



SERUM HEPCIDIN REFERENCE RANGE, GENDER DIFFERENCES, MENOPAUSAL DEPENDENCE AND BIOCHEMICAL CORRELATES IN HEALTHY SUBJECTS

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ABSTRACT:

Background: Hepcidin has emerged as the central regulatory molecule of iron homeostasis. Iron deficiency and iron overload play a major role in molecular insights of many disease states and serum hepcidin normal values and biochemical correlates are of substantial importance.

Objective: The aim of this study is to examine the serum hepcidin reference range, gender and age differences, menopausal dependence and biochemical correlates in healthy subjects.

Methods: Serum hepcidin concentration was measured with a competitive enzyme-linked immunosorbent assay (DRG Hepcidin-25 ELISA Kit) together with hemoglobin, hematocrit, serum iron, transferrin and C-reactive protein in 120 healthy subjects both men and pre- and post-menopausal women.

Results: Normal serum hepcidin values were found in the range of 1,23 – 36,46 ng/mL (mean $9,25 \pm 6,45$ ng/mL). There were statistically significant differences in measured hepcidin levels between men ($12,34 \pm 7,37$ ng/mL) and women ($6,16 \pm 3,2$ ng/mL) ($p < 0,01$) and between premenopausal ($5,51 \pm 2,8$ ng/mL) and post-menopausal women ($7,29 \pm 3,59$ ng/mL) ($p < 0,05$). Strong correlations were found with serum ferritin and hemoglobin but not with serum iron, transferrin and CRP. No 5-year age interval differences were deemed significant.

Conclusion: Serum hepcidin concentration varied substantially between subjects, which is reflected in wide reference ranges. Serum hepcidin levels were gender and menopausal status related and were in correlation with hemoglobin and serum ferritin in healthy subjects.

Key words: hepcidin, range, gender, menopause, healthy subjects,

INTRODUCTION

Hepcidin has emerged as the central regulatory molecule of systemic iron homeostasis. It is predominantly produced by hepatocytes as a 25 amino acid peptide (2789.4 Da), that is secreted into the circulation [1]. It has been shown to bind in vitro to α_2 -macroglobulin and albumin in human plasma [2]. Hepcidin is produced as an 84-amino acid precursor that subsequently undergoes proteolytic

cleavages to generate the mature form. Further hepcidin - 25 processing can result in the generation of two amino-terminal truncated isoforms, hepcidin-22 (hep-22) and hepcidin-20 (hep-20), which physiological role is still unclear [3]. Human gene named HEPC for hepcidin is constituted of 3 exons and 2 introns located on chromosome 19, in close proximity to USF2 gene [4]. Acute phase protein hepcidin is the master regulator of iron homeostasis [5]. Hepcidin binds to ferroportin, the only known iron export protein, which results in internalization and degradation of this transporter, which then blocks iron export from enterocytes and macrophages to the circulation [6].

Increased iron stores and inflammation decreases hepcidin production, whereas hypoxia, anaemia, iron deficiency, increased erythropoiesis and hepcidin synthesis. Thus, inflammation decreases the availability of iron, whereas hypoxia or anaemia increases iron release and absorption. Recent studies demonstrated that the hypoxia-inducible factor (HIF)-1 alpha contributes to (down-) regulation of hepcidin, which was suggested to be a direct transcriptional mechanism or mediated by muscle-derived soluble haemojuvelin, which may be increased by the HIF - dependent induction of furin activity. However, the molecular mechanisms of the hypoxic or anaemic regulation of hepcidin are far from being understood. Several studies demonstrated that the induction of erythropoiesis and not hypoxia or anaemia itself down-regulates hepcidin. The relationship between hepcidin production and erythropoiesis suggests presence of a regulator between the erythron and the liver, and several candidates for this role have been proposed, for example the soluble transferrin receptor (sTfR) and the growth differentiation factor (GDF)-15 [7]. Determination of serum hepcidin concentration may be a helpful tool in screening for hereditary hemochromatosis, thus preventing cumbersome procedures in the search for causative (rare) genetic variants. Furthermore, hepcidin concentrations have been suggested to negatively correlate with the severity of hemochromatosis and to determine the prognosis and need for stringency of the treatment protocol. Hepcidin concentrations may also be used in the management of patients with iron-loading anemias. In addition, hepcidin is a key in the diagnosis of iron refractory, iron deficiency anemia and might contribute for diagnosis of iron

deficiency in patients with anemia of chronic diseases. Hcpidin might be a potential marker in the prediction of erythropoietin response and to guide treatment with erythropoietin and intravenous iron. Finally, measurement of serum hepcidin concentration is important for the monitoring of novel therapies for iron disorders that target hepcidin, its upstream regulators, or its downstream receptor ferroportin [8].

METHODS

Study population

The pilot study entailed a random sample of 120 male and female above or 18 years of age. The blood samples were taken between 8 AM and 10 AM; after overnight fast (12 hours). All participants gave written informed consent for participation in the study.

The following variables were extracted from the self-administered questionnaire: length, weight, age, use of iron supplements at time of blood donation for at least 6 months, presence of anemia determined by a physician, being a blood and/or plasma donor, pregnancy, and presence of a regular menstruation, cirrhosis or chronic hepatitis B and C, alcohol consumption - women with daily consumption of alcohol >40 g / day and men with daily alcohol consumption >60 g/day, haemochromatosis, concomitant infections, malignant disease, chronic diseases, immunosuppressive therapy, acute infections or invasive procedures (operations, catheterization) in the previous 6 months, neurological, endocrine or other systemic diseases, cardiovascular incident in the previous 6 months or pregnancies.

Laboratory methods

Serum hepcidin concentration was measured with a competitive enzyme-linked immunosorbent assay (DRG Hcpidin-25 ELISA Kit). It is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The micro titer wells are coated with a monoclonal (mouse) antibody directed towards an antigenic site of the hepcidin-25 molecule. Endogenous hepcidin-25 of a specimen sample competes with a hepcidin-25-biotin conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off and a streptavidin - peroxidase enzyme complex is added to each well. After incubation, unbound enzyme complex

is washed off and substrate solution is added. The blue color development is stopped after a short incubation time, turning the color from blue to yellow. The intensity of color developed is reverse proportional to the concentration of hepcidin in the specimen sample. Hemoglobin (HGB) and hematocrit (HCT) were measured using the Sysmex analyzer. Total serum iron was measured by colorimetric measurement using ascorbate / Ferrozine reagents (Biosystems) on an Biosystems analyzer. Serum ferritin causes agglutination of latex particles coated with anti-human ferritin antibodies. The agglutination of the latex particles is proportional to the ferritin concentration was determined by turbidimetry on Biosystems analyzer. Serum C-reactive protein (CRP) causes agglutination of the latex particles coated with antihuman C-reactive protein. The agglutination of the latex particles is proportional to the CRP concentration and it was measured by turbidimetry on Biosystems analyzer.

Statistical analysis

Statistical analysis was performed with SPSS for Windows release 16.0.2, using the standard descriptive methods (mean \pm SD), and corresponding analytical tests. Levene's Test for Equality of Variances was performed to determine the equality of variances, and appropriate independent samples, while the Student's t-test was used to compare the means. The correlation between the results was tested with the Pearson's Correlation Coefficient. The data are presented as mean \pm standard deviation (SD) and $p \leq 0.05$ is considered statistically significant

RESULTS

Characteristics of the study population

Study included 120 participants (age $41,65 \pm 12,3$), 50% of them were males (age $39,73 \pm 12,25$) and 50% women (age $43,57 \pm 12,2$). HGB and HCT were increased in males, compared with women. The concentration of iron was significant increased in males compared to females. Serum ferritin concentration was considerably lower in women than in men, which is in concordance with the lower median iron concentration and transferrin observed in women compared with men.

Table 1. The characteristics of the study population.

Variables	All	Males	Females	P
N	120	60	60	
Age (years)	$41,65 \pm 12,3$	$39,73 \pm 12,25$	$43,57 \pm 12,2$	$P=0,089$ NS
Haemoglobin (g/dl)	$139,76 \pm 14,5$	$149,7 \pm 11,75$	$129,82 \pm 9,3$	$P=0,000$ $p<0,01$
Hematocrit (L/L)	$0,415 \pm 0,04$	$0,43 \pm 0,03$	$0,398 \pm 0,03$	$p = 0,000$ $p<0,01$
Ferritin(μ g/L)	$94,59 \pm 65,9$	$120,2 \pm 70,67$	$69,01 \pm 49,36$	$p= 0,00004$ $p<0,01$
Serum hepcidin (ng/mL)	$9,25 \pm 6,45$	$12,34 \pm 7,37$	$6,16 \pm 3,2$	$p = 0,000$ $p<0,01$
CRP(mg/l)	$2,87 \pm 2,8$	$2,69 \pm 2,3$	$3,06 \pm 3,2$	$p = 0,54$ NS

Table 1 present characteristics of the study population (N = 120). Data are means with std. deviation and p-values of males vs females by t-test.

Age- and sex-specific reference ranges for serum hepcidin concentration in the reference set

The concentration of hepcidin in all participants was ranged from 1,23 – 36,46 ng/mL (mean 9,25 ± 6,45 ng/mL). Statistical analysis showed that males (3,493 - 30,471 ng/mL) had statistically higher hepcidin levels than women (2,740- 13,208 ng/mL). Reference values for hepcidin for all participants were 2,933 - 21,913 ng/mL. Reference

ranges of serum hepcidin concentration in the reference set per 5-year age group are given for all participants in Table 2. Serum hepcidin concentration in men was almost constant over age. Hepcidin concentrations in women trend upwards as they progress through menopause, with median serum hepcidin concentration of 4,71 ng/mL for premenopausal women and 6,39 ng/mL for menopausal women.

Table 2. Reference ranges for serum hepcidin (ng/mL) per 5-year age group for all participants

Age	N	Median	5%	95%
18-24	10	7,9	3	15,3
25-29	20	5,76	3,03	19,1
30-34	9	6,57	2,93	16
35-39	13	14,74	3,25	30,79
40-44	14	6,072	3,42	22,57
45-49	16	6,1	1,23	30,6
50-54	14	8,55	4	20
55-59	15	7,06	2,74	22,01
60	9	10,43	2,13	36,46

The lowest median hepcidin concentration (3- 15,3 ng/mL) was found in the category 18-24 years of age, whereas the highest median concentration was observed in the category of 60 years of age (2,13 - 36,46 ng/mL). Serum hepcidin concentration varied substantially between subjects, which is reflected in wide reference ranges.

The median hepcidin values in healthy premenopausal women is 4,71 ng / mL (rank 1,23 - 14,7 ng / mL), while the group in healthy women after menopause amounts to 6,39 ng / mL (rank 2 13 - 14,67 ng / mL). Statistical analysis confirmed the difference in values between hepcidin healthy women before and after menopause as significant for $p < 0.05$ ($Z = 1,94$ $p = 0,042$).

Table. 3. Values of hepcidin in pre-menopausal and post-menopausal women

Parameter(units)	Females		p-value
	Pre-menopausal n = 38	Post-menopausal n = 22	
Hepcidin(ng/mL)			
mean±SD,	5,51 ± 2,8	7,29 ± 3,59	Z = 1,94
range	1,23 – 14,7	2,13 – 14,67	p = 0,042*

$p < 0,05$ ** $p < 0,01$

Biochemical correlates of serum hepcidin concentration

We found that hepcidin levels were strongly correlated with HGB and HCT ($R = 0,428$, $p < 0,05$; $R = 0,347$, $p < 0,05$) (Table 4). A positive association between iron and serum hepcidin concentration was observed ($R = 0,189$ $p < 0,05$) (Table 4). Additional statistically significant associations were found for increasing serum hepcidin concentra-

tion and ALT ($R=0,186$, $p < 0,05$) (Table 4).

These analyses revealed ferritin to be most strongly associated with serum hepcidin concentration. CRP and transferrin were not statistically significantly associated with serum hepcidin (Table 4).

Table 4. Results of correlations of hepcidin with: HGB, HCT, serum iron concentrations, CRP, ferritin and transferrin.

Hepcidin with	Spearman Rank (R)	p – value
HGB	R = 0,428	p < 0,05
HCT	R = 0,347	p < 0,05
Iron	R = 0,189	p < 0,05
CRP	R = 0,146	p > 0,05
Ferritin	R = 0,577	p < 0,05
Transferrin	R = 0,016	p > 0,05

DISCUSSION

In this study, we used a newly developed ELISA test from DRG (<http://www.drg-diagnostics.de>) with monoclonal antibodies against bioactive hepcidin-25, without detectable cross reactivity against pro hepcidin, α -fetoprotein, human chorionic gonadotropin, human placental HPL and follicle stimulating hormone. We set found hepcidin reference values in healthy population in the range 2,933 - 21,913 ng / ml. These results confirm data from the study in a Geert et al. [9] that use the same kit and receive reference values 20.5-66 ng / mL. Ashby et al. [10] are reference values for hepcidin in healthy volunteers in the range of 2-56 ng / ml, with a median of 11 ng / ml. In our study a statistically significant difference between sexes was found - namely hepcidin is significantly higher in men compared to women. A similar study of Ganz T et al. [11] shows that men have higher concentrations of hepcidin than women. It was also found that post-menopausal women have significantly higher levels of hepcidin compared to pre-menopausal women. These results confirmed the data of Galesloot TE et al. [8].

Still hepcidin was significantly higher in men compared to both pre-menopausal and post-menopausal women. These differences are almost double between men and women after menopause and triple between men and women in the period before menopause. When examining correlations serum concentration of hepcidin were strongly bound to ferritin, but not to transferrin. These results confirm published data from Galesloot TE et al, Roe MA et al. [8, 12]

CONCLUSION

ELISA method for hepcidin determination is characterized by high reliability and analytical selectivity for accurate quantification of hepcidin with relatively short analysis time and provide insights of hepcidin relationships with gender, age and menopausal status in women. Our study provides age- and sex-specific reference ranges of serum hepcidin concentration and indicates ferritin as the primary correlate of serum hepcidin concentration.

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