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IL-13 mRNA tissue content identifies two subsets of adult ulcerative colitis patients with different clinical and mucosa-associated microbiota profiles

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ABSTRACT

Background and Aim. A personalized approach to therapy has great promise to improve disease outcomes. To this end, the identification of different subsets of patients according with the prevalent pathogenic process might guide in the choice of therapeutic strategy. We hypothesize that UC patients might be stratified according to distinctive cytokine profiles and/or to a specific mucosa-associated microbiota **Methods.** In a cohort of clinically and endoscopic active UC patients and controls, we analyzed by qPCR the mucosal cytokine mRNA content and the mucosa-associated microbiota composition assessed by the 16SrRNA gene sequencing. **Results.** We demonstrate, by means of data-driven approach, the existence of a specific UC patient subgroup characterized by elevated IL-13mRNA tissue content separated by patients with low IL-13 mRNA tissue content. The two subsets differ in clinical-pathological characteristics. High IL-13mRNA patients are younger at diagnosis and show higher prevalence of extensive colitis than low IL-13mRNA ones. They also show a more frequent use of steroid/immunosuppressant/anti-TNFα therapy during a one-year follow-up. The two subgroups show a differential enrichment of mucosa associated microbiota genera with prevalence of *Prevotella* in patients with high IL-13mRNA tissue content and *Sutterella* and *Acidaminococcus* in patients with low IL-13mRNA tissue content. **Conclusion**. Assessment of mucosal IL-13mRNA might help in the identification of the patients' subgroup that might benefit from a therapeutic approach modulating IL-13.

Keywords: Ulcerative colitis, IL-13, microbiota

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INTRODUCTION

A personalized approach to therapy has great promise to improve disease outcomes. Selection of patients as candidates for the early introduction of highly effective therapy can both maximize treatment efficiency and prevent long-term complications. The classic pro-inflammatory cytokines (such as IL-6 and TNF-α) have an accepted role in the pathogenesis of inflammatory lesions, and recent findings showed that Th17 response also participates in the inflammatory process [1, 2]. Ulcerative Colitis (UC) has been associated with an atypical Th2 cell response mediated inflammation [3-6]. Although some evidences provided a proof of concept that IL-13 is an effector cytokine in UC [7], administration of anti-human IL-13 neutralizing monoclonal antibody, did not significantly improve clinical response vs placebo in UC patients [8]. However, in the same study, the proportion of patients who achieved clinical remission was statistically significantly higher in the anti-IL-13 treated group compared with the placebo group [8], suggesting the presence of a UC patients' subgroup responding to IL-13 neutralizing treatment.

It is increasingly evident that there is a close relationship between the microbiota composition and gut immune response, which includes both the cellular and the soluble component of the immune response. Specific components of the gut microbiota are involved in the production of pro-inflammatory cytokines and subsequent generation of Th17 cells [9, 10]. Similarly, commensal bacteria and their metabolites can also promote the generation of regulatory T cells [10-13] which, in turn, influence the microbiota composition [14].

We hypothesize that UC patients might be stratified according to distinctive cytokine profiles and/or to a specific mucosa-associated microbiota. Therefore, we analyzed the mucosal cytokine mRNA content and the mucosa-associated microbiota composition of a cohort of clinically and endoscopic active UC

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patients. We demonstrate, by means of data-driven approach, in the context of a generalized increased mRNA IL-17A tissue content in UC patients, the existence of a specific UC patient subgroup characterized by elevated IL-13 mRNA tissue levels distinct by patients with IL-13 mRNA levels not distinguishable by control subjects. Furthermore, the two patients' subgroups (high and low IL-13mRNA tissue content) show different clinical-pathological characteristics and a differential enrichment of mucosa-associated microbiota genera known to be involved in UC dysbiosis.

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METHODS

Patients. A total of 88 UC patients attending two clinical tertiary centers (IBD, GE Unit, Sandro Pertini Hospital, Rome, and Department of General Surgery, "P. Stefanini", Sapienza University, Rome) with endoscopically active disease (Mayo score ≥1 [15]) were evaluated. All the patients were not on rectal 5-ASA and/or steroids in the last 3 months, nor had assumed antibiotics in the last 3 months. A control group of 24 subjects undergoing colonoscopy for colon cancer screening or suspected functional bowel disorders was also included. In UC patients, disease extension, at the time of endoscopy, was classified using the Montreal classification [16] as follows: proctitis, involvement limited to the rectum; left-sided colitis, involvement limited to a portion of the colorectum distal to the splenic flexure; extensive colitis, involvement extending proximal to the splenic flexure. Multiple endoscopic mucosal biopsies were obtained from endoscopically involved and uninvolved areas in UC patients, and from matched areas in controls. Histopathology was quantified in H&E stained tissue sections using the Robarts histopathology index (RHI) [17]. RHI values <3 and >6 were recorded for all biopsy samples collected from uninvolved and involved tissue, respectively. Histology confirmed the absence of inflammatory changes in controls. For cytokine/microbiota analysis, multiple biopsies taken from involved and uninvolved tissue were stored in RNA later for quantification of cytokine profile and identification of mucosa-associated microbiota by metagenomics analysis. Biopsies samples for RNA extraction were available in 81 UC patients and 21 Controls. 20 controls and 77 patients (77 samples from involved tissue and 50 samples from uninvolved tissue within the same patients) gave rise to a good quality RNA to be amplified. Biopsies for microbiota analysis were available in 24 controls and 88 UC patients. 21 controls and 79 patients (79 samples from involved tissue and 57 samples from uninvolved tissue within the same patients) endowed with a sufficient quality data for sequencing analysis. In 68 patients both RNA and microbiota analysis were available.

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Clinical-pathological variables of patients contributing cytokine, microbiota or cytokine+ microbiota data were largely superimposable. Given the main focus of the study is on the immunological subtyping we report the clinical-pathological variables for the 77 patients data set (Table1).

Cytokine tissue mRNA content quantification

RNA was extracted using RNA mini Kit Plus (Qiagen, Hilden, Germany) and its quality analyzed by Agilent RNA 6000 Nano Kit using Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA). RNA with Refractive Index Detector (RID) ≥ 7 was used for subsequent analysis. cDNA was reverse transcribed from 1μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ - Thermo Fisher Scientific, Massachusetts, USA). We quantified by quantitative real-time PCR (RTqPCR) the tissue content of TNF-α, IL-6, IFN-γ, IL-17A, IL-10 and IL-13 using the following primers: TNF-α forward 5-CCCAGGGACCTCTCTCTAATCA, reverse 5-GCTACAGGCTTGTCACTCGG-3; IL-6 forward 5'-CCACTCACCTCTTCAGAACG-3', reverse 5'-GCCTCTTTGCTGCTTTCACAC-3'; IFN-γ - forward: 5'-ATAGGTAACTGACTTGAATGTCCA-3', reverse 5'-GCTCTTCGACCTCGAAACAGC-3'; IL-17A - forward: 5'-ACTACAACCGATCCACCTCAC-3', reverse 5'-ACTTTGCCTCCCAGATCACAG-3'; IL-10-forward 5'-CCCAAGCTGAGAACCAAGACC-3', reverse 5'-TCACAGGGAAGAAATCGATGAC-3'; IL-13 - commercially available primers RT² qPCR Primers Assay (Qiagen) cat.PPH00688FRPS. As housekeeping gene, we utilized RPS9 –forward: 5'-AAGCTGATCGGCGAGTATG-3', reverse 5'-TCTTCAGGCCCAGGATGTAA-3' [18]. The gene amplification was performed in duplicate at Tm-60°C using Fast SYBR™ Green Master Mix (Applied Biosystems™ - Thermo Fisher Scientific). Data are expressed as Log2 (Relative Expression) coming from the equation: Relative Expression = 40-DCt where 40 is the maximum number of cycles and DCt is the difference in number of cycles of the gene of interest with the housekeeping gene. We attributed a relative expression of 40 to samples with

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undetectable expression, (threshold cycle ≥ 40); next, we estimated the relative expression as described above.

Statistical analysis

The presence of a statistically significant difference between controls and UC patients' cytokine tissue mRNA content was assessed by Mann-Whitney's U. The optimal 'clustering properties' of IL-17A and IL-13 with respect to the other cytokines were assessed by means of pseudo F statistics [19, 20] bimodality index as well as by the correlation structure among a set of cytokines by means of Pearson correlation and Principal Component Analysis (PCA). The bi-dimensional space spanned by IL-17A and IL-13 was analyzed by K-means [21], the by far most common unsupervised non-hierarchical clustering technique maximizing the Between/Within cluster variance ratio. The choice of the number of clusters (K value) was based upon the maximal distance of the observed R-square (Between cluster variance/Total variance ratio) with the one expected by a Gaussian distribution of data. The search for relevant correlations between cluster composition and clinical-pathological variables was performed by means of Chi-Square statistics and Analysis of Variance for categorical and interval variables respectively.

Mucosal samples collection and bacterial genomic DNA extraction

Endoscopic biopsies were collected in RNAlater and stored at -80°C until extraction of nucleic acids. The bacterial genomic DNA extraction was carried out with DNeasy PowerLyzer PowerSoilKit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality was assessed by gel electrophoresis and spectrophotometry, measuring OD 260/280.

16S Ribosomal RNA Gene Amplicons preparation and Illumina MiSeq sequencing

Library of 16S rRNA gene amplicons was prepared by IGA Technology Services (Udine, Italy) through amplification of the V3-V4 hypervariable region by using specific-barcoded primers with

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overhang adapters. The standard protocol was followed according to the 16S metagenomic sequencing library preparation guide from Illumina (Part # 15044223 Rev. B; https://support.illumina.com/). Pooled V3-V4 amplicon libraries were sequenced using the Illumina MiSeq platform. Each sample was sequenced at IGA Technology (Udine, Italy) using Illumina MiSeq instrument and a 300-bp paired-end reads protocol.

Sequencing Data analysis

Demultiplexed reads were downloaded and quality checked using FastQC 0.11.5. Reads were further pre-processed by removing Illumina sequencing adaptors with CUTADAPT [22], and by trimming the low quality end of each read with SICKLE [23], with a quality threshold of 20. Trimmed forward and reverse reads were then joined with PEAR [24] and checked for chimera presence with VSEARCH [25]. OTUs picking was performed in QIIME 1.8 [26] with uclust algorithm [27] using default values, and assigning the taxonomy using the GreenGenes 13.8 database. Low coverage OTUs were filtered using filter_otus_from_otu_table.py and parameter --min_count_fraction 0.00005.

Subsequent analyses were performed in R statistical software. The file produced by QIIME was parsed in R with phyloseq package [28] and OTUs count data were normalized by cumulative sum scaling (CSS) as implemented in the metagenomeSeq package [29]. Alpha diversity was evaluated using Observed richness (the number of different OTUs in a sample), Shannon's diversity Index (a measure of community diversity, accounting for both abundance and evenness of bacterial OTUs), and Evenness index (a measure of uniformity of relative abundances of the OTUs in a sample) [30]. Beta diversity was analyzed by means of principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity index producing an explicit metric space from microbiome profiles dissimilarities. Inferential statistics on the above described metrics was performed by Analysis of Variance and Student's t-test. LEfSe (Linear discriminant analysis Effect Size) [31] on the CSS transformed abundances, was performed to identify plausible bacterial biomarker(s) able to separate different

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groups. In LEfSe, Kruskal–Wallis rank-sum test (significance threshold 0.05) was firstly used to identify significantly different taxa abundances among classes (in this case, we used patients' clusters as group variable), and then LDA (linear discriminant analysis) is used to estimate the size of the discriminating effect for each feature. A feature (i.e. a Genus-level OTU) is retained as a suitable biomarker of a class if the LDA value (Between/Within Variance ratio) was higher than 2.

Microbiota sequence data are available at http://www.ebi.ac.uk, under the accession number PRJEB31884. The script used for pre-treatment and OTUs picking, as well as the script for data analysis performed in R, are available at the following GitHub repository https://github.com/FrancescoVit/Supplementary-to-Butera-et-al.-2019

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RESULTS

IL-13 mRNA tissue content allows for a meaningful classification of UC patients

We preliminary analyzed the mRNA tissue content of different cytokines in UC patients and control subjects. As illustrated in Figure 1, in UC patients, all the cytokines evaluated show significantly increased mRNA tissue content in the involved tissue vs uninvolved tissue that in turn show values comparable to controls. However, it is worth noting IL-13 values have a clear bimodal distribution, while IL-17A values in UC involved tissue show the smallest overlap with the controls suggesting discriminatory ability in separating the two groups. Analysis of Variance as applied to controls-patients discrimination resulted statistically significant for all the analyzed cytokines with the only exception of IL-13 (Supplementary Table 1). Moreover, IL-13 distribution and the related quantile-quantile (Q-Q) distribution (Supplementary Figure 1, panel A and B, respectively) clearly show a deviation from the normal distribution for both patients and controls, thus not related to "*a priori* class discrimination" suggesting clustering relevance of IL-13 for the detection of disease sub-groups. This suggestion had a strong proof by the application of two independent computational approaches: a) Bimodality estimation by Pseudo F statistics [19, 20] and b) Pearson correlation and Principal Component Analysis as applied to cytokine profile of UC patients.

Pseudo-F statistics corresponds to the ratio of Between/Within cluster variance: the greater this ratio, the greater the relevance of the clusters. In the case of two clusters, the Pseudo-F is an index of bimodality, with high values pointing to the presence of two distinct populations in the same set.

We computed the Pseudo-F statistics on the two cluster optimal K-means solution for all the evaluated cytokines independently from the control/patient *a priori* classification. According with this analysis IL-17A and IL-13 were the two cytokines endowed with the higher values of the index and thus the two optimal variables for classification purposes (Supplementary Table 2). In the case of IL-17A, Pseudo-F

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registers the control/patient discriminatory power of this cytokine. On the contrary, the lack of any control/patient discriminant power of IL-13 makes the elevated Pseudo-F values of this cytokine the image in light of a different latent biological categorization.

Even more compelling, we found that the differences in mRNA tissue content of the involved vs uninvolved colonic area from the same patient relative to IL-6, TNF-α, IFN-γ and IL-10 were each other strongly correlated (Pearson correlation coefficient), while IL-17A and IL-13 variations were both independent from all the other cytokine mRNA concentration and independent of each other. This correlation structure gave rise to a three principal components (PCs) solution of the entire cytokine space (Supplementary Table 3). The first principal component (PC1, explaining 49% of total variance) consists in an aspecific inflammatory response including the joint variation of all the cytokines evaluated, with the exception of IL-13 and IL-17A. Notably, IL-13 and IL-17A belong to two mutually independent components (PC2 and PC3) explaining 19.4% and 16.5% of total variance respectively. The inspection of component loading, correspondent to the Pearson correlation between original variable and components (Supplementary Table 3 and accompanying Figure) identifies PC2 with IL-13 (Pearson r=0.94) and PC3 with IL-17A (Pearson r=0.96) respectively. The above results point to additional properties (the components are each other orthogonal by construction) of IL-13 and IL-17A (PC2 and PC3), independent from aspecific inflammation (PC1) that can be used to identify subgroups of UC patients by means of a clustering approach on these two cytokines. Thus, while PC1 is a 'size' component [32, 33], both PC2 and PC3 are 'shape' components independent of the 'entity' of inflammation (registered by PC1) and potentially useful for the discrimination of disease sub-groups.

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UC patients are distributed in clusters characterized by high and low IL-13 mRNA

Given the above described observations, we concentrated on IL-13 and IL-17A mRNA levels in order to single out a reliable partition of the data. As shown in Figure 2, the combination of the two cytokines indeed clearly separated UC patients from control subjects confirming the significant alteration of the two cytokine expression due to the disease. We then evaluated the distribution of subjects in the IL-17A/IL-13 plane. As shown in Figure 3A only UC patients have IL-17A values greater than the mean value of IL-17A (right quadrants), while, as far as IL-13 values, the higher than mean values belong to both UC patients and control subjects although the former are prevalent. Thus, distribution of IL-17A and IL-13mRNA values points to the existence of discrete clusters of subjects. A k-means cluster analysis on the IL-17A/IL-13 plane was computed (Figure 3B). It is worth to stress that k-means is an unsupervised technique and does not take into account the class of the subjects (patients or controls) but only their mutual relations in the IL-17A/IL-13 plane. Data represented in Supplementary Figure 2 points to a six clusters partition as optimal. The six clusters partition explains 91% of the total information present in the data set (R-square=0.91). With the only exception of cluster 1 (accommodating for two outlier observations), the classes are sufficiently populated to allow for a reliable profiling of IL-17A and IL-13 tissue content. The IL-17A mRNA tissue content varies smoothly from healthy to diseased patients: the ‘extreme’ cluster 6 and cluster 2 are devoid of any control subject, while the intermediate and ‘left side of the plane’ clusters have a mixed controls/UC populations (Figure 3B). Cluster 6 is made of patients with ‘high IL-17A/high IL-13’ pattern, while cluster 2 have a ‘high IL-17A/low IL-13’ pattern (Figure 3B). The above analysis allows us to define a bipartite profile of UC patients according to ‘high IL-13’ (Cluster 6) and ‘low IL-13’ (Cluster 2) mRNA tissue content in the context of high IL-17A mRNA tissue content.

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Clusters in the IL-13/IL-17A plane show differences as for sex, age at diagnosis, prevalence of extensive colitis and steroid/ immunosuppressant/anti-TNF-a therapy use

Having proved the existence of patient cluster distribution (further confirmed by bootstrap simulations), we analyzed the observed cytokine-based patient subgroups according to the clinical-pathological variables. We preliminary assessed the presence of differences in the clinical--pathological variable among the clusters 2, 3 and 4 (low IL-13mRNA content) and between the clusters 5 and 6 (high IL-13 mRNA content). Since we did not find any statistical differences (Supplementary Table 4) we grouped cluster 2, 3 and 4 (low IL-13mRNA content) and cluster 5 and 6 (high IL-13mRNA content) for the analysis. No differential distribution as for disease duration (139±20 *vs* 123±16 months, mean±SE; p=0.5, low and high IL-13 subset, respectively), therapy at time of endoscopy (corticosteroids, immunosuppressant, biological therapy at the time of endoscopy: 33% *vs* 32 % low and high IL-13 subset, respectively) and endoscopic Mayo score (1.9±0.12 *vs* 2.1±0.11, mean±SE; p=0.38, low and high IL-13 subset, respectively), was observed. A significantly lower prevalence of females was observed in patients belonging to low IL-13 mRNA tissue content (Figure 4A). Patients with high IL-13 mRNA tissue content show a significant lower age at diagnosis than patients with low IL-13 mRNA tissue content (Figure 4B). A significant higher prevalence of patients with extensive colitis was observed in patients with high IL-13 mRNA than in patients with low IL-13 mRNA tissue content (Figure 4C). In 42 out of the 77 patients, information regarding therapy during at least 1 year of follow-up was available. This subset of patients did not differ from the whole group as for clinical--pathological variables at entry (Supplementary Table 3). Furthermore, the distribution along different clusters of the 42 patients in which follow up was available did not differ from the whole set distribution (clusters 2+3+4: 41.86% vs 42.86%; clusters 5+6: 58.14% vs 57.14%, respectively). Therefore, we considered the 42 patients subset as an unbiased subsample of the whole set.

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In this subset of patients, the relative frequency of clinical relapses during the one-year follow-up period did not differ between patients with low and high IL-13mRNA content (27 vs 29 %, proportion of patients with clinical relapses, respectively). However, patients with high IL-13 mRNA showed, during the follow-up, but not at the time of endoscopy (corticosteroids, immunosuppressant, biological therapy: 27.78% vs 20.83% low and high IL-13 subset, respectively), increased steroid/ immunosuppressant/anti-TNF-α therapy use to reach and maintain the remission, when compared with patients with low IL-13mRNA (Figure 4D). Taken together, the above results suggest that UC patients showing high IL-13 mRNA tissue content have a more severe colitis than patients showing low IL-13 mRNA tissue content.

When males and females were separately analyzed for the clinical-pathological variables described above, it appears that the only variable influenced by gender was the age at diagnosis where males contributed to the observed significant lower age at diagnosis in patients belonging to clusters 5+6 (males clusters 5+6: 30 (4-61) *vs* males clusters 2+3+4: 42 (20-74), median (range); p= 0.0043, by Mann Whitney test).

Characterization of mucosa-associated microbiota in UC patients and controls

To investigate the ability of microbiota composition to identify different UC patients subsets or to complement cytokine profile information, we preliminary evaluated mucosal microbiota composition in UC patients (both in involved and uninvolved tissue) and in controls, by performing Illumina MiSeq sequencing of 16S rDNA. A summary of microbiota profiles (relative abundances) at family level is reported in Figure 5A.

As for α-diversity (Supplementary Figure 3) and ß-diversity (Figure 5B), we did not observe major differences in the mucosa-associated microbial profiles between UC and Controls (both involved and uninvolved tissue). Furthermore, we did not observe major differences between involved and

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uninvolved tissue in UC patients. Interestingly, PCoA performed on Bray-Curtis distances suggests a three group's ordination of mucosa-associated microbial profiles, varying independently from mucosal tissue type (involved, uninvolved, control mucosa) (Figure 5B), IL-13 and IL-17 tissue cytokine content (Supplementary Figure 4), cytokine clusters (Supplementary Figure 5), as well as clinical-pathological variables (Supplementary Table 5).

Considering the data-driven characteristic of PCoA, this implies neither disease, nor cytokine profile are relevant order parameters of the microbiota profile. Thus, the global microbiota profile stems from other biological features (e.g. diet, micro-environmental features, genetics) and we can only focus on local microbial features to try to get a 'signature' of the disease and cytokine status. This methodologically implies the shift from a data-driven (PCoA) to a supervised (Discriminant Analysis) approach. When discriminant microbial signatures were investigated by LEfSe analysis (Figure 5C-D), comparison between controls mucosa-associated microbiota (Controls, gray highlighted node of the tree) and UC mucosa-associated microbiota (both involved and uninvolved, red node of the tree) showed an enrichment of *Roseburia* genus in control mucosa compared to UC (Figure 5C), as previously reported in several studies [34-36].

UC samples (involved and uninvolved tissue) showed relative enrichment of *Bifidobacteriaceae, Gemellaceae, Enterococcaceae, Eryspelothricaceae,* and *Lactobacillales,* including also *Bifidobacterium, Enterococcus* and *Acidaminococcus* genera (Figure 5B). When we examined differential taxa between involved mucosa and uninvolved mucosa (Figure 5C), *Bifidobacterium* and *Acidaminococcus* genera and *Gemellaceae* family were enriched in uninvolved mucosa, while, *Lactobacillus, Eubacterium* and *Enterococcus* genera were enriched in the involved mucosa. These data are in agreement with the reported increase in UC fecal samples of *Enterococcaceae*, *Lactobacillaceae* [37] families and *Enterococcus* genus [38] suggesting that the contribution to the reported increase in fecal samples is mainly due to the increase observed in inflamed tissue.

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Comparative analysis of mucosal microbiota composition in CL6 (high IL-13 tissue content) and CL2 (low IL-13 tissue content) patients' clusters

Mucosal microbiota composition was further analyzed focusing on the subset of involved tissues in subjects belonging to cytokine Cluster 6 (CL) and CL 2, the two clusters populated by UC patients only characterized by high and low IL-13 mRNA, respectively. Alpha diversity analysis (i.e. richness, evenness, Shannon's index, and Faith's phylogenetic diversity index; Supplementary Figure 6A) and beta diversity (Supplementary Figure 6B) did not highlight differences between involved mucosa-associated microbiota of patients in CL6 and CL2. Interestingly, when we selected only samples belonging to CL6 and CL2 to evaluate their ß-diversity (Supplementary Figure 6B), the spatial arrangement of samples on the ordination space (PCoA based on Bray-Curtis distances) resembled the one observed in the complete samples population (Figure 5B). This evidence further confirms our previous observation that the global microbiota profile stems from other biological features than cytokine status alone.

Nevertheless, when differences between involved mucosa-associated microbiota of patients belonging to the two cluster that show the lowest (CL2) and the highest (CL6) IL-13 values were evaluated by LEfSe (Figure 6), *Prevotella* genus resulted a microbial signature associated to subjects in CL6, while *Acidaminococcus* and *Sutterella* genera, were associated to patients in CL2.

Interestingly, a more in-depth sequencing analysis at species-level revealed that 2.5% of the total number of the reads (218'557 out of 8'498'053 total number) were assigned to *Prevotella spp.* and *Prevotella copri* was present in more than 50% of the CL6 samples (Supplementary Figure 7).

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DISCUSSION

In the present study, we demonstrated the existence of two different subsets of adult endoscopically active UC patients characterized, in the context of a generalized increase of IL-17A tissue mRNA content, by high and low IL-13 tissue mRNA content. Patients in the two subsets show different clinical-pathological characteristics and some local microbial markers.

Among the cytokines explored in the present study, only IL-17A and IL-13 show classificatory ability independent from the global inflammatory status of the patients (PC1, see Results). IL-17A and IL-13 are each other linearly independent, with IL-17A marking the 'presence of disease' and IL-13 the disease sub-typing. Our observation of increased IL-17A expression in UC is in line with previous observations by our and other groups [2, 7, 39, 40], and with a recent report in which a large cohort of pediatric patients was evaluated [41]. Notably, in the last reported study, increased IL-17A and IL-13 mRNA tissue content (with no significant correlation between IL-13 and IL-17A expression) were increased in ulcerative colitis and IL-17A and IL-5 mRNA expression were able to distinguish UC from colon-only Crohn's disease [41]. It has been recently shown that the ratio of mucosal IL17A to IL17F expression correlate significantly with endoscopic disease activity in adult UC [42] and we previously demonstrated that dual expression of IL17A by CD4+CD25- regulatory T cells expressing surface transforming growth factor-β in its latent form (LAP) reduces the suppressor activity of these cells in UC [2]. In addition to IL-17A, sufficient evidence points to IL-13 as an important pathological factor in adult and pediatric UC patients [3, 4, 41,43, 44], although few reports [45, 46] did not show increased level of IL-13 in UC patients. The reason for such discrepancies may reside in both the different experimental techniques [45, 46] and the very low number of patients in which IL-13mRNA was evaluated [45] in the latter mentioned studies.

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The two subsets of UC patients observed in the present study differ in the prevalence of gender, being females more represented in the high IL-13 subset, as well as in the age at diagnosis that was significantly lower in the high IL-13 patients' subset when compared to the low IL-13 patients' subset. No significant difference between the two subsets in the disease duration from the diagnosis was observed.

As regard disease characteristics, the high IL-13 patients' subset show an increased prevalence of extensive colitis. Furthermore, in the group of 42 patients in which clinical information during the follow-up were available, the use of steroids, immunosuppressant and biological agents to achieve and maintain clinical remission, not significantly different at the time of endoscopy, was higher in the high IL-13 patients' subset than in the low IL-13 patient's subset. Taken together data suggest that UC patients showing high IL-13 mRNA tissue content have a more severe colitis than patients with low IL-13 mRNA tissue content. This finding somehow differs from the observation reported in a treatment-naive UC pediatric patients cohort characterized by concomitant increased mucosal IL-17A and IL-13 mRNA [41]. In this cohort of patients, higher IL-13 expression was associated significantly with an increased likelihood of clinical response at 6 and 12 months to first-line therapies (i.e. corticosteroids, mesalamine, and thiopurines) and steroid free remission compared with that of patients characterized by essentially undetectable IL-13 expression. Moreover, during the same period of time, these patients were less exposed to anti-TNF-biologic drugs. In our cohort, patients with high and low IL-13 mRNA did not differ in the clinical response or in the percentage of patients experiencing a clinical relapse during the one year follow-up, however the use of systemic corticosteroids, immunosuppressant and anti-TNF-α biologic drugs was higher in the high IL-13 mRNA patient subset. It is quite possible that in newly diagnosed pediatric patients in which the immune-response is not influenced by the effects of inflammatory relapses and consequent therapy, initial induction of IL-13 is adequately counteracted by the standard therapy. Alternatively, increased production of IL-13 might even have, in the initial phase

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of the disease, a protective role, as shown in some animal models [47- 49]. However, with the progression of the disease, inflammatory flares and their treatment might induce changes favoring the occurrence or amplification, in genetically predisposed individuals, of autoimmunity phenomena as trigger of IL-13 production that switch the role of IL-13 production from protective to pathogenic. Indeed, in adult UC patients, it has been recently demonstrated the presence in colonic lamina propria of increased proportion of NKT cells able to produce IL-13 in response to lysosulfatide, a sulfatide glycolipid [50]. Sulfatide glycolipids are self-antigens present in cells in many tissues including the epithelial cells in the gastrointestinal tract. A stress response of genetically conditioned epithelial cells to certain type of bacteria in the colonic microbiota may indeed promote the autoimmune-induced IL-13 production**.**

Interesting, in the present study, we observed different mucosal microbial profiles associated to the two patients' subsets. Particularly, while mucosa-associated microbiota in patients with high IL-17A and low IL-13mRNA is enriched in *Sutterella* and *Acidaminococcus*, *Prevotella* (mainly *Prevotella copri*) is enriched in the mucosa-microbiota of patients showing increased IL-13 mRNA content in addition to high IL-17A mRNA content. It is worth to note that *Sutterella* [51], *Acidaminococcus* [52] and *Prevotella copri* [53] have been reported to be increased in fecal microbiota of UC patients. All of these microorganisms are classically considered commensal bacteria due to their extensive presence in the healthy human body. However, emerging studies have linked increased *Prevotella* abundance and specific strains to inflammatory disorders, suggesting that at least some strains exhibit pathobiontic properties. In particular, it has been recently reported that expansion of *Prevotella copri* in fecal samples strongly correlate with disease in new-onset untreated patients with rheumatoid arthritis, a systemic autoimmune disease [54]. In the same study it has been described its ability, after colonization of mice, to increase sensitivity to chemically induced colitis [54]. Indeed *Prevotella* might disrupt the mucosal barrier function through production of sulfatases that actively degrade mucus oligosaccharides [55]. Thus, it is plausible that the dysbiotic community observed in the high IL-13 mRNA patients

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subsets (enriched in *Prevotella* genus), might influence the epithelial stress response, favoring the presentation of autoantigens contributing to the pathogenic role of IL-13. On the other hand, the microbial community enriched in both *Sutterella* and *Acidaminococcus,* characterizing the low IL-13 mRNA subset of patients, might contribute to a less severe disease. In fact, although *Sutterella* was described to be associated with lack of remission after fecal microbiota transplantation [(56)], recent studies, showed the ability of *Acidaminococcus* genus to produce short chain fatty acids [(57)]. Short chain fatty acids are in fact considered important metabolites as they serve as the major energy source for colonocytes, they have anti-inflammatory properties and regulate gene expression, in host cells [(58)].

The existence of the two subsets of patients observed in the present study introduces some issues about the clinical implications of the finding. It is presently unknown whether the two subsets of patients are such since the onset of the disease or if they represent different stages of the disease. Very recently, it has been reported, in 15 patients with active ulcerative, that mucosal immune profiles differ during early and late phases of the disease, with a Th1 and Th2-driven prevalent profile disease in early and late disease, respectively [(59)]. However, in the study, IL-13 expression in paired samples from early and late disease has not been evaluated. Patients evaluated in the present study have, at the time of endoscopy, a median time of 9 years of disease duration from the diagnosis thus belonging, according to the above-reported study, to the "late disease". However, we did not observe differences in the disease duration between the two patients' subsets, yet the age at diagnosis was significantly lower in patients with high IL-13 and these same patients showed more frequently an extended colitis, similar to the observations reported in pediatric patients [(41)]. Therefore, we favor the view about the existence of subsets of UC patients showing different IL-13mRNA content from the onset of the disease.

The different cytokine profile might be differentially associated to the presence of different serological markers and to their prognostic ability [(60, 61)], so that it might be useful to combine the assessment of

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tissue cytokine profile with the assessment of serological markers to obtain an increased prognostic ability.

Finally, the findings described in the present study might have important implications on therapeutic strategies in UC. The observation that IL-6, TNF-α, IFN-γ and IL-10 mRNA tissue content were each other strongly correlated, while IL-17A and IL-13 variations were both independent from all the other cytokines and independent of each other suggests that IL-17A and IL-13 mRNA contents are distinctive features of the pathogenic process of UC. As that, therapy oriented to the neutralization of inflammatory cytokines might only indirectly and differentially affect the IL-17A and IL-13 expression which in turn might influence the response to the neutralization of inflammatory cytokines. Notably, it has been shown that response to infliximab treatment was significantly associated with higher pretreatment mucosal IL-17A gene expression [(62)], and fewer Gata3+ T cells were observed in the lamina propria of anti-TNF-α responders, than in non-responders patients [(63)]. Beyond these considerations, an important therapeutic implication of the findings reported in the present study concerns the treatment of patients with high IL-13 mRNA tissue content. As recently highlighted [(64, 65)] previous results obtained with IL-13 neutralizing treatments might be reconsidered according to the IL-13mRNA colonic content of the patients that have been studied. A treatment approach that specifically target IL-13 or a different IL-13 receptor [(66)] might indeed be effective in the patients' subset with high IL-13mRNA tissue content identified in this study.

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FIGURE LEGEND

Figure 1: Cytokine mRNA tissue content in controls samples (n=20), in the uninvolved tissue (n=50) and involved tissue (n=77) of UC patients. Lines represent median and range. p= as for Mann-Whitney test.

Figure 2: Mean values location in the bi-dimensional IL-17A *vs* IL-13 plane is different in UC (n=77) and Controls (n=20). Data in a bivariate mean/SE plane for both IL-13 and IL-17A. Points represents mean±SE.

Figure 3: UC patients are distributed in six clusters characterized by high and low IL-13 mRNA and increased IL-17A mRNA tissue content. Panel A: Data distribution of subjects (UC patients and controls) in the IL-17A/IL-13 plane. Each point represent patient or control IL-17A/IL-13 mRNA values. Lines represent mean of IL-17A and IL-13. Panel B: Distribution of clusters in the IL-17A/IL-13 plane. Each point represent patient or control IL-17A/IL-13 mRNA values- associated cluster. Panel A and B: UC patients n=77; Controls n=20

Figure 4: Clusters in the IL-13/IL-17A plane show differences as for sex (A), age at diagnosis (B), prevalence of extensive colitis (C) and steroid/ immunosuppressant/biological therapy use (D). Panel A, B, and C: UC patients n=77. Panel D: UC patients n=42.

Figure 5: Explorative analyses and comparison of mucosal associated microbiota composition in UC and control subjects. (A) Barplot shows the composition at the Family level, according with the Phylum of belonging. Family with relative abundance < 1% were discarded. (B) PCoA ordination based on Bray-Curtis dissimilarities of mucosal samples from UC (involved, n=79, and uninvolved, n=57, tissue) and controls (n=21). PERMANOVA using the Adonis function with 999 permutations

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resulted not significant. (C-D) LEfSe analysis showing significantly biomarker taxa in two different comparisons, based on (C) disease status: UC (both involved and uninvolved tissue, passed as subclasses t LEfSe) compared to control samples, and (D) tissue: controls versus uninvolved versus involved tissue in UC patients. Cladograms show the most discriminative bacterial clades. Coloured regions/branches indicate differences in the bacterial population structure between the different groups. Statistically significant taxa enrichment among groups was obtained with Kruskal-Wallis test among classes (Alpha value = 0.05). The threshold for the logarithmic LDA score was 2.0.

Figure 6. Comparison of mucosal microbial profiles between UC patients belonging to Cluster 2 (CL2, n = 10) and Cluster 6 (CL6, n = 30). LEfSe analysis showing significantly enriched biomarker taxa in CL6 vs CL2 comparisons. Cladogram and barplot show the most discriminative bacterial clades. Colored regions/branches indicate differences in the bacterial population structure between the different CL2 and CL6. Statistically significant taxa enrichment among groups was obtained with Kruskal-Wallis test among classes (Alpha value = 0.05). The threshold for the logarithmic LDA score was 2.0.

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Table 1. Clinical-pathological variables of patients at time of endoscopy

	Ulcerative Colitis n. 77	**Controls** n. 20
Age at the time of endoscopy years: median (range)	51 (19-82)	57 (37-86)
Gender M/F (n)/(n)	45/32	10/10
Disease Duration from diagnosis years: median (range)	9 (0-36)	
Endoscopic Activity (Mayo Score) median (range)	2 (1-3)	
Disease Extension		
Proctitis	29	
Left Sided Colitis	29	
Extensive Colitis	19	
Therapy		
CS	2+ 10*	
5-ASA agents	50+ 23*	
Immune modulators	1+10*	
Biological Agents	2*	
No therapy	4	
*Patients with combined therapy		

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Figure 1

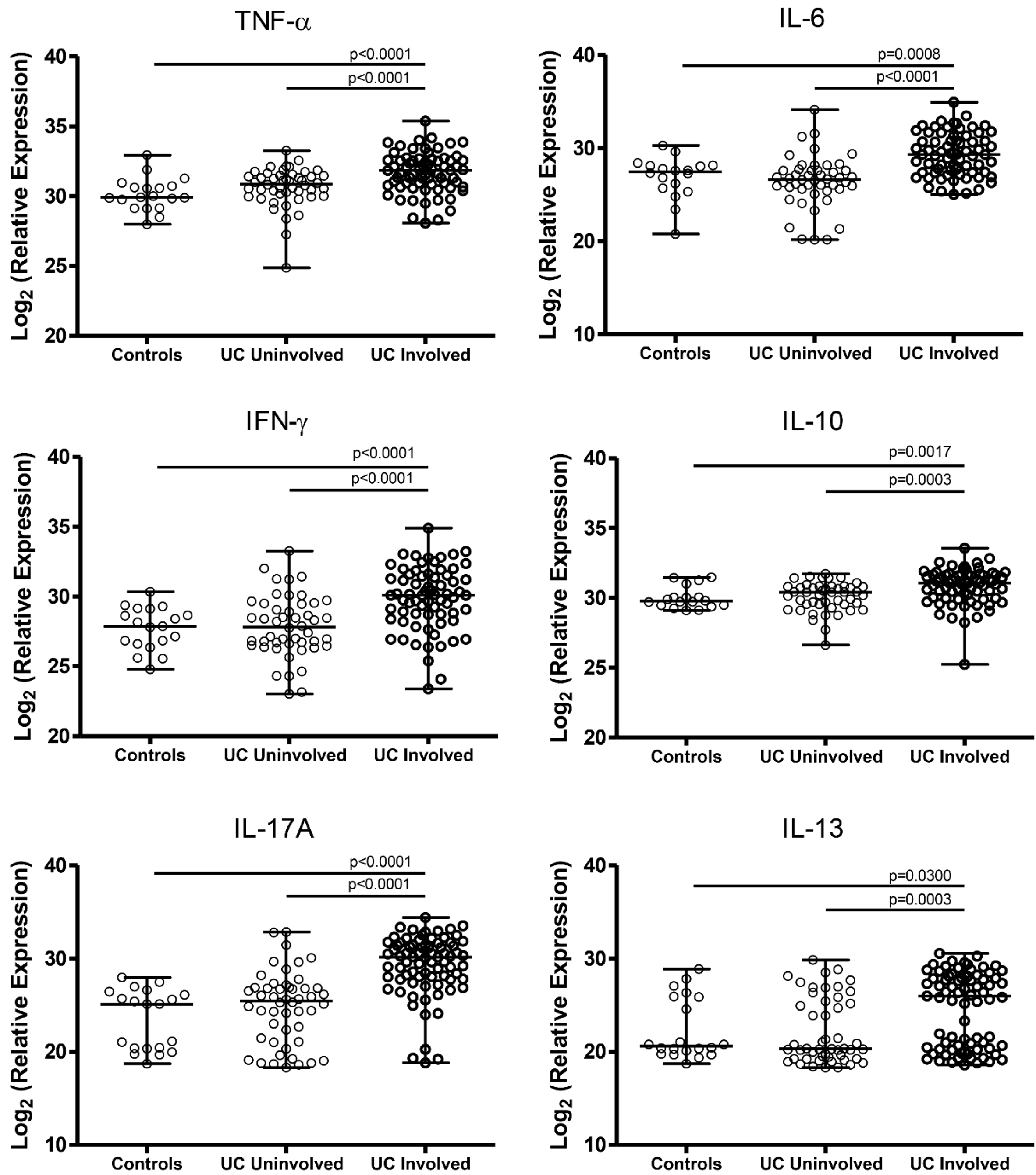

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Figure 2

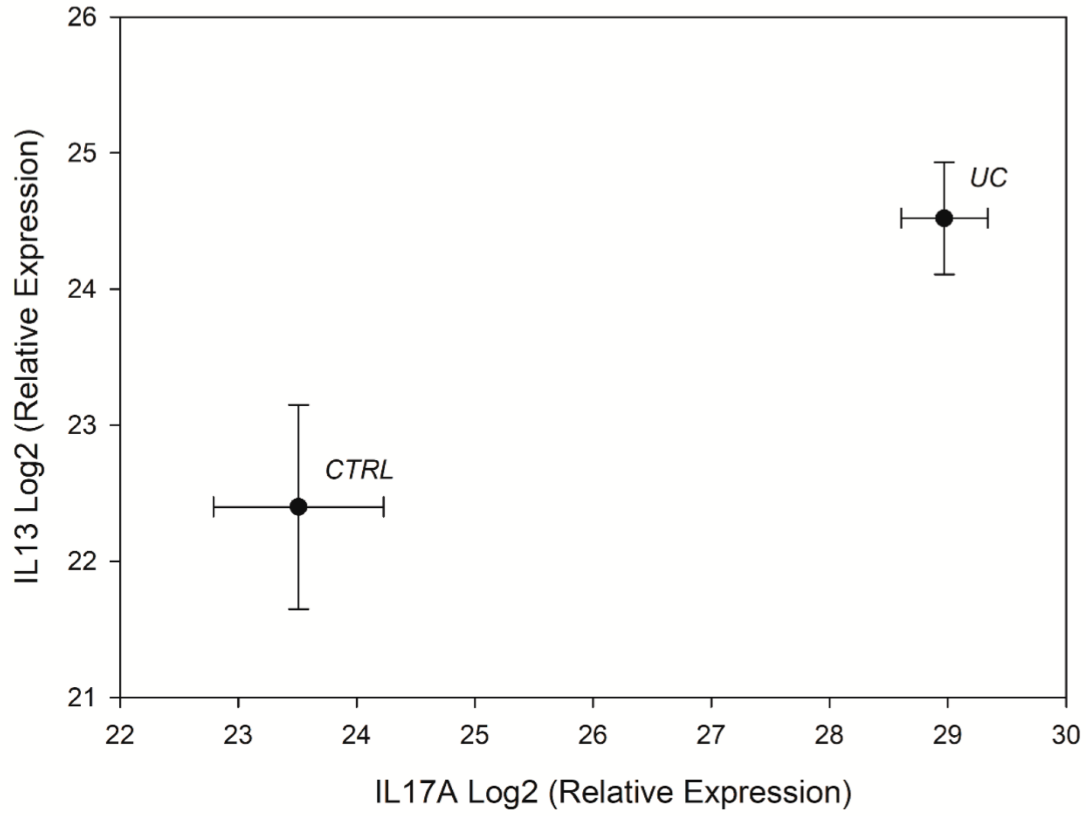

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Figure 3

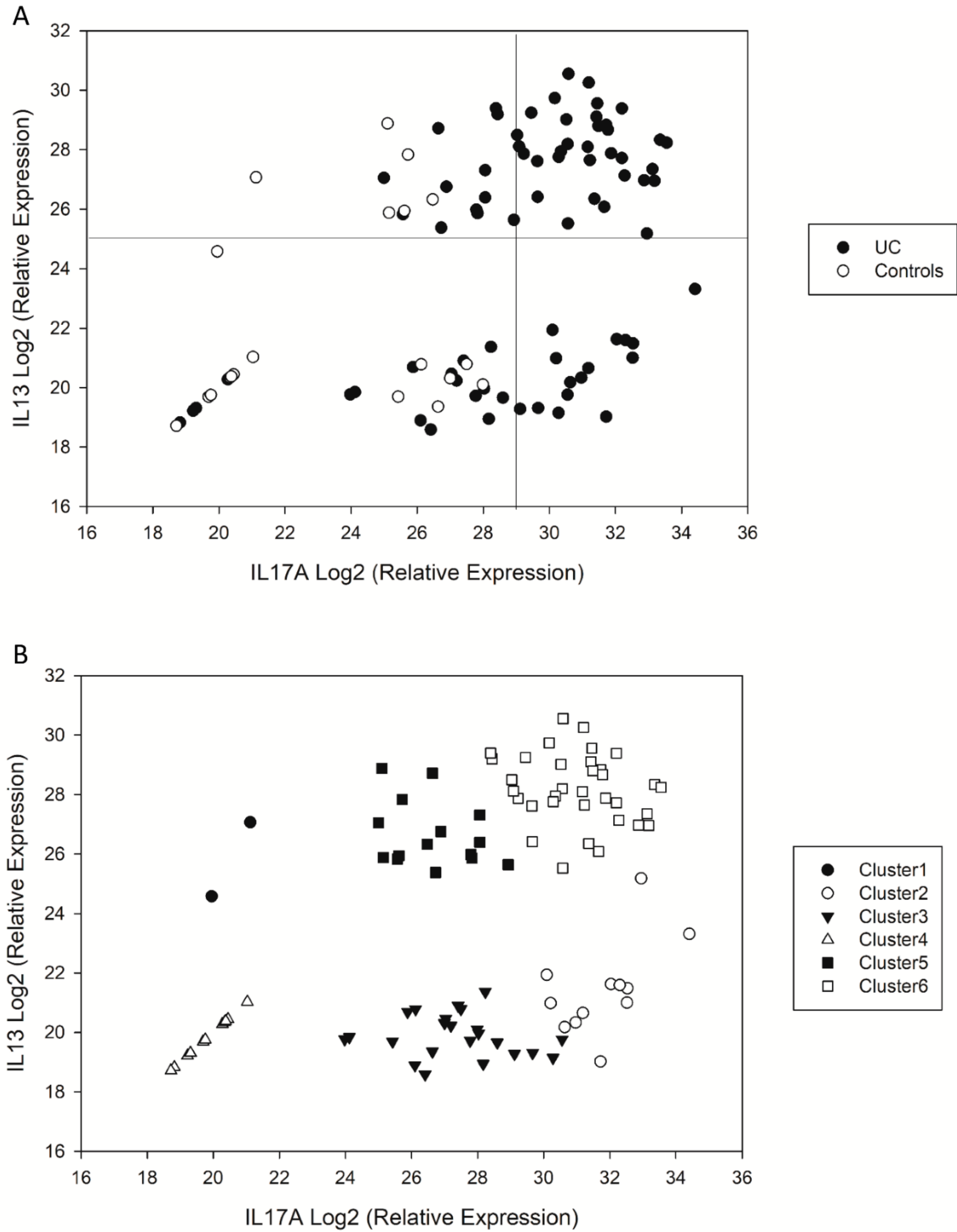

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Figure 4

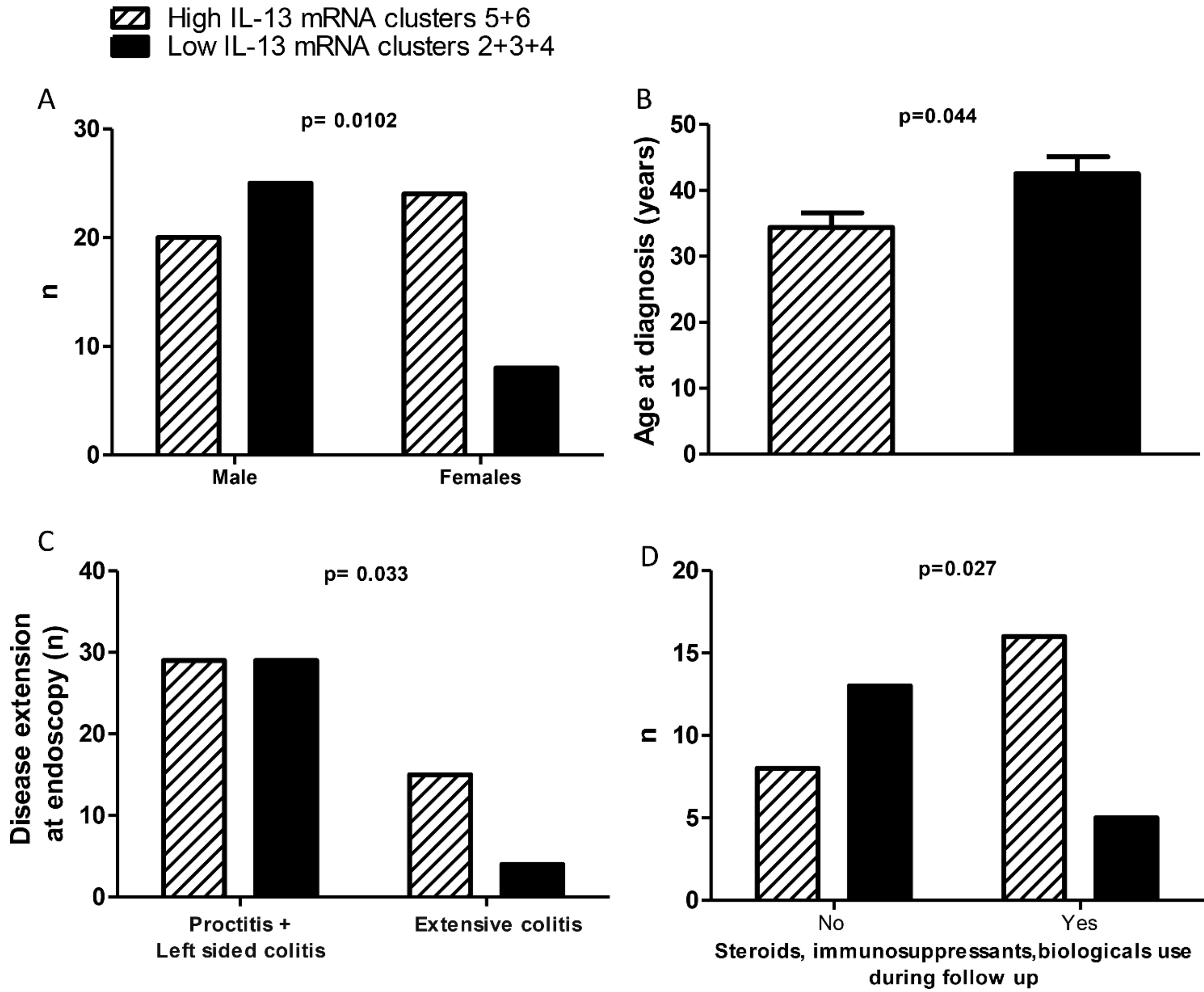

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Figure 5

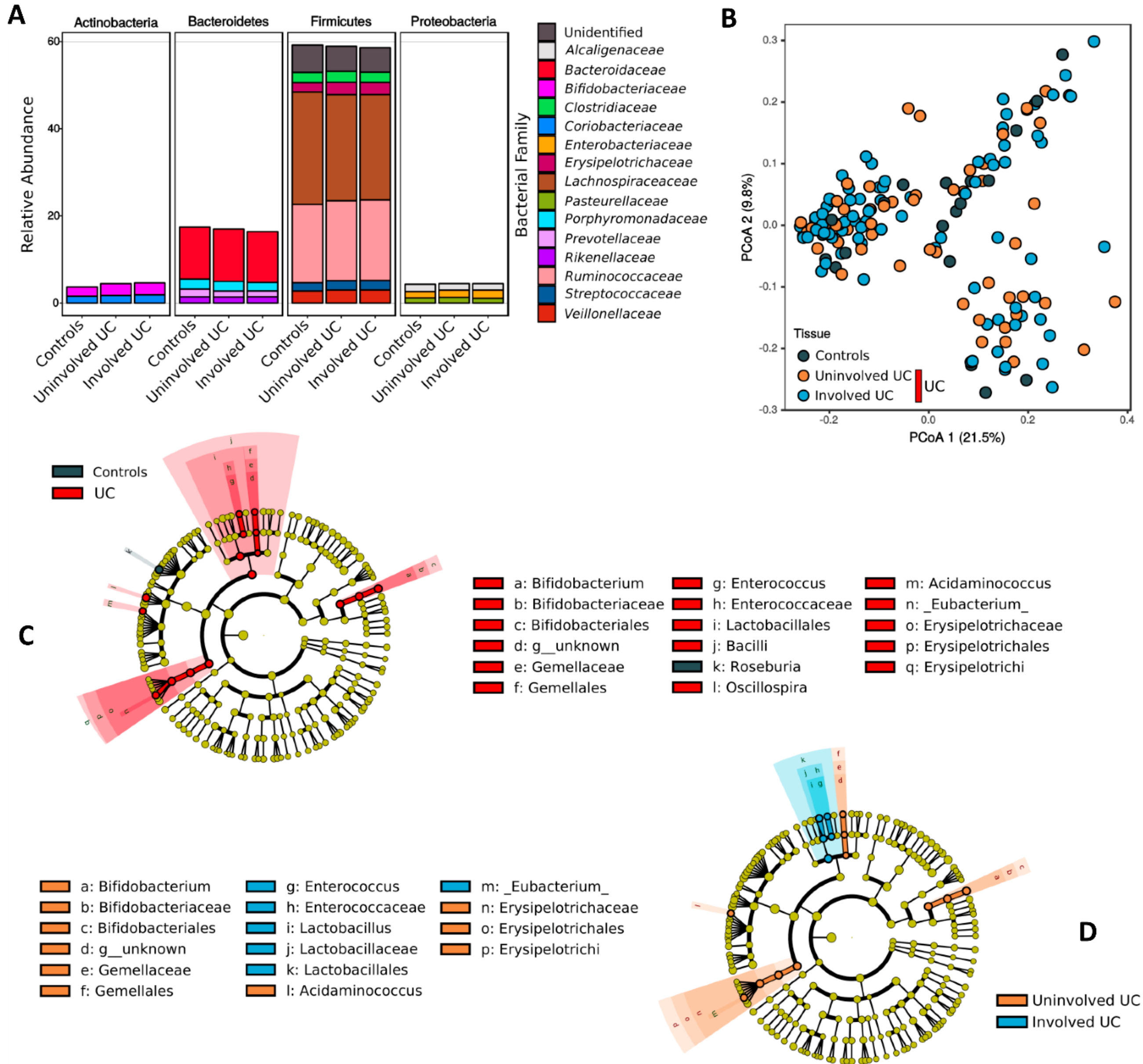

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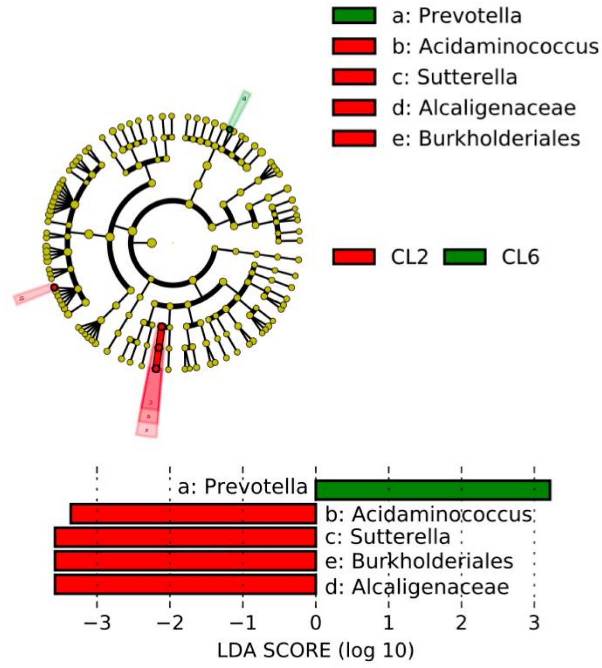
Figure 6



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