

Metabonomics in Ulcerative Colitis: Diagnostics, Biomarker Identification, And Insight into the Pathophysiology

Jacob T. Bjerrum,^{*,†} Ole H. Nielsen,[†] Fuhua Hao,[‡] Huiru Tang,[‡] Jeremy K. Nicholson,[§]
 Yulan Wang,^{*,‡} and Jørgen Olsen^{||}

Department of Gastroenterology, Medical Section, Herlev Hospital, University of Copenhagen, Denmark, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Centre for Magnetic Resonance, Wuhan Institute of Physics and Mathematics, The Chinese Academy of Sciences, PR China, Department of Biomolecular Medicine, Imperial College London, United Kingdom, and Department of Cellular and Molecular Medicine, The Panum Institute, University of Copenhagen, Denmark

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Nuclear magnetic resonance (NMR) spectroscopy and appropriate multivariate statistical analyses have been employed on mucosal colonic biopsies, colonocytes, lymphocytes, and urine from patients with ulcerative colitis (UC) and controls in order to explore the diagnostic possibilities, define new potential biomarkers, and generate a better understanding of the pathophysiology. Samples were collected from patients with active UC ($n = 41$), quiescent UC ($n = 33$), and from controls ($n = 25$) and analyzed by NMR spectroscopy. Data analysis was carried out by principal component analysis and orthogonal-projection to latent structure-discriminant analysis using the SIMCA P+11 software package (Umetrics, Umeå, Sweden) and Matlab environment. Significant differences between controls and active UC were discovered in the metabolic profiles of biopsies and colonocytes. In the biopsies from patients with active UC higher levels of antioxidants and of a range of amino acids, but lower levels of lipid, glycerophosphocholine (GPC), *myo*-inositol, and betaine were found, whereas the colonocytes only displayed low levels of GPC, *myo*-inositol and choline. Interestingly, 20% of inactive UC patients had similar profiles to those who were in an active state. This study demonstrates the possibilities of metabonomics as a diagnostic tool in active and quiescent UC and provides new insight into pathophysiological mechanisms.

Keywords: biomarker identification • diagnostics • metabonomics • NMR spectroscopy • ulcerative colitis

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two common chronic, relapsing entities belonging to inflammatory bowel disease (IBD) of which the clinical features are well-characterized. These two similar, yet distinct diseases seem to be the consequence of an inadequate or prolonged immune response to environmental factors in genetically predisposed individuals, but the etiology and exact pathophysiology are still to be fully elucidated.^{1,2}

Most recently, genomics and proteomics have shown their potential^{3–5} as innovative approaches in uncovering the exact

pathophysiology, but they only tell us what might have happened, as there are several downstream regulatory mechanisms. Metabonomics, on the other hand, tells us what actually did happen, as metabonomics is defined as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification”,⁶ and hence characterizes the end-products of biological processes.

Nuclear magnetic resonance (NMR) spectroscopy has proven to be a very efficient and a robust way of creating metabolic profiles.^{7,8} It requires only small sample amounts and is virtually the only technique available that can investigate the metabolic composition of intact tissues using the magic-angle spinning (MAS) technique. By combining this technology with an appropriate multivariate statistical analysis, the metabolic profiles can be used in the understanding of relevant metabolic variations of disease perturbations, and provide molecular insight to the variations of interest.

A few NMR spectroscopy-based metabonomic studies have been performed on humans with IBD,^{9–11} but to the best of our knowledge, no metabonomic studies have taken a systemic approach on UC in an effort to generate enhanced insight into the pathophysiology of this disease.

* To whom correspondence should be addressed. Jacob Tveiten Bjerrum, M.D., Department of Gastroenterology, Medical Section, Herlev Hospital, University of Copenhagen, Herlev Ringvej 75, DK-2730 Herlev, Denmark. Phone, +45 44884161; mobile, +45 26284661; fax, +45 44944056; e-mail, jacobje01@heh.regionh.dk. Prof. Yulan Wang, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Centre for Magnetic Resonance, Wuhan Institute of Physics and Mathematics, The Chinese Academy of Sciences, Wuhan, 430071, PR China. Phone, +86 2787197143; e-mail, yulan.wang@wipm.ac.cn.

[†] Department of Gastroenterology, University of Copenhagen.

[‡] Wuhan Institute of Physics and Mathematics, The Chinese Academy of Sciences.

[§] Imperial College London.

^{||} Department of Cellular and Molecular Medicine, University of Copenhagen.

Table 1. Clinical Details^a

	ulcerative colitis		controls <i>n</i> = 25
	active <i>n</i> = 35	inactive <i>n</i> = 33	
Gender (Male/Female)	17/18	13/20	9/16
Age, years (mean, range)	41 (18–75)	48 (23–75)	47 (19–65)
Age at diagnosis (<25 years/>25 years)	9/26	9/24	-
Years with disease (<10 years/>10 years)	10/25	14/19	-
Familial disposition (D/ND)	3/32	5/28	-
Mayo-score (mean, range)	6 (2–12)	0.3 (0–1)	-
Inflammation (P/PS/LC/PC)	7/15/10/3	-	-
Extra intestinal manifestations (present/never present)	10/25	9/24	-
Steroids (dependency/ nondependency/nonapplicable)	8/15/12	2/16/15	-
Smoking/nonsmoking	4/31	5/28	6/19
Daily Medication:			
Systemic mesalazine (1.6 mg to 3.2 g)	29	29	-
Topical mesalazine (1 g)	16	10	-
Systemic glucocorticoids (75 mg)	5	2	-
Topical glucocorticoids (100 mg)	4	2	-
Azathioprine (100–150 mg)	7	3	-
TNF- α antibody	1	-	-
None	5	2	-

^aD, disposed; ND, non-disposed to ulcerative colitis and/or Crohn's disease; P, proctitis; PS, proctosigmoiditis; LC, left-sided colitis; PC, pancolitis. Extra-intestinal manifestations from skin, eyes, liver and/or joints: present/never present. Medication is stated as daily dosage. Smoking defined as smoking zero cigarettes in the last 6 months. TNF- α : Tumour Necrosis Factor-alpha (Infliximab).

With this in mind, the aim of this study was to employ ¹H NMR spectroscopy-based metabonomics on mucosal colonic biopsies, colonocytes, lymphocytes, and urine from UC patients and controls in order to explore the diagnostic possibilities, define new potential biomarkers, and generate a better understanding of the pathophysiology of UC.

Material and Methods

Patient Population. Ninety-nine patients, subjected to flexible colonoscopy at the Department of Gastroenterology, Medical Section, Herlev Hospital, Denmark were consecutively enrolled into the study from December 2006 until May 2007: 41 UC patients with active disease, 33 UC patients without disease activity, and 25 controls (i.e., the diagnosis of irritable bowel syndrome¹² was subsequently reached). All eligible UC patients had previously had their diagnosis verified by well-established criteria¹³ and were at the time of enrolment graded in accordance with the Mayo-score;¹⁴ a score of 0–1 being regarded as inactive disease, 2–4 as mildly active, 5–8 as moderate, and 9–12 as severe disease activity. The endoscopic assessment of activity was confirmed by histological examination and any discrepancy between the observations led to an exclusion of the patient. Hence, none of the included controls or patients with quiescent UC had any signs of inflammation. Other exclusion criteria were age over 75 years or below 18 years, clinical evidence of active infections, recent (within 14 days) use of antibiotics, pregnancy, and severe mental illness. All included UC patients or controls underwent elective colonoscopy due to either surveillance of their UC or suspected gastrointestinal disorders.

Patient Information. Six patients with active UC were excluded: one as the histopathology showed signs of microscopic colitis and the remaining 5 patients, because of microgranulomas in colonic specimens. Table 1 provides clinical details.

All patients participated in this study in conformation with the principles outlined in the Declaration of Helsinki V and

with the approval of the Scientific Ethics Committee of the Copenhagen Capital Region (H-KA-20060164). Informed written consent was obtained from each patient.

Sample Collection. Six adjacent (no more than 5 mm apart) mucosal colonic biopsies (approximately 10–20 mg each) were obtained endoscopically from the descending colon in each patient using routine endoscopic forceps. The descending colon was explicitly preferred to avoid any intersegmental variation in gene expression, and because this is the typical area of involvement in UC patients. The first biopsy was used for histopathological evaluation conducted in an unblinded fashion by staff pathologists in accordance with well-established criteria¹⁵ and focused on confirming the degree of inflammation. The second biopsy was snap-frozen in liquid nitrogen and stored at –80 °C for subsequent NMR spectroscopy. The remaining four biopsies were pooled, placed on ice, and used for subsequent isolation of colonocytes.

On the day of colonoscopy, whole blood was drawn by clean venous puncture from each patient, and collected into two sterile 6 mL VACUETTE tubes (Greiner Bio-One, Denmark) coated with K3EDTA. Peripheral blood mononuclear cells were isolated under sterile conditions using Lymphoprep (Axis-Shield PoC, Roskilde, Denmark) and stored at –80 °C for subsequent NMR spectroscopy.

On the day of colonoscopy, a urine sample (1 mL) from each patient was collected in Eppendorf tubes and stored at –80 °C for subsequent NMR spectroscopy.

Isolation of Colonocytes. The colonocytes were isolated as earlier described by Seidelin et al.¹⁶ In brief, the four pooled biopsies were kept on ice in 5 mL of phosphate-buffered saline (PBS) without Mg²⁺ or Ca²⁺, containing 50 IU/mL penicillin, 50 μ g/mL streptomycin and 0.5 mg/mL gentamycin, and within 15 min of sampling were washed three times in ice-cold PBS. The biopsies were subsequently incubated in 1 mM ethylenediaminetetraacetic acid and 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid in PBS without Mg²⁺ or Ca²⁺ at 21 °C for 75 min. The chelating buffer was

replaced by 3 mL of ice-cold PBS, and crypts and surface epithelium were liberated from the biopsies by shaking. The PBS containing crypts and surface epithelium was transferred to a new tube and centrifuged at 300g for 4 min at 4 °C. The obtained loose pellet had a very high (>99%) purity of colonocytes and consisted of well-preserved colon crypts without apoptotic features devoid of basal membrane and single cells, as revealed in previous ultra structural analyses¹⁶ and phase-contrast microscopy.¹⁷ The pellet was frozen at -80 °C for later NMR spectroscopy.

¹H NMR Spectroscopy. Urine samples were prepared by mixing 55 μ L of phosphate buffer (K₂HPO₄ and NaH₂PO₄, 1.5 M in D₂O) containing 0.1% TSP (3-trimethylsilyl-2,2,3,3-tetra-deuteriosodium propionate) with 550 μ L of urine sample and followed by centrifugation. A total of 550 μ L of the mixture was transferred into a 5 mm outer diameter NMR tube.

Lymphocytes and colonocytes were sonicated with 600 μ L of D₂O for 2 s followed by 2 s of rest, and this procedure was repeated 99 times. A total of 550 μ L of lymphocyte extract was transferred after centrifugation into a 5 mm NMR tube for NMR spectroscopy, whereas colonocyte extracts were freeze-dried and reconstituted in 60 μ L of D₂O. A total of 50 μ L of the extract was transferred into a 1.7 mm outer diameter NMR tube for NMR spectroscopy.

Urine samples and lymphocyte extracts were analyzed at 600.22 MHz on a Bruker DRX 600 spectrometer (Bruker, Rheinstetten, Germany) equipped with a CryoProbe, and colonocyte extracts were analyzed using 500 MHz on a Bruker NMR spectrometer equipped with a Broadband Inverse probe. All the samples were regulated at 298 K.

The frozen biopsies were thawed and packed into 4 mm in diameter zirconia rotors with deuterium water for field lock. ¹H NMR spectra were acquired on a Bruker DRX-600 spectrometer (Bruker) and a triple-resonance-high-resolution-MAS (HRMAS) probe with a magic-angle gradient. Samples were spun at 5 kHz at the magic angle (54.7°) and regulated at 283 K to minimize temperature induced biochemical degradation. A total of 15 min was allowed for the temperature to reach equilibration for each sample before a spectrum was acquired. Previous investigations on liver tissue have shown that no obvious changes are observed in the NMR spectra if samples are kept at low temperatures for short periods of time (in this case 15 min) prior to the acquisition of spectra.¹⁸

The 90° pulse length (~10.0 μ s) was set for all the samples. A total of 128 transients were collected into 16 k data points for each tissue spectrum and 32 k data points for all other spectra with a spectral width of 20 ppm, and a recycle delay (RD) of 2.0 s. Two ¹H NMR spectra were acquired for mucosal colonic biopsies and lymphocyte samples: (1) A standard one-dimensional NMR spectrum was acquired using the first increment of the nuclear Overhauser effect spectroscopy pulse sequence to achieve water presaturation [RD-90-*t*₁-90-*t*_m-90-acq],¹⁹ 2) A spin-spin relaxation edited NMR spectrum was acquired using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [RD-90-(*T*-180-*T*)_{*n*}-acq].²⁰ Only the standard one-dimensional NMR spectra were obtained for urine and colonocyte extracts. For the standard one-dimensional experiment, 3 μ s and 100 ms were set for the inter pulse delay *t*₁ and the mixing time *t*_m, respectively. Water irradiation was applied during *t*_m and RD. For the CPMG experiment, spin-spin relaxation delays, 2*m* π , of 200 and 80 ms was used for mucosal colonic biopsy samples and lymphocyte extracts, respectively. For assignment and verification purposes, two-dimensional

¹H-¹H CORrelation Spectroscopy (COSY)²¹ and TOrtal Correlation Spectroscopy (TOCSY)²² NMR spectra were also acquired for selected intestinal samples as previously described in detail.⁷

Data Analysis. Free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor prior to Fourier transformation. Spectra were manually corrected for phase and baseline distortions using XWINNMR 3.5 (Bruker). Urine spectra were referenced to the TSP resonance at δ 0.00, spectra of lymphocytes and colonocytes to the chemical shift of lactate at δ 1.33, and spectra of mucosal colonic biopsies to the chemical shift of the anomeric proton of α -glucose at δ 5.22. The spectra over the range δ 0.5–9.5 were digitized using a Matlab script. For spectra of mucosal colonic biopsies, the region δ 4.79–5.06 was removed to avoid the effects of imperfect water suppression. Regions δ 1.07–1.21, δ 3.37–3.57, δ 3.62–3.67, and δ 3.84–3.91 were also removed due to the presence of peaks from ethanol and propylene glycol, which were introduced during sampling. Regions δ 2.12–2.22 and δ 4.70–9.50 were removed for urine spectra, and regions δ 4.70–9.50 and δ 4.40–6.70 were removed for spectra of colonocyte and lymphocyte extracts. Normalization to the total sum of the spectrum was performed prior to pattern recognition analysis.

Data analysis was conducted with principal component analysis (PCA) using SIMCA-P+11 software package (Umetrics, Umeå, Sweden) in order to observe intrinsic clusters and obvious outliers (based on the principles of Hotelling T²) within the data set. In addition, orthogonal-projection to latent structure-discriminant analysis (O-PLS-DA)²³ was carried out on the NMR spectra using unit variance scaled data in order to uncover metabolic changes induced by the inflammation using MATLAB 7.0 with scripts developed in-house at Imperial College London. O-PLS is an extension of the Partial Least Square regression method²⁴ featuring an integrated Orthogonal Signal Correction filter.²⁵ For visualization purpose, the O-PLS coefficients indicating variables contributing to the discrimination in the model were back transformed as described by Cloarec et al.²⁶ The validation of the model was conducted using 7-fold cross validation, that is, iterative construction of models by repeatedly leaving out one-seventh of the samples, and predicting them back into the model. An additional validation method, permutation test, was also conducted: 20 000 models were constructed using randomized classification for the samples and *Q*² generated from these models were compared to the *Q*² of the real model. If the maximum value of *Q*²_{max} from the permutation test was smaller than or equal to the *Q*² of the real model, the model was regarded as a predictable model. Similarly, the *R*² was used to evaluate possibly overfitted models. The significance of metabolites contributing to the discrimination in the models was further tested with the use of a simple Student's *t* test and Bonferroni corrected significance levels.

Results

¹H HRMAS NMR Spectra of Human Mucosal Colonic Biopsies, Colonocytes, and Lymphocytes. Typical ¹H HRMAS NMR spectra of mucosal colonic biopsies and extracts of colonocytes and lymphocytes are shown in Figure 1. The observed endogenous metabolites in the spectra of mucosal colonic biopsies included a number of amino acids, such as leucine, isoleucine, valine, alanine, lysine, aspartate, asparagine, taurine, glycine, glutamate and glutamine, and membrane component metabolites as well as creatine, inositols, ascorbate

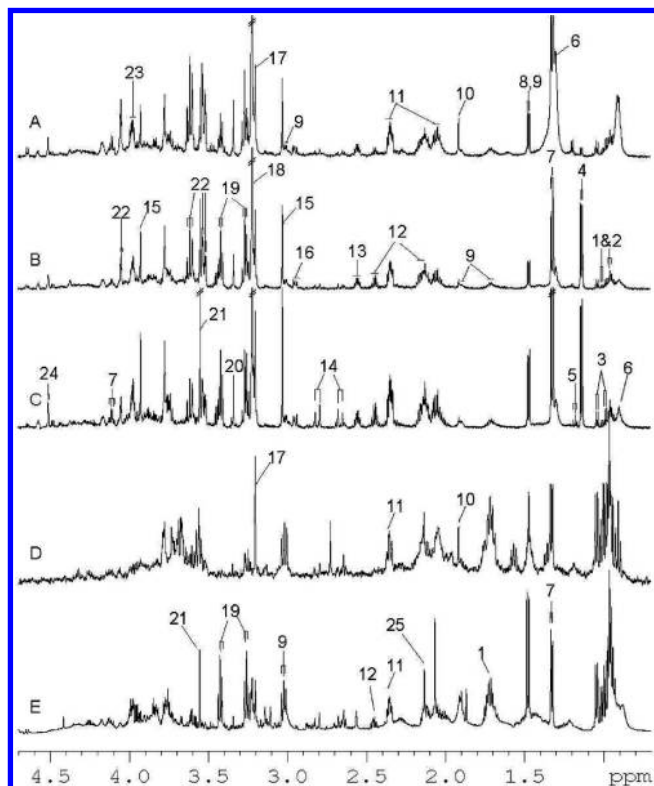


Figure 1. Typical ^1H NMR spectra from mucosal colonic biopsies obtained from controls (A), patients with inactive UC (B), active UC (C), and extracts of colonocytes (D) and lymphocytes (E); Key: 1, 2, leucine/isoleucine; 3, valine; 4, propylene glycol; 5, ethanol; 6, lipid; 7, lactate; 8, alanine; 9, lysine; 10, acetate; 11, glutamate; 12, glutamine; 13, glutathione; 14, aspartate; 15, creatine; 16, asparagine; 17, choline; 18, phosphatidylcholine; 19, taurine; 20, scyllo-inositol; 21, glycine; 22, *myo*-inositol; 23, phosphorylethanolamine; 24, ascorbate; 25, *N*-acetyl glycoprotein.

and lipids. The exogenous metabolites propylene glycol and ethanol originated from the lubricant and antiseptics used during the endoscopic procedure. Similarly, a variety of amino acids were detected in the extracts of lymphocyte and colonocyte (Figure 1). Furthermore, a signal of acetyl from *N*-acetyl glycoproteins was found in the NMR spectra of lymphocytes.

Multivariate modeling of both the standard one-dimensional NMR spectral data and the CPMG spectral data of mucosal colonic biopsies and lymphocytes showed similar results, and in the present paper, only the results generated from CPMG spectral data are presented.

Multivariate Data Analysis of NMR Spectra. PCA was applied to the NMR data obtained from mucosal colonic biopsies. On the basis of the principles of Hotelling T^2 (95% confidence limit), four patients with inactive UC, three with active UC, and one control subject were found to be outliers and consequently removed. PLS-DA models comparing controls with active UC, and controls with inactive UC were generated; CPMG NMR data as a X matrix and class information as the Y variables,²⁶ that is, control, active UC or inactive UC. A total of three PLS components were calculated for each model explaining a total of 23.7% and 29.4% of X variables (R^2X), and Q^2 describing the predictability of the models was 0.658 and 0.471, respectively. The score-plot generated from the model comparing control and active UC is shown in Figure 2, in which a clear separation between controls and active UC was observed. The O-PLS-DA strategy was subsequently applied

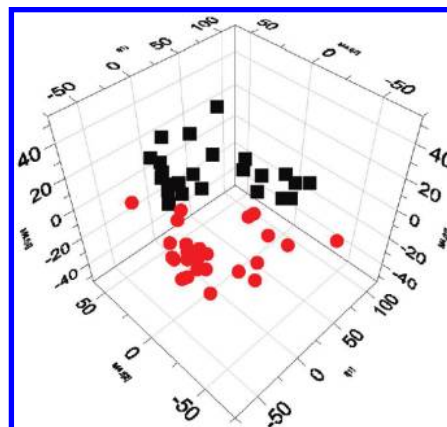


Figure 2. Score-plot obtained from PLS-DA modeling of the ^1H NMR spectra from mucosal colonic biopsies comparing controls (black) and active UC (red).

Table 2. Correlation Coefficients and p -Values of Metabolites Associated with Active UC^a

metabolites (ppm)	mucosal colonic biopsies active vs control $Q^2Y = 0.46$ $R^2X = 0.20$	colonocytes active vs control $Q^2Y = 0.53$ $R^2X = 0.20$
lipid (1.30)	-0.39 (0.0083**)	
glutamate (2.06)	+0.66 (0.02**)	
glutamine (2.13)	+0.52 (0.0002**)	
glutathione (2.54)	+0.33* (0.0002**)	
aspartate (2.67)	+0.64 (0.0001**)	
GPC (3.22)	-0.57 (0.004**)	-0.64 (0.005***)
betaine (3.28)	-0.42 (0.004**)	
taurine (3.25)	+0.54 (0.0001**)	
<i>myo</i> -inositol (4.06)	-0.42 (0.032**)	-0.77 (0.0001***)
ascorbate (4.51)	+0.51 (0.0001**)	
choline (3.21)		-0.78 (0.0001***)

^a On the basis of the multivariate model, the changes were significant at $p < 0.05$ except *: significant level $p = 0.1$. The + and - indicate the direction of the change, i.e. - for reduced concentration in active UC samples and + for increased concentration in active UC samples. Values in brackets are the p -values of individual metabolites calculated using univariate Student's t -test. Bonferroni corrected significance levels (p -value is $0.05/N$, where N is the number of metabolites used for multiple correction): ** p -value $0.05/10 = 0.005$; *** p -value $0.05/3 = 0.017$.

in order to uncover metabolic changes associated with UC in the mucosal colonic biopsies.

Two O-PLS-DA models (controls versus active UC and controls versus inactive UC) were constructed with one PLS component and one orthogonal component for each model. The quality of the models was assessed by the cross-validation parameter (Q^2Y) indicating the predictability of the model and by R^2X , which are listed in Table 2. A weak model was obtained when comparing controls and inactive UC, whereas a strong separation between controls and active UC was achieved, as suggested by the p -values from these models (0.026 and 5.3×10^{-9} , respectively). To further validate the PLS-DA models, permutation tests were performed (Figure 3). In the case of controls versus inactive UC, higher Q^2_{\max} was obtained from the permutation test than from the real model, implying lack of predictability (Figure 3A). Similarly, the resulting R^2 gives the impression of an unambiguously overfitted model. The maximum Q^2_{\max} (Figure 3B) obtained from the permuted models comparing controls and active UC did not exceed Q^2 obtained from the real model, which suggested great predictability. However, R^2 gives the impression of a possibly overfitted

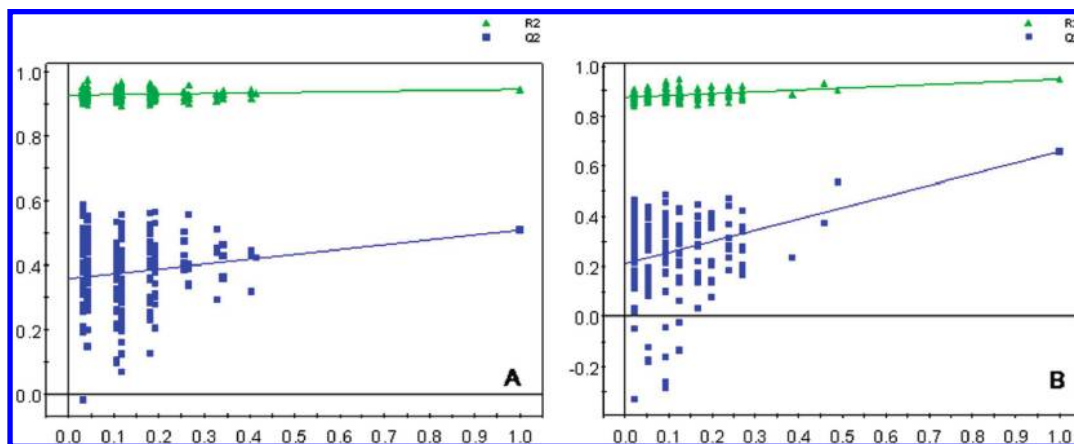


Figure 3. Plot of PLS-DA permutation tests showing an invalid model of controls vs inactive UC (A) and a valid model of controls vs active UC (B).

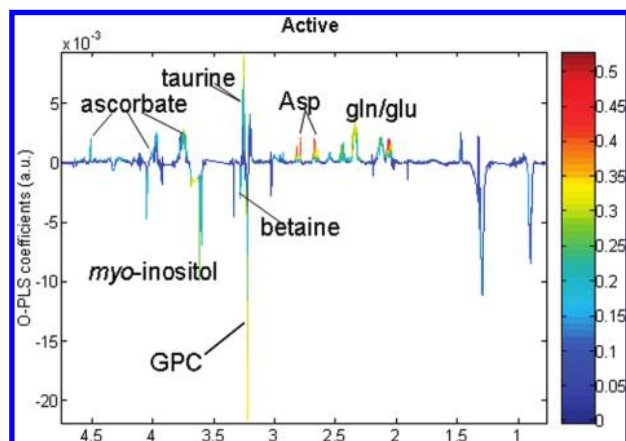


Figure 4. Coefficient plot calculated from O-PLS-DA modeling of the ^1H NMR spectra from mucosal colonic biopsies of controls vs active UC. Asp, aspartate; gln, glutamine; glu, glutamate; GPC, glycerophosphocholine.

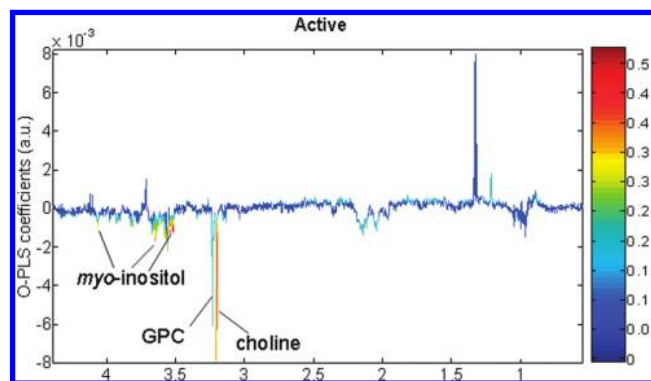


Figure 5. Coefficient plot calculated from O-PLS-DA modeling of the ^1H NMR spectra from the extracts of colonocytes of controls vs active UC. GPC: glycerophosphocholine.

model, which necessitates a validation of the loadings in terms of univariate testing.

The same procedures were applied to the NMR spectral data from urine and the extracts of lymphocytes and colonocytes. The metabolic profiles of urine were identical in all three groups of patients. No difference between the metabolic profiles of controls and inactive UC was observed in the lymphocytes and colonocytes. The metabolic profiles of lymphocytes resulted in a positive Q^2 (0.1) when the controls and active UC were compared, but the permutation test suggested that the model was without predictability, hence invalid, while the model created from profiles of colonocytes revealed a significant separation between controls and active UC patients.

Metabolites responsible for the separation of active UC patients from controls could be found in the O-PLS-DA coefficient plot, as shown in Figures 4 and 5. The coefficient plots display differences in metabolic profiles related to the metabolites in active UC. Here the direction of the resonances associates with the relative variations of metabolites in active UC compared to the controls. For example, peaks pointing upward indicate a relative increased intensity of metabolites associated with active UC, and downward peaks suggest a decreased intensity of metabolites in active UC. The colors shown on the plot are associated with the significance of metabolites in the differentiation of active UC and controls, as

scaled on the right-hand side of the plot. The coefficients indicating the significance of the metabolites found in mucosal colonic biopsies and colonocytes are summarized in Table 2 together with the cross-validation parameters. Here, based on the number of samples in each group, the coefficients are considered significant when higher than 0.39 and 0.46 for mucosal colonic biopsies and colonocytes, respectively, which corresponded to the critical value of a correlation coefficient of 5% ($p = 0.05$). To validate the findings, the integrations of the significant metabolites suggested from the correlation coefficients were further compared using Student's t test and corrected for multiple testing with the use of Bonferroni corrected significance levels. The results are listed in Table 2.

The data analysis showed that the mucosal colonic biopsies obtained from patients with active UC contained relative higher levels of antioxidants such as ascorbate and glutathione, and a range of amino acids including glutamate, glutamine, taurine and aspartate than those obtained from controls. These variations were associated with relatively lower levels of lipid, glycerophosphocholine (GPC), *myo*-inositol, and betaine. Compared to the metabolic profiles obtained from controls, colonocytes from active UC patients contained relative low levels of GPC, *myo*-inositol, and choline. Glutamine was also reduced, albeit not significantly.

No significant differences in the metabolites were found between controls and inactive UC of mucosal colonic biopsies and colonocytes, respectively, which was in accordance with the lack of predictability of the corresponding O-PLS-DA

models. However, in order to evaluate the status of patients with quiescent UC, the models from mucosal colonic biopsies and colonocytes comparing controls and active UC were used to predict those obtained from patients with inactive UC. It is interesting to note, that, in both of the models (biopsy and colonocytes), 20% (7 samples) of the samples from patients with inactive UC were predicted as active UC: two of the patients with inactive UC were predicted as active on the basis of both their biopsy and colonocyte sample, whereas 5 patients with inactive UC were predicted as active due to their biopsy sample and another 5 patients with inactive UC due to their colonocyte sample. In all, 12 patients with inactive UC were predicted as having active UC based on either their biopsy sample, colonocyte sample or both. Retrospectively, these twelve patients were characterized by either having a flare-up within 6 month prior to or after the sample collection.

A final analysis was conducted on the different phenotypes using both mucosal colonic biopsy and colonocyte samples: gender, age at diagnosis (≤ 25 years/ >25 years), years with disease (≤ 10 years/ >10 years), familiar disposition (disposed, nondisposed to IBD), Mayo-score (mild, moderate, and severe), extent of inflammation (proctitis, proctosigmoiditis, left-sided colitis, and pancolitis), extra-intestinal manifestations (present/never present), steroids (dependent/nondependent), and smoking status (smoking/nonsmoking). This was completed by creating PLS-DA models for all UC patients and either patients with active UC or inactive UC; NMR data as an X matrix and phenotype information as the Y variables. However, none of these models turned out to be predictive (data not shown).

Discussion

In accordance with previous findings,¹⁰ this study shows that the metabolic profiles of biopsies can be used to differentiate active UC from controls, and for the first time, it is also demonstrated that colonocyte samples can be employed successfully with the same purpose. Not even phenotypes or confounding factors had any effect on the discriminant capabilities, which unfortunately also made it impossible to subclassify the patients in accordance with clinical phenotypes. It was also noticed that medication did not have any impact on the metabolic profiles, which was indirectly assessed by the fact that no differences between controls and inactive UC were present in the colonic tissue. However, an interesting observation was made as 12 patients with inactive UC were classified as being active, even though they were histological graded as "normal". At the transcriptomics level, Wu et al.⁴ and Olsen et al.⁵ also found abnormal profiles of mucosal colonic biopsies from inactive UC patients. Olsen et al. consequently suggested the existence of a possible continuous inflammatory state in quiescent UC. Given the results from Bezabeh et al.,¹⁰ this might also be true at the metabonomics level as they¹⁰ found that nine out of 20 inactive UC samples were classified as "abnormal". Unfortunately, they¹⁰ did not register the individual time frames for flare-ups and remissions, which seems to be of crucial importance seeing that the quiescent UC patients that were classified as active UC in this study had either just experienced a flare-up or were about to. Hence, in accordance with the results of this study, no continuous inflammatory state exists in quiescent UC—at least not at the metabonomics level. The results, however, do show that NMR spectroscopy-based metabonomics is undoubtedly able to detect early pathogenic changes that take place before

any clinical or para-clinical evidence of a flare-up is present. This potential is of vital importance to the treatment of UC, as an imminent flare-up in an otherwise quiescent UC patient could be predicted and hence timely addressed and possibly avoided.

At this point, it is not evident why some patients with inactive UC were classified as active UC based on the metabolic profile of their mucosal biopsy, whereas others were classified as such based on their colonocyte sample; this is an issue, which needs to be further addressed and elucidated. However, at this point, it does emphasize the fact that colonocytes are not innocent bystanders in the pathogenesis of UC.

The metabolic profiles of lymphocytes and urine did not allow a differentiation between active UC, inactive UC, and controls, which means that the inflammation in the large bowel is not reflected systemically in a cohort with moderate UC. This is in contrast to recent studies. Murdoch et al.²⁷ used NMR spectra from urine of interleukin 10 (IL-10) gene-deficient mice and the resulting PLS-DA model was able to differentiate between diseased and wild-type mice. However, this was only evident with severe inflammation and apart from developing enterocolitis, IL-10 gene-deficient mice also sustain hepatic injury, which also explains some of the urinary metabolite perturbations found in IL-10 gene-deficient mice. Williams et al.²⁸ acquired NMR spectra from urine samples of CD and UC patients and controls and created PLS-DA models that were able to distinguish the cohorts. Unfortunately Q^2 is not described, and the cross-validation is based on excluding just one sample at the time, whereas this study applied a one-seventh cross-validation. Furthermore, a homogeneous cohorts was created by including only Caucasians and excluding vegetarians, patients on a therapeutic diet, and patients with an intercurrent illness. Because of the occurrence of metabolites of acetaminophen and 5-acetylsalicylate, 18 out of 86 CD patients and 17 out of 60 UC patients were excluded as outliers. None of these restrictions were imposed on this study, as it would have made the results clinically irrelevant.

The increased levels of a range of amino acids (Table 2) appear to be a logic consequence of the inflammatory process. During intestinal inflammation, glutamine reduces oxidative stress and cytokine production, which impairs the inflammatory response,²⁹ and glutamine is a major respiratory fuel for enterocytes and gut-associated immune cells through its metabolism into its intermediary metabolites: glutamate and ammonia. Glutamate by itself serves as an energy source and is also a precursor for the synthesis of the endogenous thiol antioxidant glutathione (Figure 6), which together with ascorbate³⁰ constitutes an important part of the defense mechanism in oxidative stress.³¹ In consequence, glutamine, glutamate, glutathione, and ascorbate are all required in greater quantities during catabolic conditions and corresponding high loads of oxidative stress as found in the inflammatory process of UC. This concurs with the high levels of taurine, as taurine has been shown to possess antioxidant, antiapoptotic and anti-inflammatory effects.³²

Surprisingly, glutamine, glutamate, glutathione, and ascorbate were found not to be elevated in the colonocytes from patients with active UC compared to controls, and glutamine was even found with a trend toward reduced levels. Two important notions can be made from this observation: one, the increased levels of glutamine and glutathione found in the biopsies from patients with active UC are most likely due to activated lymphocytes in the lamina propria, as these metabo-

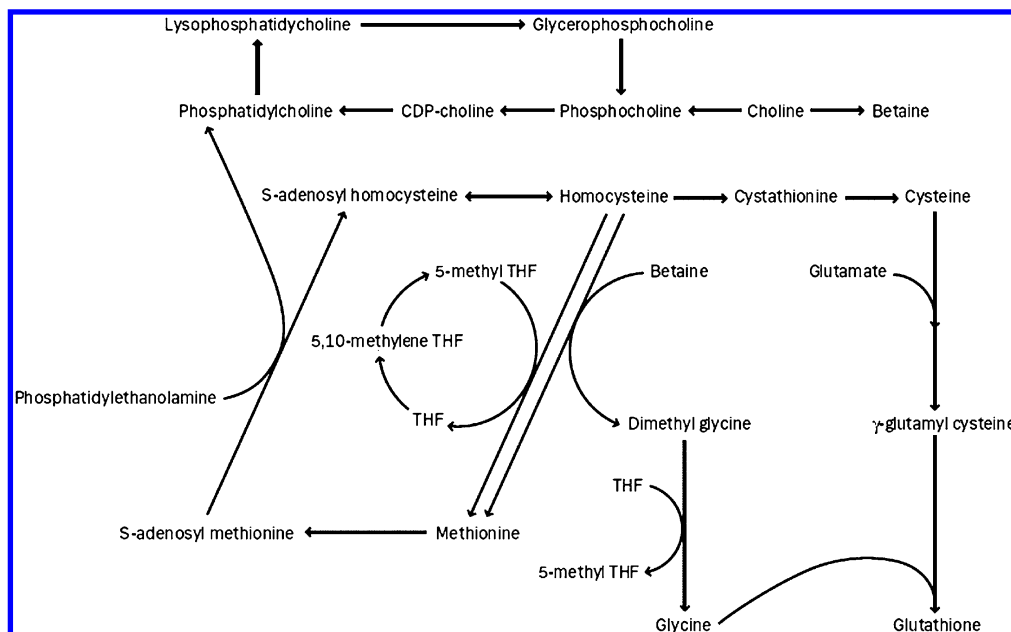


Figure 6. Schematic presentation of the interaction between phospholipid metabolism, methionine-homocysteine cycle, and glutathione synthesis. THF: tetrahydrofolate.

lites were prominent features of the metabolic profile of peripheral lymphocytes (Figure 1E); two, it raises the question of this circumstance being a part of the pathophysiology of UC, as it has been proposed that gut mucosal turnover and barrier function is compromised due, in part, to relative glutamine and glutathione deficiency.³³ Hence, the results of this study generate a hypothesis of an imbalanced antioxidant response in the lamina propria and colonocytes of patients with UC. Especially glutamine and glutathione and their part in the pathogenesis of IBD have been studied intensely, but unfortunately with inconsistent results. Animal models of IBD have found a beneficial effect of dietary glutamine supplementation,³⁴ whereas human studies have not.³⁵ Finnie et al.³⁶ demonstrated increased glutamine metabolism in the distal colon of patients with quiescent UC, whereas Chapman et al.³⁷ and Duffy et al.³⁸ found no such difference. Similar inconclusive results have been obtained with respect to glutathione,^{39,40} and these contradicting results seem to be primarily explained by the heterogeneity in study design. However, when comparing the present study with a similar designed study,¹⁰ the observed changes in metabolites (including glutamine and glutamate) are in broad agreement. Conversely, if compared with a slightly different approach as the one by Balasubramanian et al.,⁹ who pretreated the samples and employed NMR spectroscopy on perchloric acid extracts of mucosal colonic biopsies from IBD patients, an evident discrepancy with this study arises as they⁹ found low levels of a range of amino acids (including glutamine, glutamate, isoleucine, leucine, valine and alanine) in patients with active UC compared to controls. Why these two similar, yet distinct methodological approaches, result in diverging results is at this point not evident.

This study also demonstrated decreased levels of the membrane metabolites glycerophosphocholine (GPC), choline, and *myo*-inositol in colonic tissue from patients with active UC (Table 2). GPC and choline are essential constituents of the phospholipid metabolism, which is closely interrelated to the above-mentioned thiol metabolism and synthesis of glutathione through the methionine-homocysteine cycle (Figure

6).⁴¹ Sustained choline deprivation promotes cell survival *via* enhanced NF- κ B activation, which subsequently leads to continuous inflammation and oncogenesis.⁴² The oncogenesis is further augmented by the low levels of *myo*-inositol, as inositol compounds have been shown to inhibit UC-associated carcinogenesis in mice.⁴³ Furthermore, low levels of *myo*-inositol is a feature shared by spectra of malignant colon tissue specimens,⁴⁴ and under these circumstances, *myo*-inositol has an inverse relationship with taurine, which is also observed in this study. Supported by the established lipid profile and lack of crucial amino acids in the colonocytes, it is hypothesized from this study that chronic and extensive colonic inflammation and consequently a continuous high load of oxidative stress and low levels of especially choline and *myo*-inositol are central elements in the UC associated increased risk of colorectal cancer.⁴⁵

This work has illustrated the possibilities held by metabolomics as a diagnostic tool during active UC, but more importantly so also during quiescent UC. NMR spectroscopy seems to be able to detect even subtle changes taking place prior to a clinical or para-clinical evident flare-up, making more timely intervention possible in the future. Furthermore, this study has shown metabolomics to be useful in terms of hypothesis-generation, bringing about promising pathophysiological explanations to the genesis of UC and its association with the increased risk of colorectal cancer. In order to proceed with these results and hence improve the diagnostics, get a better understanding of the inflammatory state, and to validate the hypotheses, an intriguing future approach would be systems biology—combining metabolomics, proteomics, and transcriptomics.

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