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Serum hepcidin: reference ranges and biochemical correlates in the general population

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To date, concentrations of the promising biomarker hepcidin have only been assessed in serum of relatively small series of healthy volunteers and patients. We assessed age- and sex-stratified reference ranges of serum hepcidin concentration in a selected reference set and performed regression analyses to study associations between hepcidin and (biochemical) variables in a large, well-phenotyped sample of the general population (n = 2998). All participants filled out a questionnaire on lifestyle, health status, and medical history. Serum measurements of iron parameters, liver enzyme alanine aminotransferase, creatinine and C-reactive protein were available. Serum hepcidin concentrations were lower for premenopausal than for postmenopausal women (median, 4.1nM vs 8.5nM, respectively). Hepcidin concentrations in men were constant over age (median, 7.8nM). Serum hepcidin was strongly associated with serum ferritin in men and women: β-coefficient of log-transformed variables (95% confidence interval): 0.78 (0.74-0.82) and 0.83 (0.78-0.88), respectively. Additional significant, though less strong, associations were observed for C-reactive protein and total iron binding capacity in men and for total iron binding capacity, alanine aminotransferase, and glomerular filtration rate 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. (*Blood*. 2011;117(25):e218-e225)

Introduction

Hepcidin has emerged as the central regulatory molecule of systemic iron homeostasis.[1,2] It is a 25-amino acid peptide hormone that is produced and secreted predominantly by hepatocytes, circulates in the bloodstream, and is excreted by the kidneys. By binding to the cellular iron exporter ferroportin and inducing its internalization and degradation, hepcidin regulates cellular iron efflux.[3] In this way, the absorption of dietary iron from the intestine and the release of recycled iron derived from senescent erythrocytes are controlled.[1,2]

The synthesis of hepcidin is regulated by certain physiologic and pathologic processes. Hepcidin concentrations are decreased in situations that require increased concentrations of circulating iron. In case of increased erythropoiesis, for example in response to hypoxia, anemia, iron deficiency, or conditions characterized by ineffective erythropoiesis (eg, thalassemia major and intermedia), a decreased hepcidin concentration will result in the release of stored iron and in an increase in the dietary iron absorption.[4-7] On the other hand, infection and inflammation cause an increase in hepcidin synthesis,[4,8-10] resulting in decreased availability of circulating iron, which is considered to represent a defense mechanism of the human body against extracellularly proliferating (iron-dependent) pathogens.[1] In chronic (low-grade) inflammatory states, this ultimately leads to a deficiency of iron available for erythropoiesis called anemia of chronic disease.[11] Finally, hepcidin concentration is increased in situations of iron overload,[12] except for situations in which mutations in genes encoding hepcidin or its upstream positive regulators are responsible for the surplus of iron by preventing hepcidin up-regulation.[6] Although notable progression has been made in discovering the identities of hepcidin regulators involved in the aforementioned processes, we do not yet fully understand the mechanisms by which they influence hepcidin expression.

Since the discovery of hepcidin and the elucidation of its important role in iron homeostasis,[1,2] hepcidin has been suggested as a promising diagnostic marker for iron-related disorders. 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.[13] Hepcidin concentrations may also be used in the management of patients with iron-loading anemias.[5] In addition, hepcidin is key in the diagnosis of iron refractory iron deficiency anemia[14] and might contribute to the diagnosis of iron deficiency in patients with anemia of chronic diseases.[15] Hepcidin might be a potential marker in the prediction of erythropoietin response and to guide treatment with erythropoietin and intravenous iron.[16] Finally, measurement of serum hepcidin concentration is of importance in the monitoring of novel therapies for iron disorders that target

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hepcidin, its upstream regulators, or its downstream receptor ferroportin.[17]

Until now, knowledge on how hepcidin exerts its regulatory function and on the molecular processes that regulate hepcidin production is largely based on animal studies and in vitro studies, which often use hepcidin mRNA expression as a readout.[2] In addition, many studies of hepcidin in humans have relied on urinary hepcidin assays and on measurements in relatively small groups of healthy subjects or patient series with a variety of iron disorders,[2,18] predominantly without making a distinction between sex and age, factors that most probably influence hepcidin concentration. Reference ranges of serum hepcidin concentration, based on a large subset from the general population, are instructive for the use of hepcidin as a diagnostic tool and therapeutic target in the future but are currently not available. Furthermore, knowledge on the association between potential correlates, for example markers of infection, inflammation and iron status, and serum hepcidin concentration in humans is relatively scarce, although this information could greatly contribute to understanding of the mechanisms by which hepcidin expression is influenced in vivo. There are a number of studies that reported a high correlation between serum hepcidin and serum ferritin concentration in small samples of healthy persons,[19-22] but other potentially important correlates have only been investigated in animal experiments or small healthy and diseased human populations. For example, transferrin has been shown to be a major determinant of hepcidin expression in hypotransferrinemic mice,[23] and in multivariate analyses the estimated glomerular filtration rate (eGFR) was reported not to be a major independent correlate of serum hepcidin concentration in patients with chronic kidney disease.[21] However, the applicability of these and other findings to the general human population is unclear. To increase our insight in the distribution of hepcidin in human populations and to pave the way for its use in diagnostic medicine, we studied serum hepcidin concentrations and reference ranges and determined its association with selected (biochemical) parameters in a large, well-phenotyped sample of the general population.

Methods

Study population

We included 2998 participants from the Nijmegen Biomedical Study. Details of the Nijmegen Biomedical Study (NBS) have been described before.[24] Briefly, the NBS is a population-based survey conducted by the Department of Epidemiology, Biostatistics, and HTA and the Department of Laboratory Medicine of the Radboud University Nijmegen Medical Center, The Netherlands. Approval to conduct the study was obtained from the Radboud University Nijmegen Medical Center Institutional Review Board. Age- and sex-stratified randomly selected adult inhabitants of Nijmegen (n = 22 451), a city located in the eastern part of The Netherlands, received an invitation to fill out a postal questionnaire on, for example, lifestyle, health status, and medical history, and to donate an 8.5-mL blood sample in a serum separator tube and a 10-mL ethylenediaminetetraacetic acid blood sample. A total of 9350 (43%) persons filled out the questionnaire, of which 6468 (69%) responders donated blood samples between 8 AM and 9 PM; time of blood sampling was recorded. Before the procedure, described earlier in this paragraph, a pilot study was performed to optimize the logistical procedures of the NBS. The pilot study entailed a random sample of 650 male and female inhabitants of Nijmegen age more than or equal to 18 years; 342 subjects (53%) filled out the questionnaire, and 262 (77%) of the responders donated a blood sample. All participants gave written informed consent for participation in the NBS.

For this study, serum hepcidin concentration was measured in 2998 of the total 6730 available serum samples. Serum measurements of iron, ferritin, total iron binding capacity (TIBC), liver enzyme alanine aminotransferase (ALT), creatinine, and C-reactive protein (CRP) were previously performed in all available serum samples.

The following variables were extracted from the self-administered questionnaire: length, weight (used to derive body mass index [BMI]), 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.

Laboratory methods

Serum hepcidin concentration was measured with a competitive enzyme-linked immunosorbent assay as described before.[25] In short, 96-well plates were coated overnight at 4°C with goat anti–rabbit IgG (Fc) antibody, washed, and then blocked with bovine serum albumin (2 hours, ambient temperature), washed, and thereafter incubated with an in-house raised rabbit anti–human hepcidin antibody[26] (2 hours, ambient temperature) and again washed. Next, the standards, study samples (20-fold diluted), and a reference sample were pipetted into the wells. Subsequently, biotinylated hepcidin-25 calibrator was added to all wells, and the plates were incubated overnight at 4°C. After washing, the plates were incubated with streptavidin-β-peroxidase conjugate for 1 hour at ambient temperature, again washed, and then o-phenylenediamine substrate was added and incubated for 15 minutes in the dark. The color reaction was stopped with H_2SO_4, and optical density was measured at 492 nm in an automated c-enzyme-linked immunosorbent assay reader. In each plate, a standard curve and the reference preparation were present. This reference preparation was used for estimation of the accuracy of the method. In total, 86 microtiter plates were used in 15 different assay runs. The mean hepcidin concentration in this preparation was 6.87nM, whereas the intra-assay variation, the between-plates variation, and inter-assay variation were 6.3%, 5.5%, and 11.9%, respectively. The intra-assay, inter-assay, and between-plates variations were calculated from the results obtained in the reference preparation. In 86 plates, the reference preparation was measured in duplicate and the calculated concentrations in each plate were used to establish the intra-assay variation. The mean hepcidin concentration per plate was used for calculation of the between-plates (inter-day) variation and the inter-assay variation. The analytical sensitivity, defined as the minimum hepcidin concentration evoking a response significantly different from that of the zero calibrator, was 8.96pM. As the samples were 20-fold diluted, samples found to have a hepcidin concentration less than 179pM (20 × 8.96pM) were imputed with a random value out of a uniform distribution with a minimum of 0nM and a maximum of 0.18nM (n = 12). Hepcidin concentrations are expressed in nanomoles per liter; 1nM serum hepcidin equals 2.79 μg/L.

Total serum iron was measured by colorimetric measurement using ascorbate/FerroZine reagents (Roche Diagnostics) on an Abbott Aeroset analyzer. Unsaturated iron binding capacity was measured by adding a known quantity of Fe^{3+} to the serum samples, reducing it with ascorbate to Fe^{2+} and measuring it with FerroZine as described for total serum iron (Roche reagents on an Aeroset). TIBC was calculated by adding serum iron and unsaturated iron-binding capacity. Serum transferrin saturation (TS) was computed by dividing serum iron by TIBC. Serum ferritin concentration was determined by a chemiluminescent microparticle immunoassay on the Abbott Architect calibrated against the ferritin assay on the Immulite 2000 of Diagnostic Products Corporation.

Serum creatinine was measured by a kinetic alkaline picrate method on an Abbott Aeroset autoanalyzer by exploiting Bromcresol purple (Jaffe method[27]). In view of the importance of interlaboratory and methodologic differences in the creatinine assays on results of eGFR, our creatinine data obtained by the Jaffe method were calibrated against creatinine values traceable to isotope dilution mass spectrometry.[27,28] For this purpose, the eGFR was calculated using a reexpressed Modification of Diet in Renal Disease formula: $175 \times (\text{standardized serum creatinine (in } \mu\text{mol/L)}/88.4)^{-1.154} \times (\text{age in years})^{-0.203} \times 0.742$ (if female). As this formula is only valid for white persons and we did not have information about race except for country of birth, we used this variable as a proxy and calculated

the Modification of Diet in Renal Disease formula only for subjects born in The Netherlands and other Western countries. Genetic analyses revealed that country of birth was highly associated with race (data not shown), supporting our assumption.

ALT was measured using standard reagents in a reaction rate assay based on the conversion of nicotinamide adenine dinucleotide plus hydrogen to nicotinamide adenine dinucleotide (Abbott Reagent on Aeroset). Samples with a measurement result of less than 3 U/L were set at 2 U/L.

CRP was quantified by immunologic agglutination detection with latex-coupled polyclonal anti-CRP antibodies (Abbott Reagent on Aeroset).

Statistical analysis

Statistical analyses were performed with SPSS for Windows release 16.0.2. Distributions of serum hepcidin, serum ferritin, ALT, and BMI were skewed toward higher values; logarithmic transformations were applied to normalize the distributions. For all continuous variables, median and 2.5th and 97.5th percentiles (P2.5 and P97.5, respectively) were calculated from original untransformed values. Blood sampling time and CRP were categorized into 3 groups based on clinically relevant cutoffs. For blood sampling time, these were before 12 PM, 12 PM to 5 PM, and later than 5 PM, according to Dutch routine and in line with previously reported hepcidin concentration patterns throughout the day.[22,29] For CRP, cutoffs of less than 5 mg/L, 5 to 20 mg/L, and more than 20 mg/L were used. Categorical variables were expressed in numbers and corresponding percentages.

Reference ranges for serum hepcidin concentration, stratified by 5-year age groups and sex, were constructed using the median, P2.5 and P97.5 per category. A reference subset was selected by excluding subjects that passed the following criteria at time of blood sampling: pregnant, ALT more than 50 U/L, CRP more than 10 mg/L, eGFR less than 60 mL/min/1.73 m^2, use of iron supplements, presence of anemia, or BMI more than 30 kg/m^2.

Univariable and multivariable least squares linear regression analyses were used to evaluate the associations between log-transformed serum hepcidin concentrations and selected (biochemical) variables, unadjusted and adjusted for age and time of blood sampling. The assumption of linearity between serum hepcidin concentrations and independent variables was confirmed using graphic methods. Resulting regression coefficients (β) express the change in log-transformed serum hepcidin that are associated with a 1-unit change in the independent variable. Some of the independent variables were log-transformed as well; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a β% change in serum hepcidin. The R^2 (adjusted for the number of explanatory variables in the model) was obtained to indicate the amount of variance in hepcidin concentration that was explained by the included variables. All analyses were stratified by sex.

Results

Characteristics of the study population

A total of 48% of the total study population (n = 2998) was male. Median age of men was 63 years; median age of women 54 years. CRP was increased (> 20 mg/L) in 2% of the population. Serum ferritin concentration was considerably lower in women than in men, which is in concordance with the lower median iron concentration, higher TIBC, lower TS, and larger number of anemic subjects observed in women compared with men. For most subjects, blood sampling was performed between 12 PM and 5 PM. Blood sampling time distribution over the age groups was dissimilar: older persons underwent blood sampling earlier during the day (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Additional characteristics of the subjects included in the study, stratified by sex, are described in Table 1. A total of 1948 subjects (1066 men; 882 women) passed set criteria and were included in the reference set. The characteristics of the reference set are given in supplemental Table 1.

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

Reference ranges of serum hepcidin concentration in the reference set per 5-year age group are given for men and women in Table 2 and supplemental Figure 2. Serum hepcidin concentration in men was constant over age, with a median of 7.8nM (P2.5-P97.5, 0.6-23.3nM). Hepcidin concentrations in women trend upwards as they progress through menopause, with a median serum hepcidin concentration of 4.1nM (P2.5-P97.5, 0.4-19.7nM) for women younger than 55 years and 8.5nM (P2.5-P97.5, 1.2-24.8nM) for women 55 years of age and older. These results were confirmed by univariable regression analyses (supplemental Table 2): age was not associated with serum hepcidin concentration in men ($\beta = 0.003$; 95% confidence interval [CI], −0.001 to 0.007), but it was in women, whether age was defined as a continuous ($\beta = 0.029$; 95% CI, 0.025-0.032) or dichotomous variable (< 55 years vs ≥ 55 years, based on the distinction between premenopausal and postmenopausal state using age as a proxy in the absence of information on menopausal state; $\beta = 0.840$; 95% CI, 0.745-0.935).

The lowest median hepcidin concentration (2.6nM; P2.5-P97.5, 0.7-10.5nM) was found in the category of women 18 to 24 years of age, whereas the highest median concentration was observed in the category of women 80 to 84 years of age (11.9nM; P2.5-P97.5, 1.6-19.2nM). Serum hepcidin concentrations lower than the detection limit of 0.18nM were observed for both men (n = 4) and women (n = 5). Serum hepcidin concentration varied substantially between subjects, which is reflected in wide reference ranges.

As hepcidin concentrations in serum follow a clear circadian rhythm,[22,29,30] we considered the influence of time of blood sampling on serum hepcidin reference ranges by calculating reference ranges stratified for time of blood sampling divided in 3 categories: before 12 PM, 12 PM to 5 PM, and later than 5 PM (supplemental Tables 3-5). Serum hepcidin concentration in men who underwent blood sampling in the morning (before 12 PM) was lower compared with the concentration in men who donated blood after 12 PM (12 PM to 5 PM vs before 12 PM: $\beta = 0.427$; 95% CI, 0.315-0.539; after 5 PM vs before 12 PM: $\beta = 0.366$; 95% CI, 0.225-0.508; supplemental Table 2). This effect did not change by adjusting for age. In women, a similar effect was observed for hepcidin concentration in blood sampled between 12 PM and 5 PM compared with morning samples ($\beta = 0.416$; 95% CI, 0.287-0.546), but the hepcidin concentration was only slightly elevated in samples obtained after 5 PM compared with morning samples ($\beta = 0.080$; 95% CI, −0.087 to 0.247). However, adjusting for age increased the regression coefficient in women for samples obtained after 5 PM compared with morning samples to 0.305 (95% CI, 0.150-0.460; data not shown). The regression coefficient for samples obtained between 12 PM and 5 PM compared with morning samples did not change by adjusting for age. Hence, time of blood sampling has an effect on serum hepcidin concentration that is independent of age and sex.

Finally, we assessed serum hepcidin reference ranges after the additional exclusion of persons with a serum ferritin concentration less than 30 μg/L (supplemental Table 6). Hepcidin reference ranges in these non–iron-deficient subjects were slightly elevated compared with the ranges presented in Table 2, especially in premenopausal women.

Table 1. Characteristics of the study population (N = 2998)

	Men (N = 1445)				Women (N = 1553)			
Variable	**N***	**(%)**	**Median**	**P2.5-P97.5**	**N***	**(%)**	**Median**	**P2.5-P97.5**
Age, y	1445	(100)	63	29-81	1553	(100)	55	25-80
Ferritin, μg/L	1443	(100)	164.1	17.8-620.2	1553	(100)	81.6	8.7-368.6
Iron, μM	1434	(99)	18	8-32	1544	(99)	16	6-30
TIBC, μM	1434	(99)	58	43-78	1544	(99)	60	44-84
TS, %	1434	(99)	30.2	13.1-56.9	1544	(99)	26.9	9.3-50.0
ALT, U/L	1398	(97)	13	5-35	1507	(98)	10	4-34
eGFR, mL/min/1.73 m^2	1430	(99)	82.7	46.0-122.4	1530	(99)	82.0	51.1-123.7
CRP								
< 5 mg/L	1160	(80)	NA	NA	1201	(77)	NA	NA
5-20 mg/L	241	(17)	NA	NA	314	(20)	NA	NA
> 20 mg/L	29	(2)	NA	NA	29	(2)	NA	NA
Unknown	15	(1)	NA	NA	9	(1)	NA	NA
BMI								
< 18 kg/m^2	4	(0)	NA	NA	15	(1)	NA	NA
18-25 kg/m^2	628	(44)	NA	NA	825	(53)	NA	NA
25-40 kg/m^2	786	(54)	NA	NA	662	(43)	NA	NA
> 40 kg/m^2	3	(0)	NA	NA	13	(1)	NA	NA
Unknown	24	(2)	NA	NA	38	(2)	NA	NA
Current use of iron supplements								
Yes	12	(1)	NA	NA	25	(2)	NA	NA
No	1264	(87)	NA	NA	1349	(87)	NA	NA
Unknown	169	(12)	NA	NA	179	(12)	NA	NA
Pregnant								
Yes	NA	NA	NA	NA	12	(1)	NA	NA
No	NA	NA	NA	NA	1494	(96)	NA	NA
Unknown	NA	NA	NA	NA	47	(3)	NA	NA
Regular menstruation								
Yes	NA	NA	NA	NA	505	(33)	NA	NA
No	NA	NA	NA	NA	1001	(64)	NA	NA
Unknown	NA	NA	NA	NA	47	(3)	NA	NA
Self-reported anemia								
Yes	52	(4)	NA	NA	361	(23)	NA	NA
No	1283	(89)	NA	NA	1087	(70)	NA	NA
Unknown	110	(8)	NA	NA	105	(7)	NA	NA
Current blood and/or plasma donor								
Yes	496	(34)	NA	NA	399	(26)	NA	NA
No	934	(65)	NA	NA	1125	(72)	NA	NA
Unknown	15	(1)	NA	NA	29	(2)	NA	NA
Time of blood sampling								
Before 12 PM	352	(24)	NA	NA	326	(21)	NA	NA
Between 12 PM and 5 PM	812	(56)	NA	NA	956	(62)	NA	NA
After 5 PM	273	(19)	NA	NA	263	(17)	NA	NA
Unknown	8	(1)	NA	NA	8	(1)	NA	NA

NA indicates not applicable.

*Numbers are different from the total number of included persons because of missing values.

Biochemical correlates of serum hepcidin concentration

The results of the univariable linear regression analyses are presented in supplemental Table 2. Results of regression analyses after adjustment for age and time of blood sampling (Table 3) are discussed in this paragraph. These analyses revealed ferritin to be most strongly associated with serum hepcidin concentration. The relation between serum ferritin and serum hepcidin was positive for both men and women with adjusted regression coefficients of 0.806 (95% CI, 0.770-0.843) and 0.853 (95% CI, 0.813-0.892), meaning that a 1% change in serum ferritin in micrograms per liter is associated with a 0.81% and 0.85% change in serum hepcidin concentration (nanomoles per liter), respectively. TIBC demonstrated a negative association with serum hepcidin (men: $\beta = -0.033$; 95% CI, −0.038 to −0.028; women: $\beta = -0.027$; 95% CI, −0.032 to −0.022). A positive association between BMI and serum hepcidin concentration was observed, but this association was not statistically significant in men. Additional statistically significant associations in men after adjustment for age and time of blood sampling were found for increasing serum hepcidin concentration and iron, TS, and CRP (Table 3). ALT and eGFR were not statistically significantly associated. In women, additional statistically significant associations adjusted for age and time of blood sampling were observed for iron, TS, ALT, eGFR, and CRP (Table 3).

A sex-specific multivariable model was constructed that included age and time of blood sampling and those variables that showed a *P* value less than .05 for association with hepcidin concentration after adjustment for age and time of blood sampling. For men, these were ferritin, iron, TIBC, and CRP; for women, these were BMI, ferritin, iron, TIBC, ALT, eGFR, and CRP (Table 3). TS was omitted as it is derived using TIBC and iron. In men, independent correlates in the multivariable model for serum

Table 2. Reference ranges for serum hepcidin (nM) per 5-year age group for men and women in the reference population*

	Men (N = 1066)				Women (N = 882)			
			95% reference range				95% reference range	
Age, y	N (%)	Median	P2.5	P97.5	N (%)	Median	P2.5	P97.5
18-24	10 (1)	9.1	2.3	17.8	21 (2)	2.6	0.7	10.5
25-29	16 (2)	8.4	0.5	24.2	28 (3)	3.1	0.6	11.0
30-34	18 (2)	7.4	0.8	25.0	24 (3)	3.9	0.2	21.0
35-39	22 (2)	6.4	0.7	19.4	36 (4)	3.3	0.5	16.0
40-44	19 (2)	10.2	1.6	17.8	65 (7)	4.8	0.3	24.2
45-49	76 (7)	8.2	1.3	21.0	110 (12)	3.5	0.3	14.6
50-54	106 (10)	7.0	0.3	22.0	140 (16)	5.4	0.4	22.8
55-59	173 (16)	7.7	0.4	24.8	129 (15)	8.5	0.8	21.7
60-64	179 (17)	7.9	0.3	22.7	137 (16)	8.2	1.2	27.3
65-69	186 (17)	9.0	0.5	22.2	95 (11)	8.4	1.4	22.6
70-74	133 (12)	8.4	1.0	26.9	62 (7)	8.7	1.0	37.8
75-79	99 (9)	6.8	0.8	25.5	16 (2)	9.2	2.1	29.0
80-84	22 (2)	6.8	3.5	20.1	10 (1)	11.9	1.6	19.2
≥ 85	7 (1)	11.3	3.4	20.5	9 (1)	6.7	1.2	24.5
All	1066 (100)	7.8	0.6	23.3	882 (100)	6.5	0.5	23.2

*See supplemental Table 6 for serum hepcidin reference ranges after the additional exclusion of persons with a serum ferritin concentration < 30 μg/L.

hepcidin concentration were time of blood sampling, ferritin, TIBC, and CRP (Table 4). In women, independent correlates for serum hepcidin concentration were age, time of blood sampling, ferritin, TIBC, ALT, and eGFR (Table 4). ALT demonstrated a negative association with serum hepcidin concentration, which is opposite to the positive association observed by adjusting for age and time of blood sampling only, indicating confounding influences by other variables. The models explained 59.8% and 63.3% of total serum hepcidin variance in men and women, respectively. Note that unadjusted, univariable analyses revealed that serum ferritin concentration by itself explained 56.0% and 60.2% (supplemental Table 2) for men and women, respectively, indicating that almost all explained variance in the multivariable model was attributable to variation in serum ferritin.

Discussion

Our data provide age- and sex-specific reference ranges of serum hepcidin concentration in a large reference population. We demonstrated that serum hepcidin concentrations are lower for premenopausal than for postmenopausal women but are constant over age in men. In addition, our results confirm previous reports about the circadian rhythm of hepcidin. Based on regression analyses results, we report a strong association between serum hepcidin concentration and serum ferritin in both men and women, which explained approximately 60% of total hepcidin variance. This finding withstood adjustment for other (biochemical) variables: BMI, age, iron, TIBC, ALT, eGFR, CRP, and time of blood sampling.

For the calculation of serum hepcidin concentration reference ranges, we selected a reference subset excluding persons with characteristics evidently influencing hepcidin concentration and thus explicitly warranting specific serum hepcidin reference ranges, namely, characteristics that indicate conditions with (1) low hepcidin concentration associated with increased iron demands or decreased synthesis (ALT > 50 U/L, pregnancy, and anemia), and (2) increased hepcidin concentration associated with (low-grade) inflammation (CRP > 10 mg/L and BMI > 30 kg/m^2), increased iron concentration (use of iron supplements), or decreased elimination (eGFR < 60 mL/min per 1.73 m^2).[1,2,8,10,19,21,31-34]

Table 3. Results of linear regression analyses for serum hepcidin concentrations (nM) adjusted for age and time of blood sampling

	Men				Women			
		95% CI				95% CI		
Variable	β*	Lower limit	Upper limit	R^2, %	β*	Lower limit	Upper limit	R^2, %
BMI, kg/m^2†	0.307‡	−0.063	0.677	3.9	0.528‡	0.223	0.832	20.1
Ferritin, μg/L	0.806‡	0.770	0.843	58.7	0.853‡	0.813	0.892	62.6
Iron, μM	0.009	0.001	0.017	4.0	0.034	0.026	0.042	22.6
TIBC, μM	−0.033	−0.038	−0.028	13.5	−0.027	−0.032	−0.022	25.5
TS, %	0.013	0.009	0.017	6.1	0.025	0.020	0.030	24.8
ALT, U/L	0.070‡	−0.026	0.165	3.4	0.145‡	0.051	0.239	19.6
eGFR, mL/min/1.73 m^2	−0.001	−0.004	0.001	3.9	−0.005	−0.008	−0.002	20.2
CRP								
< 5 mg/L	Ref	Ref	Ref		Ref	Ref	Ref	
5-20 mg/L	0.283	0.158	0.408	6.3	0.230	0.113	0.347	20.8
> 20 mg/L	0.775	0.450	1.099		0.712	0.369	1.055	

Ref indicates reference category.

*The dependent variable hepcidin was log-transformed before inclusion in the models. Thus, the β values express the changes in log-transformed hepcidin (nM) that are associated with a 1-unit change in the dependent variable, except for β values marked with "‡."

†BMI was included as continuous variable in the regression models.

‡Both the dependent and independent variables were log-transformed before inclusion in the linear model. Interpretation of these β values is as follows: a 1% change in the independent variable corresponds to a β % change in the dependent variable.

Table 4. Results of multivariable regression models for serum hepcidin concentrations (nM) stratified by sex

	Men			Women		
		95% CI			95% CI	
Variable	β*	Lower limit	Upper limit	β*	Lower limit	Upper limit
BMI, kg/m²†	NA	NA	NA	−0.042‡	−0.274	0.190
Ferritin, μg/L	0.779‡	0.740	0.818	0.830‡	0.783	0.877
Iron, μM	−0.005	−0.010	0.001	0.003	−0.003	0.009
TIBC, μM	−0.008	−0.012	−0.005	−0.005	−0.009	−0.001
ALT, U/L	NA	NA	NA	−0.111‡	−0.180	−0.043
eGFR, mL/min/1.73 m²	NA	NA	NA	−0.003	−0.005	−0.001
CRP						
< 5 mg/L	Ref	Ref	Ref	Ref	Ref	Ref
5-20 mg/L	0.126	0.042	0.210	0.058	−0.030	0.147
> 20 mg/L	0.264	0.047	0.482	0.219	−0.023	0.462
Age, y	0.000	−0.004	0.004	−0.002	−0.007	0.002
Age, < 55 y vs ≥ 55 y	−0.101	−0.211	0.009	0.129	0.020	0.238
Time of blood sampling						
Before 12 PM	Ref	Ref	Ref	Ref	Ref	Ref
Between 12 PM and 5 PM	0.336	0.264	0.409	0.389	0.303	0.474
After 5 PM	0.374	0.277	0.470	0.419	0.306	0.532

NA indicates not applicable; and Ref, reference category.

*The dependent variable hepcidin was log-transformed before inclusion in the models. Thus, the β values express the changes in log-transformed hepcidin (nM) that are associated with a 1-unit change in the dependent variable, except for β values marked with "‡."

†BMI was included as continuous variable in the regression models.

‡Both the dependent and independent variables were log-transformed before inclusion in the linear model. Interpretation of these β values is as follows: a 1% change in the independent variable corresponds to a β % change in the dependent variable.

Variation in hepcidin concentration over age differed between men and women. Men showed a stable hepcidin concentration, although a nonsignificant trend for an age-related increase in serum hepcidin was previously reported based on 65 men.[22] In women, serum hepcidin concentration was substantially higher for postmenopausal than for premenopausal women, although we used age as a proxy in the absence of information on menopausal state of the women. Nevertheless, higher serum hepcidin concentration in postmenopausal women is in agreement with the observation that ferritin concentrations tend to increase sharply as women progress through menopause.[35,36] Serum hepcidin concentrations were elevated for men older than 85 years, supporting a role of hepcidin in anemia of the elderly.[37] However, an opposite effect was observed for women, and the number of men and women in this age category may be too low (7 and 9, for men and women, respectively) to draw conclusions.

Our serum hepcidin reference ranges are in concordance with hepcidin concentrations in healthy controls previously reported by our group and others.[20,26,29,38] However, Ganz et al[22] reported much higher median hepcidin concentrations for their series of healthy volunteers. In addition, they found a significant sex difference in serum hepcidin that was far more pronounced than the overall differences between men and women that we observed, which might be because of the lower median age for women (32.6 years) in their study compared with that in our population (55 years). The difference in absolute hepcidin concentrations between the 2 studies could have been caused by the application of different hepcidin assays.[39] In the past few years, hepcidin assays have been developed on mass spectrometry platforms, and more recently also immunochemical methods have become available. In patients with diseases associated with substantially elevated hepcidin concentrations or a decrease in renal clearance, we found that our immunochemical assay measured relatively higher concentrations than mass spectrometry methods, most probably because an immunochemical method lacks the selectivity to distinguish hepcidin-25 from its isoforms hepcidin-20 and -22, which are prevalent in these diseases.[25] However, in our experience with the exploitation of time-of-flight mass spectrometry techniques, hepcidin isoforms are rare in reference populations.[25] Moreover, the study of Ganz et al[22] and our current study used similar immunochemical methodologies, although antibodies might differ. This apparent difference in absolute hepcidin concentrations between the various assays around the world[39] clearly illustrates the need for harmonization. In this context, it is worth mentioning that in the most recent worldwide send-out of samples (the so called round robin 2), which took place in the spring of 2010 among 18 centers, including 22 methods for measuring plasma hepcidin, we obtained data that will allow us to generate regression equations for hepcidin results measured by the various methods in various laboratories and thus to define hepcidin reference ranges for universal use (J.J.K., D.W.S., et al, manuscript in preparation).

We observed a trend of increasing hepcidin concentration during the day, consistent with previous reports.[22,26,29,30,38] In addition, this is in agreement with the observation that hepcidin expression is regulated by transcription factors, such as upstream stimulatory factor and c-Myc/Max through E-boxes, as genes that are regulated through E-boxes, including the Clock genes *period*, *timeless*, and *clock*, tend to be under circadian rhythmic transcriptional control.[40] However, circadian variation in hepcidin could be secondary as well (eg, driven by variation in iron intake during the day). There is currently no evidence to support either a primary or secondary circadian variation in hepcidin. Nevertheless, because of the daily variations in serum hepcidin concentration, we adjusted for time of blood sampling in our association analyses to correct for its potential confounding influence.

Serum ferritin was shown to be the most important correlate of serum hepcidin concentration. A positive regression coefficient was found, indicating that increased serum ferritin concentration is associated with increased serum hepcidin concentration, which has consistently been reported before.[19-22] Iron, TIBC, and TS only showed moderate associations with serum hepcidin, but directions of the effects were in accordance with expectations. Increasing iron concentration and TS were associated with increased hepcidin concentrations. This may suggest that TS determines hepcidin

concentration, confirming hepcidin's proposed role in counter-regulating increased body iron concentration by decreasing iron absorption and macrophage iron release. However, the cross-sectional design of our study does not allow assessment of causality. TIBC showed a negative relation with serum hepcidin, which corresponds to the observation that TIBC increases in situations of low iron status. The association between hepcidin on one side and serum iron concentration on the other disappeared after incorporation of variables, which were significant in a model adjusted for age and time of blood sampling: ferritin, TIBC, and CRP for men and BMI, ferritin, TIBC, ALT, eGFR, and CRP for women. Similarly, the association between hepcidin and BMI in women became nonsignificant after inclusion of these variables in the model. CRP showed a positive and significant association with serum hepcidin concentration, both in men and women, which is in accordance with the theory that inflammation and infection cause an increase in hepcidin production.[1,2,4,8-11] However, this effect was substantially reduced in women after inclusion of BMI, ferritin, TIBC, ALT, and eGFR, indicating that the association between CRP and serum hepcidin was confounded by these variables. ALT and serum hepcidin concentration showed a positive association when adjusted for age and time of blood sampling only, but this effect became negative after adjusting for BMI, ferritin, iron, TIBC, eGFR, and CRP as well. ALT is a marker of hepatocyte damage; thus, it is not surprising that increasing ALT concentrations are associated with decreasing hepcidin concentrations because hepcidin is predominantly produced by these cells.[2] Notably, causal relationships cannot be deduced from the regression analyses of the current study.

Our study has some limitations. First, in the absence of a definition of "health" of a reference population, the exclusion criteria applied in this study to generate reference ranges could be debated, even though carefully selected. Moreover, hemoglobin values were not measured in our study population, and we therefore used self-reported anemia as a proxy for low hemoglobin concentrations as a exclusion criterion for the reference set. In addition to data on presence or absence of anemia, information about BMI, pregnancy, and use of iron supplements was also obtained using self-administered questionnaires. Thus, persons who were unaware of having one of the characteristics used for exclusion or who reported false answers might mistakenly be included in the reference subset. However, because we used a large study population, we expect that this potential misclassification has not influenced the hepcidin reference ranges. Furthermore, we applied age as a proxy for menopausal state using a cutoff of 55 years. Finally, we included subjects in the reference set with missing values for exclusion variables, but this did not influence reference ranges (data not shown).

We observed a high degree of interindividual variance in serum hepcidin concentration. This implicates that population-based reference ranges may have limitations when used for the interpretation of individual hepcidin concentrations. It appears that hepcidin values, like other hormones, should be interpreted as (in)appropriate in the context of indices of iron metabolism.

In conclusion, we provide age- and sex-specific reference ranges of serum hepcidin concentration. Until reliable (commutable) calibrators become available for harmonization of the current differences in hepcidin results obtained by the various assays, our reference ranges can be made appropriate for universal use by exploiting algorithms derived from regular worldwide assay comparison studies. Universal hepcidin reference ranges enable translational scientists as well as physicians in clinical practice around the world to compare hepcidin concentrations and to collectively define criteria for the use of hepcidin assays in diagnosis, staging, monitoring, and assessing treatment indication of iron disorders. Furthermore, our data provide insight in (biochemical) correlates of serum hepcidin concentration, designating serum ferritin as by far the most important associate of serum hepcidin concentration. This association was robust to adjustment for other iron parameters and biochemical variables measured in this study. However, no inference can be made about causality based on our current study; thus, it is impossible to state whether it is the serum ferritin concentration that determines the serum hepcidin concentration or the other way around. We emphasize the importance of additional studies that do allow elucidation of causality between hepcidin, ferritin, and other (biochemical) variables that may be associated with serum hepcidin concentration but were not evaluated in this study.

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Authorship

Contribution: T.E.G. analyzed the data, interpreted the results, and wrote the manuscript; S.H.V. and D.W.S. designed the research, coordinated data collection, interpreted the results, and edited the manuscript; A.J.G.-M., S.M.K., and D.v.T. performed the measurements and critically reviewed the manuscript; J.J.K. analyzed the data and critically reviewed the manuscript; and J.F.M.W., L.A.L.M.K., F.C.S., and M.d.H. coordinated data collection and critically reviewed the manuscript.

Conflict-of-interest disclosure: D.W.S. is medical director of the www.hepcidinanalysis.com initiative that serves the scientific and medical community with hepcidin measurements at a fee-for-service basis. The remaining authors declare no competing financial interests.

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References

1. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of mammalian iron metabolism. *Cell.* 2010;142(1):24-38.
2. Kemna EHJM, Tjalsma H, Willems HL, Swinkels DW. Hepcidin: from discovery to differential diagnosis. *Haematologica.* 2008;93(1):90-97.
3. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science.* 2004;306(5704):2090-2093.
4. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest.* 2002;110(7):1037-1044.
5. Nemeth E, Ganz T. Hepcidin and iron-loading anemias [editorial]. *Haematologica.* 2006;91(6):727-732.
6. Ganz T, Nemeth E. Regulation of iron acquisition

and iron distribution in mammals. *Biochim Biophys Acta.* 2006;1763(7):690-699.

7. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. *Blood.* 2006;108(12):3730-3735.
8. Nemeth E, Rivera S, Gabayan V, et al. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest.* 2004;113(9):1271-1276.
9. Kemna E, Pickkers P, Nemeth E, van der Hoeven H, Swinkels D. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. *Blood.* 2005;106(5):1864-1866.
10. Wessling-Resnick M. Iron homeostasis and the inflammatory response. *Annu Rev Nutr.* 2010;30:105-122.
11. Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med.* 2005;352(10):1011-1023.
12. Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem.* 2001;276(11):7811-7819.
13. Swinkels DW, Janssen MC, Bergmans J, Marx JJ. Hereditary hemochromatosis: genetic complexity and new diagnostic approaches. *Clin Chem.* 2006;52(6):950-968.
14. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nat Genet.* 2008;40(5):569-571.
15. Theurl I, Aigner E, Theurl M, et al. Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. *Blood.* 2009;113(21):5277-5286.
16. Swinkels DW, Wetzels JF. Hepcidin: a new tool in the management of anaemia in patients with chronic kidney disease? *Nephrol Dial Transplant.* 2008;23(8):2450-2453.
17. Nemeth E. Targeting the hepcidin-ferroportin axis in the diagnosis and treatment of anemias. *Adv Hematol.* 2010;2010:750643.
18. Piperno A, Mariani R, Trombini P, Girelli D. Hepcidin modulation in human diseases: from research to clinic. *World J Gastroenterol.* 2009;15(5):538-551.
19. Ashby DR, Gale DP, Busbridge M, et al. Plasma hepcidin levels are elevated but responsive to erythropoietin therapy in renal disease. *Kidney Int.* 2009;75(9):976-981.
20. Swinkels DW, Girelli D, Laarakkers C, et al. Advances in quantitative hepcidin measurements by time-of-flight mass spectrometry. *PLoS ONE.* 2008;3(7):e2706.
21. Peters HPE, Laarakkers CMM, Swinkels DW, Wetzels JFM. Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate. *Nephrol Dial Transplant.* 2010;25(3):848-853.
22. Ganz T, Olbina G, Girelli D, Nemeth E, Westerman M. Immunoassay for human serum hepcidin. *Blood.* 2008;112(10):4292-4297.
23. Bartnikas TB, Andrews NC, Fleming MD. Transferrin is a major determinant of hepcidin expression in hypotransferrinemic mice. *Blood.* 2010;117(2):630-637.
24. Hoogendoorn EH, Hermus AR, de Vegt F, et al. Thyroid function and prevalence of anti-thyroperoxidase antibodies in a population with borderline sufficient iodine intake: influences of age and sex. *Clin Chem.* 2006;52(1):104-111.
25. Kroot JJC, Laarakkers CMM, Geurts-Moespot AJ, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. *Clin Chem.* 2010;56(10):1570-1579.
26. Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJC, et al. High-sensitive radioimmunoassay for human serum hepcidin. *Br J Haematol.* 2009;146(3):317-325.
27. Wetzels JFM, Kiemeney LALM, Swinkels DW, Willems HL, den Heijer M. Age- and gender-specific reference values of estimated GFR in Caucasians: the Nijmegen Biomedical Study. *Kidney Int.* 2007;72(5):632-637.
28. Wetzels JFM, Willems HL, den Heijer M. Age- and gender-specific reference values of estimated glomerular filtration rate in a Caucasian population: results of the Nijmegen Biomedical Study [letter]. *Kidney Int.* 2008;73(5):657-658.
29. Kroot JJC, Hendriks JCM, Laarakkers CMM, et al. (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. *Anal Biochem.* 2009;389(2):124-129.
30. Kemna EHJM, Tjalsma H, Podust VN, Swinkels DW. Mass spectrometry-based hepcidin measurements in serum and urine: analytical aspects and clinical implications. *Clin Chem.* 2007;53(4):620-628.
31. Rehu M, Punnonen K, Ostland V, et al. Maternal serum hepcidin is low at term and independent of cord blood iron status. *Eur J Haematol.* 2010;85(4):345-352.
32. Bekri S, Gual P, Anty R, et al. Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology.* 2006;131(3):788-796.
33. Ruivard M, Lainé F, Ganz T, et al. Iron absorption in dysmetabolic iron overload syndrome is decreased and correlates with increased plasma hepcidin. *J Hepatol.* 2009;50(6):1219-1225.
34. del Giudice EM, Santoro N, Amato A, et al. Hepcidin in obese children as a potential mediator of the association between obesity and iron deficiency. *J Clin Endocrinol Metab.* 2009;94(12):5102-5107.
35. Koziol JA, Ho NJ, Felitti VJ, Beutler E. Reference centiles for serum ferritin and percentage of transferrin saturation, with application to mutations of the HFE gene. *Clin Chem.* 2001;47(10):1804-1810.
36. Cook JD, Finch CA, Smith NJ. Evaluation of the iron status of a population. *Blood.* 1976;48(3):449-455.
37. Ferrucci L, Semba RD, Guralnik JM, et al. Proinflammatory state, hepcidin, and anemia in older persons. *Blood.* 2010;115(18):3810-3816.
38. Busbridge M, Griffiths C, Ashby D, et al. Development of a novel immunoassay for the iron regulatory peptide hepcidin. *Br J Biomed Sci.* 2009;66(3):150-157.
39. Kroot JJC, Kemna EHJM, Bansal SS, et al. Results of the first international round robin for the quantification of urinary and plasma hepcidin assays: need for standardization. *Haematologica.* 2009;94(12):1748-1752.
40. Bayele HK, McArdle H, Srai SKS. Cis and trans regulation of hepcidin expression by upstream stimulatory factor. *Blood.* 2006;108(13):4237-4245.