MATERIAL AND METHODS

Symptom questionnaires

In order to assess symptoms, all patients completed a modified Italian version[1] of the Bowel Disease Questionnaire.[2] The questionnaire includes questions on the following symptoms: abdominal pain / discomfort and bloating, frequency and characteristics of bowel movement, allowing the sub-classification of patients into diarrhoea or constipation, flatulence, relief of abdominal pain / discomfort by defecation, onset of symptoms associated with a change in frequency or in form (appearance) of stool were assessed as present/absent. Number of evacuations per week were also recorded. Patients scored the severity and frequency of their symptoms over the previous two weeks before the visit. Symptom severity was graded 0 – 4 according to its influence on patients’ usual activity, as previously described.[3] Similarly, the symptom frequency was graded 0 – 4 according to the following scale: 0, absent; 1, up to 1 day per week; 2, 2 or 3 days per week; 3, 4 – 6 days per week; 4, daily. Bowel habit was further characterized into number of bowel movements per day or per week in the previous two weeks. Sub-classification of patients based on bowel habit was obtained according to predominant bowel habit, that is more than one fourth of bowel movements with one of the following stool characteristics: hard or lumpy (constipation), regular or loose/watery (diarrhoea).[3] In addition, we collected data regarding previous diseases and drug use and volupuary habits such as current or previous smoking habit, and dietary habits. In particular a fiber-rich diet (daily fiber assumption > 18 g) was assessed by a dietician based on a 24 hr recall and quantity of fibre was assessed using a software (Food Intake Dietosystem, DS Medica). In order to verify if the symptoms reported by patients with SUDD fulfilled criteria for IBS, in S UDD patients, symptoms were evaluated by the Rome III modular questionnaire.[4]

Biopsy processing, histology, immunohistochemistry and quantification of inflammatory cells

For each biopsy site, one specimen was fixed in buffered 10% formalin for histology and immunohistochemistry and one was snap-frozen and subsequently stored at -20° until gut microbiota analysis was performed. All tubes were labeled with a code and all laboratory personnel performing histological, immunological and microbiological analyses were unaware of the characteristics and name of the patients. Codes were broken only at the end of the study when all the analyses were performed.

Histological sections were evaluated for the exclusion of microscopic colitis or overt mucosal inflammation by a pathologist who was unaware of the diagnosis. For immunohistochemistry, paraffin-embedded biopsies were cut and processed as following. Mast cells were detected as previously described[3 5] using mouse monoclonal antibody directed against tryptase (1:3000, Millipore, Billerica, MA). T cells were detected using rabbit polyclonal antibody against CD3 (1:200, Dako, Glostrup, Denmark) after heat–induced antigen retrieval with 10 mmol/L Tris buffer, 1 mmol/L EDTA (pH 9.0) at 95°C for 25 min. Macrophages were detected using a mouse monoclonal antibody against CD68 (1:50, DAKO, Glostrup, Denmark) after heat–induced antigen retrieval with 10 mmol/L citrate buffer (pH 6.0) at 95°C for 25 min. For each staining after 2 hours incubation with the primary antibody, slides were washed with PBS and then incubated with secondary biotinylated anti mouse-rabbit antibody followed by streptavidin – horseradish peroxidase conjugate (Millipore, Billerica, MA). Different immune cell subtypes were quantified on immunehistochemically stained sections with a Leitz Dialux microscope in blind fashion using a previously validated computer-assisted analysis system .[3 6] Results are expressed as percentage of cells (%) over lamina propria area (mm²), as previously described.[3 5] Briefly, this method, which represents a modification of the original method developed by Lee et al.,[7] adapts morphometric point-counting technique to quantify the area covered by the different kinds of cells in the lamina propria. The results are expressed as percentage of dots landing on a given structure. In ancillary studies we have demonstrated a high intra-observer and inter-observer reproducibility.
of the method. This method has the advantage over conventional pathological evaluations to minimize bias and maximize accuracy in counting.[8]

DNA extraction from feces

Total bacterial DNA from fecal samples was extracted using DNeasy Blood & Tissue Mini Kit (Qiagen, Duesseldorf, Germany) using a modified protocol.[9] Briefly, 250 mg of feces were suspended in 1.2 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4% SDS). Four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK) were added, and samples were treated thrice in FastPrep (MP Biomedicals, Irvine, CA) at 5.5 ms for 1 min. Samples were heated at 95°C for 15 min and centrifuged for 5 min at full speed. Supernatants were added with 260µL of 10 M ammonium acetate, kept in ice for 5 min, then centrifuged at full speed for 10 min. One volume of isopropanol was added to the supernatants and incubated in ice for 30 min. DNA was precipitated by centrifugation for 15 min at full speed. Pellets were resuspended in TE buffer and treated with 2µL of DNase-free RNase (10 mg/ml) at 37°C for 15 min. Protein removal by Proteinase K treatment and DNA purification with Qiagen columns were performed.

Bacterial DNA extraction from colon biopsies

Total bacterial DNA from colonic biopsy samples was extracted using DNeasy Blood & Tissue Mini Kit (Qiagen, Duesseldorf, Germany) with a modification of a previous method.[10] Briefly, frozen biopsies were immersed in 500 μL of sterile PBS buffer and treated in ultrasound bath for 2 minutes twice to separate bacterial cells. Eucaryotic cells were precipitated by centrifugation at 700 g for 1 min, then the supernatant was transferred in a clean tube and centrifuged at 9000 g for 5 min to pellet bacteria. Collected bacteria were resuspended in 180 μL of Qiagen enzymatic lysis buffer and incubated at 37°C for 30 min, then the Qiagen protocol for Gram positive bacteria was followed. Bacterial DNA was eluted in 50 μL of PCR grade water.

Microbiota analysis by HTF-Microbi.Array

PCR products were purified by using the High Pure PCR Clean up Micro kit (Roche, Mannheim, Germany), following the manufacturer instructions, eluted in 20 µl of sterile water and quantified with NanoDrop 1000 (NanoDrrp Technologies, Wilmington, DE). The HTF-Microbi.Array utilized in this study is based on the Ligase Detection Reaction-Universal Array (LDR-UA) approach[11] developed and implemented by Candela et al.[12 13] Slide chemical treatment, array production, LDR protocol and hybridization conditions were carried out as previously reported[13 14] with probe annealing set at 60°C. The LDRs were carried out in a final volume of 20 µl with 50 fmol of PCR product. Two hundred and fifty fmol of Synthetic template (5'-AGCCGCGAACACCACGATCGAGCTGCAGCTGCTCATG-3) were used for normalization purposes. All HTF-Microbi.Array experiments were performed in independent duplicates. All arrays were scanned and processed according to the protocol and parameters previously described.[13] Mean data from two replicated experiments were obtained and utilized for statistical analysis of the probes relative contribution. The microbiota of all subjects was analyzed by using the HTF-Microbi.Array, a LDR-microarray-based tool able to reliably approximate the composition of the intestinal microbiota in terms of relative abundance of the most common groups of bacteria.

Samples preparation for 1H-NMR

Urine samples were prepared for NMR analysis through centrifugation for 15 min at 15K rpm and 4°C. 700 ml of supernatant were added to 160 μl of a D2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 6.25 mM. After pH adjustment to 7.00 with the addition of HCl or NaOH (0.5 M). Fecal samples were prepared for NMR analysis by vortex mixing for 5 min 40 mg of stool with 1 ml of deionized water. The obtained mix was then processed as explained for urine samples. 1H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz. The HOD residual signal was suppressed by applying the first increment of the NOESY pulse sequence and a spoil gradient.[15] This was done by employing the NOESYGPPR1D sequence, part of the standard pulse sequence library. Each
spectrum was acquired by summing up 256 transients using 32 K data points over a 7211.54 Hz spectral (for an acquisition time of 2.27s). The recycle delay was set to 5s, keeping into consideration the longitudinal relaxation time of the protons under investigation. The signals were assigned by comparing their chemical shift and multiplicity with the Human Metabolome Database[16] and Chenomx software data bank (Chenomx Inc., Canada, ver 7.7).

**RESULTS**

**Supplementary Table 1. Concentration (mM/g) in the feces of the molecules showing discriminative power among the three groups of patients under investigation**

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>Controls</th>
<th>Diverticulosis</th>
<th>SUDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>7.735</td>
<td>3.25E-04 ± 9.53E-05 a&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.91E-04 ± 4.93E-05 b&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uracil</td>
<td>7.534</td>
<td>1.98E-04 ± 5.77E-05 b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.62E-04 ± 7.65E-05 ab&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.329</td>
<td>5.63E-04 ± 7.68E-05 b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.17E-04 ± 1.29E-04 a&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-7.15&lt;sup&gt;**&lt;/sup&gt;</td>
<td>7.155</td>
<td>3.75E-04 ± 1.78E-04 a&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.88E-04 ± 1.42E-04 b&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-deoxyuridine</td>
<td>6.312</td>
<td>5.60E-05 ± 1.88E-05 a&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.49E-05 ± 2.29E-05 b&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-5.58&lt;sup&gt;**&lt;/sup&gt;</td>
<td>5.576</td>
<td>1.46E-05 ± 9.28E-06 b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.54E-05 ± 1.42E-05 a&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate</td>
<td>4.135</td>
<td>2.46E-03 ± 9.58E-04 b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.09E-03 ± 7.23E-04 a&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.568</td>
<td>4.28E-03 ± 1.17E-03 b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.72E-03 ± 1.42E-03 a&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methanol</td>
<td>3.364</td>
<td>2.94E-04 ± 7.90E-05 a&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.23E-04 ± 1.62E-04 b&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-aminoadipate</td>
<td>2.218</td>
<td>4.08E-04 ± 1.43E-04 b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.83E-04 ± 1.36E-04 a&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.478</td>
<td>2.41E-03 ± 4.52E-04 b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.31E-03 ± 5.62E-04 ab&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>Valine</td>
<td>1.050</td>
<td>3.30E-04 ± 6.25E-05 a&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.62E-04 ± 5.08E-05 b&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>Caprylate</td>
<td>0.872</td>
<td>9.68E-04 ± 5.87E-04 a&lt;sup&gt;6&lt;/sup&gt;</td>
<td>6.65E-04 ± 3.79E-04 b&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

SUDD, symptomatic uncomplicated diverticular disease.
* Different letters evidence statistically (P<0.05) significant differences.
** Signals pertaining to unidentified molecules are indicated with an X followed by their chemical shift.

**Supplementary Table 2. Confusion matrix for the PLS models calculated on fecal samples**

<table>
<thead>
<tr>
<th>Predicted</th>
<th>Controls</th>
<th>Diverticulosis</th>
<th>SUDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65.85±16.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.75±13.77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.40±12.96&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>18.37±11.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>57.40±12.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.23±11.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>30.65±16.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.90±13.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>57.45±16.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Correlation between metabolomics data and microbiota
We sought a correlation between microbiota, both fecal and mucosal, and the metabolome of urines. For the purpose, concerning microbiota, we restricted the observation to the relative abundance of the species that resulted as statistically different among the observed groups of patients. Concerning urinary metabolome, we focused on the molecules that were employed for LDA analysis, because characterized by the highest discriminative power among the patients. We compared the two datasets by means of coinertia analysis, a powerful tool of general applicability for highlighting common structures of a pair of datasets (Supplementary Figure 1), recently successfully employed for the characterization of the fecal microbiota-metabolome relationships caused by different diets.

The scoreplots, evidencing the samples’ clustering, show that urinary metabolome and microbiota features give rise to distributions of the groups with remarkably superimposable trends, characterized by control subject appearing at low values of the first canonical axis and diverticulosis and SUDD patients appearing at higher values. The loadingplots allow to visually appreciate which molecule (Supplementary Figure 1C) and microorganism (Supplementary Figure 1D) is mainly responsible for such clustering. Ethanolamine, more concentrated in the control subjects, seems the most important in characterizing a healthy status, followed by 3-aminoisobutyrate and 3-hydroxykynurenine. From the microbiota point of view, clostridium cl-IV and clostridium cl-IX present in the feces seem the species respectively with the highest direct and inverse correlation to a healthy status.

DISCUSSION

There is a longstanding unresolved issue on whether the SUUD patients are in fact IBS patients with diverticula or diverticular disease patients with abdominal symptoms. Indeed, this has been one of the topics of a recent consensus on Diverticular disease.[17] In this perspective, in future studies it would be interesting: 1) to include a group of IBS subjects without diverticula; 2) to design a longitudinal study assessing the eventual evolution of IBS patients into symptomatic diverticular disease. However, the present study was not designed to shed light into this complicated issue which was beyond our specific aims.

In our cases we did not find overall differences in the number of mast cells in patients with diverticular disease compared with controls. This appears to be in slight contrast with previous data.[18] However differences in patient populations and methodologies may explain this discrepancy. In particular, the quoted previous study was based on surgical resections and 44% of patients had acute disease subjected to surgical resection, and the remaining 56% were anyway severe cases thus more complicated and inflamed tissues compared to those included in our study, likely explaining the differences.

FIGURE LEGENDS

Supplementary Figure 1. Coinertia analysis combining urines metabolome and microbiota colonizing feces (F), and mucosa near diverticula (D) and far away from them (N). Subjects’ clustering in the scoreplots were done by considering the molecules employed for LDA analysis of metabolome (A) and fecal and mucosal microbiota features which appeared as statistically different among the investigated groups (B). Gray arrows begin from sample position in the metabolomics dataset and end at sample position in the microbiota dataset. Black, red and green colors indicate control, diverticulosis and SUDD subjects. The loadingplots highlight the importance of the metabolome (C) and microbiota (D) features in the subjects’ clustering.
REFERENCES


