Anemia of chronic disease and defective erythropoietin production in patients with celiac disease

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ABSTRACT

Background
Anemia due to hematinic deficiencies is common in patients with untreated celiac disease. Although celiac disease is a chronic condition characterized by an intense inflammatory response of the intestinal mucosa, scant data are available about the prevalence of anemia of chronic disease in celiac disease.

Design and Methods
One hundred and fifty-two patients with celiac disease at presentation were studied. Anemia was investigated by determining complete blood counts, body iron status, serum levels of the soluble transferrin receptor, erythropoietin, prohepcidin and interferon-γ. Genotyping for *HFE* mutations associated with hereditary hemochromatosis was performed. Fifty-three anemic patients were re-evaluated for hematologic response after 1 year on a gluten-free diet.

Results
At the time of diagnosis of celiac disease the prevalence of anemia was 34%. Fifty-three out of 65 anemic patients had either iron and/or vitamin deficiency (folate, vitamin B_{12}). Hereditary hemochromatosis mutations did not affect the prevalence of anemia. In 11 cases iron status parameters were indicative of anemia of chronic disease, sometimes in association with iron deficiency (6 patients). Patients with anemia of chronic disease had low levels of erythropoietin for the degree of anemia and increased serum interferon-γ. In most cases anemia improved following a gluten-free diet, response rates being similar in anemia of chronic disease and in anemia due to hematinic deficiencies.

Conclusions
Our study shows that, in addition to iron and vitamin deficiencies, anemia of chronic disease has a significant role in some patients with celiac disease. Suppression of intestinal inflammatory changes as a result of a gluten-free diet improves anemia by correcting iron and vitamin malabsorption as well as mechanisms contributing to anemia of chronic disease.

Key words: celiac disease, iron deficiency anemia, inflammation.

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Introduction

Celiac disease is an autoimmune disorder precipitated by the ingestion of gluten. The main feature of the disease is a gastrointestinal malabsorption syndrome, related to damage of the small bowel mucosa, which may affect many organ systems, although some patients present with subtle symptoms in the absence of severe malnutrition, weight loss and diarrhea. Anemia is the most common hematologic abnormality of celiac disease, with a prevalence ranging from 12% to 69% at diagnosis,[1] and may represent the first manifestation of an otherwise subclinical/silent disease.[2,3] The pathogenesis of anemia is usually dependent on malabsorbtion of iron or vitamins,[1] although occult blood loss from the gastrointestinal tract may occur.[4]

Anemia of chronic disease (ACD) is the most common form of anemia in hospitalized patients and occurs in association with acute or chronic inflammation caused by immune activation. In these conditions an increased production of inflammatory cytokines can directly inhibit erythropoiesis, interfere with erythropoietin (Epo) production and induce changes in iron homeostasis characterized by reductions of both iron absorption and macrophage iron release.[5] ACD is not recognized as a frequent finding in celiac disease. In fact, systemic inflammation as evidenced by increased serum levels of acute phase proteins is uncommon in celiac disease, although gliadin-dependent activation of mucosal lamina propria mononuclear cells causes overproduction of pro-inflammatory cytokines such as interferon-γ and interleukin-6,[6-8] both mediators of ACD.[9,10]

In the present study we investigated the pathogenesis of anemia in patients with celiac disease.

Design and Methods

Patients and study design

Patients with celiac disease were investigated for the presence of anemia, at the time of diagnosis, and its pathogenesis. The study was approved by the local medical ethics committee in accordance with the revised Helsinki Declaration. The diagnosis of celiac disease required a duodenal biopsy showing villous atrophy with crypt hyperplasia and intraepithelial lymphocyte infiltration, a positive anti-endomysium antibody test, and a positive response to a gluten-free diet.[11] Histopathological findings were graded according to Corazza *et al.*[12]

One hundred and thirty-two consecutive patients with celiac disease were enrolled from January 2000 to September 2005 and followed up for 1 year while on a gluten-free diet, since it has been shown that recovery from anemia in celiac disease usually requires 6 to 12 months of such a diet.[13] Twenty additional patients with celiac disease, who were anemic at diagnosis but not included in the initial investigation on the prevalence of anemia, were evaluated to study the pathogenesis of anemia in this disease. The nutritional status of the patients was evaluated by determining serum albumin concentration and calculating the body mass index (BMI). At enrollment none of the patients was receiving iron supplementation or erythropoiesis-stimulating agents, and none had recently been treated with blood transfusions.

Anemia was evaluated by determining complete blood cell counts, body iron status, and serum levels of folate, vitamin B_{12}, and C-reactive protein. These assays were performed as routine automated laboratory tests. C-reactive protein was measured by a high-sensitive C-reactive protein assay (Dade Behring), with a reference interval up to 0.6 mg/dL. In the anemic patients and in a subgroup of non-anemic patients with celiac disease serum levels of soluble transferrin receptor (sTfRc), endogenous erythropoietin, prohepcidin and interferon-γ were measured. The following commercial kits were used for these determinations: the sTfRc kit from Dade Behring (Marburg, Germany), the EPO ELISA kit from Medac (Hamburg, Germany), the Hepcidin ELISA Kit from DRG (Heidelberg, Germany) and the Endogen Human IFNγ ELISA kit from Pierce Biotechnology (Rockford, IL, USA). All blood samples were collected after overnight fasting, and serum samples for erythropoietin levels, sTfRc, prohepcidin, folate and vitamin B_{12} determinations were stored at –70°C until analysis.

Peripheral blood DNA from the patients was genotyped for the C282Y and H63D *HFE* gene mutations associated with hereditary hemochromatosis using TaqMan technology and an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA, USA).[14]

Definition of anemia

Anemia was defined by a hemoglobin (Hb) concentration <13.0 g/dL in males and <12.0 g/dL in females.[15] According to the World Health Organization (WHO) mild anemia corresponds to a Hb ≥9.5 g/dL, moderate anemia to a Hb ≥8 g/dL but <9.5 g/dL, and severe anemia to a Hb <8.0 g/dL. Patients were not eligible for the study if other conditions which could cause anemia or interfere with erythropoiesis were present (malignancy, previous chemotherapy or radiotherapy, connective tissue diseases, infections, renal failure). A hematologic response to a gluten-free diet was defined as the normalization of the Hb level or by a Hb increase ≥1.0 g/dL.

Iron-deficiency anemia was characterized by the presence of anemia associated with low serum ferritin (<10 ng/mL for females, <15 ng/mL for males) or with a transferrin saturation <16% together with serum ferritin levels <30 ng/mL. The diagnosis of ACD required the presence of reduced transferrin saturation (<16%), normal/reduced serum transferrin with normal/high serum ferritin (>100 ng/mL).[16] The association of transferrin saturation <16% with normal/reduced transferrin and a serum ferritin ≥30 ng/mL, but ≤100 ng/mL, defined a group of patients who possibly had a combination of iron deficiency and chronic disease as pathogenetic mechanisms of anemia.

To study the serum erythropoietin and the erythropoietic response to anemia, regressions between the log(Epo), sTfRc and Hb were determined in a group of 35 reference subjects with iron-deficiency anemia or β-

thalassemia intermedia but no laboratory or clinical signs of inflammation or renal failure, and compared with those found in the celiac disease patients. Reference subjects were supposed to have a normal erythropoietin response to anemia with adequate bone marrow function.

Statistical analysis

The Student's *t* test for paired and unpaired data, the F test (one-way analysis of variance), the Mann-Whitney U test and the Kruskal-Wallis test were used to compare continuous variables. Categorical variables were compared by the χ^2 test and Fisher's exact test. Correlations between continuous variables were expressed by Pearson's correlation coefficent or Spearman's R test. Regressions were tested for equality among groups using Student's t-tests in an analysis of covariance. All tests were two-sided. Data are reported as means ± 1 standard deviation (SD). *p* values less than 0.05 were considered statistically significant.

Results

Prevalence and etiology of anemia in celiac disease

Our series of 132 consecutive patients diagnosed with celiac disease comprised 85 females and 47 males. The most common symptoms at presentation were diarrhea and weight loss (49 patients); 33 patients were asymptomatic and were diagnosed during endoscopic screening of first-degree relatives of patients with celiac disease. Forty-five patients had anemia, with a prevalence that was higher among females (41% in females *vs*. 21% in males, p=0.021). In ten patients anemia was the first manifestation of the disease and was the reason for performing an endoscopy. There were no differences between anemic and non-anemic patients in terms of age at diagnosis, C-reactive protein levels and histopathological findings, although there was a trend towards a higher percentage of patients with more severe histology in the anemic group (85% grade B2 disease in the anemic group versus 71% in the non-anemic group of patients). Serum albumin and BMI were lower in the anemic population (Table 1); the BMI difference, however, was due to the different gender distribution between groups. Seven patients had a β-thalassemia trait that did not influence the development of anemia.

Table 2 shows the hematologic characteristics of the 65 anemic patients. Fifty-two patients had mild anemia, and only three had severe anemia. Most cases of anemia were accounted for by isolated iron or vitamin deficiency (45 and 2 cases, respectively). In six other patients iron deficiency was associated with folate or vitamin B_{12} deficiency. The relevance of iron deficiency was confirmed by the 50% prevalence of microcytosis among anemic subjects and by the correlation between mean corpuscular volume and Hb concentration (r=0.304; p=0.021). For one patient it was not possible to determine the cause of anemia. Five patients had iron status parameters compatible with ACD and six had iron parameters that could be associated with a combination of iron-deficiency anemia and ACD. All these patients had C-reactive protein levels within the normal range. In the 11 subjects with ACD the mean Hb level was 9.9±1.7 g/dL, compared with 10.7±1.2

Table 1. General characteristics of celiac disease patients according to the presence or absence of anemia.

Parameter	*Anemic patients (n=45)*	*Non-anemic patients (n=87)*	*Significance (p)*
Age, years	34.3±12.2	35.5±14.2	n.s.
Males/females	10/35	37/50	0.021
Hemoglobin (g/dL)	10.6±1.3	14.7±1.3	0.001
Mean corpuscular volume (fL)	82.4±12.6	88.2±4.2	0.048
Albumin (g/dL)	3.6±0.7	4.3±0.5	0.001
Body mass index	20.4±2.9	22.2±4.3	0.019
Patients with B2 histological grade (%)	38 (84)	62 (71)	n.s.
C-reactive protein (mg/dL)	0.37±0.18	0.38±0.26	n.s.

Values are reported as mean ± 1 SD; n.s.: not significant.

Table 2. Factors related to erythropoiesis and iron status in the anemic and non-anemic patients with celiac disease.

Variables (n=65)	*Anemic patients patients (n=30)*	*Non-anemic values*	*Reference*
N. of patients with			
severe anemia	3		
moderate anemia	10		
mild anemia	52		
Transferrin saturation (%)[a]	11±9 (2-52)	21±9 (8-35)	16-54
Serum ferritin (mg/L)[a]	60±236 (2-1680)	76±130 (6-476)	Males: 15-250 Females: 10-150
Folic acid (nmol/L)	6.7±15.1 (2.2-114.9)	n.d.	4.5-45.0
Vitamin B_{12} (pmol/L)	423±333 (83-1999)	n.d	179-660
Serum Epo (mU/mL)[a]	35.1±51.1 (2.1-305.0)	12.2±6.8 (4.5-26.6)	5.0-25.0
sTfRc (mg/L)	1.80±0.84 (0.41-3.50)	1.37±0.50 (0.80-2.00)	0.83-1.76
HFE mutations (allele frequencies)[b]			
C282Y	0.015	0.023	0.018
H63D	0.146	0.172	0.150

Values are reported as mean ± 1 SD (range). In the case of serum ferritin and serum erythropoietin (Epo) the log transformations were used for comparisons. [a]differences were statistically significant. [b]HFE genotyping was extended to all of the 84 non-anemic patients with celiac disease. n.d.: not determined.

Table 3. Differentiation between celiac disease patients with anemia of chronic disease, with iron-deficiency anemia or without anemia.

Variables	*Anemia of chronic disease (n=11)*	*Iron-deficiency anemia (n=45)*	*Non-anemic patients (n=30)*
sTfRc/log(ferritin)[a]	0.56±0.34	2.93±1.99	1.20±0.65
Ferritin/transferrin[a]	1.874±4.808	0.027±0.028	0.347±0.596
Log(Epo) O/P ratio[b]	0.60±0.27	1.01±0.23	n.d.
Interferon-γ (pg/mL)[c]	41.9±27.3	10.72±23.2	3.3±3.9
Prohepcidin (ng/mL)	140.1±67.8	112.8±30.1	128.2±51.1

Values are reported as mean ± 1 SD. [a]p<0.01 when comparing ACD and iron-deficiency anemia; [b]p<0.001; [c]p<0.05 when comparing ACD and the other groups; n.d.= not determined.

g/dL in the anemic patients without ACD, but the difference was not statistically significant. BMI and serum albumin did not differ between the two groups. To confirm that the observed abnormalities in iron status parameters were caused by ACD, the sTfRc/log(ferritin) ratio and the ferritin/transferrin ratio,[16] which move in opposite directions in iron-deficiency anemia and ACD, were determined. According to both tests patients with an ACD component differed significantly from the other anemic patients, and in particular from those with iron-deficiency anemia (Table 3).

Mechanisms leading to iron deficiency and anemia of chronic disease in patients with celiac disease

Allele frequencies of the C282Y and H63D *HFE* mutations among the patients with celiac disease were 2% and 16%, respectively, which are similar to those previously reported in a control population from the same geographic area,[14] with no differences between anemic and non-anemic patients (Table 2). Similarly, there were no differences in serum prohepcidin levels between groups of patients with celiac disease (Table 3). Higher prohepcidin levels were, however, observed in subjects with active Crohn's disease (199.5±78.5 ng/mL, p=0.006), confirming that prohepcidin is increased in the presence of systemic inflammation (*unpublished data*).

The adequacy of serum erythropoietin levels in relation to the degree of anemia was evaluated by plotting log(Epo) *vs*. Hb in the patient and the reference groups and deriving the corresponding linear regressions (Figure 1). The resultant equations were log(Epo) = 4.478 – (0.284xHb) for the reference group (r=–0.827; p<0.001) and log(Epo)=2.269 – (0.091xHb) for the anemic patients with celiac disease (r=–0.288; p=0.022). Regressions in the two groups were different (p<0.001), but the difference was less evident when celiac disease patients with ACD were excluded from the analysis. The reference group equation was employed to derive the predicted log(Epo) value of the anemic patients with celiac disease and the observed/predicted log(Epo), or O/P ratio.[17] The O/P ratio was lower in

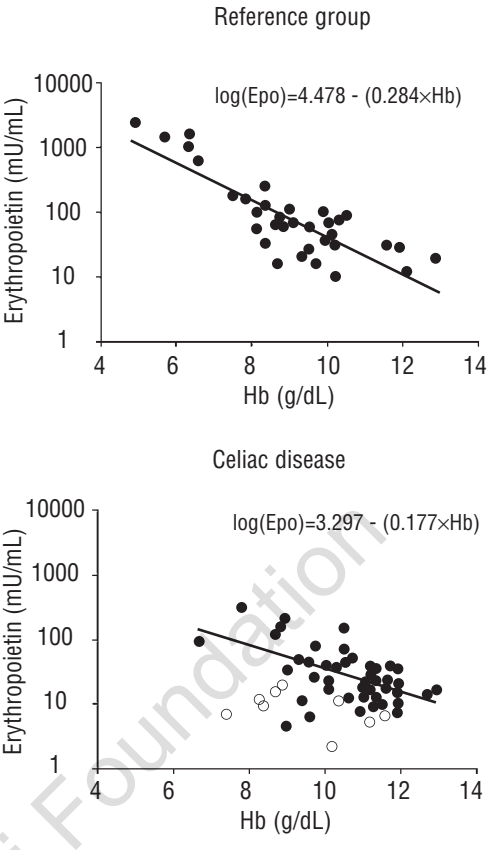

Figure 1. Regressions of serum erythropoietin (Epo) concentration to the level of hemoglobin (Hb) in anemic patients from the reference group (r=–0.827; p<0.001) and from the group with celiac disease. In the celiac disease panel empty circles correspond to patients with anemia of chronic disease, black circles to the remaining patients with anemia; the celiac disease regression line and equation (r=–0.542; p<0.001) refers only to the patients with anemia of chronic disease.

patients with ACD than in those without ACD (0.60±0.26 *vs.* 0.98±0.26, p<0.001), showing that in celiac disease with ACD the production of endogenous erythropoietin is inappropriately low for the degree of anemia (Table 3). No correlation was found between serum erythropoietin and prohepcidin levels.

The sTfRc is an index of erythropoietic activity.[18] In the anemic subjects from the reference group there was an inverse relationship between sTfRc and Hb (r=–0.590; p<0.001) and a direct correlation between sTfRc and log(Epo) (r=0.719; p<0.001), expression of the normal erythropoietic response to anemia. In celiac disease patients with anemia sTfRc levels and log(Epo) were significantly related (r=0.728; p<0.001), but the inverse relationship between sTfRc and Hb was present only when patients with ACD were not included in the analysis (r=–0.638; p<0.001). Predicted sTfRc levels, derived from the reference group regression equations, were not significantly different from the observed values, but ACD patients had a trend towards lower than expected sTfRc levels for the degree of anemia. These data suggest that in patients with anemia not related to ACD the erythroid response to anemia and the bone marrow response to erythropoietin are normal.

The Hb concentration was inversely related to the serum level of interferon-γ (r=–0.422, p=0.017), and the level of interferon-γ was higher in ACD patients than in non-anemic patients or those with iron-deficiency anemia (Table 3). The BMI and serum albumin concentration were not different in patients with ACD compared to in the other anemic subjects. The histopathological severity of the disease did not correlate with the pathogenesis of anemia (ACD versus non-ACD), with interferon-γ levels or with the response of anemia to a gluten-free diet. This may be due to the high prevalence of grade B2 disease at diagnosis.

For 53 anemic patients blood counts obtained after up to 1 year on a gluten-free diet were available; in most of them anemia either improved (8 patients) or was completely corrected (37 patients). The presence of ACD did not influence the response to the gluten-free diet. Patients with more severe degrees of anemia or who did not respond to the gluten-free diet were investigated for gastrointestinal occult blood loss or by colonoscopy with negative results.

Discussion

The results of our study confirm the high prevalence of anemia in untreated celiac disease (34%). Most cases of anemia (81%) were related to hematinic deficiencies (iron, folate, vitamin B_{12}) that occur as a consequence of intestinal malabsorption. The role of gastrointestinal blood loss in the pathogenesis of iron deficiency and anemia in celiac disease is still debated. Initial observations suggested that gastrointestinal blood loss occurs in up to 50% of patients with total villous atrophy,[4] but a subsequent study performed using a more specific method showed that bleeding is uncommon in celiac disease and is not a major contributor to iron deficiency in affected patients.[19] We did not systematically look for gastrointestinal blood loss in our patients, but those who were tested for blood in the feces gave negative results. Thus, although gastrointestinal bleeding may occur in some patients with celiac disease, we believe that its contribution to iron deficiency is limited. In our study *HFE* mutations associated with hereditary hemochromatosis did not influence the prevalence of anemia. Due to the relatively low number of patients we cannot rule out a protective effect of these mutations against iron deficiency, as was previously suggested,[20] although another group failed to find a role for *HFE* mutations in celiac disease.[21] We found a low number of patients with multiple nutrient deficiencies; perhaps additional cases of combined iron and folate deficiency might have been detected using a more sensitive red cell folate assay rather than serum folate measurements.

The main information provided by the present study is that ACD affects a significant portion of celiac disease patients at presentation, contributing to 11/65 (17%) cases of anemia in our series, sometimes in association with iron deficiency. Patients with ACD had iron status parameters similar to those usually found during inflammation, and their anemia could not be explained by isolated iron deficiency or other pathogenetic mechanisms. The assumption that they had ACD was strengthened by the observation that the ferritin/transferrin ratio was increased while the sTfRc/log(ferritin) ratio and the log(Epo) O/P ratio were low in these patients compared with the other anemic subjects and with the reference group. The reported changes in iron status parameters are typical of ACD, and are expressions of iron retention within macrophages.

The peptide hormone hepcidin is secreted by hepatocytes and regulates iron homeostasis. Hepcidin is overexpressed in ACD and causes hypoferremia during inflammation.[22] We used serum prohepcidin concentration as a surrogate measure of hepcidin concentration[16] in an attempt to clarify the mechanisms of ACD in celiac disease. Prohepcidin was not increased in patients with ACD. This may be due to the low number of patients with ACD and the wide standard deviation of prohepcidin values in our series, although it is possible that mechanisms distinct from hepcidin overexpression contribute to ACD in celiac disease. Alternatively, prohepcidin levels may not provide the same information as hepcidin levels with regards to the regulation of iron homeostasis.

A recent study suggested that the etiology of anemia in celiac disease is multifactorial, and ACD is relatively frequent;[23] in this study the diagnosis of ACD was based on serum ferritin concentrations above the 50th percentile expected for the age and gender of the patients, in the absence of folate or vitamin B_{12} deficiency. Although the study design and the diagnostic criteria for ACD were different, the prevalences of ACD in celiac disease were similar in our study and the one by Harper *et al.*[23] The finding that ACD can affect patients with celiac disease is not completely unexpected, although these patients usually lack signs of systemic inflammation. In fact, mean serum levels of inflammatory cytokines that contribute to ACD, including interleukin-1β, interleukin-6, tumor necrosis factor-α, and interferon-γ, are increased in active celiac disease.[24-27]

Our results hint at a defective production of endogenous erythropoietin, in addition to changes in iron homeostasis, as a pathogenetic mechanism of ACD. A defective production of erythropoietin for the degree of anemia has been reported in some studies on ACD,[28-30] although adequate erythropoietin levels were observed in ACD subjects with systemic juvenile chronic arthritis.[31] This could be due to differences in mechanisms and cytokines causing ACD. Interleukin-6 plays a major role in systemic juvenile chronic arthritis[32] and in many cases of ACD through its effects on iron homeostasis[22] and has been suggested to increase erythropoietin production.[33] In contrast, interferon-γ inhibits erythropoietin secretion *in vitro*,[34] and has suppressive effects on erythroid progenitors[9] and iron release from monocytes/macrophages.[10] In our patients serum levels of interferon-γ were inversely related to Hb concentration and were higher in ACD than in iron-deficiency anemia. In celiac disease interferon-γ is the dominant cytokine secreted by inflammatory cells infiltrating the intestinal mucosa upon exposure to gluten;[6-8] excessive

production of interferon-γ in response to gluten may represent the candidate mechanism responsible for the alterations in iron homeostasis and the blunted erythropoietin response to anemia that characterize ACD in patients with celiac disease. Although the erythroid response to erythropoietin, as evaluated by means of the sTfRc, was adequate in the present series of patients, no firm conclusions can be drawn about bone marrow behavior in celiac disease-associated ACD, since the low serum erythropoietin concentrations observed in these patients are associated with sTfRc in the low/normal range also in control subjects with normal bone marrow function. We did not address the influence of ACD on iron absorption. A previous study showed that the response of residual enterocytes to iron deficiency in celiac disease is characterized by an increased expression of proteins required for iron absorption, such as the iron carrier DMT1 and the iron exporter ferroportin.[35] Thus, iron malabsorption in celiac disease seems to be linked to loss of microvilli in the proximal duodenum, the main site of iron absorption, rather than to inhibition of iron transport at the cellular level. However, since tumor necrosis factor-α reduces iron transport in intestinal cell lines,[36,37] it is possible that a reduction in iron uptake capability by the enterocyte occurs in celiac disease patients with ACD.

According to the follow-up data, the response of anemic patients to a gluten-free diet was not influenced by the presence of ACD; this suggests that gluten-induced inflammatory responses in the intestinal mucosa have a primary role in determining the malabsorption of iron/vitamins and the dysregulation of iron homeostasis and ineffective erythropoietin production that, in some patients, lead to ACD.

Authorship and Disclosures

GB: designed the research, analyzed data and wrote the manuscript; KM: designed research and analyzed data; RA: performed biochemical analyses; AD: performed biochemical analyses and analyzed data; FB: designed the research and recruited patients; RC: recruited patients and participated in *HFE* genotyping; EA: performed most of the *HFE* genotyping; GRC: designed the research, analyzed data and wrote the manuscript. The authors reported no potential conflicts of interest.

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