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# Phosphatidylethanolamine is a Key Regulator of Membrane Fluidity in Eukaryotic Cells*

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*Running title: *Phosphatidylethanolamine regulates membrane fluidity*

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## ABSTRACT

**Adequate membrane fluidity is required for a variety of key cellular processes and in particular for proper function of membrane proteins. In most eukaryotic cells membrane fluidity is known to be regulated by fatty acids desaturation and cholesterol although some cells, such insect cells, are almost devoid of sterols synthesis. We show here that insect and mammalian cells present similar microviscosity at their respective physiological temperature. In order to investigate how both sterols and phospholipids control fluidity homeostasis we quantified the lipidic composition of insect SF9[b] and mammalian HEK 293T cells under normal or sterol-modified condition. As expected, insect cells show minimal sterols compared to mammalian cells. A major difference is also observed in phospholipid content as the ratio of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) is inverted (four times higher in SF9 cells). In vitro studies in liposomes confirm that both cholesterol and PE can increase rigidity of the bilayer, suggesting that both can be used by cells to maintain membrane fluidity. We then show that exogenously increasing the cholesterol amount in SF9 membranes leads to a significant decrease in PE/PC ratio while decreasing cholesterol in HEK 293T cells using statin treatment leads to an increase in the PE/PC ratio. In all cases the membrane fluidity is maintained, indicating that both cell types combine regulation by sterols and phospholipids to control proper membrane fluidity.**

The maintenance of proper cell membrane fluidity is of critical importance for adequate diffusion of membrane components, from lipids to proteins and influence dynamics and function of integral membrane proteins. It is also responsible for their remarkable flexibility and thus required for a multitude of morphological transformations necessary for cellular functions including division, differentiation and general adaptation to the environment (1,2).

In bacteria, membrane fluidity is mostly regulated by adapting the length and saturation of acyl chains (3). For instance, upon lowering of the growth temperature, bacteria will typically shift their lipid tails from polyunsaturated to monounsaturated, shorten chain length and modify branching or cyclization so as to lower the temperature of phase transition from gel to liquid-crystalline state, a process called *homeoviscous* adaptation (4). In mammalian cells, membrane fluidity is known to be also regulated by cholesterol, which has the ability to render membrane fluid by interfering with acyl-chain packing, inhibiting the transition to the solid gel state but at the same time to rigidify fluid membranes by

reducing the flexibility of neighboring unsaturated acyl chains (5,6).

Membranes of cell organelles needs to adapt their properties and thus their composition according to the function they need to fulfill (2,5). It is indeed known that total percentage of cholesterol varies extensively among the organelles implicated in cell trafficking; low in ER (5% of lipids) its amount increases in the trans-Golgi and plasma membrane allowing a tighter packing compatible with the plasma membrane function as a barrier (5,7). Thus animal cells are submitted to a fine-tuned regulation of cholesterol and fatty acids synthesis through the Sterol Regulator Element-Binding Proteins (SREBP) pathway (8). In this context, an imbalance in membrane lipid composition could affect membranes properties and could lead to pathological consequences, as it was described for erythrocytes membranes in hypercholesterolemia patients and in types 2 diabetes (9,10). Indeed, an increased ratio cholesterol/phospholipids (PL) leads to a decreased membrane fluidity and is associated with a reduced binding of insulin (9).

On the other hand, some higher eukaryotes, such as insects, do not synthesize sterols. While mass spectrometry studies have shown that *insect cell* membranes contain the main classes of PL found in mammalian cells, the presence of sterols is strictly diet dependent (11).

How cells sense and regulate the complex mix and distribution of lipids in order to preserve membranes characteristics is, therefore, an important and challenging question. In this work we compared the membrane lipid distribution regulation and its consequence on membranes fluidity of two extensively used eukaryotic cell models: insect SF9 and human HEK 293T cells. Using Liquid Chromatography system coupled to a high resolution mass spectrometer (MS), we determined the lipid composition of both membranes. We show that despite the differences in their cholesterol and phosphatidylethanolamine (PE) contents, the fluidity of both membranes was similar. We show that fluidity in SF9 and HEK 293T membranes is similarly regulated. Indeed, a change in cholesterol amounts did not affect membranes fluidity of both cells but resulted in an inverse modulation of PE content. Our findings point to a conserved compensation balance between cholesterol and PE metabolism that might be needed for membrane fluidity control.

## EXPERIMENTAL PROCEDURES

*Cell culture*- SF9 cells were grown in ESF 921 protein free medium from Expression system at 27°C with 130 rpm agitation. For cholesterol amounts enhancement, cells were cultured for 5 passages (7-10 days) in ESF921 supplemented with 10% FBS (Lonza). HEK 293T cells were grown in DMEM (Lonza) supplemented with 10% FBS, 4.5mg/ml glucose, 1mM Na pyruvate, 2mM L-glutamine at 37°C with 5% $CO_2$. For statin treatment, Simvastatin stock solution was prepared and activated by the following method: first 54 mg simvastatin was dissolved in 1.04 ml ethanol 95% then 813 µl of 1N NaOH was added. The resulting solution was neutralized with 1N HCl to a pH of 7.2 and brought up to 13ml with distilled water. The stock of 10mM was then aliquoted and stored at -20°C until used. Prior to simvastatin treatment, cells that will be treated and controls were cultured for 48h in serum free DMEM supplemented with ITS (insulin, transferrin, and selenium from Gibco), 0.1mg/ml BSA, 4.5mg/ml glucose, 1mM Na pyruvate, 2mM L-glutamine at 37°C with 5% $CO_2$. Then 75µM simvastatin were added only for statin treated cells while control cells remained statin free. Cells were then harvested after 48-50h, washed with phosphate buffer saline and pellets stored at -80°C for membrane preparation.

*Lipid Standards*-_The following standards were used: for PC, PE, PG, PS and SM (C12:0 C12:0) and (C17:0 C17:0); for PI (C18:0 C20:4) and Cholesterol-2,3,4-$^{13}$C3. Standard curves were made by MS analysis using dilution series of standards (from 0.02mg/ml to 5 $10^{-5}$mg/ml for phospholipids and from 0.2mg/ml to 0.002mg/ml for cholesterol). All phospholipid standards were purchased from Avanti® Polar lipids, Inc., Cholesterol-2,3,4-$^{13}$C3 was purchased from Sigma.

*Membrane preparation and membrane lipids extraction*- Cell pellets (~$20\times10^6$ cells for HEK 293T, $60\times10^6$ cells for SF9) were transferred into hypotonic lysis buffer (10mM Tris pH7.4, 1mM EDTA) and stirred for 15min for cell breaking. Lysed cells were centrifuged at 25000×g for 10 min and the pellets containing the membranes were resuspended in phosphate buffer saline. This procedure was carried out at 4°C. Standards for each phospholipid class and cholesterol-$^{13}$C3 were added to the membrane and lipids were then extracted from membranes by methanol/chloroform solution (50:50 v/v) as described in Bligh and Dyer (1959) with the

following modifications: 10µL HCl 6N were added to the mixture and extraction was repeated 3 times on the same sample to have maximum recovery of all types of lipids; corresponding organic phases were pooled together, the solvent evaporated under nitrogen stream and lipids then dissolved in dichloromethane: isopropanol (1:4, v/v) prior to MS analysis.

*Mass spectrometry analysis*- Two µl of each sample were injected in a RRLC system (1200 series from Agilent Technologies, CA, USA) fitted with a Zorbax XDB Eclipse Plus C18, 4.6x50 mm, 1.8 µm particle size. Run was 30 min long with the following characteristics: flow rate: 0.3 ml/min,) column temperature: 40 °C, mobile phase A was formic acid 0.1 % (positive MS analysis) or 5 mM ammonium acetate pH 5 (negative MS analysis) and B was isopropanol, gradient: started with 90 % solvent A and directly increase to 20 % in 10 min, stayed 15 min with 20 % solvent A, re-equilibration to starting conditions in 5 min. A 6520 series electrospray ion source (ESI) - quadrupole time-of-flight (Q-TOF) high resolution mass spectrometer from Agilent Technologies was used. For the MS/MS analyses, auto-MS/MS mode was used and parameters were: positive or negative mode; high resolution acquisition mode (4 GHz); gas temperature of 330 °C; drying gas of 7 L/min; nebulizer pressure of 50 psig; capillary voltage of -4500 V; Fragmentor: 210 V; Fixed collision energy (CE): 25 V; MS scan range and rate: 100-1700 at 4 spectra/s; MS/MS scan range and rate: 50-1700 at 3 spectra/s; auto-MS/MS: 3 max precursors, Precursor absolute threshold: 200 counts, active exclusion on 2 repeats and released after 0.5 min; fixed exclusion range: 100-499, 1500-1700; preferred charge state: 1 and 2. Data were acquired by the Mass Hunter Acquisition® for TOF and QTOF version B.04 SP3 (Agilent Technologies). For quantification, single MS analyses were performed with the following parameters: positive or negative mode; Extended Dynamic Range mode (2 GHz); gas temperature of 330 °C; drying gas of 7 L/min; nebulizer pressure of 50 psig; capillary voltage of 4500 V; Fragmentor: 210 V; MS scan range and rate: 100-1700 at 2 spectra/s. Data were acquired by the Mass Hunter Acquisition for TOF and QTOF® version B.04 SP3 (Agilent Technologies). Phosphatidylcholines and cholesterol were analyzed in positive mode while the other PLs were analyzed in negative mode.

*Data analysis and quantification*- The data were first analyzed by Mass Hunter Qualitative Analysis® version, B.06 SP1 (Agilent Technologies, CA, USA) based on a fragment based searching mode. Briefly, the MS/MS spectra obtained during the run time were selected according to the specific fragmentation of each phospholipid (PL). In negative mode, the search mode was focused on the fatty acid fragments (C16:0; C16:1; C14:0; C14:1, C18:0…). In positive mode, the search mode was focused on the fragments of the polar head of the PL. Maximal accepted error on detected m/z was 5ppm. Regarding quantification, samples were run in simple MS mode. Extracted ion chromatograms were extracted based on the exact masses of the lipids observed during auto MS/MS analyses. Phospholipids were quantified by the standard curves (with mass concentrations) run during the set of experiments.

*Liposomes formation and size determination by Dynamic Light Scattering (DLS)*- Lipids used for liposomes formation (POPE, POPC and cholesterol) were all purchased from Avanti® Polar lipids, Inc. Lipids mixes were prepared with the following procedure: POPC was mixed with 0, 10, 20 or 30% W/W cholesterol or with 40, 50 or 60% W/W POPE or with 10% cholesterol in addition to 30 or 60% POPE (W/W) to a final lipid concentration of 3mg/ml in HEPES 100mM pH 7.4. Liposomes were then sonicated in ice/water bath for 5 minutes and immediately used for size determination or DPH fluorescence measurements. Mean diameter of liposomes was assessed by DLS using Zetasizer, Malvern instruments limited.

*Membrane fluidity monitoring by DPH fluorescence polarization measurements*- Lipids extracted from membranes were dried in pre-weighed vials then weighed and resuspended in the right volume of 100mM HEPES pH7.4 to have 0.3mg/ml. For liposomes, we took the volume of lipids from chloroform stock corresponding to 0.3mg in total, according to the concentration provided by the manufacturer, and then dried before being resuspended in 1ml of 100mM HEPES pH7.4. Membranes and liposomes were sonicated prior to DPH fluorescence polarization measurements. Then 1.5ml of bilayer at 0.3mg/ml final concentration in 100mM HEPES pH7.4 was mixed with 0.2µg/ml final concentration DPH (from stock of 0,1 mg/ml in N-N dimethylformamide). This mix was then vortexed and heated for 15min at 45°C to allow homogeneous integration of DPH in the bilayer.

Fluorescence polarization of DPH was measured on a Photon Technology International (PTI) fluorimeter with the following protocol: time based polarization, digital mode, excitation wavelength of 358nm, emission wavelength at 429nm and 4nm bandpass, polarization was assessed on a temperature gradient from 16° to 50°C by 2°C increase, stabilization of temperature for 1 min and 3 measurement per temperature (1 measurement per every 6 seconds). Fluorescence polarization of non DPH labelled control bilayers was used for correction.

**RESULTS**

*Mammalian and insect cell membranes display similar fluidity at physiologically relevant temperatures* - We initially analyzed the viscosity of membranes using polarization measurements of the 1,6-diphenyl -1,3,5-hexatriene (DPH) dye. The probe is incorporated into membranes in parallel to the acyl chains of membrane lipids and its fluorescence polarization depends on the bilayer viscosity. We measured DPH polarization of membranes extracted from SF9 and HEK 293T cells at a range of temperature between 15 and 50°C (Fig. 1). Remarkably, we observed almost identical values (0.219 ± 0.002 for SF9 cells and 0.211 ± 0.008 for HEK 293T) when comparing the fluidity of both membranes at relevant temperatures (i.e. temperature used for cell culture), namely 27°C for SF9 and 37°C for HEK 293T (Fig. 1, respectively grey and black arrows). Interestingly, the temperature dependence of the fluidity is much less pronounced in insect cell membranes compared to mammalian cell membranes (see below).

*Lipidic composition of insect and mammalian membranes* - Quantitative analysis of the lipid composition of membranes prepared from insect SF9 and mammalian HEK 293T cells was performed by MS. For quantification purposes, internal standards for each of the phospholipid (PL) species were added prior to the lipid extraction. All main eukaryotic lipids were considered: phosphatidylcholine (PC and plasmalogen PCO-), phosphatidylethanolamine (PE and plasmalogen PEO-), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) as well as sphingomyelin (SM) and cholesterol (see material and methods). The method allows evaluating and correcting the experimental loss for each lipid class in a specific manner. Extracted lipids were then subjected to Rapid Resolution Liquid Chromatography (RRLC) followed by high resolution mass spectrometry (ESI-QTOF 6520 series from Agilent Technologies). We first analyzed the samples by auto MS/MS in order to identify the components (head groups and acyl chains) and then by single MS to quantify each of the lipids identified by auto MS/MS using the standard curves and the internal standards.

Both cell membranes contain comparable amounts of all classes of phospholipids (Fig. 2A). Total membrane lipid contents from both SF9 and HEK 293T cells were composed of 18.4-23.4% PS, 7-13% for PI, 3% for PG and around 11% for SM (Fig. 1A). As expected larger amount of cholesterol was found in mammalian membranes, expressed as cholesterol/PL ratio (9 times higher in HEK 293T membranes when compared to SF9) (Fig. 1B). The small amount of cholesterol identified in insect cells most likely comes from the cell culture medium which, indeed, was found to contain low amounts of cholesterol (about 0.2% of total medium mass, table 1). However, a significant difference was observed when comparing PC and PE amounts. PE represented 38% and PC 16% of total membrane lipids in SF9 cells (no plasmalogen were found), while HEK 293T membranes contained 16% PE, 14% PEO- and 36% for PC, 5%PCO-. Thus, the PE/PC ((PE+PEO-)/(PC+PCO-)) ratio is 4 times larger in HEK 293T than in SF9 cells (Fig. 1C).

Analysis of fatty acid chains shows, for both cell types, a large dominance of acyl chains composed of 16 and 18 carbons (Fig. 1D and table 2) with an equal distribution of 44% between C16 and C18 in SF9 membranes and a majority of 58% of C18 acyl chains versus 30% of C16 in HEK 293T. Interestingly, C16 acyl chains contained more unsaturation in in SF9 than in HEK 293T cells (77% versus 43 %) (Fig. 1E).

Our data largely agrees with published studies analyzing the lipid composition of insect cell membranes (11), in particular SF9 cells (12), especially regarding limited amounts of cholesterol as well as for their high PE content. On the other hand we found that SF9 similarly to HEK 293T cells contain all major PL species, notably PG, PS and SM, which was described for *Drosophila* but not found in previously published studies on SF9 cells. Epithelial mammalian cultured cells (MDCK) were already studied for their lipid composition by MS. The obtained results are in agreement with our data concerning overall PL distribution(13). Nevertheless, the saturation state of acyl chains differed between

both studies. Reasonable amounts of polyunsaturated fatty acids (PUFA) were found in MDCK cells while our data shows only traces of PUFA in HEK 293T cells. This difference in PUFA could be due to methodological differences (sample preparation, and quantification method), or to intrinsic differences in the cell lines used.

*Increased unsaturation of PE reduces temperature-dependence of membrane fluidity* - As shown in Figure 1 the fluidity of SF9 cells shows little or no dependence to temperature, in contrast to mammalian cells. Insects are generally ectotherms and poikilotherms organisms while potentially exposed to rapid temperature changes in their environment. From an evolutionary standpoint it would thus be sensible that membrane properties of insect cells, and fluidity in particular, do not fluctuate much with temperature changes. On the other hand, studies on a wide range of organism have shown that, upon temperature changes, metabolic control of membrane homeostasis (e.g. fluidity) in reaction typically involves modulation of the unsaturation state (4,14-17). Therefore we investigated whether differences in fatty acid unsaturation between lipids from mammalian and insect cells observed here (Figure 2E) could be correlated to the difference in temperature sensitivity. As shown in Fig. 3, the temperature dependence is lowered in the presence of DOPE compared to POPE as illustrated by the statistically significant differences in slopes of the DPH fluorescence polarization in function of temperatures. Indeed, in a linear approximation, we see that for PC:PE ratio in the mixes (40:60) the slope for DOPC:DOPE is half of that of DOPC:POPE ($p<0.001$) and the slope of the POPC:DOPE is respectively 1.5 smaller than that POPC:POPE ($p<0.005$). This demonstrates a reduced temperature-dependence in presence of di-unsaturated PE when compared to mono-unsaturated PE. The modulation of temperature-dependence by unsaturations was specific to PE since no difference in the slope of linear approximation curves were noticed between DOPC and POPC.

*Both PE and cholesterol affect microviscosity in vitro* - As we have observed that the membrane microviscosity is almost identical in both SF9 and HEK 293T cells at growth temperatures, our data argue that cholesterol and acyl chain unsaturation are not the sole regulators of membrane fluidity of higher eukaryotes and that PE/PC ratio may be involved. We thus performed microviscosity measurements on liposomes of controlled composition, varying the relative amounts of PE and PC of identical acyl chains and the cholesterol amounts (Fig. 4). As expected, DPH measurements on liposomes of POPC containing 0, 10, 20 or 30 % of cholesterol showed a clear increase in DPH polarization curves, reflecting an increase in the bilayer rigidity (Fig. 4A). Similar results were obtained when we replaced cholesterol by PE: i.e. addition of increasing amounts of POPE (40, 50, 60%) to POPC liposomes leads to a steady increase in DPH fluorescence polarization reflecting the enhancement of the bilayer viscosity (Fig. 4B), in agreement with previous studies (18,19). We also compared the effect of the inversion in PE/PC ratio as observed between SF9 and HEK 293T in presence of cholesterol and noticed a clear decrease in the fluidity when PE/PC ratio is higher (Fig. 4C). We controlled by dynamic light scattering that the size of all the liposomes was in the same range (180-300nm), excluding curvature-related effect in the observed changes (table 3). In summary, DPH polarization measurements indicates that presence of either PE or cholesterol enhance membrane viscosity indicating that a balance and a regulation between them could be involved in maintaining cell membrane properties.

*Insect cells modulate PE amounts to balance exogenous sterols* - In order to establish the biological relevance of the above observations, we analyzed how insect cells adapt their membrane upon increase of the sterol content through diet supplementation. We cultured SF9 cells with or without 10% Fetal Calf Serum (FBS) containing cholesterol for 7 days leading to >2 fold increase in membrane cholesterol (Fig. 5A). Surprisingly, despite the increase in cholesterol levels, membranes fluidity of FBS cultured cells is almost unchanged compared to the control with DPH polarization values at 27°C of $0.210 \pm 0.006$ and $0.219 \pm 0.002$ respectively (Fig. 5B).

We then measured the PL distribution of both samples by MS and observed that PE/PC ratio is significantly decreased by 1.8x (Fig. 5D) reflecting a decrease in PE content of the membranes from 40% to 32% together with an enrichment in PC from 18% to 25% in FBS cultured cells compared to controls (Fig. 5C). The amounts of the other lipid components of membranes remained unchanged. Significant differences were observed in acyl chain length distribution of total lipids, i.e. a decrease in C16 fatty acids together with an increase of C20 chains (Fig.

5E). Analysis of the acyl chains saturation state (total lipids and in each of PL classes) shows a clear shift in unsaturation distributions (Fig 5F and Fig. 6). Indeed while controls contained a majority of C16 with 1 unsaturation (85%) and only a minority of saturated C16 (15%), SF9 cells supplemented with FBS showed increased saturated C16 acyl chains (33%) along with a decrease in mono-unsaturated C16 (67%). No difference in saturation state of acyl chains of C18 was observed. These shifts towards lower saturated state were also distributed among the different individual phospholipid species i.e. PE, PG, PS, and PI (Fig. 6).

These results suggest that the increase in cholesterol in SF9 membranes could be compensated by a decrease in PE content together with a decrease in unsaturated C16 fatty acids in order to maintain similar fluidity of cell membranes.

*Cholesterol decrease leads to PE upregulation in mammalian cells* - In order to assess whether the balance between cholesterol and PE is also operating in mammalian cells, we inhibited cholesterol synthesis in HEK 293T cells using statins. We compared PL distribution and viscosity of membranes from HEK 293T cultivated in cholesterol free medium with or without addition of 75µM of simvastatin for 48h, a treatment that leads to a 2 times fold decrease of the cholesterol/PL ratio (Fig. 7A). In spite of this change, the microviscosity of the statin-treated cells is not changed compared to control cells (Fig. 7B). Remarkably, MS analysis of the PL distribution demonstrated an increase of PE and PEO- % from 14 and 4% in controls to 20 and 7% respectively in statin treated membranes leading to a change in the PE/PC ratio from 0.55 in control to 0.78 in treated membranes (Fig. 7C and D). With the exception of PC and SM that remained unchanged in both conditions, all other PLs decreased in statin treated membranes (Fig. 7C). Surprisingly, we did not observe a significant change neither in the length nor in the saturation state of total lipids (Fig. 7E and F). The saturation state of acyl chains in the different phospholipid species remained also unchanged (Fig. 8).

Taken together our data support the hypothesis that a compensatory regulation between cholesterol and PE metabolism in eukaryotic cells would be involved in the control of membrane homeostasis.

## DISCUSSION

The main finding of this study is that both mammalian and insect cells achieve membrane fluidity homeostasis not only by modulating unsaturation levels and (in mammalian cells) sterols but also by changing phosphatidylethanolamine amounts. Specifically, changes in the sterol content are compensated, in both cell types by changes in PE quantities. This was particularly surprising for SF9 cells as insect do not synthesize sterols, suggesting a complex sterol-sensitive (or viscosity-sensitive) regulation mechanism (see below).

In the case of SF9 membranes, exogenous increase of sterol led to a reduction in PE percentage along with a clear shift in acyl chains saturation state. Correspondingly, blocking cholesterol synthesis in HEK 293T cells cultured in cholesterol free medium results in an enhancement in PE amounts although with no detectable change in saturation state, suggesting that modulating PE amounts is a main mechanism for regulating fluidity. In this experiment, the extent of the effects observed in statin treated HEK 293T cells were somewhat moderate, but this is likely due to the fact that statin treatment was intentionally limited to 48h. Indeed extending such treatment over 48-60h leads to significant increase in cell death (20,21). Our data implies a metabolic control of HEK 293T and SF9 membranes lipid composition to maintain membranes properties.

DPH fluorescence polarization measurement has been traditionally used as a reliable tool to assess membrane fluidity but when applied to lipid mixtures, it is reasonable to raise the issues of portioning and sidedness. As DPH is a symmetrical hydrophobic molecule that has been shown to be deeply buried in the acyl chain region of the bilayer (22) it is likely be evenly distributed in both layers of the membranes. In this same study the authors show that the changes in DPH fluorescence polarization in the presence of cholesterol are likely to be due to change in motion and in localization of the probe.

On the other hand, in vitro studies investigating the behavior of PE/PC have shown that phase separation may occur at high molar ratio of PE (i.e. POPE >30%) suggesting that domains may form in SF9 cells where we detected about 38% of total PE (mostly DOPE). While we do see a transition effect at 60% POPE (40% POPC) below 15°C such behavior was not observed in physiologically relevant range of temperature (25-40°C). Considering these various points, we deem reasonable to use DPH

fluorescence polarization as a reliable assessment of membrane fluidity for complex lipid mixtures.

Membrane fluidity and/or lipid composition alteration can be responsible for pathological behavior of the cells. The effect of lipid composition modulation on red blood cells has been extensively studied. Indeed, the rheology of erythrocytes is altered in patients with hypercholesterolemia due to increased Cholesterol/PL ratio and impaired membrane fluidity (23,24). Statin treatment lowers the cholesterol amount in the membranes and normalizes erythrocytes membrane fluidity (10,25). Similarly insulin resistant diabetes type 2 may result from modified lipid composition of the erythrocytes membranes notably fatty acids saturation and PE/PC ratio (9). Hence, the lipid homeostasis of cell membranes is crucial for the accomplishment of their functions. Since erythrocytes are nuclei free, they might lack for regulation elements allowing the maintenance of their membrane properties although cholesterol exchange with low density proteins has been described (26).

Our data beg the question on how is membrane fluidity controlled in these cells and whether there is a direct fluidity sensor in the membrane? Considering that insect cells share similar composition in PL as mammalian cells, how do they regulate their membrane fluidity in the absence of endogenous sterol? Thermal and fluidity sensors regulated by the lipid composition have been long described in bacterial membranes. For example, the two component pathway (Desk/DesR) of *bacillus subtilis* is able to sense the change in membrane properties upon temperature change and to trigger the expression of acyl lipid desaturase in order to increase the amounts of unsaturated fatty acids in the membrane and thus maintaining the fluidity (3). Membrane fluidity control pathways were also described in yeast cells. Rho/PKC1/MAPK signaling pathway appears to be implicated in the membrane fluidity homeostasis as shown by genetic studies of yeast mutants and is regulated by the PL acyl chains (27).

IRE1 is a conserved-ER stress sensor in eukaryotes which detects –via its transmembrane domain– perturbations like increased lipid saturation and alert the lipid biosynthesis machinery to restore the ER membrane homeostasis by synthetizing unsaturated lipids. Any uncontrolled modification of the saturated/unsaturated balance (like an increase of saturated acids content in ER) can lead to liver failure in chronic diseases like obesity and diabetes (5,28).

Previous studies from the Brown and Goldstein laboratories have described that the SREP pathway (Sterol Regulator Element-Binding Proteins) that is implicated in cholesterol and fatty acids homeostasis in mammalian cells is present in insect cells where it is involved in control of fatty acid synthesis and can be regulated by a phospholipid with a palmitate (most probably POPE) (8,29,30). Indeed, in HEK 293T cells, the down-regulation of the SREBP cascade can be triggered by accumulation of cholesterol and/or unsaturated fatty acids in the cells in a way to maintain the metabolic homeostasis of both. On the other hand, studies on *Drosophila* cells show that PE, and palmitated PE in particular, controls the release of SREBP and exert feedback on other phospholipids and fatty acids synthesis (29). This regulation could be required to maintain a certain lipid composition of cell membranes and thus for the adjustment of lipid synthesis as the authors suggested. Nevertheless a direct relation or a balance between palmitated PE and cholesterol amounts in the same cells has never been established. Moreover the link between SREBP regulation by cholesterol/palmitated PE in order to adjust lipid composition and the preservation of membrane properties such as fluidity was suggested but never investigated.

The similarity in behavior observed between insect and mammalian cells could indicate that they rely on comparable molecular mechanisms to maintain membrane properties and homeostasis not only by regulating the saturation state of the newly synthetized phospholipids but also by a fine tuned balance between PL, especially PE, and cholesterol contents of the membranes. The implication of a membrane fluidity sensor in this regulation is the key question that needs to be investigated in the future.

Evidently, establishing the molecular basis of this regulation could bring new perspectives in the comprehension of the physiopathological processes related to hypercholesterolemia.

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**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article

**Author contributions:** RD performed most sample preparation and analysis with the help of CT. CD, CN and PVA performed mass spectrometry, JMR, PVA and CG designed and supervised the work and wrote the paper with RD. All authors analyzed the results and approved the final version of the manuscript

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**FOOTNOTES**

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[b] The abbreviations used are: SF9, Spodoptera frugiperda cells, HEK 293T cells, Human Embryonic Kidney 293T cells; DPH, 1,6-diphenyl -1,3,5-hexatriene; PL, phospholipid; PC, phosphatidylcholine; PCO-, phosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; PEO-, phosphatidylethanolamine plasmalogen; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

**TABLE LEGENDS**

**TABLE 1.** Quantification of Sterols in SF9 media.
Sterols present in SF9 cell culture medium (ESF921) normalized to total medium mass, and represented by Mean ± S.D. (n=3). N.F: Not Found.

**TABLE 2.** Fatty acids chain length distribution in single PL species of SF9 and HEK 293T membranes.
Fatty acids chain length of lipids extracted from SF9 and HEK 293T membranes in single PL specie (A) PC, (B) PE, (C) PG, (D) PS, (E) PI, (F) SM normalized to 100% w/w and represented by Mean ± S.D. (n=3).

**TABLE 3.** Liposomes diameter sizes of DPH tested liposomes.
Average of bilayer diameter in synthetic liposomes measured by Dynamic light scattering. represented by mean in nm ± S.D. (n=3).

**FIGURES LEGENDS**

**FIG. 1.** SF9 and HEK 293T membranes fluidity
DPH fluorescence polarization in a temperature range from 15 to 50 °C on membranes prepared from SF9 (grey) and HEK 293T (black) cells. Error bars indicates standard deviation (n=3). Arrows indicate growth temperature for SF9 (grey, 27°C) and HEK 293T (black, 37°C).

**FIG. 2.** Phospholipids and cholesterol distribution in SF9 and HEK 293T membranes. A-E, lipids extracted from SF9 (grey) and HEK 293T (black) membranes were analyzed on a RRLC coupled to ESI-QTOF mass spectrometer. Species were identified by Mass Hunter Qualitative Analysis® software of auto MSMS data with accepted maximal error of 5 ppm for detected m/z and quantification made by automatic integration of peaks from extracted ion chromatograms. Bars represent values normalized to 100% w/w. (A) Phospholipids distribution, (B) Cholesterol to Phospholipid ratio, (C) PE (including PEO-) to PC (including PCO-) ratio, (D) Fatty acids chain length distribution, (E) unsaturation(s) per acyl chain in main fatty acids chains. Error bars indicate standard deviation (n=3). *P* values were estimated by student test (n=3): *** $P < 0{,}001$, **p<0,005.

**FIG.3.** Effect of unsaturations on temperature dependence of membrane fluidity. Membrane fluidity curves were measured by DPH fluorescence polarization in a temperature range from 16 to 48°C on (A) synthetic liposomes with POPC and POPC with 60% DOPE or POPE(W/W) and (B) synthetic liposomes with DOPC and DOPC with 60% DOPE or POPE (W/W). Error bars indicate standard deviation (n=3).

**FIG. 4.** Effect of Cholesterol and PE on lipid bilayer viscosity. Membrane fluidity curves were measured by DPH fluorescence polarization in a temperature range from 10 to 50 °C on (A) synthetic liposomes with 0 , 10, 20 and 30% of cholesterol (W/W) and (B) synthetic liposomes with POPC, POPC with 40% , 50% and 60% POPE (W/W), (C) POPC with 30% POPE and 10% Cholesterol (Blue), POPC with 60% POPE and 10% Cholesterol (red) (W/W). Error bars indicate standard deviation (n=3).

**FIG. 5.** Membrane fluidity maintained by Cholesterol and PE in SF9 membranes upon FBS addition. Lipid distribution and membrane fluidity of SF9 control (grey) and SF9 cultivated with 10% FBS (black) membranes. (A) Cholesterol to Phospholipid ratio, (B) DPH fluorescence polarization, (C) Phospholipids distribution, (D)PE to PC ratio, (E) Fatty acids chain length distribution, (F) unsaturation(s) per acyl chain in main fatty acids chains. Bars represent values normalized to 100% w/w. Error bars indicate standard deviation (n=3). *P* values were estimated by student test (n=3): *** $P < 0{,}001$, **p<0,005.

**FIG. 6.** Unsaturation(s) distribution per acyl chain of different PL species in SF9 control and cultivated in 10% FBS.
Unsaturation(s) per acyl chain distribution in different PL species from SF9 control (grey) and SF9 cultivated with 10% FBS (black). (A) PC, (B) PE, (C) PG, (D) PS, (E) PI, (F) SM. Bars represent values normalized to 100% w/w. Error bars indicate standard deviation (n=3). *P* values were estimated by student test (n=3): *** $P < 0{,}001$, ** $P < 0{,}005$.

**FIG. 7.** Membrane fluidity maintained by Cholesterol and PE in HEK 293T membranes upon statin treatment. Lipid distribution and membrane fluidity of HEK 293T control (black) and HEK 293T treated with 75µM Simvastatin for 50h (grey) membranes. (A) Cholesterol to Phospholipids ratio, (B) DPH fluorescence polarization, (C)phospholipids distribution, (D)PE (including PEO-) to PC (including PCO-) ratio, (E) Fatty acids chain length distribution, (F) unsaturation(s) per acyl chain in main fatty acids chains . Bars represent values normalized to 100% w/w. Error bars indicate standard deviation (n=3). *P* values were estimated by student test (n=3): *** $P < 0{,}001$, ** $P < 0{,}005$, *$P<0{,}01$.

**FIG. 8.** Unsaturations distribution in acyl chains of different PL species in HEK 293T control and statin treated. Unsaturation(s) per acyl chain distribution in different PL species from HEK 293T Control (black) and HEK 293T treated with 75µM Simvastatin for 50h (grey). (A) PC, (B) PE, (C) PG, (D) PS, (E) PI, (F) SM. Bars represent values normalized to 100% w/w. Error bars indicate standard deviation (n=3). *P* values were estimated by student test (n=3): *** $P < 0{,}001$, ** $P < 0{,}005$, *$P < 0{,}01$.

| | **ESF 921** |
|---|---|
| **Cholesterol** | 0,18 ± 0,01 % |
| **Stigmasterol** | N.F |
| **β-Sitosterol** | N.F |
| **Ergosterol** | N.F |
| **Campesterol** | 0,01 ± 0,0% |

Table 1:

Quantification of Sterols in SF9 media

**A**

| PC | SF9 | HEK |
|---|---|---|
| **C14** | 5,3 ± 1,7 | 6,2 ± 1,6 |
| **C16** | 44,1 ± 7,8 | 48,6 ± 4,3 |
| **C18** | 48,9 ± 7,1 | 43,1 ± 0,1 |
| **C20** | 0,6 ± 0,3 | 1,5 ± 2,1 |
| **C22** | N.F | 0,1 ± 0,1 |
| **others** | 1,0 ± 1,3 | 0,5 ± 0,6 |

**B**

| PE | SF9 | HEK |
|---|---|---|
| **C14** | N.F | N.F |
| **C16** | 41,0 ± 0,2 | 32,2 ± 2,8 |
| **C18** | 45,9 ± 0,1 | 59,9 ± 4,7 |
| **C20** | 11,0 ± 0,4 | 7,5 ± 7 |
| **C22** | 2,0 ± 0,1 | 0,4 ± 0,3 |
| **others** | N.F | N.F |

**C**

| PG | SF9 | HEK |
|---|---|---|
| **C14** | N.F | N.F |
| **C16** | 80,2 ± 3,3 | 12,2 ± 4,4 |
| **C18** | 19,8 ± 3,3 | 87,8 ± 4,4 |
| **C20** | N.F | N.F |
| **C22** | N.F | N.F |
| **others** | N.F | N.F |

**D**

| PS | SF9 | HEK |
|---|---|---|
| **C14** | N.F | N.F |
| **C16** | 29,4 ± 3,7 | 24,4 ± 4,8 |
| **C18** | 33,4 ± 0,9 | 32,0 ± 3 |
| **C20** | 10,7 ± 2,3 | 18,6 ± 2,7 |
| **C22** | 20,0 ± 1,4 | 23,8 ± 0,8 |
| **others** | 6,5 ± 0,8 | 1,2 ± 1,9 |

**E**

| PI | SF9 | HEK |
|---|---|---|
| **C14** | N.F | N.F |
| **C16** | 44,8 ± 1,3 | 14,5 ± 4,3 |
| **C18** | 53,5 ± 0,7 | 76,7 ± 3,4 |
| **C20** | 1,6 ± 0,7 | 8,5 ± 0,9 |
| **C22** | 0,1 ± 0,0 | 0,3 ± 0 |
| **others** | N.F | N.F |

**F**

| SM | SF9 | HEK |
|---|---|---|
| **C14** | 2,3 ± 3,3 | 2,1 ± 2,4 |
| **C16** | 27,5 ± 0,4 | 44,7 ± 3,4 |
| **C18** | 68,0 ± 3,5 | 50,2 ± 2,7 |
| **C20** | N.F | N.F |
| **C22** | N.F | N.F |
| **others** | 2,2 ± 0,0 | 3,0 ± 3,3 |

Table 2:

Fatty acids chain length distribution in single PL species of SF9 and HEK membranes

| **Liposomes** | **Average diameter in nm** |
|---|---|
| POPC | 185,9 ± 5,2 |
| POPC Chol 10 | 210,8 ± 7,4 |
| POPC Chol 20 | 270,6 ± 4,3 |
| POPC Chol 30 | 296,4 ± 11,5 |
| POPC60 POPE40 | 193,3 ± 6,2 |
| POPC40 POPE60 | 205,4 ± 9,4 |
| POPC60 POPE40 Chol 10 | 227,3 ± 6,6 |
| POPC40 POPE60 Chol 10 | 219 ± 5,5 |

Table 3:

Liposomes diameter sizes of DPH tested liposomes

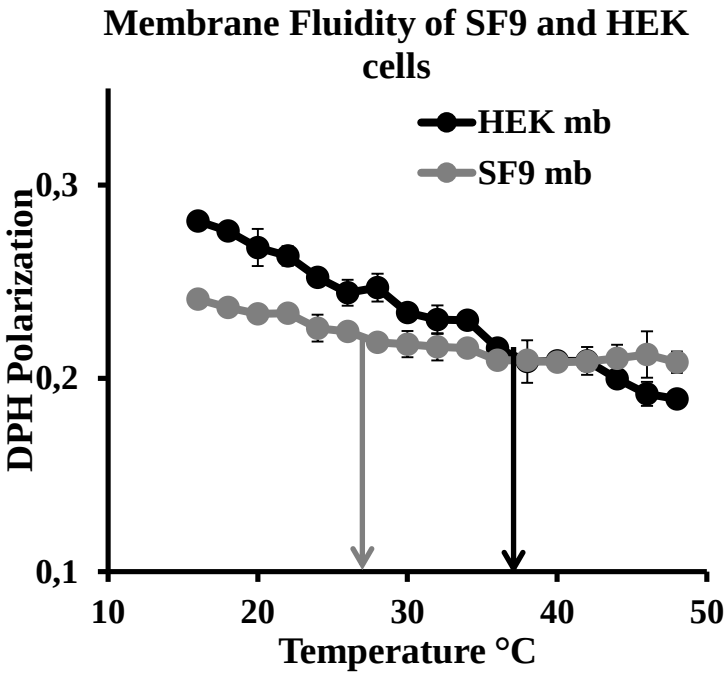


Figure 1

SF9 and HEK membranes fluidity

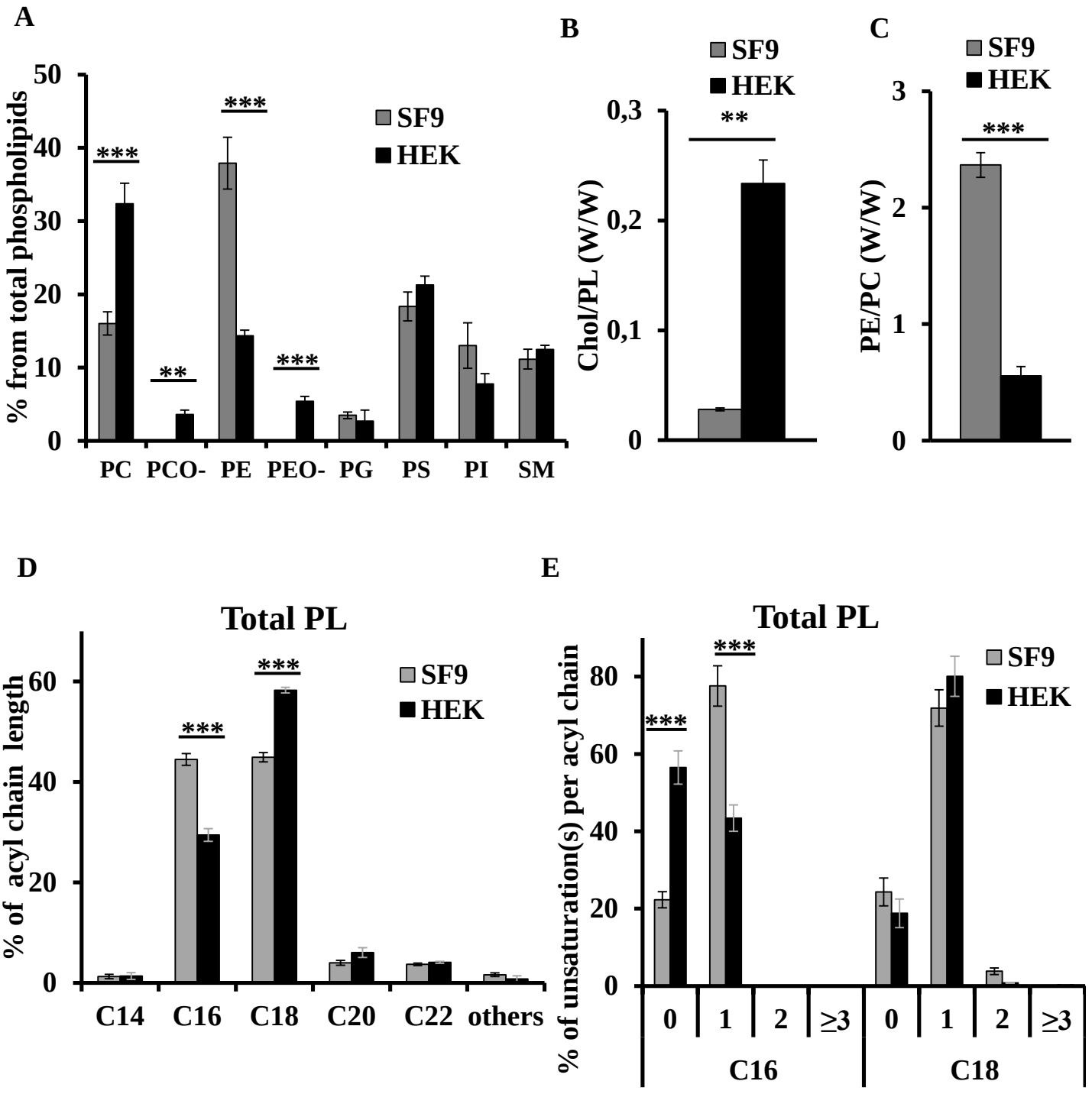


Figure 2

Phospholipids and cholesterol distribution in SF9 and HEK membranes

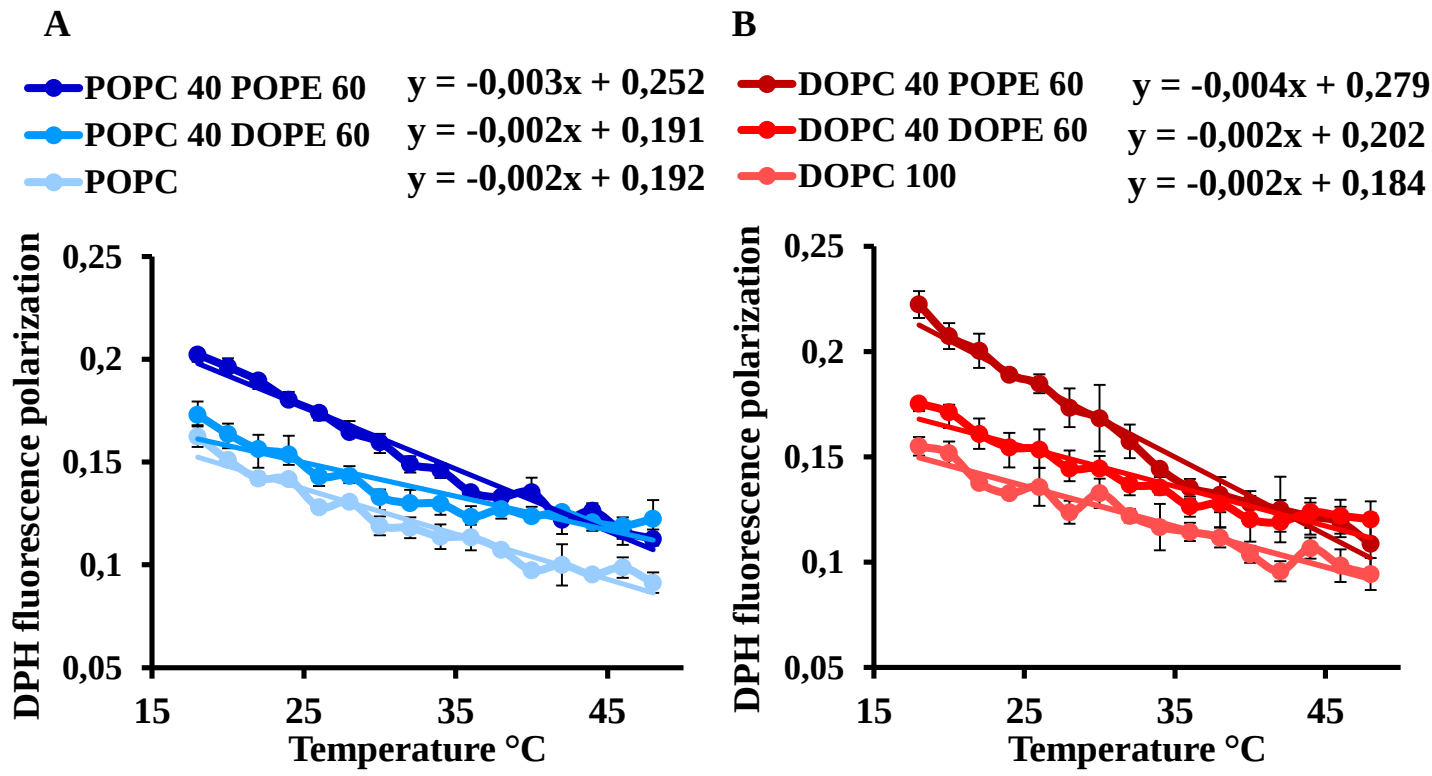


Figure 3

Effect of unsaturations on temperature dependence of membrane fluidity

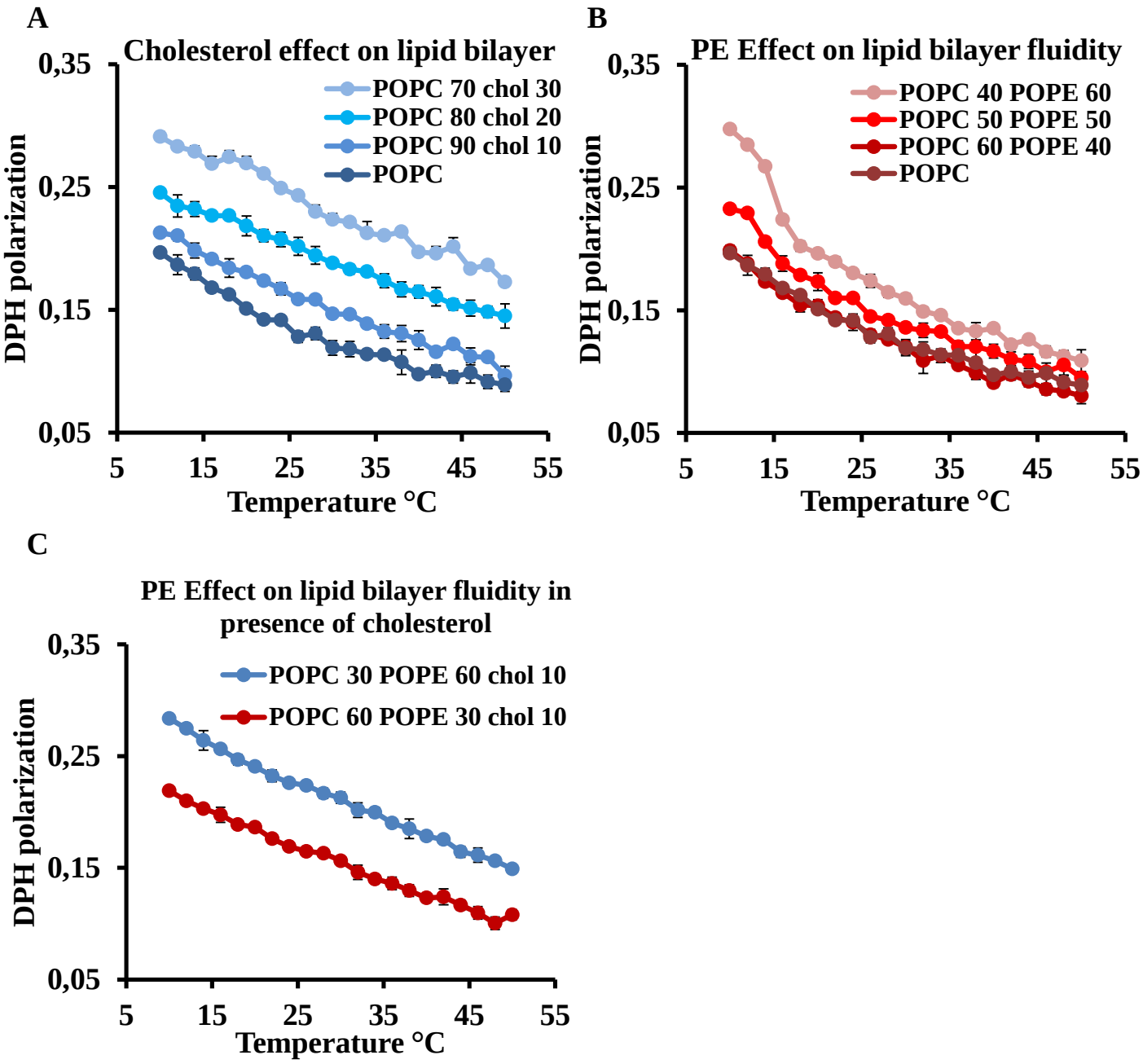


Figure 4

Effect of  Cholesterol and PE on lipid bilayer viscosity

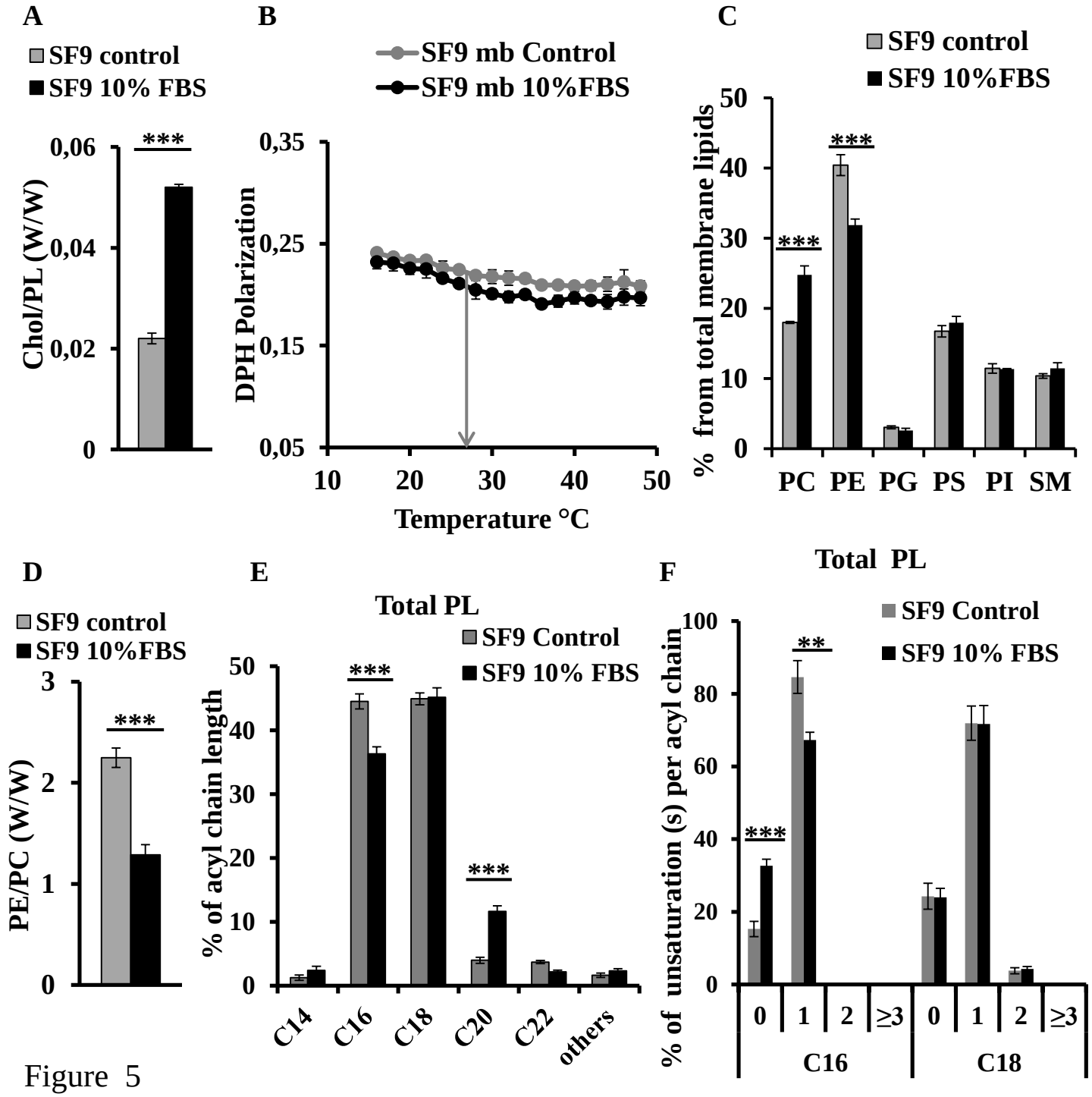


Figure 5

Membrane fluidity maintained by Cholesterol and PE in SF9 membranes upon FBS addition

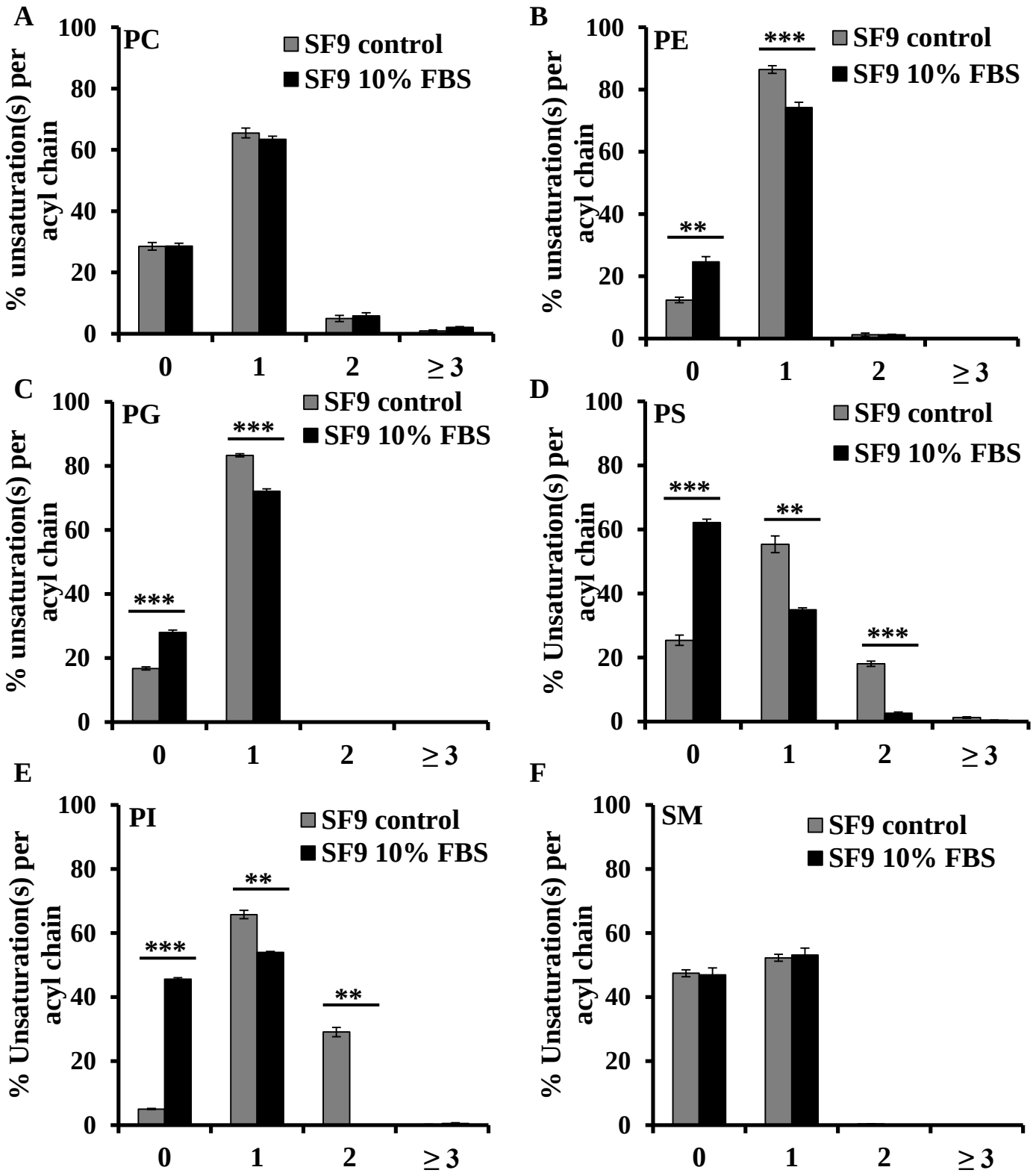


Figure 6:

Unsaturation(s) distribution per acyl chain of different PL species in SF9 control and cultivated in 10% FBS

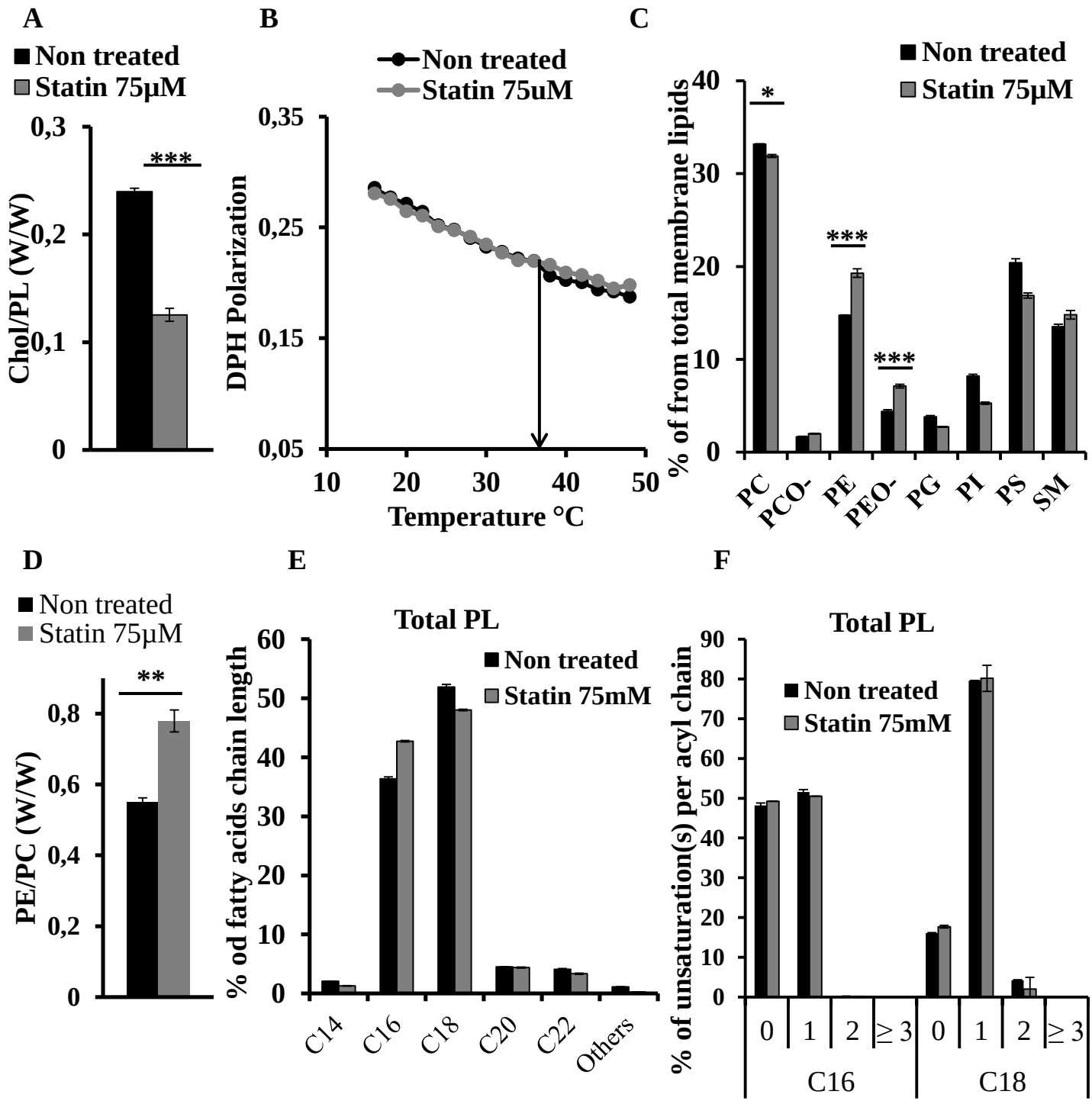


Figure 7

Membrane fluidity maintained by Cholesterol and PE in HEK membranes upon statin treatment

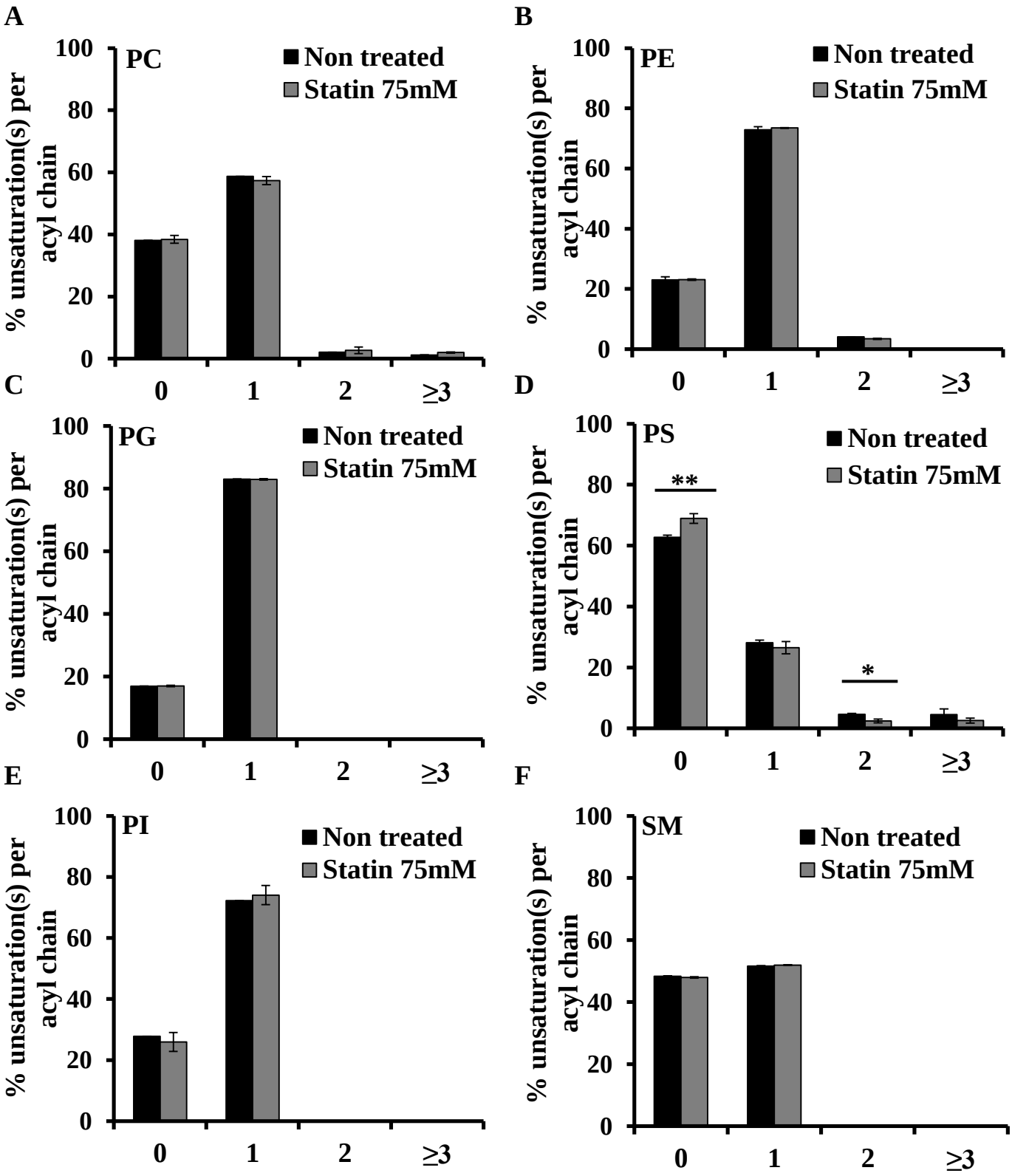


Figure 8:

Unsaturations distribution in acyl chains of different PL species in HEK control and statin treated


Membrane Biology:

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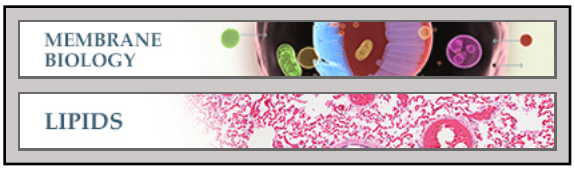


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