RESEARCH

Changes in serum hepcidin according to thyrometabolic status in patients with Graves' disease

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Abstract

Introduction: Hepcidin is an acute-phase protein and a key regulator of iron homeostasis. Anaemia frequently occurs in patients with thyroid dysfunction, and hepcidin may be a potential link.

Objectives: Prospective assessment of hepcidin serum concentration and other parameters related to Fe homeostasis in hyperthyroid patients in the course of GD at diagnosis and during remission.

Patients and Methods: Out of the 70 patients recruited, 42 (32 women, 10 men), aged 42.5 ± 15.1 years, met the inclusion criteria. Clinical and biochemical assessment, including hepcidin measurement by ELISA, was performed at baseline (T0) and after restoration of euthyroidism (T1).

Results: Hepcidin concentration at T0 in the 24 patients who completed the study was significantly higher than the value during euthyroidism (28.7 (8.1–39.4) ng/mL vs 7.9 (4.3–12.9) ng/mL, $P < 0.001$). Hepcidin level was most significantly correlated with ferritin ($\rho = 0.723$) in women at T0. In both men (377 (171–411) vs 165 (84–237) ng/mL, $P = 0.001$) and women (84 (23–104) vs 35 (16–64) ng/mL, $P = 0.001$), a significant decrease in ferritin level was demonstrated following therapy. A significant ($P < 0.001$) increase in mean corpuscular volume (MCV) (83.5 (82.5–87.1) vs 89.5 (88.8–90.0) fL) and mean concentration of haemoglobin (MCH) (29.0 (28.0–29.4) vs 30.4 (29.5–31.1) pg) was observed.

Conclusions: Hepcidin and ferritin decrease significantly during the transition from a hyperthyroid state to euthyroidism in patients with GD. The observed changes occur in parallel to iron homeostasis fluctuations. During the transition from the hyperthyroid state to euthyroidism, the improvement of haematological status is reflected mainly by the increase in MCV and MCH.

Key Words

- ▶ hepcidin
- ▶ Graves' disease
- ▶ hyperthyroidism
- ▶ anaemia
- ▶ iron

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Introduction

In the biochemical landscape of thyroid dysfunction, iron (Fe) homeostasis abnormalities are frequently observed (1, 2). The prevalence of anaemia in overt hyperthyroidism was found to be higher (14.6%) than in overt hypothyroidism (7.7%), subclinical thyroid dysfunctions (3.3–5%) and the euthyroid state (4.7%) according to a

large cohort study (3). Notably, reports indicated that complete blood count (CBC) disturbances commonly resolve after restoration of euthyroidism (4).

The mechanism responsible for anaemia in the course of hyperthyroidism is not well understood. Due to accelerated metabolism and thus higher oxygen

requirements, bone marrow presents erythroid hyperplasia, where ineffective erythropoiesis occurs. Consequently, Fe utilization is inappropriate (5). Typically, microcytic anaemia is the most representative form of thyroid hormone excess (6, 7). In a group of 235 hyperthyroid patients, microcytic anaemia was detected in 30.3%, while isolated microcytosis (decreased MCV with normal haemoglobin) was detected in 57.4% of patients (4). The most common cause of hyperthyroidism is autoimmune Graves' disease (GD). Immunologic factors, acting via inflammatory markers, might be an additional underlying cause of haematologic changes in GD, next to the thyroid hormone excess (8).

Hepcidin is a liver-derived protein responsive to Fe plasma concentration and helps maintain proper iron status. It acts mainly through blockage of Fe transporters in the duodenum. Another pathway leading to hepcidin overexpression is triggered by inflammation, particularly interleukin-6 (IL-6) (9). Thus, both mechanisms may play a role in anaemia under thyroid hormonal disturbances. We have already demonstrated that the hepcidin serum level is high at the diagnosis of other thyroid inflammatory diseases, subacute thyroiditis (SAT) and Hashimoto's thyroiditis and significantly decreases after treatment. The observed changes in hepcidin concentration take place along with the improvement of iron homeostasis parameters (10, 11). So far, data concerning the role of hepcidin in hyperthyroidism are sparse and ambiguous, which might be related to the diverse aetiology and severity of thyroid disease among the studied participants (12, 13). Thus, the aim of our study was to prospectively assess hepcidin serum concentration and other parameters related to Fe homeostasis in a well-characterized group of hyperthyroid patients exclusively in the course of GD at diagnosis and during remission.

Patients and methods

The research was conducted in accordance with the declaration of Helsinki and was approved by the Poznan University of Sciences Bioethics Committee (approval number: 386/16). All patients agreed to participate in the study by signing written informed consent.

Study design

The project was carried out in an outpatient clinic at the tertiary reference endocrine centre. This was a prospective observational study on hyperthyroid patients with GD.

The evaluation of patients was conducted twice: at the moment of diagnosis (T0) in the hyperthyroid state and after euthyroidism was restored (T1). Each time, the patients were physically examined. Then, laboratory tests (including hepcidin measurement) and thyroid ultrasound examination were performed. Following the initial evaluation (T0), patients were treated with thiamazole. The initial dose was 40 mg or 60 mg per day (depending on the severity of the disease), and the dose was gradually tapered under the systematic control of TSH and free thyroid hormone levels every 4–8 weeks. Liver aminotransferases and complete blood count were additionally controlled when side effects in the form of liver failure or granulocytopenia were suspected. The state of remission was defined as a lack of clinical symptoms of hyperthyroidism, accompanied by full normalization of TSH and free thyroid hormones without anti-thyroid therapy or with the use of a low maintenance dose of 2.5 mg of thiamazole per day.

Studied group

The studied group included adult patients with a confirmed newly diagnosed first episode of hyperthyroidism in the course of GD.

Assessment of patients

The diagnosis of GD hyperthyroidism (constituting the inclusion criterium) was based on clinical, laboratory (significant elevation of free thyroid hormones, decreased thyroid-stimulating hormone and positive anti-thyrotropin receptor autoantibodies) and sonographic (decreased inhomogeneous echogenicity and increased vascularization) features (Fig. 1). To avoid the influence of other (than thyroid disease) factors on hepcidin level, several exclusion criteria were strictly respected (Fig. 2). Patients with a positive personal history of previous episodes of hyperthyroidism were also excluded from the analysis. Moreover, to ensure homogeneity of the group, patients with thyrotoxicosis of an aetiology other than GD were also excluded. Blood was always collected in the morning after at least 8 h of fasting.

The level of hepcidin was measured by the Hepcidin 25 (bioactive) hs ELISA, a highly sensitive enzyme immunoassay for the quantitative *in vitro* diagnostic measurement (DRG Instruments GmbH, Germany). The thyroid gland status was characterized by thyroid-stimulating hormone (TSH), free thyroid hormones (free triiodothyronine – fT3 and free thyroxine – fT4) and autoantibodies (anti-thyroid peroxidase – TPOAb,

Figure 1

An ultrasound picture presenting one of the patients at diagnosis of hyperthyroidism in the course of Graves' disease, demonstrating decreased echogenicity of the thyroid parenchyma and significantly increased blood flow visualized by colour Doppler examination.

anti-thyroglobulin – TGAb and anti-TSH receptor – TRAb). To exclude chronic liver and kidney failure or acute inflammatory states, the following parameters were also measured: alanine aminotransferase (ALT), aspartate

aminotransferase (AST), creatinine with eGFR (estimated glomerular filtration rate) and C-reactive protein (CRP). The ferritin level, Fe and complete blood count (CBC) were used to evaluate iron homeostasis.

Figure 2

Factors affecting hepcidin level, constituting the exclusion criteria of our study.

The assessment of ALT, AST, Fe, creatinine and CRP was performed using a Hitachi Cobas e501 analyser (Roche Diagnostics), while the measurements of thyroid-related parameters (TSH, fT3, fT4, TPOAb and TGAb) and ferritin were done using a Hitachi Cobas e601 chemiluminescent analyser (Roche Diagnostics). The eGFR was estimated by an online medical calculator (<https://www.mdcalc.com/mdrd-gfr-equation>) based on the MDRD (Modification of Diet in Renal Disease Study) equation. The CBC was measured by an automated flow cytometer Sysmex-XN 1000 (Sysmex Europe GmbH, Born-barch, Germany). An AIXPLORER system (Supersonic Imagine, Aix en-Provence, France) was used for ultrasound examination of the thyroid gland.

Statistical methods

The acquired data were analysed statistically using STATISTICA software (StatSoft, Tulsa, Oklahoma, USA). Non-parametric tests were applied. The Wilcoxon signed-rank test was used to compare two related samples (T0 vs T1). Spearman's rank correlation coefficient was used to evaluate the correlation between hepcidin and other laboratory parameters before recovery (T0). The data are presented as median and 25–75% interquartile range (IQR). The parameters with different reference ranges in men and women were calculated separately. The level of statistical significance was set at $P < 0.05$.

Results

Seventy patients with suspicion of hyperthyroidism in the course of GD were screened for eligibility criteria. In the end, 42 patients met the inclusion criteria, were enrolled in the study and underwent initial assessment (T0). The mean age of patients who qualified for analysis was 42.5 ± 15.1 years (range 18–70), median 38 (IQR 30–50). Twenty-four patients were also assessed for a second time, after complete remission was achieved (T1), while 18 did not fulfil the remission criteria or were lost to follow-up. The time range to achieve euthyroidism (from T0 to T1) was 6 to 24 months from diagnosis.

Hepcidin

The median hepcidin concentration at T0 in 42 patients was 13.7 (5.6–38.4) ng/mL, whereas in men it reached

50.2 (33.5–66.0) ng/mL ($n = 10$) and in women it reached 8.4 (2.3–19.1) ng/mL ($n = 32$).

The level of hepcidin was correlated with all biochemical parameters measured in this study before treatment (T0) in the whole studied group and in females only for parameters with different reference ranges depending on sex. The most statistically significant correlation was observed for hepcidin and ferritin ($\rho = 0.723$) in women at T0, denoted in Fig. 3.

In 24 patients who achieved remission of the disease, the concentration of hepcidin at diagnosis of GD was compared to the level of hepcidin in remission (T0 vs T1). The data are presented in Table 1. Additional calculations were performed separately in the female and male subgroups (Table 2). A statistically significant decrease in hepcidin and ferritin concentrations was observed in men and women (Fig. 4).

Other parameters

Analysis of 24 patients who were evaluated both before (T0) and after therapy (T1) revealed that, at T1, patients presented a statistically significant improvement in thyroid status, reflected by normalisation of all parameters (TSH, fT3 and fT4) and reductions in the concentration of autoantibodies (TPOAb, TGAb and TRAb). The parameters of CBC not dependent on sex, such as MCV and MCH, were significantly higher following treatment when compared to values at diagnosis (Table 1). The level of CRP (1.1 (0.5–2.9) vs 1.2 (0.6–1.7) mg/L) did not change significantly during therapy.

Figure 3

Correlation of hepcidin and ferritin in the women at baseline (T0). The non-parametric Spearman test was used, and the result is presented as a logarithmic scale ($\rho = 0.723$).

Table 1 Biochemical parameters in patients with hyperthyroidism due to Graves' disease (GD) at baseline (T0) compared to the values obtained following complete remission of the disease (T1).

Parameter	Reference range	GD (T0) <i>n</i> = 24	GD (T1) <i>n</i> = 24	P-value
Hepcidin (ng/mL)	0.2–47.7	28.7 (8.1–39.4)	7.9 (4.3–12.9)	<0.001
WBC × 10 ³ /μl	3.9–11.0	7.1 (5.9–8.6)	6.9 (6.0–8.0)	NS
MCV (fL)	80.0–99.0	83.5 (82.5–87.1)	89.5 (88.8–90.0)	<0.001
MCH (pg)	27.0–33.5	29.0 (28.0–29.4)	30.4 (29.5–31.1)	<0.001
MCHC (g/dL)	31–38	34 (33–34)	34 (33–34)	NS
RDW-CV (%)	11.0–16.0	12.6 (11.9–13.3)	12.8 (12.5–13.1)	NS
PLT × 10 ³ /μl	130–400	266 (237–301)	254 (229–289)	NS
TSH (μIU/mL)	0.2–4.2	0.01 (0.01–0.01)	1.0 (0.6–2.0)	<0.001
ft3 (pmol/L)	3.9–6.7	14.8 (9.4–29.0)	4.7 (4.5–5.2)	<0.001
ft4 (pmol/L)	11.5–21.0	37.3 (27.8–61.8)	15.9 (14.7–17.1)	<0.001
TPOAb (IU/mL)	<34	117 (13–227)	46 (17–139)	0.01
TGAb (IU/mL)	10–115	60 (11–594)	20 (10–248)	0.01
TRAb (IU/L)	<2	6 (2–11)	1 (1–3)	<0.001

Values are expressed as median (IQR) for the nonparametric Wilcoxon signed-rank test.

ft3, free triiodothyronine; ft4, free thyroxine; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; *n*, number of patients; NS, non-significant; PLT, platelets; RDW-CV, red blood cell distribution width-coefficient of variation; TGAb, anti-thyroglobulin antibodies; TPOAb, anti-thyroid peroxidase antibodies; TRAb, thyrotropin receptor antibodies; TSH, thyroid-stimulating hormone; WBC, white blood cells.

Additional analysis was performed for the two subgroups divided according to sex. Altogether, 18 women completed the full treatment trial. Among the parameters with different reference ranges depending on sex, RBC and ferritin became significantly lower following remission than at baseline (Table 2).

Statistically significant differences in biochemical parameters in the male patients (*n*=6) who were observed until complete remission of the disease was achieved are presented in Table 2.

Discussion

Hepcidin, presumed to be a potential link in the mutual relationship between thyrometabolic and haematologic status, is a liver-derived protein encoded by the *HAMP*

gene, responsible mainly for bodily Fe distribution (9). The mechanism of hepcidin response to inflammatory stimuli comprises two possible pathways: one through bone morphogenetic protein-mothers against decapentaplegic (BMP-SMAD) and more specifically via Janus kinase (JAK) signal transducer and activator of transcription 3 (STAT3). The latter axis is triggered by cytokines, mainly IL-6, which activates JAK2 and leads to phosphorylation of STAT3, enabling its translocation to the nucleus and upregulation of *HAMP* gene transcription. IL-6 also contributes to hepcidin regulation via the BMD pathway (14). Cross-talk between two presented pathways has been postulated (15). Stimulated hepcidin affects ferroportin, which is a cellular iron transporter, causing its internalization and degradation, thus retaining Fe absorption from a lumen of duodenum, macrophages of the liver and spleen as well as hepatocytes into plasma (16).

Table 2 Iron-related parameters significantly changed in patients with Graves' disease (GD) after the transition from hyperthyroidism (T0) to euthyroidism (T1), according to sex.

Parameter	Hepcidin (ng/mL)	RBC × 10 ⁶ /μl	Ferritin (ng/mL)	Fe (μg/dL)
Reference range	0.2–47.7	F: 3.5–5.2 M: 4.2–5.8	F: 13–150 M: 30–400	F: 37–145 M: 59–158
GD _F (T0) <i>n</i> = 18	10.0 (1.7–33.1)	4.7 (4.5–5.0)	84 (23–104)	119 (79–145)
GD _F (T1) <i>n</i> = 18	5.0 (1.6–10.2)	4.5 (4.3–4.7)	35 (16–64)	103 (78–138)
<i>p_F</i> value	0.003	0.003	0.001	NS
GD _M (T0) <i>n</i> = 6	41.6 (33.5–60.1)	5.2 (4.6–5.6)	377 (171–411)	125 (104–132)
GD _M (T1) <i>n</i> = 6	10.0 (8.4–14.0)	4.7 (4.7–5.1)	165 (84–237)	89 (62–108)
<i>p_M</i> -value	0.03	NS	0.04	0.04

Values are expressed as median (IQR) for the nonparametric Wilcoxon signed-rank test.

F – females; Fe – iron; M – males; *n* – number of patients; NS – non-significant; RBC – red blood cells.

Figure 4
Changes in hepcidin and ferritin concentrations during the transition from hyperthyroidism (T0) to euthyroidism (T1).

Consequently, chronically increased hepcidin may lead to hypoferremia and iron-restricted anaemia, as in the pathogenesis of iron-refractory iron deficiency anaemia (IRIDA) (17). In contrast, hepcidin is inhibited by states characterized by increased erythropoiesis demand (anaemia, haemorrhage, haemolysis, hypoxia and β -thalassaemia). Hepcidin's mechanisms of action are considered to be attractive therapeutic targets, with several agents under study during clinical trials (18).

Notably, IL-6 was markedly ($P < 0.0001$) elevated in the hyperthyroid population with GD and dropped significantly after methimazole therapy (19). Moreover, a positive correlation was found between fT3 and IL-6 ($r = 0.35$, $P < 0.025$) as well as between TRAb and IL-6 ($r = 0.5$, $P < 0.003$) (20). Additionally, polymorphisms in the IL-6 gene were found to be correlated with GD predisposition (21). It may be presumed that IL-6 overproduction in patients with GD stimulates the JAK2-STAT3 pathway

to synthesize hepcidin, which consequently affects iron homeostasis and haematological parameters (12, 22).

Although very severe anaemia is not a common comorbidity in thyroid disorders, a better understanding of the mechanisms involved in iron homeostasis disturbances accompanying the hyperthyroid state and its restoration following antithyroid therapy may help to avoid unnecessary diagnostic procedures and supplementation (23). In view of the autoimmune background of GD and the frequent association of hyperthyroidism with haematological disturbances, we decided to evaluate the impact of thyroid function on hepcidin. To the best of our knowledge, this is the first study evaluating hepcidin concentration in a well-characterized, homogeneous group and the largest initially enrolled (42 patients) group so far, including the most representative female population with GD hyperthyroidism. The application of very strict inclusion and exclusion criteria allowed us to demonstrate for the first time that, in contrast to the foregoing attempts, a significant decrease in serum hepcidin was achieved (measured by the highly sensitive ELISA method) in patients with GD hyperthyroidism occurring after euthyroidism. The median hepcidin concentration was markedly lower after therapy, both in the whole group and in the male and female subgroups.

According to Fischli *et al.*, fT3 stimulates hepcidin mRNA expression *in vitro* in a cell culture model (HepG2 cells) (12), but there has been no *in vivo* trial on proteins confirming the influence of thyroid hormones on hepcidin regulation at the pre- or posttranscriptional level. However, in our previous reports, we found positive correlations between hepcidin and fT3 in other thyroid inflammatory diseases, for example, Hashimoto's thyroiditis ($r = 0.465$, $P = 0.033$) and SAT ($r = 0.815$, $P < 0.001$) (10, 11). Presumably, the extent of fT3 disturbances in a course of GD hyperthyroidism is too high to reveal such a relationship. In the study by Fischli *et al.*, no significant differences were noted in hepcidin concentration between hyper- and euthyroid states in patients with GD using the same method as in our study. The hepcidin level was markedly lower after recovery in males ($P < 0.001$), but not in females ($P = 0.258$), as measured by mass spectrometry (MS), a different method than in our study (12). In the whole group, a tendency for hepcidin measured using the ELISA method to decrease after treatment was noticeable ($n = 25$; before 185 vs after 115 ng/mL); however, it did not reach statistical significance ($P = 0.533$). In another study by Lehtihet *et al.*, hepcidin measured by ELISA did not change significantly during the transition from hyperthyroidism to the euthyroid state; however, a tendency for hepcidin

to drop after treatment (7.52 vs 5.47 ng/mL) was noted (13). Although MS is considered a model method for hepcidin-25 measurements, hs ELISA has the advantages of simplicity, low cost, higher throughput and modest expenditure of labour, which makes it more suitable for implementation in clinical practice, as described before (24). Additionally, a strength of our study is a strict inclusion criteria, helping us include only patients with their first episode of GD hyperthyroidism, which allowed us to maintain homogeneity of the group.

We found a strong positive correlation between hepcidin and ferritin at T0 in the female subgroup, confirming the association between hepcidin and iron status. Moreover, like hepcidin, the median ferritin significantly decreased after treatment. Presumably, the male group size was too small to achieve similar observations. This is consistent with previous research, where ferritin concentration was increased in hyperthyroid subjects and returned to the normal range after antithyroid therapy (25). The authors suggested that the increase in serum ferritin level in hyperthyroidism may be explained by the direct action of thyroid hormones on its synthesis or accompanying impaired iron utilization.

Although, among the included patients there was no diagnosed severe anaemia (exclusion criterium), and hence, the extent of disturbances might be too subtle to yield statistically significant differences. We noticed significant improvement of MCV and MCH in all patients. Although previous studies have shown a correlation between RDW and thyroid status reflected by a significant increase in RDW in the hypothyroid state, we failed to find a noticeable fluctuations of RDW during the transition from hyperthyroidism to euthyroid state (26, 27). The study by Gianoukakis *et al.* reported correction of anaemia of unknown origin in 89% of hyperthyroid patients due to GD following antithyroid therapy and in none with anaemia of other than hyperthyroidism-related causes (8).

The mechanism of anaemia in hyperthyroidism is still uncertain. Thyroid hormones increase the basal metabolic rate, which results in higher oxygen demand and the need for oxygen transporters. Thyroid hormones induce erythropoiesis by acting directly on bone marrow, causing erythroid-line hyperplasia, and indirectly by erythropoietin (EPO) production through enhancing EPO gene expression (28). However, the boosted erythropoiesis may be ineffective, so pathological sideroblasts are visible in the marrow, and Fe utilization is diminished. Additionally, thyroid hormones were found to increase the 2,3-diphosphoglycerate concentration in RBCs,

which facilitates oxygen delivery to tissues (29). This results in decreased MCV and MCH values. Moreover, enhanced erythrocyte osmotic fragility and oxidative stress lead to precocious haemolysis (30). Thus, although total red cell mass is increased in thyrotoxic patients, HGB and HTC are usually low due to a concomitant increase in plasma volume (5).

Although hepcidin is an acute inflammatory reactant, we did not find a positive correlation between this protein and CRP, as previously demonstrated in the case of SAT (10). This might be explained by the fact that GD is not an acute inflammatory process but rather a chronic autoimmune disease and is not associated with a significant increase in CRP concentration (31).

In our group, a sex difference in hepcidin concentration, as with ferritin, was observed. A higher hepcidin concentration was observed at T0 in males compared to females. Yang *et al.* found that 17- β -oestradiol suppressed hepcidin gene (*HAMP*) expression *in vitro* in human cells and therefore increased Fe uptake (32). This finding was confirmed by Lehtihet *et al.* during *in vivo* studies, where exogenous oestrogen administration caused a decrease of 40% in hepcidin-25 in females (13). This phenomenon might at least partially explain our observation. Moreover, we noticed a wide range of hepcidin concentrations in particular subjects. According to Galesloot *et al.*, the hepcidin concentration depends on the oestrogen status of the females, being the lowest in young women and higher following menopause, but independent of age in men (33). This may create potential limitations in comparisons of the studied females with the control group of healthy subjects, as the hepcidin concentration might depend on whether they are pre- or postmenopausal. In our study, most patients from the female subgroup were of premenopausal age with regular menstrual bleeding. Other limitations of the study should be noted here. The most relevant issue is the limited number of subjects who reached the T1 timepoint ($n=24$) and the overall small male cohort ($n=10$). Another is the method of hepcidin concentration assessment (ELISA), which is considered to present lower accuracy than MS. So far, measuring hepcidin-25 by LC-MS has been considered a gold standard. However, the comparison of this method with hs ELISA revealed a strong correlation between the results ($r=0.98$; $P<0.001$), demonstrating the reliability of the results and high specificity of the autoantibodies used in hs ELISA to bind almost entirely to the bioactive form of hepcidin (24).

In conclusion, hepcidin level decreases significantly during the transition from the hyperthyroid state

to euthyroidism in patients with GD. The observed changes presumably occur in parallel to iron homeostasis fluctuation, especially a ferritin level decrease. During the transition from the hyperthyroid state to euthyroidism, the improvement of haematological status is reflected mainly by an increase in MCV and MCH.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A K, E S P and M R performed study design, A K and E S P performed data acquisition and interpretation, A K performed statistical analysis, A K and D F prepared the manuscript and E S P and M R performed final revision of the manuscript.

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