Analysis of Serum Markers Under the Sandwich Elisa Method in Children Between 12 and 120 Months With Iron Deficiency Anemia in the City of Arequipa

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Abstract
Introduction: Anemia is a pathology present in the blood, which affects mainly children and pregnant women living in developing countries and has several associated factors, of which molecular factors have been little studied. Objective: To analyze different serum molecular markers related to anemia. Material and methods: Samples of blood from 40 children with iron deficiency anemia and 10 children without anemia from the city of Arequipa were analyzed as controls. Abbkine ELISA detection kits were used, including Hepcidin (HEP25), erythropoietin (EPO), vitamin B12(VITB-12), interleukin 6 (IL-6), folic acid, ferritin light chain (FTL), C-reactive protein (CRP) and transferrin (TF) to determine their serum concentration and establish their relationship with childhood anemia. Results: We found a hepcidin level of 40.88 ug/Lm lower than the control 126.06 ug/L (p = 0.019); and an average interleukin 6 level (p = 3.83E-6) of 12.55 ug/L, lower than the control of 4.30 ug/L. For the erythropoietin marker, no statistical difference was found between cases and the control group; for the Vitamin. B12, a significant statistical difference was found between cases and control. However, the variable is ambiguous since it has not yet been determined with certainty how important the lack of vitamin B12 may be for the generation of iron deficiency anemia. No significant difference was found for protein C, transferrin, erythropoietin, and folic acid. Conclusion: It was found that the molecular markers: Hepcidin, vitamin B12, IL-6, Folic Acid and C-reactive protein presented statistically significant differences with the control group. The markers for erythropoietin and transferrin showed no changes. Likewise, the vitamin B12 marker was observed to have a lower value in children with anemia. It is hoped that further work can establish the impact of vitamin B12 and the categorization of the other molecular markers for the detection of iron deficiency anemia.
Keywords: iron deficiency anemia, molecular markers, sandwich ELISA

1. Resumen

Introducción: La anemia es una patología presente en la sangre, que afecta sobre todo a niños y gestantes que viven en países en desarrollo y que tiene a diversos factores asociados, de los cuales, han sido poco estudiados los factores moleculares. Objetivo: Analizar diferentes marcadores moleculares séricos, relacionados con la anemia. Material y métodos: Se analizaron muestras de sangre de 40 niños con anemia ferropénica y como control 10 niños sin anemia, provenientes de la ciudad de Arequipa. Se emplearon kits de detección ELISA de la marca Abbkine, dentro de los cuales están: Hepcidina (HEP25), eritropoyetina (EPO), vitamina B12(VITB-12), interleuquina 6 (IL-6), ácido fólico, cadena ligera de la ferritina (FTL), proteína C reactiva (CRP) y transferrina (TF) para determinar su concentración sérica y establecer su relación con la anemia infantil. Resultados: Se encontró un nivel de hepcidina de 40.88 ug/Lm inferior al control 126.06 ug/L (p = 0,019); y un nivel de interleucina 6 (p = 3,83E-6) promedio de 12,55 ug/L, inferior con respecto al control de 4,30 ug/L. Para marcador de eritropoyetina, no se halló diferencia estadística entre los casos y el grupo control; para el marcador Vitamina. B12 se halló una diferencia estadística significativa entre los casos y el control, sin embargo, la variable es ambigua ya que aún no se ha llegado a determinar con seguridad que tan importante puede ser la

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falta de Vit. B12 para la generación de anemia ferropénica. Para la proteína C, transferrina, eritropoyetina y ácido fólico no se encontró diferencia significativa. Conclusión: Se encontró que los marcadores moleculares: Hepcidina, vitamina B12, IL-6, Ácido Fólico y proteína C reactiva presentaban diferencias estadísticamente significativas con el grupo control. Los marcadores para eritropoyetina y transferrina no presentaron cambios. Igualmente, se observó que el marcador de vitamina B12 presentaba un valor más bajo en niños con anemia. Se espera que en trabajos posteriores se pueda establecer el impacto de la vitamina B12, así como la categorización de los otros marcadores moleculares para la detección de la anemia ferropénica.

Palabras clave*: anemia ferropénica, marcadores moleculares, ELISA sándwich.*

2. Introduction

Anemia is a pathology in the blood defined by a decrease in the hemoglobin concentration in the organism [1]. Its structure possesses iron metal, so it is an essential element in the human being. One of the iron markers is the hormone hepcidin, which is expressed in liver tissue and regulates iron metabolism, controlling absorption and distribution in other tissues. Regulation of hepcidin expression occurs at the transcriptional level and affects serum iron levels, iron stores, inflammation, hypoxia and changes in erythropoietic activity. Iron deficiency is the most popular anemia caused by nutritional deficiency and is a public health problem in developing countries. The negative regulatory marker of iron deficiency anemia is hepcidin which is suppressed, inhibiting the compensatory mechanism that increases iron absorption by the intestine and reduces iron sequestration by macrophages [2].

However, iron deficiency anemia is not the only type of anemia. While 50% of anemias are due to iron deficiency, the others are due to other factors such as folic acid deficiency, vitamin B12 deficiency, vitamin A deficiency, and protein malnutrition. Other less common causes are heavy metal intoxication, either by trace amounts such as zinc or copper, and some recent studies suggest that low vitamin D levels also contribute to the onset of anemia [3]. The diagnosis of anemia involves a relationship between the clinical evaluation of the patient, looking for related symptoms and signs, and accompanied by laboratory support that consists of requesting hematological tests such as hemoglobin, erythrocyte count and corpuscular constants, from which other markers of anemia can be requested according to what is found. Peru is a country with high rates of anemia in rural areas, mostly deficiency anemia due to the lack of micronutrient intake in children; it is also a highly rugged country with significant variations in altitude; therefore, hemoglobin concentration increases as altitude increases to compensate for the decrease in available oxygen [4]. The present study aims to analyze the molecular markers expressed in deficiency anemias in children with anemia in the town of Arequipa; to know their level and behavior during the disease.

3. Material and methods

Study population

Blood and serum samples of 40 children identified with anemia were analyzed by puncture and measurement by Hemocue ® Hb301 Hemoglobinometer. Also, 10 children without anemia were selected as a control group from the city of Arequipa.

Design

This is an observational field study.

Procedures

Sera from cases and controls were used for the determinations; at the same time, 8 Abbkine kits were used. It should be considered that these are designed to work in the detection range shown below.

Hepcidin (HEP25): 2.5 ug/L - 40 ug/L
Erythropoietin (EPO): 3.75 IU/L
Vitamin B12(VITB-12): 2.5 nmol/L - 40 nmol/L
Folic acid: 22.5 ng/mL - 360 ng/mL
Ferritin light chain (FTL): 100pg/mL - 1600 pg/mL
Transferrin (TF): 3.125 ug/mL - 50ug/mL
Interleukin 6 (IL-6): 3.13pg/mL - 200 pg/mL
C-reactive protein (CRP): 15.6 pg/ mL- 1000 pg/mL

The kits corresponding to hepcidin, erythropoietin, vitamin B12, folic acid, Ferretin light chain and Transferrin follow the same protocol as mentioned in the title "Protocol 1" with a duration of at least 2 hours due to incubation times, while Interleukin 6 and C-reactive protein were made using "Protocol 2" kits had a duration of about four hours.

Protocol 1

The company's recommendations were brief: 50 ul of standard or 1/5 diluted samples were added to the corresponding well. Incubated at 37 °C for 45 minutes. Then washing was done as follows, the contents of each well were aspirated, and 250 ul of wash buffer was added after incubating for 1 minute and completely aspirated for the next step; the washing process was repeated 5 times. Add 50 ul of second HRP-conjugated antibody diluted 1:100, to each well and incubate at 37 °C for 45 minutes. Washed 5 times, the same as the previous washing step. Add 100 ul of substrate mixture A and B (1:1) and incubate at 37 °C for 15 minutes in the dark. Finally, 50 ul of stop solution was added to each well; the color should change from blue to yellow; let stand for 15 minutes and measured absorbance in an Elisa reader at 450 nm.

Protocol 2

The company's recommendations were brief: 100 ul of standard or 1/5 diluted samples were added to the corresponding well. Incubated at room temperature for 2 hours. Then washing was done as follows, the contents of each well were aspirated, and 250 ul of wash buffer was added after incubating for 1 minute and completely aspirated for the next step; the washing process was repeated 3 times. Added 100 ul of the first antibody against IL-6 h or C-reactive protein diluted 1:100 to each well and incubated for 1 hour at room temperature. Washed 3 times as in the previous washing step. Add 100 ul of Streptavidin-HRP conjugate diluted 1:100 and incubate in the dark for 30 min. Wash five times. Add 100 ul of TMB substrate to each well and incubate for 15 min at room temperature in the dark. Finally, add 50 ul of stop solution to each well. The color should change from blue to yellow; let stand for 15 minutes and measure absorbance in an Elisa reader at 450 nm.

Statistical analysis of data: The t-student test was used for statistical analysis, given that the sample evaluated presents parametric characteristics. Since the data were continuously quantitative, the t-statistic was applied, emphasizing the equality or difference between variances, and the F-test was previously performed to evaluate variances.

Ethical aspects: The study is part of the project approved by the research ethics committee, Favorable opinion 326 -2021 CEI-UCSM.

4. Results

Thirty-nine sera from patients with anemia called "cases" and 10 sera from apparently healthy patients called "control" were evaluated. To obtain more accurate data, duplicate measurements were performed, so the average of these measurements will be used for statistical analysis since they will provide a more accurate view of the evaluations.

Table 1. Mean HEPCIDIN values in children with anemia and controls.		
	Anemia	Control
Hepcidin (ug/L)	40.88	126.06
n	39	10
Standard deviation	71.11	93.63
	40.88±11.3	126.06±29.6
In order to demonstrate the difference between cases and controls, the following hypothesis was proposed: Ho= No significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 0.019 is to the left of alpha 0.05, which provides statistically significant evidence at a confidence level of 95%, thus leading to the rejection of H0 and acceptance of H1, i.e., it is possible to state that there is a significant difference between cases and controls.

Table 2. Mean EPO values in children with anemia and controls		
	Anemia	Control
EPO (IU/L)	38.54	37.6
n	39	10
Standard deviation	28.13	14.60
	38.54±4.5	37.6±4.6
In order to demonstrate the difference between cases and controls, the following hypotheses are proposed: Ho= No significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 0.885 is to the right of alpha 0.05, which allows with statistically significant evidence, at a confidence level of 95%, the Ho is accepted and the H1 is rejected, i.e., it is possible to state that there is no significant difference between cases and controls.

Table 3. Mean Vit B12 values in children with anemia and controls.		
	Anemia	Control
Vit B12 (nmol/L)	14.73	57.91
n	39	10
Standard deviation	10.60	34.97
	14.73±1.7	57.91±11.06
In order to demonstrate the difference between cases and controls, the following hypotheses are proposed: Ho= There is no significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 0.003 is to the left of alpha 0.05, which allows for statistically significant evidence at a confidence level of 95% to reject the Ho and accept H1, i.e., it is possible to state that there is a significant difference between cases and controls.

Table 4. Mean IL-6 values in children with anemia and controls.		
	Anemia	Control
IL-6 (pg/mL)	12.55	4.30
n	39	10
Standard deviation	8.72	1.51
	12.55±1.5	57.91±0.5
In order to demonstrate the difference between cases and controls, the following hypotheses are proposed: Ho= There is no significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 3.83E-06 is to the left of alpha 0.05, which allows with statistically significant evidence at a confidence level of 95%, the Ho is rejected and H1 is accepted, that is, it is possible to affirm that there is a significant difference between cases and controls.

Table 5. Average Folic Acid values in children with anemia and controls.		
	Anemia	Control
Folic Acid (ng/mL)	3.99	16.24
n	39	10
Standard deviation	2.19	3.95
	3.99±0.35	16.24±1.25
In order to demonstrate the difference between cases and controls, the following hypotheses are proposed: Ho= No significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 2.73E-06 is to the left of alpha 0.05, which allows with statistically significant evidence at a confidence level of 95%, the Ho is rejected and H1 is accepted, that is, it is possible to affirm that there is a significant difference between cases and controls.

Table 6. Mean FTL values in children with anemia and controls.		
	Anemia	Control
Folic Acid (pg/mL)	223.26	238.046
n	39	10
Standard deviation	210.10	279.53
	223.26±33.64	238.046±88.39
In order to demonstrate the difference between cases and controls, the following hypotheses are proposed: Ho= There is no significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 0.878 is to the right of alpha 0.05, which allows, with statistically significant evidence at a confidence level of 95%, to accept the Ho and reject H1, that is, it is possible to affirm that there is no significant difference between cases and controls.

Table 7. Mean CRP values in children with anemia and controls.		
	Anemia	Control
CRP (pg/mL)	321.09	393.33
n	39	10
Standard deviation	124.52	31.33
	321.09±19.9	393.33±9.9
In order to demonstrate the difference between cases and controls, the following hypotheses are proposed: Ho= There is no significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 0.00216 is to the left of alpha 0.05, which allows for statistically significant evidence at a confidence level of 95% to reject the Ho and accept H1, i.e., it is possible to state that there is a significant difference between cases and controls.

Table 8. Mean TF values in children with anemia and controls.		
	Anemia	Control
TF (ug/mL)	127.11	117.52
n	39	10
Standard deviation	26.77	41.26
	127.11±4.28	117.52±13.04
In order to demonstrate the difference between cases and controls, the following hypotheses are proposed: Ho= There is no significant difference between cases and controls. H1= There is a significant difference between cases and controls		

The two-tailed P-value of 0.49 is to the right of alpha 0.05, which allows with statistically significant evidence at a confidence level of 95% to accept the Ho and accept H1, i.e., it is possible to state that there is no significant difference between cases and controls.

5. Discussion

This study aimed to evaluate the levels of molecular markers normally used for predicting anemia and to determine if they are adequate markers. A population of 39 patients between 1 and 10 years of age was analyzed and compared versus 10 healthy controls for Hepcidin, EPO, Vit. B21, IL-6, Folic Acid, C-reactive protein and transferrin markers.

In the case of Hepcidin, it was found in an average serum level of 40.88 ug/L in comparison with the control value of 126.06 ug/L. Performing the t-student analysis, a significant difference was found ($p=0.019$). These results are similar to those of Staffan K Berglund [5] and Rita Wegmüller [6], in which low hepcidin values served as effective predictors of anemia in infant populations. Similarly, in a study conducted in China, serum hepcidin levels in patients with iron deficiency anemia were found to be significantly lower than those in the control group ($P<0.001$) and were positively correlated with serum hemoglobin concentration, serum iron, serum ferritin and transferrin saturation ($r=0.448$, $r=0.496$, $r=0.754$, $r=0.491$) [7]. In Zimbabwe, anemic infants were studied at 3 months finding higher CRP (median 0.45 vs. 0.21 mg/L; $P=0.037$) and high hepcidin (median 14.7 vs. 9.7 ng/ml; $P=0.022$) than non-anemic infants. Anemic infants at 6 months had lower hepcidin at control (median 7.9 versus 4.5 ng/mL; $P=0.016$) and high CRP (median 2.33 versus 0.32 mg/L; $P<0.001$), but less ferritin (median 13.2 versus 25.1 μg/L; $P<0.001$) than nonanemic infants. Anemic infants at 12 months had lower ferritin (median 3.2 vs. 22.2 μg/L; $P<0.001$) and low hepcidin (median 0.9 vs. 1.9 ng/mL; $P=0.019$), but similar CRP levels [8]. These results demonstrate that hepcidin levels are low during anemia, possibly due to a failed process of ferric homeostasis. High levels of hepcidin limit iron absorption at the small intestine level, while low values induce erythrocyte generation and iron release from the bone marrow [9]. It is possible that, in the case of iron deficiency anemia, low levels are present as an attempt by the body to raise hemoglobin levels; however, iron deficiency anemia caused by malnutrition would counteract the effects of low hepcidin levels [10].

On the other hand, in the erythropoietin marker, no significant difference was found between the clinical cases and the control group ($p=0.885$). This result differs from other studies, such as that of Yael Ben-David [11], which showed that high EPO values are correlated with low hepcidin values since hepcidin is mainly affected by iron status and not by inflammation, so that it could be more specific in iron deficiency anemia. The low erythropoietin response is most notable in chronic kidney disease, which is the second leading cause of iron deficiency anemia, together with inflammation levels contributing to the anemia, and is expressed by high ferritin values [12].

For the Vitamin B12 marker, a significant statistical difference was found between cases and controls. This variable is ambiguous since it has not yet been determined with certainty how important vitamin B12 deficiency can be in producing iron deficiency anemia. In a study carried out in Mexico on a population of almost 5000 pre-school and school-age infants under eleven years of age, it was found that only a low percentage of the infants with anemia were deficient in vitamin B12; however, it was noted that about 30% of the patients had consumed food before the sample was taken, which may have somewhat underestimated vitamin B12 deficiency [13]. Another study on micronutrient deficiency as a predictor of anemia found that only 29% of children were deficient in vitamin B12, which was not significantly associated with iron deficiency anemia [14]. Despite this, other studies show a contrary trend. In a study conducted in India, vitamin B12 and vitamin B9 deficiency were found in 53.7% and 68% of children, respectively [15]. In Mexico, a study was conducted in which vitamin B12 was measured in children aged 12 to 23 months, obtaining a higher prevalence of vitamin B12 deficiency compared to children aged 48 to 59 months (4 vs. 1.3%), and in a logistic regression, it was found that indigenous children had more vitamin B12 deficiency than children living in the southern urban area. In addition, no statistical difference was found between sex and body mass index [16]. The recent study by Janmejaya et al. demonstrated by spectrophotometric and computational techniques that vitamin B12 can bind to the hemoglobin molecule; although the exact mechanism of action of vitamin B12 is not known, these results would explain the significant change in serum level of the vitamin [17].

Regarding the IL-6 marker, we found one of the greatest significant statistical differences between our cases and controls; however, this is different from what was found in other studies, such as that of Athraa Abdul Kareem Mseer [18] in which IL-6 levels were not substantially altered. However, in studies in adults [19] and chronic diseases [20] it was found to be a good predictor of anemia. This is probably because IL-6 is an effective marker of inflammation and degenerative states. In one study, obesity was associated with iron deficiency anemia, taking into account inflammatory factors such as hepcidin, IL-6 and CRP, and no significant statistical difference was found between the control group and the obese anemic group [21]. In a case report of IL-6 dosage in a patient with iron deficiency anemia as a consequence of Castleman's disease, it was detailed that chronic IL-6 synthesis by the tumor is caused by iron deficiency, the mediator being hepcidin, since it allows normal iron absorption by the intestine [24].

Folic acid level is probably the most studied marker for anemia deficiency in children. In our case, the highest significant statistical value was obtained for this marker, whereby no difference was observed between cases and controls. The value found is different from other studies in large populations, such as in Brazil [23] and Georgia [24], in which the degree of correlation between iron deficiency anemia and folate was found in a considerable percentage. In general, adequate supplementary feeding practices with folic acid are important to prevent the development of anemia in infant populations. In another study carried out in Kuwait, microcytic anemia in children was not related to vitamin B12 or folic acid since none of the participants presented values below 320 nmol/L, which is considered the limit value to define it as folate deficiency 3. A multivariate relationship was also obtained in low hemoglobin levels, with three serum components that were low in the population of Kuwait. A logistic regression model found that low levels of vitamin B12, folic acid and vitamin A were associated with low hemoglobin values [25]. As for C-reactive protein, no statistical difference was found between cases and controls. Although studies try to correlate CRP with iron deficiency anemia [26], the truth is that CRP is a rather unspecific marker to determine anemia since it is not indicative of anemia by itself but of inflammation in general [27]. One study described that in children without inflammation, CRP showed a decrease, but only decreases in iron deficiency anemia were statistically significant [28]. C-reactive protein is a very good inflammatory marker, but in patients with elevated CRP it can lead to false-positive results of measured iron markers, so it is recommended to exclude its dosage until the inflammatory process is resolved and CRP is in normal values [29]. For the Transferrin marker, widely used to diagnose anemia, no significant statistical difference was found between the case group and controls in the study. Like the CRP value, this is a value that by itself is not sufficient to determine iron deficiency anemia, so its values and interpretations should be made with caution [30]. In a study conducted in Kuwait, children with iron deficiency anemia had low ferritin levels, with values below 15 ng/ml, and a low transferrin saturation level [11].

6. Conclusions

In the present study, serum values of different molecular markers in children with anemia were evaluated to determine the importance of these markers in diagnosing the disease. It was found that the molecular markers: Hepcidin, vitamin B12, IL-6, Folic Acid and C-reactive protein presented statistically significant differences with the control group. On the other hand, the markers for erythropoietin and transferrin showed no changes, which contradicts other studies. Likewise, the vitamin B12 marker was observed to have a lower value in children with anemia. Although there is not much information on vitamin B12 in the iron absorption cycle, it is known that it is structurally bound to this molecule. With the results obtained in this work, it is expected that it will be possible to establish the impact of vitamin B12 and categorize other molecular markers for the detection of iron deficiency anemia in subsequent works.

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