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A novel gas chromatography mass spectrometry-based serum diagnostic and assessment approach to ulcerative colitis

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Abstract

Background & aims: To improve the clinical course of ulcerative colitis (UC), more accurate serum diagnostic and assessment methods are required. We used serum metabolomics to develop diagnostic and assessment methods for UC.
Methods: Sera from UC patients, Crohn's disease (CD) patients, and healthy volunteers (HV) were collected at multiple institutions. The UC and HV were randomly allocated to the training or validation set, and their serum metabolites were analyzed by gas chromatography mass

Abbreviations: ASCA, Anti-*Saccharomyces cerevisiae* antibodies; AUC, Area under the ROC curve; CAI, Clinical activity index; CD, Crohn's disease; CDAI, Crohn's disease activity index; GC/MS, Gas chromatography/mass spectrometry; GSEA, Gene set enrichment analysis; HV, Healthy volunteers; IBD, Inflammatory bowel disease; IC, Indeterminate colitis; IDO, Indoleamine-2,3-dioxygenase; LC/MS, Liquid chromatography/mass spectrometry; MALDI-TOF-MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MIAI, Multivariate indexes established from plasma aminograms; MSTFA, N-Methyl-N-trimethylsilyl-trifluoroacetamide; NMR, Nuclear magnetic resonance spectroscopy; OmpC, *Escherichia coli* outer membrane porin; p-ANCA, Perinuclear anti-neutrophil cytoplasmic antibodies; ROC, Receiver operating characteristic; SELDI-TOF-MS, Surface enhanced laser desorption ionization time-of-flight mass spectrometry; TCA, T tricarboxylic acid cycle; UC, Ulcerative colitis; UCa, Ulcerative colitis patients in the active phase; UCr, Ulcerative colitis in remission; VIF, Variance inflation factors.

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spectrometry (GC/MS). Using the training set, diagnostic and assessment models for UC were established by multiple logistic regression analysis. Then, the models were assessed using the validation set. Additionally, to establish a diagnostic model for discriminating UC from CD, the CD patients' data were used.

Results: The diagnostic model for discriminating UC from HV demonstrated an AUC of 0.988, 93.33% sensitivity, and 95.00% specificity in the training set and 95.00% sensitivity and 98.33% specificity in the validation set. Another model for discriminating UC from CD exhibited an AUC of 0.965, 85.00% sensitivity, and 97.44% specificity in the training set and 83.33% sensitivity in the validation set. The model for assessing UC showed an AUC of 0.967, 84.62% sensitivity, and 88.23% specificity in the training set and 84.62% sensitivity, 91.18% specificity, and a significant correlation with the clinical activity index ($r_s = 0.7371$, $P < 0.0001$) in the validation set.

Conclusions: Our models demonstrated high performance and might lead to the development of a novel treatment selection method based on UC condition.

1. Introduction

Inflammatory bowel disease (IBD) involves chronic and recurring inflammation of the gastrointestinal tract and is composed of two major subtypes, ulcerative colitis (UC) and Crohn's disease (CD). The incidence rates of IBD are increasing in several industrialized countries, especially Asian-Pacific countries in which the population's lifestyle has become more westernized, e.g., Japan.[1] Previous studies have suggested that these increases in the incidence of IBD are associated with immunological disorders caused by genetic, environmental, and microbiological factors,[2,3] and environmental factors are probably the main causes of these increases because it is unlikely that the genetic background of the people in these countries has changed markedly within a few decades.[4,5] However, the etiology of IBD remains to be understood, and therefore, disease-specific treatments and diagnostic methods for IBD have yet to be established. At present, comprehensive diagnostic criteria based on clinical, endoscopic, histological, and radiological findings are used to diagnose IBD, but it is difficult to distinguish CD from UC in approximately 2–15% of IBD cases, and these cases are treated as indeterminate colitis (IC).[6–8] Recently, a combination of perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) and anti-*Saccharomyces cerevisiae* antibodies (ASCA) was demonstrated to be a useful biomarker for discriminating between the different types of IBD,[9] but such serological markers are not suitable for clinical application because of their low sensitivity/specificity.

Recently, IBD has started to be evaluated via proteomics and metabolomics-based methods involving nuclear magnetic resonance spectroscopy (NMR), surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), gas chromatography mass spectrometry (GC/MS), and/or liquid chromatography mass spectrometry (LC/MS).[10–14] In this study, metabolomics was used to investigate the alterations in the serum metabolite profiles of IBD patients. Metabolomics is a comprehensive method and is capable of evaluating the characteristics of interactions between the low molecular weight metabolites of a cell, tissue, organ, or organism. Metabolites are located at the endpoint of the omics cascade; i.e., the last step before the phenotype, and therefore, metabolite profiles might represent the expression patterns of regulatory factors. Thus, studying such profiles might allow the pathology of IBD to be elucidated in more detail. In our previous reports, it was demonstrated that a serum metabolite profile consisting of amino acids and tricarboxylic acid cycle (TCA)-related metabolites was able to discriminate among UC patients, CD patients, and healthy volunteers,[15,16] although the study only involved a small number of samples. Therefore, in the present study an increased number of samples were used, and both training and validation sets were employed. Using a training set composed of UC patients (N = 60) and healthy volunteers (N = 60), diagnostic and assessment models for UC were established by selecting candidate metabolites using the volcano plot and stepwise methods and then subjecting them to multiple logistic regression analysis. Then, the validity of the resultant predictive models was assessed using a validation set consisting of UC patients (N = 60) and healthy volunteers (N = 60). In addition, to establish a diagnostic model for discriminating UC from CD and to validate the diagnostic model for discriminating UC from HV, CD patients' (N = 39) data were used as a training set and additional validation set.

2. Materials and methods

2.1. Patients

This study was approved by the ethics committees at Kobe University Graduate School of Medicine (Hyogo, Japan) and Hyogo Medical University (Hyogo, Japan) and was performed between February 2009 and February 2013. The human samples were used in accordance with the guidelines of each hospital, and written informed consent was obtained from all subjects. The serum samples from the IBD patients (N = 159), UC patients (N = 120), and CD patients (N = 39) were collected at Kobe University Hospital and Hyogo Medical University. UC and CD were diagnosed according to relevant criteria based on clinical symptoms, and radiographic, endoscopic, and pathological findings. Disease activity was assessed using the Crohn's disease activity index (CDAI)[17] and Rachmilewitz index [a clinical activity index (CAI)].[18] Active disease was defined as a CDAI of $\geq$150 for CD and a CAI of $\geq$6 for UC. Remission was defined as a CDAI of $<$150 for CD and a CAI of $<$6 for UC. The serum samples from the HV (N = 120) were collected at Aijinkai

Chibune General Hospital (Osaka, Japan) and Shinkokai Shinko Hospital Health Examination Center (Hyogo, Japan). No clinical abnormalities were detected in the HV during medical check-ups involving physical, blood, urine, imaging, and/or endoscopic examinations.

2.2. Serum collection and preparation

Each whole blood sample was collected in a clean tube and immediately centrifuged at 3000 ×*g* for 10 min at 4 °C. Then, the serum was transferred to a clean tube and stored at −80 °C until use. The extraction of low molecular weight metabolites was performed according to the method described in our previous report.[19] Briefly, 50 μl of serum was mixed with 250 μl of a solvent mixture (MeOH:H_2O:$CHCl_3$ = 2.5:1:1) containing 10 μl of 0.5 mg/ml 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) dissolved in distilled water as an internal standard, and then the solution was shaken at 1200 rpm for 30 min at 37 °C, before being centrifuged at 16,000 ×*g* for 3 min at 4 °C. Two hundred and twenty-five microliters of the resultant supernatant was transferred to a clean tube, and 200 μl of distilled water was added to the tube. After being mixed, the solution was centrifuged at 16,000 ×*g* for 3 min at 4 °C, and 250 μl of the resultant supernatant was transferred to a clean tube, before being lyophilized using a freeze dryer. For oximation, 40 μl of 20 mg/ml methoxyamine hydrochloride (Sigma-Aldrich) dissolved in pyridine was mixed with a lyophilized sample, which was then shaken at 1200 rpm for 90 min at 30 °C. Next, 20 μl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (GL Science, Tokyo, Japan) was added for derivatization, and the mixture was incubated at 1200 rpm for 30 min at 37 °C. The mixture was then centrifuged at 16,000 ×*g* for 5 min at 4 °C, and the resultant supernatant was subjected to GC/MS measurement.

2.3. GC/MS analysis

According to the method described in a previous report,[20] the GC/MS analysis was performed using a GCMS-QP2010 Ultra (Shimadzu Co., Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleed/MS; inner diameter: 30 m × 0.25 mm, film thickness: 0.25 μm; Agilent Co., Palo Alto, CA). The front inlet temperature was 230 °C, and the flow rate of helium gas through the column was 39.0 cm/s. The column temperature was held at 80 °C for 2 min and then raised by 15 °C/min to 330 °C and held there for 6 min. The transfer line and ion-source temperatures were 250 °C and 200 °C, respectively. Twenty scans per second were recorded over the mass range of 85–500 *m/z* using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co.).

2.4. Data processing

Data processing was performed according to the methods described in previous reports.[20,21] Briefly, the MS data were exported in netCDF format. The peak detection and alignment were performed using the MetAlign software (Wageningen UR, The Netherlands). The resultant data were exported in CSV format and then analyzed with in-house analytical software (Aloutput) and an in-house metabolite library for peak identification and semi-quantitative analysis. For the semi-quantitative analysis, the peak height of each ion was calculated and normalized to the peak height of 2-isopropylmalic acid as an internal standard. Names were assigned to each metabolite peak based on the method of a previous report.[21]

2.5. Statistical analysis

The UC patients were randomly allocated to the training or validation set, and the HV were recruited as age- and sex-matched controls for the UC. In both the training and validation studies, the serum levels of metabolites were compared between the UC patients and HV, between UC patients in the active phase (UCa) and those in remission (UCr), and between the UCa/UCr and HV using the Wilcoxon's rank sum test. Next, volcano plots and the stepwise method were used to select metabolite biomarker candidates for the multivariate analysis according to their significance and fold change values.[22] The multiple logistic regression analysis was used to establish a diagnostic model discriminating the UC patients from HV as described previously.[19,23] The multicollinearity of the selected variables was also examined by calculating their variance inflation factors (VIF). Receiver operating characteristic (ROC) analysis was used to calculate area under the ROC curve (AUC), sensitivity, and specificity values for the model in order to evaluate its diagnostic performance, and the optimal cut-off value of the model was determined from its ROC curve. In the validation set study, the accuracy of the diagnostic model was evaluated using the cut-off value obtained in the training set study. Another diagnostic model for discriminating between the UC and CD patients was established by comparing the serum metabolite levels of the UC patients in the training set with those of the CD patients using Wilcoxon's rank sum test and then employing the abovementioned metabolite selection method. In addition, an assessment model for differentiating between the UCa and UCr patients was prepared by comparing the serum metabolite levels of the UCr and UCa patients in the training set using Wilcoxon's rank sum test and then employing the abovementioned metabolite selection method. Next, the correlation between the assessment model and the CAI score was evaluated using Spearman's rank-correlation coefficient.

In our study, 7 UC patients, including 4 UCa patients and 3 UCr patients, who were included in the abovementioned analyses were prospectively evaluated using the assessment model until remission or flare-up occurred, and the utility of the model as a tool for monitoring UC was examined. In addition, we also examined whether various factors such as body weight, body mass index (BMI), serum level of total protein (TP), albumin (Alb) and total cholesterol (T-chol), and medical treatment including medication, enteral nutrition and food restriction therapy influenced the alterations of metabolite biomarker candidates which we selected in diagnostic and assessment models for UC. At first, the correlation between the metabolite alterations and the following factors; body weight, BMI and serum level of TP, Alb and T-chol in the sera of HV was evaluated using Spearman's rank-correlation coefficient. Next, the correlation between the metabolite alterations and medical treatments; current dose of each salicylate; Pentasa, Asacol and sulfasalazine (SASP), azathioprine, and current dose and total dose within 1, 3, 6 months and 1 year of prednisolone in the sera of UCr was evaluated using Spearman's

rank-correlation coefficient. The correlation between the metabolite alterations and current total calorie of enteral nutrition; Elental and amino acid production in the sera of CDr was also evaluated using Spearman's rank-correlation coefficient. As far infliximab, the number of CD patient's samples was sufficient for statistical analysis, so we used CD patients' data. Samples of CD patients were picked up and divided into two groups; administration group and non-administration group which various points such as age, gender ratio, BMI, medicated condition and clinical activity were matched between, and the alterations of metabolites were compared between two groups using the Wilcoxon rank sum test. Similarly, samples of UC patients were divided into two groups; food restriction therapy group and non-food restriction therapy group which various points such as age, gender ratio, BMI, medicated condition and clinical activity were matched between, and the alterations of metabolites were compared between two groups using the Wilcoxon rank sum test. As far BMI and serum levels of TP and Alb in HV, we could not obtain data on all the subjects from reports of medical check-ups, and deficient data existed. Therefore, the abovementioned analysis for the correlation between the metabolite alterations and these data was performed using the obtained data only. The details were also demonstrated in the legend of Supplemental Tables 10, 11. P-values of less than 0.05 were considered to indicate a significant difference. These analyses were carried out using the default conditions of JMP10.0.2 (SAS Institute Inc., Cary, NC).

3. Results

The background information of the IBD patients and HV is summarized in Table 1 and Supplementary Table 10. In our metabolomic GC/MS-based study, which mainly targeted water-soluble metabolites, 114 metabolites were detected in the sera of IBD patients and HV, and the levels of these metabolites were compared between the UC patients and HV, between the UCa and UCr, and between the UCa/UCr and HV. In both the training and validation sets, 77 metabolites exhibited significant alterations between the UC patients and HV; 48 of the 77 metabolites displayed significantly decreased levels in the UC patients whereas 29 metabolites demonstrated significantly increased levels (Supplemental Table 1). Among these metabolites, the components of the TCA cycle; i.e., succinic acid, fumaric acid, and malic acid, and urea cycle intermediates; i.e., ornithine, citrulline, and urea, exhibited some of the most significant alterations, and all of these metabolites except urea displayed significantly decreased serum levels in the UC patients. Several amino acids such as histidine, isoleucine, leucine, lysine, and methionine, also demonstrated significantly decreased levels in the UC patients. In addition, combining the results from the training and validation sets a total of 24 metabolites exhibited characteristic alterations between the UCa and UCr and/or between the UCa/UCr and HV (Fig. 1A, B, Supplemental Table 2), and all of these metabolites except pantothenate demonstrated

Table 1 Characteristics of the patients in the training and validation sets.

	Training set		Validation set		Training & validation set
	UC	HV	UC	HV	CD
N (male/female)	60 (21/39)	60 (23/37)	60 (22/38)	60 (23/37)	39 (26/13)
Age [a)]	42.8 ± 14.1/ 16 − 72	45.4 ± 11.1/ 26 − 71	41.9 ± 15.0/ 13 − 77	45.4 ± 10.8/ 27 − 70	35.4 ± 12.0/ 13 − 58
Years with disease [a)]	8.7 ± 7.3/ 0.2 − 23	–	7.9 ± 6.0/ 0.3 − 21	–	9.8 ± 8.9/ 0.3 − 32
Disease location					
UC: P/LS/PC	7/23/30	–	7/24/29	–	–
CD: S/C/both	–	–	–	–	10/4/25
Disease activity [a)]					
CAI	3.7 ± 3.6/ 0 − 12	–	3.6 ± 3.8/ 0 − 15	–	–
CDAI	–	–	–	–	1202.1 ± 98.6/ 0 − 287
Remission/active	34/26	–	34/26	–	29/10
Daily medication					
Salicylates	56	–	50	–	29
Prednisolone	14	–	20	–	2
6-Mercaptopurine	1	–	2	–	2
Azathioprine	13	–	12	–	17
Tacrolimus	3	–	3	–	0
Anti-TNF-α agents	4	–	5	–	25
Enteral nutrition	-	–		–	21

[a)]: Age, years with disease and disease activity are shown as the mean ± standard deviation together with the range. P: proctitis, LC: left-sided colitis, PC: pan-colitis, S: small bowel, C: colon, Both: small bowel & colon, CAI: Rachmilewitz index (a clinical activity index), CDAI: Crohn's disease activity index.

significant correlations with the CAI in the UC patients (Supplemental Table 3).

In the training set study, to establish a diagnostic model for differentiating the UC patients from the HV, 22 of the 114 metabolites were selected as metabolite candidates from volcano plots using the following criteria: the serum concentration of the metabolite had to differ significantly at the $P < 0.05$ level between the UC patients and HV according to the Wilcoxon's rank sum test and exhibit a fold change of greater than 2.0 (Supplemental Fig. 1, Supplemental Table 4). Of these 22 metabolites, 4; i.e., taurine, S-benzyl-L-cysteine, maleic acid, and N-acetyl-L-glutamine, were selected using the stepwise method. On the basis of their VIF, it was confirmed that these 4 metabolites did not exhibit multicollinearity (Supplemental Table 5). Next, a diagnostic model consisting of the 4 metabolites was established using multiple logistic regression analysis as follows (Table 2): $p = 1/[1 + e - \{0.83 - 116.3(\text{taurine}) - 335.3(\text{S-benzyl-L-cysteine}) + 10801.1(\text{maleic acid}) + 2220.8(\text{N-acetyl-L-glutamine})\}]$.

The ROC curve obtained for this diagnostic model in the training set study is shown in Fig. 2. The AUC of this model was 0.988, and the optimal cut-off value was 0.659. The model's sensitivity and specificity values were 93.33% and 95.00%, respectively, whereas in the validation set study, its sensitivity and specificity values were 95.00% and 98.33%, respectively. Furthermore, as an additional validation set study, the metabolite data of the 39 CD patients were subjected to this diagnostic model. When this set was used, the model was found to have a specificity value of 0.26% (Supplemental Fig. 2, Table 3). Therefore, although this diagnostic model was able to discriminate between UC and HV with high sensitivity and specificity, it could not distinguish between UC and CD.

Next, a diagnostic model for discriminating between UC and CD was investigated using the abovementioned method. In the training set study, 9 of the 114 metabolites were selected as metabolite candidates from a volcano plot according to the same criteria as were used to develop the previous model (Supplemental Fig. 3, Supplemental Table 6). Of these, 4 metabolites; i.e., oxalate, 3-hydroxybutyrate, ribulose, and 1,6-anhydroglucose, were selected via the stepwise method, and none of them exhibited multicollinearity (Supplemental Table 7). The following diagnostic model was established using multiple logistic regression analysis (Table 4): $p = 1/[1 + e - \{1.36 - 2281.82(\text{oxalate}) - 35.00(3\text{-hydroxy-butyrate}) - 570.81(\text{ribulose}) + 279.26(1,6\text{-anhydroglucose})\}]$.

The ROC curve obtained for this diagnostic model in the training set study is shown in Fig. 3. The AUC of the model

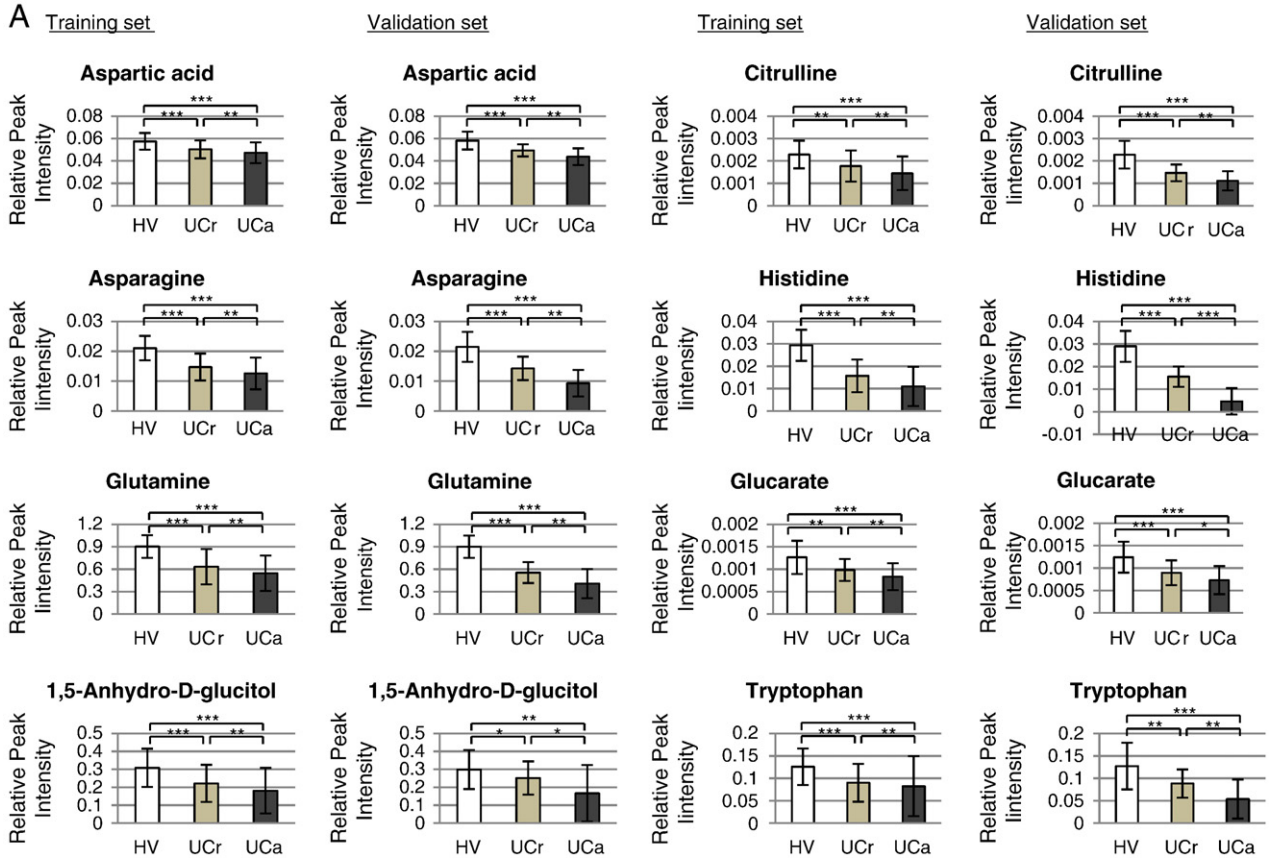

Figure 1 Metabolites that displayed altered serum levels according to the presence/absence of UC and disease activity. Twenty-four metabolites showed significantly decreased (A) or increased (B) serum levels between the UCa and UCr, and between the UCa/UCr and HV, in both the training and validation sets. Data are shown as the mean ± standard deviation. P-values were calculated using the Wilcoxon rank sum test; *$P < 0.05$, **$P < 0.01$ ***$P < 0.001$.

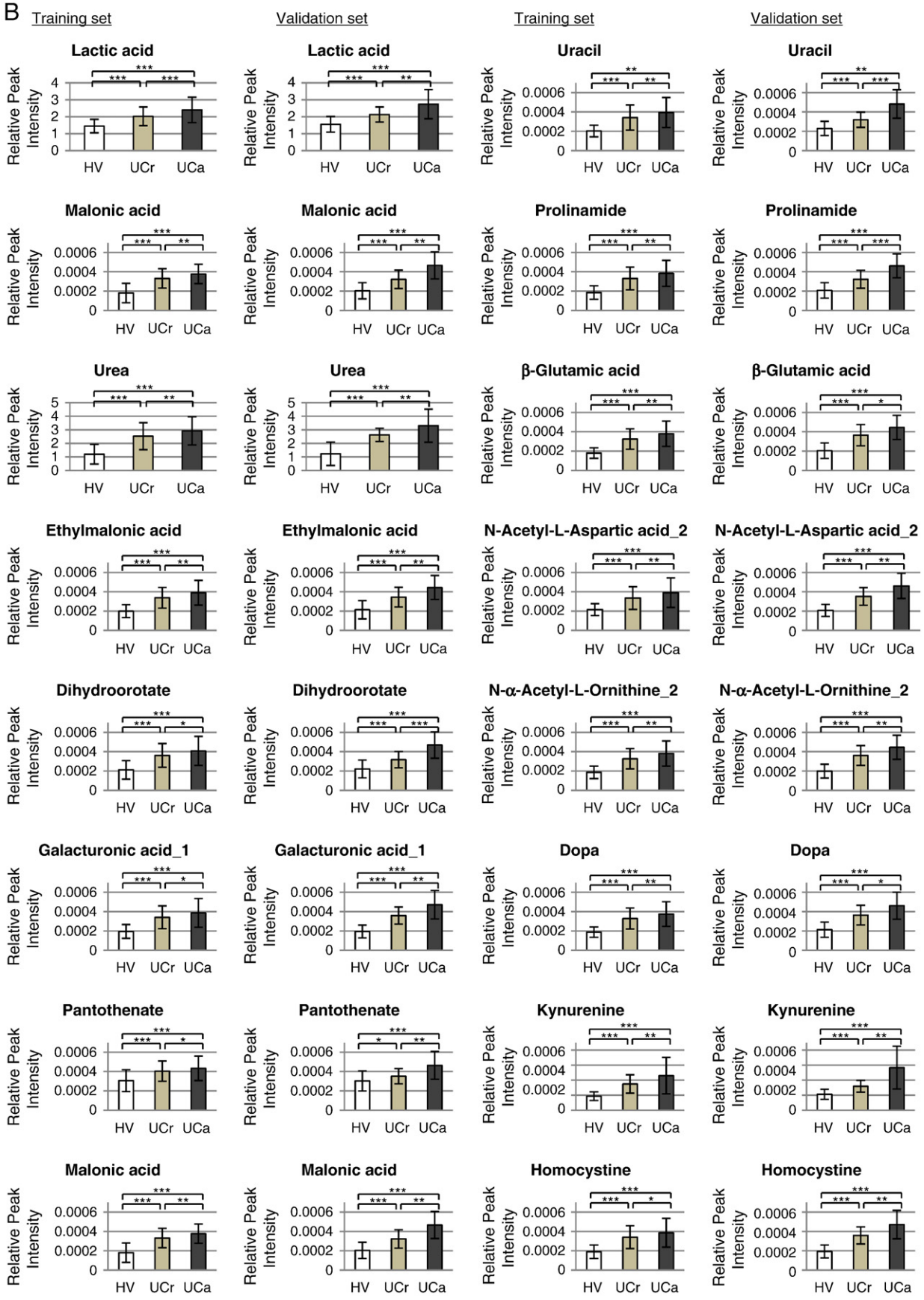

Figure 1 (*continued*).

Table 2 Variables selected by the stepwise method.

Metabolite	Coefficient	Standard error	P-value	Lower 95% CI	Upper 95% CI
(Intercept)	0.84	2.03	0.6802	−3.29	4.94
Taurine	−116.34	37.26	0.0018	−204.86	−55.76
S-Benzyl-L-cysteine	−335.31	116.46	0.004	−607.59	−142.98
Maleic acid	10801.09	5924.86	0.0683	162.81	24165.11
N-Acetyl-L-glutamine	2220.85	1371.78	0.1055	241.26	5001.77

P-values were calculated using the Wald test.

was 0.965, and its optimal cut-off value was 0.653. Its sensitivity and specificity values were 85.00% and 97.44%, respectively. In the validation set study, the metabolite data of the UC patients were subjected to this diagnostic model, which demonstrated that it displayed 83.33% sensitivity (Table 5). In addition, we examined whether disease location affected the ability of the model to discriminate between UC and CD. As a result, it was demonstrated that the model was able to discriminate between UC and CD regardless of the location of the disease (Supplemental Fig. 4).

Finally, we investigated whether an assessment model for UC could be established. In the training set study, 12 of the 114 metabolites were selected as metabolite candidates from a volcano plot according to the same criteria as were used for the previous two models (Supplemental Table 8, Supplemental Fig. 5), and 2 metabolites; i.e., histidine and p-hydroxybenzoic acid, were selected from the 12 metabolites via the stepwise method, neither of which displayed multicollinearity (Supplemental Table 9). An assessment model consisting of these 2 metabolites was established using multiple logistic regression analysis as follows (Table 6): $p = 1/[1 + e - \{-1.64 + 380.17(\text{histidine}) - 2559.38(\text{p-hydroxybenzoic acid})\}]$.

Based on the ROC curve obtained for this assessment model in the training set study (Fig. 4), its AUC was calculated as 0.967, and its optimal cut-off value was 0.421. In addition, its sensitivity and specificity values were 84.62% and 88.23%, respectively, whereas in the validation set study, its sensitivity and specificity values were 84.62% and 91.18%, respectively (Fig. 4, Table 7). Moreover, the values assigned to the patients by this assessment model exhibited a positive correlation with the CAI (Fig 5). The ability of this model to monitor disease activity was also evaluated. In this study, 7 UC patients were prospectively monitored (Fig 6). In the 4 active UC patients, the value of this model decreased when their CAI fell below 4 after treatment. In the 3 UC patients that were in remission, the model's value increased when their condition flared-up to such an extent that their CAI was elevated by more than 6 points, suggesting that our UC assessment model is useful for monitoring disease activity.

In addition, whether various factors such as body weight, BMI, serum levels of TP, Alb and T-chol, and medical treatment including medication, enteral nutrition and food restriction therapy influenced the alterations of selected metabolite biomarker candidates were examined using Spearman's rank-correlation coefficient and Wilcoxon rank sum test (Supplemental Tables 11, 12, 13, 15, 17, 18) The background information of the IBD patients who we picked up was summarized in Supplementary Tables 14 and 16. As a result, regarding 10 metabolites biomarker candidates; i.e., oxalate, 3-hydrobutyrate, maleic acid, p-hydroxybenzoic acid, ribulose, taurine, 1,6-anhydroglucose, N-acetyl-L-glutamate, histidine,

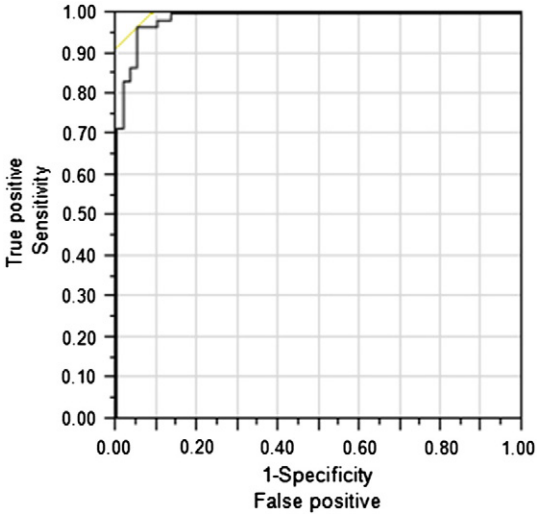

Figure 2 ROC curve for diagnostic model A. ROC curve for the diagnostic model constructed based on the training set. Its AUC and cut-off values were 0.9881 {95% confidence interval (95% CI): 0.9644–0.9961} and 0.6586, respectively.

Table 3 Diagnostic performance of our predictive model (diagnostic model A).

	Training set	Validation set	Additional validation set
	UC + HV	UC + HV	CD
Sensitivity (%)	93.33%	95.00%	–
Specificity (%)	95.00%	98.33%	0.26%
Accuracy (%)	94.17%	96.67%	–
PPV	94.91%	98.28%	–
NPV	93.44%	95.16%	–
LR+	18.67	57.00	–
LR−	0.07	0.05	–

PPV: positive predictive value, NPV: negative predictive value, LR+: positive likelihood ratio, and LR−: negative likelihood ratio. The accuracy of our diagnostic model was calculated using the cut-off value obtained by ROC analysis. The cut-off value of the model was 0.6586. In an additional validation set study, the diagnostic model was used to assign values to 39 CD patients.

Table 4 Variables selected by the stepwise method.

Metabolite	Coefficient	Standard error	P-value	Lower 95% CI	Upper 95% CI
(Intercept)	−1.36	1.15	0.2393	−3.86	0.82
Oxalate	2281.82	773.27	0.0032	1148.43	4446.28
3-Hydroxybutyrate	35.00	18.89	0.0640	8.40	84.47
Ribulose	−570.81	250.43	0.0227	−1142.15	−151.30
1,6-Anhydroglucose	−279.26	260.87	0.2844	−1051.20	−1.92

P-values were calculated using the Wald test.

S-benzyl-L cysteine_1, the significantly high correlation based on the following statistical criteria; P value < 0.05 and $r_s > 0.7$, or the significant alternation between 2 groups could not be confirmed except SASP. p-Hydroxybenzoic acid exhibited a high correlation with dose of SASP.

4. Discussion

The diagnosis of IBD is dependent on comprehensive criteria based on the results of clinical, endoscopic, histological, and radiological examinations; however, performing all of these examinations is expensive and takes a long time, making it difficult to achieve an early diagnosis. Therefore, rapid, low cost, and non-invasive diagnostic tools for IBD are required. In addition, it is also difficult to discriminate UC from CD in spite of the comprehensive criteria that are currently used to diagnose IBD. Therefore, a diagnostic tool for discriminating between the different types of IBD, which would enable clinicians to select an appropriate treatment at an earlier point, is also required. Previously, several serological biomarkers such as perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA), anti-*S. cerevisiae* antibodies (ASCA), *Escherichia coli* outer membrane porin (OmpC), *Pseudomonas fluorescens*-associated sequence (I2), and flagellin (CBir1) were suggested as diagnostic tools for IBD.[9,24–30]

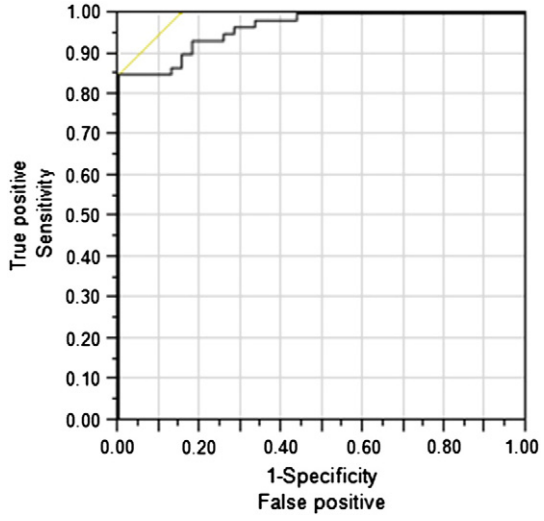

Figure 3 ROC curve for diagnostic model B. ROC curve for diagnostic model B constructed based on the training set. Its AUC and cut-off values were 0.9650 {95% confidence interval (95% CI): 0.9214–0.9848} and 0.6526, respectively.

OmpC, I2, and CBir1 display higher prevalences among CD patients (24–55%) than in UC patients (2–11%)[24–30] and so might be useful for discriminating between UC and CD. The combination of p-ANCA and ASCA is also considered to be a useful serological marker for discriminating between the different types of IBD, and pANCA+/ASCA− displays sensitivity and specificity values of 51% and 94%, respectively, for detecting UC.[9] Recently, Hisamatsu T et al. reported a new biomarker for IBD, multivariate indexes established from plasma aminograms (MIAI), which was constructed based on the plasma amino acid profiles of each type of IBD.[31] It displayed superior diagnostic utility compared with the abovementioned serological markers and was able to discriminate UC from CD in both the active (AUC for ROC = 0.879) and remission phases (AUC for ROC = 0.744). Our diagnostic model based on metabolite profiling was able to distinguish UC from CD with high sensitivity (85.00%) and specificity (97.44%) and displayed a high AUC value (0.965) (Fig. 3, Table 5).

To select appropriate treatments according to IBD status, a tool for assessing IBD that enables clinicians to evaluate disease activity is also required. The abovementioned serological biomarkers including ASCA and p-ANCA are not available for assessing disease activity, but fecal biomarkers, especially calprotectin, are suggested to be useful for monitoring UC.[32–35] These biomarkers exhibit strong correlations with not only mucosal activity according to

Table 5 Diagnostic performance of our predictive model (diagnostic model B).

	Training set	Additional validation set
	UC + CD	UC
Sensitivity (%)	85.00%	83.33%
Specificity (%)	97.44	–
Accuracy (%)	86.87%	–
PPV	98.08%	–
NPV	80.85%	–
LR+	33.15	–
LR−	0.15	–

PPV: positive predictive value, NPV: negative predictive value, LR+: positive likelihood ratio, and LR−: negative likelihood ratio. The accuracy of our diagnostic model was calculated using the cut-off value obtained from ROC analysis of the training set. The cut-off value was 0.6586. In an additional validation set study, diagnostic model B was used to assign values to the UC patients in the validation set.

Table 6 Variables selected by the stepwise method.

Metabolite	Coefficient	Standard error	P-value	Lower 95% CI	Upper 95% CI
(Intercept)	1.64	1.12	0.1439	−0.55	4.17
Histidine	−380.17	105.29	0.0003	−638.95	−208.44
p-Hydroxybenzoic acid	2559.38	1377.28	0.0631	865.61	6269.25

P-values were calculated using the Wald test.

endoscopy but also clinical activity based on UC symptoms in both adults (r_s = 0.67) and children (r_s = 0.68).[32,33] The MIAI has also been found to be a useful tool for assessing UC and is able to discriminate between UCr and UCa (AUC for ROC = 0.849). It also displayed a positive correlation with the CAI (r_s = 0.598). Our assessment model displayed a superior ability to evaluate the clinical activity of UC (r_s = 0.737; AUC for ROC = 0.967) (Figs. 4, 5A, Table 7). Therefore, both our diagnostic and assessment models are useful clinical tools for UC.

The IBD patients who we recruited in our study were administrated various medications such as salicylates, immunomodulatory agents and biological agents. The evaluation about the influence of each medication on metabolomic profiles is required for improving the accuracy of our model. In addition, it is also important to evaluate whether food intake or food restriction therapy including enteral nutrition therapy influenced the metabolite alterations or not. In our study, metabolite biomarker candidates which consisted of our diagnostic and assessment models did not have the significantly high correlation or significant alterations for the abovementioned factors except SASP, although the effects of tacrolimus, 6-mercaptopurine and adalimumab could not be evaluated due to the small number of the patients medicated with tacrolimus, 6-mercaptopurine and adalimumab (Supplemental Tables 11, 12, 13, 14, 15, 16, 17, 18). Only the dose of SASP displayed the significantly high correlation with the serum level of p-hydroxybenzoic acid. Regarding other medications, their sample numbers were small, although their evaluations were performed in this study, and therefore the more detailed evaluations about these issues may be required in the future. Then, in present study, the number of CD patients' sample was smaller than that of UC, so the further research adding more CD patients' samples may be also required to improve accuracy of our diagnostic models in the future.

The metabolite profile of UC seems to reflect the pathogenesis of the condition in more detail. Our metabolomic study detected significant alterations in the levels of 77 metabolites between HV and UC patients, and among these metabolites, 48, including several amino acids, TCA-related metabolites, and urea cycle-related metabolites, exhibited significantly decreased levels in the UC patients (Supplemental Table 1). Previously, it was demonstrated that stressors such as injury and infection enhanced glycolytic activity by increasing cellular glucose uptake, adenosine triphosphate turnover, and adenosine monophosphate production, which stimulate phosphofructokinase production[36,37]; however, a study of the global gene expression profile of the inflamed colonic tissues of UC patients using DNA microarrays detected the widespread downregulation of genes associated with the protein metabolism, the urea cycle, the citric acid cycle, and the oxidative electron transport.[38] Gene set enrichment analysis (GSEA) also demonstrated the downregulation of ribosomal and Krebs cycle proteins in the colons of experimental colitis mice,[39] and these reports seem to be in line with our findings.

Our metabolomic study identified several metabolites that showed characteristic alterations between UCa and UCr and/or between UCa/UCr and HV (Fig. 1A, B, Supplemental

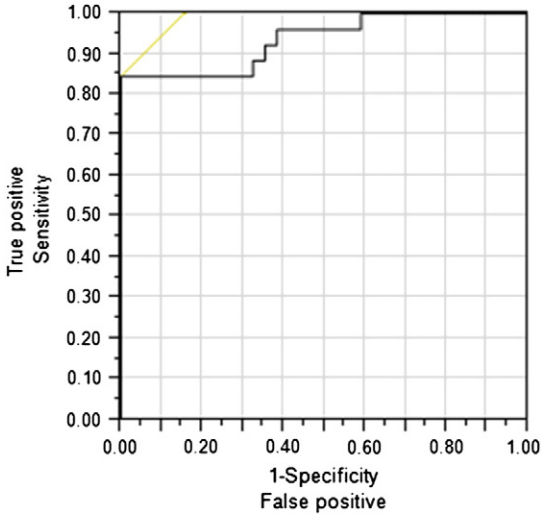

Figure 4 ROC curve for assessment model A. ROC curve for assessment model A constructed based on the training set. Its AUC and cut-off values were 0.9667 {95% confidence interval (95% CI): 0.8327–0.9777} and 0.4214, respectively.

Table 7 Diagnostic performance of our predictive model (assessment model A).

	Training set	Validation set
	UCr + UCa	UCr + UCa
Sensitivity (%)	84.62%	84.62%
Specificity (%)	88.23%	91.18%
Accuracy (%)	86.67%	88.33%
PPV	84.62%	88.57%
NPV	88.23%	95.16%
LR+	7.19	9.58
LR−	0.17	0.16

PPV: positive predictive value, NPV: negative predictive value, LR+: positive likelihood ratio, and LR−: negative likelihood ratio. The accuracy of our diagnostic model was calculated using the cut-off value obtained from ROC analysis of the training set.

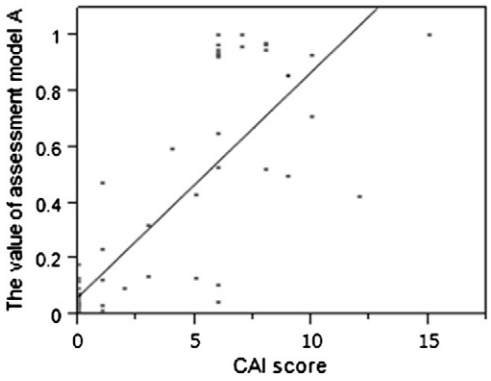

Figure 5 Correlation between the values assigned by assessment model A and the CAI in the validation set. A scatterplot with a regression line showing the correlation between the values assigned by assessment model A and the CAI in the validation set. {Spearman's rank correlation coefficient (r_s) = 0.7371 ($P < 0.001$), value assigned by assessment model A = 0.067 + 0.081 × CAI score}. The values assigned by assessment model A exhibited a positive correlation with the CAI.

Table 2) and whose levels were significantly correlated with the CAI in UC patients (Supplemental Table 3, Fig. 1A, B). IBD is regarded as the multifactorial disorder, and the complexity raises the necessary to take a careful consideration about IBD. However, these metabolites might reflect the pathogenesis of UC more closely. Of these metabolites, glutamine, histidine, and tryptophan have been well documented to have beneficial effects against inflammation. For example, the oral supplementation of an experimental colitis model with glutamine, histidine, or tryptophan exerted anti-inflammatory effects.[15,40,41] In addition, glutamine was demonstrated to play a key role in the maintenance of gut functions; i.e., it was shown that glutamine has gut-protecting effects and inhibits apoptosis by downregulating Sp3 expression in intestinal epithelial cells.[42] Glutamine also has anti-oxidative effects, and the glutamine supplementation of protein-depleted rats during inflammatory shock restored their jejunal glutathione concentrations.[43] In addition, significantly increased levels of kynurenine and decreased levels of tryptophan were detected in the UC patients in the present study (Supplemental Table 2, Fig. 1A, B), and these results might reflect the pathogenesis of UC. Tryptophan is metabolized into kynurenine by indoleamine-2,3-dioxygenase (IDO). This leads to immunomodulatory effects,[44–46] and it was reported that IDO is overexpressed in the intestinal lesions of UC patients.[47] Homocysteine and citrulline also exhibited significant alterations in their levels between the different patient groups in our study (Fig. 1A, B, Supplemental Table 2), and these metabolites are known to be associated with IBD. Homocysteine, an intermediate product in methionine metabolism, exhibited significantly increased serum levels in the UC patients (Fig. 1A, B, Supplemental Table 2). Hyperhomocysteinemia displays a prevalence of 30% in UC patients, which is higher than that seen in healthy controls.[48] Homocysteine promotes oxidative effects through its auto-oxidation and inhibition of glutathione peroxidase (GPx)[49] and was found to have thrombogenic effects on arterial endothelial cells,[50] suggesting that hyperhomocysteinemia induces vascular events. In IBD patients, the incidence of arterial and venous thromboembolic events was reported to be 1–8%,[51,52] and our results suggest that a relationship exists between homocysteine and the risk of vascular events in UC patients. In addition, significant alterations in the levels of citrulline and urea, which

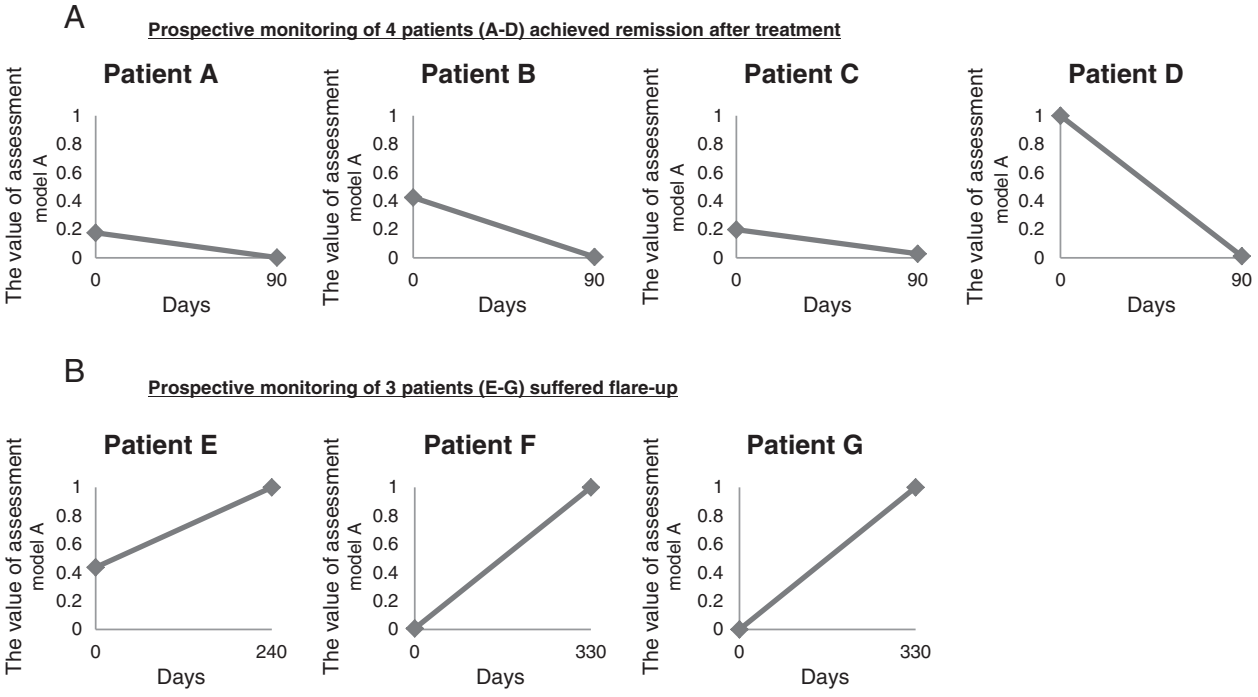

Figure 6 Prospective monitoring of UC with assessment model A. In our study, 7 UC patients were prospectively monitored. The seven upper graphs show the alterations in the values assigned to the 7 UC patients by assessment model A, respectively. Among them, 4 patients (A–D) achieved remission after treatment (A), and 3 patients (E–G) suffered flare-up (B).

are components of urea cycle-related metabolites, were also found in the UC patients in the present study (Fig 1A, B, Supplemental Table 2), and in a previous study the UC patients demonstrated significantly decreased plasma levels of ornithine and citrulline compared with the HV.[31] In the present study, the serum level of arginine, which is another component of the urea cycle, was positively correlated with the disease activity of UC, and arginine was found to exert anti-inflammatory effects in DSS-treated mice.[53,54] Therefore, our results suggest that UC patients suffer from a disorder that affects the metabolic pathways of the urea cycle.

In conclusion, using multiple logistic regression analysis we established novel and non-invasive serum metabolomics-based diagnostic and assessment models for UC. Our models demonstrated higher accuracy and/or stronger correlations with disease activity than previously reported biomarker candidates for UC. In addition, several metabolites displayed characteristic alterations in UC that varied according to disease activity. Our GC/MS based-metabolomic research will aid the elucidation of the pathogenesis of UC although IBD is one of the multifactorial disorders, and might lead to the development of a novel method for selecting appropriate treatments according to UC status.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.crohns.2014.01.024.

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