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REVIEW ARTICLE

The Role of Reticulocyte Hemoglobin Content for Diagnosis of Iron Deficiency and Iron Deficiency Anemia, and Monitoring of Iron Therapy: a Literature Review

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SUMMARY

Background: **The diagnosis of iron deficiency anemia is still complicated and most of the tests have drawbacks. Bone marrow examination, the gold standard for the diagnosis of iron deficiency and iron deficiency anemia, is a painful, invasive, and costly procedure. Other methods are also used to diagnose iron deficiency and iron deficiency anemia; soluble transferrin receptor, serum iron, serum ferritin, and transferrin saturation are most common biomarkers of iron status that are frequently affected by inflammation, chronic diseases, and in the normal aging process (except soluble transferrin receptor). All are less available compared to complete blood count with reticulocyte hemoglobin content (CHr). Reticulocytes have a normal life span of one or two days in the circulation. CHr is a good indication of iron availability and an early marker of iron deficient erythropoiesis which can be obtained readily using automated blood cell analyzers. Therefore, the main objective of the current review is to assess the role of CHr for diagnosis of iron deficiency, iron deficiency anemia, and monitoring of iron therapy.**
Methods: **Studies published in English were searched using the National Library of Medicine, PubMed, and Google scholar databases.**
Results: **According to this review, CHr has a moderate sensitivity and specificity for diagnosing iron deficiency, and is less affected by inflammation than serum iron, transferrin saturation, and ferritin and is an early predictor of treatment response. It is used in screening of iron deficiency, diagnosis of iron deficiency anemia, and diagnosis of functional iron deficiency anemia in acute or chronic diseases or inflammation. CHr is also important in treatment monitoring. It is useful for early measurement of response to iron therapy, increasing within days of the initiation of iron therapy. It helps monitoring of intravenous iron supplementation, recombinant human erythropoietin therapy, and oral iron therapy in hemodialysis and non-hemodialysis patients, and children.**
Conclusions: **It is easy to analyze, less time consuming, and less expensive than bone iron examination and iron biochemical tests. However, there is no standardized cutoff point and different researchers use varying cutoff values which affects its accuracy in diagnosing iron deficiency and it should therefore be standardized. Moreover, since CHr can be affected with any conditions that cause iron restricted erythropoiesis, further analysis may be needed.**
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KEY WORDS

Reticulocyte, Reticulocyte hemoglobin, Iron Deficiency, Iron Deficiency Anemia

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LIST OF ABBREVIATIONS

CBC - Complete Blood Count
CHr - Reticulocyte Hemoglobin Content
ESRD - End Stage Renal Diseases
FS - Forward Scattered
HD - Hemodialysis
Hb - Hemoglobin
HYPO - Hypochromic Cells
ID - Iron Deficiency
IDA - Iron Deficiency Anemia
IV - Intravenous
pg - pico gram
RBC - Red blood cell
Ret-He - Reticulocyte Hemoglobin Equivalent
RET-Y - Reticulocyte Hemoglobin Parameter
rHuEP - Recombinant Human Erythropoietin
TfR - Transferrin Receptor
TS - Transferrin Saturation

INTRODUCTION

Anemia is defined as low hemoglobin (Hb) concentration of the red blood cells (RBCs) below the lower limit of the 95% reference interval for the individual's age, gender, and geographical location [1,2]. It is a public health problem that affects low, middle, and high-income countries and has significant adverse health consequences, as well as adverse impacts on social and economic development [3,4].

There are three major pathophysiological causes of anemia. These are: blood loss, impaired red cell production, and accelerated red cell destruction. Impaired RBC production is caused by impaired function, decreased number of precursor cells, reduced bone marrow infiltration, or deficiency of nutrients [5]. Among these causes, nutritional deficiency anemia is the most common form of anemia [6], and the most common nutritional anemia is iron deficiency anemia (IDA), which mostly occurs in infants and middle-aged women [2].

Iron deficiency (ID) is defined as a condition in which the body lacks enough iron to supply its needs. The more severe stages of ID are associated with anemia [2]. Generally, 50% of the cases of anemia are due to ID [4]. It is still the most prevalent and common type of micronutrient deficiency in developing countries and results from long-term negative iron imbalance. Usually ID develops gradually and does not have clinically apparent symptoms until anemia becomes severe [7].

IDA is also a problem of reproductive aged women in Ethiopia. A study conducted in Ethiopia showed that the overall prevalence of anemia, ID, and IDA was 30.4%, 50.1%, and 18.1%, respectively [8]. IDA has detrimental public health effects and results in retarded infant development, increased morbidity and mortality at child birth, and reduced work performance [9].

An important distinction that is mostly missed is the difference between decreased stores of a certain nutrient, and the presence of clinical signs and symptoms associated with this shortage. People with asymptomatic deficiency of iron are different from people who really are suffering from clinical symptoms [6]. In normal practice, iron supplementation is only initiated in patients in whom anemia is already present i.e., microcytic anemia with Hb $\leq$ 11 g/dL. Patients are not routinely screened for earlier phases of ID, when anemia is not yet present [10]. This strategy leads to delay in evaluation, especially in young females and pregnant women who are more affected by IDA. The delay in diagnosis results in a significant delay in etiological diagnosis of anemia and treatment. In this sense, ID with or without anemia should always be investigated because it can potentially cause serious illness [11].

There are many tests for the measurement of the body's iron level. However, the diagnosis of IDA is still complicated because most of these tests have limitations. The gold standard for the diagnosis of ID and IDA is a bone marrow examination. But this test is a painful, invasive, and costly procedure [10]. In IDA, the first noted change is a decline of serum ferritin levels, which can also be detected by hemosiderin staining in bone marrow. However, in some inflammatory and chronic cases, the levels of ferritin may be falsely elevated making this test alone insufficient for diagnosis of ID and IDA. Moreover, during the normal aging process, there may be continuous low-grade chronic inflammation, causing increased levels of ferritin. For this reason, symptoms of ID appear in elderly when ferritin level is higher [12].

When iron level decreases, the delivery of iron to cells decreases and transferrin level increases as an attempt to increase iron level. This will cause cells to synthesize transferrin receptor (TfR), which is also increased in any case of hematopoietic proliferation. The elevation of TfR may indicate IDA. However, it is not yet widely available as ferritin, and the clinician must exclude other causes of elevated erythroid activity. In almost all the cases, all these tests must be done and analyzed in order to come with an accurate specific diagnosis [13]. Moreover, these tests are not readily available in many hospitals. This is because they are not routine; require a longer turnaround time, and they are more expensive than complete blood counts (CBC) with CHr [14,15].

Reticulocytes are the most immature RBCs found in circulation of our body. They exist in circulation for only a day or two before becoming fully mature RBCs. Measurement of the CHr provides a snapshot of the iron directly available for Hb synthesis and is an early indicator of the body's iron status [16].

There are different instruments that measure CHr. It is a measurement of the Hb content of reticulocytes expressed in pg/cell. CHr was first measured by Bayer H3 instruments and abbreviated as CHr [17]. It is the product of the cellular volume and the cellular Hb concentration. The volume and Hb concentration of individual reticulocytes are independently determined from measure-

ment of light scatter at two different angles (high angle, 5° - 15° and low angle, 2° - 3°), after isovolumetric sphering of stained reticulocytes. The isovolumetric sphering has great importance in the light-scattering detection at high angle and it reflects the refractive index of the cells in reticulocytes and erythrocytes, it can also be related to Hb concentration, while light scattering detected at low angle is proportional to cell volume [18]. A similar methodology is used in the ADVIA 120 and ADVIA 2120 analyzers. Reticulocyte mean cell hemoglobin has become available in other fully automated hematology analyzers that provide information regarding to reticulocyte count and maturity. The methodology developed by Sysmex for the XE and later for the XN series of automated hematology analyzers provides the reticulocyte hemoglobin equivalent (Ret-He), formerly defined as reticulocyte hemoglobin parameter (RET-Y). This parameter is measured using a photodiode through the light-scattering signals detected at the forward scatter [19]. RET-Y has been assessed as the mean channel number of the FS light signal histogram within the reticulocyte population. However, the FS signal depends on many cell characteristics, such as size and volume if the cell is spherical, shape and orientation, refractive index or density, internal structure and cell complexity, and staining. RET-Y raw data showed a better comparison with CHr ($r = 0.94$). Therefore, for simplicity purpose we used CHr for reticulocyte hemoglobin content throughout our document. The CHr is a parameter measured in the reticulocyte channel and is used to measure the incorporation of iron into erythrocyte Hb. It supports rapid, direct analysis of an earlier stage of RBC development for prompt clinical follow-up, assessment of anemia and is an established parameter used for assessing the initial iron status of kidney disease patients. It is an accurate and sensitive measurement of RBC production that supports effective monitoring of costly drug protocols for cell stimulation [20]. Therefore, the main objective of the current review is to assess role of CHr for screening of ID, IDA, and monitoring of iron therapy in IDA.

MATERIALS AND METHODS

Articles were identified by a computer-assisted search of the literature published in English using the National Library of Medicine, PubMed, Google Scholar, and Google databases. The following terms were used to search independently and in combination: reticulocyte hemoglobin, CHr, reticulocyte hemoglobin measurement, iron deficiency, ID, iron deficiency anemia, IDA and anemia diagnosis.

Role of CHr as a screening tool for ID

A normal Hb level does not rule out ID, because individuals with normal body iron stores can lose a large portion of body iron before the Hb falls below the laboratory definition of anemia [21]. CHr is an indication of iron availability and an early marker of ID erythropoiesis before anemia is presented [11]. It is less affected by inflammation than TS and ferritin [21].

A cutoff 26 pg CHr has a moderate sensitivity and specificity for diagnosing ID with sensitivity and specificity of 100% and 80%, respectively, as compared to intravenous (IV) iron dextran treatment response of 1 base reticulocyte index change as gold standard in the diagnosis of ID [22]. On the other hand, the same cutoff value has sensitivity and specificity of 70% and 78%, respectively, by using TS < 20% as gold standard [23]. Individuals with normal TS or with no iron treatment response may have low CHr (less than 26 pg). This is because CHr is the measurement of functional iron availability incorporated into the newly released imamture RBCs (reticulocyte). But this incorporation can be prevented with different diseases like thalassemia, inflammation, and other chronic diseases [22,23]. Swart et al. also supports this stating that a group of patients who have anemia will have a normal TS (failed to be diagnosed with TS), but can be diagnosed with CHr. They stated that a cutoff 29 pg is a moderate marker for the diagnosis of ID in ill infants and children, with a sensitivity of 86% and the specificity of 50%. The lower specificity may be due to the presence of a sub-group of patients who had anemia with normal TS but low CHr. Therefore, CHr is better than TS and serum ferritin for prediction of functional ID [10].

The sensitivity and specificity of CHr with cutoff value 27.2 pg to diagnose ID is higher in population confirmed with traditional parameters for ID (serum iron < 40 ug/dL, TS < 20%, ferritin < 100 ng/mL, Hb < 11 g/dL). A cutoff 27.2 pg can diagnose ID with a sensitivity of 93.3% and a specificity of 83.2%. However, in general populations (iron depleted and functional iron deficient populations), CHr is less favorable to diagnose ID [24].

CHr cutoff of 27.5 pg for the diagnosis of ID shows the prevalence of ID in toddler population was 18.8%, whereas the prevalence of anemia defined by Hb < 11 g/dL was 4.1%. The finding showed that prevalence of anemia using low reticulocyte content is higher than using Hb; this suggests that single use CHr captures individuals before they become anemic [25]. Toki et al. reported that CHr has excellent sensitivity and specificity for the diagnosis of ID. When the cutoff value decreases its sensitivity also decreases while the specificity increases. According to this study, CHr is a clinically useful marker for determining ID in the general population, and has comparable importance with TfR as both are less influenced by inflammation [26].

However, in the absence of inflammation or chronic diseases, CHr is less preferable than serum ferritin and TS for the diagnosis of ID. This is supported by Kiudelienė et al. who showed that CHr value of less than 28.55 pg had optimal sensitivity and specificity (76.6% and 78.4%, respectively), while a ferritin value of 20.45 µg/L had a sensitivity of 81.3% and a specificity of 81.9%, and TS value of 10.5% had a sensitivity of

Table 1. Some cutoff values of CHr and their sensitivity and specificity to diagnose ID.

Study No	Study area	Author and published Year	Standard	Cutoff value	Sensitivity	Specificity
1.	USA	Fishban et al. 1997 [22]	Erythropoietic response to the iron treatment.	26 pg	100%	80%
2.	USA	Carlo et al. 1999 [23]	TS < 20%	26 pg	70%	78%
3.	Japan	Toki et al. 2017 [26]	Ferritin < 12 ng/mL	30.9 pg	92%	81%
4.	Lithuanian	Rosita et al. 2008 [27]	Ferritin, transferrin, TS, and TfR	28.55 pg	76.6%	78.4%
5.	South Africa	Swart et al. 2014 [10]	TS < 25%	29 pg	86%	50%
6.	USA	Ellinor et al. 2014 [28]	TS < 20%, serum iron < 40 µg/dL	31 pg	75.8%	83.0%

Table 2. Some cutoff values of CHr and their sensitivity and specificity to diagnose IDA.

Study No	Study area	Author and published Year	Standard	Cutoff value	Sensitivity	Specificity
1.	USA	Fishban et al. 1997 [22]	Erythropoietic response to the iron treatment.	26 pg	100%	86.7%
2.	China	Cai J. et al. 2017 [30]	Bone marrow examination	27.2 pg	87.5%	92.9%
3.	Thailand	Chaipokam et al. 2016 [15]	Ferritin < 50 ng/mL, hemoglobin level rose ≥ 1 g/dL within 1 month of oral iron therapy	24.6 pg	73.6 %	74.8%,
4.	USA	Brugnara et al. 2006 [24]	Serum iron < 40 mg/dl, TS 20%, ferritin < 100 ng/ml, Hb < 11 g/dl	27.2 pg	93.3%	83.2%
5.	South Africa	Schapkaitz et al. 2016 [32]	Bone marrow examination	28 pg	75.56%	84.1%
6.	Turkey	Karagülle et al. 2013 [34]	Ferritin < 20 ng/mL and Hb < 120 g/L	29.3 pg	90.6%	66.7%
7.	Sweden	Karlsson T. et al. 2011 [31]	Bone marrow examination	30.5 pg	69%	93%
8.	USA	Ellinor et al. 2014 [28]	Hb < 11 g/dL TS < 20%, serum iron < 40 µg/dL, ferritin < 100 ng/mL	< 31 pg	100%	69.7%
9.	Japan	Kaneko et al. 2003 [36]	TS < 20%	32.5 pg	59.2%	62.7%

85.9% and a specificity of 87.9% [27].

CHr as a diagnostic tool for IDA

There are two types of ID: absolute and functional ID. A decrease of iron in the bone marrow is referred to as absolute ID. Absolute ID may occur from impaired intestinal absorption, blood loss through the dialysis circuit, vascular access surgeries, repeated phlebotomies, and increased iron utilization with erythropoietin therapy. In clinical practice, absolute ID is determined by serum ferritin and TS. Functional ID is a condition when iron reserves in the bone marrow are adequate, but iron mobilization is impaired as in the states of chronic inflammation. Interpretation of iron status using TS and serum ferritin in chronic disease patients is difficult (in approximately 48% of the cases, iron status is determined using serum ferritin and TS) [29-31].

However, CHr may assist in diagnosis of IDA and anemia of chronic diseases better than ferritin or other serum indicators of iron status. It has an excellent negative predictive value for ruling out ID, but low positive predictive value. The low positive predictive value is due to a high cutoff value for CHr and interference of anemia in chronic diseases. This is because CHr used

for the diagnosis of functional ID can be restricted not only by ID but also chronic diseases or inflammation. Since anemia of chronic disease is characterized by iron restricted hematopoiesis, CHr levels are expected to be low and to overlap with those found in ID [28].

CHr is also useful to differentiate IDA from thalassemia trait, but cannot accurately differentiate IDA from thalassemia disease in patients with microcytic anemia [15]. IDA and thalassemia groups presented inefficient erythropoiesis, because of lack of iron or globin, it is characterized by low mean CHr value [20]. According to a study by Chaipokam et al. when the cutoff value increased and thalassemia patients were excluded, the sensitivity, specificity, predictive value, and accuracy of CHr for the diagnosis of IDA among general population also increased. The false positive results were from thalassemia diseased patients. But including the reticulocyte count can differentiate IDA from thalassemia disease (reticulocyte is higher in thalassemia disease patients than IDA patients) [15]. This is also supported by Fishban et al. who stated that the specificity of CHr with a cutoff value of 26 pg increases from 80% to 86.7% with a sensitivity of 100% when the thalassemia patients are excluded from analysis. Off course this study is conducted on small number of hemodialysis (HD) patients [22,32].

The same cutoff value of 27.2 pg CHr for diagnosing IDA also has a sensitivity of 87.5% and a specificity of 92.9%. In contrast to the above stated by Chaipokam J et al., this study showed that CHr has a high positive predictive value. The reason might be because there is no anemia of chronic diseases in the study population and in these people with or without inflammation there is no effect on CHr [33].

On the other hand, Torbjorn suggested that the CHr with a cutoff value of 30.5 pg has a low specificity and is not superior to analysis of mean corpuscular hemoglobin, ferritin, soluble TfR, when used for screening of ID in a population of elderly hospitalized anemic patients. The low specificity might be because the group of patients with pure IDA was not analyzed separately from those with anemia of chronic disease, which influence CHr results. But the type of ID in this study was absolute ID as determined by bone marrow iron staining, and it is conceivable that the CHr analysis is superior to mean corpuscular hemoglobin in earlier stages of ID or functional ID [31]. In contrast to this, Schapkaitz et al. reported that CHr has good specificity for the diagnosis of IDA in hospital patients by using bone marrow examination as the gold standard. A CHr cutoff < 28 pg reliably distinguished IDA from anemia of chronic disease [32].

Generally most of the studies stated that a cutoff value of 27.2pg of CHr has a sensitivity of 84.9% - 93.3% and specificity of 83.2% - 92.9% which is moderate sensitivity and specificity for the diagnosis of IDA [15,24, 33,35]. When the cutoff value of CHr increases the sensitivity also increases, whereas the specificity decreases [23,36].

CHr as an indicator of response to iron therapy

The challenge in IDA is not only early diagnosis, but also to monitor and evaluate its response to iron therapy. Response to iron therapy is classically assessed by an increase in peripheral blood reticulocyte count which occurs in 3 - 4 days and rise in Hb within the first week. However, in certain situations like bleeding, the peripheral blood reticulocyte count will increase despite no improvement in Hb. Moreover, peripheral blood reticulocyte count does not give sufficient information on the incorporation of iron into developing red blood cells [38]. Therefore, measurement of CHr in peripheral blood samples is useful for early assessment of response to the iron therapy which shows an increment within 2 to 4 days of the initiation of iron therapy [32]. CHr and reticulocyte number increases significantly after intravenous [29] iron therapy as early as 48 hours after initiation of treatment irrespective of the status of the iron stores (deplete or functional ID). Patients who had depleted iron stores showed greater increase in CHr in response to IV iron therapy as compared to those with functional ID [22,38]. However, there is no significant difference in reticulocyte number between patients who had depleted iron stores and those with functional ID. This showed that reticulocyte number (immature reticulocyte fraction) just reflects the erythropoietic activity of bone marrow but does not show the actual incorporation of iron in developing RBCs [38]. Anemia in end stage renal disease (ESRD) is a chronic disorder and related to severe complications among patients on HD. The commonly used indicators for detecting and monitoring IDA in HD patient are TS and ferritin. However, it is well established that specificity and sensitivity of serum ferritin level may be reduced due to inflammation, infection, malnutrition or malignancy. Potentially, one of the more accurate tests is CHr and is found to be a good test to guide in iron management. Utilizing CHr in management and treatment of anemia in patients on HD would be more accurate and effective. The effect of IV iron supplementation given to patients on HD with low CHr yet adequate ferritin and TS shows functional ID [32].

CHr is also clinically useful as an iron parameter measurement in non-dialysis chronic renal failure patients. It is more sensitive in detecting functional ID [37]. The effects of recombinant human erythropoietin (rHuEPO) treatment on CHr, TS, hypochromic percentage (% HYPO), and hematocrit were observed over time in the same patients. rHuEPO administration improved the hematocrit level within 2 months, and the CHr level significantly decreased within 1 month of the start of therapy; in contrast, the TS (affected by inflammation which is common in chronic renal failure patients) and % HYPO levels did not change significantly [39]. Erythropoiesis stimulating agent administration is a potent suppressor of hepcidin expression. This increases iron entry into circulation from dietary sources, iron recycling macrophages, and hepatocyte stores and increases iron utilization or demand due to increment of

erythropoietin activity. This causes patients to be more susceptible to iron deficiencies because of maintaining high serum rHuEPO levels and so, after certain time of rHuEPO treatment without external iron supplementation, the body iron is depleted and the first noted change is CHr due to the short life span of reticulocytes in the circulation.[40].

It is also an early and accurate predictor for oral iron therapy response and pediatric patients can take advantage at diagnosis and during follow-up of IDA for assessing early erythropoietic response to iron replacement therapy. Reticulocyte count is an enumeration of newly released immature RBCs and reflects bone marrow responsiveness. It is observed in acute or chronic bleeding, hemolysis and following treatment of IDA [5, 39]. However, it does not give any information on the incorporation of iron into developing RBCs [40]. Hemoglobin level of mature RBCs cannot detect early iron-deficient erythropoiesis due to the slow turnover of erythrocytes (long life span) (~120 days) in circulation [26]. Therefore, CHr (measure of Hb content of the freshly produced RBCs) is better for early response oral iron supplementation than absolute reticulocyte count and Hb level of mature RBCs [41].

Iron overdose leads to an epidemic overload of iron in the ESRD population. IV iron bypasses the biological safeguards for the transport and handling of iron and helps to intensify chronic kidney disease-associated oxidative stress and inflammation. As a consequence, indiscriminate use of IV iron can accelerate cardiovascular disease, promote microbial infections, aggravate eventual viral hepatitis, and worsen diabetes and diabetic complications in such patients. For these reasons, IV iron should be appropriately used in this vulnerable population [42]. Therefore, the another advantage of using CHr is avoidance of over-dosage of IV iron, which can potentially damage organs and can lead to a reduction in health care direct and indirect costs [43].

CONCLUSION

Reticulocytes have a normal life span of one or two days in peripheral circulation. One of the reticulocyte parameter, CHr, is a good indicator of iron availability and an early marker of iron deficient erythropoiesis. It has a moderate sensitivity and specificity for diagnosing ID and is less affected by inflammation than serum iron, TS, and ferritin. It is used in screening of ID and in the diagnosis of IDA and functional IDA in acute or chronic diseases or inflammation. CHr is also important in treatment monitoring. CHr in peripheral blood samples is useful for early measurement of response to the iron therapy, increasing within 2 to 4 days of the initiation of iron therapy. It helps in the monitoring of IV iron supplementation, rHuEPO therapy, and oral iron therapy in HD, non-HD patients and children. It is easy to analyze, less time consuming, and less expensive than bone iron examination and iron biochemical tests. However, there is no standardized cutoff point of Chr and different researchers have been using different cutoff values for CHr which affects the accuracy of CHr to diagnose ID. It is therefore important to standardize the cutoff point of CHr.

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YG and MM: conceived the idea; YG: drafted the review manuscript; YG, BW and MM: Critically revised and edited the manuscript. All authors contributed to the writing of the manuscript and approved the submitted version of the manuscript.

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