Urinary NMR metabolomic profiles discriminate inflammatory bowel disease from healthy

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Metabonomics;
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Abstract

Background and aims: Inflammatory bowel disease, a chronic inflammation of the intestinal tract, presents in two variations, Ulcerative Colitis (UC) and Crohn's disease (CD). Given that treatment of CD differs from UC, a single test that provided strong diagnostic ability would offer great clinical value. Two previous studies have indicated that CD can be distinguished from UC, and that both can be distinguished from non-IBD-type gastrointestinal disease, based on urinary and faecal metabolite profiling.

Methods: Analysis of healthy as well as CD and UC patients attending an IBD clinic was performed. IBD patients were classified into two groups (CD or UC) based on chart review of clinical, endoscopic, and histological assessment. Urine samples were obtained and analyzed using nuclear magnetic resonance (NMR) spectroscopy combined with targeted profiling techniques, followed by univariate and multivariate statistical analysis.

Results: Based on urinary metabolomics, individuals with IBD could be differentiated from healthy. Major differences between IBD and healthy included TCA cycle intermediates, amino acids, and gut microflora metabolites. Comparison of CD and UC patients revealed discrimination, but removal of patients with the surgical intervention confounder revealed that CD could not be discriminated from UC.

Conclusions: This study highlights the potential for metabolomics to distinguish IBD from the healthy state but shows that careful consideration must be given to establishing disease-representative cohorts that are free of confounding factors.

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1. Introduction

Inflammatory bowel disease (IBD) involves chronic inflammation of the intestinal tract. The form of the IBD, either Ulcerative Colitis (UC) or Crohn’s disease (CD), is diagnosed based on the pattern of inflammation. The pathogenesis of IBD involves an inappropriate and ongoing activation of the mucosal immune system driven by the presence of intestinal microflora in a genetically predisposed patient.1 On a global scale, the incidence of IBD has been increasing, with environmental factors, diet, smoking habits, hormone drug use, and genetics now being viewed as potential multifactorial causes leading to the disease.

UC typically presents as confluent mucosal inflammation of the large bowel, virtually always involving the rectum. CD presents with a pattern of patchy transmural inflammation anywhere along the digestive tract; the terminal ileum is involved in the majority of cases. UC and CD are diagnosed from a constellation of clinical symptoms, endoscopic and radiologic findings, and histological assessment results. Unfortunately, the heterogeneity of disease presentations, the overlapping of symptoms, and the lack of truly pathognomonic findings for either condition mean that, in nine to twenty percent of cases, CD is indistinguishable from UC, and thus the diagnosis of indeterminate colitis is given.2–4 Furthermore, milder forms of IBD may not have obvious clinical findings; thus, they are virtually indistinguishable from the more commonly diagnosed condition of irritable bowel syndrome. To date, no single, non-invasive diagnostic method has been specific or sensitive enough to distinguish CD from UC. Given that treatment of CD differs from that for UC, a single test that offers strong diagnostic ability, and that can be used to provide disease surveillance and prognostic information, is a highly sought-after tool.

Metabolomics is a relatively new area of study being investigated as offering possible diagnostic and evaluative tools for use in diabetes and lipid research, multiple sclerosis, oncology research, and study of metabolite responses to cardiovascular ischemia, nutrition and diet modification, as well as pharmacologic treatment and drug use.5–11 A recent metabolomics study of fecal samples from 20 patients with IBD (10 CD and 10 UC) and 13 healthy subjects found decreased levels of butyrate, acetate, methylvamine, and trimethylamine in fecal water from both CD and UC patients, as well as increased levels of amino acids, when compared with results from a healthy control standard (Chenomx Inc., Edmonton, AB) (consisting of ~5 mM sodium azide in 99% D2O).12 A recent study indicated that there was a difference in the metabolites of the UC and CD groups. Similarly, in a study by Williams et al.,13 analysis of urine samples provided by CD and UC patients revealed metabolomic differences between IBD and healthy samples, as well as between CD and UC samples, regardless of medication usage.

In the present study, we used NMR spectroscopy to build a metabolic profile that allowed us to use a single spot urine sample to distinguish patients with IBD from healthy individuals. As with Williams’ study, we observed lower concentrations of hippurate in the urine of individuals who had CD or UC (versus healthy subjects). However, we observed no differences between the metabolomic profiles of subjects with CD and those with UC.

2. Materials and methods

2.1. Participants

Sixty prospectively-identified patients with CD or UC who were attending the inflammatory bowel disease referral clinic at the University of Alberta Hospital (Edmonton, Alberta, Canada) consented to participate in this study, which was conducted between April 2007 and May 2008. Diagnosis was confirmed by chart review of clinical, endoscopic, and histological assessments. Exclusion criteria applied at enrolment were age lower than eighteen years, the presence of urinary dysfunction or infection, and patient choice not to participate in the study.

All patients completed a questionnaire documenting age, IBD type and diagnosis date, gender, number of flare-ups requiring a visit to the physician in the prior year, co-morbid medical conditions, current medication history, and intestinal surgical history. For subjects with CD, disease activity was determined using the Harvey–Bradshaw Index questionnaire.14,15 For those with UC, disease activity was determined using the Mayo Disease Activity Score.16

Sixty self-identified healthy subjects, as described in Slupsky et al.,17 were used as the comparator group. No subjects in this healthy cohort had IBD.

2.2. Ethical considerations

The Ethics Committee for Medical Research at the University of Alberta approved this protocol. Written informed consent was obtained from study participants. The trial was performed in accordance with the International Conference on Harmonisation Good Clinical Practice guidelines, which originated in the Declaration of Helsinki.

2.3. Urine sampling

A single spot urine sample was obtained from each participant after determination of disease activity. Urine was collected and stored in containers which had enough sodium azide added to ensure a final concentration of 0.02% in 100 mL of urine in order to prevent bacterial growth. Once collected, samples were refrigerated at 4 °C; within two hours of collection, they were stored at −80 °C until the time of nuclear magnetic resonance (NMR) spectroscopy data acquisition.

Urine samples were prepared for analysis by centrifuging to remove particulate matter, and adding 65 μL of internal standard (Chenomx Inc., Edmonton, AB) consisting of ~5 mM DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate, and 0.2% sodium azide in 99% D2O) to 585 μL of supernatant, as described in Slupsky et al.17 The pH was adjusted to 6.8±0.1 by the addition of small amounts of NaOH or HCl. A 600 μL aliquot of prepared sample was placed in a 5 mm NMR tube (Wilmad, Buena, NJ) and stored at 4 °C until the time of NMR data acquisition.

2.4. NMR spectroscopy

All one-dimensional NMR spectra of urine samples were acquired using the first increment of the standard NOESY
pulse sequence on a 4-channel Varian (Varian Inc., Palo Alto, CA) INOVA 600 MHz NMR spectrometer with triaxial-gradient 5 mm HCN probe as previously described. All spectra were recorded at 25 °C with a 12 ppm sweep width, 1 s recycle delay, 100 ms $\tau_{\text{mix}}$, an acquisition time of 4 s, 4 dummy scans and 32 transients. $^1$H decoupling of the water resonance was applied for 0.9 s of the recycle delay and during the 100 ms $\tau_{\text{mix}}$. All spectra were zero-filled to 128 k data points and multiplied by an exponential weighting function corresponding to a line-broadening of 0.5 Hz. Analysis of these data was accomplished by targeted profiling using NMR Suite 6.0 (Chenomx Inc., Edmonton, Canada), which compares the integral of a known reference signal (in this case DSS) with signals derived from a database of compounds to determine metabolite concentration. A total of 68 metabolites were identified, quantified, and used for statistical analysis and included sugars (fucose, glucose, levoglucosan, mannitol, myo-inositol, sucrose, and xylose), amino acids (alanine, asparagine, glutamine, glycine, histidine, isoleucine, leucine, lysine, pyroglutamate, serine, threonine, tryptophan, tyrosine, valine, 1-methylhistidine, and 3-methylhistidine), organic acids (2-aminobutyrate, 2-hydroxyisobutyrate, 2-oxoglutarate, 3-aminosobutyrate, 3-hydroxybutyrate, 3-hydroxyisovalerate, 4-hydroxyphenylacetate, citrate, formate, fumarate, glycolate, guanidoacetate, hippurate, lactate, pyruvate, quinolinic acid, succinate, tartarate, cis-aconitate, and trans-aconitate), fatty acids (acetate, and adipate), and others (1-methylnicotinamide, 3-indoxylsulfate, acetone, allantoin, betaine, carnitine, creatine, creatinine, dimethylamine, ethanolamine, hypoxanthine, methanol, methylamine, methyguanidine, N, N-dimethylglycine, O-acetylcarnitine, pantothenate, propylene glycol, trigonelline, trimethylamine-N-oxide (TMAO), uracil, and urea). Identification and quantitation accuracy of a number of these compounds were previously verified through spiking experiments. Comparison of precision in concentration among several analysts revealed low coefficients of variation (<10%) for compounds above 100 µM, or when there was little spectral overlap for compounds lower than 100 µM. To ensure reliability and avoid systematic bias in concentration reporting, several of the normal and IBD spectra were reanalyzed and determined to have low variation in reported concentration.

2.5. Statistical analysis

NMR variables derived from targeted profiling were subjected to multivariate data analysis. Partial least-squares discriminant analyses (PLS-DA) and orthogonal partial least-squares-discriminant analyses (OPLS-DA) were performed on log$_{10}$-transformed metabolite concentrations using SIMCA-P (version 11, Umetrics, Umeå, Sweden) that had been mean-centered, and unit-variance scaling was applied as described in Slupsky et al. Scatter-dot plots and significance tests using Wilcoxon rank-sum tests were performed using GraphPad Prism (GraphPad Software, San Diego, USA). Significance was set at $\alpha=0.05$.

3. Results

Table 1 outlines baseline patient characteristics and demographics at the time of study enrolment. Overall, 30 patients with CD, 18 of whom had undergone previous surgical intervention with primary anastomosis, and 30 patients with UC, 3 with previous colectomies and ileo-anal pouch
procedure, were enrolled. The numbers of males and females were approximately equal in each group. The total numbers of patients with active disease at the time the urine samples were collected were similar for the UC and CD patient groups (Table 1). Each CD patient’s disease activity was determined using the Harvey Bradshaw Index (HBI), while each UC patient’s disease activity was determined using the Mayo Disease Activity Score (MDAS). Based on their individual HBI scores, 13 CD patients were regarded as being in clinical remission, 5 had mildly active disease, 11 had moderately active disease, and 1 had severely active disease. In the UC group, 17 patients were regarded as being in clinical remission, 7 as mildly active, 6 as moderately active, and none as severely active, based on their individual MDAS (Table 1). All patients were taking medication to manage their CD or UC at the time of urine collection (Table 1). The mean age of the CD patients was 37±11 years, of the UC group was 39±16 years, and of the healthy cohort was 41±15 years.

### 3.1. Metabolomic profiles

#### 3.1.1. Separating IBD patients from healthy participants.

Comparison of urinary metabolite profiles of healthy individuals with those of participants having either CD or UC using Principal Component Analysis (PCA) reveals that metabolomics can be used to distinguish between healthy individuals and those with IBD ($R^2 = 0.668; Q^2 = 0.482$) (data not shown). To examine which metabolites were contributing to the separation, orthogonal partial least squares-discriminate analysis (OPLS-DA) was done (Fig. 1A). Permutation testing, performed on the PLS-DA model, revealed significantly positive slopes with an $R^2$ of 0.811 and a $Q^2$ of 0.698 in the final model (Fig. 1B), indicating statistical significance for the separation of the two groups.

Important metabolites for distinguishing IBD patients from healthy (non-IBD) individuals included the TCA cycle intermediates (succinate, and citrate); amino acids (asparagine, lysine, histidine, and 1-methylhistidine); metabolites derived from gut microflora (methanol, formate, hippurate, acetate, and methylamine); as well as the other metabolites trigonelline, creatine, urea, and taurine. Trimethylamine-N-oxide (TMAO) levels were not significantly different between the cohorts. Table 2 summarizes these metabolite differences between IBD and non-IBD patient samples. Observed differences were similar to those previously published by Williams et al.

#### 3.1.2. Separating Crohn’s disease from Ulcerative Colitis.

Once it was established that the metabolomic profile could be used to separate participants with IBD from those without IBD, a comparison of the metabolomic profiles of CD and UC patients was undertaken. When the IBD patients with intestinal resection were included in the cohorts, it was observed that CD could be differentiated from UC using the metabolomic profile from a single spot urine sample (Fig. 2).

However, when the urine sample data provided by individuals with intestinal resection were removed from the analysis (17 CD patients and 3 UC patients), no differences were seen between the CD profiles and the UC metabolomic profiles (data not shown). Interestingly, comparison of those patients with resections and those with no resections did not produce a robust model. Similarly, the urine profiles of the CD patients on anti-TNF-α antibody therapy were significantly different from those of CD patients on other forms of therapy. Stratification based on drug treatments other than anti-TNF-α antibody therapy, disease duration, disease activity, and disease location did not produce models that might be used to differentiate CD from UC using either a multicomponent y-table and PLS2 or excluding observations and performing PLS. Analysis of formate and hippurate results using the Wilcoxon rank sum test revealed no significant difference between the CD and UC groups (Fig. 3).

### 4. Discussion

This study focussed on the potential applicability of metabolomics to the analysis and diagnosis of inflammatory
bowl disease. Our results differentiating IBD from healthy participants correlate well with those of a previously published study. Indeed, they verify the finding that certain urine metabolites, those that are products of metabolism or the co-metabolism of the host and the host's unique microflora, are different in IBD and normal states. These metabolites, then, can provide information about host–microbial interactions and lead to a better understanding of IBD. However, our study did not find any significant differences between the CD and UC participant group results, once the data for those who had undergone resection had been removed from the overall results.

In a recent publication, Williams et al. report that hippurate concentrations are lower for CD patients but formate levels are higher, as compared to UC results. In our study, however, a comparison of hippurate and formate results for CD and UC patients revealed no significant difference when the data from participants who had had bowel resections were removed from consideration. In addition, stratification based on drug treatments, disease duration, or disease location did not clearly differentiate CD from UC patients, except when we retained the data from those CD patients who had had surgical intervention or were on anti-TNF-α treatment. In the Williams study, the authors did not observe any differences between results from CD patients who had had bowel resections and those that had not. Marchesi et al. did not report whether any of the CD patients had undergone surgery. The differences between the results of these studies and ours, where the distinction between CD and UC could only be seen if we included data from patients with bowel resections or that from patients on anti-TNF-α therapy, strongly suggest that distinguishing CD from UC via urine metabolite profile analysis may be difficult.

Table 2  Metabolites distinguishing IBD patients from healthy controls.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% difference</th>
<th>Healthy v. IBD p-value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA cycle</td>
<td></td>
<td></td>
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<tr>
<td>Succinate</td>
<td>−77</td>
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</tr>
<tr>
<td>Trans-aconitate</td>
<td>−43</td>
<td>&lt;0.0001</td>
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<tr>
<td>Citrate</td>
<td>−35</td>
<td>0.003</td>
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<tr>
<td>Amino acids</td>
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<td></td>
</tr>
<tr>
<td>1-Methylhistidine</td>
<td>−55</td>
<td>0.0301</td>
</tr>
<tr>
<td>Histidine</td>
<td>−48</td>
<td>0.0004</td>
</tr>
<tr>
<td>Lysine</td>
<td>−41</td>
<td>0.0029</td>
</tr>
<tr>
<td>Asparagine</td>
<td>−31</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gut microflora</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippurate</td>
<td>−71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Formate</td>
<td>−60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methanol</td>
<td>−52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Acetate</td>
<td>−41</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methylamine</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Methylamine</td>
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<td>0.003</td>
</tr>
<tr>
<td>Others</td>
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<td></td>
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</tr>
<tr>
<td>Urea</td>
<td>−34</td>
<td>0.0002</td>
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a p-values were not corrected for multiple testing.

Figure 2  CD may be distinguished from UC including patients with bowel resections. (A) PLS-DA model comparing CD patients (n=30 (17 with bowel resections), black squares) and UC patients (n=30 (3 with bowel resections), open circles). In total 2 components were computed using a 7-fold cross-validation rule, with these components accounting for 76% of the total variance. (B) Model validation using 100 permutations. The original model (R²=0.598, Q²=0.498) is different from randomly permuted models.

With respect to the comparison of IBD and healthy participants, decreased concentrations of energy metabolites such as TCA intermediates (succinate, and citrate) reflect energy metabolic changes. Interestingly, similar changes were seen in the serum samples of a DSS-induced colitis mouse model study by Shiomi et al. Decreased serum levels of TCA cycle intermediates (in addition to decreases in glutamine, tryptophan, tyrosine, asparagine, and glycine) were observed. Decreased levels of some amino acids (asparagine, lysine, and histidine) were also observed in the urine of individuals who had IBD in this study, and these changes could potentially be related to the intestinal malabsorption caused by the disease, in a manner which correlates well with findings of increased amino acids in the feces (as shown by Marchesi et al.).

Changes in gut flora are considered to be an element of IBD’s pathophysiology. Enteric bacterial species such as Clostridia and Bacteroides preferentially produce acetate, butyrate, and other Short Chain Fatty Acids (SCFAs)
that are the preferred energy substrates of colonic epithelial cells and are thought to enhance epithelial barrier integrity and modulate the gastrointestinal (GI) immune response.24,25 However, the mechanism whereby SCFAs mediate their anti-inflammatory effects on gut epithelial cells remains poorly understood.26 In cultured epithelial cells and a mouse colitis model, acetate was shown to suppress inflammatory cytokine secretion, results suggesting that SCFAs contribute to the ability of the mucosa to tolerate the presence of vast numbers of living microorganisms.27–29 Not surprisingly, then, we found that the patients with IBD in our study had decreased levels of acetate as measured in the urine. Unfortunately, other SCFAs, such as butyrate, were below the detection limit, and could not be compared. Other changes in bacterially produced metabolites included lower levels of urinary methylamine, methanol, formate, and hippurate. These changes were also identified in fecal studies done by Marchesi et al.12 Indeed, significantly reduced concentrations of Clostridia have been observed in individuals with either CD or UC.1 Thus, the changes documented in this study are likely due to disruptions of normal gut microflora.

It may be that the difficulty in distinguishing CD from UC using metabolomic techniques is due to the complicated nature of the disease and the fact that it is difficult to obtain specimens from newly-diagnosed, and therefore as-yet-untreated, subjects. Drug and dietary therapies, in addition to surgical resections, appear to be confounding factors that interfere with our understanding of the metabolic changes associated with the diseases. Obtaining urine samples from patients at diagnosis, and thus prior to any drug therapies or surgical interventions, would be ideal in order to test the hypothesis that NMR metabolomic techniques can be used to clearly distinguish between, and potentially diagnose, CD and UC at the outset, thus ensuring the best treatment regimen is prescribed for the patient.

Conflict of interest

None.

Acknowledgments

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References