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RESEARCH ARTICLE

Standardized serum hepcidin values in Dutch children: Set point relative to body iron changes during childhood

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Abstract

Background: Use of serum hepcidin measurements in pediatrics would benefit from standardized age- and sex-specific reference ranges in children, in order to enable the establishment of clinical decision limits that are universally applicable.

Procedure: We measured serum hepcidin-25 levels in 266 healthy Dutch children aged 0.3-17 years, using an isotope dilution mass spectrometry assay, standardized with our commutable secondary reference material (RM), assigned by a candidate primary RM.

Results: We constructed age- and sex-specific values for serum hepcidin and its ratio with ferritin and transferrin saturation (TSAT). Serum hepcidin levels and hepcidin/ferritin and TSAT/hepcidin ratios were similar for both sexes. Serum hepcidin and hepcidin/ferritin ratio substantially declined after the age of 12 years and TSAT/hepcidin ratio gradually increased with increasing age. Serum hepcidin values for Dutch children <12 years (n = 170) and >12 years (n = 96) were 1.9 nmol/L (median); 0.1-13.1 nmol/L (p2.5-p97.5) and 0.9 nmol/L; 0.0-9.1 nmol/L, respectively. Serum ferritin was the most significant correlate of serum hepcidin in our study population, explaining 15.1% and 7.9% of variance in males and females, respectively. Multivariable linear regression analysis including age, blood sampling time, iron parameters, ALT, CRP, and body mass index as independent variables showed a statistically significant negative association between age as a dichotomous variable (≤12 vs >12 years) and log-transformed serum hepcidin levels in both sexes.

Conclusions: We demonstrate that serum hepcidin relative to indicators of body iron is age dependent in children, suggesting that the set point of serum hepcidin relative to stored and circulating iron changes during childhood.

KEYWORDS
child, ferritin, hepcidin, pediatric reference ranges, transferrin saturation

1 | INTRODUCTION

Hepcidin is a key regulatory hormone of systemic iron homeostasis by controlling iron absorption and bioavailability within the circulation.[1] In response to alterations in body iron demand, it is produced by hepatocytes and secreted into the circulation. It acts by binding the cellular iron exporter ferroportin, triggering its internalization and degradation, thereby inhibiting iron release from enterocytes and macrophages.[1]

Hepdicin production is tightly regulated to prevent both iron deficit and iron overload. Its levels are decreased by hypoxia, erythropoietic activity, and reduced levels of both circulating and stored body

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CRP, c-reactive protein; EDTA, ethylenediaminetetraacetic acid; Ht, hematocrit; ID, iron deficiency; IDA, iron deficiency anemia; IRIDA, iron refractory iron deficiency anemia; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; RM, reference material; TIBC, total iron binding capacity; TSAT, transferrin saturation.

iron.[1] In contrast, its levels are increased by inflammation and infection as a host defense mechanism. By sequestering iron within the reticulo-endothelial system, plasma iron content is reduced, which consequently limits extracellular microbial growth.[1,2]

Dysregulation of hepcidin production contributes to the pathogenesis of several iron disorders.[2] Hepcidin is inappropriately low relative to stored iron in case of (a) hereditary hemochromatosis[3] and (b) increased (especially ineffective) erythropoiesis[4] resulting in low hepcidin/ferritin ratios.[4] Conversely, hepcidin is inappropriately high relative to circulating transferrin bound iron in case of iron refractory iron deficiency anemia (IRIDA) resulting in low transferrin saturation (TSAT)/hepcidin ratios.[5,6]

Hepcidin levels reflect the integration of multiple key signals involved in iron regulation.[7] Therefore, its measurement is a promising clinical tool for the diagnosis and management of iron disorders involving iron deficiency (ID), iron loading, and iron maldistribution. Examples include (a) evaluation of suspected IRIDA[5,6,8]; (b) diagnosis of concomitant ID in patients with anemia of inflammation[9]; (c) prediction of responsiveness to oral iron therapy and guiding iron treatment under conditions of competing signals (anemia, ID, inflammation)[10–12]; (d) evaluation of suspected iron overload disorders[13]; (e) evaluation of ID, before the occurrence of iron deficiency anemia (IDA), for example in pregnant women,[14,15] in children,[16,17] and in children with cystic fibrosis.[18]

Apart from diagnostic applications, multiple agents targeting the hepcidin/ferroportin axis are under development as novel therapeutics for iron disorders in adults.[19] In order to use hepcidin as a diagnostic tool and a therapeutic target, reference values of the healthy population are crucial. For adults, reference ranges of serum hepcidin (and its ratio to ferritin and TSAT) are available for our assay.[20,21] They have been constructed based on data from the general adult population (N∼3000), and have recently been converted to standardized reference ranges.[21,22] These data have revealed that in both men and women, serum hepcidin concentrations are strongly associated with serum ferritin[20,23] and only marginally with circulating iron (TSAT).[20] Serum hepcidin concentrations in men stay stable over age while in women concentrations are lower in premenopausal than in postmenopausal women. This is in agreement with the observation that serum ferritin concentrations tend to increase as women progress through menopause.[20]

Implementation of measurement of serum hepcidin levels in clinical pediatrics is hampered by the lack of standardized reference values for healthy children from different age groups, relative to iron status.[24–26] Available studies concern either small series with limited age groups or series including children with (anemia of) inflammation.[16,27–31]

As a first step to the implementation of serum hepcidin measurements in pediatric clinical practice, we established age- and sex-specific standardized serum hepcidin values relative to body iron indicators in healthy Dutch children aged 0.3-17 years. Our observations suggest a changing set point of serum hepcidin relative to body iron indicators during childhood.

2 | METHODS

2.1 | Study population

Participants were consecutively enrolled at the Department of Pediatrics while attending the Máxima Medical Center (MMC), Veldhoven, the Netherlands for either minor surgical interventions (eg, correction of orchidopexy, removal of osteosynthesis material) or diagnostic procedures (eg, magnetic resonance imaging under sedation).

Inclusion criteria were age 0 to <18 years, need for a venipuncture or intravenous drip placement for the purpose of general anesthesia or sedation and both oral and written informed consent for inclusion in the study. Exclusion criteria were known IDA, any systemic underlying disease (malignancy, asthma, diabetes, congenital heart disease, kidney failure, congenital immunodeficiency, etc.), acute infection, trauma or operation 48 h ago, inflammation, infection (CRP >5 mg/L), ALT >40 IU/L, or treatment with iron preparations. Children admitted for eartube insertion, adenotomy, or adenotonsillectomy because of recurrent infections of the upper airways were not included in the study. All children that were enrolled in the study visited the hospital for elective surgery or diagnostic procedures. Therefore, none of the children had fever since this is a contra-indication for receiving general anesthesia or sedation.

Between July 2016 and April 2018, we obtained informed consent for participation in the study for 317 children. Forty-one children were excluded because of failed blood withdrawal (n = 23), CRP >5 mg/L (n = 14), underlying disorders (n = 2), double inclusion (n = 2), ID (n = 4), or IDA (n = 6), according to the WHO definition—ID, ferritin <12-15 μg/L; IDA, Hb <11-13 g/dL in combination with ferritin <12-15 μg/L; specific cutoffs dependent on age.[32–34] This resulted in the inclusion of 266 healthy children (157 males, 109 females) aged 0.3-17 years (Table 1).

Blood sampling was performed (0.5 mL EDTA tube, 3.5 mL serum tube) before the surgical or diagnostic procedure, between 7:30 AM and 6 PM, after 4-6 h of fasting because of perioperative fasting guidelines. Participants or their representatives filled out a questionnaire on medical history, general health, and medication use as part of the anesthesia screening procedure.

Since we aimed to cover the different key periods of human growth and development for both sexes, we divided our study population in subgroups for age and sex; this included infancy and toddler stage (0 to <2 years), early childhood (2 to <6 years), middle childhood (6 to <12 years), and adolescence (12-17 years).[35] Because of limited numbers, infants and toddlers were merged into one group.

Body mass index (BMI) was determined and interpreted according to international standards, which describe age-dependent cut-off points for underweight, normal weight, overweight, and obesity.[36] The study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethics committee of the MMC. For all participants, oral and written informed consent was obtained.

TABLE 1 Demographic, clinical, and laboratory characteristics of the study population (N = 266)

	Males (n = 157)	Females (n = 109)	*P*
Demographic and clinical characteristics			
Age (range)	9 (0-17)	11.5 (1-17)	.003
Ethnicity			
Western-European	144 (92)	105 (96)	.131
Mediterranean	13 (8)	4 (4)	.131
BMI[a]			
Underweight	3 (2)	6 (5)	.111
Normal weight	131 (83)	91 (84)	.922
Overweight	18 (12)	10 (9)	.549
Obese	5 (3)	2 (2)	.499
Time of blood sampling			
Between 7:30 AM and 12 PM	76 (48)	50 (46)	.684
Between 12 PM and 3 PM	58 (37)	44 (40)	.572
Between 3 PM and 6 PM	23 (15)	15 (14)	.839
Laboratory characteristics			
Hemoglobulin, g/dL	12.6 (10.4-15.5)	12.6 (10.5-15.1)	.837
Ht, l/L	0.4 (0.3-0.5)	0.4 (0.3-0.5)	.706
Reticulocytes, $\times 10^9$/L	47.5 (26.9-78.4)	52.0 (25.0-106.8)	.209
MCV, fL	82.0 (72.0-90.0)	84.0 (76.8-94.8)	.000
MCH, fmol	1.8 (1.5-1.9)	1.8 (1.6-2.0)	.301
Ferritin, μg/L	41.0 (15.9-98.1)	40.0 (16.5-112.8)	.937
Iron, μmol/L	16.0 (3.0-26.2)	15.5 (4.0-29.3)	.903
TIBC, μmol/L	64.5 (50.0-83.0)	66.0 (47.0-86.5)	.194
TSAT, %	24.0 (9.5-45.0)	23.0 (6.6-45.5)	.978
sTfR, mg/L	1.3 (0.9-2.0)	1.2 (0.8-1.9)	.082
ALT, IU/L	18.0 (11.0-42.3)	17.0 (9.8-31.2)	.229
CRP[b], mg/L	0.2 (0.0-3.7)	0.3 (0.0-4.4)	.209

Abbreviations: BMI, body mass index; sTfR, soluble transferrin receptor.

Note. Data are given as median for continuous variables and as n for categorical variables. For the continuous variable values in parenthesis, refer to ranges (age) or p2.5-p97.5; for the categorical variable values in parenthesis, refer to percentages.

Note. *P*-values for continuous variables were calculated with the independent sample median test; *P*-values for categorical values were calculated with the chi-square test.

[a]BMI was assessed according to the international standards established by Cole and Lobstein.[36]

[b]In a 3-year-old male, CRP was missing. Hepcidin was low (0.58 nM) in this child, arguing against inflammation. Therefore, this case was not excluded although CRP >5 mg/L was an exclusion criterion for the study.

2.2 | Laboratory methods

We measured the levels of the bioactive form of hepcidin (hepcidin-25[2]) by weak cation exchange chromatography followed by time of flight mass spectrometry, as described before.[37] Our hepcidin assay was recently standardized using a second reference material (RM) that was value assigned by a provisional primary RM.[22] For additional information on the laboratory methods, see the Supporting Information.

2.3 | Statistical analysis

Descriptive statistics were reported as medians, 2.5th to 97.5th percentiles, and/or ranges for continuous variables and as absolute frequencies and percentages for categorical variables, using original untransformed values. Normality of distributions of the continuous variables was visually checked using histograms. A logarithmic transformation was applied to normalize the nonnormal distributions of serum hepcidin, ferritin, CRP, hepcidin/ferritin ratio, and TSAT/hepcidin ratio. For hepcidin values below the lower detection limit of 0.5 nM, imputation was performed with a random value between 0 and 0.5 nM.

Reference ranges for (untransformed) serum hepcidin concentration, stratified by age groups and sex, were constructed using the median and p25 to p75. Univariable and multivariable linear regression analyses were used to evaluate the associations between (log-transformed) serum hepcidin concentrations, log-transformed

TABLE 2 Reference ranges for serum hepcidin, ferritin/hepcidin ratio, and transferrin saturation (TSAT)/hepcidin ratio per age group and sex

	Males				Females				
Hepcidin[a] (nmol/L)									
Age (years)	n (%)	Median	p25 to p75	Min-Max	n (%)	Median	p25 to p75	Min-Max	*P*
0 to <2	11 (7)	1.8	1.1-7.3	0.9-20.9	6 (5)	1.8	0.9-7.5	0.2-14.1	1.00
2 to <6	29 (19)	2.0	1.0-3.3	0.1-7.0	18 (17)	2.0	1.1-3.8	0.7-9.5	.853
6 to <12	71 (45)	1.7	0.9-3.4	0.1-15.5	35 (32)	2.0	0.9-3.9	0.0-18.6	.680
12 to <18	46 (29)	0.7	0.3-1.8	0.0-9.3	50 (46)	1.0	0.5-2.2	0.0-11.4	.409
Total	157 (100)	1.5	0.7-3.0	0.0-20.9	109 (100)	1.5	0.8-2.9	0.0-18.6	1.000
Hepcidin/ferritin (pmol/μg)									
Age (years)									
0 to <2	11 (7)	49.7	34.5-124.0	11.4-302.6	6 (6)	59.7	29.2-203.4	7.8-261.7	1.000
2 to <6	28 (18)	54.0	36.9-94.8	4.6-216.5	18 (17)	71.6	38.9-143.9	19.8-399.1	.365
6 to <12	71 (46)	43.7	23.8-82.6	2.9-249.6	35 (32)	42.5	24.9-87.6	0.5-166.9	1.000
12 to <18	46 (29)	18.2	5.6-31.3	0.5-106.0	49 (45)	22.8	12.8-40.0	0.6-151.9	.354
Total	156 (100)	35.0	18.8-67.4	0.5-302.6	108	35.7	19.0-73.2	0.5-399.1	.900
TSAT/hepcidin (%/nmol/L)									
Age (years)									
0 to <2	11 (7)	11.4	2.5-13.8	0.2-22.4	6 (6)	9.3	3.6-13.0	2.8-37.8	.620
2 to <6	28 (18)	10.9	6.6-24.3	2.5-230.9	17 (16)	13.2	4.6-20.3	2.9-44.4	.908
6 to <12	71 (46)	14.0	7.4-25.9	0.5-377.6	33 (32)	13.2	6.7-27.9	1.2-869.5	1.000
12 to <18	45 (29)	32.8	14.5-77.5	1.6-1855.2	48 (46)	24.9	14.3-44.4	0.5-765.2	.606
Total	155 (100)	15.2	8.2-31.6	0.2-1855.2	104 (100)	15.6	7.5-34.2	0.4-869.5	.666

Abbreviation: Min-Max, minimum to maximum.

[a]Hepcidin was below the detection limit of 0.5 nmol/L in 26 out of 157 males (17%)—0 to <2 years: 0 out of 11 (0%), 2 to <6 years: 2 out of 29 (7%), 6 to <12 years: 6 out of 71 (8%), 12-17 years: 18 out of 46 (39%). Hepcidin was below the detection limit in 19 out of 109 females (17%)—0 to <2 years: 1 out of 6 (2%), 2 to <6 years: 7 out of 18 (0%), 6 to <12 years: 6 out of 35 (17%), 12-17 years: 12 out of 50 (24%).

hepcidin/ferritin ratio, TSAT/hepcidin ratio, 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. The resulting regression coefficients (β) express the change in log-transformed serum hepcidin that is 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. Explained variances (R^2) were obtained to indicate the amount of variance in hepcidin concentration that was explained by the included variables.

Analyses were stratified by sex because of sex-specific differences in hepcidin levels and iron homeostasis that have been described earlier.[20,26,27,30,38,39] SPSS version 22 was used for data analysis.

3 | RESULTS

3.1 | Characteristics of the study population

Characteristics of the study population are described in Table 1. Fifty-nine percent (n = 157) of the total study population was male. Median age of males was 9 years (range, 4 months to17 years); median age of females was 11 years (range, 1-17 years).

Hb, CRP, and iron indicators for the different age groups (0 to <2 years, 2 to <6 years, 6 to <12 years, 12 to <18 years) are presented in Table S1. Data on the blood sampling time of hepcidin and the BMI of the participants are presented and discussed in Figures S1 and S2.

3.2 | Age- and sex-specific reference ranges for serum hepcidin concentrations

Reference ranges (p25 to p75) for serum hepcidin remained constant until the age of 12 years (independent samples median test, males *P* .886, females *P* .712) but were substantially lower after the age of 12 years in both males and females (independent samples median test, males *P* .003, females *P* .016) (Table 2, Table S2). These results were confirmed with unadjusted univariable linear regression analysis for <12 years versus >12 years (males: $\beta = -0.49$; 95% confidence interval [CI] −0.66 to −0.31, females: $\beta = -0.26$; 95% CI −0.46 to −0.05) (Table S3).

Hepcidin concentration was below the detection limit of 0.5 nmol/L in 26 out of 157 males (17%) and 19 out of 109 females (17%) (Table 2). The wide reference ranges reflect the substantial interindividual variation in serum hepcidin concentrations.

TABLE 3 Results of linear regression models for serum hepcidin concentrations (nM) adjusted for age and time of blood sampling and stratified by sex

		Males					Females				
			95% confidence interval (CI)					95% CI			
Variable		Beta[a]	Lower limit	Upper limit	R^2, %[b]	*P*	Beta[a]	Lower limit	Upper limit	R^2, %[c]	*P*
Hb, g/dL		0.018	−0.086	0.121	18.4	.737	0.046	−0.056	0.148	7.7	.374
Reticulocytes, $\times 10^9$/L		0.001	−0.005	0.007	18.7	.668	−0.001	−0.007	0.005	7.0	.816
MCV, fL		0.002	−0.022	0.026	18.4	.879	−0.016	−0.047	0.014	7.9	.294
Ferritin, µg/L[d]		1.313	0.946	1.679	38.8	.000	0.919	0.468	1.371	19.9	.000
Iron, µmol/L		0.005	−0.011	0.021	17.6	.517	0.008	−0.010	0.026	7.0	.402
TIBC, µmol/L		−0.009	−0.018	0.001	20.3	.071	−0.015	−0.026	−0.004	13.2	.007
TSAT, %		0.006	−0.004	0.015	18.2	.233	0.009	−0.002	0.021	8.6	.121
sTfR, mg/L		−0.216	−0.526	0.094	19.3	.170	0.211	−0.237	0.660	7.7	.352
ALT, IU/L[d]		0.522	−0.024	1.067	20.2	.061	−0.234	−1.031	0.563	7.5	.561
CRP, mg/L[d,e]		0.229	0.106	0.352	25.3	.000	0.148	−0.001	0.297	10.3	.052
BMI[f]	Normal weight	ref	ref	ref			ref	ref	ref		
	Underweight	0.484	−0.087	1.056	20.5	.096	−0.022	−0.482	0.457	5.5	.923
	Overweight	0.196	−0.050	0.442		.118	0.102	−0.259	0.461		.575
	Adipose	0.254	−0.194	0.702		.264	−0.102	−0.865	0.661		.791

Abbreviations: ref, reference category; sTfR, soluble transferrin receptor.

Note. Adjustment for age was done with age as a continuous variable; adjustment for sampling time was done with sampling time as a categorical variable (7:30 AM to 12 PM, 12-3 PM, 3-6 PM).

[a]Beta expresses the change in the dependent variable—log-transformed serum hepcidin—that is associated with a 1-unit change in the independent variable.

[b]For males, multivariate linear regression model with log-transformed hepcidin as the dependent variable and age (continuous variable) and sampling time as independent variables showed R^2 18.9%, with ß: −0.048, CI −0.065 to −0.030 (*P* .000), ß: 0.254, CI 0.081-0.426 (*P* .004), ß: 0.496, CI 0.255-0.737 (*P* .000) for age, sampling time 12-3 PM and sampling time 3-6 PM, respectively.

[c]For females, multivariate linear regression model with log-transformed hepcidin as the dependent variable and age (continuous variable) and sampling time as independent variables showed R^2 7.8%, with ß: −0.033, CI −0.055 to −0.010 (*P* .004), ß: 0.196, CI −0.023- 0.415 (*P* .079), ß: 0.246, CI −0.064-0.556 (*P* .119) for age, sampling time 12-3 PM and sampling time 3-6 PM, respectively.

[d]These 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 beta% change in serum hepcidin.

[e]CRP levels of children were between >0.1 and <5 mg/L.

[f]Body mass index was assessed according to the international standards established by Cole and Lobstein.[36]

Because of the earlier described sex-specific differences in both adults and children[20,26,27,30,39] regarding serum hepcidin levels, all analyses were stratified for sex. As shown in Table 2 and Table S2, we did not observe a difference in serum hepcidin levels between males and females. Multivariate analysis including age, sampling time, BMI, sex, iron parameters, CRP, and ALT confirmed that sex was not an independent correlate of serum hepcidin (*P* .127).

3.3 | Biochemical correlates of serum hepcidin concentrations

We also performed regression analyses adjusted for age and sampling time because of the demonstrated correlation with age and the earlier described circadian rhythm of serum hepcidin levels.[20,40] In these adjusted analyses, ferritin was most strongly associated with serum hepcidin concentration ($\beta = 1.31$, 95% CI 0.95-1.68 and ß = 0.92, 95% CI 0.47-1.77 for males and females, respectively) and was responsible for the largest amount of explained variance (adjusted R^2 38.8% and 19.9%, R^2 for age and sampling time alone 18.9% and 7.8% in males and females, respectively) (Table 3).

These results indicate that a 1% change in serum ferritin in micrograms per liter was associated with a 1.31% and 0.92% change in serum hepcidin concentration (nmol/L) in males and females, respectively. We observed a negative association between total iron binding capicity (TIBC) and serum hepcidin concentration in both sexes. CRP demonstrated a positive association with serum hepcidin in both males and females.

A sex-specific multivariable regression model was constructed including age as a dichotomous variable (<12 years vs >12 years), time of blood sampling, and those variables that were significantly ($P < .1$) associated with serum hepcidin concentrations after adjustment for age and time of blood sampling. For males, these were ferritin, TIBC, ALT, CRP, and BMI; for females, these were ferritin, TIBC, and CRP. In males, independent correlates ($P < .1$) were age, sampling time, ferritin, underweight BMI, and CRP; in females, these were age, sampling time >3 PM, and ferritin. In males, the model explained

TABLE 4 Results of multivariable linear regression analyses for serum hepcidin concentrations (nM) stratified by sex

		Males				Females			
			95% confidence interval (CI)				95% CI		
Variable		Beta[a]	Lower limit	Upper limit	*P*	Beta[a]	Lower limit	Upper limit	*P*
Age, years	<12 12 to <18	−0.579	−0.736	−0.423	.000	−0.240	−0.441	−0.040	.019
Time Blood sampling	7:30 AM to 12 PM	ref	ref	ref		ref	ref	ref	
	12-3 PM	0.143	−0.008	0.295	.063	0.157	−0.052	0.367	.140
	3-6 PM	0.369	0.158	0.580	.001	0.305	0.003	0.607	.048
Ferritin, μg/L[b]		1.014	0.604	1.425	.000	0.604	0.110	1.099	.017
TIBC, μmol/L		0.000	−0.009	0.010	.950	−0.010	−0.021	0.002	.107
ALT, IU/L[b]		0.091	−0.440	0.622	.736	na	na	na	na
CRP, mg/L[b,c]		0.107	−0.017	0.230	.091	0.095	−0.050	0.240	.197
BMI[d]	Normal weight	ref	ref	ref		ref	ref	ref	
	Underweight	0.538	0.027	1.048	.039	na	na	na	na
	Overweight	0.106	−0.125	0.337	.366	na	na	na	na
	Adipose	0.081	−0.335	0.497	.702	na	na	na	na

Abbreviations: na, not applicable; ref, reference category.

[a]Beta expresses the change in the dependent variable—log-transformed hepcidin—that is associated with a 1-unit change in the independent variable.

[b]These 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 beta% change in serum hepcidin.

[c]CRP levels of children were between >0.1 and <5 mg/L.

[d]Body mass index was assessed according to the international standards established by Cole and Lobstein.[36]

40.9% of the serum hepcidin variation, and in females it was 16.9% (Table 4).

3.4 | Age- and sex-specific reference ranges for hepcidin/ferritin and TSAT/hepcidin ratios

Reference ranges (p.25 to p75), minimum and maximum values of hepcidin/ferritin, and TSAT/hepcidin ratios are given per age group and sex in Table 2. We chose the TSAT/hepcidin ratio instead of other earlier reported variants, for example TSAT/(log)hepcidin ratio,[6] since this ratio was superior in discriminating patients with IRIDA from patients with IDA non-IRIDA in a study we recently performed in adults at our institution (unpublished results, Netherlands Trial Register, Trial NL 6845).[41]

Univarable linear regression analysis (Tables S4 and S5) showed that hepcidin/ferritin decreased and TSAT/hepcidin increased with aged in both sexes. Both effects were stronger in males compared to females.

3.5 | Biochemical correlates of hepcidin/ferritin and TSAT/hepcidin ratios

Regression analyses for the hepcidin/ferritin and TSAT/hepcidin ratios adjusted for age and time of blood sampling are presented in Tables 5 and 6, respectively. R^2 for age as a continuous variable and sampling time as a categorical variable alone was 26.8% and 13.6% and 21.3% and 6.5% for the log-transformed hepcidin/ferritin and TSAT/hepcidin ratio in males and females, respectively. Sex-specific multivariable models were constructed for both ratios, including age (as a categorical variable; age groups) and time of blood sampling and those variables that were significantly associated with these ratios after adjustment for age and time of blood sampling.

The hepcidin/ferritin ratio was significantly correlated with CRP and underweight BMI in males and with CRP in females, after correction for age and time of blood sampling (Table 5). The sex-specific model with the relevant correlates explained 32.9% of the hepcidin/ferritin ratio variation in males and in 14.3% of that in females (Table S6).

The TSAT/hepcidin ratio was statistically significantly correlated with multiple biochemical correlates in both males and females after adjustment for age and time of blood sampling (Table 6). The sex-specific multivariable models including these parameters explained 46.0% and 22.8% of the TSAT/hepcidin ratio variation in males and females, respectively (Table S7).

4 | DISCUSSION

Our data provide age- and sex-specific values for hepcidin-25 and its ratios to parameters reflecting circulating and stored iron for healthy Dutch children, as assessed by a standardized assay. These values can be used as for any other hepcidin assay worldwide that is standardized using the same secondary RM for calibration.[22]

TABLE 5 Results of linear regression models for hepcidin/ferritin ratio (pmol/μg) adjusted for age and time of blood sampling and stratified by sex

		Males					Females				
			95% confidence interval (CI)					95% CI			
Variable		Beta[a]	Lower limit	Upper limit	R^2, %[b]	*P*	Beta[a]	Lower limit	Upper limit	R^2, %[c]	*P*
Hb, g/dL		−0.007	−0.098	0.084	26.3	.885	0.014	−0.082	0.109	12.9	.775
Reticulocytes, $\times 10^9$/L		0.000	−0.005	0.005	26.3	.927	−0.003	−0.009	0.003	13.6	.339
MCV, fL		−0.007	−0.029	0.015	26.5	.515	−0.008	−0.037	0.021	13.0	.593
Iron, μmol/L		0.009	−0.005	0.023	26.0	.216	0.000	−0.016	0.017	11.7	.958
TIBC, μmol/L		−0.003	−0.012	0.005	26.5	.469	−0.005	−0.016	0.005	13.6	.331
Transferrin saturation, %		0.006	−0.002	0.014	26.2	.165	0.002	−0.009	0.013	11.9	.741
sTfR, mg/L		−0.119	−0.408	0.170	26.3	.417	0.219	−0.203	0.641	13.7	.306
ALT, IU/L[d]		0.162	−0.321	0.645	26.5	.508	−0.505	−1.241	0.230	14.3	.176
CRP, mg/L[d,e]		0.134	0.023	0.244	29.0	.018	0.119	−0.021	0.259	15.1	.094
BMI[f]	Normal weight	ref	ref	ref			ref	ref	ref		
	Underweight	0.610	0.112	1.108	29.0	.017	−0.006	−0.434	0.422	11.6	.979
	Overweight	0.169	−0.045	0.383		.121	0.135	−0.201	0.471		.427
	Adipose	0.019	−0.370	0.409		.922	−0.015	−0.726	0.696		.967

Abbreviations: ref, reference category; sTfR, soluble transferrin receptor.

Note. Adjustment for age was done with age as a continuous variable; adjustment for sampling time was done with sampling time as a categorical variable (7:30 AM to 12 PM, 12-3 PM, 3-6 PM).

[a]Beta expresses the change in the dependent variable—log-transformed serum hepcidin/ferritin ratio—that is associated with a 1-unit change in the independent variable.

[b]For males, multivariate linear regression model with log-transformed hepcidin/ferritin ratio as the dependent variable and age (continuous variable) and sampling time as independent variables showed R^2 26.8%, with ß −0.057, CI −0.072 to −0.041 (*P* .000), ß 0.175, CI 0.023-0.326 (*P* .024), ß 0.432, CI 0.221-0.643 (*P* .000) for age, sampling time 12-3 PM and sampling time 3-6 PM, respectively.

[c]For females, multivariate linear regression model with log-transformed hepcidin/ferritin ratio as the dependent variable and age (continuous variable) and sampling time as independent variables showed R^2 13.6%, with ß −0.042, CI −0.063 to −0.021 (*P* .000), ß 0.179, CI 0.026-0.384 (*P* .087), ß 0.250, CI −0.039-0.539 (*P* .089) for age, sampling time 12-3 PM and sampling time 3-6 PM, respectively.

[d]These 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 beta% change in the hepcidin/ferritin ratio.

[e]CRP levels of children were between >0.1 and <5 mg/L.

[f]Body mass index was assessed according to the international standards established by Cole and Lobstein.[36]

We found no difference between serum hepcidin levels between males and females. Serum ferritin as a reflection of iron stores was the most important correlate of serum hepcidin; however, the variance in serum hepcidin levels that could be explained by serum ferritin levels was considerably lower (R^2 15.1% for males, 7.9% for females) than that for adults (R^2 56% for men, 60% for women).[20] Although mice studies suggest that circulating iron also influences serum hepcidin concentration,[42,43] TSAT was only marginally associated with serum hepcidin levels, as seen in adults.[20]

We obtained the serum hepcidin/ferritin ratio and the TSAT/hepcidin ratio to get insight into the set off of the hepcidin regulatory pathway relative to stored iron (ferritin) and circulating iron (TSAT) with aging, as reported before.[6] Interestingly, serum hepcidin relative to ferritin and TSAT was lower for older compared to younger children and also compared to adults.[21] This was reflected in a lower hepcidin/ferritin ratio and an increased TSAT/hepcidin ratio, which implies that the extent of induction of the different regulatory pathways of hepcidin by both iron stores and circulating iron in healthy children and adolescents is dependent on age.[42,43]

These age-specific differences in hepcidin set points might be an expression of the two opposite operating forces considering iron metabolism. Iron is indispensable for the production of heme proteins and for other vital functions requiring a persistent flow of iron into the blood stream, especially during periods of rapid growth during infancy. A recent study of Armitage et al indeed observed that antecedent weight gain was negatively associated with serum hepcidin levels in Gambian infants.[39] However, the virulence of infectious organisms depends on their ability to assimilate iron from their host. Therefore, humans and other mammals have to cope with piracy of iron by pathogens in order to battle infections.[44] Since hepcidin has the ability to shut down ferroportin and limit the export of iron from the intestines and the macrophages into the circulation, it has an important role in the innate immune system.[45] We hypothesize that the high hepcidin levels in young children relative to adolescents might result

TABLE 6 Results of linear regression models for transferrin saturation (TSAT)/hepcidin ratio (%/nmol) adjusted for age and time of blood sampling and stratified by sex

		Males					Females				
			95% confidence interval (CI)					95% CI			
Variable		Beta[a]	Lower limit	Upper limit	R^2, %[b]	*P*	Beta[a]	Lower limit	Upper limit	R^2, %[c]	*P*
Hb, g/dL		−0.002	−0.112	0.108	22.9	.971	−0.008	−0.119	0.103	5.5	.885
Reticulocytes, $\times 10^9$/L		−0.004	−0.010	0.002	21.7	.184	−0.003	−0.004	0.010	6.1	.413
MCV, fL		0.012	−0.014	0.039	21.2	.354	0.027	−0.005	0.060	8.0	.102
Ferritin, µg/L[d]		−1.325	−1.716	−0.934	39.0	.000	−0.703	−1.195	−0.211	12.6	.006
Iron, µmol/L		0.024	0.007	0.040	24.9	.005	0.019	0.000	0.037	9.2	.048
TIBC, µmol/L		0.004	−0.006	0.014	21.1	.420	0.009	−0.003	0.021	7.4	.162
sTfR, mg/L		−0.050	−0.398	0.297	20.8	.775	−0.452	−0.926	0.023	8.8	.062
ALT, IU/L[d]		−0.726	−1.295	−0.158	24.0	.013	0.466	−0.392	1.324	6.6	.284
CRP, mg/L[d,e]		−0.338	−0.462	−0.213	33.5	.000	−0.217	−0.374	−0.060	12.1	.007
BMI[f]	Normal weight	ref	ref	ref			ref	ref	ref		
	Underweight	−0.674	−1.266	−0.082	25.1	.026	−0.131	−0.390	0.652	4.5	.620
	Overweight	−0.280	−0.535	−0.025		.031	−0.159	−0.539	0.220		.407
	Adipose	−0.305	−0.768	0.158		.195	−0.023	−0.824	0.788		.955

Abbreviations: ref, reference category; sTfR, soluble transferrin receptor.

Note. Adjustment for age was done with age as a continuous variable; adjustment for sampling time was done with sampling time as a categorical variable (7:30 AM to 12 PM, 12-3 PM, 3-6 PM).

[a]Beta expresses the change in the dependent variable—log transformed TSAT/hepcidin ratio—that is associated with a 1-unit change in the independent variable.

[b]For males, multivariate linear regression model with log transformed TSAT/hepcidin as the dependent variable and age (continuous variable) and sampling time as independent variables showed R^2 21.3%, with ß: 0.059, CI 0.040-0.077 (*P* .000), ß: −0.168, CI −0.350-0.015 (*P* .071), ß: −0.484, CI −0.738 to −0.231 (*P* .000) for age, sampling time 12-3 PM and sampling time 3-6 PM, respectively.

[c]For females, multivariate linear regression model with log transformed TSAT/hepcidin as the dependent variable and age (continuous variable) and sampling time as independent variables showed R^2 6.5%, with ß: 0.037, CI −0.013-0.061 (*P* .003), ß: −0.103, CI −0.340-0.133 (*P* .388), ß: −0.152, CI −0.487-0.183 (*P* .370) for age, sampling time 12-3 PM and sampling time 3-6 PM, respectively.

[d]These independent variables were log transformed; the interpretation of the regression coefficients for these variables is as follows: a 1% change in the independent variable corresponds to a beta% change in the TSAT/hepcidin ratio.

[e]CRP levels of children were between >0.1 and <5 mg/L.

[f]Body mass index was assessed according to the international standards established by Cole and Lobstein.[36]

in a survival advantage during a critical period of high vulnerability for serious infections.[44,45]

We observed a positive correlation of CRP levels (between 0.1 and 5 mg/L) with serum hepcidin levels, suggesting that, as described earlier,[46] even minor infections and/or inflammation induce hepcidin production in children, consistent with the suggested protective antimicrobial activity of hepcidin.[2] Importantly, the relatively high hepcidin levels for ferritin in young children as compared to adolescents did not result in IDA in these subjects, despite the enormous growth during infancy that is accompanied by a considerable increase of circulating blood volume and other tissues requiring iron. Studies in suckling mice suggest that enterocyte ferroportin is hyporesponsive to hepcidin during infancy.[47] Alterations to ferroportin that prevent hepcidin binding during suckling may allow iron absorption to remain sufficient regardless of hepcidin expression levels, reducing the likelihood of ID during development.[47] Whether ferroportin is still functional in the presence of increased hepcidin levels in young children, as seen in infant mice, remains to be investigated.

In both males and females, hepcidin levels were considerably lower after the age of 12 years, dropping below normal adult levels,[21] suggesting a different regulation of hepcidin production during adolescence. We suggest that the decrease in hepcidin levels in older children can be attributed to the direct influence of the gonadal hormones testosterone[48] and estrogens,[49] and also to the indirect influence of these hormones since both testosterone and estrogen stimulate growth hormone/IGF-1 secretion, which in turn inhibits hepcidin production.[50] The relatively low hepcidin levels in relation to body iron status in postpubertal compared to prepubertal children might reflect adaptation in order to guarantee sufficient iron in this period of rapid development and maturation.

Compared with adults, young children have relatively high and children >12 years relatively low hepcidin/ferritin ratios.[21] TSAT/hepcidin ratios in our population are comparable to those in adults until the age of 12 years. Thereafter, we observe a significant increase.[21] Altogether, our data suggest that the set point of serum hepcidin relative to

indicators of stored and circulating iron changes during human growth and development.

Until now, lack of harmonization and standardization of hepcidin measurements[51] has hampered comparison with other hepcidin reference studies in children.[24,26,27] To some extent, comparison is possible with the data obtained by Uijterschout et al[25] from children aged 0.5-3 years (n = 400). They used the same assay as we did, before standardization. However, standardization only slightly altered its values, that is, standardized results were found to be a factor of 1.054351 higher compared to historic results obtained without standardization (Laarakkers, Swinkels, unpublished findings). In their study, median hepcidin was slightly higher (3.6 nmol/L; p2.5 to p97.5 0.6-13.9 nmol/L) than that in our age group of 0 to <2 years (n = 17) (1.8 nmol/L; p2.5 to p75 0.2-6.3 nmol/L), with overlapping ranges. The finding of ferritin as the most important indicator of serum hepcidin concentration after adjustment for sampling time and age was consistent between the study of Uijterschout et al and our study.

The strength of our study is the unique and well-defined population (eg, no underlying disease, CRP <5 mg/L), covering the range from infancy to adolescence. We established hepcidin values relative to indicators of iron status, described in ratios, enabling diagnosis of iron loading and ID disorders. Moreover, we used an assay that was standardized using a recently validated and value assigned second RM.[22] Therefore, the values defined here can be used by all other analytically validated hepcidin assays that are standardized using the same RM.

Our study has several limitations. First, young children, children of non-Western European descent, and children with under- and overweight were underrepresented. Second, we used age >12 years as a proxy for the discrimination between prepubertal and postpubertal stage, which might not be accurate in all children. Furthermore, our study population might contain children with undiagnosed iron related disorders such as IRIDA or hereditary hemochromatosis, since we performed no genotyping of *TMPRSS6* and *HFE* in the subjects. However, given the low prevalence of these disorders,[52,53] we expect only a few of these cases, if any, among our participants.

In conclusion, we provide age- and sex-specific serum hepcidin values in healthy Dutch children as a first step to better elucidating the clinical utility of hepcidin in children with iron disorders. Our data suggest that the serum hepcidin set point relative to indicators of stored and circulating iron changes during human growth and development. Since we used a standardized hepcidin assay, the age- and sex-specific values of our assay can be applied to all validated assays that are standardized using the same RM. This paves the way for future studies needed to better elucidate the clinical utility of hepcidin in children with iron disorders.

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AUTHOR CONTRIBUTIONS

Albertine E. Donker, Dirk L. Bakkeren, and Dorine W. Swinkels designed the research. Albertine E. Donker and Dirk L. Bakkeren coordinated the data collection. Siem M. Klaver and Coby M. Laarakkers performed the hepcidin analyses. Albertine E. Donker, Dirk L. Bakkeren, Tessel E. Galesloot, and Dorine W. Swinkels analyzed the data and interpreted the results. Albertine E. Donker wrote the manuscript.

CONFLICT OF INTEREST

Siem M. Klaver, Coby M. Laarakkers, and Dorine W. Swinkels are employees of Radboudumc, which offers hepcidin assays and hepcidin reference material to the research, clinical, and pharmaceutical community at a fee for service via the Hepcidinanalysis initiative (www.hepcidinanalysis.com). All other authors have no conflict of interest to declare.

DATA SHARING STATEMENT

For original data, please contact Dorine W. Swinkels (dorine.swinkels@radboudumc.nl).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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