MMed Research Report

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Abstract

Objectives

Iron deficiency is a major cause of anaemia and differentiation from anaemia of chronic disease (ACD) can be a challenge. Iron deficiency anaemia (IDA) is a particular worry in the blood donor population. Hepcidin has been postulated as a marker capable of making the distinction. The aim of this study was to evaluate the use of hepcidin as a marker of iron status in blood donors with anaemia.

Methods

Serum hepcidin concentrations, measured using a hybrid micro-liquid chromatography triple quadrupole ion trap mass spectrometer, were categorised into three groups based on associated haemoglobin and ferritin levels (IDA, ACD, and Controls), and compared for differences. Leptin, soluble transferrin receptor (sTfR), C-reactive protein and creatinine were measured. The sTfR log ferritin index was derived and also evaluated to differentiate groups.

Results

Hepcidin showed significant differences between the ACD group [7.89 nmol/L (5.96, 10.84)] and the other two groups while IDA [1.65 nmol/L (1.06, 2.41)] and the control group [2.62 nmol/L (1.71, 3.84)] concentrations were found to overlap. Cut-offs of ≤2.88 nmol/L (AUC^{ROC} of 0.826) and >4.87 nmol/L (AUC^{ROC} of 0.93) for IDA and ACD, respectively, were derived from ROC curves. A sTfR log ferritin index of >3.04 was derived but was not found to be superior to ferritin concentrations in IDA.

Conclusion

Hepcidin measured using mass spectrometry shows benefit as a marker to differentiate blood donors with ACD from those with IDA.

Introduction

Worldwide, anaemia is a condition commonly encountered in clinical practice (1). Studies conducted by the World Health Organization (WHO) between 1995 and 2005 identified a global prevalence of anaemia of 24.8% (1,2). Africa was found to have the highest prevalence of between 47.5 – 67.6% (1), a fact reiterated in a 2011 update (3). Iron deficiency anaemia (IDA) is estimated to account for half of the cases of anaemia and once identified requires a search for the underlying cause. Anaemia of chronic disease (ACD) is the second most common cause and is multifaceted (1,2,4).

In South Africa, an estimated 7.7% of blood donors were found to be anaemic (5). Individuals from both sexes may donate every 56 days and the allowable age group is from 16 to 65 years. The high frequency allowed may be placing donors at a higher risk of iron deficiency. Van den Berg et al, estimated that up to 17.5% of all donors in South Africa had iron deficiency (5). The identification of IDA in donors may therefore present a blind spot requiring attention, to conserve the health status of this population.

Haemoglobin (Hb) concentration is used to screen donors prior to donation and a low Hb is used as a criterion for deferral. Further investigation is rarely performed. However, identifying anaemia from a haemoglobin concentration alone is inadequate and additional investigations to identify the cause of the anaemia are recommended as management of the causes differ (1).

The iron profile can be requested to further investigate anaemia and includes serum iron, serum transferrin, transferrin saturation and ferritin. Studies have shown limitations in the use of these tests in isolation when trying to distinguish IDA from

inflammatory states (4–6). These two states may even co-exist. The characteristic pattern of IDA is a low iron concentration, decreased ferritin and increased total iron binding capacity, but actual transferrin saturation is low. Inflammatory states may also present with a low iron concentration, but in contrast the total iron binding capacity is low, and ferritin is normal or even increased (2). The soluble transferrin receptor log ferritin index (sTfR/log ferritin index) has been investigated as a distinguishing marker. A high ratio is suggestive of IDA (7,8). Alternatively, hepcidin has been postulated as a marker more capable of revealing the distinction between these two causes of anaemia (2).

Role of Hepcidin

Hepcidin has gained interest as a useful marker in the diagnosis and treatment of iron related disorders and is now considered the key regulator of iron homeostasis. Bioactive hepcidin is a 25 amino acid peptide hormone produced mainly by hepatocytes and released into general circulation (9,10). Tissues such as macrophages and adipocytes also produce it, but in smaller quantities (9). Processing of hepcidin results in the formation of smaller forms, such as hepcidin-22 and hepcidin-20 that are either absent or present in low concentration in serum, but readily detected in urine (11,12). Their presence may confuse interpretation in conditions where they are increased, such as sepsis or kidney failure (13).

In an iron deficient state, hepcidin synthesis decreases, to allow for an increase in iron concentration. In infection or an inflammatory state, hepcidin expression increases, mediated by interleukin 6. As such, hepcidin has increasingly been touted as a potential target in the treatment of iron disorders and anaemia (14,15).

Methods for hepcidin analysis either fall into classical immunoassay or mass spectrometry-based techniques (9). Immunoassays are unable to differentiate active hepcidin and instead measure total hepcidin. They are, however, more accessible and allow for higher sample throughput (9,16) and have an estimated cost of R708 in our setting. Mass spectrometry is able to distinguish the isoforms, allowing for quantitation of the active form, but requires expensive equipment (9). Our research method has a basic running cost of R 587.

In this study, we aimed to assess the utility of hepcidin as a marker of iron status in anaemia identified in blood donors using Liquid Chromatography coupled with Mass Spectrometry (LCMS).

Materials and Methods

Study Design and Samples

This was a retrospective cross-sectional study using residual samples collected from South African blood donors between August 2014 and October 2014. The samples were originally collected for a multi-centre cross-sectional study conducted by van den Berg et al (5). People donating for therapeutic reasons, autologous and designated donors, donors deferred for reasons other than low Hb levels, those confirmed positive for HIV, hepatitis B and C, and syphilis and donors younger than 18, were excluded. Specimens were initially tested for full blood count (including white blood cell count, red blood cell count, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin concentration, platelets, reticulocyte count and reticulocyte percentage), serum iron, transferrin saturation, and serum ferritin. The specimens were stored in a -20°C freezer until analysis in September 2020. For the current study, hepcidin was analysed in the serum collected in serum separator

tubes, after sorting a fraction of all the samples into three categories: IDA; ACD; and Control groups. Samples were categorised based on Hb and Ferritin levels, so at an estimated prevalence of IDA of 10% with a 5% margin of error and 95% confidence interval, 135 samples from each category were sought. The control group was matched to the IDA group using age and gender.

Anaemia was defined as a donor with a haemoglobin concentration of less than or equal to 12.5 g/dL. If using the WHO Hb cut-off of 12 g/dL to define anaemia in females, one hundred and sixty seven (167) females were unintentionally excluded from the control group (1). IDA was defined as a ferritin of less than 20 µg/L for men and less than 12 µg/L in women (5). Samples with ferritin levels higher than these earlier defined values in the anaemic group were categorised as ACD. Controls were classified as donors without anaemia (Hb >12.5 g/dL) (*Fig 1*). Hepcidin was analysed on all samples. Leptin and Soluble transferrin receptor concentrations were measured to account for the presence of obesity and as an additional marker of iron deficiency, respectively. C-reactive protein was evaluated as a marker of inflammation and serum creatinine was evaluated to screen for possible kidney disease. The sTfR log ferritin index calculation was modelled on the expected increase in soluble transferrin receptor (sTfR) and decrease in ferritin normally observed in iron deficiency and expressed as a ratio: sTfR/log ferritin.

Ethics approval was obtained from the South African National Blood Services Human Research Ethics Committee with the original study's ethical clearance (2015/29) and from the Human Research Ethics Committee of the University of the Witwatersrand (M190525).

Laboratory Procedures

Hepcidin

Hepcidin was measured using a method which has been previously described (17). Briefly, the AB Sciex micro-Liquid Chromatography (UPLC) system (AB Sciex, Framingham, USA) was used, as described by Abbas et al. (2018). Hepcidin and isotopically labelled internal standard [$^{13}C_9$, $^{15}N_3$] were purchased from the Peptide Institute, Osaka, Japan. Samples were extracted using 4% trichloroacetic acid (TCA, Merck, Kenilworth, NJ, USA) in methanol and separated using reverse-phase liquid chromatography on an Eksigent Halopeptide ES 2.7 µm, 0.5 x 100 mm column (Waters, Massachusetts, USA). Analysis of hepcidin ion transitions (930.8→1144.7.0 m/z and 703.2→354.1 m/z for Hepcidin and Hepcidin (IS), respectively) were performed using triple quadrupole ion trap mass spectrometry (µLC-MS/MS QTRAP). Electron spray ionization (ESI) was used. The laboratory intra-assay coefficient of variation (CV) was 10.5% for 2.31 nmol/L and 8.3% for 9.24 nmol/L, while the inter-assay CV was 9.76% and 14.78% for 2.31 nmol/L and 9.24 nmol/L, respectively. The estimated limit of quantitation (LOQ) for hepcidin was 0.19 nmol/L and limit of detection (LOD) was 0.058 nmol/L.

Leptin

Leptin was assayed using the RD11001100 Human Leptin ELISA, Clinical Range kit (BioVendor Research and Diagnostic Products, 621 00 Brno, Czech Republic). The intra-assay CV was 9.24%, while inter-assay CV was 13.08%. The LOD was 0.2 ng/mL.

Soluble Transferrin Receptor (sTfR)

Soluble transferrin receptor was measured on the Roche Cobas 8100 system (Roche Diagnostics Ltd, D-68298 Mannheim, Germany) using the Tina-quant Soluble Transferrin Receptor kit. The inter-assay CV was 2.11% at 25 nmol/L and 1.33% at 84.2 nmol/L.

C-reactive protein

C-reactive protein was determined using the C-reactive protein Gen.3 immunoturbidimetric assay kit on the Roche Cobas 8100 system (Roche Diagnostics Ltd, D-68298 Mannheim, Germany). The inter-assay CV was 6.8% at 8.71 mg/L and 10.8% at 54.2 mg/L.

Serum creatinine

The serum creatinine was analysed enzymatically on the Roche Cobas 8100 system. (Roche Diagnostics Ltd, D-68298 Mannheim, Germany). The inter-assay CV was 5.7% at 93 µmol/L and 5.1% at 347 µmol/L. eGFR was derived from the creatinine concentration using the CKD-EPI formula.

sTfR log ferritin index

sTfR, initially measured in nmol/L, was converted to mg/L and the ratio with the common logarithm of serum ferritin (measured in µg/L) reported. The derived cut-off for IDA versus ACD was used to identify hepcidin concentration thresholds.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016® (Microsoft Corp, Redmond, CA, USA) for data capturing and comparative statistics, Tibco Statistica® (version 13.5.0.17, United States) for comparative statistics and MedCalc® (version

20.009, Belgium) for Receiver operating characteristic (ROC) curves. The mean and standard deviation (SD) were reported if data were normally distributed while median with interquartile ranges was used for skewed data. ANOVA was used to compare continuous parametric data from each group and the Kruskal-Wallis for non-parametric. Correlation analysis for the measured parameters with hepcidin was performed using the Spearman's rank correlation. The contribution of confounding factors to results were evaluated using ANCOVA. All tests were considered statistically significant if $p<0.05$. Suspected outliers were identified on Box and Whisker plot analysis (18). Hepcidin concentrations were not normally distributed, and values were log transformed prior to parametric analysis. Following back transformation, the median and interquartile ranges for hepcidin were reported. ROC curves were generated and the area under the curve (AUC^{ROC}) was determined for hepcidin concentration cut-offs as defined by the category: iron deficiency anaemia and another for anaemia of chronic disease. The Youden index [(sensitivity + specificity)-1] was calculated and used to determine the maximum point of sensitivity and specificity.

Results

Using the predefined criteria per category, 135 samples were selected for the IDA and 135 samples for the control group, respectively. One hundred and twenty-eight (128) samples met the criteria for the ACD group (*Fig 1).*

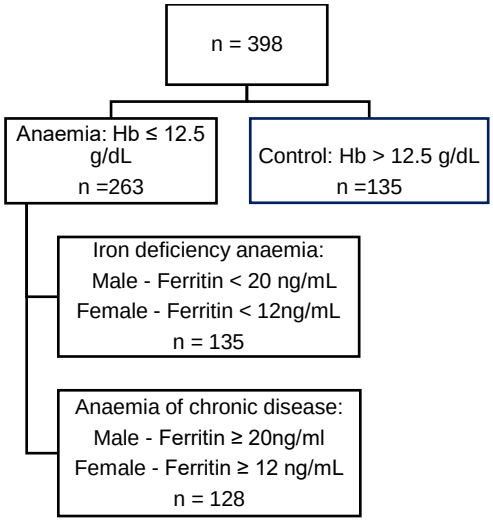

Figure 1: Patient categorisation

Study population characteristics

Of the 398 samples studied, 52 (13.1%) were from males and 346 (86.9%) from females. The mean age was 33.6 ± 13.1 years and was not significantly different (p = 0.09) between the three categories.

Analysis by category: IDA vs ACD vs Control

Measured parameters were compared between the three groups (*Table 1)*, and all except for white blood cell count and reticulocyte percentage, were significantly different ($p<0.05$). Mean corpuscular volume and mean corpuscular haemoglobin concentration were lowest in the IDA group at 85.4 ± 6.5 fL and 29.6 ± 1.3 g/dL, respectively. The same pattern was found for serum iron [6.5 µmol/L (5.2, 9.6)], transferrin saturation [7.4 % (5.7, 10.9)], and serum ferritin [8.1 µg/L (6.6, 10)]. In contrast, serum transferrin (3.5 ± 0.6 g/L) and soluble transferrin receptor [46.1 nmol/L (27.4, 74.6)] values were highest in the IDA group. Hepcidin concentrations were statistically different ($p<0.001$) between all three groups being lowest in the iron deficiency anaemia group and highest in the anaemia of chronic disease group. CRP

and leptin concentrations were highest in the ACD category, at concentrations of 2.8 mg/L (0.8, 6.0) and 15.6 ng/mL (8.4, 27.1), respectively. Most of the measured tests were highest in the control group with exceptions being platelet count, serum transferrin, CRP, hepcidin, leptin and sTfR. Previous two-year donation count was highest for the IDA group (6.1 ± 3.3) and lowest for the ACD group (4.8 ± 3.0).

The Box and Whisker plot representing hepcidin concentrations for the three categories showed the median for ACD was significantly different from IDA and the control group. Overlap was noted between the IDA and control groups. The back transformed median and interquartile ranges were 1.65 nmol/L (1.06, 2.41) for IDA, 7.89 nmol/L (5.96, 10.84) for ACD, and 2.62 nmol/L (1.71, 3.84) for controls (*Fig 2*). No true outliers were identified.

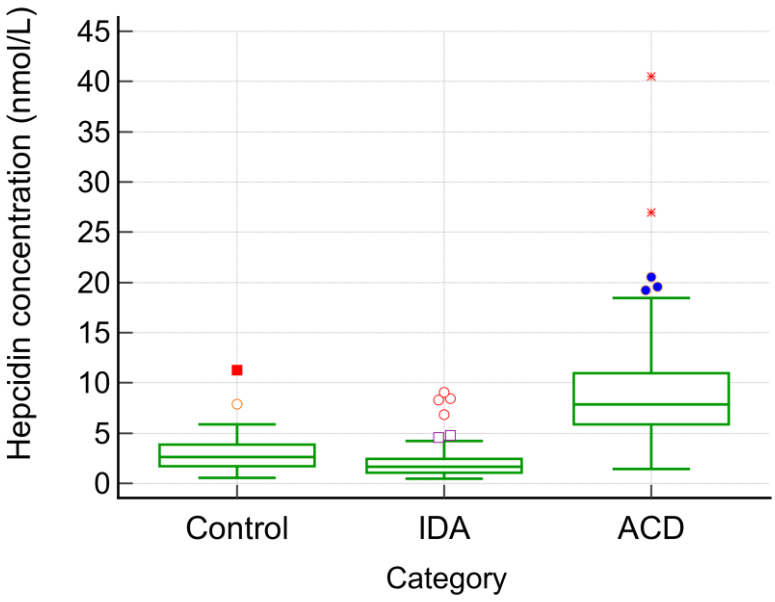

Figure 2: Box and Whisker plots of Hepcidin concentration per category

Hepcidin was positively correlated with ferritin levels ($p<0.0001$), CRP and leptin ($p<0.05$), and negatively correlated with sTfR/log ferritin index ($p<0.001$), serum transferrin ($p<0.0001$), sTfR and previous two-year donation count ($p<0.05$) as

shown in *Table 2*. It was not significant correlated with haemoglobin or eGFR. ANCOVA analysis showed that CRP, eGFR, leptin, and sTfR did not affect hepcidin concentrations significantly (p>0.05). *See Supplementary material: Table 1*.

ROC Curves

Cut-offs to differentiate IDA in the screened population using hepcidin as a marker were evaluated. ROC curve analysis was performed to assess the diagnostic value and an AUC^{ROC} of 0.826 was achieved.

The recommended cut-off for IDA was found to be ≤2.88 nmol/L (Youden index = 0.55) with a sensitivity of 85.9%, specificity of 67.3%, positive predictive value (PPV) of 22.6% and negative predictive value (NPV) of 97.7% (*Fig 3).* Improved specificity was achieved at a cut-off of ≤2.01 nmol/L with a specificity of 81.0% and sensitivity of 61.5%. Its associated PPV was 26.4% but the NPV was robust at 95.0%.

The optimal criterion proposed was ≤1.28 nmol/L with a sensitivity of 39.3%, specificity of 93.9%, PPV of 41.8 % and a NPV of 93.3%. *See Supplementary material: Table 2.*

ROC curve analysis was also performed to derive a cut-off to identify donors fitting into the ACD group (*Fig 3).* The concentration of 4.87 nmol/L (Youden index = 0.78) yielded a sensitivity of 85.0%, specificity of 93%, PPV of 57.4% and NPV of 98.2%. The AUC^{ROC} was 0.93. *See Supplementary material: Table 2.*

Using the previously mentioned ferritin cut-offs as a discriminator, a sTfR log ferritin index value was derived to identify iron deficiency. The ROC curve yielded a value of >3.04. The AUC^{ROC} was 0.819, sensitivity 69.0% and specificity 80.2%. Using this value, a hepcidin concentration cut-off was determined (*Fig 3).* The AUC^{ROC} was

only 0.623 and the cut-off of ≤2.77 nmol/L offered a sensitivity of 63.6% and a specificity of 59.5%. *See Supplementary material: Table 2*.

Discussion

In this study we measured hepcidin concentration using a hybrid micro-liquid chromatography triple quadrupole ion trap mass spectrometer to evaluate its performance as a marker of ACD or IDA in blood donors. In line with the physiological pattern predicted, hepcidin concentrations were highest in the ACD group and lowest in the IDA group. As an analyte used to identify ACD, hepcidin showed greatest utility as it was able to differentiate this population from the IDA and control groups. To our knowledge, this is the first study to investigate the utility of hepcidin for ACD in blood donors. Analysis showed that hepcidin is able to differentiate IDA from ACD but not controls.

The ability of hepcidin to identify patients having ACD demonstrated more useful value in our study, with an AUC^{ROC} of 0.93. The Youden index was identified at a concentration of 4.87nmol/L. The use of this value in practice requires scrutiny as there is a paucity of data regarding the clinical use of hepcidin concentration in ACD. In a preliminary study, Svenson et al evaluated the efficacy of hepcidin in anaemic patients from a gastroenterology clinic, and found that a concentration of 46 ng/ml (16.49 nmol/L) had a sensitivity and specificity of 100% for identifying ACD (19). This concentration is markedly greater than our findings and may be explained by the difference in populations studied and their associated disease risk.

Donors who are recognized as being anaemic are deferred and are often advised to take iron supplements. This recommendation may not be universally applicable as adverse clinical consequences may ensue. ACD is a particular concern in the South

African population certainly, considering the severe burden of chronic disease, including human immunodeficiency virus, tuberculosis, chronic kidney disease and other inflammatory conditions (20). The true prevalence of ACD is yet to be clearly elucidated and has received very little attention in the blood donor population. ACD poses a unique challenge in blood donors as they are assumed to be relatively healthy and markers of inflammation, such as CRP, may not reflect subclinical disease. In the South African population, investigation of the anaemia has relevant health benefits. Although CRP levels were lower than levels expected in inflammation (<10 mg/L), these were found to be highest in the ACD group. CRP is considered an early marker of inflammation, and may indicate low-grade inflammation(21). Even at these low levels, hepcidin analysis allowed a clear distinction as an independent marker of ACD, increasing as expected.

A South African study, published by Phatlhane et al, found iron deficiency accounted for 78% of cases of anaemia in our population (22). Previous studies have reported mixed results when evaluating the utility of hepcidin as a potential marker of donors at risk for iron deficiency (23–25). Pasricha et al. investigated iron deficiency in premenopausal female donors reporting an AUC^{ROC} of 0.87 and suggested an optimal hepcidin cut-off of 8 ng/mL equating to 2.87 nmol/L (26). These results are similar to the outcomes reported here where a cut-off concentration of 2.88 nmol/L with an AUC^{ROC} of 0.826 was noted. This concentration is also comparable to cut-offs identified in previous studies (2,8,27). However, identifying the donors who do not have iron deficiency is also important and the efficient use of iron therapy may be guided by analysing hepcidin concentration. From our study, the cut-off of 2.88 nmol/L offered a strong NPV of 97.7%.

As yet, there is no consensus on ideal hepcidin cut-off values to be used for prediction of iron deficiency anaemia. Assays are not standardized as there is no prescribed reference material, reference method or a commutable calibrator for hepcidin. The concordance between our results obtained using mass spectrometry and multiple other studies using immunoassay is encouraging, but the issue with comparability limits their common use. Efforts at harmonisation are on-going but at present absolute hepcidin concentration often varies between methods compromising comparability and efforts to establish universal reference intervals. Each method requires deriving own reference ranges and cut-offs (9).

Another marker assessed in previous studies to differentiate IDA is the sTfR/log ferritin index, although an absolute cut-off for this ratio is not universally available (7). The derivation of the cut-offs is poorly standardized and the categorisation of patients with iron deficiency most often requires interpretation of the patient's ferritin concentration. Studies of its use in Africa are also limited (7,28,29). Our study did not show increased benefit of its utility as compared to the more commonly used ferritin cut-offs, with an AUC^{ROC} of 0.623 and a similar cut-off value (2.77 nmol/L).

In literature the use of soluble transferrin receptor is most effective in patients with suspected IDA (30), and in this study, it demonstrated a negative correlation with hepcidin concentration, as expected, supporting the decrease in hepcidin seen in patients with IDA.

A few confounders were investigated in this study to improve the validity of the findings made. Obesity is a major public health concern in the South African population and iron deficiency is common in overweight people (31). Several studies have revealed a positive relationship between obesity or its surrogate, leptin, and

hepcidin concentrations. Both leptin and hepcidin can be produced by adipocytes and studies on mice and cultured cells have found leptin influences hepcidin production (32–36). In contrast, leptin was not detected as a confounder in our hepcidin analysis.

The impact of renal illness on hepcidin concentrations was also considered, as higher hepcidin concentrations have been associated (37). Although a difference was noted in absolute creatinine concentrations between the groups, the eGFR did not indicate a correlation with hepcidin concentration and it was not identified as a confounder to analysis. This is likely because the blood donor population are assumed to be relatively healthy.

Strengths of this study include the measurement of all routine analytes in the investigation of patients with iron related disorders mirroring the evaluation of patients by clinicians. The classification is based on standard clinical practice, and there was a significant correlation of hepcidin measurement with ferritin, which is currently used in the diagnosis of IDA. Use of mass spectrometry to quantify hepcidin is advantageous in ensuring the bioactive form of the protein is evaluated. Furthermore, the effect of confounders such as obesity and renal disease were investigated.

Limitations are we did not have results of bone marrow iron staining, the gold standard for iron deficiency, which is ideally used to classify patients but is not routinely done in blood donors. Other possible causes of anaemia were not considered. The stability of the remnant samples was not investigated, which may result in underestimation, compromising utility of the cut-offs' clinical relevance. Studies have shown stability of a month for samples stored at -20˚C (9,38).

In conclusion, hepcidin is still considered a novel marker, and its routine use has not been fully adopted. In South Africa, hepcidin measurement by immunoassays is exclusively available within the private sector. Our findings imply that hepcidin measured using mass spectrometry could help improve the management of blood donors identified with anaemia. It is beneficial in differentiating individuals with ACD from those with IDA, and it shows promise, particularly in the former group. Cut-offs necessitate greater investigation, and studies on fresh samples are recommended.

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Informed consent: The original study obtained a waiver for separate individual consent from the SANBS Human Research Ethics Committee.

Ethical approval: Approval for sub studies regarding investigations related to iron status was obtained from the SANBS HREC with the original study's ethical clearance (2015/29) (5). Ethics approval was obtained for the current study from the Human Research Ethics Committee of the University of the Witwatersrand. (M190525)

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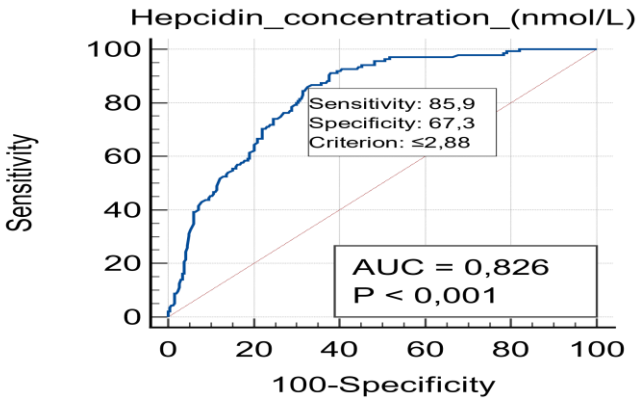
A)



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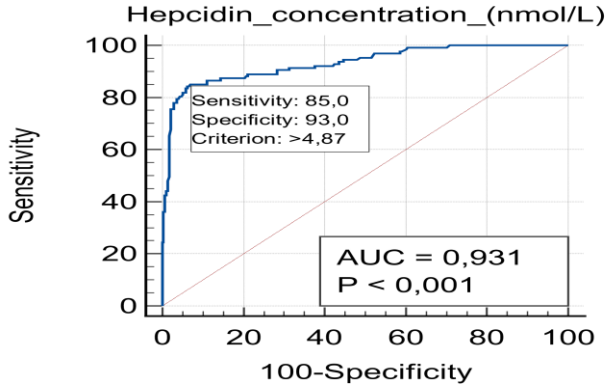

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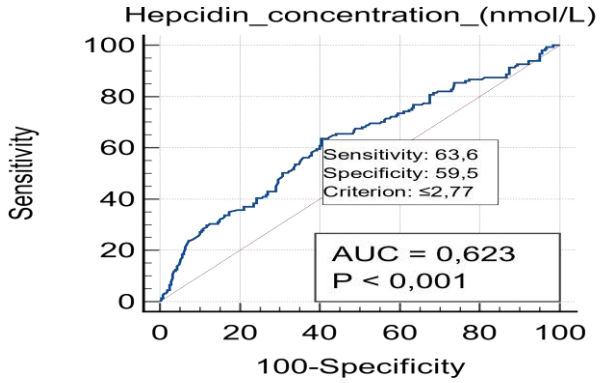

Fig 3: ROC curves for hepcidin concentration: A) Using ferritin cut-offs for Iron deficiency anaemia. B) Using ferritin cut-offs for Anaemia of chronic disease. C) Using sTfR/log ferritin index of 3.04 to define iron deficiency anaemia

Table 1: Demographics, haematological and biochemical parameters					
	IDA (n = 135)	ACD (n = 128)	Controls (n = 135)	Total (n = 398)	P-value (ANOVA)
Male (n, %)	23 (5.8%)	7 (1.8%)	22 (5.5%)	52 (13.1%)	
Female (n, %)	112 (28.1%)	121 (30.4%)	113 (28.4%)	346 (86.9%)	
Mean age ± SD (years)	32.0 ± 13.1	35.8 ± 13.6	33.3 ± 12.5	33.6 ± 13.1	0.09071
Race: White (n, %)	81 (44.5%)	31 (17.0%)	70 (38.5%)	182 (45.7%)	
Black (n, %)	60 (38.7%)	66 (42.6%)	29 (18.7%)	155 (38.9%)	
Asian (n, %)	12 (41.4%)	11 (37.9%)	6 (20.7%)	29 (7.3%)	
Coloured (n, %)	11 (45.8%)	9 (37.5%)	4 (16.7%)	24 (6%)	
Unknown (n, %)	0 (0%)	4 (50%)	4 (50%)	8 (2%)	
WBC (x 10^9/L) ± SD	6.7 ± 1.8	7.0 ± 1.7	7.2 ± 2.5	6.7 ± 2.1	0.11148
RBC (x 10^{12}/L)	4.6 (4.3, 4.9)	4.4 (4.2, 4.6)	4.8 (4.5, 5.1)	4.5 (4.3, 4.9)	**<0.0001**

Haemoglobin (g/dL)	12.0 (11.6, 12.2)	12.3 (12.0, 12.4)	14.3 (13.6,15.0)	12.4 (12,13.6)	**<0.0001**
MCV ± SD (fL)	85.4 ± 6.5	90.6 ± 6.0	92.2 ± 5.5	89.4 ± 6.7	**<0.0001**
MCHC ± SD (g/dL)	29.6 ± 1.3	30.6 ± 1.0	31.7 ± 1.2	30.6 ±1.4	**<0.0001**
Platelets ± SD (x 10^9/L)	308.8 ± 76.4	305.3 ± 75.2	261.9 ± 61.2	291.8 ± 74.2	**<0.0001**
Reticulocyte count ± SD (x 10^{12}/L)	81.4 ± 21.6	80.4 ± 21.0	87.4 ± 21.5	83.1 ± 21.5	**0.01604**
Reticulocyte percentage ± SD (%)	1.8 ± 0.4	1.8 ± 0.5	1.8 ± 0.4	1.8 ± 0.4	0.40984
Serum iron (µmol/L)	6.5 (5.2, 9.6)	10.1 (7.8, 14.5)	15.5 (10.6, 21.2)	10.1 (6.6,15.7)	**<0.0001**
Serum transferrin ± SD (g/L)	3.5 ± 0.6	3.1 ± 0.5	3.1 ± 0.5	3.3 ± 0.6	**<0.0001**
TSAT (%)	7.4 (5.7, 10.9)	12.2 (9.4, 19.9)	19.3 (13.6, 26.9)	12.1 (8.0, 20.1)	**<0.0001**
Serum ferritin (µg/L)	8.1 (6.6, 10)	20.9 (15.8, 38.9)	35.5 (24.1, 56.7)	17.3 (9.5,37.6)	**<0.0001**
CRP (mg/L)	1.1 (0.3, 2.9)	2.8 (0.8, 6.0)	1.3 (0.5, 4)	1.6 (0.5, 4.1)	**0.0001**
Creatinine ± SD (µmol/L)	68.5 ± 22.7	62.8 ± 18.2	82.6 ± 20.9	71.5 ± 22.3	**<0.0001**

Previous two-year donation count	6.1 ± 3.3	4.8 ± 3.0	5.3 ± 3.4	5.4 ± 3.3	**0.00883**
Hepcidin (nmol/L)	1.65 (1.06, 2.41)	7.89 (5.96, 10.84)	2.62 (1.71, 3.84)	3.1 (2.8, 3.4)	**<0.0001**
Leptin (ng/mL)	11.4 (7.2,17.9)	15.6 (8.4,27.1)	10.4 (7.1,19.6)	12.2 (7.5, 21.1)	**0.0035**
sTfR (nmol/L)	46.1 (27.4,74.6)	35.5 (27.1,48.1)	33.8 (27.2,42.3)	36.3 (27.2, 54.4)	**0.0022**

[1]Normally distributed continuous variables represented as mean ± SD, Non-normally distributed continuous variables represented as median (interquartile range), Categorical variables represented as n (%).

WBC: white blood cells; RBC: red blood cells; MCV: mean corpuscular volume; MCHC: mean corpuscular haemoglobin concentration; TSAT: Transferrin saturation; CRP: C-reactive protein; sTfR: soluble transferrin receptor

Table 2: Spearman's Correlation coefficient with hepcidin		
Parameter	**r_s (95% CI)**	**P-value**
Haemoglobin	0.037 (-0.062 to 0.135)	0.4629
Serum iron	0.174 (0.076 to 0.268)	**0.00053**
Serum transferrin	-0.206 (-0.298 to -0.109)	**<0.0001**
TSAT	0.221 (0.125 to 0.313)	**<0.0001**
Serum Ferritin	0.484 (0.376 to 0.532)	**<0.0001**
CRP	0.183 (0.086 to 0.276)	**0.00025**
eGFR	0.061 (-0.038 to 0.158)	0.2253
Leptin	0.164 (0.067 to 0.258)	**0.0010**
sTfR	-0.109 (-0.205 to -0.011)	**0.0291**
sTfR log ferritin index	-0.276 (-0.364 to -0.182)	**<0.0001**
Previous two-year donation count	-0.114 (-0.210 to -0.0155)	**0.0234**

Supplementary material

Table 1: Covariate analysis

Covariate analysis				
Covariate	**Sum of Squares**	**DF**	**F**	**P-value**
CRP	14.902	1	1.361	0.244
eGFR	3.167	1	0.289	0.591
Leptin	30.937	1	2.826	0.094
sTfR	6.818	1	0.623	0.430
Previous two-year donation count	6.605	1	0.512	0.475

Table 2: ROC curve analysis for hepcidin and IDA

ROC curve analysis	AUC (Confidence Interval)	Youden index (J)	J associated criterion (nmol/L)	Sensitivity (%)	Specificity (%)	Optimal cut-off (nmol/L)	Sensitivity (%)	Specificity (%)
Hepcidin and IDA	0,826 (0,785 - 0,862)	0,53	≤ 2,88	85,93	67,30	≤ 1,28	39,26	93,92
Hepcidin and ACD	0,931 (0,901 - 0,954)	0,78	> 4,87	85,04	92,99	> 5,87	75,59	97,79
sTfR/log ferritin index derivation	0,819 (0,777 - 0,855)	0,49	> 3,04	68,97	80,24	> 4,72	46,90	97,63
Hepcidin and sTfR/log ferritin index	0,623 (0,573 - 0,671)	0,23	≤ 2,77	65,58	59,51	< 0,46	0,00	100,00