Microbial changes in stool, saliva, serum, and urine between before and after anti-TNF-α therapy in patients with inflammatory bowel diseases

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Directed by Professor Jae Hee Cheon

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ABSTRACT

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(Directed by Professor Jae Hee Cheon)

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic immune-mediated intestinal inflammatory disorders associated with microbial dysbiosis at multiple sites, particularly the gut. Anti-tumor necrosis factor-α (TNF-α) agents are important treatments for IBD. This study investigated whether microbiome changes at multiple sites can predict the effectiveness of such treatment in IBD. Stool, saliva, serum, and urine biosamples were collected from 19 IBD patients (10 with CD and 9 with UC) before (V1) and 3 months after (V2) anti-TNF-α treatment and 19 healthy subjects from three University Hospitals. Microbiota analysis was performed using extracellular vesicles (EVs) for all four sample types and next generation sequencing (NGS) for stool and saliva. Microbiome diversity before and after treatment was assessed. Using NGS analysis, there were significant differences in α-diversity at V1 and V2 in stool, but not saliva, samples. Moreover, there

were no differences in α-diversity at V1 and V2 in any sample type using EV analysis. Responders to anti-TNF-α treatment had significantly higher levels of *Firmicutes* (phylum), *Clostridia* (class), and *Ruminococcaceae* (family) in V1 stool, and *Prevotella* in V1 saliva. In non-responders, serum and urine levels of *Lachnospiraceae* were significantly higher than in responders at V2 and, unlike responders, *Acidovorax caeni* was not detected in any sample type at V1 or V2. Microbiome changes at multiple sites can predict the effectiveness of anti-TNF-α treatment in IBD. As microbial analysis using EVs can be easily conducted, further research in this area would be warranted in IBD.

Key words: inflammatory bowel disease; colitis, ulcerative; crohn's disease; microbiota; tumor necrosis factor-alpha; extracellular vesicles

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I. INTRODUCTION

Inflammatory bowel diseases (IBD) are relapsing chronic inflammatory intestinal disorders that include ulcerative colitis (UC) and Crohn's disease (CD).[1] The etiology of IBD is not yet clearly identified, but it is presumed that an abnormal immune response to the damaged mucosal barrier with altered gut microbiota is caused by environmental factors in genetically vulnerable hosts.[1,2] In particular, in patients with IBD, dysbiosis, an imbalance of the gut microbiota that contributes to the host homeostasis by producing short-chain fatty acids and essential vitamins, is a crucial factor of disease development.[1] Several studies have reported altered compositions of the gut microbiota in IBD patients, with such patients having been shown to have decreased α-diversity of the gut microbiota, lower abundance of *Firmicutes*, and higher abundance of *Proteobacteria*.[1,3-5]

Studies have elucidated the pathogenesis of IBD and developed therapeutic agents for its treatment based on various immunologic and cellular biochemistry mechanisms, but the optimal treatment of IBD based on its pathogenetic mechanisms is not yet clear. Among the current treatment modalities, anti-tumor necrosis factor (TNF) agents are one of the most important therapeutic agents and are now widely used in treating IBD.[6] TNF-α

is a proinflammatory cytokine and is produced by activated macrophages, monocytes, and T lymphocytes.[7,8] IBD patients are commonly shown to have increased expressions of TNF-α protein and mRNA.[9] Therefore, several anti-TNF-α monoclonal antibodies, such as infliximab, adalimumab, golimumab, and certolizumab pegol, have been developed for the treatment of IBD.[8,10] Anti-TNF-α agents have been shown to provide higher rates of remission induction and maintenance, and to induce mucosal healing more frequently, than other conventional treatment modalities.[6] However, up to 30% of IBD patients appear to be primary non-responders who do not clinically benefit from anti-TNF-α induction therapy, while another 30-40% lose response during the first year of the treatment, leading to the need to increase their dosage or switch to another biologic agent.[11] Several reports have shown that drug treatment can improve abnormal fecal microbiota profiles to be similar to normal healthy microbiota, including re-occurrence of *Firmicutes* and *Bacteroidetes*.[1,12-14] However, studies on changes in the microbiota between groups with and without drug treatment are still lacking. In addition, most studies have been conducted using fecal samples, and there are no studies on changes in microbiota in other sites (urine, blood, etc.).

In addition, in recent years, analysis and research on extracellular vesicles (EVs) have gained a lot of attention, and EV analyses in body fluids, as well as in feces, are emerging. Bacterial EVs are nano-sized vesicles in the range of 20-400 nm[15] made of a lipid bilayer released from cells.[16] EVs contains a variety of biologically active substances, such as proteins, mRNAs, miRNAs, lipids and metabolites, that reflect the state of cells,[17] and exists in various body fluids, including blood, urine, saliva, tears, semen, breast milk, and ascites.[18] EVs act as a natural messenger involved in cell-to-cell communication between microbial populations, and also act as an immune modulator, virulence factor, and anti-bioresistance factor.[19-21] Therefore, it is possible to extract DNA and analyze microbiota using EV from various body

fluids. However, no studies on microbiota using EV analysis in body fluids such as saliva, urine, and serum have been conducted in IBD patients. Therefore, this study aimed to investigate whether microbiome changes at multiple body sites can predict the effectiveness of anti-TNF-α treatment in IBD patients. Moreover, we sought to find the most suitable sample collection site and biomarker through microbiome analysis at various sites before and after anti-TNF treatment.

II. MATERIALS AND METHODS

1. Patients

Between August 2017 and January 2020, we prospectively enrolled 19 patients with IBD and 20 healthy controls at three University Medical Centers, Seoul, Korea. The diagnosis of UC and CD was based on the clinical, endoscopic, histopathologic, and radiologic findings.[22-24] To be included in the IBD group, patients must have met the following criteria: (i) age ≥19 years; (ii) had not received antibiotics during the last 3 months; (iii) had not taken probiotics during the last 3 months; (iv) had not previously received anti-TNF-α treatment (i.e. were anti-TNF-α naïve). In addition, the following IBD patients were excluded: (i) women with suspected pregnancy or who were lactating; (ii) patients with conditions that are contraindicated for anti-TNF-α administration, such as the presence of active tuberculosis or other severe infections, such as sepsis or opportunistic infections; (iii) those with no available clinical data such as disease activity or clinical records; and (iv) those who could not be followed up during the study period. To be included in the healthy control group, they must have met the following criteria (i) age ≥19 years; (ii) had not received antibiotics during the last 3 months; (iii) had not taken probiotics during the last 3 months.

The baseline characteristics of the patients and healthy individuals were prospectively obtained from the electronic medical data collected, including

demographics, comorbid diseases, medication records, and vital signs. This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of each participating hospital. Written informed consent was obtained from the patient and healthy subjects.

The baseline characteristics of the patients and healthy individuals were prospectively obtained from the electronic medical data collected, including demographics, comorbid diseases, medication records, and vital signs. In order to evaluate the disease severity in the patients with IBD, we investigated laboratory findings such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) level, and hematocrit (Hct) level.

2. Assessment of disease activity

To evaluate changes in disease activity in IBD patients, assessments were made at visit 1 (V1), which represents the visit before initiation of anti-TNF-α treatment, and visit 2 (V2), which represents the visit 3 months after initiation of anti-TNF-α treatment.

In UC patients, disease activity was assessed using the Mayo score. The Mayo score was calculated according to the bowel frequency, rectal bleeding, endoscopic findings, and physician assessment, and each item was scored from 0 to 3 and summed to a total of 12.[25,26] Using this scale, UC was defined as mild, moderate, and severe when the score was 3-5, 6-10, and 11-12 points, respectively.[27] CD disease activity was assessed using Crohn's Disease Activity Index (CDAI) and was divided into asymptomatic remission (CDAI <150), mild-to-moderate CD (150–220), moderate-to-severe CD (220–450), and severe-fulminant disease (>450).[28]

The response to treatment was defined as clinical and endoscopic improvement, and was measured based on the activity index.[29] In patients with UC, clinical response was defined as a decrease from baseline of ≥30% and

≥3 points in the Mayo score, along with either a rectal bleeding subscore of 0 or 1 or a decrease from baseline of ≥1 in the rectal bleeding subscore, or a reduction by ≥2 points and 25% in the partial Mayo score compared with baseline.[30] In CD patients, the response to treatment was defined as a reduction in CDAI of ≥70.[31] In addition, in the patients with UC, clinical remission (CR) was defined as a Mayo score of ≤2, along with not having >1 point in any individual subscore.[32] In addition, for CD patients, CR was defined as a CDAI <150.[28]

To investigate the influence of disease activity on the microbiome at the phylum level, disease activity at V1 in patients with IBD was classified as being in remission, mild-moderate IBD or severe IBD. In addition, we investigated laboratory results assessed in V1 and V2, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, and hematocrit levels. Fecal calprotectin was also measured at V1 and V2 in IBD patients, and at baseline, as a control, in healthy individuals.

3. Sample collection and analysis methods

The healthy controls provided stool, saliva, serum, and urine samples at baseline, and IBD patients provided these four sample types at V1 and V2 (i.e. before and after anti-TNF-α treatment). Saliva was collected in a 15cc falcon tube with 5 ml of clear saliva without food intake for at least 1 hour, and 2 g of feces was collected in a stool sterilized container. Fecal calprotectin was measured in a single frozen stool sample from all subjects by using Calprotectin Bühlmann ELISA (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Experimental samples were assayed with the standards and controls included with the kit according to the manufacturer's instructions. For urine, 30 ml was collected, and 5 ml of blood was collected in a serum-separating tube.

A. Next generation sequencing, NGS

DNA extraction was performed by using FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, California, USA) on stool and saliva samples. PCR was then performed to amplify template out of the DNA samples by using V3-V4 region primers with overhang adapters attached, which were 16S_V3_F (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′) and 16S_V4_R (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′). After attaching Nextera® XT Index Kit V2, an Illumina adapter primer, sequencing was performed using an Illumina V3 600 cycle cartridge and Illlumina MiSeq equipment (San Diego, California, USA).

B. Extracellular vesicles, EVs

Nanovesicles were separated from the samples through ultracentrifugation, gDNA was extracted, then 16s rRNA sequencing was performed using Illumina Miseq (Illumina, USA). Through this process, the gut microbiota was classified, and the correlation between the clinical characteristics and the rRNA abundance derived from a specific microorganism was made. EV analysis was performed on each sample of stool, saliva, serum, and urine, and was conducted by MD healthcare, Seoul, Korea.

Bacterial EVs were boiled using a heat block for 40 min at 100 ℃ and then the remaining particles and waste were removed by centrifugation at 13,000 rpm for 30 min at 4 ℃. The DNA was extracted from supernatants using a DNeasy PowerSoil kit (QIAGEN, Germany). The DNA of bacterial EVs in each sample was quantified by QIAxpert (QIAGEN, Germany). V3-V4 regions of the 16 S rDNA gene was amplified with primers; 16S_V3_F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNG GCWGCAG-3′) and

16S_V4_R(5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAC TACHVGGGTATCTAATCC-3′). The library preparation was performed using PCR products and each amplicon was sequenced by MiSeq Paired-end reads that matched the adapter sequences were trimmed by Cutadapt version 1.1.6.[33] The resulting FASTQ files containing paired-end reads were merged with CASPER, and then quality filtered with Phred (Q) score based criteria described by Bokulich.[34,35] Any reads that were shorter than 350 bp or longer than 550 bp after merging, were also discarded. To identify the chimeric sequences, a reference-based chimera detection step was conducted with VSEARCH against the SILVA gold database.[36,37] Next, the sequence reads were clustered into Operational Taxonomic Units (OTUs) using VSEARCH with closed-reference clustering algorithm under a threshold of 97% sequence similarity. The representative sequences of the OTUs were finally classified using SILVA 132 database with UCLUST (parallel_assign_taxonomy_uclust.py script on QIIME version 1.9.1) under default parameters.[38]

4. Statistical analysis

Variables were expressed as medians (interquartile range [IQR]) or n (%). The baseline characteristics were compared using independent Student's t-test (or Mann-Whitney test) for continuous variables and the $\chi 2$ test (or Fisher's exact test) for categorical variables, as appropriate. Data were analyzed using SPSS software (version 25.0; IBM Corp., Armonk, NY, USA). *P*-values of <0.05 were considered statistically significant.

For microbiota analysis in NGS analysis, primers were trimmed by using the ChunLab program (ChunLab, Inc., Seoul, Korea), applying a similarity cut-off of 0.8. The EzBioCloud database (https://www.ezbiocloud.net/)[39] was used for taxonomic assignment by using BLAST 2.2.22, and pairwise alignments were generated to calculate similarity. The Wilcoxon rank-sum test was used to test

the difference between groups in the number of OTUs, Shannon index, and relative abundances of specific taxa. $P < 0.05$ and false discovery rate (FDR)–adjusted p value < 0.1 were considered significant. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify significantly different taxa between the groups.[40]

In the NGS analysis, α-diversity (ACE, Chao1, Jackknife, Shannon's diversity index, NP Shannon, and Simpson indexes) and β-diversity metrics (Bray-Curtis dissimilarity and generated principal coordinates analysis [PCoA]) plot were computing using MDS (Multi-dimensional Scaling) for each of the β-diversity metrics. In EV analysis, rarefaction curve of Chao1 was used for α-diversity using multiple_rarefaction.py and alpha_diversity.py QIIME package. Differences between relative abundance of microbiota were calculated by the Kruskal–Wallis test and Wilcoxon test. A *P*-value of <0.05 was considered statistically significant. Grouped comparisons of data were conducted with R software (ver 3.6.3; R Core Team 2020, Vienna, Austria) and GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

III. RESULTS

1. Baseline characteristics

Of the 20 healthy subjects who willingly decided to participate in the study, one withdrew consent; therefore, a total of 19 IBD patients and 19 healthy individuals were finally included in the study. The baseline characteristics of the control group, and the IBD group at V1 (before anti-TNF-α therapy) are summarized in Table 1. There were no significant differences between the IBD and control groups with regard to median age (33 vs 31 years; P=0.554) or proportion of males (57.9% vs. 68.4%; P=0.735).

Table 1. Baseline characteristics of control group and V1, V2

Variables	Control (n=19)	IBD, V1 (n=19)	IBD, V2 (n=19)	*P*-value *
Male sex	11 (57.9)	13 (68.4)	13 (68.4)	0.735
Age	31 (28-34)	33 (23-52)	33 (23-52)	0.554
Disease				1.000
UC	-	9 (47.4)	9 (47.4)	
CD	-	10 (52.6)	10 (52.6)	
Disease activity				
Mayo score (n=9)	-	10.0 (9.0-11.5)	3.0 (2.0-5.0)	<0.001
CDAI (n=10)	-	77.2 (45.7-153.0)	40.9 (25.0-54.4)	0.044
Laboratory findings				
ESR	-	17.5 (2.3-31.8)	12.0 (3.0-24.0)	0.270
CRP	-	5.2 (2.2-9.0)	0.8 (0.4-3.4)	0.125
Hematocrit	-	40.9 (33.0-45.8)	41.4 (36.8-45.0)	0.374
Fecal calprotectin	<100	1572 (748-1800)	378 (145-882)	0.004

Data are expressed as median (interquartile range, IQR) or n (%). **P*-value for comparing patients with IBD V1 and V2.
IBD, Inflammatory bowel disease; UC, Ulcerative colitis; CD, Crohn's disease; CDAI, Crohn's disease activity index; ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein

The baseline characteristics of CD and UC patients are summarized in Table 2. CD patients were significantly younger than UC patients (31 vs. 52 years; *P*=0.021), and more CD than UC patients used an immune-modulator (90% vs. 33.3%; *P*=0.011). However, there was no significant difference in sex, underlying diseases, or the use of other medications (Table 2).

Table 2. Baseline characteristics between CD and UC patients

Variables	CD (n=10)	UC (n=9)	*P*-value
Male sex	7 (70.0)	6 (66.7)	0.876
Age	31 (23-40)	52 (30-64)	
Underlying disease			
Hypertension	1 (10.0)	1 (11.1)	0.937

Hematologic disorder	0 (0.0)	1 (11.1)	0.279
Eye disorder (vitreous floater)	0 (0)	1 (11.1)	0.279
Hemorrhoid, anorectal disorder	1 (10.0)	0 (0)	0.330
Subdural hemorrhage	1 (10.0)	0 (0)	0.330
Viral hepatitis (HCV)	1 (10.0)	0 (0)	0.330
Medications			
5-ASA	9 (90.0)	9 (100.0)	0.330
Immunomodulators	9 (90.0)	3 (33.3)	0.011
Steroid	5 (50.0)	5 (55.6)	0.809
Anti-TNF			0.137
Infliximab (Remicade)	7 (70.0)	4 (44.4)	
Adalimumab (Humira)	3 (30.0)	2 (22.2)	
Golimumab (Simponi)	0 (0)	3 (33.3)	
Others			0.403
Iron supplement (Bolgre)	1 (10.0)	0 (0)	
Pain killer	1 (10.0)	1 (11.1)	
Functional drug (dicetel)	0 (0)	1 (11.1)	
Folic acid	1 (10.0)	0 (0)	

Data are expressed as median (interquartile range, IQR) or n (%). *P*-value for comparing patients with CD and UC.
UC, Ulcerative colitis; CD, Crohn's disease; 5-ASA, 5-aminosalicylic acid; Anti-TNF, Anti-Tumor Necrosis Factor

2. Changes in disease activity

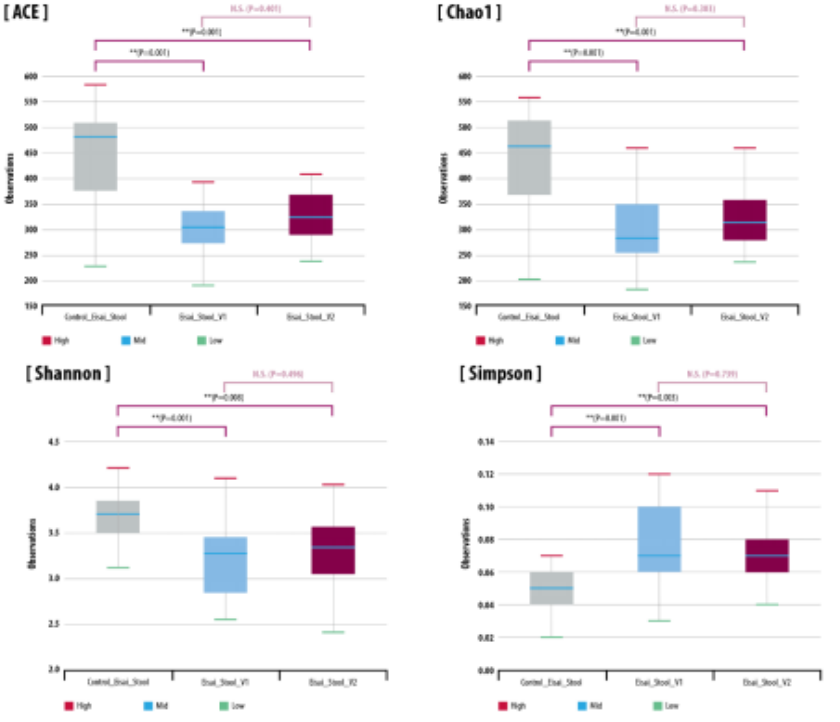
After V1, the 19 patients in the IBD group received the following anti-TNF-α agents: infliximab (n=11; 7 with CD and 4 with UC), adalimumab (n=5; 3 with CD and 2 with UC), and golimumab (n=3 with CD). Significant improvements from baseline in disease activity were seen following 3 months of anti-TNF-α treatment, with regard to median Mayo scores in UC patients (10.0 vs. 3.0; $P<0.001$), and median CDAI in CD patients (77.2 vs. 40.9; $P=0.044$), as well as median fecal calprotectin levels in all IBD patients (1572 vs. 378 μg/g; $P=0.004$) [Table 1]. However, there was no significant change from baseline in the laboratory findings of ESR, CRP, and hematocrit levels at V2 (Table 1).

3. Diversity and richness of the microbiota

A. NGS analysis in stool and saliva

In the stool samples, the α-diversity of the control group was significantly higher than that of the IBD patients at V1 and V2 in terms of all types of diversity estimators (ACE, Chao1, Jackknife, Shannon's diversity index, NP Shannon, and Simpson indexes; all P<0.05) (Figure 1 and Supplementary Figure 1). Regarding beta-diversity, using the Bray-Curtis dissimilarity and generated principal coordinates analysis (PCoA) plot of the gut microbiota was used,[41] PC 1 was 19.503%, PC 2 was 17.084%, and the IBD group at V2 after anti-TNF- α group was located close to the control group. However, there was no clear clustering, and there was an overlap in gut microbiota in the IBD group at V1 and V2.

(A)



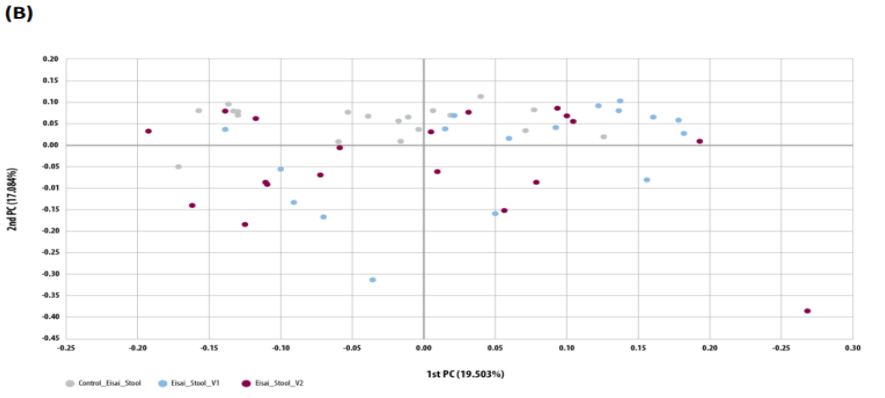
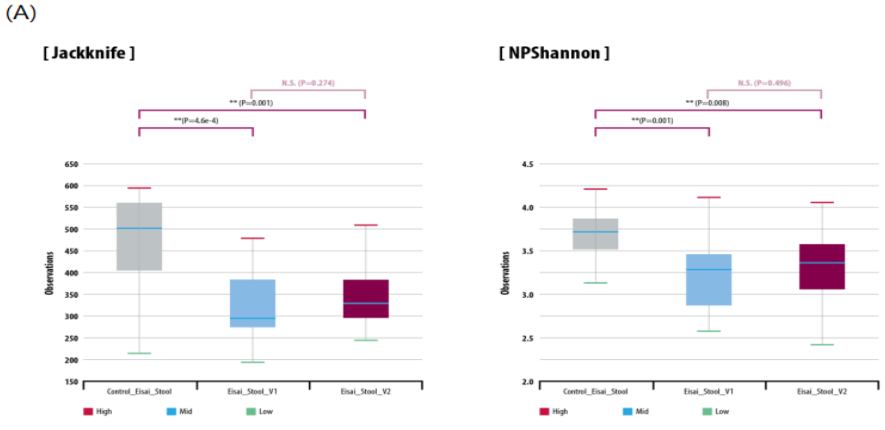
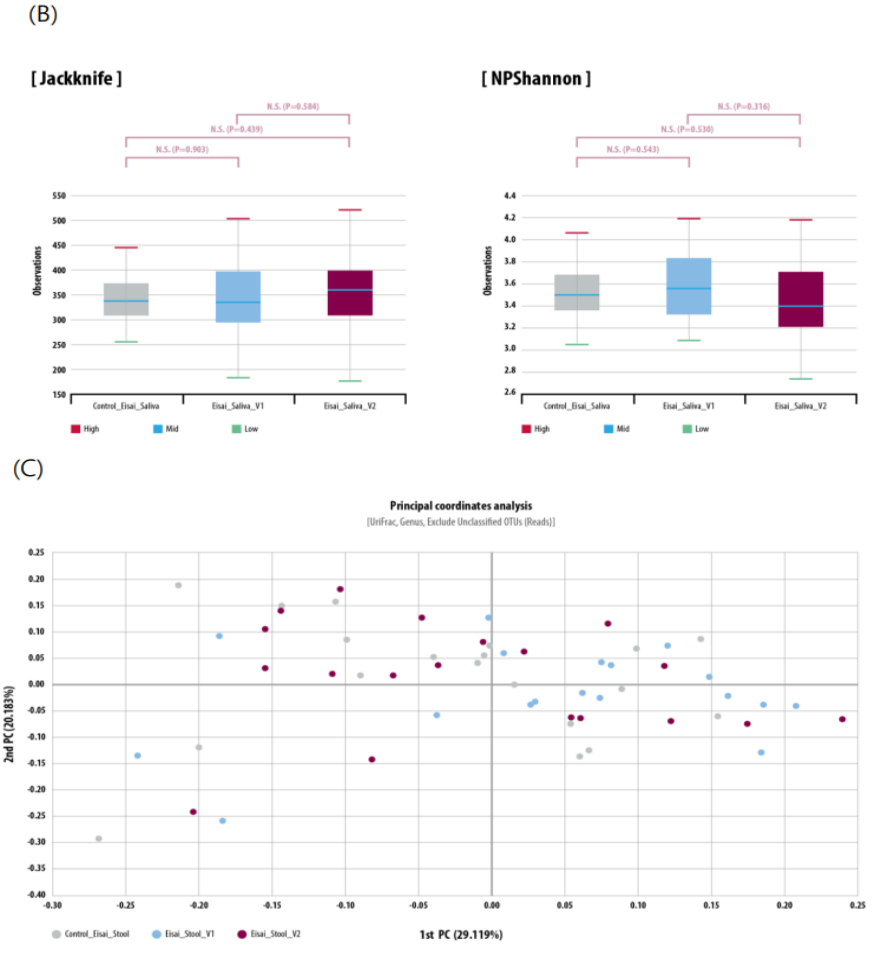


Figure 1. Stool: microbiome diversity based on 16srRNA gene sequencing in stool in the control group and the inflammatory bowel disease (IBD) group before (V1) and after (V2) anti-TNF-α treatment. (A) α-diversity analysis of gut microbiota using ACE, Chao 1, Shannon, and Simpson index, (B) β-diversity analysis of gut microbiota calculated via Principal coordinate analysis (PCoA) scatter plot

However, in the salivary microbiome, there were no significant differences in α-diversity between the control and IBD groups with regard to any of the four types of diversity index (Supplementary Figure 1). In addition, there was no significant clustering in the β-diversity of the salivary microbiota between the three groups (PC 1, 29.119%, PC 2, 20.183%) (Supplementary Figure 1).



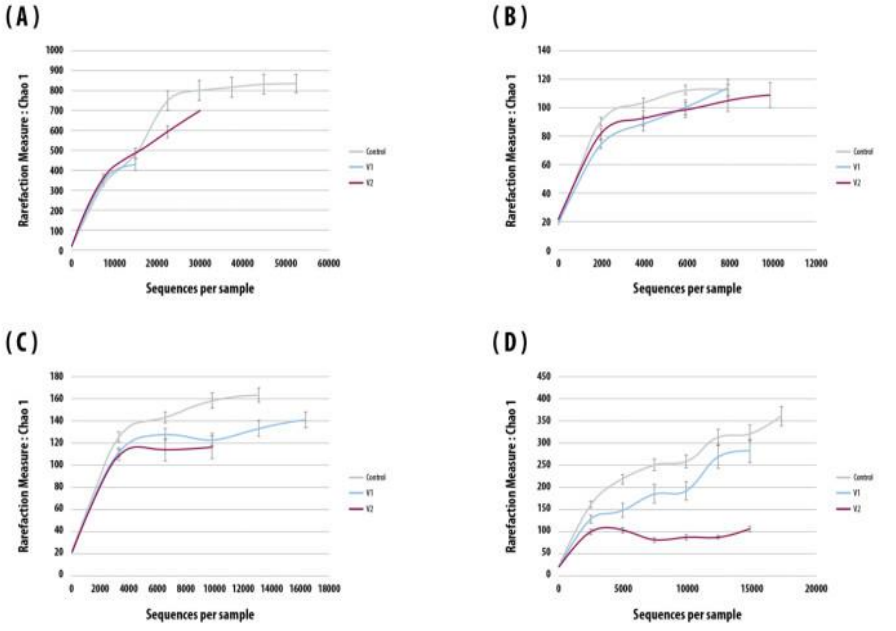


Supplementary Figure 1. α-diversity in the control group, and the inflammatory bowel disease (IBD) group before (V1) and 3 months after anti-TNF-α treatment (V2) using next generation sequencing in (A) Stool (Jackknife and NP Shannon), (B) Saliva (Jackknife and NP Shannon), (C) β-diversity analysis calculated via Principal coordinate analysis scatter plot.

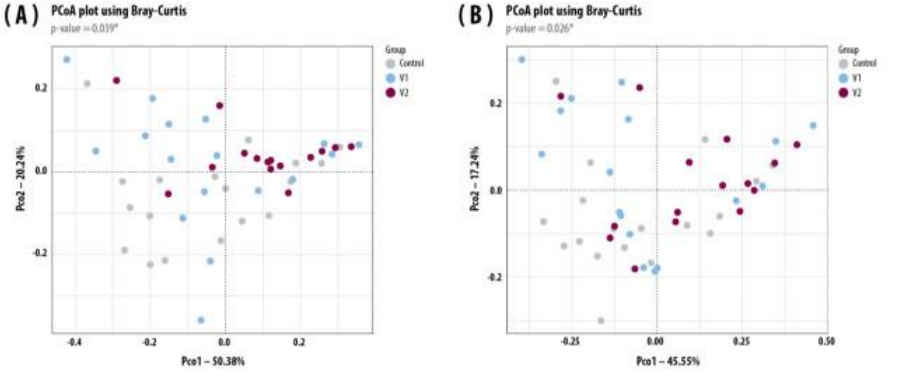
B. Extracellular vesicle analyses in stool, saliva, serum, and urine samples

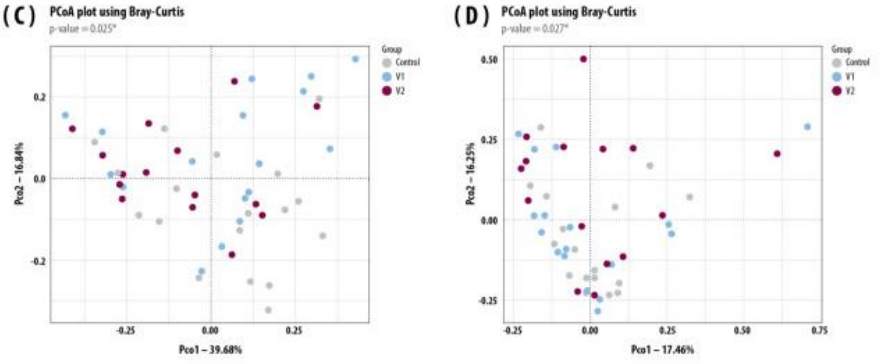
When rRNA abundance in the EVs from stool, saliva, serum, and urine samples was investigated using a rarefied Chao 1 plot, there was no significant difference in α-diversity between the control group and the IBD group at V1

and V2 (Supplementary Figure 2). However, with regard to the β-diversity of the stool microbiome, there were significant between-group differences in all of the PCoA plots for phylum, class, order, family, genus, and species (Supplementary Figure 3).



Supplementary Figure 2. α-diversity using extracellular vesicle analysis in the control group at baseline, and the inflammatory bowel disease (IBD) group before (V1) and after (V2) anti-TNF-α treatment. (A) stool, (B) serum, (C) saliva, (D) urine [rarefied Chao 1 plot] (P> 0.05)





Supplementary Figure 3. β-diversity of stool in the control group at baseline, and the inflammatory bowel disease (IBD) group before (V1) and 3 months after anti-TNF-α treatment (V2) in extracellular vesicle analysis (A) Phylum, (B) Class, (C) Order, (D) Family

4. Microbiota composition in control and IBD patients

A. NGS analysis in stool and saliva

In 16srRNA analysis of stool samples, levels of *Actinobacteria* (phylum), *Ruminococcus* (genus), *Ruminococcus faecis* (species), and *Blautia luti* (species) were higher in the control group than in the IBD patients before anti-TNF-α therapy (V1). After anti-TNF-α therapy (V2), these levels increased to become similar to those of the control group. In contrast, levels of *Enterococcaceae* (family), *Odoribacteraceae* (family), *Enterococcus* (genus), *Enterococcus faecium group* (species), *Clostridium innocuum group* (species), and *Clostridium nexile* (species), which were lower in controls than in IBD patients at V1, significantly decreased to control levels at V2 (all $P<0.05$). (Supplementary Figure 4A)

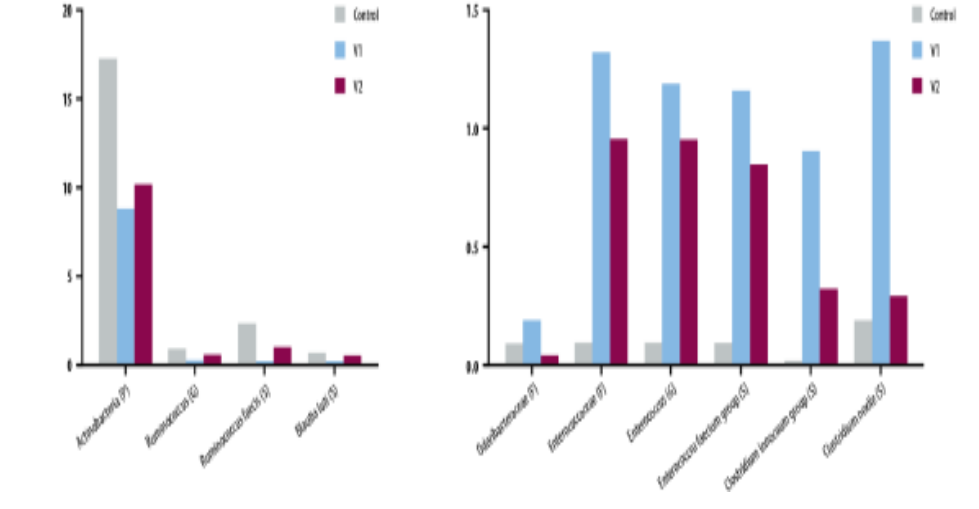
In saliva samples, bacteria levels including *Ralstonia_f* (family), *Burkholderiaceae* (family), *Ralstonia* (genus), *Paraburkholderia kururiensis* (species), *Ralstonia_uc* (species) were lower in IBD patients at V1 than in the control group, and increased at V2 (Supplementary Figure 4B).

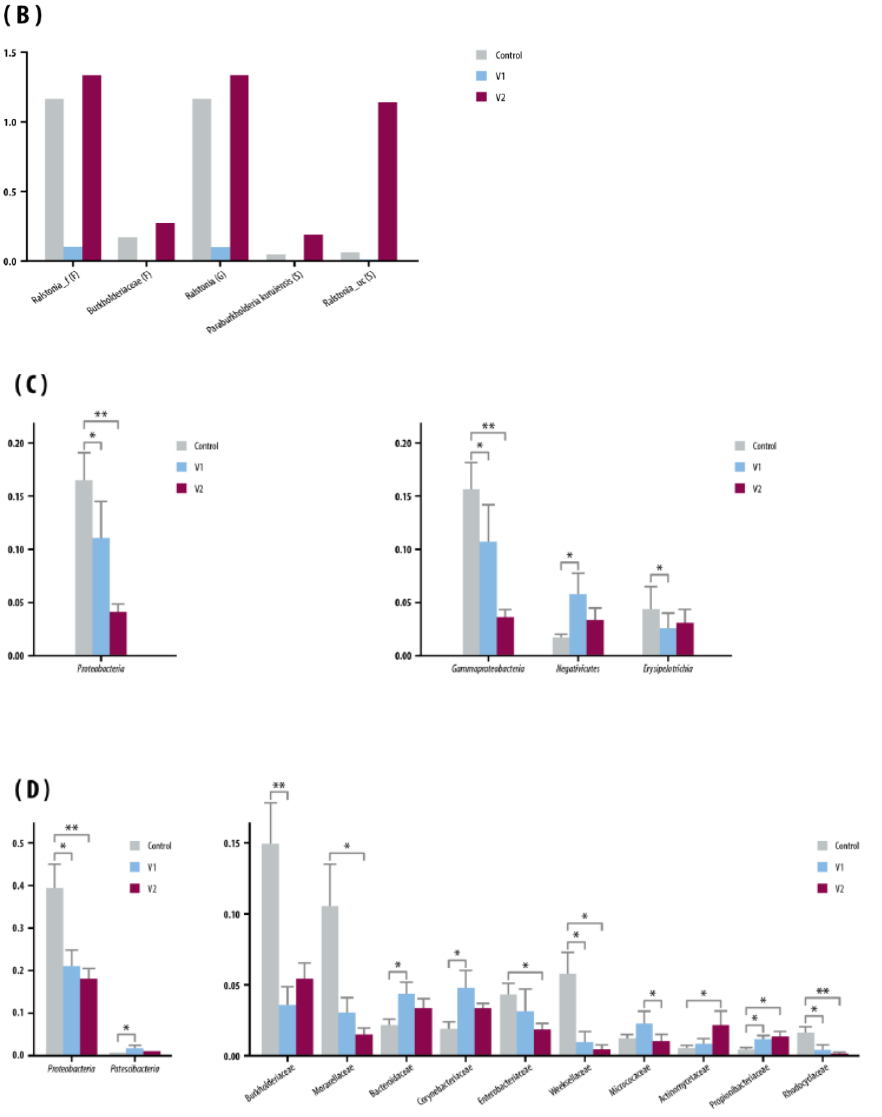
B. Extracellular vesicles analysis in stool, saliva, serum and urine

In EV analysis of the stool samples, levels of *Proteobacteria* (phylum), *Gammaproteobacteria* (class), *Ruminococcus1, 2* (genus), *Enterococcus* (genus), *Acidovorax caeni* (species), *Enterococcus faecalis* (species) were higher in the control group than in IBD patients at V1. Conversely, at V1, the IBD group had significantly higher levels of *Negativicutes* (class), *Selenomonadales* (order), *Veillonellaceae* (family), *Veillonella* (genus), *Enterococcaceae* (family), *Enterococcus* (genus), and *Clostridioides difficile* (species) than the control group (Supplementary Figure 4C).

In the saliva samples, *Proteobacteria* (phylum), *Gammaproteobacteria* (class), *Flavobacteriales* (order), *Burkholderiaceae* (family), *Moraxellaceae* (family), *Acidovorax caeni* (species), *Enterobacter cloacae* (species), *Acinetobacter calcoaceticus* (species) levels were higher in the control group than in IBD patients at V1 (Supplementary Figure 4D).

(A)





Supplemental Figure 4. Differences between in the control group, and the inflammatory bowel disease (IBD) group before (V1) and 3 months after anti TNF α treatment (V2). (A) Fecal microbial composition in next generation sequencing (NGS) analysis, (B) Salivary microbial composition in NGS analysis, (C) Fecal microbiome at the phylum and class level in extracellular vesicle (EV) analysis, (D) salivary microbiome at the phylum and family levels in EV analysis. (all $P < 0.05$)

5. Changes in microbiota with anti TNF treatment

A. Responders versus non-responders

(A) Microbiota composition in stool

ⓐ NGS

In the NGS analysis of the stool samples, *Actinobacteria* (phylum); *Coriobacteriia* (class); *Coriobacteriales* (order); *Coriobacteriaceae* (family); *Collinsella* (genus); *Collinsella aerofaciens group* (species), *Eubacterium_g21* (genus), *Dorea* (genus); *Dorea longicatena* (species), *Agathobaculum* (genus), and *Blautia* (genus); *Blautia wexlerae* (species) levels were higher at V1 in anti-TNF-α responders than in non-responders.

Moreover, *Proteobacteria* (phylum), *Gammaproteobacteria* (class), *Enterobacterales* (order), *Enterobacteriaceae* (family), *Enterobacteriaceae_g* (genus), *Enterobacteriaceae group* (species), *Odoribacter* (genus), *Odoribacter splanchnicus* (species), *Ruminococcus_g5* (genus), and *Ruminococcus gnavus* (species) levels were higher in non-responders than in responders at V1 (data not shown).

ⓑ Extracellular vesicles

When differences in microbiota between IBD patients with or without a treatment response were evaluated by EV analysis, *Firmicutes* (phylum), *Clostridia* (class), and *Ruminococcaceae* (family) were more abundant at V1 in responders than non-responders (Figure 2). Conversely, non-responders had higher levels of *Enterobacteriaceae*, *Acidaminococcaceae* and *Rikenellaceae* at the family level than responders at V1 (Figure 2).

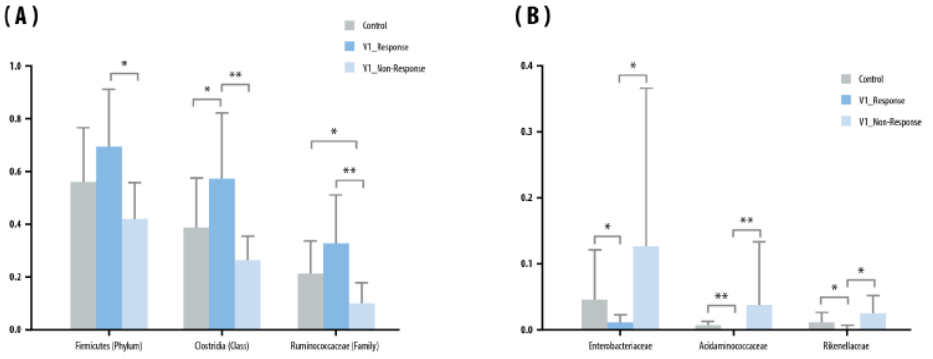


Figure 2. Stool: microbials in the stool of the control group and inflammatory bowel disease (IBD) group before anti TNF α treatment (V1). (A) Microbials abundant in responder group (phylum, class, family), (B) Microbials abundant in non-responder group (family) (all $P<0.05$)

(B) Microbiota composition in saliva

ⓐ NGS

When NGS analysis was performed on the saliva samples, levels of *Abiotrophia defective*-species (*Bacteria: Firmicutes: Bacilli: Lactobacillales: Aerococcaceae: Abiotrophia*), *FJ976422_s*-species (*Bacteria: Bacteroidetes: Bacteroidia: Bacteroidales: Prevotellaceae: Alloprevotella*), *AFQU_s*-species (*Bacteria: Firmicutes: Bacilli: Lactobacillales: Streptococcaceae: Streptococcus*), and *AF385506_s*-species (*Bacteria: Saccharibacteria_TM7: Saccharimonas_c: Saccharimonas_o: Saccharimonas_f: PAC000677_g*) were higher in responders than in non-responders at V1. However, *Ralstonia_f* (family) and *Ralstonia* (genus) levels at V1 were significantly higher in the saliva of non-responders than in that of responders (Figure 3).

ⓑ Extracellular vesicles

In the responder group, the level of *Prevotella 9* in saliva was higher than that of non-responders at V1 (Figure. 3). In addition, the level of *Ralstonia* was

higher at V2 in the non-responder group than in the responder group (Figure 3).

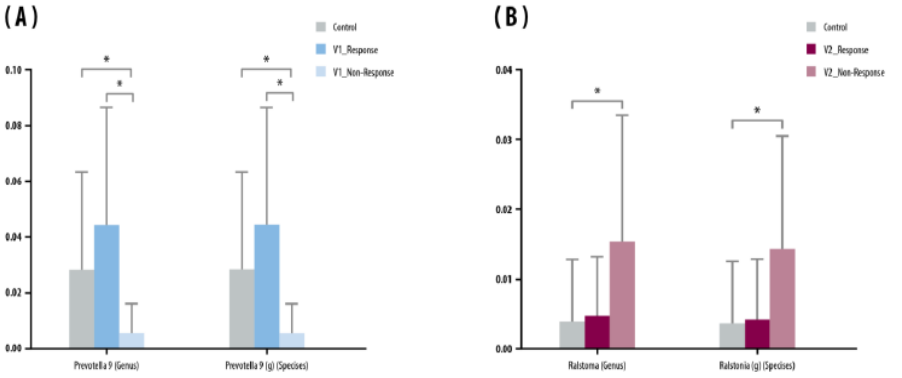


Figure 3. Saliva: microbial composition in the saliva of the control group and inflammatory bowel disease (IBD) patients grouped by clinical response/non-response. (A) Genera and species abundant before anti TNF α treatment (V1), (all P<0.05) (B) Genera and species abundant after anti TNF α treatment (V2), (all P<0.05)

(C) Microbiota composition in serum: EV analysis

In the serum samples, the levels of *Corynebacterium* were significantly higher at V1 in non-responders than in responders, and *Lachnospiraceae* was significantly more abundant in non-responders than responders at V2 (Figure 4).

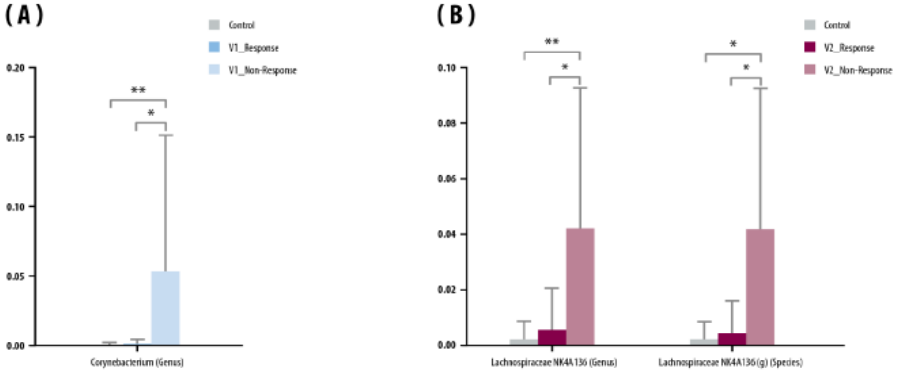


Figure 4. Serum: microbials (genus and/or species) in the serum of the control group and inflammatory bowel disease (IBD) group. (A) Microbials abundant in responder group before anti TNF α treatment (V1), (B) Microbials abundant in non-responder group after anti TNF α treatment (V2) (all $P< 0.05$)

(D) Microbiota composition in urine: EV analysis

In urine samples, the levels of *Pseudomonadales* (Order), *Moraxellaceae* (Family), and *Acinetobacter* (genus) were significantly higher in responders than in non-responders at V1 (Figure 5). However, non-responders had higher levels of *Lachnospirace* and *Ruminococcaceae* than responders at V1 (Figure 5).

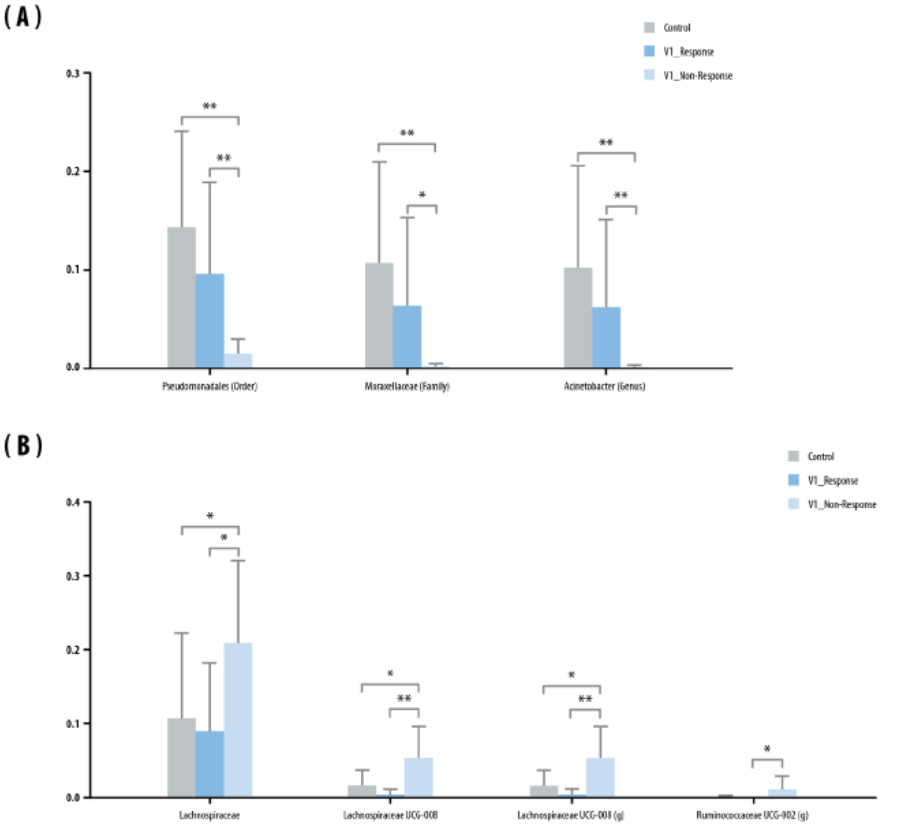
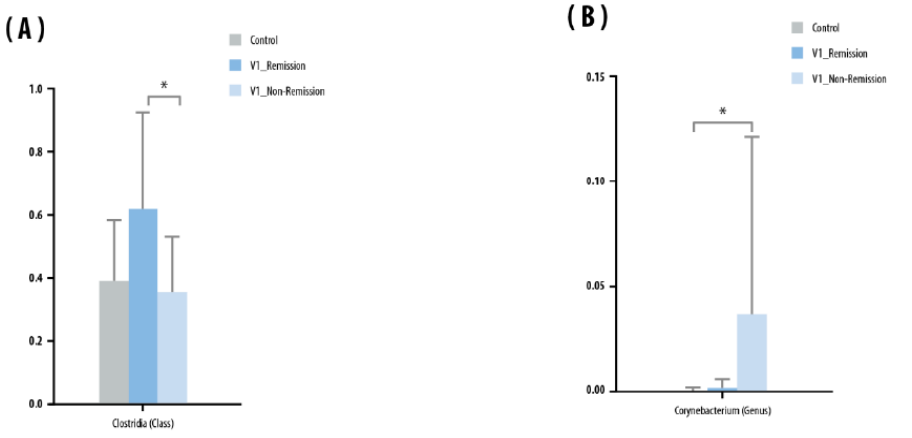


Figure 5. Urine: microbials in the urine of the control group and the inflammatory bowel disease (IBD) group before anti TNF α treatment (V1). (A) Microbials abundant in responder group: order, family and genus level, (B) Microbials abundant in non-responder group: species level (all $P < 0.05$)

B. Remission versus non-remission

When comparing the microbiomes of patients who showed remission after anti-TNF-α treatment with those who did not, the results were similar to those of responders versus non-responders. At V1, the non-remission group had higher levels of *Clostridia* (Class) in stool, V1 (Supplementary Figure 5), and *Corynebacterium* (genus) in serum than the remission group (Supplementary Figure 5).



Supplementary Figure 5. Microbials of the control group and inflammatory bowel disease (IBD) group before anti TNF α treatment (V1) between patients with and without remission. (A) Stool, microbials abundant in remission group, (B) Serum, microbials abundant in non-remission group (all $P< 0.05$)

C. Prediction of anti-TNF-α response: EV analysis

When the microbiota was analyzed using EVs, *Acidovorax caeni* (species) was found in all types of samples (stool, saliva, serum, and urine) at V1 in

responders, as well as in the control group, but not in non-responders (Figure 6).

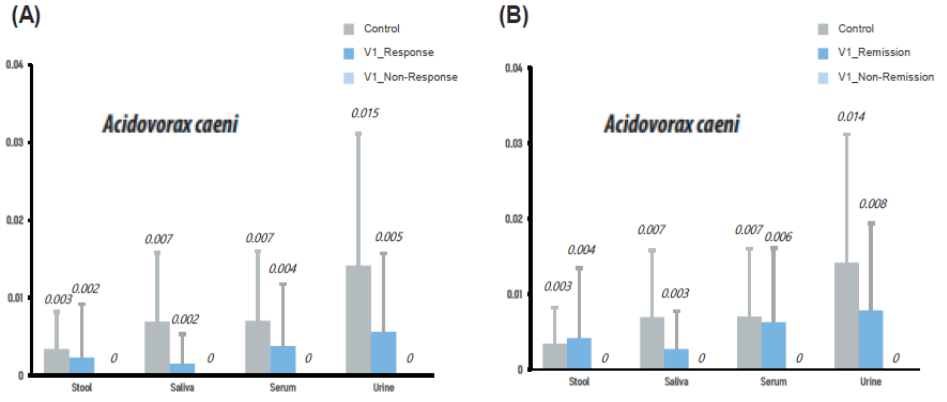


Figure 6. Microbiome (extracellular vesicles analysis) in the inflammatory bowel disease (IBD) group with significant differences in all 4 samples (stool, saliva, serum, urine) before anti-TNF-α treatment (V1) between (A) patients with and without response, (B) patients with and without remission (all P <0.05)

Additionally, when examining the *Firmicutes/Bacteroidetes* (F/B) ratio, the stool F/B ratio was relatively low in the control group, whereas, it was high before anti-TNF-α treatment in the IBD group, and decreased after treatment. Conversely, the F/B ratio in urine was relatively high in the control group, but was low in the IBD group before treatment, and then increased after treatment. Saliva F/B ratios were comparable between the control and IBD group at V1, but increased in non-responders at V2. However, there were no significant differences between responders and non-responders with regard to stool, saliva, and urine F/B ratios at either V1 or V2. In serum, the F/B ratio was significantly higher in responders than in non-responders at V1, but not at V2 (Table 3).

Table 3. *Firmicutes/Bacteroidetes* (F/B) ratio in stool, saliva, serum, and urine samples

Variables	Stool	Saliva	Serum	Urine
Control	8.78 (15.78)	3.81 (4.23)	2.00 (1.68)	26.06 (74.36)
Visit 1	624.72 (2035.31)	3.31 (2.53)	5.32 (6.69)	6.89 (6.37)
Responder	265.07 (762.87)	2.35 (0.87)	8.05 (8.07)	4.32 (4.62)
Non-responder	1029.32 (2906.89)	4.27 (3.29)	1.91 (0.97)	9.75 (7.06)
P value*	0.458	0.125	0.040	0.061
Visit 2	21.25 (51.23)	110.45 (432.06)	6.30 (7.06)	16.59 (41.77)
Responder	31.37 (63.77)	4.86 (3.17)	6.51 (8.02)	22.43 (55.59)
Non-responder	4.38 (2.30)	261.30 (672.80)	6.05 (6.42)	9.07 (11.20)
P value*	0.324	0.352	0.906	0.544
P value**	0.300	0.251	0.058	0.569

Data are expressed as mean (standard deviation, SD). * *P* value of R-NR (t-test). **P* value of control,V1,V2 (Kruskal-Wallis)

6. Microbiota composition based on disease activity

Disease activity (remission, mild-moderate, or severe) in IBD patients had some influence on the microbiome at the phylum level (Figure 7). In stool samples in patients with severe disease activity, the level of *Firmicutes* was significantly higher than that in patients in remission, and that of *Bacteroidetes* was significantly lower than those in patients in remission or mild-moderate disease activity (Figure 7). In saliva, when the disease activity was mild-moderate, *Proteobacteria* and *Fusobacteria* levels were significantly higher than those in the remission group, but there was no significant difference between those in the severe disease activity group (Figure 7). There were no significant differences between the disease severity groups in microbiota at the phylum level in the serum samples (Figure 7). In urine samples, *Bacteroidetes* was significantly higher in abundance in patients with severe disease activity than in those in remission (Figure 7).

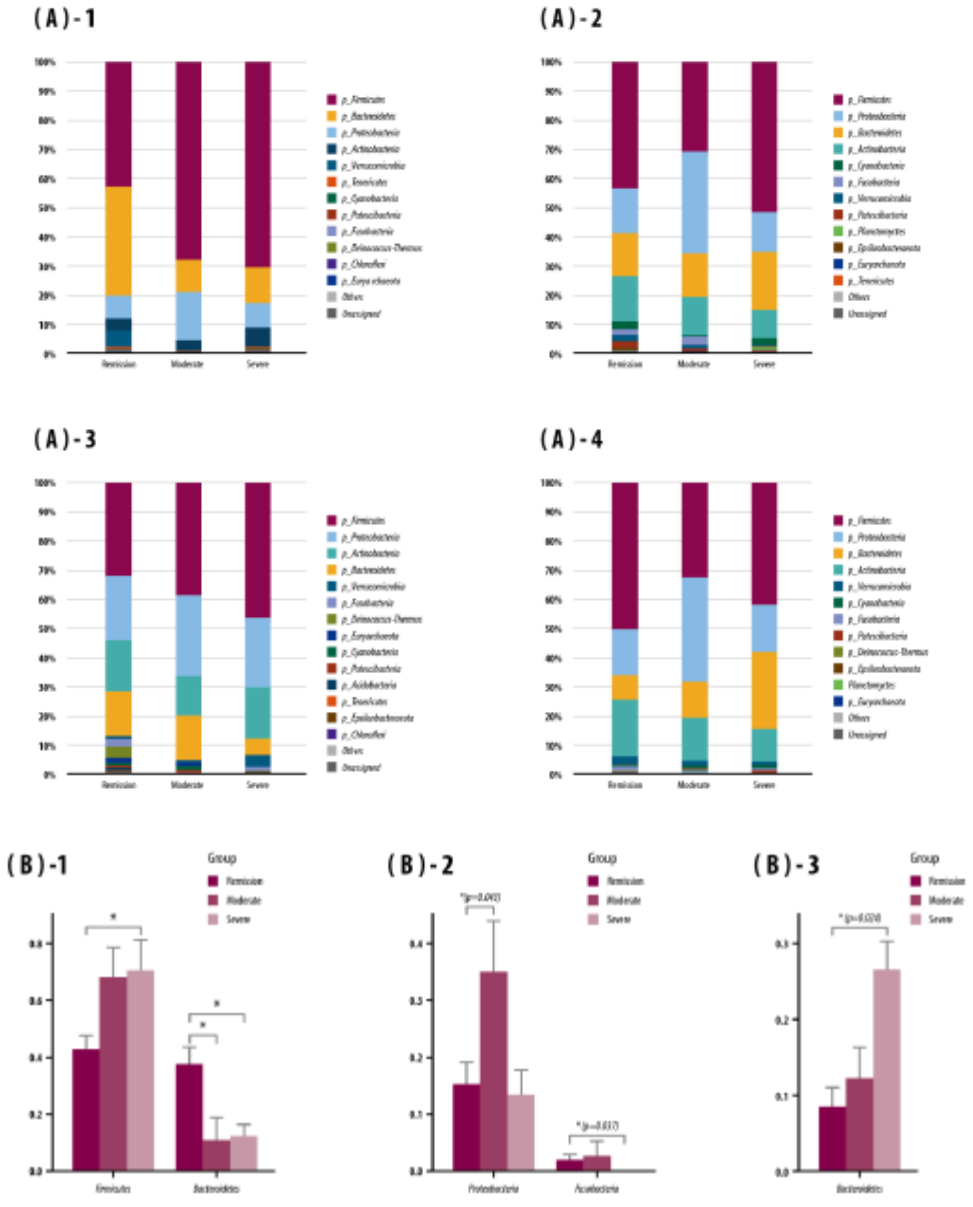


Figure 7. Composition of microbiome and bacteria showing significant differences according to baseline disease activity (remission, mild moderate, severe) at the phylum level. (A) microbial composition; 1- stool, 2- saliva, 3- serum, 4- urine, (B) microorganisms showing significant difference, 1- stool, 2-saliva, 3-urine. (all P<0.05).

IV. DISCUSSION

Since the development of anti-TNF-α therapy, the treatment of IBD patients has progressed considerably. Although the treatment needs are not yet fully met, it is still an important treatment modality. In IBD patients, the microbiota plays an important pathogenic role and is a factor that regulates inflammation.[42] It is predicted that anti-TNF-α therapeutic efficacy may be related to the microbiome not only in the intestines, but also in other body sites, but studies are still lacking. In our study, we examined the differences in the microbiome in stool, saliva, serum, and urine samples using EVs, and investigated the important microbiota by comparing taxa in stool and saliva samples using conventional NGS.

Our study was the first to analyze microbiome in various body fluids, such as stool, saliva, serum, and urine, using conventional NGS analysis and nano-particles using EV. Relative to EV analysis, NGS analysis of fecal samples showed a significant reduction in microbiome diversity in IBD patients compared to healthy controls. Although EV analysis did not show any significant difference in terms of microbiome diversity, it showed the similar results as NGS analysis with regard to significant bacterial changes in stool and saliva samples. Analysis of NGS in feces showed lower levels of *Actinobacteria*, *Ruminococcus* and increased levels of *Enterococcaceae*/ *Enterococcus* in IBD patients relative to healthy controls, whereas EV analysis showed a decrease in *Proteobacteria*, *Ruminococcus*, *Enterococaceae*/ *Enterococcus* levels, and an increase in *Clostridiodes difficile* levels in IBD patients. In some cases, such as *Enterococcus*, the results of the NGS and EV analyses were opposite, but the most important results were similar. Differences exist between the NGS and EV analyses, suggesting that EV itself may play a role related to inflammation. However, further research is needed.

EV analysis provided similar results when conducted in saliva, serum, and

urine samples in the control and IBD patients. In IBD patients relative to the control group, *Actinobacteria* and *Fusobacteria* were more abundant in saliva samples, *Firmicutes*, *Actinobacteria*, and *Fusobacteria* were more abundant in serum samples, and *Firmicutes* and *Actinobacteria* were more abundant in urine samples. In addition, saliva, serum, and urine samples in the control group showed increased levels of *Proteobacteria* and *Bacteroidetes* compared to those in IBD patients. It appears that the microbiome of feces and the microbiome of saliva, serum, and urine show some opposite tendencies with regard to these bacteria.

We showed that responders to anti-TNF-α treatment had more abundant levels of *Firmicutes* (*Clostridia*, *Ruminococcaceae*) in their stool, and *Prevotella* in their saliva before treatment than non-responders to treatment. In addition, in serum and urine samples, in which the microbiome results of IBD patients were analyzed for the first time, the level of *Lachnospiraceae* was found to be higher in the non-responder group than in the responder group after anti-TNF-α treatment. Furthermore, *Acidovorax caeni* was not detected in the stool, saliva, serum, and urine samples of non-responders before anti-TNF-α treatment (V1), but was found in all samples of responders and control.

The association of Firmicutes with IBD is well known. The reduced diversity of gut microbiota in patients with IBD is related to decreased levels of *Firmicutes*, such as the *Clostridium leptum* group and *Faecalibacterium prausnitzii*.[1] *Firmicutes*, especially *F.prausnitzii*, has anti-inflammatory effects by producing substances such as short chain fatty acids, butyrate, that can inhibit Th17 cells in IBD.[43] Studies have shown that changes in *Firmicutes* level play a role as an important marker, even during anti-TNF-α treatment. Busquets et al.[14] reported that the use of adalimumab in CD patients leads to the recovery of *Firmicutes*, *Bacteroides*, and *Actinobacteria*. In addition, Magnusson et al.[44] reported that patients with UC who were *F.prausnitzii*-rich

at baseline responded to anti-TNF-α treatment. Another study showed an increase in the levels of *Lachnospiraceae* and *Blautia* in response to infliximab in CD patients.[45] In addition, *Clostridia* was more abundant in IBD patients who responded to infliximab,[45] and Zhou Y et al. also reported higher abundance of *Clostridia* in CD patients who responded to treatment and predicted infliximab effectiveness when combined with fecal calprotectin and CDAI.[46] Similar to these previous studies, our study also showed an increase in *Firmicutes* levels (*Clostridia*, *Ruminococcaceae*, *Lachnospiraceae*) in V1 stool of IBD patients who responded to anti-TNF-α agent. *Firmicutes* levels in feces can be used as a predictive marker for anti-TNF-α treatment effectiveness in IBD.

Until recently, most microbiome research has been focused on the gut, however, new studies are investigating the non-invasive and accessible saliva microbiome. Recent studies have shown that intestinal inflammation and IBD pathogenesis are related by the oral-gut axis connection, in that oral-derived pathobiogens translocate to the intestine and cause IBD.[47-49] The major components of the saliva microbiome are *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria*.[50] Compared to the gut microbiome, the phylum level in saliva shows a similar composition, but it has been reported that there is a difference in order of abundance.[50,51] Said et al. reported that there was no difference in diversity of salivary microbiota between IBD patients and healthy subjects, with the dominant genera being *Streptococcus*, *Prevotella*, *Neisseria*, *Haemophilus*, *Veillonella*, and *Gemella* in IBD patients.[52] This is similar to the results of this study, in which there was no difference in α-diversity between IBD patients and the healthy control group, showing that in IBD patients, the salivary microbiota may not differ as much as that of the stool. Therefore, it would be difficult to use the change in saliva diversity as a follow-up test. However, our study showed that *Prevotella* was abundantly present at baseline in patients responding to anti-TNF-α

treatment, which could be an important factor in patients with IBD. *Prevotella* is a genus of Gram-negative, obligate anaerobe,[53] and reported to be associated with opportunistic infections such as vaginosis, esophagitis, and antral gastritis,[54-56] while most of them are intestinal commensal bacteria in the gut.[57] *Prevotella* can play a role in patients with IBD, as it is reported that it can induce circulation of bacteria and other inflammatory mediators by inducing inflammation of the mucosa.[58] So far, studies on the saliva microbiome are insufficient, and the association with gut microbiota and in patients with IBD has not yet been clearly identified. In addition, the status of conditions such as tooth decay and periodontitis[59,60] and diet-related lifestyle can change the composition of the oral microbiome.[61] Although more research is needed, it can be expected that salivary bacteria such as *Prevotella* could be used as predictive markers for treatment.

To the best of our knowledge, this is the first study to analyze microbiome in the serum and urine in IBD patients. Some studies have reported on metabolic interactions in patients with IBD in serum and urine.[62,63] Kolho et al. reported the serum metabolomics in pediatric IBD patients and showed changes in the serum pathways associated with the inflammatory response.[62] However, no previous study has analyzed the microbiome by extracting 16srRNA from serum and urine. In this study, the non-responder group showed a tendency for higher levels of *Lachnospiraceae* in saliva, serum, and urine samples than the responder and control groups, but the stool samples showed an increase in *Lachnospiraceae* in the control and responders, indicating that the microbiome of saliva/serum/urine and feces showed the opposite tendency. In addition, the level of *Corynebacterium* before treatment was higher in the serum of the non-responder group than in the control and responder groups. *Corynebacterium* is a genus of Gram-positive, aerobic bacteria, with Dinakaran et al.[58] reporting an increase in this bacteria in colon specimens from patients with IBD. Therefore, *Lachnospiraceae* and *Corynebacterium*

have potential as predictive serum markers for treatment. In urine samples, *Pseudomonadales* (order), *Moraxellaceae* (family), and *Acinetobacter* (genus) belonging to the *Proteobacteria* taxa were higher in abundance before treatment in responders than in non-responders to anti-TNF-α therapy.

In this study, levels of *Proteobacteria* in saliva were higher in the control group than in IBD patients before treatment, and decreased further in IBD patients after treatment. This differs from the results of previous studies[1,46] which found higher *Proteobacteria* levels in IBD patients than in control, but also showed a significant decrease after treatment, indicating that the treatment effect and *Proteobacteria* levels were related. In addition, in stool samples, patients responding to treatment had lower *Proteobacteria* levels before treatment than non-responders. This is also the opposite result of an increase in the baseline *Proteobacteria* levels in urine of the responder group. These novel findings seem to show an inverse correlation between the microbial community of feces and serum/urine. This means that the microbiota can be predicted to circulate in feces, saliva, serum, and urine through EV.

In this study, we showed that *Acidovorax caeni* was observed in the baseline analysis of the responder group, but not in the non-responder group in each of the stool, saliva, serum, and urine samples using EVs. *Acidovorax caeni* is a species of Gram-negative, aerobic bacteria with a polar flagellum;[64] its phylum level is *Proteobacteria*, and it consists of following taxa *Gammaproteobacteria* (class), *Betaproteobacteriales* (order), *Burkholderiaceae* (family), *Acidovorax* (genus). Increased levels of *Enterobacteriaceae* in IBD patients are well known,[1,65,66] with Alam et al. also showing increased abundance of *Burkholderiaceae* in both CD and UC patients.[67] It is not yet clear whether an increase in *Acidovorax caeni* levels is associated with an increase in *Burkholderiaceae* levels and the potential role it plays in patients with IBD. It may be a species that predicts the therapeutic effect in patients with IBD because all sample types showed the same results.

Further research is needed.

This is the first study in which 16srRNA was extracted with nano-particles to analyze the microbiome of stool, saliva, serum, and urine in IBD patients. The microbiome in feces and saliva were also analyzed and compared with NGS, and the results were similar to those of previous studies. This showed that it was possible to easily analyze the microbiome, even in other fluids, using EVs. In addition, if probiotics using bacteria important for anti-TNF-α treatment response are developed, it can be expected to be useful in clinical situations. However, our study has several limitations. First, a study involving only anti-TNF-α naive patients may result in selection bias. Differences depending on the type and duration of previously used drugs or differences in prevalence of disease may affect the results. However, since most of the patients used anti-TNF-α according to the clinical practice guidelines, it is believed that this should not affect the results. Secondly, the analysis was performed by two methods, EVs and NGS. The results of the two methods were not completely identical, but important results were similar between the two analyses. Intriguingly, some strains were meaningful in EVs, but not in NGS. However, even when both of these analyzes were applied, it was revealed that the strains showing the same results are important in the microbiome of actual IBD patients. Lastly, there has been no study of microbiome in serum and urine in IBD, so the implications of the results of this study are not clear. In addition, lipopolysaccharide (LPS), which can reflect intestinal barrier dysfunction and predict the degree of inflammation, [68] can be measured by EV, but this study did not analyze it and could not investigate metabolites. So further studies are needed in the future to elucidate the role of the microbiome discovered in this study.

V. CONCLUSION

In summary, this study showed that the levels of *Firmicutes* (phylum),

Clostridia (class), and *Ruminococcaceae* (family) were increased in stool, and the levels of *Prevotella* were increased in saliva at baseline in patients who responded to anti-TNF-α therapy. In serum and urine, the levels of *Lachnospiraceae* were increased in patients in the non-responder group. Finally, *Acidovorax caeni* was found, in all four sample types, only in those IBD patients who responded to anti-TNF-α treatment, so levels of this species may prove helpful in predicting the anti-TNF-α treatment response in IBD patients.

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ABSTRACT(IN KOREAN)

염증성 장질환 환자에서 항 TNF-α 치료 전후의 대변, 타액, 혈청 및 소변의 미생물 변화

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박 용 은

크론병 및 궤양성 대장염을 포함한 염증성 장질환은 여러 신체 부위, 특히 장에서 미생물 불균형 (dysbiosis)와 관련된 만성 면역 매개 장 염증 질환입니다. 항 종양 괴사 인자-알파 (항-TNF-α) 제제는 염증성 장질환 환자의 치료에서 중요한 치료제로, 이 연구는 여러 부위의 미생물 군집의 변화가 염증성 장질환 환자에서 항 TNF-α 치료의 효과를 예측할 수 있는지를 조사하고자 하였습니다.

대변, 타액, 혈청 및 소변의 검체는 3개의 대학 병원에서 19 명의 염증성 장질환 환자와 19 명의 건강한 피험자를 대상으로 수집되었습니다. Microbiota 분석은 세포 외 소포 (EV)를 사용하여 4가지 유형의 샘플 모두에 대해 수행되었으며, 대변 및 타액 샘플에는 NGS (Next Generation Sequencing) 분석을 같이 시행하였습니다. 환자 그룹에는 9명의 궤양성 대장염 환자와 10명의 크론병 환자가 있었고, 각 샘플의 미생물 총은 항 TNF-α 처리 전과 치료 3 개월 후에 분석되었습니다.

대변에서 항 TNF-α 처리 전과 후에 NGS 분석을 사용한 검사에서, α-diversity 에는 유의한 차이가 있었지만 타액 샘플에서는 차이가 없었습니다. 또한, EVs 분석을 이용한 4가지 샘플 분석에서 anti-TNF-α 처리 전후의 α-diversity는 차이가 없었습니다. *Firmicutes* (phylum), *Clostridia* (class) 및

Ruminococcaceae (family)는 치료 전 대변에서 유의하게 높았으며, *Prevotella*는 항 TNF-α 치료 후 반응이 있던 환자의 치료 전 타액에서 유의하게 높았습니다. 혈청과 소변의 추적 관찰에서 *Lachnospiraceae*은 anti-TNF-α 치료 후 비반응군에서 유의하게 증가하는 것으로 나타났습니다. 또한, 비반응자에서 치료 전후의 대변, 타액, 혈청 및 소변에서 *Acidovorax caeni*가 검출되지 않았습니다.

결론적으로, 여러 부위의 microbiome 전체 변화는 염증성 장질환 환자에서 항 TNF-α 치료의 효과를 예측할 수 있습니다. 또한 세포 외 소포를 이용한 미생물 분석은 다양한 체액에서 쉽게 검사할 수 있으므로 염증성 장질환 환자에서 더 많은 연구를 기대할 수 있습니다.

핵심되는 말 : 염증성 장질환; 궤양성 대장염; 크론병; 미생물 균총; 항 종양 괴사 인자-알파; 세포 외 소포