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Absorption of non-heme iron during gastric acid suppression in patients with hereditary hemochromatosis and healthy controls

The He-GAIA study

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29 Radboudumc that via its Hepcidinanalysis.com initiative offers high quality hepcidin
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43 List of abbreviations: HH: hereditary hemochromatosis, p.C282Y: p.Cysteine282Tyrosine, PPI:
44 Proton pump inhibitor, HC: Healthy control subject, Tsat: Transferrin saturation, Fe³⁺: ferric
45 iron, Fe²⁺: ferrous iron.

46

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Abstract

Phlebotomies are performed in hereditary hemochromatosis (HH) to maintain normal iron concentrations. Proton pump inhibitors (PPIs) can reduce the number of phlebotomies in HH patients. However, in patients without HH, the iron concentrations do not appear to be compromised when using PPIs. Therefore, we aim to explain the differences in iron absorption between patients with and without HH.

Methods: In ten p.C282Y homozygous HH patients with normalized iron stores and ten healthy control subjects (HCs), the iron parameters and hepcidin concentrations were determined before ingestion of a pharmacological dose of 50 mg iron (Fe³⁺) polymaltose, and hourly for four hours afterwards. This was repeated after seven days' treatment with pantoprazole 40 mg once daily.

Results: Serum iron concentrations and transferrin saturation percentages dropped significantly during PPI use in the HH patients, while no changes were observed in the HCs. Hepcidin concentrations were lower in the HH patients compared with the HCs both before and during PPI use. In both groups hepcidin levels did not significantly decrease during the treatment.

Conclusions: Seven day PPI use significantly reduces iron absorption in HH patients but not in HCs. Changes in hepcidin concentrations could not explain these different PPI effects on iron absorption probably due to a small sample size.

New & Noteworthy

This study confirms that lowering gastric acidity by proton pump inhibitors results in a reduction in iron absorption in hemochromatosis patients and not in healthy control subjects. The presupposition that a decrease in hepcidin concentration in healthy control

72 subjects in response to lowering gastric acidity can explain the difference in iron absorption
73 between these groups, could not be confirmed probably due to a small sample size.

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77 **Key words**

78 Iron overload, iron absorption, hepcidin, proton pump inhibitors, hereditary
79 hemochromatosis

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92   **Introduction**

93   The most prevalent form of hereditary hemochromatosis (HH) is homozygosity for the
94   p.Cys282Tyr (p.C282Y) variant in the HFE gene.¹ This condition is characterized by an
95   ineffective regulatory feedback mechanism in which circulating hepcidin concentrations are
96   disproportionally low for body iron stores. Since hepcidin is the key regulator of systemic
97   iron metabolism, persistently low hepcidin concentrations will result in excessive iron
98   absorption leading to iron accumulation.¹⁻³ Iron overload causes damage to parenchymal
99   tissues and can lead to liver cirrhosis, severe arthropathy, diabetes mellitus, cardiac disease,
100   and premature death.⁴ The standard therapy for iron overload in HH is phlebotomy.
101   However, patients can experience side effects with significant burden, reducing quality of
102   life and requiring additional hospital visits.^{5,6}
103   Proton pump inhibitors (PPIs) have been suggested as an attractive additional therapy to
104   reduce the need for phlebotomies by reducing gastric acid secretion which results in
105   decreased iron absorption.^{7,8} Studies about the occurrence of anemia during long-term use
106   of PPIs in patients without HH are contradictory.^{9,10} Furthermore, the study describing PPI
107   associated anemia in patients without HH, did not rule out a pre-existent iron deficient state
108   or possible upper gastrointestinal blood loss.¹⁰ To date, it is unclear via which mechanism
109   PPIs appear to have a different influence on iron absorption in patients with HH and without
110   HH. We hypothesized that HH patients benefit from a reduction in bioavailable iron via
111   gastric acid inhibition, because their hepcidin levels are already disproportionally low. While
112   in patients without HH the use of PPI will not result in a reduction in iron absorption because
113   their hepcidin concentrations will decrease in response to gastric acid inhibition and the
114   accompanying reduction in bioavailable iron.

116 **Methods**

117 *Patients*

118 We conducted a non-randomized proof of concept study, between January 2015 and
119 January 2016, with ten p.C282Y homozygous HH patients with normalized iron stores and
120 ten gender-matched healthy control subjects (HCs). The HH patients were recruited from the
121 outpatient department of the Zuyderland medical centre in Heerlen/Brunssum, The
122 Netherlands. The HCs were recruited among personnel of the medical centre and their
123 acquaintances. All participants gave written informed consent, in keeping with the
124 Declaration of Helsinki. The regional ethics committee Atrium-Orbis-Zuyd approved the
125 study. In both groups, men and menopausal women between the age of 18 and 65 years
126 with ferritin concentrations < 400 µg/L for at least three months were included. HH patients
127 needed to be homozygous for p.C282Y, on maintenance treatment for at least one year and
128 had their last phlebotomy ≥ six weeks before entering the study. HCs did not have *HFE*
129 mutations ([compound] heterozygosity or homozygosity for p.C282Y or p.H63D). Exclusion
130 criteria for both groups were coexistence of acute or chronic inflammatory disorders, such as
131 inflammatory bowel disease or rheumatoid arthritis, hepatitis B, hepatitis C or HIV infection.
132 Also, anemia, an active malignancy, alcohol intake of > 21 units a week for men and >14
133 units a week for women, and present PPI treatment or other gastric acid-suppressing
134 medication were criteria to exclude patients from the study. The same goes for the use of
135 medication that interfered with PPIs e.g. vitamin C supplements, the use of iron supplements
136 and previous side effects of PPIs.

137

138 *Study design*

On the first test day, after an overnight fast, baseline blood samples were drawn between 7.30 and 8.00 a.m. (T0) after which the participants ingested iron polymaltose (Ferrum Hausman, Vifor, Germany) containing a pharmacological dose of 50 mg of Fe³⁺ iron on a small piece of white bread. After ingesting the iron polymaltose, blood samples were drawn hourly for four hours (T1-T4). The participants did not receive breakfast until the 3rd blood sample (T2) was drawn, to reduce the chance of an interference with the outcome (Fig 1). They all consumed the same breakfast of +/- 300 kcal consisting of bread, coffee and water. The following day the participants started using 40 mg pantoprazole orally once daily, before breakfast, for seven days. On the seventh day, we repeated the oral iron challenge and blood sample collection.

Laboratory analyses

Blood samples for hepcidin analysis were stored at -80°C. Serum iron and transferrin were determined on the days of the test by standard laboratory analysis. Transferrin saturation was calculated from serum iron and transferrin: iron (μmol/L) x 4.5 / transferrin (g/L). Hepcidin measurements were performed in freshly thawed serum samples by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry using an internal standard for quantification.^{11,12} Hepcidin-25 concentrations were expressed as nmol/L (nM). The median reference concentrations for serum/plasma hepcidin-25 (Dutch population) are 4.5 nM for men, 2.0 for premenopausal women and 4.9 nM for postmenopausal women.¹³ The hepcidin test lower limit of detection was 0.5, in case of a hepcidin concentration below 0.5, the result is shown as <0.5.

Statistical analysis

Statistical analyses were carried out using SPSS, version 23 for Windows (IBM Statistics for Macintosh, Chicago, IL, USA). A power calculation was not possible since this is an exploratory study and no previous data regarding hepcidin concentrations in hemochromatosis were available. Data are expressed as mean (SD) for continuous variables and frequency (%) for categorical variables. Comparison of baseline values between the HH group and the HC group were performed using the independent T Test in case of continuous variables and using the Fisher's exact test for categorical variables.

The comparison of repeated measurements, before and during PPIs, within groups and between groups (HH patients and HCs), was performed using a linear mixed model with a first order autoregressive (AR1) covariate type for the repeated measurements. Fixed factors included the following variables: group (HH patient vs. HC), PPI use (before vs. during PPI use), and the time points of the blood sample collection (T0, T1, T2, T3 and T4). The random factor was the participant ID. The repeated variables included the test days (before vs. during PPI use) and the time points of the blood sample collection. Furthermore, EM means were calculated. In case of hepcidin concentrations < 0.5 , we performed statistical analyses with hepcidin concentrations of 0.5, 0.25 and 0.01 to test if this would lead to different outcomes. This was not the case, therefore in this paper we reported the value 0.25 when hepcidin analysis showed < 0.5 . A Bonferroni correction was used to correct for multiple testing. P-values were considered significant when ≤ 0.05 .

Results

Patient baseline characteristics

Of the 126 p.C282Y homozygous HH patients regularly visiting the outpatient clinic, 24 matched the inclusion criteria. Of these 24 HH patients, ten agreed to participate in the study.

Twenty-three HCs were screened for the *HFE* mutations p.C282Y and p.H63D and their ferritin concentrations were checked. In 13% (3/23) hyperferritinemia was observed and in 43.5% (10/23) heterozygosity for one of the *HFE* mutations was found (2/10 p.C282Y and 8/10 p.H63D). These HCs were then excluded leaving ten subjects who fulfilled the inclusion criteria. Baseline characteristics are summarized in table 1.

In this gender-matched study the majority of participants was male (70%). Age and BMI were not significantly different between groups.

Serum iron and transferrin saturation

In HH patients both serum iron concentrations before and during PPI treatment were significantly higher than those obtained from HCs at all-time points, with a mean difference between the two groups of 14.2 $\mu\text{mol/l}$ ($p=0.001$) before PPI use and 9.9 $\mu\text{mol/l}$ ($p=0.013$) during PPI use (Fig 2a).

In the HC group serum iron concentrations before and during PPI use showed no significant difference (mean difference of 0.02 $\mu\text{mol/l}$, $p=0.985$). In the HH patients, lower serum iron concentrations were found during PPI use compared with before PPI use with a mean difference of 4.35 $\mu\text{mol/l}$ ($p<0.001$) (Fig 2a).

Similar results were obtained for transferrin saturation (Tsat) (Fig 2b). HH patients had higher Tsat percentages at all-time points, both before (mean difference 39.18%, $p<0.001$) and during PPI use (mean difference 28.25%, $p=0.001$) compared with HCs. In the HH

patients, significantly lower T_{sat} percentages were shown during PPI use compared with before PPI use (mean difference 10.33%, $p < 0.001$). In the HC group no significant difference was found in T_{sat} comparing before and during PPI use (mean difference 0.60, $p = 0.760$).

Hepcidin

HH patients had significantly lower hepcidin concentrations at all-time points compared with HCs, both before (mean difference 2.46 nmol/L, $p = 0.002$) and during PPI use (mean difference 1.69 nmol/L, $p = 0.029$) (Fig 3a-b). The hepcidin concentration in the HC group showed lower concentrations during PPI use compared to before PPI use, however this difference was not statistically significant (mean difference 0.54 nmol/L, $p = 0.166$). Also in the HH patients no statistically significant difference in hepcidin concentrations was found during PPI use compared with before PPI use (mean difference 0.23 nmol/L, $p = 0.549$) (Fig 4).

Discussion

This proof of concept study shows that short term use of PPIs leads to significantly lower circulating iron concentrations in iron-depleted HH patients, whereas it has no effect on serum iron concentrations in HCs. These results support the finding that reducing the acidity of the gastric content with PPIs leads to a decrease in iron absorption in HH patients.^{7,8}

Iron in the food can be present in the non-heme and/or heme-bound form. Iron is absorbed mainly in the duodenum and the upper jejunum. In persons who eat meat, heme-iron may contribute to 10 – 15 % of the daily iron intake. Heme-iron is absorbed to a higher extent than non-heme iron. In contrast to non-heme iron, heme-iron is less influenced by dietary constituents and the higher pH of the small intestine.^{14,15} Heme-iron is absorbed into the enterocyte through the heme carrier protein 1. Inside the cell iron is released from heme by

233 heme oxygenase.³ Non-heme iron absorption takes place mainly on the apical membrane of
 234 the enterocyte via the divalent-metal transporter-1 (DMT-1).^{3,16} This transporter is selective
 235 for ferrous, Fe²⁺, iron. Since ferric, Fe³⁺, iron is the predominant form present in the diet a
 236 reduction step of ferric iron to ferrous iron is necessary for absorption. The reduction is
 237 catalysed by duodenal cytochrome-b, a major intestinal ferrireductase.^{3,17,18} DMT-1 is a H⁺-
 238 coupled cotransporter and it functions optimally at acidic pH.¹⁹ Proton pump inhibitors
 239 reduce the acid content of gastric secretions resulting in higher pH up to 6. As a result the
 240 DMT-1 function will decrease and the reduction of ferric iron will be diminished, with less
 241 ferrous iron available for absorption via the DMT-1.²⁰ In patients with HH, intestinal DMT-1 is
 242 upregulated which may result in increased iron absorption into the enterocyte.^{20 21} Results
 243 of a mice study suggested that in case of a more acidic pH, the ferrireductase activity is
 244 lower.²² This mechanism suggests that HH patients on PPIs would have increased iron
 245 absorption through upregulated DMT-1 and increased ferrireductase activity. However, in
 246 patients with HH treated with PPI, far less phlebotomies were needed to maintain a stable
 247 serum ferritin concentration. This indicates that the inhibitory effects of PPI use, lowering
 248 gastric acid secretion, on the uptake of iron, overruled the promoting effects (upregulated
 249 DMT1 and increased ferrireductase activity) seen in the intestinal cells in hemochromatosis
 250 .^{7,8,23} Our results also show significantly lower circulating iron concentrations in HH patients
 251 after PPI use. These results are in line with Hutchinson et al., who previously reported a
 252 significant reduction in increase of iron concentrations following an iron challenge, after
 253 seven days of PPIs, in HH patients.²³ Furthermore, our study showed no decrease in
 254 circulating iron concentrations after PPI use in HCs. An explanation for this might be the fact
 255 that iron, whether originating from heme or from non-heme sources, can only leave the
 256 enterocyte via ferroportin, a process that is regulated by hepcidin.²⁴ This protein is

disproportionally low in hereditary hemochromatosis but not in HCs, suggesting that in HCs hepcidin concentrations could be lowered to ensure enough iron uptake from the enterocyte, whereas HH patient cannot use hepcidin to regulate their iron concentrations.

We did not observe a significant decrease in serum hepcidin concentrations after one week of PPIs in HCs. As shown in figure 3 there was a wide variation in hepcidin concentrations in HCs both before and after PPI administration. Because of these wide variations and our small sample size in this study it is not possible to determine the possible effect of hepcidin on the differences in iron absorption.

It should be noted that an increase in iron concentrations after the iron challenge was not seen in our study. Previous studies measuring iron concentrations following an oral iron challenge used ferrous sulphate. These studies did observe an increase in iron parameters.^{25,26} We aimed to measure the effect of gastric acid inhibition so in our study the use of a ferric preparation was essential. Furthermore, most of the iron in our food is in the ferric form. However, the lack of rise in iron concentrations after iron administration cannot solely be related to the administration of ferric iron compared to ferrous iron.²⁷ Hutchinson et al. also used ferric chloride and showed an increase in iron concentrations.²³ A possible explanation for the lack of iron increase in our study could be that absorption of ferric preparations is up to seven times better when taken with food and our patients were fasted while the patients of Hutchison et al. received their ferric chloride combined with an iron enriched meal.²⁸

Strengths of this study include the accurate study protocol, including an hourly monitoring of serum iron parameters and the accurate selection process of the HCs to exclude acquired or genetic confounding factors, including *HFE* genotypes susceptible for

iron overload. Moreover, using the stable portion of iron polymaltose instead of an iron enriched meal allowed an exact amount of Fe^{3+} to be ingested. Furthermore, the effect of PPI use on hepcidin concentrations after an oral iron challenge has never been studied before. We recognize that the current study has limitations. First, the period of PPI use was short and no gastric pH measurements were done to check the effect of PPI. However, continually measuring gastric pH can be experienced as invasive and the decrease in acidity following use of PPI has already been clearly documented, even after seven days of PPI.²⁹ Furthermore, the study population was small. The sample size was based on previous studies researching serum ferritin concentrations. Because of the lack of data on hepcidin concentrations in hemochromatosis patients using PPI, a power calculation for hepcidin as outcome measure could not be performed. Therefore, the results on hepcidin should be interpreted with caution and it is necessary for future studies on hepcidin to include a larger study population.

In conclusion, our proof of concept study has shown that PPI use significantly reduces serum iron concentrations in HH patients but not in HCs, indicating that PPI use reduces iron absorption only in HH patients. The presupposition that PPI use in HCs will not result in a reduction in iron absorption because their hepcidin concentrations will decrease in response to lowering gastric acidity could not be confirmed. However, it should be noted that the sample size was small.

Future studies should include a larger study population and preferably also different doses of PPIs to unravel the pathophysiological mechanisms.

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Fig. 1 Study design

The baseline blood samples were drawn between 7.30 and 8.00 a.m. (T0) after which the participants ingested iron polymaltose (Ferrum Hausman, Vifor, Germany) containing a pharmacological dose of 50 mg of Fe³⁺ iron on a small piece of white bread. After ingesting the iron polymaltose, blood samples were drawn hourly for four hours (T1-T4). All patients consumed the same breakfast of +/- 300 kcal consisting of bread, coffee and water after the third blood sample (T2).

The following day the participants started using 40 mg pantoprazole orally once daily, before breakfast, for seven days. On the seventh day, we repeated the oral iron challenge and blood sample collection. In all blood samples serum iron, transferrin, transferrin saturation and hepcidin concentrations were measured.

Fig. 2 Effect of PPI on serum iron concentrations and transferrin saturation over time in patients with HH (n=10) and HC subjects (n=10) after oral iron supplementation the day before and on day 7 of PPI administration

(a) Serum iron concentrations (b) transferrin saturation

HC, healthy control subjects; PPI, proton pump inhibitor; HH, hereditary hemochromatosis patients *Values represent estimated means with a standard error of the mean calculated by mixed model analysis

Fig. 3 Serum hepcidin concentrations, measured four hours (T4) after the intake of iron polymaltose, displayed for each individual patient/healthy control subject before and during PPI.

(a) The serum hepcidin concentrations for each healthy control subject (n=10), in five subjects the hepcidin level decreased (n=4) or stayed unchanged (n=1) comparing before vs during PPI

(b) The serum hepcidin concentrations for each hemochromatosis patient (n=10) in seven patients the hepcidin level decreased (n=5) or stayed unchanged (n=2) comparing before vs during PPI use, in two other patients the measured increase was not more than 0,1nM

Fig. 4 No significant differences were seen between the hepcidin concentrations before and during PPI use within HCs (n=10) and within HH patients (n=10) (HC group mean difference of 0.54, p=0.166; HH group mean difference 0.23, p=0.549). Between group analysis showed a statistically significantly lower hepcidin concentration in HH patients for all time points (before PPI use mean difference 2.46, p=0.002; during PPI use mean difference 1.69, p=0.029)

HCs, healthy control subjects; PPI, proton pump inhibitor; HH, hereditary hemochromatosis.

*Values are estimated means with a standard error (SE) of the mean calculated by mixed model analysis

Visit 1

Visit 2

- HC before PPI
- HC after PPI
- ▲ HH before PPI
- *·- HH after PPI

Serum Hepcidin (nM)

- HC before PPI
- HC after PPI
- ▲ HH before PPI
- * HH after PPI

Table

Table 1 Baseline characteristics of the study population

Baseline features	Hemochromatosis patients (n=10)	Healthy control subjects (n=10)	P-value
Age (Year)	55.3 ± 8.2	50.1 ± 9.4	0.205
Gender (male)	7 (70%)	7 (70%)	>0.999
BMI (kg/m ²)	26.8 ± 2.8	24.5 ± 3.0	0.100
Smoking (yes)	2 (20%)	1 (10%)	>0.999
Alcohol (yes)	10 (100%)	7 (70%)	0.211
CRP (mg/l) (0-10)	1.8 ± 2.6	0.8 ± 1.1	0.282
ALT (U/L) (0-40)	25.8 ± 8.8	26.7 ± 8.8	0.821
GGT (U/L) (0-40)	52 ± 29.0	47 ± 42.2	0.761
Hb (mmol/l) (8.5-11♂; 7.5-10♀)	9.7 ± 0.8	9.3 ± 0.7	0.258
Ht (L/L) (0.41-0.51 ♂ 0.36-0.47 ♀)	0.45 ± 0.04	0.44 ± 0.03	0.500
Serum ferritin (µg/L) (30-400)	98.3 ± 110.0	153.1 ± 91.6	0.242
Transferrin saturation (%) (16-45)	61.2 ± 18.2	26.7 ± 10.6	0.000
Transferrin (g/L) (2-4.1)	2.0 ± 0.2	2.5 ± 0.3	0.000

Data are expressed as absolute numbers (percentage), mean ± SD.

CRP, C-reactive protein; ALAT, alanine aminotransferase; GGT, gamma-glutamyl transferase;

Hb, hemoglobin; HT, hematocrit; SF, serum ferritin; Tsat, Transferrin saturation.

Absorption of non-heme iron during gastric acid suppression in patients with hereditary hemochromatosis (HH) and healthy controls (HC)

METHODS

CONCLUSION

PPI use significantly reduces serum iron in HH patients but not in HCs, indicating that PPI use reduces iron absorption only in HH patients. The hypothesis that PPI use in patients without HH will not result in a reduction in iron absorption because their hepcidin levels will decrease in response to gastric acid inhibition could not be confirmed.

OUTCOME