Role of HMGB1 as a Suitable Biomarker of Subclinical Intestinal Inflammation and Mucosal Healing in Patients with Inflammatory Bowel Disease

Francesca Palone, PhD,* Roberta Vitali, PhD,* Salvatore Cucchiara, MD, PhD,[†] Maria Pierdomenico, PhD,* Anna Negroni, PhD,* Marina Aloi, MD,[†] Federica Nuti, MD,[†] Carla Felice, MD,[‡] Alessandro Armuzzi, MD,[‡] and Laura Stronati, PhD*

Background: Noninvasive biomarkers of high- and low-grade intestinal inflammation and of mucosal healing (MH) in patients with inflammatory bowel disease are currently lacking. We have recently shown that fecal high mobility group box 1 (HMGB1) protein is a novel biomarker of gut inflammation. We aimed at investigating in a mouse model if HMGB1 was able to foresee both a clinically evident and a subclinical gut inflammation and if its normalization indicated MH. We also aimed at confirming the results in patients with Crohn's disease (CD) and ulcerative colitis.

Methods: C57BL6/J mice were treated with increasing doses of dextran sodium sulphate to induce colitis of different severity degrees; 28 with CD, 23 with ulcerative colitis, and 17 controls were also enrolled. Fecal HMGB1 was analyzed by enzyme-linked immunosorbent assay and immunoblotting.

Results: Fecal HMGB1 increased by 5-, 11-, 18-, and 24-folds with dextran sodium sulphate doses of 0.25%, 0.50%, 1%, and 4%, respectively, showing that the protein detected a high-grade and a subclinical inflammation. After a recovery time of 4-week posttreatment, HMGB1 returned to control levels, paralleling MH. In patients, fecal HMGB1 significantly correlated with endoscopic indexes (Simple Endoscopic Score for Crohn's Disease [SES-CD], endoscopic Mayo subscore), but not with the disease activity indexes (Crohn's disease Activity Index, partial Mayo score).

Conclusions: Fecal HMGB1 is a robust noninvasive biomarker of clinically overt and subclinical gut inflammation; it can also be a surrogate marker of MH. We suggest the use of fecal HMGB1 to monitor the disease course and assess therapy outcomes in inflammatory bowel disease.

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Key Words: HMGB1, fecal biomarker, gut inflammation, mucosal healing, human IBD

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing and unremitting conditions that affect the gastrointestinal tract. Endoscopy is currently viewed as the gold standard for diagnosing IBD, permitting the description of mucosal inflammatory lesions, their surface extent and severity, and also contributing to assess disease activity.[1–3] Moreover, recent studies have identified mucosal healing (MH) on endoscopy as a key prognostic parameter in the management of IBD. Evidence has now accumulated that MH can alter the course of IBD, as it is associated with sustained clinical remission, and reduced rates of hospitalization and surgical resection.[3–5]

However, endoscopy is still an expensive and invasive method, and it can be troublesome in severely ill patients and in children. Thus, the need of noninvasive biomarkers for evaluating intestinal inflammation is strongly increasing.[6–9] In this frame, serum markers, such as C-reactive protein, erythrocyte sedimentation rate, antibodies against Saccharomyces cerevisiae, and perinuclear antineutrophil cytoplasmic proteins have shown a low sensitivity and specificity as markers of intestinal inflammation, mainly reflecting a set of systemic responses of the host, rather than a localized inflammation in the gut.[10,11] Instead, fecal markers have been found to be more accurate than serum markers in revealing gut inflammation. About a dozen of fecal markers have been studied so far. Among them, the lactoferrin and, mainly, the calprotectin are the most used in the management of IBD, as they may differentiate the latter from functional bowel disorders, correlate with their disease course and, finally, predict their relapse.[12–14] However, because of the complexity of IBD, the availability of ever more refined and reliable tools for the accurate and early diagnosis and for assessing the inflammatory status of the intestinal mucosa of patients is currently a growing requirement. Furthermore, novel appropriate and clinically applicable biomarkers would be helpful for detecting and

preventing colon cancer because patients with IBD are known to be at increased risk.[15–17]

In a previous study, our group showed that high mobility group box 1 (HMGB1) protein is a sensitive marker of gut inflammation as assessed in a cohort of 40 children with IBD. Indeed, the protein was found in high amount in the stools of patients, but not in the controls.[18]

HMGB1 is a DNA-binding nuclear protein that, like other endogenous molecules termed alarmins or damage-associated molecular patterns, can be released into the extracellular milieu during states of cellular stress or damage and subsequently activate the immune system and promote inflammation.[19,20] HMGB1 has been implicated in several inflammatory and auto-immune disorders, such as sepsis, rheumatoid arthritis, lupus erythematosus, myositis, diabetes, and, ultimately, IBD.[19–22]

In this study, we aimed at (1) investigating, in mouse model, the use of fecal HMGB1 as a marker of subclinical intestinal inflammation; (2) assessing that HMGB1 normalization is suggestive of MH; and (3) confirming in humans the results obtained in mouse model by analyzing a cohort of adult patients with IBD with different degrees of disease severity.

MATERIALS AND METHODS

Patients

Fifty-one consecutive patients with IBD, 28 with CD (median age: 43 yr; range: 26–68 yr), 23 with UC (median age: 44 yr; range: 21–78 yr), and 17 controls (median age: 43 yr; range: 27–86 yr), referred to the IBD Unit of the Complesso Integrato Columbus in Rome, Italy, between September and December 2011, and needed an ileocolonoscopy to reassess the intestinal disease, were included in this study. All patients had a confirmed diagnosis of IBD based on a combination of medical history, clinical evaluation, and typical endoscopic, histological and radiological findings, as suggested by international guidelines.[23] Fecal samples were collected before the patients started the ileocolonoscopy preparation and were frozen at −80°C. Serum samples, as well as all clinical data, were obtained the day of endoscopy. Patients undergoing ileocolonoscopy for no specific gastrointestinal symptoms and presenting normal endoscopy and histology were enrolled as controls. All subjects gave written informed consent to participate in the study.

The clinical activity was classified as in remission, mild, moderate, or severe using the Crohn's disease Activity Index (CDAI) for patients with CD[24] and the partial Mayo score[25] for patients with UC. The endoscopic activity was calculated using the Simple Endoscopic Score for Crohn's Disease score (SES-CD)[26] and the endoscopic Mayo subscore[27] for the evaluation of patients with CD and UC, respectively.

Animals

C57BL6/J female mice (8–9 wk of age) were purchased from the animal housing unit of the Harlan Laboratories, SRL,

(Indianapolis, Indiana) and they were housed in collective cages at 22 ± 1°C under a 12-hour light/dark cycle and with food and water provided ad libitum. The experimental procedures were previously approved by the Ministry of Health for the protection of animals used for experimental purposes, and the study was conducted in accordance with Italian regulations on animal welfare. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Italian National Agency for New Technology, Energy and Sustainable Economic Development (ENEA) (Permit Number:131/2012-B).

Animal Treatments

To induce a colitis of different degree of severity, 20 mice were divided into 4 groups (5 mice for each group) and fed with 4 increasing doses (0.25%, 0.5%, 1.0%, and 4.0%) of dextran sodium sulphate (DSS; molecular mass, 36,000–50,000 Da, MP Biomedicals, Santa Ana, California) dissolved in drinking water ad libitum for 7 days; 5 control mice received drinking water without DSS.

To induce a subclinical as well as a severe inflammation and evaluate the wound healing after a recovery time, 5 mice were given 1.5% DSS and 5 mice 4% DSS, respectively, in drinking water for 7 days followed by 28 days without treatment; stool specimens were collected at 1, 3, 5, and 7 days posttreatment and frozen at −80°C before analysis; 5 mice without DSS treatment were used as controls. Mice were euthanized on the seventh day, colons were weighed, and lengths were measured (from the anus to the top of the cecum). A small piece of distal colon was frozen in liquid nitrogen for colonic myeloperoxidase (MPO) analysis and RNA extraction. Blood samples from submandibular vein and stools were collected from each animal and stored at −80°C.

Mice were daily checked for the clinical score by assessing the following parameters: behavior, body weight, stool consistency (0 for normal stool, 1 for moist/sticky stool, 2 for soft stool, and 3 for diarrhea), presence of blood in stools (0 for no blood, 1 for evidence of blood in stools or around anus, and 2 for severe bleeding), and general appearance of the animal (0 was assigned if normal, 1 for ruffled fur or altered gait, and 2 for lethargic or moribund), according to Maxwell et al.[28] The percentage of weight loss was calculated in relation to the starting weight using the formula: ([Weight on day X − Initial weight]/Initial weight) × 100. Stool specimens were collected every day and frozen at −80°C. Mice were euthanized on the 35th day; tissue, serum, and stool specimens were collected as above. The experiments were performed according to Italian and European guidelines.

Histology

Distal colon samples were fixed in 10% formalin and embedded in paraffin for routine histology. To produce the histological score, fixed colon tissues were transversally sectioned (4 μm thickness), mounted on glass slides, deparaffinized, and stained using standard Hematoxylin and Eosin techniques. Sections were analyzed by light microscopy and scored according to the criteria of Maxwell et al.[28] Experiments were carried out in double blind.

Real-time Polymerase Chain Reaction

Total RNA was extracted from mouse colonic tissues with the RNeasy kit (QiaGen GmbH, Hilden, Germany), and 1 μg of total RNA was reverse transcribed by a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction was carried out with an ABI PRISM 7300 Sequence Detection System using the SYBR Green kit (Applied Biosystems). The following primers were identified using the Primer Express v.3.0 (Applied Biosystems) provided with the ABI Prism 7300 sequence detector: tumor necrosis factor (TNF)-α fwd primer: 5′-CAGACCCTCACACTCAGAT CATCTT-3′, rvs primer: 5′-TCGTAGCAAACCACCAAGTGG-3′; Interleukin (IL)-1β fwd primer: 5′-CGAGGCAGTATCACTCA TTG-3′, rvs primer: 5′-CGTTGCTTGGTTCTCCTTGT-3′; IL-6 fwd primer: 5′-CAAGTCGGAGGCTTAATTACACATG-3′, rvs primer: 5′-AGAAAAGAGTTGTGCAATGGCA-3′; GAPDH fwd primer 5′-AACTTTGGCATTGTGGAAGG-3′, rvs primer 5′-CACATTGGGGGTAGGAACAC-3′.

Messenger RNA (mRNA) levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the 2-ΔΔCT method. The expression level of each mRNA was reported as folds of induction as respect to controls. Experiments were repeated 3 times.

Fecal Extraction

Human and murine stool specimens, stored at −80°C, were resuspended in extraction buffer (ScheBo Biotech AG, Giessen, Germany) to a final concentration of 500 mg/mL. Samples were vortexed for 1 minute at room temperature and placed in orbital shaking for 1 hour at room temperature. After being centrifuged twice for 5 minutes at 10,000 rpm at 4°C, clear supernatants were collected and stored at −80°C. Total protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Quantification of Fecal and Serum HMGB1 by Enzyme-linked Immunosorbent Assay

Murine fecal and serum HMGB1 levels were analyzed using the HMGB1 enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource and EMELCA Bioscience, Breda, the Netherlands); samples were diluted (1:5) and (1: 200), respectively, in the kit-recommended diluent buffer. Human serum HMGB1 levels were analyzed using the HMGB1 ELISA kit (IBL International, Hamburg, Germany). Experiments were repeated 3 times.

Quantification of Fecal HMGB1 by Immunoblot Analysis

Twenty microgram of fecal extracts was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred in polyvinylidene fluoride membrane (Bio-Rad Laboratories) and blocked with TBS-Tween-20 0.1% (Tris-buffered saline with Tween-20) containing 5% nonfat dry milk. Antimurine HMGB1 (1:1000; Sigma, St. Louis, MO), antihuman HMGB1 (1:1000; R&D, Minneapolis, Minnesota) and anti-β-Actin (1:5000; Sigma) were diluted in TBS-Tween-20

containing 3% nonfat dry milk and incubated overnight at 4°C. Membranes were washed in TBS-Tween-20 0.1%, incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, California), washed in TBS-Tween-20 0.1%, and developed with ECL-Plus (GE Health-care, Buckinghamshire, United Kingdom). Densitometric analysis of the blots was performed using the Software ImageQuant Las500 (GE Healthcare Life Science, Uppsala, Sweden).

Colonic Myeloperoxidase Assay

Neutrophil influx and activation in murine colonic tissues were analyzed using a Myeloperoxidase Colorimetric Activity Assay kit (Sigma), according to manufacturer's instructions. Briefly, small pieces of murine colonic tissues were rapidly homogenized in 4 volumes of cold kit-provided MPO buffer and centrifuged at 12,000 rpm for 10 minutes at 4°C. Fifty microliter of clear supernatant were placed into a 96-well plate, and changes in optical density were measured at 450 nm at 30, 60, and 120 minutes. One unit of MPO activity was defined as the amount of enzyme that hydrolyzed the substrate and generated taurine chloramine to consume 1.0 μmol of Tris-NaCl-Blocking Buffer (TNB) per minute at 25°C. Concentrations were given as milli-units per milliliter.

Statistics

Statistical analysis for significance was determined using the GraphPad InStat software. Data were presented as mean ± SD. The Kolmogorov–Smirnov test was used to assess whether data were sampled from populations following the Gaussian distribution. Comparison among groups was performed using the analysis of variance ordinary test. Statistical differences between independent groups were calculated using the Kruskall–Wallis test followed by the Dunn test for multiple comparisons. Correlations between MPO activity and fecal HMGB1 data were assessed by the Spearman's rank correlation test. Differences were noted as significant *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

RESULTS

Administration of Increasing Doses of DSS Causes a Colitis of Varying Degrees of Severity in Mice

Although there are no animal models that effectively mimic human IBD, however, the DSS model of colitis in mice is commonly used to investigate mechanisms of intestinal inflammation. In this study, mice were treated for 7 days with 4 increasing doses of DSS: 0.25%, 0.5%, 1.0%, and 4.0% to induce a colitis of different grade of severity. The onset of mild–moderate–severe colitis was then verified by analyzing macroscopic (animal weight, colon length, clinical score), microscopic (histology, MPO assay), and molecular (mRNA expression of proinflammatory cytokines) endpoints.

As expected, results showed that the dose of 4% led to a severe colitis, which was macroscopically appreciable in terms
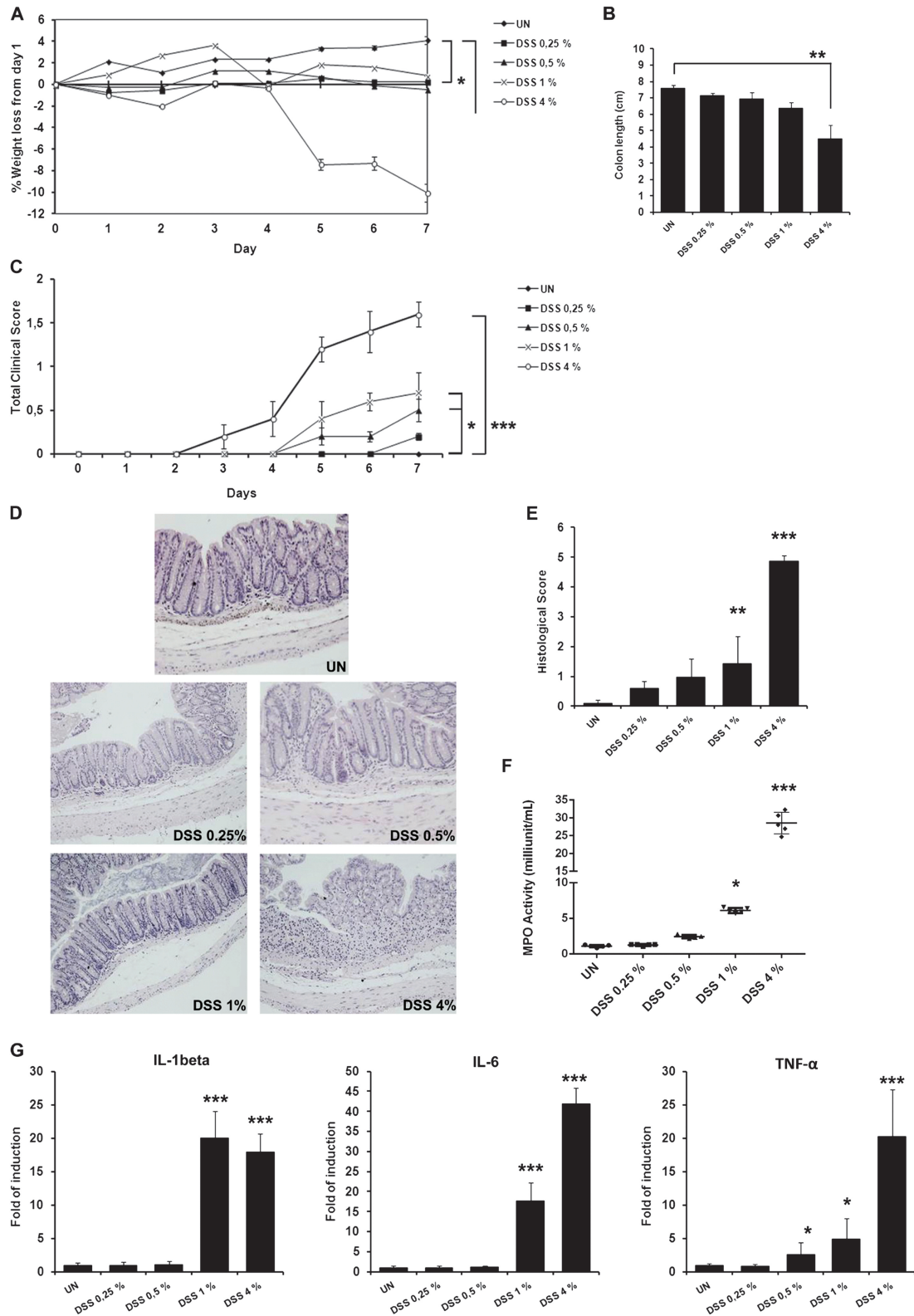
FIGURE 1. Increasing doses of DSS cause a colitis of varying degrees of severity in mice. C57BL/6 mice were administered with 4 different doses (0.25%, 0.5%, 1.0%, and 4.0%) of DSS in drinking water for 7 days and monitored every day. Following parameters were analyzed: (A) weight Loss; (B) colon Length; (C) total clinical score; (D) histology; (E) histological score; (F) MPO activity; (G) mRNA cytokine expression (IL-1β, IL-6, TNF-α). UN, untreated animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

of significant decrease of animal weight, and colon length and increase of clinical score ($P < 0.001$; $P < 0.01$; $P < 0.001$, respectively). However, at all doses, mice showed a visible growth arrest and a dose-dependent increase of clinical score, as compared with controls ($P < 0.05$) (Fig. 1A–C).

Histological score displayed a dose-dependent increase from the lowest dose (0.25%), although the effect was statistically significant from the dose of 1% ($P < 0.01$ for 1%; $P < 0.001$ for 4%) (Fig. 1D, E). This result was confirmed by the MPO assay, a measure of neutrophil infiltration, which reflects the inflammatory grade of colonic tissue ($P < 0.05$ for 1%; $P < 0.001$ for 4%) (Fig. 1F).

To investigate the occurrence of mucosal inflammation, colonic specimens of DSS-treated mice were taken, and mRNA expression levels of the proinflammatory cytokines, IL-1β, IL-6, and TNF-α were analyzed. Results showed that IL-1β and IL-6 significantly increased from the dose of 1% ($P < 0.001$). TNF-α significantly increased at increasing of DSS doses starting from the dose of 0.5% ($P < 0.05$) (Fig. 1G). These findings endorse that mice administered with increasing doses of DSS show a colitis of varying degrees of severity.

HMGB1 Is a Reliable and Early Marker of Subclinical Intestinal Inflammation in Mice

To evaluate if fecal HMGB1 is able to detect a subclinical intestinal inflammation, a time course experiment was carried out by treating mice with the same doses of DSS as above and collecting stool samples at 1, 3, 5, and 7 days posttreatment. HMGB1 levels were analyzed by immunoblotting and ELISA. Although HMGB1 was present in fecal sample of mice treated with the 2 higher doses of DSS (1% and 4%) from the third day, however, the presence of the protein was well detectable since the lowest dose (0.25%) within the seventh day. Moreover, it was shown that HMGB1 amount was DSS dose-dependent: indeed, it increased by 5-, 11-, 18-, and 24-folds in response to DSS doses of 0.25%, 0.5%, 1%, and 4%, respectively (Fig. 2A, B).

These findings show for the first time that fecal HMGB1 is an early biomarker of intestinal inflammation, and it is able to detect a high-grade and a subclinical inflammation.

We also evaluated serum level of HMGB1 by ELISA and found a positive and significant correlation between the protein and intestinal inflammation. Indeed, serum HMGB1 increased of 3.5-, 5-, 17-, and 54-folds in response to 0.25%, 0.5%, 1%,
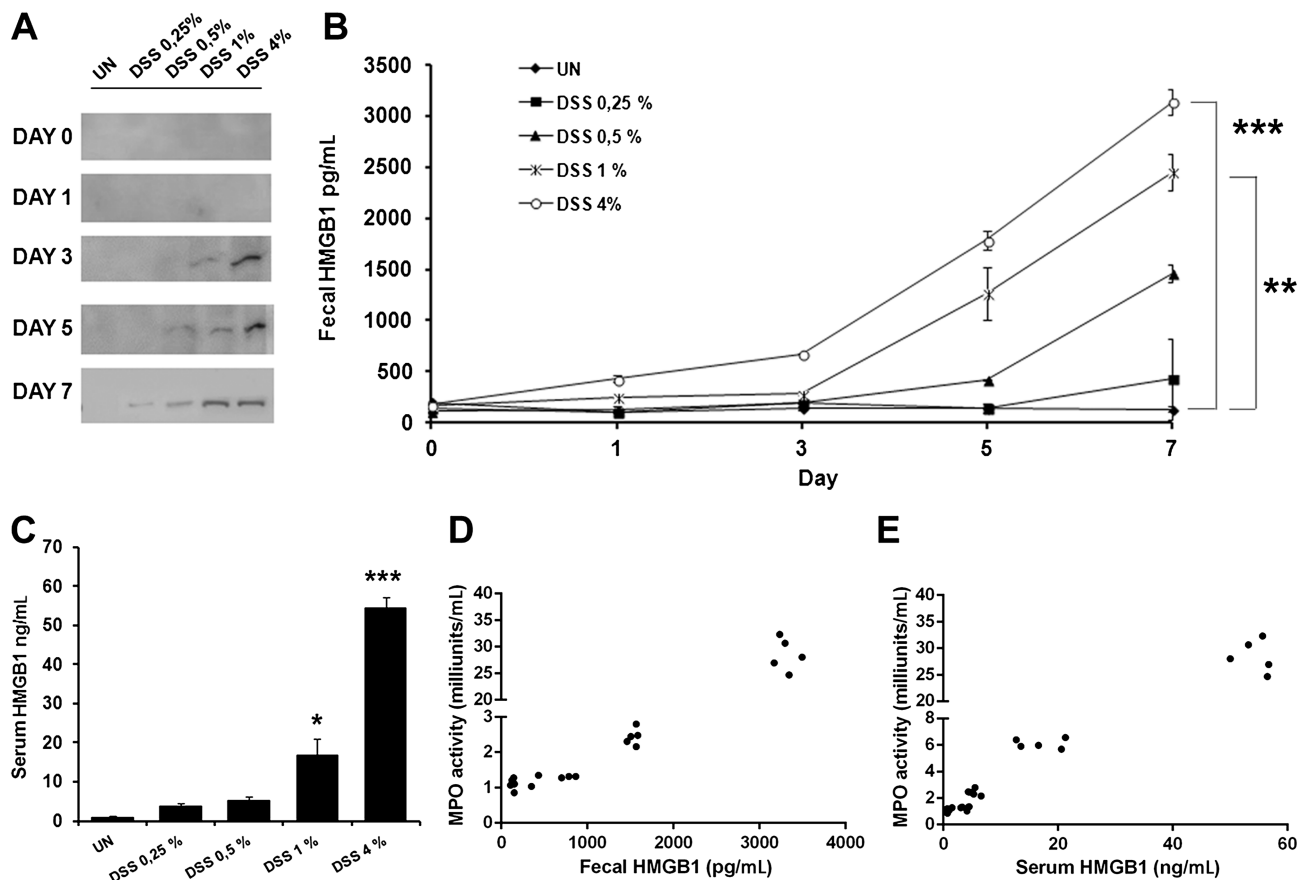

FIGURE 2. HMGB1 is a reliable and early biomarker of subclinical intestinal inflammation in mice. Fecal HMGB1 was measured in feces at day 0, 1, 3, 5, and 7 by (A) immunoblotting and (B) ELISA in mice; (C) serum HMGB1 was analyzed at the seventh day by ELISA. Correlation between MPO activity and HMGB1 levels in (D) feces ($r = 0.9544$), and (E) serum ($r = 0.9449$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

and 4% DSS doses, respectively, confirming previous results (Fig. 2C). Moreover, we correlated fecal and serum HMGB1 levels with MPO activity and found a markedly positive correlation (stools: r = 0.9544; serum: r = 0.9449) (Fig. 2D, E).

HMGB1 Normalization Is Suggestive of Colitis Amelioration and MH in Mice

We wished to demonstrate that the normalization of the protein HMGB1 was suggestive of colitis amelioration and of MH. Therefore, we treated mice with a dose of 1.5% of DSS and a dose of 4% of DSS for 7 days, to induce a moderate and a severe colitis, respectively; after treatment, mice were kept alive for other 4 weeks and then sacrificed on the 35th day. Untreated control mice were kept in the same environmental conditions of DSS-treated mice for the same period of time. Fecal samples were taken and analyzed for HMGB1 by immunoblotting and ELISA at 1, 3, 5, and 7 days of the first week (during the DSS treatment) and at the seventh day of each following week. At the end of the experiment, animal weight, colon length, clinical and histological scores, and cytokine mRNA expression of colonic specimens were measured.

All mice sacrificed the 35th day showed an almost total recovery of body weight, colon length, and clinical (data not shown) and histological scores, as compared with control mice, suggesting an improvement of colitis (Fig. 3A–C). Moreover, cytokine mRNA expression returned to control levels in colonic tissues, suggesting an occurring MH (Fig. 3D).

Interestingly, results showed that fecal HMGB1 taken from all mice, after reaching a concentration peak on the seventh day, returned to control levels at the 28th day (Fig. 3E, F).

Serum HMGB1 analyzed at the 35th day showed the same results as above (data not shown). Taken together, these findings show for the first time a close relationship between HMGB1 level normalization, colitis amelioration, and MH.

Fecal HMGB1 Levels Correlate with Mucosal Inflammation in Patients with IBD and Normalization of the Protein Is Indicative of Their MH

Fifty-one adult patients with IBD (28 with CD and 23 with UC) and 17 controls were assayed for the presence of fecal HMGB1 by immunoblotting. Patients were divided into 4 subgroups with different degrees of disease severity according to the disease activity index (CDAI and partial Mayo score): severe disease (8 patients with UC), moderate disease (13 with CD and 7 with UC), mild disease (6 with CD and 6 with UC), and inactive disease (9 with CD and 2 with UC). According to our previous study in children,[18] we found that the average value of fecal HMGB1 in adult patients was significantly increased as compared with controls, in whom the protein was undetectable ($P < 0.001$) (Fig. 4A, B). However, there was no significant correlation between the disease activity indexes and HMGB1 levels or significant difference in protein levels among all groups of patients. Considering that MH on endoscopy has become an important therapeutic goal in

IBD and that the endoscopic index is more appropriate than disease activity indexes to assess the mucosal inflammation, we tested the correlation between HMGB1 and endoscopic scores of the patients. Thus, the latter were divided into 3 subgroups with different degree of mucosal inflammation, according to the endoscopic indexes (SES-CD and endoscopic Mayo subscore)[26,27]: severe disease (5 with CD and 8 patients with UC), moderate disease (8 with CD and 10 with UC), and inactive disease (15 with CD and 5 with UC) (Fig. 4A). We showed a significant linear correlation between fecal HMGB1 levels and the endoscopic index (r = 0.763, $P < 0.001$ for SES-CD; r = 0.440, $P < 0.05$ for endoscopic Mayo subscore). Furthermore, there was a significant difference by comparing HMGB1 levels among different subgroups of patients with both CD and UC according to the endoscopic index (severe versus moderate: $P < 0.05$ for CD and UC; moderate versus inactive: $P < 0.01$ for CD and $P < 0.05$ for UC) (Fig. 4C). Interestingly, comparing fecal HMGB1 in patients classified in different subgroups (Fig 4A), it was evident a mismatch between the disease activity index and the endoscopic score in relation to patient subdivision into groups of different degree of disease severity, highlighting a certain inadequacy of the clinical score to display mucosal inflammation.

These results show for the first time the linear correlation between fecal HMGB1 and mucosal inflammation of patients with IBD and confirm that normalization of the protein is also indicative of human MH. No difference was found between serum HMGB1 of patients with IBD and controls (Fig. 4D).

DISCUSSION

Fecal markers are helpful tools in the overall management of patients with IBD, with a peculiar role in monitoring disease activity and course and in giving an ancillary information of the mucosal inflammation.[10,30] They are less invasive than colonoscopy and can be helpful in guiding management of IBD in a more cost-effective manner.

It is widely agreed that is clinically pivotal to identify the onset and the persistence of gut inflammation. Evidence is now accumulating that even a subclinical, other than a severe, intestinal inflammation may cause severe cell damage, providing a common ground in which either cancer or other disorders may develop.[31,32] Thus, it would be very welcome the availability of readily measured markers able to detect clinically evident and subclinical inflammation of the gut mucosa.

We have previously suggested the potential use of HMGB1, an alarmin abundantly secreted in the stools of pediatric patients with IBD, as a fecal biomarker of intestinal mucosal inflammation.[18] In this study, we wished to expand the use of the protein also in the adult disease, and, more interestingly, to produce evidence that the protein is able to clearly detect both a clinically evident and a subclinical gut inflammation. For this purpose, we set up an experimental protocol to induce in mice a colitis of different severity degrees (from mild to severe), by administering increasing doses of DSS. We showed for the first time that fecal HMGB1 levels were unambiguously indicative of low-grade intestinal inflammation in
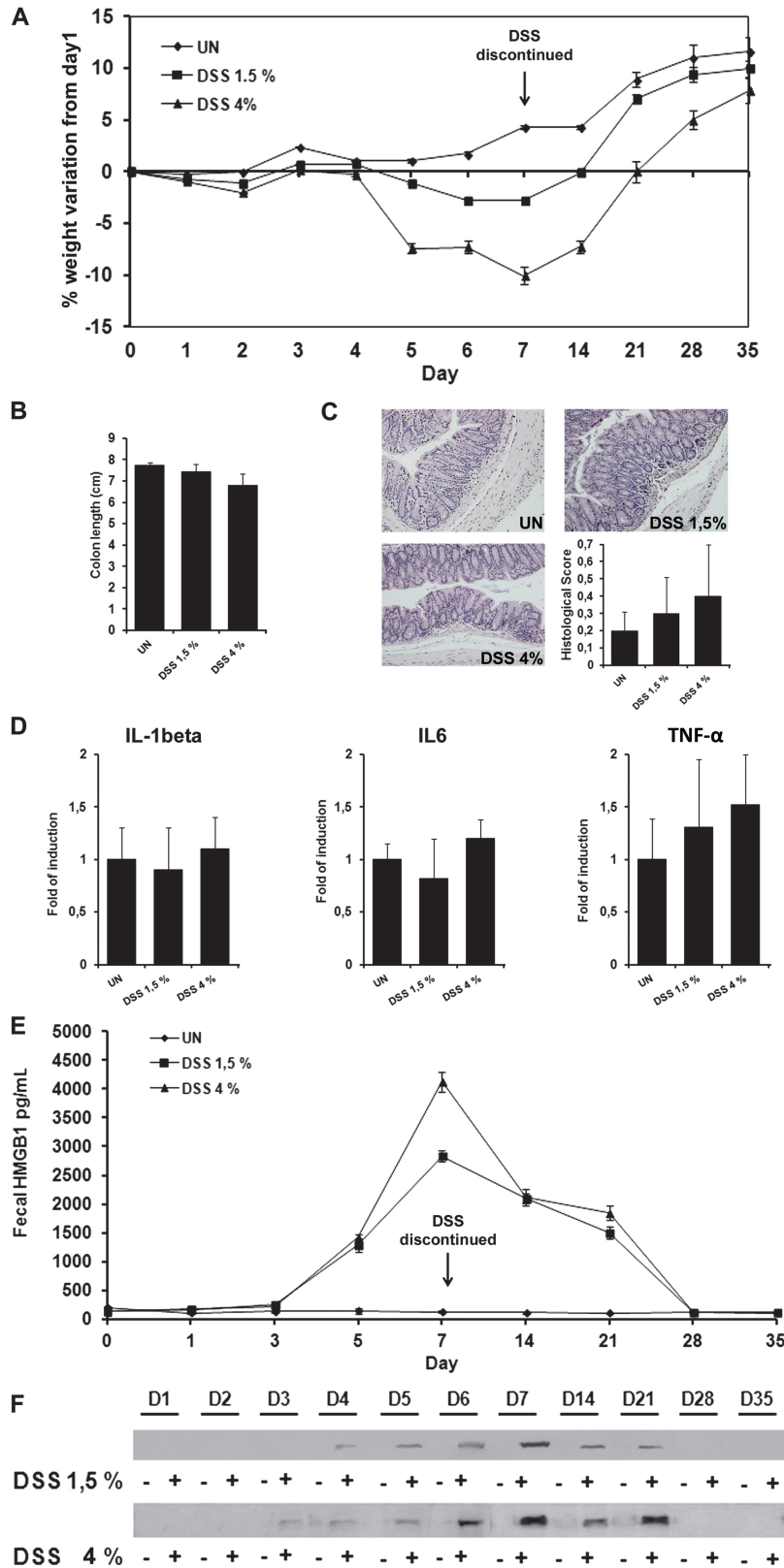
FIGURE 3. Fecal HMGB1 normalization indicates colitis amelioration and MH in mice. Mice were administered with 1.5% DSS or 4% DSS for 7 days, untreated for following 28 days and killed on the 35th day. Following parameters were analyzed: (A) weight Loss, (B) colon Length, (C) histological Score, (D) mRNA cytokine expression (IL-1β, IL-6, TNF-α); (E) fecal HMGB1 measured by ELISA, and (F) immunoblotting.
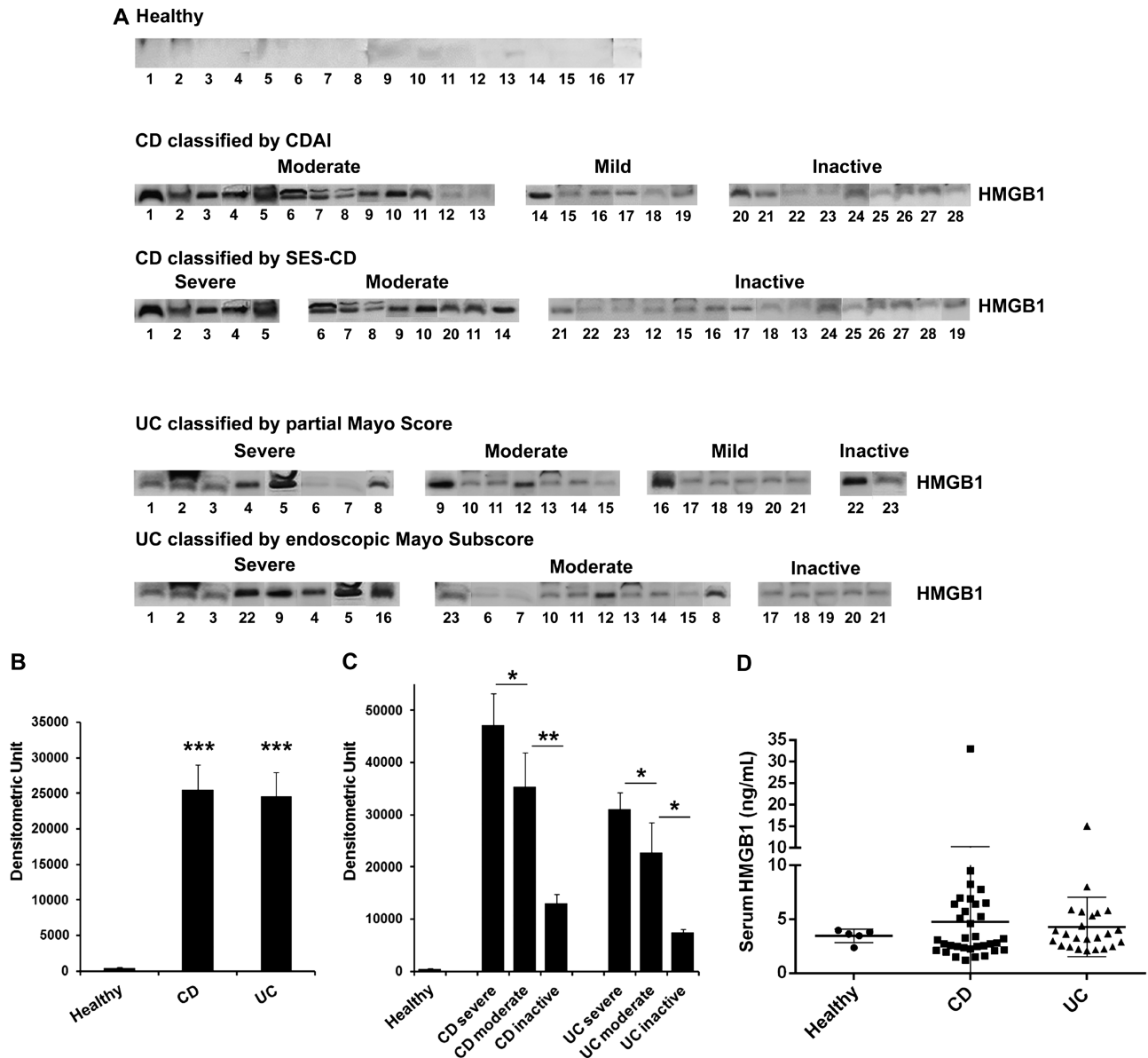
FIGURE 4. Fecal HMGB1 correlates with mucosal inflammation in patients with CD and UC. HMGB1 normalization is indicative of MH. A, Immunoblotting of fecal HMGB1 in healthy controls and patients with IBD divided into subgroups according to disease activity indexes (CDAI, partial Mayo score) and endoscopic indexes (SES-CD, endoscopic Mayo subscore); the same number identifies the same patient; (B) densitometric analysis in total CD, UC, and controls and (C) in subgroups according to endoscopic indexes; (D) serum HMGB1 analyzed by ELISA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

mouse model, suggesting its role as a trustworthy and early biomarker of subclinical intestinal inflammation.

Furthermore, we aimed at correlating the HMGB1 normalization with gut MH. The structural basis of MH is the restitution of gut epithelium that prevents translocation of commensal bacteria into the mucosa and subsequent immunostimulation. This is an intriguing point as MH has emerged as a major treatment outcome in IBD. Indeed, although the treatment of IBD has been traditionally focused on reaching clinical remission, without requiring a demonstration of MH, recent studies suggest that achieving

MH engenders better long-term results, such as less hospitalization and surgery as well better quality of life, with a critical impact on the natural history of the disease.[4,5,33,34] Remarkably, the advent of biological agents has shown that inducing and maintaining MH is a realistic outcome in a significant proportion of treated patients, thus altering dramatically the disease course.[35,36]

In our study, we induced a moderate as well as a severe colitis by treating mice with a low (1.5%) and a high (4%) dose of DSS for a week and evaluated the level of fecal HMGB1 after 4 weeks posttreatment, to allow the mucosa recovery, as assessed

by the reduced histological score, the MPO activity and the expression of potent proinflammatory mediators (TNF-α, IL-1β, IL-6) in the tissue colonic specimens. Interestingly, we found in all treated mice that fecal HMGB1 strongly increased during treatment and then decreased to control levels during the recovery time, suggesting a close relationship between HMGB1 normalization and MH in both severe and moderate colitis. These findings show for the first time that fecal HMGB1 normalization is a suitable tool to document the intestinal MH.

Results in the animal model were then assayed in humans by analyzing the stools of adult patients with both CD and UC. Patients were divided into groups with severe, moderate, mild, or inactive disease according to the activity disease indexes (CDAI and partial Mayo score), traditionally used as the gold standard in clinical trials. We found a significant difference in HMGB1 levels between stools of patients and healthy controls, confirming our previous results in the pediatric IBD population. Differently, there was no correlation between HMGB1 levels and activity index or difference among groups of patients with diverse degrees of disease severity. Considering that the activity disease indexes are mainly based on clinical symptoms and laboratory parameters, there are growing evidences that their correlation with mucosal lesions may be weak.[1,37] Therefore, we wished to use the endoscopic indexes (SES-CD and endoscopic Mayo subscore), as a more reliable measure of mucosal inflammation and to split again patients into subgroups with different severity of inflammation. Intriguingly, we found that HMGB1 levels significantly correlated with the endoscopic scores, and that differences among all patient groups were statistically significant both for CD and UC. Moreover, the fecal protein was able to detect even a very low, but persistent, inflammation, as seen in those patients belonging to the clinically inactive group. As the decreasing of the endoscopic score was always paralleled by a lowering protein expression, up to the control levels, we suggest that the progression of HMGB1 toward normalization values is indicative of a gradual MH.

These findings, all together, show that fecal HMGB1 is a suitable marker of subclinical inflammation and MH in patients with IBD. We did not find evidence that the serum HMGB1 is somewhat suggestive of human intestinal inflammation.

It is noteworthy the mismatch between the disease activity index and mucosal lesions in highlighting the mucosal inflammation: indeed, some patients in clinical remission showed a severe-to-moderate inflammation and other patients with severe clinical symptoms displayed a mild-to-moderate inflammation. The evidence that clinical symptoms, as scored by the disease activity indexes, are not a reliable measure of the underlying gut inflammation has been very recently emerged by a study,[37] showing a discrepancy between the CDAI and the endoscopic mucosal appearance in a large populations of patients with CD. Because of the clinical weight of measuring and knowing the degree of mucosal inflammation in patients with IBD, as the disease is by definition inflammatory in nature, we wish to emphasize the need for tools capable of providing prompt information about the inflammatory status of the mucosa, mostly in those patients with mild or no clinical symptoms.

In conclusion, in a previous study, we showed the role of fecal HMGB1 as a novel marker of intestinal inflammation in IBD. In this study we provide a well-supported evidence that the protein may represent a robust noninvasive biomarker of subclinical gut inflammation, in addition to clinically evident inflammation, suitable to monitor patients during the disease course and to assess the therapy outcome. Furthermore, we show as well that fecal HMGB1 normalization could be a novel biomarker of MH. This study emphasizes the importance of HMGB1 as a non-invasive tool easy and quick to use; furthermore, it highlights the need for deeper knowing the peculiatities of the protein, for example its ability to discriminate between functional and organic disorders. This question will be subject of future investigations on larger size populations with intestinal inflammatory disorders other than IBD, such as allergic colitis, irritable bowel syndrome and necrotizing enterocolitis.

ACKNOWLEDGMENTS

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Erratum

Functional Polymorphisms in the Regulatory Regions of the VNN1 Gene Are Associated with Susceptibility to Inflammatory Bowel Diseases: ERRATUM

In the article on page 2315, volume 19, issue 11, an author was mistakenly not included in the author listing. Mathias Chamaillard, PhD is a contributing author for this article. Dr. Chamaillard's affiliation is Inserm U1019 Centre d'Infection et d'Immunité de Lille, Lille, France.

The order of authors is as follows: Thomas Gensollen, Christophe Bourges, Pascal Rihet, PhD, Agathe Rostan, Virginie Millet, Tetsuro Noguchi, PhD, Violene Bourdon, PhD, Hagay Sobol, MD, PhD, Laurent Dubuquoy, PhD, Benjamin Bertin, PhD, Maturin Fumery, MD, Pierre Desreumaux, MD, PhD, Jean-Frédéric Colombel, MD, PhD, Mathias Chamaillard, PhD, Xavier Hebuterne, MD, PhD, Paul Hofman, MD, PhD, Philippe Naquet, MD, PhD, and Franck Galland, PhD.

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