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ORIGINAL ARTICLE

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지방변의 대사체 진단에 있어서의 대사체학

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Metabolomic Study of a Diagnostic Model for the Metabolites of Stool Fat

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Background/Aims: Metabolomics is a powerful tool for measuring low-molecular-weight metabolites in an organism at a specified time under specific environmental conditions. The aim of this study was to determine the usefulness of metabolomics in identifying the metabolites in stool-fat-positive specimens, and to establish whether the results could be used to predict the long-term prognosis.
Methods: Fecal specimens were collected from 52 subjects with bowel habit change. The subjects were accessed using Rome III questionnaires and Bristol stool scale form, and followed after three years. The feces samples were centrifuged and the resulting extracts reconstituted for liquid chromatography/mass spectrometry analysis. The datasets were autoscaled, log-transformed, and mean-centered in a column-wise fashion prior to principal-components analysis and partial least-squares-discrimination analysis modeling.
Results: Fecal samples from 10 of the 52 patients gave a positive stool-fat result of 30-100 μm; those of the remaining 42 contained neither fatty acids nor neutral fats. The peak intensities of lithocholic acid (p=0.001), lysophosphatidyl ethanolamine (lysoPE) 16:0 (p=0.015), and lysoPE 18:1/0:0 (p=0.014) were correlated with the size of the fatty acid. Subjects with positive stool-fat result showed higher score in Bristol stool scale form than those with negative stool-fat result at initial (p=0.040) and after three years (p=0.012).
Conclusions: The metabolomic assay of stool fatty acid revealed mainly lysoPEs and lithocholic acid. The size of the fatty acid was correlated with higher concentrations of lysoPEs and lithocholic acid in stool-fat-test-positive specimens and related to loose stool even after three years of follow-up period. **(Korean J Gastroenterol 2013;61:9-16)**

Key Words: Bristol stool scale form; Fatty acid; Metabolomics; Stool fat

INTRODUCTION

Fecal matter can produce a positive result in the stool-fat test when the excretion of fat in the stool increases.[1] Such stools may also float due to the excess lipid, have an oily appearance, and be especially foul smelling. Increased fat excretion can be measured by determining the fecal fat level, and the sizes of fatty acids and neutral fats can be measured by the stool-fat test.[2] When a malabsorption disorder or other cause disrupts the process, emulsified dietary fats are not completely absorbed in the small intestine, resulting in a positive stool-fat test result.[3-6]

Genomics has proven itself invaluable for providing vast amounts of data regarding the expression of genes and pro-

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teins, whereas metabolomics provides data regarding all metabolic processes of a cell or organism.[7] Metabolomics is defined as the quantitative measurement of low-molecular-weight metabolites in an organism at a specified time under specific environmental conditions. Metabolomics can provide a broader insight into the biochemical composition of living organisms and how this composition changes with time and processing.[8] Recent developments in plant metabolomics have enabled the simultaneous detection of several hundred metabolites which makes reliable comparisons between nonspecific samples possible in an untargeted manner.[9,10] Metabolomics is a powerful tool that can be used to differentiate between the phytochemical composition of different origins, varieties, or products, and can be used for quality assessment.[10-12]

Metabolomics is a powerful platform for studies of the metabolism of a variety of biological samples but has not been systematically evaluated for stool-fat levels in subjects who do not have definite gastrointestinal diseases. In addition, the collection of stools is invariably difficult and often incomplete in clinical practice, since three sequential days of collection are required. It would be helpful to develop an innovative test that does not require this sequential stool-collection procedure. The aim of the present study was thus to determine whether indigenous fecal metabolomic analysis was useful for discriminating stool-fat-positive and stool-fat-negative cases.

SUBJECTS AND METHODS

1. Patient recruitment and sample collection

Consecutive patients who visited the Digestive Disease Center of Konkuk University Medical Center from December 2008 to February 2009 because of bowel habit change were asked to complete the stool tests and questionnaires on Rome criteria. Subjects with infectious disease or other causes that might induce diarrhea were excluded from the beginning by (i) history taking, (ii) stool cultures including *Salmonella*, *Shigella*, *Campylobacter*, and *Vibrio* cultures, and (iii) stool occult blood test. Subjects with cholecystecomy, small bowel resection, or specific cause that may cause abnormal finding on stool fat test were also excluded from the study. This includes malabsorption (inflammatory bowel disease, celiac disease, and abetalipoproteinaemia), exocrine pancreatic insufficiency, pancreatitis, choledocholithiasis (obstruction of the bile duct by a gallstone), pancreatic cancer (if it obstructed biliary outflow), primary sclerosing cholangitis, bacterial overgrowth, short bowel syndrome, cystic fibrosis, Zollinger-Ellison syndrome, protozoan parasite infection, or a intake of certain prescribed slimming pills such as Orlistat.

Because most of the patients refused stool tests at the outpatient department, only a cohort of 52 subjects were finally included in the study. The fecal samples were collected for stool-fat quantification test in all of the subjects. Of 52 subjects, 15 subjects revealed irritable bowel syndrome (IBS) based on the Rome III criteria. Fifteen IBS patients were consisted of 11 diarrhea-dominant type, 2 constipation- dominant type, and 2 mixed type IBS. Main complaints of bowel habit change in 37 non-IBS subjects were; (i) loose stool in 34 subjects and (ii) hard stool in 3 subjects. After three years of study enrollment, the subjects were followed up again using Rome III questionnaires and Bristol stool scale form. All patients provided informed consent, and this study was approved by the Institutional Review Board of Konkuk University Medical Center.

2. Stool-fat test

Presence of stool fat in fecal material was evaluated by Sudan III stain as described in a previous study.[13] Neutral fat appeared in orange color upon ethyl alcohol, and fatty acid was stained after managing with acetic acid and heating. Of lipid droplets, largest size was reported as a result in micrometer (μm). Neutral fat which can be detected in malabsorption disorders and Vitamin B12 deficiency, was reported positive if more than 60/high power field (HPF). Fatty acid which can be detected in obstructive jaundice due to bile juice deficiency, was reported negative if less 100 (1-4 μm)/HPF. Fatty acid was reported positive if more than 100 (6-75 μm)/HPF.

3. Specimen processing

High performance liquid chromatography (HPLC) grade Methanol for extraction was purchased from Burdick & Jackson (Muskegon, MI, USA) as described in previous studies.[14,15] Acetonitrile and water used in liquid chromatography/mass spectrometry (LC/MS) were HPLC grade from Fisher Scientific (Pittsburgh, PA. USA). Formic acid was ob-

tained from Sigma Chemical Co. (St. Louis, MO, USA).

4. Preparation and extraction of fecal samples

All samples were immediately frozen at stored at −80°C before analysis. Feces (mean weight 100 mg/sample) homogenate were mixed with 300 μL of methanol, vortexed for 1 min, sonicated for 1 min and centrifuged 160 g for 15 min at 4°C. Of the upper organic phase, the extract obtained was filtered through a 0.22 μm filter. The extracts was reconstituted in water : sample (10 : 1; v/v) before LC/MS analysis.

5. Liquid chromatography–electrospray interface/mass spectrometry conditions for metabolite profiling

The LC/MS instrument were consisted of a 212-LC Binary Solvent Delivery System, a Prostar™ 410 AutoSampler, a Prostar™ 335 photodiode array detector, and coupled to a 500-ion trap mass spectrometer equipped with an electrospray interface from Varian Technologies (Palo Alto, CA, USA). Chromatographic separations were performed in a 150×2.0 mm inside diameter (i.d.), 3 μm PurSuit XRs C18 column (Varian Inc., Lake Forest, CA, USA) with a MetaGuard 2.0 PurSuit XRs C18 guard column (Varian Inc.) with flow rate at 0.2 mL/min. The column was maintained at 30°C and eluted with the mobile phase consisting of a combination of A (0.1% [v/v] formic acid in water) and B (0.1% [v/v] formic acid in acetonitrile). Samples were run with the isocratic 10% B (v/v) for 2 min, and a linear gradient of 100% B for 30 min, to heal at 100% B for 35 min, and then decreased linearly to 10% until 35.06 min and held for 40 min for the next analytical sample at a flow rate of 0.2 mL/min. A 10 μL aliquot of each fraction was subsequently injected into an HPLC system. The full scan mass spectra were recorded in the range of mass-to-charge ration (*m/z*) 100-1,000. The operating parameters in the negative ion mode for samples were as follows: a spray needle voltage of ±5 kV, a capillary voltage of 70 V, a drying gas temperature of 300°C, the drying gas pressure as nitrogen of 20 psi, a nebulizer gas pressure as air of 40 psi, and trap damping gas as helium with flow rate 0.8 mL/min. Tandem mass spectrometry analysis showing were investigated using scan type turbo data dependent scanning (DDS). The collision-induced dissociation voltage was automatically set by DDS. Data were acquired in continuous mode within a mass scan mode 15,000 u/sec. Mass scan averaged was set at 3 microscans (2.19 sec/scan).

6. Ultra performance liquid chromatography/electrospray interface–quadrupole–time–of–flight condition for identification of metabolites

Ultra performance liquid chromatography was conducted using a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) with a Waters ACQUITY BEH C18 column (100×2.1 mm i.d., 1.7 μm; Waters). The column was maintained at 37°C and eluted with the mobile phase consisting of a combination of A (0.1% [v/v] formic acid in water) and B (0.1% [v/v] formic acid in acetonitrile) at a flow rate of 0.3 mL/min with the injection of a 3 μL sample. The eluting conditions were performed as follows: linear gradient of B from 20% to 100% for 3 min, to heal at 100% B for 3.7 min, and then decreased linearly to 20% until 3.8 min and held for 5 min for the next analytical sample. The full scan mass spectra were recorded in the range of *m/z* 100-1,500. The operating parameters in the negative ion mode for samples were as follows: capillary of

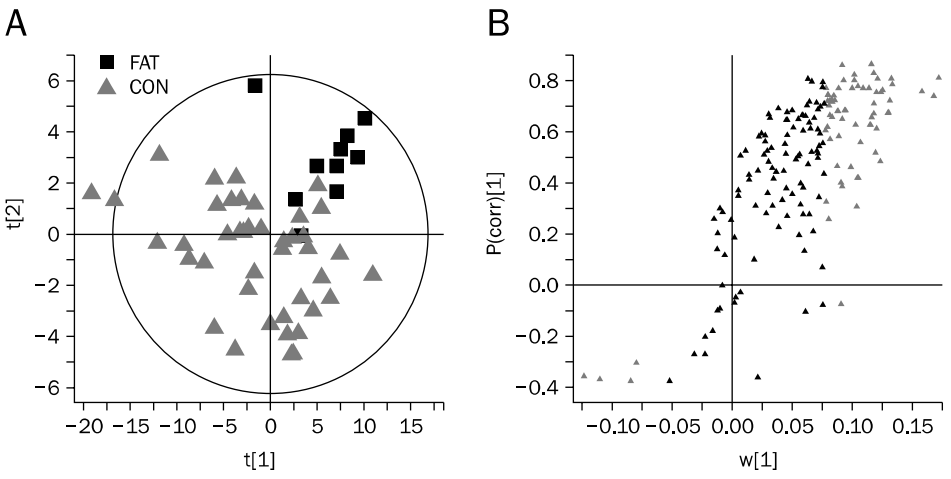

Fig. 1. Partial least-squares discrimination analysis of liquid chromatography/mass spectrometry-based metabolic profiles for the stool-fat-positive (FAT) group (n=10) and the stool-fat-negative (CON) group (n=42). (A) Score plot between FAT (square) and CON (triangle). (B) Loading plot for 66 variables selected by VIP>1. t[1], variables accounted for 31.6% of the measured variance; P (corr), positive correlation.

2.8 kV, sample cone of 20.0 V, extraction cone of 1.2 V, source temperature of 120°C, desolation temperature of 320°C, ion energy of 1.0 V, and collision energy of 5 V, respectively.

7. Data processing and multivariate analysis

LC-MS data were acquired and analyzed using MS workstation software (version 6.9; Varian Inc.). The raw (*.xms) files were subsequently converted into netCDF (*.cdf) formats using Vx Capture software (version 2.1; Adron Systems, Laporte, MN, USA). After conversion, the files were subjected to preprocessing, peak extraction, retention time correction, and alignment using various forms of chromatography mass spectrometry (XCMS).[7] The XCMS parameters were input in R 2.9.0 language (www.bioconductor.org) using simple commands as XCMS's default settings method (http://masspec.scripps.edu/xcms/documentation.php). The resulting peak list as a txt file was exported to Microsoft Excel (Microsoft, Redmond, WA, USA). A list of the detected peaks was generated using the average area, corrected retention time, and *m/z* data as the identifier for each peak. The resultant data matrix containing the sample name as observation and peak area information as variables was processed by SIMCA-P software 12.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. The datasets were auto-scaled (unit variance scaling) and log-transformed with mean-centered in a column-wise fashion prior to principal component analysis and partial least squares-discrimination analysis (PLS-DA) modeling. Univariate statistics for multiple classes were conducted via breakdown and unpaired Student's t-test using Excel (Microsoft). Statistical analyses were done using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA) for clinical data related to Bristol stool scale form. The data were reported as median with ranges for continuous variables and percentage with 95% CI for categorical variables. A difference with p

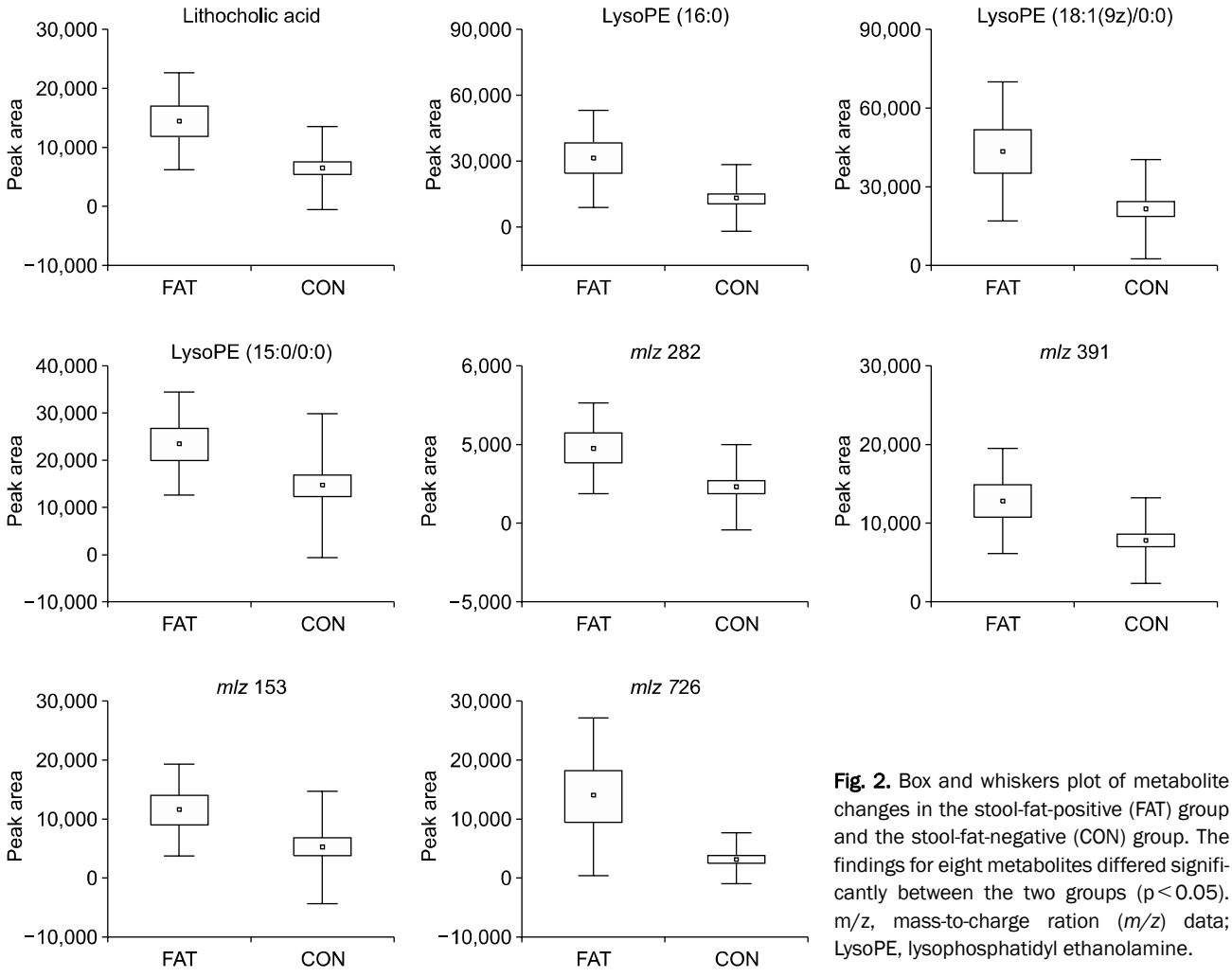

Fig. 2. Box and whiskers plot of metabolite changes in the stool-fat-positive (FAT) group and the stool-fat-negative (CON) group. The findings for eight metabolites differed significantly between the two groups ($p < 0.05$). m/z, mass-to-charge ration (*m/z*) data; LysoPE, lysophosphatidyl ethanolamine.

Table 1. Masses Defined through Analysis Using High-resolution Mass Spectrometry

Raw mass (m/z)	Retention time	Fold	p-value	TOF mass (m/z)	Compound name	HMDB formula	Error (mDa)	Matching MW
727	17.4	4.3	0.036	-	-	-	-	-
452	27.3	2.5	0.014	452.2750	LysoPE 16:0	C21H44NO7P	−2.7	453.26
375	28.7	2.2	0.015	375.2931	Lithocholic acid	C24H4003	3.2	376.30
153	4.5	2.2	0.042	152.9848	-	-	-	-
282	11.4	2.1	0.027	-	-	-	-	-
478	28.2	2.0	0.030	478.2931	LysoPE 18:1(9z)/0:0	C23H46NO7P	−0.4	479.30
438	25.3	1.6	0.048	438.2627	LysoPE 15:0/0:0	C20H42NO7P	0.6	439.27
391	25.5	1.7	0.048	391.2802	-	-	-	-

m/z, mass-to-charge ration (*m/z*) data; TOF, time-of-flight; HMDB, human metabolome database; MW, molecular weight.

< 0.05 was considered significant.

RESULTS

1. Metabolite profiles in stool-fat-positive and -negative groups

Of 52 subjects, 10 subjects showed stool fat ranging from 30-100 μm, whereas other 42 subjects revealed 0 μm. Score plot on PLS-DA revealed a significant difference between the stool-fat-positive group and -negative group (Fig. 1A). A total of 66 variables were selected for the analysis as a loading plot (Fig. 1B). PLS-DA of stool-fat-positive versus -negative group revealed an R^2X (cum) of 0.316 and a Q^2 (cum) of 0.0906. S loading plot corresponding to PLS-DA scores plot showed ($p < 0.05$). Eight raw masses were significantly increased in the stool-fat-positive group than the -negative group (Fig. 2).

2. Significant components of the stool-fat-positive group

Masses defined through the analysis assigned in HRMS are summarized in Table 1. Significant components of the stool-fat-positive group revealed lithocholic acid, lysoPE 16:0/0:0, lysoPE 18:1(9z)/0:0, and lysoPE 15:0/0:0 in raw mass 452, 375, 478, and 438, respectively. Formula was accessible only in four raw mass, whereas mass-to-charge ration (*m/z*) data was accessible in six raw mass ranging from 152 to 478. It revealed C21H44NO7P for lysoPE 16:0 (p=0.014), C24H4003 for lithocholic acid (p=0.015), C23H46NO7P for lysoPE 18:1(9z)/0:0 (p=0.003), and C20H42NO7P for lysoPE 15:0/0:0 (p=0.048).

Table 2. Link between the Size of a Fatty Acid and Its Metabolites

	Size of fatty acid			p-value
	0 μM	1-50 μM	51-100 μM	
Lithocholic acid	4,640 (301-28,648)	10,556 (4,901-22,478)	24,217 (21,085-27,348)	0.001
LysoPE (16:0)	7,355 (730-87,232)	34,642 (7,105-82,036)	37,391 (14,614-60,168)	0.015
LysoPE (18:1/0:0)	15,144 (388-90,613)	30,091 (15,829-76,560)	52,208 (22,037-82,378)	0.014
LysoPE (15:0/0:0)	9,994 (240-68,382)	19,311 (5,438-36,412)	31,674 (23,879-39,468)	0.183

Values are expressed as median (range).
LysoPE, lysophosphatidyl ethanolamine.

3. Link between the type of metabolite and the sizes of stool fat

Peak intensities of metabolites were related to sizes of fatty acid and neutral fat (Table 2). Lithocholic acid (p=0.001), lysoPE 16:0 (p=0.015), and lysoPE 18:1/0:0 (p=0.014) were increased in specimens with large-sized fatty acid. Sizes of fatty acid and neutral fat in stool-fat-positive cases are summarized in Table 3. With regard to the peak intensities of lithocholic acid, lyso PE 16:0, lysoPE 18:1(9z)/0:0, and lysoPE 15:0/0:0, four subjects that showed both fatty acid and neutral fat were not significantly different from the subjects who showed positive findings only on fatty acid without neutral fat.

4. Long-term follow up results

All of the 52 subjects were followed after three years of initial enrollment. A phone call interview was done if the patient did not visit the outpatient clinic. Among 52 subjects who have participated in this study between December 2008 and

Table 3. Sizes of Fatty Acids/Neutral Fats and Peak Intensities of Metabolites in Stool-fat-test-positive Patients

Gender/Age (yr)	Size of fatty acid (μM)	Size of neutral fat (μM)	Peak intensity of lithocholic acid	Peak intensity of lysoPE 16:0	Peak intensity of lysoPE 18:1(9z)/0:0	Peak intensity of lysoPE 15:0/0:0
M/35	30	0	16,966.96	45,049.74	76,560.70	21,759.79
F/47	30	0	22,478.13	82,036.39	56,704.74	16,863.56
F/67	30	0	5,277.719	28,120.99	75,822.84	14,606.92
F/38	40	40	4,901.545	7,105.938	34,684.49	5,438.531
F/48	45	50	18,506.04	41,164.15	24,853.82	36,412.34
M/31	50	70	6,414.613	12,602.15	25,498.29	15,581.43
M/46	50	0	13,973.96	43,429.49	20,507.02	25,836.44
M/62	50	30	7,138.484	9,633.017	15,829.03	35,252.89
F/30	80	0	21,085.16	60,168.72	82,378.52	23,879.18
F/29	100	0	27,348.93	14,614.56	22,037.56	39,468.80

LysoPE, lysophosphatidyl ethanolamine.

Table 4. Bristol Stool Form Scale in Stool-fat-test-positive and Negative Patients

Bristol stool form and metabolites	Positive stool fat test (n=10)	Negative stool fat test (n=42)	p-value
Bristol stool form at the time of enrollment			0.040
Type 1 - Separate hard lump (like nuts)	0	1 (2.4)	
Type 2 - Sausage-shaped but lumpy	0	1 (2.4)	
Type 3 - Like a snake with crack	0	14 (33.3)	
Type 4 - Like a smooth and soft snake	1 (10)	13 (30.9)	
Type 5 - Soft blobs with clear-cut edge	3 (30)	7 (16.7)	
Type 6 - Fluffy pieces with ragged edge	5 (50)	5 (11.9)	
Type 7 - Watery (no solid pieces)	1 (10)	1 (2.4)	
Bristol stool form after 3 years of follow-up			0.012
Type 1 - Separate hard lump (like nuts)	0	0	
Type 2 - Sausage-shaped but lumpy	0	2 (4.8)	
Type 3 - Like a snake with crack	1 (10)	11 (26.2)	
Type 4 - Like a smooth and soft snake	1 (10)	12 (28.6)	
Type 5 - Soft blobs with clear-cut edge	1 (10)	9 (21.4)	
Type 6 - Fluffy pieces with ragged edge	5 (50)	8 (19.0)	
Type 7 – Watery (no solid pieces)	2 (20)	0	
Lithocholic acid	15,470 (4,901-27,348)	4,640 (301-28,648)	0.004
LysoPE (16:0)	34642 (7,105-82,036)	7,355 (730-87,232)	0.004
LysoPE (18:1/0:0)	30091 (15,829-82,378)	15,144 (388-90,613)	0.004
LysoPE (15:0/0:0)	22,819 (5,438-39,468)	9,994 (240-68,382)	0.103

Values are expressed as number (%) or median (range).
LysoPE, lysophosphatidyl ethanolamine.

February 2009, only one patient (a 71 year-old man who showed normal stool fat test at the initial finding) complained of minor bowel habit change. His symptom was not consistent with IBS on Rome III criteria, and did not show any significant abnormal finding on the follow-up examination. Other 51 subjects had completely recovered from their initial symptoms, and had no abdominal discomfort after a three-year follow-up period. According to the Bristol stool scale form, subjects with positive stool-fat result showed loose stool than those with negative stool-fat result both at the time of study enrollment (p=0.040) and after three years of follow-up period (p=0.012). Mean values of the intensities of lithocholic acid, lysoPE 16:0, and lysoPE 18:1/0:0 were significantly higher in subjects with positive stool-fat result (Table 4).

DISCUSSION

Several metabolites were positively correlated with the sizes of fatty acids and neutral fats in this study, and were re-

lated to loose stool not only at the beginning of this study but also after three years. Stool-fat-positive fecal materials contained several significant components including lysoPE and lithocholic acid that were not found in stool-fat-negative fecal materials. Our findings demonstrate that the metabolomic analysis of fecal metabolite concentrations can reveal specific fecal metabolites that produce a positive stool-fat test result. A particularly interesting finding was that increases in the amount of stool fat appeared to parallel the amounts of lysoPE 16:0, lithocholic acid and lysoPE 18:1(9z)/0:0. LysoPEs are phospholipids, whereas lithocholic acid comprises mainly bile acids and fat solubilization and waste products.[16] Since lithocholic acid is a bile acid produced from bacterial action on chenodeoxycholate and usually conjugated with glycine or taurine, it can be used as a cholagogue and a choleretic.

To the best of our knowledge, this is the first study to demonstrate fecal metabolic differences between stool-fat-positive and stool-fat-negative subjects. The stool-fat test detects steatorrhea (i.e., excess fat in bowel movements) due to fat malabsorption, whereby a Sudan Red stain applied to a smear on a microscope slide is used to estimate the amount of fat in the stool. The application of metabolomics to identify the positivity and amount of fat in fecal material demonstrates the potential utility of this technique in clinical applications for detecting when less fat has been absorbed through the gut wall, indicating fat malabsorption. We expect that this tool can be applied as a noninvasive technique for the diagnosis of stool-fat-positive specimens. A better understanding of the mechanisms responsible for disease pathogenesis, as well as the development of less invasive diagnostic and disease-monitoring techniques, will improve the quality of life of the many individuals who suffer from loose stools.

Lipids were originally thought to enter the enterocytes via passive diffusion, but there is now some evidence that carriers are involved.[5,6] Lipids are rapidly esterified inside cells, maintaining a favorable concentration gradient from the lumen into the cells. The fate of the fatty acids in enterocytes depends on their size. Fatty acids containing less than around 11 carbon atoms are sufficiently water-soluble that they pass through the enterocyte unmodified and are actively transported into the portal blood, and subsequently circulate as free (unesterified) fatty acids. Fatty acids containing more than around 11 carbon atoms are too insoluble for this mechanism to occur, and are reesterified to form triglycerides within the enterocytes; some of the absorbed cholesterol is also esterified. The triglycerides and cholesterol esters are then coated with a layer of protein, cholesterol, and phospholipid to form chylomicrons. These leave the cell and enter the lymphatic vessels, because they are too large to pass through the junctions between capillary endothelial cells. Although we could not verify the exact mechanism in the present study, this mechanism supports our data since the intensities of metabolites such as lithocholic acid, lysoPE 16:0, and lysoPE 18:1/0:0 were significantly related to the sizes of fatty acids and neutral fat.

In summary, we have demonstrated the presence of metabolites in stool-fat-positive specimens. Metabolites such as lithocholic acid and lysoPE are related to the amount of fatty acid in stool that produces a positive result in the Sudan Red stool-fat testm and are related to loose stool in Bristol stool scale form. Stool-fat-test-positive subjects show higher frequency of loose stool even after three years.

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