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REFERENCE VALUES OF HEPCIDIN AMONG HEALTHY CHILDREN IN UMUAHIA, SOUTH-EASTERN NIGERIA

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ABSTRACT

There has been promising applications of hepcidin in diagnostic medicine, but these are focused on adults. The diagnostic utility of hepcidin is limited due to the lack of standardization and absence of age-specific reference ranges in children. This study was aimed at determining reference values of hepcidin among healthy children in Umuahia, South-eastern Nigeria. A total of 150 apparently healthy children from 5 primary schools across Umuahia, aged 0.5 – 17 years were enrolled in this study, using a cross-sectional study design. Venous blood was obtained from the subjects and tested for serum hepcidin, serum ferritin, and the full blood count (FBC). SPSS Windows version 20 was used for statistical analysis. The stochastic data were sorted into different age groups, gender, and times of blood sample collection (before and after 12:00pm, i.e 12:00 to 17:00pm), expressed as Median, P2.5, and P97.5 values of hepcidin concentration and hepcidin/Ft ratio. The Median, P2.5, and P97.5 values were used to construct reference ranges for the various groups. The normality of the data was tested using histograms and the Shapiro-Wilk test. Hepcidin and ferritin concentrations showed skewed distributions, hence were log-transformed prior to analysis. The unpaired T test was used to evaluate differences between gender and times of blood sample collection. ANOVA was used to assess differences between the age groups and various parameters. Data were considered significant at $p - value \leq .05$. Results showed unique reference values for the hepcidin concentration (24.68 – 202.16 $\times 10^{-6}\, g/l$ and the hepcidin:ferritin ratio (0.45 – 2.87 $\times 10^{-6}\, g/l$) among all subjects in our setting. Reference values of hepcidin among male children (32.18 – 106.70 $\times 10^{-6}\, g/l$) was significantly different from that of the females (21.21 – 177.62$\times 10^{-6}\, g/l$) (p = .02). Reference values of hepcidin obtained among healthy children in Umuahia are unique from other setting. It is thus recommended that reference values of healthy children in other setting should not be used for diagnostic purpose in Umuahia.

KEYWORDS: Hepcidin, ferritin, iron, children.

INTRODUCTION

Although iron (Fe) has been identified as a major component of human blood for more than 200 years, poor Fe status still affects billions of people around the world.[1] Despite the abundance of Fe on earth, iron deficiency (ID) and subsequently Iron Deficiency Anaemia (IDA) is the most prevalent anaemia worldwide; with IDA being more common in developing counties.[1,2] Globally, 50% of anaemia is caused by ID and results in 841, 000 deaths annually. Of this figure, Africa and parts of Asia recorded 71% of the global mortality burden; whereas North America recorded 1.4% of the total morbidity and mortality associated with IDA. In 2001, the World Health Organization (WHO) reported the prevalence of IDA among school aged children, in developed countries as 5.9%. This was by far lesser than the prevalence of 48.1% in developing countries.[3]

ID is associated with some neurological disorders. Some persons with IDA feel a compulsion to move their lower extremities while at rest (restless leg syndrome) and this has been recognized as a reversible symptom of reduced brain Fe levels that is especially prevalent in pregnancy. Another associated neurological comorbidity is Pica (or dietary compulsions for materials that may not usually be consumed in the diet of humans without ID. Geophagia, a type of Pica has been reported in a majority of pregnant African women living in regions where IDA is extremely common.[2] Individuals with ID have increased absorption of divalent toxic heavy metals like lead and

cadmium that can cause poisoning.[3] Furthermore, IDA in children retards psychomotor development and impairs cognitive performance, increase morbidity from infectious diseases, and decrease work capacity.[3,4]

Owing to the relevance of IDA in causing neurological damage during pregnancy and infancy, rapid diagnosis and subsequent aggressive treatment is expedient.[2] Diagnosis of IDA is through the detection and measurement of some cellular storage and transport proteins associated with Fe homeostasis. Ferritin (Ft) is the primary Fe storage protein and provides a reserve of Fe. It is comprised of an approximately spherical apoprotein shell with molecular weight of 480,000, surrounding a core of ferric hydroxyphosphate of about 4,000 Fe atoms.[5] Binding of exogenous Ft to cell surface receptors have been implicated in Fe delivery pathway in the brain.[6] There has also been suggestions that serum ferritin is secreted by macrophages in response to changing Fe levels.[5]

Fe exported to the serum is bound by the Fe transport protein, transferrin (Tf). It is a glycoslylated Fe-binding protein, which is found in blood plasma, lymph, and other body fluids. Tf transports Fe to all cells.[7] It also keeps free Fe at a very low concentration (about 10^{-18} M), which avoids the high potential risk of damage and deprives pathogens of Fe which they require for growth.[7] These transport proteins are sensitive to the recently discovered Fe regulatory hormone, hepcidin. Although, the contribution of dietary Fe sources to Fe homeostasis remains expedient, the discovery of hepcidin has immensely elucidated our knowledge of Fe balance and regulation.[1] Ferritin had been the central parameter for determination of significant ID as well as therapeutic response,[8,9] however, in situations of significant inflammation, serum ferritin levels may not reflect accurate Fe stores.[2] This is where the knowledge and efficient diagnosis of hepcidin comes in.

Hepcidin is a small peptide of 20 – 25 amino acids (aa) with several isoforms released from a large prepropeptide of 84 aa.[5] It is a key regulator of Fe metabolism in chordates.[10] Hepcidin inhibits Fe transport by binding to the major Fe export protein, ferroportin (FPN1) which is located on the basolateral surface of gut enterocytes and the plasma membrane of reticuloendothelial (RE) cells like macrophages. This process breaks down the transporter protein in the lysosomes. This inhibition of FPN1 prevents the export of Fe from the cells, and the Fe is hence sequestered in the cells.[10,11] This process prevents the influx of Fe into the hepatic portal system, via the enterocytes and, hence, reduces dietary Fe absorption. The reduction of Fe released from macrophages is also by the mechanism involving the inhibition of FPN1. An example is anaemia of chronic inflammation seen in renal failure.[12]

As shown in several studies, serum hepcidin levels are significantly associated with ID.[1,10,11,13] Thus, there is need to determine reliable reference ranges for hepcidin in children. Few studies have established reference ranges for hepcidin in children elsewhere,[14,15] but there is little or no documented study on determination of reference range of hepcidin among children in South-East, Nigeria.

MATERIALS AND METHODS

Subjects, Design, and Setting

The cross-sectional study design was employed in enrolling a total of 150 apparently healthy children who are residents of Umuahia. Subjects were aged between 0.5 to 17 years. 30 subjects were enrolled from each of the following primary schools: Amuzukwu Community Primary School, Umuahia, Amafor Isingwu Community School, Umuahia, Okwu Community Primary School, Olokoro, Mbonu Central School, Umuahia, and Umosu Community School, Ubaka. Children who did not submit written informed consent signed by their parents or guardian, with signs of ID, infection, or anaemia, and those older than 17 years were excluded from the study. Ethical approval to conduct this study was obtained from the ethical committee of the Federal Medical Centre, Umuahia and the Abia State Ministry of Education.

Blood Sample Collection

At least 2 ml of venous blood was collected into plain sterile evacuated tubes (serum separator tubes), from each of the subjects and allowed to clot undisturbed for about 1 hour at room temperature as described by Jurry and associates.[16] Serum was obtained from the samples by centrifugation at 1200 × g for 10 minutes. The supernatant serum was pipetted into another tube and centrifuged again at 1200 × g for 10 minutes to obtain pure serum. This was followed by storage at -20^{o}C pending analysis.

Serum Ferritin Estimation

Serum ferritin concentration was estimated using Human Ferritin Enzyme-linked Immunosorbent Assay (ELISA) kit manufactured by Elabscience Biotechnology Inc.[17] The Ferritin assay is based on the Sandwich ELISA principle. Here, the micro ELISA plate supplied in this kit is pre-coated with an antibody that has specificity to human ferritin. The addition of either sample or standard to the micro ELISA well plates results in the formation of an antigen-antibody complex. This is then followed by the successive addition of a biotinylated detection antibody, specific to human ferritin, and avidin-horseradish peroxidase (HRP) conjugate to each micro well plate, and subsequent incubation. Wells that contain human ferritin, biotinylated antibody, and avidin-horseradish peroxidase will give a blue colouration. This enzymatic reaction is then terminated by the addition of a stop solution that turns the whole preparation to yellow. The optical density (OD) of this solution is measured in a spectrophotometer at 450 nm. The OD of this solution is directly proportional to the concentration of human ferritin in the solution.[17]

Estimation of Serum Hepcidin

Serum hepcidin concentration was estimated using Human Hepcidiin 25 (Hepc25) Enzyme-linked Immunosorbent Assay (ELISA) kits manufactured by MyBioSource Inc.[18] This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for Hepc25 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Hepc25 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for Hepc25 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of Hepc25 bound in the initial step. The colour development is stopped and the intensity of the colour is measured.[18]

Statistical Analysis

SPSS Windows version 20[19] was used for statistical analysis. The stochastic data were sorted into different age groups, gender, and times of blood sample collection (before and after 12:00pm, i.e 12:00 to 17:00pm), expressed as Median, P2.5, and P97.5 values of hepcidin concentration and hepcidin/Ft ratio. The Median, P2.5, and P97.5 values were used to construct reference ranges for the various groups. The normality of the data was tested using histograms and the Shapiro-Wilk test. Hepcidin and ferritin concentrations showed skewed distributions, hence were log-transformed prior to analysis. The unpaired T test was used to evaluate differences between gender and times of blood sample collection. ANOVA was used to assess differences between the age groups and various parameters. Data were considered significant at $p - value \leq .05$.

RESULTS

Table 1: Demographic Information of Subjects.

Parameter	Statistic	Percentage (%)
Total number	150	100.00
Gender		
• Males	89	59.30
• Females	61	40.70
Time of Sample Collection		
• Before 12:00 noon	75	50.00
• After 12:00 noon	75	50.00
Age Range (y)	0.50 – 15.00	

Table 2: Reference Values of Hepcidin and Hepcidin:Ferritin Ratio among Male and Female Children Resident in Umuahia.

Parameter	Total	Male	Female	*T*	*p*
N	150	89	61		
Hepcidin $(\times 10^{-6}\ g/l)$					
• P2.5 – P97.5	24.68 – 202.16	32.18 – 106.70	21.21 – 177.62	-2.43	.02*
• *Mdn*	78.35	81.60	66.70		
Hepcidin:Ferritin Ratio					
• P2.5 – P97.5	0.45 – 2.87	0.54 – 2.81	0.39 – 3.00	-0.38	.70
• *Mdn*	1.31	1.31	1.31		

Note: Mdn = Median, N = total number of subjects, P2.5 = 2.5th percentile, P97.5 = 97.5th percentile, *T* = *T* test statistic.
*Significant difference observed between male and female hepcidin concentration, $p < .05$ by the independent *T* test.

Table 3: Reference Values of Hepcidin, Ferritin, Hepcidin:Ferritin Ratio and Serum Iron Concentration among Children of Various Age Groups.

Parameter	Age (y)				*F*	*p*-value
	<1.00	1.00 – 5.00	6.00 – 10.00	11.00 – 15.00		
N	12	22	44	72		
Hepcidin $(\times 10^{-6}\ g/l)$						
• P2.5 – P97.5	20.60 – 32.50	24.60 – 41.05	33.31 – 92.64	68.34 – 210.56		
• *Mdn*	32.50 $^{\alpha}$	41.05	64.50	133.80	163.56	.00**
Ferritin $(\times 10^{-6}\ g/l)$						
• P2.5 – P97.5	26.40 – 42.90	24.80 – 52.90	20.96 – 87.24	51.84 – 109.56		
• *Mdn*	42.90$^{\beta}$	52.90	52.00	77.95	30.60	.00**
Hepcidin:Ferritin Ratio						
• P2.5 – P97.5	0.39 – 0.70	0.38 – 0.73	0.46 – 3.15	0.91 – 2.87		
• *Mdn*	0.70$^{\delta}$	0.73	1.13	1.68	24.15	.00**
Serum Iron $(\times 10^{-5}\ g/l)$						
• P2.5 – P97.5	42.70 – 51.50	40.30 – 53.40	28.63 – 92.13	58.87 – 147.69		
• *Mdn*	51.50$^{\varepsilon}$	53.40	54.45	90.45	54.85	.00**

Key: Mdn = Median, N = total number of subjects, P2.5 = 2.5th percentile, P97.5 = 97.5th percentile, y = years, *F* = *F* statistic, NA = Not applicable. All *post hoc* analysis was done using Turkey *Honestly Significant Difference* (HSD) and Games-Howell, as applicable. $^{\alpha}$ No Significant difference was observed in hepcidin concentration between <1.00 and 1.00 – 5.00 y age groups, *p*-value = .24. Significant differences were observed for all other pairwise hepcidin comparisons, $p \leq .01$. $^{\beta}$ No significant differences were observed between each of <1.00, 1.00 – 5.00, and 6.00 – 10.00 y age groups ferritin concentrations, $p > .05$. Significant differences were observed for all other pairwise ferritin comparisons, $p \leq .01$. $^{\delta}$No significant difference was observed in the hepcidin:ferritin ratio between <1.00 and 1.00 – 5.00 y age groups, *p*-value = .85; significant differences were observed for all other hepcidin:ferritin ratio pairwise comparison, $p \leq .01$. $^{\varepsilon}$No significant differences were observed in the serum iron concentrations between each of <1.00, 1.00 – 5.00, and 6.00 – 10.00 y age groups, $p > .05$; significant differences were observed for all other pairwise serum iron comparisons, $p \leq .01$. **Significant at *p*-value <.01.

Table 1: Comparison of Hepcidin Concentration and Hepcidin/Ferritin Ratio between Different Times of Sample Collection.

Parameter	Before 12:00 noon N = 75	After 12:00 noon N = 75	*T*	*p*-value
Hepcidin $(\times 10^{-6}\ g/l)$				
• P2.5 – P97.5	21.59 – 91.63	67.51 – 208.65	17.71	.00**
• *Mdn*	46.6	132.2		
Hepcidin/Ferritin Ratio				
• P2.5 – P97.5	0.39 – 2.47	0.92 – 2.92	8.12	.00**
• *Mdn*	0.92	1.76		

Key: *Mdn* = Median, P2.5 = 2.5th percentile, P97.5 = 97.5th percentile, N = number of subjects, *T* = *T* test statistic. **Significant difference observed in the hepcidin concentration and hepcidin/ferritin ratio between samples collected before 12:00 noon and after 12:00 noon, *p-value* < .01 using the independent *T* test.

DISCUSSION

Haematological and iron parameters have been shown to vary significantly between populations.[20] Thus, there is need to determine population specific intervals for these parameters, in order to establish efficient therapy and monitoring of ID. This study was aimed at determining reference values of hepcidin in healthy children in Port Harcourt, South-South Nigeria. In order to achieve this, 150 apparently healthy children aged between 0.50 to 15.00 years were enrolled in the study. Subjects consisted of 89 (59.30%) males and 61 (40.70%) females whose parents or guardian gave written consent to the study (Table 1).

From Table 2, there was significant difference in the median hepcidin concentration between male and female children resident in Umuahia (*T*= -2.43, *p*-value = .02). This result is similar to that obtained by Ganz and colleagues[21] in adult population, and the median values for male and female children in our study (81.60 and 66.70 $\times 10^{-6}\ g/l$, respectively) were lesser than the median male but higher than the median female value in their study (112 and 65 $\times 10^{-6}\ g/l$, respectively). The difference in the age of the two populations may be the underlying factor for the different values (theirs being an adult population). Our results show unique reference values of hepcidin concentration (P2.5 – P97.5: 32.18 –

106.70 $\times 10^{-6}\ g/l$ for males and 21.21 – 177.62 $\times 10^{-6}\ g/l$ for females) in apparently healthy children in Umuahia, South-eastern Nigeria. The median values (*Mdn*: 78.35 $\times 10^{-6}\ g/l$, P2.5 – P97.5: 24.68 – 202.16 $\times 10^{-6}\ g/l$,) for hepcidin obtained in the total study population was higher than that obtained by Uijterschout and associates.[15] (*Mdn*: 22.03 $\times 10^{-6}\ g/l$, P2.5 – P97.5: 5.30 – 79.77 $\times 10^{-6}\ g/l$), for the immunochemical method used in their study.

Contrary to the results obtained for hepcidin concentration, there was, however, no significant difference in the hepcidin;ferritin ratio based on gender (T = -0.38, p = .70) (Table 2). This may be due to decreased iron storage and increased sequestration of iron in enterocytes or macrophages as seen in the similar values of median hepcidin:ferritin quotient in both gender that is >1 (1.31 each). Our subjects were all apparently healthy. Tan and colleagues[22] showed in their study that a lower hepcidin:ferritin ratio is a potential biomarker for liver cirrhosis.

In our comparison of median values of hepcidin concentration and hepcidin:ferritin ratio between children of various age groups (Table 3), we observed significant increase in values with increase in age. This is contrary to that obtained by Uijterschout and associates,[15] who showed a decrease in the median hepcidin concentration between 0.50 – 1 y and 1 – 3 y age groups among Dutch children. Also on the contrary, Uijterschout and associates[23] in their study in infants showed a progressive decrease in the median hepcidin concentration from week 1 to 4 months post-delivery.

In this study, we established separate reference ranges for blood samples collected before 12:00 noon and after 12:00 noon, consistent with the blood sample collection routine in our setting. We observed that the values for each of hepcidin concentration (P2.5 – P97.5: 67.51 – 208.65$\times 10^{-6}\ g/l$) and hepcidin:ferritin ratio (P2.5 – P97.5: 0.92 – 2.92) of samples collected after 12:00 noon were significantly higher than those before (Hepcidin: 21.59 – 91.63 $\times 10^{-6}\ g/l$, and hepcidin:ferritin ratio: 0.39 – 2.47) (p = .00, Table 4). This finding is consistent with that of several studies.[24,26] It has been observed that hepcidin expression seems to be under the regulation of circadian transcriptional factors like upstream stimulatory factor and c-Myc/Max via E-boxes of the Clock genes.[27] On the contrary, circadian oscillation in hepcidin concentration may be secondary to variations in daily iron intake.

This study has had its share of limitations. Few guardians consented to the participation of their children in this study. This caused a setback in the number of eligible samples collected for laboratory analysis. More so, longitudinal follow up on each subject to teenage age was not carried out due to limited time. This shall be done in a further research.

CONCLUSIONS

This study has obtained unique reference values for the hepcidin concentration among healthy children in Umuahia, Southeastern Nigeria. To the best of available literature, no previous study has established reference values for the hepcidin concentration in this study area. The study also showed different reference values of hepcidin for the male and female children.

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