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Changes in lipid biomarker related to trauma severity in intertrochanteric fracture patients

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ABSTRACT

This study aimed to identify whether there are elevations or declines in specific plasma lipids in intertrochanteric fracture (ITF) patients which might serve as potential biomarkers for assessing the severity of trauma, or therapeutic targets for controlling post-traumatic responses. Ten metal work removal patients were enrolled. Their preoperative blood samples served as the control group (C group). Their 24-hour postoperative blood samples served as the moderate trauma group (M group). The ITF group was composed of 12 intertrochanteric fracture patients. A total of 707 lipid species were identified from 32 plasma samples (10 controls, 10 moderate trauma and 12 ITF samples). We first identified 31 lipids that were elevated and 6 lipids that were decreased in the more severe trauma group in aged patients, with an especially strong relationship among 14 lipids that are candidates as markers for trauma severity evaluation. Fourteen lipids were identified as potential markers of bone trauma. The definition of important lipids in trauma may not only provide guidance for the formulation of optimum ITF operation time, but may also have importance in other traumatic models, and in further understanding the components of the systemic inflammatory response for new drug targets.

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Introduction

Intertrochanteric fracture (ITF) is associated with significant patient morbidity and with profound temporary and sometimes permanent impairment of independence and quality of life (1-3). The timing of surgery in patients with ITF impacts these outcomes, and is, therefore, a matter of substantial current debate (4, 5). The reasons for this are not well understood in elderly patients; however, variations in patient inflammatory response may play a major role. The response to trauma is very complicated progress that includes various endocrine, metabolic and immunological changes. Lipid synthesis and metabolism are important for maintaining cell membrane structure, energy storage and signal transduction. There is a clear relationship between trauma and lipid metabolic response. For example, free fatty acids are primary sources of energy after trauma. Triglycerides provide 50–80% of the energy consumed after trauma and critical illness. In a seminal paper, Campbell et al. (6) showed that the size of the metabolic response is associated with the severity of trauma.

Lipids have been identified as potential biomarkers for establishing preventive or therapeutic programs for human diseases (7-12). Several studies in elderly hip fracture patients have explored the time-dependent effects of fractured bone, osteoporosis and calcium metabolism on lipid metabolism using plasma and urinary markers (13-17); however, a comprehensive and systematic lipid spectrum analysis of hip fractures has not been reported in the elderly in relation to severity of trauma.

Here, we use a novel methodology, non-targeted lipidomics, to identify whether there are elevations or declines in specific plasma lipids which might serve as potential biomarkers for assessing the severity of trauma, or therapeutic targets for controlling post-traumatic responses.

Materials and Methods

Study design and patient selection

This study was performed according to the standards of the Institutional Ethical Committee and the Helsinki Declaration. It was approved by the review board of Beijing Huaxin Hospital First Hospital of Tsinghua University. Ten metal work removal patients were enrolled, all aged over 55 years. Their preoperative blood samples served as the control group (C group). Their postoperative blood samples (24 hours after the operation) served as the moderate trauma group (M group). The ITF group was composed of 12 patients, all aged over 55 years who met the following criteria: 1). Hip fracture within 24 hours and caused by simple fall; 2). Community ambulators; Exclusion criteria for patients from both groups were co-morbidities such as diabetes and other systematic disorders that might affect lipid metabolism. All patients are required to sign the relevant informed consent form prior to enrolment

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Extraction of lipids

Briefly, 100 μL of plasma was mixed with 900 μL of isopropanol (IPA). After vortexing for 30 s and sonicating for 10 min, the mixture was frozen under -20°C for 1 hour and then centrifuged at 10,000 rpm for 10 minutes. The upper layer was collected (800 μL) and transferred to a sample vial to be injected and analyzed by UPLC-QTOF/MS.

UPLC-QTOF/MS analysis

Quality control (QC) samples were prepared by pooling aliquots of all plasma samples that were representative of the plasma samples under analysis. Blank samples (IPA/water: 90/10, the same solvent as in samples) and QC samples were injected every five samples during acquisition. The UPLC-QTOF/MS analyses were performed using a UPLC system (ACQUITY UPLC I-Class, Waters, Manchester, UK) coupled to an electrospray ionization quadruple time-of-flight mass spectrometer (ESI-QTOF/MS) (Xevo G2-S Q-TOF, Waters, Manchester, UK). Waters ACQUITY UPLC CSH C18 column (particle size, 1.7 μm; 50 mm (length) x 2.1 mm (i.d.)) was used for the LC separation and the column temperature was kept at 55°C. The flow rate was 0.4 mL/min and the sample injection volume was 2 μL. The mobile phases A was 0.1% formic acid and 10 mM ammonium formate in acetonitrile /H_2O (60%/40%), and B was 0.1 % formic acid and 10 mM ammonium format in IPA/ACN (90%/10%). The linear gradient was set as follows: Initial: 40% B, 0-2 min 40% B to 43% B, 2–2.1 min: 43% B to 50 % B, 2.1–12 min: 50% B to 54% B, 12–12.1min: 54% B to 70% B, 12.1–18 min: 70% B to 99% B, 18–18.1 min: 99% B to 40% B, 18.1–20 min: 40% High-accuracy MS data were recorded by MassLynx 4.1 software (Waters, Manchester, UK). The capillary voltage was 3.0 kV for the positive mode and 2.5 kV for the negative mode, whereas the cone voltage was 40 V for both modes. The source temperature was set at 120°C with a cone gas flow of 50 L/h, and the desolvation temperature was set at 400°C with a desolvation gas flow of 800 L/h. Leucine–enkephalin (Waters Co., Manchester, UK) was used as the lock mass generating a reference ion at m/z 556.2771 in positive mode and m/z 554.2615 in negative mode, which was introduced by a locking spray at 5 μL/min for data calibration. The MS^E data were acquired in continuum mode using ramp collision energy in two scan functions. For low energy mode, scan range 50-2000 Da, scan time 0.2 s, and collision energy 6 V were set. While for high energy, a scan range of 50–2000 Da, a scan time of 0.2 s, and a collision energy ramp of 10− 40 V were employed.

Lipidomics data analysis

MassLynx4.1 Software (Waters, Massachusetts, USA) was employed to submit samples and record results. The raw data were imported into Progenesis QI software (Waters, Massachusetts, USA) for further analysis including peak alignment and annotation, and compound identification. Then, exported data were filtered and calibrated by QC using R software (version 3.4.1). EZinfo 3.0 inserted by Progenesis QI was used to perform Principal Component Analysis (PCA), orthogonal signal correction Partial Least Square Discrimination Analysis (OPLS-DA), Variable Importance in Projection (VIP) and coefficients vs. VIP spots. All statistical analyses were conducted using R software (version 3.4.1). The differences between the intensities of lipids in the control, moderated trauma group and ITF group were compared using T-test where the data follow a normal distribution or the Wilcoxon rank-sum test where otherwise. The metabolic network was built based on Correlation using Metscape in Apps of Cytoscape. The probable metabolic pathways were derived by network and the reference.

Results

Characteristics of patients

The basic clinical characteristics of the patients enrolled in the study were summarized in Table 1. The mean age was 65.7±10.3 years in the C & M group and 72.2±13.2 years in the ITF group. There were 6 males and 4 females in the C & M group, and 4 males and 8 females in the ITF group. According to the Evans & Jensen classification, the ITF group included 1 type IB, 4 type IIA, 5 type IIB and 2 types III. All fractures were unstable and displaced. In the M group, the mean blood loss was 134.00±77.36 ml and the mean operation time was 91.42±52.73 minutes. Table 2 describes the surgical characteristics of the metal-work removal group.

Table 1. General information on metal work removal patients and ITF patients.

Parameters	C & M group (10)	ITF group (12)
Mean age	65.7±10.3	72.2±13.2
Sex (Male/Female)	6/4	4/8
Fracture type Evans & Jensen classification	-	I B (1)/IIA (4)/IIB (5)/III (2)

Table 2. Operation information of metal work removal patients.

Parameters	M Group
Operative blood loss (ml)	134.00±77.36
Operation time (min)	91.42±52.73
Internal fixation mode	Tibia nail(2)/Spine screws(1)/ PFN(1)/Femur plate(1)/Femur neck screws(1)/Radius plate(2)/ Humerus nail (2)
Anesthesia Mode	general anesthesia(2) epidural anesthesia (4) brachial plexus anesthesia(4)

Lipid class composition between the C group, M group and ITF group

Lipidomic analysis by the platform of UPLC-QTOF/MS was performed for the detection and identification of lipid species. A total of 707 lipid species were identified from 32 plasma samples (10 controls, 10 moderate trauma and 12 ITF samples), including 313 glycerophospholipids (GP), 167 glycerolipids (GL), 91 sphingolipids (SP), 64 Fatty Acids (FA), 41 sterol lipids (ST), 14 prenol lipids (PR), 16 polyketides (PK), and 1 saccharolipid (SL). Compared with lipid standards, samples from this study showed intensities that were consistent with standard concentrations. The percent of lipid classes was different among C, M and ITF groups (Figure 1). Specifically; compared to the ITF group (GL increased to 24%), GL was just 19% in the C group and 22% in the M group. SP decreased to 12% in the ITF group, compared to 16% in the C and 15% in the M group.

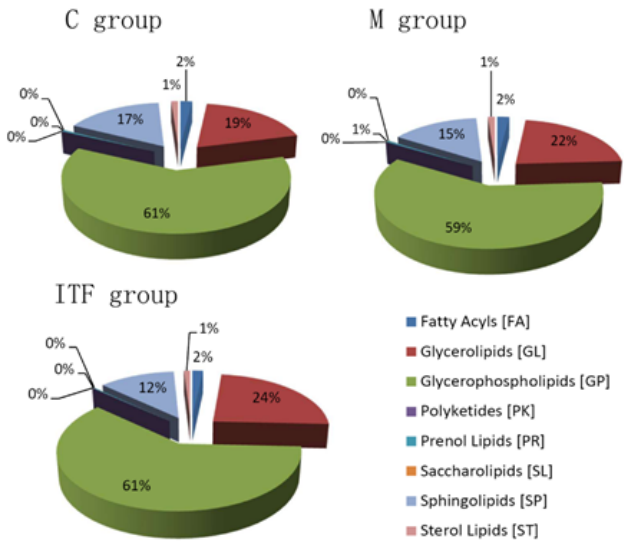

Figure 1. Difference of lipid class composition between the C group, M group and ITF group. In the C group, FA 2.00%, GL 18.97%, GP 60.91%, PK 0.05%, PR 0.45%, SL 0%, SP 16.45%, ST 1.17%; In the M group, FA 2.04%, GL 22.04%, GP 59.38%, PK 0.08%, PR 0.46%, SL 0%, SP 14.81%, ST 1.19%; In the ITF group, FA 1.70 %, GL 24.04%, GP 61.01%, PK 0.04%, PR 0.23%, SL 0%, SP 11.99%, ST 0.99%.

Multivariate and univariate analysis of lipid metabolites in three groups

The OPLS-DA classification model was used to describe the segregation between the C group and the ITF group and between the M group and the ITF group. A clear separation of the C group and ITF group was observed in the score plot, with the C group in the left half and the ITF group in the right half of the plot, while the separation showed the M group in the right and the ITF group in the left. In addition, we obtained an S-plot that showed an excellent S curve in both C vs. ITF group and M vs. ITF group. Based on the variable importance in the projection (VIP) values from the OPLS-DA model, 65 lipids were selected with VIP>1 between C and ITF groups, while 76 lipids were selected with VIP>1 between M and ITF groups. Univariate statistical analysis using the R project was performed to further validate the statistical significance ($P<0.05$) of lipid differences between the C and ITF groups and between the M and ITF groups (Figure 2).

After the screening, fifty-one lipids (VIP>1) were selected with adjusted $P<0.05$ in C vs. ITF group. These 51 differentiating lipids include 29 GP, 19 GL, and 2 FA. Forty-seven lipids (VIP>1) were selected with adjusted $P<0.05$ in M vs. ITF group. These 47 differentiating lipids include 34 GP, 11 GL, 1 FA and 1 PR.

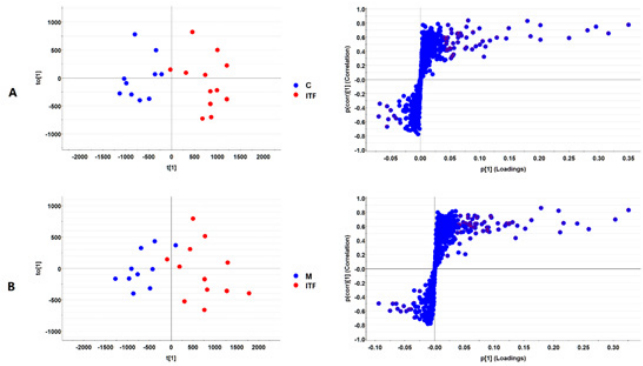

Figure 2. Group separation by OPLS-DA model based on lipidomics data of plasma by UPLC-QTOF/MS. (A) OPLS-DA score plot (left) and S-plot (right) in the plasma of the C group and ITF group. In the score plot, each red point represents one ITF sample; each blue point represents one control sample. S-plot was based on P values of correlation and covariance between metabolites in the plasma of patients with the C group and ITF group. The lipid metabolites with VIP>1 were marked with red squares. (B) OPLS-DA score plot (left) and S-plot (right) in the plasma of the M group and ITF group. In the score plot, each red point represents one ITF sample; each blue point represents one moderate sample. S-plot was based on p values of correlation and covariance between metabolites in the plasma of patients with the M group and ITF group. The lipid metabolites with VIP>1 were marked with red squares.

Similar and different changes of lipid pattern in three groups Screening of differential lipid species for assessing the severity of bone trauma

Among these differentiating lipids, we used Venn analysis to screen consistent and variant components in C vs. ITF group and M vs. ITF group. Compared to the C group, 45 lipid species demonstrated significant elevation and 6 lipid species declined in the ITF group. We also found 39 lipid species elevated and 8 lipid species declined in the ITF group compared with the M group. Finally, we identified 31 lipid species increased (U1-U31) and 6 decreased (D1-D6) in the ITF group (Figure 3 and Table 3 & Table 4) which may relate to the severity of bone trauma. These key lipids included Cardiolipin (CL), Diglyceride (DG), Phosphatidic acid (PA), Phosphatidylcholine (PC), Phosphatidyl ethanolamines (PE) and Triacylglycerols (TG).

Compared to the control group, three species of triacylglycerols demonstrated a significant elevation in the M group. These included TG (15:0/18:0/22:1), TG (16:1/18:0/22:0) and TG (16:0/18:1/22:1) and all exhibited an increase greater than 1.3-fold. No lipids decreased in the M group compared to the C group. Consequently, we selected U1-U31 and D1-D6 for further analysis.

Relationships among selected lipids and potential biomarkers analysis

The intensities of these 37 lipids selected above were analyzed to investigate their relationships. We performed

Table 3. Lipid species decreased in the ITF group compared with both the C and M groups.

No.	Lipid species	VIP		P value		*q* value	
		C vs. ITF	M vs. ITF	C vs. ITF	M vs. ITF	C vs. ITF	M vs. ITF
D1	PS(O-18:0/18:0)	1.19	1.14	0.03	0.02	0.11	0.08
D2	PS(O-18:0/18:3)	1.29	1.33	0.03	0.02	0.11	0.10
D3	PG (18:0/18:1)	1.48	1.50	0.02	0.03	0.10	0.11
D4	PC(O-14:0/2:0)	1.02	1.05	0.01	0.00	0.08	0.04
D5	PC(0:0/18:0)	1.66	1.37	0.00	0.01	0.06	0.07
D6	PC(0:0/16:0)	2.29	2.31	0.01	0.01	0.08	0.07

Table 4. Lipid species increased in the ITF group compared with both the C and M groups.

No.	Lipid species	VIP		*P* value		*q* value	
		C vs. ITF	M vs. ITF	C vs. ITF	M vs. ITF	C vs. ITF	M vs. ITF
U1	TG(18:0/18:1/20:4)	1.23	1.28	0.01	0.00	0.08	0.06
U2	TG(12:0/18:4/22:0)	1.27	1.30	0.01	0.01	0.08	0.07
U3	TG(18:1/18:2/20:4)	1.85	1.86	0.01	0.01	0.08	0.08
U4	TG(18:1/18:2/20:3)	2.17	2.22	0.01	0.01	0.08	0.08
U5	TG(16:0/16:1/22:4)	1.85	1.89	0.00	0.00	0.08	0.06
U6	PE(18:2/22:1)	1.14	1.20	0.03	0.03	0.11	0.10
U7	CL(1'-(22:1/22:1),3'-(22:1/14:1))	1.73	1.80	0.00	0.01	0.08	0.07
U8	DG(18:1/18:2/0:0)	1.40	1.34	0.01	0.05	0.09	0.13
U9	PE(21:0/20:5)	2.03	2.07	0.00	0.00	0.06	0.06
U10	PC(16:0/20:4)	8.31	7.94	0.01	0.02	0.09	0.09
U11	PC(18:0/20:4)	6.88	6.68	0.02	0.02	0.09	0.08
U12	PC(16:0/20:5)	1.47	1.41	0.00	0.00	0.06	0.06
U13	PC(16:0/20:3)	4.94	5.09	0.00	0.00	0.04	0.03
U14	PE-NMe2(18:1/18:1)	1.72	1.73	0.02	0.02	0.10	0.09
U15	CL(1'-(20:0/20:0),3'-(18:1/18:1))	2.64	2.79	0.00	0.00	0.07	0.06
U16	PA(17:0/20:2)	1.95	1.89	0.00	0.00	0.05	0.06
U17	PC(18:0/22:6)	2.60	2.60	0.00	0.00	0.06	0.06
U18	PC(20:0/18:3)	2.61	2.77	0.00	0.00	0.06	0.06
U19	TG(16:0/18:2/20:2)	4.00	3.68	0.02	0.03	0.09	0.10
U20	PE(P-18:0/19:0)	1.66	1.79	0.01	0.00	0.07	0.06
U21	PC(16:0/22:6)	4.30	4.01	0.00	0.00	0.06	0.06
U22	PC(O-18:0/20:5)	1.91	2.05	0.01	0.01	0.08	0.07
U23	PC(20:0/20:5)	1.19	1.11	0.02	0.04	0.10	0.12
U24	PA(16:0/22:1)	1.29	1.44	0.01	0.00	0.06	0.05
U25	CL(1'-(20:0/18:0),3'-(16:0/20:0))	5.13	5.59	0.00	0.00	0.06	0.06
U26	27:3	1.44	1.36	0.00	0.02	0.07	0.09
U27	PC(18:1/18:1)	7.52	8.77	0.00	0.00	0.07	0.06
U28	PC(18:0/18:3)	2.67	3.11	0.05	0.02	0.13	0.10
U29	PE(O-20:0/15:0)	1.17	1.43	0.03	0.01	0.11	0.07
U30	PC(16:0/18:1)	7.10	6.71	0.00	0.01	0.07	0.07
U31	PC(16:0/18:2)	8.79	8.40	0.00	0.00	0.06	0.07

a matrix correlation analysis of these lipids (Figure 4A). We also performed correlation network analysis (CI >0.7) to investigate the internal relationship among the 37 lipids (Figure 4B). We found 4 patterns that were significant in these lipids, which were denoted as Pattern 1: U1-2-3-4-5; Pattern 2: U6-7-10-11-14; Pattern 3: D3-U4-5-6-7-9-13-15-19-23-27; Pattern 4: D4-5-6. Bar charts of relative signal intensity in these 14 lipids (CI>0.7) are shown in Figure 5, which may be considered as potential biomarker for severity assessment of bone trauma.

Discussion

In this study, we selected patients who underwent implant removal as a model for moderate trauma compared to ITF which is more severe trauma in bone disruption. This design helps us to standardize the extent of trauma; the process of removing internal fixation involves soft-tissue and bone disruption with bleeding which may be considered as minor bone and soft-tissue trauma.

We first identified 31 lipids that were elevated and 6

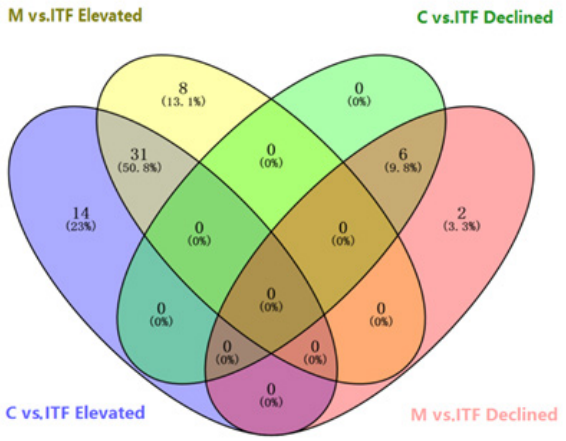

Figure 3. Similar and different changes of lipid pattern in three groups by Venn analysis. The Venn analysis showed that 31 common elements in "C vs. ITF elevated" and" M vs. ITF elevated " and 8 elements were included exclusively in "M vs. ITF elevated "; 6 common elements in "C vs. ITF declined" and "M vs. ITF declined"; 14 elements included exclusively in "C vs. ITF elevated" and 2 elements included exclusively in "M vs. ITF declined.

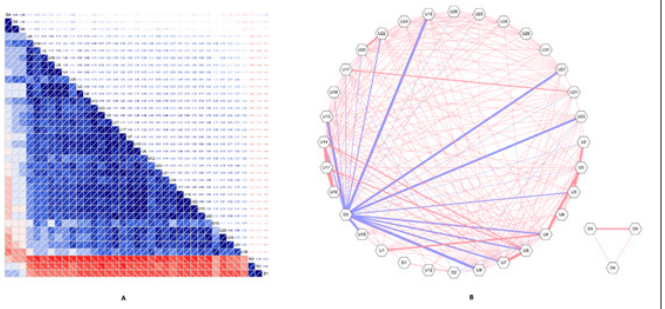

Figure 4. Correlation analysis between selected lipid species by matrix and network. (A) Correlation matrix: Red represents negative correlations, and blue represents positive correlations. The thickness and color intensity of lines represents the degree of correlation: thicker line and darker color indicate higher correlation. (B) Correlation network: Red represents negative correlations, and blue represents positive correlations. Four sets of network patterns showed a strong correlation: Pattern 1. U1-2-3-4-5 and Pattern 2. U6-7-10-11-14 are in elevated lipids; Pattern 4. D4-D5-D6 is a set of declined lipids. Pattern 3. D3-U4-5-6-7-9-13-15-19-23-27 are interactive with each other.

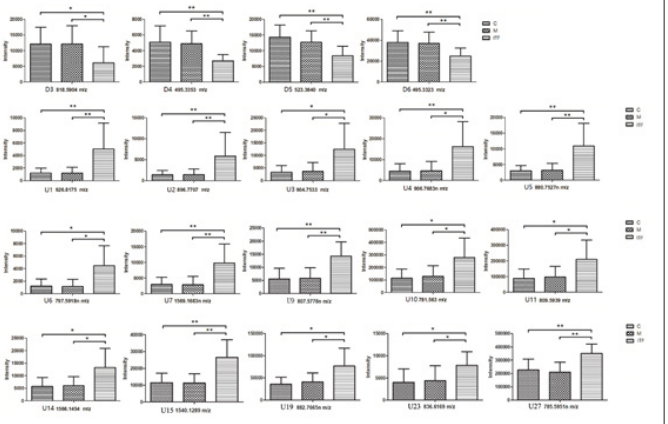

Figure 5. The relative intensity of potential lipid biomarkers of bone trauma in C group, M group and ITF group. Data are expressed as mean ± SD. All m/z of lipids are displayed. Statistically significant differences are indicated by an asterisk, where * refers to P<0.05, and ** refers to P<0.01. D3=PG (18:0/18:1), D4=PC (O-14:0/2:0), D5=PC (0:0/18:0), D6=PC (0:0/16:0), U1=TG (18:0/18:1/20:4), U2=TG (12:0/18:4/22:0), U3=TG (18:1/18:2/20:4), U4=TG (18:1/18:2/20:3), U5=TG (16:0/16:1/22:4), U6=PE (18:2/22:1)), U7=CL (1'-(22:1/22:1),3'-(22:1/14:1)), U9=PE (21:0/20:5), U10=PC (16:0/20:4), U11=PC (18:0/20:4), U13=PC (16:0/20:3), U14=PE-NMe2 (18:1/18:1), U15=CL (1'-(20:0/20:0),3'-(18:1/18:1)), U19=TG (16:0/18:2/20:2), U23=PC (20:0/20:5), U27=PC (18:1/18:1)

lipids that were decreased in the more severe trauma group in aged patients. A previous clinical research study found changing concentrations of total cholesterol, triglycerides and different lipoprotein fractions in the blood plasma of patients with severe mechanical trauma, with changing ratios of high-density lipoproteins, low-density lipoproteins and extra-low-density lipoproteins also observed in survivors by day 15. It can be stated on the basis of comparing the study results with the clinical outcome that the dynamic concentration of total cholesterol in blood plasma is an important prognostic factor (18).

These 31 elevated lipids belong to CL, DG, PA, PC, PE, and TG lipid groups. By CI analysis (CI>0.7), we obtain 10 lipids in 2 sets which showed a strong correlation. These belonged to PC and PE lipid groups. PCs are mediated by which hydrolyzes the glycerophospholipids to fatty acids and lysophosphatidylcholines (19). Mats Lindahl et al. (20) performed a prospective controlled clinical study in serious trauma patients admitted to ICU. PLA2 is significantly increased in the circulation and PLA2 levels correlate well with the severity of the disease. PEs conduct calcium signal transduction (21). Feld et al. (22) reported PEs involved in the protein kinase B/Akt signaling pathway which regulates cell survival, growth, and metabolism, and inhibits apoptosis. Akt activation was also found to be dependent on the severity of the injury.

We also identified 6 decreased lipids belonging to PS, PG and PC lipid groups. See the decrease in lipid D3 (PG (18:0/18:1)) correlated inversely more than 10 lipids which increased. D3 may act at a core position in the underlying bimolecular processes, which may include inflammation and alterations in cell membrane dynamics and metabolism. Three positive correlation lipids (CI >0.7) belong to PC. 1-alkyl, 2-acylglycerophosphocholines (PC (O-14:0/2:0)), which was found to decline in a mouse model with liver injury (23). The other two lipids PC (0:0/18:0) and PC (0:0/16:0) have no specific literature to illuminate possible changes in trauma.

Only three types of triacylglycerols were up-regulated in the M group compared with the C group. This may be due to preoperative fasting; a finding which was identified in a rat fasting model (24).

Post-traumatic pathophysiological changes are a very complex process in which metabolic changes can be summarized as a multisystem systemic inflammatory response syndrome. Both pain and bed rest after ITF are associated with a more systemic inflammatory response. The surgery itself is another traumatic insult.

In summary, we identified in elderly patients 31 elevated and 6 decreased lipids, with an especially strong relationship among 14 lipids which are candidates as markers for trauma severity evaluation. Further definition of important lipids in this process may not only provide guidance for the formulation of optimum ITF operation time,

but may also have importance in other traumatic models, and in further understanding the components of the systemic inflammatory response for new drug targets.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interests

The authors declared no conflict of interest.

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