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Original Research

Correlation Of Hba2 By HPLCand Serum Ferritin Levels in Pregnant Females With Co-Existence of Iron Deficiency Anemia

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Abstract

Background: Anemia is a condition in which a person's hemoglobin (Hb) concentration and red blood cell (RBC) count are low and insufficient to meet physiological needs; it affects around one-third of the world's population. This study aims to correlate the value of borderline HbA2 and serum ferritin to rule out the coexistence of iron deficiency anemia and Beta thalassemia trait (BTT).

Methodology: This study was done on 407 antenatal cases of any trimester without any signs and symptoms in all pregnant females recruited both from rural and urban areas of Punjab Department of Biochemistry in Government Medical College and Rajindra Hospital, Patiala, during the period of 1 and half year, with approval of the ethical committee. Hb and Red cell indices were measured on an automated hematology analyzer. HBA2 was examined by the HPLC method. Serum ferritin levels were analyzed by using a standardized chemical kit based on the ELISA technique.

Results: Blood indices such as MCV, MCH, MCHC, RDW-CV, RBC count, and PCV were within typical ranges for anemic conditions. IDA (HBA2 < 3.2%) occurred in 88% of the mothers, and serum ferritin levels ≤14 ng/mL in 92.9% of the cases. In comparison based on HBA2 levels, MCV, MCHC, RDW, HBA2, and Serum Ferritin showed significant differences, indicating varying degrees of anemia severity among the groups.

Conclusion: This study brings into perspective the complicated interaction between BTT and IDA in pregnant women. The findings put into light the prevalence of iron deficiency among the population, highlighting the need for targeted screening and management strategies to fight maternal health during pregnancy.

Keywords: Anemia, HBA2, Serum Ferritin, iron deficiency anemia, Beta thalassemia trait.
Assessment (SOFA)

Introduction

Anaemia is a worldwide public health issue affecting developing and industrialized nations. It has significant effects on both human health and social and economic development[1]. It can occur in all stages of life, however antenatal mothers and children are particularly susceptible[2]. Anemia is a condition in which a person's hemoglobin (Hb) concentration and red blood cell (RBC) count are low and insufficient to meet physiological needs; it affects around one-third of the world's population.[3]

Anaemia is associated with increased morbidity and mortality in mothers and children, along with poor birth outcomes, decreased work productivity in adults, and poorer cognitive and behavioral development in children[6,7,8,9,1011]. When Hb is less than 13 g/dL, in accordance with World Health Organization recommendations (Adult males) Hb < 12g/dl (Adult females, non-pregnant) Hb <11g/dL (females, pregnant) is considered anemia. [4,5]

Anemia can be caused by multiple factors that often coexist. Iron deficiency anemia is the most prevalent form of anemia. WHOestimates that around 30% of the world's population, or approximately 2.1 billion individuals, have iron deficiency anemia[6]. Iron deficiency is more prevalent in low and high-income countries. It is commonly believed that iron deficiency is the cause of 50 percent of cases of anemia; however,

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this proportion may vary between regions and population groups. [7]

IDA is a common nutritional disorder in developing countries in the pregnant women. Pregnancy greatly increases the overall iron demand compared to non-pregnancy, because of the exponential increase of iron needs to expand the plasma volume, produce a greater quantity of red blood cells, support the growth of the fetal-placental unit, and compensate for iron loss at delivery [8]. Thalassemia is the commonest single gene disorder representing a major health burden in India and worldwide. They are a group of autosomal recessive disorders in which there is inhibition in the production of α or β globin chains of hemoglobin resulting in varying levels of anemia [9]. Thalassemia disorders and IDA are the two most common causes of microcytic hypochromic anemia. Research studies have found that iron deficiency occurs in patients with beta-thalassemia trait (BTT). Previous studies found reduced initial hemoglobin levels in patients with IDA and BTT [10,11]. HbA2 levels have been observed to be lower in patients with coexisting IDA and BTT, but levels improve with iron therapy. The decline in HbA2 levels in patients with concomitant BTT and IDA has been suggested to interfere with the diagnosis of the former. Mostly IDA and BTT usually coexist, making it challenging to distinguish between the two due to similar findings such as microcytosis and hypochromia. [12]

The series of tests, including serum ferritin, serum iron, and hemoglobin A2 (HbA2) levels, can be used to provide an accurate diagnosis [13]. In humans, RBCs contain mostly iron (2–2.5 g out of total body iron of 3–4 g) which is present in the form of hemoglobin [14]. The principal iron-storing compound in the body is ferritin, which is primarily found in the reticuloendothelial cells of the intestines, liver, and bone marrow. Ferritin is a reliable quantitative indicator of the total iron content of the storage compartment. It is normally present in the circulating plasma ranging from 31 to 300µg/l. Therefore, it can be used as a sensitive measure to detect iron deficiency at its initial stages[15,16]. Iron stores are categorized as: a. Depleted, when serum ferritin (SF) value is < 14 µg/l b. Reduced, when value ranges between 15-30 µg/l c. Normal or replete, at a value between 31-300 µg/l d. Increased, keeping values beyond 300µg/l Iron deficiency anemia is diagnosed when the Serum ferritin (SF) level is below 12 µg/l. Serum ferritin with low values serves as a reliable. [17,18]

The purpose of the present study is early detection of BTT by HbA2 estimation and coexistence of Beta thalassemia trait and IDA during the routine screening of antenatal cases by measuring serum ferritin levels in cases who have HbA2 in the borderline range. This can be evaluated by calculating HbA2 through High-performance liquid chromatography (HPLC) and serum ferritin through Enzyme-linked immunosorbent assay (ELISA) technique.

Methodology

This study was done on 407 antenatal cases of any trimester without any signs and symptoms in all pregnant females recruited both from rural and urban areas of Punjab in the Department of Pathology and association with the Department of Biochemistry in Government Medical College and Rajindra Hospital, Patiala, during the period of 1 and half year, with approval of the ethical committee. This study was done after obtaining consent from each patient.

The inclusion criteria for the study were all antenatal cases of reproductive age group (18y- 45y) with Hb less than 10 gm% and all antenatal cases of reproductive age group with mean corpuscular volume (MCV) less than 80 fl, while the exclusion criteria were any pregnant female having Hb greater than 10 gm% or already diagnosed cases of beta thalassemia trait or any history of blood transfusion less than three months.

Blood collection and serum preparation: A history of any blood transfusions was taken and then 5mL intravenous blood sample was drawn from all patients under aseptic conditions and collected in two different vials. 3ml of blood was drawn into an EDTA vial to measure Hb, Red cell indices, and HBA2. Hb and Red cell indices were measured on an automated hematology analyzer. (Med source alpha count 60) (Figure 1) and HbA2 was studied by HPLC method (Figure 2). In the 2nd vial 2ml blood was taken and incubated for 20-25mins at room temperature after then centrifuged at 3000 rpm for 10mins. Serum was extracted from the clear supernatant to measure the serum ferritin levels in each subject. Serum samples can be stored refrigerated for 5 days at 2-8 degree celsius. If storage time exceeds 5 days, store frozen at -20 degree celsius for up to one month. Serum ferritin levels were analyzed by using a standardized chemical kit (Cal biotech) based on the ELISA technique. (Figure 3)

Statistical analysis: The SPSS software for Windows, version 26.0, was used to perform the statistical analysis (SPSS, Chicago, Illinois). Categorical data were shown as absolute numbers and percentages, whereas continuous variables were shown as mean ± SD. Before statistical analysis, the normality of the data was checked. Continuous variables were compared using the unpaired t-test, and Categorical variables were analyzed using either the chi-square test or Fisher's exact test. A p-value of <= 0.05 was considered to be significant.

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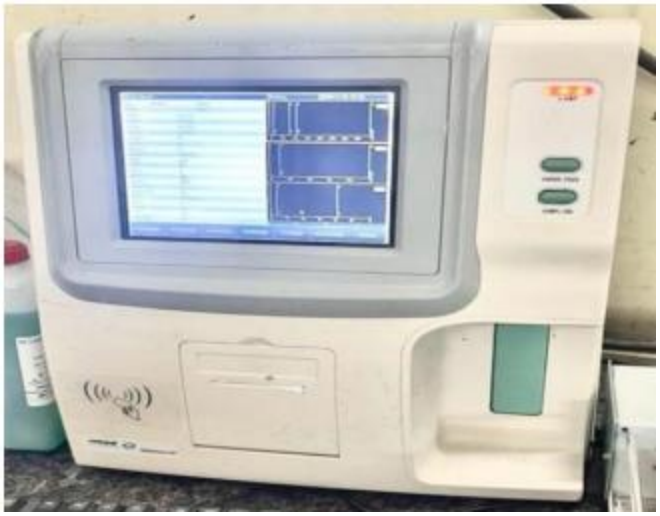

Figure 1: Automated hematology Analyzer (Medsource alpha count 60).

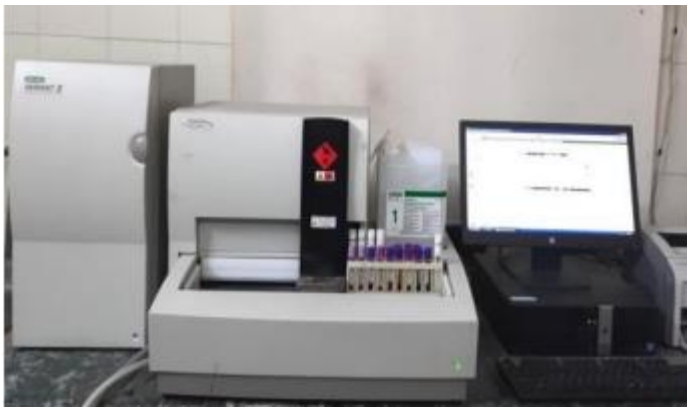

Figure 2: HPLC (Bio-Rad Variant II Hemoglobin Testing System).

Figure 3: Cali biotech kit used for the analysis of serum ferritin.

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Results

The table 1 describes demographic distribution of antenatal mothers with anemia. The age group under 20 years has 13 mothers (3.2%), while the 21 25 years group has 150 (36.9%), and the 26-30 years group was the largest with 190 (46.7%) mothers. There was a drop to 48 (11.8%) mothers in the 31 35 years group, and the smallest group was over 35 years, with only 6 (1.5%) mothers. This indicates that most antenatal mothers were between 21 and 30 years old, with significantly fewer older than 35. The distribution of antenatal mothers with respect to their previous obstetric history shows that the "Gravida" section shows that 44.7% of mothers were experiencing their first pregnancy, 36.6% their second, and smaller percentages for higher numbers of pregnancies. The "Para" section indicates that 51.6% of mothers have had no previous births, while 32.9% have had one previous birth. Most mothers (52.3%) have not had any live births, and 89.2% have not experienced any abortions. A very high percentage (98%) have not had any stillbirths.

Table 1: Demographic distribution of antenatal mothers with anemia.

		Frequency	Percent
Age group (antenatal mothers)	**<20**	13	3.2
	21-25	150	36.9
	26-30	190	46.7
	31-35	48	11.8
	>35	6	1.5
Obstetric History			
Gravida(G)	G1	182	44.7
	G2	149	36.6
	G3	68	16.7
	G4	7	1.7
	G6	1	0.2
Para(P)	P0	210	51.6
	P1	134	32.9
	P2	58	14.3
	P3	4	1
	P4	1	0.2
Live births (L)	L0	213	52.3
	L1	139	34.2
	L2	51	12.5
	L3	4	1
Abortions (A)	A0	363	89.2
	A1	37	9.1
	A2	6	1.5
	A4	1	0.2
Still Birth (S)	S0	399	98
	S1	8	2

The data on the table 2 shows the distribution of antenatal mothers with respect to any medical or surgical illnesses. The vast majority (99.28%) of mothers reported no medical or surgical illnesses. A very small percentage of (0.72%) mothers reported other chronic illnesses like Diabetes, hypertension, or placenta previa. This indicates that almost all of the antenatal mothers in this group do not have significant medical or surgical conditions.

Table 2: Distribution of antenatal mothers with respect to any medical or surgical illness

Any medical or surgical illness:	Frequency	Percent
No chronic illness	404	99.28
Chronic illness (Diabetes, hypertension, and placenta previa)	3	0.72
Total	407	100

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Table 3 describes a detailed distribution of antenatal mothers concerning various red cell indices. For RBC count, 43.5% of individuals have counts below 3.93 ×10^12/L, with a mean of 4.04 and a standard deviation of 0.50, while 56.5% have counts of 3.93 ×10^12/L or higher. For PCV, 95.8% of the individuals possess values not above 35.5%, a mean of 28.25 and a standard deviation of 3.90, and 4.2% possess only values above 35.5%. For MCV, 100% of the individuals have values not exceeding 80 fL, with a mean of 69.90 and a standard deviation of 4.89. Similarly, for MCH, the entire sample was comprised of values less than 27 pg with mean 20.25 and standard deviation 1.91, and for MCHC, all individuals have values less than 320 g/L with mean 29.03 and standard deviation 2.63. Lastly, RDW-CW distribution shows 6.67% of patients with values between 11.5% and 15.4%, with the average being 19.78 and the standard deviation being 2.33, and 93.33% with values over 15.4%.

Table 3: distribution of antenatal mothers with respect to red blood cell indices.

RED CELL INDICES	VALUES	Frequency	Percent	Mean	SD
RBC count	<3.93	177	43.5	4.04	0.50
	=>3.93	230	56.5		
PCV	<=35.5	392	95.8	28.25	3.90
	>35.5	17	4.2		
MCV	<=80	407	100%	69.90	4.89
MCH	<27	407	100%	20.25	1.91
MCHC	<320	407	100%	29.03	2.63
RDW-CW	11.5-15.4	27	6.67	19.78	2.33
	>15.4	380	93.33		

Table 4 indicates the distribution of antenatal mothers based on serum ferritin level and HBA2. Out of the 407 mothers, 92.9% of them have low levels of ferritin (<=14 ng/mL) while the rest of the mothers have serum ferritin >14ng/mL. When it comes to HBA2, most of them, 88% of the total sample (358 individuals), have HBA2 levels <3.2, IDA diagnosis confirmed. Roughly 9.3% of the mothers, a total of 38, fall into the borderline range (3.2-3.8), which may present a transitional status. Another small percentage, 2.7% of the sample (11 individuals), have HBA2 levels above 3.8, which is an indication of a thalassemia trait.

Table 4: Distribution of antenatal mothers with respect to serum ferritin levels and HBA2.

	Frequency	Percent
Serum ferritin levels(ng/mL)		
<=14	378	92.9%
>14	29	7.1%
HBA2		
IDA confirmed (<3.2)	358	88
Borderline (3.2-3.8)	38	9.3
Thalassemia trait (>3.8)	11	2.7

Table 5 describes the association of hba2 levels with serum ferritin levels.In the group with HBA2 levels <3.2%, the vast majority (99.4%) have serum ferritin levels ≤14 ng/ml, while only 0.6% have levels >14 ng/ml. In the 3.2%-3.8% HBA2 group, 44.7% have serum ferritin levels ≤14 ng/ml, and 55.3% have levels >14 ng/ml. Similarly, in the >3.8% HBA2 group, 45.5% are ≤14 ng/ml and 54.5% are >14 ng/ml. 92.9% of the entire sample are ≤14 ng/ml and 7.1% are >14 ng/ml. The Chi-square value of 193.76 with p-value <0.001 indicates a statistically significant association between HBA2 levels and serum ferritin levels, which means that greater levels of HBA2 are related to higher serum ferritin levels.

Table 5: Association of hba2 levels with serum ferritin levels.

Serum ferritin	HBA2							
	<3.2%		3.2-3.8%		>3.8		Total	
	n	%	n	%	n	%	N	%
<=14 ng/ml	356	99.4%	17	44.7%	5	45.5%	378	92.9%

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>14ng/ml	2	0.6%	21	55.3%	6	54.5%	29	7.1%
Total	358	100.0%	38	100.0%	11	100.0%	407	100.0%
Chi-square	193.76							
p-value	<0.001							

Data in table 6 describe the comparison of HBA2 with various parameters among three groups classified by HBA2 levels (3.8%). There were no variations in hemoglobin level among the groups, and therefore all had equal average values. The RBC was equal among the groups, without any statistical variation found. PCV among the group of >3.8% was increased, but the variation was not statistically significant. MCV was also higher in the >3.8% group, indicating increased mean cell size in this group. MCH is notably different between groups but not statistically different. But MCHC was notably lower in the >3.8% group, indicating decreased hemoglobin content per cell in this group. RDW was significantly higher in the <3.2% group, indicating increased variability of red cell size. Serum Ferritin was also much higher in this group, with both of these differences being statistically significant. These findings indicate large differences in MCV, MCHC, RDW, HBA2, and Serum Ferritin across the different HBA2 level groups, while other parameters are quite unchanging.

Table 6: Comparison of hba2 with various parameters among antenatal mothers.

Parameter	<3.2% (Mean ± SD)	3.2%-3.8% (Mean ± SD)	>3.8% (Mean ± SD)	t-test	pvalue
Hemoglobin (g/dL)	8.11 ± 0.90	8.37 ± 0.93	8.28 ± 0.99	1.537	0.216
RBC count (10^12/L)	4.05 ± 0.50	4.03 ± 0.52	4.05 ± 0.69	0.013	0.987
PCV (%)	28.29 ± 3.90	27.59 ± 3.09	30.25 ± 7.68	1.929	0.147
MCV (fL)	69.96 ± 4.88	68.72 ± 5.09	74.11 ± 7.53	4.983	0.007
MCH (pg)	20.17 ± 1.92	20.86 ± 1.71	20.67 ± 1.85	2.519	0.082
MCHC (g/dL)	28.90 ± 2.70	30.35 ± 1.09	28.19 ± 3.88	5.817	0.003
RDW (%)	20.02 ± 1.29	17.84 ± 6.30	17.36 ± 4.97	219.876	0.000
HBA2 (%)	2.69 ± 0.28	3.30 ± 0.13	7.44 ± 7.35	87.902	0.000
Serum ferritin (ng/mL)	9.63 ± 2.35	30.42 ± 37.27	81.51 ±105.25	82.599	0.000

Discussion

A total of 407 antenatal mothers were screened and selected based on values of Hemoglobin, MCV, MCH, MCHC, RBC count, RDW, and HbA2 was studied by the HPLC method. Cases with HbA2 more than the cut-off value of (>3.8%) were classified as Thalassemia trait and all those with HbA2 in the range of 3.2 3.8% were Borderline cases. In the present study, the mean age of the mother was 26.72±3.81 years with the majority of antenatal mothers with IDA within the age group of 26-30 years with 190(46.7%) mothers. The mean hemoglobin levels were 8.1±.90 g/dL with a range of 4.1 9.90 gm/dl. The results were in accordance with the study conducted by Verma et al who studied 30 patients with concomitant IDA and BTT. They found the mean hemoglobin level to be 9.8 ± 1.1 g/dL with a range of 8.2 11.2 g/dL although this study wasn't done on antenatal mothers. [19]. The significant burden of anemia in various population groups in India, particularly among children and pregnant women. Additionally, the prevalence of Beta thalassemia trait (BTT) in India is notable, ranging from 3.5% to 10%, according to the National Family Health Survey (NFHS-3) data from 2011.

In the present study, we found that the majority, comprising 88% of the total sample exhibit HBA2 levels below 3.2, confirming a diagnosis of IDA. About 9.3% of the mothers, fall within the borderline range (3.2-3.8). A smaller proportion, representing 2.7% of the sample, have HBA2 levels exceeding 3.8, indicative of a thalassemia trait. A study conducted by Arora et al. on 90 females with IDA, where found that 93.3% of the patients had HbA2 levels less than 2%. Additionally, 44% of the patients had HbA2 levels greater than 4.0%, which characterizes the BTT, and the remaining 2.22% had HbA2 levels between 3.0% and 4.0% [20]. Some authors have suggested that iron deficiency (ID) can interfere with the determination of HbA2 levels, leading to false-positive or false negative results. Intracellular iron deficiency reduces α-globin chain synthesis relative to non-α-globin chains.[21] However, some studies suggest that the Beta-thalassemia trait doesn't necessarily confer an advantage in maintaining iron balance, and HbA2 levels are not significantly lower in the presence of IDA, which aligns with the findings of the present study [22]. A recent study by Dolai TK et al,

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from south India, also showed lower hemoglobin levels in BTT with iron deficiency than those without [23]. In our study also, the mean hemoglobin value did not differ across three groups i.e. antenatal mothers with iron deficiency anemia, borderline, and thalassemia trait.

In the present study, serum ferritin was analyzed to confirm the diagnosis of IDA and beta-thalassemia. We observed that Serum ferritin levels also demonstrated a statistically significant difference between these groups ($p = 0.024$) i.e. patients with borderline HbA2 and with thalassemia trait with serum ferritin being more in patients with thalassemia trait. On categorizing mothers based on HBA2 levels (<3.2, 3.2-3.8 and >3.8) and serum ferritin levels (<=14, >14 ng/mL), 92.9% of the total sample have serum ferritin levels ≤14 ng/ml and 7.1% have levels >14 ng/ml. The Chi-square test value of 193.76 with a p-value of <0.001 indicates a statistically significant association between HBA2 levels and serum ferritin concentrations, suggesting that higher HBA2 levels are correlated with higher serum ferritin levels. Quereshi et al conducted a study on 135 individuals with confirmed cases of the beta-thalassemia trait. These findings revealed that 100 individuals (74%) showed serum ferritin levels within the normal range, 17 individuals (12.6%) had levels above the normal range, and 18 individuals (13.4%) had levels below normal [24]

Arshad et al conducted a study in Pakistan to investigate the impact of iron deficiency on Hb-A2 levels in individuals with the beta-thalassemia trait and to determine how frequently these individuals might be missed due to concurrent iron deficiency. The study highlights the potential for iron deficiency to mask the beta-thalassemia trait, suggesting that additional screening measures are necessary to avoid misdiagnosis. [25] In our study there were 17 borderline cases whose serum ferritin was <=14ng/mL in these cases Oral iron replacement should be given between meals to enhance absorption, and a response should be seen as an increase in hemoglobin level within two weeks. If a patient's anemia is multifactorial (for example, if iron deficiency occurs on a background of thalassemia or anemia of chronic disease), the hemoglobin level will not accurately reflect the effectiveness of iron replacement. The serum ferritin or the ratio of serum transferrin receptor (sTfR) to serum ferritin can be used to follow the response to iron replacement in these cases.[26]

Study limitation: The study limitation was the failure to evaluate iron status following iron therapy.

Conclusion

This study emphasizes the complex interaction between IDA and BTT in pregnant women. The majority of the subjects exhibited characteristics of IDA, with low levels of HBA2 and significantly low serum ferritin levels. These findings indicate the prevalence of high iron deficiency in this group, emphasizing the need for targeted screening and management strategies to ensure maternal health during pregnancy. The borderline HBA2 levels and their association with this wide spectrum of serum ferritin concentrations have been of tremendous importance for practical application. Critical practice approaches in diagnosis instead of assessment of only HBA2 might make all the differences in most cases, especially where iron deficiency coincides with BTT. By elucidating the diagnostic problems and clinical significance of the findings, this study provides us with valuable information to maximize the accuracy of diagnoses and guide individualized interventions for antenatal care in an effort to maximize maternal andfetaloutcomes.

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