Replacing carbohydrate with protein and fat in prediabetes or type-2 diabetes: greater effect on metabolites in PBMC than plasma

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Abstract

Background: Active metabolism of peripheral blood mononuclear cells (PBMC) could suggest their suitability for metabolomics studies. This study examined whether reductions in PBMCs and plasma lipoprotein-associated phospholipase A2 (Lp-PLA2) activities induced by dietary intervention affected the overall metabolic profiles of PBMC and plasma.

Methods: Eighty nonobese subjects aged 40–70 years (18.5 ≤ BMI < 30 kg/m²) with prediabetes or newly-diagnosed type-2 diabetes were assigned to consume either the usual refined-rice diet (control group, n = 40) or to replace refined rice with whole grains and legumes as carbohydrates (whole-grain group, n = 40) for three meals per day during the 12-week intervention. Fasting PBMC and plasma metabolomes were profiled using UPLC-LTQ-Orbitrap mass spectrometry.

Results: After 12 weeks, changes in fasting glucose, HbA1c, HOMA-IR, MDA, ox-LDL, LDL particle size, plasma Lp-PLA2 activity, and PBMC enzyme activity in the whole-grain group were significantly different from those in the control group before and after adjusting for baseline levels. The PBMC levels of L-leucine, oleamide, lysoPC (16:0), and lysoPC (18:0) in the whole-grain group showed greater reductions compared with those of the control group. Changes in plasma metabolites were not significantly different between the two groups. Changes in PBMC Lp-PLA2 activity positively correlated with changes in L-leucine, oleamide, lysoPC (16:0), lysoPC (18:0), glucose, and ox-LDL, and negatively correlated with changes in LDL particle size.

Conclusions: This study showed that dietary intervention in prediabetic or type-2 diabetic patients had a greater effect on PBMC Lp-PLA2 activity and metabolites compared with those of plasma metabolites.

Trial registration: NCT02191644

Keywords: Metabolites, Peripheral blood mononuclear cells, Prediabetes, Whole-grains and legumes
Background

Diabetes is an epidemic metabolic disorder; about 2.7 million Korean people (8.03%) aged 30 years or older had type-2 diabetes (T2D) and 25.0% of adults had prediabetes in 2013 according to Korean Diabetes Fact Sheet 2015. Diabetes-related mortality was steadily decreased since 2003 and ranked as the fifth leading cause of natural death [1]. Prediabetes can be indicated by either impaired fasting glucose (IFG) by the American Diabetes Association criteria [2] or impaired glucose tolerance (IGT) by World Health Organization criteria [3]. Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) independently predicts T2D incidence and may be involved in its etiology [4]. In recent study, an inverse association was observed between protein intake and circulating Lp-PLA₂ activity, suggesting that nutritional factors may influence Lp-PLA₂ activity [5]. An intervention study that replaced refined rice with whole grains and legumes reduced blood glucose, insulin, Lp-PLA₂ activity, and cardiovascular risk factors in patients with prediabetes or T2D [6]. The effects of this intervention diet on plasma and peripheral blood mononuclear cell (PBMC) metabolites have not been determined.

PBMCs include monocytes and lymphocytes which are blood cells having a round nucleus. These blood cells are a critical component in the immune system to fight infection and adapt to intruders. Monocytes have a key role in onset and development of inflammatory reactions by generating bioactive molecules such as Lp-PLA₂ in response to inflammatory stimuli [7]. Lymphocytes are consist of three major types; T cells, B cells, and natural killer cells. T cells and B cells are the major cellular components of the adaptive immune response, whereas natural killer cells are a part of the innate immune system. The production and release of Lp-PLA₂ by lymphocytes may become increased under inflammatory conditions [8]. Dietary intervention induces PBMC gene expression changes, including downregulating genes involved in inflammatory processes [9]. Therefore, changes in PBMC metabolites and Lp-PLA₂ activity after dietary intervention could reflect dynamic responses, which are not detectable in plasma metabolomics analyses. The aim of this 12-week intervention study was to examine whether reductions in PBMC and plasma Lp-PLA₂ activities induced by dietary intervention (replacement of refined rice with whole grains and legumes, and higher intake of vegetables) affected the overall metabolic profiles of PBMC and plasma in nonobese patients that exhibited IFG, IGT, or newly-diagnosed T2D.

Methods

Subjects and study design

Nonobese subjects aged 40–70 years (18.5 ≤ BMI < 30 kg/m²) were recruited from the Health Service Center (HSC) at the Ilsan Hospital, Goyang, Korea, during January–June 2013. Based on the HSC data, subjects who had IFG (100 ≤ fasting glucose < 126 mg/dL) or newly-diagnosed T2D (fasting glucose ≥126 mg/dL) were referred to the Department of Family Medicine or Internal Medicine. Exclusion criteria included: current and/or past history of cardiovascular disease; liver or kidney dysfunction; thyroid or pituitary disease. Subjects who were taking medications or supplements also were excluded. A total of 82 subjects were enrolled. The macronutrient composition of each subject’s usual diet corresponded to a typical diet with cooked refined rice. The purpose of the study was carefully explained to all participants, and written consent was obtained prior to their participation. The Institutional Review Board of the NHIC-sponsored Ilsan Hospital and Yonsei University provided ethical approval of the study protocol, which was performed according to the Helsinki Declaration.

The present study was performed in two phases, including a 2-week run-in phase consisting of the usual diet with refined rice, and a 12-week intervention phase. During the run-in period, two subjects who did not maintain their energy intake dropped out. The remaining 80 subjects were randomly subdivided into the two study groups, and were assigned to consume either the usual refined-rice diet (control group, n = 40) or to replace refined rice with whole grains and legumes as carbohydrates (whole-grain group, n = 40) for three meals per day during the 12-week intervention.

Assessment of dietary intake and physical activity level

All subjects were given written and verbal instructions by a registered dietitian on completion of a 3-day (2 week days and 1 weekend day) dietary record every 2 weeks throughout the study. On the dietary record sheet, subjects were instructed to weigh and record the food amount before and after ingestion. All participants were advised to continue their usual refined-rice diet during a 2-week run-in period. Baseline measurements were performed at the start of the run-in phase. After a run-in period, subjects in the control group maintained the usual refined-rice diet, whereas subjects in the whole-grain group replaced refined rice with a mix of 1/3 legumes, 1/3 barley, and 1/3 wild rice three times per day, and increased vegetable intake to at least 6 units (30–70 g/unit) per day for sufficient dietary fiber intake. The dietitian monitored subject compliance and body-weight changes during the whole study by performing biiweekly visits or telephone interviews and all participants were encouraged to maintain their usual lifestyles. Dietary energy values and nutrient contents from 3-day food records were calculated using the CAN-pro 3.0 (Korean Nutrition Society, Seoul, Korea). Total energy expenditures (kcal/day) were calculated from activity....
patterns including basal metabolic rate, physical activity for 24 h [10], and specific food dynamic action. Basal metabolic rate for each subject was calculated with the Harris–Benedict equation [11].

**Anthropometry and blood pressure analysis**

Body weight and height of unclothed subjects without shoes were measured in the morning for calculating body mass index (BMI, kg/m²). Waist circumference was measured on standing subjects at the umbilical level after normal expiration. Blood pressure (BP) of seated subjects after a 20-min rest was measured in the left arm with an automatic BP monitor (FT-200S, Jawon Medical, Gyeongsan, Korea). After a 12-h fasting period, venous blood specimens were collected in EDTA-treated and plain tubes and centrifuged to yield plasma or serum, respectively, which were stored at −70 °C until analysis.

**Clinical measurements**

Fasting total cholesterol and triglyceride levels were analyzed using a Hitachi 7600 Autoanalyzer (Hitachi Ltd., Tokyo, Japan). ApoB-containing lipoproteins were precipitated with dextran-magnesium sulfate, and high density lipoprotein (HDL)-cholesterol concentrations in patient serum samples were measured enzymatically. For subjects with serum triglyceride levels <400 mg/dL, low density lipoprotein system (CBS Scientific Company, San Diego, CA) on commercially available, non-denaturing gels containing a linear 2–16 % acrylamide gradient (CBS Scientific Company). Latex-bead (30 nm) conjugated thyroglobulin (17 nm), ferritin (12.2 nm), and catalase (10.4 nm) standards were used to estimate the relative band migration rates. Gels were scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). Plasma oxidized (ox)-LDL was measured using an enzyme immunoassay (Mercodia AB, Uppsala, Sweden), and the resulting color reaction was determined at 450 nm on a Wallac Victor® multilabel counter (Perkin-Elmer Life Sciences, Boston, MA).

**Global (nontargeted) metabolic profiling of PBMC and plasma**

**PBMC and plasma extract sample preparation**

Before analysis, 800 μL of 80 % acetonitrile was added to 100 μL of PBMC and plasma, mixed by vortexing, and centrifuged at 10,000 rpm for 5-min at 4 °C. The supernatant was dried with N₂ (l), dissolved in 10 % methanol, mixed by vortexing, and centrifuged at 10,000 rpm for 5-min at 4 °C. The supernatant was transferred into a vial.

**Ultra performance liquid chromatography**

PBMC and Plasma extract samples (4 μL) were injected into an Acquity UPLC-BEH-C18 column (2.1 × 50 mm, 1.7 μm; Waters, Milford, MA) that was coupled in-line with a UPLC-LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA). The injected samples were equilibrated with water containing 0.1 % formic acid. Samples were eluted with an acetonitrile gradient containing 0.1 % formic acid at a flow rate of 0.35 mL/min for 20-min. Metabolites were separated by UPLC, analyzed, and assigned by LTQ-
Orbitrap-XL. The mass spectrometer (MS) was operated in ESI-positive mode. The spray voltage was 5 kV. The flow-rate nitrogen sheath gas and the auxiliary gas were 50 and 5 (arbitrary units). The capillary voltage (V), tube-lens voltage (V), and capillary temperature (°C) were kept constant at 35 V, 80 V, and 370 °C. Orbitrap data were collected in the range of m/z 50–1,000. MS/MS spectra of metabolites were obtained by a collision-energy ramp from 55–65 eV, and conducted with Xcalibur 2.1 and MS Frontier software (Thermo Fisher Scientific).

Data processing and identification of metabolites
All MS data including retention times, m/z, and ion intensities were extracted by SIEVE software (Thermo Fisher Scientific) incorporated into the instrument, and the resulting MS data were assembled into a matrix. SIEVE parameters were set as follows: m/z range 50–1,000; m/z width 0.02; retention time width 2.5; and m/z tolerance 0.005. Metabolites were searched using the following databases: ChemSpider (www.chemspider.com), Human Metabolome (www.hmdb.ca), Lipid MAPS (www.lipidmaps.org), KEGG (www.genome.jp/kegg), and MassBank (www.massbank.jp). Selected metabolites were confirmed by retention times and mass spectra of standard samples.

Statistical analyses
Statistical analyses were performed using SPSS v. 21.0 (IBM SPSS Statistics 21, Chicago, IL). Skewed variables were logarithmically transformed for statistical analyses. A two-tailed P-value of <0.05 was considered statistically significant. Differences in biochemical variables between two groups at baseline and follow-up were tested using Student's independent t-test. General linear model tests were applied to compare parameter changes between the two groups by adjusting for baseline values. Paired t-tests were used to evaluate differences between baseline and follow-up levels in each group. Pearson's and partial correlation coefficients were used to examine the relationships between variables over time. False discovery rate corrected q-values were computed using the R package ‘fdrtool’. Heat map was created to visualize and evaluate correlations among metabolites and conventional risk factors in study populations.

Multivariate statistical analysis was performed using SIMCA-P+ software version 12.0 (Umetrics, Umeå, Sweden). Partial least-squares discriminant analysis (PLS-DA) was used as the classification method for modeling the discrimination between groups by visualizing the score scatter plot or S-plot using the first and second PLS components. The goodness-of-fit was quantified by R²Y, whereas the predictive ability was quantified by Q²Y. Generally, R²Y describes how well the data in the training set were mathematically reproduced and varied between 0 and 1 (a value of 1 indicated a model with a perfect fit). Models with Q²Y ≥0.5 were considered to have good predictive capabilities.

Results
Clinical characteristics, lipid profiles, and nutrient intake
There were no significant differences between two groups in baseline characteristics including age, gender, smoking, and drinking (data not shown). At baseline, there were no significant differences between two groups in BMI, waist:hip ratio (WHR), systolic BP, diastolic BP, serum triglyceride, total cholesterol, LDL-cholesterol, HDL-cholesterol, FFA, and hs-CRP. BMI, WHR, BP, serum lipid profiles, hs-CRP, total energy expenditure, and total energy intake were similar before and after the study in both groups (data not shown).

Replacement with whole grains and legumes caused significant increase in percent energy intake of protein and fat, and significant decrease in percent energy intake of carbohydrate. The percent energy intake of protein, fat, and carbohydrate significantly differed between the two groups before adjusting for baseline values. The whole-grain group had significant increases in fiber intake and polyunsaturated-to-saturated fatty acids ratio compared with baseline values. After 12-week, the whole-grain group had lower percent energy of carbohydrate, higher percent calorie of protein and fat, and fiber intake than control group (Table 1).

Fasting glucose, insulin, and malondialdehyde
At the end of the study, glucose, HbA1c, and MDA concentrations significantly increased in the control group, whereas glucose, glucose AUC (area under the curve), HbA1c, HOMA-IR, insulin, and MDA significantly decreased in the whole-grain group (Table 1). Changes in glucose, glucose AUC, HbA1c, HOMA-IR, and MDA in the whole-grain group were significantly different from those in the control group before and after adjusting for baseline levels. Post-treatment glucose, HOMA-IR, and MDA in the whole-grain group were significantly lower than those in the control group (Table 1).

Plasma ox-LDL, LDL particle size, Lp-PLA2 activity in plasma and unstimulated PBMC
At the end of the study, the whole-grain group had lower ox-LDL and Lp-PLA2 activity in PBMC and larger LDL particle size, whereas the control group had higher Lp-PLA2 activity in PBMC (Table 1). These changes in ox-LDL, LDL particle size, plasma Lp-PLA2 activity, and PBMC Lp-PLA2 activity in the whole-grain group were significantly different from those in the control group before and after adjusting for baseline levels. The post-treatment whole-grain group had lower ox-LDL and
PBMC Lp-PLA₂ activity, and larger LDL particle size than control group (Table 1).

Metabolic profiling of PBMC and plasma using UPLC-LTQ-orbitrap MS

**Nontargeted metabolic pattern analysis**

MS data of PBMC and plasma metabolites obtained at baseline and follow-up were analyzed with PLS-DA score scatter plot for the following two combinations: 1) control and whole-grain groups at baseline, control group at follow-up, and whole-grain group at follow-up (Fig. 1a, PBMC; Fig. 1c, plasma); and 2) control and whole-grain groups at follow-up (Fig. 1b, PBMC; Fig. 1d, plasma). The PBMC metabolite PLS-DA score scatter plot showed distinct clustering and clear separation for the following subjects: control and whole-grain groups at baseline, control group at follow-up, and whole-grain group at follow-up \([R^2X(cum) = 0.124, R^2Y(cum) = 0.34, Q^2Y(cum) = 0.218]\) (Fig. 1a). These distinct clusters indicate that PBMC profiling detects metabolic changes induced by dietary intervention. The PBMC metabolite PLS-DA score scatter plot showed distinct clustering for control and whole-grain groups at follow-up \([R^2X(cum) = 0.125, R^2Y(cum) = 0.809, Q^2Y(cum) = 0.525]\) (Fig. 1b).

The plasma metabolite PLS-DA score scatter plot were not as clearly clustered as those for PBMC metabolites \([R^2X(cum) = 0.201, R^2Y(cum) = 0.704, Q^2Y(cum) = 0.414]\) (Fig. 1d). To identify metabolites that differentially

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**Table 1** Biochemical characteristics and estimates of daily nutrient intake before and after 12-week dietary intervention

|                     | Control group (n = 40) | Whole-grain group (n = 40) | \(p^a\) | \(p^b\) | \(p^c\) | \(p^d\) |
|---------------------|------------------------|---------------------------|--------|--------|--------|--------|
| Glucose (mg/dL)     | 1198 ± 5.40            | 1266 ± 6.02***           | 0.958  | 0.005  |        |        |
| Change              | 6.78 ± 1.51            | 9.45 ± 1.43              | <0.001 | <0.001 |        |        |
| Glucose AUC (mg/dL × h) | 3768 ± 17.7          | 3904 ± 22.9              | 0.923  | 0.225  |        |        |
| Change              | 13.6 ± 9.52            | 24.7 ± 8.75              | 0.004  | 0.004  |        |        |
| HbA₁c (%)           | 6.46 ± 0.19            | 6.60 ± 0.19†             | 0.821  | 0.061  |        |        |
| Change              | 0.14 ± 0.06            | 0.23 ± 0.12              | 0.007  | 0.003  |        |        |
| HOMA-IR             | 2.24 ± 0.15            | 2.34 ± 0.17              | 0.791  | 0.006  |        |        |
| Change              | 0.10 ± 0.11            | 0.39 ± 0.10              | 0.002  | 0.001  |        |        |
| Insulin (IU/mL)     | 7.58 ± 0.35            | 7.47 ± 0.43              | 0.798  | 0.132  |        |        |
| Malondialdehyde (nmol/mL) | 9.24 ± 0.53       | 10.2 ± 0.61***           | 0.530  | 0.041  |        |        |
| Change              | 0.98 ± 0.24            | 0.71 ± 0.24              | 0.001  | <0.001 |        |        |
| Oxidized LDL (U/L)  | 49.1 ± 2.14            | 50.7 ± 2.26              | 0.846  | 0.036  |        |        |
| Change              | 1.65 ± 0.90            | 4.41 ± 0.82              | 0.001  | <0.001 |        |        |
| LDL particle size (nm) | 24.1 ± 0.12           | 24.1 ± 0.13              | 0.214  | 0.015  |        |        |
| Change              | 0.05 ± 0.06            | 0.24 ± 0.05              | 0.001  | 0.001  |        |        |
| Plasma Lp-PLA₂ activity (nmol/mL/min) | 30.1 ± 1.64     | 30.3 ± 1.61              | 0.874  | 0.701  |        |        |
| Change              | 0.24 ± 0.41            | 2.38 ± 0.55              | 0.001  | <0.001 |        |        |
| Unstimulated PBMC-Lp-PLA₂ activity (nmol/mL/min) | 2.00 ± 0.12   | 2.28 ± 0.13***           | 0.365  | 0.046  |        |        |
| Change              | 0.28 ± 0.08            | 0.26 ± 0.10              | 0.001  | <0.001 |        |        |

Estimate of daily nutrient intake

|                     | Baseline | Follow-up | \(p^a\) | \(p^b\) | \(p^c\) | \(p^d\) |
|---------------------|----------|-----------|--------|--------|--------|--------|
| Energy intake (kcal/d) | 2143 ± 40 | 2170 ± 38 | 0.665  | 0.786  |        |        |
| Carbohydrate (%)     | 61.9 ± 0.16 | 61.9 ± 0.17 | 0.833  | <0.001 |        |        |
| Protein (%)          | 16.3 ± 0.10 | 16.3 ± 0.08 | 0.851  | <0.001 |        |        |
| Fat (%)              | 22.1 ± 0.16 | 22.2 ± 0.15 | 0.731  | <0.001 |        |        |
| Crude fiber (g)      | 25.2 ± 1.20 | 22.8 ± 1.19 | 0.713  | 0.001  |        |        |
| PUFA/SFA             | 2.10 ± 0.18 | 2.10 ± 0.16 | 0.418  | <0.001 |        |        |

Mean ± SEM. *tested by logarithmic transformation, \(P^a\), values derived from independent t-test in baseline, \(P^b\), values derived from independent t-test in changed value after adjusting for baseline, \(P^c\), values derived from independent t-test in changed value before and after dietary intervention, \(P^d\), values derived from independent t-test in changed value after adjusting for baseline. \(P < 0.05, \*P < 0.01, \***P < 0.001\) derived from paired \(t\)-test. PBMC, peripheral blood mononuclear cell; AUC, area under the curve; PUFA/SFA, polyunsaturated-to-saturated fatty acids ratio.
determined data at baseline and follow-up, S-plots of $p(1)$ and $p(\text{corr})$ were generated using centroid scaling. The S-plots revealed that metabolites with higher or lower $p(\text{corr})$ values more clearly discriminated between the two groups.

**Identification of PBMC metabolites**

Of 1,923 PBMC metabolites, those that correlated with separation between the groups were identified by the variable important in the projection (VIP) parameter; VIP values >1.0 were highly relevant for group differences. 51 metabolites had VIP >1.0; 10 of these were previously identified and 41 were unknown. Those 10 PBMC metabolites at baseline and follow-up are shown in Table 2. There were no significant differences in baseline metabolites between two groups. After follow-up, the control group showed significant changes in six PBMC metabolite levels, whereas the whole-grain group showed significant changes in seven PBMC metabolite levels (Table 2).

We compared PBMC metabolite changes between two groups. The whole-grain group had greater reductions in L-leucine ($q = 0.031$), oleamide ($q = 0.032$), lysoPC (16:0) ($q = 0.003$), and lysoPC (18:0) ($q = 0.003$) (Table 2). At follow-up, the whole-grain group had higher peak intensities of L-pyroglutamic acid and ribothymidine, and lower peak intensities of palmitic amide, oleamide, and lysoPCs, compared with those of the control group (Table 2).

**Identification of plasma metabolites**

Of 4,121 plasma metabolites, those that correlated with separation between the groups were selected by VIP >1.0. 122 plasma metabolites were selected; 20 were previously identified and 102 were unknown (Table 3). There were no significant differences in baseline metabolites between two groups. After follow-up, C17 sphinganine significantly increased in the control group, also, there were no significant differences in metabolites between two groups, and no significant differences in metabolite changes with respect to baseline (Table 3).

**Correlations among fasting glucose, plasma and PBMC Lp-PLA$_2$ activities, biochemical parameters, and major PBMC metabolites**

The correlation matrix of changes in glucose, Lp-PLA$_2$ activities in plasma and PBMC, biochemical parameters, and major PBMC metabolites was computed (Fig. 2).
Table 2: Identification of PBMC metabolites at baseline and 12-week follow-up

| Metabolite                        | Formula (M + H) | Exact mass (M + H) | Control group (n = 40) | Whole-grain group (n = 40) | Variable important in the projection | Baseline vs. follow-up 12-weeks |
|-----------------------------------|-----------------|--------------------|------------------------|---------------------------|-------------------------------------|----------------------------------|
| Cyclopentanone dimethylhydrazone  | C₇H₁₄N₂        | 127.1235           | Baseline                | 82,551 ± 21,507            | 43,192 ± 11,728                     | 0.0722                           |
|                                   |                 |                    | Follow-up               | 101,931 ± 26,693           | 51,504 ± 14,505                     | 0.1523                           |
|                                   |                 |                    | Control                  | 38,283 ± 198,486           | 722,514 ± 145,341†                  | 1.0119                           |
|                                   |                 |                    | Whole-grain              | 2,410,045 ± 108,001        | 2,484,381 ± 103,907†                 | 2.6550                           |
|                                   |                 |                    | Baseline                 | 2,880,509 ± 113,266        | 2,825,355 ± 101,371‡                 | 1.1474                           |
| L-Pyroglutamic acid               | C₅H₇NO₃        | 130.0504           | Baseline                | 2,410,045 ± 108,001        | 2,484,381 ± 103,907†                 | 2.6550                           |
|                                   |                 |                    | Follow-up               | 2,075,511 ± 48,362∗        | 335,279 ± 14,924††                   | 0.4537                           |
|                                   |                 |                    | Control                  | 3,023,210 ± 22,108         | 322,971 ± 16,498†                   | 0.5962                           |
|                                   |                 |                    | Whole-grain              | 2,455,521 ± 70,667         | 2,761,323 ± 56,360                   | 1.1474                           |
|                                   |                 |                    | Baseline                 | 2,484,381 ± 103,907†       | 2,484,381 ± 103,907†                 | 2.6550                           |
| L-Leucine                         | C₆H₁₃NO₂        | 132.1025           | Baseline                | 7,884,178 ± 309,297        | 8,253,845 ± 170,803                  | 1.8209                           |
|                                   |                 |                    | Follow-up               | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
|                                   |                 |                    | Control                  | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
|                                   |                 |                    | Whole-grain              | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
| Change                            |                 |                    | Control                  | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
| L-Phenylalanine                   | C₈H₁₁NO₂        | 166.0868           | Baseline                | 2,880,509 ± 113,266        | 2,825,355 ± 101,371‡                 | 1.1474                           |
| Dihydrobiopterin                  | C₉H₁₃N₅O₃      | 239.1018           | Baseline                | 432,360 ± 23,845           | 427,487 ± 16,797**                   | 0.4537                           |
|                                   |                 |                    | Follow-up               | 354,492 ± 50,930           | 330,909 ± 44,809                     | 0.4537                           |
|                                   |                 |                    | Control                  | 320,902 ± 12,851**         | 311,114 ± 19,898†                   | 0.5962                           |
|                                   |                 |                    | Whole-grain              | 320,902 ± 12,851**         | 311,114 ± 19,898†                   | 0.5962                           |
| Palmitic amide                    | C₁₆H₃₃NO       | 256.2640           | Baseline                | 7,884,178 ± 309,297        | 8,253,845 ± 170,803                  | 1.8209                           |
|                                   |                 |                    | Follow-up               | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
|                                   |                 |                    | Control                  | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
|                                   |                 |                    | Whole-grain              | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
| Change                            |                 |                    | Control                  | 7,922,462 ± 251,819        | 7,531,331 ± 171,144††                | 5.0363                           |
| Oleamide                          | C₁₈H₃₅NO       | 282.2797           | Baseline                | 2,676,408 ± 412,881        | 2,357,688 ± 511,380                  | 18.4793                          |
|                                   |                 |                    | Follow-up               | 2,676,408 ± 412,881        | 2,357,688 ± 511,380                  | 18.4793                          |
|                                   |                 |                    | Control                  | 2,676,408 ± 412,881        | 2,357,688 ± 511,380                  | 18.4793                          |
|                                   |                 |                    | Whole-grain              | 2,676,408 ± 412,881        | 2,357,688 ± 511,380                  | 18.4793                          |
| Change                            |                 |                    | Control                  | 2,676,408 ± 412,881        | 2,357,688 ± 511,380                  | 18.4793                          |
| LysoPC(16:0)                      | C₂₄H₅₀NO₇P     | 496.3403           | Baseline                | 414,438 ± 44,544           | 645,199 ± 57,544†                    | 3.5066                           |
|                                   |                 |                    | Follow-up               | 414,438 ± 44,544           | 645,199 ± 57,544†                    | 3.5066                           |
|                                   |                 |                    | Control                  | 414,438 ± 44,544           | 645,199 ± 57,544†                    | 3.5066                           |
|                                   |                 |                    | Whole-grain              | 414,438 ± 44,544           | 645,199 ± 57,544†                    | 3.5066                           |
| Change                            |                 |                    | Control                  | 414,438 ± 44,544           | 645,199 ± 57,544†                    | 3.5066                           |
| LysoPC(18:0)                      | C₂₆H₅₄NO₇P     | 524.3716           | Baseline                | 285,044 ± 26,648           | 423,718 ± 34,129†                     | 2.7864                           |
|                                   |                 |                    | Follow-up               | 285,044 ± 26,648           | 423,718 ± 34,129†                     | 2.7864                           |
|                                   |                 |                    | Control                  | 285,044 ± 26,648           | 423,718 ± 34,129†                     | 2.7864                           |
|                                   |                 |                    | Whole-grain              | 285,044 ± 26,648           | 423,718 ± 34,129†                     | 2.7864                           |
| Change                            |                 |                    | Control                  | 285,044 ± 26,648           | 423,718 ± 34,129†                     | 2.7864                           |

Mean ± SEM. *q < 0.05, **q < 0.01, ***q < 0.001 derived from paired t-test. †q < 0.05, ‡q < 0.01, ††q < 0.001 derived from independent t-test in follow-up. †‡q < 0.05, ‡‡q < 0.01, ‡‡‡q < 0.001 derived from changed values between control and whole-grain groups.
### Table 3: Identification of plasma metabolites at baseline and 12-week follow-up

| Metabolite                          | Formula      | Exact mass (M + H) | Normalized peak intensity | Variable important in the projection |
|-------------------------------------|--------------|--------------------|--------------------------|-------------------------------------|
|                                     |              |                    |                          | Control group (n = 40) vs. follow-up | Whole-grain group (n = 40) vs. follow-up | Baseline vs. follow-up | 12-weeks Control vs. whole grain |
| L-Valine                            | C6H11NO2     | 118.0868           | 1,338,114 ± 39,666       | 0.7807                              | 0.1901                              | 1.0144                      |
| L-Leucine                           | C6H13NO2     | 132.1025           | 3,109,670 ± 99,839       | 0.4904                              | 0.5781                              | 0.7853                      |
| L-Phenylalanine                     | C9H11NO2     | 166.0868           | 1,984,169 ± 49,821       | 0.6137                              | 1.1072                              | 0.7382                      |
| Oleamide                            | C18H35NO     | 282.3797           | 11,074,949 ± 815,519     | 8.4316                              | 19.2720                             | 6.6270                      |
| C17 Sphinganine                     | C17H37NO2    | 288.2903           | 323,013 ± 20,549         | 0.9135                              | 1.3462                              | 0.1114                      |
| (4E,8E,10E-d18:3) Sphingosine       | C18H33NO2    | 420,731 ± 59,507   | 237,343 ± 31,518         | 0.1156                              | 1.4954                              | 0.5840                      |
| Anandamide (18:4, n-3)              | C20H33NO2    | 296.2590           | 170,194 ± 28,584         | 0.1156                              | 1.4954                              | 0.5840                      |
| LysoPC (14:0)                       | C20H50NO7P   | 468,0390           | 480,474 ± 33,898         | 0.1156                              | 1.4954                              | 0.5840                      |
| LysoPC (16:1)                       | C20H50NO7P   | 494,3247           | 1,133,250 ± 77,052       | 0.1156                              | 1.4954                              | 0.5840                      |
| LysoPC (16:0)                       | C20H50NO7P   | 496,3403           | 1,592,932 ± 573,566      | 0.1156                              | 1.4954                              | 0.5840                      |
| LysoPC (17:0)                       | C20H50NO7P   | 510,3560           | 689,578 ± 58,089         | 0.1156                              | 1.4954                              | 0.5840                      |
| LysoPC (18:3)                       | C20H50NO7P   | 518,3247           | 620,814 ± 20,714         | 0.1156                              | 1.4954                              | 0.5840                      |
| LysoPC (18:2)                       | C20H50NO7P   | 520,3403           | 6,388,637 ± 262,115      | 2.2535                              | 4.8170                              | 15.1889                     |
| LysoPC (18:1)                       | C20H50NO7P   | 522,3560           | 5,375,808 ± 229,029      | 4.2976                              | 3.7059                              | 4.2779                      |
| LysoPC (18:0)                       | C20H50NO7P   | 524,3716           | 5,661,866 ± 195,118      | 4.2976                              | 3.7059                              | 4.2779                      |
| LysoPC (20:5)                       | C20H50NO7P   | 542,3427           | 771,884 ± 61,530         | 2.1218                              | 0.4055                              | 1.2595                      |
| LysoPC (20:4)                       | C20H50NO7P   | 544,3403           | 2,512,566 ± 118,266      | 2.1218                              | 0.4055                              | 1.2595                      |
| LysoPC (22:6)                       | C20H50NO7P   | 568,3803           | 1,288,789 ± 89,205       | 2.1218                              | 0.4055                              | 1.2595                      |
| SM (d18O/16:1)                      | C20H25N2O5P  | 703,5754           | 918,463 ± 110,095        | 2.1218                              | 0.4055                              | 1.2595                      |
| Lactosylceramide (d18:1/120)        | C24H43N2O13  | 806,5630           | 5,977,825 ± 630,100      | 2.1218                              | 0.4055                              | 1.2595                      |

Mean ± SEM. *q* < 0.05, **q** < 0.01, ***(q*** < 0.001 derived from paired t-test. 'q' < 0.05, **'q** < 0.01, ***q*** < 0.001 derived from independent t-test in follow-up. 'q' < 0.05, **'q** < 0.01, ***q*** < 0.001 derived from changed values between control and whole-grain groups. 1PCs were detected by Orbitrap MS; therefore, all detected PC amounts were combined. SM, sphingomyelin
Analysis of metabolic changes including all subjects identified the following correlations: glucose correlated positively with insulin, HOMA-IR, plasma Lp-PLA₂ activity ($r = 0.454$, $P < 0.001$), MDA, ox-LDL, Lp-PLA₂ in PBMC ($r = 0.511$, $P < 0.001$), glucose AUC, C-peptide, HbA₁c, and PBMC lysoPCs after adjusting for age, gender, BMI, smoking, and drinking. After adjusting for confounding variables, plasma Lp-PLA₂ activity correlated positively with glucose, HOMA-IR, ox-LDL, Lp-PLA₂ activity in PBMC ($r = 0.516$, $P < 0.001$), glucose AUC, PBMC palmitic amide, and PBMC oleamide. After adjusting for confounding variables, PBMC Lp-PLA₂ activity correlated positively with glucose, HOMA-IR, plasma Lp-PLA₂ activity, ox-LDL, PBMC L-leucine, PBMC oleamide, PBMC lysoPCs, and correlated negatively with LDL particle size (Fig. 2).

**Discussion**

We identified four PBMC metabolites that had statistically significant differences after dietary intervention, including L-leucine, oleamide, lysoPC (16:0), and lysoPC (18:0); however, there were no significant differences in plasma metabolites after dietary intervention. These aspects of results were also shown in both subjects with prediabetes and T2D, respectively. PBMCs may be a useful tool for nutrigenomics and understanding the pathophysiology of chronic disease due to their active metabolism [14, 15]. These results identify PBMC metabolites as powerful metabolomics tools to detect diet-induced metabolic changes.

Improving glycemic control in the whole-grain group decreases ox-LDL, and reduces PBMC Lp-PLA₂ activity and PBMC lysoPCs. A strong correlation between PBMC Lp-PLA₂ activity and ox-LDL, but not LDL-cholesterol, is consistent with a previous report of a direct effect of ox-LDL on Lp-PLA₂ expression in THP-1 monocytes [16]. Ox-LDL may upregulate PBMC Lp-PLA₂ expression in smokers [17]. Ox-phospholipids in LDL particles are hydrolyzed by Lp-PLA₂ at the sn-2 position to produce bioactive ox-FFAs and lysoPCs. Only 1−5 % of the total non-ox-LDL PC content is lysoPC; however, up to 40−50 % of LDL PC is converted to lysoPC during LDL oxidation [18]. This study identified strongly positive correlations among ox-LDL, PBMC Lp-PLA₂ activity, PBMC lysoPCs, which may indicate that ox-LDL and PBMC Lp-PLA₂ activity are major determinants of PBMC lysoPC levels.

A negative correlation between PBMC Lp-PLA₂ activities, PBMC lysoPCs with LDL particle size is consistent with a previous report of Lp-PLA₂ binding preference.
obtained in our study could be partly due to dietary-induced effects on blood cell inflammatory processes [23]. This study detected many metabolic markers using UPLC-LTQ-Orbitrap MS, but most are currently unidentified. Endogenous biomolecule databases for use with LC-MS–based metabolomics research are still under construction [30]. Despite this limitation, UPLC-LTQ-Orbitrap MS metabolomics and multivariate data analysis identified greater reductions in PBMC L-leucine, PBMC oleamide, PBMC lysoPCs in the whole-grain group than control group; however, there were no significant differences in plasma metabolites between two groups.

Conclusion
This study demonstrates that replacing refined rice with whole grains and legumes induced greater differences in PBMC Lipoprotein-associated phospholipase A2 activity and metabolites than in plasma metabolites in nonobese patients with prediabetes or newly-diagnosed T2D. Therefore, consumption of minimally refined grains, legumes, and vegetables should be recommended to control glucose metabolism and reduce cardiovascular risk factors in patients with IFG, IGT, or newly-diagnosed T2D.

Abbreviations
Lp-PLA2: Lipoprotein-associated phospholipase A2; T2D: Type-2 diabetes; PBMC: Peripheral blood mononuclear cell; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance; BMI: Body mass index; BP: Blood pressure; HDL: High density lipoprotein; LDL: Low density lipoprotein; FFA: Free fatty acid; HbA1c: Hemoglobin A1c; IR: Insulin resistance; HOMA: Homeostasis-model assessment; FBS: Fetal bovine serum; hs-CRP: High-sensitivity C-reactive protein; MDA: Malondialdehyde; TBARS: Thiobarbituric acid-reactive substances; Ocx: Oxidized; MS: Mass spectrometer; PLS-DA: Partial least-squares discriminant analysis; AUC: Area under the curve; VIP: Variable important in the projection.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
All the authors were involved in the development of the study protocol and the experimental design. GS, MK and JHY collected samples and carried out experiments. MK performed data analysis. T-SJ, S-HL and JHL reviewed data and participated in general discussion. JHL provided the research funding and wrote the manuscript. All the authors read, commented on, and contributed to the submitted manuscript. All authors read and approved the final manuscript.

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for small dense LDL [19]. This study and other work [6] reported positive correlations among glucose, PBMC Lipoprotein-associated phospholipase A2 activity, and plasma Lipoprotein-associated phospholipase A2 activity. A strongly positive correlation between PBMC and plasma Lipoprotein-associated phospholipase A2 activities also is observed in healthy subjects [17, 20]. In a porcine diabetes model, PBMC Lipoprotein-associated phospholipase A2 expression is up-regulated in the presence of glycation end products [21]. Increases in circulating Lipoprotein-associated phospholipase A2 activity and increased ox-LDL levels in hypercholesterolemic pigs are primarily due to plaque macrophages [22]. These results indicate that the primary sources of plasma Lipoprotein-associated phospholipase A2 are plaque macrophages [22] and PBMC [17]. This could explain our observations of lower plasma Lipoprotein-associated phospholipase A2 activity changes compared with that of PBMC Lipoprotein-associated phospholipase A2 activity, and no significant differences in plasma metabolite changes between two groups.

Reduced Lipoprotein-associated phospholipase A2 activity in plasma and PBMC in the whole-grain group could be a marker of metabolic changes induced by increased consumption of protein relative to carbohydrate. Diet composition is an important factor in inflammatory processes of blood cells [23]. A study of macronutrient composition determined that increasing dietary protein from 19 % energy intake to 30 % yielded immediate and persistent downregulation of immunological genes in PBMCs [9]. Replacing 5 % of energy from carbohydrates with energy from protein and measured a 2.2 nmol/min/mL reduction in Lipoprotein-associated phospholipase A2 activity that was independent of other changes in lipid profiles [5]. Our study replaced 7 % of energy from carbohydrate with approximately 4 % energy from protein and 3 % energy from fat.

Whole grains, legumes, and vegetables contain many antioxidants, vitamins, minerals, and phytochemicals [24, 25]. Antioxidants slow the oxidation rate of reduced substrates [24, 26]. Soybean phytochemicals reduce lipid peroxidation in vivo and attenuate LDL oxidation [27]. Our observed changes in glucose and HOMA-IR strongly correlated with changes in MDA and ox-LDL in patients with prediabetes or T2D, consistent with a previous report [28]. We observed positive correlation between changes in HOMA-IR and PBMC L-leucine, but not plasma L-leucine. This may be due to a negligible effect of PBMC L-leucine on plasma L-leucine, or the 12-week dietary intervention may not be long enough to change plasma L-leucine. The whole-grain group also had greater reduction in PBMC oleamide, but not in plasma, compared with control group. We identified PBMC oleamide (VIP = 22.5845) as the most important metabolite for evaluating differences between two groups at the end of the study. Recently, Ha et al. [29] identified plasma oleamide as the most important metabolite for distinguishing nondiabetic from diabetic males. Therefore, positive correlations between changes in PBMC oleamide, Lipoprotein-associated phospholipase A2, PBMC palmitic amide, and PBMC lysoPCs
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