Plasma lipid composition and risk of developing cardiovascular disease.

Fernandez, Celine; Sandin, Marianne; Sampaio, Julio L; Almgren, Peter; Narkiewicz, Krzysztof; Hoffmann, Michal; Hedner, Thomas; Wahlstrand, Björn; Simons, Kai; Shevchenko, Andrej; James, Peter; Melander, Olle

Published in:
PLoS ONE

DOI:
10.1371/journal.pone.0071846

2013

Link to publication

Citation for published version (APA):
Fernandez, C., Sandin, M., Sampaio, J. L., Almgren, P., Narkiewicz, K., Hoffmann, M., Hedner, T., Wahlstrand, B., Simons, K., Shevchenko, A., James, P., & Melander, O. (2013). Plasma lipid composition and risk of developing cardiovascular disease. PLoS ONE, 8(8), [e71846]. https://doi.org/10.1371/journal.pone.0071846

Total number of authors:
12

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Plasma Lipid Composition and Risk of Developing Cardiovascular Disease

Celine Fernandez1*, Marianne Sandin2, Julio L. Sampaio3, Peter Almgren1, Krzysztof Narkiewicz4, Michal Hoffmann4, Thomas Hedner5, Björn Wahlstrand5, Kai Simons3, Andrej Shevchenko3, Peter James2, Olle Melander1*

1 Department of Clinical Sciences, Lund University, Malmö, Sweden, 2 Department of Immunotechnology, Lund University, Lund, Sweden, 3 Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, 4 Department of Hypertension and Diabetology, Medical University of Gdansk, Gdansk, Poland, 5 Department of Medicine, Sahlgrenska Academy, Goteborg University, Goteborg, Sweden

Abstract

Aims: We tested whether characteristic changes of the plasma lipidome in individuals with comparable total lipids level associate with future cardiovascular disease (CVD) outcome and whether 23 validated gene variants associated with coronary artery disease (CAD) affect CVD associated lipid species.

Methods and Results: Screening of the fasted plasma lipidome was performed by top-down shotgun analysis and lipidome compositions compared between incident CVD cases (n = 211) and controls (n = 216) from the prospective population-based MDC study using logistic regression adjusting for Framingham risk factors. Associations with incident CVD were seen for eight lipid species (0.21 ≤ q ≤ 0.23). Each standard deviation unit higher baseline levels of two lysophosphatidylcholine species (LPC), LPC16:0 and LPC20:4, was associated with a decreased risk for CVD (P = 0.024–0.028). Sphingomyelin SM 38:2 was associated with increased odds of CVD (P = 0.057). Five triglyceride (TAG) species were associated with protection (P = 0.031–0.049). LPC16:0 was negatively correlated with the carotid intima-media thickness (P = 0.010) and with Hba1c (P = 0.012) whereas SM38:2 was positively correlated with LDL-cholesterol (P = 0.0010) and the q-values were good (q ≤ 0.03). The risk allele of 8 CAD-associated gene variants showed significant association with the plasma level of several lipid species. However, the q-values were high for many of the associations (0.015 ≤ q ≤ 0.75). Risk allele carriers of 3 CAD-loci had reduced level of LPC16:0 and/or LPC 20:4 (P ≤ 0.056).

Conclusion: Our study suggests that CVD development is preceded by reduced levels of LPC16:0, LPC20:4 and some specific TAG species and by increased levels of SM38:2. It also indicates that certain lipid species are intermediate phenotypes between genetic susceptibility and overt CVD. But it is a preliminary study that awaits replication in a larger population because statistical significance was lost for the associations between lipid species and future cardiovascular events when correcting for multiple testing.

Introduction

Cardiovascular mortality and morbidity is a major public health problem in Western societies. Traditional cardiovascular risk factors do not fully explain future cardiovascular events [1,2] and adding modern biomarkers to the standard risk factors has, thus far, only proven to minimally improve individual risk prediction [3,4], thus underlining the need to identify new biomarkers.

Lipids are thought to play a central role in cardiovascular disease (CVD) development and total plasma triglycerides and cholesterol as well as LDL- and HDL-cholesterol are traditionally monitored as predictors of cardiovascular events. However, those are crude measurements of the sum of a complex composition of lipids and do not at all reflect other potentially atherogenic lipid species. We here hypothesized that specific plasma lipid species, rather than the rough phenotype of total triglycerides and cholesterol may be altered in subjects who develop CVD later in life, implying that they may be involved in the CVD pathogenesis.

Lipidomics, a subset within the field of metabolomics, strives to quantitatively describe the complete set of all lipids in a given cell type, tissue or biologic fluid of interest at a given time [5]. There is no single instrument or approach that can currently do so, but
instead multiple and often complementary analytical approaches can be employed. Typically, global lipid profiling is conducted by directly infusing a crude lipid extract into the mass spectrometer without prior chromatographic separation, also called shotgun technique [6], or by using on-line liquid chromatographic separation prior mass spectrometry (MS) analysis [7]. Lipidomic analyses for human biomarker discovery using either approach are now emerging [8–10].

Shotgun lipidomics which allows high-throughput, high intersample reproducibility, high sensitivity and ease of automation [11] was here used for screening of the plasma lipidome in a case-control material derived from a prospective population-based cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Materials and Methods

Ethics Statement

The Malmo¨ Diet and Cancer study was approved by the Ethics Committee at Lund University and all participants provided written informed consent.

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Materials and Methods

Ethics Statement

The Malmo¨ Diet and Cancer study was approved by the Ethics Committee at Lund University and all participants provided written informed consent.

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Materials and Methods

Ethics Statement

The Malmo¨ Diet and Cancer study was approved by the Ethics Committee at Lund University and all participants provided written informed consent.

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Materials and Methods

Ethics Statement

The Malmo¨ Diet and Cancer study was approved by the Ethics Committee at Lund University and all participants provided written informed consent.

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Materials and Methods

Ethics Statement

The Malmo¨ Diet and Cancer study was approved by the Ethics Committee at Lund University and all participants provided written informed consent.

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Materials and Methods

Ethics Statement

The Malmo¨ Diet and Cancer study was approved by the Ethics Committee at Lund University and all participants provided written informed consent.

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Materials and Methods

Ethics Statement

The Malmo¨ Diet and Cancer study was approved by the Ethics Committee at Lund University and all participants provided written informed consent.

Study Participants and Data Collection

The Malmo¨ Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort consisting of 28,449 individuals who attended a baseline examination between 1991 and 1996 [14]. From the MDC cohort, 6,103 persons were randomly selected and asked to participate in a cardiovascular cohort study with similar plasma total lipids level. A top-down approach where individual lipid species are identified by accurately determining precursor masses with no recourse to tandem MS was implemented as previously described [8,12].

Because the mechanisms underlying CVD for most of the reported CVD-associated gene variants are unknown, we also tested whether the plasma lipidome associates with 23 well-validated gene variants for risk of coronary artery disease [13].

Table 1. Baseline characteristics of the study samples.

| Characteristic                  | Control (n = 216) | CVD case (n = 211) | P value |
|--------------------------------|------------------|------------------|--------|
| Age (years)                    | 60.7±5.1         | 60.2±5.3         | 0.331  |
| Women (%)                      | 47.7             | 47.4             | 0.952  |
| BMI (kg/m²)                    | 26.3±4.3         | 26.5±4.4         | 0.558  |
| Systolic blood pressure (mm Hg)| 149.3±19.8       | 149.7±18.4       | 0.815  |
| Diastolic blood pressure (mm Hg)| 90.1±9.6        | 90.1±9.5         | 0.981  |
| Glucose (mmol/l)               | 5.2±1.2          | 5.7±2.2          | 0.008  |
| Cholesterol (mmol/l)           | 6.3±1.1          | 6.3±1.0          | 0.611  |
| Triglycerides (mmol/l)         | 1.5±0.7          | 1.4±0.6          | 0.136  |
| High density lipoprotein (mmol/l)| 1.3±0.3        | 1.3±0.3          | 0.541  |
| Low density lipoprotein (mmol/l)| 4.3±1.0          | 4.4±1.0          | 0.426  |
| Diabetes (%)                   | 8.3              | 15.2             | 0.028  |
| Current smoker (%)             | 33.3             | 33.6             | 0.945  |
| Anti-hypertensive treatment (%)| 25.9             | 23.7             | 0.594  |
| Lipid lowering drugs (%)       | 0.9              | 3.8              | 0.050  |

Values are mean±s.d. or percentage. P values were calculated using a t test for continuous variables and Pearson Chi-Square for binary variables.

doi:10.1371/journal.pone.0071846.t001
methanol 5/1.5 (v/v) providing a total of 2.7 nmol cholesteryl heptadecanoate (CE17:0), 0.7 nmol heptadecanoyl sphingomyelin (SM17:0), 3.5 nmol 1,2-di-O-hexadecyl-sn-glycero-3-phosphoethanolamine (PE-O16:0/O16:0), 3.1 nmol 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC12:0), 0.4 nmol N-heptadecanoyl-D-myricosine (Cer17:0), 3.1 nmol tri-1-laurin (TAG12:0) and 0.5 nmol dilaunin (DAG12:0). Then 550 µL of MTBE/methanol 5:1.5 were added and the samples were shaken at 4 °C for 1 h. Afterwards, 150 µL of water were added, followed by shaking at 4 °C for 10 min and centrifugation for 5 min at 4,000 rpm on Rotanta 460R centrifuge (Hettich, Tuttlingen, Germany). The upper organic phase was transferred into a 96-well plate with glass inserts and a silicone/Teflon coated sealing mat (Chromacol) and stored at −20 °C until performing the MS analysis for all the samples successively.

**Shotgun Screening of Plasma Lipidome**

Prior to the MS analysis, the lipid extracts were diluted 10 times with a mixture of chloroform/methanol/2-propanol 1/2/4 (v/v/v) containing 7.5 mM ammonium acetate and placed in a 96-well plate (Eppendorf) that was then sealed with aluminium foil (Corning). Shotgun analysis was performed on a LTQ Orbitrap (Thermo Fisher Scientific, Waltham, MA) coupled to a TriVersa NanoMate robotic nanoflow ion source (Advion BioSciences, Ithaca, NY) [8,12]. Samples were analyzed in duplicate. Lipids were identified and quantified using the LipidXplorer software [24] and lipid species of the following lipid classes were recognized: triacylglyceride (TAG), diacylglyceride (DAG), cholesteryl ester (Chol-FA), sphingomyelin (SM), phosphatidylcholine (PC), PE-ether (PE-O), lyso-PC (LPC), phosphatidylethanolamine (PE) and PE-ether (PE-O). Identification of the different lipid species was based on MS survey scans acquired in positive ion mode in the Orbitrap analyzer at a target mass resolution of 100,000 using a mass accuracy of better than 5 ppm and a signal to noise ratio of 2. Lipid species were quantified by normalizing the intensities of their peaks to the intensity of the peaks of internal standards spiked into the sample prior to lipid extraction. The internal standards were also used to monitor the quality of the MS analysis and representative mass spectra are presented (Supplementary Figure S1A and S1B). An internal standard mix was both extracted and run independently 18 times across the entire analysis to get an estimate of the coefficient of variation of the combined lipid extraction and MS analysis from the internal standards (Supplementary Table S1). The maximum value of duplicate samples was kept. Lipid species with >30% missing observations were excluded.

**Statistical Analyses**

SPSS (version 18.0) was used for all statistical analyses. Data were assessed for normality with histograms. Due to non-normality all the lipid species were log transformed prior analysis. All tests were two-sided and data were considered significant if P<0.05.

To determine the association of baseline individual lipid species with future CVD, we performed binary logistic regression adjusting for age, sex, diabetes, smoking status, LDL-cholesterol, HDL-cholesterol, systolic blood pressure (SBP), body mass index (BMI) and use of anti-hypertensive treatment.

Q-values were calculated using the QVALUE software [25]. Hierarchical clustering was performed with Euclidean distance and average linkage in MATLAB R2011a (version 7.12.0.635).
As a result of the initial matching procedure (age, gender and Framingham risk score) the baseline characteristics of the 211 incident cases of CVD and 216 control subjects were similar for most risk factors except fasted plasma glucose level and diabetes. The frequency of use of lipid lowering drugs was low (Table 1). Lipid profiling was performed on samples obtained from the baseline examination that took place between 1991 and 1994. A total of 85 lipid species belonging to 9 major lipid classes were identified and quantified by the approach used (Supplementary Table S2). The total quantities of triglycerides and cholesterol determined by mass spectrometry were correlated with the values obtained by traditional clinical chemistry analysis (Figure 1 and Supplementary Figure S2). As known from previous study, the correlation was substantially stronger for triglycerides than for cholesterol [8].

### Results

**Lipid Metabolites Profiling in the Cardiovascular Cohort of the Malmö Diet and Cancer Study**

As a result of the initial matching procedure (age, gender and Framingham risk score) the baseline characteristics of the 211 incident cases of CVD and 216 control subjects were similar for most risk factors except fasted plasma glucose level and diabetes. The frequency of use of lipid lowering drugs was low (Table 1). Lipid profiling was performed on samples obtained from the baseline examination that took place between 1991 and 1994. A total of 85 lipid species belonging to 9 major lipid classes were identified and quantified by the approach used (Supplementary Table S2). The total quantities of triglycerides and cholesterol determined by mass spectrometry were correlated with the values obtained by traditional clinical chemistry analysis (Figure 1 and Supplementary Figure S2). As known from previous study, the correlation was substantially stronger for triglycerides than for cholesterol [8].

**Selected Lipid Species Associate with Future Adverse Cardiovascular Disease Outcome**

Binary logistic regression was performed to assess the association between baseline lipid species level and future CVD adjusting for Framingham risk factors. Associations with incident CVD were seen for lipid species belonging to the lysophosphatidylcholine (LPC), sphingomyelin (SM) and triacylglyceride (TAG) lipid classes, but the q-values for the associations were rather high ($0.21 \leq q \leq 0.23$) (Tables 2, 3 and Supplementary material online, Table S3A). Similar results were obtained when only adjusting for diabetes (Supplementary Table S3B).

In the LPC class, each standard deviation (SD) unit higher baseline levels of LPC16:0 or LPC20:4 was associated with a decreased risk of developing CVD over the 12-year follow-up period (OR = 0.79; $P = 0.028$ and OR = 0.77; $P = 0.024$, respectively) (Table 2). Individuals whose plasma level of LPC16:0 or LPC20:4 was in the top quartile had decreased odds of future CVD compared with individuals in the lowest quartile (OR = 0.57; $P = 0.032$ and OR = 0.62; $P = 0.048$, respectively) (Table 2).

SM38:2, with a borderline $P$-value, was the only lipid species of its class to be associated with increased odds of future CVD.

### Table 2. Relation of baseline phospholipids level to future adverse cardiovascular outcome adjusting for Framingham risk factors.

| Model                     | LPC16:0 (n = 424) | LPC20:4 (n = 353) | SM38:2 (n = 318) |
|---------------------------|-------------------|-------------------|------------------|
| Models adjusting for sex, age, BMI, type 2 diabetes, anti-hypertension treatment, smoking, LDL, HDL and SBP |                    |                   |                  |
| Lipid specie as continuous variable |                    |                   |                  |
| Per s.d.                  | 0.79 (0.65–0.97)  | 0.77 (0.61–0.96)  | 1.28 (0.99–1.64) |
| $P$                       | 0.028             | 0.024             | 0.057            |
| q-value                   | 0.210             | 0.210             | 0.228            |
| Lipid specie as categorical variable |                    |                   |                  |
| First quartile            | 1.0 (referent)    | 1.0 (referent)    | 1.0 (referent)   |
| Second quartile           | 1.21 (0.69–2.11)  | 1.13 (0.61–2.07)  | 0.94 (0.49–1.81) |
| Third quartile            | 0.94 (0.54–1.65)  | 0.62 (0.34–1.16)  | 1.320 (0.68–2.56)|
| Fourth quartile           | 0.57 (0.32–1.00)  | 0.62 (0.33–1.17)  | 1.85 (0.92–3.71) |
| $P$ for trend             | 0.032             | 0.048             | 0.054            |

Values are odds ratios (95% confidence intervals) for cardiovascular disease from multivariate adjusted binary logistic regressions performed with the Z score of a given lipid specie obtained after log transformation. BMI, body mass index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; LPC, lysophosphatidylcholine; SBP, systolic blood pressure; SM, sphingomyelin. doi:10.1371/journal.pone.0071846.t002

### Table 3. Relation of baseline triglycerides specie level to future adverse cardiovascular outcome adjusting for Framingham risk factors.

| Model                     | TAG48:1 (n = 424) | TAG48:2 (n = 424) | TAG48:3 (n = 402) | TAG50:3 (n = 424) | TAG50:4 (n = 423) |
|---------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Models adjusting for sex, age, BMI, type 2 diabetes, anti-hypertension treatment, smoking, LDL, HDL and SBP |                    |                   |                   |                   |                   |
| Lipid specie as continuous variable |                    |                   |                   |                   |                   |
| Per s.d.                  | 0.78 (0.63–0.98)  | 0.79 (0.64–0.98)  | 0.81 (0.65–1.00)  | 0.79 (0.63–0.98)  | 0.79 (0.64–0.98)  |
| $P$                       | 0.031             | 0.034             | 0.049             | 0.036             | 0.033             |
| q-value                   | 0.210             | 0.210             | 0.228             | 0.210             | 0.210             |

Values are odds ratios (95% confidence intervals) for cardiovascular disease from multivariate adjusted binary logistic regressions performed with the Z score of a given triacylglyceride specie obtained after log transformation. BMI, body mass index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TAG, triacylglyceride. doi:10.1371/journal.pone.0071846.t003
Coronary Artery Disease (CAD) and Plasma Lipid Profile

Association between Susceptibility Gene Variants for Different Correlation Patterns between the Various Lipid Species

In the TAG class, plasma levels of TAG48:1, TAG48:2, TAG48:3, TAG50:3 and TAG50:4, were associated with decreased odds of future CVD (OR = 0.78–0.81; P = 0.031–0.049) (Table 3). However, the quartiles analysis showed poor linearity between the various TAGs and CVD risk (Supplementary material online, Table S4).

Table 4. LPC16:0 and LPC20:4 negatively correlate with CVD risk factors whereas SM38:2 positively correlates with CVD risk factors.

| Lipid specie | LPC16:0 | | | LPC20:4 | | | SM38:2 | | |
|--------------|---------|---|---|---------|---|---|---------|---|
|              | Correlation | P   | q-value | Correlation | P   | q-value | Correlation | P   |
| Imtcca0      | -0.13    | 0.010 | 0.007  | -0.03      | 0.591 | 0.218  | 0.09       | 0.112 |
| HbA1c        | -0.12    | 0.012 | 0.009  | -0.09      | 0.074 | 0.043  | -0.05      | 0.385 |
| BMI          | -0.14    | 0.004 | 0.003  | -0.19      | 2.0E-04 | 2.2E-04 | 0.04       | 0.438 |
| SBP          | -0.06    | 0.244 | 0.114  | -0.11      | 0.039 | 0.025  | -0.02      | 0.710 |
| LDL          | 0.07     | 0.154 | 0.081  | -0.05      | 0.356 | 0.153  | 0.36       | 7.4E-11 |
| HDL          | 0.12     | 0.014 | 0.011  | 0.18       | 5.5E-04 | 5.8E-04 | 0.06       | 0.262 |

Partial correlations were performed between LPC16:0, LPC20:4, or SM38:2 after log transformation and current known laboratory predictors for cardiovascular disease, adjusting for age and sex. BMI, body mass index; HbA1c, haemoglobin A1c; HDL, high-density lipoprotein cholesterol; Imtcca0, intima-media thickness of the common carotid artery at baseline; LDL, low-density lipoprotein cholesterol; LPC, lysophosphatidylcholine; SBP, systolic blood pressure; SM, sphingomyelin.

doi:10.1371/journal.pone.0071846.t004

LPC16:0 and LPC20:4 negatively correlate with CVD risk factors whereas SM38:2 positively correlates with CVD risk factors (OR = 1.28; P = 0.057). Individuals in the top quartile of baseline SM 38:2 plasma level had an increased risk of developing CVD (OR = 1.28; P = 0.054) (Table 2).

Discussion

Top-down Lipidomics, a Tool for Clinical Screens

The importance of two main lipids, i.e. triglycerides and cholesterol, as a tool for CVD prediction has long been known. But, modern lipidomics analysis shows that the human plasma lipoprotein comprises of at least several hundreds of individual lipid species and gives a glimpse of the complexity of the lipidome that has been overlooked until recently mainly because of technical limitations. We here performed a plasma lipoprotein screen in a prospective population-based cohort using top-down shotgun lipidomics. We aim to look for differences in the plasma composition in individuals with similar plasma total lipids level. We analyzed 427 samples with 2 technical replicates and identified and quantified 35 lipid species belonging to 9 different lipid classes and to our knowledge this study constitutes the first extensive lipid profiling of plasma for incident CVD in the primary preventive setting. Top-down shotgun lipidomics was the method of choice for this study because it is a quantitative and highly sensitive technique that allows high-throughput and relatively extensive lipid coverage.
Figure 2. Association between the lipid profile and the risk allele of 8 CAD-associated gene variants. Heat map of regression coefficients obtained from linear regressions performed between the CAD-associated locus (with the CAD-associated allele coded) and the lipid species after log transformation adjusting for age and sex. *P<0.05, **P<0.01, ***P<0.001.
doi:10.1371/journal.pone.0071846.g002
Refining the Dyslipidemia Phenotype

Although total increased plasma TAG concentration is considered a risk factor for CVD, we have here identified some individual TAG species that were associated with decreased odds of future CVD. LPC as a whole lipid class has previously been linked with inflammation as well as with both pro- and anti-atherogenic effects [26,27], whereas we have here shown that two specific LPC species i.e., LPC16:0 and LPC20:4, were protective for CVD. These findings demonstrate that systematic analysis of plasma lipid species rather than lipid classes as a whole may reveal opposite relationships with CVD risk and thus could help to better understand the mechanisms leading to CVD and to improve CVD risk prediction.

Recently, a plasma lipidomics analysis was conducted using a different MS platform than the one we used, in a cross sectional setting, showing that certain patterns of lipids could discriminate between patients with stable angina and those with unstable CAD as well as healthy controls [9]. The results obtained in patients with unstable CAD are supported and extended by our prospective study of subjects without prior CVD, i.e., decreased level of most measured LPC species both in CAD versus control as well as in unstable versus stable CAD, increased levels of several SM species in unstable versus stable CAD and decreased level of specific TAG species in unstable versus stable CAD, were reported. This suggests that such alterations of lipid patterns may not only be a marker of coronary atherosclerosis and plaque instability but also that it may play a role in the pathogenesis of CVD, given its presence more than 10 years before clinical disease onset.

Integrating Genomic and Lipidomics Information

Out of the 8 CAD susceptibility gene variants displaying significant association with circulating lipid species concentrations, 3 have not yet been previously reported to be involved in lipid metabolism (WDR12, ZC3HC1 and PHACTR1) and 3 are only known to affect lipoproteins levels (LPA, SORT1 and the ZNF259/APOA5-A4-C3-A1 gene region) [13,28]. However, any potential link between the genetic alteration of these lipids and CAD needs to be substantiated by mechanistic studies. Two of the 8 CAD loci are directly coding for enzymes involved in lipids biosynthesis (PPAP2B and the PEMT/RASD1/SMCR3 locus) [29,30]. The PPAP2B gene encodes a phosphatidic phosphatase that converts phosphatidic acid into diacylglycerol, the precursor for de novo synthesis of TAG, PC and PE. Moreover, PEMT encodes an enzyme which sequentially converts PE into PC. Both carriers of the PPAP2B and of the PEMT/RASD1/SMCR3 risk allele display reduced level of multiple glycerophospholipids including the CVD-protective lipid species LPC16:0 and/or LPC20:4. Overall, our findings highlight that integrating lipidomics with genomics is a promising approach to increase the understanding of mechanisms underlying the gene-CVD associations as well as CVD pathogenesis.

Study Limitations

This is an initial discovery study that needs to be replicated especially since the false discovery rate was high when looking for associations between the lipid species and future cardiovascular events or between the lipid species and most of the CAD-associated gene variants. Also, we do acknowledge that this is a case control study and not a general population study, thus the findings cannot be generalised to the whole population. Furthermore, our study could be complemented by acquiring spectra in negative ion mode to extend the lipid class coverage and by performing tandem MS for some targeted lipid species in order to get their full structural information. Another drawback of the study is the lack of a pooled quality control plasma sample run across the study. Finally, we do not know to what extent the −80 degree Celsius storage over approximately 20 years may have affected the original lipid profile.

Conclusions

This study constitutes a proof-of-concept screen that shotgun lipidomics can be used as a tool in the search for novel CVD biomarkers. Moreover, we here highlight the importance of refining the dyslipidemia phenotype and thus looking at the level of individual lipid species rather than the total sum of the different lipid classes in their relationship with CVD risk. We identified some specific lipid species as potential biomarkers of adverse cardiovascular outcome. However, statistical significance was lost for the association between the lipid species and future cardiovascular events when correcting for multiple testing. Finally, our results support the informative value in bringing together genomic and lipidomics data, suggesting that certain individual lipid species are intermediate phenotypes between genetic susceptibility and overt CVD. Overall, this is an explorative study that will need to be replicated in a larger population.

Supporting Information

Figure S1 Representative mass spectra of total lipid extracts from plasma. The most abundant peaks are annotated with m/z; the shaded areas indicate the m/z ranges where the corresponding lipid classes were detected. (PDF)

Figure S2 Absolute quantification of TAGs by top-down lipidomics correlates with the total triglyceride levels measured at baseline examination. Linear regression was performed between the total absolute TAG levels determined by MS versus the total triglyceride levels measured by traditional clinical chemistry analysis. The total TAG level measured by MS is obtained by summing the abundances of all the individual TAG species. (PPT)

Figure S3 Different correlation patterns between the various plasma lipid classes and CVD traditional risk factors. Heat map of correlations coefficients obtained from partial correlations performed between the lipid species after logarithmic transformation and traditional laboratory predictors for cardiovascular disease adjusting for age and sex. *P<0.05, **P<0.01, ***P<0.001. (TIF)

Table S1 Coefficient of variation (CV) of the combined lipid extraction and MS analysis for the 8 internal standards. (DOCX)

Table S2 Absolute levels of the lipid species. (DOCX)

Table S3 A. Relation of baseline lipid species level to future adverse cardiovascular outcome adjusting for Framingham risk factors. B. Relation of baseline lipid species level to future adverse cardiovascular outcome adjusting for type 2diabetes only. (DOCX)

Table S4 Relation of baseline triglycerides species level to future adverse cardiovascular outcome adjusting for Framingham risk factors. (DOCX)
Table S5  Estimated q-values of the tests performed to study the association between CVD risk factors and the lipid species.

Table S6  Relation between 23 validated coronary artery disease associated gene variants and baseline plasma lipid metabolites level.

Table S7  The risk allele of 8 of the validated coronary artery disease associated gene variants shows significant association with the baseline plasma level of several lipid species.

References

1. Greenland P, Knoll MD, Stamler J, Neaton JD, Dyer AR, et al. (2003) Major risk factors as antecedents of fatal and nonfatal coronary heart disease events. JAMA 290: 891–897.

2. Khot UN, Khot MB, Bajzer CT, Sapp SK, Ohman EM, et al. (2003) Prevalence of conventional risk factors in patients with coronary heart disease. JAMA 290: 898–904.

3. Melander O, Newton-Cheh C, Almgren P, Hedblad B, Berglund G, et al. (2009) Novel and conventional biomarkers for prediction of incident cardiovascular events in the community. JAMA 302: 49–57.

4. Wang TJ, Gona P, Larson MG, Levy D, et al. (2009) Top-down lipidomics reveals ether lipid deficiency in blood plasma of hypertensive patients. PLoS One 4: e5261.

5. Meikle PJ, Wong G, Tsorotes D, Barlow CK, Weir JM, et al. (2011) Plasma lipidomic analysis of stable and unstable coronary artery disease. Arterioscler Thromb Vasc Biol 31: 2723–2732.

6. Han X, Gross RW (2005) Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipids directly from crude extracts of biological samples. Mass Spectrum Rev 24: 367–412.

7. Sandra K, Pereira Ados S, Vanhoenacker G, David F, Sandra P (2010) Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 49: 1137–1146.

8. Graeser J, Schwucke D, Schwarz PE, Herzog R, Shevchenko A, et al. (2009) Lipid profiling at the interface of structure and function in systems biology. Chem Biol 16: 284–291.

9. Persson M, Hedblad B, Nelson JJ, Berglund G (2007) Elevated Lp-PLA2 levels in plasma predict cardiovascular events among middle-aged nondiabetic subjects. Arterioscler Thromb Vasc Biol 27: 1411–1416.

10. Pang D, Chen S, Tian Y, Zhang H, Pan Q, et al. (2011) A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 12: R8.

11. Arslan A, Meikle PJ, Whelan T, Toogood J, Weir JM, et al. (2011) Lipidomic analysis of stable and unstable coronary artery disease. Arterioscler Thromb Vasc Biol 31: 2723–2732.

12. Han X, Gross RW (2005) Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipids directly from crude extracts of biological samples. Mass Spectrum Rev 24: 367–412.

13. Sandra K, Pereira Ados S, Vanhoenacker G, David F, Sandra P (2010) Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 49: 1137–1146.

14. Persson M, Hedblad B, Nelson JJ, Berglund G (2007) Elevated Lp-PLA2 levels add prognostic information to the metabolic syndrome on incidence of cardiovascular events among middle-aged nondiabetic subjects. Arterioscler Thromb Vasc Biol 27: 1411–1416.

15. Persson M, Hedblad B, Nelson JJ, Berglund G (2007) Elevated Lp-PLA2 levels add prognostic information to the metabolic syndrome on incidence of cardiovascular events among middle-aged nondiabetic subjects. Arterioscler Thromb Vasc Biol 27: 1411–1416.

16. Rowell M, Jannuzzi L, Berglund G, Engstrom G, Hedblad B (2003) Incident coronary events and case fatality in relation to common carotid intima-media thickness. J Intern Med 257: 430–417.

17. Kathiresan S, Melander O, Anweski D, Giudicci C, Bartt NT, et al. (2008) Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med 358: 1240–1249.

18. Myocardial Infarction Genetics C, Kathiresan S, Voight BF, Purcell S, Musunuru K, et al. (2009) Genome-wide association of early-onset myocardial infarction with single nucleotide polymorphisms and copy number variants. Nat Genet 41: 334–341.

19. Erdmann J, Grosshennig A, Braund PS, Konig IR, Hungtenberg C, et al. (2009) New susceptibility locus for coronary artery disease on chromosome 3p22.3. Nat Genet 41: 289–292.

20. Ripatti S, Tikkanen E, Orho-Melander M, Havulinna AS, Silander K, et al. (2010) A multilocus genetic risk score for coronary heart disease: case-control and prospective cohort analyses. Lancet 376: 1393–1400.

21. Musunuru K, Orho-Melander M, Canfield MP, Li S, Salameh WA, et al. (2009) Ion mobility analysis of lipoprotein subfractions identifies three independent axes of cardiovascular risk. Arterioscler Thromb Vasc Biol 29: 1975–1980.

22. D’Agostino RB Sr., Grundy S, Sullivan LM, Wilson P (2001) Validation of the Framingham coronary heart disease prediction scores: results of a multiple ethnic groups investigation. JAMA 285: 180–187.

23. Mayya Y, Liebsch G, Kuricha FV, Shevchenko A, Schwucke D (2008) Lipid extraction by methyl-tert-butyly ether for high-throughput lipidomics. J Lipid Res 49: 1137–1146.

24. Herzog R, Schwucke D, Schulmann K, Sampaio JL, Bornstein SR, et al. (2011) A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 12: R8.

25. Graeser J, Schwucke D, Schwarz PE, Herzog R, Shevchenko A, et al. (2009) Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 49: 1137–1146.

26. Schucke D, Schulmann K, Sampaio JL, Bornstein SR, et al. (2011) A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 12: R8.

27. Sladek R, Day IN, Dina C, D’Agostino Jr RP, Ripatti S, et al. (2007) A genome-wide association study identifies diabetes susceptibility locus on chromosome 7q12. Nature 445: 881–885.

28. Musunuru K, Orho-Melander M, Canfield MP, Li S, Salameh WA, et al. (2009) Ion mobility analysis of lipoprotein subfractions identifies three independent axes of cardiovascular risk. Arterioscler Thromb Vasc Biol 29: 1975–1980.

29. Matyash V, Liebsch G, Kuricha FV, Shevchenko A, Schwucke D (2008) Lipid extraction by methyl-tert-butyly ether for high-throughput lipidomics. J Lipid Res 49: 1137–1146.

30. Schucke D, Schulmann K, Sampaio JL, Bornstein SR, et al. (2011) A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 12: R8.

31. Storey JD (2002) A direct approach to false discovery rates. Journal of the Royal Statistical Society Series B-Statistical Methodology 64: 479–498.

32. Musunuru K, Orho-Melander M, Canfield MP, Li S, Salameh WA, et al. (2009) Ion mobility analysis of lipoprotein subfractions identifies three independent axes of cardiovascular risk. Arterioscler Thromb Vasc Biol 29: 1975–1980.

33. D’Agostino Jr RP, Buring JE, Rifai N, Patscherkova J, Wilson P, et al. (2003) The metabolic syndrome and risk of cardiovascular disease among women. JAMA 289: 1959–1966.

34. Schucke D, Schulmann K, Sampaio JL, Bornstein SR, et al. (2011) A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 12: R8.

35. Sladek R, Day IN, Dina C, D’Agostino Jr RP, Ripatti S, et al. (2007) A genome-wide association study identifies diabetes susceptibility locus on chromosome 7q12. Nature 445: 881–885.

36. Sladek R, Day IN, Dina C, D’Agostino Jr RP, Ripatti S, et al. (2007) A genome-wide association study identifies diabetes susceptibility locus on chromosome 7q12. Nature 445: 881–885.

37. Schucke D, Schulmann K, Sampaio JL, Bornstein SR, et al. (2011) A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 12: R8.