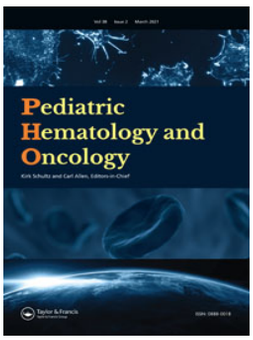

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Serum hepcidin concentrations in relation to iron status in children with type 1 diabetes

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ABSTRACT

Chronic low-grade inflammation in type 1 diabetes (T1D) might increase hepcidin synthesis, possibly resulting in functional iron deficiency (FID). We hypothesized that in T1D children with FID, hepcidin concentrations are increased compared to those with normal iron status and those with absolute iron deficiency (AID). We evaluated hepcidin concentrations in T1D children in relation to iron status, and investigated whether hepcidin is useful in assessing FID. A cross-sectional study was conducted. FID was defined as elevated zinc protoporphyrin/heme ratio and/or red blood cell distribution width, and AID as low serum ferritin concentration. Post-hoc analyses with different definitions of FID were performed, using transferrin saturation and reticulocyte hemoglobin content. Serum hepcidin concentrations were measured using mass-spectrometry. The IRODIAB-study is registered at www.trialregister.nl (NTR4642). This study included 215 T1D children with a median age of 13.7 years (Q_1–Q_3: 10.1–16.3). The median (Q_1–Q_3) hepcidin concentration in patients with normal iron status was 1.8 nmol/l (0.9–3.3), in AID-patients, 0.4 nmol/l (0.4–0.4) and in FID-patients, 1.6 nmol/l (0.7–3.5). Hepcidin concentrations in FID-patients were significantly higher than in AID-patients ($p < 0.001$). Irrespective of FID-definition used, hepcidin concentrations did not differ between FID-patients and patients with normal iron status. This might be explained by the influence of various factors on hepcidin concentrations, and/or by differences in response of iron parameters over time. Single hepcidin measurements do not seem useful in assessing FID in T1D children. Multiple hepcidin measurements over time in future studies, however, might prove to be more useful in assessing FID in children with T1D.

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Introduction

Type 1 diabetes (T1D) is one of the most common chronic diseases of childhood and is caused by immune-associated destruction of insulin-producing pancreatic beta-cells.[1] We previously reported that functional iron deficiency (ID) is common in children with T1D.[2] Since ID in children is associated with adverse effects, such as cognitive and behavioral impairment,[3,4] it is important to assess their iron status.

When assessing iron status, it is important to realize that ID can occur in two major forms: absolute and functional ID. Absolute ID (AID) refers to depleted iron stores and often develops when iron demand exceeds intestinal iron absorption rate. When iron stores are exhausted and iron availability for erythropoiesis is compromised, iron deficiency anemia occurs.[5,6] In functional ID (FID), inflammation induces changes in iron homeostasis by upregulating hepcidin synthesis. Hepcidin, a peptide hormone, is the key regulator of iron homeostasis. Hepcidin decreases intestinal iron absorption and increases iron retention in storage sites of the reticuloendothelial system, leading to reduced iron concentrations.[7,8] When iron availability is insufficient for erythropoiesis, so-called anemia of chronic disease may occur.[5,9] Hepcidin synthesis is regulated by various factors. Reduced iron concentrations and/or iron stores, increased erythropoietic activity and hypoxia, decrease hepcidin synthesis. Inflammation, infection and iron overload, increase hepcidin synthesis.[7,8] Differentiation between AID and FID, although challenging, is important because the therapeutic approach for both types of ID, varies.

There are few studies reporting on the prevalence of ID in children with T1D,[2,10,11] and only one study differentiated between AID and FID.[2] We previously showed that the prevalence rates of AID and FID in T1D children were 5.7% and 47%, respectively.[2] It remains unclear why FID was found relatively common in these children.

Since it has been reported that T1D is characterized by chronic low-grade inflammation,[12–14] we hypothesized that in T1D children with FID, hepcidin concentrations are increased compared to those with normal iron status and those with AID. We therefore evaluated serum hepcidin concentrations in the previously reported group of T1D children in relation to their iron status, and investigated whether hepcidin might be useful in assessing FID in children with T1D.

Materials and methods

Study design

Data were used from the IROn status of children with DIABetes mellitus type 1 (IRODIAB) study.[2] This study is registered at www.trialregister.nl (NTR4642) and was previously described in detail.[2] The IRODIAB-study is a cross-sectional study, conducted in the Netherlands in the Juliana Children's Hospital (JCH), The Hague and in the Reinier de Graaf Hospital (RdGH), Delft, from January 2015 to September 2016. The study was approved by the Medical Ethics Committee of South-West Holland. Written informed consent was obtained from each participant and/or their parent(s)/legal representative(s), depending on age.

Study population and procedure

Children 1 to 19 years of age were eligible for inclusion if they had been diagnosed with T1D[15] for at least 1 year and if blood was obtained at a follow-up visit during the study period. Exclusion criteria were: infection(s) in the last 4 weeks, hemoglobinopathy, congenital malformation, oncologic disorder, iron supplementation in the last 6 weeks and/or blood transfusion in the last 6 months. Patients with a high-sensitivity C-reactive protein (hsCRP) concentration $\geq$10 mg/l were registered but not included in the analyses. Children with T1D were identified through medical records. Eligible patients were approached for study participation. After signing informed consent, additional blood was drawn to assess iron status. Demographic and clinical data were collected from medical records and interviews with the participant and/or their parent(s)/legal representative(s).

Laboratory analysis

Non-fasting blood samples were taken throughout various times of the day. Blood sampling performed before and after 12 PM was registered. Blood samples were analyzed for hemoglobin (Hb), mean corpuscular volume (MCV) and red blood cell distribution width (RDW), using a Sysmex XN-1000 (JCH) or Sysmex XN-2000 (RdGH) automated hematology analyzer (Sysmex Corporation, Kobe, Japan). RDW-coefficient of variation (RDW-CV) was used for JCH-participants and RDW-standard deviation (RDW-SD) for RdGH-participants. RDW is an index of anisocytosis, and has been described as an early indicator of ID.[16] Reticulocyte hemoglobin content (Ret-He) was measured using a Sysmex XE-2100 analyzer (Sysmex Corporation, Kobe, Japan). Plasma iron, total iron binding capacity (TIBC), transferrin, transferrin saturation (TSAT), serum ferritin (SF) and hsCRP were measured using a COBAS 6000 or 8000 Clinical Chemistry and Immunochemistry Analyzer (Roche Diagnostics, Mannheim, Germany). Zinc protoporphyrin/heme ratio (ZnPP/H) was analyzed in whole blood after washing with phosphate buffered saline, using a ZP-Hematofluorometer Model 206 (AVIV Biochemical Company, Lakewood, NJ, USA). ZnPP/H increases in case of iron-restricted erythropoiesis, since zinc replaces missing iron in the protoporphyrin-ring during heme synthesis.[17] Glycated hemoglobin A1c (HbA1c) was determined using a Tosoh G8 HPLC Analyzer (Sysmex Corporation, Kobe, Japan). Finally, serum hepcidin concentrations were measured using a combination of Weak Cation eXchange chromatography and Time of Flight mass-spectrometry (WCX-TOF MS) (Hepcidinanalysis.com, Nijmegen, the Netherlands).[18] Hepcidin measurements were preceded by one freeze-thaw cycle. An internal standard (synthetic heavy hepcidin-25 stable isotope +40 Da; custom made Peptide International Inc.) was used for quantification.[19] Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOF MS platform (Bruker Daltonics, Bremen, Germany). The lower detection limit was 0.5 nmol/l; average coefficients of variation were 2.8% (intra-run) and 6.4% (inter-run).[19] For hepcidin concentrations below the detection limit, a value of 0.4 nmol/l was used.

Table 1. Age- and sex-specific cutoff values of Ret-He.

Sex	Age (years)	Ret-He (fmol)
Male	<6	<1.719
	6–12	<2.011
	12-18	<1.880
	≥18	<2.234
Female	<6	<1.818
	6-12	<1.887
	12-18	<1.856
	≥18	<1.899

Abbreviation: Ret-He, reticulocyte hemoglobin content.

Definitions

When applicable, World Health Organization (WHO) guidelines were followed to determine age- and sex-specific cutoff values for iron parameters used.[20,21] AID was defined as SF <12 µg/l in patients <5 years of age or SF <15 µg/l in patients ≥5 years of age, in the absence of infection and/or acute inflammation (hsCRP-concentration ≥10 mg/l).[20] FID was defined as ZnPP/H > 61 µmol/mol heme in patients <5 years of age or ZnPP/H > 70 µmol/mol heme in patients ≥5 years of age[20] and/or RDW-CV >14%[22] or RDW-SD >43.39 fl.[23] Patients with AID may have signs of impaired erythropoiesis (ie elevated ZnPP/H and/or RDW). Therefore, FID was determined in patients without AID. Patients with normal iron status were defined as not having anemia, AID and/or FID. Anemia was defined as Hb >2 SDs below the mean of age- and sex-matched children.[20] Ethnicity was classified as Caucasian (if both parents were Caucasian) and non-Caucasian (if at least one parent was non-Caucasian). Socioeconomic status (SES) was determined by postal code. SES-scores are available for all postal code areas in the Netherlands. Lower SES-scores represent lower socioeconomic status. Diabetes regulation was assessed by HbA1c. HbA1c-levels are presented as both mmol/mol and %.[24] Body mass index (BMI) and BMI-SD were calculated using Dutch growth charts.

Additional post-hoc analyses were performed, using different definitions of FID. In the first post-hoc analysis (PHA-1), FID was defined as TSAT <15%.[21] In the second post-hoc analysis (PHA-2), FID was defined by Ret-He using age- and sex-specific cutoff values (Table 1).[25] For converting pg to fmol, a conversion factor of 0.06206 was used.[25] In both post-hoc analyses, FID was determined in patients without AID.

Statistical analysis

For all analyses, SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) was used. Distribution of data was assessed using histograms and Shapiro-Wilk tests. Data are presented as mean with standard deviation (SD) for normally distributed variables, or median with first and third quartiles (Q_1–Q_3) for non-normally distributed variables. Categorical variables are presented as numbers with percentage. Comparisons between groups were made using the independent T-test for normally distributed variables and the Mann-Whitney U non-parametric test for non-normally distributed variables. For categorical variables, comparisons between groups were made using the Chi-squared or Fisher's exact test. Statistical significance was defined as $p < 0.05$.

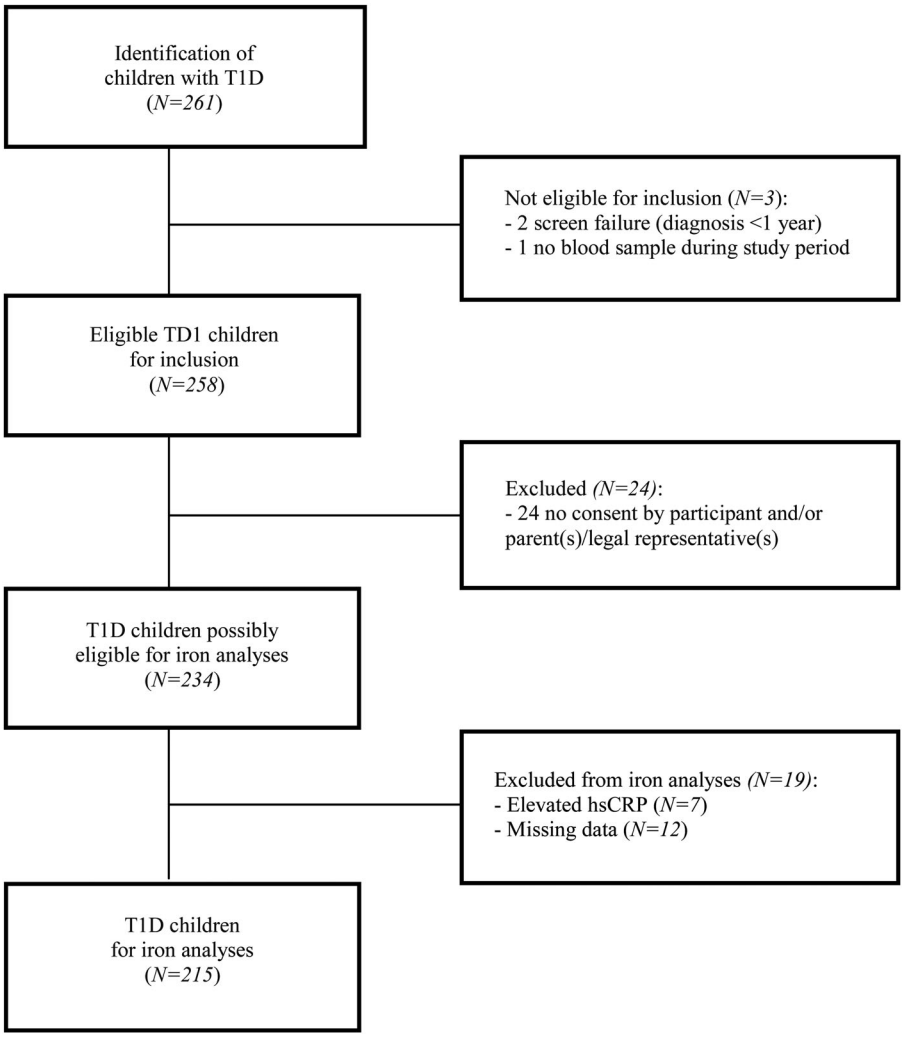

Figure 1. Flow chart of the study population. Abbreviations: T1D, type 1 diabetes; *N*, number; hsCRP, high-sensitivity C-reactive protein.

Results

Study population

In total, 261 children with T1D were identified. A total of 234 patients were included, 7 of whom had hsCRP-concentration $\geq$10 mg/l and 12 had missing data regarding iron status, and were therefore excluded from analyses. Data from the remaining 215 patients were used for analyses (Figure 1).

The median age of the total study population was 13.7 years (10.1–16.3). There was an equal sex distribution (50.7% male). Ninety-two patients (42.7%) were Caucasian. The median disease duration was 4.3 years (2.2–7.6). The median hepcidin concentration was 1.6 nmol/l (0.7–3.3); the median HbA1c-level, 62.0 mmol/mol (56.0–73.0) or

7.8% (7.3–8.8) and the median hsCRP-concentration, 0.5 mg/l (0.2–1.3). Characteristics and laboratory measurements of the study population are shown in Table 2.

Iron status – main analysis

A total of 102 patients (47.4%) had normal iron status, 11 patients (5.1%) had AID, and 101 patients (47.0%) had FID (Table 2). One patient had anemia without AID or FID, and was therefore not assigned to an iron status group. Sex distribution differed between AID-patients and FID-patients ($p = 0.008$), and patients with normal iron status ($p = 0.008$), as only one male was present in the AID-group (Table 2). AID-patients had significant lower hepcidin concentrations (0.4 nmol/l (0.4–0.4)) than FID-patients (1.6 nmol/l (0.7–3.5), $p < 0.001$) and patients with normal iron status (1.8 nmol/l (0.9–3.3), $p < 0.001$). Hepcidin concentrations did not differ between FID-patients and patients with normal iron status ($p = 0.632$). No differences in hepcidin concentrations were found between female and male patients with FID ($p = 0.832$) or with normal iron status ($p = 0.573$). SF-concentrations in AID-patients (8.0 µg/l (6.0–12.0)) were lower than in FID-patients (39.0 µg/l (28.0–59.0), $p < 0.001$) and in patients with normal iron status (41.5 µg/l (30.0–54.0), $p < 0.001$) (Table 2). No differences in HbA1c-levels or hsCRP-concentrations were found between iron status groups (Table 2).

Iron status – post-hoc analysis-1 (PHA-1)

With FID defined by TSAT, 147 patients (68.4%) had normal iron status, 11 patients (5.1%) had AID, and 51 patients (23.7%) had FID (Table 3). Six patients had anemia without AID or FID, and were therefore not assigned to an iron status group. FID-patients had a lower median age (11.6 years (8.7–15.3)) compared to AID-patients (14.0 years (13.1–16.5), $p = 0.034$) and patients with normal iron status (14.0 years (10.5–16.6), $p = 0.010$). Sex distribution differed between AID-patients and FID-patients ($p = 0.002$), and patients with normal iron status ($p = 0.010$), as only one male was present in the AID-group (Table 3). FID-patients more often were non-Caucasian and had lower SES-scores compared to patients with normal iron status ($p = 0.007$ and $p = 0.048$, respectively). AID-patients had significant lower hepcidin concentrations (0.4 nmol/l (0.4–0.4)) than FID-patients (1.4 nmol/l (0.6–2.8), $p < 0.001$) and patients with normal iron status (1.8 nmol/l (0.9–3.5), $p < 0.001$). No difference in hepcidin concentrations was found between FID-patients and patients with normal iron status ($p = 0.093$). Hepcidin concentrations did not differ between female and male patients with FID ($p = 0.169$), or with normal iron status ($p = 0.935$). SF-concentrations in AID-patients (8.0 µg/l (6.0–12.0)) were lower than in FID-patients (37.0 µg/l (28.0–54.0), $p < 0.001$) and patients with normal iron status (42.0 µg/l (31.0–58.0), $p < 0.001$). Higher hsCRP-concentrations were found in FID-patients (0.9 mg/l (0.5–2.5)) compared to AID-patients (0.3 mg/l (0.1–0.7), $p = 0.010$), and patients with normal iron status (0.4 mg/l (0.2–1.0), $p < 0.001$). No differences in HbA1c-levels were found between iron status groups (Table 3).

Table 2. Characteristics and laboratory measurements in all T1D children and in relation to iron status.

	All (N = 215)	Normal iron status (N = 102)	AID (N = 11)	FID (N = 101)	p_1	p_2	p_3
Characteristics							
Age (years)	13.7 (10.1–16.3)	14.1 (9.9–16.4)	14.0 (13.1–16.5)	12.9 (9.7–16.1)	0.427	0.343	0.173
Sex (male)	109 (50.7%)	54 (52.9%)	1 (9.1%)	54 (53.5%)	0.008*	0.940	0.008*
Caucasian ethnicity	92 (42.7%) *(N=178, M=37)*	45 (44.1%) *(N=81, M=21)*	4 (36.4%)	43 (42.6%) *(N=86, M=15)*	0.334	0.420	0.526
Disease duration (years)	4.3 (2.2–7.6)	4.5 (2.3–7.4)	8.3 (3.8–9.1)	4.0 (2.0–7.2)	0.144	0.464	0.091
SES-score	0.60 (–0.89;1.25) *(N=214, M=1)*	0.70 (–0.42;1.37) *(N=101, M=1)*	0.25 (–1.42;0.93)	0.52 (–1.42;1.18)	0.163	0.167	0.500
BMI-SD	0.79 (±0.98) *(N=202, M=13)*	0.67 (±0.99) *(N=93, M=9)*	0.75 (±0.74)	0.91 (±0.99) *(N=97, M=4)*	0.899	0.116	0.546
Menarche (female)	49 (58.3%) *(N=84, M=22)*	16 (48.5%) *(N=33, M=15)*	9 (90.0%) *(N=10, M=0)*	24 (58.5%) *(N=41, M=6)*	0.028*	0.483	0.077
Time of blood sampling (after 12 PM)	177 (82.3%)	88 (86.3%)	10 (90.9%)	75 (77.2%)	1.000	0.105	0.452
Laboratory measurements							
Hepcidin (nmol/l)	1.6 (0.7–3.3) *(N=211, M=4)*	1.8 (0.9–3.3) *(N=99, M=3)*	0.4 (0.4–0.4) *(N=10, M=1)*	1.6 (0.7–3.5)	0.000*	0.632	0.000*
SF (μg/l)	39.0 (28.0–55.0)	41.5 (30.0–54.0)	8.0 (6.0–12.0)	39.0 (28.0–59.0)	0.000*	0.496	0.000*
ZnPP/H (μmol/mol heme)	70.0 (60.0–87.5)	62.5 (55.0–65.0)	162.5 (82.5–345.0)	85.0 (75.0–110.0)	0.000*	0.000*	0.007*
RDW-CV (%)	12.5 (12.0–12.9) *(N=179, M=0)*	12.3 (12.0–12.8) *(N=87, M=0)*	14.2 (12.7–17.1) *(N=10, M=0)*	12.5 (12.0–13.1) *(N=81, M=0)*	0.000*	0.198	0.001*
RDW-SD (fl)	39.1 (37.6–41.3) *(N=36, M=0)*	38.9 (37.3–41.5) *(N=15, M=0)*	38.5 *(N=1, M=0)*	39.4 (37.4–41.0) *(N=20, M=0)*	NA	0.560	NA
MCV (fl)	85.0 (82.0–88.0)	86.0 (83.0–88.0)	76.0 (67.0–84.0)	84.0 (81.0–87.5)	0.000*	0.002*	0.004*
Hb (mmol/l)	8.4 (7.9–8.9)	8.5 (8.1–9.1)	7.2 (6.2–7.7)	8.4 (7.9–8.9)	0.000*	0.269	0.000*
Plasma iron (μmol/l)	15.0 (11.0–20.0)	15.0 (12.0–20.0)	5.0 (2.9–10.0)	15.0 (11.0–20.0)	0.000*	0.550	0.001*
TIBC (μmol/l)	76.0 (71.0–82.0)	75.0 (70.8–79.0)	94.0 (91.0–107.0)	75.0 (70.0–82.0)	0.000*	0.535	0.000*
Transferrin (g/l)	2.7 (2.5–2.9)	2.7 (2.5–2.8)	3.5 (3.3–4.0)	2.7 (2.5–2.9)	0.000*	0.762	0.000*
TSAT (%)	19.0 (14.0–26.0)	20.5 (16.0–27.3)	6.0 (3.0–11.0)	19.0 (14.0–26.0)	0.000*	0.437	0.000*
Ret-He (fmol)	1.9 (0.0–2.0) *(N=206, M = 9)*	2.0 (0.0–2.1) *(N = 97, M = 5)*	1.6 (1.2–1.9)	1.9 (0.0–2.0) *(N = 97, M = 4)*	0.032*	0.178	0.074

(*continued*)

Table 2. Continued.

	All (N = 215)	Normal iron status (N = 102)	AID (N = 11)	FID (N = 101)	p_1	p_2	p_3
HbA1c (mmol/mol)	62.0 (56.0–73.0)	62.0 (55.0–71.0)	59.0 (55.0–63.0)	62.0 (56.0–75.5)	0.473	0.632	0.411
HbA1c (%)	7.8 (7.3–8.8)	7.8 (7.2–8.6)	7.5 (7.2–7.9)	7.8 (7.3–9.1)			
hsCRP (mg/l)	0.5 (0.2–1.3)	0.5 (0.2–1.2)	0.3 (0.1–0.7)	0.5 (0.3–1.9)	0.296	0.266	0.110

Data are expressed as median (Q_1–Q_3) or numbers (percentages). In case of normal distribution, means (with standard deviation) are reported. Abbreviations: T1D, type 1 diabetes; AID, absolute iron deficiency; FID, functional iron deficiency; *N*, number; *M*, missing; SES-score, socioeconomic status score; BMI, body mass index; SD, standard deviation; SF, serum ferritin; ZnPP/H, zinc protoporhyrin/heme ratio; RDW, red blood cell distribution width; CV, coefficient of variation; NA, not applicable; MCV, mean corpuscular volume; Hb, hemoglobin; TIBC, total iron binding capacity; TSAT, transferrin saturation; Ret-He, reticulocyte hemoglobin content; HbA1c, glycated hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein. p_1 denotes p-value regarding comparisons between "normal iron status" and "AID," p_2 denotes p-value regarding comparisons between "normal iron status" and "FID," p_3 denotes p-value regarding comparisons between "AID" and "FID." *Statistically significant with $p < 0.05$.

Iron status – post-hoc analysis-2 (PHA-2)

With FID defined by Ret-He, 98 patients (45.6%) had normal iron status, 11 patients (5.1%) had AID, and 95 patients (44.2%) had FID (Table 4). Nine patients had missing Ret-He, and two patients had anemia without AID or FID, and were therefore not assigned to an iron status group. FID-patients had a lower median age (11.3 years (8.8–15.5)) compared to AID-patients (14.0 years (13.1–16.5), $p = 0.043$) and patients with normal iron status (14.6 years (12.0–16.5), $p = 0.005$). Sex distribution differed between AID-patients and FID-patients ($p = 0.002$), and patients with normal iron status ($p = 0.025$), as only one male was present in the AID-group (Table 4). Moreover, more males were present in the FID-group compared to the normal iron status group ($p = 0.025$). FID-patients more often were non-Caucasian and had lower SES-scores compared to patients with normal iron status ($p = 0.002$ and $p = 0.017$, respectively). FID-patients had a shorter median disease duration (3.9 years (2.0–6.5)) compared to AID-patients (8.3 years (3.8–9.1), $p = 0.030$). AID-patients had significant lower hepcidin concentrations (0.4 nmol/l (0.4–0.4)) than FID-patients (1.7 nmol/l (0.8–2.8), $p < 0.001$) and patients with normal iron status (1.8 nmol/l (0.9–3.6), $p < 0.001$). No difference in hepcidin concentrations was found between FID-patients and patients with normal iron status ($p = 0.235$). Hepcidin concentrations did not differ between female and male patients with FID ($p = 0.500$) or with normal iron status ($p = 0.702$). SF-concentrations in AID-patients (8.0 µg/l (6.0–12.0)) were lower than in FID-patients (41.0 µg/l (29.0–54.0), $p < 0.001$) and patients with normal iron status (41.0 µg/l (30.8–59.0), $p < 0.001$). No differences in HbA1c-levels or hsCRP-concentrations were found between iron status groups (Table 4).

Discussion

We evaluated serum hepcidin concentrations in relation to iron status in children with T1D. We hypothesized that in T1D children with FID, hepcidin concentrations are increased compared to those with normal iron status due to increased chronic low-grade inflammation. Irrespective of iron parameters used to define FID, hepcidin concentrations did not differ between patients with FID and patients with normal iron

Table 3. Post-hoc analysis-1 (PHA-1) – Characteristics and laboratory measurements in all T1D children and in relation to iron status.

	All (N = 215)	Normal iron status (N = 147)	AID (N = 11)	FID (N = 51)	p_1	p_2	p_3
Characteristics							
Age (years)	13.7 (10.1–16.3)	14.0 (10.5–16.6)	14.0 (13.1–16.5)	11.6 (8.7–15.3)	0.486	0.010*	0.034*
Sex (male)	109 (50.7%)	75 (51.0%)	1 (9.1%)	32 (62.7%)	0.010*	0.192	0.002*
Caucasian ethnicity	92 (42.7%) *(N=178, M=37)*	71 (48.3%) *(N=118, M=29)*	4 (36.4%)	15 (29.4%) *(N=43, M=8)*	0.200	0.007*	1.000
Disease duration (years)	4.3 (2.2–7.6)	4.7 (2.2–7.4)	8.3 (3.8–9.1)	3.4 (2.0–6.5)	0.153	0.231	0.064
SES-score	0.60 (–0.89;1.25) *(N=214, M=1)*	0.72 (–0.37;1.34) *(N=146, M=1)*	0.25 (–1.42;0.93)	0.25 (–1.78;0.86)	0.166	0.048*	0.912
BMI-SD	0.79 (±0.98) *(N=202, M=13)*	0.72 (±0.98) *(N=137, M=10)*	0.75 (±0.74)	1.07 (±1.01) *(N=48, M=3)*	0.936	0.083	0.360
Menarche (female)	49 (58.3%) *(N=84, M=22)*	30 (55.6%) *(N=54, M=18)*	9 (90.0%) *(N=10, M=0)*	7 (41.2%) *(N=17, M=2)*	0.074	0.406	0.018*
Time of blood sampling (after 12 PM)	177 (82.3%)	117 (79.6%)	10 (90.9%)	45 (88.2%)	0.693	0.209	1.000
Laboratory measurements							
Hepcidin (nmol/l)	1.6 (0.7–3.3) *(N=211, M=4)*	1.8 (0.9–3.5)	0.4 (0.4–0.4) *(N=10, M=1)*	1.4 (0.6–2.8) *(N=48, M=3)*	0.000*	0.093	0.000*
SF (μg/l)	39.0 (28.0–55.0)	42.0 (31.0–58.0)	8.0 (6.0–12.0)	37.0 (28.0–54.0)	0.000*	0.173	0.000*
ZnPP/H (μmol/mol heme)	70.0 (60.0–87.5)	67.5 (60.0–80.0)	162.5 (82.5–345.0)	72.5 (65.0–92.5)	0.000*	0.030*	0.001*
RDW-CV (%)	12.5 (12.0–12.9) *(N=179, M=0)*	12.3 (12.0–12.9) *(N=123, M=0)*	14.2 (12.7–17.1) *(N=10, M=0)*	12.5 (12.1–13.0) *(N=42, M=0)*	0.000*	0.210	0.001*
RDW-SD (fl)	39.1 (37.6–41.3) *(N=36, M=0)*	38.8 (37.3–40.7) *(N=24, M=0)*	38.5 *(N=1, M=0)*	40.2 (38.7–41.8) *(N=9, M=0)*	NA	0.093	NA
MCV (fl)	85.0 (82.0–88.0)	86.0 (83.0–88.0)	76.0 (67.0–84.0)	82.0 (80.0–86.0)	0.000*	0.000*	0.019*
Hb (mmol/l)	8.4 (7.9–8.9)	8.5 (8.2–9.0)	7.2 (6.2–7.7)	8.2 (7.7–8.9)	0.000*	0.007*	0.000*
Plasma iron (μmol/l)	15.0 (11.0–20.0)	17.0 (15.0–21.0)	5.0 (2.9–10.0)	9.2 (7.0–10.0)	0.000*	0.000*	0.102
TIBC (μmol/l)	76.0 (71.0–82.0)	74.0 (69.0–79.0)	94.0 (91.0–107.0)	77.0 (73.0–82.0)	0.000*	0.008*	0.000*
Transferrin (g/l)	2.7 (2.5–2.9)	2.6 (2.4–2.9)	3.5 (3.3–4.0)	2.8 (2.6–3.0)	0.000*	0.013*	0.000*
TSAT (%)	19.0 (14.0–26.0)	23.0 (19.0–29.0)	6.0 (3.0–11.0)	12.0 (9.0–13.0)	0.000*	0.000*	0.023*

(*continued*)

Table 3. Continued.

	All (N = 215)	Normal iron status (N = 147)	AID (N = 11)	FID (N = 51)	p_1	p_2	p_3
Ret-He (fmol)	1.9 (0.0–2.0) *(N=206, M=9)*	2.0 (0.0–2.0) *(N=140, M=7)*	1.6 (1.2–1.9)	1.9 (0.0–2.0) *(N=50, M=1)*	0.019*	0.008*	0.267
HbA1c (mmol/mol)	62.0 (56.0–73.0)	62.0 (55.0–73.0)	59.0 (55.0–63.0)	62.0 (57.0–69.0)	0.440	0.816	0.444
HbA1c (%)	7.8 (7.3–8.8)	7.8 (7.2–8.8)	7.5 (7.2–7.9)	7.8 (7.4–8.5)			
hsCRP (mg/l)	0.5 (0.2–1.3)	0.4 (0.2–1.0)	0.3 (0.1–0.7)	0.9 (0.5–2.5)	0.423	0.000*	0.010*

Data are expressed as median (Q_1–Q_3) or numbers (percentages). In case of normal distribution, means (with standard deviation) are reported. Abbreviations: T1D, type 1 diabetes; AID, absolute iron deficiency; FID, functional iron deficiency; *N*, number; *M*, missing; SES-score, socioeconomic status score; BMI, body mass index; SD, standard deviation; SF, serum ferritin; ZnPP/H, zinc protoporhyrin/heme ratio; RDW, red blood cell distribution width; CV, coefficient of variation; NA, not applicable; MCV, mean corpuscular volume; Hb, hemoglobin; TIBC, total iron binding capacity; TSAT, transferrin saturation; Ret-He, reticulocyte hemoglobin content; HbA1c, glycated hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein. p_1 denotes p-value regarding comparisons between "normal iron status" and "AID," p_2 denotes p-value regarding comparisons between "normal iron status" and "FID," p_3 denotes p-value regarding comparisons between "AID" and "FID." *Statistically significant with $p < 0.05$.

status. In patients with AID, we found lower hepcidin concentrations compared to patients with FID and patients with normal iron status.

This is the first study evaluating serum hepcidin concentrations in children with T1D in relation to their iron status. Recently, two studies, conducted in the same population of Polish children with T1D, reported a mean (95%confidence interval) hepcidin concentration of 0.25 ng/ml (0.20–0.29).[26,27] Both studies reported hepcidin concentrations in the total study population without distinguishing different iron status groups as we did. Moreover, different assays for hepcidin measurements were used that have not been similarly standardized. Therefore, comparison between hepcidin concentrations in our study population and those reported in the Polish studies, is hampered.

Various factors influencing hepcidin

Contrary to our hypothesis, and irrespective of iron parameters used to define FID, we found no increased serum hepcidin concentrations in T1D children with FID compared to those with normal iron status. This might be explained by the influence of various factors on hepcidin concentrations.

First, inflammation, which upregulates hepcidin synthesis,[7,8] seems to have limited influence in our study. In children with T1D, elevated hsCRP-concentrations have been found (compared to nondiabetic controls),[13,14,28] and these are similar to hsCRP-concentrations we found in our study population.[13,28] However, only in the PHA-1 group we found higher hsCRP-concentrations in FID-patients compared to patients with normal iron status, whereas hepcidin concentrations in this group were similar in both FID-patients and patients with normal iron status. Two studies showed a positive association between HbA1c and CRP in T1D children.[13,28] In our study, HbA1c-levels did not differ between iron status groups, regardless of the definition of FID, and patients had overall good glycemic control. The lack of an association between hsCRP and hepcidin suggests limited contribution of inflammation to FID in our study possibly due to good glycemic control throughout our study population.

Table 4. Post-hoc analysis-2 (PHA-2) – Characteristics and laboratory measurements in all T1D children and in relation to iron status.

	All (N = 215)	Normal iron status (N = 98)	AID (N = 11)	FID (N = 95)	p_1	p_2	p_3
Characteristics							
Age (years)	13.7 (10.1–16.3)	14.6 (12.0–16.5)	14.0 (13.1–16.5)	11.3 (8.8–15.5)	0.805	0.005*	0.043*
Sex (male)	109 (50.7%)	44 (44.9%)	1 (9.1%)	58 (61.1%)	0.025*	0.025*	0.002*
Caucasian ethnicity	92 (42.7%) *(N=178, M=37)*	52 (53.1%) *(N=81, M=17)*	4 (36.4%)	30 (31.6%) *(N=75, M=20)*	0.102	0.002*	1.000
Disease duration (years)	4.3 (2.2–7.6)	4.7 (2.7–8.8)	8.3 (3.8–9.1)	3.9 (2.0–6.5)	0.268	0.088	0.030*
SES-score	0.60 (–0.89;1.25) *(N=214, M=1)*	0.72 (–0.37;1.56)	0.25 (–1.42;0.93)	0.18 (–1.68;1.17) *(N=94, M=1)*	0.115	0.017*	0.695
BMI-SD	0.79 (±0.98) *(N=202, M=13)*	0.76 (±1.08) *(N=93, M=5)*	0.75 (±0.74)	0.91 (±0.85) *(N=87, M=8)*	0.887	0.306	0.540
Menarche (female)	49 (58.3%) *(N=84, M=22)*	30 (65.2%) *(N=46, M=8)*	9 (90.0%) *(N=10, M=0)*	9 (37.5%) *(N=24, M=13)*	0.253	0.042*	0.008*
Time of blood sampling (after 12 PM)	177 (82.3%)	80 (81.6%)	10 (90.9%)	80 (84.2%)	0.685	0.704	1.000
Laboratory measurements							
Hepcidin (nmol/l)	1.6 (0.7–3.3) *(N=211, M=4)*	1.8 (0.9–3.6)	0.4 (0.4–0.4) *(N=10, M=1)*	1.7 (0.8–2.8) *(N=92, M=3)*	0.000*	0.235	0.000*
SF (μg/l)	39.0 (28.0–55.0)	41.0 (30.8–59.0)	8.0 (6.0–12.0)	41.0 (29.0–54.0)	0.000*	0.551	0.000*
ZnPP/H (μmol/mol heme)	70.0 (60.0–87.5)	70.0 (60.0–80.0)	162.5 (82.5–345.0)	70.0 (60.0–92.5)	0.000*	0.338	0.000*
RDW-CV (%)	12.5 (12.0–12.9) *(N=179, M=0)*	12.2 (11.9–12.9) *(N=74, M=0)*	14.2 (12.7–17.1) *(N=10, M=0)*	12.5 (12.1–12.9) *(N=86, M=0)*	0.000*	0.202	0.000*
RDW-SD (fl)	39.1 (37.6–41.3) *(N=36, M=0)*	39.5 (37.9–41.5) *(N=24, M=0)*	38.5 *(N=1, M=0)*	38.9 (36.6–40.3) *(N=9, M=0)*	NA	0.332	NA
MCV (fl)	85.0 (82.0–88.0)	87.0 (83.8–89.0)	76.0 (67.0–84.0)	83.0 (80.0–87.0)	0.000*	0.000*	0.008*
Hb (mmol/l)	8.4 (7.9–8.9)	8.5 (8.1–9.1)	7.2 (6.2–7.7)	8.3 (8.0–8.8)	0.000*	0.046*	0.000*
Plasma iron (μmol/l)	15.0 (11.0–20.0)	16.0 (12.0–20.0)	5.0 (2.9–10.0)	14.0 (10.0–18.0)	0.000*	0.044*	0.001*
TIBC (μmol/l)	76.0 (71.0–82.0)	75.0 (69.8–81.0)	94.0 (91.0–107.0)	76.0 (71.0–81.0)	0.000*	0.983	0.000*
Transferrin (g/l)	2.7 (2.5–2.9)	2.7 (2.5–2.9)	3.5 (3.3–4.0)	2.7 (2.5–2.9)	0.000*	0.867	0.000*
TSAT (%)	19.0 (14.0–26.0)	20.5 (16.8–27.3)	6.0 (3.0–11.0)	19.0 (13.0–24.0)	0.000*	0.030*	0.000*
Ret-He (fmol)	1.9 (0.0–2.0) (*N=206*, *M=9*)	2.0 (2.0–2.1)	1.6 (1.2–1.9)	0.0 (0.0–1.9)	0.000*	0.000*	0.146

(*continued*)

Table 4. Continued.

	All (N = 215)	Normal iron status (N = 98)	AID (N = 11)	FID (N = 95)	p_1	p_2	p_3
HbA1c (mmol/mol)	62.0 (56.0–73.0)	62.0 (54.8–73.3)	59.0 (55.0–63.0)	62.0 (57.0–72.0)	0.475	0.773	0.359
HbA1c (%)	7.8 (7.3–8.8)	7.8 (7.2–8.9)	7.5 (7.2–7.9)	7.8 (7.4–8.7)			
hsCRP (mg/l)	0.5 (0.2–1.3)	0.5 (0.2–1.9)	0.3 (0.1–0.7)	0.5 (0.2–1.2)	0.139	0.600	0.174

Data are expressed as median (Q_1–Q_3) or numbers (percentages). In case of normal distribution, means (with standard deviation) are reported. Abbreviations: T1D, type 1 diabetes; AID, absolute iron deficiency; FID, functional iron deficiency; *N*, number; *M*, missing; SES-score, socioeconomic status score; BMI, body mass index; SD, standard deviation; SF, serum ferritin; ZnPP/H, zinc protoporhyrin/heme ratio; RDW, red blood cell distribution width; CV, coefficient of variation; NA, not applicable; MCV, mean corpuscular volume; Hb, hemoglobin; TIBC, total iron binding capacity; TSAT, transferrin saturation; Ret-He, reticulocyte hemoglobin content; HbA1c, glycated hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein. p1 denotes p-value regarding comparisons between "normal iron status" and "AID," p2 denotes p-value regarding comparisons between "normal iron status" and "FID," p_3 denotes p-value regarding comparisons between "AID" and "FID." *Statistically significant with $p < 0.05$.

Second, various stimuli with opposing effects on hepcidin synthesis may very well act at the same time.[8] For example, in our main-analysis group, it might be that in the FID-group, a combination of both AID and FID exists.[29] Both hepcidin and ferritin act as acute phase reactants. Therefore, the chronic low-grade inflammatory state in T1D children might not only cause an increase in hepcidin concentration, with subsequent elevated ZnPP/H and/or RDW (ie FID), but also an increase in SF-concentration, thus masking a co-existing AID. This might have resulted in hepcidin concentrations within the "normal range".

Third, it has been shown that hepcidin concentrations increase considerably within a few hours in response to increased iron concentrations.[30] In the PHA-1 group (defined by TSAT), we found lower plasma iron concentrations in FID-patients compared to patients with normal iron status, which might have contributed to not finding increased hepcidin concentrations in FID-patients. Plasma iron concentrations and subsequently TSAT, are subjective to dietary iron intake. Since blood samples were taken throughout various times of the day and without fasting, FID-patients in the PHA-1 group might have had less iron intake prior to blood sampling than patients with normal iron status resulting in reduced plasma iron and hepcidin concentrations.

Fourth, differences in age and ethnicity may have played a role in not finding increased hepcidin concentrations in FID-patients in both the PHA-1 and PHA-2 group. In both groups, FID-patients were considerably younger and counted more non-Caucasians than patients with normal iron status. Since significant lower hepcidin concentrations were described in children *older* than 12 years,[31] it seems unlikely that a younger age contributed to not finding increased hepcidin concentrations in T1D children with FID. Lower hemoglobin concentrations and TSAT have been described in African-Americans compared to Caucasians,[32,33] and might explain why more non-Caucasians were found among FID-patients in the PHA-1 and PHA-2 group. To our knowledge, there are no studies that clearly evaluated the influence of ethnicity on hepcidin concentrations. However, we cannot rule out that "more non-Caucasians" among FID-patients in the PHA-1 and PHA-2 group have contributed to not finding increased hepcidin concentrations in FID-patients.

Finally, it has been suggested that estrogen decreases hepcidin synthesis,[34,35] and hereby possibly causing a gender influence on hepcidin concentrations. We found a

majority of females in the AID-group compared to the FID- and normal iron status group. However, no differences in hepcidin concentrations were found between female and male patients with FID or with normal iron status (data not shown). It is therefore unlikely that the difference in sex distribution between iron status groups is responsible for the lower hepcidin concentrations found in patients with AID. We believe that blood loss, hence iron loss, due to menstruation (90% of female patients with AID attained menarche) predisposes females to the development of AID. This could explain why the majority of our T1D patients with AID is female.

Differences in response over time

Differences in response of iron parameters over time, may explain why hepcidin concentrations do not correspond with iron parameters used to define FID in T1D children. Red blood cell indices, such as MCV, hypochromic red cells, RDW and ZnPP/H, reflect the circulating population of mature red blood cells. As erythrocytes have an average lifespan of approximately 120 days, red blood cell indices may not reflect "current" iron status or changes in iron availability. Reticulocytes are the earliest forms of erythrocytes, and remain in the circulation for approximately 1–2 days. Reticulocyte-specific indices, like Ret-He, therefore are more sensitive to acute changes in iron availability, and represent a more "current" iron status than red blood cell indices. However, hepcidin acts as an acute phase reactant, and is known to decline very rapidly after an inflammatory signal has been cleared.[36] Slower reacting iron parameters such as ZnPP/H and RDW, and even Ret-He, might therefore not correspond with hepcidin concentrations in our study.

Strengths and limitations

Strengths of this study are the large patient population and the fact that acute infection is taken into account by excluding children with hsCRP-concentration $\geq$10 mg/l. The present study also has limitations. The cross-sectional design of the study might limit adequate evaluation of hepcidin concentrations in relation to iron status since iron parameters react differently to various stimuli over time. In this respect, multiple measurements of hepcidin over time might be more useful in assessing FID in children with T1D. Another limitation is that non-fasting blood samples were obtained, and therefore the impact of recent iron intake on hepcidin concentrations cannot be ruled out. Moreover, the lack of a healthy control group in our study limits the interpretation of the inflammatory state and hepcidin concentrations in T1D children with a different iron status. Finally, we primarily used ZnPP/H and RDW to define FID, because WHO approved reference values are available for children. We then performed additional post-hoc analyses using TSAT and Ret-He to define FID, although TSAT cutoff values are not specifically described for children. The use of different definitions of FID, based on different iron parameters reflecting different stages of iron metabolism, likely results in different compositions of iron status groups, as we have demonstrated. However, irrespective of FID-definition used, we did not find increased hepcidin concentrations in patients with FID compared to patients with normal iron status. We did not measure

soluble transferrin receptor (sTfR) because it is quite expensive, not widely available and reference values are not yet well established for children. However, since sTfR reflects tissue iron demand and erythropoietic activity and is not affected by inflammation, it might be a promising alternative to asses FID in children with T1D.

Conclusion

Contrary to our hypothesis, and irrespective of iron parameters used to define FID, we found no increased serum hepcidin concentrations in T1D children with FID compared to those with normal iron status. This might be explained by the different influences of various factors on hepcidin concentrations, but also by the differences in response of iron parameters over time. Based on these results, single measurements of serum hepcidin concentration do not seem to be useful in assessing FID in children with T1D. Further studies in T1D- and healthy children with multiple measurements of hepcidin concentrations over time are warranted.

Disclosure statement

The authors report no conflict of interest.

Data availability statement

Participants of this study did not agree for their data to be shared publicly, so supporting data is not available.

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