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Defective spleen function in autoimmune gastrointestinal disorders

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Corresponding Author:	Antonio Di Sabatino First Department of Internal Medicine, San Matteo Hospital Foundation, University of Pavia ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	First Department of Internal Medicine, San Matteo Hospital Foundation, University of Pavia
Corresponding Author's Secondary Institution:	
First Author Secondary Information:	
First Author:	Paolo Giuffrida
Order of Authors:	Paolo Giuffrida
	Nicola Aronico
	Matteo Rosselli
	Marco Vincenzo Lenti
	Sara Cococcia
	Davide Roccarina
	Francesca Saffioti
	Mariangela Delliponti
	Douglas Thorburn
	Emanuela Miceli
	Gino Roberto Corazza
	Massimo Pinzani
	Antonio Di Sabatino
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	Defective spleen function increases susceptibility to bacterial infections which can be prevented by vaccine prophylaxis. Splenic hypofunction can be found in a number of autoimmune disorders, however no data are available regarding autoimmune atrophic gastritis (AAG), autoimmune enteropathy (AIE) and autoimmune liver disease (AILD). Peripheral blood samples from patients with AAG (n=40), AIE (n=3) and AILD (n=40) were collected. Patients affected by autoimmune disorders already known to be associated with splenic hypofunction, i.e. coeliac disease (CD) and ulcerative colitis (UC), were included as disease controls, while splenectomised patients and healthy subjects were evaluated as positive and negative controls, respectively. Counting of

	erythrocytes with membrane abnormalities, i.e. pitted red cells, was used as an indicator of spleen function (normal upper limit 4%). Defective splenic function was observed in 22 of the 40 patients with AAG (55.0%), in two of the three patients with AIE (66.6%) and in 35 of the 40 patients with AILD (87.5%). As expected, in untreated CD, refractory CD and UC there was a high prevalence of hyposplenism (43.7%, 88.2%, and 54.4%, respectively). Due to the high prevalence of splenic hypofunction, patients with AAG, AILD and AIE should undergo pitted red cell evaluation and, if hyposplenic, they should be candidate to vaccine prophylaxis against encapsulated bacteria.
Response to Reviewers:	We thank the Editor for the opportunity to present a revised manuscript. We found all the comments very helpful and we believe that the manuscript has been further strengthened. Please find attached a revised version of our manuscript entitled "Defective spleen function in autoimmune gastrointestinal disorders" (IAEM-D-19-00287), that we would like to resubmit to be reviewed for possible publication in Internal and Emergency Medicine as an original article. COMMENTS FOR THE AUTHOR: Reviewer #1: Referee comments to the Authors The paper of the group of prof Di Sabatino aimed to search for splenic hypofunction in three different autoimmune disorders, atrophic gastritis (AAG), autoimmune enteropathy (AIE) and autoimmune liver disease (AILD); this topic has never previously investigated from this point of view, on my knowledge. The Authors found a quite high frequency of erythrocytes with membrane abnormalities (pitted red cells) in all the three autoimmune conditions: 55% in AAG, 66% in AIE and 87% in AILD. GENERAL COMMENT The data are original and interesting. The methodology sounds and the paper is well written. I have some suggestions which I hope could improve the paper. We thank the Reviewer for his/her helpful comments and for his/her appreciation of the study. We have attempted to answer all the points raised as detailed below. SPECIFIC POINTS ° Figure 1 is the main information/result of the research and, obviously, the most interesting results regard the three autoimmune conditions studied. I suggest that the numerous study groups make this figure and the comparison difficult to read. On my opinion, the data of autoimmune enteropathy can be excluded from the figure and given in the text. Similarly, the control groups are too numerous: I would limit the analysis to HC, Untreated Celiac patients and splenectomised, making a total of 6 groups. As suggested, we have now modified Figure 1 (new Figure 1). ° The four tables are detailed but do not add data which are relevant in the result section, I would suggest to take off them and to add some data to the Patients section. As suggested, we have now taken off the four Tables and added some data to the Patients section (page 4, line 21). ° Please include in the Discussion a comment about the lack of data about sepsis in the autoimmune conditions included in this study. We thank the Reviewer for raising this point. We have added a comment in the Discussion section (page 9, line 18). MINOR points Methods line 29: It should be specified that CD group comprised "Untreated CD patients" As suggested, we have now specified that.

Defective spleen function in autoimmune gastrointestinal disorders

Running head: Spleen hypofunction in autoimmune gastrointestinal diseases

Paolo Giuffrida[1], Nicola Aronico[1], Matteo Rosselli[2], Marco Vincenzo Lenti[1], Sara Cococcia[1], Davide Roccarina[2], Francesca Saffioti[2], Mariangela Delliponti[1], Douglas Thorburn[2], Emanuela Miceli[1], Gino Roberto Corazza[1], Massimo Pinzani[2], Antonio Di Sabatino[1]

From: [1]First Department of Internal Medicine, San Matteo Hospital Foundation, University of Pavia, Pavia, Italy; [2]Institute for Liver and Digestive Health, University College London, London UK.

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Abbreviations: AAG, autoimmune atrophic gastritis; AIE, autoimmune enteropathy; AIH, autoimmune hepatitis; AILD, autoimmune liver disease; CD, coeliac disease; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; RCD, refractory coeliac disease; UC, ulcerative colitis.

Keywords: autoimmune atrophic gastritis; autoimmune enteropathy; autoimmune liver disease; coeliac disease; pitted red cell; ulcerative colitis.

Address for correspondence: Prof. Antonio Di Sabatino, Clinica Medica I, Fondazione IRCCS Policlinico San Matteo, Università di Pavia, Piazzale Golgi 19, 27100 Pavia, Italy. Phone: +39 0382 502973; Fax: +39 0382 502618; E-mail: a.disabatino@smatteo.pv.it

Abstract

Defective spleen function increases susceptibility to bacterial infections which can be prevented by vaccine prophylaxis. Splenic hypofunction can be found in a number of autoimmune disorders, however no data are available regarding autoimmune atrophic gastritis (AAG), autoimmune enteropathy (AIE) and autoimmune liver disease (AILD). Peripheral blood samples from patients with AAG (n=40), AIE (n=3) and AILD (n=40) were collected. Patients affected by autoimmune disorders already known to be associated with splenic hypofunction, i.e. coeliac disease (CD) and ulcerative colitis (UC), were included as disease controls, while splenectomised patients and healthy subjects were evaluated as positive and negative controls, respectively. Counting of erythrocytes with membrane abnormalities, i.e. pitted red cells, was used as an indicator of spleen function (normal upper limit 4%). Defective splenic function was observed in 22 of the 40 patients with AAG (55.0%), in two of the three patients with AIE (66.6%) and in 35 of the 40 patients with AILD (87.5%). As expected, in untreated CD, refractory CD and UC there was a high prevalence of hyposplenism (43.7%, 88.2%, and 54.4%, respectively). Due to the high prevalence of splenic hypofunction, patients with AAG, AILD and AIE should undergo pitted red cell evaluation and, if hyposplenic, they should be candidate to vaccine prophylaxis against encapsulated bacteria.

Introduction

Defective spleen function is an acquired condition characterised by the impairment of the reticuloendothelial and immune functions of the spleen. As a consequence, individuals suffering from spleen hypofunction may develop life-threatening infections [1]. Defective spleen function has already been reported in a number of haematological, immune-mediated, infectious and gastrointestinal disorders, including coeliac disease (CD), inflammatory bowel disease and primary eosinophilic disorders [2-5]. On the contrary, spleen function has never been systematically assessed in other immune-mediated gastrointestinal conditions, including autoimmune atrophic gastritis (AAG), autoimmune enteropathy (AIE), and autoimmune liver disease (AILD). In CD, the prevalence of defective spleen function is particularly increased in patients with concomitant autoimmune disorders and in refractory CD (RCD) [6]. Patients with CD have a higher risk of overwhelming septicaemia sustained by encapsulated bacteria [7,8], and this susceptibility has been related to the depletion of IgM memory B cells which derive from the marginal zone of the spleen and are known to be crucial in opsonising encapsulated bacteria [9]. The impairment of spleen function may be reversible, as in the case of CD after gluten-free diet [2], or Crohn's disease after anti-TNF-α therapy with infliximab [10], or eosinophilic gastrointestinal disorders after corticosteroid treatment [5], and it is not necessarily associated with reduced spleen size. Conversely, the irreversible impairment of spleen function, mostly observed in RCD and its complications, i.e. ulcerative jejunoileitis, enteropathy-associated T-cell lymphoma, is often linked to splenic atrophy [6]. Recent studies [7,8] showing the significantly higher risk for pneumococcal sepsis in large cohorts of CD patients highlighted the importance of prevent overwhelming post-splenectomy infections in hyposplenic patients through vaccine prophylaxis [3].

On this basis, we aimed to investigate spleen function by counting pitted red cells in a series

of consecutively enrolled patients with AAG, AIE and AILD, including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), in comparison to patients with uncomplicated CD, RCD and ulcerative colitis (UC).

Methods

Patients. Patients were prospectively enrolled at the First Department of Internal Medicine of San Matteo Hospital Foundation (Pavia, Italy) or at the Institute for Liver and Digestive Health of University College London (London, UK). Peripheral blood samples were obtained from 40 patients affected by AAG (median age 66 years, range 31-85), three adult patients with AIE (median age 52 years, range 42-65) and 40 patients with AILD (median age 51 years, range 22-89), i.e. AIH (n=12), PBC (n=8), and PSC (n=20). As positive controls, 32 patients with uncomplicated CD (median age 34 years, range 19-58), 17 patients with RCD (median age 54 years, range 34-73), and 22 patients with UC (median age 37.5 years, range 18-60) were also included. Twenty-four healthy volunteers (median age 39.5 years, range 24-63) and 20 splenectomised patients (median age 38 years, range 22-72) were also studied as negative and positive reference groups, respectively. All diagnoses were established and reviewed according to internationally agreed criteria. In particular, diagnosis of AAG was based on both the updated Sydney-Houston criteria and the anti-parietal cell antibody positivity [11,12]. Amongst patients with AAG, nine patients (22.5%) had mild-to-moderate atrophy and 31 (77.5%) severe atrophy. The average serum chromogranin A (normal values = 19.4–98.0 ng/mL) was 173.2 ng/mL (range 8.0-1429.0 ng/mL) in patients with AAG. Diagnosis of AIE was based on villous atrophy at duodenal biopsies nonresponsive to any dietary exclusion, positivity of serum anti-enterocyte antibodies and/or associated autoimmune conditions, and lack of significant immunodeficiency [12]. Diagnoses of AIH, PBC, and PSC were based according to the

European Association for the Study of the Liver guidelines [13-15]. In all patients with AILD the average model for end-stage liver disease score, alanine aminotransferase (normal values = 11–34 U/L), aspartate aminotransferase (normal values = 11–39 U/L) and circulating platelet counts (normal values = 150–450 x $10^3/\mu L$) were 7.3 (range 6.0-14.4), 58.7 U/L (range 10.0-308.0 U/L), 47.5 U/L (range 16.0-140.0 U/L) and 253.2 x $10^3/\mu L$ (range 44.0-453.0 x $10^3/\mu L$), respectively. Diagnosis of uncomplicated CD was based on the positivity of serum anti-endomysial IgA and anti-tissue-transglutaminase IgA antibody, associated with typical histopathological lesions, namely villous atrophy, increased intraepithelial lymphocytes, and crypt hyperplasia [16]. Diagnosis of RCD was made in CD patients with persistent symptoms and villous atrophy despite a long-term gluten-free diet [16]. According to the classification of Rubio-Tapia and Murray [17], patients with RCD were subdivided in type 1 (n = 8) and type 2 (n = 9). The average aberrant intraepithelial lymphocytes were 41.9% (range 1.0-89.0%) in patients with RCD. Diagnosis of UC was established by clinical, laboratory, imaging, endoscopic and histological criteria [18]. Amongst patients with UC, ten patients (45.5%) had pancolitis, nine (40.9%) left-sided colitis and three (13.6%) proctitis.In UC patients, disease activity was assessed according to the Clinical Activity Index of Rachmilewitz [19]. Clinical remission was defined as a score below 4. In patients with UC the average C-reactive protein (normal values < 0.5 mg/dL) and faecal calprotectin (normal values < 50 mg/kg) were 1.0 mg/dL (range 0.1-7.3 mg/dL) and 177.9 mg/kg (range 18.0-866.0 mg/kg), respectively. Each patient provided informed consent to the study prior to blood draw. The study protocol conforms to the ethical guidelines of the Declaration of Helsinki.

Evaluation of splenic function. Splenic function was assessed by counting pitted red cells (i.e., red cells with membrane abnormalities visible under interference phase

microscopy as so-called *pits*), which is the most accurate and reproducible test for this purpose [1]. Briefly, a drop of fresh venous blood was mixed with 1.5 mL 3% buffered-gluteraldehyde solution, pH 7.4. One thousand red cells were examined in a wet preparation (magnification, 1000X) with a direct-interference contrast microscope (Leitz Dialux 20; Lietz, Cape Coral, FL) equipped with Nomarsky optics by an independent observer who was unaware of patients' diagnoses. The percentage of pitted red cells was calculated and taken as a measure of splenic function (upper limit of normal = 4%).

Statistical analysis. Data were analysed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) by the nonparametric Mann-Whitney *U* test. Correlations were studied by the Spearman rank correlation test. Differences in frequencies were tested using the Fisher exact test. For all statistical analyses a level of $p<0.05$ was considered as statistically significant.

Results

We evaluated splenic function by counting the proportion of pitted red cells in a peripheral blood smear. As shown in Figure 1, the median percentage of circulating pitted red cells was significantly increased in AAG patients (median 4.7%, range 0.8–13.8%; $p<0.0001$), and in AILD patients (median 7.2%, range 2.6–23.2%; $p<0.0001$) in comparison to healthy volunteers (median 1.9%, range 0.6–4.0%). In the three patients with AIE the median percentage of pitted red cells was 9.0 (range 3.8–17.0%). As expected, the median percentage of circulating pitted red cells was significantly ($p<0.0001$) higher in uncomplicated CD (median 3.7%, range 1.8–12.0%), RCD (median 8.8%, range 3.4–25.8%) and UC (median 4.9%, range 1.0–15.0%) than in healthy volunteers. In addition, the median percentage of circulating pitted red cells was significantly ($p<0.05$) higher in patients with

AILD than in those with AAG, uncomplicated CD and UC. The median percentage of circulating pitted red cells was also significantly ($p<0.01$) higher in patients with RCD than in those with AAG, uncomplicated CD and UC. No significant difference was found between AIH (median 7.9%, range 2.8–14.8%), PBC (median 6.3%, range 3.2–10.6%) and PSC (median 7.1%, range 2.6–23.2%). The median percentage of circulating pitted red cells was higher in patients with RCD type 2 (median 11.0%, range 7.0–25.8%) than in those with RCD type 1 (median 6.0%, range 3.4–18.0%), though at limit of statistical significance ($p=0.0519$). As expected, the median percentage of circulating pitted red cells was significantly ($p<0.01$) higher in splenectomised patients (median 21.1%, range 14.8–27.4%) compared to all the other groups.

The prevalence of spleen hypofunction (pitted red cells > 4%) was significantly ($p<0.0001$) higher in all groups in comparison to healthy volunteers, namely AAG (55.0%), AIE (66.6%), AILD (87.5%) uncomplicated CD (43.7%), RCD (88.2%) and UC (54.4%). Of note, AILD had a significantly ($p<0.01$) higher prevalence of spleen hypofunction than AAG, uncomplicated CD and UC. Likewise, RCD had a significantly ($p<0.05$) higher prevalence of spleen hypofunction than AAG, uncomplicated CD and UC.

In patients with AAG no significant correlation was identified between circulating pitted red cells and age at diagnosis, circulating platelet count, serum 17-gastrin, or serum chromogranin A. The median percentage of circulating pitted red cells was higher in AAG patients with severe atrophy (median 5.0%, range 1.8–13.8%) than in those with mild-to-moderate atrophy (median 3.6%, range 0.8–11.6%) though at the limit of statistical significance ($p=0.0579$). No significant difference was found between AAG patients with increased serum chromogranin A (median 4.7%, range 1.8–13.8%) and those with normal serum chromogranin A (median 4.2%, range 2.2–9.6%). We found a significant positive

correlation (r_s=0.5534, p=0.0230) between circulating pitted red cells and aberrant intraepithelial lymphocytes in patients with RCD. No significant differences were found in UC patients as regards disease activity (increased C-reactive protein and faecal calprotectin *vs* no increase) and disease extension (pancolitis *vs* left-sided colitis or proctitis). In patients with AILD no significant correlation was found between circulating pitted red cells and circulating platelet counts or liver function tests, while a significant positive correlation (r_s=0.34, p=0.028) was found between PRC and model for end-stage liver disease score.

Discussion

We here showed a significant association between defective spleen function and three autoimmune diseases of the gastrointestinal tract, i.e. AAG, AIE and AILD, the latter showing the highest prevalence of spleen hypofunction. In particular, in patients with AILD, spleen function impairment correlated with the degree of liver disease severity as shown by the significant correlation with model for end-stage liver disease score. Our findings are in keeping with previous papers [20,21], showing defective splenic function through high pitted red cell count and reduced tuftsin activity in patients affected by liver cirrhosis of different aetiologies, including PBC. The correlation between pitted red cells and model for end-stage liver disease in our study is in line with the correlation between tuftsin activity and Child-Pugh score previously described [21]. An association between the degree of spleen function impairment and the disease severity was also observed in patients with AAG, although in this disorder the overall degree of spleen function impairment was quite mild. As regards AIE, the low sample size of patients due to rarity of this disorder did not allow us to draw a firm conclusion, although the degree of hyposplenism seems to be quite high in these patients.

The increased prevalence of spleen hypofunction in RCD in comparison to uncomplicated CD is in agreement with our previous data [6]. In this study we have further shown a significant positive correlation between circulating pitted red cells and aberrant intraepithelial lymphocytes in RCD. Thus, the degree of hyposplenism seems to be more severe in RCD type 2 than in RCD type 1. The prevalence of hyposplenism in UC patients in this study (54.4%) is in line with that demonstrated previously (44.8%) [4]. Circulating pitted red cells did not correlate with levels of disease activity biomarkers in UC, such as C-reactive protein, faecal calprotectin, and Clinical Activity Index. This is in agreement with the lack of correlation between disease activity in inflammatory bowel disease and circulating IgM memory B cells [4], which require the spleen for their survival and/or generation [22]. In addition, circulating pitted red cells did not differ in UC patients according to different disease extension, pancolitis *versus* left-sided colitis or proctitis.

There are some limitations of the present study that should be mentioned. First, no data are available regarding the actual incidence of defective spleen function-related complications in patients with AAG, AIE and AILD. To the best of our knowledge neither sepsis nor overwhelming post-splenectomy infections have been reported so far in large cohorts of patients with AAG, AIE and AILD. Furthermore, the relatively small sample size has limited the strength of our findings. Nonetheless, this is the first systematic description of the prevalence of defective spleen function in AAG, AIE and AILD patients. Despite the evidence of an increased risk of infectious complications in hyposplenic and asplenic patients, especially in those unvaccinated, a proper management and prompt recognition are still awaited. The results of a large, nationwide, “spleen registry” set up in Australia and including asplenic and hyposplenic individuals have recently been published, showing a significant reduction of the infectious risk in registered patients, and highlighting the need for proper patient and physician education [23]. As this regards, a case finding strategy is crucial in

high risk groups, such as patients affected by AAG, AIE and AILD, through the assessment of pitted red cells, a rather inexpensive and quick test, albeit operator-dependent [24].

In conclusion, further studies are required to assess the pathophysiological relationship between spleen, gut and liver in hyposplenic patients with autoimmune gastrointestinal and liver disorders and the long-term impact of defective spleen function. The clinical relevance of our findings is the recommendation of an appropriate prophylactic management of selected hyposplenic patients, through anti-pneumococcal, anti-meningococcal, and anti-*Haemophilus influenzae* type B vaccines, due to the increased risk of overwhelming septicemia sustained by encapsulated bacteria.

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Conflicts of interest: None to declare.

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Figure legend

Figure 1. Percentages of circulating pitted red cells in 40 patients with autoimmune atrophic gastritis (AAG), 40 patients with autoimmune liver disease (AILD), 32 patients with uncomplicated celiac disease (UCD), 20 patients who had a splenectomy (SP) and 24 healthy volunteers (HV). ●, patients with primary biliary cholangitis; ●, patients with primary sclerosing cholangitis. The dashed line indicates the upper limit of the control range (4%). Horizontal bars represent median values. *p<0.005 *versus* AAG and UCD; **p<0.0001 *versus* AAG, AILD, UCD and HV; ***p<0.0001 *versus* AAG, AILD and UCD.

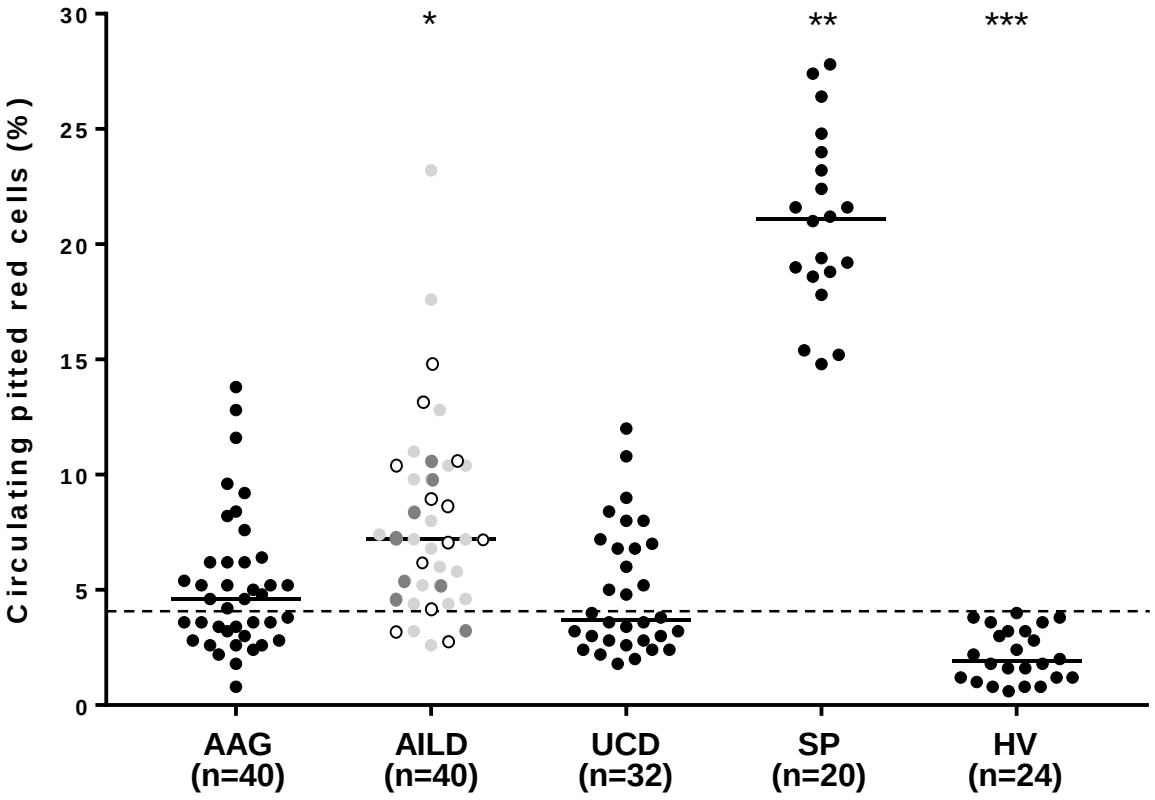
Circulating pitted red cells (%)
30
25
20
15
10
5
0
*
**
***
AAG
(n=40)
AILD
(n=40)
UCD
(n=32)
SP
(n=20)
HV
(n=24)

Figure 1