Iron Deficiency in Inflammatory Bowel Disease Is Associated With Low Levels of Vitamin D Modulating Serum Hepcidin and Intestinal Ceruloplasmin Expression

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INTRODUCTION: **Iron deficiency and vitamin D deficiency are common comorbidities in inflammatory bowel disease (IBD). Accumulating evidence indicates that active 1,25-dihydroxyvitamin D (1,25(OH)D) may enhance iron absorption by suppressing hepcidin. We investigated the influence of vitamin D on iron metabolism in patients with IBD and on the expression of genes facilitating intestinal epithelial iron absorption.**

METHODS: **Iron parameters and serum levels of 25-hydroxyvitamin D (25(OH)D), 1,25(OH)D, and hepcidin were measured in 104 adult patients with IBD (67 with Crohn's disease and 37 with ulcerative colitis). Genes involved in iron absorption were tested for induction by 1,25(OH)D in Caco-2 cells, which resemble the small intestinal epithelium.**

RESULTS: **In multiple regression models controlling for age, sex, body mass index, smoking status, disease activity, and C-reactive protein levels, low 25(OH)D levels were associated with iron deficiency in patients with IBD (β [SE] = −0.064 [0.030], $P = 0.029$). Vitamin D sufficiency was associated with increased levels of ferritin (β [SE] = 0.25 [0.11], $P = 0.024$) and transferrin saturation (β [SE] = 8.41 [4.07], $P = 0.044$). Higher 1,25(OH)D:25(OH)D ratios were associated with lower hepcidin levels (β [SE] = −4.31 [1.67], $P = 0.012$). Especially in Crohn's disease, increased 1,25(OH)D correlated with higher transferrin saturation (β [SE] = 0.43 [0.18], $P = 0.027$). Furthermore, 1,25(OH)D strongly induced the expression of the ferroxidase ceruloplasmin in Caco-2 cells.**

DISCUSSION: **Low vitamin D levels in IBD correlate with iron deficiency. Vitamin D may ameliorate iron deficiency, potentially by downregulating hepcidin and upregulating ceruloplasmin, enhancing intestinal iron absorption.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A741

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the 2 main types of inflammatory bowel disease (IBD), are chronic relapsing inflammatory disorders of the gastrointestinal tract that have rapidly increased in prevalence worldwide over the past few decades, especially in industrialized countries (1). Forecasts for Canada predict that, by 2030, almost 1% of the population could be affected by IBD, impairing patients' quality of life (2) and placing greater stress on health-care systems (1). Despite major research efforts, the etiology of IBD is still not completely understood. It is assumed that components of the gastrointestinal microbiota and various environmental factors trigger a defect in the epithelial barrier, which results in an exaggerated mucosal immune response in genetically susceptible individuals (3). A recent umbrella review highlighted the importance of vitamin D deficiency as 1 of 9 major environmental risk factors that significantly increase the risk of IBD according to robust epidemiological evidence (4).

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With a prevalence ranging between 16% and 95%, vitamin D deficiency seems to occur more frequently in patients with IBD than in the general population (3). There is increasing evidence that vitamin D deficiency also predisposes to anemia (5,6), the most common systemic complication of IBD (7). Anemia affects up to two-thirds of patients, especially at first diagnosis and in active disease (8), and considerably impairs quality of life for patients with IBD (7). The 2 major forms of IBD-associated anemia are iron deficiency anemia and anemia of chronic disease (7). Iron deficiency affects 36%–90% of patients with IBD (9). Even in the absence of concomitant anemia, iron deficiency can cause various clinical symptoms, including fatigue, impaired physical performance, impaired cognitive function, headache, paresthesia, sleeping disorders, hair loss, angular stomatitis, discontentment, agitation, or female infertility (7,10). Anemia of chronic disease arises from a hepcidin-mediated functional iron deficiency, which accompanies chronic inflammation. Proinflammatory cytokines upregulate mainly the hepatic production of hepcidin, which reduces the amount of systemically available iron by blocking ferroportin. Subsequently, basolateral iron export from macrophages in the reticuloendothelial system, representing the iron storage, and from intestinal epithelial cells during iron absorption from the duodenum is hindered (7). Recent data indicate that the hormonally active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)D), also known as calcitriol, suppresses hepcidin synthesis in hepatocytes and macrophages *via* direct transcriptional suppression of the hepcidin gene (*HAMP*) (11,12). In addition, by downregulating proinflammatory cytokines, vitamin D may enhance iron availability and counteract iron deficiency and the resulting anemia in IBD (5). Therefore, we aimed to investigate the potential association of iron deficiency and iron deficiency anemia with vitamin D deficiency in IBD. We correlated iron biomarkers, anemia, and hepcidin levels with the concentrations of the 2 main vitamin D metabolites, 25-hydroxyvitamin D (25(OH)D) and 1,25(OH)D, in a well-characterized cohort of adult patients with IBD. These 2 metabolites represent the storage form and the hormonally active form of vitamin D, respectively. We also analyzed potential iron-mobilizing functions of 1,25(OH)D other than hepcidin downregulation. The expression levels of 18 different genes known to be involved in intestinal iron absorption were tested for their induction by 1,25(OH)D in human Caco-2 cells, which resemble small intestinal epithelium.

METHODS

Study population

Patients included in this analysis were part of a larger cross-sectional serum biomarker study in patients with IBD. For this biomarker study, 254 adult patients with IBD presenting to the outpatient IBD clinics of the Department of Medicine II, University Hospital, LMU Munich, Germany, were consecutively recruited between March 2015 and July 2016. Our inclusion criteria were age 18 years or older and confirmed diagnosis of IBD (CD or UC) based on established endoscopic, histological, and clinical criteria according to the guidelines of the German Society for Gastroenterology, Digestive and Metabolic Diseases (13,14). Exclusion criteria included pregnancy, chronic kidney failure, liver cirrhosis, or clinical evidence of active infection. To address the potential association of iron deficiency markers with vitamin D levels, an additional inclusion criterion was the availability of a complete laboratory assessment of iron status. Owing to clinical considerations, this assessment was routinely performed in a subgroup of patients (n = 109). Additional exclusion criteria were an unknown dosage of vitamin D supplementation, vitamin D supplementation >2,000 IU per day or oral and intravenous iron substitution, and red blood cell transfusion within the 30 days before enrollment. Considering these criteria, 104 patients with IBD were included in the analysis. Clinical disease activity was determined in patients with CD according to the CD activity index (CDAI). A CDAI score ≤ 150 indicated disease remission and >150 indicated disease activity (15). The disease activity of patients with UC was measured by the clinical activity index developed by Rachmilewitz (16), with a score ≤ 3 defining disease remission and >3 indicating disease activity. Clinical data were obtained from patient charts and interviews at the time of enrollment. Detailed demographic and clinical characteristics of the patients are summarized in Table 1, comparing the vitamin D–deficient and vitamin D–sufficient IBD cohorts.

Definition of iron deficiency, anemia, and vitamin D deficiency

Iron deficiency was diagnosed in agreement with the European Crohn's and Colitis Organization consensus guideline based on either ferritin levels < 30 ng/mL or transferrin saturation < 20%, or by ferritin levels > 30 and < 100 ng/mL in the presence of inflammation (C-reactive protein [CRP] ≥ 0.5 mg/dL, CDAI > 150, clinical activity index > 3, leukocytes > 9 g/L, neutrophils > 70%) (7). Anemia was defined according to the criteria defined by the World Health Organization (men: hemoglobin ≤ 13 g/dL or hematocrit ≤ 39%; women: hemoglobin ≤ 12 g/dL or hematocrit ≤ 36%) (7). Serum 25(OH)D < 20 ng/mL was taken as indicative of vitamin D deficiency following the recommendations of the Institute of Medicine. This cutoff is widely used in research and in respective guidelines (17).

Sample collection and laboratory analyses

Venous blood samples were collected from all subjects at the time of enrollment in a K3 EDTA-coated or clot activator–coated tube (S-Monovette, Sarstedt, Nuembrecht, Germany). Routine laboratory testing was performed by standard procedures in the Institute of Laboratory Medicine, University Hospital, LMU Munich. Erythrocyte count, hemoglobin, and hematocrit were analyzed by the XN 9000 automated hematology system (Sysmex Europe, Norderstedt, Germany). CRP, iron, transferrin, and ferritin were determined using an automated clinical chemistry analyzer (Beckman Coulter, Krefeld, Germany). For additional serum measurements, clot activator–coated tubes were centrifuged for 15 minutes at 1,000*g* in a ROTOFIX 32A centrifuge (Hettich, Tuttlingen, Germany) immediately after collection, and the obtained serum was stored at −80 °C in a HFU 586 Basic freezer (Thermo Fisher Scientific, Langenselbold, Germany) until assayed. To determine the serum 25(OH)D and 1,25(OH)D concentrations, frozen serum samples were sent to the Central Facility for Clinical Chemistry, Ulm University Medical Center, Germany. Serum 25(OH)D was quantified using the ELECSYS Vitamin D total II assay in a COBAS E 801 analyzer (Roche Diagnostics, Mannheim, Germany) and serum 1,25(OH)D using the automated chemiluminescence immunoassay IDS-iSYS 1,25 VitD^{Xp} in an IDS-iSYS Multi-Discipline Automated System (Immunodiagnostic Systems, Tyne & Wear, United Kingdom). For quantification of serum hepcidin levels, we used a commercially available sandwich enzyme-linked immunosorbent assay (Human Hepcidin Quantikine ELISA Kit; R&D Systems Europe,

Table 1. Demographic and clinical characteristics of the study population

	Total IBD population (n = 104)	25(OH)D <20 ng/mL (n = 40)	25(OH)D ≥20 ng/mL (n = 64)
Demographics			
Age, yr[a]	41 ± 12	39 ± 11	43 ± 13
Sex, female	45 (43)	13 (33)	32 (50)
BMI, kg/m^2	24 ± 3	24 ± 4	24 ± 3
Smokers	23 (25)	9 (26)	14 (25)
Positive family history of IBD	17 (21)	8 (25)	9 (18)
Disease type			
Crohn's disease	67 (64)	25 (63)	42 (66)
Ulcerative colitis	37 (36)	15 (37)	22 (34)
Disease duration, yr	15 ± 10	15 ± 8	15 ± 11
Disease activity			
Active	42 (40)	15 (37)	27 (42)
Remission	62 (60)	25 (63)	37 (58)
CDAI	110 ± 101	92 ± 82	120 ± 111
CAI	5 ± 4	5 ± 4	5 ± 3
Crohn's disease location			
Ileum isolated	8 (8)	2 (5)	6 (10)
Colon isolated	4 (4)	1 (3)	3 (5)
Ileocolic	28 (28)	14 (37)	14 (24)
Ileocolic + jejunum	11 (11)	6 (16)	5 (8)
Ileocolic + upper gastrointestinal tract	11 (11)	4 (11)	7 (12)
Extent of ulcerative colitis			
Proctitis	6 (6)	2 (5)	4 (7)
Left-sided colitis	12 (12)	4 (11)	8 (14)
Pancolitis	17 (16)	5 (13)	12 (20)
Extraintestinal manifestations	30 (29)	15 (38)	15 (23)
Fistula	29 (28)	16 (40)	13 (20)
Stenosis	36 (35)	14 (35)	22 (34)
Abscess	15 (14)	8 (20)	7 (11)
Previous surgery	35 (24)	15 (38)	20 (31)
Current IBD medication			
No IBD medication	7 (7)	4 (10)	3 (5)
Oral steroids	3 (3)	1 (3)	2 (3)
Oral steroids + 5-ASA	6 (6)	2 (5)	4 (6)
Immunosuppressants	3 (3)	1 (3)	2 (3)
Immunosuppressant + steroids	1 (1)	0	1 (2)
Immunosuppressants + 5-ASA	2 (2)	0	2 (3)
Anti-TNF	39 (38)	18 (45)	21 (33)
Anti-TNF + steroids	4 (4)	2 (5)	2 (3)
Anti-TNF + steroids + 5-ASA	2 (2)	1 (3)	1 (2)
Anti-TNF + immunosuppressants	1 (1)	0	1 (2)
Anti-TNF + immunosuppressants + steroids	4 (4)	2 (5)	2 (3)
Anti-TNF + 5-ASA	14 (4)	4 (10)	10 (15)
Vedolizumab	5 (5)	3 (8)	2 (3)
Vedolizumab + steroids	3 (3)	0	3 (5)
Vedolizumab + 5-ASA	3 (3)	2 (5)	1 (2)
5-ASA monotherapy	7 (7)	0	7 (11)
Vitamin D supplementation, yes	51 (49)	17 (44)	36 (56)
If yes: mean dosage (IE)[a]	1,019 ± 229	1,088 ± 364	986 ± 117

Data are presented as n (%) or mean ± SD.
25(OH)D, 25-hydroxyvitamin D; 5-ASA, 5-aminosalicylic acid; BMI, body mass index; CAI, clinical activity index; CDAI, Chron's disease activity index; IBD, inflammatory bowel disease; TNF, tumor necrosis factor.
[a]Not normally distributed continuous variable

Abingdon, United Kingdom) according to the manufacturer's guidelines.

Caco-2 cell culture, reverse transcriptase polymerase chain reaction, and quantitative real-time polymerase chain reaction

Intestinal epithelial Caco-2 cells (LGC Standards, Wesel, Germany; obtained in passage 43) were cultivated in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, Taufkirchen, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich) in a humidified 5% CO_2 atmosphere at 37 °C. When grown on filter inserts, Caco-2 cells spontaneously differentiate to express morphological and functional characteristics of mature small intestinal enterocytes, including a functional vitamin D receptor (18). Thus, 5 × 10 (5) Caco-2 cells (used between passage 46 and 51) in 0.5 mL of complete medium were seeded on polycarbonate filter inserts (Corning Transwell, pore size 0.4 μM; Merck, Darmstadt, Germany). The lower chamber was filled with 1.5 mL complete Dulbecco modified Eagle medium. After cultivation for 21 days (medium changed twice a week), the cells were stimulated in triplicate with equal volumes of vehicle (100% ethanol) or 10 or 100 nM 1,25(OH)D (Merck, Darmstadt, Germany), resulting in final concentrations of 0.1% and 1% ethanol, respectively. The chosen concentrations and incubation times for 1,25(OH)D were similar to previously published studies (19–22). After 24 and 48 hours, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and 500 ng of RNA from each sample was

reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Differential expression of 18 genes known to be involved in intestinal iron absorption and metabolism (*CYBRD1, DMT1, PCBP2, SLC46A1 [HCP1], SLC48A1 [HRG1], HMOX1, HMOX2, FTL, FTH1, IRP1, IREB2, HFE, TFR2, TFRC, SLC40A1 [FPN1], HEPH, CP, TF*) (23,24) was measured by real-time quantitative polymerase chain reaction using LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany) on a LightCycler 480 instrument. polymerase chain reaction primers (TIB Molbiol, Berlin, Germany; see Supplemental Table 1, Supplementary Digital Content 1, http://links.lww.com/CTG/A741) were designed not to amplify genomic DNA. Gene expression was calculated using the 2–ΔΔCt method with *RPL13A* as the housekeeping gene. Overall, 3 independent stimulation experiments (each in triplicate) were performed.

Statistical analysis

Statistical analyses were performed with XLSTAT (Addinsoft, Paris, France) for Microsoft Excel (Microsoft, Redmond). Descriptive statistics were computed for all variables and presented as the mean values with SDs for normally distributed continuous variables, medians and interquartile range (IQR) for non-normally distributed continuous variables, and as number and proportion for categorical variables. The Shapiro-Wilk test was used to test for a normal distribution of quantitative variables. Because ferritin, transferrin saturation, hepcidin, and hemoglobin were not normally distributed, the nonparametric Mann-Whitney *U* test was generally applied for comparisons of all continuous biomarkers of iron deficiency and anemia between vitamin D–deficient and vitamin D–sufficient patients with IBD. Results were graphically visualized by boxplots including the minimum, maximum, median, and IQR. Kendall τ was calculated to test for correlations between quantitative variables.

Multiple logistic and linear regression models (i.e., ANCOVA) were used to further evaluate the association between vitamin D status (independent variable) and serum iron, ferritin, transferrin saturation, hepcidin, hemoglobin, iron deficiency, anemia, and iron deficiency anemia (dependent variables). Sex, age, body mass index, smoking status, disease activity (active disease vs remission), and CRP levels were included as covariates. Because smoking status was not documented for 12 (6 with CD and 6 with UC) of the 104 patients with IBD, these patients were excluded from the multiple regression analysis, leaving 92 patients with IBD for this analysis. All multivariate analyses were performed assuring normal distribution of the residuals as assessed by the Shapiro-Wilk test. For the examination of ferritin, hepcidin, and hemoglobin as dependent variables in the multivariate models, data transformation was necessary to fulfill the requirement of a normal distribution of the residuals for significance testing. For ferritin, this was achieved by log transformation. For hepcidin, the third root had to be extracted, whereas hemoglobin had to be squared. Vitamin D status, representing the independent variable, was calculated as a categorical variable (vitamin D sufficiency, defined by serum 25(OH)D ≥ 20 ng/mL), as continuous 25(OH)D or 1,25(OH)D concentrations, or as the 1,25(OH)D:25(OH)D ratio. In Caco-2 cell culture experiments, the mean fold changes in gene expression under nonstimulated or 1,25(OH)D-stimulated conditions were compared by the Student *t* test. In all tests, 2-tailed *P* values were calculated, and $P < 0.05$ was considered significant.

Table 2. Association of serum 25(OH)D levels with iron deficiency, anemia, and iron deficiency anemia, and of vitamin D status with serum iron, ferritin, transferrin saturation, and hepcidin in patients with inflammatory bowel disease (n = 92) according to multiple logistic and linear regression analyses

Dependent variable	β	SE	*P*
Serum 25(OH)D, independent variable			
Iron deficiency	−0.314	0.144	**0.029**
Anemia	−0.364	0.200	0.069
Iron deficiency anemia	−0.385	0.199	0.054
Serum 25(OH)D ≥ 20 ng/mL (Y vs N), independent variable			
Serum iron	0.180	0.096	0.065
Ferritin	0.248	0.107	**0.024**
Transferrin saturation	0.261	0.126	**0.044**
Hemoglobin	0.097	0.085	0.255
Hepcidin	0.231	0.104	**0.028**

Covariates: sex, age, body mass index, smoking status, disease activity, and C-reactive protein level

25(OH)D, 25-hydroxyvitamin D; β, standardized regression coefficient; SE, standard error.
Bold values represents significant $P < 0.05$.

Ethical considerations

Written informed consent was obtained from all patients before participation in the study. The study was approved by the local Ethics Committee of the Medical Faculty of LMU Munich as the responsible institutional review board (approval code 343-09) and adhered to the ethical principles of the Helsinki Declaration.

RESULTS

Association of serum 25(OH)D levels with iron deficiency in patients with IBD

In a multiple logistic regression model, serum 25(OH)D concentrations were inversely associated with iron deficiency (β standard error [SE] = −0.314 [0.144], $P = 0.029$) in patients with IBD (Table 2). Sex, age, body mass index, smoking status, disease activity, and CRP were included as covariates. Furthermore, we identified a clear trend toward an inverse association of serum 25(OH)D levels with iron deficiency anemia (β [SE] = −0.385 [0.199], $P = 0.054$) and anemia in general (β [SE] = −0.364 [0.200], $P = 0.069$) in patients with IBD. However, this association did not reach significance (Table 2). Regarding the covariates in the multivariate model with 25(OH)D as the independent variable, disease remission was associated with a lower prevalence of anemia (β [SE] = −1.805 [0.745], $P = 0.015$) and iron deficiency anemia (β [SE] = −1.653 [0.720], $P = 0.022$). As expected, anemia was associated with female sex (β [SE] = 1.574 [0.756], $P = 0.037$). Results of the multiple logistic regression models, including 1,25(OH)D or the 1,25(OH)D:25(OH)D ratio as the independent variable and iron deficiency, iron deficiency anemia, or anemia as dependent variables, accounting for the covariates listed earlier, are presented in Supplemental Table 2, Supplementary Digital Content 1, http://links.lww.com/CTG/A741. The 1,25(OH)D and the 1,25(OH)D:25(OH)D ratio were not associated with these dichotomous outcomes.

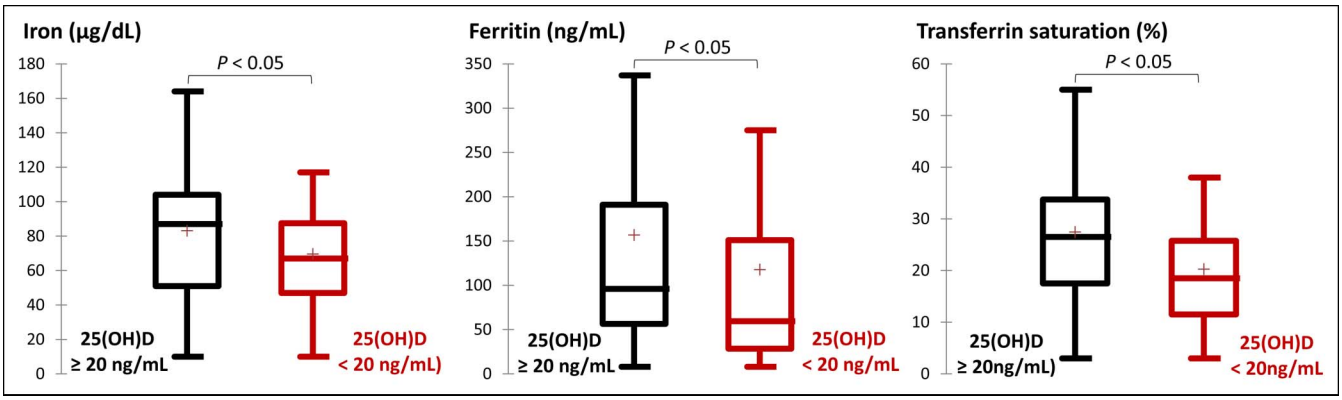

Figure 1. Markers of iron deficiency in 104 patients with inflammatory bowel disease with sufficient vitamin D (n = 64) and vitamin D deficiency (n = 40).

Vitamin D sufficiency in IBD is associated with better levels of ferritin and transferrin saturation

When investigating vitamin D sufficiency vs vitamin D deficiency in patients with IBD as an independent dichotomous variable and serum iron, ferritin, transferrin saturation, hemoglobin, and hepcidin as dependent variables (Table 2), ferritin (β [SE] = 0.248 [0.107], P = 0.024) and transferrin saturation (β [SE] = 0.261 [0.126], P = 0.044) were positively associated with vitamin D sufficiency in multiple linear regression models. Covariates included sex, age, body mass index, smoking status, disease activity, and CRP. Surprisingly, serum hepcidin (β [SE] = 0.231 [0.104], P = 0.028) was also positively associated with vitamin D sufficiency. Regarding ferritin, the studied covariates showed no further influence. When transferrin saturation was the dependent variable in the multivariate model, CRP (β [SE] = −0.302 [0.131], P = 0.026) was the only covariate with an additional negative effect. Regarding hepcidin as the dependent variable, CRP was a positively associated covariate (β [SE] = 0.352 [0.108], P = 0.002). Serum iron (β [SE] = 0.180 [0.096], P = 0.065) showed a trend toward a positive multivariate association with vitamin D sufficiency. Concerning covariates in this model, serum iron was negatively associated with CRP (β [SE] = −0.431 [0.100], P < 0.01).

Higher levels of serum iron, ferritin, and transferrin saturation in vitamin D–sufficient patients with IBD

Vitamin D–sufficient patients with IBD had significantly higher levels of serum iron (median [IQR] 87 µg/dL [51–104 µg/dL] vs 67 µg/dL [47–87 µg/dL], P = 0.042, normal range 33–193 µg/dL), ferritin (96 ng/mL [56.5–191 ng/mL] vs 59.5 ng/mL [28.5–151 ng/mL], P = 0.042, normal range 15–225 ng/mL for women and 30–600 ng/mL for men), and transferrin saturation (26.5% [17.5%–33.75%] vs 18.5% [11.5%–25.75%], P = 0.033, normal range 16%–45%) when compared with vitamin D–deficient patients with IBD (Figure 1). The separate results for CD and UC are provided in Supplemental Table 3, Supplementary Digital Content 1, http://links.lww.com/CTG/A741.

Positive correlation of 25(OH)D levels with serum iron, ferritin, and transferrin saturation in CD

As shown in Figure 2, for patients with CD, there was a direct positive correlation of serum 25(OH)D levels with serum iron (Kendall τ 0.17, P = 0.042), ferritin (Kendall τ 0.18, P = 0.035), and transferrin saturation (Kendall τ 0.21, P = 0.041). This correlation could not be demonstrated for patients with UC (serum iron: Kendall τ 0.12, P = 0.30; ferritin: Kendall τ 0.065, P = 0.57; and transferrin saturation: Kendall τ 0.065, P = 0.65). For the whole subset of patients with IBD, a correlation of 25(OH)D concentration with serum iron (Kendall τ 0.15, P = 0.026) and ferritin (Kendall τ 0.15, P = 0.027) and a trend toward a correlation with transferrin saturation (Kendall τ 0.15, P = 0.065) was found.

1,25(OH)D concentrations are associated with transferrin saturation in CD

Multiple linear regression analyses with serum 1,25(OH)D as the independent variable and serum iron, ferritin, transferrin saturation, hemoglobin, and hepcidin as dependent variables revealed a positive

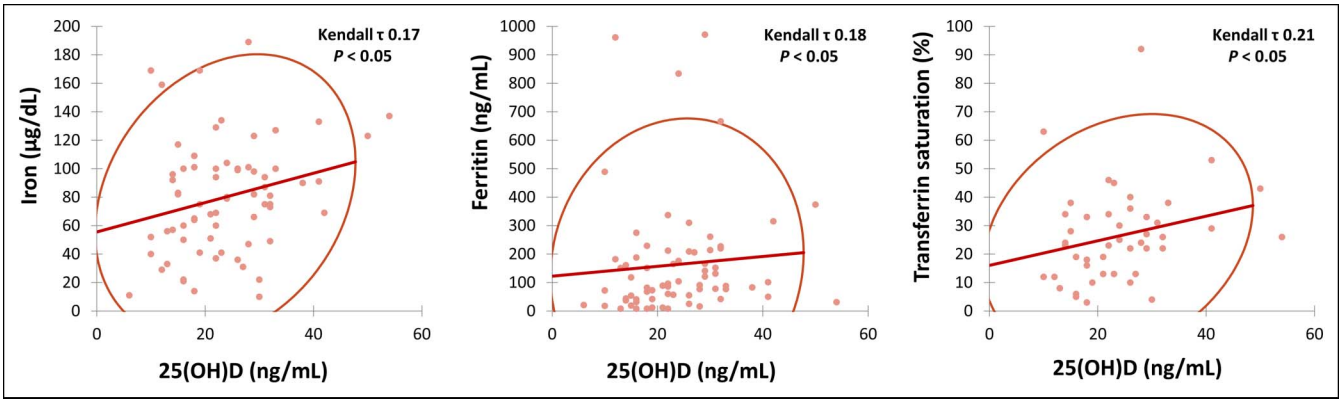

Figure 2. Correlation of markers of iron deficiency with serum levels of 25-hydroxyvitamin D in patients with Crohn's disease (n = 67).

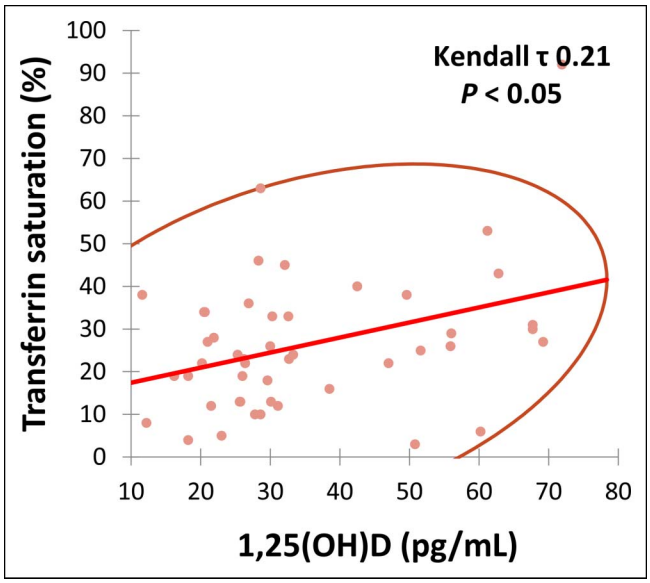

Figure 3. Correlation of serum levels of 1,25-dihydroxyvitamin D with transferrin saturation in patients with Crohn's disease (n = 67).

association of 1,25(OH)D concentration with transferrin saturation (β [SE] = 0.405 [0.175], $P = 0.027$) in patients with CD. Sex, age, body mass index, smoking status, disease activity, and CRP were included as covariates. For patients with UC, no such association could be demonstrated (β [SE] = 0.164 [0.277], $P = 0.565$). Taking into account the entire IBD population, the result was almost significant (β [SE] = 0.258 [0.131], $P = 0.055$). The other quantitative variables showed no association with 1,25(OH)D for CD, UC, or IBD in this multivariate model (see Supplemental Table 4, Supplementary Digital Content 1, http://links.lww.com/CTG/A741). In patients with CD, 1,25(OH)D concentrations directly correlated with transferrin saturation (Kendall τ 0.21, $P < 0.05$; Figure 3).

Correlation of higher 1,25(OH)D:25(OH)D ratios with lower hepcidin concentrations in IBD

The main circulating form of vitamin D is 25(OH)D; it can be stored in the liver and adipose tissue and defines vitamin D status (3). However, 25(OH)D is not the active form of vitamin D and has to be converted to active 1,25(OH)D by the enzyme 1-α-hydroxylase (3). Therefore, we calculated the serum 1,25(OH)D:25(OH)D ratio to assess the systemic effects of active vitamin D corrected for the vitamin D status. The 1,25(OH)D:25(OH)D ratio showed a highly significant inverse correlation with serum hepcidin concentrations in patients with IBD (Kendall τ 0.19, $P = 0.005$; Figure 4a). Furthermore, the serum 1,25(OH)D:25(OH)D ratio as an independent variable was negatively associated with serum hepcidin levels in IBD (β [SE] = −0.272 [0.105], $P = 0.012$; see Supplemental Table 4, Supplementary Digital Content 1, http://links.lww.com/CTG/A741) in the multiple linear regression analysis. The multiple regression analyses included sex, age, body mass index, smoking status, disease activity, and CRP as covariates. CRP (β [SE] = 0.316 [0.108], $P = 0.004$) was a significant covariate and positively associated with hepcidin, another acute phase protein. The subset of patients with IBD with a high 1,25(OH)D:25(OH)D ratio (greater than the median ratio of 1.48×10^{-3}) exhibited significantly lower serum hepcidin levels (Figure 4b) compared with patients with IBD with a low 1,25(OH)D:25(OH)D ratio (below the median; $P < 0.05$). Surprisingly, as already shown for 25(OH)D levels ≥ 20 ng/mL (Table 2), multiple linear regression analysis with 25(OH)D as the independent variable showed a positive correlation with serum hepcidin levels (β [SE] = 0.273 [0.103], $P = 0.010$) in IBD. Again, CRP (β [SE] = 0.371 [0.107], $P = 0.001$) was a modulating covariate in this model. The complete results of the multiple linear regression analyses with 25(OH)D, 1,25(OH)D, or the 1,25(OH)D:25(OH)D ratio as the independent variable and serum iron, ferritin, transferrin saturation, hemoglobin, and hepcidin as dependent quantitative variables are summarized in Supplemental Table 4, Supplementary Digital Content 1, http://links.lww.com/CTG/A741.

Strong induction of ceruloplasmin by 1,25(OH)D in Caco-2 cells

To test whether 1,25(OH)D influences intestinal iron absorption and transport across the cell layer, enterocyte-like differentiated Caco-2 cells were stimulated with 100 nM 1,25(OH)D for 48 hours for the initial screening. Among 18 intestinal iron absorption–related genes, *CP* was upregulated up to 15.7-fold compared with control cells treated with vehicle only ($P = 2 \times 10^{-5}$). This

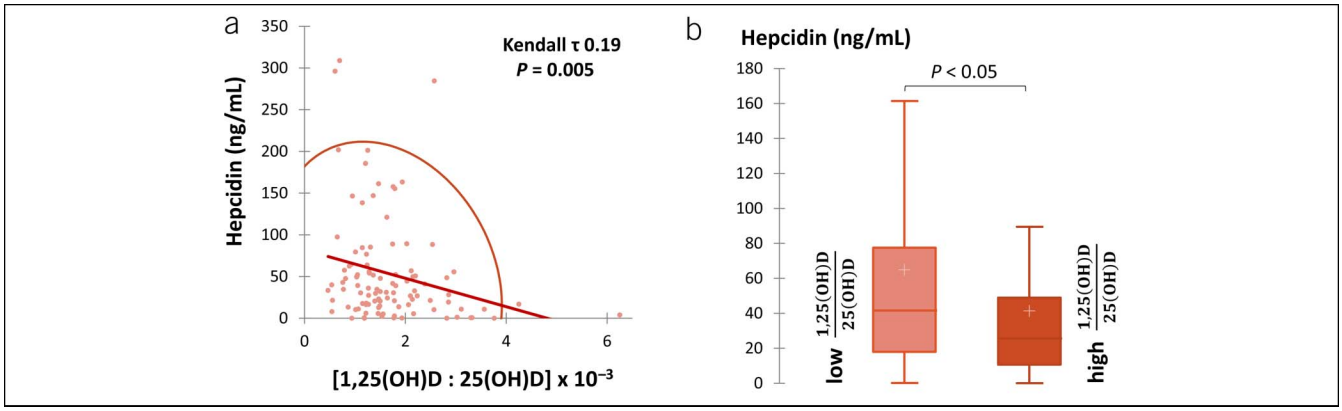

Figure 4. The serum 1,25(OH)D:25(OH)D ratio and hepcidin in IBD. (a) The ratio inversely correlated with serum hepcidin concentrations in patients with IBD (n = 104). (b) Significantly lower serum hepcidin concentrations in patients with IBD with a high (i.e., greater than median) 1,25(OH)D:25(OH)D ratio (n = 52) vs patients with IBD with a low (i.e., lower than median) ratio (n = 52). 1,25(OH)D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; IBD, inflammatory bowel disease.

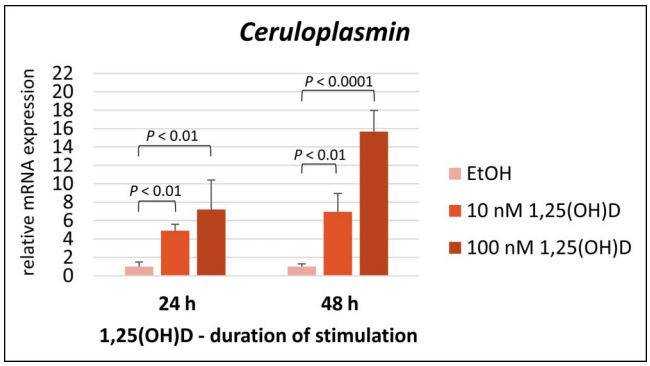

Figure 5. Strong induction of ceruloplasmin gene expression in Caco-2 cells after stimulation with 1,25-dihydroxyvitamin D. The expression in unstimulated control cells was set to 1.0. Results are from 3 independent experiments. EtOH, ethanol.

upregulation was dose-dependent and time-dependent as determined in additional experiments using 10 and 100 nM 1,25(OH)D and 24 and 48 hours of stimulation (Figure 5). The complete results of the stimulation experiments are summarized in Supplemental Table 5, Supplementary Digital Content 1, http://links.lww.com/CTG/A741. Apart from the striking induction of ceruloplasmin, we observed only a slight downregulation of transferrin by a factor of 0.76 ($P = 0.002$ vs control). The other genes were not significantly affected by 1,25(OH)D.

DISCUSSION

In this study, we demonstrate for the first time that low serum 25(OH)D levels, reflecting poor vitamin D status, are associated with iron deficiency in patients with IBD. By applying multivariate regression analyses, we showed that this association is independent of sex (25), age (25,26), body mass index (obesity) (26), smoking status (27), disease activity (3), and CRP levels (3,26). These factors, which are known to influence vitamin D status, were included as covariates in the multivariate models. To exclude diminished physiological hepatic 25-hydroxylation of cholecalciferol or reduced renal 1-α-hydroxylation as cofounding factors, patients with liver cirrhosis or chronic kidney failure were excluded from the study. Vitamin D supplementation was equally distributed in the vitamin D–deficient and vitamin D–sufficient IBD subgroups.

Of interest, serum iron, ferritin, and transferrin saturation were consistently and significantly reduced in vitamin D–deficient patients with IBD. By contrast, vitamin D–sufficient patients with IBD had significantly higher levels of the iron sufficiency parameters ferritin and transferrin saturation in the multivariate regression models. In particular, for patients with CD, higher serum 25(OH)D levels directly correlate with higher levels of serum iron, ferritin, and transferrin saturation. A direct correlation of the hormonally active form of vitamin D, 1,25(OH)D, with transferrin saturation in CD could be verified in the multivariate analysis. This points to a potential role of 1,25(OH)D in inducing enzymes that are involved in loading transferrin with iron. Physiologically, this process is coordinated by 1 of the 2 multicopper ferroxidases, hephaestin and ceruloplasmin. These enzymes promote the oxidization of Fe(2+), which is provided by the iron exporter ferroportin, for delivery to the circulating Fe(3+) carrier transferrin on the basolateral side of intestinal epithelial cells or other iron-exporting cells (28,29). Accordingly, we demonstrated that ceruloplasmin is strongly induced by 1,25(OH)D in Caco-2 cells when analyzing all 18 enzymes known to be or believed to be involved in intestinal epithelial absorption of iron (18,23,29,30). Experiments in ceruloplasmin knockout ($Cp^{-/-}$) mice with iron pathways activated by acute bleeding stress have shown that intestinal iron absorption is markedly impaired in the absence of ceruloplasmin (30). Phlebotomy of wild-type mice led to a notable shift in ceruloplasmin from the duodenal epithelium to the underlying lamina propria. This suggests a critical function of ceruloplasmin in basolateral intestinal export of iron into the blood circulation during iron absorption (30). Under bleeding stress, the mice may, to some extent, serve as an appropriate model for iron–deficient patients with IBD. Thus, we hypothesize that one possible mechanism by which 1,25(OH)D counteracts iron deficiency in patients with IBD is the induction of the intestinal ceruloplasmin expression critical for intestinal iron absorption.

In addition, active 1,25(OH)D directly transcriptionally suppresses hepcidin expression in hepatocytes and macrophages (5,11,12). The liver peptide hepcidin serves as a master regulator of iron homeostasis by diminishing intestinal iron absorption and iron recycling from macrophages in the reticuloendothelial system under inflammatory conditions, reflecting the evolutionarily conserved struggle for essential iron between host and pathogens (29,31). Proinflammatory cytokines, such as interleukin-6, increase hepcidin levels in acute and chronic inflammation. This causes anemia of inflammation or anemia of chronic disease in infectious or chronic immune-mediated diseases, such as IBD (7,29). Hepcidin exerts its negative regulation of iron availability through blockade of the iron exporter ferroportin (29). The downregulation of hepcidin and resulting resolution of the iron blockade by 1,25(OH)D is reflected in our data. We saw a highly significant negative correlation of the 1,25(OH)D:25(OH)D ratio with serum hepcidin levels in patients with IBD. Surprisingly, 25(OH)D concentrations positively correlated with hepcidin levels, and vitamin D–sufficient patients with IBD had higher hepcidin levels than vitamin D–deficient patients with IBD.

This may be due to other beneficial functions of the antimicrobial peptide and acute phase protein hepcidin from non-hepatic sources in IBD, which are not iron homoeostasis related. Of interest, dendritic cell–derived hepcidin was recently revealed to be essential for intestinal tissue repair, independent of hepatocyte-derived hepcidin or systemic iron levels (32). In this regard, hepcidin promotes mucosal healing by sequestering iron from the microbiota (32). This could be another reason why hepcidin is elevated in patients with IBD (33,34) and correlates with disease activity (33). It would also explain why patients with a sufficient supply of the generally anticolitic (35) vitamin D exhibit higher hepcidin levels.

Serum 25(OH)D is the preferred clinical parameter for assessing vitamin D sufficiency because it represents the overall body storage of vitamin D. However, it is the active form of vitamin D, 1,25(OH)D, which specifically binds to the vitamin D receptor and regulates vitamin D–dependent gene expression. The hormone-to-prohormone vitamin D activation ratio reflects the proportion of systemic vitamin D reserves being processed and can quantify the amount of vitamin D mobilized for use in endocrine signaling (36). This metabolic ratio, normalized to the total vitamin D store measured by 25(OH)D levels, may serve as a superior predictor of relevant clinical outcomes (36,37). For

example, higher 1,25(OH)D:25(OH)D activation ratios have been demonstrated to be associated with higher percentages of regulatory T cells in patients with multiple sclerosis (38) and that men with higher 1,25(OH)D:25(OH)D ratios are more likely to harbor butyrate-producing bacteria, which are associated with better microbial health in the gastrointestinal tract where the vitamin D receptor is highly expressed (36,39). Regarding IBD, remarkable extrarenal 1a-hydroxylase activity of intestinal epithelial cells and lamina propria mononuclear cells has been postulated to lead to excess 1,25(OH)D from the inflamed gut. The surplus 1,25(OH)D enters the blood circulation and may contribute to systemic metabolic effects (40). One of those could be the suppression of hepatic hepcidin synthesis, cooperating with intestinal epithelial ceruloplasmin induction as part of the local autocrine and paracrine effects of intestinally produced 1,25(OH)D. Very recently, 2 randomized controlled trials demonstrated that intravenous iron substitution rapidly decreases circulating 1,25(OH)D levels, whereas 25(OH)D levels remain unchanged (41). This effect is responsible, at least partly, for the severe hypophosphatemia that can occur as a side effect of intravenous iron preparations. It also makes it plausible for increasing 1,25(OH)D levels to be an attempt of the body to facilitate iron availability (41). This attempt may be reregulated after the reconstitution of iron status.

The downregulation of hepcidin by vitamin D, as reflected in our data, has not only been shown *in vitro* (11,12) but is also supported by clinical data. Recently, a prospective interventional study in children with newly diagnosed mild to moderate IBD demonstrated that treatment with 4,000 units of vitamin D per day for 2 weeks can significantly reduce the initial disease-specific elevated serum hepcidin levels by 81% (34). Furthermore, another cross-sectional study in children with IBD showed that vitamin D sufficiency is associated with lower hepcidin and higher hemoglobin levels. This highlights the role of vitamin D in preventing anemia of inflammation by suppressing hepcidin (42). Despite a statistical trend, hemoglobin levels and diagnosis of anemia were not significantly influenced by vitamin D status in our cohort of adult patients with IBD. However, the correlation of vitamin D metabolites with parameters related to iron status was significant and in line with a recent meta-analysis of 14 randomized controlled trials investigating the effect of vitamin D supplements on hemoglobin concentrations in adult participants without IBD (43). In this meta-analysis, supplementation with vitamin D had no significant effect on hemoglobin levels, whereas a positive effect on transferrin saturation and iron status was observed (43). A recent study of 9,590 adults who presented for periodic medical examination in a sunny Mediterranean city affirmed that 25(OH)D deficiency is significantly associated with iron deficiency (6). The considerable expression of the vitamin D receptor and 1-α-hydroxylase in the inflamed intestine (39) and elevated hepcidin levels in the inflammatory state leading to anemia of chronic disease (29,33,34) point toward a disease-specific mechanism by which vitamin D counteracts iron deficiency in chronic inflammatory diseases, such as IBD. This hypothesis is supported by the intestinal induction of ceruloplasmin expression and systemic suppression of hepcidin by vitamin D in our study. Therefore, we suggest that optimized vitamin D supplementation may improve oral iron bioavailability by partly antagonizing the blockade of iron resorption mediated by hepcidin. High hepcidin levels are known to predict nonresponsiveness to oral iron therapy in patients with iron deficiency anemia (44), and low bioavailability of oral iron supplements or dietary iron is a challenge in daily IBD clinics, leading to widespread application of expensive intravenous iron preparations in IBD (7,45).

In summary, this study provides new evidence indicating that iron deficiency in patients with IBD is associated with low 25(OH)D levels. Active 1,25(OH)D may ameliorate iron deficiency by suppressing hepcidin and inducing intestinal ceruloplasmin expression, thereby increasing intestinal iron absorption. Iron absorption experiments in gut-on-chip models (46) and further prospective and interventional clinical studies are warranted to determine whether escalated vitamin D supplementation in IBD can help overcome the limited oral bioavailability of iron caused by the mainly hepcidin-mediated blockade of intestinal iron absorption in IBD (7).

CONFLICTS OF INTEREST

Guarantor of the article: Johannes Stallhofer, MD.

Specific author contributions: J.S. and F.B. conceived and designed the research project. J.S., L.V., and J.D. performed the research and acquired the data. J.S., L.V., J.D., and P.P. analyzed the data. J.S., L.V., and F.B. interpreted the data. J.S., F.S., T.O., H.T., and F.B. recruited the patients and substantially contributed to the acquisition of clinical data. J.S. wrote the manuscript. S.B., J.M., A.S., and F.B. critically revised the manuscript regarding important intellectual content. All authors gave final approval of the version to be published.

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Study Highlights

WHAT IS KNOWN

- ✓ Iron deficiency and vitamin D deficiency are common in patients with inflammatory bowel disease (IBD).
- ✓ Active 1,25-dihydroxyvitamin D (1,25(OH)D) suppresses expression of hepcidin, the key negative regulator of iron absorption, *in vitro*.

WHAT IS NEW HERE

- ✓ Iron deficiency in adult patients with IBD is associated with poor vitamin D status.
- ✓ Vitamin D sufficiency is associated with higher ferritin and transferrin saturation levels in patients with IBD.
- ✓ A higher ratio of active 1,25(OH)D to prohormone 25-hydroxyvitamin D correlates with lower serum hepcidin concentrations in patients with IBD *in vivo*.
- ✓ Active 1,25(OH)D induces intestinal ceruloplasmin expression, potentially facilitating intestinal iron absorption.

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