Diagnostic utility of low hemoglobin density to detect iron deficiency in patients with inflammatory bowel disease

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Abstract

Background In the absence of a feasible noninvasive gold standard, iron deficiency (ID) anemia (IDA) is best measured using multiple indicators. However, the choice of an appropriate single iron biomarker for ID screening continues to be debated. Low hemoglobin density (LHD%) from Coulter counters has been suggested as a useful tool to detect ID. This study investigated the reliability of LHD% for the assessment of iron status in patients with inflammatory bowel disease (IBD) and IDA, anemia of chronic disease (ACD) or mixed anemia (MIX).

Methods The study population consisted of 143 patients with IBD (aged 39.03±12.53 years, 61.5% female). Blood count, transferrin saturation, serum ferritin, and C-reactive protein were determined by routine assays. Patients with anemia were divided into 3 groups: IDA, ACD and MIX, according to specific criteria. Receiver operator characteristic (ROC) curves were constructed.

Results ROC analysis for LHD% in the detection of ID yielded a cutoff value of 3.8%. In anemic patients, LHD% values did not differ statistically significantly between groups (IDA, ACD, MIX) and no significant difference in LHD% values was observed between patients with IDA and ID.

Conclusions These results demonstrate that LHD% is a reliable biomarker for the detection of iron deficiency in patients with IBD and anemia, regardless of whether inflammation is present. Our findings indicate that LHD% can provide added value in diagnosing iron deficiency.

Keywords Inflammatory bowel disease, iron deficiency, low hemoglobin density

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Introduction

The prevalence of iron deficiency (ID) in patients with inflammatory bowel disease (IBD) has been reported at 36-76%, with approximately 50% of patients having isolated ID without anemia. Since ID, even without manifest anemia, can substantially impact quality of life and healthcare costs, IBD-associated ID requires appropriate diagnostic and therapeutic management [1-4].

Two different types of impaired iron homeostasis may be differentiated: absolute ID and functional ID. In absolute ID, iron stores are depleted as a result of insufficient dietary iron intake, iron malabsorption and/or chronic gastrointestinal blood loss [5,6]. ID anemia (IDA) occurs when iron stores are fully depleted and iron supply is insufficient for hemoglobin (Hb) synthesis [7]. In functional ID, iron supply is inadequate to ensure sufficient hemoglobinization of reticulocytes and mature erythrocytes. This imbalance between erythroid

marrow iron requirements and actual iron supply inhibits hemoglobinization, resulting in hypochromic mature red cells and reticulocytes [8,9]. Despite a plethora of iron status parameters, screening for ID remains challenging. Serum ferritin (s-ferritin) indirectly estimates body iron stores and is the most specific biomarker of absolute ID. Absolute ID in patients with IBD may, however, be obscured by the acute-phase response induced by inflammation and/or infection, and as a result of anemia of chronic disease (ACD), characterized by apparently normal or increased iron stores but limited iron available for erythropoiesis (iron-restricted erythropoiesis) [10].

The dual "face" of ID in IBD is a conundrum for the IBD physician, as its 2 main subgroups (IDA and ACD) frequently mix and overlap. Conventional iron status parameters, such as mean cell volume, transferrin saturation (TSAT) and s-ferritin [10-12], are of limited diagnostic value in this setting because of dynamic changes (e.g., acute-phase response), and thus fail to accurately reflect changes in the red blood cell population because of the lack of temporal coincidence [13]. Higher cutoff levels for s-ferritin in the presence of infection/inflammation have been investigated, though these are not evidence-based [14]. In the absence of a feasible noninvasive gold standard, European guidelines recommend the use of multiple indicators to detect IDA [15]. Thus, there is a demand for markers suitable for the early and reliable diagnosis of ID, but uninfluenced by inflammation.

Low Hb density (LHD%), derived from the traditional marker mean corpuscular Hb concentration (MCHC), has been shown to be a highly sensitive biomarker for iron availability during erythropoiesis. MCHC is an all-encompassing parameter for the availability of iron during the previous 90-120 days, as well as for the regular use of iron in intracellular Hb. Similarly, LHD% reflects iron availability and hemoglobinization of mature red blood cells [16-18].

Despite its potential clinical utility, no previous studies have evaluated LHD% in patients with IBD. We therefore aimed to investigate its diagnostic performance in the detection of iron-restricted erythropoiesis in patients with IBD.

Patients and methods

This study was conducted as a comparative, cross-sectional, retrospective study according to the ethical principles of the Declaration of Helsinki in adult patients previously diagnosed with IBD and healthy controls. The study design was approved by the local ethics committee, the Landesärztekammer Hessen (2019-1317-evBO). All patients were required to give informed written consent prior to participation.

Study population

The data from eligible patients from the Interdisciplinary Crohn Colitis Center (ICCC) Rhein-Main (Frankfurt am Main, Germany) who had been diagnosed with IBD according to standard clinical, endoscopic, radiological and pathological criteria were retrospectively analyzed. The control group consisted of healthy adults attending the same center for routine consultations. Inclusion criteria were age 18-65 years and body mass index (BMI) <30 kg/m². Exclusion criteria were malignant underlying disease, severe metabolic disease, cardiac, pulmonary, renal or hepatic dysfunction or disease, substance abuse (alcohol, drugs), oral or intravenous iron supplementation within 3 months prior to baseline, azathioprine-induced cytopenia, and blood donation, blood loss or plasmapheresis within the last 3 months prior to the laboratory tests. Also excluded were patients for whom laboratory values were missing.

Study design

Eligible patients and controls were identified retrospectively. Prior to data extraction, the patients were contacted, fully informed about all aspects of the study and, if they were willing to participate, asked to give written informed consent. Data were then drawn from electronic patient records. The documented information included demographics, a full blood count including Hb, s-ferritin, TSAT, erythrocyte sedimentation rate (ESR), and serum high-sensitivity C-reactive protein (hsCRP). All blood samples were collected in the morning, after 12-h fasting, and were analyzed the same day. All laboratory findings were determined by routine assays.

Disease location was defined according to the Montreal Classification [19]. ID was defined as s-ferritin <30 µg/L and TSAT <20%. Functional ID was defined as hsCRP >5 mg/L, s-ferritin between 30-300 mg/L and TSAT <20% [15]. Anemia was defined as an Hb concentration below 13 g/dL for males and 12 g/dL for females, according to World Health Organization criteria [20,21], and subclassified by type: as IDA if hsCRP <5 mg/L, TSAT <20% and s-ferritin <30 µg/L; as ACD if hsCRP ≥5 mg/L, TSAT <20%, and s-ferritin ≥100 µg/L; and as mixed IDA and ACD (MIX) if hsCRP ≥5 mg/L, TSAT <20% and s-ferritin level 30-100 µg/L [22,23].

LHD% was calculated from the traditional MCHC using the mathematical sigmoid transformation recently proposed as a new ID parameter by Beckman-Coulter (Beckman Coulter Inc., Miami, FL, USA) [16]:

$$LHD\% = 100\sqrt{1 - [1 / (1 + e^{1.8(30 - MCHC)})]}$$

Statistical analysis

Statistical analysis was performed using IBM SPSS version 25.0. LHD% was calculated using the Excel formula function. All variables were investigated for normal distribution using both visual and analytical methods. Descriptive parameters were presented as median and range. Appropriate parametric or non-parametric tests were performed based on a normal distribution. Receiver operating characteristic (ROC) curve

analysis was used to evaluate the diagnostic performance of LHD% for the assessment of iron status. Statistical significance was predetermined as P<0.05.

Results

The study population consisted of 192 subjects: 143 patients with IBD (78 Crohn's disease, 65 ulcerative colitis, mean age 39.03±12.53 years, 88/143 female) and 49 healthy controls (mean age 42.37±12.23 years, 34/49 female). Of the patients with IBD, 38 were in a non-inflammatory and non-anemic state (mean age 39.47±11.86 years, 24/38 female) based on their hsCRP and Hb levels (Table 1). There was no statistical significance between the groups with regard to sex, age, age at diagnosis, disease location, disease behavior or drug therapies.

Baseline laboratory parameters of the IBD group according to iron status are shown in Table 2. Of the 143 patients with IBD, 105 (73.4%) were iron deficient; of these, 79 (75.2%) had absolute and 26 (24.8%) functional ID. Sixty-five of these iron deficient patients (62.0%) were anemic, of whom 44 (67.7%), 8 (12.3%) and 13 (20%) fulfilled the predefined criteria for IDA, MIX, and ACD, respectively. In accordance with the selection criteria, median hsCRP and ESR values were significantly higher in the MIX and ACD groups compared to the IDA group, and in the patients with functional ID compared to those with absolute ID.

A ROC analysis was performed for LHD%, and the optimal cutoff and area under the curve (AUC) for defining ID (absolute and functional) were calculated. Functional and absolute ID are presented in Table 3, together with results of the ROC analysis for ferritin (cutoff: 30 ng/mL for ID and absolute ID, 100 ng/mL for functional ID) and TSAT (cutoff: 20%). The optimal cutoff for LHD% was

Table 1 Subject characteristics

Characteristics	Patients with IBD (n=143)			Control Group (n=49)	P-value[2]	P-value[3]
	Iron deficient and/or anemic	Non-inflammatory and non-iron deficient	P-value[1]			
N (♀)	105 (64)	38 (24)	0.811	49 (34)	0.311	0.541
Age (mean ± SD)	39.02 ± 12.90	39.47 ± 11.86	0.791	42.37 ± 12.23	0.098	0.221
Disease type						
CD	59 (56.2%)	19 (50.0%)	0.511			
UC	46 (43.8%)	19 (50.0%)				
Age at diagnosis						
A1=below 16 years	1 (1.0%)	-				
A2=17 to 40 years	66 (62.9%)	21 (55.3%)	0.526			
A3=over 40 years	37 (35.2%)	17 (44.7%)				
Disease location						
L1=Terminal ileum (CD)	21 (37.3%)	9 (47.4%)				
L2=Colon (CD)	17 (30.5%)	5 (26.3%)	0.770			
L3=Ileocolon (CD)	16 (28.6%)	5 (26.3%)				
L4=Upper gastrointestinal tract (CD)	2 (3.6%)	-				
E1=Proctitis (UC)	4 (8.4%)	4 (21.1%)				
E2=Left-sided colitis (UC)	28 (58.3%)	11 (57.8%)	0.282			
E3=Pancolitis (UC)	16 (33.3%)	4 (21.1%)				
Disease behavior (CD)						
B1=non-stricturing, non-penetrating	29 (51.8%)	12 (63.2%)				
B2=stricturing	11 (19.6%)	6 (31.6%)	0.099			
B3=penetrating	16 (28.6%)	1 (5.2%)				
B4=perianal	-	-				
IBD treatments						
5-ASA	17 (16.2%)	13 (34.2%)				
Steroids	32 (30.5%)	7 (18.4%)				
Azathioprine	18 (17.2%)	5 (13.2%)				
Methotrexate	6 (5.7%)	0 (0.0%)	0.055			
Anti-TNF	38 (36.2%)	14 (36.8%)				
Ustekinumab	11 (10.5%)	3 (7.9%)				

One sample t-test for age, chi-square test for all the other comparisons; P-value[1]: comparison of iron deficient and/or anemic IBD patients and non-inflammatory and non-iron deficient IBD patients; P-value[2]: comparison of iron deficient and/or anemic IBD patients and control group; P-value[3]: comparison of non-inflammatory and non-iron deficient IBD patients and control group

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; 5-ASA, 5-aminosalicylic acid; TNF, tumor necrosis factor; SD, standard deviation

Table 2 Laboratory markers according to iron status in patients with IBD

Markers	Absolute ID (n=79)	Functional ID (n=26)	P-value[1]	IDA (n=44)	ACD or MIX (n=21)	P-value[2]
Hb (g/dL)	12.5 (7.4-16.2)	11.4 (9.5-130)	0.012*	11.0 (7.4-13.3)	11.8 (9.2-12.9)	0.311
RDW (%)	14.6 (11.9-22.5)	14.8 (11.7-21.2)	0.894	15.2 (12.5-20.8)	15.0 (11.7-21.2)	0.776
MCHC (g/dL)	32.8 (28.6-35.7)	32.3 (29.9-34.3)	0.190	31.9 (28.6-34.0)	32.2 (29.9-34.3)	0.424
Ferritin (ng/mL)	15.1 (5.0-29.1)	94.1 (30.5-1028.0)	<0.001	13.9 (5.0-28.4)	124.0 (30.5-1028.0)	<0.001
TSAT (%)	9.5 (2.5-36.1)	14.1 (2.4-25.6)	0.123	5.7 (2.5-21.9)	12.2 (2.4-25.6)	<0.001
LHD (%)	8.0 (0.6-96.2)	13.1 (2.1-73.8)	0.184	17.8 (2.7-96.2)	13.7 (2.1-73.8)	0.424
ESR (%)	14.0 (1.0-79.0)	25.5 (2.0-89.0)	<0.001	20.0 (2.0-79.0)	26.0 (2.0-89.0)	0.018*
HsCRP (mg/L)	2.7 (1.0-24.4)	13.4 (1.4-181.0)	<0.001	2.7 (1.0-24.4)	27.5 (1.4-181.0)	<0.001

*P<0.05, **P<0.001, Mann-Whitney U test

P-value[1]: difference between absolute ID group and functional ID group; P-value[2]: difference between IDA group and ACD or MIX group

IBD, inflammatory bowel disease; Hb, hemoglobin; RDW, red cell distribution width; MCHC, mean corpuscular hemoglobin concentration; TSAT, transferrin saturation; LHD, low hemoglobin density; ESR, erythrocyte sedimentation rate; HsCRP, high-sensitivity C-reactive protein; ID, iron deficiency; IDA, iron deficiency anemia; ACD, anemia of chronic disease; MIX, mixed anemia

Table includes only classified anemia – there were patients with unclassified ID (n=1) and IDA (n=8)

Table 3 Analytical performance of iron status parameters to detect patients with ID, absolute ID, and functional ID as sole biomarker

Iron status	Ferritin	TSAT	LHD
ID			
Sensitivity	75%	78%	82%
Specificity	89%	84%	85%
AUC^ROC	0.860	0.905	0.872
Cutoff	30 ng/mL	20%	3.8%
Absolute ID			
Sensitivity	100%	77%	79%
Specificity	91%	69%	70%
AUC^ROC	0.989	0.830	0.754
Cutoff	30 ng/mL	20%	3.8%
Functional ID			
Sensitivity	50%	80%	89%
Specificity	24%	55%	70%
AUC^ROC	0.250	0.675	0.763
Cutoff	100 ng/mL	20%	3.8%

ID, iron deficiency; TSAT, transferrin saturation; LHD, low hemoglobin density; AUC^ROC area under curve receiver operating characteristic

identified to be 3.8%, regardless of inflammatory status. This cutoff value was confirmed to be valid in all 3 analyses, with different sensitivity and specificity levels (Table 3). For ID (both absolute and functional), the AUC was 0.872 with 82% sensitivity and 85% specificity; for absolute ID, the AUC was 0.754 with 79% sensitivity and 70% specificity; and for functional ID, the AUC was 0.763 with 89% sensitivity and 70% specificity. Both ferritin and TSAT were poor predictors of functional ID in comparison to LHD%.

Taking 3.8% as an optimal cutoff point for LHD%, as suggested by the ROC analysis (sensitivity 82%, specificity 85%) for ID detection, 82.3% and 88.5% of patients with absolute ID and functional ID, respectively, had elevated LHD% values. In the anemic group, 97% of patients with IDA, 100% with MIX, and 84.6% with ACD had elevated levels of LHD% (Fig. 1).

Discussion

ID, with or without anemia, is recognized as one of the most common complications and extraintestinal manifestations of IBD. ID, even without anemia, can be debilitating, affecting overall health and daily work and social life.

Laboratory diagnostics of ID are currently based largely on low levels of TSAT and s-ferritin. However, inflammation is well known to mimic some aspects of ID by impairing the utilization of existing iron stores for red cell production and inducing an iron-sequestration syndrome and hypoferremia [24]. Therefore, diagnosing ID in patients affected by chronic inflammatory diseases such as IBD is a major diagnostic challenge. Additionally, chronic inflammation hinders the interpretability of other parameters of low iron stores [13,25]. Inflammation-independent diagnosis of true ID is of clinical importance when choosing the treatment approach and obviates unnecessary treatment-related adverse events that may occur as a result of unnecessary iron supplementation therapy [26].

Hepcidin has been proposed as a possible tool in this context. However, the antibodies used in diagnostic immunoassays can cross-react with biologically inactive hepcidin and thus overestimate true bioactive hepcidin, while there are additional *in vivo* factors that induce or prevent iron-restricted hemopoiesis with or without the expected hepcidin reaction [27].

In recent years, a variety of other biomarkers have been studied for their utility as indicators of true ID in the context of inflammation. One of these laboratory parameters is the serum concentration of soluble transferrin receptor (sTfR), an indicator of the iron requirement for iron erythropoiesis. However, transferrin receptor expression on cells is also influenced by inflammation, which may negatively affect the sensitivity of sTfR levels as an indicator of true ID in patients with inflammatory disorders [22,28]. The calculated ratio, transferrin receptor:$\log_{10}$ferritin (TfR-F), has been also proposed as a reliable marker and has been shown to offer superior discrimination compared with either sTfR or ferritin
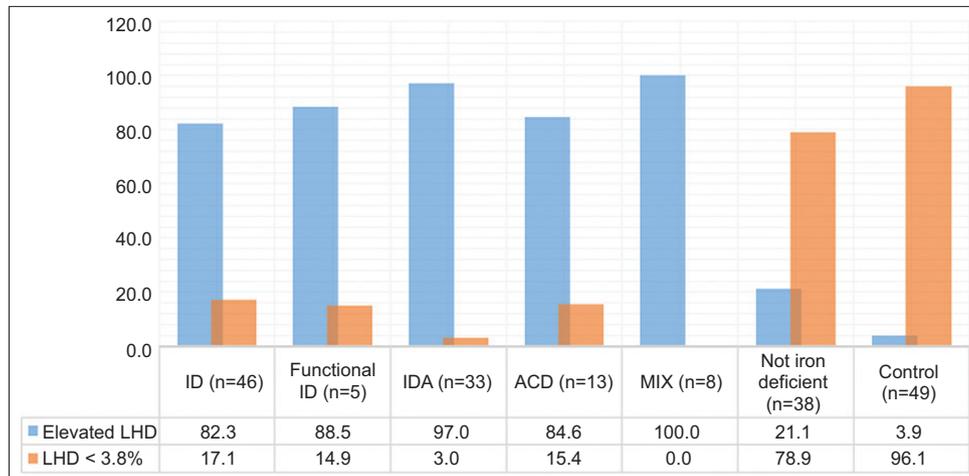
Figure 1 Percentage of elevated LHD% in the study population
ID, iron deficiency; IDA, iron deficiency anemia; ACD, anemia of chronic disease; MIX, mixture of IDA and ACD; LHD, low hemoglobin density

alone, particularly in patients with chronic inflammatory disease [29-31]. However, TfR-F has limited availability and cost constraints [32]. Moreover, current assays are hampered by a lack of external quality controls and limited availability within the test repertoire of hospital-based laboratories [27,33,34].

Hypochromic red blood cells (%HYPO), a measure of the percentage of erythrocytes with decreased hemoglobinization, has also been considered as a possible marker in this context. %HYPO and reticulocyte indices perform very well in differentiating IDA and ACD [35,36]. However, patients with ACD may also have higher %HYPO and lower reticulocytes than controls. This is due to iron-restricted erythropoiesis caused by the reduced availability of iron from body stores to red blood cells developing in the bone marrow. Furthermore, this approach is restricted by the fact that analyzers are available only from 2 manufacturers, Siemens and Sysmex.

The current gold standard in ID diagnosis, the determination of stainable iron strains in a bone marrow aspirate sample by Perls staining [37], is limited by factors such as incorrect aspiration of the bone marrow or insufficient sample size. Non-stainable iron stores seem to correlate well with true ID in patients with IDA. However, they can be misleading, since iron stores can even be stained in the bone marrow of patients with iron-restricted erythropoiesis, if adequately sampled. This reinforces current opinion among hematologists that a lack of detectable iron in bone marrow cannot be equated with ID, and that despite stringent criteria, bone marrow biopsy is not representative of a patient's true iron stores [38].

There is therefore a need to seek alternative iron status markers widely available. LHD% was recently proposed as a potential indicator of iron available for erythropoiesis [16,17,39]. LHD% is a time-averaged measurement of the degree of hemoglobinization in mature erythrocytes. The analysis can be performed concurrently with routine blood counts at no additional cost and without additional blood collection [16]. MCHC, the parameter from which LHD% is calculated, is a stable laboratory parameter that can be reliably analyzed in samples stored for up to 36 h [40].

To our knowledge, this is the first study to highlight the value of LHD% as an additional parameter to aid ID diagnosis in patients with IBD by evaluating iron status while incorporating the differentiation of absolute and functional ID. The results of our study confirmed that LHD% is a reliable diagnostic marker of iron status in patients with IBD. LHD% was found to be a more sensitive marker than hepcidin or bone marrow-stainable iron stores for identifying patients with iron-restricted erythropoiesis, especially if inflammation is present [18]. Our results indicate that LHD% is suitable for the diagnosis of ID in patients with or without active inflammation. Furthermore, LHD% was found to be superior to TSAT and s-ferritin in its diagnostic performance with regard to functional ID. Therefore, we propose that LHD% may be a worthwhile additional diagnostic biomarker of ID in patients with IBD, especially when TSAT and s-ferritin are insufficiently accurate because of the presence of inflammation, offering additional diagnostic accuracy at no extra cost and without delay. Our ROC analysis identified 3.8% as an optimal cutoff point for LHD%, similar to the value of 4.0% suggested by Urrechaga *et al* [16,17] for patients with chronic kidney disease. LHD% has clinical utility, not only as a marker of iron-restricted erythropoiesis, but also in the discrimination between IDA and thalassemia, as shown by Urrechaga *et al* [16] and more recently by Ng *et al* [41].

As a general limitation, it must be kept in mind that, as a marker of cellular hypochromia, LHD% is sensitive to the temperature and storage duration of the sample: LHD% may be falsely elevated as a result of a reduction in MCHC associated with storage at room temperature or above. Therefore, if samples are kept at room temperature, LHD% should be assessed within 6 h of collection. Refrigeration minimizes volume changes induced by sample storage, with hypochromia markers showing only minimal changes during the first 24 h after sample collection [42].

Our study has some limitations: Firstly, the study included a relatively small number of patients. Despite the relatively small number of samples, we determined a cutoff value close to that

found in previous studies, and therefore see no restrictions in terms of reliability. Secondly, since LHD% was not monitored after iron therapy, no statement can be made as to whether LHD% may be a marker of therapeutic success. The lack of data on sTfR can be considered a further limitation; this was due to the fact that sTfR is not included in routine iron status assessments in the treatment center from which our patient data were obtained. Finally, no details are available concerning signs of clinical disease activity or disease duration at the time of blood sampling. Unfortunately, given the retrospective character of the study, it was not possible to collect these missing data.

In conclusion, in this study we demonstrated that LHD% can verify the presence of ID, regardless of the presence of inflammation. LHD% can be determined as part of routine laboratory tests at no extra cost and without additional blood collection, and thus offers a rapid, accurate and convenient additional diagnostic tool for ID in patients with IBD. We therefore propose that, using a cutoff value of 3.8%, LHD% holds promise as a simple, inexpensive and sensitive tool that allows an accurate and reliable diagnosis of ID with or without anemia in patients with inflammatory disorders.

Summary Box

What is already known:

- No reliable biochemical markers exist for the differentiation between iron deficiency anemia (IDA) and anemia of chronic disease in patients with inflammatory bowel disease (IBD)
- Low hemoglobin density (LHD%) from Coulter counters has been suggested as a useful tool to detect iron deficiency, even in the context of inflammation
- Literature data suggest that LHD% is more sensitive compared to ferritin, serum iron, transferrin saturation (TSAT), hepcidin or bone marrow iron stores in identifying patients with associated iron deficiency

What the new findings are:

- LHD% had a better discriminating power than TSAT and s-ferritin in the diagnosis of IDA
- LHD% was a reliable biomarker for the detection of iron deficiency in patients with IBD and anemia, regardless of whether inflammation is present
- LHD% could provide added value in identifying patients with associated iron deficiency who could potentially benefit from parenteral iron replacement

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