New evidence from plasma ceramides links apoE polymorphism to greater risk of coronary artery disease in Finnish adults

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Abbreviations:

CAD: Coronary artery disease; **CerS:** Ceramide synthase; **FDR:** False discovery rate; **GWAS:** Genome-wide association study; **LDL-C:** LDL cholesterol; **SD:** Standard deviation; **YFS:** The Cardiovascular Risk in Young Finns Study

Abstract

ApoE, a key regulator of plasma lipids, mediates altered functionalities in lipoprotein metabolism and thus affects the risk of coronary artery disease (CAD). The significance of different apoE polymorphisms remains unclear; although the ε4 allele is clearly associated with increased cholesterol levels (which inform CAD risk), direct studies about apoE polymorphisms on CAD risk and development have controversial results. Furthermore, certain species of ceramides—complex lipids abundant in plasma LDL—are markers of increased risk of myocardial infarction and cardiovascular death. Using a high-throughput MS approach, we quantified 30 molecular plasma ceramide species from a cohort of 2,160 apoE-genotyped (rs7412, rs429358) young adults enrolled in the population-based Cardiovascular Risk in Young Finns Study. We then searched this lipidome dataset to identify new indications of pathways influenced by apoE polymorphisms and possibly related to CAD risk. This approach revealed a previously unreported association between apoE polymorphism and a consistently documented high-risk CAD marker, Cer(d18:1/16:0). Compared with the apoE ε3/3 reference group, plasma levels of apoE ε4 were elevated, and those of apoE ε2 were lowered, in all subjects without evidence of apoE-by-sex interactions. ApoE associated with seven ceramides that are connected to atherogenically potent macrophages and/or lipoprotein particles; these associations could indicate a plausible linkage between apoE polymorphism and ceramide metabolism, leading to adverse plasma LDL metabolism and atherogenesis. In conclusion, new evidence from plasma ceramides links apoE polymorphism with an increased risk of CAD and extends our understanding about the role of apoE in health and disease.

Keywords:

Atherosclerosis, Dyslipidaemias, Genes in lipid dysfunction, LDL/Metabolism, Lipidomics

Introduction

Apolipoprotein E (apoE) is a glycoprotein of 299 amino acids with a key role in regulating plasma lipid levels. The three common allelic variants of the apoE gene, ε2, ε3 and ε4, determined by two single-nucleotide polymorphisms (SNP) rs429358 and rs7412 at locus 19q13.31, code respectively three protein isoforms E2, E3 and E4. Of the six forming apoE genotypes (ε2/2, ε3/2, ε3/3, ε4/2, ε4/3 and ε4/4) the homozygote parent genotype ε3/3 is the most prevalent and the protein isoform E3 associates with normal/average plasma lipid levels.[1] Despite the early established association of ε4 allele with elevated serum total cholesterol and LDL cholesterol (LDL-C)[2,3], as well as the generally recognized causality of high serum total cholesterol and LDL-C with coronary artery disease (CAD)[4], studies investigating the significance of apoE polymorphism on the susceptibility and development of CAD have led to controversial results[5-8]. However, it has been shown that genetic components affect atherosclerosis and CAD risk[9,10]. In Finnish population with higher than average incidence of atherosclerosis and nationwide prevalence of ε4 allele[2], indications of ε4 linkage to the risk of atherosclerosis and CAD have been reported[11]. On the other hand, ε2 has been shown to e.g., reduce carotid artery intima-media thickness and coronary artery calcification across multiple ethnicities, including Finns[12,13]. Altogether, further research is required to clarify the role of apoE in the metabolic pathways and mechanisms of atherogenesis.

In predicting atherosclerosis and its complications, previous studies have concluded that the traditionally applied standard lipid panel parameters offer inadequate means to identify patients at different risks – therefore, novel, more specific risk markers have been investigated and found with state-of-art lipidomic and metabolomic methods[4]. In this present work we concentrate on the effects of apoE polymorphism on plasma ceramides with an emphasis on predicted CAD risk. Unlike with basic lipids, evidence of the associations of apoE polymorphism with plasma ceramides, or lipidomics in general, is very limited.

To allow a comprehensive analysis, we quantified 30 molecular plasma ceramide species. Certain ceramides have consistently been reported as high-risk markers of CAD and adverse cardiac outcomes, indicated among CAD patients with an established disease[14-18], as well as at population level in incident events[19,20]. The aim of our

study is to search for novel linkages between apoE polymorphism and risk of CAD by means of plasma ceramides. By implementing a population-based cohort of basically healthy, relatively young adults we pursue to recognize prevailing genotype-related differences prior to adverse manifestations – especially in the markers previously associated to atherogenesis. With our findings we expect to extend our current understanding about the role of apoE in health and disease.

Materials and Methods

Study population and data sources. The Cardiovascular Risk in Young Finns Study (YFS) is a Finnish longitudinal general population study on the evolution of cardiovascular risk factors from childhood to adulthood[21]. The study began in 1980, when 3,596 children and adolescents aged 3, 6, 9, 12, 15 and 18 years were randomly selected from five university hospital catchment areas in Finland. In 2007, 2,200 participants aged 30-45 years attended the 27-year follow-up. Of these subjects, we included those for whom the apoE genotype data and at least 80% of the lipidomic parameters were available. Therefore, 2,160 participants contributed to the cross-sectional association analyses of apoE genotype and plasma ceramide profile. The YFS was approved by the 1st ethical committee of the Hospital District of Southwest Finland and by local ethical committees (1st Ethical Committee of the Hospital District of Southwest Finland, Regional Ethics Committee of the Expert Responsibility area of Tampere University Hospital, Helsinki University Hospital Ethical Committee of Medicine, The Research Ethics Committee of the Northern Savo Hospital District and Ethics Committee of the Northern Ostrobothnia Hospital District). The study protocol of each study phase corresponded to the proposal by the World Health Organization. All present subjects gave written informed consent and the study was conducted in accordance with the Helsinki declaration. At prior follow-ups of YFS, informed consent of every participant under the age of 18 was obtained from a parent and/or legal guardian.

Clinical and biochemical measurements and their use in statistical standardization. To eliminate effects of the most probable error sources, a comprehensive set of clinical background information was analysed as confounding candidates. The effect of body mass index (BMI, measured weight [kg]/ measured height squared [m^2]) was considered by including it in the final regression models as a covariate. Based on questionnaires, daily smoking (yes/no), hormonal birth control of women (yes/no), cholesterol lowering medication (yes/no) and socio-economic status based on occupation (manual/ lower non-manual/ upper non-manual) were all tested as covariates to have negligible or zero effect on the apoE β-values. The distributions of alcohol consumption (daily portions based on a questionnaire, one portion equalling 12 g of pure alcohol) and physical activity index (graded 5-15, the higher the value the more physically active, evaluation method described elsewhere[22]) were well comparable in

every analysed apoE subgroup and therefore not confounding, which was also confirmed with covariate analyses. Also, there were only a few pregnant women and persons with diagnosed diabetes (evaluated with questionnaires) and their distributions did not differ in the analysed subgroups. Parameters describing the cardiovascular status, such as systolic blood pressure (defined as an average of three consecutive measurements with random-zero mercury sphygmomanometer), hypertension (based on a questionnaire for medically diagnosed hypertension, yes/no) and high-sensitivity CRP (measured with an automatic analyser Olympus AU400) might well reflect variations in lipidomics, i.e., could be considered more as consequent rather than confounding factors. Nevertheless, differences in these measures between the subgroups were also found to be minor – a significant majority of the study population being basically healthy. Based on the described background analyses and to avoid unnecessary selection error, additional exclusions were not made in the final analyses. The effect of possible information bias may well be neglected in our results, when comparing the subgroups to each other – bias (if any) being presumably distributed comparably.

Serum concentrations of standard lipids. Levels of total cholesterol, LDL-C, high density lipoprotein cholesterol (HDL-C), triglycerides, as well as apolipoproteins A-I (apoA-I) and B (apoB) were measured as background descriptive data with methods described elsewhere[23].

ApoE genotyping. ApoE alleles (ε2, ε3, ε4) were determined based on SNPs rs7412 and rs429358 haplotypes. Genomic DNA was extracted from peripheral blood leukocytes by using QIAamp DNA Blood Minikit and automated biorobot M48 extraction (Qiagen, Hilden, Germany). Genotyping was performed by using Taqman SNP Genotyping Assays (C__904973_10, C_3084793_20) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). As a quality control, water controls, random duplicates and known control samples were run in parallel with unknown DNA samples.

Plasma lipidomic profiling. Ceramide quantification for the stored plasma samples was performed at Zora Biosciences Oy (Espoo, Finland). Lipid extraction was based on a previously described method[24]. In brief, 10 µl of 10 mM 2,6-di-tert-butyl-4-methylphenol in methanol was added to 10 µL of sample, followed by 20 µl of

internal standards (Avanti Polar Lipids Inc., Alabaster, AL) and 300 µl of chloroform:methanol (2:1, v:v) (Sigma-Aldrich GmbH, Steinheim, Germany). Samples were mixed and sonicated in a water bath for 10 min, followed by a 40-minute incubation and centrifugation (15 min at 5700xg). The upper phase was transferred and evaporated under nitrogen. Extracted lipids were resuspended in 100 µl of water saturated butanol and sonicated in a water bath for 5 minutes. 100 µl of methanol was added to the samples before the extracts were centrifuged for 5 min at 3500xg, and finally the supernatants were transferred to the analysis plate for mass spectrometric analysis. Details of mass spectrometric analyses have also been described in detail previously[25]. The analyses were performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB Sciex, Concord, Canada) equipped with ultra-high-performance liquid chromatography (Nexera-X2, Shimadzu, Kyoto, Japan). Chromatographic separation of the lipidomic screening platform was performed on an Acquity BEH C18, 2.1 × 50 mm id. 1.7 µm column (Waters Corporation, Milford, MA, USA). The data were collected using a scheduled multiple reaction monitoring algorithm and the data were processed using Analyst and MultiQuant 3.0 software (AB Sciex).

Statistical methods. All statistical analyses were conducted with R program version >3.1.2 (https://www.r-project.org/) using PC. To facilitate comparisons across all lipidomic measures, association magnitudes (β-values) are reported in scaled standard deviation (SD) fractions (units) of normalized, ln-transformed lipid concentrations. In sex-stratified cross-sectional analyses, separate sex-specific scaling was applied to lipidomic measures.

Cross-sectional associations of apoE genotype and serum ceramide profile were analysed using linear multivariable regression models, with each lipidomic measure as the outcome and apoE genotype as the main explanatory value. Regression models were adjusted for age and BMI. The effect of sex was analysed in both ways with models stratified by sex and models with sex as a covariate. Interaction of apoE and BMI (apoE x BMI) was tested to have a negligible or zero effect on the apoE β-values and was therefore excluded from the final models. Prior to testing the interaction effects of BMI, measures of BMI were mean-centred. Similarly, interactions of apoE and sex (apoE x sex), as well as apoE and age (apoE x age) were excluded from the final models for not showing an effect within any lipidomic measure ($p>>0.05$). ApoE genotype was included in the model as a categorical variable. First, the overall effect of apoE genotype on the linear fit was F-tested with implementing the

Benjamini-Hochberg procedure[26] in false discovery rate (FDR) correction and setting the limit at $p<0.05$. After that, apoE genotypes were post-hoc compared with each other with t-tests (implementing FDR correction), the values of which were inherited from the linear regression function (lm) in R. 95% confidence intervals were also calculated, allowing a better comparison of the apoE groups.

Data Availability Statement.

The datasets generated and/or analysed during the current study are not publicly available due to restrictions imposed by Finnish legislation, as the contained information could compromise research participant privacy and consent. However, data sharing is possible upon reasonable request and all decisions are made by the YFS Publication and Data Sharing board.

Results

Characteristics of cross-sectional analyses. The frequencies of different apoE genotypes, as well as the summary of the descriptive data in the YFS study population in the 2007 follow-up are presented in Table 1. The p-values across apoE genotypes are given in Supplementary Table S1.

Cross-sectional associations of apoE genotype within measured ceramides in YFS. Due to small subgroups of apoE ε2/2 and ε4/2, as well as ε4/4 in our population-based cohort (see Table 1), apoE genotypes were clustered into three subgroups for better statistical comparison: The reference group of ε3/3, as well as ε2+ (consisting of ε2/2 and ε3/2) and ε4+ (including all ε4 carriers: ε4/2, ε4/3 and ε4/4). The cross-sectional associations of apoE genotypes within 30 quantified plasma ceramides (and three calculated ceramide ratios) are illustrated in Figure 1, in all subjects. The complete statistics of Figure 1 including exact p-values are given in Supplementary Table S2. As illustrated in Supplementary Table S3, exclusion of the ε4/2 subgroup from the analyses did not have a major effect on the results. Also, the complete results of apoB-, LDL-C- and HDL-C-concentration-adjusted analyses are provided in Supplementary tables S4-S6, respectively. Furthermore, the results from inflammatory marker association analyses (high-sensitivity CRP) are shown in Supplementary Table S7. There were no apoE x sex interactions found in relation to any studied measure ($p>>0.05$), justifying the main analyses being performed with all subjects combined.

Based on multivariate regression analyses of all subjects, six ceramide species (see Figure 1) showed an association over apoE genotypes with F-tested $p<0.05$ after FDR correction. In post-hoc comparisons of all subjects against the reference group ε3/3, with FDR corrected $p<0.05$, high-risk CAD ceramide Cer(d18:1/16:0) differed both in apoE ε2+ and ε4+ subgroups – indicating lower level in the ε2+ and elevated in the ε4+ subgroup as compared with the reference group ε3/3. In addition, three ceramides Cer(d16:1/16:0), Cer(d16:1/18:0) and Cer(d18:2/16:0) differed in the ε2+ subgroup showing lower plasma levels than in the ε3/3 and ε4+ subgroups. Moreover, one ceramide, Cer(d18:2/22:0), had an elevated plasma level in the ε4+ subgroup as compared with the most common ε3/3 group.

In sex-stratified analyses, the high-risk CAD ceramide Cer(d18:1/16:0) showed an association with apoE genotypes both in women and men (see Figure 2). In addition, the post-hoc analysis compared with ε3/3 showed differences of Cer(d18:1/16:0) in ε2+ women, as well as ε4+ men ($p=0.005$/ $p=0.01$ prior to FDR correction respectively) – as also indicative by the 95% CI in Figure 2 (see exact FDR-corrected p-values in Supplementary Table S8). Moreover, the F-tested association ($p<0.05$ after FDR correction) of another well-established high-risk CAD ceramide Cer(d18:1/18:0) was found in women only (Figure 2), not in all subjects (Figure 1) or men.

Cross-sectional associations of apoE genotype within measured sphingomyelins in YFS. The cross-sectional associations of 41 plasma sphingomyelin species over apoE genotypes in all subjects were also analysed. The complete statistics of these analyses are presented in Supplementary Table S9. 21 sphingomyelin species associated with apoE genotypes, with F-tested $p<0.05$ after FDR correction – SM(34:1) showing the strongest association. In the post-hoc tests compared with ε3/3 and with FDR corrected $p<0.05$, the levels of 14 sphingomyelin species were lower in the ε2 carriers – again, SM(34:1) as the top hit. None of the sphingomyelins did quite reach the same statistical limit after FDR correction in the ε4+ subgroup.

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Discussion

In the present work we investigated the associations of apoE polymorphism with plasma ceramide species with an emphasis on previously reported CAD-risk-related ceramide species. The results of this study show for the first time an association between apoE genotypes and a high-risk CAD ceramide Cer(d18:1/16:0). Cer(d18:1/16:0) has consistently been connected to elevated risk of cardiovascular death[16-18], major adverse cardiovascular events[14,15] and vulnerable plaque characteristics[15] among patients with established CAD, as well as to incident major adverse cardiovascular events among healthy population[19,20]. Moreover, elevated plasma levels of Cer(d18:1/16:0) have earlier been reported among patients with stable CAD compared with healthy controls[27]. Reported data on subclinical atherosclerotic associations of Cer(d18:1/16:0), or ceramides in general, is very limited. Nevertheless, Cer(d18:1/16:0) has been linked to an increased risk of carotid artery plaques[28], and sphingomyelin SM(34:1), a precursor molecule of Cer(d18:1/16:0), to an increased carotid artery intima-media thickness[29]. Our Supplementary analyses (S7) also indicate an association of Cer(d18:1/16:0) with elevated high-sensitivity CRP levels. In addition to Cer(d18:1/16:0), associations over apoE genotypes were indicated in six other ceramide species as well. The present results suggest a novel linkage between apoE polymorphism, lipid metabolism and CAD, as discussed briefly in the following. A more detailed review of previously presented, relevant pathophysiological mechanisms has been provided in an extended Supplementary Discussion S10.

Previous GWAS has mapped apoE locus to (Ingenuity) sphingolipid metabolism pathway[30], which is the major hub also for ceramide metabolism[31]. Inflammatory conditions such as atherosclerosis may increase the production of ceramides[32]. Ceramides are mainly generated through sphingomyelinase (SMase) pathway, which breaks downs sphingomyelin in the cell membrane and releases ceramides[32]. Alternatively, ceramides can be generated via de novo pathway from palmitate, serine and a covalently linked fatty acid, which is added to the molecule by a ceramide synthase (CerS) – in all cell types, liver being an active site of circulating ceramide production[33,34]. A variety of cell types present in atherosclerotic lesions, including macrophages and endothelial cells, secretes SMase[35]. In plasma, ceramides are bound to lipoproteins and are particularly abundant in LDL[36]. Moreover,

LDL isolated from human plasma has also been reported to possess SMase activity[37], being able to catalyse the formation of ceramides in the contacting, adjacent lipoproteins, as well[38].

Ceramides have been found to enrich in LDL of atherosclerotic lesions compared with plasma LDL[39]. Elevated sphingolipid content (including certain risk ceramides) of LDL particles[40], as well as macrophage-derived SMase[32,41] have been shown to promote aggregation of LDL and very-low-density lipoproteins (VLDL). Aggregated lipoprotein particles are prone to accumulate within the arterial intima, increase their uptake by macrophages, and thus promote atherosclerotic plaque formation, as well as future CAD death[32,40]. Also, macrophages loaded with oxidized LDL are demonstrated to contain more intracellular ceramides – Cer(d18:1/16:0) and Cer(d18:1/18:0) among reported species[42]. On the other hand, deficiency and/or inhibition of LDL-receptor degrading proprotein convertase subtilisin/kexin type 9 (PCSK9) enzyme has been found to decrease certain plasma ceramide levels, including Cer(d18:1/16:0) and Cer(d18:1/18:0)[43,44], as well as lower certain ceramide contents in LDL and VLDL particles, including all the seven ceramide species we found to associate over apoE genotypes[43]. Our supplementary analyses (Supplementary tables S4 and S5) are further indicating a strong connection between these ceramides and apoB-containing LDL, as well as VLDL (+ IDL) particles, whereas associations with HDL are weak to none (Supplementary Table S6). A consistent association of apoE polymorphism with all the apoB-containing lipoproteins along with their subfractions have been shown recently in the same YFS cohort[45]. Altogether, ceramides would seem as a plausible and novel common nominator in the abundantly investigated chain of apoE polymorphism, plasma LDL metabolism and the risk of CAD.

In addition to SMase pathway, it may be possible that the altered concentrations of certain ceramide species could also result from changes in de novo pathway due to altered CerS activities. For example, de novo pathway has been reported to be influenced by high-fat-diet administration (especially saturated fatty acids) and/or palmitate treatment, which in several reference studies have been shown to increase molecular ceramide contents, most consistently C16:0 and C18:0 species, independent of tissue or cell type[46].

To our knowledge, previous studies of apoE interactions with sphingolipid metabolism in atherogenesis have not investigated the effect of apoE polymorphism. However, it has been noticed that apoE increases the macrophage uptake of ceramides[47]. Moreover, apoE has been reported to prevent SMase-induced hydrophobic interaction and aggregation of lipoproteins[48]. In addition, it has been demonstrated that majority of apoE in atherosclerotic lesions is synthesized locally by lesion macrophages, and the local apoE expression by macrophages is atheroprotective[49]. On the other hand, increased cellular ceramides have been reported to reduce macrophage-derived apoE secretion (demonstrated with E3 phenotype) without increased cell retention of nascent apoE[50]. Furthermore, apoE has been demonstrated to prefer binding on oxidized-LDL-loading-induced, ceramide-enriched microdomain surfaces of human macrophages, which could contribute to atherogenesis[42]. ApoE has also been shown to prefer binding to ceramides compared with sphingomyelins[48], and SMase stimulated ceramide formation can, therefore, induce the membrane remodelling activity of apoE and free cholesterol. If hypothesized, all these reported findings would be related to the apoE parent isoform E3, and E2 or E4 might, therefore, present altered interactions worth investigating – possibly affecting atherogenic processes via e.g., lipoprotein particle aggregation, or adjustment of sphingolipid transfer balance in macrophages. In any case, apoE E4 (but not E3) in macrophages has already been demonstrated to enhance atherosclerotic plaque development in an LDL-receptor-dependent manner[51].

Our study has several important strengths. Our study cohort has a long and systematic follow-up, offering a representative cross-sectional sample of relatively young Finnish adults. Also, the comprehensive background information gathered from our cohort allowed a thorough analysis and consideration of the most expected confounding factors. Furthermore, our analyses reflect genotype-related differences in a basically healthy general population – avoiding confounding/confusing expressions of illnesses expected when using morbid study subjects. In addition, the modern and sophisticated mass spectrometry technology enabled the measurement of circulating ceramides with good accuracy. We also acknowledge certain limitations in our cross-sectional study. Our present scope did not allow direct analyses of subclinical atherosclerosis measures. As previously reported in Finnish general population[2,52], subgroups of apoE ε2/2, ε4/2, as well as ε4/4 were small also in our population-based setting and did not allow complete analyses of all the six genotypes separately. Also, a completely comprehensive

comparison between (sex-stratified) men and women might require even a bigger sample size for increased statistical power – albeit, the absence of apoE x sex interactions was confirmed in the analyses of all subjects. Nevertheless, based on reference studies (e.g.,[2,3]) these recognized limitations are not expected to alter the effect directions related to different apoE alleles, and are thus less likely to have any major impact on our main discoveries presented and discussed here.

In conclusion, based on quantified molecular ceramide species from plasma, apoE polymorphism is associated with risk of developing CAD, especially by indicating altered levels of previously reported clinical high-risk marker Cer(d18:1/16:0) in all subjects. Furthermore, the concentration of every apoE-associating ceramide species in our study has been reported to be reduced in LDL- and VLDL-particles by PCSK9 inhibition[44], whereas excessive sphingolipid loading has been reported to promote CAD death[40]. These findings suggest a plausible linkage between ceramide metabolism, apoE polymorphism, plasma LDL metabolism and atherogenesis. Discovered changes are already evident in a basically healthy general population and extend our understanding about the role of apoE in health and disease. For further work, similar analyses could be performed with patients at incident CAD and established CAD to discover, whether the observed associations of apoE polymorphism would change. Associations with subclinical atherosclerosis measures (e.g., carotid artery intima-media thickness or plaques) should also be investigated for further indications. In addition, longitudinal follow-up studies could be performed, with older test subjects included. Furthermore, effects of apoE polymorphism on sphingolipid/ceramide metabolism pathway might be worth investigating – whether e.g., the macrophage functions or lipoprotein aggregation tendency would become different depending on the apoE isoform. Additional inflammatory marker correlations might also be worth investigating, and the ceramide levels could also be correlated with sphingomyelinase activities.

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Author Contributions

J.-P.K. wrote the main manuscript text and analysed the data. N.M. contributed to genotype profiling, reviewing and editing the manuscript. N.H.-K., M.J. and J.V. participated in cohort collection and reviewed the manuscript. M.L. contributed to data analyses and reviewed the manuscript. M.H. and D.K. contributed to the lipidomics profiling and reviewed the manuscript. M.K. participated in cohort collection and reviewed the manuscript. O.R. handled funding, participated in cohort collection and reviewed the manuscript. R.L. contributed to the lipidomics profiling, funding and reviewed/edited the manuscript. T.L. handled funding and supervision, participated in cohort collection and contributed to the discussion, in addition to reviewing/editing the manuscript. All authors contributed to and have approved the final manuscript.

Competing interests:

Reijo Laaksonen is an employee and shareholder of Zora Biosciences Oy, Espoo, Finland. Mika Hilvo and Dimple Kauhanen are employees of Zora Biosciences Oy, Espoo, Finland. Other authors declare no conflict of interest.

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Tables

Table 1. Frequencies of different apoE genotypes (ε2/2, ε3/2, ε3/3, ε4/2, ε4/3 and ε4/4) with summary descriptive data in the YFS cohort in the 2007 follow-up. Values are mean (SD) or n (%). The p-values across apoE genotypes are given in Supplementary Table S1.

	All	ε2/2	ε3/2	ε3/3	ε4/2	ε4/3	ε4/4
Number of subjects	2160	4 (0.2)	142 (6.6)	1241 (57.5)	41 (1.9)	651 (30.1)	81 (3.8)
Number of males	974 (45.1)	2 (0.2)	56 (5.7)	568 (58.3)	17 (1.7)	296 (30.4)	35 (3.6)
Number of females	1186 (54.9)	2 (0.2)	86 (7.3)	673 (56.7)	24 (2.0)	355 (29.9)	46 (3.9)
Age [years]	37.6 (5.0)	36.0 (7.4)	36.9 (5.1)	37.7 (5.0)	38.6 (4.0)	37.5 (5.1)	38.0 (4.8)
Body mass index [kg/m²]	26.0 (4.8)	36.8 (17.2)	25.5 (4.8)	26.0 (4.6)	25.9 (4.5)	26.0 (4.8)	26.3 (5.3)
Daily smokers	398 (18.4)	1 (25)	21 (14.8)	229 (18.5)	6 (14.6)	127 (19.5)	14 (17.3)
Daily alcohol consumption [ethanol g]	11.3 (17.0)	4.7 (6.5)	12.4 (18.2)	11.9 (18.8)	9.8 (13.9)	10.0 (13.1)	11.5 (13.7)
Cholesterol lowering medication	45 (2.1)	0 (0.0)	2 (1.4)	23 (1.9)	2 (4.9)	15 (2.3)	3 (3.7)
Diabetes mellitus type 2	22 (1.0)	0 (0.0)	2 (1.4)	15 (1.2)	0 (0.0)	5 (0.8)	0 (0.0)
Hypertension	123 (5.7)	2 (50.0)	4 (2.8)	67 (5.4)	1 (2.4)	45 (6.9)	4 (4.9)
Total cholesterol [mmol/L]	5.05 (0.90)	4.12 (0.76)	4.49 (0.82)	5.03 (0.89)	4.68 (0.82)	5.22 (0.89)	5.28 (0.84)
LDL-C [mmol/L]	3.15 (0.81)	1.94 (0.30)	2.48 (0.65)	3.12 (0.79)	2.76 (0.68)	3.36 (0.80)	3.30 (0.76)
HDL-C [mmol/L]	1.34 (0.33)	1.17 (0.17)	1.41 (0.37)	1.35 (0.33)	1.35 (0.37)	1.31 (0.31)	1.35 (0.41)
Triglycerides [mmol/L]	1.40 (0.93)	2.22 (0.87)	1.45 (1.00)	1.38 (0.93)	1.36 (0.97)	1.39 (0.82)	1.70 (1.43)
apoB [g/L]	1.02 (0.26)	0.79 (0.16)	0.87 (0.26)	1.01 (0.26)	0.91 (0.23)	1.07 (0.26)	1.09 (0.25)
apoA-I [g/L]	1.60 (0.26)	1.60 (0.22)	1.66 (0.30)	1.61 (0.26)	1.59 (0.25)	1.57 (0.24)	1.60 (0.28)

Abbreviations: LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; apoB, Apolipoprotein B; apoA-I, Apolipoprotein A-I.

Figures and figure legends

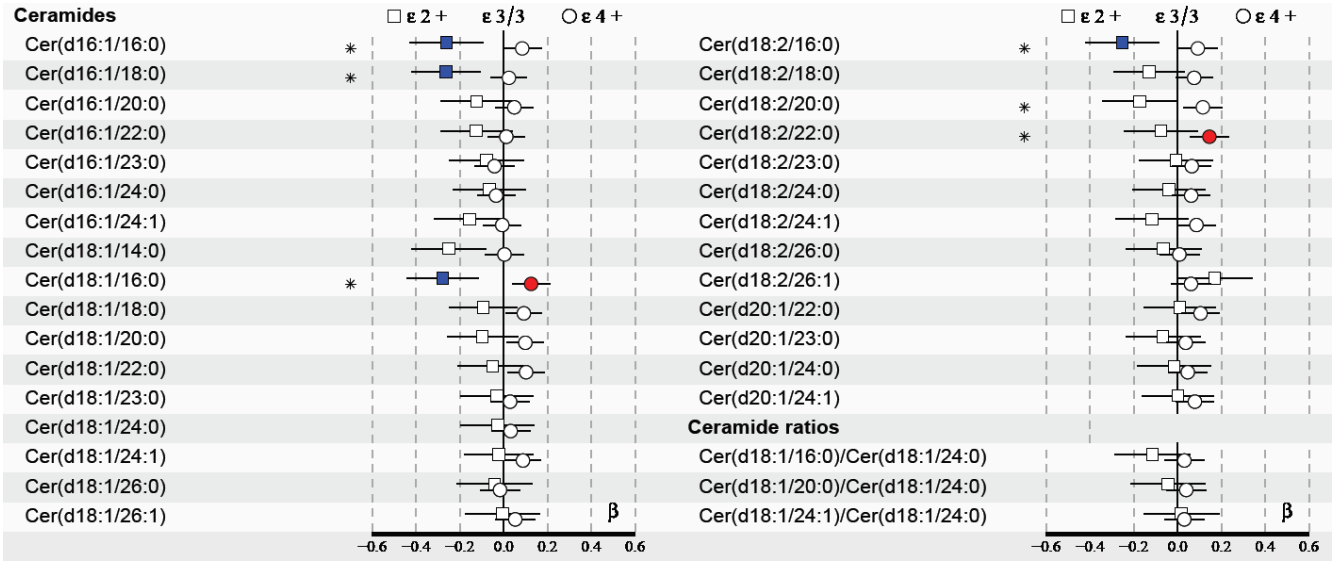

Figure 1. ApoE effects on 30 plasma ceramides and three ceramide ratios in all subjects (n=2160) of YFS cohort participated in 2007. **Statistics:** Regression models are adjusted for age, BMI and sex. Regression β-coefficients (x-axis) indicate in standard deviation (SD) units the change in lipid measure over apoE genotype subgroups (ε2+, ε3/3, ε4+). The most common ε3/3 subgroup (n=1241) is set at the origin (zero SD) and post-hoc compared with ε2+ (squares) and ε4+ subgroups (circles). β-values with 95% CI are scaled to SD increments from normalized, i.e. ln-transformed, lipid measures. F-tested effect of apoE with $p<0.05$ after false discovery rate (FDR) correction in the regression model has been indicated with (*). Post-hoc difference with $p<0.05$ after FDR correction from ε3/3 is indicated with colours (blue/ε2+, red/ε4+). **Definitions:** apoE ε2+ subgroup (ε2/2, ε3/2 combined; n=146) and apoE ε4+ subgroup (ε4/2, ε4/3, ε4/4 combined; n=773).

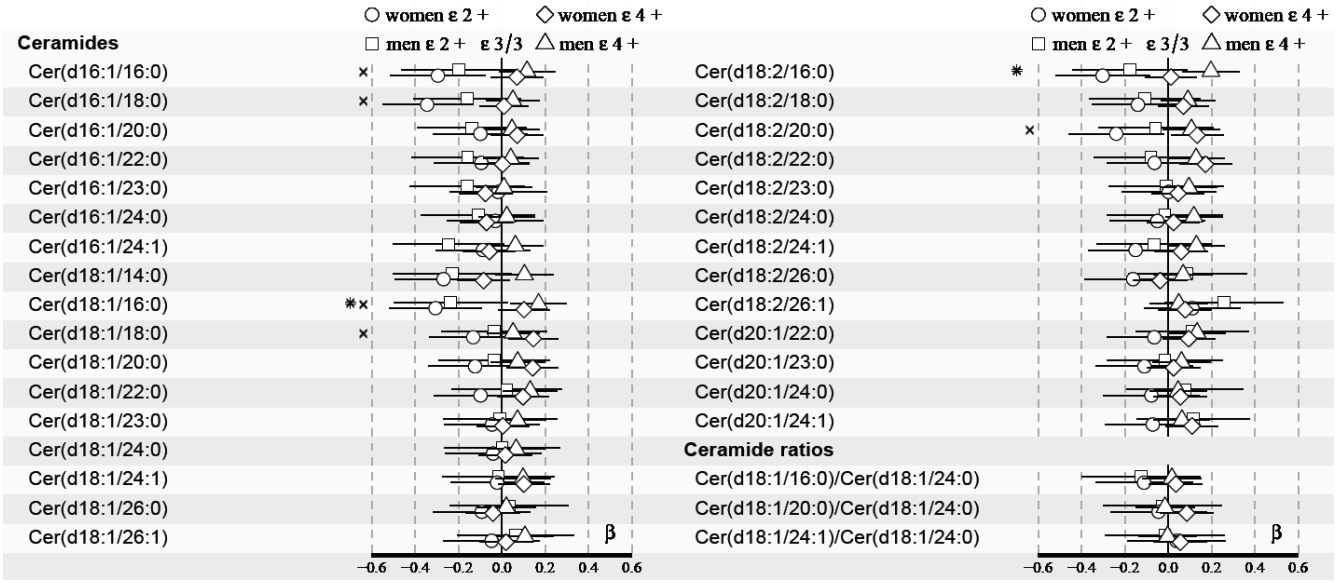

Figure 2. ApoE effects on 30 plasma ceramides and three ceramide ratios in men (n=974) and women (n=1186) of YFS cohort participated in 2007. **Statistics:** Regression models are adjusted for age and BMI. Regression β-coefficients (x-axis) indicate in standard deviation (SD) units the change in lipid measure over apoE genotype subgroups (ε2+, ε3/3, ε4+). The most common ε3/3 subgroup (n=568 for men, n=673 for women) is set at the origin (zero SD) and post-hoc compared with ε2+ (squares, men; circles, women) and ε4+ subgroups (triangles, men; diamonds, women). β-values with 95% CI are scaled to SD increments from normalized, i.e. ln-transformed, lipid measures. F-tested effect of apoE with $p<0.05$ after false discovery rate (FDR) correction in the regression model has been indicated with (*) in men, and (x) in women. **Definitions:** apoE ε2+ subgroup (ε2/2, ε3/2 combined; n=58 for men, n=88 for women) and apoE ε4+ subgroup (ε4/2, ε4/3, ε4/4 combined; n=348 for men, n=425 for women).