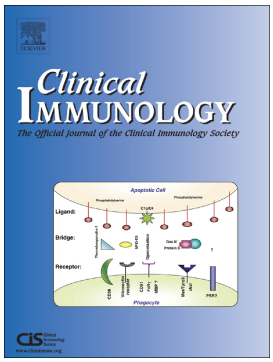

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Importance of TLR9-IL23-IL17 axis in inflammatory bowel disease development: gene expression profiling study

Sanja Dragasevic[a], Biljana Stankovic[b], Aleksandra Sokic-Milutinovic[a,c], Tomica Milosavljevic[a,c], Tamara Milovanovic[a,c], Snezana Lukic[a,c], Sanja Srzentic Drazilov[b], Kristel Klaassen[b], Nikola Kotur[b], Sonja Pavlovic[b], Dragan Popovic[a,c]

[a] Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia, Koste Todorovica 2, 11000 Belgrade, Serbia.

[b] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11000 Belgrade, Serbia.

[c] School of Medicine, University of Belgrade, Dr Subotica 8, 11000 Belgrade, Serbia.

Sanja Dragasevic MD, e-mail: dragasevicsanja@gmail.com; Biljana Stankovic PhD, e-mail: bi.stankovic@gmail.com; Prof.dr Aleksandra Sokic-Milutinovic, e-mail:asokicmilutinovic@gmail.com; Snezana Lukic, MD e-mail:lukic.snezana@gmail.com; Prof.dr Tamara Milovanovic, email:alempijevic.tamara@gmail.com; Prof.dr Tomica Milosavljevic e-mail:tommilos@hotmail.com; Sanja Srzentic Drazilov PhD, e-mail: sanja.srzentic@imgge.bg.ac.rs; Kristel Klaassen PhD, email:kristel.klaassen@imgge.bg.ac.rs; Nikola Kotur PhD, e-mail: nikola.kotur@imgge.bg.ac.rs; Sonja Pavlovic PhD, e-mail: sonya@sezampro.rs; Prof.dr Dragan Popovic, e-mail: dragan.drendo23@gmail.com

Corresponding author: Prof. Dr Dragan Popovic, Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia, Koste Todorovica 2, Belgrade, Serbia; School of Medicine, University of Belgrade, Dr Subotica 8, Belgrade, Serbia;

Email: dragan.drendo23@gmail.com; Tel: +381 63 276339; Fax: +381 11 3628582

ABSTRACT

Background and aims: Mucosal gene expression have not been fully enlightened in inflammatory bowel disease (IBD). Aim of this study was to define *IL23A*, *IL17A*, *IL17F* and *TLR9* expression in different IBD phenotypes.

Methods: Evaluation of mRNA levels was performed in paired non-inflamed and inflamed mucosal biopsies of newly diagnosed 50 Crohn's disease (CD) and 54 ulcerative colitis (UC) patients by quantitative real-time PCR analysis.

Results: *IL17A* and *IL17F* expression levels were significantly increased in inflamed IBD mucosa. Inflamed CD ileal and UC mucosa showed increased *IL23A*, while only inflamed CD ileal samples showed increased *TLR9* mRNA level. Correlation between analysed mRNAs levels and endoscopic and clinical disease activity were found in UC, but only with clinical activity in CD.

Conclusion: Both CD and UC presented expression of Th17-associated genes. Nevertheless, expression profiles between different disease forms varies which should be taken into account for future research and therapeutics strategies.

Keywords: Expression profiling, IL23/ IL17/TLR9, inflammatory bowel disease

1. INTRODUCTION

Interactions in the gut human microbiota are enabled by host's sensors such as toll like receptors (TLRs) that can recognize bacterial molecular patterns and induce activation of either pro- or anti-inflammatory response in order to sustain intestinal barrier function [1]. Nevertheless, under some circumstances influenced by various external factors, genetic susceptibility and immune response of the host, intestinal

bacteria may disturb immune homeostasis in the gut and lead to development of inflammatory bowel disease (IBD) [1, 2].

Crohn's disease (CD) and ulcerative colitis (UC) are two major forms of IBD which demonstrate different location and inflammation patterns. Precisely, CD can affect any part of gastrointestinal tract with patchy distribution of disease, while UC affects only colon with clear distinction between involved and non-involved mucosa. For long it was believed that chronic inflammation in these two IBD phenotypes is dominantly driven by different subsets of T helper (Th) cells, where CD was associated with Th1 and UC with Th2 immune responses. Meanwhile, this paradigm has changed since a lot of data indicated involvement of Th17 cells in pathogenesis of both IBD forms [3,4,5,6]. Th17 cells expand in response to interleukin (IL)-23 and further mediate inflammation by producing proinflammatory IL-17 [7,8]. This IL-23/Th17 pathway is emphasized as an important adjuster of intestinal homeostasis and defences-proinflammatory feedback to gut microbial infection and it is considered important in pathogenesis of IBD [9,10].

IL-23 is a proinflammatory mediator mainly produced by antigen-presenting cells (APC) including dendritic cells (DCs), monocytes and macrophages [11,12]. It is a heterodimeric cytokine composed of two subunits: IL-12p40, same as in IL-12 and encoded by *IL12B*, and IL-23p19, encoded by *IL23A* gene [10,11]. The activation of functional receptor for IL-23 (IL-23R) identified on various T cells induces JAK2 tyrosine kinase phosphorylation of STAT3, STAT3/STAT4 dimerization and transcription of inflammatory genes [10,11]. Th17 cell subset is enriched for IL-23R expression with production of effector cytokines IL-17A and IL-17F, although IL-23 has also been highlighted as an inhibitor for the development of regulatory T (Treg) cells in the intestine [13]. Number of studies reported elevated levels of IL-17A and IL-17F in the inflamed intestinal mucosa of human IBD and experimental murine models of the disease [14]. Since the two proteins share 50% sequence homology and the same heterodimeric receptor complex, there is a considerable overlap in their biologic functions. However, it is suggested that roles of IL-17A and IL-17F in immune regulation and disease may not be equivalent [15,16]. The

involvement of IL-23/Th17 pathway in development of CD and UC was also supported by genome wide association studies where *IL23R*, *IL12B*, *JAK2*, and *STAT3* were marked as IBD susceptibility genes [17]. Drug targeting of IL-23/IL-17 axis has been studied in several autoimmune diseases, including IBD. According to conducted studies, blockade of IL-23R activation in murine models prevents the development of colitis, while in clinical studies monoclonal antibody targeting p40 has been used in treatment of CD as an inhibitor for IL-12 and IL-23 [18,19,20]. On the other hand, studies which tested efficacy and safety of AIN457, a monoclonal anti-IL-17A antibody in treatment of CD, showed that inactivation of IL-17A is not beneficial to patients, with a tendency of worsening of the disease [14].

The importance of TLRs in pathogenesis of IBD is for long well recognized [21]. TLRs are transmembrane receptors that bind different pathogen-associated molecular patterns (PAMPs) resulting in activation of the host's immune system. In the gastrointestinal tract TLRs are expressed in different cell types including intestinal epithelial cells (IECs), monocytes/macrophages and dendritic cells [22]. TLR9 is a member of this family which function is triggered by un-methylated CpG motifs of microbes' DNA. Number of studies demonstrated increased expression level of *TLR9* in intestinal mucosa of IBD patients, but nevertheless, several studies did not show this [23,24]. In murine and animal studies of ulcerative colitis, a positive correlation was found between TLR9 expression and severity of endoscopic and histological findings [24,25,26]. Activated TLRs and among them TLR9, can induce IL-12 and IL-23 expression and Th17 cells expansion [27,28,29,30]. It has been showed that $TLR9^{-/-}$ mice displayed increased number of Treg cells within intestinal effector sites and reduced number of Th17 and Th1 effector cells [31]. Additionally, presence of genetic variants in *TLR9* gene were linked to CD-associated variants in *IL23R* and *CARD15* genes which suggested that TLR9 could be related to IL23 pathway in terms of modulation of CD susceptibility [31,32].

This work represents gene expression profiling study of TLR9-IL23-IL17 network in IBD using biopsies from non-inflamed and inflamed parts of CD and UC intestinal mucosa. The roles of these important inflammatory players, as well as their interplay in the development of both diseases have not been completely clarified, specifically, the TLR9-IL23-IL17 axis has not been yet addressed. Also, current

studies were not fully consistent with the role of these inflammatory factors in pathogenesis of IBD as a consequence of small or non-homogenous groups. Further, these studies did not always cover both colonic and ileal IBD phenotypes, or they focused either on CD or UC [3, 4, 5, 23]. It is of great interest in IBD research to define disease biomarkers in clinically well-defined IBD groups which could better distinct between different phenotypic subsets and improve therapeutic strategies.

Therefore, the aim of this study was to perform coordinated analysis of the *TLR9*, *IL23A*, *IL17A* and *IL17F* expression levels in different parts of involved intestinum (ileum and colon of CD and colon of UC patients) using large group of newly diagnosed IBD patients. To the best of our knowledge, the combined expression analysis of these genes has not been investigated in IBD. Also, we attempted to examine correlation of analysed expression patterns with clinical activity, extension and severity of the disease. Our objective was to delineate expression milieu in well-defined clinical phenotypes which could be of high importance in precise diagnosis and disease treatment. Understanding molecular mechanisms involved in IBD development and unfolding similarity or distinction in pathways underlying CD and UC may enable more personalized and effective pharmacological therapies.

2. MATERIAL AND METHODS

2.1 Patients

A cross sectional study was performed in Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia, Belgrade and included 104 newly diagnosed IBD patients (50 CD, 54 UC).

The diagnosis of CD and UC was made based on conventional clinical, endoscopic and histopathological evidence. In 9 patients diagnosis of CD was made intraoperatively due to bowel obstruction symptoms. The exclusion criteria were patients with indeterminate IBD, younger than 18 years of age. None of the patients received previous IBD treatment including corticosteroids, azathioprine/6-mercaptopurine or methotrexate. The Montreal's classification was used to determine IBD phenotype according to age at disease onset, localization and disease behavior [33]. Clinical disease activity of IBD patients were assessed by Crohn Disease Activity Score (CDAI) and Mayo score [34,35]. Endoscopic activity was

evaluated using Simple Endoscopic Score for Crohn's disease (SES-CD) and Mayo scoring system (MSc) in patients with UC [36,37,38].

This study was conducted after the approval of The Ethic Committee of Clinical Center of Serbia, School of Medicine, University of Belgrade, in accordance with the Helsinki Declaration. Written informed consent was obtained from each participant.

2.2 Endoscopic biopsy specimen collection

All IBD patients included in the study underwent ileocolonoscopy using Olympus endoscopes (model CF-H180AL video colonoscope with High Definition Television-HDTV). Biopsy specimens were obtained for routine histology and reviewed by an independent gastrointestinal (GI) pathologist. Mucosal specimens were taken from the ileum or colon, depending on the localization of the disease, and from each patient samples were collected from two sites – from area with evident endoscopic lesions (further noted as inflamed samples) and from macroscopically lesion-free region (further noted as non-inflamed samples). Additionally histopathology report confirmed inflammation in endoscopically active regions of the intestine and the absence of inflammatory processes in lesion-free areas. In our study we used the term inflamed mucosa as active inflammatory bowel disease histologically defined by presence of neutrophilic inflammation, cryptitis or crypt abscesses. Non-inflamed mucosa presented biopsy samples from intestinum free from endoscopically and histologically active or chronic inflammation. Biopsies collected for mRNA analysis were immediately submerged in All Protect Tissue Reagent (Qiagen, Germany), following manufacturer's instructions, transported to the Laboratory for Molecular Biomedicine Institute of Molecular Genetics and Genetic Engineering and stored at -20^{o}C until processing.

2.3 Gene expression analysis

Prior to RNA isolation, biopsies were disrupted and homogenised using Tissue Ruptor (Qiagen, Germany). Total RNA was then extracted with the RNeasy kit (Qiagen, Germany), following the manufacturer's recommendations. Concentration and purity of RNA was assessed spectrophotometrically at 260 and 280 nm. One microgram of total RNA was reverse-transcribed into cDNA using RevertAid Reverse Transcriptase (Thermo Scientific, USA), according to the manufacturer's instructions.

Quantification of mRNA expression was performed on ABI PRISM 7500 Real Time PCR System (Applied Biosystems, USA) using TaqMan approach with KAPA PROBE FAST qPCR Kit (Kapa Biosystems, USA). Primers were either designed (*TLR9* forward 5'-GGACCTCTGGTACTGCTTCCA-3', reverse 5'-AAGCTCGTTGTACACCCAGTCT-3', probe FAM-5'-ACGATGCCTTCGTGGTCTTCGACAAA-3'-TAMRA) or commercially available as TaqMan® gene expression assays (*IL23A* Hs00900828_g1, *IL17A* Hs00174383_m1 and *IL17F* Hs00369400_m1) (Applied Biosystems, USA). In order to normalize the obtained results, glyceraldehyde 3-phosphate dehydrogenase was used as an internal control (*GAPDH* forward 5'-GAAGGTGAAGGTCGGAGT-3', reverse 5'-GAAGATGGTGATGGGATTTC,-3', probe FAM-5'-CAAGCTTCCCGTTCTCAGCC-3'-TAMRA). Cycling conditions were as follows: 95°C for 3 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression level was calculated using the ddCt method, where median of the dCt values obtained for unaffected mucosa was used as a calibrator.

3.1 Theory

Combined gene expression analysis of *IL23A*, *IL17A*, *IL17F* and *TLR9* genes have not been performed in previous studies. Published data on IBD regarding these genes indicated their involvement in development of the disease, but there is a need for comprehensive study in which, besides their individual association, also their mutual interaction in the IBD will be analysed on a clinically well-characterized subgroups of IBD patients. Since IBD represent complex and heterogeneous disease, this "more personalized" approach could be beneficial to patients making their diagnosis, prognosis and therapy much more efficient.

3.2 Calculation

Analyses of differences in gene expression levels between paired non-inflamed and inflamed patients' mucosal samples were tested using Wilcoxon signed-rank test. Differences in gene expression levels between specific IBD phenotypes were tested by Mann-Whitney U test. The correlations between analysed gene expressions levels were examined using Spearman's correlation coefficient. All tests were

two-tailed and p values less than 0.05 indicated significant differences. Statistical analyses were performed using SPSS software v.21.0 (IBM, USA) and R 3.4.3. software.

4. RESULTS

4.1 Characteristics of the studied IBD patient group

This study enrolled 50 newly diagnosed patients with CD and 54 newly diagnosed patients with UC. Demographic and clinical data regarding study patients are presented in the Table 1. Expression levels of the selected genes were studied in non-inflamed and inflamed intestinal mucosa obtained from ileum of CD or colon of CD and UC patients depending on the disease localization.

4.2 *IL17A, IL17F, IL23A* and *TLR9* expression levels in paired non-inflamed and inflamed mucosal samples of CD and UC patients

Expression levels of *IL17A*, *IL17F*, *IL23A* and *TLR9* were analysed between paired samples of non-inflamed and inflamed ileal mucosa of CD patients as well as colonic mucosa of CD and UC patients (Figure 1).

Results showed significantly increased expression of *IL17A* and *IL17F* in inflamed mucosa when compared with non-inflamed in both ileal (p = 0.0001 and p = 0.0002, respectively) and colonic samples (p = 0.00004 and p = 0.001, respectively) of CD patients. Expression levels of *IL17A* and *IL17F* was also significantly increased in inflamed compared to non-inflamed UC colonic samples (***p*** = 0.000001 and p = 0.000005, respectively).

Significantly higher *IL23A* expression level was observed in inflamed compared to non-inflamed ileal mucosa of CD patients (p = 0.007). The *IL23A* expression level between involved and uninvolved colonic CD mucosa showed tendency to be higher in inflamed compared to non-inflamed samples (p = 0.08). In UC samples, inflamed mucosa had significantly higher *IL23A* expression level in comparison with non-inflamed mucosa (p = 0.00004).

Regarding the expression level of *TLR9*, significantly higher level of this cytokine was detected in inflamed compared to non-inflamed ileal samples of CD patients (p = 0.03), while in colonic CD and UC samples this difference was not observed.

These results indicate differential *IL17A* and *IL17F* expression profile between non-inflamed and involved CD and UC mucosa. Furthermore, results indicate probably more prominent role of *IL23A* in chronic inflammation of UC than in CD patients with colonic localization.

To further define inflammatory milieu in CD and UC mucosa, we performed correlation analyses of cytokine expression profiles in sampled intestinal biopsies (Table 2). According to our results, strong positive correlations were observed between *IL17A* and *IL17F* expression levels, and between *IL23A* and *TLR9* expression levels, in all analysed types of IBD mucosa (non-inflamed and inflamed samples of CD ileal, CD colonic and UC colonic mucosa). This may suggests *TLR9* regulated activation of *IL23* expression and coordinated involvement of *IL17A* and *IL17F* in pathogenesis of both CD and UC.

However, there were some differences in correlation patterns between IBD phenotypes. Namely, significant and moderately positive correlations were observed between *IL17A* and *IL23A*, *IL17F* and *IL23A* and between *IL17F* and *TLR9* expression levels only in UC mucosa, but not in ileal nor colonic CD mucosa.

Correlation of gene expression levels was examined also between non-inflamed and inflamed mucosa. Positive correlation of *IL17A* and *IL17F* expression levels was observed only in colonic CD mucosa, but not in ileal CD nor colonic UC mucosa. We found moderately positive correlation of *IL23A* expression levels between non-inflamed and inflamed colonic UC samples, but not in ileal and colonic CD samples. Moderately positive correlation of *TLR9* expression level was detected in ileal and colonic CD as well as in colonic UC samples.

Next, we examined differences in cytokine expression profiles between colonic CD and UC mucosa, since these two phenotypic entities are sometimes hard to distinguish. Except for better understanding of molecular pathogenesis of each disease, this type of molecular IBD classification could potentially help in more precise diagnosis and better therapy decision regarding treatment of CD and UC. Results showed

that expression levels of *IL17A* in both healthy and inflamed colonic mucosa of UC patients were significantly higher in comparison with *IL17A* expression in CD mucosa (p = 0.007 between CD and UC in non-inflamed and p = 0.001 between CD and UC in inflamed colonic mucosa) (Figure 2A). *IL17F*, *IL23A* and *TLR9* expression levels did not show significant difference between non-inflamed and inflamed colonic CD and UC mucosa (Figure 2B, C and D). However, median values of *IL17F* and *IL23A* mRNA levels were higher in inflamed UC compared to inflamed CD samples (*IL17F* 5.91 *vs* 4.42 and *IL23A* 2.01 *vs* 1.43, respectively). These results indicate more dominant role of Th17 mediators in UC inflammatory processes.

4.3 Expression levels of *IL17A*, *IL17F*, *IL23A* and *TLR9* in relation to IBD clinical parameters

In order to search for potential clinically relevant biomarkers of CD and UC we examined associations between gene expression levels and clinical parameters of analysed diseases.

First we investigated correlation of intestinal *IL17A*, *IL17F*, *IL23A* and *TLR9* expression levels with endoscopic and clinical CD scores, such as Simple Endoscopic Score for Crohn's Disease (SES-CD) and Crohn's disease activity index (CDAI), and UC scores, endoscopic Mayo and total Mayo, used in the evaluation of disease activity (Table 3).

In case of CD samples, no correlations of analysed gene expression levels with Simple Endoscopic Score for Crohn's Disease (SES-CD) were observed, but only with Crohn's disease activity index (CDAI). In UC samples, when gene expression level correlated with Mayo endoscopic score, the same correlation was observed with total Mayo score.

In ileal CD samples, expression of analysed genes did not correlate with clinical activity scores. In colonic CD samples, *IL17A* and *IL17F* expression levels measured in non-inflamed mucosa positively correlated with CDAI, while *IL23* and *TLR9* expression levels measured in inflamed mucosa negatively correlated with CDAI.

Regarding UC, results showed a positive correlation of *IL17A* expression level in colonic non-inflamed and inflamed samples with Mayo endoscopic and total Mayo scores. Positive correlation was detected between *IL17F* expression level and Mayo endoscopic and total Mayo scores in non-inflamed colonic

mucosa. *IL23* expression level in inflamed mucosa also showed positive correlation with both clinical activity scores.

We observed all significant correlations as moderate.

Next, we assessed whether expression levels of selected genes associated with more severe disease phenotypes. For that reason we compared gene expression levels between CD patients with non-stricturing/non-penetrating (NS/NP) and stricturing/penetrating (S/P) disease and between UC patients with mild and moderate/severe disease.

Significantly higher expression levels of *IL17F* and *IL23A* were observed in non-inflamed ileal mucosa of S/P compared to NS/NP CD patients (p = 0.008 and p = 0.015, respectively). In non-inflamed colonic samples of CD patients *IL17A* expression level was significantly lower in S/P compared to NS/NP CD patients (p = 0.041). Generally, in colonic CD samples median *IL17A* and *IL17F* expression levels were lower in S/P than in NS/NP patients, in non-inflamed as well as in inflamed mucosa. Difference in *TLR9* expression level between these two phenotypes was not observed (Figure 3).

Further analysis showed that UC patients with moderate and severe disease had significantly higher level of both *IL17A* and *IL17F* compared to patients with mild disease, but only in non-inflamed colonic mucosa (p = 0.001 and p = 0.013, respectively). Also, moderate/severe patients had higher *IL23A* expression level in non-inflamed and inflamed colonic mucosa in comparison with mild UC patients (p = 0.078 and p = 0.035, respectively). Expression level of *TLR9* was not different between moderate/severe and mild type of UC (Figure 4).

Finally, we examined if there is a difference in gene expression levels between CD patients who were not operated and patients who were operated at diagnosis (Figure 5). We detected significantly higher *IL17A* and *IL17F* expression levels in ileal non-inflamed samples of operated patients compared to the patients who did not undergo intervention (p = 0.0003 and p = 0.03, respectively). In colonic CD samples, *IL23* and *TLR9* expression levels were higher in inflamed mucosa of operated in comparison with not operated CD patients (p = 0.023 and p = 0.05, respectively).

Based on the presented results, we have to point out that *IL17A* and *IL17F* expression profiles in patient's currently healthy, non-inflamed intestinal setting showed promising clinical potential and could be more discriminative than expression profile in mucosa which is already affected by increased inflammatory processes.

5. DISCUSSION:

Growing number of data have put in the focus Th17 role in development of chronic intestinal inflammation seen in IBD. It is widely accepted that IBD develops as a consequence of dysregulated epithelial barrier function and permeability of intestinum in association with increased bacterial translocation [22]. As a response to microbial invasion, TLRs subsequently induce activation of specific effector cytokines, such as IL17 [30]. However, it is not yet elucidated how these pathways are regulated in the IBD environment and which pro-inflammatory mediators will influence the site and type of the disease. In some cases, it is hard to distinguish CD from UC having an impact on therapy efficacy and disease treatment. Thus, it is important to define molecular pathways specific for different IBD phenotypes.

In the presented study, mRNA profiling was conducted in ileal and colonic CD as well as colonic UC paired non-inflamed and inflamed samples for the Th17 pathway-associated genes, *IL23A*, *IL17A* and *IL17F*, and for the *TLR9* gene. Results showed increased *IL17A* and *IL17F* expression levels in inflamed mucosa of all analysed disease phenotypes, indicating the importance of Th17 effector cells in both IBD forms which is in concordance with other literature data [3,4,5,6]. Some studies point out that imbalance between IL-17A and IL-17F could be important in IBD pathogenesis, with protective effect of IL-17A and pathogenic of IL-17F, as shown in murine models [39]. Our results did not validate this assumption, since we showed increased expression level of both *IL17A* and *IL17F* in inflamed ileal CD and colonic CD and UC mucosa. Also, results showed strong positive correlation between expression levels of *IL17A* and *IL17F*, indicating their associated transcriptional regulation in IBD. The *IL17A* and *IL17F* genes are located at the same chromosomal locus in both humans and mice and their transcription is likely

coordinated, but a recent study suggested that expression of these two closely related genes could be differently regulated depending on proinflammatory milieu in various disease states [39].

In contrast with *IL17A* and *IL17F*, the expression signatures of *IL23A* and *TLR9* were not equally significant in different sites of inflammation and different disease types. In inflamed CD ileal environment *IL23A* and *TLR9* expression levels showed a significant increment compared to paired non-inflamed mucosa, but this was not observed in CD colonic setting, even though the tendency of *IL23A* mRNA was to be higher. On the other hand, UC patients had significantly higher *IL23A* expression level in inflamed compared to non-inflamed mucosa while *TLR9* expression was not considerably altered. Distinct *TLR9* expression level in affected ileal CD samples could be associated with the presence of Paneth cells which prominently express TLR9. It is showed that the administration of oligonucleotides containing CpG sequence can lead to degranulation of Paneth cells and release of different antimicrobial molecules [11]. Furthermore, TLR9 activation leads to a production of predominantly Th1 cytokines [24]. Several studies demonstrated elevated *TLR9* mRNA in UC inflamed mucosa, however in those studies the expression levels were compared between the healthy individuals and UC patients' mucosa, while in this study the paired samples approach was used [23].Yet, overall data regarding TLR9 involvement in IBD are still scarce and further research is needed.

Even though the level of *IL23A* and *TLR9* mRNA increase was specific for the location and disease type, strong positive correlation between aforementioned genes was observed in all analysed samples. This result points towards the mechanism of TLRs induced IL23/Th17 pathway, which has not been fully elucidated, but it is discussed in the literature [40,41,42]. Studies on murine dendritic cells demonstrated TLR9-dependent IL-23/IL-17 response during granulomatous inflammation in the lung and activation of *IL23A* expression after TLR9 antigens stimulation via nuclear factor kappa B pathway [29]. Nevertheless, it should be emphasized that Th17 response could be also activated independent of TLR activation [43,44]. In our study, *TLR9* did not correlate with *IL17A* and *IL17F* expression levels, except for the inflamed UC mucosa, suggesting different regulation of Th17 response between IBD forms.

We observed a positive correlation of *IL23A* with *IL17A* as well as with *IL17F* mRNA only in UC and not in CD samples. Moderate level of correlation in UC and lack of correlation in CD between *IL23A* and *IL17A*, *IL17F* could be explained by the fact that IL23 is associated with Th17 growth and expansion but not with Th17 differentiation from naïve CD4+ cells, including other cytokines such as IL-6 and TGF-β in this complex interplay [15]. When colonic UC and CD samples were compared, significantly higher *IL17A* expression level in both inflamed and non-inflamed mucosa and generally higher *IL23A* and *IL17F* expression levels in inflamed mucosa of UC patients were detected, effect observed in other studies as well [3,4]. Interestingly, recent transcriptome meta-analysis showed that the expression of Th1 and Th17-related genes is very similar between CD and UC, except for *IL23A* which was more highly expressed in UC than CD mucosa [45]. Moreover, Kobayashi *et al.*[3] suggested that IL-23 may direct production of distinct cytokines between UC and CD, inducing more prominent Th17 response in UC and Th1/Th17 response in CD. The results from our study support this observation.

In addition, presented study evaluated analysed mRNAs as potential markers of disease activity and disease severity. There were no correlations between genes' expression levels and SES-CD and CDAI activity scores in ileal non-inflamed and inflamed CD samples. In colonic CD samples non-inflamed mucosal expression levels of *IL17A* and *IL17F* positively correlated while inflamed mucosal expression levels of *IL23A* and *TLR9* negatively correlated with CDAI. In contrast with CDAI, there were no correlations of mRNA expression with endoscopic activity of the disease in overall CD setting, which is in line with reports showing that the CD endoscopic disease activity correlates poorly with the CDAI [46]. In UC samples, non-inflamed mucosal expression levels of *IL17A* and *IL17F* and inflamed mucosal expression levels of *IL17A* and *IL23A* positively correlated with both endoscopic and total Mayo score.

In the transcriptomic study by Roman *et al* [47], among a number of mucosal gene transcripts that correlated with UC total Mayo score, *IL17A* and *IL23A* mRNAs were also identified, which is in agreement with our results. Furthermore, Olsen *et al* [4] demonstrated positive correlations between *IL17A* and *IL23A* mRNA with disease activity score and endoscopic sub-score in affected mucosal samples of UC patients, but also with CDAI-score in inflamed colonic samples of CD patients. It should

be taken into account that assessment of disease activity differs across studies, particularly evaluation of endoscopic activity, therefore yielding various results. Another study showed positive correlation between transcript levels of *IL23A* in CD with severity of endoscopic lesions (in terms of Rutgeert score) [49]. Iboshi Y *et al* [5] emphasized that not individual mucosal *IL17A* or *IL17F* expression level but ratio of these two genes mRNAs is a key gene signature that correlate with endoscopic score in UC patients using Rachmilewitz endoscopic index. Our study did not show correlation between UC disease activity scores and intestinal *TLR9* mRNA expression, although this was demonstrated previously [24,49,50].

Analysing the severity of IBD, we found that significantly higher *IL17F* and *IL23A* expression levels in non-inflamed ileal mucosa of CD patients associated with stricturing/penetrating phenotype (S/P), whereas in CD colonic non-inflamed mucosa of S/P patients were characterized with significantly lower level of *IL17A* mRNA compared to non-stricturing/non-penetrating (NS/NP) disease. According to murine model studies, it is believed that IL-17A has a role in strengthening tight-junctions formation by inducing the expression of claudins in intestinal epithelial cells, stimulating mucin production and increasing the mucosal barrier, while IL-17F exacerbates inflammation [51].

Regarding UC, patients with moderate and severe disease had significantly higher level of both *IL17A* and *IL17F* transcripts compared to patients with mild disease, but only in non-inflamed colonic mucosa. It has been shown that *IL17A* mRNA level was elevated in inflamed mucosal samples of moderate to severe UC [52]. Further, we found higher *IL23A* expression level in non-inflamed and inflamed colonic mucosa of UC patients with moderate/severe disease in comparison with patients with mild disease. These findings can be explained by IL-23 contribution to intestinal inflammation from restricting Treg cell activity to inducing expression of other Th17 type proinflammatory cytokines from both T cells and non-T sources [51].

Finally, we demonstrated association between operation occurrence and higher *IL17A* and *IL17F* expression levels in ileal non-inflamed samples of CD patients, whereas in colonic inflamed CD samples surgery was related to increased *IL23A* and *TLR9* transcript levels. However, this result should be verified on larger subject group since the number of operated patients was small.

We want to underline interesting observation related to disease markers measured in non-inflamed mucosa. Summarizing results, levels of analysed mRNAs in the currently non-involved IBD mucosa were more discriminative in terms of disease phenotype and severity than in mucosa already affected with inflammation. Indeed, it is intuitive to assume that in inflamed intestinal mucosa between distinct disease forms no significant variations in gene expression levels exist and that levels of the mRNA transcripts in non-inflamed mucosa could better portrait these phenotypic differences. However, future research will require patients follow up to confirm this observation.

Analysing gene expression patterns in biopsy samples is important for revealing molecular pathogenesis of IBD and targeting proinflammatory pathways specific for CD and UC. Our study reports that expression of TLR9-IL23-IL17 pathway related genes are of importance for IBD development and disease presentation. Still, expression profiles of the analysed genes are specific for disease type, localization and phenotype, with indication that UC is more "Th17 disease" type than CD. Although expression levels of selected genes in context of IBD were previously analysed, their role in development of different IBD subtypes was not fully established. Therefore, we think that this study could contribute to the better understanding and defining gene expression patterns in specific IBD phenotypes. Also, our findings emphasize the importance of mRNAs analysis in non-inflamed IBD mucosa since the results indicated their association with different disease extent and severity. Given that efficacy of IL-23 and IL-17 antagonist are currently being evaluated in clinical trials, better understanding of the proinflammatory setting of differently presented IBD could direct clinicians to optimizing current treatments to achieve better outcomes.

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Figure Legends:

Figure 1. Relative expression levels of IL17A, IL17F, IL23A and TLR9 genes in paired samples of non-inflamed and inflamed CD and UC intestinal mucosa. Expression levels in non-inflamed and inflamed mucosa from the same patients are connected with a line. Black line represents increase and grey line decrease in gene expression level between non-inflamed and inflamed mucosa. Differences between paired samples were tested by non-parametric Wilcoxon sign rank test and $p < 0.05$ was considered significant. NI - non-inflamed, I – inflamed, RU –relative units. * $p < 0.05$, ** $p < 0.0$.

Figure 2. Difference in gene expression levels between CD (n = 24) and UC (n = 54) non-inflamed (NI) and inflamed (I) colonic mucosa. For analysis of difference between the groups Mann-Whitney U non-parametric test was used and $p < 0.05$ was considered significant.

Figure 3. Mucosal gene expression levels in CD patients with non-stricturing/non-penetrating (NS/NP) and stricturing/penetrating (S/P) disease. For analysis of difference between NS/NP and S/P groups Man-Whitney U test was applied, and $p < 0.05$ was considered significant

Figure 4. Mucosal gene expression levels between UC patients with mild and moderate/severe disease. For analysis of difference between mild and moderate/severe groups Man-Whitney U test was applied, and $p < 0.05$ was considered significant.

Figure 5. Mucosal gene expression levels between at diagnosis not operated and operated CD patients. For analysis of difference between not operated and operated groups Man-Whitney U test was applied, and $p < 0.05$ was considered significant

Table 1. Demographic and clinical characteristic of analyzed CD and UC patients

Characteristics	CDi (n = 26)	CDc (n = 24)	UC (n = 54)
Median (IQR) age, years	36.0 (25.0 - 44.3)	34.0 (28.3 - 46.8)	43.5 (33.8 - 57.0)
Gender, male/female (%)	14 / 12 (53.8 / 46.2)	19 / 5 (79.2 / 20.8)	24 / 30 (44.4 / 55.6)
Median (IQR) BMI	24.6 (22.5 - 26.8)	24.4 (20.5 - 26.1)	23.7 (21.2 - 24.7)
Median (IQR) duration of symptoms prior diagnosis, months	9.5 (4.5 - 13.0)	7.5 (6.0 - 12.0)	6.5 (6.0 - 12.0)
Localization of disease			
L1[a] / E1[b] (%)	16 (61.5)	NA	16 (29.6)
L2[a] / E2[b] (%)	NA	15 (62.5)	19 (35.2)
L3[a] / E3[b] (%)	10 (38.5)	9 (37.5)	19 (35.2)
Phenotype of disease			
B1[c] / S1[d] (%)	11 (42.3)	15 (62.5)	36 (66.7)
B2[c] / S2[d] (%)	11 (42.3)	6 (25.0)	15 (27.8)
B3[c] / S3[d] (%)	4 (15.4)*	3 (12.5)[#]	3 (5.6)
Median (IQR) CRP level, mg/l	11.5 (8.0 - 16.0)	14.5 (9.0 - 30.0)	12.0 (8.0 - 18.8)
Median (IQR) CDAI score	180.0 (137.5 - 222.5)	180.0 (154.0 - 246.3)	NA
Median (IQR) Mayo score	NA	NA	6.0 (4.0 - 8.0)
Operation at diagnosis (%)	6 (23.1)	3 (12.5)	0 (0.0)
Previous NSAID usage (%)	4 (15.4)	6 (25.0)	18 (33.3)
Extraintestinal manifestation (%)	3 (11.5)	3 (12.5)	8 (14.8)
Smoking (%)	8 (30.8)	11 (45.8)	21 (38.9)
Family history of the IBD	6 (23.1)	1 (4.2)	2 (3.7)

CDi – samples obtained from ileal CD mucosa, CDc – samples obtained from colonic CD mucosa, UC – samples obtained from colonic UC mucosa. * all penetrating CDi patients had perianal modifier, # two CDc patients with penetrating phenotype had also perianal disease , [a] Localisation of CD according to Montreal classification, B1 - ileum, B2 - colon, B3 –ileocolon, [b] Extent of UC according to Montreal classification, E1 - ulcerative proctitis, E2 - left side UC, E3 -extensive UC, [c] Behaviour of CD according to Montreal classification, B1 - non-stricturing, non-penetrating disease, B2 - stricturing, B3 – penetrating, [d] Severity of UC according to Montreal classification, S1 -mild, S2 - moderate, S3 – severe.

IQR – interquartile range, CRP – C reactive protein, CDAI – Crohn's disease activity index, NSAID – nonsteroidal anti-inflammatory drugs

Table 2. Correlation analysis of gene expression level in intestinal CD and UC mucosa

		CDi (n = 26)		CDc (n = 24)		UC (n = 54)	
		Non-inflamed	Inflamed	Non-inflamed	Inflamed	Non-inflamed	Inflamed
IL17A	***IL17A***	ns‡		rs = 0.657‡ $p < 0.001$		ns‡	
	IL17F	rs = 0.813 $p < 0.001$	rs = 0.745 $p < 0.001$	rs = 0.834 $p < 0.001$	rs = 0.923 $p < 0.001$	rs = 0.871 $p < 0.001$	rs = 0.771 $p < 0.001$
	IL23A	ns	ns	ns	ns	rs = 0.370 $p = 0.006$	rs = 0.370 $p = 0.006$
	TLR9	ns	ns	ns	ns	ns	ns
IL17F	***IL17F***	ns‡		rs = 0.764‡ $p < 0.001$		ns‡	
	IL23A	ns	ns	ns	ns	rs = 0.332 $p = 0.014$	rs = 0.311 $p = 0.022$
	TLR9	ns	ns	ns	ns	ns	rs = 0.292 $p = 0.032$
IL23	***IL23A***	ns‡		ns‡		rs = 0.464‡ $p = 0.0004$	
	TLR9	rs = 0.751 $p < 0.001$	rs = 0.815 $p < 0.001$	rs = 0.820 $p < 0.001$	rs = 0.786 $p < 0.001$	rs = 0.771 $p < 0.001$	rs = 0.686 $p < 0.001$
TLR9	***TLR9***	rs = 0.420‡ $p = 0.03$		rs = 0.417‡ $p = 0.04$		rs = 0.543‡ $p < 0.001$	

Correlation level was measured using non-parametric Spearman's test and presented with correlation coefficient (rs). $p < 0.05$ was considered significant. ‡ Expression level of the specific gene was correlated between non-inflamed and inflamed mucosa. CDi – samples obtained from ileal CD mucosa, CDc – samples obtained from colonic CD mucosa, UC – samples obtained from colonic UC mucosa

Table 3. Correlation of intestinal gene expression levels with clinical and endoscopic activity of CD and UC

			SES-CD* or Mayo endoscopic score#	CDAI* or total Mayo score#
IL17A	CDi	Non-inflamed	ns	ns
		Inflamed	ns	ns
	CDc	Non-inflamed	ns	rs = 0.405 *p* = 0.049
		Inflamed	ns	ns
	UC	Non-inflamed	rs = 0.405 *p* = 0.002	rs = 0.384 *p* = 0.004
		Inflamed	rs = 0.359 *p* = 0.008	rs = 0.351 *p* = 0.009
IL17F	CDi	Non-inflamed	ns	ns
		Inflamed	ns	ns
	CDc	Non-inflamed	ns	rs = 0.506 *p* = 0.012
		Inflamed	ns	ns
	UC	Non-inflamed	rs = 0.327 *p* = 0.016	rs = 0.290 *p* = 0.034
		Inflamed	ns	ns
IL23A	CDi	Non-inflamed	ns	ns
		Inflamed	ns	ns

	CDc	Non-inflamed	ns	ns
		Inflamed	ns	rs = - 0.413 p = 0.045
	UC	Non-inflamed	ns	ns
		Inflamed	rs = 0.359 p = 0.008	rs = 0.311 p = 0.022
TLR9	CDi	Non-inflamed	ns	ns
		Inflamed	ns	ns
	CDc	Non-inflamed	ns	ns
		Inflamed	ns	rs = - 0.406 p = 0.049
	UC	Non-inflamed	ns	ns
		Inflamed	ns	ns

* SES-CD (Simple Endoscopic Score for Crohn's Disease) and CDAI (Crohn's disease activity index) – clinical CD activity scores, # Endoscopic and Total Mayo score – clinical UC activity scores. ns – not significant, rs = Spearman's rho correlation coefficient. CDi – samples obtained from ileal CD mucosa, CDc – samples obtained from colonic CD mucosa, UC – samples obtained from colonic UC mucosa

HIGHLIGHTS

- Upregulated *IL17A* and *IL17F* expression was demonstrated in inflamed IBD mucosa.
- Upregulated *TLR9* and *IL23A* expression was observed for inflamed CD ileum.
- Upregulated *IL23A* expression was found in inflamed UC colon.
- Analysed mRNA levels correlated with clinical and endoscopic disease activity scores.
- Analysed mRNA levels in non-inflamed gut mucosa may indicate severe IBD course.

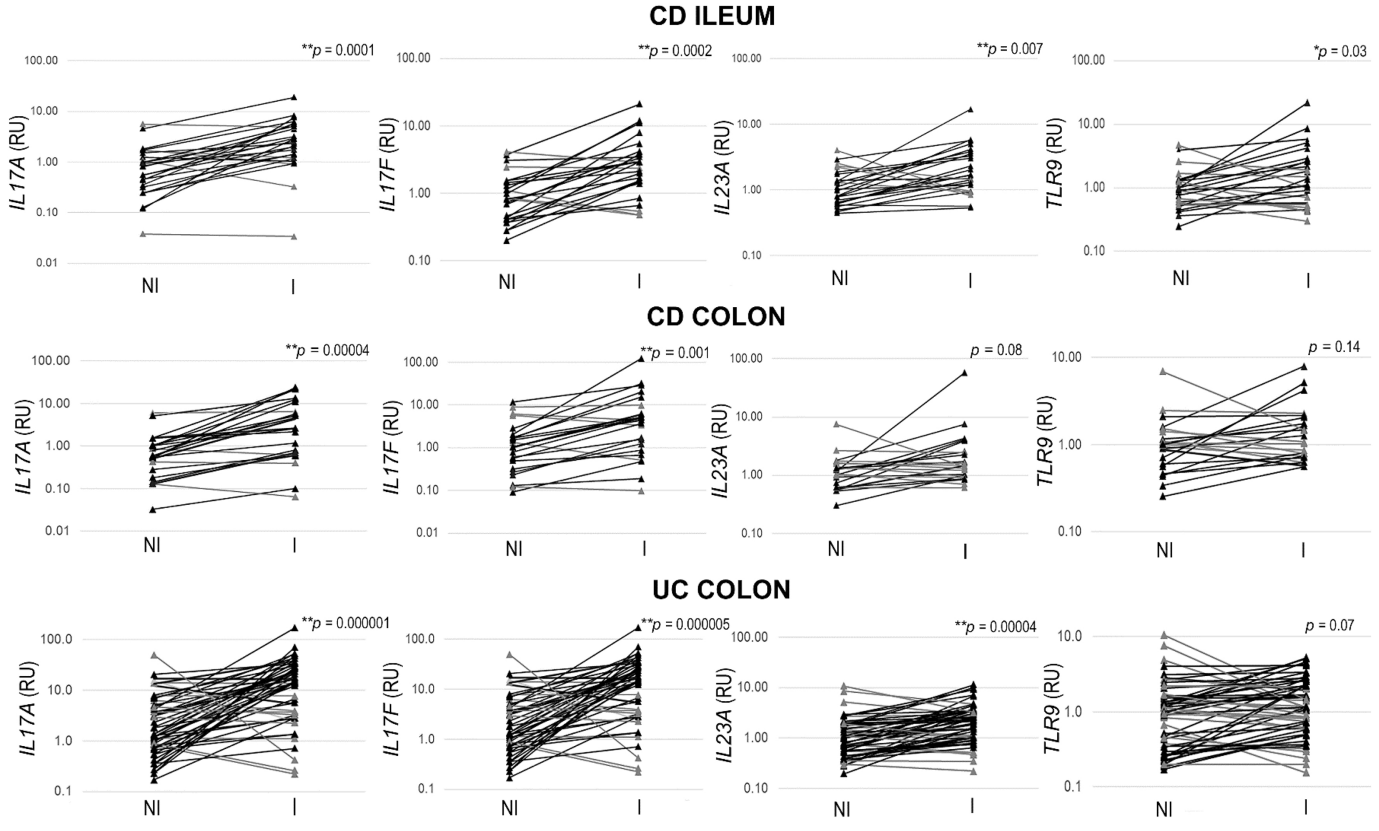
CD ILEUM
**p = 0.0001
**p = 0.0002
**p = 0.007
*p = 0.03
IL17A (RU)
IL17F (RU)
IL23A (RU)
TLR9 (RU)
NI
I
CD COLON
**p = 0.00004
**p = 0.001
p = 0.08
p = 0.14
UC COLON
**p = 0.000001
**p = 0.000005
**p = 0.00004
p = 0.07

Figure 1

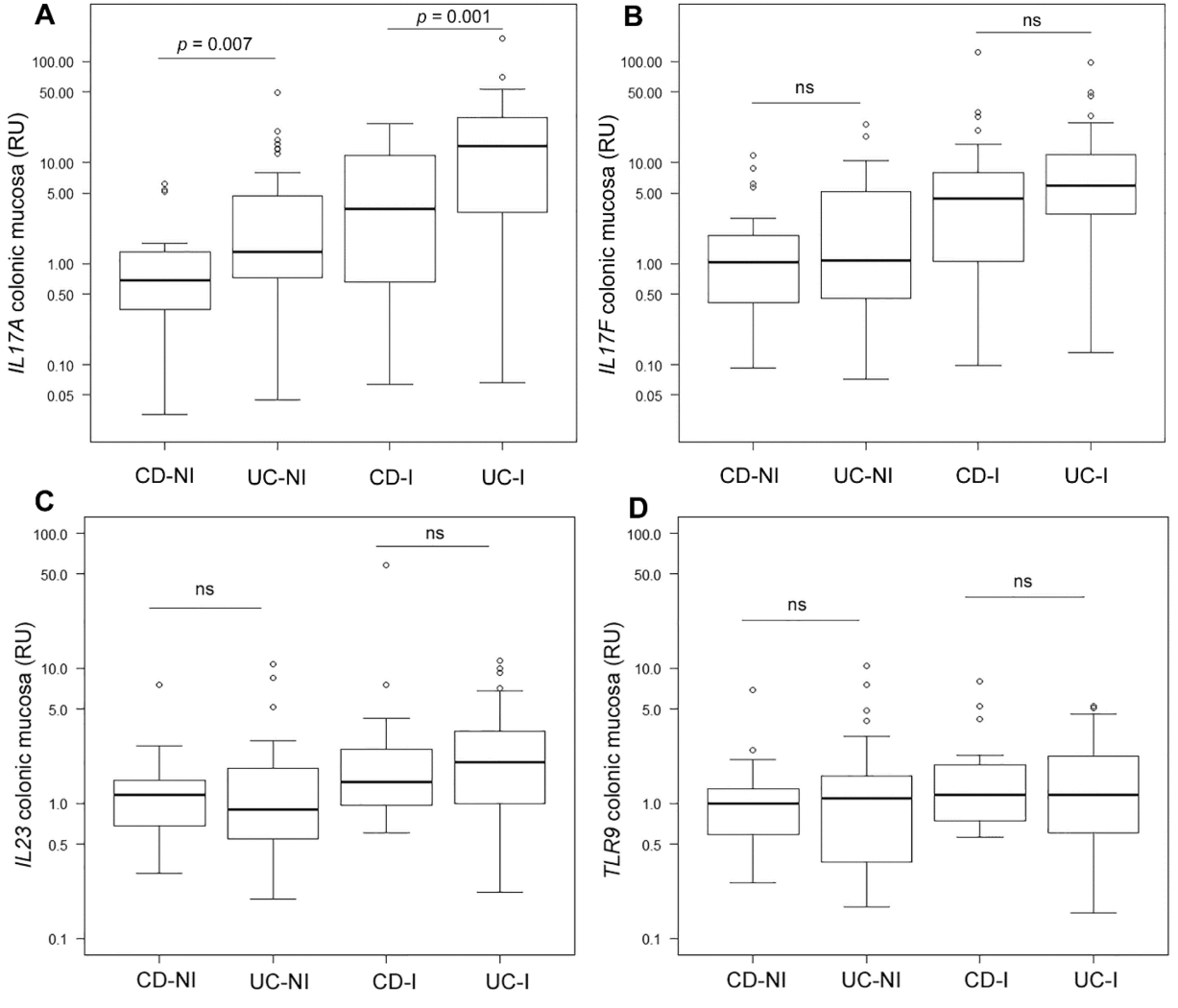
A
p = 0.007
p = 0.001
IL17A colonic mucosa (RU)
100.00
50.00
10.00
5.00
1.00
0.50
0.10
0.05
CD-NI
UC-NI
CD-I
UC-I
B
ns
ns
IL17F colonic mucosa (RU)
C
ns
ns
IL23 colonic mucosa (RU)
100.0
50.0
10.0
5.0
1.0
0.5
0.1
D
ns
ns
TLR9 colonic mucosa (RU)

Figure 2

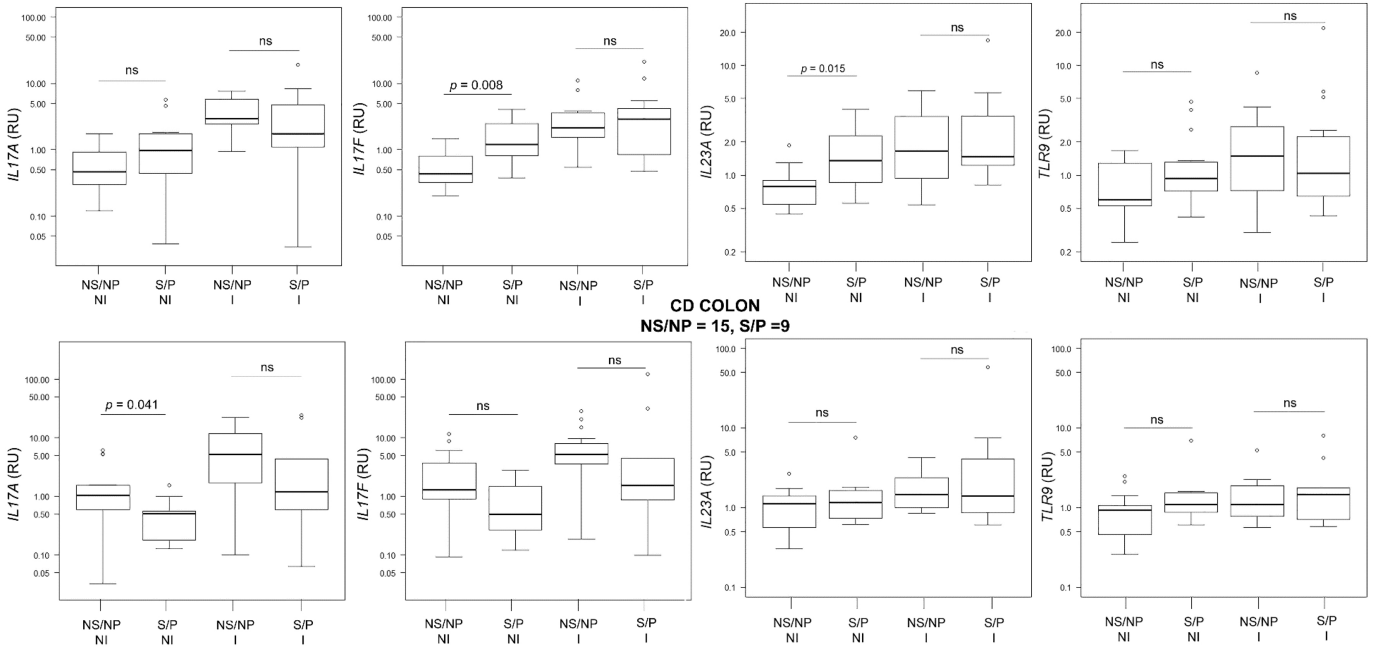
CD ILEUM
NS/NP= 11, S/P = 15
IL17A (RU)
IL17F (RU)
IL23A (RU)
TLR9 (RU)
ns
p = 0.008
p = 0.015
NS/NP NI
S/P NI
NS/NP I
S/P I
CD COLON
NS/NP = 15, S/P =9
p = 0.041

Figure 3

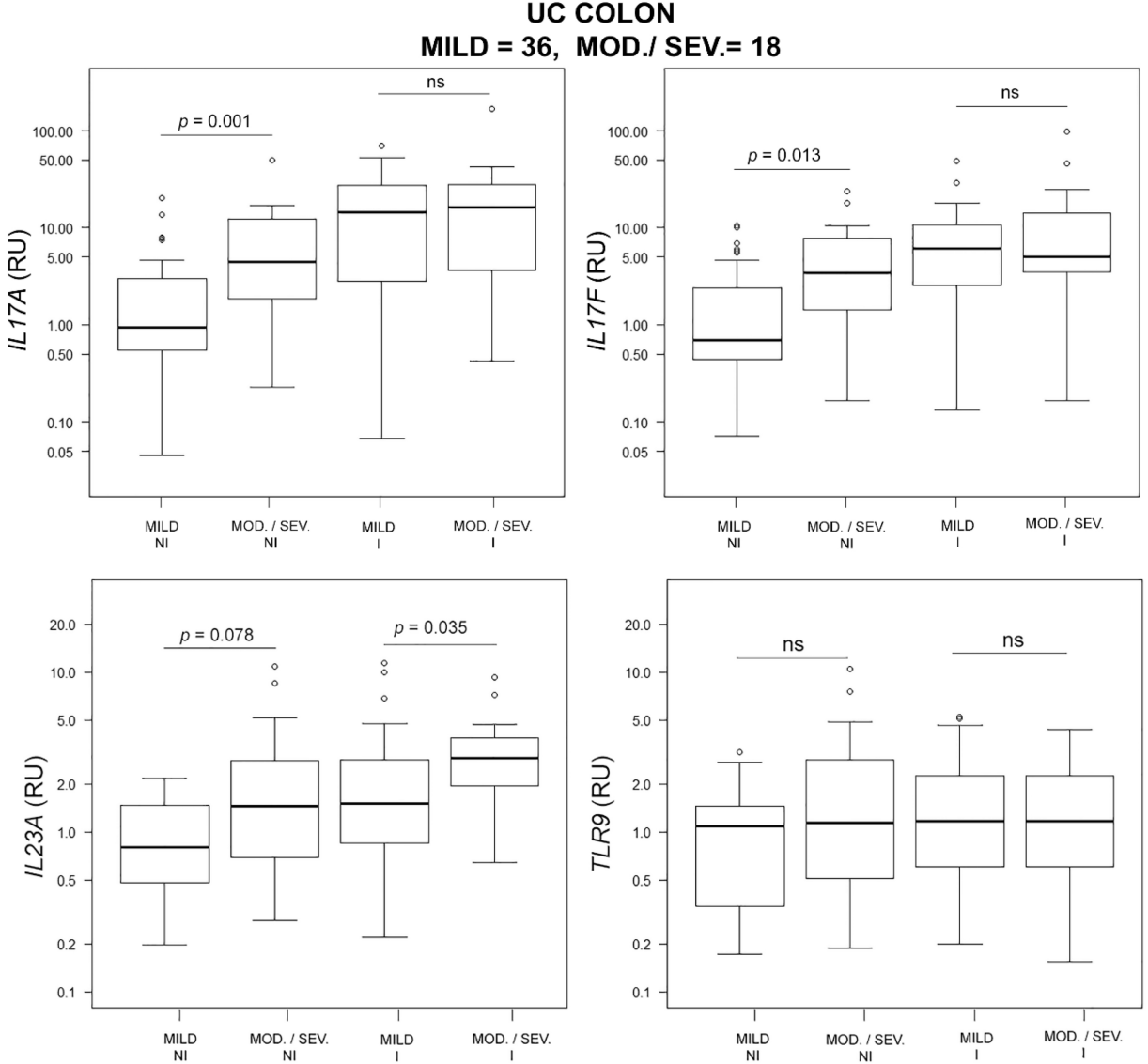
UC COLON
MILD = 36, MOD./ SEV.= 18
IL17A (RU)
p = 0.001
ns
100.00
50.00
10.00
5.00
1.00
0.50
0.10
0.05
MILD NI
MOD. / SEV. NI
MILD I
MOD. / SEV. I
IL17F (RU)
p = 0.013
ns
IL23A (RU)
p = 0.078
p = 0.035
20.0
10.0
5.0
2.0
1.0
0.5
0.2
0.1
TLR9 (RU)
ns
ns

Figure 4

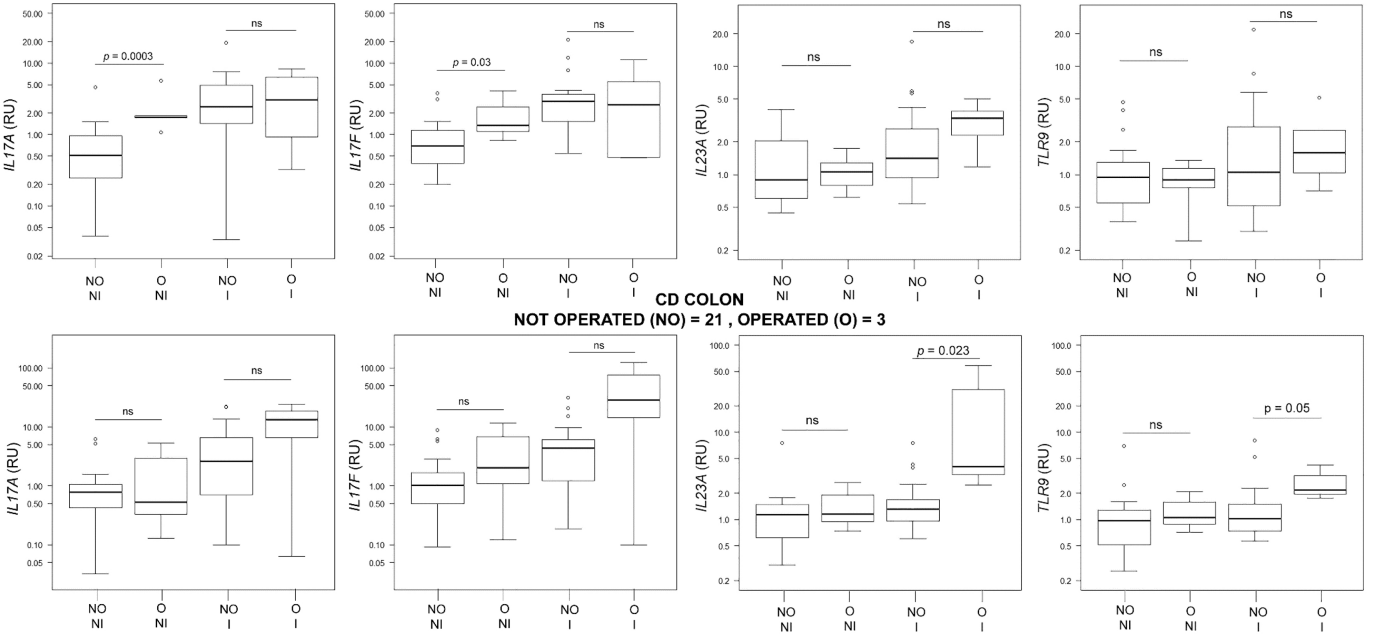
CD ILEUM
NOT OPERATED (NO) = 20, OPERATED (O) = 6
IL17A (RU)
p = 0.0003
ns
IL17F (RU)
p = 0.03
ns
IL23A (RU)
ns
ns
TLR9 (RU)
ns
ns
NO NI
O NI
NO I
O I
CD COLON
NOT OPERATED (NO) = 21 , OPERATED (O) = 3
IL17A (RU)
ns
ns
IL17F (RU)
ns
ns
IL23A (RU)
ns
p = 0.023
TLR9 (RU)
ns
p = 0.05
NO NI
O NI
NO I
O I

Figure 5