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Update on Iron Deficiency Anemia and Iron Metabolism. Laboratory perspective and differential diagnosis.

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Iron deficiency and iron deficiency anemia are global health problems and common conditions seen daily in clinical practice. Iron deficiency is the leading cause of anemia worldwide, and iron deficiency anemia has a substantial impact on the lives of children and premenopausal women in both developed and developing countries.

The prevalence of iron deficiency without dietary supplementation worldwide is 40% in preschool children, 30% in women of reproductive age, and 38% in pregnant women. These rates reflect the high iron requirements at certain ages and in specific situations.

Iron is essential in the human body for various functions, including DNA synthesis, respiration, energy production, and cell proliferation. The body contains an average of 3-5g of iron distributed in erythrocytes, macrophages of the reticuloendothelial system (RES), liver, bone marrow, muscles, and other tissues.

Iron conservation and recycling mechanisms exist after the breakdown of old or defective red blood cells, but, since iron can be toxic to the body, its absorption is limited to 1-2 mg daily (daily requirements are around 25 mg, mostly obtained through recycling). These mechanisms are controlled by the hormone hepcidin, which maintains iron levels within normal parameters, preventing both deficiency and excess.

Important definitions:

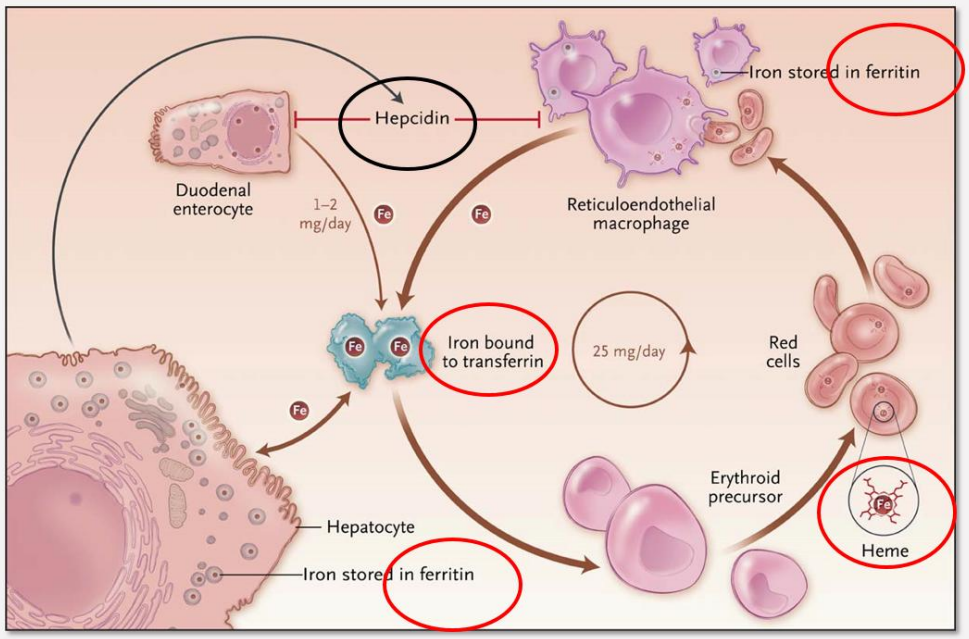
- **Iron deficiency** : decreased levels of total body iron, especially iron stores, with preserved levels of red blood cell iron.
- **Iron deficiency anemia** : decreased levels of total body iron in the presence of anemia.
- **Iron-restricted erythropoiesis** : the incorporation of iron into erythrocytes is compromised, regardless of the amount of stored iron (usually high).
- **Anemia of chronic disease or anemia of inflammation** : multifactorial, it is common in cases of inflammation, autoimmune diseases, cancer, infections, and chronic kidney failure. It is associated with high cytokine production, hepcidin overproduction, and abnormal iron balance.
- **Functional iron deficiency** : insufficient mobilization of iron in response to high requirements, such as occurs after treatment with erythropoietin.

The etiology of iron deficiency is an imbalance between the incorporation and loss of iron in the body.

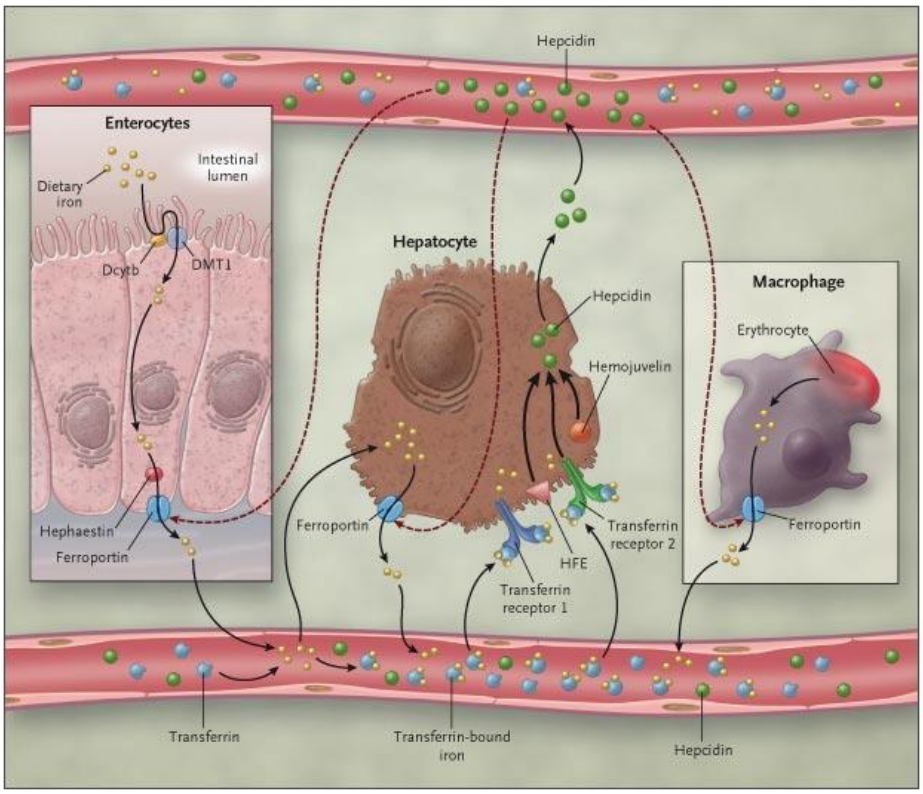
Causes:

- Physiological: increased demand
  - Childhood
  - Rapid growth (adolescence)
  - Menstrual bleeding
  - Pregnancy (second and third trimesters)
  - Blood donors
- Environmental
  - Insufficient contribution due to poverty, malnutrition
  - Diet (vegetarian, vegan, low iron)
- Pathological
  - Decreased absorption:
    - Gastrectomy
    - Duodenal bypass
    - Bariatric surgery
    - H pylori infection
    - Celiac disease
    - Atrophic gastritis
    - Inflammatory bowel disease (Crohn's disease, ulcerative colitis)
  - Chronic blood loss:
    - Gastrointestinal tract: esophagitis, erosive gastritis, peptic ulcer, diverticulitis, benign or malignant tumors, inflammatory bowel disease, angiodysplasia, hemorrhoids, parasitic infestation….
    - Genitourinary system: heavy menstruation, menorrhagia
    - Intravascular hemolysis (PNH, cold antibody AIHA, march hemoglobinuria, cardiac valvular dysfunction, microangiopathic hemolysis)
    - Systemic bleeding: hemorrhagic telangiectasia, chronic schistosomiasis, Munchausen syndrome (self-induced bleeding)
- Drug-related: glucocorticoids, salicylates, NSAIDs, proton pump inhibitors
- Genetic: IRIDA (iron deficiency anemia refractory to iron treatment)
- Iron-restricted erythropoiesis:
  - treatment with erythropoiesis-stimulating agents (EPO)
  - anemia of chronic disease
  - chronic kidney disease

Schematic of iron metabolism: **Interaction of key proteins in iron homeostasis.**



Fleming RE, Ponka P. N Engl J Med 2012;366:348-359



Fleming RE, Bacon BR. N Engl J Med 2005; 352:1741-1744.

In the duodenal enterocyte, dietary iron is reduced to the ferrous state ($Fe3^{+}$) by duodenal ferric reductase (Dcytb), transported into the cell by divalent metal transporter 1 (DMT1), and

released into the circulation via ferroportin (FPN). Hephaestin (HFE) also influences iron release from the enterocyte.

Hepatocytes take up iron from the circulation either as free iron or bound to transferrin (via transferrin receptor 1 and transferrin receptor 2). Transferrin receptor 2 can act as a sensor of circulating transferrin-bound iron, thereby influencing the expression of the iron-regulating hormone, hepcidin.

The hepcidin response is also modulated by HFE and hemojuvelin . Hepcidin is secreted into the circulation, where it negatively regulates ferroportin- mediated iron release from enterocytes , macrophages, and hepatocytes. Iron stores in the form of ferritin are present in these macrophages and hepatocytes.

Insufficient intake, malabsorption, digestive disorders, and blood loss (gynecological, digestive, urological, etc.) can lead to iron deficiency. If these causes persist, iron stores are depleted, making it difficult to maintain red blood cell production and resulting in iron deficiency anemia.

Laboratory diagnosis of iron metabolism disorders (deficiencies)

There is no laboratory test that can definitively diagnose iron deficiency in all situations.

Classical parameters: (chemical form, synthesis or location, reference values, values in iron deficiency, diagnostic value, interferences).

- ### Blood count parameters
    - Hemoglobin: its level defines anemia. According to the WHO, in men it is hemoglobin <13 g/dL, in non-pregnant women <12 g/dL, and in pregnant women <11 g/dL in the first and third trimesters of pregnancy and <10.5 g/dL in the second trimester.
    - Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC): in iron deficiency and iron deficiency anemia, their values are usually decreased.
    - VCM: direct measurement, by impedance.
        - Units: fL.
        - Normal values vary according to age and sex. In adults, they range from 83.6 to 97 fL. Lower values are observed in children.
    - HCM: Hb/RBC Calculation
        - Units: pg
        - Adult reference values: 27-32 pg
    - CHCM: Hb/hematocrit calculation
        - Units: g/dL
        - Reference values for adults: 31.5-34.5 g/dL

    - The differential diagnosis of microcytosis must include hemoglobinopathies (thalassemias), in which the values of MCV, MCH and MCHC are constantly decreased throughout the individual's life, except when coexisting with deficiencies of maturation factors (folic and/or vitamin B12) or the use of some drugs.

- Reticulocytes : Reticulocytes are young red blood cells, released into the bloodstream from the bone marrow in recent days. Their cytoplasm contains varying amounts of RNA that can be stained and quantified. This provides a measure of the bone marrow's capacity to produce red blood cells. Reticulocytes are decreased in deficiency states and bone marrow aplasia, and increased in hemolytic anemias and recent bleeding, among other conditions.

  Units:

- reticulocytes/1000 red blood cells: 5-25 x/1000
- absolute values: 20-80 x10e9/L

    - Reticulocyte hemoglobin: This is the concentration of hemoglobin present in reticulocytes. It gives us an idea of the iron available in the bone marrow for hematopoiesis. It is a good parameter of the iron response in iron deficiency anemia. It is not useful for differentiating iron deficiency from thalassemia.

- Serum iron:
    - Measures circulating iron ($Fe3^{+}$) bound to transferrin. Used to calculate transferrin saturation. Not affected by hemolysis. Limitations: Subject to circadian fluctuations. Increases after iron ingestion, parenteral administration, in the morning, and in patients receiving chelation therapy.
    - Units μ mol/L
    - Reference values 5.83-34.5 μ mol/L (32.56-192.68 μ g/dL)
- Ferritin:
    - A macromolecule that encapsulates ferrous ions. Located in tissues (liver, spleen, muscle, and bone marrow). Decreased in iron deficiency, it is generally the most useful parameter. In normal individuals, it reflects stored iron. Values < 15 $\propto\gamma$ /L have high specificity for iron deficiency but low sensitivity; higher cut-offs are proposed for screening. Lower values are found in children and premenopausal women. In the elderly, elevated values are associated with pathologies. Limitations: an acute-phase reactant (positive), elevated in inflammation, infection, neoplasms, chronic kidney disease, liver diseases, alcoholism, and obesity. Decreased in hypothyroidism, ascorbic acid, and TNF-alpha inhibitors.
    - Units: ng/mL
    - Adult reference values:
        - Women of reproductive age: 15-150 ng/mL
        - Postmenopausal woman: 30-400 ng/mL
        - Male : 30-400 ng/mL

      Cut -off for iron deficiency: values below 15 ng/mL indicate a lack of iron stores. Some authors consider iron deficiency to be present in all cases where ferritin is below 30 ng/mL. In chronic and inflammatory conditions, the ferritin cut -off for considering iron deficiency is raised to values of 100 or 200 ng/mL.
- Transferrin :

- A glycoprotein synthesized in the liver in accordance with the body's iron requirements and iron stores. It increases in iron deficiency. Used to calculate transferrin saturation index and total iron-binding capacity. It distinguishes iron deficiency anemia from anemia of chronic disease. Limitations: it is a negative acute-phase reactant. It decreases in inflammation, infection, neoplasms, autoimmune disease, nephrotic syndrome, liver disease, and malnutrition.
- Units: g/dL
- Normal values: 2-3.6 g/dL
- Values around or above 3 g/dL suggest iron deficiency. And values below 2, in the presence of anemia, suggest iron blockage.

- Transferrin saturation index :
  - Calculated from transferrin and iron. The total iron-binding capacity (TIBC) is obtained, and from it the transferrin saturation index or transferrin saturation.
  - Units: %
  - Normal values: 20-45%
  - It has decreased values in situations of iron deficiency. Values below 16% have been associated with iron deficiency anemia, and values between 16 and 20% with functional iron deficiency, anemia of chronic disease.
  - Extremely low values may be indicative of IRIDA.
- Soluble transferrin receptor (sTfR):
  - Truncated dimer of the erythrocyte membrane transferrin receptor. It is postulated as a differentiator between iron deficiency and iron blockade, that is, between iron deficiency anemia and anemia of chronic disease.
  - In iron deficiency and iron-deficiency anemia, their values are elevated. In anemia of chronic disease, the values are within the normal range. In mixed situations, involving both iron deficiency and iron blockade, the values are elevated.
  - Disadvantages: lower availability, higher cost, delay in issuing results reports. Different techniques from different providers make standardization difficult.
- Combination of sTfR and ferritin:
  - sTfR/ferritin ratio: elevated in iron deficiency, decreased in anemia of chronic disease. It combines two important parameters for studying iron metabolism.
  - sTfR/log ferritin ratio: similar to the previous one. It is used in the Thomas plot in combination with reticulocyte hemoglobin (Ret -he).
- Hepcidin:
  - Due to technical challenges, comprehensive validation of hepcidin for daily clinical use remains elusive, confining its analysis largely to the research realm. For hepcidin's potential in clinical care and research to be fully realised, consistency and analytical accuracy are crucial to ensure reliable clinical decisions and standardised reference intervals.

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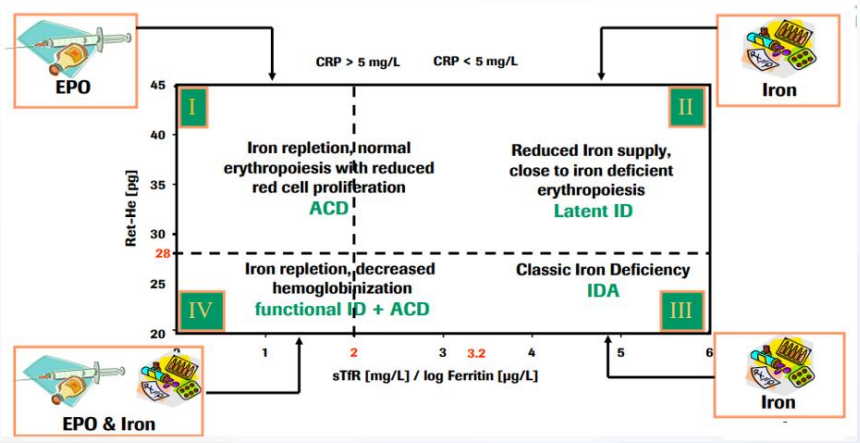
Summary of iron metabolism parameter values in anemia of chronic diseases (APC), iron deficiency (AF) or mixed anemia (AF + APC)

Analito	APC	AF	AF+APC
Hierro	↓	↓	↓
Transferrina	↔ ↓	↑	↓ ↔
Saturación de transferrina	↓	↓	↓
Ferritina	↔ ↑	↓	↓ ↔
sTfR	↔	↑	↔ ↑
Índice sTfT/log ferritina	↓	↑	↑ ↔

To better differentiate these types of anemia and provide a therapeutic indication in 2002, Lothar Thomas published a chart in which he correlated reticulocyte hemoglobin values (indicating the iron available for erythropoiesis) and the sTfR index.

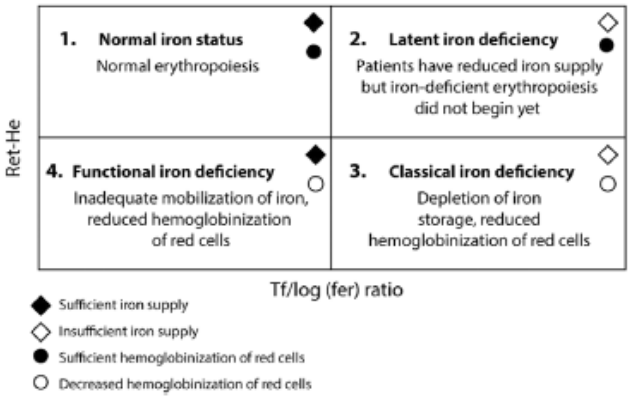
- sTfR/log ferritin index: Thomas plot
  - It established 4 quadrants:
    - I: Adequate iron stores, normal erythropoiesis with decreased erythroid proliferation: anemia of chronic disease
    - II: reduced iron deposits, latent iron deficiency
    - III: Classic iron deficiency, iron deficiency anemia
    - IV: adequate iron stores, decreased hemoglobinization (functional iron deficiency + anemia of chronic disease)
  - And he proposed treatments with iron, erythropoietin, or both.



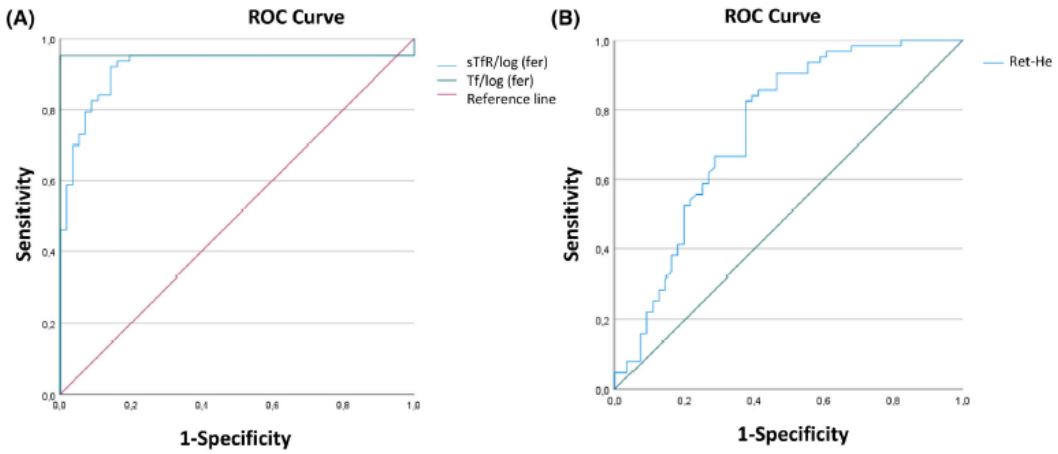
Its limitations are the same as sTfR: reduced availability, cost, and delayed results.

- Transferrin/log ferritin index: alternative Thomas plot

In 2023 from Leur et al, Int J Lab Hematol . 2023; 45:96-103 published an alternative to this Thomas plot using the ratio between transferrin and the logarithm of ferritin, compared with the reticulocyte hemoglobin value.



They established the cut-offs for the different parameters and obtained better results, with greater sensitivity and specificity for the Tf /Log Ferr index.



(C)

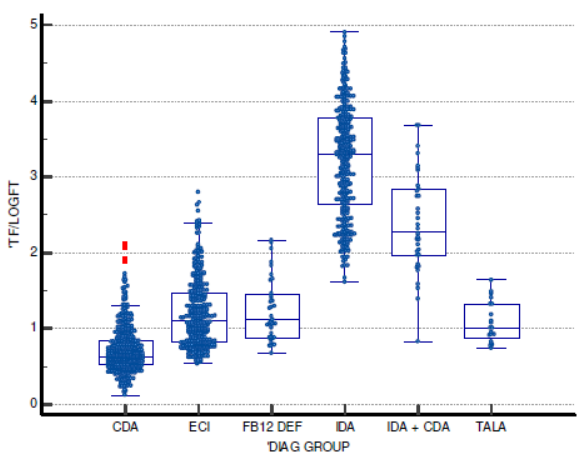
Parameter	Optimal cut-off	AUC	95% CI	*P* value	Sensitivity (%)	Specificity (%)
Ret-He	31.2 pg	0.75	0.66 – 0.84	<0.001	83	63
sTfR/log (fer)	0.91	0.92	0.86 – 0.98	<0.001	94	84
Tf/log (fer)	1.71	0.95	0.90 – 1.0	<0.001	95	100

FIGURE 3 Receiver operating characteristic (ROC)-curves generated with the study set. (A) ROC curve of the sTfR/(log) fer ratio and the Tf/(log) fer ratio. (B) ROC curve of the Ret-He. (C) Summarized optimal cut-off values, 95% confidence interval (CI), *p*-value (null hypothesis: Area = 0.5), sensitivity and specificity of the different parameters used to generate the classical and alternative Thomas-plot

Our data

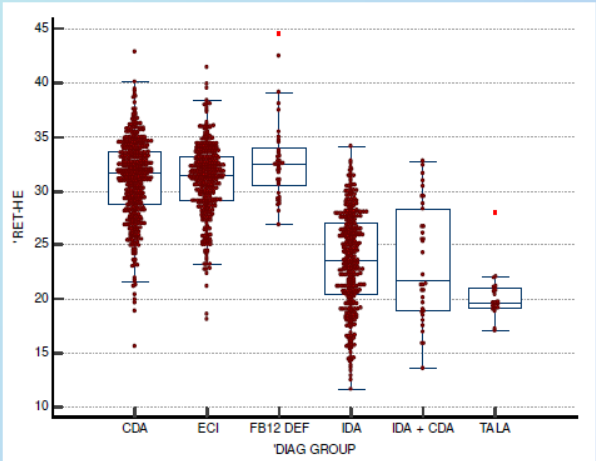
In 2024, we decided to incorporate this index into our computer system and analyze our data from november and december 2024. We obtained 1186 adult patients with anemia in whom all parameters of iron metabolism, folic acid, and vitamin B12, creatinine, and CRP or ESR had been determined to assess the chronic disease component. We divided them into the following groups: CDA (anemia of chronic disease), ECI (anemia of unknown cause), FB12 def (folic and/or B12 deficiency), IDA (iron deficiency anemia), IDA + CDA, and Tala (thalassemia).

First, we analyzed the values obtained from the Tf /Log ferr index for the different groups.



TF/Log Ferr index

GROUP	CDA	ECI	FB12 DEF	IDA	IDA+CDA	TALA
N	422	341	37	332	34	20
MEAN	0,72	1,20	1,24	3,22	2,38	1,08
SD	0,31	0,46	0,41	0,73	0,67	0,27
P5	0,34	0,65	0,77	2,06	1,48	0,77
P95	1,37	2,05	2,07	4,37	3,50	1,50

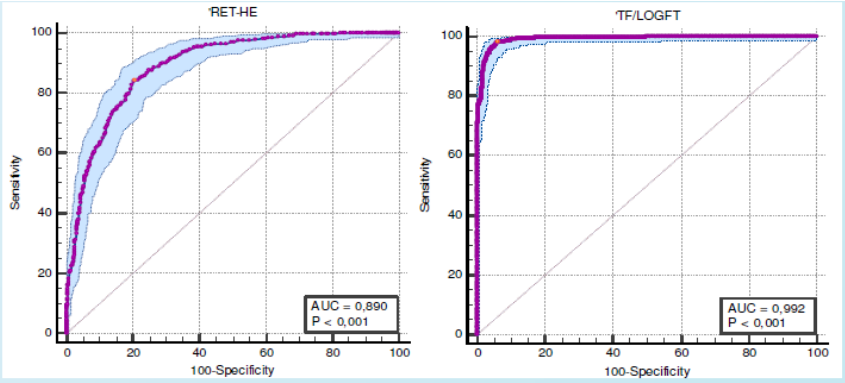


Ret-He

GROUP	CDA	ECI	FB12 DEF	IDA	IDA+CDA	TALA
N	422	341	37	332	34	20
MEAN	31,08	31,02	32,79	23,50	23,38	20,20
SD	3,76	3,40	3,76	4,52	5,40	2,24
P5	24,62	25,00	28,58	15,70	15,90	17,19
P95	36,20	35,90	39,78	30,29	31,88	22,39

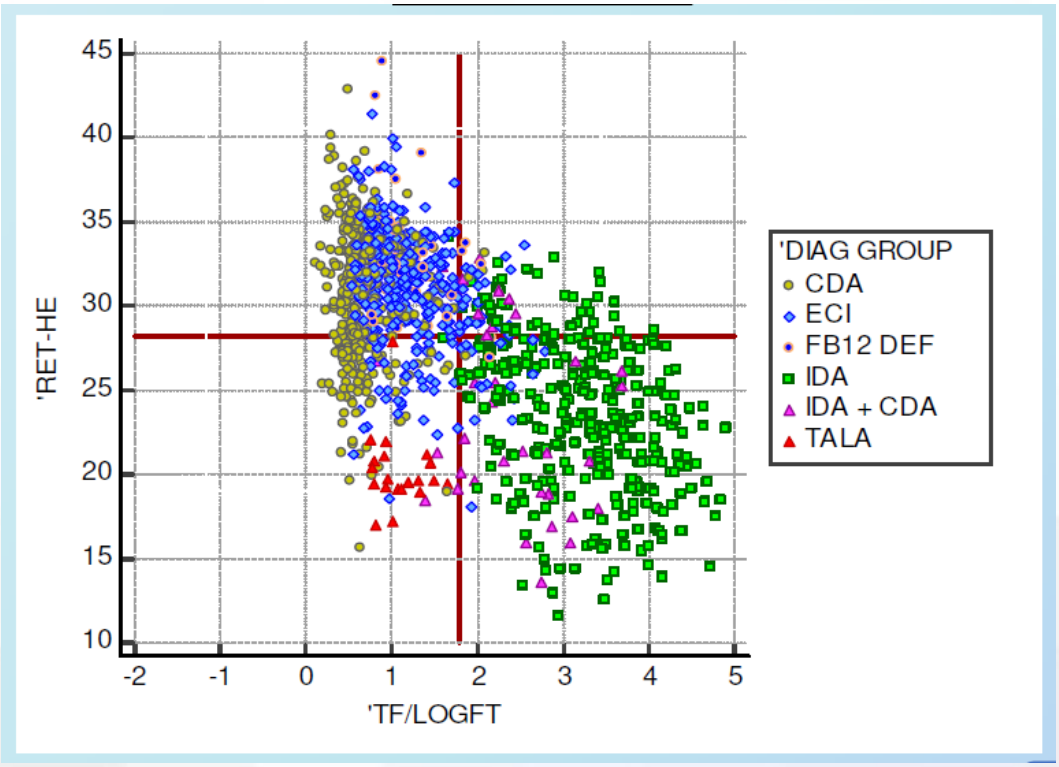
We observe good discrimination in the Tf /Log ferr index between iron deficiency anemia and the other groups. In patients with a mixed component (iron deficiency and chronic conditions), the values are closer to those of iron deficiency anemia than to those of chronic conditions. There is also good discrimination in red blood cell hemoglobin values, but as mentioned previously, it does not discriminate well between iron deficiency and thalassemia.

Then, we proceed to determine the cut-offs in our series for these parameters:



INDEX	CUTOFF	SENSITIVITY (%)	SPECIFICITY (%)	AUC
TF/Log ferr index	1,79	98,09	96,83	0,992
Reticulocyte Hemoglobin	28,1 pg	84,15	79,63	0,890

And finally, in this alternative Thomas plot, we analyze the distribution of our patients



Most samples with anemia of chronic processes have a Tf /log ferr index less than 1, and present a correct hemoglobin content in the reticulocytes, but as the Thomas plot indicated, there is a decrease in erythroid proliferation.

We use these parameters in the evaluation of anemia studies in the laboratory to generate our reports. In the future, they will be included in laboratory reports. We believe they can be helpful in interpreting the results of anemia and iron metabolism studies.

Limitations: This study did not analyze pediatric patients, and this alternative Thomas plot is not validated for pregnant women.

FINAL CONSIDERATIONS:

- Pre-analytical: Iron metabolism study results are influenced by iron treatments. It is recommended that, in follow-up studies, patients discontinue treatment at least one week before sample collection. When reviewing results with patients, inquire whether the patient was on treatment at the time of the analysis.
- Do not order iron metabolism studies after intravenous iron administration; wait at least one month before taking samples.
- In the follow-up of iron deficiency anemia after initiating iron therapy, monitoring with complete blood counts is recommended, keeping in mind that microcytosis may persist for at least two months due to the persistence of microcytic red blood cells (red blood cell lifespan of 3 months). After three months, iron metabolism studies can be requested again, with treatment discontinued at least one week prior.
- If microcytosis persists after iron deficiency is resolved, consider requesting hemoglobin studies, specifically requesting Hemoglobin A2 and F.

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