**Lipidomic analysis of plasma from healthy men and women shows phospholipid class and molecular species differences between sexes**

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**Abstract**

The phospholipid composition of lipoproteins is determined by the specificity of hepatic phospholipid biosynthesis. Plasma phospholipid 20:4n-6 and 22:6n-3 concentrations are higher in women than in men. We used this sex difference in a lipidomics analysis of the impact of endocrine factors on the phospholipid class and molecular species composition of fasting plasma from young men and women. Diester species predominated in all lipid classes measured. 20/54 PC species were alkyl ester, 15/48 PE species were alkyl ester, and 12/48 PE species were alkenyl ester. There were no significant differences between sexes in the proportions of alkyl PC species. The proportion of alkyl ester PE species was greater in women than men, while the proportion of alkenyl ester PE species was greater in men than women. None of the PI or PS molecular species contained ether linked fatty acids. The proportion of PC16:0\_22:6, and the proportions of PE O-16:0\_20:4 and PE O-18:2\_20:4 were greater in women than men. There were no sex differences in PI and PS molecular species compositions. These findings show that plasma phospholipids can be modified by sex. Such differences in lipoprotein phospholipid composition could contribute to sexual dimorphism in patterns of health and disease.

**Key Words:** Phospholipid molecular species; docosahexaenoic acid; arachidonic acid; sex differences; plasma

**Abbreviations**

DAG, diacylglycerol; HR/AM, high resolution/ accurate mass; LC/MS, liquidchromatography/ mass spectrometry; LPAT, lyso phospholipid acyltransferase; MTBE, methyl-tert-butyl-ether; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, PLA, phospholipase A; phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; UPLC, ultra-performance liquid chromatography

**Introduction**

The molecular species composition of phospholipids differs between tissues and phospholipid classes (Inoue *et al.* 2017) and is regulated by biochemical and genetic processes (Zhang & Rock 2008). The combination of fatty acids bound at the *sn*-1 and *sn*-2 positions, at least in part, determines the biophysical properties of cell membranes which influence the activities of integral membrane proteins (Dymond *et al.* 2013; Ces 2006) and provide substrates for signaling processes (Heung & Postle 1995a; Heung & Postle 1995b). Thus, factors that confer membrane phospholipid homeostasis are important for maintaining cell function (Calder 2008). Fasting plasma phospholipid molecular species composition primarily reflects that of the liver and hepatic phospholipid synthesis (Burdge *et al.* 1994; Pynn *et al.* 2011; Chalil *et al.* 2018). Therefore, plasma phospholipid composition can be a proxy for hepatic phospholipid composition and can provide insights into phospholipid metabolism in the liver.

Synthesis of phosphatidylcholine (PC), which is the predominant component of the lipoprotein phospholipid monolayer, is required for the formation and secretion of very low density lipoproteins (VLDL) (Yao & Vance 1988). Inhibition of PC synthesis can alter the phospholipid composition and structure of VLDL particles (Fast & Vance 1995). Hepatic PC and phosphatidylethanolamine (PE) are synthesized via the Kennedy pathway from CDP-choline and CDP-ethanolamine, respectively (Weiss *et al.* 1958; Gibellini & Smith 2010; Kanoh & Ohno 1975). The composition of newly synthesized phospholipids reflects that of the metabolically and compositionally distinct diacylglycerol (DAG) substrate pools (Rustow & Kunze 1985; Rustow *et al.* 1985; Burdge *et al.* 1994). Approximately 30% of hepatic PC synthesis occurs by the PE N-methylation pathway (Vance *et al.* 1997; Sundler & Akesson 1975), which yields mainly polyunsaturated fatty acid (PUFA) -containing PC species (Burdge *et al.* 1994; Chalil *et al.* 2018). The minor plasma phospholipids, phosphatidylinositol (PI) and phosphatidylserine (PS) are synthesized by PI synthase via CDP-DAG (Blunsom & Cockcroft 2020) and by base exchange with PC (PS synthase I) or with PE (PS synthase II) (Leventis & Grinstein 2010), respectively. The roles of PI and PS in lipoprotein function are not known. Newly synthesized phospholipids can undergo acyl remodeling processes via the Lands cycle (Lands 1960) to generate the molecular species composition of the mature phospholipid pool. This involves phospholipase A2 (PLA2) or PLA1, and lyso phospholipid acyltransferase (LPAT) activities (Schmid *et al.* 1991; Wang & Tontonoz 2019). Specificity of acyl remodeling, and hence the composition of the final phospholipid pool, is conferred by LPAT substrate selectivity (Wang & Tontonoz 2019). For example, in rat and guinea pig liver, newly synthesized PC undergoes acyl exchange at the *sn*-1 position, which results in partial conversion of *sn*-1 16:0 to *sn*-2 18:0 species (Burdge *et al.* 1994; Burdge *et al.* 1993).

It is well-established that phospholipid biosynthesis and membrane composition are under genetic and biochemical control (Sugimoto *et al.* 2008). However, the molecular species composition of hepatic and plasma phospholipids appears to also be under endocrine control. In pregnant women, increasing gestational age is accompanied by changes in plasma PC 18:1n-9, 18:2n-6, 20:4n-6 and 22:6n-3 concentrations (Meyer *et al.* 2016; Al *et al.* 2000), specifically in *sn*-1 16:0 or *sn*-1 18:1n-9 PC molecular species (Postle *et al.* 1995). Similarly, pregnancy in rats involves a differential increase in hepatic and plasma PC16:0/22:6 and 16:0/20:4 concentrations compared to 18:0/22:6 and 18:0/20:4 concentrations (Burdge *et al.* 1994; Chalil *et al.* 2018; Childs *et al.* 2012), which reflects the increased *sn*-1 16:0 content of the DAG substrate pools destined for PC and PE synthesis, and reduced flux through the acyl remodeling pathway. Together adaptations result in enrichment *sn*-1 16:0 PC and PE molecular species without a change in the specificity of the Lands cycle (Burdge *et al.* 1994). Administration of sex hormones to gonadectomized rats induced reciprocal changes in the PUFA content of liver phospholipids (Eden *et al.* 1987). One implication of these findings is that regulation of hepatic and plasma phospholipid compositions may be important for sex-related tissue and lipoprotein functions.

The proportions of 20:4n-6 and 22:6n-3 are typically 20% higher in total fasting plasma lipids and in phospholipids from women compared to men (Lohner *et al.* 2013). This sex difference is independent of dietary fatty acid intakes (Bakewell *et al.* 2006), and may be explained, at least in part, by greater capacity for PUFA biosynthesis in young women than in men (Burdge *et al.* 2002; Burdge & Wootton 2002). Here, we used the differences in the proportions and/or concentrations of PUFA between men and women (Lohner *et al.* 2013) as a model to investigate the specificity of sex-related differences in plasma phospholipid composition. We analyzed the PC, PE, PI and PS molecular species compositions of plasma collected from fasting healthy men and women.

**Materials and Methods**

***Ethics Statement***

The study was reviewed by the South Central – Hampshire B Research Ethics Committee (REC reference 15/SC/0627) who approved the study and participants gave written informed consent. The study is registered at ClinicalTrials.gov (Identifier: NCT03477045).

***Participants and Sample Collection***

Blood samples were collected at baseline from a postprandial lipid metabolism study that has been reported in detail previously (West *et al.* 2019). Briefly, participants were ten healthy men aged 25 ± 1 year and ten healthy women aged 25 ± 1 year, both with body-mass-index of 24 ± 1 kg/m2. Participants had blood pressure, and fasting total triacylglycerol, cholesterol, and glucose concentrations within normal ranges (West *et al.* 2019). The habitual diets of the participants were not matched or modified for the purpose of the study. Volunteers were excluded if they consumed oily fish more than once per week, took fish oil or other dietary supplements and/or smoked tobacco. Venous blood was collected at approximately 08:00 after fasting overnight for approximately 12 hours into tubes containing lithium heparin anticoagulant. Cells were removed from blood by centrifugation at 4oC (West *et al.* 2016) and the plasma then stored at -80oC.

***Analysis of Plasma Phospholipid Molecular Species Compositions***

The methods for lipid extraction and analysis by liquid chromatography-mass spectrometry have been described previously (West *et al.* 2020). Briefly, total lipids were extracted from plasma (200 μl) with methyl-tert-butyl-ether (MTBE) (Matyash *et al.* 2008). The organic phase was dried under a stream of nitrogen, dissolved in chloroform/methanol (9:1, v/v) and stored at -20oC in a nitrogen atmosphere.

Phospholipid molecular species compositions were analyzed by high resolution / accurate mass (HR/AM) lipidomics using a Vanquish - Q Exactive Plus UPLC-MS/MS system (Thermo Fisher Scientific) as described (West *et al.* 2020). Briefly, plasma total lipids were resuspended in 500 µl chloroform : methanol (1:1, v/v) and along with the internal standard PC24:1/24:1 (0.857µM). The autosampler tray was maintained at 10oC and 20 µl of each sample was injected into the UPLC/MS system. Chromatographic separation was achieved using an Accucore C18 (2.1µm x 150 mm, 2.6 mm) column (Thermo Fisher Scientific) at 35oC with a flow rate of 400 µl min-1. An elution gradient was applied to column of mobile phase A (10 mM ammonium formate in 50% acetonitrile + 0.1% (v/v) formic acid) and B (2 mM ammonium formate in acetonitrile : propan-2-ol : water (10:88:2 v/v/v) + 0.02% (v/v) formic acid) over 28 minutes; starting at 35% (v/v) B, then 60% B at 4 minutes, 85% B at 12 minutes and 100% (v/v) B at 21 mins which was held for 3 minutes before equilibrating for 4 minutes at 35% B prior to the next run. The elution system was based on Bird *et al*. (2011), but was modified such that ammonium formate concentration was varied in order to optimize the ionization conditions for the different lipid classes (Constantinou *et al*. 2020).

The Thermo Q Exactive HESI II sweep plate set in position C. Conditions were adjusted for separate positive and negative runs; replicate runs of samples in a single polarity increased the number of identifications. LC/MS at 140K full scan data HCD MS2 experiments (35K resolution) were performed in positive and negative ion modes. Full Scan was operated at 140,000 resolution across m/z 150-1200, with Top 15 selection MS/MS at 35,000 resolution. The stepped collision energy used was 25, 30, 40 and the dynamic exclusion set to 8s. The sheath gas set to 60 (arbitrary units), auxillary gas 20, sweep gas 1, spray voltage 3 KV in positive ion mode and 3.2KV negative ion mode, S-lens at 50 for +ve ion and 60 in -ve ion, capillary temperature 320 oC and aux gas heater set to 370oC. Automatic gain targets of 16 (full scan MS) and 15 (MS/MS) were used. This protocol was optimised to fragment as many of the detected peaks as possible to allow them to be identified by comparison to MS/MS libraries. The samples were run on the instrument in a random order and analysed blind using a number coding system. Blanks were run every four samples to check for carry over and background effects. The first sample of the batch was re-run at the end of the batch to check for any difference in performance.

LipidSearch 4.2 experimental workflow (Thermo Fisher Scientific) was used for lipid characterization and putative lipid species were identified separately from positive or negative ion adducts. The data for each replicate were aligned within a chromatographic retention time window by combining the positive and negative ion annotations and merging these into a single annotation. The MS/MS fragmentation spectrum was used to identify the class and fatty chain composition of the lipid species. Experimental MS/MS spectra were searched against all lipid classes in the LipidSearch database using a precursor mass tolerance of 5 ppm and a product mass tolerance of 8 ppm, and the quality of the annotation was graded A-D by the software. Only those graded A or B were used in this analysis. In grade A, all fatty acyl chains and the class were identified; in grade B, some fatty acyl chains and the class were identified. Final assignment was based on UPLC separation. Any isomers that could not be adequately separated based on retention time were then subject to additional fragmentation analysis of the sodium adducts in positive ionization mode and of the deprotonated species in negative ionization mode (Huynh *et al*. 2019).

Peak areas were normalized to the internal standard; peak areas corresponding to individual phospholipid molecular species were expressed as a proportion of the total species in each lipid class. Raw lipid molecular species composition data is deposited at EMBL-EBI MetaboLights database (http://www.ebi.ac.uk/metabolights; Identifier: MTBLS1348) (Haug *et al.* 2013).

Putative assignment of some fatty acids to *sn*-1 and *sn*-2 positions was based on previous analyses of human plasma phospholipid molecular species (Postle *et al.* 1995; Pynn *et al.* 2011; Heung & Postle 1995b; West *et al.* 2020). However, since the positions of fatty acids were not determined for all the phospholipid molecular species reported here, combinations of fatty acids are separated by an underscore (Murphy 2018) and do not indicate *sn* positions. Lysophospholipids and phosphatidic acid were excluded from the analyses because they arise as a result of lipase actions in plasma or as a consequence of sample degradation, rather than being the product of hepatic synthesis. Phosphatidic acid was not detected in these samples.

The samples were analysed blind using a number code system and in a random order. Blanks were run every 4 samples to check for carry over and background effects. The first sample of the batch was re-run at the end of the batch to check for any difference in the technical performance of the instrument and sample stability in the autosampler. The internal standard, PC 24:1/24:1, was used to correct for instrument analytical variation.

***Statistical Analysis***

Molecular species that individually contributed less than 0.01% of total molecular species in each class were excluded from analysis for differences between sexes. Analysis of residuals by the Shapiro-Wilks test showed that all data approximated a normal distribution and hence are expressed as mean ± standard error of the mean (SEM). Comparisons between men and women were by Student’s unpaired t test with adjustment for multiple testing using the Holm-Šídák method. Statistical significance after adjustment was assumed at P < 0.05.

**Results**

***Effect of Sex on the Proportions of Phosphatidylcholine Molecular Species in Plasma***

Thirty-four diester PC molecular species and twenty alkyl-ester PC molecular species were identified consistently in plasma from men and women (Tables 1 and 2). The molecular species profile of plasma PC for all participants is shown in Fig. 1. *sn*-1,2 ester linked PC species accounted for greater than 95% of all PC molecular species in men and women (Table 1). The most abundant species, namely, PC16:0\_18:2, PC16:0\_18:1 and PC18:0\_18:2 together accounted for 49.3 ± 1.4% and 47.7 ± 1.2% of total PC molecular species in men and women, respectively (Table 1). Eighteen diester PC species in women and 17 diester PC species in men each contributed less than 0.5% each of the total PC species, and combined Chart

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**Fig. 1.** Plasma phosphatidylcholine molecular species composition. Values are proportions in descending order of individual molecular species for n = 20 participants (men plus women, n = 10 per sex). Data points represent individual participants. Bar = mean (min – max) O, *sn*-1 alkyl, *sn*-2 ester species; P, *sn*-1 alkenyl, *sn*-2 ester species

men and women showed that the proportion of PC16:0\_22:6 was significantly greater (64.4%) in women than in men (Table 1). Linear regression analysis showed that differences in sex accounted for 41.9% (adjusted r2 = 0.419; P = 0.001) of the variation in the proportion of PC16:0\_22:6. The proportions of other PC molecular species did not differ significantly between men and women.

Alkyl-ester PC species each accounted for less than 4% of total PC molecular species (Table 2). PC O-18:1\_20:4, PC O-16:0\_20:4 and PC O-16:1\_20:4 were the most abundant alkyl-ester PC species in men and women, and accounted for less than 1.5% of total PC molecular species in both sexes. There were no significant differences in the proportions of alkyl-ester PC species between men and women (Table 2).

***Effect of Sex on the Proportions of Phosphatidylethanolamine Molecular Species in Plasma***

Plasma from men and women contained 15 diester PE molecular species (Table 3), twenty-one alkyl-ester PE molecular species and twelve alkenyl-ester PE molecular species (Table 4). The molecular species profile of plasma PE for all participants is shown in Fig. 2. The proportion of total diester PE molecular species did not differ significantly between sexes, while the proportion of total alkyl-ester species was significantly greater in women than in men. The proportion of total alkenyl-ester molecular species was significantly greater in men than in women (Table 4).

PE18:0\_20:4, PE18:0\_18:1 and PE16:0\_22:6 together accounted for more than 9.5% A picture containing object, antenna

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**Fig. 2.** Plasma phosphatidylethanolamine molecular species composition of plasma. Values are proportions in descending order of individual molecular species for n = 20 participants (men plus women, n = 10 per sex). Data points represent individual participants. Bar = mean (min – max). O, *sn*-1 alkyl, *sn*-2 ester species; P, *sn*-1 alkenyl, *sn*-2 ester species.

of total PE molecular species in men and women, which did not differ between sexes (Table 3). There were no significant differences between men and women in the proportions of individual diester PE molecular species (Table 3). The three most abundant alkyl ester PE molecular species were PE O-18:1\_20:4, PE O-18:2 \_20:4 and PE O-16: \_20:4 which combined accounted for 13.2 ± 1.5% of total PE molecular species in men and 18.6 ± 1.1% of total PE molecular species in women, which did not differ significantly between sexes (Table 4). The three most abundant alkenyl-ester PE molecular species were PE P-18:1\_20:4, PE P-16:0 \_20:4 and PE P-18:0\_20:4 in both sexes (Table 3). Together these species accounted for 28.1 ± 2.5% of total PE molecular species in men and 24.5 ± 0.9% of total molecular species in women, which did not differ significantly between sexes (Table 4). The proportion of PE O-16:1\_20:4 and PE O-18:2\_20:4 were significantly greater in women (69.9% and 36.7%, respectively) than in men. Sex accounted for 41.8% of the variation in the proportion of PE O-16:1 \_20:4 (adjusted r2 = 0.418; P = 0.002) and 25.4% of the variation in the proportion of PE O-18:2\_20:4 (adjusted r2 = 0.0.254; P = 0.014). There were no significant differences between sexes in the proportions of other alkenyl ester PE species (Table 4).

***Effect of Sex on the Proportions of Phosphatidylserine and Phosphatidylinositol Molecular Species in Plasma***

Three phosphatidylserine (PS) molecular species were identified in plasma from both sexes; PS18:0\_18:1, PS18:0\_20:4 and PS20:2\_20:4 (Table 5). There were no significant differences in the proportions of these molecular species between sexes.

Chart, box and whisker chart

Description automatically generated Fourteen phosphatidylinositol (PI) molecular species were identified in men and women (Table 5). The molecular species profile of plasma PI for all participants is shown in

Fig. 3. The major PI molecular species in men and women was PI18:0\_20:4, which alone accounted for over 42% of total PI molecular species in both sexes. Bar = mean (min – max). There were no significant differences in the proportions of PI molecular species between sexes (Table 5).

**Discussion**

As described previously, the present findings show that the plasma phospholipid pool is composed of a complex mixture of phospholipid classes and molecular species (Quehenberger *et al.* 2010; Burla *et al.* 2018), although the total number of molecular species detected in each phospholipid class differed to some extent from those reported here. It is possible that this may reflect the participants in the earlier study, who were of undisclosed sex, geographical differences in habitual diet, and being older (40 - 50 years old) than those in the current study.

Each phospholipid class was composed of a relatively small number of molecular species that accounted for the majority of each phospholipid class and a larger number of molecular species that individually accounted for less than 1% of total species. As described previously (Inoue *et al.* 2017), each phospholipid class had a distinct molecular species profile that differed in the diversity of the type and combinations of fatty acids, the presence of *sn*-1 ether linked fatty acids and the number of molecular species (Kawanishi *et al.* 2018). For example, PC and PE contained *sn*-1 alkyl species, while PE, but not PC, contained *sn*-1 alkenyl species. Ether linked fatty acids were absent from PS and PI. The PC molecular species composition reported here is in agreement with the partial analysis reported previously, for example PC16:0/18:2, PC16:0/18:1 and PC18:0/18:2 were the most abundant PC molecular species in pregnant women (Postle *et al.* 1995). However, there are marked differences in PC molecular species composition between the current study and Quehenberger *et al.* 2010. For example, the three most abundant PC species in the present study were PC16:0\_18:2, PC16:1\_18:1 and PC18:0\_18:2, while the three most abundant PC species in Quehenberger *et al.* 2010 were PC(38:4), PC(36:2) and PC(34:2) which we suggest for comparison were PC18:0\_20:4, PC18:1\_18:1 or PC18:0\_18:2, and PC16:0\_18:2 or 16:1\_18:1, respectively. The PE and PI molecular species profiles resembled those reported previously (Gardner *et al.* 2019; Kawanishi *et al.* 2018; Quehenberger *et al.* 2010). However, these studies only reported the total number of carbons and level of unsaturation of each molecular species instead of individual fatty acid combinations thus preventing direct comparison with the present data (Gardner *et al.* 2019; Kawanishi *et al.* 2018). Quehenberger *et al.* (2010) reported 20 PS molecular species compared to three PS molecular species in the present study. However, PS (40:6), which we suggest may have been PS20:2\_20:4 or PS18:0\_22:6, was the predominant species in both studies. Overall, there is broad agreement between studies about the molecular species compositions of plasma phospholipids and their complexity. However, differences between study designs, including selection of participants and analytical methods, and reporting of molecular species in terms of numbers of total fatty acid carbons and double bonds which suggest alternative combinations of fatty acids for each species, may account, at least in part, for inconsistencies in findings between reports.

The proportions of 20:4n-6 and 22:6n-3 are typically 20% higher in total plasma lipids and phospholipids from women compared to men (Lohner *et al.* 2013). Such sex differences in 20:4n-6 and 22:6n-3 concentrations were associated with differences in the concentrations of individual PC and PE molecular species. In particular, higher 22:6n-3 concentration in women than men was due entirely to a greater proportion of PC16:0\_22:6. The proportion of this species was approximately 64% greater in women than in men. The proportions of other PC species that contained 22:6n-3 did not differ between sexes. The sum of all PC molecular species that contained 22:6n-3 showed that the proportion of this fatty acid was significantly greater in women (7.6 ± 0.6 mol%) than in men (6.2 ± 0.5 mol%; adjusted P = 0.005). The magnitude of the difference between sexes for total PC 22:6n-3 was lower (22.3%) than for PC16:0\_22:6 and is similar to that estimated based on analysis of studies that measured total plasma or total phospholipids by Lohner *et al*. (2013). Thus, it is plausible that PC16:0\_22:6 alone may account for the difference in the concentration or proportion of 22:6n-3 between sexes, but that the magnitude of this difference is reduced by the presence in blood of other 22:6n-6 -containing molecular species that do not exhibit sexual dimorphism.

One previous study investigated the phospholipid composition of young and older men and women (Ishikawa *et al.* 2014). The findings showed relatively few differences in plasma phospholipid composition between sexes in the young participant group compared to the present study. The primary differences between sexes were higher proportions of individual sphingomyelin molecular species in young women. The proportions of a greater number of phospholipid molecular species differed between sexes in the older participant group (Ishikawa *et al.* 2014). The only finding that agreed with the present study was that the proportion of PC18:1\_22:6 was greater in men than women. The reason for the differences in findings between these studies is not clear, although differences in methodologies and in the ethnicity of the participants may be important.

The concentration of plasma PC16:0/22:6 has been shown to increase significantly during pregnancy in women (Postle *et al.* 1995) and rodents (Burdge *et al.* 1993; Childs *et al.* 2012). In rats, conversion of PC16:0/22:6 to 18:0/22:6 by acyl remodelling decreased with increasing gestational age indicating that this process is regulated by sex hormones. One study has reported that the flux through the PE N-methylation pathway was increased in pregnant rats (Chalil *et al.* 2018), although others have not found this (Burdge *et al.* 1994). There was no significant difference between sexes in the proportion of PE16:0\_22:6 in the present study. This suggests that unlike rats (Burdge *et al.* 1994), sex hormones do not appear to alter the composition of the DAG pool destined for incorporation into PE in humans and that increased N-methylation of PE16:0\_22:6 is unlikely to account for the greater proportion of PC16:0\_22:6 in women than in men. Hepatic acyl remodelling of newly synthesised PC has also been shown to be important in humans (Pynn *et al.* 2011). Flux through the Lands pathway is reduced in pregnant compared to non-pregnant rats (Burdge *et al.* 1994). This suggests that one possible explanation for a higher proportion of PC16:0\_22:6 in women than in men, is lower hepatic activity of the Lands cycle in women. Moreover, LPAT activity, which confers specificity on acyl-remodeling (Wang & Tontonoz 2019), may be a locus of metabolic regulation by sex hormones. If so, greater capacity for polyunsaturated fatty acid (PUFA) biosynthesis in young women compared to men (Burdge *et al.* 2002; Burdge & Wootton 2002) appears to be coordinated with PC biosynthesis by female sex hormones.

A systematic review of 51 studies found that the concentration of 20:4n-6 was higher in plasma phospholipids from women than men (Lohner *et al.* 2013). The present findings show that the proportions of PE O-16:1\_20:4, PE O-18:2\_20:4, but not of other 20:4n-6 -containing PE species, were significantly greater in women than men. This may explain why previous studies that analyzed either plasma total lipids or total phospholipids found a sex difference in 20:4n-6 concentration (Lohner *et al.* 2013), but this was not detected when the fatty acid composition of purified plasma PC was analyzed (Bakewell *et al.* 2006). Ether-linked PE molecular species are involved in stabilizing the structures of cell membranes (Dean & Lodhi 2018), although it is not known if they also stabilize the phospholipid monolayer of lipoproteins. Ether linked PS species have been suggested to act as antioxidants (Meikle *et al.* 2011). If so, association of 20:4n-6 with ether-linked PE species may reduce free radical-mediated oxidation. The greater overall proportion of *sn*-1 alkyl-linked PE species in women compared to men may confer a higher level of antioxidant protection in the presence of a greater potential for oxidation due to increased amounts of 20:4n-6 and 22:6n-3. Synthesis of ether lipids in mammals involves a multi-step pathway that is incompletely understood (Watschinger & Werner 2013). To our knowledge, sexual dimorphism in the activity of this pathway has not been reported previously.

PI has been shown to be associated with a VLDL/LDL fraction which suggests that it is specific component of at least some lipoproteins although the molecular species composition was not reported (Sun *et al.* 2019). It is also possible that plasma PI may reflect contamination of plasma with cell debris. One study reported 14 PI molecular species in human platelets, of which the major species were C38:4 > C32:0 > C38:3 (Mujalli *et al.* 2018) which can be deduced tentatively to be PI18:0\_20:4, PI16:0\_16:0 or PI18:0\_14:0 and PI18:0\_20:3, respectively. Although PI18:0\_20:4 is the most abundant species in most cell membranes (Holub 1986), PI18:0\_18:2 and PI16:0\_20:4 were the second and third most abundant plasma PI molecular species in the present study. Furthermore, no disaturated PI species were detected in plasma. Thus, although contamination of plasma with membrane PI from platelets or cells cannot be completely excluded, we suggest that any such contamination would be a minor artefact in the analysis and that plasma PI is probably associated primarily with lipoproteins.

PS has been detected in microparticles in blood that have been implicated in hypercoagulation in diabetic kidney disease (Yu *et al.* 2018), cancer (Liu *et al.* 2019; Zhao *et al.* 2016; Lea *et al.* 2017), and inflammation (Zhao *et al.* 2016). Whether PS is also an integral component of lipoproteins in humans is not known. PS was not detected in the only previous study which attempted to measured PS in VLDL (Sun *et al.* 2019) and the present data show that PS accounted for less than 0.02% of plasma total phospholipids. The role of PS as a focus for coagulation may preclude its incorporation into the surface monolayer of lipoproteins.

Although the diets of the participants were not controlled, the rank order of molecular species within each phospholipid class was broadly consistent between individuals thus producing a characteristic molecular species profile for each phospholipid class. Plasma phospholipid molecular species composition is regulated by the specificity of the hepatic synthesis of each phospholipid class and differential incorporation of each class into nascent lipoproteins (Pynn *et al.* 2011). For example, PC is the major phospholipid class associated with liver-derived lipoproteins which form the phospholipid monolayer (Nelson & Freeman 1960), while nascent VLDL particles are enriched in PE which is progressively removed between hepatocyte Golgi apparatus and secretion into blood (Hamilton & Fielding 1989). The diversity of molecular species between phospholipid classes, while retaining characteristic profiles between individuals, is consistent with the specificity of hepatic phospholipid biosynthesis being a primary determinant of plasma phospholipid molecular species composition (Burdge *et al.* 1994; Pynn *et al.* 2011). Demonstration that the proportions of individual plasma molecular species can be influenced by sex, ageing (Kawanishi *et al.* 2018), pregnancy (Postle *et al.* 1995) and obesity (Donovan *et al.* 2013) suggests that endocrine factors are an important influence on hepatic phospholipid and lipoprotein metabolism.

The main limitation of this observational study is that it only allowed speculation about the underlying processes instead of providing direct mechanistic insights, for example based on tracer technologies. Such interpretations were based on measurements of total plasma, while it would have been preferable to have analyzed isolated VLDL particles as a more direct representation of hepatic phospholipid metabolism. It was not possible to test directly the effects of differences in phospholipid composition on VLDL structure and function. Additional limitations of this exploratory study include the relatively small sample size, and limited range of ages and ethnicities which constrain the extent to which the the findings can be extrapolated to the wider population. Nevertheless, the findings show for the first time that plasma phospholipid composition can be influenced by sex. One future challenge will be to test whether such sexual dimorphism in phospholipid molecular species composition contributes to sex differences in lipoprotein metabolism and health outcomes.

It has been proposed that specific phospholipid molecular species in cell membranes act as ‘pivot’ species which are resistant to hydrolysis and are required for maintaining membrane structure (Dymond *et al.* 2013). We have suggested that such pivot species may be present in lipoproteins and are required to maintain the structure of the phospholipid monolayer and/or facilitate the activities of lipases (West *et al.* 2020). Hepatic and/or plasma PC16:0/22:6 concentration in humans and rodents can be modified by sex hormones (Burdge *et al.* 1994; Childs *et al.* 2012; Postle *et al.* 1995; Chalil *et al.* 2018) and appears to be resistant to postprandial hydrolysis (West *et al.* 2020). Thus PC16:0/22:6 may be a pivot molecular species in lipoproteins. PE regulates the curvature of in the inner mitochondrial membranes and is involved in points of membrane contact, by disrupting the PC bilayer due to the inverted hexagonal packing of PE molecules (Daum & Vance 1997). PI has also been shown to promote membrane curvature (Mulet *et al.* 2008), although whether PE and PI are involved in regulating membrane shape in lipoproteins is not known.

One possible explanation for the large number of plasma phospholipid molecular species is that the overall effect on the biophysical properties of the phospholipid monolayer produces an optimal environment for lipoprotein function including the activities of integral proteins. If so, sex differences in plasma phospholipid classes and molecular species compositions may modify lipoprotein function in a manner that contributes to different patterns of health and disease between men and women.

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**Author contributions**

GCB, PCC, EAM, KAL and JAN designed and supervised the study; LH, RPH, LVM, RG and ALW conducted the experiments; GCB, ALW and LVM analyzed the data; GCB wrote the first draft of the manuscript with input from all authors.

**Conflicts of interest** The Authors declare that they have no conflicts of interest

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**Fig. 3.** Plasma phosphatidylinositol molecular species composition. Values are proportions in descending order of individual molecular species for n = 20 participants (men plus women, n = 10 per sex). Data points represent individual participants. Bar = mean. O, *sn*-1 alkyl, *sn*-2 ester species; P, *sn*-1 alkenyl, *sn*-2 ester species.

**Table 1.** Plasma phosphatidylcholine *sn*-1,2 ester linked molecular species composition in men and women

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Proportion of total PC molecular species (%) | | T-test | |
| Molecular species | Men | Women | P value | Adjusted P value |
| PC14:0\_18:2 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.184 | >0.999 |
| PC14:0\_20:4 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.700 | >0.999 |
| PC14:0\_22:6 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.443 | >0.999 |
| PC15:0\_20:4 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.804 | 0.131 |
| PC15:0\_22:6 | 0.01 ± 0.01 | 0.02 ± 0.01 | 0.196 | >0.999 |
| PC16:0\_18:1 | 12.89 ± 0.33 | 13.41 ± 0.53 | 0.413 | >0.999 |
| PC16:0\_18:2 | 23.80 ± 0.81 | 22.30 ± 0.60 | 0.155 | >0.999 |
| PC16:0\_20:3 | 7.05 ± 0.23 | 6.73 ± 0.19 | 0.305 | >0.999 |
| PC16:0\_20:4 | 7.29 ± 0.43 | 7.50 ± 0.41 | 0.728 | >0.999 |
| PC16:0\_20:5 | 0.96 ± 0.12 | 0.91 ± 0.06 | 0.706 | >0.999 |
| PC16:0\_22:6 | 3.71 ± 0.33 | 6.10 ± 0.53 | 0.002 | 0.013 |
| PC17:0\_18:1 | 0.21 ± 0.01 | 0.17 ± 0.01 | 0.004 | >0.999 |
| PC17:0\_18:2 | 0.60 ± 0.03 | 0.43 ± 0.01 | 0.001 | >0.999 |
| PC17:0\_20:3 | 0.10 ± 0.0 | 0.09 ± 0.01 | 0.109 | >0.230 |
| PC17:0\_20:4 | 0.22 ± 0.02 | 0.20 ± 0.02 | 0.558 | >0.999 |
| PC17:0\_22:6 | 0.08 ± 0.01 | 0.05 ± 0.01 | 0.071 | 0.963 |
| PC18:0\_16:0 | 0.11 ± 0.01 | 0.09 ±0.01 | 0.153 | >0.999 |
| PC18:0\_18:1 | 1.54 ± 0.12 | 1.55 ± 0.12 | 0.990 | >0.999 |
| PC18:0\_18:2 | 12.65 ± 0.56 | 11.75 ± 0.56 | 0.275 | >0.999 |
| PC18:0\_20:1 | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.236 | >0.999 |
| PC18:0\_20:2 | 0.14 ± 0.01 | 0.13 ±0.01 | 0.383 | 0.969 |
| PC18:0\_20:3 | 1.14 ± 0.13 | 1.29 ± 0.19 | 0.511 | 0.927 |
| PC18:0\_20:4 | 4.19 ± 0.28 | 4.51 ± 0.22 | 0.374 | 0.832 |
| PC18:0\_20:5 | 0.66 ± 0.06 | 0.70 ± 0.04 | 0.634 | 0.996 |
| PC18:0\_22:4 | 0.09 ± 0.01 | 0.10 ±0.01 | 0.435 | >0.999 |
| PC18:0\_22:5 | 0.38 ± 0.03 | 0.39 ± 0.03 | 0.100 | 0.826 |
| PC18:0\_22:6 | 1.07 ± 0.09 | 1.12 ± 0.09 | 0.728 | >0.999 |
| PC18:1\_18:2 | 7.19 ± 0.23 | 6.84 ± 0.19 | 0.254 | >0.999 |
| PC18:1\_20:4 | 1.58 ± 0.08 | 1.43 ± 0.06 | 0.179 | >0.999 |
| PC18:1\_22:6 | 0.25 ± 0.02 | 0.17 ± 0.01 | 0.007 | >0.999 |
| PC18:2\_18:2 | 7.47 ± 0.44 | 7.65 ± 0.42 | 0.759 | >0.999 |
| PC19:0\_18:2 | 0.06 ± 0.01 | 0.05 ± 0.01 | 0.012 | >0.999 |
| PC20:0\_16:0 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.818 | >0.999 |
| PC20:4\_14:1 | 0.01 ± 0.01 | 0.01± 0.01 | 0.908 | >0.999 |
| Total | 95.60 ± 0.22 | 95.86 ± 0.21 | 0.401 | >0.999 |

## Values are mean ± SEM (n = 10 per sex) proportions of individual molecular species in total plasma PC. Comparisons of means between sexes were by Student’s unpaired t test after adjustment for multiple testing using the Holm-Šídák method. Molecular species are ranked by increasing number of carbons and degree of unsaturation of the putative *sn*-1 fatty acid.

**Table 2** Plasma phosphatidylcholine *sn*-1 alkyl, *sn*-2 ester linked molecular species composition in men and women

|  |  |  |  |
| --- | --- | --- | --- |
|  | Proportion of total PC molecular species (%) | | T-test |
| Molecular species | Men | Women | P value |
| PC O-16:0\_18:2 | 0.27 ± 0.03 | 0.25 ± 0.03 | 0.719 |
| PC O-16:0\_20:4 | 0.48 ± 0.05 | 0.52 ± 0.04 | 0.432 |
| PC O-16:1\_16:0 | 0.09 ± 0.01 | 0.09 ± 0.01 | 0.829 |
| PC O-16:1\_18:0 | 0.25 ± 0.02 | 0.22 ± 0.02 | 0.296 |
| PC O-16:1\_18:1 | 0.13 ± 0.01 | 0.13 ± 0.02 | 0.990 |
| PC O-16:1\_20:5 | 0.02 ± 0.00 | 0.02 ± 0.01 | 0.871 |
| PC O-16:1\_22:6 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.839 |
| PC O-16:1\_18:2 | 0.37 ± 0.03 | 0.30 ± 0.02 | 0.071 |
| PC O-16:1\_20:4 | 0.37 ± 0.04 | 0.34 ± 0.03 | 0.563 |
| PC O-18:0\_18:2 | 0.11 ± 0.01 | 0.09 ± 0.01 | 0.248 |
| PC O-18:0\_20:3 | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.681 |
| PC O-18:0\_20:4 | 0.20 ± 0.02 | 0.21 ± 0.02 | 0.828 |
| PC O-18:0\_22:6 | 0.04 ± 0.00 | 0.04 ± 0.01 | 0.921 |
| PC O-18:1\_18:2 | 0.17 ± 0.01 | 0.15 ± 0.02 | 0.421 |
| PC O-18:1\_18:2 | 0.08 ± 0.02 | 0.07 ± 0.02 | 0.888 |
| PC O-18:1\_20:4 | 0.64 ± 0.04 | 0.59 ± 0.04 | 0.389 |
| PC O-18:1\_20:5 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.969 |
| PC O-18:2\_20:4 | 0.22 ± 0.02 | 0.19 ± 0.01 | 0.196 |
| PC O-20:0\_20:4 | 0.04 ± 0.01 | 0.04 ±0.00 | 0.967 |
| PC O-20:1\_20:4 | 0.10 ± 0.01 | 0.09 ±0.01 | 0.424 |
| Total | 3.72 ± 0.26 | 3.48 ± 0.23 | 0.500 |

Values are mean ± SEM (n = 10 per sex) proportions of individual molecular species in total plasma PC. Comparisons of means between sexes were by Student’s unpaired t test with adjustment for multiple testing using the Holm-Šídák method (all adjusted P values were non-significant and are not shown). Molecular species are ranked by increasing number of carbons and degree of unsaturation of the putative *sn*-1 fatty acid.

**Table 3** Plasma phosphatidylethanolamine *sn*-1, 2 ester linked molecular species composition in men and women

|  |  |  |  |
| --- | --- | --- | --- |
|  | Proportion of total PE molecular species (%) | | T-test |
| Molecular species | Men | Women | P |
| PE16:0\_18:1 | 0.63 ± 0.09 | 0.84 ± 0.12 | 0.162 |
| PE16:0\_18:2 | 0.99 ± 0.20 | 0.99 ± 0.07 | 0.984 |
| PE16:0\_20:4 | 1.17 ± 0.19 | 1.43 ± 0.18 | 0.319 |
| PE16:0\_22:6 | 1.81 ± 0.34 | 1.82 ± 0.35 | 0.988 |
| PE18:0\_18:1 | 0.75 ± 0.11 | 0.92 ± 0.10 | 0.274 |
| PE18:0\_18:2 | 3.38 ± 0.61 | 3.33 ± 0.32 | 0.948 |
| PE18:0\_20:3 | 0.31 ± 0.06 | 0.37 ± 0.05 | 0.446 |
| PE18:0\_20:4 | 4.29 ± 0.62 | 5.03 ± 0.84 | 0.491 |
| PE18:0\_20:5 | 0.23 ± 0.04 | 0.30 ± 0.03 | 0.187 |
| PE18:0\_22:4 | 0.05 ± 0.01 | 0.12 ± 0.03 | 0.076 |
| PE18:0\_22:5 | 0.13 ± 0.02 | 0.16 ± 0.02 | 0.296 |
| PE18:0\_22:6 | 0.94 ± 0.18 | 1.17 ± 0.18 | 0.373 |
| PE18:1\_18:2 | 0.91 ± 0.17 | 0.78 ± 0.07 | 0.483 |
| PE18:1\_20:4 | 0.77 ± 0.11 | 0.72 ± 0.08 | 0.763 |
| PE18:1\_22:6 | 0.18 ± 0.03 | 0.16 ± 0.03 | 0.715 |
| Total | 16.54 ± 2.37 | 18.50 ± 1.86 | 0.601 |

Values are mean ± SEM (n = 10 per sex) proportions of individual molecular species in total plasma PE (*sn*-1 fatty acid / *sn*-2 fatty acid). Comparisons of means between sexes were by Student’s unpaired t test with adjustment for multiple testing using the Holm-Šídák method (all adjusted P values were non-significant and are not shown). Molecular species are ranked by increasing number of carbons and degree of unsaturation of the putative *sn*-1 fatty acid.

**Table 4** Plasma *sn*-1 alkyl or *sn*-1 alkenyl phosphatidylethanolamine molecular species composition in men and women

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Proportion of total PE molecular species (%) | | T-test | |
| Molecular species | Men | Women | P  Value | Adjusted P value |
|  | Alkyl molecular species | |  |  |
| PE O-16:1\_18:1 | 0.72 ± 0.09 | 0.92 ± 0.08 | 0.118 | 0.745 |
| PE O-16:1\_18:2 | 1.27 ± 0.14 | 1.50 ± 0.17 | 0.315 | 0.923 |
| PE O-16:1\_20:4 | 3.22 ± 0.43 | 5.47 ± 0.46 | 0.002 | 0.035 |
| PE O-16:1\_20:5 | 0.25 ± 0.04 | 0.35 ± 0.08 | 0.279 | 0.566 |
| PE O-16:1\_22:6 | 2.01 ± 0.28 | 2.60 ± 0.23 | 0.126 | 0.747 |
| PE O-18:0\_20:4 | 0.40 ± 0.05 | 0.55 ± 0.04 | 0.035 | 0.451 |
| PE O-18:1\_18:1 | 0.75 ± 0.11 | 0.81 ± 0.06 | 0.643 | 0.994 |
| PE O-18:1\_18:2 | 3.29 ± 0.41 | 3.17 ± 0.31 | 0.834 | 0.993 |
| PE O-18:1\_20:3 | 0.98 ± 0.14 | 1.30 ± 0.04 | 0.041 | 0.515 |
| PE O-18:1\_20:4 | 5.95 ± 0.90 | 7.68 ± 0.49 | 0.111 | 0.739 |
| PE O-18:1\_20:5 | 0.58 ± 0.10 | 0.83 ± 0.07 | 0.056 | 0.565 |
| PE O-18:1\_22:4 | 0.07 ± 0.01 | 0.31 ± 0.10 | 0.041 | 0.432 |
| PE O-18:1\_22:5 | 1.01 ± 0.12 | 1.28 ± 0.05 | 0.065 | 0.566 |
| PE O-18:1\_22:6 | 2.31 ± 0.35 | 2.49 ± 0.26 | 0.686 | 0.994 |
| PE O-18:2\_18:2 | 1.54 ± 0.15 | 1.80 ± 0.17 | 0.275 | 0.924 |
| PE O-18:2\_20:4 | 4.01 ± 0.36 | 5.48 ± 0.41 | 0.014 | 0.025 |
| PE O-18:2\_20:5 | 0.67 ± 0.05 | 0.80 ± 0.05 | 0.079 | 0.657 |
| PE O-18:2\_22:6 | 1.19 ± 0.08 | 1.41 ± 0.12 | 0.161 | 0.788 |
| PE O-20:1\_18:2 | 0.30 ± 0.03 | 0.30 ± 0.04 | 0.966 | 0.994 |
| PE O-20:1\_20:4 | 0.23 ± 0.03 | 0.36 ± 0.06 | 0.055 | 0.992 |
| PE O-20:1\_22:6 | 0.09 ± 0.02 | 0.10 ± 0.01 | 0.745 | 0.997 |
| Total | 30.83 ± 2.99 | 39.51 ± 1.32 | 0.021 | 0.991 |
|  |  |  |  |  |
|  | Alkenyl molecular species | |  |  |
| PE P-16:0\_18:1 | 1.06 ± 0.17 | 0.63 ± 0.08 | 0.041 | >0.999 |
| PE P-16:0\_18:2 | 2.73 ± 0.30 | 1.92 ± 0.21 | 0.040 | >0.999 |
| PE P-16:0\_20:4 | 8.28 ± 0.73 | 8.20 ± 0.51 | 0.931 | >0.999 |
| PE P-16:0\_22:6 | 4.61 ± 0.36 | 3.88 ± 0.25 | 0.114 | >0.999 |
| PE P-16:0\_22:6 | 1.17 ± 0.21 | 1.09 ± 0.24 | 0.816 | >0.999 |
| PE P-18:0\_18:1 | 0.95 ± 0.17 | 0.60 ± 0.08 | 0.098 | >0.999 |
| PE P-18:0\_18:2 | 4.05 ± 0.48 | 2.67 ± 0.32 | 0.029 | >0.999 |
| PE P-18:0\_20:4 | 9.70 ± 1.28 | 7.73 ± 0.81 | 0.214 | >0.999 |
| PE P-18:0\_20:5 | 0.77 ± 0.07 | 0.57 ± 0.07 | 0.071 | >0.999 |
| PE P-18:0\_22:6 | 3.41 ± 0.48 | 2.34 ± 0.30 | 0.077 | >0.999 |
| PE P-18:1\_18:2 | 2.81 ± 0.26 | 2.05 ± 0.18 | 0.028 | >0.999 |
| PE P-18:1\_20:4 | 10.11 ± 0.78 | 8.56 ± 0.39 | 0.100 | >0.999 |
| Total | 52.63 ± 4.29 | 42.34 ± 1.11 | 0.042 | >0.999 |

Values are mean ± SEM (n = 10 per sex) proportions of individual molecular species in total plasma PE (*sn*-1 fatty acid / *sn*-2 fatty acid). Comparisons of means between sexes were by Student’s unpaired t test with adjustment for multiple testing using the Holm-Šídák method. Molecular species are ranked by increasing number of carbons and degree of unsaturation of the *sn*-1 fatty acid.

**Table 5** Plasma phosphatidylserine and phosphatidylinositol molecular species compositions in men and women

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | |  |
| Molecular species | Men | Women | T test |
|  | Proportion of total PS molecular species (%) | | P |
| PS18:0\_18:1 | 9.53 ± 2.16 | 21.52 ± 6.56 | 0.111 |
| PS18:0\_20:4 | 12.74 ± 3.16 | 13.09 ± 3.35 | 0.940 |
| PS20:2\_20:4 | 77.73 ± 4.32 | 65.39 ± 7.85 | 0.190 |
|  |  |  |  |
|  | Proportion of total PI molecular species (%) | |  |
| PI16:0\_16:1 | 0.57 ± 0.05 | 0.60 ± 0.12 | 0.227 |
| PI16:0\_18:1 | 4.51 ± 0.51 | 5.48 ± 0.60 | 0.238 |
| PI16:0\_18:2 | 3.36 ± 0.17 | 3.57 ± 0.28 | 0.540 |
| PI16:0\_20:3 | 1.07 ± 0.06 | 1.08 ± 0.06 | 0.918 |
| PI16:0\_20:4 | 7.24 ± 0.68 | 7.08 ± 0.53 | 0.859 |
| PI16:0\_20:4 | 3.79 ± 0.24 | 4.33 ± 0.34 | 0.208 |
| PI18:0\_18:1 | 6.37 ± 0.76 | 8.26 ± 1.01 | 0.154 |
| PI18:0\_18:2 | 13.55 ± 1.04 | 12.67 ± 0.75 | 0.503 |
| PI18:0\_20:3 | 7.00 ± 0.67 | 6.77 ± 0.38 | 0.778 |
| PI18:0\_20:4 | 45.24 ± 1.60 | 42.91 ± 1.98 | 0.372 |
| PI18:0\_22:5 | 0.93 ± 0.08 | 0.88 ± 0.06 | 0.655 |
| PI18:0\_22:6 | 1.78 ± 0.26 | 2.09 ± 0.19 | 0.342 |
| PI18:1\_18:2 | 2.64 ± 0.18 | 2.27 ± 0.08 | 0.089 |
| PI18:1\_20:4 | 2.26 ± 0.12 | 2.00 ± 0.11 | 0.141 |

Values are mean ± SEM (n = 10 per sex) proportions of individual molecular species (*sn*-1 fatty acid / *sn*-2 fatty acid) in each lipid class. Comparisons of means between sexes were by Student’s unpaired t test with adjustment for multiple testing using the Holm-Šídák method (all adjusted P values were non-significant and are not shown). Molecular species are ranked by increasing number of carbons and degree of unsaturation of the putative *sn*-1 fatty acid.