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"ROLE OF RETICULOCYTE HEMOGLOBIN PARAMETER IN EVALUATION OF MICROCYTIC HYPOCHROMIC ANEMIA"

By

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Dissertation submitted to the

BLDE (DEEMED TO BE UNIVERSITY),

Vijayapura, Karnataka

In partial fulfillment of the requirements for the award of the degree of

DOCTOR OF MEDICINE

IN

PATHOLOGY

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To my Parents...

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LIST OF ABBREVIATIONS USED

Hb	Hemoglobin
RBC	Red blood cell
PCV/Hct	Packed cell volume/Hematocrit
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
RDW	Red cell distribution width
RPI	Reticulocyte Production Index
IRF	Immature Reticulocyte Fraction
Ret-He	Reticulocyte Hemoglobin equivalent
S Iron	Serum Iron
TIBC	Total Iron Binding Capacity
S Ferritin	Serum Ferritin
WHO	World health organization
ID	Iron deficiency
IDA	Iron deficiency anemia

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ABSTRACT

Introduction

Reticulocytes are RBC precursors with an average lifespan of 1-2 days. Information regarding its hemoglobin gives a good indication of Iron availability. Ret-He is an early marker of Iron deficiency erythropoiesis and reflects real-time information regarding the synthesis of young erythrocytes in the bone marrow. It provides an initial measure of functional Iron deficiency

Objectives

To determine the diagnostic utility of Ret-He in patients having Microcytic Hypochromic anemia in comparison with Serum ferritin and Iron studies.

Methods

A hospital-based observational study was carried out in Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka.

Hematological parameters like RBC count, Hb, PCV, MCV, MCH, MCHC, RDW, Reticulocyte count, Absolute Reticulocyte Count, RPI, IRF, Ret-He processed in the Sysmex XN1000 (Sysmex Corporation, Kobe, Japan) analyzer were studied and compared with Biochemical parameters of assessing body Iron stores and Iron availability such as TIBC, Serum Iron and Serum Ferritin using Biochemistry analyzer (VITROS® 250 Chemistry System) from 201 subjects, after fulfilling all the inclusion and exclusion criteria's.

Observations

When S Ferritin is taken as 'the gold standard' to detect ID, after statistical evaluation, we found the cut-off value of Ret-He to detect ID state was

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27.15pg/cell, and there was a statistically good correlation between Ret-He and S Ferritin with P <0.001. When the S Ferritin cutoff of <15ng/mL was taken as ID state, we found a Sensitivity of 57.37% and Specificity of 75.95% with AUC of 0.681 for Ret-He. PPV of 100%, NPV of 3.8%, and accuracy of 62.19% for Ret-He was found. When S Ferritin <30ng/mL was taken as ID, which supposedly increases its sensitivity and PPV in detecting ID states; we found a significant increase in sensitivity to 80.42% at the cost of decrease in specificity to 56.52% for Ret-He.

In our study, we found that Ret-He was showing significant statistical positive correlation with Hb with P <0.001. There was also a moderate positive correlation which is statistically significant between Ret-He and MCV, MCH, MCHC.

A significant statistical positive correlation was found between Ret-He and S Ferritin levels p <0.001 which suggested Ret-He as a comparable hematological parameter. Also found a statistically significant negative correlation between Ret-He and TIBC p<0.001. There was no statistical correlation between Ret-He and S Iron levels in our study which may be attributed various causes.

Conclusions

The present study suggests Ret-He is one of the better and reliable hematological parameters indicating ID and, when used along with biochemical parameters such as S Ferritin, can give valuable inputs in a better screening of ID states and diagnosis of IDA and hence proper treatment is possible.

Keywords: Ret-He, Iron deficiency, Anemia

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"ROLE OF RETICULOCYTE HEMOGLOBIN PARAMETER IN EVALUATION OF MICROCYTIC HYPOCHROMIC ANEMIA"

INTRODUCTION

Anemia is functionally defined as a decrease in the oxygen-carrying capacity of the blood to peripheral tissue, thereby causing hypoxia. It is either due to insufficient hemoglobin or impaired or inefficient hemoglobin content in the body [1,2,3,4].

For all practical purposes to establish the presence of anemia; Hemoglobin (Hb) (g/dL), Hematocrit/Packed cell volume (Hct/PCV) (%), and Red blood cell (RBC) count (10^6/uL) are used [1,4]. Anything below the reference range for these parameters for a healthy individual of similar age, sex, and race, under similar environmental conditions is considered as an anemic [2].

Anemia is a serious global public health problem that significantly affects young children and pregnant women [5]. It affects roughly 1/3rd of the world's population - approximately 2 billion people in which over 800 million are women and children globally [6,7]. The burden of this disease is enormous but poorly estimated, particularly in the developing countries [6].

Among anemia, Iron deficiency anemia (IDA) is a significant and global public health problem [8]. It is assumed that at least 50% of the cases of anemia of about 1.48 billion people are due to the insufficient Iron content in the diet, particularly

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in women in child-bearing age with increased menstrual blood loss or during pregnancy, young children, and vegetarians [(6,8,9,10,11)].

Microcytic Hypochromic anemia (MCHC anemia) is caused by any factor which reduces the body's Iron stores. Reduced Iron stores halt the production of hemoglobin chains, and its concentration and amount begin to decrease in the newly formed RBCs; hence cells show hypochromia and microcytosis [(12)]. The four major causes of microcytic red blood cells are ID, anemia of chronic disease (ACD), Thalassemia, Sideroblastic anemia, and lead poisoning. IDA and ACD are by far the most common [(13,14)].

Newer parameters introduced in the 6-part automated cell counters have changed the approach to diagnosing anemia with possible causes. With additions of automated reticulocyte analysis has led to the addition of newer Retic parameters like Reticulocyte count, Absolute Reticulocyte Count, Reticulocyte Production Index (RPI), Immature Reticulocyte Fraction (IRF), Reticulocyte Hemoglobin equivalent (Ret-He). Reticulocytes are the RBC precursors with the normal lifespan of 1-2 days. Information regarding its hemoglobin gives a good indication of Iron availability. Ret-He reflects real-time information regarding the synthesis of young erythrocytes in the bone marrow and acts as an early marker of Iron-deficient erythropoiesis [(12,14)].

This study has been undertaken to evaluate the role of these newer reticulocyte parameters in the patients having Microcytic hypochromic anemia (MCHC anemia) and comparing it with Serum Iron parameters and Ferritin levels.

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OBJECTIVES OF THE STUDY:

To determine the diagnostic utility of Ret-He and other reticulocyte indices in patients having Microcytic Hypochromic anemia in comparison with Serum Iron studies and S Ferritin levels.

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REVIEW OF LITERATURE

World health organization (WHO) defined anemia when the Hb concentration in blood lower than the normal [(5)]; that is, <11g/dL (Age: Children,6-59 months), <11.5 g/dL (Age: Children,5-11years), <12 g/dL (Age: Children,12-14 years and Non pregnant women, ≥ 15years), <11 g/dL (Age: Pregnant women), <13 g/dL (Age: Men, >/= 15years) [(6)].

According to WHO estimates in 2004, IDA resulted in 2,73,000 deaths and the loss of 19.7 million disability-adjusted life years, accounting for about 1.3% of the global population, with 97% occurring in low and middle-income countries [(15)]. It is the most common nutritional deficiency disorder globally, which is well recognized [(16)].

In children of 1-3 years of age, 9% are diagnosed with Iron deficiency (ID). In the U.S.A., IDA is the most common nutritional deficiency among children affecting about 2-3% of children [(17)]. In developing countries, 39% of children < 5 years, and 48% of children between 5-14 years suffer from anemia. In Asia, it is estimated that anemia in children of <2 years of age will possibly cover 90% of the children population. In Southeast Asia, 66% of the children are anemic, which results in 3,24,000 deaths and 1,25,00,000 Disability Adjusted Life-Years (DALYs) [(18)].

It is estimated in 10 developing countries that the median value of annual physical productivity losses due to Iron deficiency is around $2.32 per capita or 0.57% of gross domestic product (G.D.P.). Median total losses (physical and cognitive combined) are $16.78 per capita, 4.05% of G.D.P. and hence the economic implications of IDA are also massive [(16)].

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Burden of anemia in India

According to National Family Health Survey – 4 (NFHS-4), which was published in December 2017 by the ministry of health and family welfare, the Government of India has the compiled data till 2015-16 regarding the prevalence of various health conditions according to age, sex, urban-rural and in the various socio-economic status of the population. It also gives data regarding population distribution among various socio-economic strata of the society, quality of life, education, health data, and much more among the population according to the whole country, state, and even district-wise distribution [(19,20,21)].

Anemia in Children-

About 59% of children (Age: 6-59 months) have some degree of anemia; 28%- Mild, 29%- Moderate, 2%- Severe anemia. In 10years, the prevalence has declined from 70% to 59% and is more prevalent among children <2yrs than older ones and peak of 71% prevalence at 12-17 months and highest in Haryana (72%) and lowest in north-eastern states. The prevalence of 56% and 59.5% is found in urban and rural, respectively [(19)].

Anemia in adults –

About 50% of women (among them 40% - mild, 12%- Moderate, 1%- Severe) and 23% men (among them 12%- Mild, 10%- Moderate and 1%-Severe) of age 15-49years are anemic. In 10years, the prevalence has declined from 55% to 53% among women and 24%-23% among men [(19)].

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Anemia and maternity –

WHO/World Health Statistics data shows that 40.1% of pregnant women worldwide were anemic in 2016 [(22)]. It is predominant in Southeast Asian countries. Half of all global maternal deaths are due to anemia, and India contributes to about 80% of maternal death due to anemia in South Asia [(23)]. In India, it is estimated that about 20% of maternal deaths are directly related to anemia, and another 50% of maternal deaths are associated with it [(16)]. 58% of women who are breastfeeding, 50% of pregnant women, and 52% of women who neither pregnant and breastfeeding are anemic [(19,24)].

Age group	Intervention/Dose	Regime
6-60 months	1ml of IFA syrup- 20mg elemental Iron & 100µg FA	Biweekly-6-60months of age & Biannual deworming of children aged 12months and above
5-10 yrs	45mg elemental Iron & 400µg FA	Weekly- 5-10yrs of age and biannual deworming
10-19 yrs	100mg elemental Iron & 500µg FA	Weekly- 10-19yrs of age and biannual deworming
Pregnant and lactating women	100mg elemental Iron & 500µg FA	1 tab/day for 100days starting after 1st trimester, at 14-16weeks of gestation; repeated for 100 days postpartum
Women of reproductive age (15-49yrs)	100mg elemental Iron & 500µg FA	Weekly throughout the reproductive period

Table 1: National Iron+ Initiative Iron-Folic acid supplementation programme [(25)]

(IFA- Iron folic acid, FA- Folic acid)

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Anemia during pregnancy is associated with many maternal and fetal complications. It decreases the woman's reserve to tolerate bleeding (either during or after childbirth) and makes them prone to infections. It also has been associated with increased risk of intrauterine growth restriction, premature delivery, low birth weight (LBW), maternal and child mortality. 'The Ministry of Health and Family Welfare, Government of India has emphasized to prevent anemia under RMNCH+A services. National Health Policy 2017 also addressed malnutrition and micronutrient deficiencies interventions. "National Iron Plus Initiative" launched in 2013, is a comprehensive strategy to combat the public health challenge of Iron deficiency anemia (IDA)' [Table 1] [(23)].

Anemia and educational status – 56% with no schooling and 49% with 12 or more years of schooling among women whereas it is 29% and 18% among men respectively [(19)].

Anemia and socioeconomic status- Prevalence steadily decreases as the wealth of the household increases - from 59% in the lowest wealth quintile to 48% in the highest wealth quintile among women and from 32% in the lowest wealth quintile to 17% in the highest wealth quintile among men [(19)].

Anemia in rural and urban – 54% and 51% among women; 25% and 19% among men in rural and urban areas respectively [(19)].

Prevalence of anemia among adults - 60% or more population in states of union territories of Dadra & Nagar Haveli, Chandigarh, Haryana, West Bengal, Bihar, and Andhra Pradesh are anemic and < 1/3rd in north-eastern states are anemic [(19)].

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Karnataka state: 60.9% - Children (Age: 6-59 months), 44.8% - Non Pregnant women (Age: 15-49 Years), 45.4% - Pregnant women (Age: 15-49 Years), 44.8%- All women (Age: 15-49 Years) and 18.2% - Men (Age: 15-49Years) are anemic [(20)] .

Vijayapura District: 68% (68.3% in rural) - Children (Age: 6-59 months), 41.2% (40.3% in rural) - Non Pregnant women (Age: 15-49 Years), 57.5% (54.2% in rural) - Pregnant women (Age: 15-49 Years), 41.9% (40.9% in rural) - All women (Age: 15-49 Years) and 20.5% (21.6% in rural) - Men (Age: 15-49Years) are anemic [(21)].

On 1 December 2017, the Union Cabinet approved setting up of the National Nutrition Mission under the oversight of the Ministry of Women and Child Development. Among many targets, the National Nutrition Mission aims to reduce anemia among young children, adolescent girls, and women of reproductive age (15–49 years) by 1/3rd of NFHS-4 levels by 2022 [(26)].

Anemia

Anemia can be classified according to the size of RBCs. Microcytic anemia is considered when Mean corpuscular volume (MCV) is < 80 fl and hypochromia when Mean corpuscular hemoglobin (MCH) is < 27 pg [(27,28,29)]. Normocytic anemia when MCV falls in 80-100fL range and Macrocytic anemia when MCV is > 100fL [(1,2)].

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Clinical effects of Anemia

Symptoms due to anemia are based on their degree and are usually non-specific. It might be relative, and people with anemia can present late to the hospital too.

Symptoms (1,3,30)

1. Decreased work or exercise tolerance (Fatigue)
2. Shortness of breath
3. Palpitations or other signs of cardiorespiratory adjustments to anemia
4. Headache, vertigo, syncope, tinnitus, faintness, scotoma, lack of mental concentration, drowsiness, restlessness, and muscular weakness, sometimes associated with paraesthesia along with other signs and symptoms of peripheral neuropathy.
5. Dark or red urine/stool, etc.,

Signs (1,3)

1. Pallor – mucous membranes of mouth & pharynx, Conjunctive, lips & nail beds.
2. Nails – loss of luster, brittle, koilonychia.
3. Cardiovascular and pulmonary - Hypotension, tachycardia, dyspnoea, Systolic murmur
4. Jaundice - Icterus
5. Neurological dysfunction – paraesthesia's, peripheral neuropathy
6. Hepatomegaly
7. Splenomegaly
8. Bone deformities in congenital anemias

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9. Gallstones
10. Extramedullary hematopoietic masses
11. Petechiae and ecchymosis
12. Skin and mucosal changes – Thinning, loss of lustre, early greying of hair. alopecia, chronic leg ulcers, symmetric dermatitis, fissure at the angle of the mouth, mucosal bleeding.
13. Ophthalmic – flame-shaped hemorrhages, hard exudates, cottonwood spots, or tortuous retinal veins, hemorrhages, papilledema, etc.
14. Gastrointestinal – Glossitis and atrophy of tongue papillae. Painful, ulcerative, and necrotic lesions in mouth and pharynx. Dysphagia is also seen.

Symptoms of Iron deficiency anemia [8,31]

Very frequent

- Paleness (Seen in about 45–50% of people)
- Fatigue (Seen in 44% of people)
- Dyspnoea
- Headache (Seen in 63% of people)

Frequent

- Diffuse and moderate alopecia (Seen in 30% of people)
- Atrophic glossitis (Seen in 27% of people)
- Restless legs syndrome (Seen in 24% of people)
- Dry and rough skin

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- Dry and damaged hair
- Cardiac murmur (Seen in 10% of people)
- Tachycardia (Seen in 9% of people)
- Neurocognitive dysfunction
- Angina pectoris
- Vertigo

Rare

- Hemodynamic instability (Seen in 2% of people)
- Syncope (Seen in only 0·3% of people)
- Koilonychia
- Plummer-Vinson syndrome (Seen in <0·1% of people)

Laboratory Investigations available [3]

1. Erythrocyte count
2. Hemoglobin
3. Hematocrit / Packed cell volume
4. Erythrocyte indices: MCV, MCH, MCHC
5. Reticulocyte count, reticulocyte production index (RPI), corrected reticulocyte count, CHr or Ret-He, IRF
6. Blood smear examination
7. Leukocyte and platelet quantitative and qualitative examination
8. Peripheral blood smear evaluation for the presence of spherocytes, schistocytes, and other poikilocytes, and erythrocyte inclusions

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9. Tests to measure erythrocyte destruction depending on other information available: serum bilirubin, haptoglobin, hemopexin, lactate dehydrogenase (LDH), methemalbumin, urine hemosiderin, fecal and urine urobilinogen, blood in urine, Hb variant analysis by High-performance liquid chromatography (HPLC), Hb electrophoresis, Osmotic fragility test, expired CO, G6PD enzyme assay, etc.,
10. Serum Iron, Vitamin B12, Folic acid studies, etc.,
11. Bone marrow examination (depending upon results of other laboratory tests and patient clinical data)

Approach to Microcytic Anemia

Pathogenic classification of Microcytic Anemia [(1)]

1. Disorders of Iron metabolism.
2. Iron deficiency anemia.
3. Anemia of inflammation/ anemia of chronic disease.
4. Disorders of globin synthesis.
5. Alpha and beta thalassemias.
6. Hemoglobin E syndromes.
7. Hemoglobin C syndromes.
8. Sideroblastic anemias.
9. Hereditary sideroblastic anemia.
10. Acquired sideroblastic anemia.
11. Refractory anemia with ringed sideroblasts.
12. Malignancies.
13. Myeloproliferative disorders.
14. Lead intoxication (usually normocytic).

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It is essential to keep in mind that although the sensitivity of MCV is high, IDA can also present with normocytic anemia up to 40% of the cases [(31)]. IDA, the most common cause of microcytic anemia and is a nutritional problem worldwide [(27)]. IDA is anemia when the Iron is deficient in Iron studies and usually occurs in 2–5% of men and postmenopausal women. It is essential to diagnose the cause of anemia, as patients with IDA deserve urgent investigation; since 8–15% of these patients will be diagnosed with gastrointestinal cancer [(32)].

Effects of Iron deficiency on the body [(33)]

Iron is an essential component of hemoglobin in RBCs and of myoglobin in muscles, which are the major Iron-containing proteins, contains around 60% of total body Iron. It is also necessary for various cellular mechanisms, including enzyme activities, DNA synthesis, and mitochondrial energy generation [(8)].

Iron-containing proteins – In ID, hemosiderin and ferritin virtually disappear from marrow and other storage sites. Further, Hb synthesis in the marrow decreases, resulting in anemia, first due to fewer erythroblasts, but eventually resulting in the synthesis of Hb-deficient erythrocytes. Cytochromes and other mitochondrial ferroproteins are depleted but selectively so. The synthesis of many ferroproteins is regulated in an Iron-dependent manner, mainly via the Iron-responsive element (IRE)/Iron-regulatory protein (IRP) system.

Muscular function and exercise tolerance – Due to decreased oxygen delivery, performance in high-intensity exercise decreases due to ID and worsens with increasing. There is also reduced spontaneous activity, decreased ventilatory threshold, reduced endurance, and increased muscle fatigue.

Neurologic Changes – ID is associated with developmental abnormalities in children and with restless leg syndrome in adults. The substantia nigra is a

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particularly Iron-rich region of the brain and contains dopaminergic neurons, suspected of involvement in restless leg syndrome.

Host Defense and Inflammation – ID reported impairing various immune functions. Evidence for a narrowly protective and proinflammatory effect of Iron deficiency has been established. It has been studied in malaria; ID decreases the risk and severity of malaria.

Growth and Metabolism – ID in children suffer from growth retardation. Decreased thermoregulation in response to cold exposure is also seen.

Histologic Findings – The rapidly proliferating cells of the upper part of the alimentary tract seem to be particularly susceptible to the effect of ID Atrophy of the mucosa of the tongue and esophagus, stomach, and small intestine is also seen in ID Reduced thickness of the epithelium of the lateral margins of the tongue is seen in ID and is attributed to accelerated exfoliation of cells. Buccal mucosa has shown thinning and keratinization of epithelium and increased mitotic activity [(33)].

Risk factors for Iron deficiency and Iron deficiency anemia [(8,31)].

1. Physiological and pathological conditions can promote IDA. The maximum absorption of Iron from the diet is less than the body's requirements for Iron, resulting in a risk of Iron deficiency.
2. In infants and young children (Age: 0–15 years), rapid growth consumes the Iron stores that accumulate during gestation, leading to an absolute deficiency. After childhood, adolescent girls are particularly at risk of Iron deficiency anemia because of menstrual Iron losses. During pregnancy, Iron needs are tripled because of the expansion of maternal red cell mass and growth of the fetus and placenta. Daily Iron supplementation is significantly associated with reduced risk of anemia at term.

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3. Mothers who breastfeed are less likely to be Iron deficient than pregnant women because the Iron concentration in mature breastmilk is only 0.20–0.80 mg/L, and most breast-feeders are amenorrhoeic.
4. Regular blood donors are at increased risk of Iron deficiency.
5. Concerning diet, ferritin concentrations do not seem to differ between omnivores and vegetarians. Some components of the diet directly affect Iron bioavailability. Phytates (found in cereals and vegetables), polyphenols (found in vegetables, fruits, some grains and legumes, tea, coffee, and wine), calcium, and proteins inhibit Iron absorption. By contrast, ascorbic acid and muscle tissue enhance absorption.
6. Various abnormalities can lead to IDA, including blood loss, malabsorption, chronic disease (so-called IDA associated with ACD), and genetic alterations. Blood loss is the most common, especially from the digestive tract. The common causes of blood loss are colonic carcinoma, gastric carcinoma, benign gastric ulceration, and angiodysplasia. In developing countries, gastrointestinal parasites such as Trichuris Trichiura (whipworm) and Necator Americanus (hookworm) account for about a third of IDA.
7. Gynecological loss is the next most frequent cause.
8. Excessive surgical blood loss without replacement can also lead to IDA. Other blood losses, such as hematuria, epistaxis, or hemoptysis, occur much less frequently. The most common causes of Iron malabsorption are coeliac disease, gastrectomy, gastric bypass surgery, and Helicobacter pylori colonization.
9. Uncommon causes of malabsorption are substantial gut resection, atrophic gastritis, and bacterial overgrowth, each of which causes < 1% of IDA.

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10. Pica is a compulsive disorder characterized by an appetite for substances with no significant nutritional values (such as paper, clay, soil, glass, or sand), leading to Iron malabsorption. A craving for ice, pagophagia, has similar effects.
11. Many drugs are associated with IDA, either by increasing blood loss (e.g., non-steroidal anti-inflammatory drugs) or decreasing Iron absorption (e.g., proton-pump inhibitors and H2 receptor antagonists). IDA is frequently reported in chronic disorders, including inflammatory bowel diseases (IBD), chronic heart failure, chronic kidney disease (CKD), cancer, rheumatoid arthritis, and obesity.
12. Anemias caused by genetic defects are a large group of rare, heterogeneous disorders. The European Network of Rare Congenital Anemias lists 62 rare anemia subtypes, including hemolytic anemias and anemias arising from mutations in genes that control duodenal Iron absorption (e.g., SLC11A2), systemic Iron homeostasis (e.g., TMPRSS6), or erythroid Iron absorption and utilization.
13. Iron refractory IDA is a microcytic anemia that affects < one per million people. It is caused by a defect in the TMPRSS6 gene encoding matriptase-2, which has a vital role in the downregulation of hepcidin. Hepcidin concentrations are increased in disorders leading to ID. Oral Iron is ineffective. Correction of Iron refractory IDA by parenteral Iron is partial and much slower than that in patients with acquired Iron deficiency.

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Physiological conditions and pathological disorders associated with Iron deficiency anemia

Physiological [8]

- Infancy
- Adolescence in girls
- Pregnancy
- Regular blood donation
- Being an elite athlete

Pathological

Blood loss [8,33]

- Digestive tract: ulcers, hiatus hernia, varices, gastritis, colonic carcinoma, gastric carcinoma, Inflammatory bowel diseases, angiodysplasia, hemangioma, leiomyoma (Menetrier disease), mucosal hypertrophy, hypergastrinoma, antral vascular ectasia, meckel's diverticulum, polyps, hemorrhoids, telangiectasia, parasites, etc.,
- Biliary Tract: Intrahepatic bleeding, carcinomas, cholelithiasis, trauma, ruptured aneurysm, aberrant pancreas.
- Gynecological loss- menorrhagia, uterine fibroids, endometriosis, carcinoma, etc.,
- Surgery
- Hematuria, epistaxis, hemoptysis, Idiopathic pulmonary hemosiderosis
- Hemodialysis
- Non-steroidal anti-inflammatory drugs, aspirin

Malabsorption [8]

- Coeliac disease

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- Gastrectomy
- Helicobacter pylori
- Gut resection, atrophic gastritis, bypass gastric surgery, bacterial overgrowth
- Interaction with food elements: tea, coffee, calcium, flavonoids, oxalates, phytates
- Pica syndrome, pagophagia
- Antacids, Proton-pump inhibitors, and H2 antagonists

Iron deficiency anemia associated with anemia of chronic disease (8)

- Chronic heart failure
- Cancer
- Chronic kidney disease
- Rheumatoid arthritis
- Obesity
- Inflammatory bowel diseases

Genetic disorders (8)

- Iron-refractory Iron deficiency anemia
- Others (divalent metal transporter 1 deficiency anemia, Fanconi anemia, pyruvate kinase deficiency, etc.)

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Iron Kinetics

Iron is absorbed in the duodenum [1,2]. Dietary Iron is available in two forms: heme and non-heme Iron. In heme form, Iron is complexed as Fe^{2+} (ferrous Iron) in Hb, which is present in animal food sources such as meat, poultry, and seafood. Non-heme Iron (Fe^{3+} or ferric Iron) is present in the vegetarian diet (black tea, cacao, cereals, dried fruit, etc.,) [1,17]. Heme Iron is estimated to contribute 10–15% of total Iron intake in meat-eating populations, and it is generally better absorbed—with a rate of absorption is about 15–35%—than non-heme Iron, it can account for more than 40% of total absorbed Iron [8]. Heme protein of animal origin, when exposed to acid and proteases in the stomach (gastric juices), free the heme from its apoprotein, and then heme is taken up by mucosal cells [1,2]. Non-heme Iron is mainly in ferric hydroxide form or loosely bound to organic molecules such as phytates, oxalates, sugars, citrate, lactate, and amino acids. Acidic gastric Ph is essential for the solubility of these inorganic Iron. Ascorbate, animal tissues, keto sugars, organic acids, and amino acids enhance inorganic Iron absorption, whereas phytates, polyphenols, and calcium inhibit it. Depending on various combinations of enhancing and inhibitory factors, dietary Iron assimilation can vary as much as 10-fold [1].

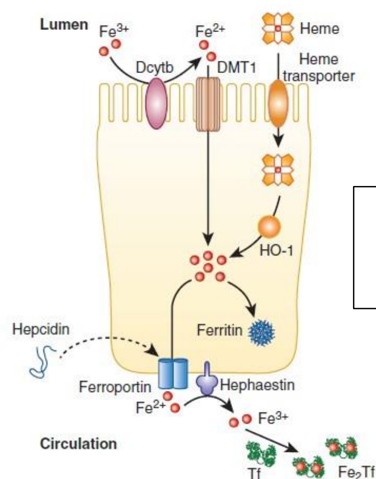

Figure 1: Absorption of Iron in small intestine [2]

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Several regulatory proteins must act and interact in a coordinated fashion to maintain Iron stores. To start with, Fe^{3+} should be converted into Fe^{2+} by the ferrireductase called Duodenal cytochrome b (Dcytb) to absorb dietary Iron; it is then transported via the divalent metal transporter 1 (DMT1, SLC11A2)- protein has 12 predicted transmembrane segments, present on the apical surface of absorptive enterocytes and it mediates intestinal absorption of Iron. Iron can also be absorbed as heme by an enterocyte membrane heme transporter, and Iron is removed from heme by heme oxygenase-1 (HO-1) [(1,2,17)].

In the synthesis of heme, Iron enters the pathway after binding transferrin to transferrin receptor 1 (TfR 1), then the internalization of this complex happens via clathrin-mediated endocytosis. After that, these endocytic vesicles will get acidified with the influx of H^{+} ions, following which the Iron is released from transferrin. DMT-1 will get reduced by STEAP-3 oxidoreductase so that the Iron is exported from the vesicle. These cytoplasmic Iron can either be stored by binding to ferritin as ferritin-bound Iron or can be transported into the mitochondria via the mitochondrial Iron importer, mitoferritin (MFR1), and later it is used in the synthesis of heme and FeS clusters. The heme responsive gene 1 protein (HRG-1)/ SLC48A1, plays a crucial role in heme-Iron recycling in macrophages [(1,2,17)].

A transport protein called Ferroportin (SLC40A1) is the only known protein that is specific that exports Iron across the cell membranes. It plays a vital role in the movement of Iron- from the enterocytes into the circulation and the release of Iron from macrophages. A multicopper oxidase, which is cell membrane-bound, oxidizes Fe^{2+} to Fe^{3+} form and then binds to transferrin protein, the primary carrier of Iron in plasma [(1,2,8,17)].

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The protein Hepcidin plays a crucial role in Iron homeostasis. It is a small peptide containing 25 amino acids [34]. Hepatocytes mainly secrete it. It was first described in 2001 in mice with Iron overload, has a vital role in the control of Iron availability to tissues. Other than the liver, macrophages, adipocytes, the heart, and the kidneys, can produce hepcidin. In plasma, hepcidin is bound to α2-macroglobulin and albumin and is cleared via the kidney [8]. Other proteins which are influenced by and involved are transferrin receptor 1 (TfR1), the hemochromatosis receptor (H.F.E.), TfR2, hemojuvelin (H.J.V.), bone morphogenic protein (B.M.P.) and its receptor (BMPR), sons of mothers against decapentaplegic (SMAD), and matriptase-2 [2].

The primary role of hepcidin is to control the surface expression of Ferroportin [1,2,34]. By binding to the protein, it is then internalized and degraded by lysosomes. So after its degradation, enterocytes, macrophages, and hepatocytes can no longer export Iron, which is sequestrated/stored in these cells. Hence high hepcidin expression will lead to decreased plasma Iron concentrations, and low levels will increase plasma Iron concentrations. Its expression is controlled by various factors, upregulated by high concentrations of Iron in the liver and plasma, inflammation, and physical activity. In contrast, it is downregulated by Iron deficiency, erythropoiesis, hypoxia, and endocrine signals (testosterone, estrogen, and growth factors) [8].

There is a new regulatory hormone called erythroferrone, which was identified in 2014. It is produced by RBCs, mainly in polychromatophilic normoblasts in response to anemia and elevated erythropoietin, and it mediates suppression of hepcidin, and in turn, helps in increased Iron absorption during stress erythropoiesis [2,8].

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Iron exported from enterocytes into the blood is Fe^{2+}, and it should be converted into Fe^{3+}. Fe^{3+} is the transport form, and this conversion is aided by Hephaestin, which oxidizes Fe^{2+} to Fe^{3+}. This Hephaestin is present on the basolaminal enterocyte membrane, and later Iron is carried by a specific protein called Apotransferrin. When Iron binds, it is known as Transferrin. Since Apotransferrin binds to two molecules of Fe^{3+} when it is fully loaded, it is often referred to as diferric transferrin or holotransferrin (1,2).

Iron can also be stored in the cytoplasm. Fe^{3+} Iron is stored in a cage-like protein called Apoferritin. Once Iron binds, it is known as ferritin, which provides a reserve of Iron. Ferritin is chiefly an intracellular Iron storage protein. It has been established that mitochondria contain ferritin as well, but trace amounts also are secreted into plasma (1,17). It is made of 24 H and L subunits (21 kDa and 19.7kDa respectively) and is spherically structured. This spherical structure has a central core and can store> 4000 Iron atoms (2,17,34). The ferroxidase activity of H-ferritin is crucial for Iron uptake by ferritin, while L-ferritin promotes Iron core formation inside the ferritin shell (17).

Partially degraded ferritin is known as Hemosiderin, which is water-insoluble, crystalline, protein-Iron complex, and is considered to be less metabolically available than ferritin. To regulate the amount of Iron inside the cell and avoid free radicals, cells can control the amount of TfR1 on their surface (2,34).

Iron recycling

The largest percentage of recycled Iron comes from red blood cells. Macrophages ingest senescent RBCs in the spleen. The hemoglobin will be degraded, and the macrophages hold iron as ferritin. Haptoglobin and hemopexin are plasma proteins that salvage plasma hemoglobin or heme, respectively, which are freed during

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fragmentation hemolysis. Macrophages carry a receptor for the haptoglobin-hemoglobin complex, whereas hepatocytes have a hemopexin-heme receptor, making them both essential cells in Iron salvage. Hepatocytes also possess ferroportin so that the salvaged Iron can be exported to transferrin and ultimately to other body cells [1,2].

Total body Iron

Protein	Function	Iron-Oxidation state	Amount (g)	%
Hb	RBC O2 transport	2	2.6	65.0
Myoglobin	Muscle O2 storage	2	0.130	6.0
Transferrin	Plasma Fe transport	3	0.003	0.1
Ferritin	Intracellular Fe storage	3	1.000	25.0
Others (Enzymes)			0.150	3.9
Catalase, Peroxidase	H2O2 degradation	2		
Cytochromes	Electron transport	2/3		
Aconitase	Tricarboxylix acid cycle	4Fe-4S cluster		
Ferrochelatase	Heme biosynthesis	2Fe-2S clustr		
HIF prolyl hydroxylases	Oxygen sensing	2		
Ribonucleotide reductase	DNA synthesis	3		

Table 2: Important Iron containing compounds in humans [1]

Full-term infants have approximately 75-80 mg/kg body weight of Iron acquired primarily from their mothers during the third trimester. This body Iron is dependent on blood volume, Hb concentration, birth weight, and delay in the clamping of the cord, which increases red cell mass by placental transfusion. This body Iron will

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be depleted in the first few months, and most young children will have sparse Iron stores throughout [1,34].

The body Iron content of normal adult men is about 50 mg/kg body weight or more. When compared with women who have body Iron averaging at 35 mg/kg, especially in post-pubertal women have increased losses of Iron due to menstruation, pregnancy, and childbirth. After menopause, women accumulate Iron linearly in parallel with adult men so that body Iron levels will be more or less similar to that of men [1].

Iron Balance

Iron is not actively excreted from the body [1,2,8]; it is eliminated by shedding or loss of epithelial cells from the gastrointestinal tract, epidermal cells of the skin, and in menstruating women, RBCs. The average loss of Iron has been estimated to be around 1- 2 mg/day in normal adult men and non-menstruating women [1,34]. Iron is also a component of sweat; a tiny amount of Iron of about 22.5 μg/L is lost via this route. Urinary Iron excretion is estimated to <0.05 mg/d of the loss and is largely due to sloughed cells. Menstruating women lose approximately 30ml/day which is around 118ml/month [34] an additional on an average of 0.006 mg/kg/day to > 0.025 mg/kg/day is lost even though it is highly variable [1].

Usually, these Iron losses are balanced naturally as the equivalent amount is absorbed from the diet, around 1-2mg/day. However, only a fraction of dietary Iron is absorbed. So, the bioavailability of Iron in industrialized and developed countries is estimated at 14% to 18%, although less may be absorbed if Iron stores are adequate. The bioavailability of Iron in a purely vegetarian diet is far lower, ranging from 5% to 12% compared to non-vegetarian and mixed diets. Absorption of Iron from the diet can increase up to 3 times, which is around 3-5 mg/d if Iron stores are

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decreased. Thus, Iron balance is primarily, if not exclusively, achieved by control of absorption [(1)].

The state of Iron overload, which causes a decrease in the utilization of Iron efficiently. It produces hydroxyl radicals, which is the most toxic type of active oxygen species formed via the Fenton and Haber–Weiss reactions. This is implicated as one of the causes in the formation of arteriosclerosis, carcinogenesis, hepatopathy, and diabetes. Therefore, it is essential to maintain body Iron at a proper level, as a specific excretion pathway for Iron is not present, any inappropriate administration of Iron supplements must be avoided; hence the real cause of anemia should be identified via thorough investigations before treatment, and thus empirical treatment is avoided [(35)].

Iron Deficiency and Anemia

Historical aspect

Since medieval times Iron deficiency is recognized. In the 15th century *Chlorosis*, which is derived from the Greek word meaning 'green, was used by Varandaeus to a disorder which was later described as "De morbo virgineo" in 1554. Later this disease became well known as "green-sickness" which is due to a greenish pallor, which occurred almost exclusively in teenaged girls. This condition was also depicted in many paintings by the Dutch masters and described even by Shakespeare in his works of literature. Other clinical features found in this green sickness were breathlessness, palpitations, slight ankle edema, gastrointestinal symptoms, emotional disturbances, depression, irritability, and moodiness were common finding then. It became especially common at the end of the 19th century, and then the incidence decreased [(1)].

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In the present day, most people believe that chlorosis term, which was described back then, could be resulted from a combination of various factors affecting adolescent girls; the demands of growth and the onset of menses, an inadequate diet, and low Iron stores from very early childhood. In the 1830s, the anemia with hypochromia and lack of Iron in the blood or Iron deficiency in the body were linked to this disorder. In 1832, Pierre Blaud had described the response of chlorosis with his famous pills - ferrous sulfate plus potassium carbonate. Many observers, including Niemeyer and Osler, confirmed his findings. Even today Ferrous sulfate remains a cornerstone of the modern treatment of ID [(1)].

In the late 1920s and early 1930s, distinct hypochromic anemia was described. Just like chlorosis, this chronic hypochromic anemia chiefly affected the women population. It differed from chlorosis in a way it was detected later in life, especially in the 4th/5th decades of life. Other clinical features found were changes involving the tongue and nails and achlorhydria. It was found that this anemia was mostly affected in women with poor dietary habits, multiple pregnancies, or menstrual irregularities. ID is the most common condition associated with microcytic hypochromic anemia worldwide, even though other causes should be ruled out before the initiation of treatment [(1)].

Iron Pathway Disorders

The principal source of Iron for Hb production is transferrin. Typically, transferrin can supply all the Iron required for normal or accelerated production rates. However, in IDA, the storage sites or forms of Iron in macrophages are depleted. The plasma Iron concentration falls when transferrin saturation with Iron falls to < 16%, the red cell production rate decreases, and hypochromic, microcytic cells are manufactured. This state is known as Iron-deficient erythropoiesis. When

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compared to Anemia of inflammation (AI)/ACD, the macrophage Iron level will be normal or may be increased, but the export of Iron from macrophages is downregulated. Thus, marrow is deprived of adequate Iron supplies due to the accumulation of Iron in the macrophages and hence fall in plasma Iron levels. Together, IDA and AI are among the most common causes of anemia [(1)].

ID is the common cause of anemia in pregnant women. Menstruating women continue to be the majority of the population who are prone to develop ID, along with young children whose growth outstrips their Iron supply. On the other hand, AI is more common among older adults, but it also can occur in younger individuals affected by certain chronic inflammatory states [(1,36)].

Anemia, hypochromia, and microcytosis are typically more pronounced in ID; the degree of anisocytosis and poikilocytosis is comparatively more in ID AI is usually normocytic. However, when ID is early and mild, the morphologic findings in the two conditions may be similar, so it will be difficult to distinguish. History examination and investigations play a crucial role [(1)].

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ID is typically defined using three progressive stages – Table 3 [1,2,17].

Laboratory Investigations	Normal Iron status	Stage 1 (Storage Iron Depletion)	Stage 2 (Transport Iron Depletion)	Stage 3 (Functional Iron Depletion)
Hb	N	N	N	↓
S Iron	N	N	↓	↓
TIBC	N	N	↑	↑
S Ferritin	N	↓	↓	↓
Soluble transferrin receptor (sTfR)	N	↑	↑	↑
Hb content of reticulocytes (RetHe)	N	N	↓	↓
Condition		Latent Iron Deficiency	Latent Iron Deficiency	Iron Deficiency Anemia

Table 3: Stages of Iron deficiency [1,2,17].

(↑ - Increased, ↓ - Decreased, N- Normal levels)

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Investigations

Peripheral smear

Peripheral smear confirms the electronically determined values and classification of RBC size. It also allows for recognizing many variations in RBC size and shape-anisopoikilocytosis seen in various conditions. Normal red cells are approximately the size of the nucleus of a small lymphocyte, and the area of central pallor is 1/3rd to ½ of the diameter of the red cell [(1)].

In IDA, RBC morphology gives the most information. Erythrocytes reveal various stages of anisocytosis, poikilocytosis, microcytosis, and an increase in the area of the central pallor of erythrocytes on the blood smear is indicative of hypochromia. When the change is pronounced, little more than a faint ring of color in the periphery- leptocytes and may be apparent with occasional target cells, usually WBCs are not affected, and platelet count may be raised in cases of IDA [(1,17)]. Hypochromia and microcytosis almost always occur together [(1,2,33)].

Abnormal RBC morphologies also include pencil cells, target cells, elliptocytes, and prekeratocytes, which have been described in cases of Iron deficiency. Pencil cells are hypochromic elliptocytes, in which the long axis is at least 3 times the length of the short axis. At the same time, prekeratocytes display both - central pallor and one or > sub-membrane vacuoles. But these findings are not exclusive to IDA alone and can be seen other conditions such as in β- thalassemia trait and the ACD [(17,33)].

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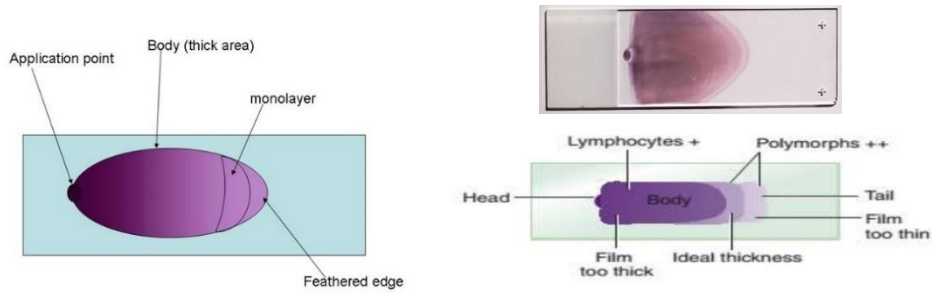

Figure 2 A – Zones of blood smear, 2B and C- Distribution of cells in peripheral smear

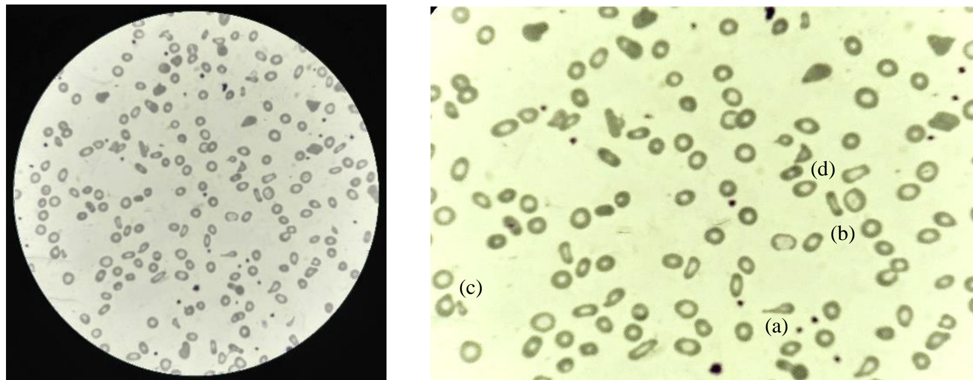

Figure 3A: Low power view of Peripheral smear in microcytic hypochromic anemia

Figure 3B: High power view of peripheral smear in microcytic hypochromic anemia showing anisopoikilocytosis with (a)- Tear drop cell, (b)- Pencil shaped cell, (c)- Microcytic and hypochromic cells, (d)- Schistocyte

The automated analysis of the blood has made the erythrocyte indices more accurate and reproducible. However, the evaluation of the blood smear remains essential because it may reveal abnormal cell populations too small to affect the erythrocyte indices [1].

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Modern Hematology analyzers and Hematological parameters

The essential components of the modern analyzers include hydraulics, pneumatics, and electrical systems. The hydraulics system includes an aspirating unit, dispensers, diluters, mixing chambers, aperture baths or flow cells or both, and a hemoglobinometer. The pneumatics system generates the vacuums and pressures required for operating the valves and moving the sample through the hydraulics system. The electrical system controls operational sequences of the total system and includes electronic analyzers and computing circuitry for processing the data generated. A data display unit receives information from the analyzer and prints results, histograms, or cytograms [(2)].

In Sysmex XN-1000 automated hematology analyzer (Sysmex Corporation, Kobe, Japan), which gives C.B.C. with six-part differential: neutrophils, lymphocytes, monocytes, eosinophils, basophils, and immature granulocytes. It also gives fully automated reticulocyte count, including nRBCs, Ret-He, and IRF Fluorescent flow cytometry is used for the WBC count, WBC differential, and to detect nRBCs.

The WBC, RBC, platelet counts, hemoglobin, and Hct. are measured directly. Three hydraulic subsystems are used for determining the hemogram: the WBC channel, the RBC/platelet channel, and a separate hemoglobin channel [(2)].

In the RBC/platelet channel, a sheathed stream with hydrodynamic focusing is used to direct cells through the aperture, which reduces the co-incident passage, particle volume distortion, and recirculation of blood cells around the aperture; and in the WBC and RBC/platelet channels, floating thresholds are used to discriminate each cell population. As cells pass through the aperture, signals are transmitted in sequence to the analog circuit and particle volume distribution analysis circuits for conversion to cumulative cell volume distribution data. The lower platelet

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threshold is automatically adjusted in the 2 to 6fL volume range, and the upper threshold is adjusted in the 12 to 30fL range, based on particle volume distribution. Likewise, the RBC lower and upper thresholds may be set in the 25 to 75 fL and 200 to 250fL volume ranges. This floating threshold circuitry allows for discrimination of cell populations on a specimen-by-specimen basis. Cell counts are based on pulses between the lower and upper auto-discriminator levels, with dilution ratio, volume counted, and coincident passage error accounted for in the final computer-generated numbers. In the RBC channel, the floating discriminator is particularly useful in separating platelets from small RBCs. Subpopulations of red cells can be measured using the upper area and lower areas of the RBC histogram to identify macrocytic cells (MacroR) and microcytic cells (MicroR) [2].

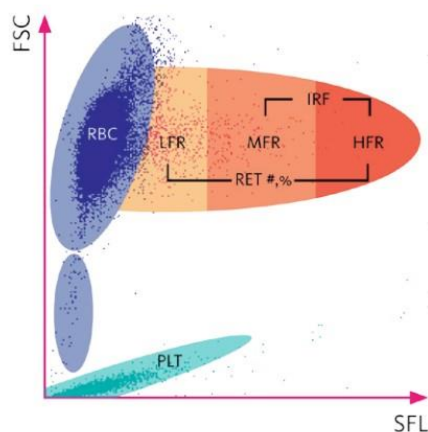

Figure 4A: Diagrammatic representation of scatter gram showing forward and side scatter in reticulocyte channel [37]

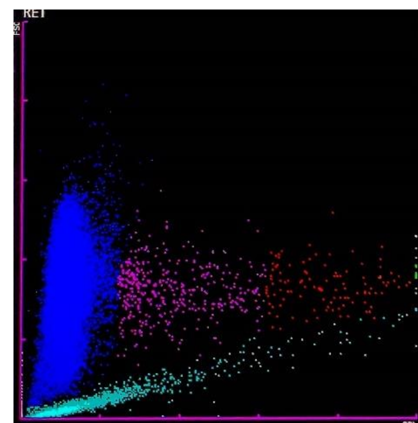

Figure 4B: Scatter gram showing forward and side scatter in reticulocyte channel obtained from Sysmex XN-1000

To accurately measure reticulocyte counts, automated counters use a combination of laser excitation, detectors, and a fluorescence marker that labels RNA and DNA (such as thiazole orange or polymethines). The sample is incubated with an RNA-binding fluorescence marker and counted by flow cytometry. Automated

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reticulocyte counters use objective thresholds for the classification of cells. This ensures a high level of reproducibility of the results. In automated counts, the measurement signals of up to 30,000 red blood cells are evaluated. This results in both high count rates and a high degree of precision [(37)].

In the Reticulocyte channel, fluorescence flow cytometry in conjunction with a nucleic acid staining dye to measure the amount of hemoglobin obtained within the reticulocytes. Sysmex X.N. series analyzer uses a Laser beam of wavelength = 633nm for analysis [(37,38,39)]. In the reticulocyte scattergram, forward scatter a measure of individual cell size, on the y-axis is plotted against fluorescence intensity, a measure of RNA content, on the x-axis [(4,16)]. The average value of the forward-scattered light intensity of reticulocytes is expressed as a log transformation of Ret-Y. The results are presented as picograms (pg) of Hb per reticulocyte. This obtained reticulocyte hemoglobin content is termed the Ret-He parameter [(16,38,40,41)]. Flow cytometry assesses the maturity of reticulocytes, separating them into 3 areas according to the degree of fluorescence, i.e. low-fluorescence reticulocytes (LFR), middle- fluorescence reticulocytes (MFR) & high-fluorescence reticulocytes (HFR); LFR being the most mature one and HFR having more RNA that corresponds to the earliest of reticulocytes and IRF is MFR + HFR [(2,42,43,44)].

The Hematocrit/Packed cell volume (HCT/PCV) also is determined from the RBC/platelet channel, based on the principle that the pulse height generated by the RBC is proportional to cell volume. The H.C.T. is the RBC cumulative pulse height and is considered a true relative percentage volume of erythrocytes [(2)].

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In the hemoglobin flow cell, hemoglobin is oxidized and binds to sodium lauryl sulfate (SLS), forming a stable SLS–hemoglobin complex, which is measured photometrically at 555 nm [(2)].

In the W.D.F. channel, RBCs are lysed, WBC membranes are perforated, and the DNA and RNA in the WBCs are stained with a fluorescent dye. Plotting side scatter on the x-axis and side fluorescent light on the y-axis enables separation and enumeration of neutrophils, eosinophils, lymphocytes, monocytes, and immature granulocytes. In the W.N.R. channel, the RBCs are lysed, including nucleated RBCs, and WBC membranes are perforated. A fluorescent polymethine dye stains the nucleus and organelles of the WBCs with high fluorescence intensity and stains the released nuclei of the nucleated RBCs with low intensity. Plotting side fluorescent light on the x-axis and forward scatter on the y-axis enables separation and enumeration of the total WBC count, basophils, and nucleated RBCs. The WBC count is automatically corrected when nucleated RBCs are present in the specimen. A WPC channel similarly detects blasts and abnormal lymphocytes using a lysing agent and fluorescent dye and plotting side scatter on the x-axis and side fluorescent light on the y-axis [(2)].

The platelet analysis on this analyzer also uses a fluorescent count (using fluorocell fluorescent dye-oxazine, with an extended platelet counting volume and time) in addition to the impedance count and optical count, called the PLT-F, performed by optical measurement. The platelets can be differentiated from other cells based on differences in intensity of the fluorescence combined with forward scattered light [(2)].

The parameters which are calculated are MCV, MCH, MCHC, RDW-SD, RDW-CV, MPV, and plateletcrit. The hemoglobin content of reticulocytes (Reticulocyte

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hemoglobin equivalent Ret-He), which is analogous to the MCH for reticulocytes, can be calculated [2].

Mean corpuscular hemoglobin (MCH) is the average weight of hemoglobin per cell. It is across the entire RBC population, in which some are 1-2 days old, while others may be 10/20/30days or even older. If ID develops, MCH does not change much to an extent or as accepted until the substantial population of RBCs produced are Iron deficient. So, the diagnosis is effectively delayed for weeks or months after Iron restricted erythropoiesis begins [2]. Hb is a late marker of ID Low Hb is considered anemia according to WHO guidelines when age and other factors are considered [6,17].

ID patients usually display normal levels of Hb, but when RBC indices are studied, they may show lower than typical values of MCV, MCH, and RDW might be increased. In cases of IDA, RDW is elevated noticeably. Increased RDW suggests anisocytosis and is seen with Iron-deficient erythropoiesis. There is a definitive relationship between MCV/MCH and the availability of Iron in the marrow for erythropoiesis. It is decreased when there is a decrease in Iron supply and increased when there is an increase in Iron supply for marrow erythropoiesis [17].

MCH is the best indicator to detect the amount of hemoglobin synthesized during erythroid differentiation. It is not impacted by factors such as temperature or

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storage time, or by the limitation of technology in the estimation of MCV and MCHC. Hence in ID, MCH displays increased heterogeneity (17).

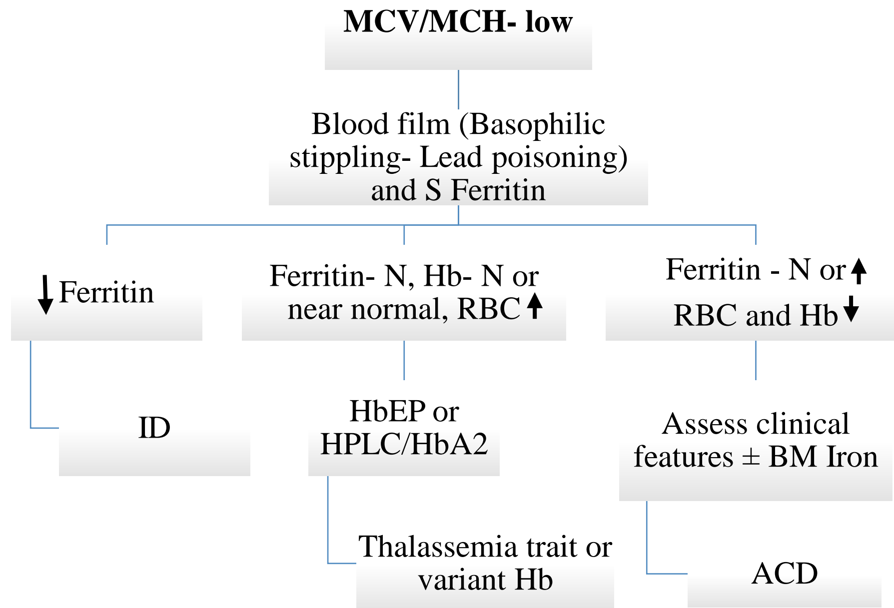

Figure 5: Approach to MCHC anemia (45)

(N- Normal, ↓- Decreased, ↑- Increased, HbEP- Hemoglobin electrophoresis, HPLC- High performance liquid chromatography)

The MCHC is useful in detecting severe hypochromia, but it is rarely abnormal when the MCV is normal. A reduced value for MCHC is observed most often in association with ID and is the last index to fall as Iron deficiency worsens. In the past, because of plasma trapping, centrifugal Hct methods overestimate the volume of packed red cells and, therefore, underestimate MCHC (1).

Other research parameters obtained in the Sysmex X.N. series are - % Hypochromic red blood cells (%HYPO): an increased %HYPO can be an early indicator of ID (17). Hypo-He : Hypochromic cells are erythrocytes with Hb content <17 pg (46). Micro/hypo RBC ratio- can help distinguish ID from thalassemias, the

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ratio is usually more than 1.0 in thalassemia where microcytosis prevails, and the ratio is less than 1.0 in ID, where hypochromia is more pronounced compared to thalassemia. These parameters are limited to research purposes and need further studies to validate their utilities [17].

Reticulocyte count and reticulocyte hemoglobin content/ Reticulocyte hemoglobin equivalent (CHr or RetHb)

The youngest erythrocyte released from bone marrow to the peripheral blood is Reticulocytes [47]. Each day approximately 1% of the RBC pool is replaced by the new young erythrocytes released from bone marrow [1]. They mature in 1 to 3 days within the bone marrow and circulate for 1 to 2 days before maturing to erythrocytes [7,40,43,47].

Due to normal homeostatic mechanisms, the body recovers from anemia by accelerating erythropoiesis, and in the marrow, this is brought about through the release of Erythropoietin (EPO). At maximum stimulation, the bone marrow can produce erythrocytes at 6-8 times the standard rate [1].

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Absolute reticulocyte counts and measuring Hb content in reticulocytes that is Ret-He - which is analogous to the MCH (2,38), is one of the potential newer markers obtained from modern hematology analyzers offers to provide a real-time picture of the bone marrow regarding its functional status (17). Its measurement is not only rapid but also convenient and cost-effective (16).

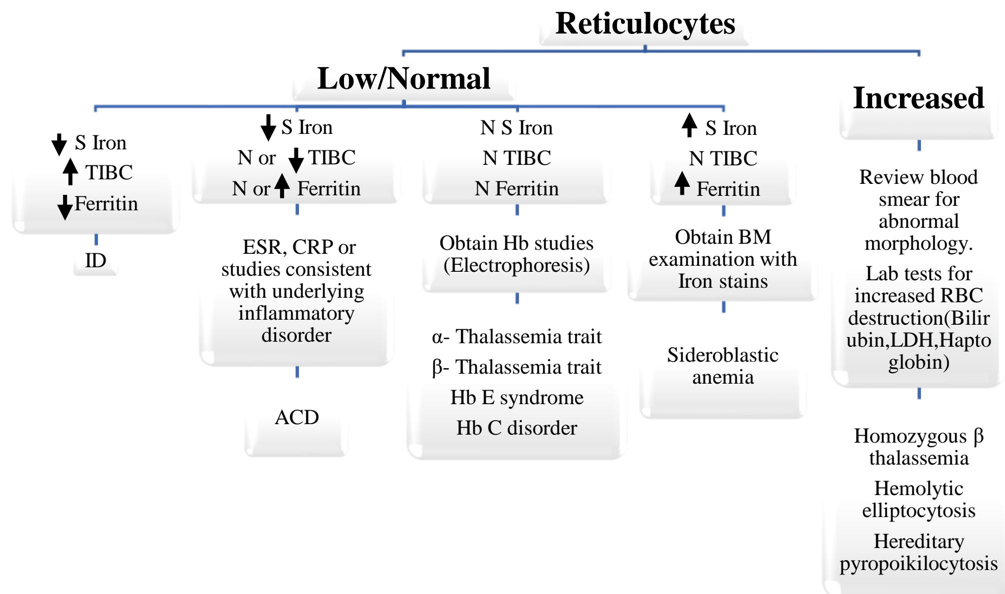

Figure 6: Diagnostic approach to a patient with microcytic anemia by using reticulocyte count (1).

(N- Normal, ↓ - Decreased, ↑- Increased)

The reticulocyte count provides an initial assessment of whether the cause of anemia is due to impaired RBC production or increased loss in the peripheral circulation (1). Previously, the reticulocyte count was done via microscopic examination of a smear prepared from fresh blood stained with a supravital stain, such as new methylene blue. The normal reticulocyte count by light microscopy is 0.5% - 1.5% of the total red cells (1,40). Automated methods count a larger number of cells, and exhibit a greater degree of reproducibility than manual methods (1).

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In the presence of anemia, the reticulocyte count must be corrected because it is spuriously elevated when it is related to the reduced number of RBCs in an anemic patient [1]. In moderate anemia cases, erythrocyte generation is expected to increase 2–3 fold within ten days in healthy bone marrow [40]. Additional correction of this index needs to be made because reticulocytes released under intense EPO stimulation remain in the peripheral blood for more than the usual 1-day survival time of non-stress reticulocytes [1].

- **Reticulocyte count** = % reticulocytes in RBC population
- **Corrected reticulocyte count** = % reticulocytes × (patient Hct/normal Hct for the age)
- **Reticulocyte production index** = Corrected reticulocyte count ÷ maturation time in peripheral blood in days*

*(Reticulocyte maturation time = 1 day for Hct ≥ 40%; 1.5 days for Hct 30%-40%; 2.0 days for Hct 20%-30%; 2.5 days for Hct <20%.)

In IDA, reticulocyte count is usually low, as the Iron availability is decreased, marrow cannot maintain the production to the extent required to compensate [31].

A reduced IRF is an early sign of red cell underproduction. An increase may be the first marker of response to therapy in patients receiving B12, Iron, or EPO engraftment in bone marrow or hematopoietic stem cell transplantation and bone marrow regeneration following chemotherapy; in patients of myelodysplastic syndrome and dyserythropoietic anemias, IRF is increased without reticulocytosis. In hereditary spherocytosis (HS), there is high reticulocyte count without equally elevated IRF [1,44].

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Ret-He can let you look at the current state of Iron supply in the marrow for erythropoiesis and can help to judge the 'quality' of the newly produced cells. It lets you detect changes in Iron status far earlier than through the hemoglobin content of mature red blood cells, and direct assessment of the Iron used for the biosynthesis of hemoglobin. It can indicate whether there is enough Iron available for erythropoiesis and provides information on the current bioavailability of Iron (1,7,10,16,37,48). It had shown stable results even when samples for Ret-He analyses were stored at room temperature, 4°C or 8°C, for up to 72 hours when compared to other erythrocyte parameters, which are unreliable after 6 hours due to osmotic swelling and other factors (35,49).

A low Ret-He (<28 pg) suggests there is a lack of Iron or lack of bioavailable Iron for erythropoiesis and hence ID (1,37). It predicts response to Iron replacement, and its increase is an early sign of response to Iron like the IRF parameter. A decreased Ret-He is a strong indicator of ineffective erythropoiesis, particularly in dialysis patients (1).

Measuring Ret-He enables detection of Iron restricted erythropoiesis within days, and it is a sensitive, reliable, specific, real-time, and early indicator of ID (2,49,50,51,52,53). Ret-He can be easily measured by merely adding the test item reticulocyte count to C.B.C. The reagents required for measuring Ret-He are cheaper, which is approximate 1/5th the amount required for measuring serum ferritin (35,52). It is an early predictor of Iron-deficient erythropoiesis compared to traditional parameters, which take approximately- MCV: 21 days, RDW: 30 days, and Hb: 60 days, respectively, to show the Iron deficiency changes (54). Currently, it is the recommended alternate laboratory screening test by the American Academy of Pediatrics (54,58). It acts as an indirect measurement of the functional

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availability of Iron for erythropoiesis in the marrow over the past 3 to 4 days [41,42,47].

It is often used together with ferritin; a high or normal ferritin value together with a low Ret-He value can suggest *functional Iron deficiency*, while low ferritin values together with a low Ret-He indicate a *classic Iron deficiency*. Since ferritin is falsely increased during the acute phase of diseases, the presence of inflammation should be checked, e.g., by CRP[37].

Ret-He from Sysmex Corporation (Kobe, Japan) correlates with CHr from Advia analyzers (Siemens Bayer Diagnostics, Tarrytown, NY, U.S.A.) and has the same clinical interpretation [42,46,56]; specifically, 29.0–30.0pg is the threshold for defining Iron-deficient erythropoiesis [46].

It can also be used for monitoring EPO and/ or IV Iron therapy. If the value increases, it indicates the treatment is having a positive effect [11,16,37]. It generally increases within 1-2 days of treatment [45,61]. When compared to ferritin, the first response occurs in 1-2 weeks [54].

A significant advantage over the ferritin or transferrin parameters is that Ret-He is not affected by the acute phase reaction and in the absence of hemoglobinopathy. A less than normal Ret-He is an indication of inadequate Iron supply relative to demand [37,52].

Serum Iron and Transferrin

Almost exclusively, Iron circulates in plasma as transferrin bound Iron [17]. Serum Iron levels measure the amount of Iron bound to transferrin [1]. It is calculated using spectrophotometric methods usually. It is measured by assessing the complex which Iron forms with a chromogen (ferrozine, bathophenanthroline, or ferene) in

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acidic pH. In acidic pH, there is a reduction of Fe^{3+} to Fe^{2+} and dissociation from transferrin. Samples collected in EDTA are unsuitable for Iron determination, and hence serum or heparinized plasma is used. Iron can be measured directly by atomic absorption, but this methodology is not widely used [(17)].

A limitation in the use of serum Iron determinations is the considerable diurnal variability in values [(1,16,33,35,40)]. It will be highest in the morning and the lowest in the evening [(17)]. Also, it can vary from 10% - 40% within a single day or from day to day in the individual [(1,17)]. Serum Iron may be transiently elevated after the ingestion of Iron-rich food or dietary supplements [(17,53)]. Hence its measurements are optimally made after 48hrs of fasting in the morning to avoid diurnal variability. Serum Iron also decreases in states of infection and inflammation [(17)]. The Iron regulatory protein hepcidin has a diurnal pattern too, and this provides a potential mechanism for the diurnal serum Iron variation [(1)].

Transferrin (Tf), which is the Iron transport protein, which is an 80kDa glycoprotein, is produced and secreted by the liver. The plasma half-life of 8-11 days [(34)]. It can bind Iron in two domains: N- and C-terminals of the molecule, so two Fe^{3+} molecules can bind to it. It can be measured directly with immunoassays. At any point in time, Tf contains only about 4mg of body Iron, but it is vital to transport Iron, with over 30mg Iron pass-through this compartment every day. A deficiency of Iron may raise its synthesis and its values in plasma, in cases of increased need for Iron (e.g., pregnancy), use of oral contraceptives, and decrease in cases of inflammation/infection. It is elevated in parenchymal liver damage [(17,34)].

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Total Iron-binding capacity (TIBC) is an indirect measurement of transferrin. It gives an idea to which Iron will bind to transferrin (1). It is determined either directly or indirectly by measuring both the unsaturated Iron-binding capacity (UIBC) and serum Iron. It is calculated from the immunologic assay of transferrin using a conversion factor that considers the molecular weight of transferrin and the binding of two Iron atoms per molecule of transferrin (1,17). In contrast to serum Iron, TIBC (or transferrin) values show less significant diurnal variation (1).

TIBC saturation is calculated with the following formula:

- **TIBC saturation (%)** = (serum Iron × 100)/ TIBC.

The normal value is 20% to 45%. Values of < 16% are noted in association with both ID and AI

The extent to which transferrin saturation decreases tends to be more in ID than in chronic disorders, but considerable overlap exists between these two conditions. In both children and adults, however, a value of < 5% is almost certainly due to IDA. In sideroblastic anemias and transfused thalassemia patients, the TIBC saturation invariably is increased and often approaches 100% (1,26).

The absolute value for TIBC may help distinguish between ID, and the ACD TIBC is increased in cases of ID and decreased in AI However, in hospitalized patients, elevated TIBC is an insensitive marker of Iron deficiency (1).

Serum Ferritin

Serum ferritin is either secreted from Iron storage sites or is freed after lysis of cells or cell death. Immunoassays measure it. These assays usually are not affected by

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mild hemolysis; if the sample is wholly hemolysed, it results in significantly increased levels. Serum ferritin has no circadian variation (17).

In most clinical circumstances, the serum ferritin concentration is proportional to total body Iron stores. In contrast to serum Iron measurements, ferritin values are not influenced by recent Iron therapy (1). Serum ferritin values in men tend to rise steadily with age (1,17). Iron regulatory protein (IRP) regulates ferritin production; which in turn, responds to a reduced cytoplasmic Iron pool. It binds to 5'-Iron responsive element in ferritin producing mRNAs to subdue its translation (17).

Even though ferritin is the best indicator of ID and low ferritin alone is diagnostic of IDA (26). S Ferritin levels of <15ng/dL corresponds to Grade 0 of marrow Iron stores (Table 4) and ID (25,36,45,58). A ferritin level of ≤ 30ng/mL could improve the sensitivity to 92% and with a positive predictive value of 83% (1) and with ferritin levels of <45 mg/L is 85% sensitive and 92% specific for IDA (31); as long as the patient suffers no acute or chronic inflammation, malignancy as it is an acute-phase reactant that could be increased in the presence of inflammation (1,16,17,40,47). The sensitivity and specificity of ferritin once the level rises above 45 ng/mL (45–100 ng/mL) and decreases significantly to 9.4% and 80.0%, respectively (31). Therefore, the diagnosis of IDA is challenging when there is coexisting inflammation, as the ferritin can be up to 100 μg/L, even in the presence of ID (1,26).

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Iron Stores	Marrow Iron stain, 0-4+	S Ferritin (µg/L)
0	0	<15
1-300mg	Trace to 1+	15-30
300-800mg	2+	30-60
800-1000mg	3+	60-150
1-2g	4+	>150
Iron overload	-	>500-1000

Table 4: Comparative chart showing Iron store measurements [30]

Certain conditions like hypothyroidism and Vitamin C deficiency can cause a decrease in S Ferritin levels irrespective of Iron levels. In some other illnesses, the serum ferritin level increases because of factors other than increased Iron stores. S Ferritin levels increase when ferritin-rich tissues are damaged and reflect cellular damage in acute or chronic processes. Hyperthyroidism, liver disease (mostly due to the hepatitis C virus)- damage to the hepatic cell causes the release of intracellular ferritin (non-glycosylated and Iron-rich). In various malignancies S ferritin levels are increased inappropriately, especially in hematologic malignancies, pancreatic, bronchial cancers, and neuroblastomas. Hyperferritinemia-cataract syndrome, alcohol consumption, and oral contraceptives are known to elevate serum ferritin independently of Iron status [1,17].

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Tests	ID	Inflammation	Thalassemia	Sideroblastic anemia
Smear	Micro/hypo	Normal micro/hypo	Micro/hypo with targeting	Variable
S Iron (µg/dL)	<30	<50	Normal to high	Normal to high
TIBC (µg/dL)	>360	<300	Normal	Normal
Transferin saturation	<10	10-20	30-80	30-80
Ferritin (µg/L)	<15	30-200	50-300	50-300
Hb pattern on Electrophoresis	Normal	Normal	Abnormal with β thalassemia; can be normal with α thalassemia	Normal

Table 5: Biochemical parameters in various microcytic anemia [30]

Evaluation of Bone Marrow Iron Stores

Iron staining of a bone marrow biopsy or aspirate is considered 'the gold standard' to assess Iron stores [16,17,41,68]. In bone marrow aspirates, hemosiderin appears as golden-yellow refractile granules [1]. To identify the same Prussian Blue reaction (Pearls' stain) is used. It appears as intracellular blue deposits that appear as spherical, clustered granules, and are absent in cases of ID. But the reliability is markedly variable since the distribution of Iron stores within the marrow shows considerable variation. This staining does not reveal Iron stored as ferritin Iron, which is a significant contributor for Iron supply for erythropoiesis [17].

Experienced observers can grade the marrow hemosiderin stores from 0 to 6+. In ID, marrow hemosiderin is absent (grade 0) compared to normal (grade 1+ to 3+).

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Iron is always present in AI (grade 2+ to 5+) and is greatly increased in sideroblastic anemia, thalassemia major, and other Iron-loading anemias (grade 5+ to 6+) [1]. One disadvantage is inter-observer variations in interpreting marrow Iron stores, which significantly contribute to the 30% of reports of absent marrow stores; which subsequently proved otherwise [17].

Soluble Transferrin Receptors

Transferrin receptors (TfRs) are disulfide-linked transmembrane proteins that facilitate the entry of transferrin bound Iron into cells. sTfR, a soluble and prune form of TfR, consists primarily of the extracellular transferrin binding domain of the molecule. It is increased in hyperproliferative anemias and using Erythropoiesis stimulating agents (ESAs), independent of Iron status, and decreased in hypo proliferative states due to EPO deficiency (e.g., in chronic renal failure) or marrow aplasia. sTfR is not directly affected by inflammation [1,17].

sTfR values are abnormally elevated in Iron-deficient states, which reflect an up regulatory response triggered by the restricted or decreased availability of Iron for erythropoiesis. In ACD, sTfR levels are normal or below normal when compared to IDA and hence helps in distinguishing the two conditions. sTfR values are decreased in Iron overload [1,17].

IRP1/2 regulates the expression of TfR in erythroid cells. In Iron deficiency binds to five 3'-Iron-responsive elements in the TfR1 producing mRNA, stabilizes it, and promotes translation [17]. It must be interpreted in association with the ferritin concentration [1]. It has been proposed that the ratio of S TfR concentration to the logarithm of the S ferritin concentration has a better capacity to distinguish ID from

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ACD than does the unadjusted serum TfR concentration [1,17]. S TfR levels also vary with the total mass of red cell precursors and serve as a marker of the degree of erythropoiesis. Therefore, the value increases in hemolytic anemia, thalassemia, and polycythemia and decreases in hypoplastic anemia and renal failure [1]. Since it is expensive and not routinely available, it is rarely used [47].

Other Tests to Assess Iron status and metabolism

Erythrocyte Zinc Protoporphyrin

Incorporation of Iron into protoporphyrin IX is the final step in the biosynthesis of heme [17]. RBC precursors typically synthesize slightly more protoporphyrin than required for heme synthesis. The excess remains with the cell throughout its lifespan and has been called free erythrocyte protoporphyrin [1]. When Iron is not available for heme synthesis, Zinc (Zn) is inserted into the molecule to form zinc protoporphyrin (ZPP), and it accumulates in excess [1,17,31]. ZPP increases dramatically in ID. It is an effective population screening test for ID, particularly in children and in resource-poor environments. The erythrocyte ZPP content is also significantly increased in lead poisoning, but it is normal in thalassemia [1,17]. But in the era of more reliable and readily available RBC and reticulocyte parameters, the diagnostic value of ZPP is limited [17].

Erythrocyte ferritin

A minor amount of ferritin is present inside the erythrocytes, and it is present in two forms – Acid and alkaline. After thorough removal of WBCs as it contains 1000 times more ferritin than RBCs, both types of ferritin present are measured

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after lysing erythrocytes. It is rarely used as it does not reflect the dynamic changes of Iron status in the body and also not readily available everywhere [1,17].

Liver Iron Stores

Iron stores can also be evaluated by liver biopsy using both histochemical and chemical methods of analysis. Measurement of hepatic Iron stores has been used mainly to assess Iron overload states and is not generally helpful in determining Iron stores for erythropoiesis. However, magnetic resonance imaging using T2* technology replaces liver biopsy as a non-invasive way to measure liver Iron content [1].

Serum hepcidin

The liver secretes hepcidin. It is 25 amino acid containing protein. It acts on ferroportin by internalization and its breakdown and thus affects the Iron availability. It is a negative regulator of Iron absorption and transport [17,35]. When the hepcidin levels are high, it stops the duodenal absorption of Iron, controls the release of Iron from macrophages. Low levels of hepcidin results and promotes Iron absorption and heme-iron recycling/mobilization from macrophages. In states of Iron overload, oral Iron therapy/intake hepcidin levels are expected to increase, whereas in ID states, it will be decreased. EPO therapy causes a decrease in hepcidin levels and thus acts as a potential tool indicative of EPO-responsiveness. It is measured via mass spectrometry or immunoassays [17]. Iron and inflammatory signals act to upregulate hepcidin production; functional Iron deficiency occurs when the Iron supply to the blood is insufficient even though body Iron content is adequate during Iron overload or inflammation, which is also a contributing factor

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in anemia of inflammation [35]. It has a diagnostic value in Iron refractory Iron deficiency anemia (IRIDA), where there will be lower serum ferritin values and increased serum hepcidin [17].

Erythroferrone, transferrin receptor 2, growth differentiation factor 15, and transmembrane serine protease 6

Erythroferrone – When the erythropoietic activity is increased erythroferrone down-regulates hepcidin production and may also contribute to the Iron overload found during ineffective erythropoiesis. Erythroferrone also plays a crucial role in mobilizing Iron stores [17].

Erythroid Transferrin receptor 2 (TfR2) – a key player that is newly discovered. Based on Iron availability, it aids in the Iron-sensing mechanisms that regulate erythropoiesis, most likely by regulating the EPO sensitivity of erythroblasts [17].

Growth differentiation factor 15 (GDF15) – Hypoxia and Iron depletion upregulate the expression of GDF 15. GDF 15 in turn, downregulates hepcidin and following which increases Iron absorption and mobilization. Its concentration in serum is extremely increased in severe thalassemias and congenital dyserythropoietic anemia type I. It also plays a vital role in the Iron overload associated with abnormal expansions of the erythropoietic marrow [17].

Transmembrane serine protease 6 (TMPRSS6)- is a crucial regulator Iron sensing receptor complex of the cell membrane. In decreased Iron availability states, it suppresses hepcidin production and promotes the intestinal absorption of Iron [17].

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Other microcytic anemias

Disorders of Hemoglobin Synthesis

The Hb disorders associated with microcytosis include the thalassemias and certain structural Hb variants [(1)].

The thalassemias are inherited disorders in which synthesis of one of the normal polypeptide chains of globin is deficient. In mild forms of the disease (thalassemia minor), hypochromia and microcytosis are prominent, but anemia may be absent or mild. In other thalassemic disorders, including homozygous β-thalassemia (β-thalassemia major) and Hb E β-thalassemia, where hypochromic, microcytic anemia is usually quite severe [(1)].

Some structurally abnormal Hb may also be associated with moderate microcytosis. This is particularly characteristic of patients carrying a Hb E gene. Normochromic microcytosis also occurs in homozygous Hb C disease. Target cells are also common in these disorders [(1)].

The possibility of thalassemia minor is often suspected when the microcytosis is more severe than the expected degree for the mild degree of anemia. Also, basophilic stippling and target cells tend to be more prominent in thalassemia than in ID. An essential feature of thalassemia trait-like conditions (which include the Hb E syndromes) is that microcytosis often is associated with very high normal to elevated RBC counts despite having little or no anemia [(1)].

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One of the most useful is a modification of the *Mentzer index*, which is based on the MCV and RBC count:

MCV/RBC - > 14 (suggestive of Iron deficiency)

MCV/RBC - 12 to 14 (indeterminate)

MCV/RBC - <12 (suggestive of thalassemia trait disorders)

RDW, a measure of anisocytosis derived from erythrocyte volume distribution, has been advocated for distinguishing ID from thalassemia minor in patients with microcytosis. Anisocytosis is an early and prominent finding in ID, often detectable before significant microcytosis, hypochromia, or even anemia is apparent. In contrast to ID, anisocytosis tends to be absent or mild in thalassemia minor, and consequently, the RDW usually is normal, although occasionally it is slightly increased. It is a typical diagnostic problem to distinguish patients with β-thalassemia trait from those with ID. In almost all cases of β-thalassemia trait, the fraction of Hb A2 is increased, whereas the value for Hb A2 is normal or decreased in Iron deficiency [(1)].

α-Thalassemia syndromes result from decreased α-globin chain synthesis that is directed by four structural genes on chromosome 16. α-Thalassemia trait (α-thalassemia I) individuals lack two α-globin genes, α-globin synthesis is impaired, and there is a mild hypochromic microcytic anemia. The deletion of three α-globin genes causes Hb H disease, and the consequence of this is mild-to-moderate hemolytic anemia. Homozygous α-thalassemia (hydrops) is due to a deletion of all four α-globin genes, resulting in severe anemia due to the complete absence of α-globin chains. This disorder is incompatible with life, and fetuses are aborted early,

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are stillborn, or die within the first few hours of life. The common clinical problem is identifying the α-thalassemia trait, which has a similar presentation to β-thalassemia trait and ID. The diagnosis is made after excluding Iron deficiency, β-thalassemia trait, and any other abnormal Hb [1].

Thalassemia and hemoglobinopathy syndromes are suspected in patients with microcytosis, in patients with unexplained hemolytic anemia, and patients with the red cell abnormalities suggestive of hemoglobinopathies, such as sickle cells or the characteristic inclusions of Hb C disease. The general approach to the identification of these disorders is quantitative Hb analysis, and the principal tool has been hemoglobin electrophoresis and hemoglobin variant analysis by High Performance Liquid Chromatography (HPLC) and capillary zone electrophoresis. Hb C, Hb E, Hb S, and Hb H can be detected by electrophoresis. The presence of increased amounts of Hb A2 and/or Hb F indicates β-thalassemia variants, and DNA sequencing is often employed in the analysis of rare or novel Hb variants [1].

Sideroblastic Anemias

The sideroblastic anemias are due to acquired and hereditary disorders of heme synthesis. A classic morphologic feature of this type of anemia, as seen in the peripheral blood smear, is the presence of erythrocyte dimorphism, with a microcytic population of cells mixed with a normal red cell population and the presence of occasional heavily stippled hypochromic cells. In hereditary (X-linked) sideroblastic anemia, the anemia and microcytosis are pronounced and these changes are accompanied by considerable anisocytosis and poikilocytosis. The serum Iron concentration usually is elevated, and the TIBC is increased. Most commonly, sideroblastic anemias occur in middle age and later life, and these

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acquired disorders can be idiopathic, secondary to drugs, alcohol, or myeloproliferative disorders [(1)].

Literature review from other studies,

In the study done by Chinudomwong P *et al* (2020), they studied a total of 938 subjects. In that 253 were of IDA group. Their study showed Ret-He >30 pg is a potential marker in ruling out IDA, and they conveyed Ret-He as a rapid, convenient, and cost-effective parameter and can be used in conjunction with ferritin studies when IDA co-exists with non ID conditions [(54)].

In the study by Andriastuti M *et al* (2019), a cross-sectional study comprises 207 children aged between 6-18yrs. They suggested that Ret-He as an alternative screening parameter to detect ID and IDA in children aged 6-18 years [(55)].

A study done by Khan N *et al* (2019), 165 female patients with anemia were studied comparing Ret-He with S Ferritin (taking it as the gold standard for ID). They concluded Ret-He as a reliable marker in diagnosis ID in the female population [(51)].

Toki Y *et al* (2017), they studied blood samples obtained from 211 patients, in that 72 were IDA group. From their study, they suggested that Ret-He as a clinically useful marker for determining ID in the general population [(71)].

A study was done by Sanyoto A *et al* (2017), which was an observational study with a cross-sectional study in 87 subjects. Thirty-six were IDA in the study population. With good sensitivity for Ret-He to detect IDA, they suggested it as a useful screening test from their study [(60)].

Agarwal MB *et al* (2017), in their study they concluded that CHr with good sensitivity and specificity could be called the gold standard, replacing both marrow Iron studies and soluble transferrin receptor (sTfR)-Ferritin index [(44)].

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In the study conducted by Rungngu S *et al* (2016), they included 50 children aged 6-12 years, of which 16 had IDA. They concluded that with Ret-He having high specificity, it might be useful as a screening tool for early detection of IDA in children [(61)].

In the study by Karagülle M *et al* (2013), a total of 32 female patients with IDA. They suggested CHr as a useful parameter that can be confidently used in the diagnosis of IDA [(63)].

In the study by Ageeli A *et al* (2012), they studied 260 adults with 100 subjects having. They suggested that the CHr parameter as a good predictor of ID, and also, when CHr is used to screen for IDA, it can increase the accuracy of diagnosis and help in early detection and treatment of ID [(62)].

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MATERIALS AND METHODS:

Source of data

A hospital-based observational study was carried out on the patients attending either outpatient or inpatient departments and who were referred to the Central Laboratory in BLDE (Deemed to be University) Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura.

Study period: 1st December 2018 to 30th August 2020

Inclusion criteria:

- All the cases with Hemoglobin less than 11g/dl, and having microcytic hypochromic blood anemia during the study period will be included.
- MCV < 75fL on CBC parameter

Exclusion criteria:

- Patients of MCHC who are associated with a systemic illness like diabetes mellitus, CKD and hypertension.
- Blood transfusion in the last 3months.

Sample collection

After informed consent, venous blood samples under aseptic precautions were collected in EDTA anticoagulated vacutainer to analyze hematological parameters and in a Plain vacutainer for Iron studies.

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Methods of collection of data

The study included a total of 201 patients who were diagnosed with microcytic hypochromic anemia. Hematological parameters will be processed in the Sysmex XN1000 (Sysmex Corporation, Kobe, Japan), which is a fully automated blood cell counter and parameters such as Red blood cell count(RBC count), Hemoglobin (Hb), Hemeatocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration(MCHC), Red cell distribution width (RDW), Reticulocyte count, Absolute Reticulocyte Count, Reticulocyte Production Index (RPI), Immature Reticulocyte Fraction (IRF), Reticulocyte Hemoglobin equivalent (Ret-He) were studied.

Figure 7: Automated Hematology Analyzer (Sysmex XN-1000)

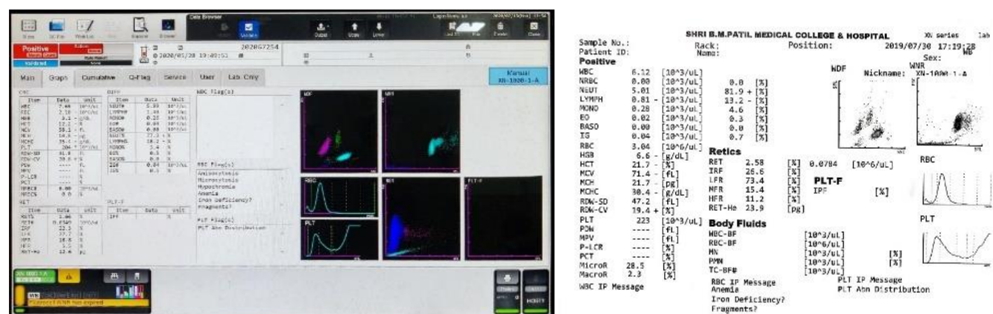

Figure 8 A and 8B: Sample report obtained using Sysmex XN-1000

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Biochemical estimation of Total Iron-binding capacity (TIBC), Serum Iron (Fe), Serum Ferritin levels were measured using a Biochemistry analyzer (VITROS® 250 Chemistry System).

Figure 9: Biochemistry analyzer (VITROS® 250 Chemistry System)

Sample size:

In the study conducted by Brugnara C et al, the anticipated sensitivity and specificity of reticulocyte hemoglobin equivalent in correlation with traditions parameters 93% and 83%, respectively [(64)], at a 99% confidence level and precision of 0.1 the sample size of minimum 189 was calculated using the formula,

$$N = \frac{Z^2 P(1-p)}{\Delta^2}$$

N will be (a+c) if we use sensitivity as p

N= (a+c)/Prevalence

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Statistical analysis

The data obtained were entered in a Microsoft Excel sheet, and statistical analysis was performed using a statistical package for the social sciences (Version 17). Results are presented as drawings, Mean ±standard deviation (SD), counts, and percentages. Results were compared using independent t-test, Chi-square test, the correlation between variables will found using correlation coefficient, Positive predictive value (PPV), Negative predictive value (NPV), ROC Curve, Sensitivity, and Specificity is be used, significance was achieved at $p < 0.001$ using IBM SPSS statistics version 23 and Microsoft Excel 2016.

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REFERENCE RANGE [45]

Parameters	Reference Range
RBC	3.8-4.8 millions/c mm
Hb	11.6-14.5 g/dl
PCV	36-46 %
MCV	80-100 fL
MCH	27-32 pg
MCHC	31.5-34.5 %
Reticulocyte count	Infants – 2-5%, Adults- 0.5-2.55%
Corrected Reticulocyte count	0.5-2.5%
RPI	0.5-2.5%
IRF	0.02-0.11%
RetHe	27-34 pg/cell
S Ferritin	**Male-** 17.9-464 ng/ml, **Female- <50 years-** 6.24-137 ng/ml and **≥50years-** 11.1-264 ng/ml
S Iron	**Male-** 49-188 mcg/dL, **Female-** 37.0-177.0 mcg/dL
TIBC	**Male-** 261-462 mcg/dL, **Female-** 265-497 mcg/dL

Table 6: Reference values [45]

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RESULTS

Our study was done at the Department of Pathology and Biochemistry, BLDE (Deemed to be University), Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka. In our study, we studied 201 patients (inpatients and outpatients) who presented with microcytic hypochromic anemia. Hematological and biochemical analysis was done in the Department of Pathology and the Department of Biochemistry after fulfilling the inclusion and exclusion criteria.

Here, we present an evaluation of the results of our study.

- **Distribution of subjects according to age, sex, and grade of anemia**

Age (Years)	No. of patients	Percentage
< 10	34	16.9
10 - 19	33	16.4
20 - 29	42	20.9
30 - 39	26	12.9
40 - 49	24	11.9
50 - 59	11	5.5
60 - 69	23	11.4
70+	8	4.0
Total	201	100.0

Table 7: Distribution of patients according to Age (Years)

- In our study, most of the population belonged to the age group 20-29years accounting for 20.9% in 201 subjects, and the average age was 31.51years. (Table 7)

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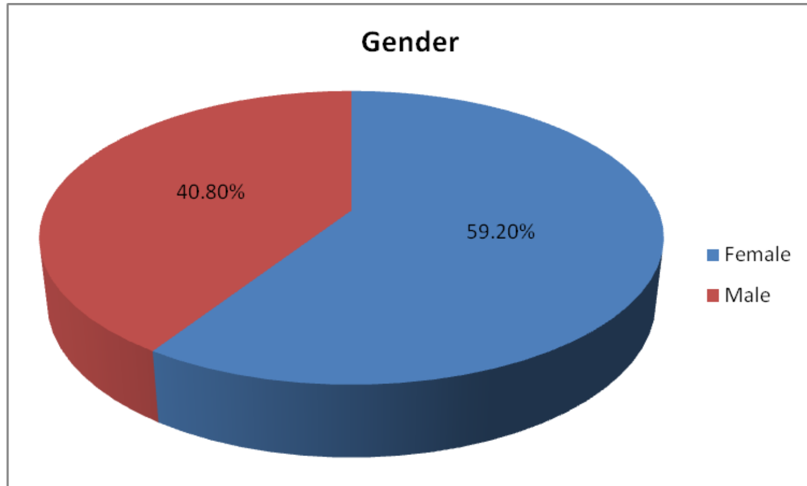

Figure 10: Distribution of patients according to Gender

- In our study, 59.20% of the study population were females, and 40.80% were males in the total study population of 201 subjects. (Figure 10)

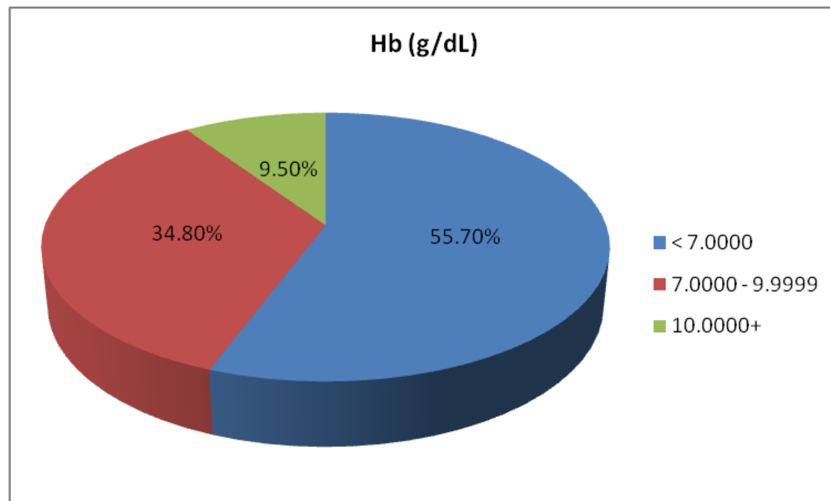

Figure 11: Distribution of patients according to Hb (g/dL)-
Anemia grading Mild, Moderate and Severe

- We found about 55.70% of the study population belonged to the severe anemia group, 34.80% were moderate anemic, and 9.50% had mild anemia (Figure 11)

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- **Descriptive statistics showing range and mean in the study population**

Parameters	Minimum	Maximum	Mean	Std. Deviation
RBC (10*6/uL)	1.6400	5.9700	3.922239	0.8611861
Hb (g/dL)	2.4000	10.9000	6.831841	1.9436515
PCV (%)	10.2	36.5	24.200	5.7021
MCV (fL)	45.9000	75.0000	61.910945	7.7245051
MCH (pg)	10.9	30.5	17.615	3.8004
MCHC (g/dL)	16.9	50.0	28.061	3.7111
RDW-CV (%)	13.8000	38.0000	21.895025	4.4269487
Reticulocyte count (%)	0.1000	13.9200	1.628465	1.5775366
Absolute Reticulocyte Count (10*3/uL)	3.6000	361.9000	59.648259	45.3458411
RPI (%)	0.001	2.300	0.44043	0.372070
IRF (%)	0.001	48.3000	20.374627	10.0445011
Ret-He (pg)	9.6000	28.9000	15.024378	4.0505620
LFR (%)	51.7000	100.0000	79.408000	10.2398638
MFR (%)	0.00001	23.9000	14.042500	4.8814842
HFR (%)	0.00001	26.1000	6.549500	6.2974510
S Iron (µg/dL)	2	431	45.3134	79.652
TIBC (µg/dL)	120	>800	462.5124	126.047
S Ferritin (ng/mL)	<1	>1000	53.597	138.479

Table 8: Descriptive statistics of Hematological findings

- In our study, we found a wide distribution of values in hematological parameters RBC count, Hb, PCV, MCV, MCH, Reticulocyte count, IRF, Ret-He. With the mean value for Hb of 6.83 g/dL, MCV of 61.91fL, MCH of 17.61pg, RDW of 21.89%.
- Mean values for reticulocyte parameters were RPI of 0.44%, IRF of 20.37%, Ret-He 15.02pg/cell.

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- In biochemical parameters, we found mean for Sr. Iron (µg/dL) was 45.31. We found a wide distribution of values in TIBC and S Ferritin with a mean value of 462.51µg/dL and 53.59ng/mL, respectively. (Table 8)

- **<u>Distribution of subjects according to Ret-He reference value</u>**

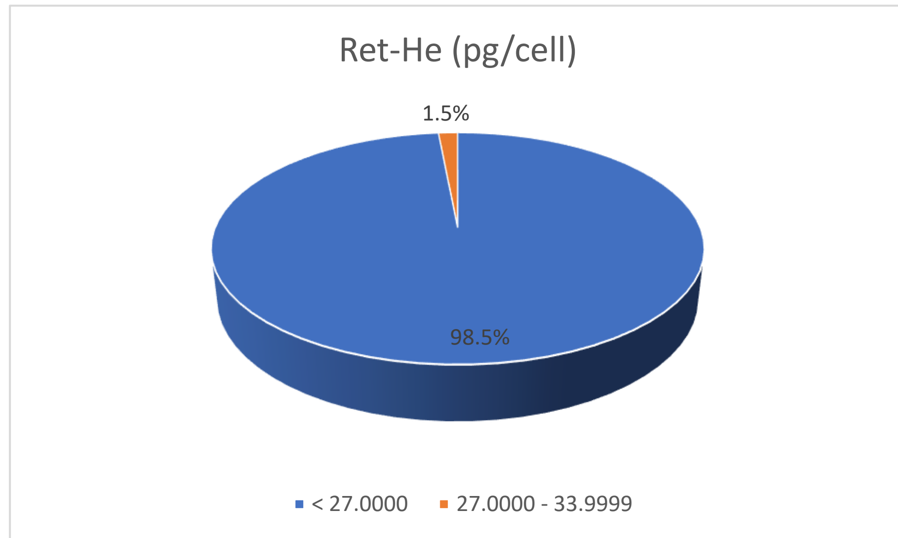

Figure 12: Distribution of patients according to Reticulocyte Hemoglobin equivalent (Ret-He) (pg/cell)

- In our study, most of the subjects (98.5%) fell under the lower cutoff of Ret-He – 27pg/cell, and only 1.5% had Ret-He levels in the normal range (Figure 12).

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- **Distribution of subjects according to S Ferritin values in male and female population studied**

S Ferritin level (ng/mL)	Male		S Ferritin level (ng/mL)	Female (Age<50 years)		S Ferritin level (ng/mL)	Female (Age≥50 years)	
	N	%		N	%		N	%
< 18	47	57.3	**<6.24**	49	49.50	**<11.1**	11	55
18 – 464	31	37.8	**6.24-137**	45	45.45	**11.1-264**	08	40
465+	4	4.9	**>137**	05	5.05	**>264**	01	5
Total	82	100	**Total**	99	100	**Total**	20	100

Table 9: Distribution of S Ferritin Level (ng/mL) among male and female

- In our study, in the male population- 57.3% had S Ferritin levels of <18ng/mL suggestive of ID group. Whereas in females <50yrs of age – 49.5% had S ferritin levels below lower cutoff levels of <6.24ng/mL. In females ≥ 50yrs of age – 55% had S ferritin levels below lower cutoff levels that are <11.1ng/mL suggestive of ID group. (Table 9)

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- **Distribution of subjects according to S TIBC values in male and female population studied**

TIBC (µg/dL)	Male		TIBC (µg/mL)	Females	
	N	%		N	%
< 261	6	7.3	**< 265**	8	6.7
261 – 462	33	40.2	**265 – 497**	62	52.1
463+	43	52.4	**498+**	49	41.2
Total	2	100.0	**Total**	119	100

Table 10: Distribution of S Total Iron Binding Capacity (µg/dL) among male and female

- In our study, we found 52.4% of the male population falling above the cutoff levels of 462 µg/dL, suggestive of Iron deficiency group. Whereas 41.2% of the female population falling above the cutoff levels of 497 µg/dL, suggestive of Iron deficiency group. (Table 10)

- **Distribution of subjects according to S Iron values in the male and female population studied**

S Iron (µg/dL)	Male		S Iron (µg/dL)	Female	
	N	%		N	%
< 49	67	81.7	**< 37**	91	76.5
49 – 188	7	8.5	**37 – 177**	19	16.0
189+	8	9.8	**178+**	9	7.6
Total	82	100.0	**Total**	119	100.0

Table 11: Distribution of S Iron (µg/dL) between male and female

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- In our study, 81.7% of the male population was found in the Iron deficiency group that is S Iron levels of <49µg/dL. Whereas 76.5% of the female population was falling below the reference range that is an Iron deficiency group, which is <37µg/dL. (Table 11)
- **<u>Descriptive statistics showing range and mean in the study population</u>**

	Male		**Female**	
	Mean	**Std. Deviation**	**Mean**	**Std. Deviation**
S FERITIN (ng/mL)	62.92	131.912	34.99	76.425
S IRON (mcg/dL)	51.41	86.705	41.11	74.492
TIBC (mcg/dL)	454.79	124.460	471.80	120.323

Table 12: Descriptive statistics when compared between male and female of biochemical findings in Iron Profile

- As per works of literature data, lower Iron stores are found in the female population. Its biochemical evaluation confirms this, suggests a significant part of their lives spent in a negative Iron balance, which can be attributed to a combination of poor diet and menstrual blood loss. The values improve in the post-menopausal age group [(1,25)]. In our study, the values are consistent with those findings. (Table 9-12)

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- **<u>Correlation between Hb and other RBC parameters. Hb and Reticulocyte parameters. Hb and S Iron studies</u>**

Correlation	Correlation coefficient	P value
Hb & PCV	r=0.933	P=0.0001*
Hb & MCV	r=0.467	P=0.0001*
Hb & MCH	r=0.632	P=0.0001*
Hb & MCHC	r=0.630	P=0.0001*
Hb & RDW-CV	r=-0.453	P=0.0001*
Hb & Reticulocyte count	r=-0.266	P=0.0001*
Hb & Ret-He	r=0.605	P=0.0001*
Hb & RPI	r=0.323	P=0.0001*
Hb & IRF	r=-0.225	P=0.001*
Hb & S Iron	r=-0.136	P=0.053
Hb & S Ferritin	r=-0.064	P=0.365
Hb & TIBC	r=-0.107	P=0.132

Table 13: Correlation between Hb and other hematological and biochemical parameters

[* = Correlation is significant at the 0.01 level (2-tailed)].

- In our study, we found a statistically significant correlation between Hb and other RBC parameters like PCV, MCV, MCH, MCHC and RDW-CV. The correlation was significant statistically between Hb and reticulocyte parameters, especially with Ret-He with p <0.00. The correlation was insignificant between Hb and serum Iron parameters (Table 13)

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- **Distribution of subjects according to IDA and Non-IDA**

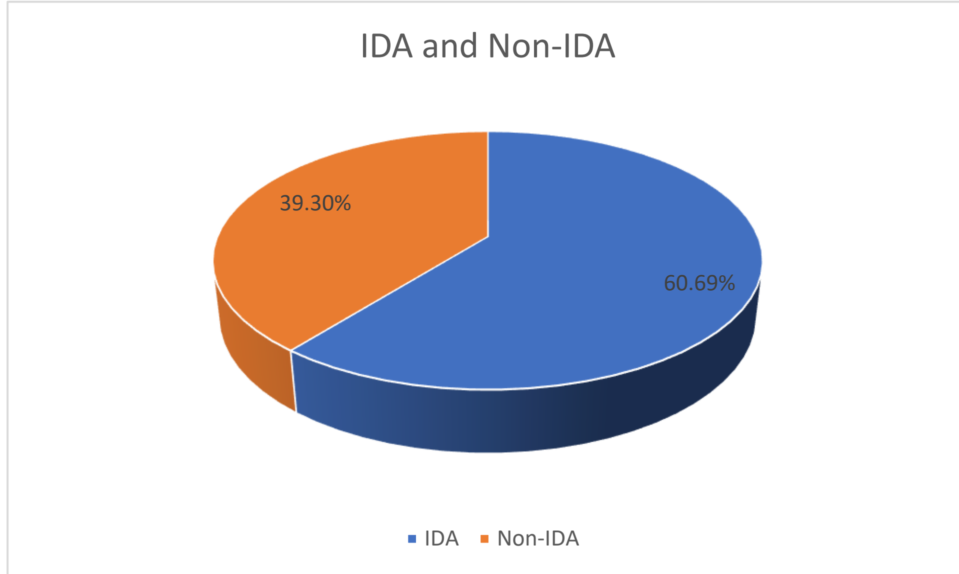

Figure 13: Distribution of subjects in IDA and non-IDA

- In our study, we found the majority of the subjects that is 122 subjects (60.69%) in the study population belonged to the IDA group and 79 subjects belonging to the non-IDA group out of 201 microcytic hypochromic anemia subjects. (Figure 14)

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- **Descriptive statistics showing mean values of hematological and biochemical parameters in IDA and Non-IDA group**

Parameters	In IDA (Mean)	Non IDA (Mean)
Age (yrs)	31.52	30.57
RBC (10*6/uL)	4.01	3.78
Hb (g/dL)	6.78	6.9
PCV (%)	24.46	23.78
MCV (fL)	61.00	63.30
MCH (pg)	16.889	18.73
MCHC (g/dL)	27.442	29.01
RDW-CV (%)	20.87	23.465
Reticulocyte count (%)	1.29	2.14
Absolute Reticulocyte Count (10*3/uL)	50.68	73.49
RPI (%)	0.37	0.54
IFR (%)	19.66	21.467
Ret-He (pg/cell)	13.92	16.72
S Iron (µg/dL)	27.418	72.94
TIBC (µg/dL)	505.09	401.89
S Ferritin (ng/mL)	5.32	128.145

Table 14: Descriptive statistics from IDA and Non-IDA population.

- In our study, we found 122 (60.69%) subjects with microcytic anemia subjects having ID. In those subjects, we found RBC, Hb, MCV, MCH, Reticulocyte count, Absolute reticulocyte count, RPI, IRF, Ret-He, S Iron, TIBC, and S Ferritin parameters were significantly less compared to the non-ID subjects with the microcytic anemia. (Table 14)

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- **Tables and scatter plot graphs showing a correlation between Ret-He and RBC parameters. Ret-He and other Reticulocyte parameters. Ret-He and S Iron parameters**

Correlation	Correlation coefficient	P-value
Ret-He & Hb	r= 0.605	P=0.0001*
Ret-He & PCV	r=0.503	P=0.0001*
Ret-He & MCV	r=0.729	P=0.0001*
Ret-He & MCH	r=0.759	P=0.0001*
Ret-He & MCHC	r=0.560	P=0.0001*
Ret-He & RDW	r=-0.277	P=0.0001*
Ret-He & Reticulocyte count	r=-0.020	P=0.782
Ret-He & RPI	r=0.305	P=0.449
Ret-He & IRF	r=-0.064	P=0.363
Ret-He & Ferritin level	r=0.419	P=0.0001*
Ret-He & S Iron	r=0.068	P=0.340
Ret-He & TIBC	r=-0.309	P=0.0001*

Table 15: Correlation between Ret-He and other hematological and biochemical parameters

[* = Correlation is significant at the 0.01 level (2-tailed)]

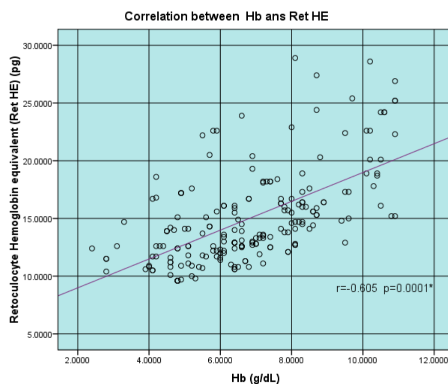

Figure 14: Correlation between Hb and Ret-He

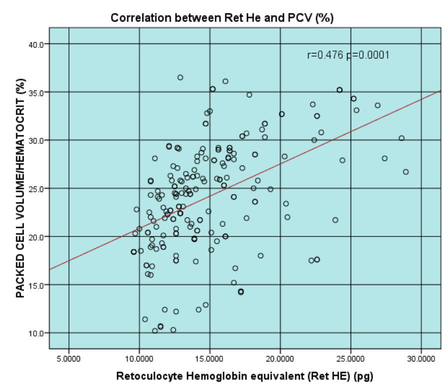

Figure 15: Correlation between Ret-He and PCV

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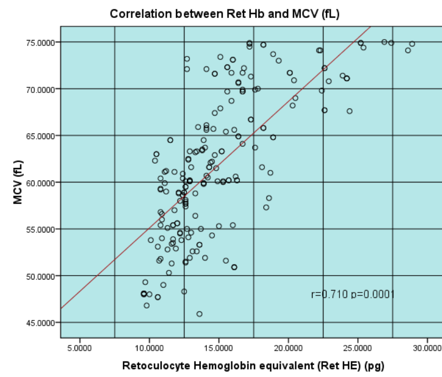


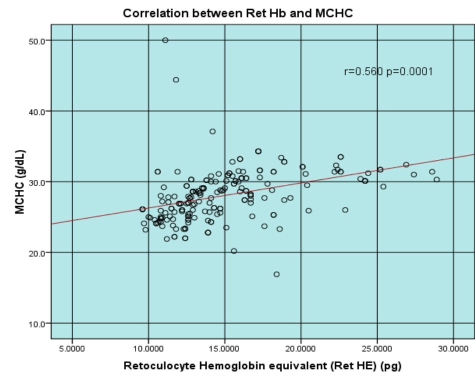
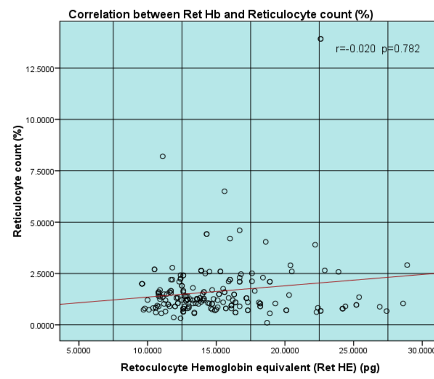


Figure 16: Correlation between Ret-He and MCV

Figure 17: Correlation between Ret-He and MCHC



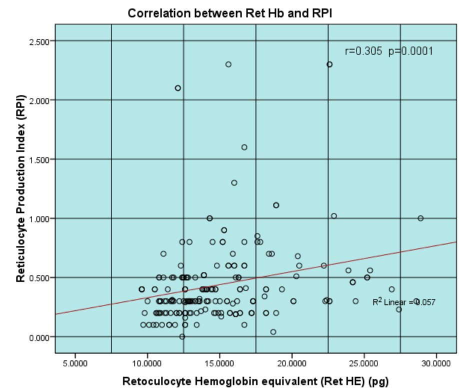
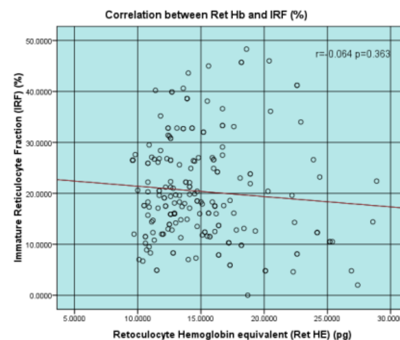


Figure 18: Correlation between Ret-He and Reticulocyte count

Figure 19: Correlation between Ret-He and RPI



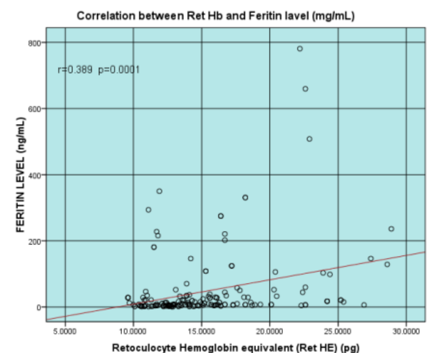


Figure 20: Correlation between Ret-He and IRF

Figure 21: Correlation between Ret-He and S Ferritin

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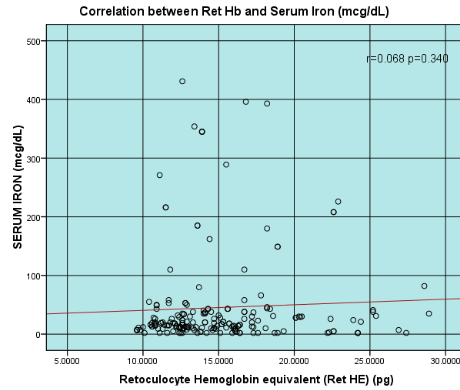

Figure 22: Correlation between Ret-He and S Iron

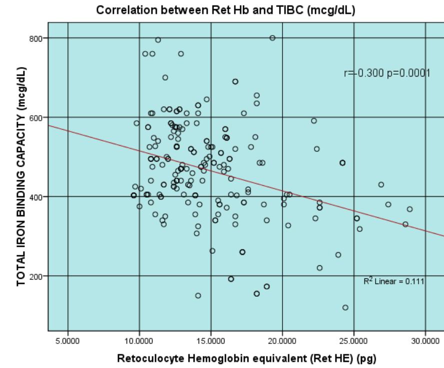

Figure 23: Correlation between Ret-He and TIBC

- In our study, we found that Ret-He showing significant statistical positive correlation with RBC parameters with p <0.001.
- The correlation was insignificant between Ret-He and other reticulocyte parameters in the subjects studied.
- A significant statistical positive correlation was found between Ret-He and S Ferritin levels p <0.001, and a statistically significant negative correlation was found between Ret-He and TIBC with P <0.001. The correlation was insignificant between Ret-He and S Iron in our study, which may be attributed to various factors (Table 15, Figure 14 to 23)

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- **<u>ROC curve when Ret-He and S Ferritin parameters compared</u>**

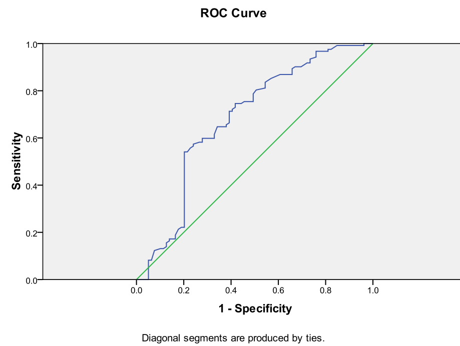

Figure 24: ROC curve when S Ferritin cut-off of <15ng/mL taken as ID with Ret-He cut off of 27.15 pg/cell- AUC 0.681, Sensitivity= 57.37% and Specificity= 75.95%

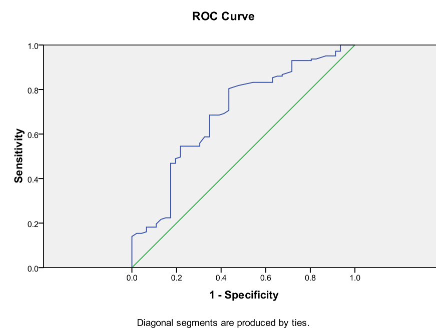

Figure 25: ROC curve when S Ferritin cut-off of <30ng/mL taken as ID with Ret-He cut off of 27.15 pg/cell. AUC 0.709

Sensitivity= 80.42% and Specificity= 56.52%

- With ROC curve analysis, the cut-off value of 27.15ng/mL was appropriate for Ret-He when S Ferritin is taken as the gold standard to detect ID states.
- In the present study we found, the sensitivity of 57.37%, the specificity of 75.95%, and PPV of 100% for Ret-He when compared with S Ferritin when its cut-off of <15ng/mL was taken to detect ID states and accuracy was found to be 62.19% for Ret-He. (Figure 24, Table 16).
- When S Ferritin cut-off of <30ng/mL was taken to detect ID states, the sensitivity of Ret-He significantly improved to 80.42%, but its specificity decreased to 56.52% (Figure 25).

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- Hence, Ret-He is more specific and less sensitive when S Ferritin cut-off of <15ng/dL, and sensitivity significantly increases when S Ferritin cut-off of <30ng/dL taken for ID at the cost of decreased specificity.

- **<u>ROC curve when Ret-He and Other Serum Iron parameters compared</u>**

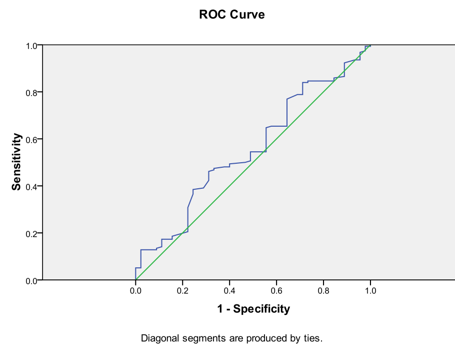

Figure 26: ROC curve when S Iron cut-off of <40µg/dL taken as ID with Ret-He cut off of 27.15 pg/cell- AUC 0.557, Sensitivity= 46.15% and Specificity= 68.89%

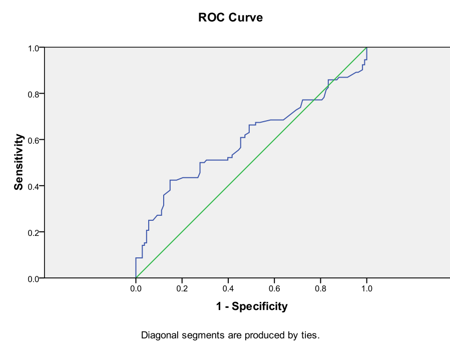

Figure 27: ROC curve when TIBC cut-off of >460µg/dL taken as ID with Ret-He cut off of 27.15 pg/cell- AUC 0.402, Sensitivity= 98.15% and Specificity= 9.78%

- When IDA is determined with S Iron lower cut-off as ID levels, we found a specificity of 68.89% for Ret-He to rule out ID. When TIBC is taken to detect IDA, sensitivity was found to be good for Ret-He, which is 98.15%. (Figure 26,27)

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Statistical parameters	Values
Cutoff of Ret-He for IDA	27.15pg/cell
Association	P=0.0001
Correlation	r=0.389 (moderate positive correlation)
AUC	0.681
Sensitivity	When S Ferritin cutoff of <15ng/mL = 57.37%
	When S Ferritin cutoff of <30ng/mL = 80.42%
Specificity	When S Ferritin cutoff of <15ng/mL = 75.95%
	When S Ferritin cutoff of <30ng/mL = 56.52%
PPV	100%
NPV	3.8%
Accuracy	62.19%

Table 16: Summary of statistical analysis in the present study when S Ferritin is taken as 'the gold standard' to determine ID state

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DISCUSSION

IDA is a major global health problem affecting about 1.48 billion world population [3,24]. It is mainly prevalent in developing countries and affects the quality of life and significant economic implications on the country [16,18]. ID is the common cause of anemia in pregnant women [36], menstruating women continue to be the majority of the population who are prone to develop ID, along with young children whose growth outstrips their Iron supply [1].

There are various causes of Microcytic Hypochromic anemia (MCHC anemia). The four major causes of microcytic red blood cells are ID, anemia of chronic disease (ACD), thalassemia's, sideroblastic anemia, and lead poisoning. IDA and ACD are by far the most common [13,14].

Newer parameters introduced in the 6-part automated cell counters like Sysmex XN series has changed the approach to the diagnosis of anemia with possible causes. With additions of automated reticulocyte analysis have led to the addition of newer Retic parameters like Reticulocyte count, Absolute Reticulocyte Count, RPI, IRF, Ret-He, etc., [12,14].

Reticulocytes are the youngest erythrocytes released to peripheral blood from bone marrow [47]. Information regarding its hemoglobin gives a good indication of Iron availability, and an early marker of Iron-deficient erythropoiesis and Ret-He reflects current and real-time information regarding the synthesis of young erythrocytes in the bone marrow and provides an early measure of Functional- ID [12,14].

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Ret-He can be easily measured by merely adding the test item reticulocyte count to CBC, and the reagents required for measuring Ret-He are cheaper, which is approximate 1/5th the amount of that required for measuring serum ferritin [(35,52)].

This study was done at the Department of Pathology and Biochemistry, BLDE (Deemed to be University), Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka.

We studied hematological parameters like RBC count, Hb, PCV, MCV, MCH, MCHC, RDW, Reticulocyte count, Absolute Reticulocyte Count, RPI, IRF, Ret-He processed in the Sysmex XN1000 (Sysmex Corporation, Kobe, Japan) analyzer and compared with Biochemical parameters of assessing body Iron stores and Iron availability such as TIBC, Serum Iron, Serum Ferritin using Biochemistry analyzer (VITROS® 250 Chemistry System) from 201 subjects, after fulfilling all the inclusion and exclusion criteria's.

In our study, when S Ferritin is taken as the gold standard to detect ID, after statistical evaluation, we found the cut-off value of Ret-He to detect ID state was 27.15pg/cell. We found an excellent statistically significant correlation between Ret-He and RBC parameters with $p < 0.001$.

There was a statistically significant correlation between Ret-He and S Ferritin with $r=0.389$ and $P=0.0001$, which is < 0.001. When the S Ferritin cut-off of <15ng/mL was taken as ID state [(30,45)], we found a Sensitivity of 57.37% and Specificity of 75.95% with AUC of 0.681 for Ret-He. And PPV of 100% and NPV of 3.8%, and accuracy of 62.19% for Ret-He. When S Ferritin <30ng/mL was taken as ID, which

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supposedly increases its sensitivity and PPV in detecting ID states [(1)], we found a sensitivity of 80.42% and specificity of 56.52% with Ret-He.

- **Comparison of various parameters between our study and other studies**

Our study showed association with various other studies (Table 16, 17, and 18)

Parameters	**Present study**		**Ageeli A *et al* (2012) [(62)]**	
Ret-He vs	Correlation	P value	Correlation	P-value
Hb	0.605	<0.001	0.789	< 0.001
PCV	0.476	<0.001	0.630	< 0.001
MCV	0.710	<0.001	0.775	< 0.001
RDW	-0.166	0.018	-.0561	<0.001
MCH	0.729	<0.001	0.667	< 0.001
MCHC	0.560	0.001	0.323	< 0.001
S Iron	0.068	0.340	0.836	< 0.001
TIBC	-0.300	<0.001	-0.723	< 0.001
S Ferritin	0.389	<0.001	0.938	< 0.001

Table 17: Comparison of correlation of various parameters between present study and study done by Ageeli A *et al* (2012) [(62)]

- Ret-He values are compared with RBC parameters like Hb, PCV, MCV, MCH, MCHC, RDW, and serum Iron parameters like S Iron, TIBC, and S Ferritin in the above table. Ageeli A *et al* also did similar comparison.
- The above table shows a statistically significant correlation between Ret-He and RBC parameters, serum Iron parameters like S Ferritin and TIBC (p <0.001) except S Iron, which did not correlate in a statistically significant manner in our study when compared with a study done by Ageeli A *et al* as S Iron levels which may be attributed to various factors like the varied

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population in our study, diurnal variation of S Iron levels, dietary factors etc.,(Table 17)

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Parameters	Present study	Khan N *et al* (2019) [(51)]	Toki Y *et al* (2017) [(71)]	Karagülle M *et al* (2013) [(63)]
Country	India	Pakistan	Japan	Turkey
Hematology analyzer	Sysmex XN 1000(Sysmex Corporation, Kobe, Japan)	Sysmex XE 5000(Sysmex Corporation, Kobe, Japan)	Sysmex XN 3000/Sysmex XE 5000(Sysmex Corporation, Kobe, Japan)	ADVIA 2120i (Bayer Diagnostics, Tarrytown, NY)
Number	122	75	72	32
Mean Age (yrs)	31.52	-	48.7	35.888
Mean RBC (10*6/uL)	4.01	-	-	4.46
Mean Hb (g/dL)	6.78	9.17	9.5	9.86
Mean MCV (fL)	61.00	-	76	71.51
Mean MCH (pg)	16.889	-	-	22.65
Mean MCHC (g/dL)	27.442	-	-	31.36
Mean Ret-He (pg/cell)	13.92	23.34	23.4	Chr=24.95

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Ret-He (pg/cell) cutoff taken for ID	<27.15	<27.6	30.9	Chr= <28
Mean S Iron (µg/dL)	27.418	-	29	21.34
Mean TIBC (µg/dL)	505.09	-	423.2	361.26
Mean S Ferritin (ng/mL)	5.32	15.2	5.4	4.56

Table 18: Comparison of various hematological and biochemical parameters between the present study with various other studies

- The above table shows a comparison of the mean values of various hematological parameters and serum Iron parameters in the IDA group in the present study with the studies done by Khan N *et al*, Toki Y *et al*, Karagülle M *et al*.
- We found the mean Hb, MCV, MCH, and Ret-He values 6.78g/dL, 61fL, 16.889pg, and 13.92pg/cell, respectively; which were significantly lower compared to the various other studies mentioned above. The lower values may be attributed to various factors like the study population, dietary habits, analyzers etc., which are different compared to the other studies. (Table 18)

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Studies	**Correlation**	**Sensitivity**	**Specificity**	**Positive predictive value (PPV) with 95% CI**	**Negative predictive value (NPV) with 95% CI**	**Accuracy**
Present study with RetHe cutoff of <27.15pg	**r=0.389** **P=0.0001** **AUC= 0.681**	**When S Ferritin <15ng/mL as cutoff = 57.37%** **When S Ferritin <30ng/mL as cutoff = 80.42%**	**When S Ferritin <15ng/mL as cutoff = 75.95%** **When S Ferritin <30ng/mL as cutoff = 56.52%**	**100%**	**3.8%**	**62.19%**
Chinudomwong P *et al* (2020) [54] with RetHe cut-off ≤30 pg for IDA	AUC = 0.876	74.2%	97.4%	80%	99.6%	-
Andriastuti M *et al* (2019) [55] with Ret-He cut-off of <27pg for IDA	r=0.336 P = 0.001 AUC = 0.700	75%	80%	18.7%	98.1%	-
Khan N *et al* (2019) [51] with Ret He cut-off <27.6 pg	P= <0.0001 AUC = 0.922	93.33%	83.33%	82.35%	93.75%	87.88%
Toki Y *et al* (2017) [71] Ret-He cut-off 30.9 pg	r = 0.654 p = 0.033 AUC= 0.902	92%	81%	-	-	-
Sanyoto A *et al* (2017) [60] Ret-	-	97.2%	66.67%	67.30%	97.14%	-

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He cut-off 25 pg						
Rungngu S *et al* (2016) [61] Ret-He cut-off 27.8 pg/L	P=0.005	43.8%	85.3%	58.3%	76.3%	-

Table 19: Comparison of the sensitivity and specificity between present study with various other studies

- The above table shows the analysis of the sensitivity, the specificity, and predictive values of Ret-He and its comparison with the studies by Chinudomwong P *et al*, Andriastuti M *et al*, Khan N *et al*, Toki Y *et al*, Sanyoto A *et al* and Rungngu S *et al*.
- The sensitivity of Ret-He of the present study when S Ferritin <15ng/ml taken as a cut-off was comparable to the values from the study done by Rungngu S *et al*. The sensitive significantly increased to 80.42% with S Ferritin cut-off of <30ng/mL which is comparable to the sensitivity derived from studies done by Chinudomwong P *et al* and Andriastuti M *et al*.
- The specificity of Ret-He of 75.95% from the present study when S Ferritin <15ng/ml taken as a cut-off was comparable to the values from the study done by Sanyoto A *et al* and Andriastuti M *et al*. The specificity significantly decreases to 56.52% with S Ferritin cut-off of <30ng/mL which is comparable to the sensitivity derived from a study done by Sanyoto A *et al*.
- The positive predictive values of the Ret-He from the present study were comparable to the values derived from studies by Khan N *et al* and Chinudomwong P *et al*. (Table 19)

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SUMMARY

- This study was done at the Department of Pathology and Biochemistry, BLDE (Deemed to be University), Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka.
- In our study, we studied 201 patients (inpatients and outpatients) who presented with microcytic hypochromic anemia who fulfilled inclusion and exclusion criteria.
- We studied hematological parameters processed in the Sysmex XN1000 (Sysmex Corporation, Kobe, Japan) analyzer, and biochemical parameters were analyzed in the VITROS® 250 Chemistry System.
- Salient observations from our study are, we found 122 subjects with IDA and 79 subjects belonging to Non-IDA microcytic hypochromic anemia.
- We found an excellent statistically significant correlation between Ret-He parameter and other RBC parameters ($p < 0.001$). A statistically significant positive correlation was found with Ret-He and S Ferritin ($p < 0.001$); a statistically significant negative correlation was found with Ret-He and TIBC ($p < 0.001$), suggesting the Ret-He as a comparable parameter in the detection of ID.
- We found no statistical significance between Ret-He and S Iron, which may be attributed to various factors.
- In our study, we found, Ret-He more specific (specificity= 75.95%) and less sensitive when S Ferritin cut-off of <15ng/dL and sensitivity significantly increase to 80.42% when S Ferritin cut-off of <30ng/dL taken for ID at the cost of decreased specificity.

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- Ret-He as a parameter can aid in detecting ID states much easier, faster, and in a cost-effective way.
- Hence Ret-He parameter can be used in the screening of microcytic hypochromic anemia states to tule out ID. Ret-He, when used along with biochemical parameters such as S Ferritin, can give valuable inputs in better and accurate diagnosis of IDA. Hence, over-treatment with Iron supplements can be avoided.

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LIMITATIONS

- In our study, we found 79 subjects who are Non-ID but showing microcytic hypochromic anemia, so further investigations like hemolytic profile, hemoglobin variant estimation etc., are needed to detect the anemia with a specific cause.
- Ret-He cut-off and reference values for different study populations need to be standardized and established, as the reference ranges taken in our study are of adults'.
- Follow-up with treatment was not done in our study, which would have given valuable input on how Ret-He values respond to Iron therapy.

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CONCLUSION

- Ret-He can be easily measured by simply adding the test item reticulocyte count to CBC in the modern hematology analyzers and hence better patient compliance. The cost per test is also significantly lower than Serum Iron parameters like S Ferritin because of lower reagent costs.
- The present study suggests Ret-He is one of the better and reliable hematological parameters having a good correlation with the S Ferritin parameter in detecting ID.
- With a reasonable specificity, it can rule out Iron deficiency, suggesting it as a better hematological screening tool to detect IDA.
- When the Ret-He parameter is used along with biochemical parameters such as S Ferritin, it can give valuable inputs in ID states and diagnosis of IDA, and hence proper treatment is possible.

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ANNEXURE - I

B.L.D.E (Deemed to be University)
SHRI.B.M.PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE
VIJAYAPUR – 586103

IEC/NO:286/2018
17-11-2018

INSTITUTIONAL ETHICAL COMMITTEE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 13-11-2018 at 03-15 PM scrutinize the Synopsis of Postgraduate Students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected and revised version synopsis of the Thesis has accorded Ethical Clearance.

Title : Role of reticulocyte haemoglobin parameter in evaluation of microcytic hypochromic anaemia.

Name of P.G. Student : Dr Sohan Rao H R.
Department of Pathology.

Name of Guide/Co-investigator: Dr.Mahesh.H.Karigoudar, Professor of Pathology.

DR RAGHAVENDRA KULKARNI
CHAIRMAN
Institutional Ethical Committee
BLDEU's Shri B.M. Patil
Medical College,BIJAPUR-586103.

Following documents were placed before E.C. for Scrutinization:

1) Copy of Synopsis/Research Project
2) Copy of informed consent form.
3) Any other relevant documents.

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BLDE

(DEEMED TO BE UNIVERSITY)

Declared as Deemed-to-be-University u/s 3 of UGC Act, 1956

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE(DU)/REG/PG-Guide/2020-21/ 674 July 11, 2020

To,
The Professor and HOD
Department of Pathology,
BLDE (DU)'s Shri B. M. Patil Medical College,
Hospital and Research Centre,
Vijayapura

Madam,

Sub: Regarding change of PG Guide.
Ref: Your letter no. Path/2020/530 dated 1st July, 2020.

With reference to the subject and letter cited above, on approval of the Hon'ble Vice-Chancellor, the change of PG Guide is permitted in respect of PG Student of your department as per below:

Sl. No.	Name of the Student	Previous Guide	New Guide	Batch/ Year
1.	Dr. Sohan Rao	Dr. Mahesh Karigoudar	Dr. R. M.Potekar	2018-19
2.	Dr. Bithika Dey	Dr. Mahesh Karigoudar	Dr. Surekha B. Hipparagi	2018-19
3.	Dr. Saswati S.	Dr. Mahesh Karigoudar	Dr. R. M. Potekar	2019-20

This is for your information and needful.

REGISTRAR

REGISTRAR
BLDE (Deemed to be University)
Vijayapura-586103. Karnataka

Copy to:

- The Dean, Faculty of Medicine and Principal
- The Controller of Examinations
- The Concerned PG Teacher

Smt. Bangaramma Sajjan Campus, Sholapur Road, Vijayapura – 586103, Karnataka, India.
University: Phone: +918352-262770, Fax: +918352-263303, Website: www.bldedu.ac.in, E-mail: office@bldedu.ac.in
College: Phone: +918352-262770, Fax: +918352-263019, Website: www.bldedu.ac.in, E-mail: bmpmc.principal@bldedu.ac.in

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ANNEXURE-II

BLDE (DEEMED TO BE UNIVERSITY)

SHRI B.M.PATIL MEDICAL COLLEGE HOSPITAL AND RESEARCH CENTRE,

VIJAYPURA-586103

RESEARCH INFORMED CONSENT FORM

TITLE OF THE PROJECT: "ROLE OF RETICULOCYTE HEMOGLOBIN PARAMETER IN EVALUATION OF MICROCYTIC HYPOCHROMIC ANEMIA"

PRINCIPAL INVESTIGATOR: DR. SOHAN RAO H. R
P.G. DEPARTMENT OF PATHOLOGY

P.G GUIDE : DR. RATNAKAR M POTEKAR MD
PROFESSOR, DEPT OF PATHOLOGY.

PURPOSE OF RESEARCH:

I have been informed that the present study is a study of the utility of Reticulocyte hemoglobin equivalent in the evaluation of microcytic hypochromic anemia.

PROCEDURE:

I understand that I undergo a detailed history and, after which necessary investigations will be done.

RISK AND DISCOMFORTS:

I understand that there is no risk involved for me being a part of the study.

BENEFITS:

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I understand that my participation in the study will help to know the role of the utility of Reticulocyte Hemoglobin equivalent in the evaluation of microcytic hypochromic anemia.

CONFIDENTIALITY:

I understand that the medical information produced by the study will become a part of the hospital record and will be subjected to confidentiality and privacy regulations of the hospital. If data is used for publications, the identity of the patient will not be revealed.

REQUEST FOR MORE INFORMATION:

I understand that I might be asked for more information about my disease at any time.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw from the study at any time

INJURY STATEMENT:

I understand that in the unlikely event of injury to me during the study, I will get medical treatment but no further compensations.

I have read the fully understood this consent form. Therefore, I agree to participate in the present study.

____

Participant/Guardian Date:

____

Signature of Witness Date:

I have explained the patient the purpose of study, the procedure required, and possible risk and benefit of my ability in the vernacular language.

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____

Investigator/P.G Date:

____

Witness to Signature Date:

DocuSign Envelope ID: 5E1FBDE2-4FD7-460A-B1D1-726CEED8EC0E

ANNEXURE - III

PROFORMA

NAME : OP/IP No.:

AGE :

SEX : D.O.A :

RELIGION : D.O.D:

OCCUPATION :

RESIDENCE :

Presenting Complaints :

Past history :

Personal history :

Family history :

Treatment history :

General physical examination:

Pallor present/absent

Icterus present/absent

Clubbing present/absent

Lymphadenopathy present/absent

Edema present/absent

Built poor/average/well

VITALS: PR: RR:

BP: TEMPERATURE:

WEIGHT:

DocuSign Envelope ID: 5E1FBDE2-4FD7-460A-B1D1-726CEED8EC0E

SYSTEMIC EXAMINATION:

Cardiovascular system:

Respiratory system:

Per Abdomen:

Central nervous system:

Clinical Diagnosis:

INVESTIGATIONS:

Red blood cell count (RBC count):

Hemoglobin (Hb):

Haematocrit / Packed cell volume (HCT/PCV):

Mean corpuscular volume (MCV):

Mean corpuscular hemoglobin (MCH):

Mean corpuscular hemoglobin concentration (MCHC):

Red cell distribution width (RDW):

Reticulocyte count:

Absolute Reticulocyte Count:

Reticulocyte Production Index (RPI):

Immature Reticulocyte Fraction (IRF):

Reticulocyte Hemoglobin equivalent (RET-He):

Peripheral smear study: RBCs:

WBCS

Platelets:

Abnormal cells:

DocuSign Envelope ID: 5E1FBDE2-4FD7-460A-B1D1-726CEED8EC0E

Impression:

Total Iron binding capacity (TIBC):

Serum Iron (Fe):

Serum Ferritin:

Other Investigations (If any):

DocuSign Envelope ID: 5E1FBDE2-4FD7-460A-B1D1-726CEED8EC0E

MASTER CHART

Sl No	Patient Id	Age	Sex	RBC	Hb	PCV	MCV	MCH	MCHC	RDW	ReticCount	RPI	IRF	Ret HE	S Fe	TIBC	S Ferritin
1	OP 163132/19	68	M	3.46	5.8	20.8	60.1	16.8	27.9	22.1	0.63	0.4	19.4	12.6	8	420	6.38
2	OP 163177/19	66	M	2.94	4.3	17.5	59.5	14.6	24.6	22.8	2.41	0.4	30.8	12.6	431	615	3.98
3	OP 162353/19	0.8	F	4.19	5.9	29.2	69.7	14.1	20.2	35.7	6.5	2.3	45	15.6	43	475	38.54
4	OP 162381/19	24	F	3.85	8.3	30.3	73.7	21.6	27.4	18.6	0.56	0.2	21.9	18.9	2	340	6.26
5	OP 162480/19	62	M	4.13	8.7	27.9	67.6	21.1	31.2	28.2	0.9	0.3	23.2	24.4	21	120	98.58
6	OP 162324/19	24	F	4.33	7.2	28.5	65.8	16.6	25.3	28	1.04	0.4	45.7	18.2	393	155	330.93
7	OP 162444/19	1.5	F	4.49	6.1	24.5	54.6	13.6	24.9	22	0.83	0.2	14.2	13	11	570	8.68
8	OP 162373/19	21	F	3.44	4.6	18.5	53.8	13.4	24.9	22.5	0.73	0.1	7	10.1	2	420	1.36
9	OP 163211/19	32	F	4.57	10.5	32.7	71.7	23	32.1	16	0.71	0.3	4.8	20.1	28	380	7.26
10	OP 163152/19	37	F	4.56	9.6	30.4	66.7	21.1	31.6	15.7	0.71	0.3	5.9	17.3	2	385	6.04
11	OP 162564/19	28	F	4.37	10.9	33.7	74.1	24.9	32.3	13.8	0.62	0.3	4.6	22.3	3	345	4.35
12	OP 163217/19	13	M	3.72	4.8	21.8	58.6	12.9	22	26	2.24	0.5	32.8	12.4	11	425	13.72
13	OP 162396/19	0.6	F	4.52	6.4	27.3	60.4	14.2	23.4	23.9	2.42	0.8	31.4	12.4	29	565	5.62
14	OP 162573/19	26	F	4.35	10.9	33.6	75	25.1	32.4	14.3	0.88	0.4	4.8	26.9	7	430	6.05
15	OP 162618/19	30	F	4.5	10.2	32.5	72.2	22.7	31.4	15.5	0.68	0.3	8.1	22.6	5	372	6.42
16	OP 162482/19	50	F	4.34	10.9	34.3	74.9	24.8	31.7	15.2	0.97	0.5	10.5	25.2	41	345	20.85
17	OP 162402/19	40	M	3.09	4.2	18	58.3	13.6	23.3	23.1	4.04	0.7	48.3	18.6	31	485	13.56
18	OP 162399/19	44	F	4.07	6.1	26.3	58	15	25.8	19.3	1.32	0.3	21.2	12.2	11	550	1.47
19	OP 162393/19	35	F	4.58	7.1	24.4	53.3	15.5	29.1	18.3	1.27	0.3	32.7	13.6	185	520	20.96
20	OP 162368/19	32	M	2.6	5.9	17.6	67.7	22.7	33.5	31.4	13.92	2.3	41.2	22.6	208	220	659.66
21	OP 162251/19	21	F	3.76	6.5	22.6	60.1	17.3	28.8	23.3	0.7	0.2	16	14.9	14	501	15.52
22	OP 162613/19	18	F	4.39	8.5	30.7	69.9	19.4	27.7	26.4	2.12	0.8	16.1	17.6	2	418	57.91
23	OP 162215/19	50	F	4.5	7.6	25.8	57.3	25.8	16.9	26.4	2.3	0.7	13.1	18.4	43	485	28.93
24	OP 162440/19	4	M	1.69	2.4	10.3	60.9	14.2	23.3	28.6	0.32	0	13	12.4	2	435	2.69
25	OP 162142/19	44	M	5.07	6.6	24.5	48.3	13	26.9	24.7	1.21	0.3	31.2	12.5	2	455	4.07
26	OP 162385/19	3	F	5.97	9.6	33	55.3	16.1	29.1	18.2	0.75	0.3	18.3	15	32	525	9.1
27	OP 162361/19	4	F	5.04	8	26	51.6	15.9	30.8	20.4	1.1	0.3	11.6	15.5	11	355	31.87
28	IP 41714/18	23	F	4.45	9.7	33.1	74.4	21.8	29.3	25.7	1.35	0.56	10.5	25.4	31	318	15.3
29	OP 172330/19	25	F	4.05	8.1	29.2	72.1	20	27.7	30	1.4	0.5	39.9	12.7	34	560	3.83
30	OP 172365/19	50	M	3.13	4	16.9	54	12.8	23.7	23.2	1.7	0.3	29.5	10.9	49	610	21.64
31	OP 172480/19	35	F	4.27	5.9	23	53.9	13.8	25.7	21.5	2.2	0.5	26.8	11.7	53	620	9.16
32	OP 172533/19	48	F	3.43	4.6	21	61.2	13.4	21.9	22.9	1.5	0.3	14.7	11.2	19	495	1
33	OP 172605/19	66	M	4.37	7.7	28	64.1	17.6	27.5	19.1	2.3	0.8	29.1	16.7	110	350	220.91
34	OP 173374/19	26	F	2.74	5.6	18.6	67.9	20.4	30.1	16.3	1.2	0.2	11.8	15.1	28	525	24.54
35	OP 173342/19	26	F	3.46	8.4	25.3	73.1	24.3	33.2	14.3	2.2	0.6	26.6	16	14	570	5.61
36	OP 173379/19	65	M	2.24	5.5	17.5	74.1	24.6	31.4	18.2	3.9	0.6	19.6	22.2	2	591	781.1
37	OP 172680/19	19	F	2.05	3.3	12.9	62.9	16.1	25.6	26.1	1.7	0.2	20.4	14.7	26	645	1
38	OP 172290/19	36	F	4.19	7.2	26.5	63.2	17.2	27.2	22.6	1.04	0.3	19.6	13.3	38	585	16.31
39	OP 172370/19	41	F	3.87	5.4	22	56.8	14.7	25.9	21.8	1.51	0.3	15.7	10.8	26	385	20.87
40	OP 173355/19	30	M	5.38	8.1	29.1	54.1	15.1	27.8	21.2	1.4	0.5	21	12.8	13	575	1.5
41	OP 172460/19	66	M	4.31	8.2	26.1	60.6	19	31.4	18.3	1.2	0.3	16	16.2	4	470	6.59
42	OP 172298/19	27	M	1.66	2.8	10.7	64.5	16.9	26.2	32.3	1	0.1	4.9	11.5	216	399	181

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43	OP 173359/19	65	F	3	7.4	23.6	74.7	24.7	31.4	17.2	0.9	0.2	9.8	18.2	44	635	7.13
44	OP 172462/19	19	F	3.36	10.2	30.2	74.1	23.7	31.4	18.2	1.04	0.3	14.4	28.6	82	330	128.87
45	OP 172363/19	47	F	5.77	9.5	36.5	63.3	16.5	26	20.2	1.4	0.8	18.1	12.9	21	440	1.01
46	OP 172619/19	63	F	3.19	5.7	22	69	17.9	25.9	24	2.6	0.6	36.1	20.5	30	405	32.59
47	OP 172554/19	49	M	4.09	8	28.6	69.9	19.6	28	23.4	4.6	1.6	36.6	16.7	26	520	44.41
48	OP 172301/19	32	F	4.3	7.8	25.9	60.2	18.1	30.1	21.7	0.8	0.2	11.5	15.7	18	510	7.11
49	OP 172348/19	24	F	4.68	6.7	24.7	52.8	14.3	27.1	22.9	1.2	0.3	26.7	11.3	9	540	2.12
50	OP 173371/19	72	M	4.17	6.9	25.7	61.6	16.5	26.8	21.7	0.9	0.3	19.7	13	13	480	5.89
51	OP 172525/19	21	F	2.7	4.1	17	63	15.2	24.1	21.3	2.7	0.4	17.6	10.5	16	405	3.1
52	OP 172630/19	2	M	3.84	5.1	21.9	57	13.3	23.3	23.2	2.2	0.5	35.2	11.8	20	700	5.1
53	OP 172661/19	53	F	3.8	8.3	27.1	71.3	21.8	30.6	18.9	1.1	0.3	18.6	17.3	37	610	5.66
54	OP 172656/19	5	F	1.83	2.8	11.4	62.3	15.3	24.6	24	0.82	0.1	6.7	10.4	55	760	8.14
55	IP 17268/19	55	F	3.41	6.9	24.9	73	20.2	27.7	20.9	1.04	0.4	12.6	19.3	5	800	7.59
56	IP 17375/19	50	M	3.44	5.2	21.9	63.7	15.1	23.7	26.4	2.51	0.85	33.1	17.6	23	411	9.85
57	IP 17368/19	65	M	3.35	4.7	17.4	51.9	14	27	23.7	1.22	0.23	7	14	35	307	9.42
58	IP 17355/19	26	F	4.09	8.3	28.9	70.7	20.3	28.7	17.9	0.6	0.2	7.9	16.4	2	192	275
59	IP 18090/19	45	F	3.41	4.2	18.9	55.4	12.3	22.2	23.4	1.66	0.31	20.5	11.7	15	430	4.32
60	IP 18444/19	22	F	3.29	4.5	19.7	59.9	13.7	22.8	27.9	2.64	0.52	38.6	13.9	345	403	34.3
61	IP 19795/19	60	F	2.78	4.8	20.4	73.4	17.3	23.5	19.9	0.86	0.17	17.9	15.1	2	263	5.05
62	IP 19898/19	35	F	3.11	4.4	18	57.9	14.1	24.2	21.2	1.6	0.29	21.2	12.6	2	527	3.4
63	IP 25037/19	14	F	3.93	6.1	20	50.9	15.5	30.5	20.8	0.94	0.19	17.5	16.1	9	548	28.05
64	IP 25017/19	48	F	3.1	6.4	21.3	68.7	20.6	30	18.9	1.31	0.28	18.3	15.9	2	463	4.18
65	IP 25049/19	80	F	3.04	6.6	21.7	71.4	21.7	30.4	19.4	2.58	0.56	26.6	23.9	24	253	103.28
66	IP 24606/19	45	F	3.87	5.5	21.3	55	14.2	25.8	21.2	1.02	0.22	18	13.7	80	459	20
67	OP 211096/19	35	M	4.79	6.6	26	54.3	13.8	25.4	23.1	2.591	0.8	26.3	14.5	38	485	15.2
68	OP 212718/19	40	F	3.91	8	25.7	65.7	20.5	31.1	17.6	0.8	0.2	7.3	14.6	7	480	1
69	OP 211020/19	50	F	2.89	4	16	55.4	13.8	25	20	1.6	0.2	20.3	10.8	14	440	2.56
70	OP 211136/19	51	M	5.33	9.4	32.8	61.5	17.6	28.7	17.4	1.26	0.6	27	14.8	9	525	3.21
71	OP 211241/19	37	F	4.48	7.7	27.8	60.7	16.8	27.7	20.8	1.27	0.4	19.8	14.1	35	325	9.04
72	OP 210043/19	2	M	4.97	6.9	25.8	51.9	13.9	26.7	23	0.8	0.2	16.1	12.9	50	760	7.8
73	OP 211208/19	40	F	4.15	7	24.4	58.8	16.9	28.7	19.5	1.8	0.5	25.6	13.3	10	470	1.36
74	OP 211265/19	27	F	4.5	8.9	29.2	64.9	19.8	30.5	16.8	1.1	0.4	13.7	16.4	16	405	3.2
75	OP 211376/19	22	M	4.01	6	22.3	55.6	15	26.9	21.3	0.9	0.2	12	12	19	495	1
76	OP 211234/19	20	F	3.91	6.4	24.9	63.7	16.4	25.7	26	1.5	0.4	43.6	14	40	585	13.75
77	OP 211286/19	64	M	3.46	6.8	24.1	69.7	19.7	28.2	20.1	2.1	0.6	33.3	16.7	38	690	7.54
78	OP 209978/19	5	F	3.76	4.6	19.3	51.3	12.2	23.8	22.5	1.6	0.3	26.1	11.6	15	480	3.44
79	OP 209971/19	2	F	5.1	10.4	31.1	61	20.4	33.4	16.6	0.1	0.04	0	18.7	2	380	4.87
80	OP 211280/19	19	M	4.67	7.7	28.1	60.2	16.5	27.4	22.6	1.48	0.5	24.2	16.3	14	495	13.31
81	OP 212803/19	8	M	4.02	6.8	24.3	60.4	16.9	28	25.1	0.9	0.2	22.2	10.8	20	610	1.74
82	OP 211365/19	20	M	4.65	8.7	27.9	60	18.7	31.2	21.1	2.6	0.9	12.4	15.3	21	340	108.37
83	OP 212809	4	M	3.03	3.9	16.1	53.1	12.9	24.2	23.2	0.6	0.1	10.2	10.6	19	405	1.11
84	OP 211328/19	27	F	5.5	10.5	36.1	65.6	19.1	29.1	27.4	0.8	0.5	12.5	16.1	5	372	6.94
85	OP 211292/19	27	F	2.12	4.1	15.2	71.7	19.3	27	22.6	0.9	0.1	27.5	16.7	58	330	202.04
86	OP 212789/19	1	M	1.67	5.1	10.2	61.1	30.5	50	32	8.2	0.7	27.1	11.1	271	355	293.92
87	OP 210021/19	1	F	4.75	6	23.9	50.3	12.6	25.1	23.9	1.5	0.4	40.2	11.4	16	405	2.36

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88	OP 209963/19	3	M	4.8	6	25.8	53.8	12.5	23.3	25.4	2.1	0.6	26.8	12.3	32	580	12.56
89	OP 210106/19	4	F	4.96	7.4	26.1	52.6	14.9	28.4	23	0.6	0.2	14.8	13.4	7	385	32.56
90	OP 212816/19	2	F	4.93	6.9	27.1	55	14	25.5	21.8	0.9	0.3	12.8	12.7	10	465	3
91	OP 211199/19	65	M	3.95	5.1	20.3	51.4	12.9	25.1	24.3	0.65	0.1	26.5	12.6	24	525	6.08
92	OP 211215/19	18	F	4.87	5.3	22.8	46.8	10.9	23.2	25.7	0.8	0.2	27.6	9.8	7	585	1
93	OP 211363/19	58	F	3.5	8.1	26.7	74.8	23.1	30.3	24.9	2.91	1	22.4	28.9	35	368	236.18
94	OP 211045/19	34	F	4.16	5.9	22.7	54.6	14.2	26	19.4	1.05	0.2	17.5	12.2	14	585	4.19
95	OP 212806/19	2	F	3.65	4.6	18.9	51.8	12.6	24.3	24.8	1.4	0.3	18.3	10.8	19	495	2.06
96	OP 211303/19	29	F	4.93	7.8	27.3	55.4	15.8	28.6	27	4.2	1.3	24.9	16	10	550	29.47
97	OP 210144/19	12	M	3.13	5	20.6	65.8	16	24.3	22.2	1.6	0.3	21.3	14.1	37	630	5.26
98	IP 42537/19	55	F	4.35	8	30.8	70.8	18.4	26	35.4	2.65	1.02	34	22.9	226	>800	508
99	IP 42736/19, OP 470705	77	M	4.89	10.4	31.7	64.8	21.3	32.8	19.6	2.1	1.11	23.8	18.9	149	173	1000
100	IP 42696/19	33	M	4.95	10.6	35.2	71.1	21.4	30.1	18.4	0.79	0.46	12.3	24.2	2	485	17.5
101	IP 43346/19	85	M	2.1	4.2	16.7	72.2	20	25.1	21.8	2.47	0.41	17.6	16.8	396	403	34.3
102	OP 11097	25	F	3.75	7.2	24.7	65.9	19.2	29.1	22.5	1.05	0.3	17.4	13.5	12	428	3.9
103	OP 11103/19	39	F	4.62	8.6	28.7	62.1	18.6	30	16.5	1.06	0.4	26.4	14.4	162	440	1.74
104	OP 11100/19	40	F	4.43	8.3	31.7	71.6	18.7	26.2	19.3	1.02	0.4	18.5	14.7	10	540	4.59
105	OP 26755/19	48	M	5.58	10.9	35.3	63.3	19.5	30.9	20.8	1.49	0.8	32	15.2	17	485	11.18
106	OP 27662/19	18	F	4.28	7.4	25.2	58.9	17.3	29.4	21.2	1.9	0.5	13.9	12.5	12	575	1
107	OP 159043/20	0.6	M	2.05	4.6	12.4	60.5	22.4	37.1	38	2.5	0.3	24.9	14.2	2	380	146.41
108	OP 159010/20	1	M	4.39	6.6	23.1	52.6	15	28.6	24.7	1.25	0.3	32.8	13.1	30	405	52.28
109	OP 159012/20	2	M	4.55	6	24.3	53.4	13.2	24.7	22.5	0.9	0.2	17.1	11.6	2	340	5.06
110	OP 159013/20	1	M	4.33	5.2	20.8	48	12	25	22.8	1.21	0.3	20.6	10	12	375	6.78
111	OP 159014/20	6	M	4.43	7.1	25	56.4	16	28.4	19.3	1.19	0.3	18.3	13.3	20	610	4.65
112	OP 53638/19	24	M	3.52	5.7	21.7	61.6	16.2	26.3	30.5	4.42	1	32.8	14.3	43	475	19.21
113	OP 159015/20	1.5	F	4.37	6.7	24.1	55.1	15.3	27.8	21.3	0.65	0.2	10.8	11.3	20	795	20.94
114	OP 53636/19	66	F	3.73	6.4	22.4	60.1	17.2	28.6	20.1	1.23	0.3	16	12.9	12	470	6.39
115	OP 159017/20	1	M	4.27	7.1	22.6	52.9	16.6	31.4	21.4	0.36	0.1	12	11.9	26	500	350
116	OP 53637/19	25	F	4.28	6.4	20.4	47.7	15	31.4	21	0.84	0.2	11.5	10.6	13	575	3.18
117	op 53635/19	17	F	4.68	8.3	29.1	62.2	17.7	28.5	19.5	1.04	0.4	19.9	14.5	10	465	3.21
118	OP 159018/20	4	M	5.79	6.6	26.9	64.5	15.8	24.5	22.4	1.12	0.4	14.9	13.9	11	355	70.45
119	OP 159019/20	14	F	4.23	6.6	24.4	57.7	15.6	27	18.3	0.79	0.2	15.9	12.6	16	440	3.02
120	OP 159020/20	2	M	4.36	5.5	22.5	51.6	12.6	24.4	25.9	0.94	0.2	8.9	10.7	28	525	28.52
121	OP 53634/19	60	M	4.01	8.6	29	72.3	21.4	29.7	33.9	1.59	0.6	16.1	15.6	43	380	26.96
122	OP 53633/19	18	F	1.89	4.9	14.3	74.9	25.9	34.3	17.4	1.3	0.2	10.3	17.2	13	260	124.44
123	OP 53632/19	35	F	4.99	7.9	29.4	58.9	15.8	26.9	22	1.31	2.1	19.1	12.1	18	620	10.52
124	OP 159022/20	0.6	M	2.03	5.5	12.4	61.1	27.1	44.4	32.9	2.78	0.3	28.4	11.8	110	350	215.56
125	OP 159023/20	1	M	4.12	4.9	20.3	49.3	11.9	24.1	22.4	0.73	0.1	12	9.7	11	425	12.14
126	OP 53626/19	25	F	2.98	6.4	19.5	65.4	21.5	32.8	22	1.76	0.3	38.1	15.5	289	390	9.89
127	OP 53625/19	29	F	3.43	6.1	21.7	63.3	17.8	28.1	18.6	0.56	0.1	11.3	13.4	354	430	8.34
128	OP 53622/19	30	M	4.36	6.4	25.8	59.2	14.7	24.8	20.9	1.58	0.5	25.9	10.8	16	495	2.93
129	OP 159025/20	13	M	3.83	4.8	18.4	48	12.5	26.1	20.9	2	0.4	26.5	9.6	7	403	28.27
130	OP 53621/19	38	F	4.17	8.1	28.1	67.4	19.4	28.8	18.2	0.75	0.3	13.5	14.7	19	495	1
131	OP 159026/20	0.8	M	3.48	4.9	19.7	56.6	14.1	24.9	19.7	1.25	0.2	9.5	10.9	43	475	46.6

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132	OP 136704/19	19	F	3.7	7	23.1	62.4	18.9	30.3	18	1.17	0.3	8.3	12.8	53	620	1.39
133	OP 159028/20	3	M	3.76	5.6	20.1	53.5	14.9	27.9	18.9	1.55	0.3	19.6	11.7	58	330	227.96
134	OP 136714/19	24	F	4.28	8.5	28.3	66.1	19.9	30	18.6	1.19	0.4	17.1	14.1	20	610	1.5
135	OP 138827/19	25	M	3.17	4.6	18.7	59	14.5	24.6	23.6	1.02	0.2	12.3	11.2	19	495	1
136	OP 144322/19	40	F	4.13	7.9	26.2	63.4	19.1	30.2	15.4	1.3	0.4	22.2	13.8	4	512	6.05
137	OP 146126/19	23	M	4.69	7.2	28.1	59.9	15.4	25.6	19.4	1.35	0.5	14.4	11.1	2	527	1.25
138	OP 146283/19	62	F	3.85	8.7	27.6	71.7	22.6	31.5	15.6	2.11	0.7	27	15.9	13	480	4.78
139	OP 159029/20	1.8	F	4.41	6.3	21.6	49	14.3	29.2	22.8	0.56	0.1	8.3	11	9	548	34.68
140	OP 168126/19	60	M	3.65	8.1	26.3	72.1	22.2	30.8	20.6	1.28	0.4	22.1	14.1	20	150	36.73
141	OP 168128/19	72	F	3.41	5.2	19.1	56	15.2	27.2	21.5	1.37	0.3	17.3	10.9	50	760	5.16
142	OP 168130/19	22	F	4.96	10.3	34.7	70	20.8	29.7	25.1	1.65	0.8	12.8	17.8	66	525	50.12
143	IP 13800/20	39	M	2.1	3.1	12.2	58.1	14.8	25.4	20.8	1.66	0.16	22.3	12.6	19	575	2.29
144	IP13837/20	2	F	4.58	6.1	21	45.9	13.3	29	25.9	1.24	0.32	40.6	13.6	2	440	9.49
145	IP 13698/20	24	F	3.75	8.7	28.1	74.9	23.2	31	21.3	0.67	0.23	2	27.4	2	380	146.41
146	IP 13862/20	37	F	4.15	8.8	28.3	68.2	21.2	31.1	17.2	1.45	0.51	20.4	20.3	30	405	52.28
147	IP 13859/20	55	F	4.3	9.5	30	69.8	22.1	31.7	18.6	0.83	0.31	14.3	22.4	26	520	44.41
148	IP 13909/20	45	F	4.06	7.2	25	61.6	17.7	28.8	19.8	1.09	0.34	23	18.1	10	550	29.47
149	OP 168131/19	28	F	4.05	8.1	29.2	73.2	20	27.7	30	1.4	0.5	39.9	12.7	34	545	3.72
150	OP 168133/19	48	M	4.56	9.5	30.4	66.7	21.1	31.6	15.7	0.71	0.3	5.9	17.3	12	385	6.04
151	OP 168132/19	32	M	4.57	10.2	32.7	71.7	23	32.1	16	0.71	0.3	4.8	20.1	28	395	7.26
152	OP 168129/19	24	F	4.33	7.4	28.5	65.8	16.6	25.3	28	1.04	0.4	45.7	18.2	180	155	330.93
153	OP 183780/19	78	M	2.94	4.2	17.5	59.5	14.6	24.6	22.8	2.41	0.4	30.8	12.6	35	615	2.93
154	OP 184061/19	65	F	3.01	7.3	23.6	74.7	24.7	31.4	17.2	0.9	0.2	9.8	18.2	46	655	7.13
155	OP 184114/19	28	F	4.51	10.1	32.5	72.2	22.7	31.4	15.5	0.68	0.3	8.1	22.6	5	372	6.42
156	OP 184227/19	22	M	2.61	5.8	17.6	67.7	22.7	33.5	31.4	13.92	2.3	41.2	22.6	208	385	59.66
157	OP 184126/19	43	F	3.41	4.1	18.9	55.4	12.3	22.2	23.4	1.66	0.31	20.5	11.7	15	430	4.32
158	OP 159031/20	14	M	3.93	6.4	20	50.9	15.5	30.5	20.8	0.94	0.19	17.5	16.1	6	548	28.05
159	OP 184287/19	38	F	4.3	7.9	25.9	60.2	18.1	30.1	21.7	0.8	0.2	11.5	15.7	18	510	7.11
160	OP 184005/19	38	F	4.57	7.2	24.4	53.3	15.5	29.1	18.3	1.27	0.3	32.7	13.6	185	520	20.96
161	OP 184080/19	16	F	4.28	7.4	25.2	58.9	17.3	29.4	21.2	1.9	0.5	13.9	12.5	12	575	1
162	OP 188062/19	19	F	1.88	4.9	14.3	74.8	25.9	34.3	17.4	1.3	0.2	10.3	17.2	30	260	124.44
163	OP 188066/19	65	F	3.72	6.4	22.4	60.1	17.2	28.6	20.1	1.23	0.3	16	12.9	8	470	6.39
164	OP 188329/19	16	M	3.74	4.8	21.8	58.6	12.9	22	26	2.24	0.5	32.8	12.4	11	425	13.72
165	OP 188344/19	18	F	3.46	8.3	25.3	73.1	24.3	33.2	14.3	2.2	0.6	26.6	16	14	570	5.61
166	OP 188596/19	22	F	2.69	4.1	17	63	15.2	24.1	21.3	2.7	0.4	17.6	10.5	16	405	3.1
167	OP 190710/19	24	F	4.52	8.9	29.2	64.9	19.8	30.5	16.8	1.1	0.4	13.7	16.4	16	445	3.2
168	OP 159032/20	12	M	3.83	4.8	18.4	48	12.5	26.1	20.9	2	0.4	26.5	9.6	7	403	28.27
169	OP 193834/19	60	F	4.34	10.9	34.3	74.9	24.8	31.7	15.2	0.97	0.5	10.5	25.2	38	345	20.85
170	OP 194290/19	27	M	4.01	6.1	22.3	55.6	15	26.9	21.3	0.9	0.2	12	12	5	495	1
171	OP 194350/19	20	F	1.87	4.9	14.2	74.5	25.9	34.3	17.4	1.3	0.2	10.3	17.2	20	260	124.44
172	OP 194431/19	60	M	3.46	6.8	24.1	69.7	19.7	28.2	20.1	2.1	0.6	33.3	16.7	38	690	7.54
173	OP 194671/19	30	M	1.64	2.8	10.6	64.5	16.9	26.2	32.3	1	0.1	4.9	11.5	216	399	181
174	OP 194676/19	42	M	4.93	10.5	35.2	71.1	21.4	30.1	18.4	0.79	0.46	12.3	24.2	2	485	17.5
175	OP 194675/19	18	Y	3.29	4.5	19.7	59.9	13.7	22.8	27.9	2.64	0.52	38.6	13.9	345	403	34.3
176	OP 194678/19	26	M	4.35	6.5	25.7	59.3	14.7	24.8	20.9	1.58	0.5	25.9	10.8	16	495	2.93

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177	OP 11097/19	70	F	3.72	6.4	22.4	60.2	17.2	28.6	20.1	1.23	0.3	16	12.9	12	470	6.39
178	OP 11103/19	48	F	4.43	8.2	31.7	71.6	18.7	26.2	19.3	1.02	0.4	18.5	14.7	10	540	4.59
179	OP 52622/19	14	F	4.26	6.6	24.4	57.7	15.6	27	18.3	0.79	0.2	15.9	12.6	16	440	3.02
180	OP 53624/19	33	F	4.16	5.8	22.7	54.5	14.2	26	19.4	1.05	0.2	17.5	12.2	14	585	4.19
181	OP 53627/19	68	M	4.03	8.6	29	72.3	21.4	29.7	33.9	1.59	0.6	16.1	15.6	43	380	26.96
182	OP 53629/19	40	F	4.96	7.9	29.3	58.8	15.8	26.9	22	1.31	2.1	19.1	12.1	18	620	10.52
183	OP 53630/19	78	M	4.89	10.1	31.7	64.8	21.3	32.8	19.6	2.1	1.11	23.8	18.9	149	173	1000
184	OP 53631/19	42	M	4.91	10.6	35.2	71.1	21.4	30.1	18.4	0.79	0.46	12.3	24.2	2	485	17.5
185	OP 53628/19	18	M	3.52	5.7	21.7	61.6	16.2	26.3	30.5	4.42	1	32.8	14.3	43	475	19.21
186	OP 159034/20	14	M	4.23	6.6	24.1	57.4	15.6	27	18.3	0.79	0.2	15.9	12.6	16	440	3.02
187	OP 53623/19	16	M	4.65	8.7	27.9	60.1	18.7	31.2	21.1	2.6	0.9	12.4	15.3	21	340	108.37
188	OP 159035/20	12	F	3.82	4.8	18.4	48.1	12.5	26.1	20.9	2	0.4	26.5	9.6	7	403	28.27
189	OP 65931/19	15	M	3.93	6.1	20	50.9	15.5	30.5	20.8	0.94	0.19	17.5	16.1	9	548	28.05
190	OP 66692/19	45	M	5.56	10.8	35.3	63.3	19.5	30.9	20.8	1.49	0.8	32	15.2	17	485	11.18
191	OP 106579/19	48	F	3.73	6.4	22.4	60.1	17.2	28.6	20.1	1.23	0.3	16	12.9	12	470	6.39
192	OP 429068/19	39	F	4.12	7.8	26.2	63.5	19.1	30.2	15.4	1.3	0.4	22.2	13.8	4	512	6.05
193	OP 429067/19	65	M	3.94	5.1	20.3	51.5	12.9	25.1	24.3	0.65	0.1	26.5	12.6	24	525	6.08
194	OP 429066/19	18	F	3.28	4.5	19.8	59.8	13.7	22.8	27.9	2.64	0.52	38.6	13.9	345	403	34.3
195	OP 429065/19	18	F	3.71	7	23.1	62.5	18.9	30.3	18	1.17	0.3	8.3	12.8	53	620	1.39
196	OP 159038/20	14	M	3.82	4.8	18.4	48.1	12.5	26.1	20.9	2	0.4	26.5	9.6	7	403	28.27
197	OP 429061/19	18	F	4.28	6.4	20.4	47.7	15	31.4	21	0.84	0.2	11.5	10.6	13	575	3.18
198	OP 429058/19	16	M	4.67	7.7	28.2	60.2	16.5	27.4	22.6	1.48	0.5	24.2	16.3	14	495	13.31
199	OP 159041/20	14	M	3.16	5.1	20.6	65.6	16	24.3	22.2	1.6	0.3	21.3	14.1	37	630	5.26
200	OP 429057/19	28	M	4.1	8.3	28.9	70.6	20.3	28.7	17.9	0.6	0.2	7.9	16.4	2	192	275
201	IP 43346/19	60	F	3.3	6.9	23.4	70.9	20.9	29.5	26.4	2.9	0.68	46	20.4	29	327	106

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Key to master chart

Sl No	Serial number
OP	Outpatient
IP	Inpatient
RBC	Red blood cell count (10*6/uL)
Hb	Hemoglobin (g/dL)
PCV	Packed cell volume (%)
MCV	Mean corpuscular volume (fL)
MCH	Mean corpuscular hemoglobin (pg)
MCHC	Mean corpuscular hemoglobin concentration (%)
RDW-CV	Red cell distribution width (%)
ReticCount	Reticulocyte count (%)
RPI	Reticulocyte Production Index (%)
IRF	Immature Reticulocyte Fraction (%)
RetHe	Reticulocyte Hemoglobin equivalent (pg/cell)
S Fe	Serum Iron (µg/dL)
TIBC	Total Iron binding capacity (µg/dL)
S Ferritin	Serum Ferritin (ng/mL)