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Genome-Wide Association Study Identifies Novel Loci Associated with Circulating Phospho- and Sphingolipid Concentrations

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Abstract

Phospho- and sphingolipids are crucial cellular and intracellular compounds. These lipids are required for active transport, a number of enzymatic processes, membrane formation, and cell signalling. Disruption of their metabolism leads to several diseases, with diverse neurological, psychiatric, and metabolic consequences. A large number of phospholipid and sphingolipid species can be detected and measured in human plasma. We conducted a meta-analysis of five European family-based genome-wide association studies (N=4034) on plasma levels of 24 sphingomyelins (SPM), 9 ceramides (CER), 57 phosphatidylcholines (PC), 20 lysophosphatidylcholines (LPC), 27 phosphatidylethanolamines (PE), and 16 PE-based plasmalogens (PLPE), as well as their proportions in each major class. This effort yielded 25 genome-wide significant loci for phospho- and sphingolipid loci, substantially increasing our knowledge of the genetic basis for these traits.
Introduction

Phospho- and sphingolipids are present in all eukaryotic cell membranes and contribute to organelle structure and signalling events that influence cell behaviour and function [1–3]. Phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), lysophosphatidylcholines (LPCs) and PE-based plasmalogens (PLPEs) are major classes of phospholipids that play an important role in several key processes such as cell survival and inflammation [4–6]. Sphingolipids are also essential components of plasma membranes and endosomes and are believed to play critical roles in cell surface protection, protein and lipid transport and sorting, and cellular signalling cascades [7]. In plasma, PC, PE and sphingomyelin (SPM) are included in the structure of lipoproteins; they constitute more than two-thirds of the total phospholipid content in HDL-C and LDL-C, as well as in platelets [8,9]. Remarkable differences in plasma lipoprotein acceptor affinities for the phospholipids exist (LDL-C is the major acceptor for SPM, whereas HDL-C is the predominant acceptor for PC) [9]. Altered concentrations of circulating phospholipids have been implicated in the pathology of type 2 diabetes, dyslipidemia and cardiovascular disease [10–15], as well as a wide range of other common diseases including dementia and depression [16].

Identifying genetic variants that influence phospho- and sphingolipid concentrations will be an important step towards understanding pathways contributing to common human disease. Earlier studies of these metabolites identified a number of genetic loci associated with their levels in blood [17–19]. We conducted a meta-analysis of genome-wide association studies (GWAS) on plasma levels of 24 SPMs, 9 ceramides (CERs), 57 PCs, 20 LPCs, 27 PEs and 16 PLPEs in five European populations: (1) the Erasmus Rucphen Family (ERF) study, conducted in the Netherlands, (2) the MICROS study from the Tyrol region in Italy, (3) the Northern Swedish Population Health Survey (NSPHS) in Norrbotten, Sweden, (4) the Orkney Complex Disease Study (ORCADES) in Scotland, and (5) the CROAS (CROATIA_Vis) study conducted on Vis Island, Croatia.

The top findings were further analysed by adjusting for plasma HDL-C, LDL-C, TG and TC levels. The influences of these top hits on within class lipid ratios were also assessed, to help elucidate potential mechanisms. Finally, the variants that were associated with plasma phospho- and sphingolipid levels were tested for association with carotid intima media thickness (IMT), type 2 diabetes (T2DM), and coronary-artery disease (CAD) using large consortia meta-analysis results.

Results

Table 1 provides an overview of the study populations. The mean age, gender ratio and mean values of major classes of phospho- and sphingolipids were comparable among the 5 populations. Means for the individual species are presented in Table S1. Figure 1A and 1B shows the combined Manhattan plot for the meta-analyses of the absolute values and proportions of all phospholipid traits, respectively; Figure 2A and 2B provides the same for the sphingolipids. Out of 357 meta-analyses performed, 202 outcomes yielded genome-wide significant findings, most of which were located around two genes, FAIS and LIPC, which were identified previously [17,19] as key lipid regulators and are associated with a large number of species (Table 2 and Table 3). Q-Q plots for the lipid GWAS that yielded significant associations are provided in Figure S1.

Phospholipids

As shown in Table 2, 25 loci were nominally associated (P-value<5×10⁻⁸) with absolute plasma levels and/or proportions of the phospholipid species. Among those loci, previously reported relationships between the FAIS1, LIPC, PLEKHI1, GCKR, APOA1-5, and ELOVL2 loci and phospholipids were successfully replicated [17,19]. Four novel genome-wide significant loci were also detected after a multiple testing correction to adjust for the approximate number of independent genotypes and phenotypes (n=23) studied (P-value<2.2×10⁻⁸). These included PAQR9 on 3q23 (associated with %PE 34:1 and %PE 36:1), AGPAT1 on 6p21.32 (associated with PC 32:0), PKD2L1 on 10q24.31 (LPC 16:1), and PDXDC1 on 16p13.11 (LPC 20:3, PC 34:2, PC 36:3 and PC 38:3). Fifteen additional regions provided suggestive evidence of association (2.2×10⁻⁸<P-value<5×10⁻⁶) with phospholipids including the PNPLA2 locus, associated with %PC 36:1; NF600 with PC/LPC ratio; ALG1 with PC 30:1; ABHD3 with %PC 32:2; KLF12 and DLG2, both associated with PC O 42:5; ILKAP with PC 40:3 and %PC 40:3; ITGA9 with PLPE.
Author Summary

Phospho- and sphingolipids are integral to membrane formation and are involved in crucial cellular functions such as signalling, membrane fluidity, membrane protein trafficking, neurotransmission, and receptor trafficking. In addition to severe monogenic diseases resulting from defective phospho- and sphingolipid function and metabolism, the evidence suggests that variations in these lipid levels at the population level are involved in the determination of cardiovascular and neurologic traits and subsequent disease. We took advantage of modern laboratory methods, including microarray-based genotyping and electrospray ionization tandem mass spectrometry, to hunt for genetic variation influencing the levels of more than 350 phospho- and sphingolipid phenotypes. We identified nine novel loci, in addition to confirming a number of previously described loci. Several other genetic regions provided substantial evidence of their involvement in these traits. All of these loci are strong candidates for further research in the field of lipid biology and are likely to yield considerable insights into the complex metabolic pathways underlying circulating phospho- and sphingolipid levels. Understanding these mechanisms might help to illuminate factors leading to the development of common cardiovascular and neurological diseases and might provide molecular targets for the development of new therapies.

18:0/22:6; ORB12 with %PC 26:0; PCDH20 with PC 32:1; CDK17 and ST79, both associated with PC O 42:6; CDBH with the proportion of saturated LPC; KCNH7 with %PC O 36:5; and ALEG4 with %LPC 18:0. Regional association plots for all phospholipid loci are presented in Figure S2.

Many of the genome-wide significant and suggestive loci in Table 2 were associated with the percentage of each lipid molecule within its own class (mol%) rather than to absolute values. Single SNP analysis of ratios showed that rs4500751 (PDXDC1) was strongly associated with PC 36:3/PC 34:2 (P-value = 4.37 × 10^{-25}) and LPC 20:3/LPC 16:1 (P-value = 6.84 × 10^{-25}) (Table S2). Further, rs11662721 (ABHD5) was associated with the ratio of PC 32:2 to PC 36:2 (P-value = 9.35 × 10^{-10}), but also to PC 36:3 (P-value = 1.80 × 10^{-8}) and PC 38:3 (P-value = 6.71 × 10^{-6}). rs9437689 (ALG14) and rs603424 (PDK2L1) were associated with the ratios of LPC 16:0 to LPC 18:0 (P-value = 2.70 × 10^{-5}) and LPC 16:1 (P-value = 2.25 × 10^{-15}), respectively. SNP rs10885997 (PNLIPRP2) was associated with PC 36:1/PC 34:1 (P-value = 3.28 × 10^{-10}) and PC 36:1/PC 34:3 (P-value = 1.15 × 10^{-9}). SNP rs7337583 (PCDH20) was associated with the ratio of PC 32:1 to several ether-bound PC species (the strongest association was with PC 32:1/PC O 32:0; P-value = 1.92 × 10^{-14}), and, finally, rs2943016 (ORB12) was associated with the ratio of PC 26:0 to several long chain PCs (the strongest association was with PC 26:0/PC 36:1; P-value = 2.93 × 10^{-5}).

Sphingolipids

Table 3 shows the 10 loci that were associated with either absolute plasma levels (panel A) or percentages (panel B) of sphingomyelin species or ceramides. Among those loci, 5 (ATP10D, FADS3-1, SGPP1, SPILC3, LASS4) were previously described in genome-wide analyses [18,19]. These loci retained significance after adjustment for the number of genotypes and phenotypes tested. In addition, five novel loci were identified at a nominal P-value of 5 × 10^{-8} (PAPD7, CNTNAP4, PDL2, LPAR2, and AP08). Two of these, APOE on 19q13.32 (associated with SPM 24:0 and SPM 22:0) and PDL2 on 17p13.2 (associated with SPM 23:0), remained significant after correction for the number of phenotypes tested. The other three showed suggestive evidence of association (2.2 × 10^{-6} < P-value < 5 × 10^{-5}) to either sphingomyelins or ceramides: PAPD7 on 3p15.31 (SPM 17:0), the CNTNAP4 region on 16q23.1 (% Glu-CER 24:1, %Glu-CER) and LPAR2 on 19p13.11 (% C 18:0). Regional association plots for the sphingolipid loci are presented in Figure S3.

When studying the ratios of the index lipid to the other lipids within the same class, the strongest association for rs12051548 (PLD2) was found with the SPM 23:0/SPM 16:1 ratio (P-value = 2.43 × 10^{-10}). SNP rs7259004 in the APOE locus was strongly associated with the ratio of SPM 24:0 to SPM 24:2 (P-value = 5.11 × 10^{-9}) and SPM 16:1 (P-value = 4.79 × 10^{-6}) but also with the ratio of SPM 22:0 to the same lipid species (SPM 24:2: P-value = 2.91 × 10^{-8} and SPM 16:1: P-value = 1.90 × 10^{-6}).

HDL-C, LDL-C, TG, and TC

As a point of reference, the genome-wide significant findings (P-value < 5 × 10^{-5}) from the GWAS of TC, LDL-C, HDL-C, and TG in these samples are provided in Table S3. CETP was associated with HDL-C levels (P-value = 8.5 × 10^{-9}), APOE was associated with LDL-C (P-value = 9.2 × 10^{-9}) and TC levels (P-value = 4.6 × 10^{-11}). APOAI (P-value = 1.6 × 10^{-6}) and PDCD11 (P-value = 2.7 × 10^{-10}) were associated with TG levels. Except for the PDCD11 locus, these associations have all been previously reported [20]. To determine if the associations of the phospho- and sphingolipid loci were mediated by these major classes of plasma lipids, we used ELISA to determine levels of HDL-C, LDL-C, TC, and TG in our samples. For HDL-C, LDL-C, TG, and TC, we used a two-sample t-test with the default settings in GenABEL.
Figure 1. Genome-wide association results for 115 phospholipid species. (A) Genome-wide association results for plasma levels of 115 phospholipid species. (B) Genome-wide association results for within-class proportions of 115 plasma phospholipid species. Manhattan plots show the combined association signals ($-\log_{10}(p\text{-value})$) on the y-axis versus SNPs according to their position in the genome on the x-axis (NCBI build 36). Novel genes are represented in red, while previously known loci are represented in black.

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lipoproteins, conditional analyses were performed. Table S4 shows the effect size, standard error, and P-values for the genome-wide significant loci when adjusted for HDL-C, LDL-C, TG and TC. Only the association of the \textit{APOE} locus (rs7259004) with SPMs was greatly affected by the incorporation of LDL-C and TC. No other major differences were observed in effect size or P-value.

Pathway analyses

Additionally, we investigated whether the genes from the GWAS fit into previously known sphingolipid and glycerophospholipid pathways, which are available among the canonical pathways from various data bases provided by ConsensusPathDB [21]. By testing for enrichment of known pathways, glycerophospholipid metabolism (P-value = 0.002; KEGG), chylomicron-mediated lipid transport (P-value = 0.003; Reactome), triglyceride biosynthesis (P-value = 0.006; Reactome), metabolism of lipids and lipoproteins (P-value = 0.002; Reactome) and biosynthesis of the N-glycan precursor (P-value = 0.003; Reactome) were found to be significantly enriched among the phospholipid related loci. Considering the sphingolipid associated loci, the same analysis implicated the sphingolipid metabolism (P-value = 1.0×10^{-5}; Reactome), metabolism of lipids and lipoproteins (P-value = 1.0×10^{-5}; Reactome), and LPA receptor mediated events (P-value = 0.002; PID) pathways. These analyses suggested that, among genes from the same locus, \textit{SRD5A1} is a more likely candidate than \textit{PAPD7} and \textit{LPAR2} is a more likely candidate than neighbouring \textit{ZNF101} and \textit{ATP13A1} (Tables S5 and S6).

Figure S4 places all of the nearest, or most likely, genes from genome-wide significant and suggestive loci in the Ingenuity glycerophospholipid metabolism pathway [22]. Of the 25 loci associated with phospholipids at a nominal P-value<5×10^{-8}, 13 genes (\textit{KCNH7}, \textit{AGPAT1}, \textit{PNLIPRP2}, \textit{SYT9}, \textit{FADS2}, \textit{DAGLA}, \textit{DLG2}, \textit{APOA1}, \textit{APOC3}, \textit{ELOVL2}, \textit{CDK17}, \textit{LIPC} and \textit{PLA2G10}) from 11 loci can be mapped to the glycerophospholipid metabolism pathway; among the 10 loci associated with sphingomyelins or ceramides, 6 genes (\textit{FADS2}, \textit{DAGLA}, \textit{PLD2}, \textit{LASS4}, \textit{APOE}, \textit{APOC2}) from 4 loci can be mapped to the same pathway (Figure S4). Figure S5 maps the same genes onto the Ingenuity sphingolipid metabolism pathway. Of the 10 sphingomyelin or ceramide loci, 9 genes from 5 loci (\textit{FADS1}, \textit{FADS2}, \textit{C11orf10}, \textit{SGPPI}, \textit{APOE}, \textit{APOC1}, \textit{APOC2}, \textit{LASS4}, and \textit{PLD2}) can be placed in this pathway, as was the case for 12 genes from 8 loci implicated in phospholipids (\textit{ILKAP}, \textit{ITGA9}, \textit{AGPAT1}, \textit{FADS1}, \textit{FADS2}, \textit{C11orf10}, \textit{APOA1}, \textit{APOA5}, \textit{APOC3}, \textit{PCDH20}, \textit{LIPC}, and \textit{PDXDC1}).

Association with IMT, T2DM, and CAD risk

The top 35 SNPs were assessed for association with IMT, T2DM, and CAD using the GWAS results from the CHARGE [23], DIABEGRAM [24] and CARDIoGRAM [25] consortia, respectively. For IMT, we observed a significant association (P-value = 7×10^{-5}) with the \textit{FADS1}-2-3 locus SNP rs102275 (Table S7). rs1061808, located in the \textit{HLA} region on chromosome 6, and two SNPs from the \textit{FADS1}-2-3 region (rs174479 and rs102275) were associated with T2DM risk (nominal P-value<0.05) (Table S8). rs964184 from the \textit{APOA1}-3 region was previously reported to be associated with CAD risk (P-value = 8.0×10^{-10}) by the CARDIoGRAM meta-analysis study (Table S9). For all three outcomes, the observed P-value distribution differed significantly from that expected under the null hypothesis (Kolmogorov-Smirnov P-value<3.3×10^{-16}; Figure S6).

Discussion

This genome-wide association study of more than 350 phospho- and sphingolipid measurements in five European populations yielded 25 loci associated with phospholipids and 10 loci associated with sphingolipids using a nominal P-value of 5×10^{-8}. After correction for the number of independent phenotypes, the novel genome-wide significant loci included: \textit{PAQR9}, \textit{AGPAT1}, \textit{PKD2L1}, \textit{PDXDC1}, \textit{APOE} and \textit{PLD2}. In addition, further analysis of suggestive SNPs with lipid ratios showed significant association for an additional 3 loci (\textit{ABHD3}, \textit{PNLIPRP2}, and \textit{PDXH}).

The strongest association in the PAQR9 locus was observed between rs9832727 and the proportion of mono-unsaturated PEs, especially with the ratios PE 34:1/PE 34:2 and PE 36:1/PE 36:2. The protein coded by\textit{PAQR9} is an integral membrane receptor and functions as receptor for the hormone adiponectin, suggesting a molecular link with obesity and T2DM [26]. However, we did not observe an association between T2DM risk and this variant.

In the \textit{AGPAT1} locus, rs1061808 was associated with the proportion of PC 32:0, and, especially, with the ratio of PC 32:0/PC 34:1. \textit{AGPAT1} is directly connected to phospholipid metabolism (Figures S4 and S5), as the product of this gene converts lysophosphatic acid (LPA) into phosphatic acid (PA) [27]. The locus lies 400 kb distant from the \textit{HLA-DRB1} gene which was previously associated with insulin secretion [28]. A suggestive association between rs1061808 and increased T2DM risk was observed in the DIAGRAM consortium meta-analysis results.

We found two loci that strongly influence plasma LPC levels: \textit{PKD2L1} and \textit{PDXDC1}. An intronic variant, rs603424 in the \textit{PKD2L1} gene, was strongly associated with LPC 16:0. Pathway analyses suggest that another gene in the same region, \textit{SCD} (\textit{FADS}-5), 25 kb away, may be a better candidate since it encodes the stearoyl-CoA desaturase (delta-9-desaturase) enzyme which is involved in fatty acid desaturation. Other members of the \textit{FADS} family are the strongest genetic regulators of phospholipid metabolism identified to date. In the \textit{PDXDC1} locus, the strongest association was observed for intronic SNP rs4100751. This variant is 300 kb distant from \textit{PLA2G10}, a gene that plays a major role in releasing arachidonic acid from cell membrane phospholipids [29] and the protein can be mapped to both the glycerophospholipid and the sphingolipid metabolism pathways by Ingenuity (Figures S4 and S5). In our study, the variant was strongly associated with the ratios of 20:3 fatty acid carrying LPCs, as well as PEs, and PCs, but not with the others, suggesting a fatty acid-specific mechanism for this enzyme.

Another index SNP (rs7259004), associated with SPMs, maps to the well known \textit{APOE} locus, which also includes three other lipid genes (\textit{APOC1}, \textit{APOC2} and \textit{APOC4}). Results from the conditional analyses (Table S4) suggest that the effect of this variant on SPM 22:0 levels is dependent on plasma LDL-C levels and that SPM 22:0 and SPM 24:0 are likely abundant in LDL-C particles, which can also be inferred from their high phenotypic correlations with LDL-C (r = 0.6, P-value = 2.8×10^{-16} for SPM 22:0 and r = 0.6, P-value = 2.8×10^{-16} for SPM 24:0).
Table 2. Variants significantly associated with circulating phospholipid levels and proportions.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chromosome</th>
<th>Position</th>
<th>Gene</th>
<th>Distance (kb)</th>
<th>$P_{value_{Nominal}}$</th>
<th>$P_{value_{Corrected}}$</th>
<th>% Explained variance</th>
<th>MAF</th>
<th>Species associated</th>
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<tr>
<td>rs466002</td>
<td>2p23.3</td>
<td>27840640</td>
<td>GCKR</td>
<td>97</td>
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<td>3.49×10^{-07}</td>
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<td>0.18</td>
<td>PC 403</td>
</tr>
<tr>
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<td>ITGA9 intonic</td>
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<td>4.49×10^{-07}</td>
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<tr>
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<td>1.47×10^{-12}</td>
<td>1.4</td>
<td>0.17</td>
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<td>73670959</td>
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<td>0.38</td>
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<td>2.27×10^{-202}</td>
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<td>0.30</td>
<td>PE 321, PE 341, PE 342, PE 343, See Text S2 for the full list.</td>
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<tr>
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<td>2.27×10^{-202}</td>
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See Text S2 for the full list.
A second locus associated with the SPMs is PLD2 (phospholipase D2). PLD2 catalyzes the hydrolysis of PC to produce phosphatidic acid and choline and the PLD2 signalling pathway is involved in the destabilization of ABCA1 and, therefore, plays a role in the generation of plasma HDL-C particles [30]. PLD2-related processes may be responsible, in part, for determining the SPM content of HDL-C. Unexpectedly, we did not observe an association between PC levels and the PLD2 locus.

The analysis of the ratios of the phospholipids uncovered three additional associations significant at the adjusted genome-wide threshold ($P$-value<2.2×10^{-8}). $ABDH3$, PNLIPRP2 and PCDH20.

The exact function of the $ABDH3$ and PCDH20 proteins, and how they relate to phospholipid metabolism, has not been determined. PNLIPRP2 (pancreatic lipase-related protein 2) fulfills a key function in dietary fat absorption by hydrolyzing triglycerides into diglycerides and, subsequently, into monoglycerides and free fatty acids (Figure S4) [31]. We found that a synonymous coding SNP (rs10885997) in PNLIPRP2 was associated with the ratios PC:36:1/PC:34:1 and PC:36:1/PC:34:3, suggesting a fatty-acid specific turnover between these lipids.

A closer examination of the findings published by Illig et al., supports the association signals within 100 kb of loci $PDXDC1$ (same SNP, $P$-value = 2.8×10^{-7}), $AGPAT7$ ($P$-value = 4.9×10^{-8}), PNLIPRP2 ($P$-value = 2.7×10^{-7}), KLPI2 ($P$-value = 5.9×10^{-7}), $ALG1$ ($P$-value = 4.7×10^{-7}), $CDH8$ ($P$-value = 7.6×10^{-7}), PLD2 ($P$-value = 9.4×10^{-6}) and $ZNF600$ ($P$-value = 3.3×10^{-5}) for various phospho- and sphingolipid outcomes. SNP rs6034242 in $PDXDC1$ was previously associated with acylcarnitine C 16:1, although this result was not replicated [19].

The significant hits from the current study were further studied for potential associations with IMT, T2DM, and CAD. For all three outcomes, the $P$-value distributions differed significantly from the expected null distribution even after exclusion of nominally significant SNPs, suggesting that some of these variants contribute to these outcomes even when they do not achieve statistical significance.

Among our top hits, rs102275 from the FADS cluster was associated with IMT in the CHARGE meta-analysis results [25]. This finding demonstrates the involvement of the FADS locus in the development of atherosclerosis.

In addition, the top SNP from the APOA1-5 locus was implicated in CAD risk in the CARDIoGRAM study [25]. This locus, previously associated with TG levels [20], influenced two other-bound PCs and the PC/SPM ratio in our study. APOA1 and APOA2 are the predominant proteins in HDL-C particles, which also transport TG. The association between the phospholipids and rs964184 remained significant after adjustment for TG levels, suggesting that this signal is not due solely to TG mediated effects. APOA1 is also a cofactor for lecithin cholesterol acyltransferase (LCAT) which converts cholesterol and PC to cholesteryl esters and LPC on the surface of HDL-C [32] and it is possible that the association we observe here is due to LCAT mediated phospholipid cleavage.

Mapping the findings into the glycerophospholipid and sphingolipid metabolism pathways uncovered several enzymes, kinases, peptidases and G-protein coupled receptors that may also be relevant for phospho- and sphingolipid metabolism. Among those involved in sphingolipid metabolism (Figure S5), HNF4A (hepatocyte nuclear factor-4) appears to be a common interacting factor for several genes ($PCDH20$, $APOC1$, $AGPAT1$, $ITGA9$, $PLD2$, $C11ORF10$, $APOC2$, $GCK$, $APOE$, $APOC3$ and $LIPC$) from our GWAS. It is already known that the extinction of many hepatic functions and their expression are correlated with expression of

---

**Table 2.** Continued.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chromosome</th>
<th>Position</th>
<th>Gene</th>
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<th>Genotype</th>
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<td>19q11.2</td>
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<td>20</td>
<td>1.29×10^{-7}</td>
<td>0.04</td>
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<td>3.3×10^{-10}</td>
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<td>0.24</td>
<td>%LPC 20:3, %PC 34:2, %PC 36:3, %PC 38:3, %PE 38:3</td>
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</table>

$Loci$ significantly associated with lipid levels.

$Loci$ associated with phospholipids for the first time.

GWAS. It is already known that the extinction of many hepatic functions and their expression are correlated with expression of

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<td>%LPC 20:3, %PC 34:2, %PC 36:3, %PC 38:3, %PE 38:3</td>
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$Loci$ significantly associated with circulating phospholipid proportions.

$Loci$ associated with phospholipids for the first time.
**Table 3.** Variants significantly associated with circulating sphingolipid levels and proportions.

### Variants significantly associated with circulating sphingolipid levels.

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<tr>
<th>SNP</th>
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<th>Position</th>
<th>Gene</th>
<th>Distance (kb)</th>
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<th>$P$-value_{corrected}</th>
<th>% Explained variance</th>
<th>MAF</th>
<th>Species associated</th>
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<td>rs13106975*</td>
<td>4p12</td>
<td>47551863</td>
<td>ATP10D</td>
<td>47551863</td>
<td>1.93×10^{-19}</td>
<td>4.45×10^{-18}</td>
<td>0.7–2.0</td>
<td>0.20</td>
<td>Glu-CER 16:0, Glu-CER 24:1, Glu-CER</td>
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<td>rs1560039</td>
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<td>PAPD7</td>
<td>62</td>
<td>1.09×10^{-08}</td>
<td>2.51×10^{-07}</td>
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<td>0.40</td>
<td>SPM 17.0</td>
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<td>rs17479*</td>
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<td>FADS-1-2-3</td>
<td>17</td>
<td>1.99×10^{-14}</td>
<td>4.58×10^{-13}</td>
<td>0.8–1.5</td>
<td>0.49</td>
<td>SPM 161, SPM 181, SPM 201, SPM 221</td>
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<td>rs17101394*</td>
<td>14q23.2</td>
<td>64232386</td>
<td>SGPP1</td>
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<td>7.13×10^{-06}</td>
<td>0.7–6.3</td>
<td>0.15</td>
<td>SPM 140, SPM 150, SPM dih16:0</td>
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<td>PLD2</td>
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<tr>
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<td>4.89×10^{-10}</td>
<td>1.12×10^{-08}</td>
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<td>0.11</td>
<td>SPM 240, SPM 220</td>
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<tr>
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<td>SPTLC3</td>
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<td>0.8–1.7</td>
<td>0.29</td>
<td>CER 220, CER 230, CER 24:1, CER 24:0, saturated-CER, total CER, unsaturated CER, Glu-CER, SPM 17:0</td>
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</tbody>
</table>

### Variants significantly associated with circulating sphingolipid proportions.

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<th>SNP</th>
<th>Chromosome</th>
<th>Position</th>
<th>Gene</th>
<th>Distance (kb)</th>
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<tr>
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<td>4.45×10^{-18}</td>
<td>0.7–2.0</td>
<td>0.20</td>
<td>%Glu-CER 16:0, %Glu-CER</td>
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<tr>
<td>rs17479*</td>
<td>1q12.2</td>
<td>61678754</td>
<td>FADS-1-2-3</td>
<td>17</td>
<td>1.99×10^{-14}</td>
<td>4.58×10^{-13}</td>
<td>0.8–1.5</td>
<td>0.49</td>
<td>%SPM 161, %SPM 181, %SPM 201, %SPM 221</td>
</tr>
<tr>
<td>rs17101394*</td>
<td>14q23.2</td>
<td>64232386</td>
<td>SGPP1</td>
<td>37</td>
<td>3.10×10^{-07}</td>
<td>7.13×10^{-06}</td>
<td>0.7–6.3</td>
<td>0.15</td>
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<td>3.68×10^{-07}</td>
<td>0.8</td>
<td>0.45</td>
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<tr>
<td>rs12051548*</td>
<td>17p13.2</td>
<td>4683035</td>
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<td>%SPM 23.0</td>
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<td>0.11</td>
<td>%SPM 240, SPM 220</td>
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<tr>
<td>rs680379*</td>
<td>20p12.1</td>
<td>12969400</td>
<td>SPTLC3</td>
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<td>1.61×10^{-16}</td>
<td>3.70×10^{-15}</td>
<td>0.8–1.7</td>
<td>0.29</td>
<td>%CER 160, %Glu-CER 160, SPM/CER, %SPM 20:0, %SPM 16:1, %SPM 17:0</td>
</tr>
</tbody>
</table>

* $P$-value_{Corrected}: Genome-wide association p-value after adjustment for number of independent vectors; MAF: Minor Allele Frequency.

* Loci significantly associated to lipid levels after Bonferroni correction.

** Loci associated to sphingolipids for the first time.

**Loci significantly associated to sphingolipid levels after Bonferroni correction.

doi:10.1371/journal.pgen.1002490.t003

**P-loci** significantly associated to within class sphingolipid ratios.
HNF4A which is a candidate transcription factor for further research on lipidomics [33].

In conclusion, we identified 15 previously undescribed loci that were suggestively associated (2.2 × 10^{-9} < P-value < 5 × 10^{-8}) with phospho- and sphingolipid levels. These included interesting candidate genes such as LEAR2. These loci will require follow-up to definitively establish their relationship with these phenotypes. We also identified nine novel loci below the corrected genome-wide significance threshold (P-value < 2.2 × 10^{-9}). These loci considerably expand our knowledge of genes/regions involved in the determination of phospho- and sphingolipid concentrations and provide interesting avenues for future research into this important topic.

Materials and Methods

All studies were approved by the local ethical committees. Detailed descriptions of the study populations that contributed to the meta-analysis, as well as detailed information on ethical statements, genotyping, lipid measurements and pathway analysis, are presented in Text S1. Briefly, lipid species were quantified by electrospray ionization tandem mass spectrometry (ESIMS/MS) using methods validated and described previously [34,35]. For each lipid molecule, we adopted the naming system where lipid side chain composition is abbreviated as Cx:y, where x denotes the number of carbons in the side chain and y the number of double bonds. For example, PC 34:4 denotes an acyl-acyl phosphatidylcholine with 34 carbons in the two fatty acid side chains and 4 double bonds in one of them. Lipid traits were analysed individually as well as aggregated into groups of species with similar characteristics (e.g. unsaturated ceramides). These were then analyzed as both absolute concentrations (μM) and as molar percentages within lipid sub-classes (mol%) (calculated as the proportion of each lipid molecule among its own class (e.g. PC, PE, PLPE, LPC). The additive value of the analyses of molar proportions is that it may bring to light genes involved in the transition of one species to another, such as through fatty acid chain elongation or (de)saturation. We also performed single SNP association analyses for each novel locus and the ratio of the index lipid (for example, PC 34:4) to the other lipids in the same class (in this example, PC 34:1/PC 36:1, PC 34:1/PC 38:1) so that we could determine whether the SNP might be involved in elongation or (de)saturation.

DNA samples were genotyped according to the manufacturer’s instructions on Illumina Infinium HumanHap300v2, HumanHap500v1 or HumanCNV370v1 SNP bead microarrays. Genotype data for these five populations were imputed using MACH 1.0 (v1.0.16) [36,37] using the HapMap CEU population (release 22, build 36).

As all of the studies included related individuals, testing for association between lipid and allele dosage were performed using a mixed model approach as implemented with the ‘nmscore’ option in the GenABEL software [38]. Results from the five populations were combined using inverse variance weighted fixed-effects model meta-analyses using the METAL software [39]. To correct for multiple testing, we adopted a Bonferroni correction for the number of phenotypes studied. Since most of the lipid values are correlated with each other, we used the number of principal components (n = 23) that accounted for 79% of the phenotypic variance for this correction and applied it to the classical genome-wide significance threshold (5 × 10^{-8}).

Supporting Information

Figure S1 Q-Q Plots from the GWAS of phospho- and sphingolipid traits with genome-wide significant findings. The x-axis shows the expected chi-square value, the y-axis shows the observed. Lambda (λ); Genomic control inflation factor.

Figure S2 Regional plots of phospholipid related loci. Regional association plots covering a 1 Mb window around the top SNPs were created using Locus Zoom (https://statgen.sph.umich.edu/locuszoom). Diamonds denote the index SNPs (with the smallest P-value).

Figure S3 Regional plots of sphingolipid related loci. Regional association plots covering a 1 Mb window around the top SNPs were created using Locus Zoom (https://statgen.sph.umich.edu/locuszoom). Diamonds denote the index SNPs (with the smallest P-value).

Figure S4 Lysophospholipid Pathways by Ingenuity (glycero-phospholipid metabolism). Genes discovered in the GWAS are shown in orange. 1. Phosphatidylcholine-sterol O-acyltransferase has member mouse Lcat. 2. ApoA-I [APOA1] increases activation of LCAT. Binding of apoA-I [APOA1] and LCAT occurs. 3. Association of human APOA1 protein and human APOLI protein occurs. 4. The affinity of binding of human APO1 protein and cardiolipin in a system of purified components is greater than the affinity of binding of human APO1 protein and phosphatidylglycerol.

Figure S5 Lysophospholipid Pathways by Enzymes (glycero-phospholipid metabolism).

Figure S6 Panel showing recombination rates. The color scale on the right refers to the linkage disequilibrium (R^2) between each SNP and the index SNP. Blue peaks show recombination rates.

Figure S7 Panel showing recombination rates. The color scale on the right refers to the linkage disequilibrium (R^2) between each SNP and the index SNP. Blue peaks show recombination rates.
LAMB2 protein and human ZNF512B protein occurs. 26. Binding of human PLCG2 protein and human ZNF512B protein occurs. 27. Interaction of cytochrome b5 [CYB5] and phosphatidylcholine bilayers occurs. 28. Binding of rat Cytb5 [Cybt5a] protein and rat Delta 6 desaturase [Fads2] protein occurs in Cos-7 cells. 29. In CHO-k1A7 cells, binding of a protein-protein complex consisting of mutant human APOA1 [R160V;H162A with its amphipathic helix 6 mutated] and of cholesterol and of phosphatidylcholine and mouse Sr-bi [Scarb1] protein is the same as binding of a protein-protein complex consisting of mutant human APOA1 [R160V;H162A with its amphipathic helix 6 mutated] and of cholesterol and of phosphatidylcholine and mutant mouse Sr-bi [Scarb1] protein [M158R]. 30. Interaction of C-reactive protein (CRP) and artificial phosphatidylcholine bilayers occurs. 31. Binding of 10 kd mutant human APOE protein (N-terminal truncation 1–222 with its carboxy terminal domain retained) and phosphatidylcholine and triolein occurs in a system of purified components. 32. Binding of phosphatidylcholine and PRNP protein occurs in detergent-resistant membrane fraction from FRT cells. 33. Brefeldin A decreases association of phosphatidylcholine and a protein fragment containing a N-terminal domain from human Apob protein. 34. Binding of phosphatidylcholine and Phospholipase c [Plc] protein(s) occurs. Phospholipase C catalyzes the following reaction: 1 phosphatidylcholine+ 1 water→1,2-diacylglycerol+1 phosphorylcholine. 35. In NCI-H295r cells, binding of human APOE3 protein and human SR-BI [SCARB1] protein increases uptake of cholesteryl ester. 36. Interaction of apolipoprotein E [APOE] and rat CRP occurs. 37. Association of apoE and phospholipid occurs. 38. Association of apoE and phospholipid occurs. 39. Binding of phospholipid and Phospholipase c [Plc] protein(s) occurs. Human PLC has member human PLCB1. 40. Binding of human APOB protein and human LIPC protein occurs. 41. Triacylglycerol lipase has member mouse Lipc. 42. Triacylglycerol lipase has member human DLAGA. 43. Triacylglycerol lipase has member rat Phlprr2. 44. Triacylglycerol lipase catalyzes the following reaction: 1 fatty acid+1 sn-2-monooacylglycerol→1,2-diacylglycerol+1 water. 45. Carnitine O-palmitoyltransferase catalyzes the following reaction: 3 coenzyme A+1 triglyceric acid→3 acyl-coenzyme A+1 glycerol. 46. Phospholipid:diacylglycerol acyltransferase catalyzes the following reaction: 1 lysophospholipids+1 triglyceric acid→1,2-diacylglycerol+1 phospholipid. 47. Sphingosine N-acyltransferase catalyzes the following reaction: 1 acyl-coenzyme A+1 sphingosylphospholipid→1 acyl-coenzyme A+1 sphingomyelin. 48. Sphingosine N-acyltransferase has member rat Lars4. 49. Non-amylo-glycuron group acyltransferase catalyzes the following reaction: 1 2-acyl-sn-glycerol 3-phosphate+1 coenzyme A→1 acyl-coenzyme A+1 glycerocephosphoric acid. 50. Non-amylo-glycuron group acyltransferase has member rat Elov12. 51. Association of apoG-III [APOC3] and triglyceric acid occurs. 52. Lipoprotein lipase has member human APOC2. 53. 1-acylcglycerol-3-phosphate O-acyltransferase has member rat Agpat1. 54. Lipoprotein lipase has member human APOC2. 55. 1-acylcglycerol-3-phosphate O-acyltransferase has member rat Agpat1. 56. 1-acylcglycerol-3-phosphate O-acyltransferase catalyzes the following reaction: 1 coenzyme A+1 phosphatidic acid→1 1-acylcglycerol 3-phosphate+1 2,3-dihydroxyacyl-coenzyme A. 57. Binding of purified rat Pcth1 protein and a protein fragment containing a phox homology domain from human PTD2 protein occurs in a cell free system. 58. EGF protein increases dissociation of human PLD2 protein and mouse Munc-18a [Stxbp1] protein. Binding of a protein fragment containing a PX domain from human PLD2 protein and mouse Munc-18a [Stxbp1] protein occurs in a system of purified components. 60. Binding of PLD protein(s) and rat Munc-18 [Stxbp1] protein occurs in rat brain. 61. GRB2 protein increases activation of rat Ptd protein(s) that is increased by Pdgf complex(es). Association of growth factor receptor-bound protein 2 [GRB2] and PLD occurs. 62. Binding of human GRB2 protein and human KCHN7 protein occurs. 63. Mouse Plkd2 protein increases activation of human Pld protein(s) in HPAEC cells that is increased by hyperoxia of HPAEC cells. Phospholipase D has member mouse Plkd2. 64. In cytoplasm, PLD protein(s) catalyzes the following reaction: phosphatidylcholine→phosphatidic acid. 65. Phospholipase D has member rat Gpd1. 66. Binding of transgenic human APOA-1 [APOA1] protein and cholesteryl ester and mouse HDL in plasma from blood of mutant mouse with a homozygous knockout of mouse Apoae1 occurs. 67. Binding of ARF1 protein and phosphatidic acid occurs in a cell fraction from Cos cells. 68. Binding of ARF1 protein and a protein fragment containing a CA2 domain from rat Svt9 protein occurs in lysate from RBL-2H3 cells. 69. Binding of phosphatidic acid and PKC ALPHTA [PKRCA] protein occurs in a system of purified components. 70. In cytoplasm, DGK ζ [DGKZ] protein catalyzes the following reaction: DAG→αPA. 71. Association of rat Dgkz protein and rat Dlg1 protein and rat Dlg2 protein and rat Dlg3 protein and rat Dlg4 protein occurs. 72. Binding of human PLD2 protein and human PRKA protein occurs. 73. Mousse Pla2g1br [Pla2r1] protein decreases binding of mousse Pla2g10 protein and PLA2R [PLA2R1] protein. (PDF)
Figure S6  Q-Q plots for the association between the top loci and disease end points. $P_{KS}$: $P$-value from a one sample Kolmogorov-Smirnov test comparing the observed $P$-value distribution to that expected under the null. (PDF)

Table S1  Mean phospho- and sphingolipid concentrations of the study participants. SD: standard deviation; $P$-value: $P$-value for the test comparing means by gender. (PDF)

Table S2  Association findings on selected lipid-lipid ratios. Effect: regression coefficient; seEffect: standard error of the regression coefficient. (PDF)

Table S3  GWAS of standard plasma lipid measures in the EUROSPAN Consortium. Allele1: Effect allele; Effect: regression coefficient; StdErr: standard error of the regression coefficient. (PDF)

Table S4  Conditional analysis of the genome-wide significant loci. Effect: regression coefficient; StdErr: standard error of the regression coefficient. (PDF)

Table S5  ConsensusPathDB pathway enrichment for sphingoethanolamine cytidylyltransferase (Pcyt2). Biochem Cell Biol 85: 381–385.

Table S6  Lipidomic analysis, metabolism and roles in membrane structure, dynamics, and high-density lipoprotein to human platelets. Biochem J 315(Pt 3): 781–789.

Table S7  Genome wide significant SNPs and their associations with type 2 diabetes risk (DIAGRAM Consortium, Voight et al, Nat Genet 42:579–589, 2010). 95% CI: 95% Confidence Interval. (PDF)

Table S8  Genome wide significant SNPs and their associations with coronary artery disease risk (CARDiogram Consortium, Schunkert et al, Nat Genet 43: 333–338, 2011). 95% CI: 95% Confidence Interval. (PDF)

Table S9  Genome wide significant SNPs and their associations with coronary artery disease risk (CARDiogram Consortium, Schunkert et al, Nat Genet 43: 333–338, 2011). 95% CI: 95% Confidence Interval. (PDF)

Text S1  Extensive materials and methods. (DOC)

Text S2  Full list of phospholipids that are significantly associated to FADS1-2-3 and LIPC region SNPs. (DOC)

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Author Contributions

Conceived and designed the experiments: GS HC UG AFW CH AAH TM BAO NDH IR JFW PPI CMvD AI. Performed the experiments: GL CG IR PU JJ CH SC OP IK GZ ZB IP SHW TM UG JFW HC AJ. Analyzed the data: AD AI YSA AKWJ IJ V TA MS LF LCK FSD LB CSF VV. Contributed reagents/materials/analysis tools: RCJ CP OP IK GZ ZB IP SHW TM UG JFW HC AI. Performed the experiments: GL CG IR PU JJ CH SC OP IK GZ ZB IP SHW TM UG JFW HC AJ. Analyzed the data: AD AI YSA AKWJ IJ V TA MS LF LCK FSD LB CSF VV. Contributed reagents/materials/analysis tools: RCJ CP OP AH JF WI AP FR JK NH NDH AU JCMW CM PN MCB CJ O NF DIAGRAM CARDiogram CHARGE. Wrote the paper: AD AI CMvD AAH CH HC BAO FSD.

References


