Metabolomics analysis reveals an effect of homocysteine on arachidonic acid and linoleic acid metabolism pathway

BIN LI[1*], GUANGQIANG GAO[1*], WANYING ZHANG[1], BOWEN LI[1], CHUN YANG[1], XIAOFENG JIANG[1], YAPING TIAN[2] and HONGYAN LIANG[1]

[1]Department of Clinical Laboratory, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150001; [2]Department of Clinical Biochemistry, Chinese PLA General Hospital, Beijing 100853, P.R. China

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Abstract. An increase in serum homocysteine level has been associated with an increased risk of vascular disease; however, the biochemical mechanisms that underlie these effects remain largely unknown. The present study aimed to use high-performance liquid chromatography-mass spectrometry (HPLC-MS) to demonstrate the effects of serum homocysteine on human blood metabolites. A total of 75 fasting serum samples were investigated in the present study. Using a threshold of 15 μmol/l serum homocysteine level, samples were divided into high- and low-homocysteine groups, and the serum extracts were analyzed with an HPLC-MS-based method. A total of 269 features exhibited significant differences and correlation with serum homocysteine levels in the electrospray ionization-positive [ESI(+)] mode, and 69 features were identified in the ESI(-) mode between the two groups. The principal component analysis plot revealed a separation between the high- and the low-homocysteine groups. Metabolite set enrichment analysis identified arachidonic acid metabolism and linoleic acid metabolism as the two pathways with significantly enriched differences. These results revealed that arachidonic acid and linoleic acid metabolism may be associated with serum homocysteine levels and may be involved in homocysteine-induced vascular disease.

Introduction

The non-protein α-amino acid homocysteine is an intermediate product that occurs during the normal biosynthesis of cysteine and methionine. Hyperhomocysteinemia has been proposed to be an independent risk factor for cardiovascular disease (1). Several previous studies have reported that an increase in the level of serum homocysteine may be involved in the pathogenesis of vascular diseases, such as coronary artery disease (2), coronary heart disease (3,4), atherosclerotic vascular disease (5,6), stroke (7,8) and ischemic heart disease (9). A meta-analysis reported that for every 5 μmol increase in serum homocysteine level the danger of coronary heart disease increased by 60-80% in adults (10); compared with healthy subjects, there was a sevenfold increase in the mortality rate in patients with high levels of serum homocysteine (11). A recent report indicated that an elevation in serum homocysteine levels may lead to vascular disease in the general population (12).

Although it has long been considered that an increased serum homocysteine level was associated with an increased risk of vascular disease, the biochemical mechanisms that underlie these effects in serum remain unclear. The present study aimed to investigate the effects of serum homocysteine levels on human blood metabolites in humans. To avoid any potential influences on the results, the samples in which vascular diseases were identified were not included in the present study.

Metabolomics analyses have previously been demonstrated to provide a dynamic depiction of metabolic status (13), and have been successfully applied in several fields of research, including disease diagnosis (14), biomarker screening (15,16) and in characterizing biological pathways (17,18). Therefore, the present study used a high-performance liquid chromatography-mass spectrometry (HPLC-MS)-based metabolomics approach to detect the biochemical alterations associated with homocysteine levels in human serum.

Materials and methods

Serum sample collection and preparation. The present study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University (Harbin, China). All participating subjects were informed of their rights and written informed consent was obtained. A total of 161 fasting blood samples were obtained from patients aged 25-79 years during routine physical examination at the Department of Physical Examination Center of the Fourth Affiliated Hospital

Correspondence to: Professor Hongyan Liang, Department of Clinical Laboratory, The Fourth Affiliated Hospital of Harbin Medical University, 37 Yiyuan Street, Harbin, Heilongjiang 150001, P.R. China
E-mail: aileenliang2005@163.com

*Contributed equally

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of Harbin Medical University between April 5 and April 18, 2015. The samples were divided into high- and low-serum homocysteine groups based on a threshold of 15.0 μmol/l serum homocysteine level. Patients were questioned about their lifestyles, including smoking status (non-smokers were identified as having never smoked or stopped smoking >1 year ago) and alcohol intake (non-drinkers were identified as having not consumed alcohol in the previous 2 weeks and having not consumed >38% alcohol v/v and >200 ml in the previous month). Patients were also asked whether they had already been diagnosed with vascular disease. In order to prevent vascular disease and unhealthy habits affecting the results of the present study, patients adhering to the following criteria were excluded from the study: Hypertension, diabetes, hyperlipemia, obesity, coronary heart disease, atherosclerosis, stroke, cerebral embolism and recipients of folic acid and/or vitamin B12 supplements. Hypertension was diagnosed when the systolic blood pressure (SBP) was ≥140 mmHg and/or diastolic blood pressure (DBP) was ≥90 mmHg. Diabetes was diagnosed when the fasting plasma glucose level was ≥6.1 mmol/l. Hypercholesterolemia and hyperlipemia were defined as total cholesterol ≥6.22 mmol/l and triglycerides ≥2.26 mmol/l, respectively, according to the 2007 Dyslipidemia Prevention Guide in Chinese Adults (19). Obesity was defined as having a body mass index (BMI) ≥28.00 kg/m^2, according to the 2006 Guidelines for Prevention and Control of Obesity in Chinese Adults (20). Coronary heart disease was confirmed by coronary angiography. Stroke, atherosclerosis and cerebral embolism were diagnosed by computed tomography scanning. A total of 86 samples were excluded and 75 samples were retained for further investigation.

All fasting blood samples were centrifuged at 1,408 x g for 5 min immediately at room temperature, then the serum was collected. Levels of fasting serum glucose (FSG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG) and serum homocysteine were determined using Hitachi 7600 Automatic Biochemistry Analyzer (Hitachi Instrument Service, Tokyo, Japan). All reagents and calibrators were purchased from Roche Diagnostics (Basel, Switzerland). All quality controls (serum homocysteine, FSG, TC, HDL-C, LDL-C and TG) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Hyperhomocysteinemia was defined as having an abnormally high level (>15 μmol/l) of homocysteine in the serum sample (21).

Each serum sample was used for metabolite extraction prior to HPLC-MS analysis. Briefly, acetonitrile (400 μl; Chromasolv chromatographic grade; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the serum (200 μl), and the mixture was vortexed for 1 min and incubated at room temperature for 10 min, followed by centrifugation at 18,500 x g at room temperature for 10 min. The supernatant (400 μl) was removed and completely evaporated using ultra-high-purity (99.9%) nitrogen gas in water bath at 40˚C (22), and 100 μl of a mixture of acetonitrile and water (3:1) was added into each tube. The solution was passed through a syringe filter (0.22 μm) into a 2 ml glass vial prior to HPLC-MS analysis.

Metabolomics profiling with ESI. HPLC-MS analysis was performed with a Shimadzu HPLC System (Shimadzu Corporation, Kyoto, Japan) coupled to an AB Sciex API4000+ mass spectrometer with electrospray ionization (ESI) in the positive (+) and negative (-) modes. The sample solution (5 μl) was injected into an HPLC InertSustain C18 Column (2.1x150 mm; 3 μm; GL Sciences Inc., Torrance CA, USA). The flow rate of the mobile phase was 0.35 ml/min, and analytes were eluted from the column under a gradient (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile). The optimal conditions for HPLC separation and ESI detection are shown in Table I.

Data and statistical analysis. Raw data was in an instrument specific format (.wiff) and were converted to common data format (.mzXML) format, using the Wiff to mzXML translator software (version 1.3; Applied Biosystems; Thermo Fisher Scientific, Inc.). The program XCMS was used for nonlinear alignment of the raw data (.mzXML files) in the time domain and automatic integration and extraction of the peak intensities (23). Accurate masses of features that were identified as significantly different and correlated with serum homocysteine levels were searched against the Metlin databases. MetaboAnalyst 3.0 software (24,25) was used for multivariate statistical calculations and plotting and metabolic pathway enrichment analysis (26). Differences between high- and low-serum homocysteine groups were determined using a two-tailed Student's t-test with 5% false discovery rate (FDR). Correlations between the level of serum homocysteine and accurate masses of features were analyzed using Pearson's correlation coefficient. Serum biochemical indicators are presented as the mean ± standard deviation. FDR-adjusted $P<0.05$ was considered to indicate a statistically significant difference. These analyses were performed using R Bioconductor (version 2.15.3; http:www.r-project.org/). The experimental workflow for the metabolomics studies performed is depicted in Fig. 1.

Results

The mean values of serum homocysteine concentration in the high- and low-serum homocysteine groups were 29.4 and 11.9 μmol/l, respectively. The clinical biochemical characteristics of the samples are summarized in Table II. No significant differences were identified between the two groups for the following characteristics: Sex, age, FSG, TC, HDL-C, LDL-C, DBP, SBP and BMI.

A total of 75 serum samples were analyzed by HPLC coupled to ESI(+)- or ESI(-)-MS. Raw data from the individual analyses were subjected to nonlinear data alignment. Univariate statistics were used to screen the differential features between the high- and low-serum homocysteine groups, and multivariate statistics were used to determine group separation. In the ESI(+) mode, a total of 1,949 features were detected, of which 695 (35.7%) exhibited significant differences between the two groups (FDR-adjusted $P<0.05$; Table III). Correlation analysis revealed that 269 (13.8%) of the 1,949 different features are correlated with the level of serum homocysteine (FDR <0.05), ranging from 0.41 to 0.65 for 59 positive correlation features and -0.68 to -0.41 for 210 negative correlation features. In the ESI(-) mode, a total of 1,721 features were detected, of which 157 (9.1%) exhibited significant differences between

Table I. HPLC separation and ESI-mass spectrometry detection conditions.

Component	Condition
HPLC InertSustain C18 Column	150x2.1 mm; 3 μm
Mobile phase A	0.1% HCOOH in H_2O
Mobile phase B	0.1% HCOOH in CH_3CN
Gradient elution	B%=2% maintained (0-2 min), increased to 20% in 4 min, 70% linearly increased (4-8 min), 100% in a further 4 min, and 100% maintained 2 min (14-18 min), followed by re-equilibration to the initial conditions in 6 min (18-25 min)
Flow rate	0.35 ml/min
Injection volume	5 μl
Polarity	ESI(+) and ESI(-)
Gas1	15 l/h
Curtain gas	10 l/h
DP	45 V
Source temperature	375˚C
MS range	m/z 40-1,500

ESI, electrospray ionization; HPLC, high-performance liquid chromatography.

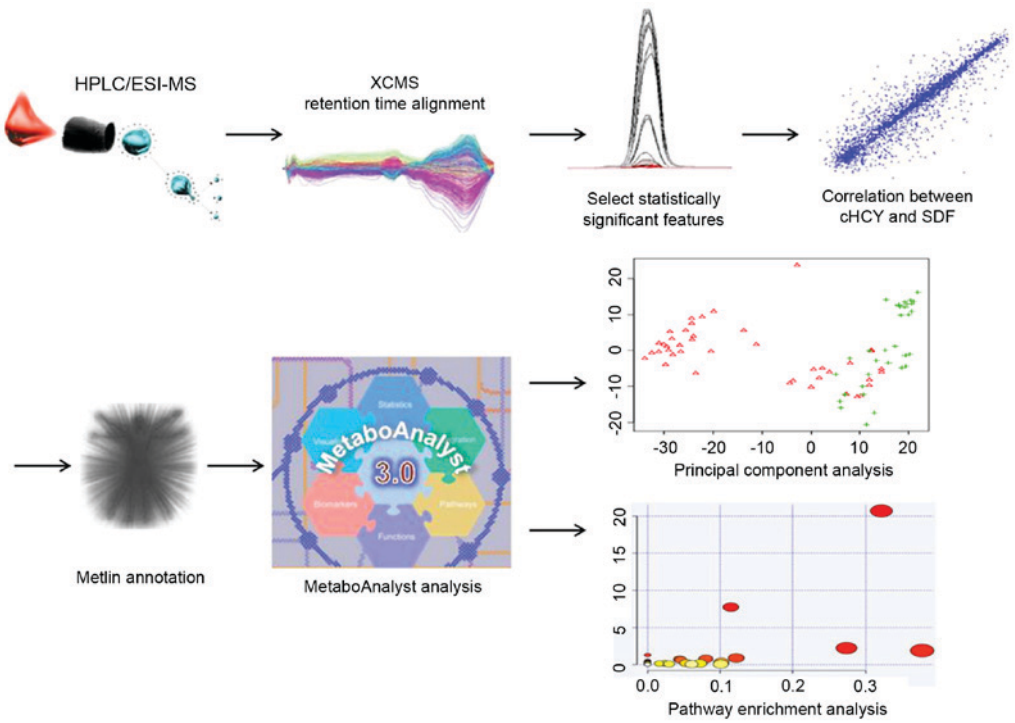

Figure 1. Experimental workflow for the serum metabolomics of different homocysteine level in human. Serum samples were detected with ESI in the positive and negative modes. The program XCMS was used for nonlinear alignment of raw data and the extraction of peak intensities. The SDF between high- and low-serum homocysteine groups were selected based on 75 serum samples and following this, the correlation between the SDF values and serum homocysteine levels were analyzed. The different and correlated features selected were searched against the Metlin database, and the corresponding compounds that were matched in the Metlin database were further analyzed by principal component analysis and metabolic pathway enrichment analysis methods in MetaboAnalyst software. cHCY, homocysteine concentration; HPLC/ESI-MS, high-performance liquid chromatography/electrospray ionization-mass spectrometry; SDF, significantly different features.

the two groups (FDR <0.05), and a total of 69 (4.0%) of the 1,721 features had clear correlations with the level of serum homocysteine (FDR <0.05), ranging from 0.40 to 0.68 for 32 positive correlation features and -0.63 to -0.39 for 37 negative correlation features. Matlin database searches revealed that only 36 accurate masses that represented significant differences and correlated features were matched (Table IV), and the corresponding 77 compounds were selected.

Principal component analysis of the 36 features aforementioned (that is, plotting principal component 1

Table II. Biochemical characteristics in the high- and low-serum HCY groups.

Characteristic	High-HCY group	Low-HCY group	P-value
Number	33	42	
Sex (female/male)	15/18	20/22	0.69
Age, year	47±10.7	44±8.6	0.25
FSG, mmol/l	5.5±1.4	5.5±1.3	0.86
TC, mmol/l	4.9±0.8	4.7±0.9	0.12
HDL-C, mmol/l	1.3±0.4	1.3±0.3	0.63
LDL-C, mmol/l	3.2±0.7	3.0±0.6	0.14
TG, mmol/l	1.91±0.5	1.78±0.7	0.43
DBP, mmHg	81±5.3	79±7.1	0.16
SBP, mmHg	131±9.5	128±6.7	0.22
BMI, kg/m^2	27.2±4.9	28.3±5.2	0.58

Data are presented as the mean ± standard deviation; P<0.05 were considered to indicate a statistically significant difference. BMI, body mass index; DBP, diastolic blood pressure; FSG, fasting serum glucose; HCY, serum homocysteine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

Table III. Summary of the metabolomics profiles using two different MS-based methods.

Method	Total peaks	Significant changed peaks (%)	Significant changed and correlated peaks (%)
HPLC/ESI(+)-MS	1,949	695 (35.7)	269 (13.8)
HPLC/ESI(-)-MS	1,721	157 (9.1)	69 (4.0)

HPLC/ESI(+/-)-MS, high-performance liquid chromatography/electrospray ionization-mass spectrometry in the positive or negative mode, respectively.

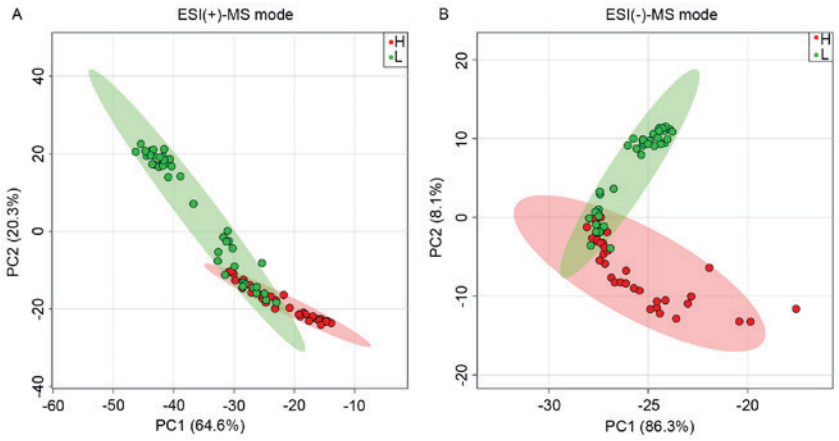

Figure 2. PCA of the high- and low-serum homocysteine groups in ESI(+)-MS and ESI(-)-MS modes. PCA plots were generated from serum sample data sets of two groups, high- vs. low-serum homocysteine. (A) PCA plot of the two sample groups in ESI(+)-MS mode. (B) PCA plot of the two sample groups in ESI(-)-MS mode. Green circles represent the low-serum homocysteine group; Red circles indicate the high serum homocysteine group. ESI(+/-)-MS, electrospray ionization-mass spectrometry in the positive/negative mode; PCA, principal component analysis.

vs. principal component 2) revealed a separation of the high- and the low-serum homocysteine sample groups (Fig. 2). In the ESI(+)-MS mode, the mean value of the samples in the low-level group was 10.8 μmol/l compared with that of the high-level groups (39.1 μmol/l) in the non-close-knit area (Fig. 2A). However, a portion of the samples in one group was dispersed to the other group in close-knit areas and exhibited similar levels of serum homocysteine: The mean value of the low-level samples in the close-knit area was 14.3 μmol/l, compared with the high-level samples (16.6 μmol/l). Similar separation was found in the ESI(-)-MS mode (Fig. 2B). In the ESI(-)-MS mode, the mean value of the samples in the low-level group was 11.2 μmol/l compared with that of the high-level groups (36.8 μmol/l) in the non-close-knit area (Fig. 2B).

Table IV. Detailed information of 36 accurate masses.

Feature	FC	P-value1	Cor	P-value2
M110T182	7.42	$2.19x10^{-07}$	0.64	$6.19x10^{-07}$
M305T140	1.50	$9.70x10^{-08}$	-0.54	$6.44x10^{-05}$
M311T139	1.53	$9.25x10^{-09}$	-0.49	$4.90x10^{-04}$
M313T131	1.45	$1.19x10^{-06}$	-0.58	$1.10x10^{-05}$
M315T127	1.53	$5.81x10^{-06}$	-0.52	$1.76x10^{-04}$
M317T125	1.68	$1.37x10^{-08}$	-0.52	$1.52x10^{-04}$
M321T139	1.70	$4.77x10^{-05}$	-0.48	$7.44x10^{-04}$
M335T120	1.46	$5.84x10^{-06}$	-0.59	$6.44x10^{-06}$
M367T135	1.62	$5.49x10^{-08}$	-0.52	$1.65x10^{-04}$
M373T128	1.27	$2.71x10^{-04}$	-0.57	$2.40x10^{-05}$
M385T122	1.41	$7.76x10^{-04}$	-0.54	$7.55x10^{-05}$
M418T138	1.31	$2.39x10^{-05}$	-0.50	$4.16x10^{-04}$
M423T137	1.25	$3.53x10^{-05}$	-0.57	$1.98x10^{-05}$
M427T139	1.42	$8.16x10^{-06}$	-0.49	$4.68x10^{-04}$
M429T132	1.50	$9.97x10^{-08}$	-0.51	$2.30x10^{-04}$
M438T135	1.30	$1.86x10^{-05}$	-0.62	$1.76x10^{-06}$
M441T118	1.55	$2.61x10^{-07}$	-0.61	$2.41x10^{-06}$
M445T126	1.38	$3.56x10^{-04}$	-0.60	$4.56x10^{-06}$
M451T121	1.64	$3.33x10^{-08}$	-0.59	$9.07x10^{-06}$
M482T134	1.34	$8.26x10^{-06}$	-0.64	$5.52x10^{-07}$
M483T134	1.42	$1.24x10^{-05}$	-0.56	$3.68x10^{-05}$
M489T128	1.59	$2.06x10^{-06}$	-0.65	$2.69x10^{-07}$
M623T115	1.65	$5.46x10^{-08}$	-0.50	$3.80x10^{-04}$
M631T132	1.57	$6.29x10^{-08}$	-0.58	$1.50x10^{-05}$
M658T131	1.49	$2.33x10^{-08}$	-0.65	$3.25x10^{-07}$
M672T472	3.90	$1.85x10^{-06}$	0.50	$4.17x10^{-04}$
M703T130_1	1.66	$8.86x10^{-10}$	-0.54	$8.58x10^{-05}$
M704T131	1.56	$4.21x10^{-05}$	-0.52	$1.93x10^{-04}$
M757T668	3.01	$1.35x10^{-06}$	0.51	$2.51x10^{-04}$
M777T418	3.89	$2.64x10^{-08}$	0.51	$2.69x10^{-04}$
M801T471	3.34	$4.14x10^{-07}$	0.48	$7.36x10^{-04}$
M819T421	3.56	$1.66x10^{-09}$	0.50	$3.93x10^{-04}$
M849T462	4.12	$4.70x10^{-11}$	0.51	$2.28x10^{-04}$
M865T412	3.04	$6.13x10^{-07}$	0.47	$9.55x10^{-04}$
M879T417	3.09	$3.64x10^{-07}$	0.47	$9.91x10^{-04}$
M923T419	3.12	$1.89x10^{-07}$	0.49	$5.58x10^{-04}$

Cor, coefficient of association; FC, fold-change; P-value1, P-value of FC; P-value2, P-value of cor.

Table V. Metabolic pathway enrichment analysis.

Pathway name	Total	Hits	P-value	FDR
Arachidonic acid metabolism	62	17	$1.00x10^{-9}$	$8.01x10^{-8}$
Linoleic acid metabolism	15	4	$4.29x10^{-4}$	$1.72x10^{-2}$

FDR, false discovery rate.

Table VI. Details of the 17 compounds in arachidonic acid metabolism pathway.

Feature	KEGG ID	Name
M321T139	C04742	15(S)-HETE
M321T139	C14770	11,12-EET
M321T139	C14771	14,15-epoxy-5,8,11-eicosatrienoic acid
M321T139	C14778	16(R)-HETE
M321T139	C14749	19(S)-HETE
M321T139	C14748	20-HETE
M321T139	C14768	5,6-epoxy-8,11,14-eicosatrienoic acid
M321T139	C04805	5-HETE
M321T139	C14769	8,9-EET
M367T135	C05961	6-keto-prostaglandin F1α
M305T140	C00219	Arachidonic acid
M335T120	C05958	Δ12-Prostaglandin J2
M335T120	C05953	Prostaglandin A2
M335T120	C05954	Prostaglandin B2
M335T120	C05957	Prostaglandin J2
M335T120	C05955	Prostaglandin C2
M819T421	C00157	Phosphatidylcholine

EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid.

Table VII. Details of the 4 compounds in linoleic acid metabolism pathway.

Feature	KEGG ID	Name
M819T421	C00157	Phosphatidylcholine
M313T131	C04717	13-L-Hydroperoxylinoleic acid
M313T131	C07354	(7S,8S)-DiHODE
M313T131	C14831	8(R)-Hydroperoxylinoleic acid

The mean value of the low-level samples in the close-knit area was 14.5 μmol/l, compared with the high-level samples (17.0 μmol/l). In general, there may be an overlap between classes owing to the subjective classification thresholds of serum homocysteine, which was set at 15.0 μmol/l.

The metabolic pathways that were significantly enriched for the aforementioned 77 compounds matched in Matlin database were analyzed and two pathways were identified (Table V; FDR <0.05). The first enriched pathway was arachidonic acid metabolism (FDR=$8.01x10^{-8}$), which serves an important role in vascular homeostasis; a total of 17 compounds that were matched in the Matlin database were revealed to be involved in this pathway (Fig. 3A; Table VI). The second pathway was linoleic acid metabolism (FDR=$1.72x10^{-2}$), and a total of 4 compounds that were matched in the Matlin database were involved in this pathway (Fig. 3B; Table VII). However,

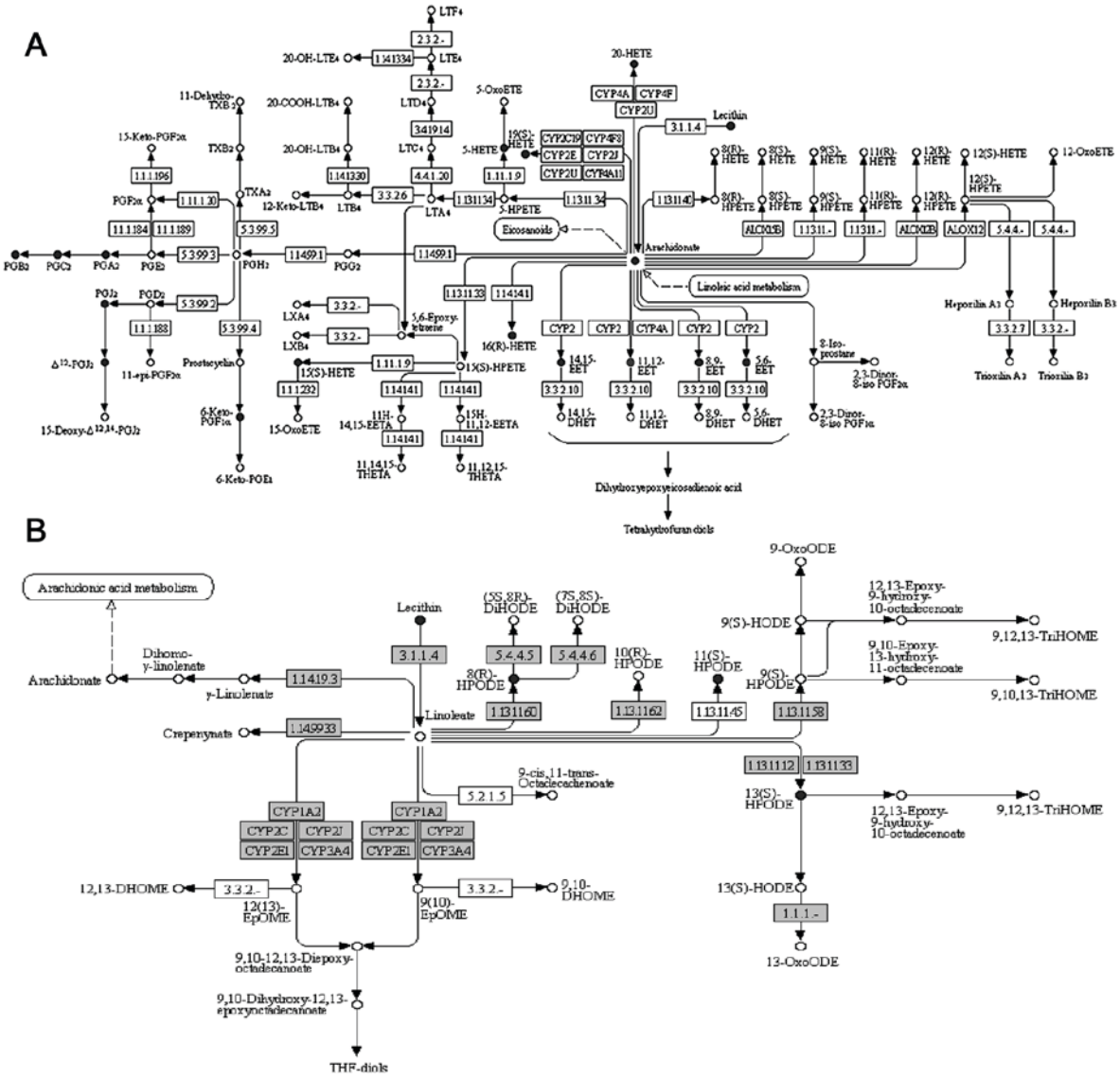

Figure 3. Arachidonic acid and linoleic acid metabolism signaling pathways. (A) The 17 compounds that were matched in Metlin database are annotated (filled black circles) in the arachidonic acid pathway. (B) The 4 compounds that were matched in Metlin database are annotated (filled black circles) in the linoleic acid metabolism signaling pathway.

a 'one-to-many' type of relationship was noted between the accurate mass and the identified compounds in the Matlin database. M321T139, for example, matched 9 compounds in the Matlin database, and all of the 9 compounds were annotated in the arachidonic acid metabolism pathway. This suggests that at least one 1 of the 9 compounds was abnormally metabolized in the arachidonic acid pathway. In this case, the false-positive rate of the pathway enrichment analysis may have been magnified. In order to obtain more reliable results in the pathway analysis, the one-to-many relationships between the accurate mass and the compounds were eliminated in the two pathways revealed to be significant, and only one compound was retained randomly in each one-to-many relationships. Regardless of which one compound was retained in the one-to-many relationships, there was no effect on the significance in the MetaboAnalyst 3.0 analysis. Subsequently, metabolic pathway enrichment was reanalyzed using MetaboAnalyst 3.0, and it was revealed that the arachidonic acid metabolism (FDR=0.02) and linoleic acid metabolism (FDR=0.12) remained the top two pathways significantly enriched with the 21 retained compounds.

Discussion

Results from the present study demonstrated a separation between the high- and the low-serum homocysteine groups using the correlated features detected in ESI(+) and ESI(-) modes. Furthermore, metabolic pathway analysis revealed that arachidonic acid metabolism and linoleic acid metabolism were significantly enriched for the compounds matched in the Matlin database. These results indicated that serum homocysteine levels may affect arachidonic acid and linoleic acid metabolism, and may subsequently contribute to the development of vascular diseases. Homocysteine was previously reported to have the potential to disrupt arachidonic acid metabolism through DNA demethylation *in vitro* (27).

A number of previous studies have suggested that arachidonic acid metabolism served an important role in vascular

diseases (28-30). Arachidonic acid is a free fatty acid derived from membrane phospholipids (31), which is catalyzed by phospholipase A2 and metabolized into hundreds of metabolites by three pathways: The cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450) pathways (32). The various arachidonic acid metabolites serve different roles in vascular diseases pathogenesis, and a total of 17 compounds annotated in arachidonic acid metabolism pathway maybe divided into three main groups: Prostaglandins, hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs). Prostaglandins are crucial bioactive molecules that are derived from the COX pathway and subsequent prostaglandin synthesis, and have been implicated in inflammation (33,34). The HETEs are metabolites in the LOX pathway, and have been implicated in numerous biological processes, such as angiogenesis (35) and platelet activation (36). The EETs are metabolites in the CYP450 pathway, and may act as antihypertensive and antiatherosclerotic (37) mediators for vasculature. Therefore, an imbalanced arachidonic acid metabolism in vascular may lead to an impairment in vascular homeostasis and the subsequent development of vascular disease.

In conclusion, the present study provided novel insights into the effects of homocysteine on metabolic alterations in human serum. The results suggested that arachidonic acid and linoleic acid metabolic pathways may be involved in homocysteine-induced vascular disease. These data revealed a novel effect of homocysteine in vascular disease and may have clinical significance for the treatment of these diseases.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author on reasonable request.

Authors' contributions

The work presented here was performed in collaboration between all authors. HL, YT and XJ defined the research theme. BL, GG, WZ, BL and CY designed the methods and experiments, performed the laboratory experiments and analyzed the data. BL and WZ wrote the manuscript. All authors have contributed to, read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University (Harbin, China; 2018-SCILLSC-02). All participating subjects were informed of their rights and written informed consent was obtained.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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