Relationship between Plasma Lipidomics and Carotid Atherosclerotic Plaque

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Abstract

**Background:** The rapidly growing discipline of lipidomics allows the study of a wide spectrum of lipid species in human plasma and provides new insights into the pathogenesis of atherosclerosis. **Objective:** To explore the roles of plasma lipid components in relation to atherosclerotic plaques.

**Methods:** Ten non-symptomatic persons (age 62.42±3.45) with 2 or more carotid plaques (soft or mixed) and ten apparently healthy controls without any carotid plaque (age 62.82±4.38), were randomly selected as cases and controls from a community-based sample of 1312 persons. Ultra-performance liquid chromatography coupled with electrospray ionization quadrupole mass spectrometry (UPLC-ESI-QTOF MS)-based lipidomics was used to measure lipid components. The difference of lipid species between cases and controls was analyzed by t-test and general linear regression.

**Results:** Among 53 lipid components, significantly elevated plasma levels of one novel individual ceramide species (ceramide 22: 0) were observed in cases compared to the controls (0.59±0.18 versus 0.41±0.10 μg/mL, p values<0.05). After adjusting for confounding factors, such as total cholesterol and low-density lipoprotein cholesterol, the results remained significant (p<0.05). 22:0 ceramide was significantly negatively correlated with vegetable intake (r=-0.74, p=0.014).

**Conclusion:** High level of plasma ceramide 22:0 may be a novel risk factor for carotid atherosclerosis in human which independent of cholesterol and low-density lipoprotein cholesterol, it deserves future studies with larger sample size to confirm.

Introduction

Atherosclerosis (AS) remains one of the major health problems globally, yet its underlying mechanisms are still unclear. Known risk factors for AS include hypercholesterolemia, hypertension, diabetes, smoking, obesity, family history, and unhealthy dietary habits. However, the mechanisms by which these risk factors lead to the development of AS are less well understood.

There are several proposed mechanisms including lipid deposition theory, inflammatory theory, and stress (mechanical) damage theory. These hypotheses involve a complex series of reactions of biomolecules, including low-density lipoprotein, oxidized low-density lipoprotein, cytokines (C-reactive protein, intercellular adhesion molecule-1, colony-stimulating factor-1, tumor necrosis factor, Interleukin-1, 6, 10, 18), nitric oxide, and signaling molecules, all of which play an important role in the development of atherosclerosis.

Whilst it is well known that abnormal lipid metabolism plays a crucial role in the development of atherosclerosis, previous studies have focused on atherosclerosis plaques that have already occurred. Inside human carotid plaques, the sphingolipids and particularly glucosylceramide are associated with and are possible inducers of plaque inflammation and instability. Another recent study found that elevated plasma levels of C16:0 and C24:1 ceramide, correlating with immune activation and inflammation, were associated with antiretroviral therapy use and progression of carotid artery atherosclerosis in 2 HIV cohorts. The specific roles of biomolecules in early stages of AS development in general population, however, remained unknown. The study of a wide spectrum of lipid species in human plasma may provide potentially new insights into the pathogenesis of atherosclerosis.

Therefore, the present study aims to explore the role of plasma lipid components as potential risk factors of atherosclerotic plaque development in patients with carotid atherosclerotic plaques and their healthy controls.

Methods
Study population

The flow chart of carotid plaque group and control group were shown in Figure 1. In 2018, 10-year Follow-up Study of Subclinical Atherosclerosis Cohort were performed in 11 communities in Beijing in 2012\textsuperscript{15}. Among 1312 participants, 250 were <69 years old, free of diseases (angina/myocardial infarction, stroke, and other symptomatic diseases, such as cancer, liver disease, kidney disease, respiratory disease, connective tissue disease, thyropathy, etc.), abnormal ECG, surgery in recent two weeks, cardiovascular surgery in recent 10 years, lipid lowering drug in recent 2 weeks, but having necessary analysis data, in which 161 had carotid plaque, and 89 were free of carotid plaque. Among these 161 with carotid plaque, 85 had 2 or more soft/mixed plaque, in which ten participants were randomly (using SURVEYSELECT procedure in SAS\textsuperscript{®} 9.4 software) selected as carotid plaque group (cases). Among these 89 free of carotid plaque, ten were randomly selected as control group (controls).

Study protocol

Data on lifestyle factors and medical history were obtained using a standard questionnaire by an interview in 2012\textsuperscript{15,16}. Smoking was defined as at least 1 cigarette per day for more than 1 year. The frequency and weight of food were collected in the validated questionnaire (Supplement A in File S1). Body Mass Index (BMI) = weight (kg) / height 2 (m2). Blood pressure was measured on the right arm by a sphygmomanometer with two consecutive measurements that were at least 30s apart. The average of the two readings was used for analysis. Laboratory tests include fasting blood glucose (FBG), serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), interleukin-6 (IL-6), interleukin-10 (IL-10), high-sensitivity C-reactive protein (CRP), P-selectin, and soluble intercellular adhesion molecule-1 (s-ICAM-1)\textsuperscript{8,9}. In the morning, an empty 12 h venous blood sample was taken for testing, and all tests were performed in the central laboratory.

At the same time (2012), carotid plaque measurements were performed using a standardized protocol as described previously\textsuperscript{15,17}. The participant was placed in the supine position with the head tilted back and to the opposite side of the inspection. The probe was placed in the running area of the corresponding body surface of the blood vessel to be examined, and scanning was performed from the proximal end to the distal side. Plaques were defined as more than or equal to 1.3 mm of local intima thickening or more than or equal to 1.3 mm of local bulging into the lumen. The examination included bilateral carotid artery and carotid sinus. According to the ultrasonic echo level, the plaque was divided into: 1 = hard plaque (high echo); 2 = mixed plaque (mixed echo); 3 = soft plaque (low echo). The mixed/soft plaques usually occur newly and later than hard plaques. At same time, bigger differences of lipid molecules and statistic power were expected to be observed between severer patients and healthy controls. Thus, in the present study, we selected those with 2 or more mixed/soft plaque as cases with atherosclerosis. The repeatability evaluation of carotid ultrasound examination were shown in previous published papers\textsuperscript{15,18}.

Blood samples were collected in 2012. Then the plasma samples were separated from blood samples and stored at -80°C. In 2018, the plasma samples of the ten randomly selected persons with 2 or more soft or mixed carotid plaques and ten healthy controls were taken out and thawed at 4 °C. Lipid species of these plasma samples were measured using Ultra-performance liquid chromatography coupled with electrospray ionization quadrupole mass spectrometry (UPLC-ESI-QTOF MS) system in Tianjin University. The application studies of this system were shown in previous published papers\textsuperscript{19,20}. Exsient LC100 instrument was used for liquid chromatography. AB SCIEX Triple TOF 5600 LC/MS (AB SCIEX) instrument was used for mass spectrometry. WatersXBridge Peptide BEH C18 3.5 μm, 2.1 x 100 mm chromatography column (Waters) was used. Other instruments included Allegra 64R Centrifuge (BECKMAN COULTER, US), nitrogen purifier (VSD150-2) (Wuxi Woxin Instrument Co., Ltd.). Reagents included ammonium acetate (content ≥ 99%) (Fluka), formic acid (content ≥ 98%) (Fluka), acetonitrile (Fisher Scientific), water (Fisher Scientific), isopropyl Alcohol (Fisher Scientific), and normal saline (Jilin DuPont Pharmaceutical Co., Ltd.).
Firstly, 10 μl of plasma was added to 10 μl of mixed standard solution (containing 2 μg/mL 19:0-19:0 PC, 10 μg/mL 17:0-17:0 PE, 15 μg/mL 12:0 SM, 2μg/mL 19:0 Lyso PC, or 10μg/mL 19:0 ceramide dissolved in isopropanol-acetonitrile solution). 10μl 0.9% NaCl and 100μl chloroform-methanol (2:1) extract were then added to the mixture. The mixture was vortexed for 60s, left to settle at 4 °C for 30 min, and then centrifuged at 13,000 g/min for 3 min. A 1 mL syringe was used to transfer 50 μl of the lower layer to a 1.5 mL EP tube, which was blown dry with nitrogen, reconstituted with 25 μl of acetonitrile-isopropanol (1:1), and vortexed for 60 s. The lipids were detected by Eksigent LC100 and AB SCIEX Triple TOF 5600 instruments. The conditions of Eksigent LC100 were as follows: mobile phase A was 10 mM ammonium acetate + 0.1% formic acid + 99.9% water, mobile phase B phase was 10 mM ammonium acetate + 0.1% formic acid + 49.95% acetonitrile + 49.95% isopropanol, the column temperature was 40 ° C, the injection chamber temperature was 4 ° C, the flow rate was 0.4 mL / min, the injection volume was 2 μl. The gradient elution conditions were shown in Supplement B in File S1. The AB SCIEX Triple TOF 5600 instrument was measured in negative ion mode and the test parameters were shown in Supplement C in File S1.

Lipid molecule data processing: according to lipid standard database, lipids in plasma samples were characterized by PeakView 1.2, identified lipids were quantified by MultiQuant2.1 to obtain peak area data and concentration according to the corresponding standard concentrations of lipid molecular area. We measured a total of 53 plasma lipid metabolomics species, including 7 ceramides, 12 phosphorylethanolamine, 7 sphingomyelin, 26 phosphatidylcholine, and 1 phosphatidylinositol. We also calculated ratio of ceramides C22:0/C16:0, C24:0/C16:0, C24:0/C24:1, C22:0/C24:1, which were reported by other previous studies21-23.

Quality Control

First, calibration standards of known concentrations were used in measurement of lipid molecules. Second, based on the research protocol, a comprehensive manual was developed, including standard operating procedures for questionnaire, height, weight, blood pressure, blood collection, specimen transportation, and laboratory testing. Third, the survey database was built using EpiData 3.1 (EpiData Association, Odense, Denmark). Double entry was performed independently by two people and all raw data were confirmed to be completely consistent. Finally, data verification was performed, and all issues were resolved before locking the database.

Statistical Analysis

Statistical analysis was performed using SAS® 9.4 software (SAS Institute Inc., Cary, NC). T-test or Wilcoxon rank test was conducted to determine the differences of the mean or median values of continuous variables between the groups. The normality of the distribution of variables was assessed using the Shapiroe-Wilk test. Chi-square test or Fisher’s exact test was conducted to determine the differences of the percentages of categorical variable between the groups. Confounding factors were controlled using two general linear models: model 1) for adjustment of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c); and model 2) for adjustment of interleukin-6(IL-6).

Model 1: Concentration of each lipid component=a+ b1*group +b2*TC+b3*LDL-c;

Model 2: Concentration of each lipid component=a+ b1*group +b2*IL-6

Here group was case/control (case=1; control=0). In order to reduce the effect of multiple comparison, we limited the models to those lipid components which showed significant association with carotid plaque by t-test. Correlations between ceramide and risk factors/dietary intake were assessed by Spearman Correlation.

The significance level of all analyses was set at P < 0.05. Due to the exploratory study design, p cut-point value adjustment for multiple comparison were not performed.
## Results

### Comparison of Key Factors between patients and healthy controls

Between carotid plaque group and healthy control group, no significant differences were found for age, sex, smoking frequency, BMI, systolic blood pressure, diastolic blood pressure, fasting blood glucose, triglyceride, high-density lipoprotein cholesterol, interleukin-6, interleukin-10, C-reactive protein, P-selectin, or soluble intercellular adhesion molecule-1 (s-ICAM-1). However, higher total cholesterol and low-density lipoprotein cholesterol levels were significantly higher in the carotid plaque group than the healthy control group (p<0.05), and thus were adjusted to assess the relationship of lipids index with atherosclerosis plaque (Table 1).

### The Relationship between Plasma Lipid Index and Atherosclerotic Plaque

Compared with the healthy controls, the carotid plaque cases showed significantly higher levels of 22:0, 24:0, 24:1 ceramide, 18:0 PE, and 24:0 SM. After adjusting for total cholesterol and low-density lipoprotein cholesterol, only 22:0 ceramide level remained statistically significant (Table 2). The model as following.

\[
22:0 \text{ceramide}=0.33+ 0.16 \times \text{group} -0.15 \times \text{TC}+0.11 \times \text{LDL-c};
\]

When type error is set as 0.05, the statistic power 0.734 for 22:0 ceramide by PASS11 software.

Because the interleukin-6 was not significantly different between cases and controls, it may not be a confounder and adjustment for interleukin-6 did not change the significance (data not shown).

### The Relationship between 22:0 Ceramide and Cardiovascular Risk Factors (Table 3)

In order to explain why 22:0 ceramide was associated with atherosclerosis, we explore the relationship between 22:0 ceramide and risk factors of cardiovascular disease using Spearman Correlation. 22:0 ceramide was significantly negatively correlated with vegetable intake (r=-0.74, p=0.014). The other correlations did not reach a significant level (Table 3).

### Discussion

In this study, ten non-symptomatic persons with carotid plaque and ten clinical healthy controls were randomly selected from community residents to analyze the association between blood lipid species and carotid atherosclerosis (AS). Among the 53 individual lipid species examined, after adjusting for confounding factors, 22:0 ceramide in carotid plaque group was significantly higher than the healthy controls.

Ceramide is a kind of sphingolipids which is made up of long-chain bases of sphingosine and fatty acids. For a long time, it was believed that the only function of ceramide was to combine with choline phosphate and hence forming sphingomyelin, which constitutes the lipid bilayer structure of mammalian cell membrane. A recent study found that higher levels of C16:0, C22:0, and C24:0 ceramides were associated with higher risk of ischemic stroke. However, the mechanism is unclear. Whereas this study provided a preliminary scientific basis for the atherogenic effect of ceramide using a population epidemiology approach. We identified 22:0 ceramide molecular significantly associated with mixed or soft carotid atherosclerosis plaques. Moreover, there was a tendency for higher ceramide (24:0) in plaque subjects. Ischemic stroke due to artery embolism is mostly due to carotid plaque formation. Thus the impact of ceramide on carotid plaque may play key role in pathophysiology of ischemic stroke due to artery-artery thromboembolism.

More important was that the associations of 22:0 ceramide with carotid plaque were independent of total cholesterol and LDL-c. In the present study, total cholesterol and LDL-c were the first and second strongest macro lipid associated with
atherosclerosis, in line with current literatures\textsuperscript{25,26}. It was previously shown that ceramides (micro lipid) was significantly associated with LDL aggregation, and significantly associated with mortality coronary artery disease (CAD)\textsuperscript{27}. Thus, ceramide may have independent extra effect on the development of atherosclerosis outside of the effect of LDL and total cholesterol.

It is worth highlighting that ceramide showed a significant negative correlation with vegetable intake ($p=0.01$). Previously, it was shown that sphingomyelin (SM) levels in LDL were reduced with healthy, vegetable containing diet suggesting that LDL with lower amount of SM is not a good substrate for sphingomyelinase in arterial wall and thereby affect ceramide formation\textsuperscript{27}. The observed association between vegetable intake and ceramide provides a potential explanation on the previous finding that vegetable intake reduce risk of subclinical carotid atherosclerosis\textsuperscript{28}. If our results were confirmed by larger studies in the future, population level strategies to incentivize higher vegetable intake may lead to reduction of ceramide levels and hence prevent atherosclerosis at population level.

There are some limitations. Firstly, the sample size was small. Thus, we performed a retrospective power-analysis. When type error is set as 0.05, the statistic power is calculated as 0.734 to check out the difference of 22:0 ceramide in patient and control group using PASS11 software. Therefore, detected associations between 22:0 ceramide and the development of AS still needs to be verified in larger sample populations. If this association can be confirmed in a large sample cohort, it will promote the understanding of 22:0 ceramide as a new risk factor as well as an important therapeutic target for cardiovascular diseases. Secondly, this study measured 22:0 ceramide and atherosclerotic plaque in a time section. If there is a causal relationship still needs to be confirmed by cohort studies. Thirdly, blood samples were collected in 2012 and plasma samples were stored at -80°C, and were thawed in 2018 to be used for lipidomics. Some lipid molecules were prone to degrade during storage. However, the degree of degradation may be limited because the lipid molecules, such as ceramide, were at same order of magnitude in our study as another published study\textsuperscript{29}. Moreover, because the blood samples were stored at same condition and were tested by same methods, the degradation (if any) is likely to be similar in both cases and controls. Thus, whilst limited degradation of sample may weaken the strength of the association, it is unlikely to have impact on the direction of results. Fourthly, we used whole plasma. Because different lipoprotein fractions contain varying amounts of ceramide especially LDL and HDL, when using whole plasma and especially here with small number of samples the plasma values are likely to be diluted\textsuperscript{30}. These limitations may weaken the association but very unlikely to change the associations observed and thus study conclusion. Fifthly, we did not match lipid profile (total cholesterol, LDL-c, etc.).

In summary, the plasma 22:0 ceramide was significantly associated with atherosclerotic plaque in the present study. The result suggested that 22:0 ceramide might be a new risk factor of atherosclerosis, but further validation is needed using larger sample cohorts.

**Declarations**

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**Availability of data and materials**
The authors declare that the data supporting the findings of this study are available within the article.

**Authors’ contributions**

GX: concept development, data cleaning analysis, and interpretation, and writing of the manuscript; PKM: critical input in interpretation of results and writing of the manuscript; ZF and YY: critical input in interpretation of results and writing of the manuscript; WM: data analyses; YH and WG: review of manuscript; YW: concept development, critical input in interpretation of results, and review and approval of the manuscript.

**Ethics approval**

Peking University Health Science Center Ethics Committee approved the study. Informed written consent was obtained from all participants in all surveys and examinations. De-identified data were used for data analysis.

**Consent for publication**

Not applicable.

**Conflict of interests**

The authors declare that they have no conflict of interests.

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Tables

Table 1. The characteristics of cases with carotid plaque and control
| Variables          | All       | With plaque | with no plaque | P Value | Statistical Test |
|--------------------|-----------|-------------|----------------|---------|-----------------|
| n                  | 20        | 10          | 10             |         |                 |
| Age, year          | 62.62(3.84)| 62.42(3.45) | 62.82(4.38)    | 0.821   | t-test          |
| Male, n (%)        | 11(55.00%)| 6(60.00%)   | 5(50.00%)      | 1.000   | Chi-sq         |
| Smoking, n (%)     | 4(20.00%) | 2(20.00%)   | 2(20.00%)      | 1.000   | (Fisher's exact test) |
| BMI, kg/m2*        | 25.30(2.99)| 24.98(2.75) | 25.62(3.32)    | 0.643   | t-test          |
| SBP, mmHg*         | 134.90(19.44)| 141.50(22.41)| 128.30(14.11) | 0.132   | t-test          |
| DBP, mmHg*         | 81.10(13.77)| 85.53(15.80)| 76.67(10.34)   | 0.155   | t-test          |
| Glucose, mmol/L    | 5.94(1.34) | 5.68(0.71)  | 6.20(1.77)     | 0.623   | Wilcoxon rank  |
| TC, mmol/L*        | 5.62(1.28) | 6.37(0.92)  | 4.86(1.15)     | 0.004   | t-test          |
| TG, mmol/L*        | 1.68(1.10) | 1.99(1.38)  | 1.38(0.65)     | 0.121   | Wilcoxon rank  |
| HDL-c, mmol/L*     | 1.48(0.39) | 1.55(0.39)  | 1.40(0.40)     | 0.407   | t-test          |
| LDL-c, mmol/L*     | 3.64(1.13) | 4.16(1.16)  | 3.13(0.88)     | 0.040   | t-test          |
| IL-6, pg/ml*       | 17.19(12.70)| 19.12(14.17)| 15.26(11.47)   | 0.512   | t-test          |
| IL-10, pg/ml*      | 32.93(20.70)| 29.16(18.23)| 36.70(23.26)   | 0.430   | t-test          |
| CRP, ug/ml*        | 2.61(2.29) | 3.24(2.49)  | 1.99(2.01)     | 0.473   | Wilcoxon rank  |
| p-selectin, ng/ml  | 0.14(0.09) | 0.17(0.10)  | 0.11(0.07)     | 0.189   | t-test          |
| s-ICAM-1, ng/ml*   | 568.75(179.52)| 604.88(198.73)| 532.62(160.11)| 0.382   | t-test          |
| History of CVD, n (%) |         |             |                |         |                 |
| Hypertension       | 11(55.0%) | 7(70%)      | 4(40%)         | 0.370   | Fisher Exact probability |
| Diabetes           | 5(25.0%)  | 2(20%)      | 3(30%)         | 1       | Fisher Exact probability |
| Dyslipidemia*      | 11(55%)   | 8(80%)      | 3(30%)         | 0.070   | Fisher Exact probability |
| Angina/MI          | 0         | 0           | 0              | -       | -               |
| Stroke             | 0         | 0           | 0              | -       | -               |
| Treatment          |           |             |                |         |                 |
| Antihypertensive   | 6(30%)    | 3(30%)      | 3(30%)         | 1.0     | Fisher Exact probability |
| Lipid lowering     | 0(0)      | 0(0)        | 0(0)           | -       | -               |
| Insulin            | 3(15%)    | 0           | 3(30%)         | 0.211   | Fisher Exact probability |
| Oral hypoglycemic drugs | 3(15%) | 2(20%)    | 1(10%)         | 1       | Fisher Exact probability |
| Aspirin            | 4(40%)    | 3(30%)      | 1(10%)         | 0.582   | Fisher Exact probability |
* TC: total cholesterol; TG: triglycerides; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; IL-6: interleukin-6; IL-10: interleukin-10; CRP: high-sensitivity C-reactive protein; and s-ICAM-1: soluble intercellular adhesion molecule-1. Dyslipidemia: TC≥6.2mmol/L or TG≥2.3mmol/L or LDL-c≥4.1mmol/L or HDL-c<1mmol/L.

Table 2. Differences in mean (standard deviation) of lipidomics between atherosclerotic plaque and non-plaque groups
| Classification | Lipidomics Index | All                  | With plaque                  | with no plaque                  | P of t-test | P α        |
|----------------|------------------|----------------------|-----------------------------|-------------------------------|------------|------------|
| Ceramide       | 22:0 ceramide, μg/mL | 0.50(0.17)           | 0.59(0.18)                  | 0.41(0.10)                    | 0.014      | 0.033      |
|                | 24:0 ceramide, μg/mL | 1.55(0.54)           | 1.82(0.56)                  | 1.28(0.38)                    | 0.020      | 0.061      |
|                | 20:0 ceramide, μg/mL | 0.09(0.04)           | 0.10(0.04)                  | 0.08(0.02)                    | 0.068      | -          |
|                | 24:1 ceramide, μg/mL | 0.63(0.26)           | 0.76(0.29)                  | 0.51(0.16)                    | 0.032      | 0.198      |
|                | 18:1 ceramide, μg/mL | 0.03(0.01)           | 0.03(0.01)                  | 0.02(0.01)                    | 0.169      | -          |
|                | 18:0 ceramide, μg/mL | 0.24(0.10)           | 0.26(0.08)                  | 0.22(0.12)                    | 0.457      | -          |
|                | 16:0 ceramide, μg/mL | 0.42(0.13)           | 0.45(0.16)                  | 0.40(0.09)                    | 0.433      | -          |
|                | Ratio of ceramide C22:0/C16:0 | 1.27(0.55)          | 1.50(0.67)                  | 1.05(0.27)                    | 0.076      | 0.084      |
|                | Ratio of ceramide C24:0/C16:0 | 3.94(1.69)          | 4.58(1.99)                  | 3.30(1.08)                    | 0.092      | 0.123      |
|                | Ratio of ceramide C24:1/C16:0 | 1.59(0.63)          | 1.85(0.71)                  | 1.32(0.41)                    | 0.058      | 0.196      |
|                | Ratio of ceramide C22:0/C24:1 | 0.83(0.21)          | 0.83(0.21)                  | 0.84(0.22)                    | 0.899      | 0.328      |
|                | Ratio of ceramide C24:0/C24:1 | 2.59(0.73)          | 2.54(0.55)                  | 2.65(0.89)                    | 0.742      | 0.285      |
| Phosphorylethanolamine | 18:0 PE          | 0.02(0.03)           | 0.03(0.03)                  | 0.01(0.01)                    | 0.045      | 0.339      |
|                | PE               | 18:0-18:1 PE         | 0.40(0.27)                  | 0.50(0.34)                    | 0.30(0.13) | 0.100      | -          |
|                |                 | 18:0-18:2 PE         | 1.93(0.83)                  | 2.15(0.59)                    | 1.71(0.99) | 0.244      | -          |
|                |                 | 16:0-22:6 PE         | 1.73(1.07)                  | 2.01(1.21)                    | 1.45(0.89) | 0.260      | -          |
|                |                 | 18:0-20:4 PE         | 2.05(1.02)                  | 2.26(1.10)                    | 1.84(0.93) | 0.371      | -          |
|                |                 | 18:0-22:6 PE         | 0.73(0.42)                  | 0.81(0.38)                    | 0.65(0.47) | 0.407      | -          |
|                |                 | 16:0 Lyso PE         | 0.56(0.29)                  | 0.52(0.26)                    | 0.61(0.33) | 0.514      | -          |
|                |                 | 18:0 Lyso PE         | 0.68(0.32)                  | 0.72(0.36)                    | 0.64(0.27) | 0.597      | -          |
|                |                 | 18:1 Lyso PE         | 0.56(0.27)                  | 0.58(0.33)                    | 0.53(0.21) | 0.702      | -          |
|                |                 | 16:0-18:2 PE         | 0.74(0.37)                  | 0.78(0.22)                    | 0.70(0.48) | 0.613      | -          |
|                |                 | 16:0-18:1 PE         | 0.31(0.16)                  | 0.30(0.11)                    | 0.32(0.21) | 0.829      | -          |
|                |                 | 16:0-20:4 PE         | 0.79(0.42)                  | 0.79(0.34)                    | 0.79(0.50) | 0.989      | -          |
| Sphingomyelin  | 24:0 SM          | 12.08(2.56)          | 13.45(1.93)                 | 10.70(2.44)                   | 0.012      | 0.142      |
| SM            | 16:0 SM (d18:1/16:0) | 30.78(0)             | 30.11(0)                    | 31.45(0)                      | 0.792      | -          |
|                | 24:1 SM          | 22.35(7.05)          | 24.58(6.93)                 | 20.12(6.76)                   | 0.162      | -          |
|                | 17:0 SM (d18:1/17:0) | 0.82(0)              | 0.87(0)                     | 0.76(0)                       | 0.224      | -          |
|                | 18:1SM(d18:1/18:1(9Z) | 1.94(0)              | 1.89(0)                     | 1.99(0)                       | 0.807      | -          |
|                                |               |               |               |               |
|--------------------------------|---------------|---------------|---------------|---------------|
| 18:0 SM (d18:1/18:0)          | 6.43(0.9)     | 6.44(0.9)     | 6.42(0.9)     | 0.989         |
| 02:0 SM (d18:1/2:0)           | 0.05(0.03)    | 0.04(0.03)    | 0.05(0.03)    | 0.418         |
| Phosphatidylcholine PC        | 18:2 (Cis) PC (DLPC)  | 9.89(3.52)    | 11.41(2.79)   | 8.38(3.64)    | 0.051         |
| 18:0 PC (DSPC)                | 0.15(0.06)    | 0.17(0.05)    | 0.12(0.05)    | 0.054         |
| 16:1 (Δ9-Cis) PC              | 1.30(0.48)    | 1.49(0.40)    | 1.11(0.50)    | 0.076         |
| 18:0-22:6 PC                  | 7.86(3.29)    | 7.92(1.62)    | 7.81(4.50)    | 0.162         |
| 17:0 PC                       | 1.36(0.32)    | 1.46(0.31)    | 1.26(0.31)    | 0.174         |
| 18:0 Lyso PC                  | 26.45(6.90)   | 28.39(6.46)   | 24.50(7.09)   | 0.216         |
| 20:4 (Cis) PC                 | 0.22(0.10)    | 0.25(0.09)    | 0.20(0.11)    | 0.255         |
| 18:0-18:2 PC                  | 74.43(22.04)  | 79.89(21.13)  | 68.98(22.66)  | 0.280         |
| 18:1 (Δ6-Cis) PC              | 55.57(16.36)  | 59.50(15.65)  | 51.63(16.89)  | 0.294         |
| 18:1-18:0 PC                  | 7.00(1.95)    | 7.38(1.47)    | 6.62(2.35)    | 0.397         |
| 20:0 Lyso PC                  | 0.12(0.05)    | 0.13(0.05)    | 0.11(0.06)    | 0.455         |
| 16:0 PC (DPPC)                | 3.61(1.32)    | 3.45(1.48)    | 3.77(1.19)    | 0.521         |
| 18:1-14:0 PC                  | 0.21(0.10)    | 0.23(0.10)    | 0.20(0.10)    | 0.585         |
| 14:0 Lyso PC                  | 0.59(0.25)    | 0.62(0.19)    | 0.56(0.31)    | 0.597         |
| 24:0 Lyso PC                  | 0.02(0.02)    | 0.03(0.02)    | 0.02(0.01)    | 0.607         |
| 13:0 Lyso PC                  | 0.11(0.09)    | 0.12(0.12)    | 0.10(0.06)    | 0.645         |
| 18:1-16:0 PC                  | 39.38(13.94)  | 37.96(10.99)  | 40.80(16.89)  | 0.662         |
| 16:0-18:1 PC                  | 52.69(18.74)  | 50.88(14.72)  | 54.51(22.74)  | 0.677         |
| 18:0-20:4 PC                  | 40.63(13.40)  | 41.82(12.86)  | 39.44(14.51)  | 0.702         |
| 16:0-14:0 PC                  | 3.32(1.99)    | 3.16(1.16)    | 3.49(2.65)    | 0.722         |
| 18:1 Lyso PC                  | 12.70(2.90)   | 12.47(2.75)   | 12.93(3.17)   | 0.736         |
| Egg Lyso PC                   | 79.60(16.44)  | 80.76(13.72)  | 78.44(19.48)  | 0.762         |
| 22:0 Lyso PC                  | 0.02(0.01)    | 0.02(0.01)    | 0.02(0.01)    | 0.782         |
| 15:0 Lyso PC                  | 0.29(0.11)    | 0.30(0.10)    | 0.29(0.12)    | 0.817         |
| 15:0 PC                       | 0.62(0.35)    | 0.61(0.24)    | 0.64(0.44)    | 0.867         |
| 17:0 Lyso PC                  | 0.61(0.19)    | 0.62(0.20)    | 0.60(0.19)    | 0.896         |
| Phosphatidylinositol (PI)     | 20:4 Lyso PI  | 0.06(0.04)    | 0.06(0.03)    | 0.06(0.04)    | 0.698         |

*a* Adjusted for low-density lipoprotein cholesterol and total cholesterol.

*b* p value was calculated using Wilcoxon rank test;
Table 3. Spearman correlation coefficients to measure the relationship between 22:0 ceramide and cardiovascular risk factors in ten healthy controls after adjusted for age and sex

| Cardiovascular risk factors                                      | 22:0 ceramide |
|------------------------------------------------------------------|--------------|
| LDL-c                                                            | -0.37        |
| Total Cholesterol                                               | -0.28        |
| Triglyceride                                                    | 0.08         |
| High density lipoprotein cholesterol                            | 0.01         |
| Smoking count                                                   | -0.17        |
| Drinking                                                         | -0.34        |
| BMI                                                              | 0.31         |
| Systolic blood pressure                                         | 0.10         |
| Diastolic blood pressure                                        | 0.08         |
| Fasting blood sugar                                             | 0.07         |
| High-sensitivity C-reactive protein                             | 0.18         |
| Interleukin-6                                                   | 0.19         |
| Interleukin-10                                                  | 0.38         |
| P-selectin                                                      | 0.09         |
| Intercellular adhesion molecule-1 (s-ICAM-1)                    | -0.33        |
| Dietary                                                         |              |
| Staple food                                                     | -0.59        |
| Bean products                                                   | -0.55        |
| Chicken and duck                                                | -0.15        |
| Beef, pork, and lamb                                            | -0.11        |
| Fish                                                            | -0.27        |
| Eggs                                                            | -0.23        |
| Milk and dairy products                                         | 0.18         |
| Vegetables                                                      | -0.74*       |
| Fruit                                                           | 0.57         |
| Nut                                                             | -0.10        |
| Pickle                                                          | 0.21         |

*p=0.014;

Figures
Figure 1

Flow chart of carotid plaque cases and controls

Supplementary Files

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