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A simplified diagnostic pathway for the differential diagnosis of iron deficiency anaemia and anaemia of chronic disease

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Abstract

Introduction: Iron deficiency anaemia (IDA) and anaemia of chronic disease (ACD) are common causes of anaemia with similar clinical and laboratory features. IDA is caused by low iron stores while ACD is due to iron-restricted erythropoiesis occurring in inflammatory states. Differential diagnosis requires analysis of multiple biochemical and haematological parameters. IDA can occur simultaneously to ACD (mixed aetiology). It is essential that true iron deficiency is identified, as these patients will require iron therapy. This preliminary study investigated whether hepcidin, the master regulator of iron homeostasis, in conjunction with reticulocyte haemoglobin equivalent (RetHe) has the potential to differentiate IDA from ACD, and to exclude IDA in patients with mixed aetiology.

Methods: Hepcidin concentration (measured using a commercially available ELISA method), RetHe, and iron parameters along with C-reactive protein (CRP) were analysed in 77 Gastroenterology patients with anaemia in a secondary care setting.

Results: Receiver operator characteristic (ROC) analysis showed that hepcidin at an optimal cut-off concentration of <6ng/ml could identify IDA with a sensitivity and specificity of 88.9% and 90.6% respectively and could distinguish ACD from IDA with both a sensitivity and specificity of 100% at a cut-off of >46ng/ml. Identifying true IDA in mixed aetiology patients could be achieved by RetHe analysis and applying an optimal cut-off of <30pg.

Conclusion: Hepcidin, in conjunction with RetHe, offers a new simplified diagnostic pathway for differential diagnosis of IDA and ACD, thereby reducing the diagnostic turnaround time and allowing appropriate treatment of patients with a true iron deficiency.

KEYWORDS

anaemia of chronic disease, hepcidin, Iron deficiency anaemia, reticulocyte haemoglobin equivalent

1 | INTRODUCTION

Iron deficiency anaemia (IDA) and anaemia of chronic disease (ACD) are the most prevalent causes of iron-related anaemia in subjects with gastrointestinal disorders contributing significantly to morbidity and mortality.[1-3] IDA is the result of a true iron-deficient state leading to ineffective erythropoiesis. In contrast, the iron-restricted erythropoiesis observed in ACD is due to an inflammation-induced block in intestinal iron absorption and iron retention in reticuloendothelial (RE) cells.[4] The diagnosis of IDA and ACD currently requires a combination of multiple haematological and biochemical tests. Furthermore, diagnosis is not always straight forward, and patients can present with an iron deficiency alongside ACD (mixed aetiology), usually resulting from chronic blood loss. Mixed aetiology patients present a diagnostic quandary as traditional biochemical iron parameters, such as ferritin, serum iron and transferrin, are affected by inflammation making them less suitable indicators.[5] Indeed, although low serum ferritin and transferrin (a negative acute response protein) levels may imply IDA due to lack of iron storage and transport in a truly iron-deficient patient, these results may also be seen in ACD due to abnormal retention of the iron within the RE system. It is important that true iron deficiency in patients with ACD is identified, since these are the patients that will require iron therapy to allow for optimal erythropoiesis.[6] In contrast, administering iron supplements to ACD patients who have normal iron stores may be inappropriate and potentially harmful.[7]

The mechanism of iron homeostasis has long been established and is a complex process, involving absorption of iron via enterocytes in the duodenum, passage of the iron into plasma and transport via the carrier protein apotransferrin.[8] Iron may be stored within the RE system, in hepatocytes, or within macrophages in the bone marrow, or may be utilized during red cell production. Movement of iron out of enterocytes, hepatocytes and macrophages occurs via ferroportin.[9] The 25 amino-acid antimicrobial peptide hormone, hepcidin, is a regulator of iron homeostasis with levels reported as being high in individuals with inflammation, and low in those with IDA.[10] Produced in the liver, hepcidin internalizes ferroportin, blocking the release of iron from the RE system and reducing absorption of dietary iron, thereby limiting iron availability for erythropoiesis.[9] Indeed, during inflammation, high circulating levels of interleukin-6 (IL-6) promote hepcidin production.[11] Responsive changes in hepcidin concentration make it an ideal real-time indicator of iron supply for erythropoiesis, with changes to haematological status seen within hours.[12] However, identifying subjects with mixed aetiology is difficult as serum hepcidin values tend to appear within the normal reference interval.[7]

The concept of hepcidin analysis in the diagnosis of IDA and ACD is not new, but the majority of assay methods have centred on mass-spectrometry (MS) and radioimmunoassay techniques. While these methods have good specificity, their sensitivity is poor.[13] To overcome this, the development and introduction of an Enzyme-linked Immunosorbent Assay (ELISA)-based method has been widely researched,[14-19] and although very few commercial assays are currently available, the Hepcidin-25 (bioactive) ELISA immunoassay does have future potential in a routine clinical laboratory due to the high sensitivity, high specificity and relative cost efficiency.

Reticulocytes are the precursors of mature erythrocytes and circulate in peripheral blood for 1-2 days before maturing.[20] The measurement of reticulocyte haemoglobin parameters such as reticulocyte haemoglobin content (CHr) or reticulocyte haemoglobin equivalent (RetHe) allows assessment of the actual availability of iron and the quality of erythropoiesis[21] and has been used to monitor iron status in several patient cohorts and settings by serving as an indicator of the early stages of iron demand in erythropoiesis, before the development of anaemia.[22-24] Changes in these parameters may be seen within a couple of days of altered iron availability. Such studies have revealed the cost-effectiveness of introducing such parameters into routine diagnostic workup of anaemias.

This preliminary study aimed to investigate whether the use of a commercially available hepcidin-25 bioactive ELISA in conjunction with RetHe measurement could reduce the number of tests required during anaemia investigations. The study focussed on Gastroenterology patients, since there is a relatively high incidence of mixed aetiology anaemia in this cohort due to prevalence of chronic disease in such patients. The study was therefore also able to evaluate whether true iron deficiency could be identified in mixed aetiology patients using this Hepcidin-RetHe combination, thereby more easily identifying a subgroup of patients who require iron therapy.

2 | MATERIALS AND METHODS

2.1 | Ethics

Local approval for the study was obtained from the Clinical Audit and Effectiveness Team. This was a preliminary study, and the research was conducted using excess biological material (whole blood or serum) collected during normal clinical care. All collected material was anonymized for the purposes of the research. It involved no deviation from standard clinical care, and the data obtained were not used in the clinical management of patients. Blood samples were also collected from normal healthy volunteers with approval from NRES (16/NE/0291) and in accordance with the declaration of Helsinki. These samples acted as the "control" group. Consent was obtained from the healthy volunteers after the nature of the procedure(s) had been fully explained.

2.2 | Routine haematology testing

A total of 77 adult patients ≥18 years of age were identified for the study. These were hospital inpatients under the Gastrointestinal team or patients attending Gastrointestinal outpatient clinics at Hull Royal Infirmary, Hull, United Kingdom. Samples were identified using the World Health Organisation (WHO) definition of anaemia;

TABLE 1 Haematological and biochemical profile of subjects involved in the study

	Normal Healthy Volunteers	All Patient Data	Iron Deficiency Anaemia (IDA)	Anaemia of Chronic Disease (ACD)	Mixed Aetiology (IDA/ACD)	Patients with Normal iron status
Sample size	32	77	36	4	16	21
Patient Age	46 (19-64)	69 (18-89)	65.5 (18-89)	70.5 (63-84)	69.5 (19-89)	74 (19-84)
Hb (g/L)	138 (125.0-159.0)	108.0 (80.0-130.0)	105.5 (82.0-130.0)	106.0 (96.0-120.0)	108.0 (81.0-130.0)	117.0 (80.0-130.0)
MCV (fL)	88.05 (82.2-98.6)	80.4 (66.0-99.7)	77.0 (66.0-92.2)	89 0.0 (791-91.5)	83.6 (70.9-91.2)	87.1 (70.2-21.6)
MCH (pg)	29.6 (27.4-33.9)	26.1 (19.4-33.2)	24.5 (19.4-32.47)	28.5 (24.8-29.0)	26.3 (20.6-30.3)	28.8 (21.6-33.2)
CRP (mg/L)	1.2 (0.3-9.7)	4.3 (0.4-178.0)	2.8 (0.4-18.0)	26.0 (9.4-178.0)	19.0 (8.3-92.0)	2.8 (0.7-10.0)
Ferritin (µg/L)	39.5 (8-224)	17 (3-916)	8.5 (3-82)	303.5 (106-916)	26 (6-72)	38 (5-388)
Transferrin (g/L)	2.75 (2.1-4.3)	3.10 (1.4-3.1)	3.50 (2.3-8.1)	1.99 (1.4-4.1)	2.60 (1.7-3.6)	2.70 (2.0-4.0)
TSAT (%)	29 (10-85)	8 (1-83)	5.89 (1-83)	13 (8-21)	6.82 (1-15)	21.6 (9-62)
Iron (µmol/L)	20 (9-51)	6.00 (1.0-62.0)	5.00 (1.0-52.0)	7.55 (2.9-17.4)	4.75 (1.00-8.0)	14.00 (8.0-62.0)
Hepcidin (ng/ml)	17.15 (4.5-43.6)	4.98 (1.6-136.0)	3.76 (1.63-18.9)	79.5 (73.7-136.0)	13.15 (2.6-41.2)	15.30 (1.6-79.0)
RetHe (pg)	33.2 (30.3-39.5)	30.4 (19.1-38.5)	28.3 (19.1-38.5)	33.0 (25.4-34.6)	29.7 (19.9-36.7)	35.6 (26.1-37.5)

Note: Values are presented as median (range).

Abbreviations: CRP, C-Reactive Protein; Hb, Haemoglobin; MCH, Mean Cell Haemoglobin; MCV, Mean Cell Volume; RetHe, Reticulocyte Haemoglobin Equivalent;TSAT Transferrin Saturation.

haemoglobin (Hb) <130 g/L for males and <120 g/L for females. Each sample retrieved included a request for a full blood count (FBC) analysis, iron-related parameters (serum ferritin, serum iron or transferrin) and C-reactive protein (CRP). They were retrieved from the routine laboratory workload with the study being performed on excess biological material after all analyses requested by the clinical team had been performed. The FBC analysis was performed on the Sysmex XE-2100 FBC analyser, which was quality controlled daily using e-CHECK (XE) quality control samples. RetHe parameters were performed using corresponding Ethylenediaminetetraacetic acid (K2 EDTA) samples within 24 hours of sample collection. Samples used for the routine biochemistry, iron and CRP were collected, and residual serum sample material was divided into 3 aliquots and frozen at −20°C until required for Hepcidin-25 analysis. This is in line with the manufacturer's recommendations. A further 32 blood samples were collected from healthy volunteers. The same testing profile was performed on the healthy samples as was performed on the samples from the gastrointestinal patients.

2.3 | Biochemistry analysis

Analysis of serum ferritin was performed using a Beckman Coulter UniCel DxL 800 Access Immunoassay System (Beckman Coulter Inc) and analysed using an in vitro chemiluminescent immunoassay method for quantitative determination. Transferrin, serum iron and CRP analyses were performed using a Beckman Coulter AU5800 Clinical Chemistry System (Beckman Coulter Inc). Transferrin and CRP were measured in vitro using an immune-turbidimetric method for quantitative determination. Serum iron was measured in vitro using a photometric colour test method for quantitative determination. Transferrin saturation % was calculated from the iron and transferrin measurement by the following calculation.

Transferrin saturation (TSAT) = Serum Iron/Transferrin x 100.

2.4 | Hepcidin C-ELISA

Hepcidin analysis was performed using a commercially available, CE-marked, fully validated competitive Hepcidin ELISA[25] (EIA-5258) kit (DRG Diagnostic GmbH) according to the manufacturer's instructions. The kit has a measuring range of 0.15-81.0 ng/ml and an analytical sensitivity of 0.153 ng/ml. Standard and patient serum samples were run in duplicate.

2.5 | Statistical analysis

Concentration of hepcidin (ng/ml) in patient samples was calculated from the standard curve. The statistical software "Analyse-it" (Version 2.11, Analyse-it Software Ltd) was used to analyse distribution and correlation. All test parameters were analysed for normality using a Kolmogorov-Smirnov D test. Nonparametric data

were presented as median ± range. Kruskal-Wallis analysis with Dunn's post hoc test was performed to identify significant differences in ferritin, hepcidin and RetHe between patient subgroups. Correlation studies were conducted between serum hepcidin, serum ferritin, serum iron, transferrin and TSAT using the nonparametric Spearman's Rank Correlation. All parameters were expressed in logarithmic value. All statistical comparisons were performed using a 95% confidence interval, and statistical significance was defined as $P < .05$. Receiver operator curves (ROC) were produced using GraphPad Prism (version 6.0), and the area under the curves (AUCs) reported. Youden's index was calculated to report on the cut-off values for optimal specificity and sensitivity. MedCalc® Diagnostic Test Evaluation Calculator was used to determine the positive predictive value (PPV) and negative predictive value (NPV) for each test at the optimal cut-off value.

3 | RESULTS

3.1 | Patient demographics

Data collected from 77 patients (36 males and 41 females) and 32 healthy volunteers (6 males and 26 females) were analysed. Based upon the results from the traditional diagnostic testing pathway and established practice for diagnosis (see Figure S1), patients were diagnosed with IDA, ACD, IDA/ACD (mixed aetiology) or normal iron status. Of the 77 patients, 36 patients (47%) were shown to have IDA, 4 (5%) ACD, 16 mixed aetiology (ACD/IDA) (21%) and 21 (27%) normal iron status, despite an initial low Hb result. The Haematological and biochemical profile of the subjects are summarized in Table 1.

3.2 | Differential diagnostic utility of serum hepcidin and RetHe

The median levels of hepcidin in the different patient subgroups were compared to determine whether it allowed for discrimination between IDA, ACD and mixed aetiology (Figure 1A). Kruskal-Wallis analysis revealed that although the IDA and ACD groups were significantly different ($P < .001$), hepcidin levels were not significantly different between the mixed aetiology and ACD subgroups. When RetHe was considered in isolation, it was also unable to differentiate mixed aetiology and ACD patients (Figure 1B).

When Spearman Rank Correlation (Table S1) was used to assess the correlation between serum hepcidin, traditional iron parameters and CRP, there was very strong positive correlation between hepcidin and ferritin (R2 = 0.79, 2-tailed $P < .0001$). As expected, transferrin showed an inverse correlation with hepcidin (R2 = −0.55, 2-tailed $P < .0001$). Positive correlations were seen between hepcidin and transferrin saturation (R2 = 0.46, 2-tailed $P < .0001$), hepcidin and iron (R2 = 0.31, 2-tailed p 0.0063) and hepcidin and CRP (R2 = 0.23, 2-tailed p 0.0488). These results indicated that hepcidin could successfully replace ferritin for identification of IDA and ACD.

3.3 | Diagnostic cut-off concentrations

In order to assess the true diagnostic value of hepcidin and RetHe for determination of IDA and ACD, ROC analyses were performed and the area under the curve (AUC) was calculated (Table 2). The positive and negative predictive powers of each parameter at the optimal cut-off value (derived from the maximal Youden's index) were determined (Table 2). For diagnosis of IDA, the AUC for hepcidin was 0.9488, and at a cut-off value of <6 ng/ml, the test provided a sensitivity of 88.89% and a specificity of 90.63%. For differentiation of ACD from IDA, the AUC for Hepcidin was 1.0, and at a cut-off value of >46 ng/ml, both a sensitivity and specificity of 100% were achieved. Further, the sensitivity and specificity remained stable above a hepcidin cut-off of 12 ng/ml (7.5% variation from maximal Youden index). Hepcidin was unable to differentiate mixed aetiology patients from those with ACD. The diagnostic utility of RetHe for IDA and ACD was assessed. ROC analysis revealed an AUC of

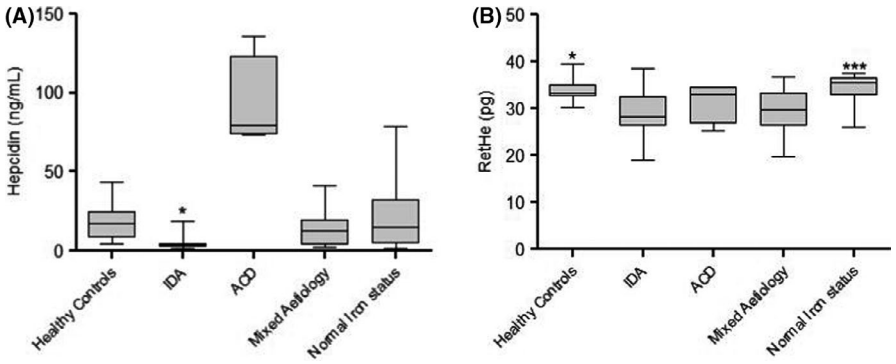

FIGURE 1 (A) Hepcidin and (B) reticulocyte haemoglobin equivalent (RetHe) in healthy controls and the four patient subgroups (Iron Deficiency Anaemia (IDA), Anaemia of Chronic Disease (ACD), Mixed Aetiology & Normal Iron Status). Data are presented as Median ± Range. Kruskal-Wallis with Dunn's *post hoc* test determined significant differences between the mixed aetiology group and all other groups. *represents $P < .05$; ***represents $P < .001$

TABLE 2 Determination of the diagnostic accuracy of Hepcidin and RetHe for IDA and ACD

		AUC (95% CI)	Optimal Cut-off value	Sensitivity (95% CI)	Specificity (95% CI)	Negative Predictive Value (95% CI)	Positive Predictive Value (95% CI)
Hepcidin	IDA	0.9488 (0.9006 to 0.9970)	<6 ng/ml	88.89% (73.94 to 96.89)	90.63% (74.98 to 98.02)	90.62% (76.48% to 96.64%)	88.89% (76.05% to 95.27%)
	ACD from IDA	1.00	>46 ng/ml	100% (39.76 to 100)	100% (90.26 to 100)	100.00%	100.00%
RetHe	ACD from IDA	0.6632 (0.3845 to 0.9419)	>30.8 pg	75.00% (0.1941 to 0.9937)	69.44% (0.5189 to 0.8365)	95.65% (79.80% to 99.19%)	17.65% (9.63% to 30.11%)

Abbreviations: ACD, Anaemia of Chronic Disease. Healthy Controls, n = 32; *ACD*, n = 4;*AUC, Area Under the Curve; IDA, Iron Deficiency Anaemia; IDA*, n = 36.

0.6632 and an optimal cut-off of >30 pg for differentiating ACD from IDA.

3.4 | Combining hepcidin and reticulocyte haemoglobin equivalent

Of the 36 patients with IDA, 69% (n = 25) had a ReHe <30 pg confirming inadequate iron availability. The remaining 11 patients had RetHe >30 pg, implying adequacy of iron availability for developing cells. All 4 ACD subjects had RetHe >30 pg inferring sufficiency of iron stores. All 21 subjects with normal iron status had RetHe concentrations >30 pg. Of the sixteen patients with mixed aetiology (according to the current diagnostic pathway (Figure S1)), 10 had a RetHe <30 pg implying true IDA, and therefore patients who would benefit from iron therapy.

3.5 | Re-classification of patient data using the new simplified diagnostic pathway

Subjects were reclassified using a new proposed two-step pathway with serum hepcidin being the primary indicator and RetHe as the predictor of iron deficiency and response to oral iron (Figure 2). All 77 gastroenterology patients were defined as either IDA or ACD, with definitive diagnosis of those 16 cases originally defined as mixed aetiology anaemia. The re-classification of subjects is summarized in Table 3, based on the optimum cut-off values derived from the ROC curves.

4 | DISCUSSION

Anaemia is a frequent and significant clinical problem in Gastroenterology patients. Many patients suffer from an anaemia to which several factors contribute, with the most common being iron deficiency and inflammation.[26] At present, a collection of tests is required to provide a differential diagnosis. Even using the existing extensive testing profile, it is difficult to identify true iron deficiency if there is a concomitant ACD, and yet, it is important to ensure that these patients are identified as iron supplementation will be required.[6] Assessment of iron status has typically focussed on iron stores, indicated by the serum ferritin concentration. However, stored iron is metabolically inactive and is not available for immediate use within the bone marrow. Assessment of active iron metabolism and its availability to the red cell pool is a much more useful indicator.[27]

In this single-centre, preliminary study, 77 adult secondary care Gastroenterology subjects, with gastrointestinal related disease and anaemia, were identified. Currently, ferritin concentration is considered one of the most robust markers of iron stores and may be used to monitor and assess the impact of interventions on iron status. Serum hepcidin showed a strong positive correlation with serum ferritin ($R^2 = 0.79$; 2-tailed $P < .0001$), as well as strong correlations

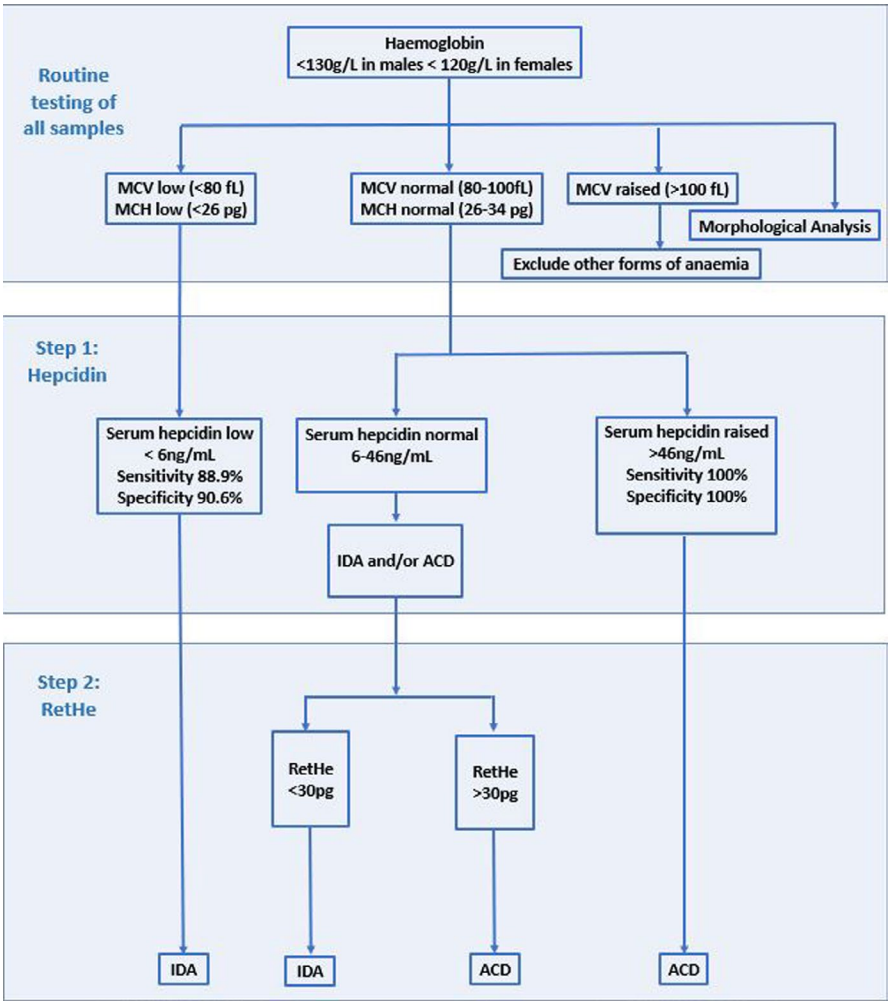

FIGURE 2 Suggestion of new two-step diagnostic testing pathway with serum hepcidin as the primary indicator and reticulocyte haemoglobin equivalent (RetHe) as the predictor of response to iron therapy. Abbreviations: MCV, Mean Cell Volume; MCH, Mean Cell Haemoglobin; IDA, Iron Deficiency Anaemia; ACD, Anaemia of Chronic Disease; RetHe, Reticulocyte Haemoglobin Equivalent

TABLE 3 Classification of subjects based upon the new 2-step diagnostic pathway

	Total	Iron Deficiency Anaemia (IDA)	Anaemia of Chronic Disease (ACD)	Mixed Aetiology (IDA/ACD)
Sample size	77	52	25	0
Hb (g/L)	108.0 (80.0-130.0)	107.0 (81.0-131.0)	111.0 (80.0- 130.0)	-
MCV (fL)	80.4 (66.0-99.7)	77.5 (66.0-92.2)	86.8 (72.4-99.7)	-
MCH (pg)	26.1 (19.4-33.2)	24.7 (19.4-32.7)	28.0 (22.5-33.2)	-
CRP (mg/L)	4.3 (0.4-178.0)	4.7 (0.4-92.0)	4.0 (0.7-178)	-
Ferritin (μg/L)	17 (3-916)	9.5 (3.0-82.0)	42.0 (9.0-916.0)	-
Transferrin (g/L)	3.10 (1.40-3.10)	3.3 (1.7-8.10)	2.6 (1.40-4.13)	-
TSAT (%)	8 (1-83)	6.0 (1.0-83.0)	13.9 (7.0-62.0)	-
Iron (μmol/L)	6.00 (1.00-62.00)	5.0 (1.0-52.0)	9.0 (2.0-62.0)	-
Hepcidin (ng/ml)	4.98 (1.57-136.0)	3.8 (1.57-41.21)	26.0 (6.32-136.0)	-
RetHe (pg)	30.4 (19.1-38.5)	28.4 (19.1-38.5)	34.4 (25.4-37.5)	-

Note: Values are presented as median (range).

Abbreviations: CRP, C-Reactive Protein; Hb, Haemoglobin; MCH, Mean Cell Haemoglobin; MCV, Mean Cell Volume; RetHe, Reticulocyte Haemoglobin Equivalent;TSAT Transferrin Saturation.

with the other tested parameters (Transferrin, TSAT, Iron, CRP) (Table S1). This is in agreement with previous studies using C-ELISA methods[15,28,29] and indicates that hepcidin could successfully replace these markers for identification of IDA and ACD. However, in patients with mixed aetiology, the ferritin values were not significantly different from those seen in healthy volunteers or anaemic gastroenterology patients with normal iron status. As previously stated, a normal ferritin concentration alone cannot exclude iron

deficiency, since the presence of the concomitant chronic inflammation may have a masking effect.[30]

Since the study involved a group of normal healthy participants, we were able to derive our own reference interval for hepcidin. Despite the small sample size, the derived reference interval was in line with that published by the manufacturer of the C-ELISA assay and those derived from studies focussing on other patient cohorts or using other analytical methods.[31] When hepcidin was analysed in the 77 Gastroenterology patients, significantly lower concentrations were seen in IDA patients when compared with all other patient cohorts, a finding supported by several other studies.[10,12,15,32] Unsurprisingly, individuals with low serum ferritin <30 μg/L had a serum hepcidin level <6 ng/ml, as previously reported, verifying a true iron-deficient state. Patients with mixed aetiology and normal iron status had serum hepcidin levels similar to those seen in healthy controls.

The optimal hepcidin cut-off concentrations derived from the present data set for diagnosis of IDA and ACD of 6 ng/ml and 46 ng/ml respectively demonstrated excellent sensitivities and specificities and are in agreement with those derived from analysis of hepcidin by C-ELISA in paediatric, geriatric and also female blood donor populations.[31,33,34] In other studies, it has been shown that nonresponsiveness to oral iron can be predicted from patients' baseline hepcidin levels, and that these have a better positive predictive value than TSAT or ferritin concentrations.[35,36]

In agreement with other published work,[12] hepcidin alone was unable to differentiate mixed aetiology patients as these patients showed varying levels of hepcidin, falling into the reference interval with normal and iron-deficient subjects. The ability of RetHe to identify these mixed aetiology patients and therefore predict which of these patients are likely to respond to iron therapy was assessed and an optimal RetHe cut-off for diagnosis of IDA of <30 pg was derived. The use of RetHe in the assessment of anaemia is fairly novel and, so far, has been relatively understudied. However, from the limited literature available, suggested thresholds for predicting iron deficiency appear to vary between 25 pg and 30 pg. A cut-off of 28 pg has been shown to predict iron response in paediatric cancer patients,[37] while cut-offs of 25 pg[38] and 30 pg[39] have been reported to be diagnostic of IDA in separate studies encompassing patients with a wide range of iron-deficient and inflammatory states. The British Society of Haematology (BSH) working group for "the laboratory diagnosis of iron deficiency in adults (excluding pregnancy) and children" guidelines, are shortly due to state that "Until further data is available a threshold of 29 pg is a pragmatic recommendation."

Studies of hepcidin, and certainly RetHe, in gastroenterology-related disorders are very limited, despite anaemia patients frequently presenting with iron deficiency and accounting for 4%-13% of all gastroenterology referrals,[40] and so in that respect, our small study provides a useful platform for future study. Larger studies in this area are clearly warranted, taking into account other limitations that were present in the current study such as specimen collection times and effect of diurnal and circadian differences on these markers. Response to iron supplementation post-testing would also provide evidence of effectiveness of the proposed testing protocol. Indeed, in the present work, samples were retrieved from the routine workload at varying times. The half-life of hepcidin is much shorter than CRP (19 hours) and this may contribute to the lower concentrations seen in the mixed aetiology group. Time of collection of samples has also been documented as affecting the serum hepcidin concentration, due to nonfasted specimens, circadian differences and diurnal variation.[34,41]

Key advantages of the RetHe and hepcidin assays are the ease of use meaning they could easily be incorporated into a routine clinical laboratory. Indeed, despite the ability to analyse hepcidin by MS techniques, use of these is limited in routine clinical laboratories mainly due to the expense of the equipment, availability and unsuitability for high throughput testing.[13] It has been suggested that such techniques may be more appropriate in establishing a reference standard.[42] Work is on-going to improve standardization material and normal ranges.[43] Hepcidin responds within hours to changes in haematological status, making it more useful than ferritin which requires several days for changes in iron status to be reflected. However, It should be noted that since the kidneys are involved in both the synthesis and clearance of hepcidin, renal function will influence hepcidin concentrations, and as such, this would need to be considered in the investigation of anaemia in patients with concomitant renal failure.[44] For hepcidin, the ELISA method is simple and only small amounts of serum are required (20 μl), while for RetHe, this is analysed alongside all other parameters on the FBC. It is important to note that RetHe is a proprietary method of Sysmex. Many studies have shown strong correlation between RetHe and CHr, a parameter provided by Bayer Diagnostics analysers,[38,45,46] and so the exact parameter to be used in our proposed diagnostic pathway will be dependent upon the laboratory analyser supplier. Disadvantages of the hepcidin ELISA are the current lack of availability of a universal hepcidin standard and the time taken to perform the assay (110 minutes), although this may be shortened by the use of semi-automated methods and the development of high throughput automated ELISA.

In conclusion, our data, although derived from a small cohort, show that using a combination of serum hepcidin and RetHe offers the potential development of a clearer clinical pathway for the investigation of anaemia in patients with inflammatory disease, especially those with mixed aetiology. This includes a reduction in the number of investigations required from the traditional FBC plus five additional tests (Figure S1) to FBC plus just two additional tests (Figure 2). From our new diagnostic pathway, individuals with a serum hepcidin <46 ng/ml and with a RetHe <30 pg are those predicted to respond to iron therapy, which allows administration of treatment before the onset of severe symptoms, thereby having the potential to improve quality of life in these patients.

CONFLICTS OF INTEREST

There are no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Contribution: NS designed the research study, performed the experiments and analysed the data. S.D and ND-H were also involved in data analysis. JB and N.D-H. supervised the study. All authors drafted and edited the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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