

Original Paper

Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease

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Abstract

Phagocyte-derived S100 proteins are endogenous activators of innate immune responses. S100A12 binds to the receptor for advanced glycation end-products, while complexes of S100A8/S100A9 (myeloid-related proteins, MRP8/14; calprotectin) are ligands of toll-like receptor 4. These S100 proteins can be detected in stool. In the present study we analyse the release of S100A12 and MRP8/14 from intestinal tissue. Specimens from patients with Crohn's disease (CD; $n = 30$), ulcerative colitis (UC; $n = 30$), irritable bowel syndrome (IBS; $n = 30$) or without inflammation ($n = 30$) were obtained during endoscopy. After 24 h culture, S100A12 and MRP8/14 were analysed in supernatants. Endoscopic, histological, laboratory and clinical disease activity measures were documented. We found an increased spontaneous release of S100A12 from tissue in inflammatory bowel disease (IBD). The release of S100A12 into the supernatants was 28-fold enhanced in inflamed tissue when compared to non-inflamed tissue (mean 46.9 vs. 1.7 ng/ml, $p < 0.0001$). In active CD, release of S100A12 and MRP8/14 was strongly dependent on localization, with little release from sites of active ileal inflammation compared to colonic inflammation. This difference was more pronounced for S100A12 than for MRP8/14. S100A12 and MRP8/14 provoked up-regulation of adhesion molecules and chemokines on human intestinal microvascular endothelial cells (HIMECs) isolated from normal colonic tissue. The direct release of phagocyte-derived S100 proteins from inflamed tissues may reflect secretion from infiltrating neutrophils (S100A12) and also monocytes or epithelial cells (MRP8/14). Via activation of pattern recognition receptors, these proteins promote inflammation in intestinal tissue. The enhanced mucosal release can explain the correlation of fecal markers with disease activity in IBD.

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Introduction

Both Crohn's disease (CD) and ulcerative colitis (UC) are the consequence of chronic inflammatory reactions in the gastrointestinal tissue and are collectively defined as inflammatory bowel disease (IBD). IBD results from a disturbed interplay of the environment — with various exogenous factors, such as nutrition — and the intestinal microflora with the immune system of a genetically susceptible host. The resulting inflammatory processes involve both adaptive and innate immune mechanisms, such as the activation of

neutrophils, production of pro-inflammatory mediators and, finally, tissue damage [1–3]. In recent years the role of innate immunity in IBD has been emphasized, as an example of the association of CD with abnormalities in pattern recognition receptors (PRRs) such as NOD2, which play a key role in innate host defence [4]. Therapies targeting cytokines mainly produced by phagocytes improve the clinical course of IBD. However, there is a growing body of evidence that factors other than classical cytokines contribute to the activation of innate immune responses, including responses towards self molecules. Recently, a novel group of

important pro-inflammatory molecules has been introduced to the concept of innate immunity. In parallel to pathogen-associated molecular patterns (PAMPs) as exogenous factors initiating inflammation, the term 'damage associated molecular pattern (DAMP) proteins', for endogenous molecules that are released by activated or damaged cells under conditions of cell stress, has been established. Examples of this group are phagocytic S100 proteins, which mediate inflammatory responses via interaction with PRRs [5,6].

DAMP molecules accumulating in the intestinal mucosa represent excellent markers for mucosal inflammation. These proteins may also spill over into the gut lumen, and the additional release by inflammatory cells in crypt abscesses results in significant amounts that mix with other secretions and gut contents, which ultimately appear in the patients' stool. Therefore, assays which determine intestinal inflammation by detecting neutrophil-derived DAMPs in stool bear a great diagnostic potential [7].

Among the factors released by infiltrating neutrophils are DAMP proteins of the S100 family [8]. One example is a complex of two distinct S100 proteins, S100A8 (myeloid-related protein 8, MRP8) and S100A9 (MRP14) [9–11]. In recent years, the MRP8/14 complex has been proposed as a fecal marker of gut inflammation, termed 'fecal calprotectin' [7,12–18]. MRP8/14 is found in large amounts in granulocytes as well as monocytes but it is also inducible in epithelial cells, e.g. in lactose intolerance [13,14,19,20]. S100A12, as a marker of neutrophil activation, is more restricted to granulocytes and is released during inflammatory conditions at the site of inflammation and abundant in the intestinal mucosa of patients with IBD [8,21,22]. Over-expression at the site of inflammation and correlation with disease activity in a variety of inflammatory disorders underscore the role of this granulocytic protein as pro-inflammatory molecule [23]. The phagocyte specific S100 proteins represent classical DAMPs which promote inflammation by specific interactions with pattern recognition receptors, MRP8/14 via activation of the Toll-like receptor 4 (TLR4) complex and S100A12 via binding to the receptor for advanced glycation end products (RAGE) [24,25]. Data on murine models of colitis as well as human IBD point to an important role of these DAMPs during the pathogenesis of IBD [8,25,26].

In previous studies, we have already demonstrated that S100A12 is over-expressed during chronic active IBD and serves as a useful serum and stool marker of inflammation [8,22]. In the present study we investigated whether the release of phagocyte-related S100 proteins from intestinal mucosal specimens correlates to the intestinal inflammation status, which would be relevant for the diagnostic usefulness of these proteins as fecal biomarkers. We investigated S100A12 and MRP8/14 concentrations in supernatants of intestinal tissue obtained at endoscopies from adult patients with active IBD and compared them to non-inflamed

tissue specimens. The biological activity of these DAMP molecules on primary intestinal microvascular endothelial cells was investigated to confirm the relevance of their local release for the induction of inflammatory responses in bowel tissue.

Methods

Patients and controls

We included patients with CD and UC into the IBD group. The non-inflamed group consisted of patients with irritable bowel syndrome (IBS) and individuals without inflammation. Subjects in screening programmes for early detection of polyps and colon cancer and having normal colonoscopic finding were chosen for normal controls. Random colonic biopsies were collected in healthy subjects, and histological examination was normal in all these individuals. All patients underwent endoscopic examination, and biopsies were taken for histological investigations at all colonoscopies. Each patient gave signed informed consent. The study was approved by the Centre for Digestive Diseases Human Research Ethics Committee, Sydney, and Human Research Ethics Committee, University of Newcastle, Australia. All patients with IBS fulfilled the ROME II criteria [27]. Endoscopic, histological and clinical disease activity measures were documented.

Determination of disease activity

To define inflammatory activity in the gut, macroscopic signs of inflammation were recorded during colonoscopy by the experienced gastroenterologist, using the simple endoscopic score for CD (SES-CD) or a similar index for UC [28,29]. In addition, biopsies were taken from multiple sites, including the terminal ileum. Biopsy sections were encoded and analysed by independent observers who were blinded to diagnosis and clinical data. As a further inclusion criterion, the histology score had to be in accordance with endoscopic signs of inflammation. Endoscopic and histological appearance was used to categorize patients into groups of disease severity (0 = no inflammation, 1 = mild inflammation; 2 = moderate to severe inflammation), as previously described [22]. This inflammation score served as the reference standard to determine absence of inflammation or active IBD in our study cohort. Levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood count (WBC), haemoglobin (Hb), platelets (Plt) and haematocrit (Hct) were determined in all patients. Mucosal specimens were considered non-inflamed ('inactive') if there were no endoscopic and histological signs of inflammation.

Tissue cultures

Biopsy specimens were transferred to the laboratory and handled within a maximal lag of 2 h after biopsy.

Each biopsy weighted about 10 mg (with little variation). Two intestinal biopsy tissues were gently washed in AIM-V medium (Life Technology, Melbourne, Victoria, Australia), weighed and cultured in 1 ml of serum-free AIM-V medium for 24 h at 37 °C in an incubator with 5% CO₂. The culture supernatants were collected and centrifuged. Aliquots were stored at -20 °C until assay [30]. Lactate dehydrogenase levels were measured to control for the absence of necrosis at the end of the culture period and histological analyses were performed in parallel to demonstrate structural integrity.

Isolation of human intestinal microvascular endothelial cells (HIMECs)

For HIMEC isolation, macroscopically normal colonic specimens were obtained from patients undergoing scheduled colonic resection. The use of human tissues for immunohistochemistry and isolation of endothelial cells was approved by the ethical committee of the University of Münster. HIMEC were isolated as previously described [31]. In brief, mucosal strips from resected normal colon were washed, minced and digested in collagenase type II solution (Worthington, Lakewood, NJ, USA; 2 mg/ml). ECs were extruded by mechanical compression and plated onto tissue culture dishes coated with collagen type I from rat tail (Upstate Biotechnology Inc., Waltham, MA, USA) in endothelial cell growth medium MV (PromoCell, Heidelberg, Germany) containing antibiotics and antimycotics. Following 7–10 days of culture, microvascular EC clusters were physically isolated, and a pure culture was obtained. HIMEC cultures were recognized by microscopic phenotype and expression of factor VIII-associated antigen. All experiments were carried out using HIMEC cultures between passages 6 and 12.

Expression of pro-inflammatory genes by endothelial cells

Purification of recombinant human S100A12 and native human MRP8/14 was performed as described. HIMECs were cultivated as previously reported and either left unstimulated with only vehicle control or stimulated with either 10 ng/ml TNF, 20 µg/ml S100A12, or 200 µg/ml MRP8/14 for 4 h, respectively [32–34]. RNA expression of vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) was analysed by RT-PCR [33].

FACS analyses

FACS analysis of ICAM-1 surface expression on HIMECs was performed after 16 h stimulation with 20 µg/ml S100A12, as reported previously [33].

Immunoassays

The analyses for S100A12 and MRP8/14 were performed using aliquots from identical samples. Concentrations of S100A12 were determined by a double sandwich enzyme-linked immunosorbent assay (ELISA) system established in our laboratory, as described previously [8]. Concentrations of MRP8/14 were determined by a double sandwich ELISA system established in our laboratory, as described previously [19]. The readers of the laboratory assay were blinded for the diagnosis and the inflammation score.

Immunohistochemistry

Expression of S100A12 and MRP14 was detected on paraffin-embedded bowel specimens by staining with specific antibodies, as described previously [8,35]. To identify neutrophils in the sections, murine anti-human CD15 or alternatively anti-human elastase antibodies (DAKO, Glostrup, Denmark) were employed. Co-expression of neutrophil markers was analysed on serial sections. Secondary antibodies and substrates for colour reaction were used as described before [35]. Sections were analysed using a Zeiss Axioskop connected to an AxioCam camera supply with software Axiovision 3.0 for Windows (Zeiss, Goettingen, Germany).

Statistical analyses

All statistical analyses were performed with SPSS software package version 14 (SPSS Inc., Chicago, IL, USA). Differences for parameters found to be significant in Kruskal–Wallace tests were further compared group-to-group by Mann–Whitney *U*-test. Correlation of different markers and scores with disease activity was analysed using Spearman's rho-test. All tests of significance were two-tailed. A *p* value of <0.05 was considered significant. Data are presented as mean ± standard error of the mean (SEM) except otherwise stated.

Results

Characteristics of the study population

We included 30 patients with CD, 30 patients with UC, 30 patients with IBS and 30 healthy control individuals. The characteristics of our cohort are listed in Table 1.

Mucosal release of phagocytic S100 proteins

We found a strongly increased spontaneous release of the neutrophil activation marker S100A12 from tissue in IBD compared to IBS and healthy controls (*p* < 0.0001). The release of S100A12 into the supernatants was 28-fold enhanced in inflamed tissue (mean 46.9 ± 13.9 ng/ml) when compared to non-inflamed tissue (1.7 ± 0.8 ng/ml; *p* < 0.0001). An

Table 1. Patient characteristics

Patient group	CD-active			CD-inactive			UC-active			UC-inactive			IBS			Controls			
	All	Ileum	Colon	All	Ileum	Colon	All	Ileum	Colon	All	Ileum	Colon	All	Ileum	Colon	All	Ileum	Colon	
Number	23	12	11	35	12	11	7	19	11	30	30	30	47	30	46	30	46	30	
Age (years) [†]	36 (21–62)**	37 (22–53)	36 (21–62)	35 (19–47)*	37 (22–53)	36 (21–62)	35 (19–47)*	37 (19–72)	51 (18–69)	47 (21–65)	47 (21–65)	46 (25–74)	47 (21–65)	47 (21–65)	46 (25–74)	46 (25–74)	46 (25–74)	46 (25–74)	46 (25–74)
Gender (m/f)	12/11	6/6	6/5	3/4	6/6	6/5	3/4	10/9	6/5	11/19	11/19	18/12	11/19	11/19	18/12	18/12	18/12	18/12	18/12
Therapy																			
Aminosalicylates	8	4	4	1	4	4	1	9	4	2	1	1	4	2	1	1	1	1	1
Steroids	5	2	3	0	2	3	0	3	0	0	0	0	0	0	0	0	0	0	0
Azathioprine	4	1	3	3	1	3	3	2	1	0	0	0	1	0	0	0	0	0	0
Methotrexate	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Tissue inflammation ^{††}	1.2 (1–2)	1.1 (1–2)	1.4 (1–2)	0 (0)	1.1 (1–2)	1.4 (1–2)	0 (0)	1.6 (1–2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Hb (g/l)	138 (125–154)	141 (125–154)	136 (128–146)	145 (138–152)	141 (125–154)	136 (128–146)	145 (138–152)	138 (123–162)	143 (120–165)	130 (117–144)	130 (117–144)	149 (126–160)	130 (117–144)	130 (117–144)	149 (126–160)	149 (126–160)	149 (126–160)	149 (126–160)	149 (126–160)
Hct (%)	42 (37–45)	42 (39–45)	41 (37–44)	44 (42–45)	42 (39–45)	41 (37–44)	44 (42–45)	42 (39–47)	41 (34–47)	39 (34–44)	39 (34–44)	44 (4–48)	39 (34–44)	39 (34–44)	44 (4–48)	44 (4–48)	44 (4–48)	44 (4–48)	44 (4–48)
WBC (×1000/μl)	6.7 (4.1–9.0)	7.2 (5.8–8.4)	6.4 (4.1–9.0)	10.0 (4.4–15.6)	7.2 (5.8–8.4)	6.4 (4.1–9.0)	10.0 (4.4–15.6)	7.4 (5.8–12.1)	7.9 (5–11.6)	6.4 (4.6–9.8)	6.4 (4.6–9.8)	5.93 (4.4–8.0)	6.4 (4.6–9.8)	6.4 (4.6–9.8)	5.93 (4.4–8.0)	5.93 (4.4–8.0)	5.93 (4.4–8.0)	5.93 (4.4–8.0)	5.93 (4.4–8.0)
Plt (×1000/μl)	260 (160–332)	250 (198–284)	268 (160–332)	356 (281–430)	250 (198–284)	268 (160–332)	356 (281–430)	243 (166–317)	248 (194–338)	263 (172–343)	263 (172–343)	280 (154–379)	263 (172–343)	263 (172–343)	280 (154–379)	280 (154–379)	280 (154–379)	280 (154–379)	280 (154–379)
Albumin (mg/dl)	41 (36–44)	42 (39–44)	40 (36–43)	38 (35–41)	42 (39–44)	40 (36–43)	38 (35–41)	44 (42–46)	47 (46–48)	43 (39–46)	43 (39–46)	45 (39–48)	43 (39–46)	43 (39–46)	45 (39–48)	45 (39–48)	45 (39–48)	45 (39–48)	45 (39–48)
ESR (mm/h)	21 (3–60)	16 (4–33)	24 (3–60)	8 (5–14)	16 (4–33)	24 (3–60)	8 (5–14)	15 (2–44)	3 (1–5)	7 (1–25)	7 (1–25)	6 (3–12)	7 (1–25)	7 (1–25)	6 (3–12)	6 (3–12)	6 (3–12)	6 (3–12)	6 (3–12)
CRP (mg/l)	9.2 (0.5–23)	10.8 (0.5–18)	8.8 (5.6–23)	5.6 (0.5–10.2)	10.8 (0.5–18)	8.8 (5.6–23)	5.6 (0.5–10.2)	6.5 (0.5–7.9)	3.8 (0.5–6.6)	2.7 (0–2.1)	2.7 (0–2.1)	2.6 (0–18)	2.7 (0–2.1)	2.7 (0–2.1)	2.6 (0–18)	2.6 (0–18)	2.6 (0–18)	2.6 (0–18)	2.6 (0–18)

* $p < 0.05$; ** $p < 0.01$ (Kruskal–Wallace test).

† Mean (range) except where stated; †† median (range); ††† score 0–3.

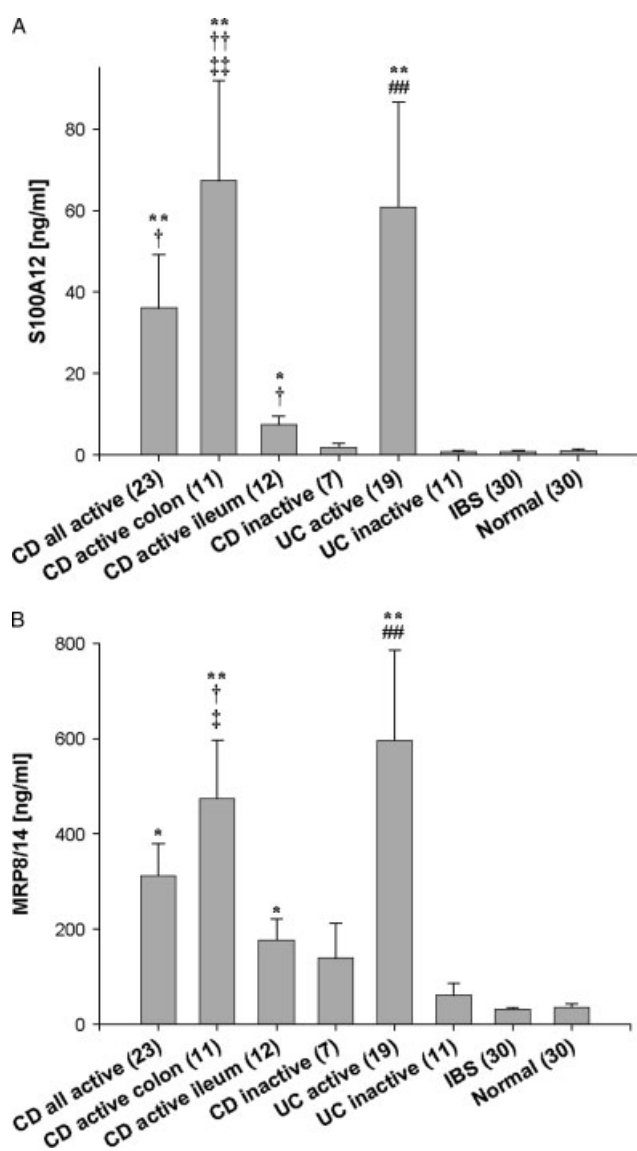


Figure 1. Release of S100 proteins from intestinal tissue samples. Concentrations of S100A12 (A) and MRP8/14 (B) were determined by ELISA in supernatants from intestinal tissue biopsies cultured for 24 h. S100A12 was significantly higher in active CD and UC compared to all other groups (** $p < 0.01$ versus healthy controls; † $p < 0.05$ versus inactive CD; †† $p < 0.01$ versus inactive CD; ††† $p < 0.01$ versus inactive UC). MRP8/14 was also higher in active IBD compared to IBS and healthy controls, but differences between patients with active and inactive CD did not reach statistical significance. In active CD, release of S100A12 and MRP8/14 was strongly dependent on localization, with little release from sites of active ileal inflammation compared to colonic inflammation. This difference was more pronounced for S100A12 than for MRP8/14 (** $p < 0.01$ versus healthy controls; † $p < 0.05$ versus inactive CD; †† $p < 0.01$ versus inactive CD; ††† $p < 0.05$ between ileal and colonic CD; †† $p < 0.01$ between ileal and colonic CD)

eight-fold difference was found for MRP8/14, this protein complex was also found at significantly higher concentrations in supernatants from inflamed tissue (459 ± 102 ng/ml) when compared to non-inflamed tissue (60 ± 13 ng/ml; $p < 0.0001$). Mucosal release of S100A12 was significantly elevated in active CD and UC when compared to inactive IBD, IBS and healthy controls. When analysing active CD patients

as a whole group, MRP8/14 release did not differ significantly between active and inactive disease. However, the release of S100A12 and MRP8/14 was strongly dependent on localization, with little release from sites of active ileal inflammation compared to colonic inflammation. This difference was more pronounced for S100A12 ($p < 0.01$) than for MRP8/14 ($p < 0.05$). For both proteins, levels in inactive disease were not dependent on the former disease localization. The S100A12 levels in active ileal CD differed from inactive CD ($p < 0.05$), while MRP8/14 concentrations in active ileal CD and inactive CD were not significantly different. For both proteins, levels in active UC were significantly different from inactive UC, and levels in active IBD as a whole differed significantly from IBS and healthy controls (Figure 1).

Correlation to disease activity

There was a significant correlation of S100A12 release from intestinal tissue to the tissue inflammation in IBD patients (Figure 2). There was a difference between MRP8/14 levels in inactive CD on the one hand side and IBS or healthy controls on the other. Otherwise, there were no differences in the 'non-inflamed' group. The S100A12 concentrations in supernatants correlated also positively with MRP8/14 release and ESR, while a negative correlation to albumin was found (Table 2). Concentrations of MRP8/14 in supernatants also correlated well with tissue inflammation, but correlation to other parameters of disease activity was weaker. No significant correlation to age, Hb, Hct, WBC, platelets or CRP was found. It is possible that medication was influencing the release of S100A12 and MRP8/14 from tissue samples in active IBD, even though the inflammation score did not differ between the groups (indicating that the degree of inflammation was similar regardless of therapies in our cases). The release of S100 proteins was significantly more pronounced when the patients did not receive any therapy (Figure 3). The difference was more pronounced for S100A12 than for MRP8/14, which may point to the fact that therapies targeting immune cells attenuate release of S100A12 and MRP8/14 from phagocytes, while some additional release of MRP8/14 from epithelial cells is unaffected. However, our study was not designed to accurately demonstrate the impact of medical therapies upon the mucosal release, which would only be possible by looking serially within the same patient.

Detection of S100 proteins in intestinal tissue

Staining of tissue from patients with active IBD showed a specific distribution of S100A12 expression by infiltrating cells in inflamed areas of the gut. Epithelial cells were S100A12-negative in all cases, while profound subepithelial expression of S100A12 in granulocytes was found in IBD. In gut biopsies with a high degree of inflammation,

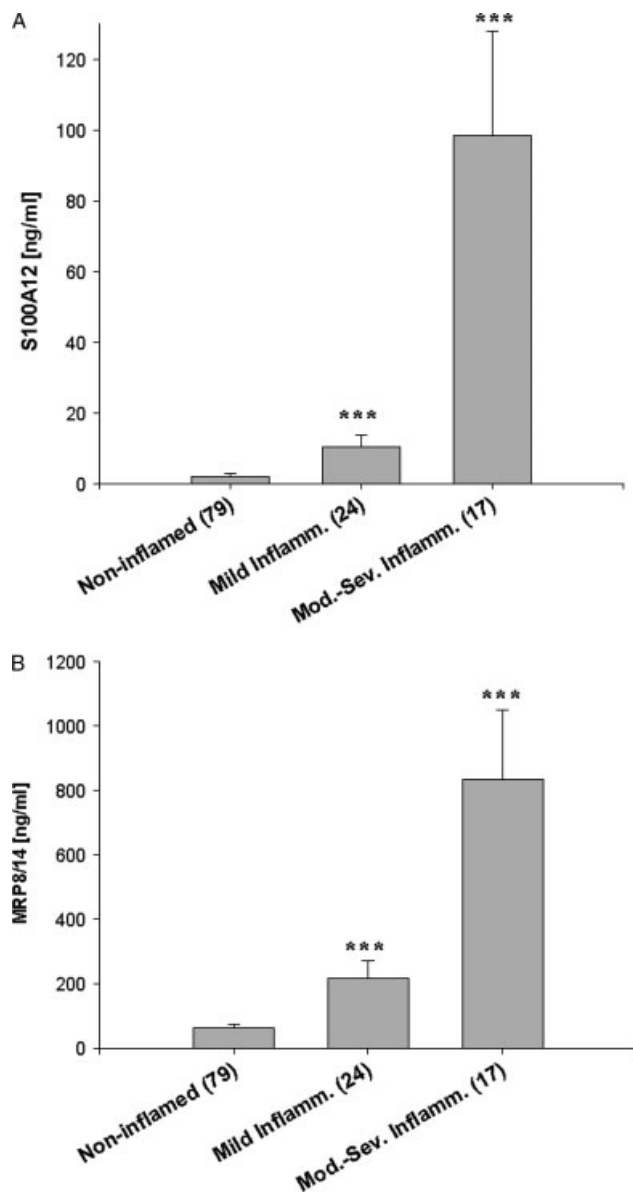


Figure 2. Correlation of S100 protein release to tissue inflammation. An inflammation score was calculated by combining endoscopic and histological information in all patients and controls. The non-inflamed group consists of patients with inactive IBD, IBS and healthy individuals. There were significant differences for S100A12 (A) and MRP8/14 (B) levels between the groups of individuals (** $p < 0.001$). The differences were more pronounced for S100A12.

Table 2. Correlation coefficients

	Supernatant S100A12	Supernatant MRP8/14
Supernatant MRP8/14	0.79**	1
Tissue inflammation	0.70**	0.67**
ESR	0.59**	0.43*
Albumin	-0.42*	-0.25

* $p < 0.05$; ** $p < 0.01$ (Spearman rho-test).

S100A12 was present in an extracellular distribution surrounding S100A12-positive cells, reflecting secretion of the protein (Figure 4A). There was only

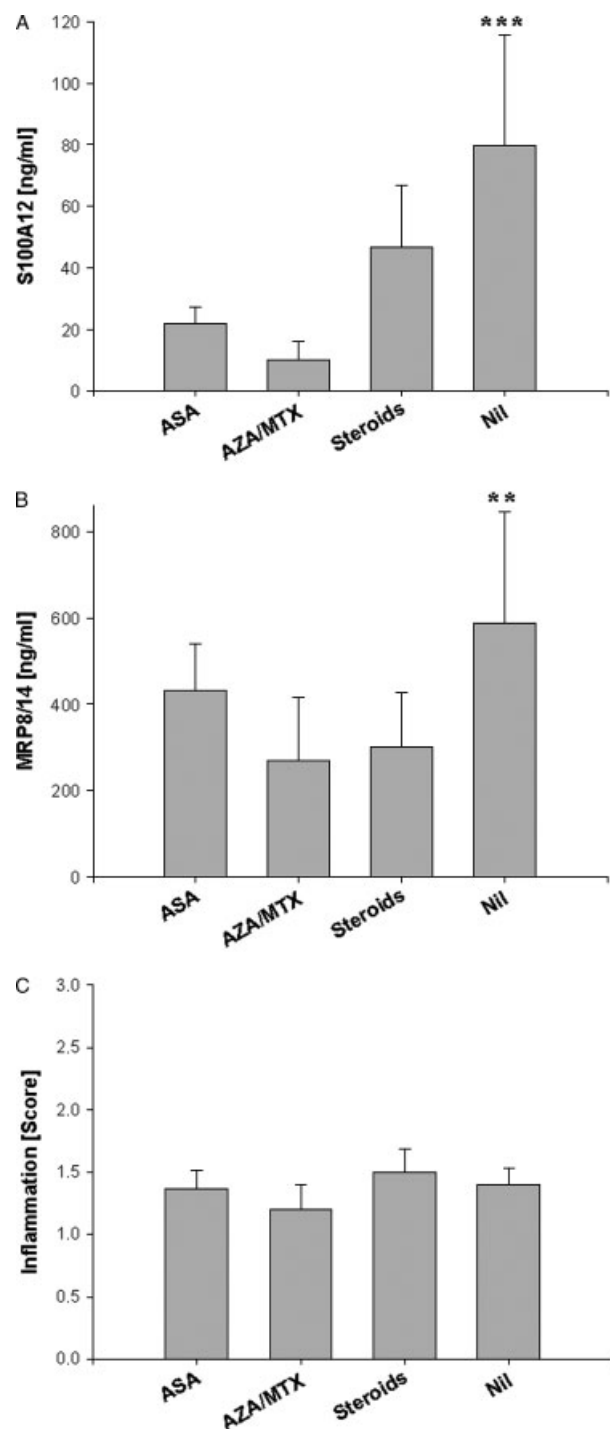


Figure 3. Impact of medication on S100 protein release. We analysed whether medication was influencing the release of S100A12 (A) and MRP8/14 (B) from tissue samples in active IBD. While the inflammation score (C) did not differ between the groups, the release of S100 proteins was significantly more pronounced when the patients did not receive any medication (Nil) compared to patients receiving either aminosalicylates (ASA), azathioprine or methotrexate (AZA/MTX), as well as steroids. The difference was more pronounced for S100A12 (** $p < 0.001$) than for MRP8/14 (** $p < 0.01$).

sparse S100A12 expression in tissue samples from patients with IBS or healthy controls and only few S100A12 positive cells in samples of IBD patients with no evidence of inflammation in endoscopic or

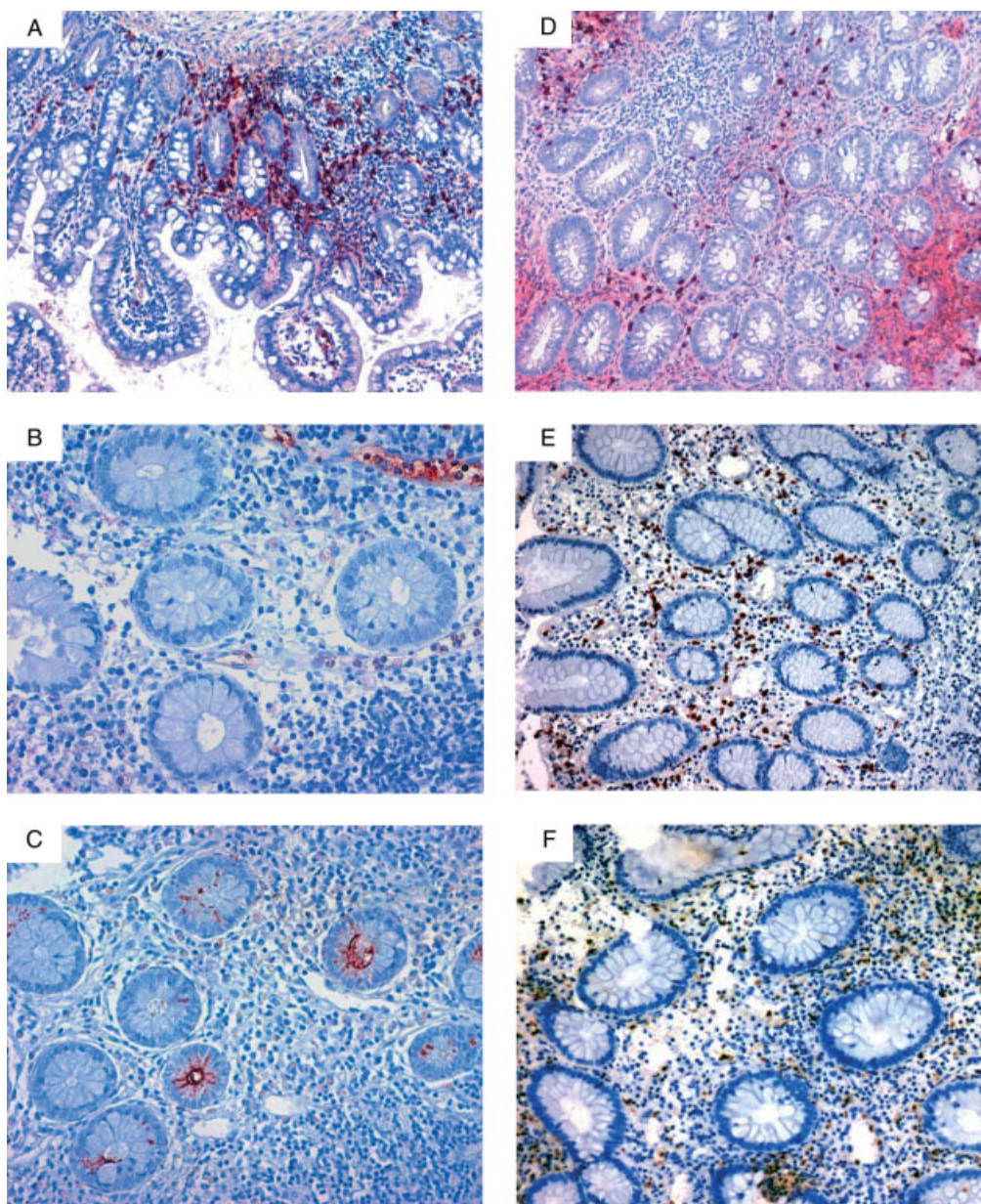


Figure 4. Immunohistochemical staining. S100A12 and MRP14 were stained in biopsies obtained at colonoscopy. Epithelial cells were S100A12-negative in all cases, while profound subepithelial expression of S100A12 in granulocytes was found in gut biopsies with a high degree of inflammation. S100A12 was also present in an extracellular distribution surrounding S100A12-positive cells, reflecting secretion of the protein (A; terminal ileum in CD with severe inflammation; anti-S100A12, magnification $\times 50$). In patients with mild inflammation, the cellular infiltrate was dominated by S100A12-negative cells, while S100A12-positive neutrophils adhered to the endothelium of small blood vessels (B; terminal ileum in CD with mild inflammation, anti-S100A12; magnification $\times 100$). In the same patient, staining for MRP14 was not only found in infiltrating phagocytes but also in epithelial cells (C; terminal ileum in CD with mild inflammation; anti-MRP14; magnification $\times 100$). Staining for MRP8 was similar to MRP14 (data not shown). In CD patients with severe colonic inflammation, there was an extensive staining for S100A12 (D; ascending colon, anti-S100A12, magnification $\times 50$). In the colon, neither S100A12 (E) nor MRP14 (F) was found in epithelial cells (magnification $\times 100$)

histological examination (not shown). In patients with mild inflammation, the cellular infiltrate was dominated by S100A12-negative cells, while S100A12-positive neutrophils adhered to the endothelium of small blood vessels or were found scattered within the tissue (Figure 4B). In contrast to S100A12, staining for MRP8/14 was not only found in infiltrating phagocytes but also in epithelial cells. This staining was also found in some cases with mild activity of CD, which may explain the still enhanced release of

MRP8/14 from tissue samples of patients with inactive CD (Figure 4C).

S100A12 and MRP8/14 induce pro-inflammatory responses in HIMECs

Finally, we analysed the effects of S100A12 and MRP8/14 on human intestinal endothelium *in vitro*. S100A12 and MRP8/14 increased PCR transcripts for VCAM-1, ICAM-1, IL-8 and MCP-1 in HIMECs. The

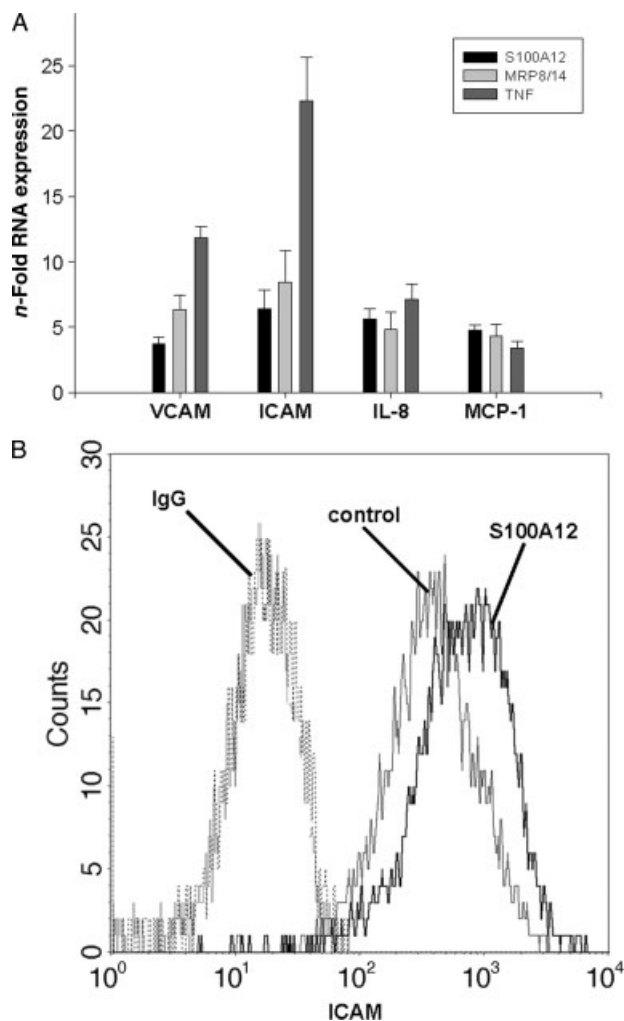


Figure 5. Effects of S100A12 and MRP8/14 on endothelium *in vitro*. Stimulation with either S100A12 (20 μ g/ml) or MRP8/14 (200 μ g/ml) for 4 h resulted in an increased RNA expression of VCAM-1, ICAM-1, IL-8 and MCP-1 on HIMECs. Stimulation with TNF (10 ng/ml) served as a positive control. Gene expression was normalized to the endogenous housekeeping control gene GAPDH and analysed as *n*-fold RNA expression relative to unstimulated controls (A). The enhanced expression of ICAM-1 on HIMECs after stimulation with S100A12 for 16 h was confirmed on the protein level by FACS analyses (B). Data are representative of three independent experiments

enhanced expression of adhesion molecules was confirmed on the protein level for ICAM-1 by FACS analyses of HIMECs (Figure 5). Thus, a pro-inflammatory response may be induced on intestinal endothelium by S100 proteins directly released at the site of inflammation, i.e. within the intestinal mucosa in the case of IBD.

Discussion

Various cytokines, such as IL-12 and IFN- γ in CD, IL-5 in UC, or IL-6, IL-8 and TNF in both CD and UC are present at abnormal levels in the mucosa of IBD patients [3,36,37]. However, these cytokines have limited utility as non-invasive markers of disease activity because serum and stool levels often remain within

normal limits in patients with IBD. In contrast to these cytokines, DAMP molecules, which may accumulate in the intestinal mucosa, represent excellent markers for mucosal inflammation. Especially assays which determine intestinal inflammation by detecting neutrophil-derived products in stool bear a great potential [7].

An important mechanism in the initiation and perturbation of inflammation in IBD is the activation of innate immune mechanisms [2,3]. Among the factors released by infiltrating neutrophils are proteins of the S100 family [8]. One example is 'calprotectin', which is detectable in serum and stool during intestinal inflammation [18]. Calprotectin was initially described as a protein of 36 kDa but later characterized as a complex of two distinct S100 proteins, S100A8 and S100A9 (MRP8 and MRP14) [9–11]. In recent years, MRP8/14 has been proposed as a fecal marker of gut inflammation, reflecting the degree of phagocyte activation [7,12–17]. On the other hand, the broad expression pattern of MRP8/14, which is found in granulocytes as well as monocytes and is also inducible in epithelial cells under certain circumstances, may be influencing the results [19,38]. In this context, the elevation of fecal levels in lactose intolerance and the increased release from tissue in coeliac disease is notable, although the absolute level is typically still lower than in colonic CD [13,14,20,39]. The expression of MRP8/14 in epithelial cells in specimens obtained from ileal CD may point to the fact that small bowel epithelium can directly contribute to MRP8/14 release. It has previously been noted that 'fecal calprotectin' tends to be lower in ileal-only CD and that the correlation to disease activity is better in colonic inflammation [39]. It was hypothesized that the S100 proteins may be degraded during the passage from the ileum through the whole colon, thus giving falsely low levels for small-bowel inflammation. Our data indicate that there is already less release of these proteins from the ileum even in patients with equivalent disease severity. Especially MRP8/14 is therefore rather unreliable to monitor ileal CD, since its release seems to be even prolonged in patients with inactive disease, due to factors not related to phagocytic activation, thus leading to non-significant differences between active and inactive ileal CD. The direct release of phagocyte-derived S100 proteins from local tissues under inflammatory conditions may reflect secretion from infiltrating phagocytes during active disease, which can explain the correlation of fecal S100 levels with disease activity. In addition, the potential release of MRP8/14 from epithelial cells may be induced in conditions where the intestinal mucosa is irritated both by inflammatory and non-inflammatory stimulants [34].

In contrast to MRP8/14, S100A12 is exclusively expressed in relevant amounts by granulocytes, which play an important role in the pathogenesis of IBD. S100A12 is secreted by activated neutrophils and promotes inflammation through activation of RAGE [25].

Since neutrophil influx into the intestinal mucosa is closely linked to IBD activity, especially during early steps of inflammation, neutrophil-derived S100A12 in tissue and exudates strongly correlates to inflammatory activity [8,40]. Excretion of the protein into the gut lumen could reflect the number of neutrophils infiltrating the mucosa, as well as their activation status [34,41]. Therefore, S100A12 may be more specific for neutrophil activation in inflammatory bowel disease, while MRP8/14 may be more sensitive in conditions with mucosal irritation, which could have been present in our patients with inactive or mildly affected CD.

Previous studies demonstrated that S100A12 and MRP8/14 are over-expressed during chronic active IBD and may serve as useful serum and stool markers of inflammation [8,22,42]. In a recent study it was also shown that phagocyte-specific S100 proteins are released from tissue specimens obtained from children with IBD [40]. However, it is not known to date whether the release of phagocyte-related S100 proteins from intestinal mucosal specimens correlates to intestinal inflammation status. We now provide novel evidence for an increased spontaneous release of S100 proteins from tissue in adult IBD patients compared to IBS and healthy controls, and we show for the first time that this release correlates with tissue inflammation. The release of S100A12 into the supernatants was more significantly enhanced than that found for MRP8/14. Our study reveals a strong pro-inflammatory response of intestinal endothelium induced by phagocyte-derived S100 proteins which are directly released at the site of inflammation within the intestinal mucosa. S100A12 activates endothelium via interaction with RAGE, while MRP8/14 has pro-inflammatory effects on endothelium as ligands of TLR4 [24,25]. These pro-inflammatory responses induced on intestinal endothelium promote infiltration of inflamed mucosa by additionally recruited immune cells [33,34]. On the other hand, these proteins may also promote epithelial healing. While beyond the scope of the current study, it might therefore be of interest in the future to also determine the effect of MRP8/14 and S100A12 upon intestinal epithelial cell gene expression and wound healing responses in greater detail.

As in previous studies, we decided to correlate the S100 data in the present study directly to tissue inflammation parameters, rather than to clinical indices. This approach is crucial, taking into account the observed inaccuracy of clinical indices. The CAI and the CDAI correlate poorly with invasive assessment of inflammation by endoscopy and histology (which was our gold standard) and depend upon interobserver variations [43,44]. These clinical scores have been found to be of little use in the monitoring of disease activity in a number of studies, including ours [22,45]. It was our aim to include only well-characterized IBD patients, in whom endoscopic investigation with collection of bowel biopsies was performed, allowing the use of a histology inflammation score as a gold standard.

In a previous study it was shown that the fecal concentrations of these proteins correlate to tissue inflammation also determined by analysing biopsies [22]. It is thus conceivable that the release of these proteins from inflamed tissue into the gut lumen correlates to concentrations in stool. Indeed, the results found in tissue supernatants correlate well with our results obtained in stool samples [22]. In addition, the more pronounced elevation of S100A12 release from inflamed versus non-inflamed mucosa as compared to MRP8/14 fits the observation that S100A12 is also a more specific fecal marker of intestinal inflammation than 'fecal calprotectin' [22].

Taken together, DAMP molecules released from phagocytes may be involved in tissue inflammation. Remarkable pro-inflammatory properties have been described for S100A12 and MRP8/14 in this context [33,34]. The direct release of phagocyte-derived S100 proteins from local tissues under inflammatory conditions may reflect secretion from infiltrating neutrophils (S100A12) and also monocytes or epithelial cells (MRP8/14) during active disease. This release can explain the correlation of fecal S100 levels with disease activity in IBD, thus further validating the use of these proteins as markers of intestinal inflammation.

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References

- Hanauer SB. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm Bowel Dis* 2006;**12**(suppl 1):S3–9.
- Brandtzaeg P, Haraldsen G, Rugtveit J. Immunopathology of human inflammatory bowel disease. *Springer Semin Immunopathol* 1997;**18**:555–589.
- Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003;**3**:521–533.
- Eckmann L, Karin M. NOD2 and Crohn's disease: loss or gain of function? *Immunity* 2005;**22**:661–667.
- Foell D, Wittkowski H, Roth J. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat Clin Pract Rheumatol* 2007;**3**:382–390.
- Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J Leukoc Biol* 2007;**81**:28–37.
- Langhorst J, Elsenbruch S, Mueller T, Rueffer A, Spahn G, Michalsen A, *et al.* Comparison of four neutrophil-derived proteins in feces as indicators of disease activity in ulcerative colitis. *Inflamm Bowel Dis* 2005;**11**:1085–1091.
- Foell D, Kucharzik T, Kraft M, Vogl T, Sorg C, Domschke W, *et al.* Neutrophil derived human S100A12 (EN-RAGE) is strongly expressed during chronic active inflammatory bowel disease. *Gut* 2003;**52**:847–853.
- Hunter MJ, Chazin WJ. High level expression and dimer characterization of the S100 EF-hand proteins, migration inhibitory factor-related proteins 8 and 14. *J Biol Chem* 1998;**273**:12427–12435.

10. Roth J, Goebeler M, Sorg C. S100A8 and S100A9 in inflammatory diseases. *Lancet* 2001;**357**:1041.
11. Roth J, Vogl T, Sorg C, Sunderkotter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends Immunol* 2003;**24**:155–158.
12. Luger N, Stoll R, Kucharzik T, Schmid KW, Rohlmann G, Burmeister G, et al. Immunohistochemical distribution and serum levels of the Ca²⁺-binding proteins MRP8, MRP14 and their heterodimeric form MRP8/14 in Crohn's disease. *Digestion* 1995;**56**:406–414.
13. Olafsdottir E, Aksnes L, Fluge G, Berstad A. Faecal calprotectin levels in infants with infantile colic, healthy infants, children with inflammatory bowel disease, children with recurrent abdominal pain and healthy children. *Acta Paediatr* 2002;**91**:45–50.
14. Nissen AC, van Gils CE, Menheere PP, Van den Neucker AM, van der Hoeven MA, Forget PP. Fecal calprotectin in healthy term and preterm infants. *J Pediatr Gastroenterol Nutr* 2004;**38**:107–108.
15. Pardi DS, Sandborn WJ. Predicting relapse in patients with inflammatory bowel disease: what is the role of biomarkers? *Gut* 2005;**54**:321–322.
16. Tibble JA, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* 2000;**119**:15–22.
17. Tibble JA, Sigthorsson G, Foster R, Forgacs I, Bjarnason I. Use of surrogate markers of inflammation and Rome criteria to distinguish organic from nonorganic intestinal disease. *Gastroenterology* 2002;**123**:450–460.
18. Fagerhol MK. Calprotectin, a faecal marker of organic gastrointestinal abnormality. *Lancet* 2000;**356**:1783–1784.
19. Foell D, Frosch M, Sorg C, Roth J. Phagocyte-specific calcium-binding S100 proteins as clinical laboratory markers of inflammation. *Clin Chim Acta* 2004;**344**:37–51.
20. Campeotto F, Butel MJ, Kalach N, Derrieux S, Aubert-Jacquin C, Barbot L, et al. High faecal calprotectin concentrations in newborn infants. *Arch Dis Child Fetal Neonatal Ed* 2004;**89**:F353–355.
21. Vogl T, Propper C, Hartmann M, Strey A, Strupat K, van den Bos C, et al. S100A12 is expressed exclusively by granulocytes and acts independently from MRP8 and MRP14. *J Biol Chem* 1999;**274**:25291–25296.
22. Kaiser T, Langhorst J, Wittkowski H, Becker K, Friedrich AW, Rueffer A, et al. Faecal S100A12 as a non-invasive marker distinguishing inflammatory bowel disease from irritable bowel syndrome. *Gut* 2007;**56**:1706–1713.
23. Foell D, Roth J. Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis Rheum* 2004;**50**:3762–3771.
24. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med* 2007;**13**:1042–1049.
25. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 1999;**97**:889–901.
26. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest* 2001;**108**:949–955.
27. Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999;**45**(suppl 2):II43–47.
28. Daperno M, D'Haens G, Van Assche G, Baert F, Bulois P, Maunoury V, et al. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest Endosc* 2004;**60**:505–512.
29. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987;**317**:1625–1629.
30. Clancy R, Ren Z, Turton J, Pang G, Wettstein A. Molecular evidence for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Crohn's disease correlates with enhanced TNF α secretion. *Dig Liver Dis* 2007;**39**:445–451.
31. Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. *J Biol Chem* 2003;**278**:8508–8515.
32. Viemann D, Goebeler M, Schmid S, Nordhues U, Klimmek K, Sorg C, et al. TNF induces distinct gene expression programs in microvascular and macrovascular human endothelial cells. *J Leukoc Biol* 2006;**80**:174–185.
33. Viemann D, Strey A, Janning A, Jurk K, Klimmek K, Vogl T, et al. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. *Blood* 2005;**105**:2955–2962.
34. Wittkowski H, Sturrock A, van Zoelen MA, Viemann D, van der Poll T, Hoidal JR, et al. Neutrophil-derived S100A12 in acute lung injury and respiratory distress syndrome. *Crit Care Med* 2007;**35**:1369–1375.
35. Foell D, Hernandez-Rodriguez J, Sanchez M, Vogl T, Cid MC, Roth J. Early recruitment of phagocytes contributes to the vascular inflammation of giant cell arteritis. *J Pathol* 2004;**204**:311–316.
36. Papadakis KA, Targan SR. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annu Rev Med* 2000;**51**:289–298.
37. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, Stepankova R, et al. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 2006;**25**:309–318.
38. Frosch M, Metz D, Foell D, Vogl T, Sorg C, Sunderkotter C, et al. Early activation of cutaneous vessels and epithelial cells is characteristic of acute systemic onset juvenile idiopathic arthritis. *Exp Dermatol* 2005;**14**:259–265.
39. Costa F, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, et al. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 2005;**54**:364–368.
40. Leach ST, Yang Z, Messina I, Song C, Geczy CL, Cunningham AM, et al. Serum and mucosal S100 proteins, calprotectin (S100A8/S100A9) and S100A12, are elevated at diagnosis in children with inflammatory bowel disease. *Scand J Gastroenterol* 2007;**42**:1321–1331.
41. Carlson M, Raab Y, Seveus L, Xu S, Hallgren R, Venge P. Human neutrophil lipocalin is a unique marker of neutrophil inflammation in ulcerative colitis and proctitis. *Gut* 2002;**50**:501–506.
42. Wittkowski H, Foell D, af Klint E, De Rycke L, De Keyser F, Frosch M, et al. Effects of intra-articular corticosteroids and anti-TNF therapy on neutrophil activation in rheumatoid arthritis. *Ann Rheum Dis* 2007;**66**:1020–1025.
43. Gomes P, du Boulay C, Smith CL, Holdstock G. Relationship between disease activity indices and colonoscopic findings in patients with colonic inflammatory bowel disease. *Gut* 1986;**27**:92–95.
44. Fischbach W, Becker W. Clinical relevance of activity parameters in Crohn's disease estimated by the faecal excretion of ¹¹¹In-labeled granulocytes. *Digestion* 1991;**50**:149–152.
45. Brignola C, Lanfranchi GA, Campieri M, Bazzocchi G, Devoto M, Boni P, et al. Importance of laboratory parameters in the evaluation of Crohn's disease activity. *J Clin Gastroenterol* 1986;**8**:245–248.