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Fecal calprotectin as a biomarker of intestinal graft versus host disease after allogeneic hematopoietic stem cell transplantation

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The diagnosis of gastrointestinal graft versus host disease (GI-GVHD) is based on clinical symptoms and histological findings. In clinical practice, it is often difficult to decide whether abdominal symptoms in an allogeneic transplant recipient are caused by GVHD or other disorders. Endoscopic biopsies are helpful in establishing the diagnosis, but endoscopy is not always possible to perform due to poor general condition of the patients. No biomarkers are routinely used to predict GVHD. The aim of fecal calprotectin and alpha-1 antitrypsin testing in our study was to find out whether determination of the concentrations of these proteins may be used as a screening method for enteric GVHD. We studied prospectively 51 patients, 8 of whom developed GI-GVHD. Our data demonstrate that elevated fecal calprotectin levels were significantly associated with presence of GI-GVHD. We found a positive association between high F-calprotectin and severe gastrointestinal GVHD. In bivariate analysis, only calprotectin but not alpha-1 antitrypsin was independently associated with GI-GVHD. Testing for fecal calprotectin after allogeneic stem cell transplantation may be a useful screening tool.

Cytotoxic T cells emanating from the donor are capable of exerting an immunologic attack on malignant cells in leukemia patients who undergo allogeneic stem cell transplantation (ASCT)¹. The possibility of obtaining a graft versus leukemia effect is the most important reason for performing allogeneic transplantations in leukemia patients. However, the immunologic attack is not confined to malignant cells only, but normal cells and tissues are regularly affected by graft versus host disease (GVHD) that contributes significantly to morbidity and mortality in allogeneic transplant recipients, limiting the use of this important therapy². 40–50% of patients receiving allogeneic grafts suffer from gastro-intestinal GVHD (GI-GVHD)³. Symptoms of GVHD involving the GI tract are anorexia, nausea, vomiting, abdominal pain, and diarrhea. Gastrointestinal tract involvement is a severe form of GVHD and it is also difficult to treat. Intestinal bleeding carries a poor prognosis and occurs as a result of mucosal ulceration. Endoscopic findings in patients with GI-GVHD range from normal mucosa to extensive edema, mucosal sloughing, and diffuse bleeding⁴.

The diagnosis of GVHD is based on histological findings in biopsies. The histological hallmark of GI-GVHD is epithelial apoptotic cell death followed by loss of crypts^{5,6}. Endoscopic biopsies are helpful in establishing the diagnosis, but endoscopy is not always possible to perform due to poor general condition of the patients. In clinical practice, it is often difficult to decide whether abdominal symptoms in an allo-transplant patient are caused by GVHD or other disorders, such as infection by cytomegalovirus (CMV), Epstein-Barr virus (EBV), fungal infection, or mucosal damage due to conditioning therapy. Means for helping to decide whether a patient in this situation suffers from GVHD would be valuable, because an erroneous decision to treat a patient for GVHD may have fatal consequences and omission to treat an infection can have similar consequences.

Calprotectin is a protein that has antibacterial and antifungal activities and induces apoptosis in cell cultures. Excessive increment may, however, induce cell damage⁷. Calprotectin is found in macrophages and neutrophils⁸. The fecal content of calprotectin depends on migration of neutrophils into the intestinal lumen and has proven to

SUBJECT AREAS:
DIAGNOSTIC MARKERS
HAEMATOLOGICAL DISEASES

Received
22 May 2014

Accepted
5 December 2014

Published
21 January 2015

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be a sensitive marker of disease activity for inflammatory intestinal diseases such as Crohn's disease and ulcerative colitis⁹.

The plasma protein alpha-1 antitrypsin increases in inflammation and is likewise robust and protease resistant. The fecal content of alpha-1 antitrypsin can be used as a marker for loss of plasma proteins to the gastrointestinal lumen. Increased losses into feces can be caused by inflammatory diseases leading to enhanced vascular wall permeability, gut erosions causing loss of interstitial fluid, increased venous pressure, and lymphatic obstruction. Since alpha-1 antitrypsin is degraded at low pH in the stomach, only enteric losses below the stomach can be detected¹⁰.

The aim of fecal calprotectin and alpha-1 antitrypsin testing was to find out whether determination of the concentrations of these proteins may be used as a screening method for diagnosis of enteric GVHD.

Patients and Methods

We studied 51 patients who underwent allogeneic hematopoietic stem cell transplantation at the Department of Hematology, Umeå University Hospital, May 2007 through May 2011 for hematological disorders. The project was approved by the regional ethical committee in Umeå (dnr 07-014 M) and all patients gave their written informed consent to participate in the study. The methods used were carried out in accordance with approved guidelines and regulations.

Thirty-eight males and 13 females were included in the study. Their median age was 53 years, range 18–70. Demographic information is provided in Table 1. HLA-matching of donor and recipient showed that 40 were matched 10/10, six were 9/10, and five were 8/10 antigen matched.

Stool samples for fecal calprotectin and alpha-1 antitrypsin were collected before transplantation, weekly during hospitalization, three and six months after transplantation, and also when GI-GVHD was suspected on clinical grounds.

Measurements of fecal calprotectin and alpha-1 antitrypsin were performed at the Department of Clinical Chemistry, Umeå University Hospital. Calprotectin was analyzed with ELISA PhiCal reagent kit from Calpro AS, Norway. The upper limit of normal with this assay was 100 mg/kg. The content of alpha-1 antitrypsin in extracts of feces was determined with a turbidimetric method using antibodies from DAKO AS, Denmark. The normal reference interval used was < 1.5 g/kg.

Stool samples were tested for *Clostridium difficile* toxin, and blood cultures were tested for bacteria and fungi if the patients had any sign of infection. Blood samples were taken for PCR of CMV and EBV twice weekly.

Endoscopy was performed before transplantation and four weeks after transplantation if coagulation tests were normal and platelet counts were greater than 40×10^9 . Platelet transfusions were given before endoscopy if the platelet count was lower than 40×10^9 . Endoscopy was initially performed in all patients before conditioning, but from February 2010 only when patients had gastrointestinal symptoms of GVHD. Histological grading of GI-GVHD was performed according to Lerner; grade 1 GVHD; isolated apoptotic epithelial cells, without crypt loss, grade 2; loss of isolated crypts, without loss of contiguous crypts, grade 3; loss of two or more contiguous crypts, and grade 4; extensive crypt loss with mucosal denudation¹¹.

Differences in calprotectin and alpha-1 antitrypsin levels between groups were tested by the Wilcoxon rank-sum test or the Mann-Whitney test. Bivariate analysis was performed using logistic regression.

Acute GVHD was treated with methylprednisolone 1–2 mg/kg BID for at least 7 days.

Results

Out of the first fourteen asymptomatic patients who were examined with endoscopy and mucosal biopsies before transplantation, ten underwent sigmoidoscopy, one gastroscopy, and three both gastroscopy and sigmoidoscopy. Fecal calprotectin and alpha-1 antitrypsin were normal or slightly elevated in some patients before transplantation, 20–272 mg/kg, and 0.4–4.7 g/kg, respectively. There were no signs of GVHD, neither at endoscopy nor in the biopsies. When these results were obtained, the protocol was changed in February 2010 and endoscopy was thereafter performed only in patients with symptoms suggestive of GI-GVHD.

Eight of 51 patients (16%) were diagnosed clinically with GI-GVHD. Four patients had both diarrhea and abdominal pain and one of these four patients had also melena. Two other patients had only diarrhea, one patient had only nausea, and one patient had only rectal pain (Table 2). The severity of GI-GVHD, based on volume of diarrhea and abdominal pain, was stage 2 in one patient, stage 3 in one patient, and stage 4 in four patients. One patient had only rectal pain and one had only nausea so these patients could not be staged.

Table 1 | Patient characteristics

Characteristic	Number of patients (%)
Underlying disease	
AML	17 (33,3)
ALL	8 (15,7)
MDS	4 (7,8)
Refractory lymphoma	9 (17,6)
AA	1 (2)
CLL	3 (5,9)
CML	1 (2)
MF	6 (11,7)
Myelosarcoma	1 (2)
HLLH	1 (2)
Stem cell source	
peripheral blood	47 (92,2)
bone marrow	4 (7,8)
Conditioning regimen	
myeloablative	12 (23,5)
reduced intensity	39 (76,5)
HLA matching	
10/10	40 (78,5)
9/10	6 (11,7)
8/10	5 (9,8)
GVHD prophylaxis	
Antithymocyte globulin ATG	before SCT
Cyclosporine	2 mg/kg bw x2, IV, from day -1
Methotrexate	8 mg/m ² IV, day +2, +4, +8.

These two patients had calprotectin levels within the normal range. The other six patients with distinct clinical intestinal GVHD had elevated calprotectin and alpha-1 antitrypsin (Table 2).

Endoscopic and histological findings. None of the first 14 asymptomatic patients who were examined before transplantation had any endoscopic or histological evidence of GVHD changes. Eight of 51 patients developed clinical symptom of GI-GVHD. A summary of observations concerning these eight patients is presented in Table 1. Seven of these eight patients had endoscopic signs of GI-GVHD, but no endoscopic abnormalities were observed in one patient who was biopsy-positive for GVHD. The endoscopic appearance varied from subtle mucosal edema, hyperemia, erythema and erosion to ulceration and active bleeding. The clinical diagnosis was confirmed histologically by endoscopic biopsies in all eight patients; one gastric, six sigmoidal, and one rectal biopsy. The histological changes in GI-GVHD included apoptosis of crypt epithelial cells, dropout of crypts, and lymphocytic infiltration in epithelium and lamina propria.

Endoscopy of patient 1 with stage 4 acute GVHD showed normal conditions in the ventricle and colon. Histological examination of the ventricle, duodenum and colon biopsy specimens showed dropout of crypt epithelial cells, variable lymphocytic infiltration of the epithelium and lamina propria, especially in the duodenum and less pronounced in the antrum and colon. The changes were consistent with GVHD. Calprotectin was significantly elevated at 2345 mg/kg, but alpha-1 antitrypsin was normal. Serum PCR for CMV was positive.

In patient 2 with stage 4 acute GVHD, the upper endoscopic examination showed mucosal edema with erythema in the duodenum, but the appearance of the esophagus and ventricle was normal. Sigmoidoscopy showed localized erythema with a significant ulcer. Biopsies from the duodenum and sigmoidum disclosed lymphocytic and neutrophil infiltration, crypts with multiple apoptotic cells in duodenum, lymphocytic infiltration, and apoptosis of crypt cells and dropout of crypts in sigmoidum (GVHD). Gastric biopsy was normal. Calprotectin was elevated above 2500 mg/kg and alpha-1 antitrypsin was elevated at 5.9 g/kg. Serum PCR for CMV was negative.



Table 2 | Patients with GI-GVHD

Patient	nausea	vomiting	abdominal pain	diarrhea	melena	GVHD stage	calprotectin	α -1 AT	gastroscopy	sigmoideoscopy	colonoscopy	histology
1	YES	YES	YES	YES	NO	4	2345	1.5	Normal	Normal	Normal	GVHD vdc
2	YES	YES	YES	YES	NO	4	>2500	5.9	GVHD d	GVHD s	ND	GVHD ds
3	YES	NO	NO	NO	NO	NA	57	0.6	GVHD vd	ND	ND	GVHD vd
4	YES	YES	YES	YES	YES	4	2500	6.9	ND	GVHD s	ND	GVHD s
5	YES	YES	NO	YES	NO	2	204	9	GVHD v	GVHD s	GVHD c	GVHD cr
6	YES	YES	NO	YES	NO	3	731	2.2	GVHD v	GVHD s	ND	GVHD v
7	NO	NO	Rectal pain	NO	NO	NA	32	0.8	ND	GVHD s	ND	GVHD s
8	YES	YES	YES	YES	NO	4	968	15.8	Normal	GVHD s	ND	GVHD vds

Abbreviations: v-ventricle, d-duodenum, c-colon, s-sigmoideum, r-rectum, NA-not applicable, ND-not done, α -1 AT- α -1 antitrypsin.

In patient 3 with nausea only, gastroscopic examination disclosed shallow erosions in antrum and duodenum. Antrum and duodenum biopsies showed sporadic foci with necrotic areas indicating mild GVHD. Both calprotectin and alpha-1 antitrypsin were within the normal range. Serum PCR for CMV was positive.

Patient 4 with stage 4 acute GVHD had mucosal bleeding at sigmoideoscopy. The endoscopist found diffuse bleeding from the sigmoidal mucosa. Sigmoideum biopsies showed evidence of acute GVHD grade 3. Calprotectin was elevated at > 2500 mg/kg, alpha-1 antitrypsin was high at 6.9 g/kg. Serum PCR for CMV and EBV was positive and the patient also had *Escherichia coli* infection.

Patient 5 with stage 2 GVHD showed hemorrhagic spots in the ventricle and erythema and edema both in the ventricle and the colon at endoscopy.

Biopsies showed discrete apoptosis of crypt epithelial cells in colon and rectum but normal findings in gastric and duodenal biopsies. Calprotectin was marginally raised at 204 mg/kg. Alpha-1 antitrypsin was high at 9 g/kg.

Serum PCR for CMV was positive.

Patient 6 with stage 3 acute GVHD had erythema in the ventricle and sigmoideum at endoscopy. Biopsies showed apoptosis in the ventricle reflecting GVHD grade 1. Biopsies from the duodenum and sigmoideum were normal.

F-calprotectin was elevated at 731 mg/kg, alpha-1 antitrypsin was marginally elevated at 2.2 g/kg. Serum PCR for CMV was positive.

In patient 7 with rectal pain only, sigmoideoscopy disclosed erythema and signs of inflammation in the sigmoideum. Sigmoideum biopsies disclosed inflammatory infiltration in lamina propria and apoptosis of crypt cells compatible with GVHD grade I. Both calprotectin and alpha-1 antitrypsin were normal. Serum PCR for CMV was negative.

In patient 8 with stage 4 acute GVHD, endoscopy showed erythema and edema in the sigmoideum but normal mucosa in the ventricle. This patient was also suffering from hepatic GVHD. Gastric, duodenal and sigmoideum biopsy specimens showed apoptosis of crypt cells. There were signs of GVHD grade I in the ventricle and duodenum, grade II in sigmoideum. Calprotectin was elevated at 968 mg/kg and alpha-1 antitrypsin was elevated at 15.8 g/kg. PCR for CMV was positive and the patient also had *Enterococcus faecium* sepsis and positive *Aspergillus* antigen.

The maximal fecal calprotectin levels were markedly higher in the eight patients who developed GI-GVHD than in those who did not (median 850 mg/kg vs 119 mg/kg; $p = 0.016$; Figure 1). Four out of five patients with fecal calprotectin > 800 mg/kg developed GI-GVHD, but one did not. This patient had acute liver GVHD grade III and was simultaneously PCR positive for CMV. Two other patients with elevated calprotectin levels, 770 and 348 mg/kg, respectively, also had liver GVHD. None of the GVHD patients without liver-GVHD or GI-GVHD had calprotectin exceeding 200 mg/kg. Also, when only looking at the 25 patients who underwent endoscopy, the

difference in calprotectin between the eight patients with verified GI-GVHD and the other seventeen was significant ($p = 0.031$).

Maximal levels of alpha-1 antitrypsin showed a non-significant trend for increase in GI-GVHD patients ($p = 0.058$; Figure 1). When calprotectin and alpha-1 antitrypsin competed in bivariate analysis with respect to the occurrence of GI-GVHD, only calprotectin was independent ($p = 0.019$; $N = 46$).

Discussion

It is often difficult to distinguish between GI-GVHD and other common complications such as gastro-intestinal infections after allogeneic stem cell transplantation. However, correct diagnosis is extremely important because corticosteroid treatment may worsen infection, and omission to treat GVHD may also have fatal consequences. At present, endoscopy with biopsies remains the gold standard for the diagnosis of acute GI-GVHD. There is no specific non-invasive tool for the diagnosis of GVHD. Fecal calprotectin is used in clinical practice for estimation of disease activity of inflammatory bowel disease, but it is not routinely used for diagnostic purposes in transplant patients. There are a few recently published studies where calprotectin estimation was analysed in patients with GVHD. Bastos Oreiro et al found that calprotectin appears to be a promising non-invasive biomarker of acute GVHD¹². The level of calprotectin also correlated with the stage of GVHD. Calprotectin was low in patients without GI-GVHD and only slightly elevated in patients with CMV infection or in stage 1-2 GI-GVHD. Rodriguez-Otero et al found that fecal calprotectin and alpha-1 antitrypsin were higher in patients with severe GVHD (stage 2-3) but concluded that these proteins are no sensitive indicators of GVHD stage¹³. Calprotectin and alpha-1 antitrypsin may be used to predict the response to steroids¹³. Chiusolo et al reported that fecal calprotectin was higher in patients with acute or chronic GVHD with intestinal involvement than in non-GI-GVHD¹⁴. Calprotectin was lower in infective colitis compared with acute GI-GVHD¹⁴.

Montalto et al found that patients with chronic gastritis do not have increased fecal calprotectin¹⁵. The inflammatory process in gastritis may be less severe than in intestinal bowel disorders, or degradation of calprotectin may occur in the upper GI tract. Sipponen et al¹⁶ reported that patients with ileal Crohn's disease had lower fecal calprotectin compared to subjects with colonic involvement. This finding is in agreement with the observation in the present study that one patient who had gastric GVHD and one patient with rectal pain only and confirmed GVHD in the sigmoideum had normal fecal calprotectin levels.

Our data demonstrate that asymptomatic patients who were examined before transplantation did not have macroscopic changes at endoscopy nor histopathological changes in mucosal biopsies, so our protocol was changed and endoscopy was then performed only in patients with gastrointestinal symptoms. Endoscopic findings varied markedly among patients. Disparity between endoscopic and

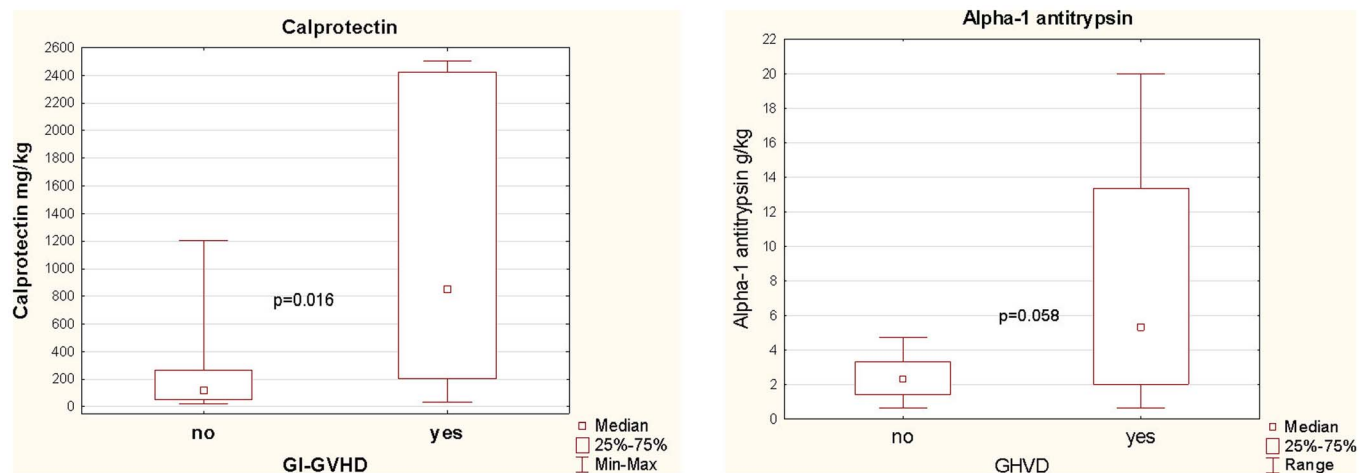


Figure 1 | Calprotectin was significantly higher in patients with GI-GVHD than in patients without GI-GVHD ($p = 0.016$). Alpha-1 antitrypsin was also higher among patients with GI-GVHD ($p = 0.058$).

histological assessment of GVHD grade was seen in one case. This patient had clinical signs of GI-GVHD with high calprotectin but no endoscopic signs of GVHD, although biopsies verified GI-GVHD.

We found a significant association between high F-calprotectin and severe gastrointestinal GVHD, although calprotectin was elevated also in patients with liver GVHD. Patients without any GVHD symptoms had normal to slightly raised calprotectin and alpha-1 antitrypsin, with the exception of one patient with high fecal calprotectin who did not develop GI-GVHD. In analysis, calprotectin but not alpha-1 antitrypsin was associated with GI-GVHD. We need larger studies to verify the value of testing for fecal calprotectin after allogeneic stem cell transplantation as a screening tool for identifying those patients who are most likely to benefit from endoscopy and biopsy for diagnosis of GI-GVHD.

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Acknowledgments

This study was supported by grants (ALF) from the Department of Internal Medicine and the Cancer Centre, University Hospital of Umeå.

Author contributions

F.L. and A.W. contributed to the design of the study and wrote the manuscript text. F.L. prepared Table 1. M.W. performed scopies. R.P. performed histological assessment. B.E.W. did the statistical analysis. S.M. was responsible for assessment of calprotectin and alpha-1 antitrypsin. All authors reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Lorenz, F. *et al.* Fecal calprotectin as a biomarker of intestinal graft versus host disease after allogeneic hematopoietic stem cell transplantation. *Sci. Rep.* **5**, 7920; DOI:10.1038/srep07920 (2015).



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