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Mucosal *CCR1* gene expression as a marker of molecular activity in Crohn's disease: preliminary data

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Abstract

Aim: A series of mechanisms of immune response, inflammation and apoptosis have been demonstrated to contribute to the appearance and evolution of Crohn's disease (CD) through the overexpression of several cytokines and chemokines in a susceptible host. The aim of this study was to identify the differences in gene expression profiles analyzing a panel of candidate genes in the mucosa from patients with active CD (CD-A), patients in remission (CD-R), and normal controls. *Patients, Materials and Methods*: Nine individuals were enrolled in the study: six CD patients (three with active lesions, three with mucosal healing) and three controls without inflammatory bowel disease (IBD) seen on endoscopy. All the individuals underwent mucosal biopsy during colonoscopy. Gene expression levels of 84 genes previously associated with CD were evaluated by polymerase chain reaction (PCR) array. *Results*: Ten genes out of 84 were found significantly differentially expressed in CD-A (*CCL11*, *CCL25*, *DEFA5*, *GCG*, *IL17A*, *LCN2*, *REG1A*, *STAT3*, *MUC1*, *CCR1*) and eight genes in CD-R (*CASP1*, *IL23A*, *STAT1*, *STAT3*, *TNF*, *CCR1*, *CCL5*, and *HSP90B1*) when compared to controls. A quantitative gene expression analysis revealed that *CCR1* gene was more expressed in CD-A than in CD-R. *Conclusions*: Our data suggest that *CCR1* gene may be a putative marker of molecular activity of Crohn's disease. Following these preliminary data, a confirmation in larger cohort studies could represent a useful method in order to identify new therapeutic targets.

Keywords: Crohn's disease, gene expression, PCR array, *CCR1*, cytokines, chemokines.

⊟ Introduction

Crohn's disease is an immune-mediated chronic disease involving all segments of the gastrointestinal tract in genetically susceptible individuals following the interaction with gut microbiota. As a consequence, the overexpressed immune response in the gut contributes to complications such as strictures, fistulas and perforations. The etiology of inflammatory bowel diseases (IBDs), mainly represented by ulcerative colitis (UC) and Crohn's disease (CD) remains undetermined. Thus, no specific drug targets have been identified and available biological molecules (targeting the immune response mechanisms) might induce remission, but are associated with various side effects.

In the last years, promising advances in research related to genetic risk factors associated with IBD have been made by using genome-wide association studies (GWAS) and more than 160 susceptible loci have been identified [1]. However, the obtained results suggest that other factors may interfere with disease evolution, including several environmental factors like lifestyle habits (*i.e.*, smoking, oral contraceptive pills, diet, infections, vaccinations) [2–6]. Recently, the discovery of innate lymphoid cells (ILCs) along with the integration of data

regarding autophagy, oxidative stress and microbial sensing, have contributed to elucidate the mechanisms of the mucosal immune system and its relationship with environmental factors [7].

Traditionally, T-helper 1 (Th1) cells have been linked to chronic inflammation in relation to CD through the overproduction of interferon-gamma (IFNγ), interleukin (IL)-17, IL-21, IL-22, CXCL8, while Th2 cells have been connected to disease activity in UC through IL-5 and IL-13 [8]. However, data that are more recent have also shown Th17 cells and their imbalance with Th17 regulatory (Treg) cells as an important actor involved in the development of intestinal inflammation [9].

Practically, for UC patients, multiple epithelial mechanisms have already been described, such as Goblet cells depletion altering the mucus layer, impaired secretion of alpha- and beta-defensins, which cause an endothelial reticulum stress, finally contributing to the development of colitis [10–14].

In CD, it seems to be an inflammatory cascade that starts with the driver, represented by intestinal pathogens in a genetically susceptible host (*NOD2* genetic variants, *ATG16L1* genetic variants) and is followed by a deficient clearance of bacterial debris with continuous activation

of Th17 cells and secretion of cytokines and chemokines, contributing to recruitment of other circulating leukocytes perpetuating the inflammatory process in a "vicious circle".

For the past decades, there has been a florid development of antibodies targeting molecules implicated in different inflammation pathways as treatment for CD. The primary outcome for drug efficacy in CD clinical trials has been modified throughout time. Initially, clinical response and remission were the basis and progressively biological and endoscopic remission became the gold standard. This shift has been based on the observation that relapse is influenced by achieving remission after certain treatment agents (*e.g.*, endoscopic remission has a much lower risk of relapse than clinical remission) [15]. Histological and putative molecular remission might represent the next targets to be considered, but data regarding their role in clinical practice are scarce.

The aim of our study was to obtain preliminary data regarding the molecular signature of CD correlating with endoscopic lesions/remission compared to healthy controls in a small cohort of Romanian patients investigating the expression of a panel of genes that might define the molecular activity/remission status.

⊟ Patients, Materials and Methods

Study groups

Six adult patients having been diagnosed with CD (either active or in remission on endoscopy), and three healthy controls were evaluated in the Department of Gastroenterology and Hepatology, "Elias" Emergency University Hospital, Bucharest, Romania and enrolled in the study. The controls (two males and one female of 26, 33 and 35 years, respectively) were selected from a cohort of patients undergoing colonoscopy for screening. All the patients and controls were of Romanian origin. The diagnosis was made on clinical, endoscopic and histological criteria according to *European Crohn's and Colitis Organization* Guidelines [16] and the ongoing treatment was noted. Written informed consent was obtained from all participants prior to biopsy collection. The working protocol was approved by the local ethics committee. Biopsy samples were obtained during colonoscopy from actively inflamed areas (CD-A), from previously active area (now in remission) (CD-R) and from controls.

Score descriptions

Montreal Classification was used to phenotypically define the disease considering the age at diagnosis (A1<16 years, A2: 16–40 years, and A3>40 years), disease behavior (B1: inflammatory, B2: structuring, B3: penetrating) and localization (L1: ileal, L2: colonic, L3: ileo-colonic, L4: isolated upper disease, and p: perianal disease) [17].

Crohn's Disease Activity Index of severity (CDAI) was used to define clinical flare severity (remission with CDAI<150, mild with CDAI: 150–219, moderate with CDAI: 220–449, and severe with CDAI>450).

Simple Endoscopic Score for Crohn's Disease (SES-CD) and Rutgeerts score in patients with anastomosis after intestinal resection were used to define the severity of endoscopic lesions [18–20]. Endoscopic severity assessed by SES-CD takes into account the size of mucosal ulcers, the ulcerated surface, the endoscopic extension and the presence of stenosis, with point added for each item, the higher the score the higher the endoscopic severity. The Rutgeerts score was used to evaluate postoperative recurrence as follows: I0 no recurrence with I1 to I4 showing progressive severity of lesions.

For the histological evaluation, we chose the *Global Histological Activity index of Severity* (GHAS) [21]. The GHAS score incorporates epithelial damage, architectural changes, mononuclear or polymorphonuclear cells in the lamina propria and epithelium, presence of erosions/ulcers and granulomas, as well as the number of segmental biopsy specimens, by adding points for each parameter in order to define severity. A score above "2" indicates histological activity of the disease.

RNA isolation and cDNA preparation

RNA isolation from tissues preserved in RNA later was performed using miRNeasy mini Kit (Qiagen), according to the manufacturer's protocols. The concentration of RNA was quantified using the Nanodrop 2000 (Thermo Scientific) by measuring the absorbance at 260 nm. Moreover, the OD260/230 and OD260/280 ratios were determined to assess RNA purity. Both 260/280 nm and 260/230 nm parameters were >1.9. Next, 600 ng of RNA were reverse transcribed to cDNA. The cDNA was synthesized using the RT^2 First Strand Kit (Qiagen) following the manufacturer's instructions.

RT^2 Profiler polymerase chain reaction (PCR) Array

The Human Crohn's Disease RT^2 Profiler PCR Array (PAHS 169Z, Qiagen) was used to profile the expression of 84 key genes differentially expressed during IBD, according to the manufacturer's protocol. One hundred and two μL of cDNA were mixed with 2×RT^2 SYBR Green Mastermix and RNase-free water to obtain a total volume of 2700 μL. Subsequently, 25 μL of the mix were placed into each well of the 96-well PCR array. The three steps of the cycling program were 95°C for 10 minutes for one cycle, followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute. This process was performed on the ABI-7500 fast instrument (Applied Biosystems). The expression levels of each gene were normalized on the geometric mean values of five housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*).

Statistical analysis

Gene expression data were analyzed by the RT^2 Profiler PCR Array software package. This package uses *ΔΔCT*-based fold change calculations and the Student's *t*-test to calculate two-tail, equal variance *p*-values. The other statistical analyses were conducted using the Statistical Package for Social Sciences ver. 17.0 (SPSS Inc.).

⊟ Results

Control and patients group were homogeneous for age and gender (average of age in controls: 31.33±4.72, average of age in patients: 28±5.96, *p*=0.431; gender *p*=0.635, X^2=0.225). Patients were divided in two groups, based on endoscopic findings. Clinical and phenotypic characteristics of the patients are summarized in Table 1.

The two groups were homogenous for age (CD-R: 28±5.29, CD-A: 28±7.81, *p*=1), gender (*p*=0.414, X^2=0.667) and disease duration (CD-R: 5.66±3.21, CD-A: 5.33±4.04, *p*=0.916).

Table 1 – *Clinical and phenotypic characteristics of patients*

Group type	Group A (active disease)			Group B (remission)		
Patient No.	*P 1*	*P 2*	*P 3*	*P 4*	*P 5*	*P 6*
Age at recruitment [years]	33	19	32	26	34	24
Gender	M	F	F	M	M	F
Age at diagnosis	A2	A1	A2	A2	A2	A1
Disease duration [years]	3	3	10	2	8	7
Disease behavior	B2	B2	B1	B2	B1	B1
Disease activity (CDAI)	Remission	Remission	Mild	Remission	Remission	Remission
Rutgeerts score	I4	I1	N/A	I0	N/A	N/A
SES-CD	N/A	N/A	12	N/A	0	0
GHAS	2	10	10	2	2	3
Medication	ADA	ADA	ADA	ADA	ADA	IFX

P: Patient; M: Male; F: Female; Age at diagnosis: A1<16 years, A2: 16–40 years, A3>40 years; Disease behavior: B1 – Inflammatory, B2 – Structuring, B3 – Penetrating; CDAI: Crohn's Disease Activity Index of severity; N/A: Not available; SES-CD: Simple Endoscopic Score for Crohn's Disease; GHAS: Global Histological Activity index of Severity; ADA: Adalimumab; IFX: Infliximab.

Five patients were in clinical remission and one had mild clinical activity. All patients had biological treatment at the time of biopsy sampling – five were treated with Adalimumab (ADA) and one with Infliximab (IFX).

Endoscopic lesions were variable in terms of severity in the Group A, ranging from mild to severe as reported in Table 1.

Histological evaluation of biopsies from the two groups showed either very high GHAS score (10 points) characterized by atrophic crypts, basal plasmacytosis, dense mononuclear and polymorphonuclear (PMN) cells infiltration, erosions and mucosal granuloma (Figure 1a), either low GHAS score (2 points) characterized by mild architectural changes and minimal, focal, infiltration of the lamina propria by mononuclear cells (Figure 1b). In one case, the GHAS score was "3", characterized by mild architectural changes, infiltration of mononuclear cells in the lamina propria and rare cryptitis (Figure 1c). No intermediate severity scores have been identified in our patients.
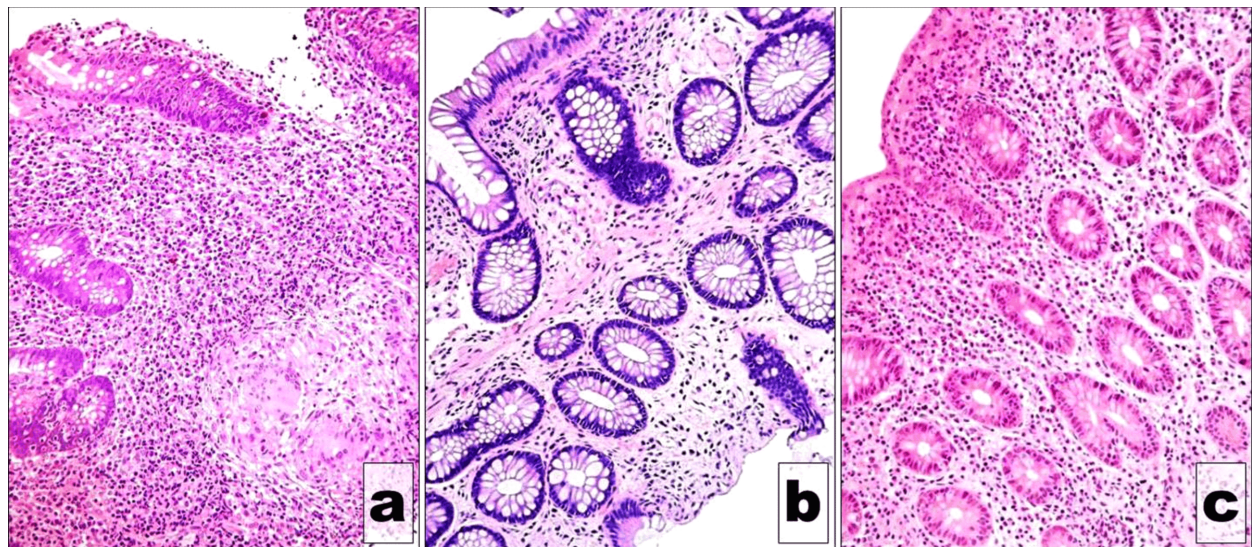

Figure 1 – *(a) GHAS score 10; (b) GHAS score 2; (c) GHAS score 3. Hematoxylin–Eosin (HE) staining, ×200. GHAS: Global Histological Activity index of Severity.*

When analyzing patients individually, we found a discrepancy between the severity of endoscopic lesions and histological activity score in just one patient having Rutgeerts I4 and a GHAS of 2 points.

PCR array analysis showed 48 genes differentially expressed in terms of Fold Regulation (2<FR<-2) in CD-R (46 up-regulated and two down-regulated) and 64 in CD-A (61 up-regulated and three down-regulated) compared to controls. However, only eight and 10 genes in CD-R and CD-A respectively, reached a statistically significant difference (*p*<0.05).

Figure 2 shows the mean of Log_2(fold change) ± standard error of the mean (SEM) (CD-A *vs.* controls in red and CD-R *vs.* controls in blue) for the differentially expressed genes. When comparing CD-R *vs.* CD-A, a difference in *CCR1* and *HSP90B1* levels between the groups (*p*=0.043; *p*=0.019, respectively) was noted. No correlation was observed between disease duration and gene expression levels of the studied genes and no difference in terms of gene expression was observed when comparing different disease behaviors (B1 *vs.* B2 phenotypes).

Moreover, a significant Pearson's correlation between *CCR1* and *IL23A* levels [Log_2(fold change)] and severity of the lesions evaluated by GHAS score was found (*CCR1*: *p*=0.024, *r*=0.870; IL23A: *p*=0.026, *r*=0.865).
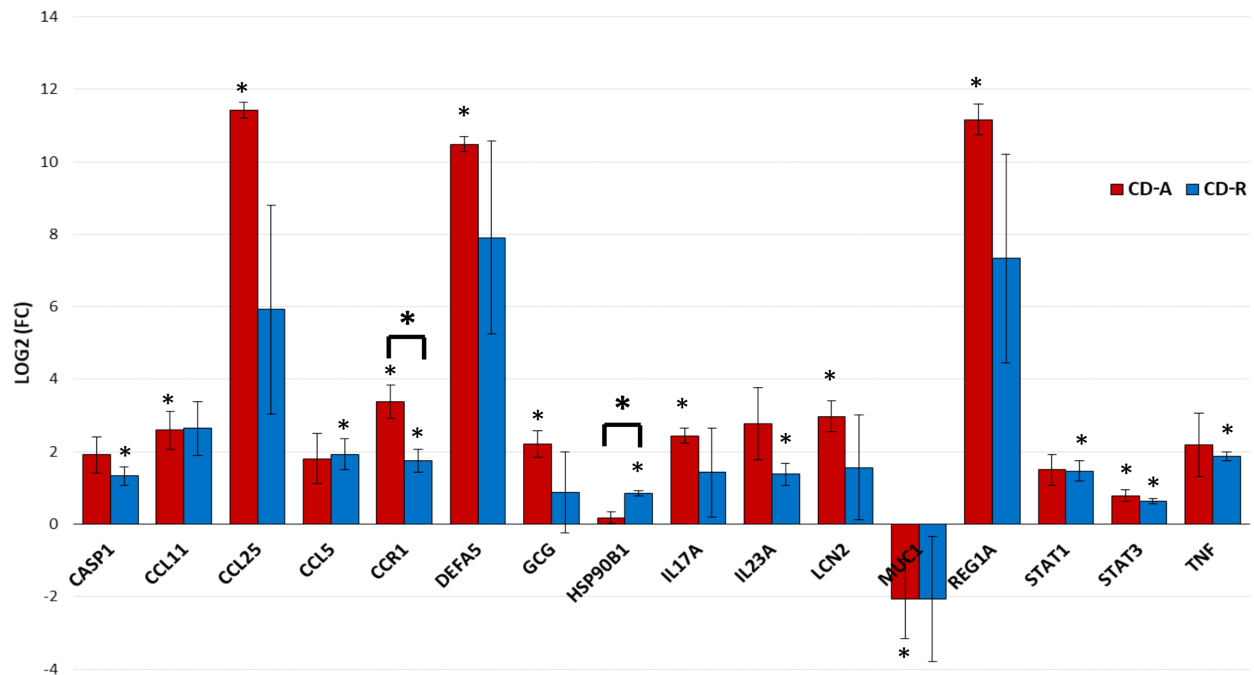
Figure 2 – *mRNA expression of the significant genes (*p<0.05). CD-A: Active Crohn's disease; CD-R: Crohn's disease in remission; FC: Fold change.*

Discussion

This is the first Romanian study investigating gene expression and regulation of a large panel of genes implicated in inflammation, apoptosis, immune response, cellular adhesion, tissue remodeling and mucous secretion isolated in tissue samples from CD patients.

Our results showed that 10 genes in the CD-A group and eight genes in the CD-R group were differentially expressed compared to controls. However, when comparing gene levels between CD-A and CD-R, the only statistically significant difference was the change in *CCR1* and *HSP90B1* expression. Moreover, we found a positive correlation between the expression of *CCR1* and the histological severity of the disease, suggesting that *CCR1* levels could represent a marker of activity.

CCR1 gene encodes a member of the beta-chemokine receptor family, which is predicted to be a seven trans-membrane protein similar to G protein-coupled receptors. The ligands of this receptor include macrophage inflammatory protein-1alpha (MIP-1α), regulated on activation normal T expressed and secreted (RANTES) protein, monocyte chemoattractant protein-3 (MCP-3) and myeloid progenitor inhibitory factor-1 (MPIF-1). Our results showing an up-regulation of *CCR1* in CD-A patients were expected since *CCR1* is a part of the chemokine receptor family implicated in leukocyte trafficking and homing at the inflammation site. The role of the relationship chemokine receptor/ligand has been widely studied and well depicted by Zimmerman *et al.* [22].

Intriguingly, we found lower expression levels of *HSP90B1* in CD-A than in CD-R, contrasting the current literature [23, 24]. This may be due to the high individual variability of *HSP90B1* levels among the patients from both groups.

Regarding the other up-/down-regulated genes differentially expressed between controls and CD-A or CD-R that did not differ between the patient groups, a brief discussion for the most relevant genes is reported below.

Genes differentially expressed between CD-A and controls

Genes up-regulated

REG1A is a type I subclass member of the *REG* gene family expressed by metaplastic colonic Paneth cells. The *REG* gene family is a multigene family divided into four subclasses, types I, II, III and IV, based on the primary structures of the encoded proteins. This gene family encodes a group of proteins highly expressed in several pathologies, many of which are associated with epithelial inflammation. Indeed, the regenerating (*REG*) family member genes were found to be expressed in both inflamed and non-inflamed biopsies [25]. Moreover, in line with our results, an up-regulation of this gene was observed in children with CD (compared to age- and sex-matched controls without IBD) and in colitis mouse model [26, 27].

CCL11 is a member of eosinophil-selective chemokines (eotaxins). The involvement of eotaxin-1 in IBD has been previously suggested and increased levels have been described in both ulcerative colitis and in CD. A study on pediatric UC demonstrated that higher levels of *CCL11* can be found in patients with active disease and this levels correlates with the number of eosinophils seen on histology [28]. We found *CCL11* significantly overexpressed only in CD-A compared to controls even though the mean values were not different when comparing CD-A and CD-R.

The **CCL25** mediating *CCR9* activation plays a crucial role for mucosal lymphocyte recruitment in the gut. Trivedi *et al.* showed that mucosal *CCR9* is elevated in patients with active UC and stricturing CD and that the level of expression correlates with endoscopic scores of disease activity [29]. We found an up-regulation of *CCL25* in CD-A reaching statistical significance when compared to controls. In CD-R patients, the mean values were elevated even though it did not reach statistical significance.

IL-17 is the pivotal cytokine secreted by the Th17 cells following activation by IL-23. This pathway that has been widely studied in the pathogenesis of IBD. Animal studies targeting the IL-23/IL-17 pathway suggested a possible therapeutic approach for CD [30]. However, Secukinumab, an IL-17 monoclonal antibody, has failed in a phase II study [31, 32].

Lipocalin-2 (LCN2), an antimicrobial peptide regulated by IL-17A, IL-22 and tumor necrosis factor-alpha (TNF-*α*), has been suggested to be a biomarker of active UC and a fecal marker in CD [33, 34].

STAT (signal transducer and activator of transcription) family proteins are cytoplasmic transcription factors activated after Janus kinases (JAKs) phosphorylation in response to cytokine binding. Schreiber *et al.* showed that STAT expression is elevated in IBD, predominantly in UC and that glucocorticoid treatment can normalize the expression [35]. We found similar mean values for STAT-1 and STAT-3 in CD-A and CD-R, compared with controls.

Genes down-regulated

MUC1 depletion has been linked to the development of IBD through GWAS. Decreased expression of the mucin gene in inflamed terminal ileum suggests that the mucin layer protecting the intestinal epithelium from pathogens may become insufficient [5, 36]. Our results also show a downregulation of *MUC1* in both CD-A and CD-R compared to controls with statistical significant reached only for CD-A. This may suggest that mucin depletion persists even in remission.

Genes differentially expressed between CD-R and controls

Regarding the results in CD-R patients, we found a significant up-regulation of *CASP1*, *TNF-α*, *IL23A* and *CCL5*, compared to controls. *TNF-α* and *IL23A* encode for cytokines related to inflammation and are currently used in daily practice (Infliximab, Ustekinumab). Their up-regulation compared to healthy controls might denote a persistent inflammation, undetected by endoscopy and even histology. Caspase-1 (*CASP1*) regulates host defense along with lysozyme (LYZ) through synthesis of IL-1*β* and IL-18. These genes are expressed at different levels in colonic biopsies of IBD and irritable bowel syndrome (IBS) patients [37].

We also found higher mean levels in CD-A *vs.* CD-R, but statistical significance was reached only for the CD-R group. This suggests a persistent altered host defense even in endoscopic remission.

Our results should be interpreted with caution as they can be influenced by the small cohort size of the study, and by the heterogeneity in disease localization – small bowel *vs.* colonic disease (gene expression is different throughout the digestive segments).

Gene profiling between inflamed and uninflamed mucosa could be useful for identifying new physio-pathological pathways and future drug targets. Moreover, mucosal gene expression might be used as a tool for evaluating treatment efficacy, predicting early relapse and for defining profound remission.

⊟ Conclusions

Even though this is a small case-to-case study, we identified many genes up-regulated in both CD-A and CD-R showing patterns of overexpression indicative of chronic inflammation. Of note, *CCR1* was more expressed in the active condition than in remission, suggesting that it may be a putative marker of molecular activity of Crohn's disease. Our results need to be reproduced for confirmation on larger cohorts.

Conflict of interests

The authors declare that they have no conflict of interests.

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