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Rapid and Noninvasive Metabonomic Characterization of Inflammatory Bowel Disease

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Inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) have a major impact on the health of individuals and populations. Accurate diagnosis of inflammatory bowel disease (IBD) at an early stage, and correct differentiation between Crohn's disease (CD) and ulcerative colitis (UC), is important for optimum treatment and prognosis. We present here the first characterization of fecal extracts obtained from patients with CD and UC by employing a noninvasive metabonomics approach, which combines high resolution ^{1}H NMR spectroscopy and multivariate pattern recognition techniques. The fecal extracts of both CD and UC patients were characterized by reduced levels of butyrate, acetate, methylamine, and trimethylamine in comparison with a control population, suggesting changes in the gut microbial community. Also, elevated quantities of amino acids were present in the feces from both disease groups, implying malabsorption caused by the inflammatory disease or an element of protein losing enteropathy. Metabolic differences in fecal profiles were more marked in the CD group in comparison with the control group, indicating that the inflammation caused by CD is more extensive in comparison with UC and involves the whole intestine. Furthermore, glycerol resonances were a dominant feature of fecal spectra from patients with CD but were present in much lower intensity in the control and UC groups. This work illustrates the potential of metabonomics to generate novel noninvasive diagnostics for gastrointestinal diseases and may further our understanding of disease mechanisms.

Keywords: Crohn's disease • ulcerative colitis • metabonomics • feces • NMR spectroscopy

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two major types of chronic inflammatory bowel disease (IBD). CD can involve any part of the gastrointestinal tract from the mouth to the anus and affects the entire bowel wall, whereas UC is typically restricted to the colon and affects only the mucosa (i.e., inner lining) of the colon.[1] The incidence of these diseases ranges from 37 to 246 cases per 100 000 people for UC and from 26 to 199 cases per 100 000 people for CD depending on population, with the incidence being at the higher end of the scale in developed countries.[2] Several recent reports have documented an increasing prevalence of both these diseases in developing countries.[3] The cause of IBD is unknown, but both genetic and environmental factors are thought to contribute to the pathogenesis of the disease. Studies have implied that several genomic regions contain genes indicative of the likelihood of IBD susceptibility, with some that are unique to CD or UC, and others common to both. For example, NOD2/CARD15 gene mutations are associated with the localization of CD in the small intestine.[4−6] In addition to genetic factors, the host intestinal microbiota is another key factor related to the inflammatory process in IBD.[7−11] Patients with IBD have an altered gut microbiota with levels of *Clostridia*, especially those involved in short chain fatty acid production, being reduced in both CD and UC.[12,13] No conclusive evidence has been provided to suggest that there are significant differences between bacteria associated with ulcerated and non-ulcerated mucosa in CD, and the bacterial changes seem to be localized to the luminal communities.[14,15] Although the onset of the disease requires the presence of

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bacteria, no specific species has been shown to cause IBD.[16] This has led to the conclusion that a dysbiosis of the microbiota is involved either in initiating the disease or maintaining it; however, further work is required to elucidate the exact role that microbes play in IBD.

Accurate diagnosis of IBD, and correct differentiation between CD and UC, is of great importance for optimum treatment and prognosis. Currently, standard diagnosis of IBD is based on clinical radiological, endoscopic, and histopathological evidence[17] all of which are invasive techniques. In addition, a diagnosis can only be made when a clinical phenotype is manifested. Therefore, accurate diagnosis at an early stage is required for effective disease diagnosis and surveillance. Metabonomics represents an unexploited approach to the identification of disease biomarkers in the fecal extracts of patients with IBD and could potentially be used for disease surveillance.

Metabonomics is defined as "the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification".[18] The combination of NMR spectroscopy with chemometric data analysis techniques is one of the most efficient and robust methods of generating metabolite profiles for biological samples.[18] NMR spectra provide a "metabolic fingerprint" of biofluids and tissues and contain a wealth of information concerning the end products of biological processes.[19] Multivariate statistical analysis can be applied to reduce the dimensionality of the spectral data, establish patterns relating to physiological or pathological perturbation and to aid biological interpretation. Metabonomics has found successful applications in many fields (e.g., the study of disease processes,[20−22] drug toxicity,[23−26] and the detection of metabolites of inborn errors of metabolism).[27] Recently, the application of metabonomics has been extended to characterize the development of the intestine[28] and more subtle metabolic signatures of dietary interventions such as soy and chamomile ingestion or alcohol consumption.[29−31]

In the current study, we report an investigation of fecal extracts from patients suffering from UC and CD, employing ^{1}H NMR spectroscopy in conjunction with multivariate data analysis techniques. The aim of the investigation was to characterize fecal extracts of IBD patients and to exploit the potential of metabonomics as a diagnostic tool for differentiating patients affected with UC and CD, hence improving disease surveillance.

Materials and Methods

Sample Collection and Preparation. The study was approved by the Cork University Hospital medical ethics committee (Cork, Ireland), and consent was obtained from all the volunteers. Stool samples were obtained from individuals with CD (n = 10, 4 male, 6 female, age = 30 ± 14), UC (n = 10, 3 male, 7 female, age = 49 ± 12), and a control group of healthy individuals (n = 13, 6 male, 7 female, age = 33 ± 9). Both CD and UC patients were being treated with the drug prednisolone (40 mg/day) and 5-aminosalicylic (0.8−1.5 g/day) as part of disease management. All stool samples were taken immediately after being voided and stored at −80 °C until required for further extraction. Fecal water was extracted by taking a weighed sample of thawed stool material and adding 2 volumes (w/v) of sterile phosphate buffered saline (PBS; 1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). The mixture was homogenized by vortex mixing for 60 s. The fecal slurry was centrifuged at 3000g_{av} for 15 min, and the supernatant was filtered through a cellulose filter (Whatman 113V, approximate pore size 30 μm). The filtrate was centrifuged at 16 000g_{av} for 15 min, and the supernatant filtered through a Whatman 25 mm GD/X PES sterile syringe filter of 0.2 μm pore size. The filtered fecal water was stored at −80 °C prior to analysis. Control samples of the same batch of PBS, used to create the fecal water, were also filtered through the same batch of filters used previously, either separately (i.e., only through one filter and not all filters) or through both sets of filters. These controls were subjected to exactly the same metabonomic analysis to determine whether any contamination may have been introduced from the PBS or filters.

^{1}H NMR Spectroscopy. A volume of 400 μL of fecal extract was added to 200 μL of water containing 10% D_2O (which acted as a field frequency lock for the spectrometer) and 0.01% sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4, which was used as a chemical shift reference. The resulting samples were centrifuged at 14 000g_{av} for 10 min to remove particulates. A 580 μL aliquot of the supernatant was then pipetted into a 5 mm NMR tube for spectroscopic analysis. ^{1}H NMR spectra were acquired for each sample using a Bruker DRX 600 NMR spectrometer operating at 600.13 MHz for ^{1}H equipped with a 5 mm triple resonance probe with an inverse detection (Bruker, Germany). A standard one-dimensional NMR spectrum was acquired using the first increment of pulse sequence [RD−90°−t_1−90°−t_m−90°−ACQ]. Water suppression was achieved by irradiation of the water peak during the recycle delay (RD = 2 s) and mixing time, t_m (100 ms). t_1 was set to 3 μs. The 90° pulse length was adjusted to approximately 10 μs. The spectral width was set to 20 ppm, and a total of 64 transients was collected into 32k data points for each spectrum. For assignment purposes, standard two-dimensional (2-D) correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) NMR spectra were also acquired for a selected sample following the method described by Hurd and Bax and Davis.[32,33]

Data Analysis. All free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor prior to Fourier transformation. The spectra were corrected for phase and baseline distortions and referenced according to a MATLAB script developed at Imperial College London (Dr. T. Ebbels, Imperial College London). The spectra over the range of δ 0.5−10.0 were digitized using a MATLAB script developed in-house (Dr. O. Cloarec, Imperial College London). The region of δ 4.68−5.10 was removed from the analysis to avoid the effect of imperfect water suppression. Normalization to the sum of the spectrum was carried out on the data prior to pattern recognition analyses. Initial principal component analysis (PCA) was performed blinded on the dataset. Resonances from the drug 5-aminosalicylic acid and its metabolite *N*-acetyl-5-aminosalicylic acid[34] discriminated between the disease classes and the control group. The entire aromatic region and the region between δ 2.12−2.26 were removed to eliminate signals resulting from drug treatment. The drug and all of its major metabolites were characterized by Novak et al.[34] Renormalization was carried out subsequently. PCA (SIMCA-P + 11, Umetrics; Umeå, Sweden) was employed for the data analysis using mean centered data. Data were visualized by means of principal component (PC) scores plots where each point represents an individual sample. Projection to latent structure with an inbuilt orthogonal signal correction filter developed by Trygg (O-PLS-DA)[35] was also used to analyze the NMR spectra using a unit variance scaling method in a

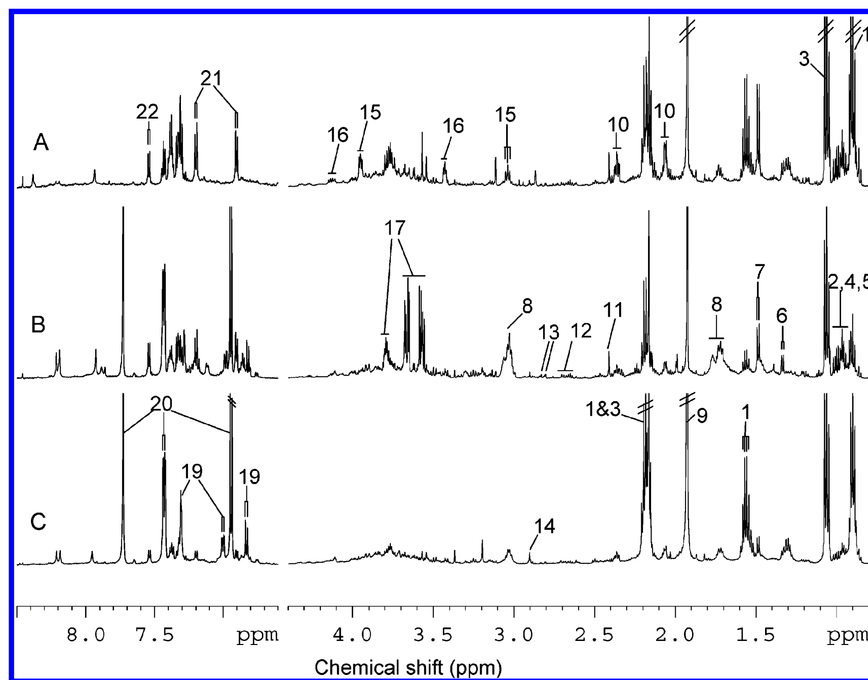

Figure 1. Typical 600 MHz ^{1}H NMR spectra of fecal extracts obtained from healthy volunteers (A), patients with Crohn's disease (B), and ulcerative colitis (C). The spectra in the aromatic region (δ 6.5–8.5) were magnified 4 times as compared to the aliphatic region. Keys: 1, butyrate; 2, leucine; 3, propionate; 4, valine; 5, isoleucine; 6, threonine; 7, alanine; 8, lysine; 9, acetate; 10, glutamate; 11, succinate; 12, aspartic acid; 13, asparagine; 14, trimethylamine; 15, cysteine; 16, proline; 17, glycerol; 18, methylamine; 19, 5-aminosalicylic acid; 20, *N*-acetyl-5-aminosalicylic acid; 21, tyrosine; and 22, uracil.

MATLAB 7.0 environment with a MATLAB script developed in-house (Dr. O. Cloarec, Imperial College London).[36] This procedure involved the combination of orthogonal signal correction and PLS discriminant analysis and utilized back transformation of the coefficient incorporating the weight of the variable contributing the discrimination in the models for displaying the loadings. The statistical total correlation spectroscopy (STOCSY)[37] method developed in-house (Dr. O. Cloarec, Imperial College London) was also applied to aid metabolite assignment.

Results

^{1}H NMR Spectroscopy of Fecal Extracts. Typical examples of the ^{1}H NMR spectra of fecal extracts obtained from a healthy control (A) and from patients with CD (B) or UC (C) are shown in Figure 1. Resonances were assigned with the aid of the 2-D ^{1}H NMR methods COSY and TOCSY. Signals for 5-aminosalicylic acid and the corresponding metabolite, *N*-acetyl-5-aminosalicylic acid, identified from the literature were present in all UC and CD samples as a result of drug treatment.[38,39] The endogenous compounds detected in the spectra were comprised of a number of amino acids including leucine, isoleucine, valine, alanine, lysine, asparagine, aspartic acid, tyrosine, and glutamate, and a range of short chain fatty acids, such as acetate, butyrate, and propionate. In addition, dimethylamine, trimethylamine, glycerol, uracil, and succinate could all be directly assigned. It was clear that a markedly reduced amount of butyrate, but an elevated level of glycerol, was present in the feces of patients with CD. To eliminate the possibility that the observed glycerol had been introduced into the sample during the sample workup procedure as a contaminant, blank extractions were performed using the same batch of filter paper and filters used for extraction of the feces samples. Subsequent NMR analysis of these blank extracts showed that no glycerol resonance was present. To establish a global overview of characteristics of the inflammatory disease, multivariate data analysis was applied to the NMR data.

Data Analysis. PCA was first carried out on the mean-centered normalized ^{1}H NMR spectra acquired from the fecal extracts to generate an overview of the variations between the control volunteers and those with CD or UC. Two principal components were calculated for the models of comparing the control class with CD and UC with a total of 69 and 77% of variance being expressed, respectively. While complete discrimination was not achieved, a clear indication of some degree of separation between the control group and those with Crohn's disease (Figure 2A) and between the control volunteers and those with ulcerative colitis (Figure 2B) was observed. To determine the nature of those metabolites contributing to these differences associated with IBD, further analysis was performed.

O-PLS-DA comparison between spectra obtained from the healthy controls and patients with IBD was carried out with a unit variance scaling strategy. In addition, a comparison between the NMR spectra obtained from patients with CD and with UC was also performed. The O-PLS-DA algorithm is derived from the projection to latent structure regression method.[35] The O-PLS-DA model was constructed using the NMR data as the X matrix and the class information identifier for the disease category as the Y variables.[36] One orthogonal component was calculated for the model to remove the variation in the NMR spectra unrelated to the variation between the different disease groups. The validation of the model was conducted using a 7-fold cross validation method (i.e., constructing models repeatedly by leaving out one-seventh of the samples and predicting them back into the model).[36] The quality of the model was described by the cross-validation parameter Q^2, indicating the predictability of the model, and R^2, which represents the total explained variation for the X

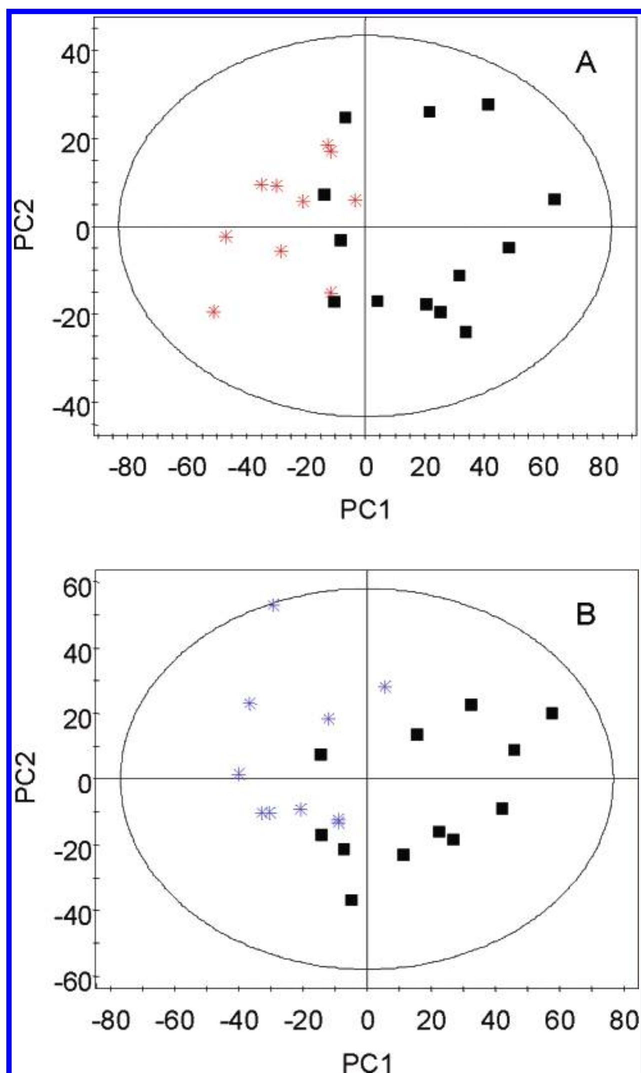

Figure 2. PCA score plots generated from ^{1}H NMR spectra of fecal extracts showing separation between samples obtained from healthy controls (black boxes) and those with Crohn's disease (red stars) (A) and between control volunteers (black boxes) and those with ulcerative colitis (blue stars) (B).

matrix. The values derived from the models indicating the quality of models are tabulated in Table 1. A clear separation was achieved between the ^{1}H NMR spectra of the healthy controls and from patients with IBD (Figure 3A,B). In addition, good differentiation between patients with CD and patients with UC (Figure 3C) was also obtained as illustrated in the cross validated score plot and corresponding O-PLS-DA coefficient plot. The O-PLS-DA coefficient plot reflects class differences in the NMR spectra. Here, the direction of the signals relates to the relative concentration of the metabolites in the class of interest with respect to the other classes in the model as calculated from the covariance matrix. The color coding of signals in the spectrum is associated with the significance of metabolites in characterizing NMR data for a given class, as calculated from the unit variance scaled data. A key defining the correlation coefficients is shown as a color scaling map on the right-hand side of each coefficient plot. For example, red indicates a more significant contribution to the separation between classes than blue. The values indicating the significance of the metabolites contributing to the separation for each model are summarized in Table 1. Here, the coefficient of 0.42

Table 1. Coefficients of Metabolites Contributing to Separation between Feces Obtained from Patients with Crohn's Disease, Ulcerative Colitis, and Healthy Volunteers[a]

metabolite	CD vs Control	UC vs Control	CD vs UC
	$R^2 = 0.92$	$R^2 = 0.77$	$R^2 = 0.93$
	$Q^2 = 0.80$	$Q^2 = 0.52$	$Q^2 = 0.70$
acetate	−0.67		−0.50
alanine	+0.64		+0.72
butyrate	−0.64	−0.36[b]	
glutamate		+0.68	
glycerol			+0.82
isoleucine	+0.69		+0.66
leucine	+0.74		+0.74
lysine	+0.72	+0.62	+0.67
methylamine	−0.73	−0.39	
trimethylamine	−0.52	−0.35	
unknown (1.33)	−0.82	−0.69	
valine	+0.66		+0.71

[a] The + and − indicate the direction of the change (i.e., + increase and − decrease) in the concentration in feces obtained from patients with IBD as compared to those obtained from healthy volunteers or in feces obtained from patients with CD as compared to those from patients with UC. [b] Not significant at the level of 0.05.

was used as the cut-off value that was calculated based on discrimination significance at the level of 0.05 ($p \leq 0.05$).

The coefficient suggests that feces obtained from patients with CD contained relatively lower amounts of butyrate, acetate, methylamine, and trimethylamine than that obtained from the healthy controls (coefficient > 0.5). These metabolites were also reduced in concentration in feces obtained from UC patients, although to a lesser extent (coefficients 0.35 to ~0.39) as summarized in Table 1. Significantly higher quantities of amino acids were found in feces obtained from patients with CD and with UC in relation to the control group. Furthermore, the relative amounts of amino acids, glycerol (increased), butyrate, and acetate (decreased) in the feces of CD patients contributed to the differentiation of patients with CD and UC.

Discussion

In the present study, metabonomics was utilized to classify the fecal extracts from patients suffering from IBD to assess the potential of the technique as a diagnostic tool for such diseases and to identify characteristic metabolites in the feces of participants with specific types of IBD.

The depletion of short chain fatty acids (SCFA), including acetate and butyrate, was a prominent feature of CD patients when compared with healthy subjects. These SCFA are normally produced by the gut bacteria via the fermentation of complex carbohydrates such as cellulose, fiber, and starches. The SCFA, especially butyrate, provide energy to the intestinal cell wall and promote epithelial cell growth.[40] In addition, amounts of methylamine and trimethylamine were decreased in the aqueous fecal water extracts of patients with CD. These two compounds are derived from intestinal degradation of food components such as choline and carnitine by microbiotia.[41,42] The depletion of these microbiota-related metabolites in feces suggests that there has been a disruption, or dysbiosis, of the normal bacterial ecology in patients with IBD. Several culture independent analyses of the diversity of microbiota associated with IBD have shown that members of the *Clostridium coccoides* and *C. leptum* groups are significantly reduced when compared to healthy subjects.[12,43] These bacterial groups

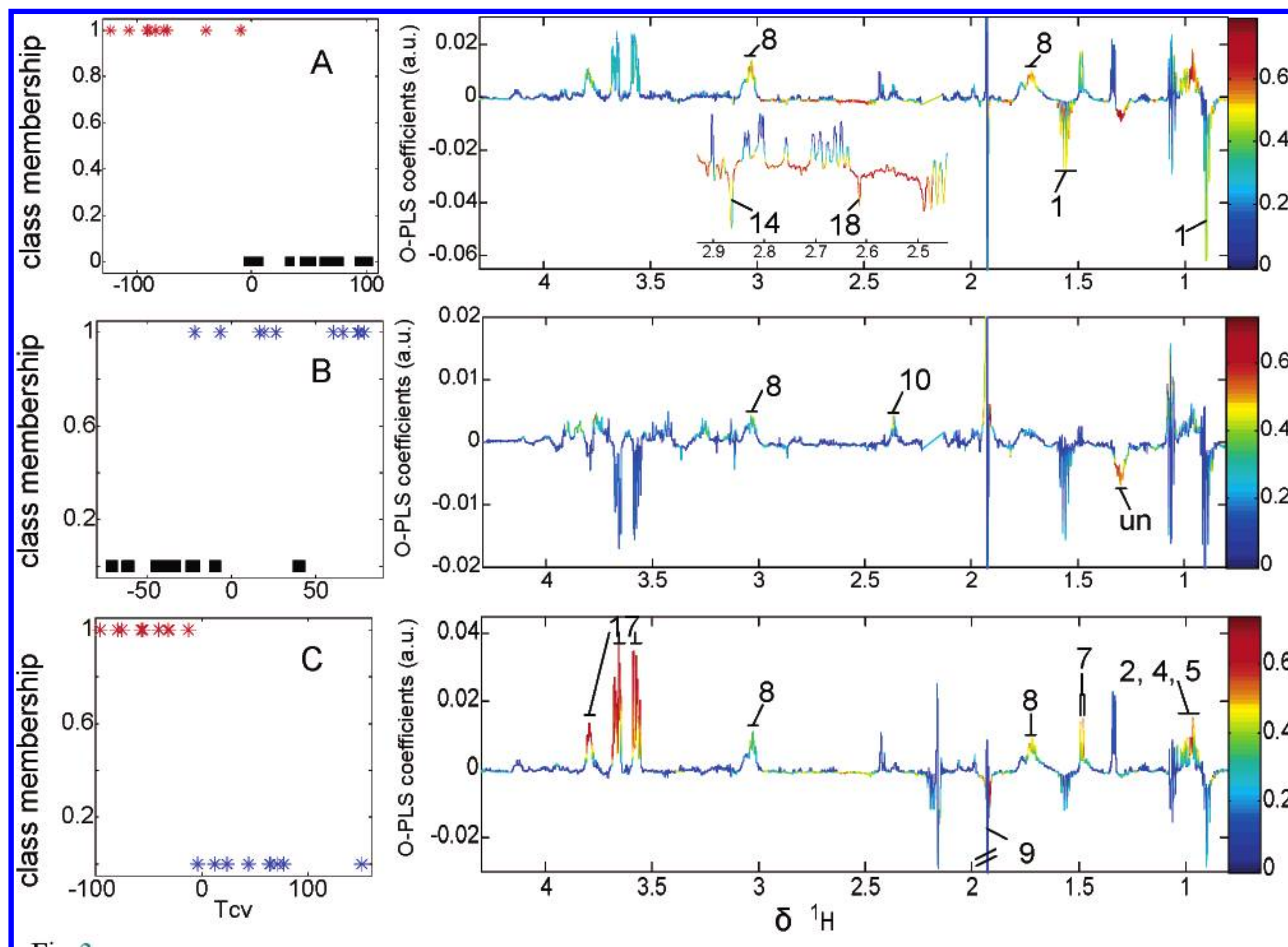

Figure 3. Cross-validated score plots (left) and corresponding back-transformed coefficient plots calculated from O-PLS-DA modeling of the ^{1}H NMR spectra of fecal extracts. (A) Crohn's disease (red stars) as compared with the control (black boxes); (B) ulcerative colitis as compared (blue stars) with control (black boxes); and (C) Crohn's disease (red stars) as compared with ulcerative colitis (blue stars). For key, see Figure 1 caption.

constitute functionally important clades in the gut as they are principally responsible for production of SCFA, which is consistent with the results presented here. This dysbiosis in the gut bacterial ecology, either quantitatively or qualitatively, could be due to the destruction of microbiota by the T lymphocytes that are hyperactive against bacterial antigens and promote inflammatory destruction of the intestinal brush border.[44] As a result, inflammatory epithelium barrier protection is compromised, which could lead to malabsorption of nutrients. Higher amounts of amino acids were found in the feces of patients with CD and UC as compared to the healthy controls that could be the consequence of a malabsorption caused by the inflammation.

As compared to the healthy subjects, the fecal samples obtained from CD and UC patients manifested similar global differences in metabolic profiles. However, the changes in metabolic profiles observed for UC patients were not as marked as those observed with the CD group. This suggests that the metabolic consequences caused by inflammation are more severe in CD patients than in UC patients. In particular, a high concentration of glycerol was found in the feces of CD patients in comparison with UC patients. The origin and role of glycerol in the feces of these CD patients is unclear.

Currently, the standard diagnosis of IBD is based on clinical phenotypes observed using radiological, endoscopic, and histopathological techniques,[17] which are expensive, labor intensive, and invasive. We have demonstrated that differentiation between IBD patients and healthy volunteers was made possible based on the ^{1}H NMR profiles of fecal extracts. More importantly, patients with CD and UC could also be discriminated using this approach and metabonomics holds promise as a novel diagnostic technique for further disease recognition and surveillance. Ongoing studies are in place to validate the initial findings.

Abbreviations: CD, Crohn's disease; COSY, ^{1}H−^{1}H correlation spectroscopy; IBD, inflammatory bowel disease; NMR, nuclear magnetic resonance; O-PLS-DA, orthogonal-projection on latent structure-discriminate analysis; PCA, principal component analysis; SCFA, short chain fatty acid; STOCSY, statistical total correlation spectroscopy; TOCSY, ^{1}H−^{1}H total correlation spectroscopy; UC, ulcerative colitis.

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