODUCTION

1.1. Background of study

Chronic kidney disease (CKD) is defined by "persistent urine abnormalities, structural abnormalities or impaired excretory renal function suggestive of a loss of functional nephrons" (1), also defined by KDIGO in 2012 as "abnormalities of kidney structure or function present for >3 months, with implications for health" (2). CKD is a global health burden with a high economic cost to health care and is an independent risk factor for cardiovascular disease (CVD) (3).

The burden of non-communicable diseases (NCDs) has increased exponentially over the past decade, and NCDs account for the majority of health-related morbidity and mortality worldwide (4). Likewise, the global prevalence of (CKD) has increased in the last decade (5). Hence, morbidity and premature mortality from CKD almost doubled between 1990 and 2016 (6). Furthermore, a study on the global burden of the disease estimated that the number of disability-adjusted life-loss years (DALYS) attributable to kidney disease increased by 62 % between 1990 (19 million) and 2016 (35 million) (7). The regional pattern also suggests increasing trends in CKD morbidity and mortality.

Data from the American National Health and Nutrition Examination Survey demonstrated that over the period 1999 to 2004 the prevalence of CKD increased by 3 %, as opposed to the increase over the survey period 1988 to 1994 (13.1 % vs 10.0 %) (8). The global burden of CKD is estimated to be 19.2 million. This accounts for 11 % of the adult population in the United States (US), with about 385 000 people with end stage kidney disease (ESKD) (9, 10). The burden of CKD stages 1 to 5 is 11.9 % in Taiwan, and 13 % in China (11). Patients in low- and middle-income countries (LMIC) also contribute largely to these increasing trends. This can be attributed to increasing awareness and improved diagnostic capabilities. However, the human and infrastructural resources

to support the management of these growing populations of CKD patients in developing countries such as South Africa are inadequate (3).

The burden of CKD in sub-Saharan African countries has not been well documented, probably on account of the non-availability of registry records in most African countries (12). Likewise, the prevalence of CKD in South Africa was reported to be 25 % (13) and the Fourth Annual Report of the South African Renal Registry showed that the prevalence of patients with (ESKD) increased from 167 per million in 2013 to 189 per million in 2015 (14). The population of pre-dialysis CKD patients is also increasing because of the awareness of the disease. However, there is a marked difference in the management and prognosis of pre-dialysis and haemodialysis patients. South Africa is a multi-ethnic country with considerable variations and inequalities in its social and health conditions. No doubt, such inequities in access to care and socio-economic circumstances, coupled with other local characteristics and patient attributes could modify the prognosis and quality of life of pre-dialysis CKD patients. Despite the statistics relating to the increasing trends in the prevalence of CKD and premature mortality from this disease, global interest in research aimed at preventing the outcomes of this disease, and at improving the quality of life of CKD patients has been relatively limited. This is evident when other non - communicable diseases (NCDs), such as CVD and cancer, that enjoy so much more attention globally, are considered. Similarly, there is a paucity of robust data in South Africa on the prevalence of the CKD and aspects of its management. One key clinical condition that impacts on the prognosis and quality of life of CKD patients - especially in a low-resource country such as South Africa, is anaemia, especially iron deficiency anaemia (IDA) (15).

Anaemia is twice as prevalent among patients with CKD as opposed to the general population (15.4 % vs 7.6 %) (16). Although diminished erythropoietin production by the kidney is a common

cause of anaemia in patients with CKD, iron deficiency is also a leading contributor to morbidity and mortality from CKD (17). There is currently the need to develop novel, less-invasive markers for the early and prompt identification and management of IDA among pre-dialysis CKD patients, and to improve their quality of care. Currently, transferrin saturation and serum ferritin are the main parameters for screening and diagnosing IDA, but these parameters have their limitations and do not identify IDA in its early stages (17).

Human diets contain iron as haem or non-haem iron. Non-haem iron refers to various forms of inorganic iron and is usually associated with iron in plants. Cellular iron homeostasis is maintained by iron and haem transport proteins that work in concert with ferrireductase, ferroxidase, and chaperones to direct the movement of iron into, within, and out of cells (18). Systemic iron homeostasis is regulated by the liver-derived peptide hormone, hepcidin. Hepcidin and its receptor, the cellular iron exporter ferroportin, constitute a feedback-regulated mechanism that maintain adequate plasma concentrations of iron-transferrin for erythropoiesis and other functions, ensures sufficient iron stores, and avoids iron toxicity and iron-dependent microbial pathogenesis (19, 20).

The gold standard for diagnosing IDA is to assess the bone marrow iron stores, an invasive and impractical method for routine testing (21). Bone marrow iron staining lacks accuracy in detecting functional iron deficiency in patients with CKD. The laboratory diagnosis of iron deficiency in patients with CKD has not been perfected because a true gold standard is lacking (22). Thus, there is the need for newer novel markers with similar or better predictive abilities than the conventional markers. We hypothesize that some biomarkers, such as hepcidin, growth differentiation factor (GDF)-15, CHr, and %HYPO may have greater potential in the screening and diagnosis of IDA in the CKD population.

The diagnostic usefulness of biomarkers is related to the prevalence of the disease in the defined population. Hence, the sensitivity, specificity, or negative and positive predictive value of a biomarker may not be extrapolated to other populations (23). In view of the paucity of data from Africa, there is therefore the need to study the prevalence of anaemia and IDA, the utility value of novel biomarkers in the diagnosis of IDA as compared to conventional biomarkers, and to assess the role of these biomarkers in the progression of CKD and mortality.

It is well established that genetic polymorphisms and mutations play significant roles in iron overload (24). However, much less is reported on the genetic basis of IDA. Understanding the genetics of iron status is key to optimizing strategies to prevent and manage IDA, as well as to discovering appropriate biomarkers to monitor iron status in the CKD population.

Based on the knowledge gaps identified, we set out to assess the prevalence of anaemia and IDA among a cohort of CKD patients in Johannesburg, South Africa. This study compared the respective performances of novel and conventional biomarkers in the diagnosis of IDA in a CKD population. We further examined for the first time in a sub-Saharan African cohort the role of these novel biomarkers in CKD progression and mortality. Lastly, this study evaluated the role of variants in TMPRSS6 gene in the genetic susceptibility of black South African CKD patients to the development of IDA.

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2. CHAPTER TWO: LITERATURE REVIEW

2.1. Introduction

Chronic kidney disease (CKD) is defined as "kidney damage or a glomerular filtration rate (eGFR) less than 60mls/min/1.73m2 for three months or more, or structural or radiological abnormalities for more than three months (1, 2)," also defined by KDIGO in 2012 as "abnormalities of kidney structure or function present for >3 months, with implications for health (3)" . Globally, 10% of the population worldwide was reported as having CKD in 2015 (4). In a systematic review and meta-analysis by Stanifer et al, the overall prevalence of CKD from 21 medium-quality and high-quality studies was 13.9%. Kaze et al, (5) reviewed 98,432 individuals and found a prevalence of 15.8% for CKD stages 1-5 in the general adult population living on the African continent; the prevalence of CKD was higher in Sub-Saharan Africa than North Africa (6) The crude prevalence of CKD in South Africa was reported by Matsha et al (7) as 14.8% for Cockcroft-Gault, 7.6% and 23.9% respectively for the MDRD with and without correction for ethnicity, and 7.4% and 17.3% for the CKD-EPI equations with and without ethnicity correction. Prevalence of CKD among teachers in Cape Town, South Africa was found to be 6% by Adeniyi et al (8).The burden of CKD in sub-Saharan Africa is three to four times higher than in developed countries (9, 10). CKD is associated with adverse cardiovascular morbidity and mortality, while the risk of death through cardiac causes has increased by 46 % in patients with CKD stages 3 to 5. It should, however, be noted that CKD is influenced by traditional cardiovascular risk factors such as hypertension, anaemia, and diabetes (10, 11).

Richard Bright first linked anaemia to CKD over 170 years ago (12). As kidney disease progresses, anaemia increases in prevalence, affecting nearly all patients with CKD stage 5. Anaemia in CKD is associated with reduced quality of life and increased risk of CVD, hospitalisation, cognitive

impairment, and death (13). Iron deficiency refers to the reduction of iron stores that precedes overt IDA or persists without progression. IDA is a more severe condition in which low levels of iron are associated with anaemia (14). The principal mechanism of anaemia prevalent in CKD is triggered by a reduction in the production of erythropoietin by the failing kidneys, although other mechanisms such, as amongst others, shortened red blood cell (RBC) survival, chronic blood loss, hyperparathyroidism, bone marrow depression, iron, folate and vitamin B12 deficiency may be implicated (9, 10).

Functional iron deficiency is a state of iron-starved erythropoiesis (15), in which insufficient iron is mobilised from the stores as a result of increased demands, as observed after treatment with erythropoiesis-stimulating agents. Iron deficiency can be caused by acquired or inherited defects. Acquired defects are mostly due to iron deficiency resulting from poor dietary intake or impaired absorption, blood loss or gastrointestinal loss (16). Genetic causes of IDA in humans include defects in the divalent metal-iron transporter (DMT1), a deficiency of ceruloplasmin, and the recently described mutations in the matripas-2 (TMPRSS6) gene (16). Very few studies have investigated the genetics of IDA in a CKD population; hence the need to evaluate the genetics of IDA in a black CKD population. Advances in molecular biology have substantially improved our understanding of the anaemia of chronic diseases. The anaemia of CKD is among them, with the added burden of erythropoietin deficiency. Recent elucidation of specifically disrupted erythroid marrow function by inflammatory mediators, pro-inflammatory cytokines and inflammation-mediated induction of hepcidin has also improved our understanding of hypo-responsiveness to the erythropoietin-stimulating agent (ESA) (17).

Previous studies that have used bone marrow aspiration for diagnosis have also reported a high prevalence of iron deficiency in the CKD population (18, 19). For example, iron stores were depleted in 48 % of their study population (19); iron deficiency was present in 68.6% of the women (20) and in 53.8 % of the men (2, 21) and 36 % of the non-dialysis CKD patients presented with iron deficiency (2, 22). The burden of IDA among black CKD patients in South Africa is not known; hence the need to determine this prevalence.

Previous researchers have looked at IDA in CKD patients, mostly in those on haemodialysis, where the cause is usually related to erythropoietin deficiency, or replacement therapy, blood loss, and chronic inflammation. However, there is a paucity of data on iron balance and the prevalence of functional iron deficiency in patients with CKD that are not on dialysis. Hence, the aim of our study was to evaluate the magnitude of iron deficiency in a non-dialysis CKD population (2).

This research explores the link between CKD, IDA, the utility of novel biomarkers as diagnostic tools or markers of IDA and compares the conventional parameters with other newer parameters and predicts their utility in the diagnosis of IDA in non-dialysis CKD patients, and the association of genetic variants with the development of IDA in the CKD population. For instance, this study evaluates the associations of hepcidin and GDF-15 with IDA, and further ascertains the novel markers (hepcidin, GDF-15, reticulocyte haemoglobin content, the proportion of hypochromic red cells) and compares them with other old traditional markers of iron deficiency (transferrin saturation (TSAT) and Ferritin).

2.2. Epidemiology of anaemia in CKD

In the National Health and Nutrition Examination Survey (NHANES) III (2007-2010), a national representative survey in the United States (US), the prevalence of anaemia (Hb <12 g/dl in men and 11 g/dl in women) was about (5%) in individuals with CKD stages 1 and 2, 15 to 20 in those with CKD stage 3, and more than 60 % in patients with CKD stage 4 (23). In this study, anaemia was present in 40 % of patients with stage 3 CKD, 50 to 55 % of those with stage 4 CKD, and 80 % in those with stage five CKD (24). In a pre-dialysis survey of anaemia involving 21 European countries, the prevalence of anaemia was 68 % in 4 337 pre-dialysis patients in Israel and South Africa (25).

There was a higher prevalence of anaemia associated with CKD in patients older than 60 years, as compared to those aged between 46 and 60 years. This may be due to the lower estimated GFR associated with ageing (26). Bone marrow biopsies performed on 47 patients with CKD and with (Hb) levels of lower than 12 g/dl. Severe iron deficiency was present in 46 of the 47 patients tested (2).

In Romania, bone marrow biopsies performed on 100 pre-dialysis CKD patients showed 48 % of the patients to be iron-depleted (12). This is one of the largest studies to evaluate the central iron indices in non-dialysis patients with CKD. It confirmed that bone marrow iron stores are depleted in a large proportion of non-dialysis patients with CKD and further supported the therapeutic use of iron in this group of patients.

Rahman, Dutta (27) conducted an observational study on 52 pre-dialysis CKD patients with chronic anaemia. Bone marrow iron stains were taken and compared with the serum iron profiles, and the conclusion was reached that an absence of stainable iron in the bone marrow presents better evidence of iron depletion than the serum iron profile (TSAT and ferritin) which correlates less well with the bone marrow iron status in patients with CKD. Moreover, the study by Syid, Mohmmed (28) reported that there was no stainable iron in the bone marrow of 23 of the 52 pre-dialysis patients. Variations in their findings could be explained by differences in methods used for examinations and the fact that the bone marrow iron evaluation results were not detailed enough (26, 27). Additionally, Stancu, Bârsan (19) used only the number of macrophages sideroblast particles and carried out a more complex examination involving not only macrophage iron but also iron in sideroblast.

Black individuals have four times greater risk of developing CKD than white individuals (29). There is also an increased prevalence of anaemia in the general black population (30). A study carried out on an urban black South African population reported a 13 % prevalence of anaemia (31). Prevalence of IDA in ND-CKD population varies from 15 %-48 % (Figure 2.1).

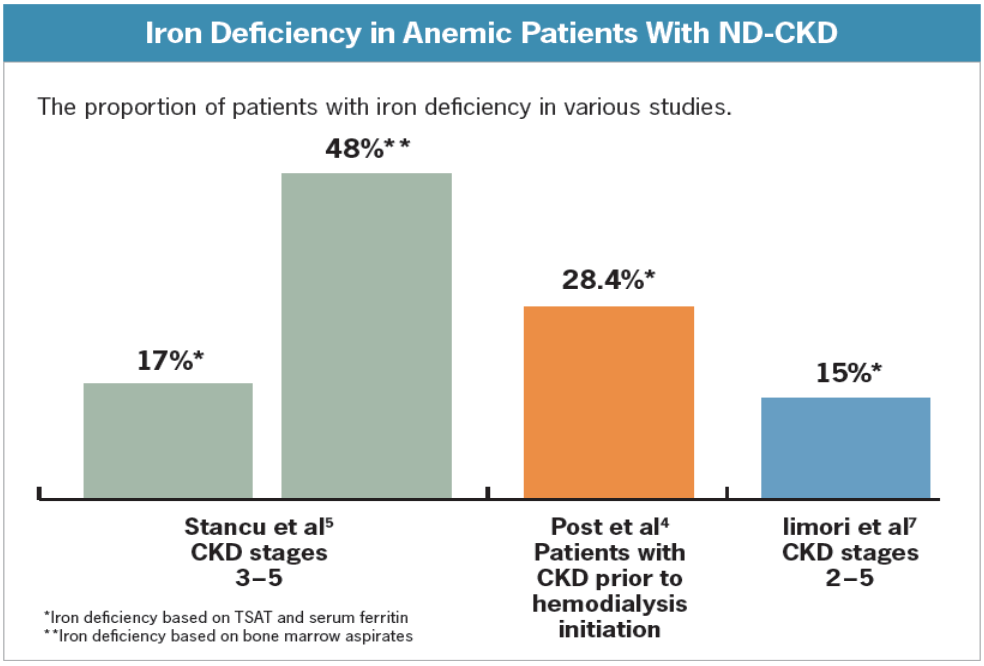


Figure 2:1: Iron deficiency in anaemic patients with ND-CKD

(*Reproduced from Renal and Urology News. July 19, 2017; free, open access)*

Hence, there is a need to carry out more studies to investigate the burden of anaemia in black patients with CKD. The few studies carried out in the black population have yet not revealed the prevalence of anaemia in this population group.

2.3. Pathogenesis of renal anaemia

Anaemia in CKD is typically normocytic, normochromic, and hypo-proliferative. The kidneys are the main source of erythropoietin, a circulating factor responsible for stimulating erythropoiesis described in a study by Jacobson et al in the 1950s (32). It is hypothesized that erythropoietin deficiency is a predominant cause of anaemia in CKD. Erythropoietin levels are generally normal or slightly increased in the anaemia of CKD; however, levels are inappropriately low relative to the degree of anaemia. Anaemic patients with normal kidney function have 10 to 100 times higher EPO levels (33). Anaemia associated with chronic illness traditionally includes any inflammatory,

infectious, or malignant diseases, or long-standing diseases such as CKD. Under these conditions, there is a relatively low level of erythropoietin, and a mild reduction in the life span of red blood cells (RBCs) from 120 days to 70 to 80 days (34). Insight into the multiple factors involved in the pathogenesis of anaemia has improved recently and has led to important developments in treatment options. The dominant contributing factors are the inadequate production of EPO and iron deficiency however, other factors important to clinicians should be considered (35).

Recently, hepcidin, which is an as important antimicrobial peptide, has been identified as the peptide controlling the level of plasma iron and the transfer of iron stored in the hepatocytes. Hence, increased levels of hepcidin have been associated with inflammatory diseases and are a significant mediator of the accompanying anaemia (36). Cytokines, such as interleukin (IL1&6) and tumour necrosis factor (TNF∝) which causes the destruction of the red blood cell (RBC) precursors and reduces the number of erythropoietin receptors on progenitor cells (37), may be associated with elevated hepcidin levels (Figure 2.2).

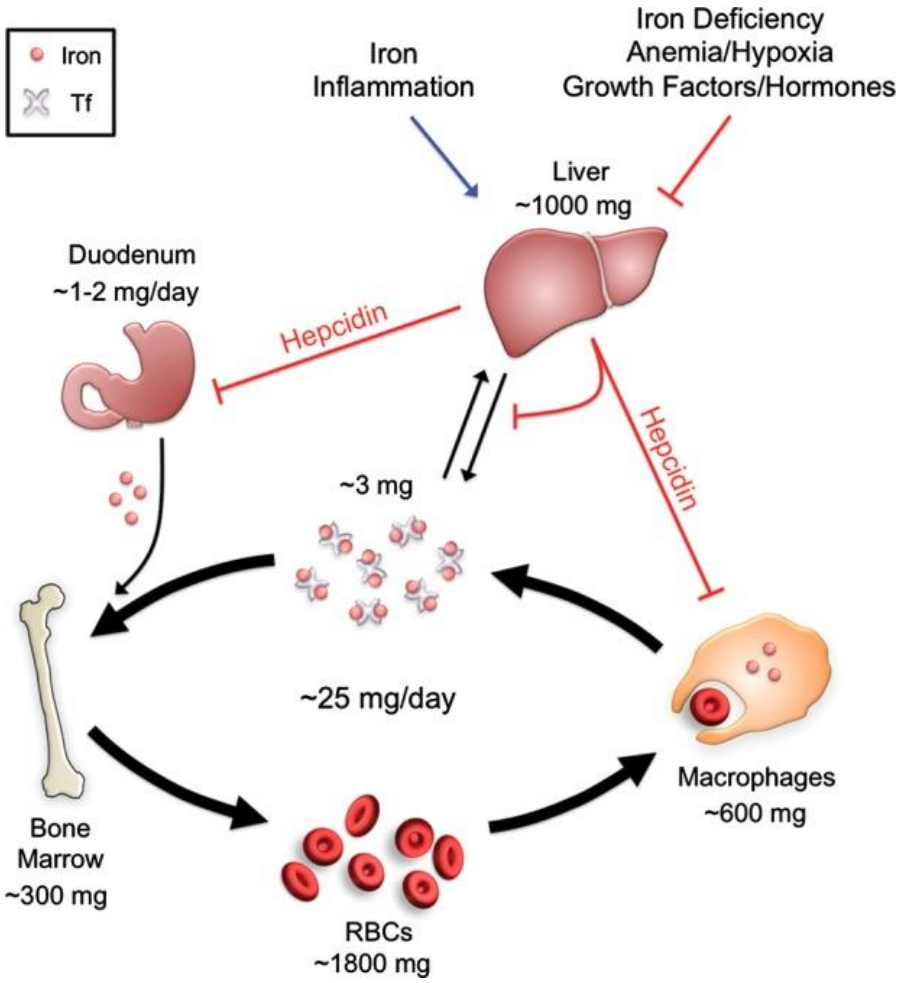


Figure 2:2: Diagram illustrating the pathogenesis of CKD anaemia

(*Reproduced with permission from Zumbrennen-Bullough and Babitt (38), Appendix E)*

Hypoxia in the presence of normal kidney function stimulates erythropoietin gene transcription, and hence increases the RBC levels. In the anaemia of CKD, there is a primary deficiency of erythropoietin which is produced by interstitial fibroblasts in the kidney, which leads to anaemia. Numerous studies suggest that circulating uraemic-induced inhibitors of erythropoiesis contribute to anaemia. On the other hand, some studies have reported the lack of such inhibitors (39, 40). Nutritional deficiencies of for example folate and vitamin B12, due to anorexia or the loss of blood are currently uncommon as supplements are provided.

Iron regulation is another important factor in the pathogenesis of renal anaemia. Patients on haemodialysis lose one to three grams of iron per year as a result of chronic bleeding from uraemic-associated platelet dysfunction, frequent phlebotomies and blood tapping during the dialysis process (41). Kidney disease patients present with poor dietary iron absorption. However, while oral iron has proved to be a well-tolerated and effective form of iron supplementation in long term dialysis patients in a study by Lenga et al (42), it is less effective than intravenous iron in improving anaemia, mitigating or preventing iron deficiency, and in reducing the ESA dosage in haemodialysis patients (39). Many CKD patients receive ESA, which depletes the circulating iron pool by increasing erythropoiesis, thus making iron supplementation an integral part of CKD anaemia management. In addition to iron deficiency, many CKD patients present with functional iron deficiency, which is characterised by impaired iron release from body stores that are unable to meet the demands of the erythropoietic process. It is, therefore, important to conduct more research that will explore further the relationship between absolute and functional IDA especially in pre-dialysis patients, where the relevant data are limited. Excess hepcidin may account for impaired dietary iron absorption and reticulo-endothelial cell iron blockade present in many CKD patients (36) Hepcidin is the main hormone responsible for maintaining systemic iron homeostasis. Inflammatory cytokines directly induce hepcidin transcription (36), presumably as a mechanism to isolate iron from invading pathogens, leading to iron sequestration, hypoferaremia, and anaemia all of which are hallmarks of any chronic disease, including CKD (43).

The most important uraemic toxins in the pathophysiology of renal anaemia are polyamines such as spermine, and spermidine. Polyamines are organic cations involved in cell growth and maturation that reduce the proliferative activity of erythroid cells in the bone marrow through their

direct toxic effect that is independent of the interaction with EPO (44). This study explores the relationship of hepcidin to iron status in pre-dialysis CKD patients.

Reduced haemoglobin occurs in aluminium intoxication, first described by Alfrey (1972) (45) and subsequently by Elliott, Mishler (46) and Macdougall, Cavill (47). Anaemia caused by aluminium toxicity is of a microcytic and hypochromic nature. Aluminium competes with iron for a binding site for transferrin molecules, while free receptors increase aluminium and also suppress cytosolic uroporphyrinogens, decarboxylase and mitochondrial ferrochelatase (48). Hypo-proliferative anaemia may be exacerbated by fibrous osteodystrophy, usually caused by severe secondary hypoparathyroidism (49). Excessive parathyroid hormone levels are known to reduce EPO production, increase haemolysis, and suppress the stem cells of the red cell line in the bone marrow (49).

Anaemia may be aggravated by drugs such as ACE inhibitors, which suppress the synthesis of erythropoietin through enhanced oxygenation in peritubular regions via a decrease in vascular resistance in efferent arterioles, decreased serum concentration of growth factor-1 (GF-1) and increased apoptosis of the progenitor erythroid cells, CD4 (50). Malnutrition also plays a role in renal anaemia by causing severe hypoalbuminemia, which is associated with significant impairment of the erythropoietic response. There is a link between malnutrition and anaemia in the malnutrition- inflammatory (MIA) complex syndrome (51).

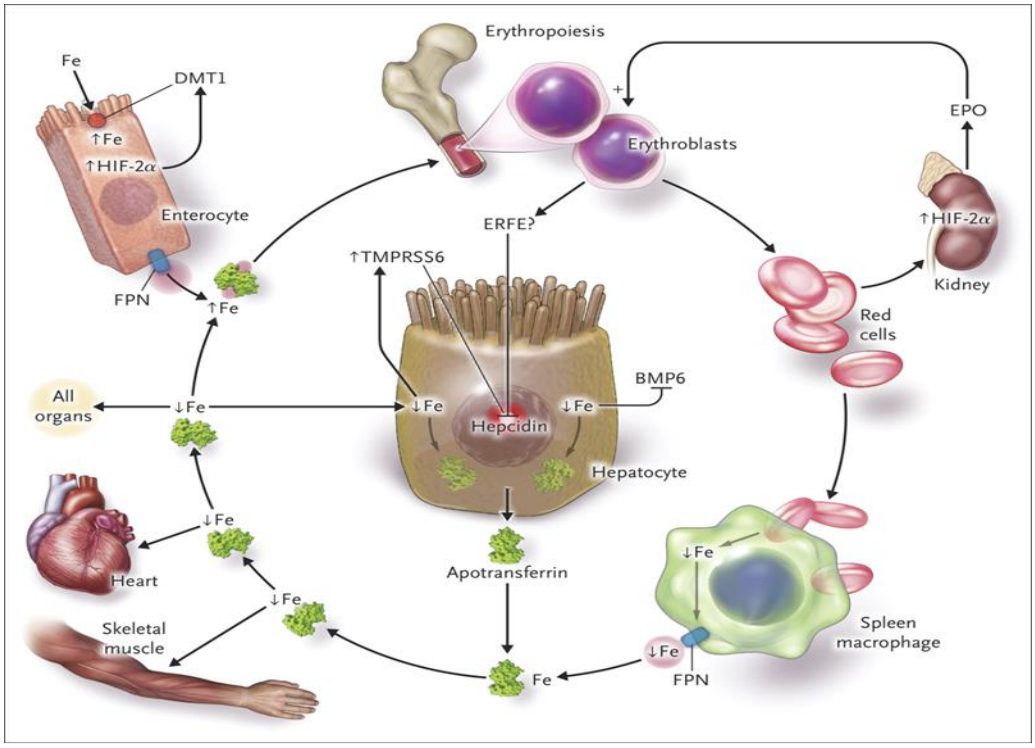
2.4. Iron deficiency anaemia

Iron deficiency is a state of altered iron balance that results in an insufficient supply of iron to the body cells and organs (14). More than approximately 2 billion people worldwide are affected by iron deficiency (52). In CKD, knowledge regarding iron deficiency is mainly associated with research in the haemodialysis population (53). Observations have been made to the effect that iron

deficiency is a common problem that interferes with the effectiveness of erythropoietin stimulating agent (ESA). Hence, researchers have concluded that the iron balance cannot be maintained without supplementation. There is, however, a paucity of information on iron balance in pre-dialysis CKD patients (54, 55). The National Health and Nutritional Examination Survey (NHANES) for 1988 to 2004 demonstrated that low iron stores are generally present in most patients with reduced kidney function (54). The NHANES III Survey (1988 to 1994) studied iron status in CKD patients, and found the presence of iron indices suggestive of iron deficiency contribute to anaemia in many CKD patients. Hsu, McCulloch (17) demonstrated that relative iron deficiency was an independent predictor for anaemia, and concluded that several CKD patients, in the US may not have enough iron stores to support erythropoiesis.

Iron deficiency develop in stages, sequential changes in iron status occurs before the diagnosis of iron deficiency anaemia (56). In the first stage, iron requirement exceeds intake, causing progressive depletion of bone marrow iron stores. As stores decrease, absorption of dietary iron increases in compensation. During later stages, deficiency impairs red blood cell synthesis, ultimately leading to iron deficiency anaemia (57). The mechanism of iron acquisition is tightly regulated by hepcidin-based homeostatic controls; its expansion increases in response to high circulating iron levels in the tissues and in persons with systemic inflammation or infections (58). The production of hepcidin is inhibited by the expression of erythropoiesis, iron deficiency, and tissue hypoxia in response to signals from the bone marrow. In iron deficiency, the transcription of hepcidin is suppressed, and adaptive mechanisms facilitate the absorption of iron and its release from the body stores. Intestinal iron (non-haem iron) uptake from the duodenum is mediated through a divalent metal transporter (DMT1). The uptake of iron is increased by the cation of hypoxia-inducible factor 2 (Figure 2.3) (52, 59). The degree of depletion of iron stores determines

the rapidity with which iron deficiency develops in the case of blood loss or a reduction in iron absorption. Hepatocytes are the key reservoirs.



The mechanisms of adaptation to iron deficiency are centered on the suppression of the hepatic hormone hepcidin and the tissue hypoxia that develops consequently to anaemia. The production of erythropoietin (EPO) by the kidney increases in response to enhanced levels of hypoxia-inducible factor 2α (HIF-2α). As a consequence of the stimulation of erythropoietin, erythropoiesis is increased and hypochromic microcytic red cells are produced because of the low availability of iron. Senescent red cells are destroyed by macrophages, and their iron is recycled. The increase in erythropoiesis suppresses the production of hepcidin. In mice, this function is mediated by erythroferrone (ERFE), which is secreted by the erythroblasts to maintain adequate iron absorption and efficiency in erythropoiesis (44). HIF-2α increases the expression of the duodenal divalent metal transporter 1 (DMT1)22 on the apical surface of enterocytes to increase the transfer of dietary iron from the lumen to enterocytes. Hepcidin levels are depressed in response to a reduction in the physiologic signals that maintain its production (e.g., increases in levels of iron-bound transferrin and in the iron content of the liver) (23, 40) to the increased activity of the inhibitor transmembrane protease, serine 6 (TMPRSS6) (46) to the reduction in levels of the activator bone morphogenetic protein 6 (BMP6), and to increased inhibition from erythropoietin-stimulated erythropoiesis. Ferroportin (FPN), which is no longer being degraded because of the low levels of hepcidin, exports the available iron across the enterocyte basal membrane and from macrophage stores to the circulation (39). Once stores are exhausted, levels of circulating iron decrease, even if absorption from the lumen is increased. Reduced levels of iron in the liver trigger increases in the synthesis of the iron carrier transferrin (referred to as apotransferrin when not bound to iron), further decreasing levels of iron-bound transferrin, the ligand of the transferrin receptor. Consequently, the uptake of iron from transferrin receptors by all cells and organs (e.g. skeletal muscles and the heart) is reduced.

Figure 2:3: The Iron-Cycle Mechanisms of Adaptation to Iron Deficiency

(*Reproduced with permission from (scientific reference and citation), Copyright Massachusetts Medical Society) (60).*

Furthermore, owing to a lack of oxygen and reduced haemoglobin synthesis, the RBCs become more fragile, which can result in haemolysis and a further worsening of the anaemia. Disease conditions with functional iron deficiency can occur when iron is not available for haemoglobin synthesis despite normal or increased availability of iron stores. The anaemia of inflammation can be mistaken for iron deficiency based on the heamogram machine (53)

Rare genetic defects in the regulation of ferroportin and hepcidin have been reported to cause refractory IDA. A defect in the hepcidin regulator TMPRSS6 has been recently implicated in humans (61). Although pre-dialysis CKD patients are not exposed to dialysis-related blood loss, as outlined below, iron deficiency can still arise from a variety of causes (14, 53, 62).

Anorexia is due to uraemia and diets containing low protein levels (especially animal protein), the increased consumption of iron, erythropoiesis-stimulating therapy, poor gastrointestinal iron absorption, impaired iron transport owing to renal failure (and mediated by the regulation of hepcidin). Platelet dysfunction leading to the increased tendency to bleed in renal failure, the concomitant use of prophylactic aspirin or anticoagulation medication, repeated venepuncture, the loss of iron-containing proteins in the urine of patients with proteinuria, and hookworm infestations, particularly in the tissues (63).

Iron deficiency anaemia is a chronic and frequently asymptomatic condition. Clinical signs due to anaemia do not occur until the condition is severe and includes lethargy, decreased exercise tolerance, difficulty in concentrating, weight loss, and low delivery levels of oxygen to the tissues. These signs are typical of any anaemia. The development of pica is unique to iron deficiency (64). Evidence of blood losses such as melaena, haematuria, or bleeding from other sites, may also be noticed in patients.

Physical examination outcomes may be normal, except for pallor, or may reflect the underlying disease processes. If anaemia is severe, a bounding pulse, arrhythmia, and systolic heart murmurs may be noted. Melaena or haematochezia may be noted on examination of faeces or on digital rectal examinations. However, tachypnoea and tachycardia are unusual with IDA.

2.5. Diagnostic approach to iron deficiency anaemia

The diagnostic approach to IDA includes identifying the underlying disease through a detailed review of the medical history of the patient, examination, and diagnostic evaluation. The medical history should include a review of the medications, diet, concurrent medications, faecal abnormalities, exposure to fleas and ticks, and a history of possible blood loss. Diagnostic evaluation, including a complete blood count (CBC), is necessary. See table 2.1 the expected parameters in iron deficiency.

Table 2:1: Expected serum parameters in iron deficiency

Table 1

Expected serum parameters in iron deficiency anemia and anemia of inflammatory disease

	Iron deficiency anemia	Anemia of inflammatory disease
Hematocrit	↓ to ↓ ↓ ↓	↓ to ↓ ↓
MCV	↓ to ↓ ↓ ↓	Normal to ↓
MCHC	↓	Normal
Serum iron	↓ to ↓ ↓ ↓	Normal to ↓ ↓
Serum TIBC	Normal to ↑	Normal to ↓
Serum ferritin	↓ to ↓ ↓	Normal to ↑ ↑
Stainable iron in marrow	Absent	Normal to ↑
Reticulocytes	Normal to ↓	↓

MCV — mean corpuscular volume, MCHC — mean corpuscular hemoglobin concentration, TIBC — total iron binding capacity.

(Table reproduced from renal and urology news, free open access)

Extensive research has resulted in a wealth of information about iron status and its association with anaemia in dialysis patients. However, this association is less defined in the pre-dialysis population. Studies suggest that substantial numbers of patients with non-dialysis CKD may have iron deficiency, which would be based on the findings of bone marrow biopsies - the gold standard for the diagnosis of IDA, but which is usually not carried out owing to the invasive nature of the procedure. Furthermore, in the light of the conventional iron indices (TSAT and ferritin), which have their own shortcomings, there is a need to explore other parameters (26, 65).

Three recent clinical trials regarding the treatment of anaemia in patients with non- dialysis CKD, namely the Convention of Haemoglobin and Outcome in Renal Insufficiency (CHOIR) (66), Cardiovascular Risk Reduction by Early Anaemia Treatment with the Epoetin Beta Trial (CREATE), and the Trial to Reduce Cardiovascular Events and Aranesp Therapy (TREAT) have provided much needed data (67).

CHOIR CREATE recruited patients with stage 4 CKD and tested the hypothesis that a normal haemoglobin level (Hb) (13-15 g/dl) would lead to improved outcomes, compared with a lower Hb target. None of these studies could confirm the hypothesis. Furthermore, a trend for an increased risk of cardiovascular death and events in the high Hb target arm of the study were reported. Important to note is that the CHOIR and CREATE trials have ignited intense debate about what the target Hb level in both dialysis and non-dialysis populations should be (11-12 g/dl), and that maintaining haemoglobin at 13 g/dl or higher is not advisable (68).

The TREAT trial was conducted to look at patients who were anaemic (Hb less than 11 g/dl), and had diabetic CKD stages 3 and 4 (eGFR 20-60 mls/min/1.73m2). At the end of the trial, there

were no differences in the primary cardiovascular composite endpoints. However, among those treated with dabapoetin alfa, a significantly higher proportion of patients suffered a fatal or non-fatal stroke (68). This necessitate an exploration into the possible reasons for an increase in the incidence of strokes.

2.6. Functional iron deficiency anaemia

Functional IDA is a state in which there is an insufficient supply of iron (low plasma iron levels, or low serum ferritin levels) for incorporation into erythroid precursors in the face of normal or increased body iron stores (63). This condition usually occurs in patients with anaemia of chronic inflammation, and in patients being treated with erythropoietin-stimulating agents (ESA). Bohn (69) defined the basis of erythropoietin response in three individuals and later through this observation, he was able to discover "relative iron deficiency", also known as functional iron deficiency, which he defined as "when the increased erythron iron requirement exceeds the available supply of iron".

Hepcidin plays a key role in iron- restricted erythropoiesis and is released in response to raised serum iron levels, infections, and inflammation. Inflammation and iron deficiency have opposite effects on hepcidin expression (70). The suppression of hepcidin during anaemia or hypoxia is an indirect effect dependent on increased erythropoietin production and the erythroid precursor's expansion in the bone marrow. Hepcidin inhibits iron efflux by directly binding to ferroportin to presumably induce a conformational change, and to trigger the endocytosis of both molecules, with consequent lysosomal degradation. The prolonged effect of hepcidin may reflect the time required to re-synthesise ferroportin and deliver it to the cell membranes (65).

Functional iron deficiency is common in haemodialysis patients. Not many studies have been carried out on functional IDA in the CKD population. In Spain, functional iron deficiency has been reported in 22 % of the haemodialysis patients (24). We set out to assess the presence and prevalence of absolute and functional iron deficiency in black pre-dialysis patients and to determine the sensitivity and specificity of various biomarkers in the diagnosis of iron deficiency anaemia.

2.7. Biochemical Markers and Haematological Indices in the Diagnosis of Iron Deficiency

Clinicians focus on the following:

a) early recognition of sub-clinical iron deficiency to prevent systemic complications;
b) recognition of iron-deficient anaemia caused by diet, malabsorption, and bleeding, as this condition responds to therapy;
c) the distinction between iron deficiency and other entities with different diagnoses (e.g. infections, inflammation, cancers);
d) distinguishing the anaemia of chronic diseases from functional IDA. Haemoglobinisation of erythrocytes is used to detect functional iron deficiency because the haemoglobin content of reticulocytes and erythrocytes provides an evaluation of bone marrow activity (53)

Biochemical markers measure the iron supply to the bone marrow and are indirect indicators of the balance between iron and erythropoiesis (71).

Biochemical markers include:

- Serum or plasma iron
- Transferrin (Tf)
- Transferrin saturation (TfS)

- Ferritin
- Serum soluble transferrin receptor (STfR)

Haematological markers

- Hypochromic red cells (% HYPO)
- Reticulocyte haemoglobin content (CHr)

2.7.1. Serum Ferritin

Ferritin is a storage protein complex usually seen in cells of the reticulo-endothelial system in the liver, spleen, and bone marrow (72). Serum ferritin correlates well with whole body iron stores (73). A level below 15-30 ng/ml is predictive of iron deficiency anaemia in non-uraemic subjects (68). In patients with ESKD, there are limitations in using serum ferritin as a marker of the body's iron storage compartment and is not a direct reflection of the iron available for erythropoiesis. Hence, it allows for the accurate estimation of functional iron requirements in Recombinant Human Erythropoietin (rHu EPO) treated patients. It also acts as an acute phase reactant in many clinical states associated with inflammation, infection, liver disease, smoking, and kidney disease. Hence, it increases independently of iron status (68). In rare conditions, hypothyroidism and vitamin C deficiency may lower the serum ferritin level independently of iron depletion; hyperthyroidism, liver disease (especially caused by the hepatitis-C virus), the hyperferritinaemia-cataract syndrome (74), alcohol consumption, and oral contraceptives, are all known to elevate serum ferritin independently of iron status (75).

Kidney disease is an inflammatory disease; hence, it will raise the level of ferritin independent of the iron status. Using a functional response, Mittal, Agarwal (76) found that serum ferritin of less

than 100 ng/ml, has low sensitivity and specificity (48 %, and 75 % respectively), and Kalantar-Zadeh, Rhee (77) that sensitivity was 41 % and specificity 100 % in haemodialysis patients with a serum ferritin level of 200 ng/ml. Currently, most centres use serum ferritin at levels of less than 100 ng/ml, as an indicator of deficient iron status in renal failure patients (78). Serum ferritin is not a reliable marker of iron stores in CKD since it is elevated by concomitant inflammation; hence the need for newer biomarkers, which will be explored in this study (79).

In a review by Guyatt, Oxman (71), the authors concluded that a serum ferritin concentration of 30 ng/ml is associated with iron deficiency with an odds ratio (OR) of 2 in the general population and of 4 in a population of patients with inflammatory disease (78). Serum ferritin and TSAT are readily available and inexpensive, and levels vary by up to 62 % in normal individuals when measured over a long period of time. They also vary technically, thus leading to significant analytical variability. As such, it was concluded that a change in the serum ferritin levels may not necessarily reflect a change in iron status Dignass, Farrag (80) and thus the need to explore other newer parameters, with less variability.

2.7.2. Serum Soluble Transferrin Receptor Assay (STfR)

The soluble transferrin receptor (STfR) is normally found on all cells that require iron. Transferrin in circulation, which carries iron, binds to the transferrin receptor and the iron is internalised into the cells. A part of the receptor is released from the cells into the circulation in a concentration proportional to the alteration of the cellular receptor expression. An increase in the production of the transferrin receptor occurs as a response to iron deficiency, which reflects an up regulatory response to the restricted availability of iron to the erythron (81). Beguin, Loo (81) found that a

small increase in STfR occurs with functional iron deficiency in ESKD patients. The ratio of the transferrin receptor and the log-ferritin (TFR-F) index is useful in identifying patients with depleted iron stores. Ferguson, Skikne (82) found that STfR could be used to diagnose IDA but would appear normal in acute infections and inflammation (similar to serum ferritin) (83, 84). However, as stated by Leggett, Brown (75), in some studies, the estimation of the amount of STfR did not prove to be superior to that of serum ferritin in the detection of iron deficiency (67). A recent systematic review concluded that the use of this test improves the diagnosis of iron deficiency, especially in the presence of chronic diseases or gastrointestinal malignancy (76). A study of 145 patients with IDA or anaemia of chronic disease showed that the STfR index is superior to the STfR alone in differentiating between the anaemia of chronic disease (ACD) and IDA (85). As described by Fernández-Rodríguez, Guindeo-Casasús (86) in patients with stable kidney disease, who were not on dialysis and not receiving iron or ESAs, the STfR concentration alone proved to be inferior to serum ferritin levels in identifying those with co-existing IDA. However, with stable erythropoiesis, this test proved to be superior to the zinc protoporphyrin (ZPP) and serum ferritin tests, but inferior to the %HYPO and CHr tests in differentiating between iron-deficient and iron-sufficient individuals (87). STfR has been successfully measured through various methods using immunoassays (85, 88).

A major challenge in using STfR is the lack of proper standardisation for the assay. There are several commercial assays available but no common reference materials. This test has proved to be very useful in differentiating between IDA and anaemia of chronic diseases (89). The soluble transferrin receptor is the best available circulating indicator of iron deficiency in diseases with an inflammatory component such as CKD. This hypothesis will be further confirmed through this

study. Although further studies might be needed to shed more light on the diagnostic utility value and performance of STfR, the evidence currently available suggests that this marker is best used in combination with other iron indices (90). Our research further explores the clinical utility of the combination of markers in the diagnosis of IDA.

2.7.3. Serum Iron, TIBC, and % Saturation (%sat)

The saturation (%sat) value is derived from serum iron and TIBC and is the one most frequently used in clinical practice. The serum iron level falls within hours of the onset of inflammation, and the TIBC level also falls, but to a lesser degree, thus leading to a reduced %sat. It remains low throughout the illness. The %sat is a measure of iron in transport, but not of iron in storage, and in functional iron deficiency, iron in storage is needed. Thus, this measure may not be ideal in situations where the measurement of iron is required in terms of its storage mass. Furthermore, a %sat level of less than 20 % indicates the need for parenteral iron in the setting of anaemia treated with ESA, as described by Roger (91) the %sat level had a low sensitivity and specificity in those participants who responded to intravenous iron. Markedly lower values for serum iron, TIBC, and transferrin (Tf) saturation are generally observed in normal males with the CD phenotype (92). Single nucleotide polymorphisms (SNPs) in the Tf gene and the C282Y mutation of HFE explain approximately 40 % of the genetic variability for this parameter (93, 94).

2.7.4. Reticulocyte haemoglobin content (CHr)

CHr is defined by the formula CHr= (**CHr** = **MCVr X CHCMr**) (95), where MCVr is the mean reticulocyte cell volume and CHCMr is the mean haemoglobin concentration of the reticulocyte, which is obtained by measuring the cell haemoglobin (82). CHr levels have been measured

recently in numerous studies as a test for iron deficiency and functional iron deficiency. CHr has been found to be highly specific and sensitive and its exact threshold values are 28 to 30 pg. This measure is a direct assessment of the incorporation of iron into erythrocyte haemoglobin and thus an estimate of the recent functional availability of iron to the erythrocyte (96). In a study by Thomas and Thomas (96), CHr has been shown to be a sensitive and specific indicator of functional iron deficiency in healthy individuals and in patients with ESKD who are being treated with ESA. After administering EPO to healthy individuals, it was found that a reduction in CHr was inversely correlated to baseline serum ferritin levels. This experiment also showed that CHr is a useful indicator of iron-deficient erythropoiesis. CHr proved to be a more stable indicator of functional iron deficiency than either serum ferritin or STfR (97, 98).

In a study by Vidyashankar P (99), newer parameters such as CHr were evaluated in the diagnosis of iron deficiency in non-dialysis CKD patients, and the validity of these tests was established in the presence and absence of inflammation. These tests concluded that the newer parameters have variable sensitivity and specificity when applied only in the case of CKD patients, while their use in combination with iron parameters such as serum ferritin, and TSAT was found to increase specificity and sensitivity in the presence of inflammation.

In a study by Fishbane, Galgano (53), the CHr levels were found to be highly accurate for the diagnosis of iron deficiency in haemodialysis patients, with a value of less than 26 pg indicating iron deficiency with a sensitivity of 100 % and a specificity of 50 %. Furthermore, reticulocyte correction occurred very rapidly after intravenous iron was administered. In fact, within days of receiving iron, the reticulocyte haemoglobin level improved.

Different studies have detected the diagnostic accuracy of using CHr at different cut-off values in the diagnosis of IDA. A study by Lorenz, Arand (100) reported higher diagnostic accuracy in detecting IDA than serum ferritin and TSAT. In a study by Swartz et al (48), CHr at a cut-off value of 29 pg was found to be an accurate marker for the diagnosis of IDA with a sensitivity of 86 % (101); a lower cut-off value of 28 pg had a sensitivity level of 76%. Ullrich, Wu (102) used a cut-off value of 27.5 pg with a sensitivity of 83 %. The performance of CHr in the diagnosis of IDA in black South Africans is not well established and will therefore be evaluated in this study.

2.7.5. Percentage Hypochromic Red Cells (%HYPO)

Hypochromic red cells are those with haemoglobin levels of less than 280 g/l. The utility of this test is variable in detecting functional iron deficiency in patients with CKD treated with ESA. A variety of studies have shown that an increased % HYPO can be a sensitive and early indicator of iron deficiency. Macdougall, Cavill (47) reported that a value of greater than six percent (6 %) is superior to measurements of other traditional parameters such as serum ferritin and TSAT.

Two other methods are available for the assessment of hypochromia. One is where the percentage of hypochromic red cells is measured by means of certain Siemens blood auto analysers (XE 5000 and XN 200, Washington, USA). As described by Urrechaga (103) it defines those red cells with mean corpuscular haemoglobin (MCH) levels of less than 17 g and has proved its utility in the differential diagnosis of anaemia. The second method, which involves the measurement of a low haemoglobin density (LHD) level, is based on a mathematical transformation of the mean corpuscular haemoglobin concentration (MCHC) value and is available on some Beckman

instruments. The values in this case correlate strongly with those of the hypochromic red cells (103).

Schaefer and Schaefer (104) found that 22 % of the RBCs among ESKD patients with iron deficiency are hypochromic and that on being treated with intravenous iron, the percentage of hypochromic red cells will decrease significantly. Macdougall, Cavill (47) found that a hypochromic red cell level of more than 10 % is the predicted response to an intravenous injection of iron into dialysis patients. Braun, Lindner (105) treated 70 haemodialysis patients with 500 mg of intravenous ferrous gluconate and studied the erythropoietic response. These authors also found that %HYPO was a good indicator for predicting a reduction in the EPO dosage after iron treatment. However, they also found that the %HYPO was low, despite the presence of serum ferritin at a level of less than 100 ng/ml. A European study of dialysis patients found the %HYPO to be the sole predictor of mortality, among various iron status parameters, that the risk of mortality increased two-fold for values greater than 10%, as opposed to values of less than 5 % and that patients with high hypochromatic red cell counts were 6 % more likely to respond to intravenous iron therapy (106, 107). North American studies have not been able to demonstrate the value of %HYPO in assessing iron availability in dialysis patients, (53, 108). The reason for this difference is not yet fully understood. The general limitations around the parameters assessing cellular hypochromia are their sensitivity to temperature and the storage of the samples. These markers may be falsely elevated because of a simultaneous increase in MCV and a reduction in MCHC associated with storage at temperatures greater than or equal to room temperature. All hypochromic markers will increase with reticulocytosis, because reticulocytes have lower MCHCs than mature RBCs (109).

The reticulocyte haemoglobin content (CHr) and the proportion of hypochromic red cells (% HYPO) have been used together with biochemical markers as diagnostic tools to distinguish IDA from ACD. They have now been incorporated into the guidelines for the monitoring of human erythropoietin therapy (110-112). These indices have also been found to be reliable markers of iron deficiency under physiological conditions, where the demand for iron increases (e.g. in children and in women of reproductive age) (113). However, their diagnostic utility in patients with CKD has been explored to a lesser extent, and as such will be evaluated in this study.

There is insufficient evidence to determine the performance of combinations of the newer biomarkers, or of combinations of the newer and classical biomarkers, in diagnosing IDA. However, it may be that the CHr and the % HYPO have better predictive ability. As such, this combination will be explored in this study (Figure 2.4).

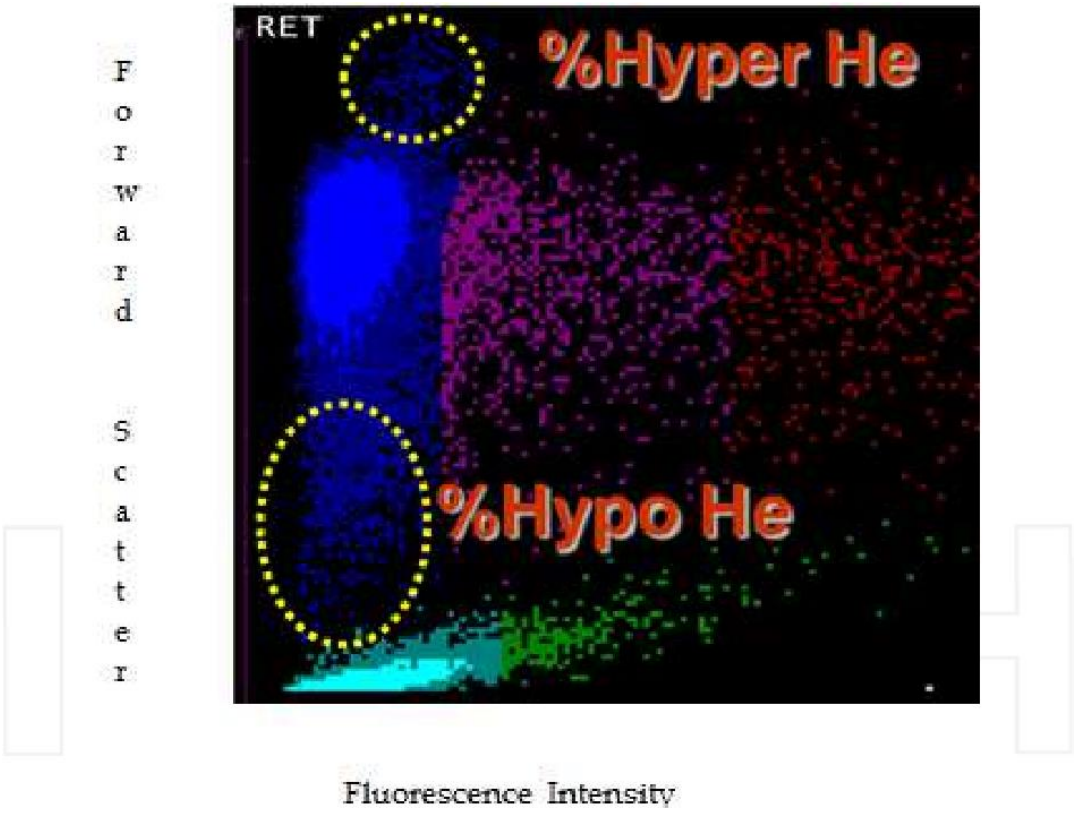


Figure 2:4: Bi-dimensional distribution of forward scattered light and florescence (114).

Bi-dimensional distribution of forward scattered light and florescence is presented as a scattergram, indicating mature red cells and reticulocytes. Forward scatter correlates with the haemoglobin content. *(Reproduce with permission from creative commons attribution 3.0 license, which permits unrestricted use in production in any medium provided is cited)*

2.7.6. Zinc Protoporphyrin (ZPP)

Zinc protoporphyrin (ZPP) is a trace by-product of haemoglobin synthesis and any condition that limits iron supply to the erythroid marrow or stimulates porphyrin synthesis leads to an increased concentration of ZPP in the circulating red cells. Incorporation of iron into Protoporphyrin 1X is the final stage of haemoglobin synthesis, and an increase in ZPP is an indicator of a defect in the pathway. Elinor A. Graham (115) reported that ZPP is non-specific, with levels increasing in cases of iron deficiency, functional iron deficiency, and in lead poisoning.

This measurement has two limitations owing to the methods used in its measurement. The plasma constituents contribute to the magnitude of the ZPP values. Hence ZPP levels are raised by hyperbilirubinaemia (116) and in CKD (15, 117). Secondly, an increase is generally observed as the Hb falls below the 100 g/l level, individuals with anaemia have reduced levels of haemoglobin irrespective of their iron status (15). The ZPP reflects the entire red cell population and is less sensitive than the %HRC or the CHr to changes in the availability of iron.

Mwangi, Maskey (118) studied the utility value of ZPP as a diagnostic tool to detect iron deficiency in Kenyan pregnant women and found that ZPP is limited in ruling out iron deficiency when used for screening in conditions of low iron prevalence. Thus, it was concluded that ZPP is unreliable as a marker in discriminating between pregnant women with and without iron deficiency. In another study by WongA, Qutishat (119) among hospitalized patients, the predictive value of ZPP in the diagnosis of iron deficiency at a cut-off value of 170 mol/mol had a sensitivity of 93 % and a specificity of 90 %. These values are not in agreement with the findings of Thomas and Thomas (96). Zinc protoporphyrin is elevated in cases of iron deficiency and it should be noted that such elevated levels may be present in the anaemia of chronic disease, lead toxicity, and myelodysplasia (120, 121). These observations should be interpreted with caution, especially in patients with CKD.

2.7.7. Growth Differentiation Factor 15 (GDF-15)

GDF-15 is a member of the transforming growth factor superfamily, also known as macrophage inhibitory cytokine-1. This protein is expressed in almost all tissues, which suggests its importance

in general and basic cellular functions. Its exact biological function is still under investigation, but it has clearly been shown to be involved in regulating inflammatory and apoptotic pathways (122). GDF-15 has been shown to be a strong and independent predictor of disease progression and mortality in patients with kidney disease, heart disease, stroke and other conditions. Zhang, Moszczynski (123) confirmed its role in the development and progression of CKD. In a cohort community of elderly patients, it was shown that GDF-15 concentrations increased by 11 % over a five-year period (124).

Recently, GDF-15 has been identified as one of the regulators of iron status. Furthermore, as described by Lukaszyk, Lukaszyk (125), oxidative stress and inflammation are factors that increase the expression of GDF-15. These authors in 2016 illustrated that GDF-15 was higher in patients with anaemia although the sample size was small, thus limiting statistical analysis (125). Therefore, more studies are needed to establish whether GDF-15 is a clinically useful biomarker and whether it can be used to identify the group of patients who will benefit from various interventions or treatment. Several studies have found an association between elevated levels of GDF-15 and cardiovascular injury (124, 126, 127). It is possible that GDF-15 could signal intra-renal injury, either from a systemic process or from intra-renal patho-physiological processes. Significant associations between higher levels of GDF-15 and the development of CKD, a rapid decline in kidney function, and albuminuria, were reported among participants in the Framingham study (128). GDF-15 was recently identified as a strong prognostic marker in patients with cardiovascular disease, including coronary artery disease, acute coronary syndromes (127, 129) and heart failure (130). The expression of GDF-15 in conjunction with other inflammatory markers

is up regulated by oxidative stress, tissue ischaemia, and cancer in organ tissues, including the heart and kidneys (131, 132).

There has been increasing interest in the relationship between GDF-15 and kidney disease in diseases such as diabetes and IgA nephropathy (133, 134). Lajer, Jorsal (135) reported that elevated GDF-15 levels are significantly associated with a worse renal outcome in insulin-dependent diabetic patients with microalbuminuria, the hypothesis being that elevations in the GDF-15 levels may aggravate outcomes in patients with kidney disease. This biomarker will be investigated in this study.

2.7.8. Hepcidin

Hepcidin is a new iron marker which was discovered in 2001. It was noticed that pro-hepcidin has no impact on iron metabolism in healthy individuals or in patients with CKD (136). Hepcidin is a critical inhibitor of iron export from macrophages, enterocytes, and hepatocytes. It is filtered and degraded by the kidneys. Elevated levels of hepcidin in renal failure have been suggested to play a role in the disordered iron metabolism of uraemia (137). Production by hepatocytes is modulated in response to anaemia, hypoxia, or inflammation figure 2.5. Iron is essential for life, and excesses of iron can cause organ and tissue damage. To prevent iron overload, the iron balance is regulated by hepcidin. Iron deficiency lowers hepcidin, leading to enhanced iron absorption and the mobilisation of iron from stores. The hepcidin levels also increase with inflammation and play a major role in the anaemia of chronic diseases, which are associated with increased hepcidin levels. This factor is likely to contribute to the incidence and severity of anaemia, and the ESA.

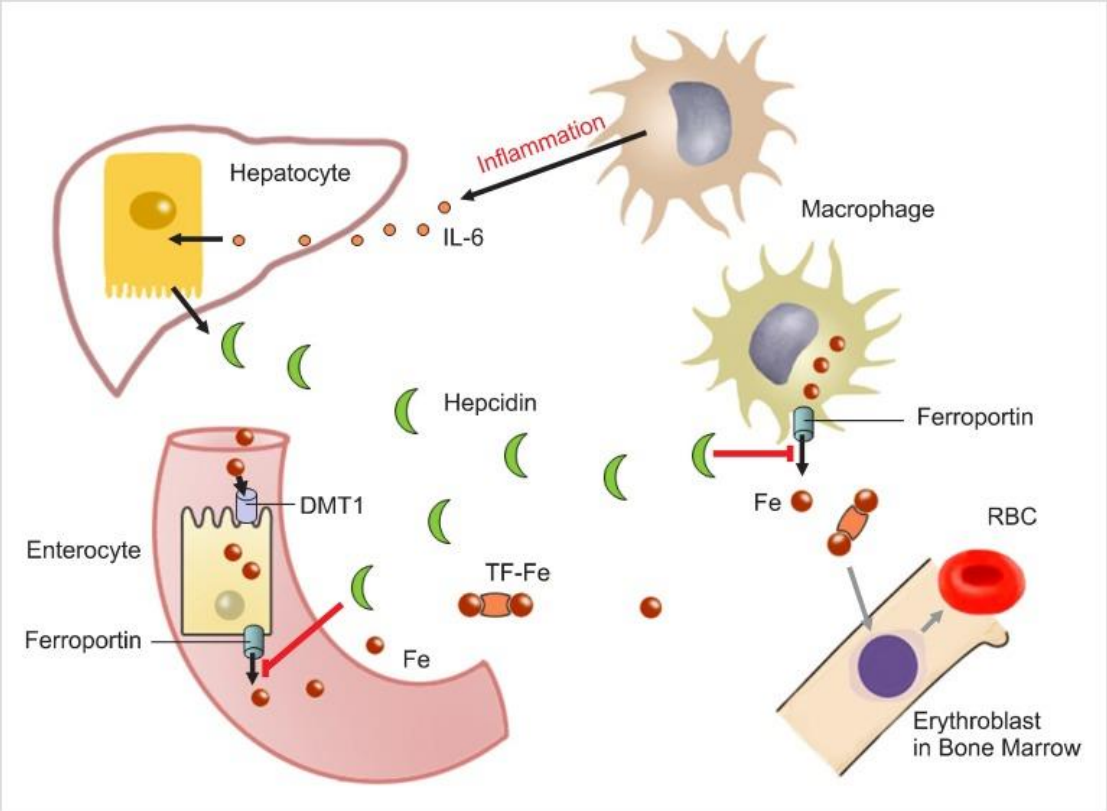


Figure 2:5: Iron sufficiency and inflammation enhance hepcidin production in the liver

Inflammation increases the production of interleukin-6. The consequent increase in hepcidin blocks the release of macrophage iron, as well as the intestinal absorption of iron, resulting in hypoferremia. [Abbreviations: TF: transferrin; Fe: iron; DMT1: divalent metal transporter (138)] ***(****With permission to reproduce from Kidney International publisher, Elsevier, appendix E)*

Hepcidin could also act as an indicator of FID in CKD patients (137), hence the need to explore the mechanism by which hepcidin is able to indicate functional iron deficiency. The production of hepcidin in patients with CKD depends on iron status, anaemia, inflammation, hypoxia, and erythropoietin (139). Elevated levels of hepcidin in patients with CKD and who are on dialysis may be related to the occurrence of high triglycerides and high IL-6 serum concentrations (140). This probably suggests that hepcidin may play a role in the progression of atherosclerosis and inflammation. However, this hypothesis should be further evaluated.

Previous studies have attempted to measure the serum levels of pro-hepcidin, the peptide precursor of hepcidin, but these studies are difficult to interpret because the relationship between pro-hepcidin, hepcidin, and the iron parameters remains unclear (139). In the current study, we evaluated the relationship between these iron parameters and the underlying genetics of IDA. Mass spectrometry is capable of reliably measuring the serum hepcidin levels. There is a positive correlation between the hepcidin and ferritin levels in CKD. However, it should be noted that mass spectrometry has its limitations as it is semi-quantitative by nature and the equipment is not widely available (141).

Hepcidin, an acute phase reactant, is currently thought to be the major regulator of iron homeostasis, and according to certain studies such as that by Goyal, Mohanty (142), hepcidin was found to have the potential to provide information beyond the iron profile. These authors found that hepcidin levels are higher in CKD patients when they are compared to their age- and gender-matched healthy controls. Furthermore, hepcidin levels have also been found to increase through stages 3 to 5 of CKD (40).

There is a positive correlation between the hepcidin and TSAT levels. This relationship has been reported in previous studies (143, 144). Other studies have found median hepcidin levels to be significantly higher in patients with normal iron status, followed by a relative deficiency of iron as opposed to an absolute deficiency of iron (142, 144), thus supporting the hypothesis that hepcidin levels increase in patients with the anaemia of CKD, with normal iron status and with functional iron deficiency. Hepcidin levels were down regulated in patients with absolute iron deficiency. Furthermore, the positive correlation between the hepcidin and ferritin levels raises controversy as to the performance of hepcidin and ferritin, in the diagnosis of iron disturbances in

haemodialysis patients (136). Though many studies have been carried out in haemodialysis patients, this suggests that hepcidin and ferritin are no better than traditional iron markers (145).

A study carried out by Uehata, Tomosugi (143) among non-dialysis CKD patients demonstrated that hepcidin levels are negatively associated with haemoglobin concentrations in non- dialysis CKD patients with sufficient iron stores. The authors concluded that ferritin modifies the association between hepcidin and haemoglobin (143). However, their finding of elevated hepcidin levels is in keeping with the findings of other researchers (136, 139). Other studies have shown that erythropoietic activity is required in order to suppress hepcidin production (146). The association between hepcidin levels and the glomerular filtration rate (GFR) remains to be determined. Hence there is a need to further explore the relationship between hepcidin levels and the GFR and iron status in non-dialysis CKD patients.

Hepcidin assays have been used in the diagnosis of both iron deficiency and overload, which are key regulators in iron homeostasis (147). Hepcidin can be measured by means of either mass spectrometry or immunoassays (142). There has been progress in recent years towards the standardisation of such investigations (148), but still there is no common reference range. A reference range cut-off for our environment is was evaluated in this study.

Using mass spectrometry, lower serum hepcidin levels were reported in blood donors who donated 13 units of blood over a two-year time period (149). At present, no approved hepcidin assay is readily available for clinical use (79). There is still a paucity of knowledge on how the determination of serum hepcidin levels would fit into diagnostic algorithms for iron states outside iron refractory iron deficiency anaemia (IRIDA) (150).

Serum hepcidin levels may open a new door in predicting therapeutic responses. Concerns have been raised in developing countries about implementing widespread iron supplementation since it can aggravate the incidence and severity of malaria (135). Hepcidin has been shown to be the most consistent predictor of erythrocyte iron incorporation (151). Data indicate that the level of serum hepcidin in a patient might also reflect his/her responsiveness to oral iron therapy, with higher hepcidin values conveying poor or no response to oral iron supplements (152). More research is required to ascertain the role of hepcidin in CKD patients.

2.8. Management of Iron Deficiency Anaemia and Functional Iron Deficiency

2.8.1. Treatment with Iron Supplements in Patients with CKD who are not on Dialysis (CKD stages 1-4)

The role of iron supplementation in patients with CKD who are not on dialysis therapy is still not well established (93). Zhou, Laszik (153), in separate randomised controlled trials, compared oral iron with intravenous iron. The results were inconclusive as to whether any iron treatment, regardless of the route of administration, would be necessary. In the setting of absolute iron deficiency, oral iron treatment should be initiated in tandem with an evaluation for unknown blood loss. In patients with functional iron deficiency, supplementation should be guided by treat-to-goal iron targets to support ESA therapy in patients with CKD who are not yet on dialysis (154). An ideal study would be a double-blind randomised controlled trial to compare oral or intravenous treatment with a placebo, in order to ascertain the efficacy, safety and tolerability of such a treatment. However, there is a paucity of such studies.

2.8.2. Iron replacement therapy in CKD

The treatment of iron deficiency with oral or intravenous supplementation can reduce the severity of anaemia in patients with CKD (34). Iron supplementation is being used widely in patients with

CKD to treat iron deficiency, to prevent its development in ESA-treated patients, to raise the haemoglobin level in the presence or absence of ESA treatment, and to reduce ESA doses in patients receiving ESA treatment (155). There are limited data on the long-term clinical benefits of iron supplementation other than the direct effect on haemoglobin concentration, and there is little information on the long-term adverse effects of iron supplementation in excess of that necessary to provide adequate bone marrow stores (1, 156). Since bone marrow aspiration studies are rarely done in clinical practice, iron supplementation is assessed by blood–based iron indices, which have their shortcomings (157, 158).

Several randomised control trials of small sample size in patients with ESKD who were on dialysis examined the effects of intravenous versus oral iron administration or of classical versus novel intravenous iron formulation on the correction of anaemia (159, 160). The conclusion reached was that intravenous iron is more effective than oral iron for replenishing iron stores, improving anaemia, and reducing ESA dosage requirements. There is still a paucity of data regarding randomised controlled trials for optimal iron management in the treatment of anaemia in pre-dialysis CKD.

The recent study by Macdougall, Bock (161), the FIND- CKD study, a randomised controlled trial (RCT), involved 626 patients with CKD stages 3-5 and IDA. They were not receiving ESAs for the three treatment arms, namely intravenous ferric carboxymaltosr (FCM) targeting a high ferritin range (400-600 g/l, intravenous FCM targeting a lower ferritin range (100-200 ng/l), or oral iron (200 mg ferrous sulphate daily). Furthermore, the primary endpoint indicated the time at which to initiate other anaemia management practices (e.g. ESA, other iron therapy methods, or blood transfusion) or haemoglobin triggers of two consecutive values (<10 g/dl) over the period eight to 52 weeks. The secondary end points were the necessity for blood transfusion and an increase in

haemoglobin to the order of at least 10 g/dl (160). The primary end point occurred in 36 patients (23.5 %), 49 patients (32.2 %), and 98 patients (31.8 %) in the high-ferritin-target, low-ferritin-target, and oral-iron group respectively, with a significant difference only between the high-ferritin-target and the oral-iron groups (160, 161). Although the authors found no renal toxicity, this trial was not able to assess the safety end points, which was a limitation.

2.8.3. Intravenous Iron in CKD

Parenteral iron was first introduced in the early 20th century - in 1932 (162). Ferric hydroxide solution was injected subcutaneously and intramuscularly into anaemic patients and, intravenous iron was introduced later (163). In patients with non-dialysis-dependent CKD (CKD-ND), this experiment suggested that the selection of the iron administration route (oral versus intravenous,) should be made on the basis of the severity of the anaemia, the availability of venous access, the response or lack of response to prior oral iron therapy, the previously observed adverse effects, the patient's adherence to the treatment and the associated costs (163). There are several intravenous iron preparations that are currently in use in CKD patients (164, 165).

Some pre-dialysis patients, who do not respond to oral iron therapy, may respond to intravenous iron. A prospective study by Silverberg, Wexler (166) investigated the effects of intravenous iron sucrose (200 ng of iron per month for five months) in pre-dialysis patients with anaemia who had proven non-responsiveness to oral iron and found that none of the patients was receiving ESA. The mean haemoglobin level increased by 1.2 g/dl versus the baseline ($p= 0.08$), with 22/23 patients showing responses that ranged from 0.4 to 3.6 g/dl. Similar results were also observed by Bhowmik, Modi (167), in a small study of 11 patients, where the mean haemoglobin level of 5.6 g/dl increased to 8.0 g/dl in one month after a single dose of intravenous iron dextran. This was achieved in spite of the small sample size and the fact that the patients had to rely on oral iron. A

study in India also based on the intravenous administration of iron dextran to 56 pre-dialysis CKD patients, led to an increase in haemoglobin levels from 8.28+ 0.57 at baseline to 9.12+ 0.44 g/dl in 12 weeks (168).

In a prospective single-arm study, intravenous iron sucrose administered at a rate of 200 mg monthly over a period of twelve months showed a significant increase in haemoglobin levels from 9.7 + 1.1 at baseline to 11.3 + 2.5 g/dl after 12 months, and a mean increase in haemoglobin of 1.6 g/dl (169). Intravenous iron therapy in pre-dialysis patients without EPO seems often to ameliorate the anaemia, thus avoiding the necessity for EPO or blood transfusions in one third of the pre-dialysis, non- diabetic patients (170).

Clinical trials in which different intravenous iron formulations were compared directly for safety and efficacy mostly involved a small number of patients and short follow-up periods. A randomised study of CKD patients who were not on dialysis showed a higher risk of adverse drug reactions associated with low molecular weight iron dextran as opposed to iron sucrose and sodium ferric gluconate (171). On the other hand, randomised trials comparing iron isomaltoside (172) and feumoxytol levels (161) to iron sucrose levels showed equivalent efficacies with respect to increases in haemoglobin, and no significant differences in the adverse effect profiles.

There is still a need for more randomised control trials to improve guidelines for iron management in CKD patients who are not on dialysis. Although the two recent trials (FIND-CKD and REVOKE) (161, 173) have improved the quality of evidence, their results have proved to be conflicting. The KDIGO Guideline in 2012 (174), which was based on observational data, also showed conflicting results. As such, there is a need for more observational and randomised control trials, with sufficient duration of follow up. This need has been partly fulfilled by the Proactive IV

Iron Therapy in haemodialysis patients (PIVOTAL) trial (175), which was designed to provide insight on how these iron therapy approaches affect ESA dose requirements, red blood cell transfusions, complications of haemodialysis treatment, quality of life, and relevant laboratory biomarkers. The results of this trial are awaited and will provide information relevant to dialysis physicians and dialysis providers worldwide, for the benefit of these patients.

2.9. Iron deficiency anaemia and its genetics

The acquired defects resulting from an iron deficiency are those emanating from either abnormal iron absorption from the diet, gastrointestinal tract or blood loss. Genetic causes of IDA in humans are mostly due to a defect in the divalent metal-iron transporter (DMT1) and in the recently identified trans-membrane protease serine 6 (TMPRSS6), a gene that controls hepcidin expression (61).

Several genes regulate iron metabolism, and a key role is played by hepcidin, a peptide that controls intestinal iron absorption and macrophage iron release. So far, the only mutation in TMPRSS6, which encodes a negative regulator of hepcidin expression, has been associated with chronic IDA. Genome-wide Association Studies including a recent meta-analysis, indicate that TMPRSS6 genetic variants are associated with red cell and iron traits in Caucasian and Asian populations (176).

Hepcidin levels are usually low, undetectable, or both in the urine and serum in the presence of iron deficiency. They are usually due to the suppressive effect of iron absorption and its release from macrophages (177, 178). In contrast, in the case of IDA, and owing to a defect in TMPRSS6, the urinary and serum hepcidin levels are inappropriately high for the degree of anaemia.

Du, She (16) reported the discovery of a protein with non-redundant function in HAMP (hepcidin antimicrobial peptide) suppression, which permits the normal uptake of enteric iron in response to iron depletion and is capable of converting all known hamp activity stimuli to stimulate 57BL/65 background. Low iron levels were observed which, coupled with high hamp stimulated transcript levels in masked mutants, suggest that TMPRSS6 might negatively regulate hamp activation to promote iron uptake (16).

In a study by Melis, Cau (61), TMPRSS6 mutations were found to lead to the overproduction of hepcidin and in turn to defective iron absorption and utilisation. The authors later confirmed that in humans there is an inhibitory effect of TMPRSS6 on the synthesis of hepcidin (61). As the research was demonstrated on mice, the role of TMPRSS6 in pre-dialysis CKD patients could not be determined (179). In another study by Beutler, Van Geet (180), although they did not find any of the common polymorphisms of TMPRSS6 to be risk factors for iron deficiency, the authors identified three exons in the anaemic population. Because these studies were limited to the white population only, they cannot be considered representative of the black population, which is the sector under consideration in this study.

In a study by Danquah, Gahutu (181), the TMPRSS6 (736) (V) allele which was previously reported to be associated with iron deficiency and anaemia, was found to be less common in Rwanda than in non-African populations and did not fully explain the low haemoglobin levels of the patients. Though their sample size should have been larger, and they used ferritin, which has limited variability as an acute phase reactant, and as a marker of iron status, there may have been abundant infections and inflammatory processes among the children in the study, which should therefore not be ruled out.

Various studies have observed a geographical variation in iron status, which has led to the hypothesis that differences in genetic adaptations across different ethnicities may contribute to differences in iron status. Studies on the genetics of iron status in African populations are important for the ultimate goal of having to characterise risk variants beyond what can be determined from European populations alone. This information is useful in informing strategies to address iron deficiency. Understanding the genetics of low iron status is important in populations of African ancestry, where there are disproportionately higher burdens of iron deficiency (182, 183).

Genome-wide association (GWA) studies have identified common variants that are associated with iron status in populations of largely European ancestry (179, 184, 185). Whether these findings can be generalised to apply to African ancestry populations is not known. We hypothesise that single nucleotide polymorphisms (SNPs) tag larger regions of the genome in Caucasians than they would normally do among individuals of African ancestry. Therefore, index SNPs in European populations may not necessarily tag causal variants within the African population.

In recent decades, GWA studies have identified several single nucleotide polymorphisms (SNPs) that influence red blood cell phenotypes (184, 186, 187). Among the SNPs, rs855791 has the strongest association with red blood cell indices and iron parameters in the general population (94, 184), but the role of rs855791 and its association with iron parameters in CKD patients have not been studied. Its role is evaluated in this present study. TMPRSS6 is located in the functional part of TMPRSS6 and the rs855791 (2321 C>T) causes a non-synonymous substitution that reduces the ability of the enzyme to inhibit hepcidin transcription (188). It is our objective in this study to investigate the role of the genetic variant rs855791 on the susceptibility of pre-dialysis CKD patients to IDA.

2.10. Ethnic Variations in Haematological Markers of Iron Deficiency Anaemia

Anaemia is a common haematological condition among patients with CKD, with prevalence increasing as a function of age after the fifth decade of life (189). Several studies have reported the differential distribution of anaemia by age and sex (190). Less attention has been devoted to disparities in anaemia by race. A study in the United States by Patel, Harris (189) showed that anaemia, and severe anaemias, are serious health concerns for specific subgroups such as blacks, Hispanics, older adults, non-pregnant women of reproductive age, and pregnant women. The finding that anaemia is more common among blacks is consistent with other reports (191, 192). Among individuals in the Duke Established Population for Epidemiologic Studies of the Elderly cohort, blacks had a three-fold higher prevalence of anaemia than whites after adjustments were made for age, educational level, body mass index, and a composite health condition score (193). It should, however, be noted that this study did not cover racial differences and individual associations of risk factors with anaemia. There is a need for studies to identify the magnitude and the implications of apparent racial differences in haemoglobin levels.

2.11. Justification for Study

Anaemia is a common complication of CKD and contributes to adverse outcomes. Early identification and treatment improve quality of life and reduce cardiovascular morbidity and mortality. In sub-Saharan Africa, there is a paucity of information on the prevalence and genetics of IDA (absolute and functional) in CKD patients.

Bone marrow biopsy is the gold standard for diagnosing IDA in CKD patients. However, it is not routinely done in clinical practice owing to its invasive and cumbersome nature. Therefore, there is a need to use other non-invasive techniques in diagnosing IDA. Various iron parameters such as serum ferritin, transferrin saturation, and soluble transferrin receptors have been used to

diagnose iron deficiency but have their shortcomings. Thus, this study has been carried out to explore other parameters, including the percentage of hypochromic red cells and the reticulocyte haemoglobin content, hepcidin, and GDF-15 and to compare their performance with more routinely-used conventional parameters and possibly determine the best non-invasive parameters in the diagnosis of iron deficiency anaemia in non-dialysis CKD patients.

There has been no study that has investigated the susceptibility gene, and the prevalence of the single nucleotide polymorphism in a black population with CKD, and the possibility that certain variants of the TMPRSS6 serine gene increase the susceptibility to develop iron deficiency anaemia in patients with CKD. Additionally, the role of the biomarker GDF-15 in the diagnosis of IDA is unknown among non-dialysis CKD patients. Identifying non-invasive parameters in the diagnosis of iron deficiency and genetically susceptible individuals, as well as the genes involved, will add knowledge to the nephrology speciality and may aid in reducing morbidity and mortality.

It is for these reasons that the study was undertaken to determine the prevalence of IDA, to explore the performance of various non-invasive iron parameters and the genetics associated with IDA in a black CKD population, not on dialysis.

2.12. Research hypotheses

The research hypotheses for this study were as follows:

- Anaemia is a common complication of CKD and its aetiology is multifactorial; a substantial proportion of non-dialysis CKD patients may have IDA across all stages of CKD.

- Novel parameters such as reticulocyte haemoglobin content, the percentage of hypochromic red cells and GDF-15 may perform better than the older conventional parameters in the diagnosis of IDA in non-dialysis CKD patients.
- Novel biomarkers may predict the progression of the disease and mortality in pre-dialysis CKD patients.
- Single nucleotide polymorphisms (SNPs) of the TMPRSS6 serine gene and its variant (rs855791) may be positively associated with an increased susceptibility to the development of IDA.

2.13. Aims

The aim of this study was to evaluate the haematological, biochemical and genetic markers of IDA in black South Africans with CKD.

2.14. Specific objectives

- To determine the prevalence of iron deficiency (both absolute and functional) anaemia in patients with CKD stages 1 to 5.
- To compare the performance of the percentage of hypochromic red cells and the reticulocyte haemoglobin content with conventional parameters to determine iron status.
- To ascertain the association of hepcidin and GDF-15 in patients with IDA.
- To determine the association of novel biomarkers with CKD progression and mortality.
- To determine the prevalence of SNPs of the TMPRSS6 gene and its association with IDA.

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3. CHAPTER THREE: MATERIALS AND METHODS

3.1. Study design and population

This thesis entails cross-sectional studies (Studies 1 to 4), diagnostic test validity studies (studies 2 and 4) and a cohort study (Study five) which were conducted from May 2016 to May 2018. Consenting consecutive chronic kidney disease (CKD) patients attending the Renal Outpatient Clinic of the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), Johannesburg, South Africa, and a comparative group of age- and sex matched apparently healthy controls were recruited after obtaining ethical clearance from the Human Research Ethics Committee (HREC) of the University of the Witwatersrand (Appendix number A) and informed written consent. (Appendix number B). The CKD participants were followed up for a period of 24 months (two years).

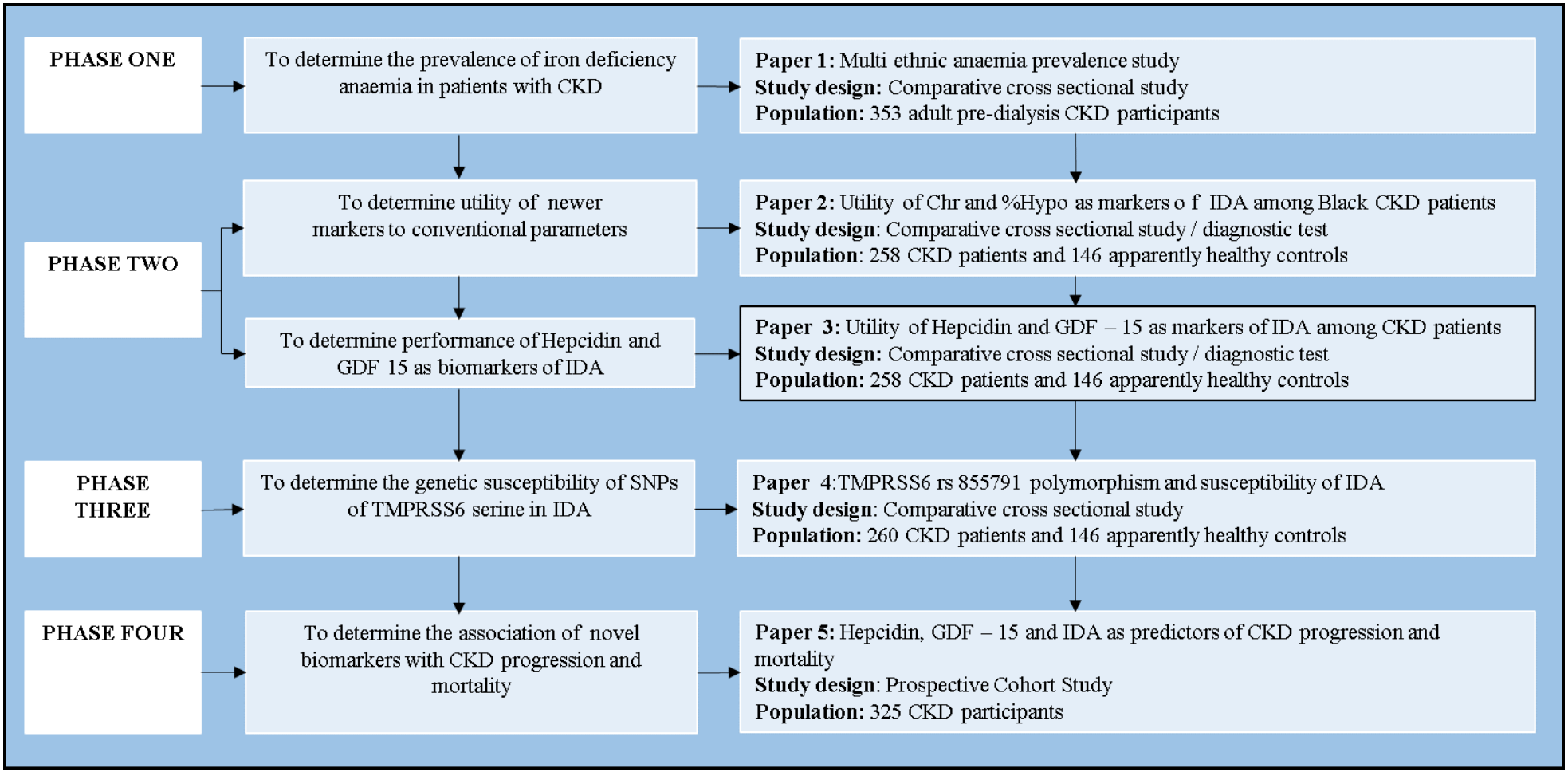

Figure 3:1: Algorithm of various studies conducted

3.2. Sample size determination

For studies 1 and 4 (cross-sectional study):

An estimation of the sample size was done using the formula: $N = Z^2PQ/D^2$

Z =1.96 (i.e. the normal standard deviation at a 95% confidence interval) P = Prevalence of iron deficiency

Q = 1-P

D = 0.05 precision

The prevalence (P) of iron deficiency anaemia was 64.7% (1). Accordingly, prevalence of 64.7% was used.

N= 〖1.96〗^2 x 0.647 x (1-0.647)/ 〖0.05〗^2= 350.96 ~ 351

Hence, the estimated sample size was 351 participants. However, 353 participants were recruited for Studies 1, 2 and 5.

For studies 3 and 4 (cross sectional study and diagnostic test), the sample size was estimated using the diagnostic formula (2): P (1-p)/α2

Where

Z = 1.96 (a constant)

p = assumed sensitivity.

For a good clinical diagnostic value, we assumed a sensitivity level of 80% and α = margin of error = 0.05 for a 95% confidence interval.

Therefore N = (1.96)2 X 0.8 X (1- 0.8) / $(0.05)^2$ = 245.9

Hence, a minimum sample size of 246 was adequate for the diagnostic tests. However, we recruited 258 participants for studies 3 and 4.

The matched healthy control group for the genetics study consisted of individuals without CKD and without any condition as listed in the exclusion criteria. These participants included CMJAH staff members, students and attendees at the Renal Donor Clinic prior to donation.

The ratio of the study population to controls was set at 3:1; thus, the number of controls was 120.

The control group was matched with the study population according to age, gender and ethnicity.

3.3. Eligibility criteria

3.3.1. Inclusion Criteria

Stage two to five CKD patients who attended the renal clinic, who had not been on iron medication two weeks prior to enrolment, who had not received a blood transfusion three months prior to enrolment, who were no older than 18 years, and who agreed to participate in the study were recruited.

3.3.2. Exclusion Criteria

Patients with the following conditions were excluded: HIV positive patients, those with known haemoglobinopathies, active infections, active bleeding, inflammation, active malignancy, those on immunosuppressant drugs and those who had been on iron therapy two weeks before enrolment.

3.4. Ethical considerations

The research protocol was approved by the Health Research and Ethics Committee (HREC) of the University of the Witwatersrand, Clearance Certificate Number M 150929. The clearance was in

accordance with the Helsinki Declaration of 1975, as revised in 2000. Written informed consent form was obtained from each participant before his/her enrolment in the study.

3.5. Study Procedure

The study physician administered the questionnaire and examined the participants using a semi-structured questionnaire which included demographic history and the history of aetiology of kidney disease and other co-morbidities such as diabetes, and medications.

3.5.1. Blood sample preparation

Study phlebotomists collected blood samples. Whole blood (5mls) was collected in ethylenediaminetetraacetic (EDTA) tubes and stored at -20 ℃ for genomic DNA extractions. Blood was taken from the participants for measurement of the haemoglobin concentration, creatinine, albumin, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, calcium, phosphate, red blood cell indices, including reticulocyte haemoglobin content (CHr), and the percentage hypochromic red cells (%HYP). All tests were analysed in the National Health Laboratory as part of the standard of care using ADVIAR auto-analysers (Siemens Health Diagnostics Inc, Tarrytown, NY, USA). The blood for measuring hepcidin, the growth differentiation factor-15 (GDF-15), soluble transferrin receptor, and C-reactive protein (CRP) was collected in anticoagulant-tubes. Samples were kept on ice until the serum was separated within thirty minutes of collection and centrifuged at 2800 revolutions per minute for 15 minutes at room temperature (U-320R, Boeco, Germany). The serum was separated and stored in aliquots in 1.5ml micro-centrifuge tubes at -70°C until ready for assay. Measurements were carried out by solid-phase enzyme-linked immunosorbent and luminex assays from the commercial suppliers. Highly sensitive CRP (hsCRP) levels were determined using the

HycultBiotech human HK369 Elisa kit, (HycultBiotech, Netherlands). Samples were thawed only once, at the time of the assay.

3.5.2. Laboratory Measurements

Routine Methods

Early morning venous blood samples were drawn from the patients. Their biochemical iron status, total iron-binding capacity (TIBC), and serum iron and serum ferritin levels were measured. Serum iron was determined by means of the ferrozine calometric method, their TIBC levels through the colorimetric chromazurol dye-binding method using ADVIA 1800 (Siemen Medical Solutions Diagnostic, USA), while their serum ferritin levels were determined by using the two-site chemiluminescent immunometric assay devised by the IMMULITE®2000 system (Siemens Medical Solutions Diagnostics, USA). Transferrin saturation was calculated by means of the formula: serum iron/TIBC. Complete blood counts and CHr and %HYPO levels were obtained after processing the blood samples using the Siemens ADVIA 2120, Technion H3 RTX and RTC systems analysers (Siemens Medical Solutions Diagnostics, Tarrytown, NY). Haematological measurements were made on fresh venous blood with EDTA and clotted blood samples. Haemoglobin concentrations (Hb), red blood cell count (RBC) and platelet count were measured using a haematology analyzer (Siemens Diagnostics, Tarrytown, USA).

Growth Differentiation Factor 15 (GDF-15)

The plasma growth differentiation factor (GDF-15) was measured using the human GDF-15 DuoSet ELISA (R&D Systems), which is a sandwich-based enzyme-linked immunosorbent assay kit with a lower limit of detection of 7.81pg/ml. Plates were prepared by adding 100µl of captured antibody in phosphate-buffered saline (PBS) to each sample well and incubating the solution overnight at room temperature. The following day the plates were washed three times with wash

buffer (0.05% Tween20 in PBS) and blocked with 100µl dilution reagent and [1%] bovine serum albumin in PBS) per well, and incubated at room temperature for a minimum of one hour. After the plate was washed three times again, 100ul of plasma sample or an appropriate prepared standard (one in two serial dilutions in reagent diluent starting at 500 pg/ml down to 7.81 pg/ml) were added to the appropriate wells in addition to the 100µl of conjugate antibody. The plate was sealed and allowed to incubate for two hours on a micro-plate shaker, following which the solutions were decanted and the plate washed three times with wash buffer. A measure of 100µl of detection antibody was then added to the wells and further incubated for two hours on a plate shaker. The solutions were decanted from the wells and the plate washed again three times with wash buffer. A measure of 100µl of streptavidin-HRP was then added to each well, the plate covered and incubated for 20 minutes at room temperature. The solutions were decanted from the wells and the plate washed three times with wash buffer. A measure of 100µl of substrate solution (1:1 mix of hydrogen peroxide and tetramethylbenzidine) was added to each well and was allowed to incubate for 20 minutes. Finally, a measure of 50µl of stop solution was added, and the plate tapped gently to ensure thorough mixing. The optical density at 450nm was measured on an ELx800 microplate reader (Bio Tek, Winooski, VT, USA).

Soluble Transferrin Receptor Assay sTfR

The soluble transferrin receptor (sTfR) was measured using the Quantikine IVD Human sTfR immunoassay from R & D systems (St. Louis, MO, USA); the lower limit of detection was 3nmol/L. A measure of 100µl of sTfR assay diluent was added to each well along with 20µl of the sample or standard. The plate was covered with an adhesive strip and allowed to incubate for one hour. The solutions were decanted from the wells and the plate washed four times with wash buffer. A measure of 100µl of sTfR conjugate was added to each well, covered and left to incubate for

one hour at room temperature. The solutions were decanted from the wells and the plate washed four times with a wash buffer. A measure of 100µl of substrate was added to each well and allowed to incubate for 30 minutes at room temperature. Finally, a measure of 100µl of stop solution was added to each well. The wells were gently tapped until a uniform colour change appeared to ensure that thorough mixing had taken place. The optical density at 450nm was measured on an ELx 800 microplate reader (Bio Tek, Winooski, VT, USA).

Hepcidin assay

The sample was thawed at room temperature and the vortex was then mixed. Measures of 100µL for each sample were then aliquoted into microfuge tubes, and followed by the addition of 100µL of internal standard solutions and 200µL of cold 4% trichloroacetic acid (TCA). The samples were vortex mixed. They were then reconstituted with 50/50/0.1 (v/v/v) methanol/deionized water/formic acid and subsequently centrifuged at 217revolutions per minute for five minutes, with 20µL of the supernatant aliquoted into vials.

As described and optimised by Liet al (3), hepcidin levels were analysed using an AB Sciex-micro-liquid Chromatography (UPLC) system (Ab Sciex, Framingham, USA). Samples were separated using reverse-phased liquid chromatography on a HALO Peptides-ES 0.5 50mm column (Waters, Massachusetts, United States). The mobile phases included 0.1% of formic acid in de-ionised water (Mobile phase A) and 0.1% formic acid methanol (Mobile phase B). A gradient at a flow rate of 30µL/min was used. As such, Mobile phase B was maintained at 15% for the first two minutes, then ramped up to 98% over two minutes, and then dropped to 50% for one minute and reduced further to 15% for one minute.

Hepcidin and isotope-labelled internal standards [13C9,15N3] were purchased from the Peptide Institute (Osaka, Japan). The serum hepcidin-25 and isotope-labelled internal standards [13C9,15N3] were extracted and separated, as previously described by Li (3). Briefly, samples were extracted with (4%) HPLC grade trichloroacetic acid (TCA, Merck, Kenilworth, NJ, U.S.A) and separated using reverse-phased liquid chromatography using a 0.5x50mm Halo Peptide- ES column (Sciex, Framingham, USA) with a total run time of five minutes per sample. The analysis was performed on an AB Sciex 5500 QTRAP (Sciex, Framingham, USA) coupled to a micro-liquid chromatography (Exigent M3) system with a Turbo IonSpray ionization source operated in positive ion mode. Multiple reactive monitoring (MRM) detection was applied using the Sciex 5500 for identification and quantification on the basis of ion transitions (m/z) of 698.0 up to 354.0 and 703.2 up to 354.1 for hepcidin and stable isotope-labelled hepcidin [13C9,15N3] respectively.

Concentrations of serum hepcidin were expressed in nanograms per millilitre (ng/ml). The intra- and inter-assay coefficients of variation (CV) were less than 6.7% and less than 8.8% respectively. The lower limit of detection was 0.5ng/ml. The median reference level for serum hepcidin in the healthy controls was 5.7ng/ml.

Determination of serum creatinine and the glomerular filtration rate

The creatinine in serum was measured through a modified Jaffe reaction, whereby creatinine produces an orange colour which is based on the formation of colour from a reaction of creatinine with a picric acid/alkaline medium (4). The creatinine assay itself is traceable to isotope dilution mass spectrometry (IDMS). The glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease (MDRD) equation (R), involving four variables, namely GFR (in ml/min per $1.73m^2$) = 175 x SCr (exp[-1.154]) x Age (exp[-0.203])x(0.742 if female)x(1.21 if black) (5).

The reason why MDRD was chosen in this study was based on the reference laboratory, and the fact that our patient population had already been diagnosed with CKD based on the eGFR. The MDRD equation is used for patients with CKD stages 2 to 4 (5), in spite of the fact that it may underestimate the GFR in the early stages of CKD. The other formula used was the CKD-Epidemiology Collaboration (CKD-EPI) (6), which is more reliable at higher GFRs.

DNA extractions

Genomic DNA was extracted from whole blood using the Maxwell DNA purification kit (Promega AS1010, USA) as per the manufacturer's protocol. DNA concentrations were determined by the NanoDropTM 2000 spectrophotometer (Thermo Scientific, USA) and the quality was assessed by means of the A260/280 ratios. A 260/280 ratio of 1.8 to 2.0 was considered as indicative of DNA of a good quality. Samples that had suboptimal DNA concentrations (<10ng/ul) were re-extracted using a modified salting-out method (Miller et al, 1988). For the salting-out method, whole blood obtained via anti-coagulated blood (EDTA) was placed in a 50ml polypropylene tube with approximately 40ml of an ice-cold lysis buffer (10mM Tris-HCl; 5mM MgCl2, 1% Triton X-100, 320mM Sucrose). Cells were pelleted by centrifugation at 2 300 revolutions per minute for 10 minutes, the supernatant was discarded, and the pellet fully re-suspended in 20ml of an ice-cold lysis buffer. The cells were again pelleted through centrifugation at 2 300 revolutions per minute for 10 minutes, the supernatant discarded, and the pellet re-suspended in three millimetres (3ml) of T20E5 (20mM Tris-HCl, 5mM EDTA). The samples were digested overnight at 42°C with a proteinase k-solution (2mg/ml proteinase k, 2mM EDTA, 1% SDS). The following day, one millilitre (1ml) of saturated sodium chloride (NaCl) was added to each sample and the samples allowed to incubate on ice for five minutes. The protein precipitate was pelleted through centrifugation at 4°C for 30 minutes at 2500 revolutions per minute. The supernatant was

transferred to a clean 50ml polypropylene tube and 20ml of absolute ethanol was added to precipitate the DNA. The resulting DNA was washed with 70% ethanol to remove the excess salt, allowed to air-dry and finally re-suspended in approximately 100µl of a Tris-EDTA (TE) buffer. The DNA was quantified using a NanoDropTM 2000 spectrophotometer and the quality assessed by means of the 260/280 ratio.

Genotyping of TMPRSS6 rs855791 polymorphism

The region of the TMPRSS6 gene containing the rs855791 C>T polymorphism was amplified using the polymerase chain reaction (PCR). Primers were as described in Pei et al (2014): TMPRSS6F 5'-TAG AGA ACA GGG GCT CCA GG-3'; TMPRSS6R 5'-ATG TGG GCA GCA TCC TTT C-3'. The PCR reactions each contained 1x KAPA2G Robust HotStart Ready Mix (KAPABiosystems, Massachusetts, USA), 0.125µM of each of the forward and reverse primers, and 50ng of the extracted DNA. The thermocycling conditions were 95°C for three minutes, 40 cycles of 95°C for 15 seconds, 65°C for 15 seconds, 72°C for 20 seconds and a final extension of 72°C for one minute. This resulted in the amplification of a single 249bp fragment.

The rs855791 SNP was genotyped using restriction fragment length polymorphism (RFLP) analysis. The PCR products were restricted overnight at 37°C with two units of StuI (New England Biolabs) and then resolved on a one percent (1%) agarose gel. The genotypes were determined in terms of fragment size (CC - a single 249bp fragment; CT - a 249bp fragment; and TT - a 125 bp fragment).

3.6. References

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4. CHAPTER FOUR - PAPER ONE: ETHNIC PREVALENCE OF ANAEMIA, AND PREDICTORS OF ANAEMIA AMONG CHRONIC KIDNEY DISEASE PATIENTS AT A TERTIARY HOSPITAL IN JOHANNESBURG, SOUTH AFRICA

Nalado, A.M., Mahlangu, J.N., Waziri, B., Duarte, R., Paget, G., Olorunfemi, G. and Naicker, S., 2019. Ethnic prevalence of anemia and predictors of anemia among chronic kidney disease patients at a tertiary hospital in Johannesburg, South Africa. *International Journal of Nephrology and Renovascular Disease, 12*, p.19.

4.1.Abstract

Introduction: Anaemia is a complication of chronic kidney disease (CKD) that can greatly impact on its prognosis. However, the risk factors for anaemia, including the influence of ethnicity, are not well established for the CKD population in Johannesburg.

Methods: This was a cross-sectional study of 353 adult CKD patients attending the Renal Outpatient Clinic of the Charlotte Maxeke Johannesburg Academic Hospital, in Johannesburg, South Africa from 1 June 2016 to 30 December 2016. Socio-demographic and clinical characteristics were obtained using a proforma. Blood samples were collected for serum electrolytes and haematological parameters. The prevalence and predictors of low haemoglobin and iron deficiency anaemia were evaluated using multivariable binary logistic regression.

Results: The mean age of the CKD participants was 55.3 ±15.0 years. The prevalence of anaemia (Hb<13g/dl in males and <12g/dl in females) among black CKD patients was 46.9%, with 26.1% presenting with iron deficiency anaemia, which was three times more frequent among CKD patients than among the controls (35.3% vs 9.2%). On the other hand, 32.3% (95%CI; 27.9%-37.1%) of the study population suffered from iron deficiency without anaemia. Blacks (46.9%) had the highest prevalence of anaemia, while Indians/Asians (18.2%) had the lowest. Although the odds of anaemia were 3.8-fold higher (OR=3.8, P-value=0.059) among CKD stage 5 participants as compared to CKD stage I participants, the relationship between anaemia and the stage of CKD proved to be non-linear. Diabetes mellitus (DM) (OR=2.31, P-value=0.005) had a strong association with anaemia among the CKD participants.

Conclusion: Almost half of the CKD participants were anaemic, but noteworthy is the fact that the anaemia did not increase linearly with an increase in the severity of CKD. There was a

marked ethnic disparity in anaemia prevalence. Our study highlights the need for risk-based management of anaemia among CKD patients.

Keywords: anaemia, predictors, chronic kidney disease, haemoglobin, risk factors, iron deficiency anaemia

4.2. Introduction

Anaemia is a major cause of morbidity and mortality among chronic kidney disease (CKD) patients (1). Anaemia is defined as a low level of haemoglobin (Hb), less than 12g/dl in females and less than 13g/dl in males among CKD patients (2). About 75% of CKD patients were reported to be anaemic in low and middle-income countries, as opposed to a lower anaemia prevalence of 22.2% among CKD patients in high-income countries (3). Anaemia occurs early in the course of kidney disease and may worsen and become intractable with declining kidney function (2, 4, 5).

Although the development of anaemia in CKD patients is mainly due to an absolute or relative deficiency of erythropoietin, other possible causes of anaemia include blood loss, a decreased half-life of red blood cells, iron deficiency, inflammation, nutritional deficiency (on account of an inadequate diet and defective iron absorption) and the accumulation of uraemic toxins that inhibit erythropoiesis (4, 6).

Iron deficiency anaemia (IDA) is also a major cause of anaemia in CKD. Several causes of IDA in CKD patients include blood loss (from frequent laboratory testing, occult gastrointestinal bleeding, access bleeding), decreased duodenal iron absorption (resulting from inflammation), interference with iron absorption (resulting from medications such as gastric acid inhibitors, phosphate binders) (7).

Some of the adverse clinical outcomes of anaemia in CKD patients include sleep disturbance, exercise intolerance, and increased mortality (8). Some studies have shown that early identification and prompt treatment of anaemia through near normalisation of haemoglobin and iron levels in CKD patients are associated with slower progression to end-stage kidney disease (ESKD), and reduced cardiovascular morbidity and mortality, as reported in the Cardiovascular Risk Reduction by Early Treatment with Epoietin Beta (CREATE) trial (9, 10).

Anaemia in CKD patients is the product of a complex interplay between patient-specific attributes, CKD stage, treatment modalities, and socio-economic and environmental factors. Therefore, it is being advocated that individualised risk-based management of anaemia in CKD patients should be promoted because of the multiple factors responsible for the evolution of anaemia in them (11). Oral iron is usually preferred because of its convenience and low cost. However, its utility value is often limited by gastrointestinal side effects, poor absorption and low efficacy in CKD patients (12, 13).

South Africa is a multi-ethnic country with about 80% of the population being black and most of the people likely to be of low socio-economic status. According to Statistics South Africa's Living Conditions Survey, (2016) the annual household income for blacks stood at an average of R92, 893 Rands (about 7000 US dollars) compared to R444,446 (29 000 US dollars) for whites. Furthermore, the median monthly earnings of white (R9 500), Indian/ Asian (R6 000), mixed race (R2 652) and black (R2 167) South Africans showed a marked ethnic disparity that is reflected in the variations in the socio-economic attributes of the population (14-16). Hence, socio-economic circumstances among CKD patients may impact on the prevalence and outcomes of anaemia in them (17, 18). However, little is currently known of the interactions of various predictors of anaemia among the different ethnic groups to which CKD patients, who are yet to commence dialysis in South Africa, belong. Therefore, the aim of this study was to assess the overall and ethnic prevalence of iron deficiency anaemia, and stage-specific prevalence and possible predictors of anaemia among pre-dialysis CKD patients in Johannesburg (17, 18).

4.3. Patients and methods

This cross-sectional study was conducted on 353 pre-dialysis CKD patients from 1 June to 31 December 2016. The study population comprised of patients attending the Renal Out-Patient Clinic of Charlotte Maxeke Academic Hospital, Johannesburg. All consecutively consenting

pre-dialysis CKD participants (>18 years of age) were recruited for the study. All patients with active bleeding (such as upper gastrointestinal bleeding), infection and inflammation, who had undergone a blood transfusion within three months of enrolment, who had been placed on oral iron therapy within two weeks of enrolment, who were being treated with erythropoietin stimulating agents (ESA), within four weeks of enrolment, who were presenting with active malignancy, a human immunodeficiency virus infection, who were using immunosuppressive drugs, and those with known haemoglobinopathies were excluded from the study.

The socio-demographic attributes of the sample of CKD patients were obtained using a proforma. In this study, employment status and educational level were used as proxies for socio-economic status (19). In addition, weight, height and blood pressure were measured using a stadiometer and a mercury sphygmomanometer respectively. Blood pressure can be defined as an average based on greater than two readings obtained from two separate blood pressure measurements that are greater than 140/90 mm of haemoglobin (Hg), as defined by JNC 7 (20).

Blood samples were collected for serum electrolytes and haematological parameters. The biochemical iron status, serum iron, total iron-binding capacity (TIBC), and serum ferritin were also measured. Serum iron was determined by means of the ferrozine calometric method, TIBC through the colorimetric chromazurol dye-binding method using ADVIA 1800 (Siemen Medical Solutions Diagnostic, USA), and serum ferritin by using two-site chemiluminescent immunometric assays presented by the IMMULITE®2000 system (Siemens Medical Solutions Diagnostics, USA). Transferrin saturation was calculated through the formula: serum iron/TIBC. Complete blood counts were obtained after processing the blood samples using the Siemens ADVIA 2120, Technion H3 RTX and RTC systems analyzers (Siemens Medical Solutions Diagnostics, Tarrytown, NY).

Participants were classified as anaemic based on haemoglobin (Hb) level of less than 13g/dl in men and less than 12g/dl in women. Mild anaemia was defined as presenting with an Hb value greater than 11g/dl, moderate as presenting with an Hb of nine (9) to 11g/dl, and severe anaemia as presenting with an Hb greater than 9g/dl (21). Iron deficiency was defined as a serum ferritin level of less than 100ug/l or a ferritin level of 100 to 300ug/l, and a transferrin saturation level (TSAT) of less than 20% (18). The glomerular filtration rate (GFR) was determined by means of the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation for eGFR (22) stage 1 with an eGFR greater than 90mls per minute; stage 2 with an e GFR equivalent to 60 to 89 mls per minute; stage 3 with an eGFR equivalent to 30 to59 mls per minute; stage 4 with an eGFR equivalent to 15 to29 mls per minute; and stage 5 with an eGFR less than 15mls per minute.

Ethical approval for the study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, ethics certificate number M150929. All participants provided forms showing their written informed consent.

4.4. Statistical analysis

Normally distributed continuous variables were presented as means ± standard deviations (SD), while non-normally distributed continuous variables were presented as medians (interquartile range, IQR). Categorical variables were presented as numbers, percentages and charts. The prevalence of anaemia (with a 95% confidence interval) was estimated for the CKD participants and in terms of socio-demographic factors. All these values were stratified according to the four ethnic groups (blacks, whites, the mixed-race group and Indians/Asians). Anaemic and non-anaemic patients were compared in terms of socio-demographic and clinical parameters using Pearson's Chi-square and student's t-test as the appropriate statistical methods. The relationship between anaemia status and the stage of CKD was determined using Pearson's Chi-Square (Fisher's exact test was used when the expected cells were fewer than

5). Furthermore, the difference in mean levels of haematological parameters by stage of CKD was determined using the analysis of variance (ANOVA) test. The Post hoc Bonferroni Pairwise Test was conducted to determine where the statistically significant difference between the relevant variables lies. This analysis was conducted for each ethnic group. Univariate logistic regression analysis was conducted for all the explanatory variables with anaemia status as the outcome of interest. Variables with a P-value of less than 0.2 were added according to the backward elimination method to arrive at the final multivariable model of the predictors of anaemia.

The CKD stage and ethnicity were the primary explanatory variables. However, age, gender and a history of diabetes mellitus were added to the model because they were selected a priori. Crude and adjusted odds ratios (ORs) and a 95% confidence interval (95%CI) were obtained from the multivariable model. Post regression diagnostics was conducted using the Hosmer-Lemeshow Goodness of Fit Test and the discriminatory value of the model for predicting anaemia status in the CKD participants was conducted. It was based on the area under the curve (AUC) of the receiver operator characteristics curve (ROC). A two–tailed test of the hypothesis was assumed. The statistically significant level was set at a 95% confidence interval (or a P-value of less than 0.05). Data were analysed using Stata version 14 (Stata Corp, USA).

4.5. Results

Of the 379 eligible participants, 26 declined to participate in the study; 353 were therefore enrolled in the study. The mean age of the participants was 55.3 ±15.0 years. The majority of participants were blacks (n=258/353, 73.1%), followed by whites (n=52/353, 14.7%), Indians/Asians (n=22/353, 6.2%) and those of a mixed race (n=21/353, 6.0%).

The overall prevalence of anaemia was 43.1% (95%CI: 38.1 - 48.4 %.) (Figure 3.1). The Indian/Asian population had the lowest prevalence of anaemia, while the blacks had the

highest. The prevalence of anaemia in the black, mixed race, white and Indian/Asian groups was 46.9% (95%CI: 40.8 - 53.0), 45% (95%CI: 23.8 – 68.2), 34.6% (95%CI: 22.7 - 48.9), and 18.2% (95%CI: 6.4 - 41.9) respectively. Furthermore, the prevalence of IDA among black, white, mixed and Indian/Asian groups was 35.3% (95%CI: 29.6 - 41.3%), 23.1% (95%CI: 13.3% - 36.9%), 14.3% (95%CI: 4.2% – 38.7%) and 9.1% (95%CI: 2.0% - 3.3%) respectively.

The majority (79.0%) of the participants were in the late stages of CKD (stages 3 to 5) (Table 3.1). About 44.6% (91/204) of the hypertensive participants were anaemic, while 40.4% (38/94) of the diabetic participants were anaemic (Figure 4. 2). The mean CRP level of the anaemic participants was higher than that of the non-anaemic patients (33.6±4.32 *vs* 14.80±1.7, P-value<0.001). There were no significant ethnic differences in the clinical characteristics among the anaemic participants. However, the blacks had the lowest mean age at recruitment, namely 52.40±14.0 years, P-value<0.001 (Table 3.1). None of the 92 smokers in the cohort had anaemia (Table 3.1).

Table 4.2 shows the statistically significant association between the stages of CKD and anaemia status (P-value<0.001). The proportion of participants with anaemia decreased from 39.1% in CKD stage I to 21.9% in stage 3a, and then increased to 91.4% among black participants in CKD stage 5. There was a statistically significant difference in the mean levels of Hb, MCH and MCV across the five stages of CKD (Table 4. 2).

The mean CRP fluctuated between 10±1.5 mg/l and 21.9±5.0 mg/l between stage 1 and stage 3a and increased from 13mg/l in stage 3b to 51.3mg/l in stage 5 (P-value <0.0001). Furthermore, the prevalence of inflammation decreased from stage I (26.1%) to stage 3 (9.4%) and subsequently increased, reaching the highest levels in stage 5 (86.2%); (Table 2A). The *post hoc* Bonferroni Analysis is shown in Table 2B.

Among the white ethnic group, haemoglobin (Hb) levels were statistically different across the stages of CKD (P-value<0.0001) (Table 3). However, there was no statistically significant difference in the parameters among the stages in the mixed and Indian/Asian races respectively (Supplementary Table 3.1 and Supplementary Table 2). Furthermore, there was a positive correlation between haemoglobin levels and eGFR (r= 0.334, P-value <0.0001). There was also a positive correlation between haemoglobin and eGFR among males and females and among the black, white, and mixed ethnic groups (Table 3).

As shown in Figure 3, globally, there was a J- shaped relationship between CKD stage and the odds of contracting anaemia Thus, the odds of anaemia decreased from 1.0 in stage I to 0.17 in stage 3 and subsequently increased abruptly from stage 4 (adjusted odds ratio: 0.66) to Stage V (adjusted odds ratio : 3.83). Furthermore, the likelihood for Stage 3 participants to contract anaemia was 83% less than that for stage I participants (adjusted OR: 0.17, 95% CI: 0.05 - 0.54, P-value = 0.003). On the other hand, after correcting for confounding variables, the odds for stage 5 participants to develop anaemia amounted to a 3.8-fold increase as opposed to the odds for the Stage one participants (Table 4). Furthermore, the odds for CKD participants with diabetes mellitus to develop anaemia were twice those for CKD participants without diabetes mellitus. However, important to note is that a history of hypertension did not impact on the odds among the CKD participants of developing anaemia. There was no statistically significant difference in the odds of developing anaemia by gender (P-value = 0.388). The odd of developing anaemia among the Indian/Asian group was 96% less than the odds for the blacks (Table 4).

Although the odds for developing anaemia among participants with inflammation (as defined by CRP levels) showed a fivefold increase over those without inflammation, the univariate analysis did not, however, prove this relationship to be statistically significant after corrections were made for confounders. Patients with hypalbuminaemia had a three times greater chance

of developing anaemia as opposed to those with lower TSAT levels who were less likely to do so (Table 4). Based on the Hosmer-Lemeshow Goodness of Fitness Test (P-value= 0.9939), the final model shows that the model did in fact fit the data. Also, the area under the ROC curve of the final model amounted to 8.6%, which shows that the final model proved to be highly discriminatory in terms of estimating the odds of developing anaemia among the participants.

4.6. Discussion

Anaemia is a major complication of CKD. It is also a risk factor for cardiovascular disease among CKD patients (23) while some studies from South Africa have reported on the prevalence of anaemia in the healthy population (24), the prevalence of anaemia in South African CKD patients is largely unknown. In this present study involving a multi-ethnic CKD population, almost half (43.18%, 95%CI: 38.1 - 48.4%) of the participants had anaemia. As expected, our study showed a higher prevalence of anaemia as compared to the prevalence of anaemia of 12.5 % that was reported in the total adult population of South Africa (24). This prevalence percentage is lower than those listed in previous reports pertaining to most low- and middle-income countries (LMIC) where the anaemia prevalence was set at 75% to 79% among CKD patients (25-27). A slightly higher anaemia prevalence of 58.5% was reported among patients with CKD stages 3 to 5 in Catalonia (21, 28). Furthermore, our study showed that the prevalence of anaemia among South African CKD patients was two to three times higher than the anaemia prevalence among CKD patients in the United Kingdom and the United States of America (USA) (29).

In Singapore, a lower prevalence of anaemia (35.4%) was reported among CKD patients (30), while in Nepal, similar to our study, a prevalence of 47.5% was reported (31). Geographical location (altitude), life style, racial and genetic make-up may have played significant roles in the observed variations in the prevalence of anaemia among our cohort of CKD patients as compared to CKD patients from other regions. The relatively lower prevalence of anaemia

among CKD patients in Johannesburg as compared to other low- and middle-income countries (LMICs) may be related to the relatively higher level of access to health care services among CKD patients in South Africa. South African CKD patients have access to free medical care as opposed to the majority of CKD patients from other developing countries that pay out of their own pockets for their medical services. This suggests the need for global advocacy to design policies that will reduce the cost and burden of CKD on patients and their relatives. This can impact positively on prognostic factors such as anaemia and thereby improve outcomes.

There was a marked ethnic variation in the prevalence of anaemia in our study population with the black and mixed ethnic groups having a similarly high prevalence (45%-47%), while the whites and Indians/Asians had a lower prevalence (18-35%). This ethnic disparity in prevalence of anaemia may be related to the general socio-economic status and dietary patterns of the ethnic groups. Socioeconomic status (SES) has been shown to be a predictor of nutritional status in several studies (32, 33). Pathways through which SES may be associated with nutritional status include income, education, and occupation (34).

Some studies conducted in developing counties are in agreement with our findings that low SES is associated with a high prevalence of anaemia (35-37). Most of these studies were conducted in developing countries with limited sample sizes and were not on CKD patients. Our study revealed that almost half of our black cohorts have low levels of education. The low educational attainment could be a proxy for low socio-economic status among them. The blacks are generally of a lower socio-economic class mainly because of the deprivation that was suffered during the apartheid era. Moreover, the Indian/Asians' vegetarian lifestyle might increase the rate of anaemia among them. However, this was not a finding in this study. A possible explanation could be that most of the participants may not have been on a strict vegetarian diet (18, 38). Our observation also showed that tailored management may be necessary. Thus, in addition to routine CKD care, nutritional support may be more frequently

indicated among the blacks and mixed-race South Africans as compared to the whites and Indian/Asians.

Despite the fact that about four-fifths of our cohort of CKD patients were in the late stage of the disease, almost half of the anaemic patients had mild anaemia. A similar predominance of mild anaemia among CKD patients was also reported in other studies (39, 40). In contrast to studies from both high-income and LMICs, the prevalence of anaemia in our cohort did not increase linearly with increasing stages of CKD. Furthermore, we surprisingly found that the odds of anaemia were lowest in CKD patients in stages 2 and 3 but higher in those in stages one and five.

A study conducted in the Kingdom of Saudi Arabia (KSA) showed a similar prevalence pattern of anaemia among pre-dialysis patients according to the stages of CKD with prevalence percentages of 42%, 33%, 48%, 71%, and 82%, respectively among CKD participants in stages 1 to 5 of the disease (39). The J-shaped pattern of the prevalence of inflammation among CKD stages was similar to the pattern of anaemia across the stages of CKD in this study, as there was a decreasing prevalence of inflammation (26.1%) in stage I to a nadir of 9.4% in stage three, followed by an abrupt rise in indicators of inflammation up to stage 5 (86.6%). Furthermore, we found on univariate analysis that there was a five-fold increase in the odds of developing anaemia among participants with inflammation. This may suggest a link between inflammation and anaemia among CKD participants. From the foregoing, although the link between anaemia and inflammation across CKD stages is poorly understood, our study further suggests that inflammatory processes may play a role in the development of anaemia among CKD patients (and possibly across the stages of CKD), as shown in other studies (41)

In the present study, the increased odds of anaemia in CKD stage 5 are consistent with findings from previous studies (39, 42). For example, Lau (30). showed that in comparison to patients

with CKD stage one, stage five CKD patients are more likely to develop anaemia (OR = 16.8). Anaemia is believed to traditionally intensify with the progressive decline in kidney function, as it is believed that erythropoietin production decreases as kidney function worsens (43). A few hypotheses may be proposed to partly explain the J-shaped pattern of anaemia risk among varying stages of CKD as observed in our study participants. There may be some unrecognised perturbations that are responsible for this initial improvement in the haemoglobin levels as kidney disease worsens from stage I to stage 3 before the eventual decline in haemoglobin concentrations from stage three to stage three.

Firstly, the initial inflammatory process in the early stages of CKD may cause the hyper stimulation of erythropoietin that then produces higher levels of haemoglobin (4, 44). This mechanism may not be sustained with the continuing deterioration in kidney function during the later stages of CKD. In fact, hepcidin pathways and hypoxia inducible factors may play a role in this mechanism.

Secondly, there might be aggressive management of anaemia in the early stages of the disease, or patients may respond to the anaemia treatment modalities (such as oral iron therapy and the erythropoietin-stimulating agent) that are employed in the early stages of the disease, especially in stages one and two. Thus, patients may not respond properly to similar modalities of treatment at the later stages of the disease. Such intractable anaemic states at the later stages may be related to poor nutritional status or there may be the need to employ parenteral erythropoietin therapy that may not be readily available for use during the earlier stages. Thus, there may be the need to closely monitor anaemia in all CKD patients as the disease progresses.

Another hypothesis for the pattern of anaemia progression in the stages of kidney failure may be that there may be some unrecognised mechanisms that support haemoglobin concentration in stages two and three of CKD. All of the aforementioned hypotheses still need further

evaluation. Thus, CKD staging alone may not be appropriate for screening or for the determination of the risk and severity of anaemia among CKD patients.

The glomerular filtration rate, gender, ethnicity, iron therapy, and other co-morbidities such as diabetes mellitus were identified as risk factors of anaemia among CKD patients by other researchers (30, 45, 46). However, some differences were observed in our study, contrary to the findings of previous studies (45, 47), we did not find a significant association between gender and the development of anaemia (P-value=0.6). McClellan (29) found that female patients had higher odds of developing anaemia. Similarly, Fishbane (47) reported lower rates of iron deficiency anaemia among adult men (57.8 to 58.8%) as opposed to women (69.9 to72.8%) with CKD stages 3 to 5 in the National Health and Nutrition Examination Survey (NHANESII (1988-1994) and 1999 to 2004 surveys (29, 48).

Our study tends to support the reports of previous researchers that increasing age is an independent risk factor for anaemia among CKD patients, as we found that patients older than 50 years have double the risk of developing anaemia as compared to younger patients (3, 49). Thus, the process of ageing may greatly contribute to poor outcomes of anaemia in CKD patients. Older patients with anaemia may also have intractable forms of anaemia and should therefore be managed more intensively.

Consistent with previous studies, CKD patients with diabetes mellitus had a three-fold increased risk of anaemia (p=0.007) (50, 51). Thus, a high index of suspicion for anaemia should be instituted among diabetic CKD patients to improve outcomes in them. There is evidence that anaemia is more severe and occurs at an earlier stage of kidney disease in patients with diabetes as opposed to non-diabetic patients (29, 52). Some studies suggest that renal abnormalities and possibly interstitial fibrosis may play a role in the pathophysiology of anaemia in diabetes (52, 53). Less well-known causes of anaemia in diabetes may include

interrelated mechanisms, such as ultra-filtration, proteinuria, chronic inflammation, damage to the interstitial kidney tissue, autonomic nephropathy, uraemic toxins, damage to the renin-angiotensin system, increased tubular sodium reabsorption, and disorders of erythrocytes which have all been implicated in the pathogenic mechanism of anaemia in diabetic patients (54-56).

This present study found that smoking is not a predictor of anaemia, but it is associated with an increase in haemoglobin levels. This finding is in agreement with those of previous studies (57, 58). A possible explanation for this is that cigarette smoking causes an upward shift of the haemoglobin distribution curve which reduces the ability of the haemoglobin levels to detect anaemia. Hence it causes increased haemoglobin concentration that is likely to be mediated by exposure to carbon monoxide (58). Furthermore, our study found that hypoalbuminemia is associated with anaemia, which is in agreement with the results of earlier studies of CKD and haemodialysis patients (59). Low serum albumin is used as a marker of inflammation, and the relationships between anaemia and inflammation and between anaemia and malnutrition have been shown in several studies (60, 61).

As in previous studies, we found other predictors of anaemia, including transferrin saturation (TSAT) and hypocalcaemia (9, 62). Also, in agreement with the findings of other researchers, we found hypocalcaemia to be a worthwhile predictor of anaemia among our cohort of CKD participants (53, 63). Several mechanisms have been postulated to explain this observed relationship. For example, hyperphosphatemia in CKD can cause a decrease in vitamin D synthesis, which will result in hypocalcaemia and elevated parathyroid hormone levels (PTH) (53, 64). Elevated PTH, in turn, has been shown to directly inhibit erythropoiesis, induce haemolysis and cause bone marrow fibrosis in CKD patients (65, 66). Although serum PTH levels were not ascertained in our study, the mechanism underlying the occurrence of serum phosphorous, calcium and anaemia in CKD is still unclear.

In our study, angiotensin-converting enzyme inhibitors (ACEIs), and angiotensin receptor blockers (ARBs) predicted anaemia. This finding is in agreement with those of previous studies (67-69). The mechanism of action of ACEI and ARBs and their effect on haemoglobin levels in patients with kidney disease is still not well established. Possible proposed mechanisms include interference with the production of erythropoietin (69) and the modulation of multiple factors interacting with the erythroid marrow progenitor. Mruget al (70) another possible mechanism may be that angiotensin 2 may increase the proliferation of erythroid progenitors, but not the progenitors of other cell lines, and that ARBs completely abolish this effect. All these observations may suggest a possible inhibitory effect of these medications on the bone marrow (69, 70). Further research is required to establish the exact mechanism explaining how these medications work.

In conclusion, we found that almost half of the CKD participants in our study were anaemic which confirms that anaemia is a leading co-morbidity factor among CKD patients in Johannesburg. Also, as was previously observed by other researchers, the risk of anaemia does not increase linearly with increasing degrees of renal deterioration. Thus, CKD staging alone may not be appropriate for screening and monitoring anaemia. In addition, diabetes mellitus and calcium levels are strong predictors of anaemia among CKD patients. Our study therefore highlights the need for personalised, risk-based prevention and treatment strategies for anaemia among CKD patients in low-resource settings such as ours. The relationship between the severity of kidney deterioration and anaemia still needs further evaluation.

This study is not without limitations. Firstly, the cross-sectional design of the study limited us from demonstrating causality between anaemia and the respective stages of CKD. As only one set of haemoglobin results was determined, trends and fluctuations in haemoglobin could not be monitored. Secondly, our study sample may not reflect a generalised population of South African CKD patients, since the study was conducted in an urban setting in South Africa.

Thirdly, we did not evaluate the serum folate and vitamin B12 levels of our participants which might have assisted us in providing the nutritional status of the participants. Furthermore, the vitamin D and parathyroid hormone levels (PTH) were not evaluated in this study. The drug history of the chronic use of non-steroidal anti-inflammatory drugs (NSAIDS) was also not elicited. However, CKD patients in the hospital are generally counselled to avoid NSAIDS, since it can further aggravate kidney function. Because patients on oral iron and ESA therapy were excluded during the patient enrolments, this issue could affect the true prevalence of anaemia in the studied population. Thus, we were unable to adequately stratify our patients in terms of nutritional status.

Despite these limitations, this study has contributed to the existing evidence on the prevalence of anaemia across CKD stages in pre-dialysis CKD patients.

Conflict of interest

All authors declare that they have no conflict of interest.

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We would like to acknowledge the participants for participating in our study.

Table 4:1: Predictors of anaemia among chronic kidney disease patients

Variable	OR	95%CI	P-value	Adjusted OR	95%CI	P-value
GFR (ml/min/1.73m$^{2)}$						
Stage 1	1.00	Reference	Reference	1.00	Reference	Reference
Stage 2	0.37	0.13 -1.07	0.067	0.20	0.05 - 0.81	0.024
Stage 3	0.53	0.23 – 1.22	0.138	0.17	0.05 - 0.54	0.003
Stage 4	1.25	0.54 - 2.90	0.598	0.66	0.21 - 2.08	0.479
Stage 5	14.25	4.93- 41.29	<0.001	3.83	0.95 - 15.39	0.059
Age (years)						
<50	1.00	Reference	Reference	1.00	Reference	Reference
≥ 50	1.20	0.77 - 1.87	0.425	2.33	1.05 - 5.16	0.037
Race						
Blacks	1.00	Reference	Reference	1.00	Reference	Reference
Indian/Asian	0.60	0.32 - 1.12	0.106	0.04	0.01 - 0.28	0.001
Mixed	0.93	0.37 - 2.31	0.870	0.45	0.09 - 2.36	0.347
White	0.25	0.08 - 0.76	0.015	0.41	0.14 - 1.23	0.111
Gender						
Male	1.00	Reference	Reference	1.00	Reference	Reference
Female	1.26	0.83 - 1.93	0.280	0.74	0.38 - 1.46	0.388
History of Diabetes Mellitus						
No	1.00	Reference	Reference	1.00	Reference	Reference
Yes	2.07	1.32 - 3.25	0.002	2.06	1.00 - 4.25	0.050
History of Hypertension						
No	1.00	Reference	Reference	1.00	Reference	Reference
Yes	0.90	0.30 - 2.74	0.852	-	-	-
Hypoalbuminemia (g/l)						
No (>35)	1.00	Reference	Reference	1.00	Reference	Reference
Yes (≤35)	5.33	2.61 - 10.89	<0.001	3.24	1.10 - 9.53	0.033

Table 4.1b: Predictors of anaemia among chronic kidney disease patients

Variable	OR	95%CI	P-value	Adjusted OR	95%CI	P-value
Hypocalcaemia						
No (>2.5)	1.00	Reference	Reference	1.00	Reference	Reference
Yes (≤2.5)	6.52	3.55 - 11.98	<0.001	-	-	-
Hyperphosphatemia						
No (<1.45mmol/l)	1.00	Reference	Reference	1.00	Reference	Reference
Yes (>1.45mmol/l)	1.40	0.63 – 3.14	0.227	-	-	-
Dyslipidaemia						
No	1.00	Reference	Reference	1.00	Reference	Reference
Yes	1.25	0.58 - 2.68	0.568	-	-	-
TSAT (%)						
>20	1.00	Reference	Reference	1.00	Reference	Reference
<20	0.38	0.24-0.60	<0.001	0.40	0.19 - 0.83	0.014
CRP (mg/l)						
Normal (CRP<10)	1.00	Reference	Reference	1.00	Reference	Reference
Inflammation (CRP≥ 10)	3.93	2.51 – 6.16	<0.001	1.16	0.57 - 2.34	
ACE-I/ARB						
No	1.00	Reference	Reference	1.00	Reference	Reference
Yes	44.46	18.55 - 106.53	<0.0001	75.68	21.34 - 268.35	<0.001
Serum ferritin						
>100	1.00	Reference	Reference	1.00	Reference	Reference
≤100	1.50	0.98- 2.31	0.065	-	-	-
Obesity (Kg/m^2)						
Normal (18.5 – 24.9)	1.00	Reference	Reference	1.00	Reference	Reference
Obese (BMI: 25 – 34.9)	0.68	0.25-0.98	0.195	-	-	-
Morbid obesity (BMI>35)	0.78	0.28 – 1.06	0.391	-	-	--
Serum creatinine, (mg/dl)						
<2.5	1.00	Reference	Reference	1.00	Reference	Reference
>2.5	5.58	3.50 - 8.88	<0.0001	-	-	-

95%CI: 95% confidence interval; OR: Odds ratio; Multivariable model corrected for stage of the disease, age, gender, history of Diabetes Mellitus, hypalbuminaemia, ACE-I/ARB use.
Hosmer and Lemeshow Chi-square = 204.89and its P-value = 0.9939
ACE-I: Angiotensin converting enzyme inhibitor use; ARB: Angiotensin receptor blocker

Table 4:2: Distribution of anaemia and its parameters among Mixed Race South Africans at different stages of chronic kidney disease

Anaemia status (%)	Stage 1 (n, %)	Stage 2 (n, %)	Stage 3a (n, %)	Stage 3b (n, %)	Stage 4 (n, %)	Stage 5 (n,%)	Total (n=258)	P-value
Anaemic	0 (0.0)	3 (100)	2 (66.7)	2 (50.0)	4(80.0)	0 (0.0)	11 (55.0)	0.063
Non-anaemic	1 (100.0)	0 (0.0)	1 (37.3)	2 (50.0)	1 (20.0)	4 (100.0)	9 (45.0%)	
Hb (g/dl) mean± SD	12.5 ± 0.57	14.3 ± 0.51	12.1 ± 2.8	11.7 ± 0.8	12.9± 2.7	10.0± 1.64	12.2± 2.2	0.155#
MCV(fl) mean± SD)	90.4 ± 0.42	90.6 ± 0.15	91.5± 7.4	84.8± 10.7	90.0 ± 5.9	91.2 ± 3.1	89.6 ± 6.1	0.23#
MCH(pg) mean± SD	32.1 ± 0.7	29.3 ± 0.4	29.1 ± 1.8	27.0± 4.7	29.7 ± 2.6	27.5 ± 1.8	28.9± 2.8	0.33#
MCHC mean± SD	30.7 ± 1.2	32.4 ± 1.0	31.7± 1.5	31.8 ± 3.0	32.5 ± 1.2	31.3 ± 1.2	31.8 ± 1.7	0.394#
CRP (mg/l) mean± SD	10.0±0.0	10.0±0.0	18± 13.9	18 ±14.7	15.8± 8.6	17.5±6.8	15.5 ±9.1	0.179#
CRP groups								
Inflammation (CRP≥10)	0 (0.0)	0(0.0)	1 (33.3)	2 (50.0)	2 (40.0)	3 (75.0)	8 (36.1)	0.496$
No inflammation (CRP<10)	2 (100.0)	3 (100.0)	2 (66.7)	2 (50.0)	3 (60.0)	1 (25.0)	13 (61.9)	
# ANOVA ; ^ Pearson's Chi-Square; $ Fischer's exact test ; CRP: C-reactive protein								

Table 4.2b: Distribution of anaemia and its parameters among Indian/ Asian South Africans at different stages of chronic kidney disease

Anaemia status (%)	Stage 1 (n, %)	Stage 2 (n, %)	Stage 3a (n, %)	Stage 3b (n, %)	Stage 4 (n, %)	Stage 5 (n,%)	Total (n=258)	P-value
Anaemic	4 (80.4)	2 (100.0)	1 (50.0)	5 (100)	4 (100.0)	2 (50.0)	18 (81.8%)	0.26
Non-anaemic	1 (20.0)	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	2 (50.0)	4 (18.2%)	
Hb (g/dl) mean± SD	12.8± 1.7	14.7± 1.4	12.7 ± 1.2	13.3 ± 0.9	14.4± 1.4	11.5 ± 3.7	13.1± 2.1	0.40#
MCV(fl) mean± SD)	90.7 ± 1.6	90.9 ± 3.5	91.2± 5.7	91.8± 8.6	90.5 ± 11.8	87.5 ± 4.1	90.0 ± 8.7	0.98#
MCH(pg) mean± SD	30.5 ± 0.6	22.1 ± 6.6	28.7 ± 2.1	27.1± 2.2	27.7 ± 1.9	29.3 ± 2.7	28.0 ± 3.2	0.024#
MCHC mean± SD	33.5 ± 0.6	32.2 ± 2.7	32.6± 1.1	31.5 ± 0.7	32.7 ± 1.6	32.4 ± 0.5	32.4 ± 1.2	0.28#
CRP (mg/l) mean± SD	15.8±12.9	14.5 ±6.4	15.5± 7.8	15.6±5.9	11.3± 2.5	10.5±1.0	13.8±7.1	0.179#
CRP groups								
Inflammation (CRP≥10)	1 (20.0)	1 (50.0)	1 (50.0)	4 (80.0)	1 (25.0)	1 (25.0)	9 (40.9)	0.411$
No inflammation (CRP<10)	4 (80.0)	1 (50.0)	1 (50.0)	1 (50.0)	3 (75.0)	3 (75.0)	13 (59.1)	
# ANOVA ; ^ Pearson's Chi-Square; $ Fischer's exact test ; CRP: C-reactive protein								

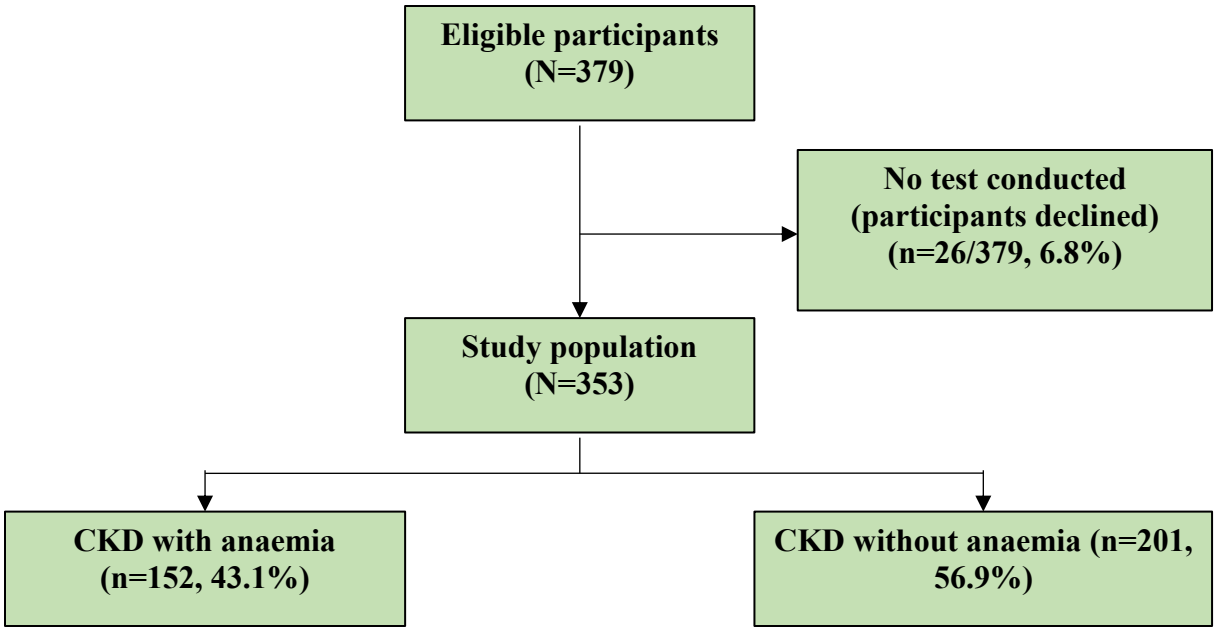

Figure 4:1: Flow chart of the participants

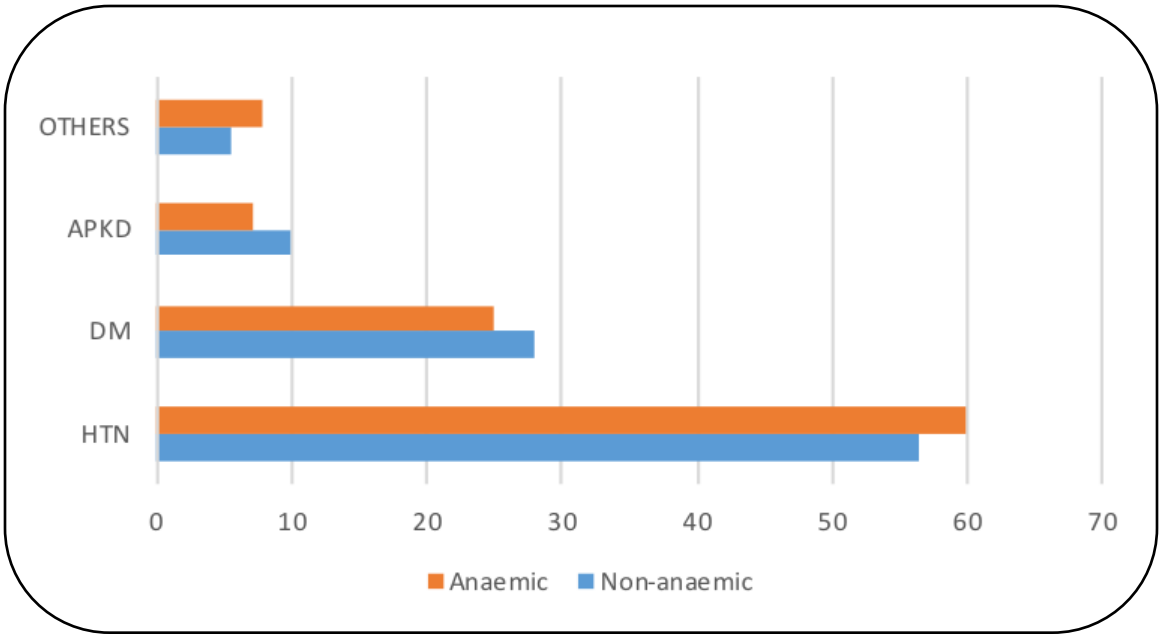

Figure 4:2: The aetiology of chronic kidney disease by anaemic status

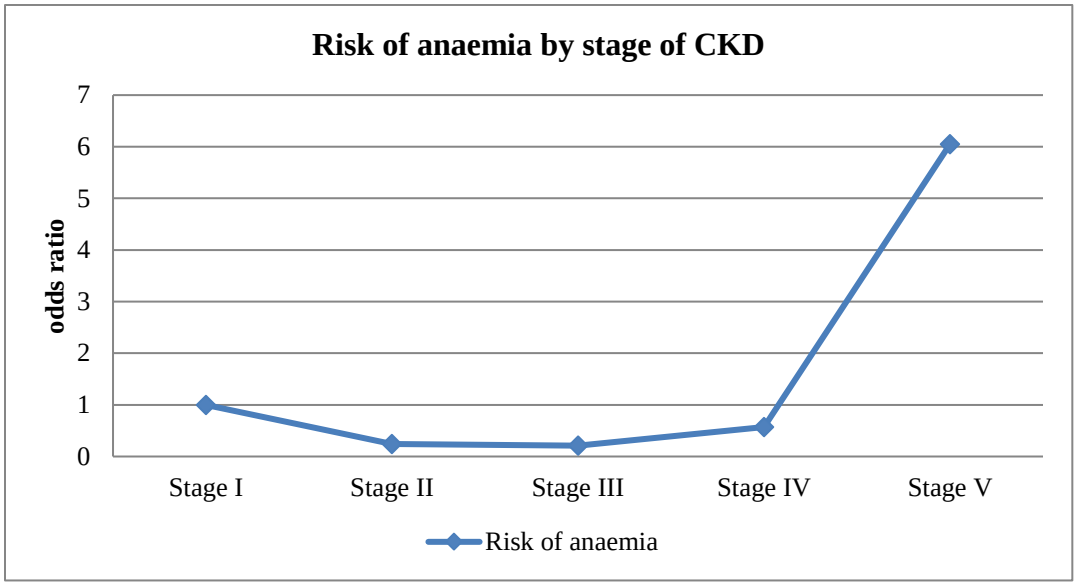

Figure 4:3: Odds ratio of anaemia by CKD stage

4.7. References

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5. CHAPTER FIVE - PAPER TWO: UTILITY OF RETICULOCYTE HAEMOGLOBIN CONTENT AND PERCENTAGE HYPOCHRONIC RED CELLS AS MARKERS OF IRON DEFICIENCY ANAEMIA AMONG BLACK CKD PATIENTS IN SOUTH AFRICA

Nalado, A.M., Mahlangu, J.N., Duarte, R., Paget, G., Olorunfemi, G., Jacobson, B.F. and Naicker, S., 2018. Utility of reticulocyte haemoglobin content and percentage hypochromic red cells as markers of iron deficiency anaemia among black CKD patients in South Africa. *PloS one*, *13*(10), p.e0204899.

5.1. Abstract

Introduction

Iron deficiency anaemia (IDA) worsens the prognosis and outcomes of chronic kidney disease (CKD). However, while the haemoglobin level is unreliable for early detection of IDA, reticulocyte haemoglobin content (CHr) and hypochromic red cells (%HYPO) are early markers of IDA.

Methods

This was a cross-sectional study of black adult participants (n=258) with CKD and apparently healthy members of staff at the Charlotte Maxeke Johannesburg Academic Hospital, South Africa, and the patients' relatives (n=141). It was conducted between 1 June 2016 and 31 December 2016. Serum iron, serum ferritin and transferrin were measured using standard laboratory methods, while the haematology analyser was employed to measure CHr and %HYPO. The validity of CHr and %HYPO as markers of IDA was evaluated. Multivariable binary logistic regression was conducted to determine the predictors of the relationships between IDA, CHr and %HYPO. The area under the receiver operator characteristics (ROC) curve (AUC) of the final models was used to evaluate the discriminatory value of CHr and %HYPO respectively.

Results

About one-quarter (26.1%) of the participants in this study had IDA, which was also found to be more than three times more prevalent among CKD patients than in the controls (35.3% vs 9.2%). Furthermore, 32.3% (95%CI: 27.90% – 37.10%) of the non-anaemic study population had an iron deficiency and the prevalence of iron deficiency without anaemia was lower in CKD patients than

in the controls (29.5% vs 37.6%). The mean age of the CKD patients was higher than in the controls (52.7 ±14.3 vs 40.4 ±12.6 years, P-value<0.001). The sensitivity and specificity for diagnosing IDA among CKD participants was 62.6% and 80.2% respectively for CHr (at a cut-off value of <28pg) and 63.3% and 79.8% respectively for %HYPO. CKD participants with CHr levels lower than 28pg were 82% less likely to be diagnosed as having IDA than those with CHr levels in the region of 28pg or less) (adjusted odds ratio= 0.18, 95% CI: 0.09 – 0.37). The AUC for CHr (0.81, 95% CI: 0.76 – 0.87) was higher than the AUC for %HYPO (0.76, 95%CI: 0.70 – 0.82).

Conclusion

The diagnostic usefulness of CHr and the screening performance of %HYPO in predicting IDA among CKD patients proved to be significantly high. Furthermore, their lower cost in comparison with those associated with conventional markers of IDA recommends their use in clinical practice. Further cost-effectiveness studies of these parameters are therefore warranted.

Key words: chronic kidney disease, iron deficiency anaemia, reticulocyte haemoglobin content, percentage hypochromic red cells, validity

5.2. Introduction

The prevalence of anaemia is high among chronic kidney disease (CKD) patients owing to multiple factors (1). Anaemia also impacts on the morbidity and mortality of CKD patients by accelerating the progression of the disease and reducing the chances of survival (2). The importance of anaemia prevention, monitoring, and management in CKD patients cannot be overemphasised, since an intricate balance must be maintained between the stimulating effects of erythropoiesis and the prevention of an iron overload among CKD patients (3).

Moreover, the treatment of iron deficiency anaemia (IDA) is an important component of care in CKD patients, with numerous benefits, such as a higher tolerance for physical activity, improved cognitive and cardiovascular functioning, an improved quality of life, and lower mortality (1). The monitoring of haemoglobin (Hb) levels alone may not be adequate for evaluating IDA among CKD patients as the decline in haemoglobin levels occurs late in IDA (4). Furthermore, changes in the serum levels of traditional iron parameters such as iron, transferrin saturation, and ferritin may not always correlate with the functional iron deficiency (FID) status of the patient (4). However, FID status is clinically relevant since it defines the instantaneous iron deficiency state of the patient and guides management (4). Monitoring of FID status is also important among CKD patients who are on recombinant erythropoietin therapy (5).

Changes in the morphology and other indices of red blood cells and reticulocytes correlate with the dynamic state of iron deficiency (ID) (2, 3, 5). Hence, the evaluation of IDA among CKD patients (especially those on treatment for IDA) can be enhanced by monitoring their reticulocyte haemoglobin content (CHr) and their hypochromic red cell levels (%HYPO) (6, 7) CHr and %Hypo tests are four times cheaper than the conventional haematological tests and they can be used to detect ID before clinical manifestations of anaemia are observed (6). CHr and %HYPO

can also be reliable for detecting IDA among CKD patients because inflammatory conditions do not affect the levels of reticulocyte haemoglobin content.

Bone marrow iron stores are often regarded as the best indicator (gold standard) of iron status (8). However, a bone marrow biopsy is invasive and predisposes the patient to the risk of infection, or bleeding at the puncture site (9). The bone marrow biopsy procedure is also relatively costly (9). Thus, a bone marrow biopsy is not generally advocated for the routine monitoring of IDA in CKD patients. The determination of the functional iron status of the patient involves measuring the proportion of hypochromic red cells (%HYPO). %HYPO is an index that provides information about the functional iron status of the patient several months before the manifestations of clinical anaemia become evident. Furthermore, it is a late indicator of iron-restricted erythropoiesis (7, 10). Likewise, CHr is an early marker of functional iron deficiency, as reticulocytes exist in the circulation for only one to two days. Thus, CHr can be useful in monitoring the early stage of IDA and responses to erythropoietin therapy (4). Since classical laboratory biomarkers of ID exhibit wide biological variability in CKD patients (10), there is a need to evaluate other novel and consistent markers. The validity of a screening or diagnostic tool is related to the prevalence of the disease and the attributes of the study population. Hence, it is not recommended to extrapolate the results of a validity test on a particular study population onto a different population. Furthermore, since the severity of anaemia also varies across the spectrum of CKD stages, the efficiency of markers of IDA may be affected by the CKD stage in question. Thus, we aimed to evaluate the usefulness of CHr and %HYPO in the diagnosis of IDA and their performance across the CKD stages, and to compare them with other traditional markers of iron deficiency in pre-dialysis black CKD patients.

5.3. Patients and methods

This cross-sectional analytical study was conducted at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) from July to December 2016. The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand. All the study participants signed informed consent forms prior to their enrolment in the study.

Three hundred and ninety-nine (399) adult participants were recruited for the study. The CKD cases (n=258) were black non-dialysis-requiring patients at the Renal Out-Patient Clinic of CMJAH, while the comparator group consisted of the apparently healthy members of staff and the relatives of the patient participants (n=141).

The inclusion criteria for the study were patients aged 18 years and above, who had already been diagnosed with CKD on the basis of an estimated glomerular filtration rate (eGFR) of less than 60ml per minute. The GFR was calculated using the CKD EPI formula (11). Patients who were on immunosuppressant therapy, who were infected with the Human Immuno Virus and with other active infections, and who had known haemoglobinopathies, those who had received a blood transfusion in the three months preceding recruitment, as well as those with malignancies, gastrointestinal bleeding and active inflammation, were excluded from the study.

Early morning venous blood samples were drawn from the patients. Their biochemical iron status, total iron-binding capacity (TIBC), and serum iron and serum ferritin levels were measured. Serum iron was determined by means of the ferrozine calometric method, their TIBC levels through the colorimetric chromazurol dye-binding method using ADVIA 1800 (Siemen Medical Solutions Diagnostic, USA), while their serum ferritin levels were determined by using the two-site chemiluminescent immunometric assay devised by the IMMULITE®2000 system (Siemens

Medical Solutions Diagnostics, USA). Transferrin saturation was calculated by means of the formula: serum iron/TIBC. Complete blood counts and CHr and %HYPO levels were obtained after processing the blood samples using the Siemens ADVIA 2120, Technion H3 RTX and RTC systems analysers (Siemens Medical Solutions Diagnostics, Tarrytown, NY).

Haematological deficiencies were also defined. They were based on the internationally- accepted iron parameters for CKD patients (5). Thus, based on the Kidney Disease Outcomes and Quality Initiative target-to-treat (KDOQI) cut-offs and the National Institute for Health and Care Excellence (NICE) guidelines, absolute iron deficiency was defined when serum ferritin levels were recorded at less than 100µg/L and transferrin saturation (TSAT) levels at less than 20% (12, 13).

Functional iron deficiency was defined as a serum ferritin level of greater than 100ug/L and TSAT at less than 20%. Iron deficiency anaemia was defined at a TSAT level lower than 20%, a ferritin level lower than 100µg/L, and a haemoglobin level (Hb) lower than 12g/dl in women, and lower than 13g/dl in men (4, 5, 13).

Although the Kidney Disease Improving Global Outcomes (KDIGO) defines iron deficiency as a TSAT level lower than 30% and a ferritin level lower than 500ug/L (14, 15), this definition was not used in this study. The prevalence of iron deficiency in CKD patients without anaemia (with Hb levels greater than 12g/dl in women and greater than 13g/dl in men) was also ascertained in our study population.

5.4. Statistical analysis

Continuous variables with a normal distribution were reported as means □ standard deviations, continuous variables with a non-normal distribution were reported as medians, and interquartile

ranges. Categorical variables were described as frequencies and percentages. The correlation of each parameter with other haematological indices was performed using Spearman's correlation coefficients. Among the CKD participants, the specificity, sensitivity, negative predictive and positive predictive value of the CHr (at cut-off <28pg) and %HYPO (at cut-off >5%), as markers of IDA were evaluated using the TSAT and ferritin levels as the reference points. Multiple logistic regression analyses were conducted to determine the associations between CHr and IDA; %HYPO and IDA; and combined CHr/%HYPO and IDA among the CKD participants. Variables with P-values of less than 0.2 on univariable analysis were eligible for inclusion in the multivariable models. Thus, three models were built: one each for: CHr and IDA; %HYPO and IDA; and a combined CHr/%HYPO and IDA. Receiver operator characteristic (ROC) curves, as well as the area under the curve (AUC), for each of the final models were used to further evaluate the discriminatory value of CHr, %HYPO and a combination of CHr and %HYPO in diagnosing IDA. A comparison of the performance of CHr, %HYPO and a combination of the two in predicting anaemia was conducted in terms of the stages of CKD and based on the AUC values. The association between gender and the CKD stages was assessed by means of Pearson's Chi-square test. A P-value of less than 0.05 was taken as the level of statistical significance and we assumed a two-tailed test of hypothesis. All statistical analysis was performed using STATA 14.0 software (Stata Corp, USA).

5.5. Results

The combined model of CHr and %HYPO (Supplementary Table 1) shows that while the odds ratio of CHr did not change significantly (the adjusted odds ratio changed from 0.18 in Table 5 to 0.2 in Supplement 1), the odds ratio for %HYPO was also not significant. There was no statistically significant relationship between gender and the respective CKD stages (P-value = 0.101)

(Supplementary Table 2). A comparison of the performance of CHr and hypochromic red cells in diagnosing iron deficiency anaemia showed, the AUC of the ROC curve was higher for CHr than that for %HYPO (81.3%% vs 76.0%, P= 0.0149) in diagnosing IDA (Table 6, Figure 2 . Thus, CHr has a higher discriminatory value for diagnosing IDA as opposed to that of %HYPO. No additional value emanated from combining CHr and %HYPO for diagnosing IDA as opposed to using only CHr (P-value = 0.94) as an iron marker in diagnosing IDA among our cohort. Furthermore, the CHr marker performed better in the early stages of CKD (stages I and II) than in the later stages (4 and 5) (82% vs 76%) of CKD.

5.6. Discussion

Over the past few decades, studies from developed countries have assessed the diagnostic utility values of non-conventional iron markers such as CHr and %HYPO in predialysis and dialysis patients. However, little is known about the performance of these tests in African CKD populations. Hence, we evaluated the validity and performance of CHr and %HYPO in diagnosing IDA among black CKD patients to provide evidence for their utility value in a low-resource clinical setting such as ours.

We found that the sensitivity and specificity of CHr at a cut-off of 28pg was 62.6% and 80.2% respectively. This finding was higher than that of Fishbaneet al (16) who reported a specificity of 75.5% and a relatively lower sensitivity of 41.9% with the use of CHr in identifying IDA among patients on maintenance haemodialysis. A possible explanation for this finding could be attributed to the relatively long lifespan of erythrocytes. Since information about the status of iron stores in the body over a long period of time can be obtained from changes in erythrocytes levels, CHr can be potentially useful in the early detection of chronic IDA (9). Diagnostic tools should normally have high specificity levels so that positive cases can be determined with a high level of certainty.

Our study revealed that the specificity of CHr was high (80.2%) and those participants with CHr levels of higher than 28pg were 82% less likely to be diagnosed with IDA than those with CHr levels adjusted to about 28pg or less. In other words, those diagnosed with IDA at a cut-off of 28pg would have odds of about 5.6 of being diagnosed with IDA as opposed to those with higher CHr levels. Therefore, in terms of our study population, CHr can be safely considered for use as a diagnostic tool for diagnosing IDA. In addition, we found that the performance of a combination of %HYPO and CHr in detecting IDA did not prove to be superior to using CHr alone as a marker. Thus, the single less-costly test of CHr can be safely used for diagnosis and for monitoring IDA in our communities.

We further assessed and compared the predictive ability of CHr in detecting IDA across all five of the stages of CKD and found that CHr performed better in the early stages of CKD than in the later stages (82% vs 76%), and that this difference was statistically significant (p= 0.0162). To our knowledge, this is the first time a relationship between CHr and its performance in diagnosing IDA according to the stages of CKD, has been evaluated. Our results may be partly explained by the fact that IDA is more frequent in the earlier than the later stages of CKD. Our findings were at variance with those of Aoun (15), who reported a significant difference in iron deficiency by gender and across the different stages of CKD. The differences in our findings could be explained in terms of the differences in the studied populations. However, further studies are required to further explore these findings. Our study highlights the fact that there may be a need to use different cut-off values for CHr levels at different stages in CKD.

Some of the advantages of CHr as an iron marker include its ability to provide a snapshot view of the iron available for erythropoiesis, which can be used in the early detection of IDA (9). It is also a cheaper method for measurement than the conventional iron markers. From our experience, the

unit cost of running CHr is approximately five to seven US dollars (US$5 US$7, as opposed to US$25- US$30dollars for the cost of assessing the TSAT and ferritin levels in CKD patients. Therefore, CHr can serve as an alternative to conventional markers (TSAT and ferritin) in evaluating iron status in resource- impoverished countries.

Furthermore, CHr is free from the biological variability that affects serum iron, ferritin, and other biochemical parameters. CHr also reflects the iron that is made available at the time that the reticulocyte is produced in the bone marrow. CHr levels also highlight abnormalities that are related to the reticulocyte stage of erythropoiesis which may be missed because of the short or transient nature of the reticulocyte stage (10, 11)

Consistent with other studies, the prevalence of IDA in our CKD population was more than three times higher than its prevalence in the control group (35 % versus 9%) (10). Thus, anaemia remains as a major cause of morbidity in our cohort. Previous studies such as the National Health and Nutritional Examination Survey (2004) also reported a high prevalence of iron deficiency in 58% of men and 72% of women with CKD (17). The Dialysis Outcomes and Practice Pattern Study [DOPPS] (2003) reported that iron deficiency was present in 31 to 38% of CKD patients on haemodialysis (18). Another study reported the prevalence of IDA in pre-dialysis CKD to be 29% (19).

The use of various CHr cut-off values in different studies for distinguishing between IDA in non-dialysis and dialysis populations has led to variations in the specificities and sensitivities of this ID marker. For example, studies have defined functional iron deficiency as CHr being less than 28pg (10, 16) and reported that a CHr-cut-off level of less than 28pg is a better predictor of IDA than serum ferritin and transferrin saturation in a cohort of dialysis patients on erythropoietin

therapy (10, 16). However, Kimet al (20) reported a cut-off value of 32pg for predicting IDA among haemodialysis patients. Therefore, interpretations and comparisons in respect of the predictive ability of CHr should be carried out in the contexts of ethnic variations, study populations and whether the participants are on erythropoietin therapy.

A study of non-CKD hospitalised patients in South Africa also used CHr to predict iron deficiency as opposed to other parameters, such as bone marrow aspirates. They established that a CHr of less than 28pg can predict IDA with a sensitivity of 75.8% and a specificity of 84.1%, which is similar to our findings (21).

Another study at Pelonomi Hospital, South Africa, among infants and children showed the optimal CHr cut-off value for the diagnosis of IDA to be 29pg, corresponding to a sensitivity level of 86% and a specificity level of 50%. This cut-off value is slightly higher than our cut-off value of 28pg, and this variation could be due to differences in age among the study population (22).

Consistent with some previous studies, we found that the sensitivity of using CHr to predict iron deficiency is equivalent to that of the conventional parameters (TSAT, ferritin) (6, 7). A further advantage of CHr, which includes its lower and affordable cost, makes it more attractive in low-resource settings. Furthermore, CHr is not influenced by inflammation and infection.

5.7. Study limitations

Our results should be interpreted in the context of the limitations of the study. Our point of reference was serum ferritin/TSAT instead of bone marrow aspirate. However, we believe that our results are valid and would contribute to the growing literature on the subject. Bone marrow aspiration was not feasible on account of its high cost and invasive nature.

There might also be certain diagnostic limitations in respect of CHr as an iron marker, as not all haematology analysers are capable of measuring CHr and the proportion of hypochromic red cells. Furthermore, CHr can also be falsely low in cases of haemoglobinopathies, thus causing microcytic anaemias, or alternatively, falsely high in megaloblastic anaemias. However, this group of patients was excluded from our current study population.

In conclusion, CHr appears to be a useful tool for predicting IDA among black CKD patients not on dialysis. There was no added advantage of combining the test with %HYPO for this purpose. Further studies are required to confirm the usefulness of CHr in this group of patients to improve practice. Furthermore, CHr could be a potential parameter to monitor early response to intravenous iron supplementation in pre-dialysis patients.

Acknowledgement

We thank the participants for participating in this research study.

Declaration of conflict of interest

The authors declare that there is no conflict of interest.

Table 5:1: Comparison of the socio-demographic, haematological and biochemical characteristics of CKD patients and controls

Characteristics	CKD patients	Controls	Total	P-value
Age (years)				
Mean (±SD)	52.7 (±14.3)	40.4 (±12.6)	48.3 (±14.9)	<0.0001
<25	6 (2.3)	18 (12.8)	24 (6.0)	<0.0001
25-34	22 (8.5)	34 (24.1)	56 (10.0)	
35-44	43 (16.7)	43 (30.5)	86 (21.6)	
45-54	65 (25.2)	23(16.3)	88 (22.1)	
55-64	69 (26.7)	16 (11.4)	85 (21.3)	
≥ 65	53 (20.5)	7 (5.0)	60 (15.0)	
Gender, n (%)				
Male	134 (51.9)	59 (41.8)	193 (48.4)	0.054
Female	124 (48.1)	82 (58.2)	206 (51.6)	
Stage of CKD				
stage1/normal	23 (8.9)	97 (68.8)	120 (30.1)	<0.001
stage 2	34 (13.2)	43 (30.5)	77 (19.3)	
stage 3a	32 (12.4)	1 (0.71)	33 (8.3)	
stage 3b	52 (20.2)	0 (0.0)	52 (13.0)	
stage 4	59 (22.9)	0 (0.0)	59 (14.8)	
stage 5	58 (22.5)	0 (0.0)	58 (14.5)	
Systolic Blood Pressure (mmHg), Mean (SD)	143.6 (± 22.8)	135.2 (± 11.3)	140.6 (± 19.9)	<0.0001
Diastolic Blood Pressure (mmHg), Mean (SD)	81.8 (± 15.55)	82.2 (± 10.4)	82.0 (± 13.9)	0.78
Haemoglobin, Hb (g/dl)Mean (SD)	12.2 (±2.7)	14.1 (±1.8)	12.9 (±2.6)	<0.0001
Reticulocyte Hb Median (IQR)	29.33(27.66-31.22)	30.2(28.6–31.2)	29.88(27.88 – 31.22)	0. 0.0256
Hypochromic Red Cells Median (IQR)	7.7(3.1 – 14.2)	3.8 (1.9 - 5.9)	5.3 (2.3 – 12.3)	<0.0001
#MCHC, Mean (SD)	32.4 (± 1.9)	32.6 (± 1.3)	32.4 (± 1.9)	0.307
MCV , Mean (SD)	87.8 (± 6.3)	88.2 (± 5.2)	87.9 (± 5.9)	0.4788
Serum Ferritin, Median (IQR)	103 (58-197)	76.0 (56-144)	92 (56–177)	0.0015
TSAT , Median (IQR)	18 (13-22)	20 (16-24)	18 (14-23)	0.0021
eGFR(ml/min), Mean (SD)	41.2 (±35.0)	99.6 (±18.7)	61.8(±41.2)	<0.0001

^TSAT: Transferrin saturation; eGFR: estimated Glomerular filtration rate; #MCHC: Mean corpuscular haemoglobin concentration; MCV: Mean corpuscular volume; IQR: Inter-quartile range

Table 5:2: Iron status in the study participants

	CKD n (%)	Controls n (%)	Total	P-value
Iron Deficiency Anaemia	91 (35.3)	13 (9.2)	104(26.1)	<0.001
Functional Iron Deficiency Anaemia	48 (18.6)	2 (1.4)	50 (12.5)	<0.001^
Absolute Iron Deficiency Anaemia	43 (16.7)	11 (7.8)	54 (13.5)	0.013#
^Fischer's exact test # Chi-square				

Table 5:3: Correlation of reticulocyte haemoglobin content and percentage hypochromic red cells with other haematological parameters among CKD participants and healthy controls

Haemato-logical parameters	**Chronic Kidney Disease patients**				**Healthy Controls**			
	Hypochromic level		CHr		Hypochromic level		CHr	
	A	P-value	A	P-value	A	P-value	A	P-value
HB	-0.27	<0.0001	0.56	<0.0001	-0.30	0.0004	0.18	0.0372
MCV	0.009	0.8999	0.33	<0.0001	-0.42	<0.0001	0.59	<0.0001
MCHC	-0.42	<0.0001	0.38	<0.0001	-0.54	<0.0001	0.37	<0.0001
α: Spearman's rank order correlation coefficients								

Table 5:4: Predictive ability of reticulocyte haemoglobin content and percentage hypochromic red cells using TSAT and ferritin levels as reference parameters among chronic kidney disease participants and healthy controls

		CKD patients		Total	Healthy Controls		Total
		NIDA	IDA		NIDA	IDA	
CHr n (%)	≤28pg	33(19.76)	57(62.64)	90 (34.88)	22(17.19)	6 (46.15)	28 (19.86)
	>28pg	134(80.24)	34(37.36)	168(65.12)	106(82.81)	7(53.85)	113(80.14)
%HYPO	>5%	93 (55.69)	67(73.63)	160(62.02)	34 (26.56)	9 69.23)	43 (30.50)
	≤5%	74 (44.31)	24(26.37)	98(37.98)	94 (73.44)	4 (30.77)	98 (69.50)
	Total	167	91	258	128	13	141

IDA: Iron deficiency anaemia; NIDA: Non-iron deficiency anaemia

Table 5:5: Relationship between reticulocyte haemoglobin content, percentage hypochromic red cells and iron deficiency anaemia among chronic kidney disease participants

	[1]Multivariable logistic regression analysis for CHr			[2]Multivariable regression analysis for %HYPO		
Factors	**OR**	**95% CI**	**P-value**	**OR**	**95%CI**	P-value
CHr						
≤ 28 (ID)	1.00	Ref	Ref	-	-	-
>28 (NID)	0.18	0.09 - 0.37	<0.001	-	-	--
Percentage hypochromic red cells						
>5% (ID)				1.00	Ref	Ref
≤5% (NID)				0.52	0.28-0.96	0.037
Gender						
Male	1.00	Ref	Ref	1.00	Ref	Ref
Female	2.12	1.16 - 3.89	0.015	2.25	1.27- 4.01	0.006
Age group (years)						
<25	1.00	Ref	Ref	1.00	Ref	Ref
25 -34	0.25	0.04 – 1.63	0.147	0.29	0.05 - 2.11	0.192
35 -44	0.15	0.03 – 0.88	0.036	0.21	0.04 - 1.35	0.069
45 -54	0.20	0.04 – 1.06	0.058	0.26	0.06 - 1.48	0.101
55 -64	0.86	0.18 – 4.22	0.855	1.06	0.25 - 6.04	0.941
65 and above	0.41	0.08 – 2.03	0.272	0.37	0.08 - 2.03	0.230
Stage of kidney disease						
Early stage	1.00	Ref	Ref	1.00	Ref	Ref
Late stage	2.64	1.34 -5.21	0.005	4.35	2.36 - 8.03	<0.001

[1]Multivariable logistic regression of the model of the relationship between CHr and iron deficiency anaemia (CHr only).
[2]Multivariable logistic regression of the model of the relationship between %HYPO and iron deficiency anaemia (%HYPO only).
The two models corrected for gender, age, stage of disease.
OR: Adjusted odds ratio.
Early stage: Stages 1 to 3;
Late stage: Stages 4 and 5.
ID: Iron deficiency NID: Non-iron deficiency

Table 5:6: Performance of reticulocyte haemoglobin content and percentage hypochromic red cells in the diagnosis of iron deficiency anaemia in chronic kidney disease participants

Parameter	**AUC of ROC**	**(95%CI)**
CHr	0.81	0.76 - 0.87
Hypochromic red cells	0.76	0.70 - 0.82
CHr + Hypochromic red cells	0.81	0.76 - 0.87
CHr by stage		
Early	0.82	0.72 - 0.84
Late	0.76	0.70 - 0.82
Hypochromic red cells by stage		
Early	0.77	0.70 – 0.82
Late	0.72	0.58 – 0.72
CHr+Hypochromic Red cells by stage		
Early	0.85	0.71- 0.84
Late	0.76	0.68 - 0.81

AUC: Area under curve; \

ROC: Receiver operator characteristics curve

95% CI: 95% Confidence Interval.

Comparison of Performance:

CHr Vs % HYPO, P-value= 0.0149,

CHr Vs (CHr+%HYPO), P-value = 0.94;

%Hypo Vs (CHr+%HYPO), P-value = 0.0162

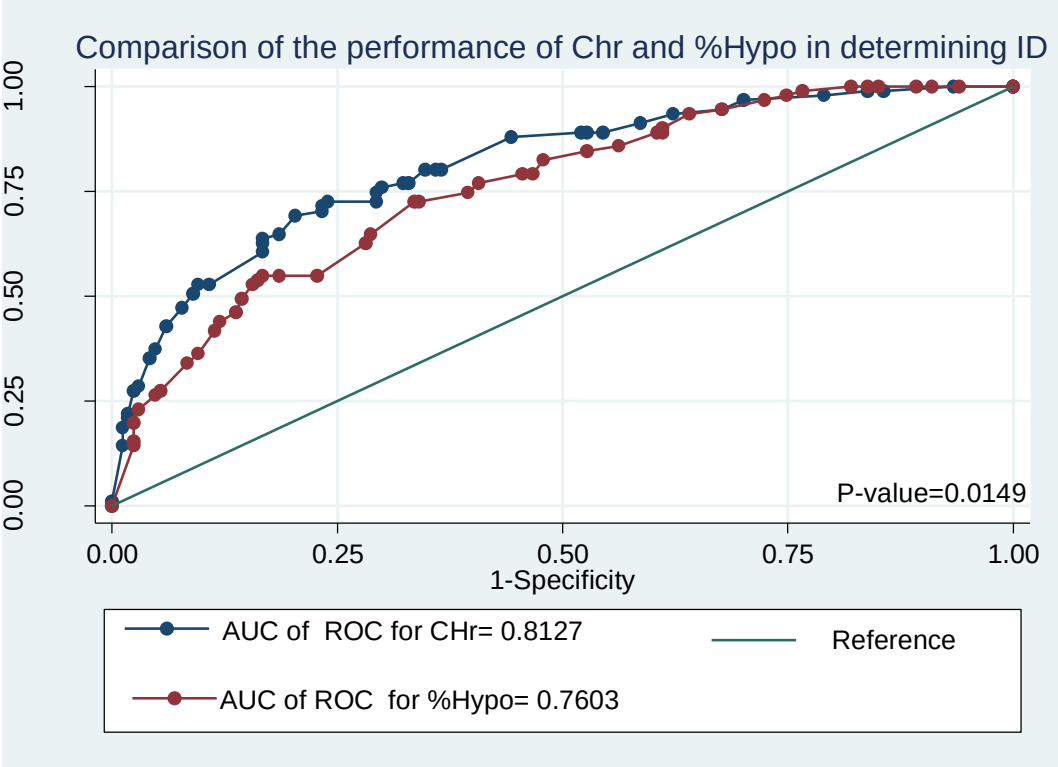

Figure 5:1: Comparison of the receiver operator characteristics (ROC) of reticulocyte haemoglobin content (CHr) and percentage hypochromic red cells

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6. CHAPTER SIX - PAPER THREE: HEPCIDIN AND GDF-15 ARE POTENTIAL BIOMARKERS OF IRON DEFICIENCY ANAEMIA IN CHRONIC KIDNEY DISEASE PATIENTS IN SOUTH AFRICA

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Status: Under review

6.1. Abstract

Background

Iron deficiency anaemia is a major cause of morbidity and mortality among chronic kidney disease (CKD) patients. There is paucity of information on the role of hepcidin and growth differentiation factor-15 (GDF-15) as potential biomarkers of iron deficiency anaemia among non-dialysis CKD patients. The aim of this study was to determine the utility of hepcidin and GDF-15 as biomarkers of iron deficiency among non-dialysis CKD patients at an academic hospital in Johannesburg, South Africa.

Method

A cross-sectional study of 312 consecutive consenting non-dialysis CKD patients, and 184 controls at Charlotte Maxeke Academic Hospital was conducted from June 2016 to December 2016. Socio-demographic and clinical characteristics were recorded. Plasma hepcidin and GDF-15 were measured using mass spectrometry and ELISA, respectively. Spearman rank correlation, linear and logistic regression and receiver operator curves were utilised to evaluate the predictive and diagnostic/reference values of hepcidin and GDF-15 in absolute and functional iron deficiency anaemia.

Results

The mean age of participants was 49.7 ±15.8 years, and 50.6% of them were females. The predictive value of diagnosing iron deficiency anaemia among CKD patients using GDF-15 and hepcidin was high (AUC=0.723 and 0.714, respectively). There was a weak negative correlation between hepcidin levels and GFR (r=-0.19, p=0.04) in anaemic CKD patients, and between serum GDF-15 and haemoglobin (r=-0.34, p=0.001). Serum ferritin (β=0.005, P-value<0.001), MCV (β=0.0276, P-value=0.029) and gender (β=-0.2188, P-value=0.042) were predictors of log

hepcidin. Urea (β=0.0062, P-value=0.044), MCHC (β= -0.0816, P-value<0.001) and gender (β=-0.0755, P-value=0.001) were predictors of logGDF-15. Both GDF-15 (P=0.028) and hepcidin (P=0.049) were associated with iron deficiency anaemia. Subgroup analysis showed that GDF-15 predicted absolute iron deficiency, while hepcidin predicted functional iron deficiency anaemia

Conclusion

GDF-15 and hepcidin are potential predictors of iron deficiency anaemia among CKD patients.

Keywords: iron deficiency anaemia, GDF-15, hepcidin, utility, chronic kidney disease

6.2. Introduction

Anaemia is a common presenting feature among patients with chronic kidney disease (CKD) and is associated with poor quality of life and attendant poor clinical outcomes (1). The pathogenetic mechanisms of anaemia in CKD include reduced erythropoietin production and reticuloendothelial iron blockade secondary to chronic kidney inflammation (2). Whilst largely responsive to erythropoietin replacement, anaemia due to chronic inflammation may be resistant to erythropoietin (EPO) treatment (3). Thus, the management of iron homeostasis should be optimized to enhance the effect of EPO treatment on CKD-associated anaemia (4).

Hepcidin is a peptide hormone that regulates iron balance in the body (5). It is synthesized by hepatocytes and reduces iron absorption and cellular release of iron, through binding to ferroportin (6). Thus, in chronic diseases, elevated levels of hepcidin can decrease absorption of dietary iron and impair the release of iron from its reservoir in the hepatocytes and macrophages (7). Further, increased hepcidin production leads to a decline in plasma iron levels, with an attendant negative impact on erythropoiesis (2). Studies have also shown that patients with chronic infections and inflammatory disease are predisposed to having increased levels of hepcidin (6, 8). Elevated hepcidin levels may therefore play a role in the anaemia of chronic kidney disease (5). The impact of hepcidin on iron metabolism may be increased among CKD patients who have chronic underlying inflammation (5). Hepcidin inhibits iron release from macrophages as well as intestinal iron absorption. In inflammatory states, hepcidin production is no longer regulated by iron burden (i.e., if the iron level is low, hepcidin synthesis should be downregulated) but is rather increased through IL-6 stimulation (9). Moreover, higher levels of hepcidin may occur among CKD patients who are likely to have poor renal clearance of plasma hepcidin, as their kidney function declines

(2). The hepcidin and Growth differentiation factor-15 (GDF-15 levels) in CKD patients may not be of greater diagnostic value than conventional parameters, but further studies on these biomarkers are needed (10). Moreover, detection of the association of GDF-15 expression with serum iron parameters in patients with IDA maybe helpful for the diagnostic and pathogenic impact of GDF-15 in anaemia, the role of GDF-15 in IDA is still controversial (11). It is also plausible that increased hepcidin concentrations may cause iron-restricted erythropoiesis in CKD associated anaemia (12). From the foregoing observations, we postulate that serum hepcidin level may be a useful biomarker of iron status among CKD patients. Hepcidin inhibitors or hepcidin lowering agents may be effective stand-alone or adjunctive therapy for maintaining normal iron homeostasis, thereby improving anaemia among CKD patients (13). These inhibitors of hepcidin production or action have shown promise in the treatment of anaemia of inflammation in pre-clinical studies and are now entering human clinical trial (14). However, the utility of hepcidin as a biomarker in the diagnosis of iron deficiency anaemia (IDA) among non-dialysis CKD patients is unclear, especially in low resource settings.

Growth Differentiation Factor-15 (GDF-15), an anti-inflammatory cytokine, has been suggested as a major regulator of hepcidin (15). Oxidative stress and inflammatory conditions can stimulate secretion of GDF-15 by macrophages (16), and GDF-15 has been implicated in the evolution of tumours (17, 18) and atherosclerosis (18). In addition, a strong positive correlation has been reported between hepcidin and GDF-15 in some anaemic patients (19). Emerging evidence shows that in response to anaemia (20), erythroblasts secrete GDF-15, which in turn suppresses hepcidin expression and decreases iron stores (16, 21, 22). It is also plausible that GDF-15 could signal intra-renal injury as circulating GDF-15 levels strongly correlated with intrarenal expression of

GDF-15, and GDF-15 was found to be associated with increased risk of CKD progression in two independent cohorts of CKD patients in the Clinical Phenotyping and Resource Biobank (C-PROBE), and the Seattle Kidney Study (SKS).(23, 24). Little is known about the relationship between GDF-15, hepcidin, and IDA among patients with non-dialysis requiring CKD. The aim of this study was to evaluate serum GDF-15 and hepcidin as surrogates or diagnostic markers of IDA among non-dialysis requiring CKD patients.

6.3. Materials and methods

We conducted a comparative cross-sectional study among 312 consecutive patients with CKD attending the renal outpatient clinic of Charlotte Maxeke Johannesburg Academic Hospital, South Africa from 1 June 2016 to 31 December 2016, and 184 healthy controls (which included patients' relatives and staff members). The inclusion criterion was all adult stable consenting non-dialysis CKD patients aged 18 years and above. This study was approved by the University of the Witwatersrand Human Research Ethics Committee, certificate number 150929. All patients gave written informed consent.

Exclusion criteria included active inflammation, active infection, haemoglobinopathies, and blood transfusion within 3 months preceding the study, immunosuppressive therapy, oral iron treatment, and use of intravenous iron therapy at least 2 weeks before enrolment, and persons currently on erythropoietin stimulating agents (ESA).

We defined absolute iron deficiency as serum ferritin <100ug/l and transferrin saturation (TSAT) <20%, while functional iron deficiency was defined as serum ferritin >100ug/l and TSAT <20%, according to Capellini et al (25). Anaemia was defined as haemoglobin <13g/l in men and <12g/l

in women. Patients were considered to have IDA if they presented with absolute iron deficiency, functional iron deficiency and anaemia with low mean corpuscular volume (MCV) (26, 27). Glomerular filtration rate (GFR) was determined by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation for eGFR (28).

6.3.1. Laboratory Measurements

All collected venous blood samples were immediately centrifuged, separated into aliquots, and stored at -80 degrees Celsius for future assays. Haematological measurements were made on fresh venous blood with EDTA and clotted blood samples. Haemoglobin concentrations (Hb), red blood cell count (RBC), platelet count, creatinine and urea were measured using a haematology analyzer (Siemens Diagnostics, Tarrytown, USA). Serum iron was determined by the ferrozine calometric method, total iron binding capacity (TIBC) by a colorimetric chromazurol dye binding method using ADVIA 1800 (Siemen Medical Solutions Diagnostic, USA), and serum ferritin was determined by using a two-site chemiluminescent immunometric assay by IMMULITE®2000 system (Siemens Medical Solutions Diagnostics, USA). Transferrin saturation (TSAT) was calculated as the ratio of serum iron and TIBC and expressed as a percentage.

Hepcidin and isotope labelled internal standards [$^{13}C_9$,$^{15}N_3$] were purchased from Peptide Institute (Osaka JPN). The serum hepcidin-25 and isotope labelled internal standards [$^{13}C_9$,$^{15}N_3$] were extracted and separated as previously described by Li et al (29) Briefly, samples were extracted with 4% HPLC grade trichloroacetic acid (TCA, Merck, Kenilworth, NJ, U.S.A) and separated using reverse-phase liquid chromatography using a 0.5x50mm Halo Peptide- ES column (Sciex, Framingham, USA) with a total run time of 5 min per sample. The analysis was performed on an

AB Sciex 5500 QTRAP (Sciex, Framingham, USA), coupled to a micro-Liquid Chromatography (Exigent M3) system with Turbo IonSpray ionization source operated in positive ion mode Multiple reactive monitoring (MRM) detection was applied using the Sciex 5500 for identification and quantification using the ion transitions (m/z) of 698.0>354.0 and 703.2>354.1 for hepcidin and stable isotope labelled hepcidin [$^{13}C_9$,$^{15}N_3$] respectively.

Concentrations of serum hepcidin were expressed in nanograms per millilitre (ng/ml). The intra- and inter-assay coefficients of variation (CV) were <6.7% and < 8.8%, respectively. The lower limit of detection was 0.5ng/ml. The median reference level for plasma hepcidin in healthy controls was 5.7 ng/ml. Serum levels of GDF-15 were measured by ELISA (R&D Systems, Minneapolis, MN, USA) with intra- and inter-assay CVs of <2.8 and <6, respectively.

6.4. Statistical analysis

Statistical analysis was performed using Stata version 14 (Stata Corp., TX, USA) software. Normally distributed continuous variables were presented as mean (± standard deviation), and non-normally distributed continuous variables were presented as median (interquartile range). Categorical variables were presented as numbers and percentages. The 5th and 95th percentile of the hepcidin and GDF-15 levels among non-anaemic apparently healthy controls were determined as the reference range for the study population. Student's t-test or Mann-Whitney U tests were used to compare continuous variables among anaemic and non-anaemic groups. Association between anaemic status and categorical variables were assessed using Pearson's Chi-square test. Spearman's rank correlation was used to determine the linear relationship between hepcidin or GDF-15 and other linear variables among anaemic and non-anaemic participants. Univariable and

multivariable linear regression was conducted with log hepcidin and logGDF-15 as outcome variables respectively. Univariable and multivariable logistic regression was conducted to determine the association between IDA and hepcidin. Confounding variables based on the literature and a univariable p-value of <0.2 were utilised to build the multivariable model using backward stepwise regression. Age and gender were chosen a priori. A post-estimation receiver operator characteristic curve (ROC) with area under the curve (AUC) was then utilised to determine the predictive value of hepcidin for IDA among CKD participants. Similar regression analysis was conducted between IDA and GDF-15. The AUC of ROC for hepcidin and GDF-15 were then compared. A non-covariate table of cut-offs with corresponding sensitivity and specificity was generated for hepcidin as a diagnostic marker for IDA among the CKD population. The optimum cut-off point of hepcidin was then calculated based on the maximal value of the Younden Index (= (sensitivity + specificity) -1). Similarly, an optimum cut-off value for GDF-15 was obtained. The above-mentioned analysis (regression, ROC and cut-offs) was conducted to determine the predictive value of hepcidin and then GDF-15 for functional and absolute IDA. Sub-group analysis was conducted to see if there was disparity in the performance of hepcidin and GDF-15 in diagnosis of absolute and functional IDA. For all analyses, a 2-tailed test of hypothesis was assumed, and the level of statistical significance was set at P-value <0.05 (95% confidence interval).

6.5. Results

The mean age of the participants was 49.7 (± 15.8) years and there was an almost equal proportion of male (49.4%, n= 245/496) and female (50.6%, n=251/496) participants. The prevalence of anaemia among CKD cases was about three times that of controls, 33.0% (95%CI: 27.99% – 38.45%) vs 9.78% (95%CI: 6.22 % - 15.05%) respectively.

The median levels of serum GDF-15 [1024.5 (429.4 - 1489.5pg/ml) vs. 447.25 (188.25 - 1192.3pg/ml), P-value<0.0001] and hepcidin [7.1 (3.9 - 36.2) vs 3.1(2.1 – 10.9), P-value <0.0001] were more than doubled among the CKD participants as compared to the controls. Among the CKD participants, the median GDF-15 level was higher among the anaemic as compared to the non-anaemic participants (P-value <0.0001, Table 1). Similarly, median GDF-15 levels were higher among the anaemic controls as compared to the non-anaemic controls (P= 0.0155). In contrast, there was no difference in the serum levels of hepcidin by anaemia status among the CKD participants (P-value =0.2790) and the controls (P-value = 0.8357)

The reference values (5% - 95% range) among the non-anaemic controls of this study for hepcidin and GDF-15 were 1.5 - 28.1 ng/ml and 108.2 – 2833 pg/ml, respectively. There was a significant negative linear relationship between hepcidin and GDF-15 among anaemic CKD patients (r=-0.28, P-value = 0.0037); supplementary Table 1.

Serum ferritin (r= 0.5, P-value <0.0001), and serum creatinine levels (r= 0.21, P-value = 0.0368) were positively correlated with hepcidin, while GFR (r= - 0.19, P-value = 0.0493) was negatively correlated with hepcidin among the participants with CKD, supplementary table 1.GDF-15 was negatively correlated with serum ferritin and haemoglobin levels, while TSAT level correlated with GDF-15 , especially among the anaemic CKD cases (supplementary table 2).

For every ng/ml increase in serum ferritin level among CKD patients, log hepcidin increased by 0.0058 (β=0.0058, P-value<0.001). In other words, hepcidin levels increased by $10^{0.0058}$ units for every ng/ml increase in serum ferritin. Similarly, for every g/dl increase in MCV, log hepcidin increased by 0.0276 (β=0.0276, P-value=0.029). Similarly, logGDF-15 increased with every unit

increase of urea (β=0.0062, P-value=0.044) but decreased with every unit increase of MCH (β= -0.0816, P-value<0.001), Table 2

The predictive value of GDF-15 and hepcidin for diagnosing IDA among CKD participants was 72% (95%CI: 66% – 78%) and 71% (95%CI: 65% – 78%), respectively. There was no statistically significant difference between the AUC of the ROC curves of GDF-15 and that of hepcidin, (P-value = 0.49, Figure 1A). A combination of the two parameters did not improve the diagnostic value of either of the 2 tests, as the AUC of the ROC of the model with the combination was 73%, 95%CI: 67% – 79%; P-value = 0.29.

Using the non-covariate analysis and Younden's index, the optimum cut-off value among CKD participants for GDF-15 was 1,030 pg/ml (at a sensitivity of 72.8% and specificity of 61.24%). Similarly, the optimum cut-off for hepcidin was 22.5ng/ml (at a sensitivity of 38.8% and specificity of 70.8%); (Data not shown).

The predictive value of GDF-15 for diagnosing absolute IDA among CKD participants was 81.9%. (Table 6, Figure 1). Using the non-covariate analysis and Younden's index, the optimum cut-off value of GDF-15 for diagnosing absolute IDA among CKD participants was 1129.3 mg/dl (at a sensitivity of 83.64%and specificity of 66.03%). Similarly, the optimum cut-off for hepcidin was 22.5ng/dl (at a sensitivity of 66.7% and specificity of 70.8%); (Table 6).

6.6. Discussion

To our knowledge, this is the first study to evaluate the clinical utility of hepcidin and GDF-15 as markers of IDA, among persons with non-dialysis CKD. This study demonstrates that in a cohort of predialysis CKD patients, serum hepcidin and GDF-15 predicted IDA among patients with CKD, with a predictive value of 71.4% and 72.3%, respectively, and GDF-15 predicted absolute

IDA, while hepcidin predicted functional IDA (83.6% vs 66.7%). In addition, we showed a negative correlation between hepcidin and eGFR among CKD patients. There was no significant correlation between eGFR and GDF-15 among anaemic patients with CKD. Our observation of a negative correlation of hepcidin with GFR agrees with findings by others (12, 30). Ashby et al, after correcting for ferritin levels, concluded hepcidin inversely correlated with eGFR (31). In contrast with our study, Zaritsky et al (32) and Lee et al (33) found that eGFR had a positive correlation with hepcidin in adult CKD patients, despite using similar assay methods (mass spectrometry) as with our study; however, hepcidin levels in their paediatric CKD patients did not correlate with eGFR. Sung Woo Lee et al (33) findings are also at variance with our findings; they found eGFR to positively correlate with hepcidin levels in patients with advanced CKD (stage 3b-5). This suggests that the relationship between hepcidin and renal function is still unclear. Thus, different aetio-pathogenic pathways may play varying roles among the CKD stages. Furthermore, the mechanism of action of hepcidin in CKD may differ from other low molecular weight proteins such as β_2 microglobulin or cystatin-c. Hepcidin pathways may be largely dependent on ferritin metabolism, with renal function playing a minimal role. The smaller sample size of previous studies may affect their conclusions. Our study recruited a relatively larger sample size. The sensitivity of the methods used may also play a role in the observed differences between our findings and others as we used mass spectrometry, in contrast to other studies where ELISA was used (30, 34).

Our data supports published studies reporting higher levels of hepcidin in CKD and among haemodialysis patients (31, 35). The increase in serum hepcidin levels in CKD patients compared to controls might be due to increased inflammation and decreased renal clearance of hepcidin attributable to renal impairment in CKD patients (36-38).

We found that serum ferritin the primary storage molecule for cellular iron positively correlated with hepcidin in our CKD patients, as previously documented among patients with CKD and those on dialysis (31, 35, 39-41). This finding is also consistent with studies in non-CKD populations (42-44). In the setting of CKD, the direct relationship of hepcidin with ferritin may represent a protective effect of hepcidin against systemic iron overload (32). However, there was no correlation between hepcidin and inflammatory markers such as hsCRP. The possible explanation may be that patients with active infections and inflammation were excluded from our study. The reference range (5%-95%) of serum hepcidin levels among healthy controls in this study was 1.5-28.1ng/ml. This value was lower than the reference range reported among healthy male and female adults [median and 5-95% reference range, 112ng/ml (29-254ng/ml) for men and 65ng/ml (17-286ng/ml) for women] (39). A possible explanation may be differences in assay used, and racial differences. Thus, there is need to establish different reference ranges for different populations.

We determined a cut-off value for serum hepcidin of <22.5ng/ml, (AUC= 0.71) at a sensitivity of 38.8%, and specificity of 70.8%, to predict IDA, which supports the literature that hepcidin levels are below the reference range in iron deficient states (45). In 261 premenopausal female blood donors, serum hepcidin <8ng/ml (with a 95% confidence range of serum hepcidin levels from 8.2-199.7ng/ml) had a sensitivity of 41.5% and specificity of 97.6% for diagnosing IDA, while serum hepcidin <18ng/ml had a sensitivity of 79.2% and specificity of 85.6% (46). A study by Vyas et al (47) determined hepcidin cut-off points to diagnose iron deficiency of <34.55, with AUC 0.845, sensitivity of 98.33%, specificity 52.94%. Choi et al reported that hepcidin levels <6.895 ng/ml had a sensitivity of 79.2% and specificity of 82.8% for diagnosis of iron deficiency in children (48). Sanad et al found urinary hepcidin at a cut-off value of <0.94 nmol/mmol to predict IDA with a sensitivity of 88% and specificity of 88% (49). These findings differ from the study by Jonker et

al(50), who reported hepcidin to be a poor predictor of bone marrow iron stores (sensitivity of 66.7% and specificity 48.5%). However, their study population included children, and intra-individual variability in serum hepcidin could account for differences in our findings. This is the first study that showed in a sub-group analysis that hepcidin was predictive of functional IDA in pre-dialysis CKD. Although levels of hepcidin are elevated in CKD, other factors may affect hepcidin levels. Further research is needed to determine whether this biomarker has advantages over conventional markers (ferritin, TSAT) (10).

We found GDF-15 levels to be significantly higher in CKD patients with IDA as compared to CKD patients without IDA, consistent with findings by Yilmaz et al (51). Although Yang Li et al (52) found an increase in GDF-15 levels in their dialysis patients, they were unable to demonstrate a correlation between GDF-15 and iron indices, a finding that was prominent in our study. There are possible mechanisms that may explain the findings of increased GDF-15 in patients with IDA. First, GDF-15 may be an important mediator in a negative feedback loop whereby it increases to supress high hepcidin levels in CKD patients with iron deficiency. Second, GDF-15 expression could be upregulated in response to chronic inflammatory processes, which often occur in patients with IDA and CKD (53, 54). Another explanation is that iron depletion could independently cause GDF-15 induction in the erythroid precursor cells as a result of iron sequestration in macrophages (55). All these are speculative, however, as the exact mechanism underlying IDA and GDF-15 levels require further investigation.

Another important finding in this study was GDF-15 predicting IDA, at a cut-off value <1,030 pg/ml with a predictive value of 72% (AUC = 0.723), sensitivity of 72.8%, and specificity of 61.24%. Lakhal et al (22) also observed a significant increase in GDF-15 in persons with iron deficiency, which was similar to our findings. In contrast, Tanno et al (56) found among blood

donors that there was no association between iron deficiency due to blood loss and serum GDF-15 levels. Our study therefore suggests that GDF-15 can be a useful diagnostic tool in patients with IDA, and may relate to the fact that erythroid cells from patients with IDA may secrete a GDF-15- specific stimulator that is induced during iron deficiency (55).However, inflammation-mediated changes seen in iron homoeostasis may not be likely to induce the increased GDF-15 levels in patients with IDA, as serum ferritin levels did not corelate with GDF-15 levels in this study. Mast et al(57) also found absence of correlation of GDF-15 with ferritin, which was similar to our finding .GDF-15 in this study was found to be predictive of absolute IDA in sub-group analysis, this finding is in contrast with findings of Yilmaz et al (51) where GDF-15 was predictive of functional IDA among haemodialysis and CKD patients. Differences in these findings could be due to differences in the study population, as their population included a dialysis population, and our study population was pre-dialysis. Another explanation could be due to different cut-off values to define functional IDA; their cut-off value was >800, while ours was >100. Further research is required to explore the puzzle of GDF-15 in IDA in CKD patients.

We found a negative correlation between participants' age and GDF-15 levels, which is at variance with reports by other researchers (58-61). The difference in these findings could be attributed to differences in the study population as our study was carried out in a pre-dialysis population, and their studies involved dialysis patients, and a normal healthy population. The association of GDF-15 with age requires further prospective, multicentre studies.

Another important finding in this study is the negative correlation of GDF-15 with haemoglobin in both CKD patients and controls. This finding is consistent with reports by others (52, 58, 62), but is in disagreement with findings in non-CKD populations, where GDF-15 was positively

correlated with haemoglobin (63-65). The difference in findings between our study and others could be explained by differences in the study population.

Our study has limitations. First, the cross-sectional study design makes it difficult to infer causality between hepcidin, GDF-15 levels, and the risk of anaemia. The cross-sectional study design also precluded us from conducting follow up serial measurements of both hepcidin and GDF-15. The intra-patient variability and diurnal variations in hepcidin assays, may interfere with serum hepcidin measurement..

The strengths of our study include the large sample size of pre-dialysis CKD patients, and our use of mass spectrometry, the gold standard for hepcidin assay. In addition, our study defined iron deficiency based on absolute and functional IDA, whereas prior studies focused mostly on functional IDA, and mainly recruited dialysis patients. Finally, to our knowledge, this is the first study to predict the performance of GDF-15 as a diagnostic tool for IDA in non-dialysis CKD patients.

In conclusion, our preliminary data indicates that GDF-15 and hepcidin predict IDA in pre-dialysis CKD patients, and could be promising tools in the diagnosis of IDA in CKD, and they could predict absolute and functional IDA, among pre-dialysis CKD. These assays are expensive, not readily available, and require technical know-how, which may preclude their use in resource constrained environments. Larger randomized prospective studies are required to confirm our findings and to help determine a reliable cut-off value of serum hepcidin, and GDF-15 in the diagnosis of IDA.

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Declaration of conflicting interest

None of the authors have any conflict of interest to declare.

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Table 6:1: Socio-demographic, haematological and biochemical characteristics of participants by iron-deficiency-anaemia status

Characteristics	CKD (n= 312)			Controls (n=184)		
	IDA n= 103 (%)	**Non-anaemic n=209 (%)**	**P-value**	**IDA n=18 (%)**	**Non-anaemic n=166 (%)**	**P-value**
Hepcidin (ng/ml) (median, IQR)	8.4 (4-45.5)	6.8 (3.9 -33.6)	0.2790	3.1 (2.3 – 7.9)	3.1 (2.1- 10.9)	0.836
GDF-15 (pg/ml) (median, IQR)	1256.8 (919.1 - 1618)	700 (335.1- 1327.8)	<0.0001	1175.9 (708.9 - 1267.1)	397.95 (183.2- 1101)	0.016
Age (mean ±SD) years	54.5 ± 15.2	54.3 ± 14.2	0.9340	45.2± 17.5	41.3 ±13.6	0.255
<40	15 (14.6)	37 (17.7)	0.484	8 (44.4)	79 (47.6)	0.800
≥40	88 (82.3)	172 (85.4)		10 (55.6)	87 (52.4)	
Race						
Blacks	95 (92.2)	165 (79.0)	0.003	12 (66.7)	133 (80.1)	0.185
Whites	8 (7.8)	44 (21.1)		6 (33.3)	33 (19.9)	
Gender						
Male	41 (39.8)	126 (60.3)	0.001	7 (38.9)	71 (42.8)	0.752
Female	62 (60.2)	83 (39.7)		11 (61.1)	95 (57.2)	
Systolic Blood Pressure (median, IQR) (mmHg)	139 (125 – 157)	140 (130 - 160)	0.1994	132 (120 – 140)	132 (130 - 140)	0.998
Diastolic Blood Pressure (median, IQR) (mmHg)	80 (70- 91)	80 (70 – 90)	0.6234	81 (70 -90)	80 (72 – 90)	0.7707
Serum Urea (median, IQR) (mmol/L)	17.1 (9.8 - 25.7)	11 (7.1 – 15.9)	0.0001	4 (3-4.9)	4.35 (3.6 – 5.2)	0.4486
Serum Creatinine (median, IQR), μmol /L	265 (158- 520)	171 (120 – 255)	<0.0001	70.5 (64 – 78)	78.5 69 – 91)	<0.0001
GFR (median, IQR), mls/min/1.73m^2	27.0(12.6 – 27.0)	43.6 (27.7 – 66.0	<0.0001	129.8(96.3 - 139.5)	114.4(96.8 – 133.0)	0.3490

Table 6:2: Multiple linear predictors of log Hepcidin and logGDF-15 among CKD patients

Variable	Log Hepcidin			Log GDF-15		
	Coefficient	**SE**	**P-value**	**Coefficient**	**SE**	**P-value**
Ferritin	0.0058	0.00045	<0.0001	0.000018	0.0003	0.953
Age	0.0010	0.0037	0.787	-0.00103	0.0027	0.704
Race						
Blacks	Reference	Reference	Reference	Reference	Reference	Reference
Whites	0.0948	0.1353	0.484	0.08541	0.1037	0.411
Gender						
Male	Reference	Reference	Reference	Reference	Reference	Reference
Female	-0.2188	0.1071	0.042	0.2621	0.0755	0.001
GFR	-0.000041	0.0009	0.965	-0.0014	0.0009	0.136
MCHC	-0.0780	0.0335	0.021	-0.0816	0.0164	<0.001
MCV	0.0276	0.0126	0.029	-	-	-
Urea	-	-	-	0.0126	0.0062	0.0440

Table 6:3: Predictors of iron deficiency anaemia with Hepcidin or GF-15 as the primary biomarker among CKD participants

Variable	[1]Multivariable logistic regression analysis with Hepcidin as the primary factor			[2]Multivariable logistic regression analysis with GFD15 as the primary factor		
	Odds ratio	**95%CI**	**P-value**	**Odds ratio**	**95%CI**	**P-value**
Hepcidin	1.0023	1.0000-1.0045	0.049	-	-	-
GDF-15	-	-	-	1.00024	1.000026 - 1.000455	0.028
Age	1.00	0.98-1.02	0.964	1.00	0.98-1.02	0.947
Gender						
Male	Reference	Reference	Reference	Reference	Reference	Reference
Female	2.80	1.65 – 4.74	<0.0001	2.73	1.60 – 4.67	<0.001
eGFR	0.99	0..98 – 1.00	0.028	0.99	0..98 – 1.00	0.33
CRP	1.01	1.00 - 1.01	0.042	1.01	1.001 - 1.015	0.025
MCV	0.94	0.90 - 0.98	0.004	0.94	0.90 - 0.98	0.006

[1]Multivariable logistic regression of the model of the relationship between Hepcidin and iron deficiency anaemia

[2]Multivariable logistic regression of model of the relationship between GDF-15 and iron deficiency anaemia.

The two models corrected for gender, age, glomerular filtration rate, C-reactive protein and mean corpuscular volume. OR: Adjusted odds ratio.

Table 6:4: Predictors of Absolute iron deficiency anaemia with Hepcidin or GF15 as the primary biomarker among CKD participants

Variable	[1]Multivariable logistic regression analysis with Hepcidin as the primary factor			[2]Multivariable logistic regression analysis with GFD15 as the primary factor		
	Odds ratio	**95%CI**	**P-value**	**Odds ratio**	**95%CI**	**P-value**
Hepcidin	1.01	0.99-1.01	0.30	-	-	-
GDF-15	-	-	-	1.001	1.001 -1.01	0.0016
Age	1.01	0.98-1.03	0.54	1.006	0.98-1.03	0.65
Race						
White	Reference	Reference	Reference	Reference	Reference	Reference
Black	0.23	0.78 – 0.66	0.007	0.35	0.14 – 0.91	0.30
Gender						
Male	Reference	Reference	Reference	Reference	Reference	Reference
Female	2.86	1.38 – 5.91	0.005	2.95	1.37 – 6.34	0.006
eGFR	0.99	0..98 – 1.01	0.503	0.99	0..98 – 1.01	0.318
CRP ()	1.01	0.99- 1.01	0.436	1.01	0.99 - 1.02	0.160
MCV()	0.91	0.88-0.96	0.002	0.92	0.87 - 0.97	0.002
MCHC ()	0.73	0.52-1.01	0.056	0.71	0.48-1.03	0.069
Phosphate ()	-	-	-	0.50	0.26 - 0.99	0.046
DM	1.87	0.94-3.71	0.008	1.77	0.86-3.64	0.012

[1]Multivariable logistic regression of the model of the relationship between Hepcidin and Absolute iron deficiency anaemia

[2]Multivariable logistic regression of model of the relationship between GDF-15 and Absolute iron deficiency anaemia.

The 2 models corrected for gender, age, glomerular filtration rate, C-reactive protein, MCV, Phosphate, Diabetes Mellitus and mean corpuscular volume.

OR: Adjusted odds ratio.

Table 6:5: A Predictors of Functional iron deficiency anaemia with Hepcidin or GF15 as the primary biomarker among CKD participants

Variable	[1]Multivariable logistic regression analysis with Hepcidin as the primary factor			[2]Multivariable logistic regression analysis with GFD15 as the primary factor		
	Odds ratio	**95%CI**	**P-value**	**Odds ratio**	**95%CI**	**P-value**
Hepcidin	1.01	1.01- 1.01	0.013	-	-	-
GDF-15	-	-	-	1.01	0.99 -1.01	0.761
Age	0.98	0.95-1.01	0.123	0.99	0.96-1.01	0.169
Gender						
Male	Reference	Reference	Reference	Reference	Reference	Reference
Female	3.23	1.47– 7.29	0.004	2.9	1.29 – 6.29	0.009
Race						
White	Reference	Reference	Reference	Reference	Reference	Reference
Black	15.09	1.20 - 188.96	0.035	15.88	1.16 - 216.46	0.038
eGFR	0.99	0.98 – 0.99	0.007	0.98	0..98 – 0.99	0.008
CRP	1.01	1.00 - 1.01	0.002	1.01	1.01 - 1.02	0.001
MCHC	0.79	0.62- 0.99	0.041	0.78	0.61- 0.99	0.044
DM	1.35	0.55-4.78	0.070	1.31	0.56-4.79	0.00

[1]Multivariable logistic regression of the model of the relationship between Hepcidin and iron deficiency anaemia

[2]Multivariable logistic regression of model of the relationship between GDF-15 and iron deficiency anaemia.

The 2 models corrected for gender, age, glomerular filtration rate, C-reactive protein and mean corpuscular volume. OR: Adjusted odds ratio.

Table 6:6: Cut-off and validity of GDF-15 and Hepcidin in diagnosing Absolute and functional Iron deficiency Anaemia among CKD patients.

Test Parameter	**Predictive value of GDF 15 for AID among CKD patients**	**Predictive value of Hepcidin for FID among CKD patients**
	Value	**Value**
Cut-off	1129.3 mg/dl	22.5ng/dl
AUC	81.9%	81.4
Sensitivity	83.64%	66.7%
Specificity	66.03%	70.8%
Likelihood ratio of positive result	2.462	2.28
Likelihood ratio of negative result	0.2478	0.47
Younden's index	49.67%	37.48%

AID, Absolute iron deficiency anaemia; FID, Functional iron deficiency anaemia; AUC, Area Under Curve.

Supplementary tables

Supplementary Table 1: Correlations of hepcidin levels with haematological and other parameters

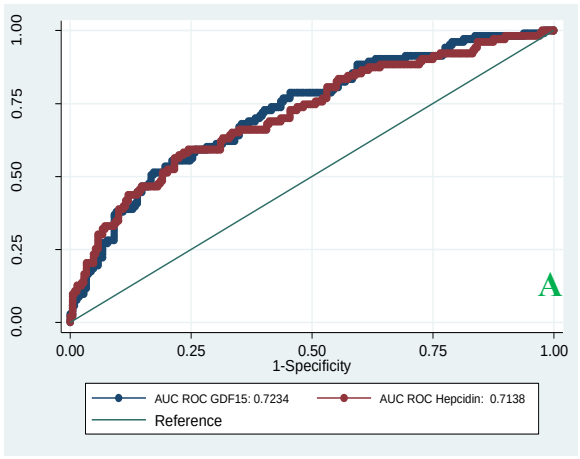
Characteristics	**Hepcidin levels among CKD patients**				**Hepcidin levels among Controls**			
	IDA		**NIDA**		**IDA**		**NIDA**	
	R	**p-value**	r	**p-value**	R	**p-value**	R	**p-value**
GDF -15	-0.28	0.0037	-0.07	0.289	-0.36	0.141	0.057	0.469
Serum Ferritin	0.50	<0.0001	0.57	<0.0001	0.25	0.325	0.45	<0.0001
TSAT	-0.031	0.759	0.21	0.002	0.48	0.046	0.23	0.003
Haemoglobin	0.017	0.862	-0.08	0.238	0.29	0.237	0.28	0.0003
GFR	-0.19	0.049	-0.17	0.012	-0.20	0.418	-0.26	0.006
Age	-0.18	0.064	0.004	0.957	0.004	0.987	0.02	0.817
hsCRP	-0.007	0.947	0.01	0.846	-0.25	0.321	-0.02	0.804
MCV	0.02	0.841	0.09	0.178	0.38	0.124	0.04	0.616
Serum Urea	0.23	0.019	0.13	0.069	0.42	0.083	0.03	0.709
Serum Creatinine	0.21	0.037	0.17	0.016	0.30	0.234	0.25	0.001
Serum Albumin	-0.18	0.070	-0.10	0.146	0.03	0.911	0.06	0.418
MCHC	0.003	0.976	-0.072	0.302	0.26	0.295	0.23	0.003
Calcium	-0.21	0.034	-0.068	0.325	0.23	0.350	0.13	0.0942
Phosphate	0.26	0.008	0.04	0.546	-0.008	0.976	-0.26	<0.0001

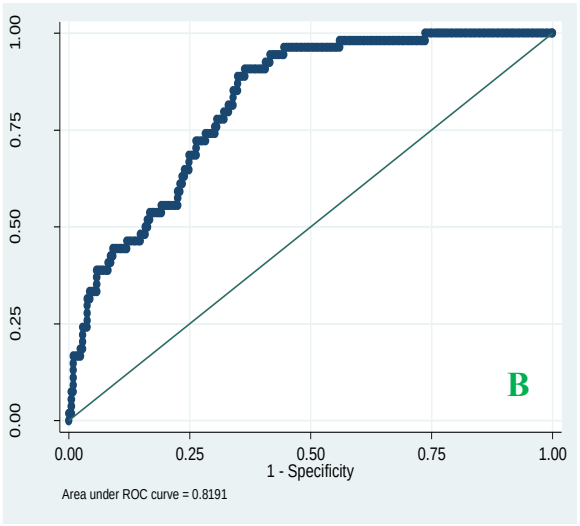
Abbreviations: IDA iron deficiency anaemia; NIDA: non- iron deficiency anaemia; TSAT: transferrin saturation; hsCRP: highly sensitive C-reactive protein; MCV: mean corpuscular volume; MCHC: mean corpuscular haemoglobin concentration

Supplementary table 2: Correlation of GDF-15 levels and iron parameters among the study population

Parameters	GDF-15 levels among CKD patients				GDF-15 levels among Controls			
	IDA		NIDA		IDA		NIDA	
	R	p-value	R	p-value	R	p-value	r	p-value
Serum Ferritin	-0.29	0.003	-0.009	0.902	-0.03	0.918	-0.09	0.229
TSAT	0.13	0.192	0.09	0.219	-0.02	0.938	0.017	0.824
Haemoglobin	-0.10	0.314	-0.34	<0.0001	-0.55	0.018	-0.23	0.003
eGFR	-0.11	0.290	-0.17	0.01	-0.06	0.817	0.09	0.225
Age	0.16	0.108	0.001	0.988	0.01	0.955	0.06	0.472
hsCRP	-0.03	0.772	0.08	0.247	-0.04	0.876	-0.13	0.100
MCV	-0.16	0.110	-0.08	0.223	-0.42	0.086	-0.04	0.573
MCHC	-0.05	0.616	-0.19	0.005	-0.16	0.525	-0.025	0.751
Serum Creatinine	0.10	0.336	0.16	0.025	0.19	0.440	-0.11	0.173
Serum Albumin	0.07	0.504	-0.04	0.564	0.13	0.595	-0.09	0.255
Calcium	0.07	0.460	-0.11	0.119	0.31	0.214	-0.11	0.178
Phosphate	-0.02	0.807	0.15	0.035	-0.11	0.650	-0.05	0.519
Serum Urea	0.07	0.489	0.19	0.006	-0.04	0.877	0.03	0.710

Abbreviations: IDA: iron deficiency anaemia; NIDA: non- iron deficiency anaemia; TSAT: transferrin saturation, hsCRP: highly sensitive C - reactive protein; MCV: mean corpuscular volume; MCHC: mean corpuscular haemoglobin concentration





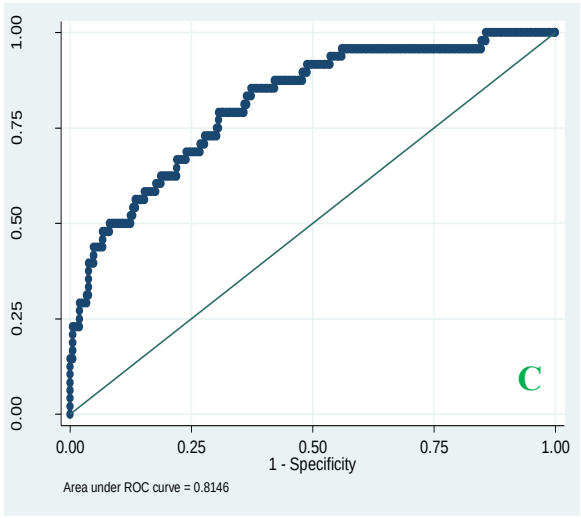


Table 6:7: Receiver Operator Characteristics curves (ROC) of: (A). Comparison of the predictive value of Hepcidin and GDF- 15 for diagnosing iron deficiency anaemia, (B): GDF-15 predicting Absolute IDA, (C). Hepcidin predicting Functional IDA

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7. CHAPTER SEVEN - PAPER FOUR: TMPRSS6 RS 855791 POLYMORPHISM AND SUSCEPTIBILITY TO IRON DEFICIENCY ANAEMIA IN NON-DIALYSIS CHRONIC KIDNEY DISEASE PATIENTS IN SOUTH AFRICA

Nalado, A.M., Dickens, C., Dix-Peek, T., Mahlangu, J.N., Olorunfemi, G., Paget, G., Duarte, R. and Naicker, S., 2019. TMPRSS6 rs855791 polymorphism and susceptibility to iron deficiency anaemia in non-dialysis chronic kidney disease patients in South Africa. *International Journal of Molecular Epidemiology and Genetics*, *10*(1), p.1.

7.1. Abstract

Background: In genome-wide studies, there is a strong association between the TMPRSS6 allele A736V (rs 855791) and significantly lower levels of serum iron, transferrin saturation, haemoglobin, and mean corpuscular volumes. The influence of this genetic variant on susceptibility to iron deficiency anaemia (IDA) in chronic kidney disease (CKD) patients is unknown.

Methods: In this cross-sectional study, we measured the full blood count and TMPRSS6 C>T polymorphism in black adult participants (n=260) with CKD and healthy controls comprising members of staff and patients' relatives (n=146) at the Charlotte Maxeke Johannesburg Academic Hospital, South Africa.

Results: The overall prevalence of anaemia in the CKD and control population was 46.9% and 19.6% respectively. Twenty-six per cent of CKD participants were iron deficient. The prevalence of rs 855791 C homozygosity was similar among iron deficient and non-iron deficient anaemia groups (86.1% vs 84.2%, p=0.723). When the analysis was confined to subjects with or without functional iron deficiency anaemia, C homozygote (88.3% vs 84.4%, p=0.425) was similar for both groups.

Conclusions: Our study suggests that homozygosity for TMPRSS6 rs855791 C genotype does not influence IDA in non-dialysis CKD patients in our population.

Keywords: iron deficiency anaemia, CKD, TMPRSS6, rs 855791.

7.2. Introduction

Anaemia is present in the majority of patients with chronic kidney disease (CKD), occurring in 15.4% of patients with stages 3-5 CKD (1, 2). While the major cause of anaemia in CKD is a relative deficiency of erythropoietin, iron deficiency anaemia contributes to anaemia of CKD (1, 2). Thus, there is a need to identify genetic factors that may be associated with iron deficiency anaemia. Iron is essential for multiple biological functions in all tissues, but especially for synthesis of haemoglobin, as shown by anaemia that results from iron deficiency (3). Over the past decade, heritable overtly pathological iron deficiencies have been accredited to mutations in several key genes that regulate iron homoeostasis (4) and thus the need to identify genetic factors that may be associated with iron deficiency anaemia.

The TMPRSS6 (transmembrane serine protease 6) gene encodes matriptase-2. Matripase-2 is an essential component of a pathway that detects iron deficiency, represses hepcidin transcription in the liver by cleaving membrane-bound hemojuvelin, and permits enhanced dietary iron absorption (5). The TMPRSS6 allele A736V (rs 855791) has been found to be related to lower levels of serum iron, transferrin saturation, haemoglobin, and mean corpuscular volume in genome-wide association studies conducted in the general population (5). Heritability estimates suggest that genetic factors contribute 20-30% of the variation in blood iron concentration (6). This single nucleotide polymorphism (SNP) rs855791 2321(C >T) is located in the functional part of TMPRSS6 and causes a non-synonymous substitution that reduces the ability of the enzyme to inhibit hepcidin transcription (7). Thus, TMPRSS6 A736V influences iron homoeostasis and erythropoiesis in normal subjects, although in another study conducted in patients with iron deficiency, based on presence or absence of anaemia, TMPRSS6 was found not to differ in women with iron deficiency with or without anaemia in the studied population (8, 9). It remains uncertain whether the association is mediated by iron or is through a direct effect of the variants on erythropoiesis.

Most studies conducted on the genetics of iron metabolism were conducted in persons of European ancestry. The few data available from the Asian population pointed to a potential role of ethnic variability in the heritability of specific measures of iron status (10, 11). There are few studies that analysed the genetics of iron metabolism in African populations, where the burden of iron deficiency is immense (12), Furthermore, it has been documented that the highest level of genetic diversity (both nuclear and mitochondrial) in the global human population is within the African population (13-15). Genetic risk of disease in the African population would likely be more complex because of increased levels of possible interactions between the genetic diversity and numerous differential environmental factors that impact iron absorption (16).

The aim of this study was to investigate the role of the genetic variant rs855791 on susceptibility to iron deficiency anaemia in pre-dialysis CKD patients and to determine whether this variant influence iron status in CKD patients.

7.3. Materials and methods

Venous blood samples were collected from patients (n=265) attending the renal outpatient clinic of the Charlotte Maxeke Johannesburg Academic Hospital, South Africa, and apparently healthy controls (n=141) which comprised of patients' relatives and hospital staff, from 1 May 2016 to 31 December 2016. Venous blood samples were collected, and standard methods were used for haematological measurements. Written informed consent was obtained from all participants, and the study protocol was approved by the Human Research Ethics Committee of the University of the Witwatersrand (MD 150929).

7.3.1. DNA extraction

Genomic DNA was extracted from whole blood using a Maxwell DNA purification kit (Promega AS1010, USA), as per manufacturers protocol. DNA concentrations were

determined by NanoDrop^{TM} 2000 spectrophotometer (Thermo Scientific, USA) and quality assessed with the A260/280 ratios. A 260/280 ratio of 1.8-2.0 was considered indicative of good quality DNA. Samples that had suboptimal DNA concentrations (<10ng/ul) were re-extracted using a modified salting out method (80).

7.3.2. Genotyping

The region of the TMPRSS6 gene containing the rs855791 C>T polymorphism was amplified using the polymerase chain reaction (PCR). Primers were as described in Jorde (77) and Peiet al (81): TMPRSS6F 5'-TAG AGA ACA GGG GCT CCA GG-3'; TMPRSS6R 5'-ATG TGG GCA GCA TCC TTT C-3'. The PCR reactions each contained 1x KAPA2G Robust HotStart Ready Mix (KAPA Biosystems, Massachusetts, USA), 0.125µM of each of the forward and reverse primers and 50ng extracted DNA. Thermo-cycling conditions were 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds, 65°C for 15 seconds, 72°C for 20 seconds and a final extension of 72°C for 1 minute. This resulted in the amplification of a single 249bp fragment. The PCR products were digested with the restriction endonuclease Stu 1 (New England Biolabs, Inc. Hitchin, Herts, UK). Genotype was determined by fragment size, under UV light in gel documentation system (Bio-Rad) and 10% of the samples were directly sequenced to confirm the genotyping results.

7.4. Statistical analysis

Normally distributed continuous variables were presented as means ± standard deviations, while non-normally distributed variables were presented as medians (interquartile ranges). Categorical variables were presented as numbers and percentages. The relationship between categorical variables and TMPRSS6 categories were tested using the Chi-square or Fisher's exact test. Analysis of variance (ANOVA) or Kruskal Wallis' test was used to determine the association between continuous socio-demographic, biochemical and haematological

characteristics and TMPRSS6 alleles. The relationship between TMPRSS6 polymorphisms and iron deficiency anaemia was evaluated using univariable and multivariable binary logistic regression and controlling for potential confounders. Statistically significant level was set at P-value <0.05 (confidence interval of 95%). Stata version 14 (Stata Corp, USA) statistical software was used for analysis.

7.5. Results

Among the CKD groups, there was no statistically significant relationship between the prevalence of the TMPRSS6 alleles and most of the demographic characteristics (Table 7.1) only gender has a statistically significant relationship with TMPRSS6 alleles (P-value = 0.005). Thus, while the majority (n=5/7) of TT alleles were found in males, only one-third (32.4%, n = 11/53) of the TC alleles were found in males; (P-value = 0.005) (Table 7.1).

Among the controls, there was no statistically significant difference in the proportion, mean or median variables of the demographic and clinical characteristics and among the TMPRSS6 alleles (Table 7.1).

From Figure 7.1A, in the control arm, there was no statistically significant difference in the prevalence of each category of TMPRSS6 rs855791 among the anaemic and non-anaemic groups (See Supplementary Table 1). Also, from Figure 7.1B, in the CKD arm, there was no statistically significant difference in the prevalence of each category of TMPRSS6 rs855791 among the anaemic and non-anaemic groups (See also Supplementary Table 1).

Table 7.2 showed that on bivariate analysis, there was no statistically significant difference in the proportions of IDA Vs non-IDA among CC (86.1% Vs 84.2%), TC (11.9% Vs 14.4%), and TT (1.9% Vs 1.5%) categories of the TMPRSS6 rs 855791 gene. Figure 7.2 also showed that ferritin and haemoglobin levels among the TMPRSS6 rs 855791 categories were not statistically different.

Supplementary Table 1 showed that on bivariate analysis, in the CKD group, there was no statistically significant difference (P-value = 0.995) in the proportions of IDA Vs non-IDA among CC (59.7% Vs 40.3%), TC (58.8% Vs 41.2%), and TT (60.0% Vs 40.0%) categories of the TMPRSS6 rs 855791 gene. Similarly, in the controls, there was no statistically significant difference in proportion of IDA and non-IDA among the categories of TMPRSS6 rs 855791 gene.

From the multivariable analysis (Table 7.3), there was a trend towards statistical significance for the relationship between TMPRSS6 rs 855791 genotypes and iron deficiency anaemia. The TC genotype tends to be protective against iron deficiency anaemia as there was a 45% lesser odds of iron deficiency anaemia among participants who had TC genotypes as compared to participants who had CC genotypes (Adj OR: 0.55, 95%CI: 0.29 – 1.06, P-value = 0.074). The CKD participants had about 3-fold higher odds of iron deficiency as compared to the controls (Adj OR: 3.2, 95% CI: 1.95 – 5.26, P-value <0.001). Similarly, participants who were 50 years or older had about 2-fold higher odds of iron deficiency anaemia as compared to participants younger than 50 years. (Adj OR: 1.81, 95% CI: 1.13 – 2.89, P-value <0.014)

Females also had about 2-fold higher odds of iron deficiency anaemia as compared to males. (Adj OR: 1.81, 95% CI: 1.13 – 2.89, P-value <0.014). Furthermore, there was a 54% lesser odds of iron deficiency anaemia among participants who had high reticulocyte haemoglobin (>28) or hepcidin (≥50) level. The area under receiver operator characteristic (ROC) curve (AUC) of the regression model was about 77.0%. Thus, the model can discriminate iron deficiency anaemia in 77% of times (see supplementary Figure 1).

7.6. Discussion

We report the first study focussing on the genetic aetiology of iron status in black non-dialysis CKD patients in South Africa. The prevalence of iron deficiency anaemia (IDA) among non-

pregnant females in South Africa was 24.3% (19). We found no association between the TMPRSS6 (rs855791) SNP, and parameters of iron status including IDA in our CKD population, whereas previous researchers have found an association in European and Asian populations (20-22). The exact mechanism through which TMPRSS6 is related to iron deficiency is still under investigation.

We found iron deficiency anaemia to be commoner (about 3-fold) among patients with CKD compared to controls; this finding is in agreement with previous studies (23-25). There are several factors that may explain the higher prevalence of iron deficiency among CKD patients; compliance with oral supplements is difficult, as iron supplements need to be taken in between meals, and they cause gastrointestinal side effects, and intestinal absorption of oral iron may be impaired in CKD, and reduced iron absorption caused by use of phosphate binders.

Our study has also provided the first report of rs855791 allelic frequency in South African black non- dialysis CKD population. The C allele frequency in our IDA and control groups was 92% and 91.3% respectively. These values are much higher than the findings in the Taiwanese population (48.6% vs 50%), the European population (39-47%) and among other Asian population (18, 21, 26). However, the C allele frequency has been reported to be 82-93% in African population (18), which is like our findings. The high prevalence of the CC genotype may have an advantage of enhanced iron absorption during food shortages or may provide some protection against infective parasites like malaria which invade the red blood cells (27, 28). These results further strengthen the hypothesis that genetic variations of the TMPRSS6 gene may contribute to variations in iron status depending on different racial and environmental factors, and more studies are needed in the African population to further ascertain the association between TMPRSS6 and iron deficiency anaemia.

In our study we found that TC genotype tended to have a protective role against the development of iron deficiency anaemia, and TT did not have an association with the development of iron deficiency anaemia. This finding is at variance with findings of, Kumar, Srivastava (29) where the TT allele had a pathological role, and the CC genotype was protective against iron deficiency anaemia. Gonçalves, Nobre Jesus (30) also found TT genotype to be significantly higher in the IDA group among Portuguese women. Differences in our findings could be explained by differences in the studied population as our study was conducted in patients with kidney disease while their studied population included pregnant women. In addition, environmental and racial factors could also contribute to these differences.

Polymorphisms of TMPRSS6 were found to be associated with a variety of iron parameters, including lower serum iron, haemoglobin, ferritin, mean corpuscular volume (20, 31-33); our study is at variance with these findings. While Danquah, Gahutu (28) findings were in agreement with our findings, a possible explanation for differences in our finding could be explained by differences in sample size as other researchers had a larger sample sizes. Other reasons could be abundant infections, and inflammatory processes affecting African CKD patients, which affect the validity of these markers on iron status. Nevertheless, more studies are needed to further explore these findings.

In this present study, increased levels of hepcidin were protective against iron deficiency anaemia; patients with hepcidin level >50ng/ml are 56% less likely to develop iron deficiency anaemia. This finding supports the literature that lower levels of serum hepcidin are associated with iron deficiency anaemia (34, 35). We found that serum hepcidin was not significantly associated with TMPRSS6 rs55791 (SNPs), in the fully adjusted models, and there was no decrease in strength of the association between this SNP and iron parameters, hence our data does not support an intermediate role of hepcidin in the rs85571 polymorphism iron parameter association in CKD patients. This finding is like findings of Galesloot, Geurts-Moespot (36).

Traglia, Girelli (37) found no association between rs85571 and hepcidin in an apparently healthy population. However, other studies were in disagreement with our findings (26, 38). A possible explanation for a negative association of TMPRSS6 variants with iron deficiency anaemia and iron parameters may be explained by a likely omission of environmental and genetic factors that may alter hepcidin concentrations independent of iron regulation, and different methods used to measure hepcidin; we used mass spectrometry while others used enzyme-linked immunoassay (ELISA). Further research with larger sample size is needed to further explore this finding.

7.7. Conclusion

The present study has shown that the TMPRSS6 rs855791 CC genotype is frequent in a South African Black population (both CKD and controls). The TMPRSS6 rs855791 SNP previously reported to be associated with iron deficiency anaemia in the general population was not associated with iron deficiency anaemia in our CKD population. The SNPs rs855791 was not associated with serum hepcidin in this study, and this further confirms that serum hepcidin, whether corrected for iron stores or not, is not the intermediate variable in the association of SNPs with iron parameters. Further studies are needed to elucidate the role of iron deficiency anaemia and hepcidin between the SNPs and iron parameters in CKD patients.

Ethics approval and consent

Ethical approval for the study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg (reference number MD150929), and participants gave informed consent at the beginning of each data collection session throughout the study.

Consent to publish

Not applicable

Competing interests

The authors declare that they have no competing interests.

Abbreviations

SNP: Single Nucleotide Polymorphisms. CKD; Chronic Kidney Disease. TMPRSS6; Transmembrane serine protease6. IDA; Iron deficiency anaemia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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None to declare

Authors Contributions

AMN designed the study, developed instruments, collected data, and data entry, participated in data analysis and manuscript writing. CD participate in study design, supervised instruments, and contributed to manuscript editing. TD participated in data analysis, study design, and manuscript editing. JM participated in study design, instrument design, and manuscript editing. GO participated in data entry, and analysis, and manuscript editing. RD participated in study

design, supervised instrument development, and manuscript editing. GP participated in study design, supervised instrument development, and manuscript editing. RD participated in study design, supervised instrument development, and manuscript-editing SN participated in study design, instrument design, and manuscript editing. All authors have read and approved the final manuscript.

Table 7:1: Comparison of demographic, biochemical and haematological characteristics of participants by TMPRSS6 gene

Parameter	CKD patients				Apparently healthy controls			
	TT (n=5)	TC (n=34)	CC (n= 221)	P-value	TT (n=2)	TC (n= 19	CC (n=122)	P-value
Age (mean ± SD)	53.8 (±17.8)	52.8 ±15.5	52.6 ±14.1	0.983[#]	37.5±3.5	43.6±16.2	40.3±11.9	0.528[#]
Gender n (%)								
Male	5 (100)	11 (32.4)	121 (54.8)	0.005	0 (0.0)	7(36.8)	53 (43.4)	0.415
Female	0 (100)	23 (67.7)	100 (45.3)		2 (100.0)	12 (63.2)	69 (56.6)	
Iron umol/Median (IQR)	10.3 (7.4-12.9)	10.8 (8.5-12.9)	9.9 (5-13.9)	0.814[$]	8.95 (7.7 – 10.2)	11.7 (8.7 – 19.6)	12.6 (10.9 – 14.5)	0.223[$]
TSAT % median (IQR)	18 (15-22)	19 (12-20)	16.5 (13-21)	0.479[$]	17 (13 - 21)	22 (19 – 28)	21 (17 – 23)	0.279[$]
Ferritin ng/ml median (IQR)	104 (58-198)	92 (70-100)	99 (44-140)	0.266[$]	69.5 (15 - 124)	66 (51 - 144)	77 (56 - 144)	0.776[$]
Haemoglobin g/l median (IQR)	12.5 (10.7-14)	14.3 (12.9-14.8)	12.5 (10.9-14.2)	0.310[$]	13.2 (12.6 – 13.8)	13.6 (12.3 – 15.1)	13.95 (13 – 15.5)	0.540[$]
Mean corpuscular volume fll (MCV) median (IQR)	88.1 (83.5-91.6)	92.9 (83.4-93.2)	88.2 (84.6-90.6)	0.856[$]	90.7 (88.2 – 93.2)	90 (86.8 – 90.9)	88.2 (84.2 – 91.6)	0.592[$]
Mean corpuscular haemoglobin pg/cell (MCH) median (IQR)	28.7 (26.9-30.1)	30.3 (26.8-31.8)	28.8 (27.6-30.1)	0.856[$]	32 (29.2 – 34.8)	29.6 (27.9 - 30.1)	29.7 (27.2 - 30.4)	0.525[$]

Mean corpuscular haemoglobin concentration (MCHC)g/dL median (IQR)	32.5 (31.4-33.4)	32.6 (32.2-33.2)	32.7 (31.4-33.7)	0.748[$]	34 (33.2 – 34.8)	32.5(31.9 – 32.9)	32.6 (31.9 – 33.5)	0.229[$]
%Hypochromic red cells median (IQR)	7.9 (3.4-15.7)	10.5 (5.5-10.8)	9.9 (1.7-22.1)	0.940[$]	5.1 (3.0 – 7.2)	4.1 (2.1 – 11.9)	3.3(1.8 – 5.6)	0.826[$]
Reticulocyte haemoglobin content (CHr) median (IQR)	27.9 (27.1-30.6)	29.8 (27.9-30.6)	27.8 (26.9-29.9)	0.412[$]	27.8(26.8 – 28.7)	29.1 (27.9 – 31.6)	28.5(27.2 - 30.6)	0.390[$]
GDF-15 median (IQR)	1012 (406.9-1458.3)	549.9 (303-598)	1170.3 (335.7-1636)	0.188[$]	978.95(648.1 – 1309.8)	309.9(159.9 – 1101)	438.7(175.5 – 1183.4)	0.519[$]
Hepcidin	8.1 (4-35.8)	4.2 (3.9-5.1)	4.8 (4-15.2)	0.403[$]	3.2(3.1 – 3.2)	2.9(1.9 – 13.1)	2.9(2.1 – 11.3)	0.960[$]
[#] P-value for analysis of variance (ANOVA). [$] P-value for Kruskal Wallis' test								

Table 7:2: TMPRSS6 rs855791 genotypes distribution between IDA and Non- IDA groups

Anaemia status	TMPRSS6 rs 855791 (n, %)			P-Value
	TT	TC	CC	
IDA n (%)	4 (1.9)	24 (11.9)	173 (86.1)	0.73
Non-IDA n (%)	3 (1.5)	29 (14.4)	170 (84.2)	

Table 7:3: Multiple logistic regression of the relationship between TMPRSS6 rs 855791 polymorphism and iron deficiency anaemia

Factor	Odds Ratio	95%CI	P-value	Adj Odds Ratio^	95%CI	P-value
TMPRSS6 rs 855791						
CC	1.00	Ref	Ref	1.00	Ref	Ref
TT	1.31	0.29 - 5.95	0.726	1.83	0.39 - 8.48	0.442
TC	0.81	0.45 - 1.45	0.486	0.55	0.29 - 1.06	0.074
Study group						
Control	1.00	Ref	Ref	1.00	Ref	Ref
CKD	3.18	2.07 - 4.88	<0.001	3.20	1.95 - 5.26	<0.001
Age (years)						
<50	1.00	Ref	Ref	1.00	Ref	Ref
≥50	2.26	1.51 - 3.37	<0.001	1.81	1.13 - 2.89	0.014
Gender						
Male	1.00	Ref	Ref	1.00	Ref	Ref
Female	2.03	1.37 - 3.02	<0.001	2.12	1.33 - 3.38	0.002
Iron(umol/L)						
<7	1.00	Ref	Ref	1.00	Ref	Ref
≥7	0.23	0.13 - 0.43	<0.001	0.34	0.17 - 0.68	0.002
Reticulocyte haemoglobin (pg/ml)						
<28	1.00	Ref	Ref	1.00	Ref	Ref
≥28	0.34	0.23 - 0.51	<0.001	0.46	0.29 - 0.73	0.001
Hepcidin level (ng/ml)						
<50	1.00	Ref	Ref	1.00	Ref	Ref
≥50	0.76	0.48 - 1.20	0.240	0.46	0.26 - 0.80	0.006
MCHC (g/dl)	0.87	0.74 - 1.02	0.083	1.11	0.96 - 1.28	0.157
MCH (pg/cell)	0.85	0.77 - 0.93	<0.001	0.88	0.79 - 0.98	0.018
^Model corrected for age, gender, study group, serum iron level, reticulocyte haemoglobin and hepcidin level. Mean-Variance inflation factor = 1.2. Hosmer-Lemeshow P-value = 0.2401						

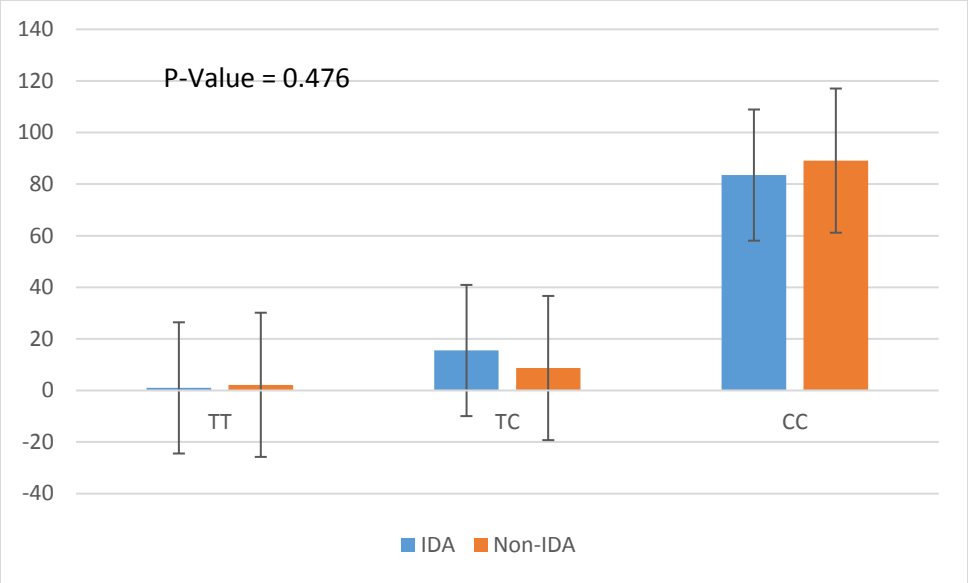

Figure 7:1: Prevalence of polymorphism by anaemic status among the controls

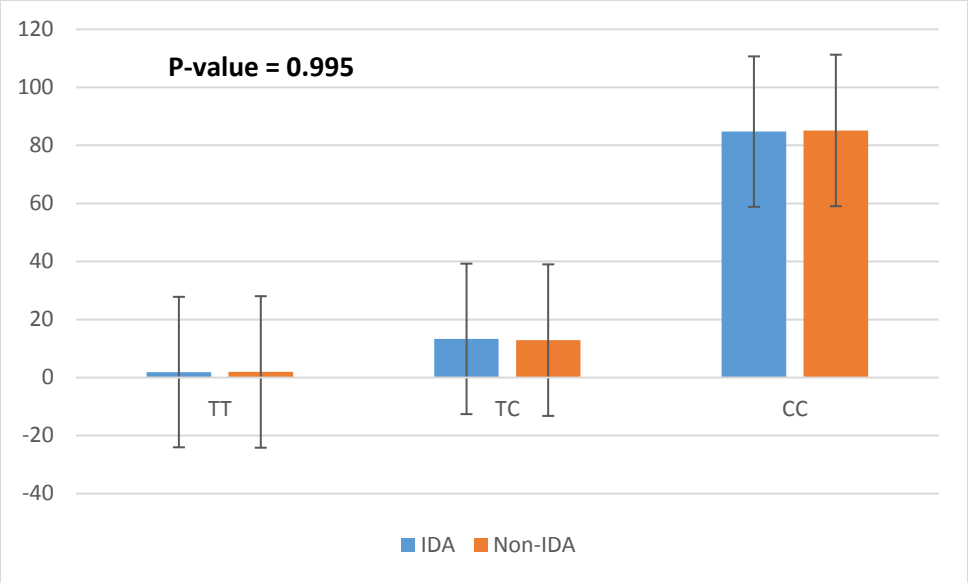

Figure 7:2: Prevalence of polymorphism by anaemic status among the CKD patients

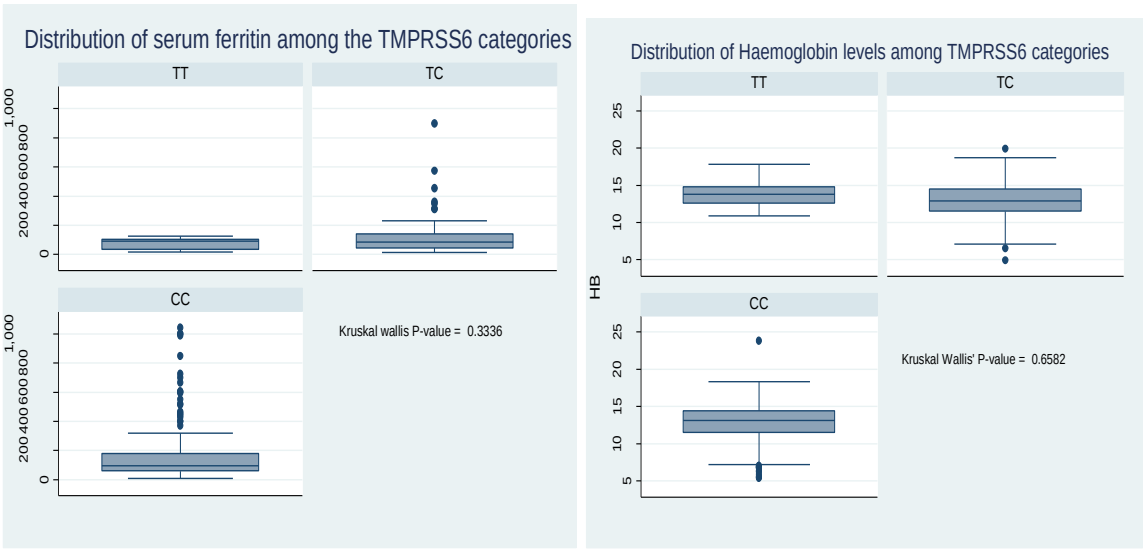

Figure 7:3: Showing distribution of haematological parameters among TMPRSS6 rs855791 categories.

7.8. References

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8. CHAPTER EIGHT - PAPER FIVE: ASSOCIATIONS OF HEPCIDIN AND GDF-15 LEVELS WITH IRON DEFICIENCY ANAEMIA AND CKD PROGRESSION AND MORTALITY IN A SOUTH AFRICAN CKD POPULATION

Journal: Kidney International Reports

Authors: AM Nalado, JN Mahlangu, G Paget, MK Muteba, B Waziri, C Dickens, T Dix-Peek, J George , R Duarte, S Naicker

Status: Under review

8.1. Abstract

Introduction

The interplay between inflammation and anaemia has been shown to play a vital role in the increased risk of cardiovascular disease (CVD) in patients with chronic kidney disease (CKD). This association may be partly responsible for the increased risk of mortality despite the correction of anaemia in CKD patients. The relationships between hepcidin, growth differentiation factor-15 (GDF-15) and iron deficiency anaemia, and disease progression and mortality in CKD are not well understood.

Methods

We prospectively studied a cohort of 325 CKD patients attending the Renal Outpatient Clinic of Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) in Johannesburg, South Africa, over a period of two years, between 1 June 2016 and 31 May 2018. The associations between baseline plasma levels of hepcidin, GDF-15, iron deficiency anaemia and clinical variables with mortality and a composite endpoint of CKD progression were investigated using Cox proportional hazard models.

Results

The mean age of the patients was 54.5±15 years. During a median follow-up period of 24 months, 77 patients died (23.7%). Patients with high levels of hepcidin >55ng/ml (median) 4.1-105.9 (IQR) were at six times greater risk of dying than patients with normal levels (1-55ng/ml) of hepcidin (HR: 6.14; 95 % CI 2.40-15.67; P<0.001). Higher plasma levels of hepcidin were strongly associated with CKD progression when compared with normal hepcidin levels (HR: 3.09; 95 %

CI; P=0.002). The risk of CKD progression was two times higher in patients with hypocalcaemia as opposed to those with normal calcium levels (HR: 2.24; 95 % CI P=0.021).

Increased levels of GDF-15 were not associated with disease progression or mortality.

Conclusion

Elevated levels of hepcidin and anaemia, together with hypocalcaemia, were independent predictors of disease progression and all-cause mortality in CKD patients.

Keywords

Hepcidin, GDF-15, iron deficiency anaemia, mortality, disease progression

8.2. Introduction

The rising prevalence of chronic kidney disease (CKD) has made it a worldwide public health problem (1). Most of these patients will ultimately progress to end-stage kidney disease (ESKD) requiring renal replacement therapy (RRT) in the form of dialysis or kidney transplantation or die prematurely before reaching ESKD. However, in Africa, the facilities for RRT are not readily accessible; hence the need to detect and correct factors associated with progression to ESKD. A major goal of CKD care is to delay the progression of patients to ESKD and the need for RRT, and to avoid cardiovascular events while doing so (1).

The risk of progression from earlier stages of CKD to ESKD is poorly understood within the context of novel biological prognostic markers, such as hepcidin and GDF-15 (2). The Framingham Risk Factor Study by Shlipak et al (3) reported that cardiovascular mortality risk was approximately 15 to 30 times higher among CKD patients than in the general population (4). The traditional risk factors that were elaborated upon in this study contributed only partially to the high incidence of CVD and death among CKD patients (5). This has led to increasing interest in identifying more novel prognostic biomarkers that can further identify those at high risk who could possibly benefit from more intensive management.

Growth differentiation factor 15 (GDF-15) is a protein belonging to the transforming growth factor 15 family, that has emerged as a potential marker of CVD and mortality (6). GDF-15 has clinical utility in the diagnosis and prediction of the course of chronic inflammatory and malignant diseases (7). GDF-15 has been identified as a novel appetite regulator that causes anorexia and weight loss when over-expressed in malignancy (8) and in cancer-associated cachexia. GDF-15 levels correlate with weight loss, lower lean body and fat mass, and decreased survival (9). These findings

are of particular importance in relation to the CKD population, where weight loss and malnutrition are among the most potent predictors of mortality, including cardiovascular death (10, 11).

Hepcidin controls systemic iron homeostasis. Iron and inflammation induce hepcidin expression, while iron deficiency and hypoxia reduce hepcidin levels (12). Hepcidin restricts systemically-available iron by regulating dietary iron absorption as well as by recycling iron from senescent erythrocytes and mobilising iron from stores (13). However, evidence on the prognostic implications of hepcidin is sparse, as only a few studies have described its association with clinical outcomes (14, 15) and importantly, have not considered its role in pre-dialysis patients.

We hypothesised that iron deficiency anaemia (IDA) is clinically important in non-dialysis CKD patients, and that increased hepcidin and GDF-15, possibly related to dysfunctional iron regulatory pathways, may contribute to disease progression and mortality in CKD patients.

We therefore aimed to determine the association of GDF-15, hepcidin and IDA with disease progression and mortality in non-dialysis CKD patients.

8.3. Materials and Methods

We prospectively studied 325 CKD patients attending the Renal Outpatient Clinic of Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) over a period of two years, from 1 June 2016 to 31 May 2018. Data was missing for 19 patients; therefore 306 patients were included in the analysis. Following approval from the Human Research Ethics Committee of the University of the Witwatersrand (clearance certificate number 150929), socio-demographic and clinical information was obtained via a self-administered proforma. Participants were followed up monthly, or every three months, depending on their clinical condition during their clinic visits, while those that did not attend the clinic were contacted telephonically to elicit information about their health status.

When necessary, patients' relatives were also contacted to ascertain whether death had occurred, its probable cause and to further ascertain when it had occurred. Hospital mortality records were reviewed to ascertain the details of those who had died in hospital. Participants who were older than 18 years were included in the study.

Exclusion criteria included patients currently on dialysis, or with active infections and inflammation, active bleeding, and malignancy; those who had received blood transfusions less than three months prior to enrolment in this project, those on immunosuppressant therapy, and those unable to provide informed consent. Data collected included demographic characteristics, blood pressure measurements, and medication history. Self-reported smoking status was classified into current smokers, ex-smokers, or non-smokers. Those who had stopped smoking during the last year before enrolment were categorised as current smokers.

The primary outcomes of this study were the progression of CKD and death. Other events such as transplantation and still being alive at the end of the study were right censored. Laboratory values and other clinical parameters were collected at six monthly intervals over a period of two years to assess the progression of CKD. The progression of CKD was defined as the requirement for dialysis or a doubling in serum creatinine (from baseline creatinine to the most recent creatinine measured), or a change in the annual glomerular filtration rate (GFR) as a percentage (more than 30%). The annual GFR percentage change was calculated as (Last eGFR - eGFR at baseline) *12/(eGFR) at baseline*time to outcome in months (16). The GFR was estimated according to the CKD-EPI formula (17). The cohort was divided according to CKD stage using the National Kidney Foundation's modified Kidney Disease Outcomes Quality Initiative (K/DOQI) classification of

CKD (20), with an eGFR of more than 90ml/min/1.73 m^2 representing stage 1; eGFR of 60 to 89ml/min/1. 73 m^2 stage 2; 45 to 59 ml/min/1.73 m^2 stage 3a; 30 to 44 ml/min/1.73 m^2 stage 3b; 15-29 ml/min/ 1.73 m^2, stage 4; and less than 15 ml/min/1.73 m^2, stage 5.

Early morning venous fasting blood samples were drawn from the patients. Serum creatinine was measured by means of an IDMS traceable enzymatic creatinine assay on the Roche auto-analyser, the method based on the Jaffe reaction. Complete blood counts were obtained after processing the blood samples using the Siemens ADVIA 2120, Technion H3 RTX and RTC systems analysers (Siemens Medical Solutions Diagnostics, Tarrytown, NY). Biochemical iron status, serum iron, total iron-binding capacity (TIBC), and the serum ferritin levels were also measured. Serum iron was determined by means of the ferrozine calorimetric method, TIBC through the colourimetric chromazurol dye-binding method using ADVIA 1800 (Siemens Medical Solutions Diagnostic, USA), and the serum ferritin level was determined by using the two-site chemiluminescent immunometric assays devised by the IMMULITE®2000 system (Siemens Medical Solutions Diagnostics, USA). Transferrin saturation was calculated by means of the formula: serum iron/TIBC.

Hepcidin and the isotopically-labelled internal standard [13C9,15N3] were purchased from the Peptide Institute (Osaka JPN). The serum levels of hepcidin-25 and the isotopically-labelled internal standard [13C9,15N3] were extracted and separated, as previously described by Li et al. (2009) (21). Briefly, samples were extracted with 4% HPLC grade trichloroacetic acid (TCA, Merck, Kenilworth, NJ, USA), and separated using reversed-phase liquid chromatography based on a Halo Peptide- ES 0.5x50mm column (Sciex, Framingham, USA) with a total running time of

five minutes per sample. The analysis was performed on an AB Sciex 5500 QTRAP (Sciex, Framingham, USA), coupled to a micro-liquid chromatography (Exigent M3) system with a Turbo IonSpray ionization source operated in positive ion mode. Multiple reactive monitoring (MRM) detection was applied using the Sciex 5500for identification and quantification using the ion transitions (m/z) of 698.0>354.0 and 703.2>354.1 for hepcidin.

Concentrations of serum hepcidin were expressed in nanograms per millilitre (ng/ml). The intra- and inter-assay coefficients of variation were smaller than 6.7% and smaller than 8.8% respectively. The lower limit of detection was 0.5ng/ml. The median reference level for serum hepcidin in healthy controls was 5.7 ng/ml. Hepcidin levels were defined as low when less than 25 ng/ml; normal when between 25 and 55 ng/ml, and high when greater than 55 ng/ml (18).
Serum levels of GDF-15 were measured by means of ELISA (R&D Systems, Minneapolis, MN, USA) with intra- and inter-assay CVs of less than 2.8 and less than 6 respectively. GDF-15 was grouped into three categories: less than 1200pg/ml, 1200- 1800pg/ml, and > 1800pg/ml (19, 20). Iron deficiency anaemia (IDA) was defined in the presence of anaemia when TSAT was equal to or less than 20% and serum ferritin was equal to or less than100ng/ml (19). Mild anaemia was defined as Hb>11g/dl, moderate Hb of 9-11g/dl. And severe Hb <9g/dl (21).

8.4. Statistical analysis

Data analysis was conducted using Stata 15.0 software (StataCorp.2017. Stata Statistical Software: Release 15. College Station, TX: Stata Corp LLC). The baseline characteristics of the participants were described and summarized across outcome groups (died, dialysis, no dialysis) using, where appropriate, medians and interquartile ranges or frequencies and percentages.

A stepwise Cox proportional hazards regression analysis with the Efron method for ties was used to identify factors associated with mortality and CKD progression. The proportional hazards assumption based on the unscaled and scaled Schoenfeld residuals was tested to fit the final model. Unadjusted and adjusted hazard ratios with their 95% confidence intervals were calculated. Kaplan-Meier graphs were used to display the association of hepcidin, GDF-15, and IDA with mortality and disease progression over the 24-month study period. All analyses were done at a five percent (5%) level of significance.

8.5. Results

As shown in Tables 8.1 and 2, 54% of the 306 participants were males. The mean age of both male and female participants was 54.5±15 years. The majority were Black (83%); 40% of the women and 63% of the men were married, 20% were unemployed, and 12% had a history of smoking cigarettes. CKD was found to be predominantly due to hypertension (53.3%) and diabetes mellitus (32.7%). During the follow-up period, 77 patients died (23.7%) at a median time of 11 months, and the earliest was within one month of the initial assessment. The death incidence rate was 0.09/month. The major cause of death was renal failure. It is important to note that the patients could not afford renal replacement therapy and that the majority were in an advanced stage of CKD.

Table 8.3 shows the increasing proportion of severe anaemia across the CKD stages (P-value <0.001). Furthermore, the proportion of stage 5 CKD participants increased among the mild, moderate and severe anaemia categories.

8.5.1. Factors associated with mortality

In the final model, the presence of anaemia was found to be strongly associated with mortality (HR: 3.21, 95% CI; 1.53-6.74; P=0.01). High levels of urea, low eGFRs and CRPs were also significantly associated with an increased risk of mortality. However, no significant association was observed between IDA and mortality among the patients (Figure8. 1).

As shown in Table8.4 4, high levels of hepcidin were found to be strongly associated with mortality, even after adjustments were made for other haematological and biochemical characteristics. Patients with high levels of hepcidin were at six times greater risk of dying as opposed to patients with normal levels of hepcidin (HR: 6.14, 95 CI; 2.40-15.67, P<0.001).Increased HDL-C levels (>1.5mmol/l) in the study population were found to be associated with higher mortality rates (HR 3.28, 95 CI; 1.43-7.54; P<0.001).

The association between GDF-15 and mortality was only marginally significant; the higher the level of GDF-15, the lower the risk of dying, However, this association did not attain statistical significance in the adjusted model (P=0.08).

8.5.2. Disease Progression

Of the 306 patients followed over the 24-month period, 100 (33%) progressed to later stages of CKD. The presence of anaemia was strongly associated with disease progression (HR: 2.45 95% CI; 1.44-4.17; P=0.001). Low levels of serum iron, transferrin saturation (TSAT), increased mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were strongly associated with the progression of CKD.

As shown in Table 8. 5, high levels of hepcidin were found to be strongly associated with CKD progression when compared to the normal hepcidin levels (HR: 3.24, 95% CI; 1.60-6.59; P=0.001).

No significant associations were observed between GDF-15, IDA and disease progression (Figure 8.2). On the other hand, the risk of disease progression was found to be two times higher in the presence of hypocalcaemia as opposed to normocalcaemia (HR:2.24 95%CI; 1.20-4.62; P=0.021). However, parathyroid hormone levels were not measured in this study.

8.6. Discussion

This prospective cohort study of a non-dialysis CKD population describes the significant burden of CKD in terms of high mortality, and the risk of progression to ESKD. Also, in this cohort, advanced stages of CKD were associated with an increased risk of mortality. The identifiable independent predictors of disease progression were anaemia, increased hepcidin, low serum iron, low TSAT levels, and high MCV levels and hypocalcaemia.

Our finding of anaemia as a predictor of disease progression is in agreement with findings from previous studies (22-24). The possible mechanisms that have been proposed to explain the effect of anaemia on CKD progression include tissue hypoxia, which will enhance ischaemic changes and increase endothelial injury, which will stimulate the production of cytokines that in their turn induce fibrosis, thereby accelerating CKD progression (25). This hypothesis was further supported by previous studies that reported the retardation of the CKD progression through corrections of anaemia (26, 27).

Our study found that a higher level of hepcidin is associated with CKD progression. Our results are consistent with those of previous studies, which found an association between increased levels of hepcidin-25 and renal anaemia and disease progression in non-dialysis CKD patients (15, 28)

and the association of hepcidin with disease progression in diabetic CKD patients (29). More recently, studies have suggested the role of hepcidin in the development of CVD, and as a prognostic biomarker of poor clinical outcomes (30, 31). The possible explanation for an increased hepcidin level and its association with disease progression may be an interaction between inflammation and anaemia that is mediated by hepcidin (32), via hypoferemia, iron –restricted erythropoiesis from cytokine-stimulated hepcidin increase.

In this current study, higher mean corpuscular volumes (MCVs) were also found to be associated with disease progression. Similar findings were reported by Kor, Hsieh (33), where patients with higher MCV levels had the greatest risk of negative clinical outcomes. A possible explanation linking increased MCV levels to negative clinical outcomes may be through the relationship of macrocytosis, nutrient deficiency, and malnutrition. Another explanation may be that increased oxidative stress can impair erythropoiesis, but such mechanisms are still not fully understood (34). We however found a negative association of high MCV with mortality, which was contrary to the findings of previous studies (34, 35). The inconsistency with these studies could be explained in terms of the differences in the study populations since their study populations included Chinese sample while our study focused on an African population.

Lower TSAT levels significantly predicted disease progression but not mortality, like previous studies (35, 36). The possible mechanisms for this association may involve the propagation of oxidative stress and parenchymal iron deposition with subsequent tissue damage (36-38). Low TSAT levels would have been expected to be associated with mortality via such mechanisms; however, this was shown in this study. Further prospective studies trials are needed to explore this issue.

We demonstrated that lower baseline serum calcium is associated with disease progression, which confirms and expands upon previous studies (39, 40). Current knowledge of CKD- mineral bone disease (CKD-MBD) favours the hypothesis that hypocalcaemia usually develops with the progression of untreated CKD and is associated with secondary hyperparathyroidism (39).

Our finding that GDF-15 is not associated with mortality is at variance with the findings of previous studies (41-43). For example, it has been reported that serum GDF-15 is a predictor of mortality in patients with advanced CKD (44). As we did not evaluate protein-energy wasting in our study population, we cannot comment on the nutritional status of our study population. Although GDF-15 has been linked to protein-energy wasting, oxidative stress and mortality, further studies are needed to explore this finding, especially in the black population.

Our study found that IDA is not associated with disease progression or mortality. This finding is contrary to the findings of Eisenga, Minovic (45) in respect of renal transplant recipients, where iron deficiency independent of anaemia was found to be associated with an increased risk of mortality. This difference could be explained in terms of the differences in the study populations, where the patients were transplant recipients and their definition of iron deficiency did not include anaemia.

The association of high levels of hepcidin with mortality in our study found endorses that of Wagner, Ashby (29), who reported that increased levels of hepcidin can be considered to be a predictor of mortality in diabetic CKD patients. However, the latter finding is at variance with the study of Eisenga, Dullaart (46) in kidney transplant patients. A possible explanation for this finding is that under certain conditions where increased hepcidin levels are not related to hypoxia, anaemia and inflammation (as is the case in renal transplant recipients), hepcidin may have a protective role

and may behave differently under conditions of hypoxia, anaemia and inflammation, as was found in our study. Thus, more research is needed to explore the relationship between hepcidin and mortality in different categories of CKD patients (45-47).

Our study found that anaemia is associated with mortality; the relative risk for death is 3.2 times greater in anaemic patients than in non- anaemic patients (48-50). In a large dialysis study involving 96,369 participants, Ma, Ebben (51) reported that lower haemoglobin levels are associated with increased mortality. Sandgren, Murray (52), investigating the mortality risk in Medicare beneficiaries, reported that patients with CKD and anaemia presented with a 27% increased risk for mortality within two years as opposed to patients without anaemia and CKD.

We found that anaemia prevalence increased in frequency and severity in the more advanced stages of CKD (stages 4 and 5), as in other studies (53). More severe anaemia was present with eGFR levels lower than 30ml/min/1.73m^2 in women and lower than 20ml/min/1.73m^2 in men (53, 54). Hsu et al (2001), in the Third National Health and Nutrition Examination Survey (NHANES III-1988-1994), found that anaemia was more common among 15, 971 adults with creatinine clearance/ eGFR levels of less than 70mls/min, and 50mls/min in men and women respectively (53).

As previously reported, we observed an increase in mortality risk with low eGFRs (55-57). Possible explanations for our finding may be due to the association of inflammatory markers and other risk factors for cardiovascular events which might contribute directly to adverse outcomes observed in patients with reduced kidney function (55), as well as the non-availability of dialysis for patients in the very advanced CKD (stage 5).

In this study, we show for the first time that higher levels of high-density lipoprotein cholesterol (HDL-C) are associated with an increased risk of mortality in patients with pre-dialysis CKD over a two-year follow up period. These findings differ from those of patients with normal kidney function, in whom higher HDL-C levels are associated with a reduced risk of mortality and cardiovascular disease; a higher risk of mortality was reported in patients with very low and very high HDL-C levels (3, 58-60).

After conducting a spline analysis, Bowe, Xie (59) reported that the relationship between HDL-C and risk of death is not linear but that it take a U-shape graphically, where the risk is highest in those with very low and very high HDL-C levels, which is similar to our finding. A possible explanation for this finding is experimental evidence suggesting that HDL-C at high levels paradoxically enhances senescence and impairs endothelial progenitor cell tube formation and angiogenesis, thus suggesting a loss of protective effect (59, 61). This hypothesis is further supported by the fact that pharmaceutical intervention strategies that aim to raise HDL-C levels do not improve cardiovascular outcomes and reduce mortality (62, 63). Therefore, there may be a range of HDL-C values, some of which might have beneficial effects, whereas others, namely the higher values, may be associated with deleterious effects (59). Additional research is required to explore this finding.

The strengths of our study include a relatively large sample size, the serial collection of baseline variables, a follow-up time of two years, and hepcidin measurement using gold standard mass spectrometry. However, the limitations of this study include the following: The linear regressions and outcome analyses had a number of explanatory variables included in the models. Hence, the overfitting of the models and finding an association by chance would be possibilities. Hepcidin was only measured at baseline. Thus, we could not determine a longitudinal relationship between

hepcidin and the outcome variables. We did not measure parathyroid hormone levels. Finally, we did not collect information on clinical events during secondary end-points, as most of the patients died at home. Examples of such information would be on cardiovascular events or stroke, and information on the cause of death for some patients was available from relatives telephonically.

8.7. Conclusions

This study has shown that hepcidin is a novel biomarker that could help identify newly- diagnosed CKD patients who might progress to ESKD or die prematurely. Human studies should evaluate the efficacious role of novel therapies to lower hepcidin levels in CKD patients safely, and to determine whether hepcidin reduction strategies will improve clinical endpoints, especially in CKD anaemia.

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Disclosures

None of the authors has any conflict of interest to declare

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Table 8:1: Baseline socio-demographic and clinical characteristics of study participants

Baseline Characteristics		**Dialysis (n=8)**	**Died (n=77)**	**Lost to follow up (24)**	**No dialysis (n=197)**	**Total (n=306)**
Age in years n (%)	median (IQR)	44(37.5-48.5)	54(45-63)	54(40-59)	57(45-67)	55(45-65)
	18-29	0(0)	3(3.9)	2(8.3)	9(4.6)	14(4.6)
	30-49	6(75)	28(36.4)	9(37.5)	59(29.9)	102(33.3)
	50-69	2(25)	37(48.1)	11(45.8)	91(46.2)	141(46.1)
	70+	0(0)	9(11.7)	2(8.3)	38(19.3)	49(16)
Sex n (%)	Male	5(62.5)	43(55.8)	14(58.3)	102(51.8)	164(53.6)
	Female	3(37.5)	34(44.2)	10(41.7)	95(48.2)	142(46.4)
Race n (%)	Black	6(75)	69(89.6)	22(91.7)	157(79.7)	254(83)
	White	2(25)	8(10.4)	2(8.3)	40(20.3)	52(17)
Marital status n (%)	Married	4(50)	41(53.2)	17(70.8)	99(50.3)	161(52.6)
	Single	4(50)	25(32.5)	4(16.7)	50(25.4)	83(27.1)
	Divorced	0(0)	3(3.9)	0(0)	13(6.6)	16(5.2)
	Widowed	0(0)	8(10.4)	3(12.5)	35(17.8)	46(15)
Occupa-tion n (%)	civil servant	3(37.5)	24(31.2)	10(41.7)	63(32)	100(32.7)
	Student	4(50)	26(33.8)	9(37.5)	62(31.5)	101(33)
	unemployed	0(0)	18(23.4)	4(16.7)	41(20.8)	63(20.6)
	Retired	0(0)	6(7.8)	1(4.2)	28(14.2)	35(11.4)
	self-employed	1(12.5)	3(3.9)	0(0)	3(1.5)	7(2.3)
Cigarette smoking history n (%)	No	4(57.1)	66(91.7)	22(95.7)	159(86.9)	251(88.1)
	Yes	3(42.9)	6(8.3)	1(4.3)	24(13.1)	34(11.9)
HTN, n (%)	No	0(0)	2(2.6)	1(4.3)	10(5.1)	13(4.3)
	Yes	8(100)	74(97.4)	22(95.7)	187(94.9)	291(95.7)

Table 8.1b. Baseline socio-demographic and clinical characteristics of study participants

Baseline Characteristics	Dialysis (n=8)	Died (n=77)	Lost to follow up (24)	No dialysis (n=197)	Total (n=306)
CKD aetiology n (%)					
Hypertension	5(62.5)	49(63.6)	9(37.5)	100(50.8)	163(53.3)
Diabetes mellitus	2(25)	20(26)	12(50)	66(33.5)	100(32.7)
Adults PKD	0(0)	5(6.5)	2(8.3)	15(7.6)	22(7.2)
Others	1(12.5)	3(3.9)	1(4.2)	16(8.1)	21(6.9)
BMI median (IQR)	28.59(23.4-30.8)	28.51(25-33)	29.9(25.8-33.5)	28.6(25.9-33)	28.6(25.5-33)
SBP (mmHg)	162(135-175)	143(130-165)	139.5(122.5-159.5)	140(129-156)	140(129-160)
DBP (mmHg)	96(81.5-103.5)	82(70-93)	85.5(71.5-96.5)	79(70-90)	80(70-91)

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; CKD: chronic kidney disease;. APKD: Adult polycystic kidney disease; HTN; Hypertension

Table 8:2: Haematological and biochemical characteristics of the study population

Characteristics	Dialysis (n=8)	Died (n=77)	Lost (24)	No dialysis (n=197)	Total (n=306)
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Urea	20(17-24.7)	24.9(15.5-37.8)	12(5.5-19.9)	9.9(6.8-13.1)	12.2(7.8-20)
S-Creatinine	400.5(325-744)	497(295-827)	203.5(120-275)	163(125-216)	193(134-358)
Creatinine doubled					
No n (%)	2(25)	58(75.3)	23(95.8)	148(75.1)	231(75.5)
Yes n (%)	6(75)	19(24.7)	1(4.2)	49(24.9)	75(24.5)
Median eGFR*	14.87(8.5-19.1)	10.93(5.6-20.3)	35(20.6-74.8)	42.2(28.1-55.3)	33.1(17.4-50.9)
CKD stages					
Stage 1	0 (0)	2 (2.60)	5 (20.83)	14 (7.11)	21 (6.86)
Stage 2	0 (0)	3 (3.90)	2 (8.33)	28 (14.21)	33 (10.78)
Stage 3	0 (0)	5 (6.49)	7 (29.17)	100 (50.76)	112 (36.60)
Stage 4	4 (50)	20 (25.97)	8 (33.33)	44 (22.34)	76 (24.84)
Stage 5	4 (50)	47 (31.04)	2 (8.33)	11 (5.58)	64 (20.92)
Calcium	2.2(2-2.4)	2.17(1.9-2.3)	2.2(2.1-2.4)	2.3(2.2-2.4)	2.2(2.1-2.4)
Phosphate	1.01(1-1.1)	1.35(1.1-1.7)	1.1(0.9-1.3)	1(0.9-1.2)	1.1(0.9-1.3)
Albumin	40.5(37.5-45.5)	39(35-41)	40(38.5-44)	40(39-43)	40(38-43)
T-cholesterol	5.3(4-5.9)	3.97(3.2-5.1)	4.3(3.4-4.8)	4.2(3.5-5)	4.2(3.4-5)
CRP*	10(10-10)	10(10-22)	10(10-11)	10(10-13)	10(10-14)
Iron	12.45(9.8-15.9)	8.9(6.7-12.7)	12.5(9.8-14.8)	10.7(7.8-13.1)	10.7(7.5-13.2)
TSAT*	19.5(17-25)	17(13-23)	20.5(17.5-26.5)	19(13-22)	18(13-23)
Serum ferritin	83.5(47-149)	143(81-274)	97(60-185)	101(59-180)	108.5(63-198)
Haemoglobin	13.45(12-14.7)	10.3(8-11.9)	13.2(11.6-14.3)	13.2(11.9-14.4)	12.8(10.7-14.1)
MCV*	86.1(81.2-92.9)	93.2(87.3-99.2)	89.4(84-90.3)	89.1(85-92.9)	89.7(85.2-94.3)
MCH*	28.75(25.8-31.8)	28.2(26.2-29.9)	29.4(27.7-30.1)	29.4(27.8-30.4)	28.9(27.5-30.3)
MCHC*	32.7(31.4-34.2)	32(30.9-32.7)	32.5(31.7-33.4)	32.5(31.5-33.3)	32.4(31.4-33.3)
HYP*	13.4(1.1-37.5)	8.5(5.4-15.8)	5.6(2.4-9.8)	6.9(2.8-12.8)	7.7(3.3-13.7)
GDF-15*	798.4(338-1273)	608.7(285.2-1220)	831.9(192.9-1091.5)	591.8(323-1215)	605.3(317-1209)

S-hepcidin assay	4.15(3.9-106.7)	139.8(120-155.1)	9.1(3.5-24.4)	7.9(3.9-25.7)	14.3(4.1-105.9)

*eGFR: estimated glomerular filtration rate; CRP: c-reactive protein; TSAT: transferrin saturation; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; HYP: percentage hypochromic red cells, GDF-15: growth differentiation factor-15

Table 8:3: CKD stages by severity of anaemia

CKD stage	Normal (n= 135)	Mild anaemia (n=66)	Moderate anaemia (n=43)	Severe anaemia (n=42)	Total	P- value
Stage 1	18 (11.6%)	3 (2.6%)	0 (0%)	0(0%)	21 (6.9%)	<0.001
Stage 2	24 (15.4%)	6 (9.1%)	2 (4.7%)	1 (2.4%)	33 (10.8%)	
Stage 3	77 (49.7%)	28 (42.4%)	5 (11.6%)	2 (4.8%)	112 (36.6%)	
Stage 4	30 (19.4%)	22 (33.3%)	19 (44.2%)	5 (11.9%)	76 (24.8%)	
Stage 5	6 (3.9%)	7 (10.6%)	17 (39.5%)	34 (80.9%)	64 (20.9%)	

CKD: Chronic Kidney Disease

.

Table 8:4: Factors associated with mortality

Parameters		Unadjusted Hazard Ratio (95% CI)	P-value	Adjusted Hazard Ratio (95% CI)	P-value
Hepcidin level	Low	1.11 (0.03-.43)	0.002	0.08 (0.02-0.35)	0.016
	Normal	1	reference		
	High	7.30 (3.16-16.87)	<0.001	6.14 (2.40-15.67)	<0.001
IDA	No	1	reference		
	Yes	1.53 (0.94-2.46)	0.084	0.97 (0.61-1.53)	0.895
HDL range	Normal	1	reference		
	Low	1.68 (0.97-2.91)	0.065	1.69 (0.97-2.93)	0.062
	High	3.28 (1.43-7.54)	0.005	4.32 (1.86-10.03)	<0.001
Urea		1.04 (1.01-1.06)	0.001	1.03 (1.01-1.05)	0.002
CRP		1.00 (1.00-1.007)	0.196	1.002 (0.998-1.007)	0.251
GDF-15	<1200	1	Reference		
	1200-1800	0.89 (0.44-1.79)	0.735	0.36 (0.17-0.77)	0.08
	>1800	0.12 (0.63-2.28)	0.587	0.53 (0.27-1.05)	0.067
eGFR (low)		1.001 (1.001-1.002)	<0.001	1.001 (1.001-1.002	<0.001)
Anaemia	No	1	Reference		
	Yes	2.66 (1.30-5.48)	<0.008	3.21 (1.53-6.74)	0.0140

eGFR; estimated glomerular filtration rate, CRP: c-reactive protein; Cox regression analysis. IDA; Iron deficiency anaemia, HDL; High density lipoprotein, GDF-15; Growth differentiation factor-15.

Table 8:5: Factors associated with CKD progression

Parameters		Unadjusted Hazard Ratio (95% CI)	p-value	Adjusted Hazard Ratio (95% CI)	p-value
GDF-15	<1200	1			
	1200-1800	1.14 (0.66-1.97)	0.634	0.81 (0.46-1.44)	0.473
	>1800	0.75 (0.39-1.46)	0.75	0.96 (0.50-1.84)	0.903 0
Hepcidin	Low	0.70 (0.36-1.37)	0.303	0.70 (0.36-1.38)	.306
	Normal	1	Reference		
	High	2.94 (1.51-5.71)	0.001	3.25(1.60-6.59)	0.001
IDA	No	1			
	Yes	2.07 (1.34-3.17)	0.001	0.90 (0.53-1.54)	0.711
Urea		1.02 (1.00-1.04)	0.022	0.97 (0.94-1.00)	0.06
Calcium	Hypocalcaemia	2.35 (1.30-4.25)	0.005	2.36 (1.20-4.62)	0.012
	Normal	1	Reference		
	Hypercalcaemia	0.72 (0.23-2.29)	0.583	0.45 (0.14-1.45)	0.180
Low Iron		0.96 (0.92-1.01)	0.118	1.07 (1.02-1.12)	0.003
Low TSAT		0.96 (0.93-0.99)	0.007	0.95 (0.91-0.99)	0.007
High MCV		1.02 (0.99-1.05)	0.239	1.05 (1.01-1.9)	0.028
MCH		0.90 (0.83-0.97)	0.009	0.85 (0.76-0.96)	0.007
Anaemia	No	1	Reference		
	Yes	2.64 (1.72-4.04)	<0.001	2.45 (1.44-4.17)	0.001

Cox regression analysis; IDA: iron deficiency anaemia; GDF-15; growth differentiation factor-15, TSAT: transferrin saturation; MCV: mean corpuscular volume; MCH: mean haemoglobin concentration; GDF-15; Growth differentiation factor-15

Supplementary tables

Table 1. Estimated mortality rates (per 1000) and lower/upper bounds of 95% confidence intervals

Parameters		Rate	Lower	Upper
Hepcidin level	Low	0.79	0.26	2.46
	Normal	7.36	3.31	16.39
	High	51.48	40.59	65.29
HDL range	Poor	16.11	11.86	21.88
	Better	10.99	7.54	16.03
	Best	9.85	5.12	18.92
GDF-15	<1200	12.80	9.85	16.63
	1200-1800	12.41	6.68	23.06
	>1800	15.01	8.31	27.10
Anaemia	No	3.29	1.77	6.12
	Yes	23.28	18.32	29.58

Table 2. Estimated disease progression rates (per 1000) and lower/upper bounds of 95% confidence intervals

Parameters		Rate	Lower	Upper
Hepcidin	Low	10.85	7.99	14.73
	Normal	13.50	7.47	24.37
	High	33.33	24.81	44.79
Calcium	Hypocalcaemia	25.95	15.07	44.69
	Normal	15.40	12.37	19.18
	Hypercalcaemia	13.70	4.42	42.47
Anaemia	No	10.54	7.45	14.90

Table 3. Mortality incidence rate per 1000 CKD patients per year

Parameters		Rate	Lower	Upper
Hepcidin_cat	Low	0.79	0.26	2.46
	Normal	7.36	3.31	16.39
	High	51.48	40.59	65.29
HDL range	Poor	16.11	11.86	21.88
	Better	10.99	7.54	16.03
	Best	9.85	5.12	18.92
GDF-15 cat	<1200	12.80	9.85	16.63
	1200-1800	12.41	6.68	23.06
	>1800	15.01	8.31	27.10
Anaemia	No	3.29	1.77	6.12
	Yes	23.28	18.32	29.58

HDL; High density lipoprotein, GDF-15; Growth differentiation factor-15

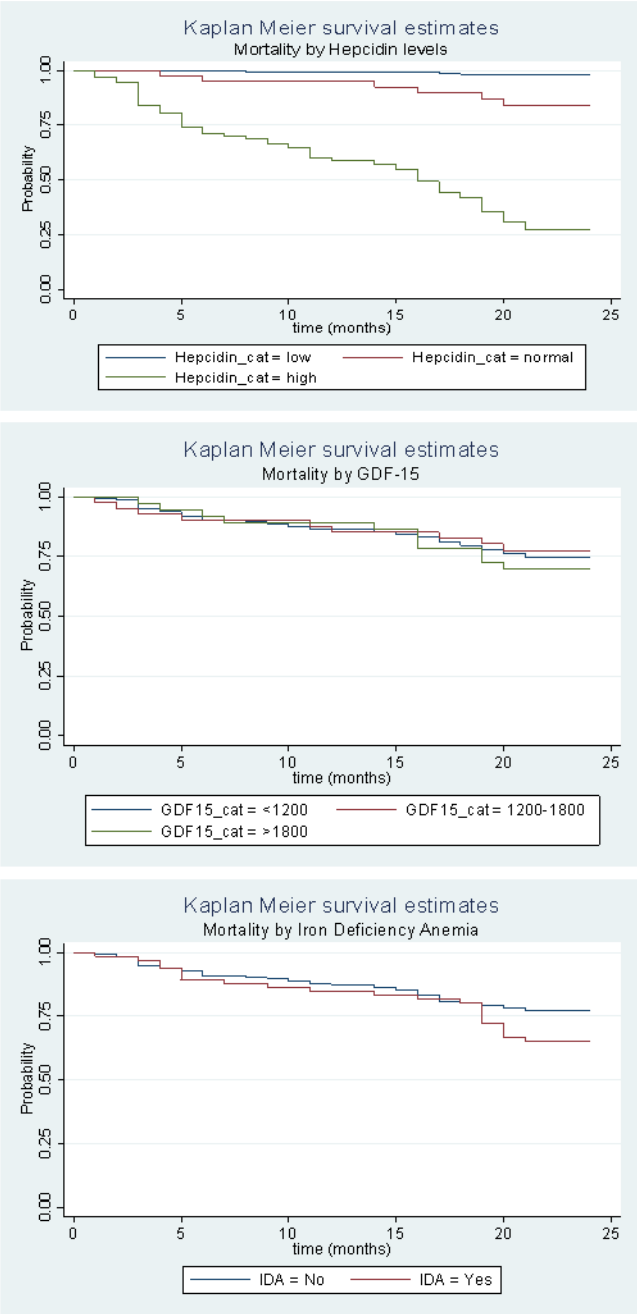

Figure 8:1: Survival probabilities according to hepcidin and GDF-15 levels, and iron deficiency anaemia

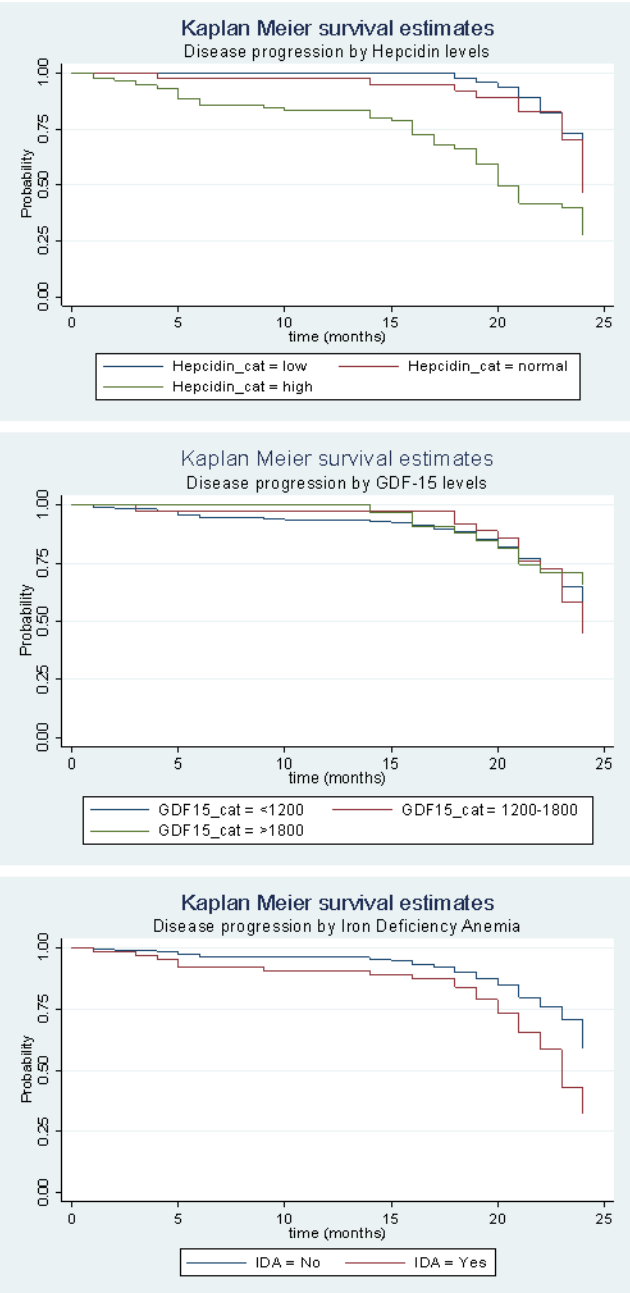

Figure 8:2: Survival probabilities according to hepcidin, GDF-15 levels, and iron deficiency anaemia

8.8. References

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9. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

9.1. Discussion

Anaemia and iron deficiency anaemia (IDA) are major and global public health problems. Several chronic diseases such as chronic kidney disease (CKD) are frequently associated with anaemia and IDA. The burden of this disease, especially in a pre-dialysis population, is not fully explored. Conventional parameters used in the diagnosis of IDA are highly variable, hence the need for newer parameters with less variability and greater sensitivity and specificity. A large proportion of the variation in iron status results from non-genetic factors; furthermore, the role of genetic factors in influencing the susceptibility to IDA is not well understood. This study was conducted to determine the prevalence of IDA, the utility of novel biomarkers in diagnosing IDA; to determine predictors of disease progression and mortality and to understand the genetics of IDA in a CKD population.

Several interventional studies have tested the hypothesis that treating the anaemia of chronic kidney disease with erythropoietic agents may reduce or reverse cardiac complications and retard the rate of progression of the disease. Ayus et al (1) determined that in anaemic patients the left ventricular mass index (LVMI) tends to decrease in relation to the baseline LVMI (142 vs 157 g/m2P = 0.007) as the haemoglobin level increases (from 9.1 to 11.3 g/dL (P = 0.001). Levin, Thompson (2) also found that the mean LVMI tends to change from the baseline after treatment of anaemia.

This study revealed a high prevalence of anaemia (43.1%) in patients with non-dialysis CKD in Johannesburg, with an increased prevalence of anaemia with advancing CKD stages. Anaemia was present in more than 80% in the late stages of CKD. The prevalence of anaemia was similar among Blacks and Mixed race but was slightly lower among whites. However, the prevalence of anaemia

among Blacks and mixed race was more than double the prevalence among Indian/Asians. Previous researchers also reported a greater prevalence of anaemia in black populations (3, 4). They observed that blacks have lower haemoglobin concentrations than other ethnic groups (5, 6), and this finding was confirmed in our studied population. It is likely that gene mutations, resulting from different environmental pressures emanating from Africa and Europe contribute to racial differences in the concentrations of haemoglobin (7)

Our observations have corroborated the previous reports of a US multicentre survey in 2004, where the prevalence of anaemia in CKD patients was found to be 47.7% (8); 51% among 2278 patients from the Health Care Financing Administration data (9); and 46% among CKD stage 1 to 5 patients in the Chronic Renal Insufficiency Cohort (CRIC) study (10). In Korea, Ryu et al (11) reported a prevalence of 49.9%, while prevalence's of 58% and 79% were reported in Catalonia (12). The prevalence of anaemia was 79% in Cameroon as reported by Kaze et al (13). These prevalence were higher than our findings, and were most likely due to the differences in the respective populations and geographical variations.

These results confirm the burden of anaemia in a CKD population, which may be attributed to a reduction in the production of endogenous erythropoietin due to a decrease in the eGFR, as well as metabolic disturbances such as the presence of uraemic toxins and electrolyte disturbances, and acid-based imbalances (14).

In our study, anaemia was found to worsen with declining kidney function, with a J- pattern observed in a non-linear correlation between anaemia and CKD stages. A few hypotheses could be proposed to partly explain the J-shaped pattern of anaemia associated with the varying stages of CKD that we observed in our study participants. Some unknown perturbations responsible for

the initial improvement in the haemoglobin levels may be observed at first, but as the kidney disease progressed from stage 1 to stage 3, there was eventual decline in the haemoglobin concentrations from stage 3 to stage 5.

The initial inflammatory process in the early stages of CKD could be caused by the hyper-stimulation of erythropoietin that would produce higher levels of haemoglobin. This mechanism is not, however, sustained with the continuing deterioration of kidney function during the later stages of CKD, and hepcidin pathways and hypoxia-inducible factors would then play a role in this mechanism.

Secondly, there could also be the possibility of an aggressive management of anaemia in the early stages of the disease, or of the patients responding to anaemia treatment modalities (such as oral iron therapy and ESA) that may be employed in the early stages of the disease, especially during stages 1 and 2.

In our study, we found the prevalence of absolute iron deficiency to be 16.7% and functional iron deficiency 18.6%, which was three times more frequent among CKD patients than among the controls (35.3% vs 9.2%) (15). Previous studies reported the prevalence of IDA to be 15-34% across all stages of CKD in the USA (16), 48% among CKD patients in Romania (17), 39% in India (18), while Talib et al (19) reported a prevalence of 42.6% of IDA among CKD patients in India. Other researchers also reported IDA to be more common among CKD patients (20, 21). This work emphasizes the severity of IDA in advanced stages of CKD (stage 3-5), which is similar to findings of other researchers (22). Further studies on the prevalence of IDA among various ethnic groups will be required to ascertain the heterogeneity of IDA across CKD populations of different ethnicities.

Reticulocyte haemoglobin content (CHr) and the proportion of hypochromic red cells (% HYPO) were found to be potential biomarkers for IDA in this study. This is the first study to be carried out in an African black CKD population, and we found both markers to be reliable in the diagnosis of IDA in CKD patients. The sensitivity and specificity of CHr at a cut-off value of lower than 28pg were found to be 62.6% and 80.2% respectively (area under the curve (AUC) = 0.81). Our study showed CHr to be a better predictor of IDA in the early stages of CKD, as opposed to serum ferritin and TSAT. The %HYPO at more than 5% had a sensitivity of 73.6% and a specificity of 44.3% (AUC=0.76) for the diagnosis of IDA. However, combining these two biomarkers did not yield any additional benefits when compared with CHr alone. Thus, CHr alone can be used to diagnose IDA in black CKD patients, with several advantages in the mix, namely: rapid measurements in less than 2 minutes, automatic processing, along with a complete blood count, no variability with inflammation, and the fact that these methods that are relatively cheaper than the conventional parameters used in diagnosing IDA (23).

The role of CHr as a diagnostic marker of IDA has been documented in American and European studies (23-26). Mittman, Sreedhara (27) assessed the efficacy of CHr in predicting functional IDA and found that a CHr value of less than 28pg is able to predict IDA more accurately than conventional parameters, and Fishbane et al (28) also showed that CHr is a more reliable marker of IDA than the conventional iron indices; this was in agreement with our findings at similar cut-off values. A study by Stancu, Stanciu (29) also showed the limited diagnostic utility value of conventional indices in identifying IDA; hence the need for newer biomarkers.

Based on these studies, the 2006 National Kidney Foundation's Kidney Disease Outcomes Quality Initiative Guidelines (KDOQI) (30) recommends maintaining a CHr value greater than 26pg in a dialysis-driven CKD population, along with a serum ferritin concentration of more than 200ng/ml.

Based on the findings from our study, we suggest a review of the KDOQI Guidelines so that a CHr value that is lower than 28pg should be considered as a marker of IDA in pre-dialysis CKD patients.

The proportion of hypochromic red blood cells (%HYPO) was found to be a predictive marker of IDA in CKD patients in this study, and is in agreement with other studies (31, 32). The measurement of circulating hypochromic red cells as a proportion of the total red blood cell count has proved to be the most sensitive marker of iron deficiency in CKD patients, with a cut-off point of six percent (6%) (33), but is limited by the fact that increased storage time results in red blood cells swelling and causes a reduction in the mean corpuscular haemoglobin concentration and an increase in % HYPO (33). This is more so in developing countries, where laboratories may be remote and storage time may be longer. There is a need for further multicentre and prospective studies on the performance of %HYPO as a marker of IDA in our communities, before it can be recommended as part of the work-up of IDA in CKD patients.

Hepcidin and Growth Differentiation Factor-15 (GDF-15) levels were found to be increased in CKD patients as opposed to the controls. This finding is consistent with the findings of other researchers (34, 35) and may indicate ineffective erythropoiesis and increased erythroid activity (36, 37). Our study showed both biomarkers to be potential predictors of IDA in pre-dialysis CKD in our patients; and to the best of our knowledge, this is the first study to evaluate the clinical utility of these biomarkers in the diagnosis of IDA in CKD.

Hepcidin has been used as a marker of IDA in different study populations (38-40), but this is the first study focusing on a CKD population. GDF-15 was found to predict iron deficiency anaemia in our pre-dialysis CKD population and this is consistent with previous studies in dialysis

populations (34, 41, 42). GDF-15 may play a role, possibly through the aetio-pathogenetic mechanism of hepcidin-independent or hepcidin-dependent pathways, but further studies on the clinical utility of hepcidin and GDF-15 in CKD patients will be required to establish this relationship.

We found anaemia to be a marker of poor outcome and mortality in pre-dialysis CKD patients; and the association between anaemia and adverse outcomes has been well established in previous studies (43-47). Our study found that there was a higher death risk for patients with haemoglobin levels that were lower than 10g/dl, as opposed to those with haemoglobin levels lower than 12g/dl. This corroborated the findings of other studies (48, 49). Observational studies have continuously demonstrated a strong association between lower haemoglobin levels and an increased risk of death. However, interventional studies have failed to establish that erythroprotien treatment to achieve near normal haemoglobin levels mitigates the risk of mortality (50-52).

Our study suggests that anaemia is associated with an increased risk of all-cause mortality in a cohort of CKD patients, and this is independent of the presence of potential confounders. However, the advantage of treating anaemia in CKD patients to improve cardiovascular outcomes remains to be demonstrated.

Our study investigated the association between novel biomarkers of anaemia and disease progression and mortality. High GDF-15 levels in this study were not associated with an increased risk of death or of disease progression. This is at variance with the findings of Nair et al (53), where GDF-15 was significantly associated with an increased risk of CKD progression in two independent cohorts. Previous studies have reported an important link between elevated GDF-15 concentrations and cardiovascular diseases (54, 55) and between elevated GDF-15 levels and

mortality in haemodialysis patients (56, 57). The difference in our findings could be explained in terms of differences in the study populations, as previous studies were conducted in heart failure and in CKD patients on haemodialysis, whereas our study was conducted amongst a non-dialysis CKD population. Although GDF-15 may be a risk factor and an outcome marker in CKD patients on dialysis, it may not be a direct participant in the disease processes pertaining to non-dialysis CKD patients.

Hepcidin was found to be independently associated with mortality (hazard ratio, 6.10; 95% CI 2.4-15.43, P=<0.001) and disease progression (hazard ratio, 3.25; 95% CI 1.60-6.59, P=0.001) even after adjustment was made for baseline estimated GFR, albumin, and haemoglobin in the present study.

Data on hepcidin as a biomarker and prognostic factor for clinical outcomes remain sparse. The hypothesis that hepcidin might be associated with clinical events seems promising. Because anaemia and chronic inflammation are common in CKD and ESKD, and both are related to worse clinical outcomes, hepcidin may play a role in clinical outcomes through its link to anaemia, and chronic inflammation in CKD patients. Other factors associated with disease progression in this study include hypocalcaemia, TSAT, and MCV. These finding are similar to previous findings by other researchers (58-60).

Increased urea levels, high density lipoprotein cholesterol (HDL-C) levels, and low estimated GFRs were associated with mortality in our study population. As reported in previous studies, low eGFRs and high urea levels are factors that are associated with mortality (61-64). However, the data pointing to increased HDL-C levels and an increased risk of mortality in patients with cardiovascular disease are limited (65, 66). This is the first study to show that the risk of mortality

is increased with elevated HDL-C levels in CKD patients. This may probably be as a result of, firstly, genetic mutations (62) leading to very high levels of HDL-C that may confer an adverse cardiovascular risk (67) or may be confounded by factors associated with both mortality and cardiovascular risk. Secondly, extreme elevations in HDL-C may directly represent dysfunctional HDL levels in some individuals, which may in turn promote cardiovascular risk (65). Further studies on the relationship between elevated HDL-C levels and mortality risk among CKD patients will be required to establish the nature of this association.

Our study is the first to focus on the genetic aetiology of IDA in a population of black non-dialysis CKD patients. Our study showed a general lack of association between investigated SNPs and iron status indicators in our population sample, whereas other researchers have reported such associations in European and Asian population samples (68). TMPRSS6 serine is likely to be involved in iron metabolism through its pleiotropic effect on hepcidin concentrations. Previous studies have reported that the rs855791 gene is associated with lower concentrations of serum ferritin and with anaemia (69-71). A possible reason for the lack of association between TMPRSS6 and IDA in our cohort may be due to our smaller population sample as opposed to the larger samples researched in previous GWA studies.

Our study also provides the first report of the frequency of rs855791 alleles in black South African CKD patients. The CC allele frequency in our IDA patients and controls was found to be 86.1% and 84.2% respectively. The CC allele has been reported to be present in 82 to 93% of African populations (123), which corroborates our findings, but is in contrast to reports from Taiwan (48.6%) (72), and in Europe (50%) (39-47%) (73). This variation could be explained in terms of

differences in ethnicity and environmental factors. Our results further strengthen the hypothesis that genetic variations in the TMPRSS6 gene may contribute to variations in iron status, depending on different racial and environmental factors. More studies are needed in the African population to further ascertain the association between TMPRSS6 and IDA. The TC allele trend has played a protective role in preventing IDA in CKD patients in other studies (72-74).

Associations between variants in the TMPRSS6 genes and iron parameters were found in individuals of Indian and Asian ancestry, as well as in those of European ancestry (75, 76). We did not find an association between TMPRSS6 and iron parameters in our study population, as was the case in the study of Beutler et al (77). This could be related to the smaller size of our study population as opposed to those of other studies.

The diagnosis of IDA in CKD populations continues to be challenging. In addition to their shortcomings in clinical use, there is discordance when measuring conventional indices (transferrin saturation, ferritin). Studies like ours that use alternate iron indices which are more accessible and cheaper are encouraging, especially in resource-constrained environments. Further research is required before these markers can have widespread use, availability and applicability.

9.2. Recommendations

Despite the associations of renal anaemia and IDA with CKD morbidity and mortality, there is a paucity of data on the burden of anaemia and its novel biomarkers in African pre-dialysis CKD patients. Our study has provided important insights into the spectrum of CKD anaemia and IDA in African CKD patients. The high prevalence of anaemia and IDA among the pre-dialysis CKD patients in our study further confirms the fact that anaemia and IDA are important complications

experienced by patients with kidney disease, and that there is a need to treat anaemia and IDA in the pre-dialysis patients.

We therefore have the following recommendations:

- Further studies on the prevalence of IDA among various ethnic groups will be required to ascertain the heterogeneity of IDA across CKD populations of different ethnicities.
- A review of the KDOQI Guidelines so that a CHr value that is lower than 28pg should be considered as a marker of IDA in pre-dialysis CKD patients.
- Multicentre prospective studies on the performance of %HYPO as a marker of IDA in different communities, before it can be recommended as part of the work-up of IDA in CKD patients.
- As the older, more conventional parameters generally used to diagnose IDA in CKD patients have been found to be suboptimal, we recommend the incorporation of these novel biomarkers into guidelines such as the KDOQI and their implementation in clinical practice to facilitate the diagnosis of IDA in pre-dialysis CKD patients.
- Prospective controlled studies are needed to elucidate the causal implications of GDF-15 in CKD populations, and to establish whether GDF-15 may represent a novel therapeutic target for the potentially fatal complications of cardiovascular disease.
- We recommend a randomised controlled trial to establish the usefulness or otherwise of a hepcidin-reducing agent, to determine whether hepcidin-reducing agents could be considered in the treatment of iron disorders. Strategies targeting the hepcidin-ferroportin axis may open up a new avenue for hepcidin regulation in an endogenous physiological way by avoiding secondary iron overload and other complications.

- Future research is required to investigate the clinical utility of hepcidin as a predictor of clinical outcomes. A larger sample size should be sought in order to determine the incremental prognostic value hepcidin carries in addition to the established risk factors associated with it.
- The findings of ethnic differences in the prevalence of anaemia and IDA in our CKD patients, and the determination of different cut-off values for the diagnosis of IDA suggest the need for a review of the KDOQI guidelines to be appropriate for different races and to establish cut-off values for the different races.
- We have shown that TMPRSS6 SNPs rs855791 polymorphism, previously associated with iron deficiency in populations of European and Asian ancestry, is not replicated in African populations. We recommend GWA studies for the African population to discover novel loci associated with iron deficiency. Once the GWA studies have been completed, candidate gene studies could be used to validate the relevant findings, as well as to explore the interactions between genetics and demographic factors.
- We also recommend fine mapping studies to identify genetic markers associated with iron deficiency in CKD populations. The potential underlying the discovery of novel variants associated with iron deficiency in CKD patients would offer opportunities for combating iron deficiency in African CKD patients. Studies analysing additional IDA phenotypes in larger samples would enable us to confirm possible associations between the genetic TMPRSS6 polymorphism and IDA in CKD patients.

9.3. Study limitations

Our findings should be considered in the context of the following limitations. Firstly, on account of the cross-sectional study design, we could not determine longitudinal changes in the biomarkers of CKD anaemia; nor could we identify circadian variations in hepcidin levels. Secondly, we

excluded patients who had received oral iron or erythroprotien-stimulating agents two to four weeks prior to recruitment. This may have underestimated the prevalence of anaemia in our CKD cohort. Although we did not perform bone marrow aspiration biopsies to establish the diagnosis of IDA among our study cohort, because of ethical considerations. Previous studies have shown the predictive capacity of iron markers and bone marrow aspiration biopsies. Newer biomarkers such as CHr, hepcidin, and GDF-15 have the potential to diagnose IDA better in CKD patients than the conventional markers (Iron, TSAT, TIBC, Transferrin, Ferritin). Further studies using tissue samples are needed to confirm these findings. However, despite these limitations, our study has strengths and has assisted in bridging the knowledge gaps in the field of CKD anaemia and IDA and has allowed for comparisons to be made with data from other CKD populations.

9.4. Conclusion

Anaemia and IDA are prevalent among pre-dialysis black South African CKD patients. Newer biomarkers have the potential to accurately diagnose IDA in CKD patients compared with the conventional markers. Anaemia was found to be a risk factor for mortality, thus the need to adequately treat anaemia in CKD patients. Our study revealed significant associations between anaemia and high levels of hepcidin, and between anaemia and disease progression and mortality in pre-dialysis CKD patients. Hence there is a need to pay more attention to treating these risk factors in the management of renal anaemia. Hepcidin antagonist therapy should be considered as a future target approach in treating anaemia in patients with CKD. The lack of association between TMPRSS6 polymorphism and iron deficiency anaemia in African CKD patients may be related to the limited size of our research sample population, and further research with a larger sample size may be required.

9.5. References

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APPENDIX A: ETHICAL APPROVAL

R14/49 Dr Aishatu Muhammad Nalado et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150929

NAME: (Principal Investigator)	Dr Aishatu Muhammad Nalado et al
DEPARTMENT:	Internal Medine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Functional Iron Deficiency Anaema and its Genetics in a Black South African Chronc Kidney Disease Population
DATE CONSIDERED:	02/10/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Saraladevi Naicker
APPROVED BY:	Professor P. Cleaton-Jones,Chairperson, HREC (Medical)
DATE OF APPROVAL:	15/06/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated,from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in September and will therefore be due in the month of September each year.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B: INFORMATION SHEET

Participant information and consent form (patient)

Section A

STUDY TITLE: Functional Iron Deficiency Anemia and its genetics in a Black South African CKD Population.

INVESTIGATOR: Dr Aishatu Muhammad Nalado

SITE: Charlotte Maxeke Johannesburg Academic Hospital

TELEPHONE: Cell: 0789984733; Land line (Department): 011-488-3672

Section B

Introduction

Good day, my name is Aisha Nalado. I am a PhD student doing research at the University of the Witwatersrand. Research is a study carried out in order to establish facts and reach new conclusions. I am conducting a study on **"Functional Iron Deficiency Anemia** and its Genetics in Black South Africans with Chronic Kidney Disease".

The information that I will collect from this research project will be kept confidential. You are hereby invited to participate in this voluntary research.

It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this hospital will continue and nothing will change. You may change your mind later and stop participating even if you agreed earlier.

If you have any questions you may ask them now or later, even after the study has started.

It is important that you read and fully understand information on this leaflet as it will help you in making the decision.

Purpose of the study

I am conducting a study to look at people with chronic kidney disease and why they develop Anemia which is one of the complications of chronic kidney disease. Anaemia is a condition in which a person has lower than normal red blood cells. Each human being has two kidneys and they function to remove waste products and produce urine, and also assist in making red blood cells. One of the objectives of this study is to determine the frequency of blood deficiency (anemia) in people with chronic kidney disease. I will also look for inheritable factors called genes that regulate the development of anaemia.

Genes are basically a collection of information or instructions that form the makeup of a human being and decide how he/she behaves.

Some people with genetic changes have high risk of developing anemia compared to those with normal genes. One way to demonstrate this relationship is though DNA testing. DNA is the chemical compound which genes are made of and is often referred to as the building blocks of life. Genetic testing may help in the diagnosis of a disorder. Genetic testing can also be performed for research.
DNA testing is optional and should you agree to participate, a separate DNA consent form will be given to read and sign.

Procedures of the study

If you agree to participate in the study, your medical records will be reviewed. You will be examined by a medical doctor and also interviewed to collect information regarding Anemia. Blood and urine samples will be collected. A total of 10mls (2 teaspoons of blood) will be drawn to measure the markers of anemia and analyze for the genetic changes.

The blood will be spun very quickly and the serum separated out. The serum which appears as a clear fluid will be kept frozen in a small test-tube. Your coded blood samples will be sent to Department of Internal Medicine research laboratory for the above mentioned tests. The remaining samples will be frozen and stored in a designated freezer for an unlimited period of time for future use in research related to diseases. However, if you decide later that you do not want the specimens

collected from you to be used for future research, please notify me or any other person in the research team.

Benefits of the study

The benefits of participating in this study are that we will be able to identify the magnitude of functional deficiency anemia and its association with genes responsible for iron absorption in people with chronic kidney disease. If you are found to have anaemia which is a common complication of kidney disease, it will be beneficial to know if you are anemia is due to abnormal genes as this will assist in correcting your anaemia. Information that will be obtained from this study will guide our decision in prompt management of anemia which will subsequently reduce the high rate of deaths associated with anaemia..

Possible risks

Possible side effects from drawing the blood sample include mild pain, bleeding, and bruising at the site of the needle insertion.

Qualified personnel will collect the blood samples to prevent such complications.

Financial arrangements

You will not be paid for participating in the study. However, there will be no costs to you for any related study visits and procedures, as any costs incurred will be compensated by research funds.

Confidentiality

All information obtained during the course of this study will be kept strictly confidential. Your records will be given unique identification numbers and the initial identification details won't be used. All physical records will be kept in a locked locker with access limited to the research team. Electronic data will be password protected.

Source of information

If you have any questions, queries and clarifications, please contact; Dr Aisha Nalado she will be reachable 24 hours every day on the number, 0789984733.

Additional information can be obtained from the chairperson of Witwatersrand University Human Research Ethics Committee, Professor Cleaton Jones on 011-717-2301

APPENDIX C: INFORMED CONSENT FORMS

Informed Consent Form: General (patient)

I confirm that I have been informed about the study by Dr Aisha M Nalado. I understand that my personal details will be kept strictly confidential and that I may at any stage withdraw my consent and participation in the study and continue to receive the appropriate treatment. I have also received, read and understood the study as explained in the participant information sheet and consent to taking part in this research study.

PARTICIPANT (printed name)……..

Signature or thumb print …… **Date** …**……..**

Witness (printed name)……

Signature……...Date…….

I, Dr Aishatu Nalado confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

…….. Signature……Date……..

Printed Name

Section D

Informed consent form: DNA Storage (Patient)

I hereby confirm that I have been informed about the study by Dr. Aisha Nalado about the nature, benefits and risks of the genes study.

I understand that my blood sample will be stored for future testing.

I understand that my personal details (any identifying data) will kept strictly confidential.

I have had the opportunity to ask questions and I have also received, read and understood the study as explained in the participant information sheet and consent to taking part in this research study.

PARTICIPANT (printed name)……..

Signature or thumb print …… **Date** …**……..**

Witness (printed name)……

Signature……...Date…….

I, Dr Aisha Nalado confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

Name …….. Signature……Date……

Section E

Informed consent form: DNA testing (patient)

I hereby confirm that I have been informed about the study by Dr. Aisha Nalado about the nature, benefits and risks of the genes study. I understand that my personal details (any identifying data) will kept strictly confidential. I have had the opportunity to ask questions and I have also received, read and understood the study as explained in the participant information sheet and consent to taking part in this research study.

PARTICIPANT (printed name)……..

Signature or thumb print …… **Date** …**……..**

Witness (printed name)……

Signature……...Date…….

I, Dr Aisha Nalado confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

Name …….. Signature……Date……

APPENDIX D: QUESTIONNAIRE

Study: Functional Iron Deficiency Anaemia and its Genetics in a Black South African CKD Population

Patients Data Sheet

Serial Number: ____

1. Age at last Birthday (Years) _____

2. Sex: Male ☐ Female ☐

3. Ethnicity_____

4. Marital status: Single ☐ Married ☐ Widow/Widower ☐

Separated/Divorced ☐

5. Occupation _____

6. How long do you have CKD? _____

7. Presence of Hypertension: (a) Yes ☐ N O

8. Do you have diabetes mellitus? (a) Yes ☐ (b) No ☐

9. Dietary History dietary recall food rich in irons

a) Spinach Yes ☐ No ☐ quantity/frequency Daily ☐ Weekly ☐ Monthly ☐ Never ☐

b) Tomatoes Yes ☐ No ☐ quantity/frequency Daily ☐ Weekly ☐ Monthly ☐ Never ☐

c) Liver Yes ☐ No ☐ quantity/frequency Daily ☐ Weekly ☐ Monthly ☐ Never ☐

d) Cereals fortified with iron Yes ☐ No ☐ quantity/frequency Daily ☐ Weekly ☐

Monthly ☐ Never ☐

e) Meat Yes ☐ No ☐ quantity/frequency Daily ☐ Weekly ☐ Monthly ☐ Never ☐

f) Legumes Yes ☐ No ☐ quantity/frequency Daily ☐ Weekly ☐ Monthly ☐ Never ☐

g) Egg yolk Yes ☐ No ☐ quantity/frequency Daily ☐ Weekly ☐ Monthly ☐ Never ☐

ii) Are you on iron tablet (a) Yes (b) No

If Yes for how long _____ ☐ ☐

10. Aetiology of CKD_____

11. Do you smoke cigarette? (a) Yes ☐ (b) No ☐

12. History of Blood loss? (a) Yes ☐ (b) No ☐

13. History of black stool (a) Yes ☐ (b) No ☐

14. History of Blood Transfusion in the proceeding 3 months (a) Yes ☐ (b) No ☐

Clinical Parameters

1) Weight (Kg) _____

2) Height **(**m**)** _____

3) BMI (Kg/m^2) _____

4) Blood Pressure (mmHg) _____

Investigations.

Haemoglobin level_____

MCV _____ MCH_____MCHC_____

Albumin _____ TIBIC _____Serum Ferritin_____

TSAT _____ Serum Iron._____(HYP Red Cells%)_____

Reticulocyte hemoglobin content _____ Serum creatinine_____

TNF- α_____ CRP_____IL-6_____

Urea_____Ca_____Phosphate_____

Stool for Occult blood_____ Serum Hepcidin Assay_____

Total Cholesterol_____

LDL _____HDL_____TG _____

Estimated GFR: _____

APPENDIX E: PERMISSION TO USE COPYRIGHT CONTENT

From: Babitt, Jodie Lynn,M.D. <Babitt.Jodie@mgh.harvard.edu>
To: aisha nalado <aishnld@yahoo.co.uk>
Sent: Friday, 30 November 2018, 18:02:29 GMT+2
Subject: Re: PERMISSION TO USE FIGURE

Dear Aisha,

Depends what you are using it for. You are welcome to use it in a scientific presentation, with appropriate citation. For other uses (e.g. some sort of publication or other commercial use), you should check the journal policies to see what is allowable and what requires additional permission from the journal

Best,
Jodie

Jodie L. Babitt, M.D.
Associate Professor of Medicine
Harvard Medical School
Massachusetts General Hospital
Division of Nephrology
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Center for Systems Biology
185 Cambridge St., CPZN 8208
Boston, MA 02114
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fax 617-643-3182
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APPENDIX F: MANUSCRIPT - ETHNIC PREVALENCE OF ANEMIA AND PREDICTORS OF ANEMIA AMONG CHRONIC KIDNEY DISEASE PATIENTS AT A TERTIARY HOSPITAL IN JOHANNESBURG, SOUTH AFRICA

International Journal of Nephrology and Renovascular Disease

Dovepress

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Open Access Full Text Article

ORIGINAL RESEARCH

Ethnic prevalence of anemia and predictors of anemia among chronic kidney disease patients at a tertiary hospital in Johannesburg, South Africa

This article was published in the following Dove Medical Press journal:
International Journal of Nephrology and Renovascular Disease

Aishatu Mohammed Nalado[1,2]
Johnny N Mahlangu[3]
Bala Waziri[1]
Raquel Duarte[1]
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Gbenga Olorunfemi[4]
Saraladevi Naicker[1]

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Introduction: Anemia is a complication of chronic kidney disease (CKD) that can greatly impact on its prognosis. However, the risk factors for anemia, including the influence of ethnicity, are not well established among the CKD population in Johannesburg.

Methods: This was a cross-sectional study of 353 adult CKD patients attending the renal outpatient clinic of the Charlotte Maxeke Johannesburg Academic Hospital (Johannesburg, South Africa) from June 1, 2016 to December 30, 2016. Sociodemographic and clinical characteristics were obtained using a proforma. Blood samples were collected for serum electrolytes and hematological parameters. Predictors of low hemoglobin and iron deficiency anemia (IDA) were evaluated using multivariable binary logistic regression.

Results: The mean age and prevalence of anemia among the CKD participants were 55.3±15.0 years and 43.18% (95% CI: 38.1%–48.4%), respectively. Blacks had the highest prevalence of anemia (46.9%), while Indians/Asians had the lowest (18.2%). Although the odds of anemia was 3.8-fold higher (odds ratio =3.8, *P*-value =0.059) among CKD stage V participants as compared to CKD stage I, the relationship between anemia and stages of CKD was non-linear. Diabetes mellitus (odds ratio =2.31, *P*-value =0.005) had a strong association with anemia among the CKD participants.

Conclusion: Almost half of the CKD participants were anemic, and the odds of anemia did not increase linearly with increasing severity of CKD. There was a marked ethnic disparity in anemia prevalence. Our study highlights the need for risk-based management of anemia among CKD patients.

Keywords: chronic kidney disease, hemoglobin, risk factors, iron deficiency anemia, ethnicity, Johannesburg, South Africa, kidney stage

Introduction

Anemia is a major cause of morbidity and mortality among chronic kidney disease (CKD) patients.[1] It is defined as a low level of hemoglobin (Hb), <12 g/dL in females and <13 g/dL in males among CKD patients.[2] About 75% of CKD patients were reported[3] to be anemic in low- and middle-income countries (LMICs), as compared to a lower anemia prevalence of 22.2% among CKD patients in high-income countries.[3] Anemia occurs early in the course of kidney disease and may worsen and become intractable with declining kidney function.[1–4]

Although the development of anemia in CKD patients is mainly due to an absolute or relative deficiency of erythropoietin, other possible causes of anemia include blood loss, decreased half-life of red blood cells, iron deficiency, inflammation, nutritional

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APPENDIX G: MANUSCRIPT -UTILITY OF RETICULOCYTE HAEMOGLOBIN CONTENT AND PERCENTAGE HYPOCHROMIC RED CELLS AS MARKERS OF IRON DEFICIENCY ANAEMIA AMONG BLACK CKD PATIENTS IN SOUTH AFRICA

PLOS ONE

RESEARCH ARTICLE

Utility of reticulocyte haemoglobin content and percentage hypochromic red cells as markers of iron deficiency anaemia among black CKD patients in South Africa

Aishatu Muhammad Nalado[1,2]*, Johnny N. Mahlangu[3], Raquel Duarte[1], Graham Paget[1], Gbenga Olorunfemi[4], Barry F. Jacobson[1], Saraladevi Naicker[1]

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Citation: Nalado AM, Mahlangu JN, Duarte R, Paget G, Olorunfemi G, Jacobson BF, et al. (2018) Utility of reticulocyte haemoglobin content and percentage hypochromic red cells as markers of iron deficiency anaemia among black CKD patients in South Africa. PLoS ONE 13(10): e0204899. https://doi.org/10.1371/journal.pone.0204899

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Data Availability Statement: Data for this study contain confidential patient information. The ethics committee of Charlotte Maxeke Academic Hospital, Johannesburg, South Africa, has imposed restrictions on sharing our data, with ethics number (M150929). We have attached the anonymized data that was used for this analysis as supporting information and confirm that the anonymized data represents the minimal data set required to replicate the findings of our study. Further data access are available for researchers

Abstract

Introduction

Iron deficiency anaemia (IDA) worsens the prognosis and outcomes of chronic kidney disease (CKD). However, while the haemoglobin level is unreliable for early detection of IDA, reticulocyte haemoglobin content (CHr) and hypochromic red cells (%HYPO) are early markers of IDA.

Methods

This was a cross sectional study of black adult participants (n = 258) with CKD and apparently healthy members of staff and patients' relatives (n = 141) at the Charlotte Maxeke Johannesburg Academic Hospital, South Africa, between 1 June 2016 and 31 December 2016. Serum iron, serum ferritin and transferrin were measured using standard laboratory methods, while the haematology analyser was employed to measure CHr and %HYPO. The validity of CHr and %HYPO as markers of IDA were evaluated. Multivariable binary logistic regression was conducted to determine predictors of the relationship between IDA, CHr and %HYPO. The area under the receiver operator characteristics (ROC) curve (AUC) of the final models were utilised to evaluate the discriminatory value of CHr and %HYPO respectively.

Results

About one-quarter (26.1%) of the participants had IDA which was more than three times more frequent among CKD patients, compared to controls (35.3% vs 9.2%); 32.3% (95%CI: 27.90%– 37.10%) of the study population had iron deficiency without anaemia and the prevalence of iron deficiency without anaemia was lower in CKD patients compared to controls (29.5% vs 37.6%). The mean age of CKD patients was higher than in controls (52.7 ±14.3

APPENDIX H: MANUSCRIPT – TMPRSS6 rs855791 POLYMORPHISM AND SUSCEPTIBILITY TO IRON DEFICIENCY ANAEMIA IN NON DIALYSIS CHRONIC KIDNEY DISEASE PATIENTS IN SOUTH AFRICA

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Original Article

TMPRSS6 rs855791 polymorphism and susceptibility to iron deficiency anaemia in non-dialysis chronic kidney disease patients in South Africa

Aishatu Muhammad Nalado[1,2], Caroline Dickens[1], Therese Dix-Peek[1], Johnny N Mahlangu[3], Gbenga Olorunfemi[4], Graham Paget[1], Raquel Duarte[1], Saraladevi Naicker[1]

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Abstract: Background: In genome-wide studies, there is a strong association between the TMPRSS6 allele A736V (rs855791) and significantly lower levels of serum iron, transferrin saturation, haemoglobin, and mean corpuscular volumes. The influence of this genetic variant on susceptibility to iron deficiency anaemia (IDA) in chronic kidney disease (CKD) patients is unknown. Methods: In this cross-sectional study, we measured the full blood count and TMPRSS6 T>C polymorphism in black adult participants (n=260) with CKD and healthy controls (n=146) at the Charlotte Maxeke Johannesburg Academic Hospital, South Africa. Results: The overall prevalence of anaemia in the CKD and control population was 46.9% and 19.6% respectively. Twenty-six per cent of CKD participants were iron deficient. The prevalence of rs855791 C homozygosity was similar among iron deficient and non-iron deficient anaemia groups (86.1% vs 84.2%, P=0.723). When the analysis was confined to subjects with or without functional iron deficiency anaemia, C homozygote (88.3% vs 84.4%, P=0.425) was similar for both groups. Conclusions: Our study suggests that homozygosity for TMPRSS6 rs855791 C genotype does not influence IDA in non-dialysis CKD patients in our population.

Keywords: Iron deficiency anaemia, CKD, TMPRSS6, rs855791

Introduction

Anaemia is present in the majority of patients with chronic kidney disease (CKD), occurring in 15.4% of patients with CKD [1, 2]. While the major cause of anaemia in CKD is a relative deficiency of erythropoietin, iron deficiency anaemia contributes to anaemia of CKD [1, 2]. Thus there is a need to identify genetic factors that may be associated with iron deficiency anaemia. Iron is essential for multiple biological functions in all tissues, but especially for synthesis of haemoglobin, as shown by anaemia that results from iron deficiency [3]. Over the past decade, heritable overtly pathological iron deficiencies have been accredited to mutations in several key genes that regulate iron homoeostasis [4] and thus the need to identify genetic factors that may be associated with iron deficiency anaemia.

The TMPRSS6 (transmembrane serine protease 6) gene encodes matriptase-2. Matripase-2 is an essential component of a pathway that detects iron deficiency, represses hepcidin transcription in the liver by cleaving membrane-bound hemojuvelin, and permits enhanced dietary iron absorption [5]. The TMPRSS6 SNP A736V (rs855791) has been found to be related to lower levels of serum iron, transferrin saturation, haemoglobin, and mean corpuscular volume in genome-wide association studies conducted in the general population [5]. Heritability estimates suggest that genetic factors contribute 20-30% of the variation in blood iron concentration [6]. This single nucleotide