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Molecular Signature of Persistent Histological Inflammation in Ulcerative Colitis with Mucosal Healing

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ABSTRACT

Background & Aims: Therapeutic targets in ulcerative colitis (UC) have evolved over time from clinical remission to biological and endoscopic remission. Histologic remission remains a debatable outcome due to lack of data regarding its impact on long-term evolution. The development of histologic activity scores has brought standardization. We aimed to identify mucosal markers differentiating histological inflammation from histological remission in UC patients.

Methods: The gene expression levels of 84 genes associated with inflammatory bowel diseases have been analyzed in 43 colonic mucosa samples from 30 patients with UC. The gene expression levels have been correlated with histological inflammation score of Geboes. Patients with endoscopic remission were divided by histological activity into two groups and molecular results were compared in order to identify differences in the mucosal gene expression.

Results: We found a significant Pearson correlation ($p<0.001$ and $r>0.5$) between the Geboes score and the expression of 29 genes, whereas negative correlation ($p<0.001$ and $r<-0.50$) was observed with two genes in the entire UC cohort. In the subgroup of patients with endoscopic remission three transcripts: formyl-peptide receptor 1 (FPR1), matrix metalloproteinases 1 (MMP1) and mucine 1 (MUC1) were significantly up-regulated in patients with histological inflammation compared to patients with histologic remission.

Conclusion: Our study further emphasizes the importance of histological assessment when endoscopic mucosal healing is present, as FPR1, MMP-1 and MUC1 were all significantly upregulated in patients with histological alterations.

Key words: ulcerative colitis - gene expression - mucosal healing – inflammation.

Abbreviations: CD: Crohn disease; ECCO: European Crohn's and Colitis Organization; End+: macroscopic inflammation; End-: mucosal healing; FRP1: formyl-peptide receptor 1; Hist-: histologic remission; Hist+: histologic activity; IBD: inflammatory bowel disease; MMP1: metalloproteinases 1; MUC1: mucine 1; RHI: Robarts Histopathology Index; STRIDE: Selecting Therapeutic Targets in Inflammatory Bowel Disease; T2T: treat-to-target; UC: ulcerative colitis.

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease affecting the colon to different extents from only a few centimeters of the rectum to extensive pancolitis. The clinical picture of patients suffering from UC varies from asymptomatic inflammation to severe bloody diarrhea, motility dysfunction of the colon, potentially tissue damage with colonic fibrosis, systemic symptoms and finally, the need for surgery [1, 2]. Almost a third of the patients (31%) with UC and limited disease extension at diagnosis will suffer from disease extension after 10 years of evolution, with 10-15% of patients finally requiring surgery [3, 4]. The achievement of mucosal healing has been shown to lower the need for surgery in UC patients, as colectomy is not curable and is associated with complications in approximately a third of the patients [4-7].

In 2015, the Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE) committee proposed the treat-to-target (T2T) approach for inflammatory bowel disease (IBD) changing the goal of treatment and aiming to prevent disease complications as dysplasia/cancer, hospitalizations and colectomy by monitoring objective disease activity parameters (e.g. fecal biomarkers, endoscopic evidence of inflammation) [8–10]. Using the T2T approach in UC, histologic remission

is not currently recommended as a therapeutic target due to lack of validation in terms of impact on disease evolution [8].

Histologic remission in UC is defined as normalization of the microscopic image of colonic mucosa [11]. There are more histological activity scores available, with the Nancy index and the Robarts Histopathology Index (RHI) being the most studied ones [12]. However, recent data comparing all four available histological scores have shown similar reliability and responsiveness [13]. Histological remission has been shown to be associated with clinical remission in 87% of patients at 1 year from baseline scoring in a prospective observational study [14]. Moreover, achieving histological remission in UC correlates with corticosteroid-free remission and absence of clinical recurrence after 3 years of follow-up, being associated to lower hospitalization and corticosteroid use rates after a medium 6 years of follow-up [15–17]. Another retrospective study showed that histological remission was a better predictor for relapse-free survival than endoscopic healing or histologic quiescence [18]. Furthermore, data form an observational cohort showed a strong association between the Geboes score at baseline and the risk of clinical relapse in UC patients who are in clinical remission at 12 months [19].

Even though these results show a good association between histology and disease outcomes, their lack of validation limit their use in clinical practice. In the future, molecular studies may offer supplementary tissue information to complement the histologic examination in UC. In fact, preliminary data from our cohort has identified mucosal mRNA levels of interferon-stimulated gene 15 (*ISG15*) to be associated with clinical remission in UC [20].

In this study, we evaluated the correlation between gene expression profile and the histologic activity assessed by the Geboes score. We aimed to identify specific genes differentiating histological inflammation from histological remission in UC patients with endoscopic healing.

METHODS

Patients

We analyzed 43 colonic mucosal samples from 30 Romanian UC patients admitted to the Gastroenterology Departments of the Elias Emergency University Hospital and Fundeni Clinical Institute in Bucharest, Romania. The colon biopsies were performed in the sigmoid colon in the case of patients with Montreal E2 and E3 and for the three E1cases were taken from the rectum.

The present study has been approved by the local Ethics Committee from the Elias Emergency University Hospital (Registration number 6598 of 11th of May 2015), and from the Fundeni Clinical Institute (Registration number 8007 of 23rd February 2018). All the recruited patients signed a written informed consent prior to biopsy sampling. For some patients, multiple endoscopic evaluation and biopsies were performed during different time points. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and its later amendments. Disease diagnosis was made following the available guidelines from the European Crohn's and Colitis Organization (ECCO) [21].

Macroscopic inflammation (End+) was defined using the endoscopic Mayo subscore, while mucosal healing (End-) was defined as a Mayo subscore of 0 [22].

Histological inflammatory activity was evaluated using the Geboes index, with histologic remission (Hist-) defined as a Geboes index of 0 and histologic activity with a Geboes > 0 (Hist+) [23].

Gene expression analysis

The RNA isolation from fresh-frozen biopsies, preserved in RNA later solution, was performed using RNeasy mini kit (Qiagen, Germany). RNA was quantified by the spectrophotometric method (NanoDrop 2000, Thermo Scientific); only samples with both 260/280 nm and 260/230 nm parameters > 1.8 were included in the study. Reverse transcription was performed with the RT2 First Strand Kit (Qiagen) using 600 ng of RNA and qPCR (performed with Human Crohn's Disease RT2 Profiler PCR Array -PAHS-169Z, Qiagen) on on the ABI-7500 fast instrument (Applied Biosystems) as previously described [20]. Genomic DNA contamination detection was performed using the dedicated wells in the array. The normalization on the geometric mean values of two selected housekeeping genes (GAPDH and HPRT1) was performed after the analysis of five candidate reference genes (ACTB, B2M, GAPDH, HPRT1, and RPLP0) on RefFinder algorithm, as previously described by our research group [24]

Statistical analysis

Correlations between gene expression levels and the Geboes score were calculated using the Pearson coefficient. The Shapiro–Wilk normality test was performed for each gene level. Since data were not normally distributed, differences in gene expression between the groups were evaluated using the nonparametric Mann-Whitney U test. Statistical analysis was performed using the Statistical Package for Social Science (SPSS version 17.0).

RESULTS

The demographic and clinical data of the patients are summarized in Supplementary Table I. Fig. 1 illustrates the study design and a summary of the main findings. In this study the gene expression levels of 84 target genes (Supplementary Table II) were evaluated in 43 colonic mucosal samples categorized in three groups: End+/Hist+ (n=25), End-/Hist+ (n=5) and End-/Hist- (n=13). The three groups were homogenous for age (with an age average of 44.4±12.7; 42.6±14.8 and 44.9±13.2, respectively, p>0.05) and gender (% of male 80%, 80% and 77%, respectively, χ^2=0.052, p=0.974). The therapeutic regimen was heterogeneous among the groups at the biopsy recruitment. However, the majority of the patients were in treatment with 5-ASA (Supplementary Table I). Images with the histological evaluation of two UC mucosal biopsies (GEBOES 0.1 and GEBOES 5.2) are shown in Figs. 2a and 2b. The gene expression results showed that the expression of 29 genes was positively correlated with the Geboes score (r>0.050, p<0.001), whereas for two genes a negative correlation was observed (r<-0.050, p<0.001). The majority of the genes found positively correlated with the Geboes, are involved as expected

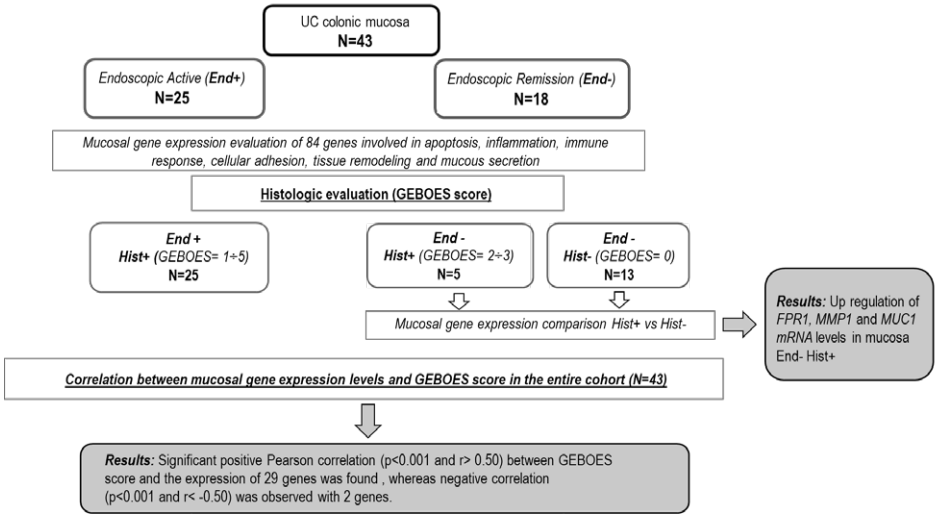


Fig. 1. Flowchart of the study design. The Geboes reported in the figure refers to Geboes grading without a sub score that is specified for each patient in Supplementary Table I.

in inflammation and many of them belong to extracellular matrix and cell adhesion mechanisms (ITGB2, SELL, VWF, CCR1, MMP10, MMP3 and MMP7). The strongest correlation, with a Pearson coefficient above 0.70 was observed with a signal transducer and activator of transcription 1 (STAT1). The results reporting the characteristics of genes and correlations are shown in Fig. 3a; the correlation plots of the most significant genes in each category are shown in Fig. 3b. When considering the subgroup of biopsies with endoscopic remission, we found that formyl-peptide receptor 1 (FPR1), matrix metalloproteinases 1 (MMP1) and mucine 1 (MUC1) were significantly up-regulated in patients with histological inflammation compared to patients with histologic remission. (Fig. 4). The principal component analysis (PCA) plot based on the expression profile of the significant genes is shown in Fig. 5.

DISCUSSION

Mucosal gene expression has yielded interesting results regarding the pattern of inflammation both in UC and Crohns' disease (CD) [24, 25]. Guided by the growing interest towards achieving histologic remission, we steered towards the molecular basis underneath.

Firstly, the aim of our study was to identify differences in gene expression profiles based on histological mucosal characteristics in UC (the severity of histological inflammation evaluated through the Geboes score). Furthermore, we wanted to emphasize the importance of histological assessment in ulcerative colitis even in endoscopic remission. Therefore, we compared the gene expression levels between tissues with and without histological inflammation in 18 UC patients with normal endoscopic mucosa.

We identified thirty-one genes with significant correlation with the Geboes score of histological activity. Even though this could be perceived as an inherent finding, obtaining quantifiable results underlines the sensibility of the method. Additionally, if the severity of histological lesions parallels the magnitude of gene expression, molecular pathways could be conjectured as part of the pathophysiological process.

Isolating the subgroup without endoscopic lesions, we found three transcripts: FPR1, MMP1 and MUC1, with significantly higher expression in the Histo+ subgroup of patients. The persistence of mucosal gene abnormalities and histological inflammation despite endoscopic healing is proof of the necessity for long-term maintenance therapy (to prevent relapses).

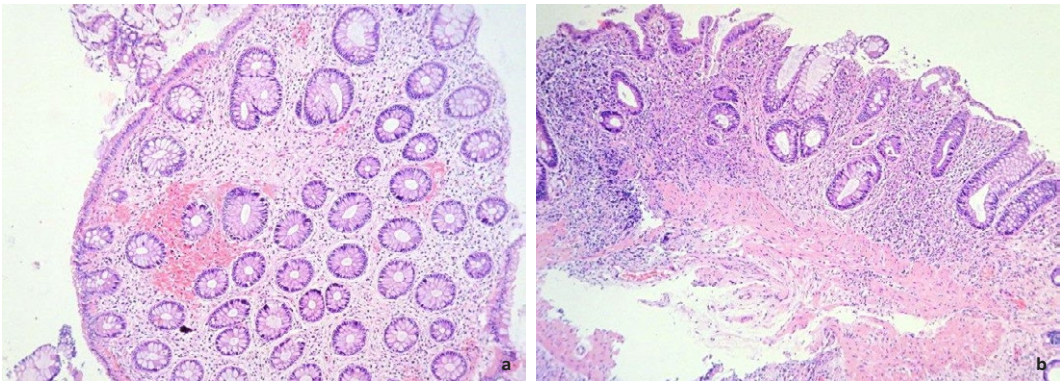
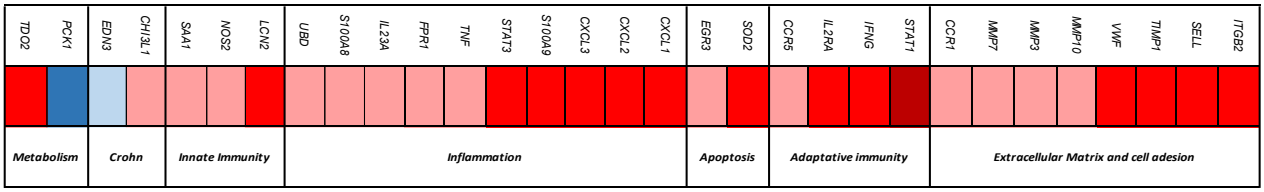
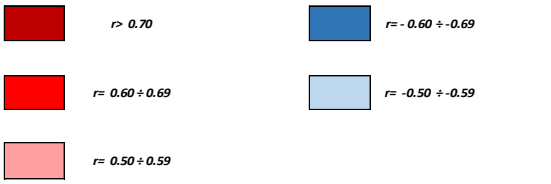
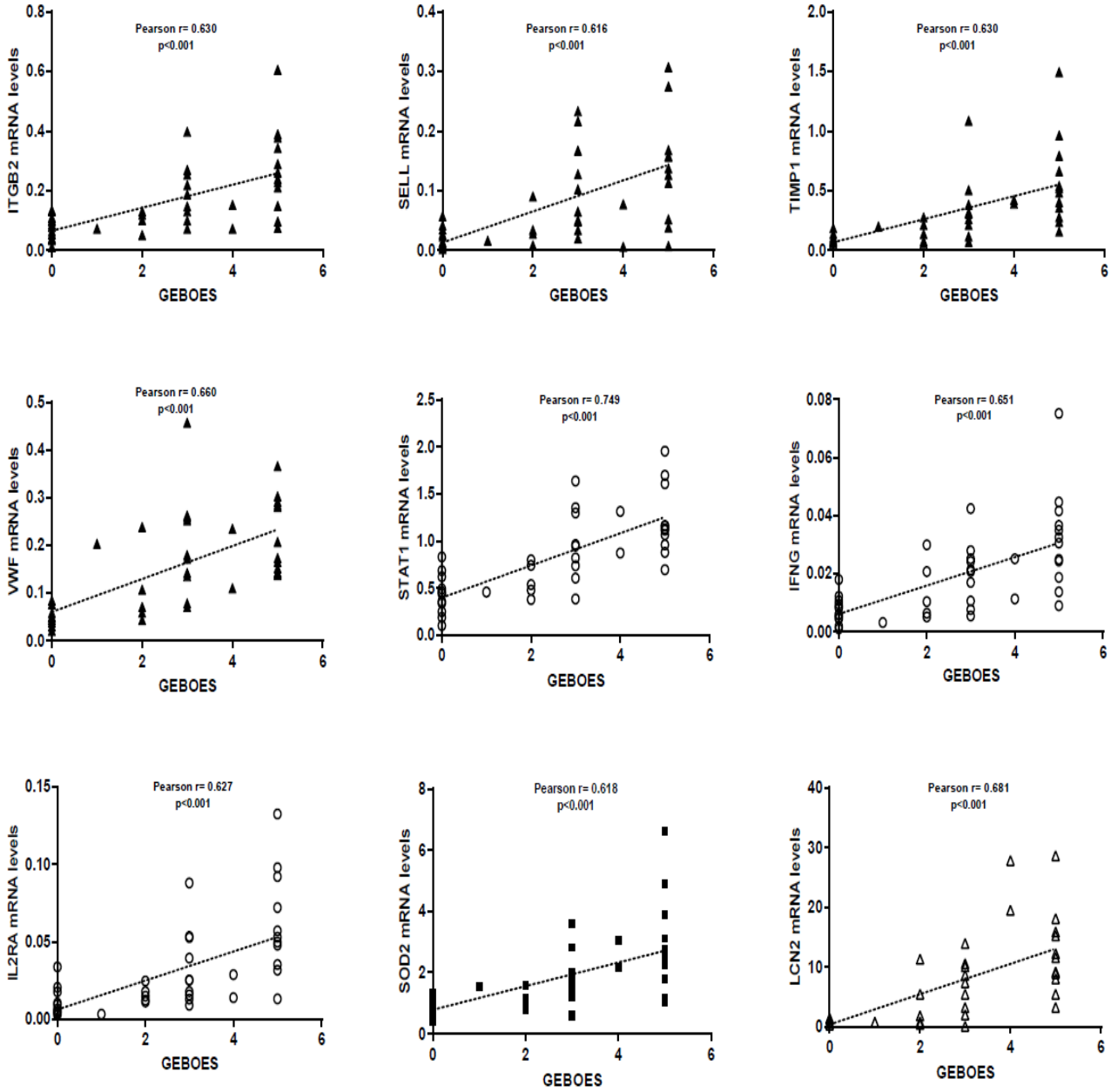


Fig. 2. (a) Geboes score 0.1; (b) Geboes score 5.2. Hematoxylin–Eosin (HE) staining, ×200.


TDO2
PCK1
EDN3
CHI3L1
SAA1
NOS2
LCN2
UBD
S100A8
IL23A
FPR1
TNF
STAT3
S100A9
CXCL3
CXCL2
CXCL1
EGR3
SOD2
CCR5
IL2RA
IFNG
STAT1
CCR1
MMP7
MMP3
MMP10
VWF
TIMP1
SELL
ITGB2
Metabolism
Crohn
Innate Immunity
Inflammation
Apoptosis
Adaptative immunity
Extracellular Matrix and cell adesion


r> 0.70
r= 0.60 ÷ 0.69
r= 0.50 ÷ 0.59
r= - 0.60 ÷ -0.69
r= -0.50 ÷ -0.59

a


Pearson r= 0.630
p<0.001
ITGB2 mRNA levels
GEBOES
Pearson r= 0.616
p<0.001
SELL mRNA levels
GEBOES
Pearson r= 0.630
p<0.001
TIMP1 mRNA levels
GEBOES
Pearson r= 0.660
p<0.001
VWF mRNA levels
GEBOES
Pearson r= 0.749
p<0.001
STAT1 mRNA levels
GEBOES
Pearson r= 0.651
p<0.001
IFNG mRNA levels
GEBOES
Pearson r= 0.627
p<0.001
IL2RA mRNA levels
GEBOES
Pearson r= 0.618
p<0.001
SOD2 mRNA levels
GEBOES
Pearson r= 0.681
p<0.001
LCN2 mRNA levels
GEBOES

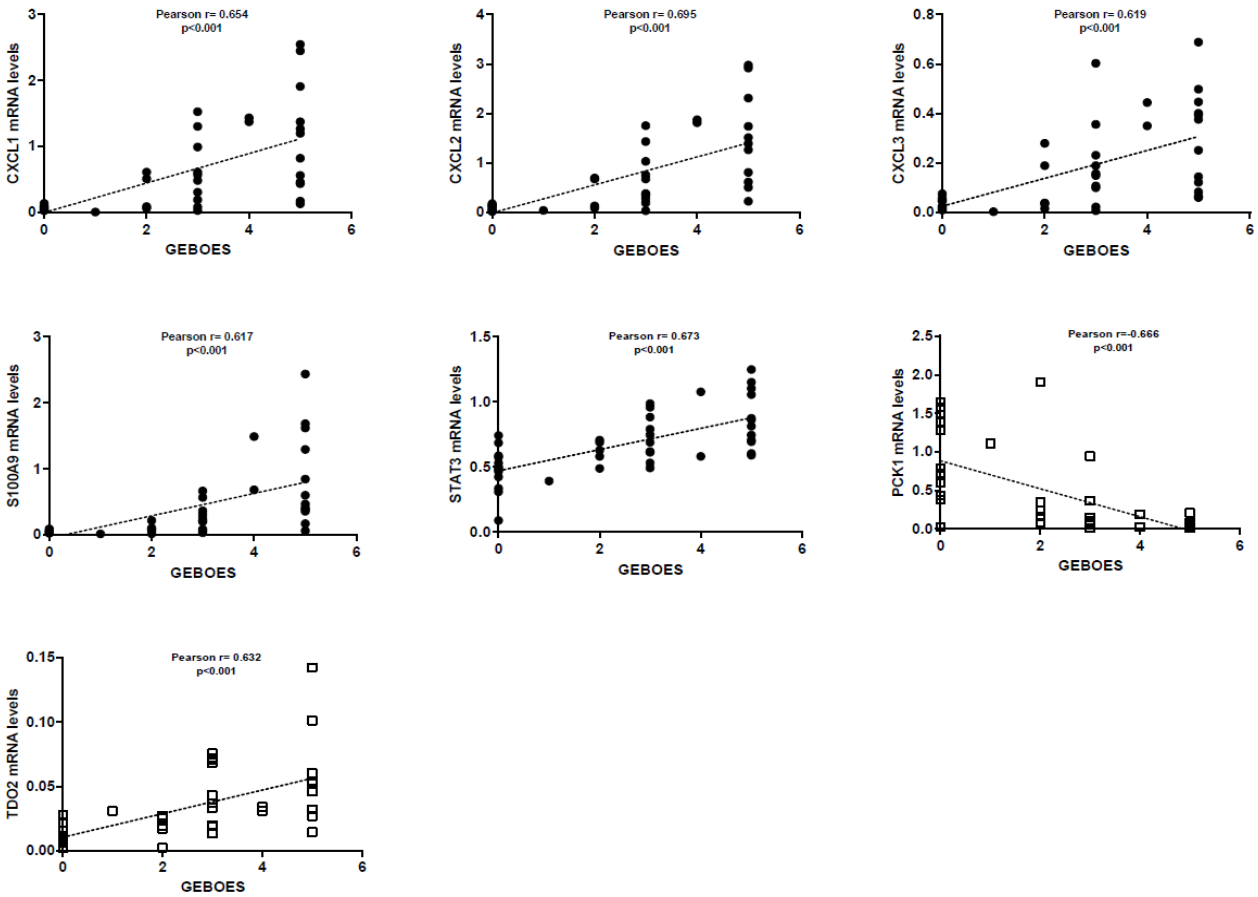


Fig. 3. (a) The heat map shows the significant correlations between the Geboes score and the expression of genes (p< 0.001). Pearson r coefficient is reported with different colors according to the strength and the direction of the association. (b) Correlation plots between mRNA levels (expressed as 2^-ΔCT) and Geboes of the most significantly correlated genes for each category. The black triangles represent genes involved in Extracellular Matrix mechanisms; the white circles refer to the genes involved in Adaptive immunity; the black squares refer to the genes involved in Apoptosis; the black circles refer to the genes implicated in inflammation, while the white triangles refer to genes of the innate immunity and the white squares refer to the genes which have a role in the metabolism.
Legend: CCR: chemokine (C-C motif) receptor; CHI3L1: chitinase 3-like 1; CXCL1: chemokine (C-X-C motif) ligand; EGR3: early growth response 3; EDN3: endothelin 3; FPR1: formyl peptide receptor 1; IL2RA: interleukin 2 receptor, alpha; IL23A: interleukin 23, alpha subunit p19; INFG: interferon gamma; ITGB2: integrin, beta 2 (complement component 3 receptor 3 and 4 subunit); LCN2: lipocalin 2; MMP: matrix metallopeptidase; NOS2: nitric oxide synthase 2, inducible; PCK1: phosphoenolpyruvate carboxykinase 1; SAA1: serum amyloid A1; SELL: selectin L; SOD2: superoxide dismutase 2, mitochondrial; STAT: signal transducer and activator of transcription; S100A8: S100 calcium binding protein A8; S100A9: S100 calcium binding protein A9; TDO2: Tryptophan 2,3-dioxygenase; TIMP1: TIMP metallopeptidase inhibitor 1; TNF: tumor necrosis factor; UBD: Ubiquitin D; VWF: Von Willebrand factor.

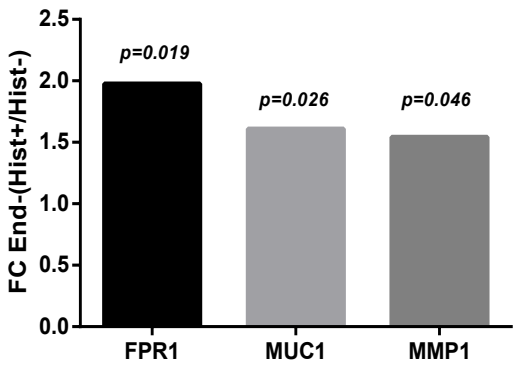


Fig. 4. The bar graphs represent the Fold Change showing the upregulation (FC>1.5) of the three genes differentially expressed in the biopsies with endoscopic remission (End-), comparing biopsies with histological inflammation (End- Hist+) with those with histologic remission (End- Hist-)

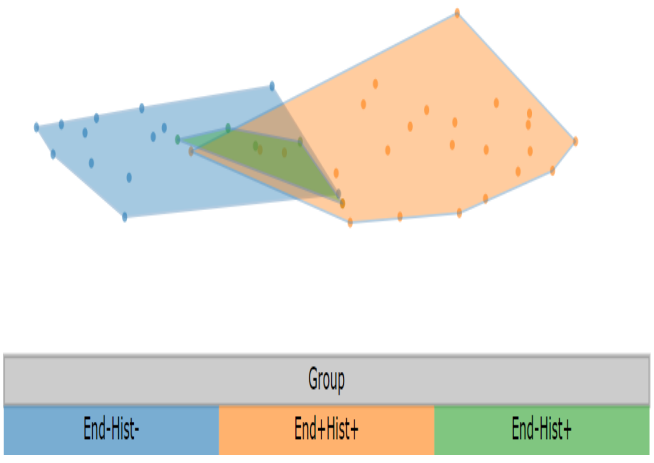


Fig. 5. PCA plot based on the expression profile of the significant genes.

As we consider these findings noteworthy for further research in the field of IBD, we will address each transcript individually.

FPR1 encodes a G-coupled protein receptor (GCPRs) which plays a crucial role in inflammation, through its implications in neutrophil sensing and chemotaxis. FPR1 is the first member described in this family, being responsible for multiple functions upon activation such as degranulation, reactive oxygen species (ROS) synthesis and phagocytosis [26, 27].

Studies targeting FPR families have revealed their role in rapid neutrophil mobilization in murine models, with increased wound healing capacities in sterile skin lesions by neutrophil infiltration [28]. In the intestinal lumen, where N-formyl peptides are elevated due to bacterial burden, the neutrophyls mediated response is of particular importance. Increased intramucosal levels of these peptides, possibly as a consequence of epithelial barrier damage, can contribute to the activation of FPR1 and the development of intestinal crypt abscesses in IBD [25]. In our study, increased FPR1 gene expression was obtained in UC patients with endoscopic mucosal healing but with persistent inflammation on histology as compared to patients with histological remission. Consequently, this emphasizes that FPR1 might be involved in wound healing. This is, to our knowledge, the first literature report linking FPR1 to UC in humans.

MMP-1 is part of a family of 24 zinc dependent endopeptidases (The Human Matrix Metalloproteinase family) [29]. Most MMPs have been found to be upregulated in response to proinflammatory cytokines, with a variety of cellular sources including epithelial cells, mesenchymal cells and leukocytes [30]. MMP-1 has been found to be upregulated in inflamed mucosa from patients with UC and its levels of expression correlated with the severity of inflammation [31]. MMP-1 and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels in plasma and colonic mucosa of patients with UC are correlated with disease severity and MMP-1/TIMP-1 ratio can be used as a quantitative parameter for the severity of the inflammatory process [32]. Most of the studies on MMPs have shown their increased levels in injured mucosa of IBD patients, but our study demonstrates increased mucosal levels in macroscopically normal mucosa with microscopic inflammation, making it a possible surrogate marker to be further evaluated and validated. In fact, fecal levels of MMP-9 have been shown to be capable of differentiating between functional diarrhea and UC, and also to correlate with fecal calprotectin with regard to inflammation severity [33]. Moreover, MMP-1 has already been established as a serologic biomarker for chronic progressive lung disease and as a urinary biomarker for urothelial cancer, thus confirming its stability as a secretory protein and making it a possible candidate for fecal testing in UC [34, 35].

MUC1 is part of the produced by the intestinal Goblet cells. It is mostly found in small intestinal cells, but it can also be expressed on the apical membrane of colonic Goblet cells [36–38]. Mucins are secreted either basal or stimulated by microbes, toxins, proinflammatory cytokines or neuropeptides, these factors contributing to a change in the consistency of the mucosal colonic barrier with increased intestinal permeability, mucosal damage and finally, systemic inflammatory response seen in patients with ulcerative colitis [39, 40]. In murine studies, MUC1 has been shown to be linked with the interleukin-17 (IL-17) pathway, being up-regulated by the T-helper 17 cells and acting through a negative feedback thus preventing an excessive Th-17 response in colons of mice [41]. Furthermore, vaccines using MUC1 as antigen in murine models have been shown to be able to delay the appearance of IBD and to prevent progression of IBD to colitis associated colorectal cancer [42, 43]. Our findings are consistent with the literature regarding the link between the inflammatory process associated to UC and the mucosal upregulation of MUC1.

There are some limitations in this study. Firstly, disease duration might contribute to changes of the mucosal inflammatory pathways with an impact on the gene expression profiles. Secondly, the differences in previous and current treatment of the disease, especially anti-TNF interfere with the level of expression for some of the studied genes [44]. Another limitation is represented by the small sample size of the subgroup of the endoscopically normal mucosa samples in which we compared the gene expression levels between biopsies with histological inflammation to those with histologic remission. Moreover, examining associations with histologic remission, the correction for multiple comparisons has not been applied.

CONCLUSIONS

We identified thirty-one genes that were correlated with the Geboes score of inflammation in UC mucosa. Furthermore, in the endoscopically normal mucosa we isolated three transcripts whose levels were differently expressed between histologically active and inactive UC. FPR1, MMP1 and MUC1 were all significantly upregulated in patients with histological alterations. Our study further emphasizes the importance of histological assessment even when endoscopic mucosal healing is present. This is a convincing argument that disease management should also take into account histological remission for therapeutic de-escalation or discontinuation of therapy, as there is a risk of disease recurrence. Despite the limitations in our study, we find the results attractive for further research on larger cohorts of patients in order to confirm the results, elaborate on physiopathological mechanisms and possibly develop new biomarkers in order to evaluate histological activity in UC patients.

Conflicts of interest: None to declare.

Authors' contribution: M.M. and E.M.I.: study design and management; E.M. and M.D.: gene expression study, data analysis and manuscript preparation. I.T.: edited and reviewed the manuscript. C.M.P., T.E.M., C.G.T., M.M.D.: endoscopy, clinical evaluation and mucosal samples collection. G.B.: histological evaluation. All the authors approved the final revision of the manuscript.

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Supplementary material: To access the supplementary material visit the online version of the *J Gastrointestin Liver Dis* at http://dx.doi.org/10.15403/jgld-576

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