

Opinion

Plasma membranes: does one model fit all?

Tore Skotland ^{1,2,*} and Kirsten Sandvig^{1,2,3}

Biological membranes consist of a lipid bilayer with a highly asymmetric distribution of lipids in the two leaflets. This asymmetry is maintained by enzymes whose activities depend on cytosolic ATP and Ca²⁺ concentrations. Recently, a new model for plasma membranes (PMs) was introduced. It is based on studies with human erythrocytes and suggests that there is a 50% overabundance of lipids with two hydrophobic chains in the cytosolic leaflet, compensated by the presence of three times more cholesterol in the outer leaflet. In this opinion article, we discuss the large differences in lipid composition between erythrocytes and other cell types, the assumptions used to reach the new membrane model, and whether this model would fit PMs of other cell types.

Biological membranes and main mammalian membrane lipids

The plasma membrane (PM), which consists of both proteins and lipids, separates the cell interior from its surroundings. It has been more than 50 years since Singer and Nicholson described that biological membranes consist of a lipid bilayer, where the headgroups of phospholipids (PLs) and sphingolipids face the hydrophilic surroundings, and the hydrophobic chains avoid contact with the hydrophilic surroundings by sequestering together inside the bilayer [4]. At the same time, Bretscher described that the two leaflets have an asymmetric distribution of lipids [5]. The names and abbreviations of the main lipid classes in mammalian membranes are listed in Table 1. Until recently, the view was that all or most of the sphingomyelin (SM) and phosphatidylcholine (PC) in the PM are in the outer (exoplasmic) leaflet, and all or most of the phosphatidylserine (PS) and phosphatidylethanolamine (PE) are localized in the inner (cytoplasmic) leaflet. This highly asymmetric distribution of lipids is maintained using enzymes, that is, **flippases** (see Glossary) and **floppases**, in energy-dependent processes requiring ATP. In contrast, **scramblases** transfer lipids nonspecifically between leaflets in an energy-independent manner. Mammalian membranes also contain cholesterol (CHOL), which constitutes approximately 40% of the total lipids in PMs. The distribution of CHOL in the two leaflets is controversial. In some articles, CHOL is reported to be mainly in the outer leaflet, whereas others report that it is mainly in the inner leaflet. For a review of the challenges in determining the distribution of CHOL in the two leaflets, see the study by Steck and Lange [8].

PMs of different cell types contain different amounts of the various lipid classes. Each class contains fatty acyl (FA) chains with different chain lengths and numbers of double bonds; thus, cells and PMs contain a large number of lipid species. We here refer to an excellent review regarding the diversity of membrane lipid composition [9]. The present picture is that PMs have a dynamic and complex mixture of lipid species and that membrane-bound or transmembrane proteins are also involved in cellular functions such as cellular uptake and signaling. The view that the two leaflets of PMs contain nearly equal amounts of CHOL and lipids with two hydrophobic chains has recently been challenged in articles from the Levental group [1,2]. The authors conclude that there is a major imbalance between lipids with two hydrophobic tails and CHOL in the two leaflets and that this imbalance has been overlooked by the scientific community. They propose that the view that there is a similar amount of lipids in the two leaflets of PMs is generally invalid. There are,

Highlights

A recently published model for the leaflet distribution of lipids in plasma membranes describes a 50% overabundance of lipids with two hydrophobic chains in the inner leaflet, compensated by three times more cholesterol in the outer leaflet.

This model was reached by mass spectrometry analysis after treatment of human red blood cells with two enzymes: (i) sphingomyelinase, which splits off the headgroup of sphingomyelins, and (ii) phospholipase A2, which removes fatty acyl chains at the *sn*-2 position of phospholipids.

We discuss key questions related to this strategy: Are the enzymes able to cleave all substrates in the outer leaflet? Can phospholipase A2 treatment induce transmembrane transfer of lipids and Ca²⁺ leakage, resulting in erroneous results?

We discuss whether the large differences in lipid composition between red blood cells and other cell types fit with one general plasma membrane model.

¹Department of Molecular Cell Biology, Institute for Cancer Research, Oslo University Hospital—The Norwegian Radium Hospital, Oslo 0379, Norway

²Centre for Cancer Cell Reprogramming, Faculty of Medicine, University of Oslo, Oslo 0379, Norway

³Department of Biosciences, University of Oslo, Oslo 0316, Norway

*Correspondence: toresko@uio.no (T. Skotland).

Table 1. Names and abbreviations for some of the most common lipid classes in mammalian membranes^a

Lipid classes/abbreviations	<i>sn-1</i>	<i>sn-2</i>	Headgroups
Phosphatidylcholine/PC	FA	FA	Choline
Lysophosphatidylcholine/lysoPC	FA	OH	
Ether-linked PC (PC O or PC P)	Alkyl or alkenyl	FA	
Phosphatidylserine/PS	FA	FA	Serine
Phosphatidylethanolamine/PE	FA	FA	Ethanolamine
Phosphatidylinositol/PI ^b	FA	FA	Inositol
Phosphatidylglycerol/PG	FA	FA	Glycerol
Phosphatidic acid/PA	FA	FA	H
Ceramide/Cer	LCB + FA		H
Sphingomyelin/SM	LCB + FA		Phosphocholine
Glycosphingolipids/GSLs ^b	LCB + FA		Carbohydrates

^aPLs most often contain two FA chains bound as esters at the *sn-1* and *sn-2* positions of glycerol, whereas headgroups bound to phosphate at the *sn-3* position give names to the various PL classes. Phospholipids with only one FA chain are called lysolipids. Phospholipids may also have hydrophobic chains bound to glycerol as an alkyl or alkenyl ether. For simplicity, both lysolipids and ether-containing lipids are shown only for PC in the table. Ceramides and sphingolipids do not contain *sn-1* and *sn-2* groups, but a long-chain sphingoid base (LCB) and only one FA chain, which is bound by an amide bond to nitrogen atoms originating from serine.

^bPI can be phosphorylated at three sites, giving rise to seven different forms called PIPs [6]. The glycosphingolipids' classes have a large variation in their carbohydrate structures [7].

however, several methodological aspects of these studies, which deserve attention and are discussed in this opinion article. Furthermore, we describe the large differences in the lipid composition between red blood cells (RBCs) and other cells and discuss to what extent a model obtained with RBCs is applicable for PMs of other cell types.

Strategy used to study the plasma membrane of human red blood cells

The PM of RBCs has, for many years, been popular to study since these cells lose all intracellular organelles during their maturation and therefore contain only one lipid bilayer. It should be mentioned that during the maturation to RBCs, reticulocytes undergo various modifications of their membrane [10], and as recently published, their lipidome undergoes further modifications during their lifetime of approximately 120 days [11]. The strategy used by the Levental group to analyze the lipid composition of the two leaflets of the RBC membrane is based on enzymatic hydrolysis of lipids in the outer leaflet, combined with mass spectrometry (MS) analysis before and after such degradation. Two enzymes were used for this purpose: **sphingomyelinase (SMase)** and **phospholipase A2 (PLA2)**. SMase removes the phosphocholine headgroup of SM, thus converting SM into ceramide (Cer), which contains one H-atom instead of phosphocholine, while PLA2 cuts off the FA groups at the *sn-2* position of PLs. The conclusions drawn from these studies are based on the assumption that these enzymes are 100% effective, as lipids that are not degraded are concluded to be present in the inner leaflet. A similar strategy, using enzymes to study the distribution of lipids between the two leaflets of RBCs, has been used for many years by several groups, and such data for human RBCs are summarized in Table 2.

There is a large variation in the data in Table 2, showing how much PS is present in the outer leaflet in the two studies using MS analyses [1,2]. There is also a remarkably large variation in the relative abundance of total lipids with two hydrophobic chains in the two leaflets, as this ratio varies between 1.4 and 2.2 [2]. It should be noted that the MS analyses in these studies were performed in the same commercial laboratory using the same equipment, lipid standards,

Glossary

Exosomes: small vesicles (mainly 50–100 nm in diameter) consisting of only one membrane bilayer. They are formed inside cells in organelles called multivesicular bodies and are excreted from cells following the fusion of the multivesicular bodies with the PM. Exosomes have the same asymmetry as PMs at the time of secretion.

Flippases: ATP-dependent enzymes that transfer PLs (mainly PS and PE) from the outer to the inner leaflet.

Flippases: In.

Floppases: ATP-dependent enzymes which transfer PLs (mainly PC) from the inner to the outer leaflet. **Floppases:** Out.

Giant unilamellar vesicles: these vesicles, often abbreviated as GUVs, have a size in the same range as cells. They are often used as simple synthetic models to study interactions between lipids and proteins. Due to their large size, they can be studied using light microscopy methods.

Phospholipase A2 (PLA2): splits off the FA group at the *sn-2* position of PLs. PLA2 thus transforms PLs with two FA chains into a lipid with only one chain. PLs with only one chain are called lysolipids; they interact more weakly with the surrounding membrane lipids and can move between the two leaflets more easily than lipids with two FA chains.

Scramblases: contribute to the loss of PM asymmetry by mediating a nonselective redistribution of lipids across the bilayer in an ATP-independent but Ca²⁺-dependent manner.

Sphingomyelinase (SMase): splits off the phosphocholine headgroup of SMs. The product of this reaction is Cer, which has just an H-atom instead of the phosphocholine group. Due to its small headgroup, Cer can move to the opposite leaflet more easily than SM. Since SM interacts more strongly than Cer with CHOL, the use of SMase is expected to influence PM stability.

Table 2. Comparison of SM, PC, PE, and PS in four studies of human RBCs using the enzymatic strategy with SMase and PLA2^a

Study	Percentage of lipid classes in the outer leaflet of human RBCs				
	SM	PC	PE	PS	Analyses
Verkleij <i>et al.</i> (1973) [12]	85	68	0	0	TLC
Zwaal <i>et al.</i> (1975) [13]	85	68	0	0	TLC
Lorent <i>et al.</i> (2020) [1] ^b	89	48	4	15	MS
Doktorova <i>et al.</i> (2025) [2] ^c	~95	~45	~0	~3	MS
Murate <i>et al.</i> (2015) [14]	98.5	98.1	0.2	0.7 ^d	Freeze etching

^aLipid analyses were performed using thin-layer chromatography (TLC) in two studies by the van Deenen group [12,13] and MS in the two studies by the Levental group [1,2]. For comparison, data from a study with freeze etching and detection with gold-labeled antibodies are also shown. The distribution of CHOL was not analyzed in any of these studies.

^bData from Supplementary Table S1 in the study by Doktorova *et al.* [2].

^cData estimated from Fig. 1 in [2]; the PC range was 37–53%.

^dSum of PS and PI, as the antibody did not discriminate between these lipids.

and software tools. It is unlikely that these differences are due to analytical uncertainty in the MS analyses; they are most likely due to challenges in obtaining reproducible samples when using the enzymatic strategy with PLA2 and SMase.

Based on the enzymatic strategy and MS analyses, the authors concluded [1,2] that the cytoplasmic leaflet of RBCs has more than a 50% overabundance of lipids with two hydrophobic chains, that is, PLs and sphingolipids, compared with what is found in the exoplasmic leaflet, and that this imbalance is compensated by approximately three times more CHOL in the exoplasmic leaflet (~60% vs. ~20%) [2].

Methodological aspects and assumptions made to obtain the new PM model

Key questions related to the assumptions made to draw the conclusions summarized in the previous chapter are as follows: (1) Is SMase treatment able to act on all SMs originally present in the outer leaflet? (2) Is PLA2 able to quantitatively remove FA chains from the *sn*-2 positions in all PLs originally present in the outer leaflet? (3) Will the treatment with these enzymes result in the transfer of lipids between the two leaflets, either making lipids originally present in the outer leaflet not available to these enzymes or making lipids from the inner leaflet available for PLA2?

The data shown for the two MS-based studies in Table 2 indicate that 11 and 5% of total SMs are present in the inner leaflet. Could the SMase treatment, by removing a large headgroup, result in bending of the membrane and formation of internal vesicles such that SM from the outer leaflet becomes unavailable to SMase? Such vesiculation has been reported following SMase treatment of RBCs [15], ATP-depleted macrophages and fibroblasts [16], and **giant unilamellar vesicles** [17].

The data in Table 2 indicate that approximately 50% of all PC species are present in the inner leaflet. Could this be due to incomplete removal of FAs at the *sn*-2 position in the outer leaflet? It is remarkable that all PC species with two saturated FA chains are reported to be localized in the cytoplasmic leaflet. PCs with two saturated FA chains might be localized in lipid rafts with tightly packed lipids, raising the question of whether PLA2 can efficiently cleave the *sn*-2 chain in such highly ordered biological membrane areas. Heiner *et al.* reported in 2008 that by treating RBCs with methyl- β -cyclodextrin, thus removing defined amounts of CHOL, they obtained increased PLA2 activity, by increasing both the hydrolysis rate and the total lipids hydrolyzed [18]. They

also reported that the treatment with methyl- β -cyclodextrin reduced the lipid order, as measured with the fluorescent probe Laurdan. If the enzyme fails to act efficiently in domains with a high lipid order in the two studies discussed here, species not hydrolyzed are wrongly concluded to be localized in the inner leaflet. Furthermore, could removal of one FA chain from many PC species result in the transfer of PLs between the two leaflets? PC originally present in the outer leaflet may then end up in the inner leaflet, and PLs originally present in the inner leaflet may end up in the outer leaflet and be degraded by PLA2.

A very important question is whether PLA2 treatment makes the membrane leaky to Ca^{2+} ions. It is discussed in [Box 1](#) how an intracellular increase in the Ca^{2+} concentration may change the lipid distribution between the two leaflets [3]. Thus, it would be an advantage to measure whether the treatments induce Ca^{2+} influx in the RBCs. The methodological issues discussed earlier are not easy to control or validate but should be discussed since they are essential for the conclusions drawn from studies using PLA2. However, such discussions are lacking in the articles presenting these data [1,2].

The authors state that data obtained for the amounts of triacylglycerols and CHOL esters were removed before performing the calculations for the RBC membrane lipids. However, it was not described how much of these lipids were present and thus removed from the calculations. Since neither triacylglycerols nor CHOL esters are expected to be present in RBC membranes, they might indicate the presence of impurities. Could such impurities (e.g., lipoproteins) contain sufficient PLs to affect the lipid data reported for the two leaflets of RBCs?

Large differences in lipid composition of red blood cells and other cells

We here discuss variations in cellular lipid composition and the consequences for membrane structure. Leidl *et al.* performed lipid analyses of human RBCs, monocytes, lymphocytes, granulocytes, and platelets and showed that RBCs have a very different lipid composition (both lipid classes and their species) compared to other blood cells [19]. A very similar lipid composition of human RBCs (CHOL not analyzed) was later reported by another group [20]. We have previously discussed the importance of interactions between PS species and CHOL, especially one specific PS species (PS 18:0/18:1), for membrane structure and signaling [21]. Data from 37 different human cell and tissue samples [22] reveal major differences between the PS species of

Box 1. Does PLA2 treatment make RBCs leaky to Ca^{2+} ions?

The authors Lorent *et al.* and Doktorova *et al.* report that no hemolysis, that is, release of hemoglobin, was observed at the time the samples for lipidomic analyses were collected [1,2]. They do not state how close the time of sampling was to when hemolysis could be observed. Hemoglobin is a tetramer of 64 kDa, and it would not be surprising if pores much smaller than those giving release of hemoglobin may affect flipping between the two leaflets. Even though fluorescein isothiocyanate (FITC)-labelled dextran (3 kDa) was used in some experiments with SMase and no fluorescence was observed in RBCs using confocal microscopy, such a dextran molecule is still large compared to ions such as Ca^{2+} . There is a large Ca^{2+} gradient across the PM in these experiments since the buffer contained 0.25 mM Ca^{2+} and the normal intracellular levels are 30–60 nM [3]. It has been known for many years that an increase of free Ca^{2+} inside RBCs will affect many reactions important for their membrane structure. RBCs are normally stored in Ca^{2+} -free, glucose-containing citrate buffer at low temperature as these storage conditions favor Ca^{2+} depletion of the cytosol (keeping it close to its normal levels) and reduce oxidative stress and ATP deprivation [3]. Increased Ca^{2+} can stimulate scramblase (half maximal activation at 30–70 μM Ca^{2+}), which transfers lipids nonspecifically between the two leaflets [3]. Furthermore, and perhaps most important for the present discussion, is that flippases are almost completely inhibited at a Ca^{2+} concentration as low as of 400 nM [3]. Flippases use ATP to correct the lipid distribution, and the ATP level may be low in RBCs having an influx of Ca^{2+} as ATP is used to pump Ca^{2+} out [3]. Flippases correct the lipid distribution such that inner leaflet lipids that for some reason have moved to the outer leaflet are flipped back to their 'correct' position. Thus, with a complete inhibition of flippases, PS or PE in the exoplasmic leaflet will not be flipped back. It should be noted that the PLA2 used (from *Apis mellifera*) is Ca^{2+} dependent, such that a Ca^{2+} -independent PLA2 might be useful to control some of the issues discussed.

RBCs and other cells. On average, there is more than twice as much PS 18:0/18:1 as the sum of polyunsaturated fatty acyl (PUFA)-containing PS species in the 37 samples analyzed, whereas there is more than 12 times as much PUFA-containing PS as PS 18:0/18:1 in RBCs (Table 3, Key table). As discussed later, we believe the PS species composition to be a key mechanistic point for CHOL partitioning in the two leaflets, and the differences shown in Table 3 to be a key issue when considering whether the membrane model published for RBCs can also be used to describe the PMs of other cell types. Our own studies of **exosomes** and PC-3 cells, from which these exosomes were released, are also relevant to the present discussion. Exosomes have (like RBCs) only one bilayer membrane and are excreted from cells after fusion of multivesicular bodies with the PM. The exosome analyses [27] revealed 44% CHOL and amounts of SM, PC, PE, PS, and other lipid classes fitting well with a model where all sphingolipids and PC are in the outer leaflet and all other classes are in the inner leaflet [28,29]. In agreement with the idea that these vesicles have an asymmetric lipid distribution at the time of secretion are several reports about the lack of PS species on the surface. Thus, there are many studies where PS on the surface of extracellular vesicles (EVs) has been identified and quantified using PS-binding substances, such as lactadherin, fragments of lactadherin, or Annexin A5. The most extensive of such studies was recently published by Jin *et al.*, who reported that out of 10 000 EVs positive for the exosome marker CD63, only 2.0–6.0 EVs (0.02–0.06%) were positive for PS when testing five nontumorigenic cell lines; there were five times more PS-positive EVs (0.1–0.3%) when testing eight tumorigenic cell lines [30]. Thus, very few EVs with PS on the surface were found in EV samples from these 13 cell lines. Furthermore, it would be surprising if the membrane asymmetry of the EVs differs from that of the PM.

Based on lipid analyses of exosomes, molecular dynamic simulation studies performed with 14 symmetric and 14 asymmetric models showed much higher interactions between the two leaflets (interdigitation) when using the asymmetric models. The interdigitation was much stronger with SM d18:1/24:0 than with SM d18:1/16:0 and also stronger with PS 18:0/18:1 than with PS 16:0/18:1. The very strong interaction observed between SM d18:1/24:0 and PS 18:0/18:1 was the only interaction that increased after adding CHOL to the system [23]. CHOL obviously had an important effect on the interactions between these lipids in the two opposing leaflets, and the highest effect was obtained with a little more CHOL in the SM-containing leaflet. In agreement with these data, Seo *et al.* [31] reported simulation studies showing that interdigitation with SM d18:1/24:0 was stronger than with SM d18:1/16:0 and was important for interactions with lipids in the opposing leaflet when using asymmetric membranes.

The group of Fairn has reported several studies describing that the interactions between CHOL and PS species, to a large extent, depend on the FA chains in PS, with PS 18:0/18:1 being much better than other PS species (PS 16:0/18:1, PS 16:0/18:2, and PS 18:1/18:1) in shielding CHOL from CHOL oxidase and clustering with CHOL in liposomes [24]. They also showed that

Key table

Table 3. Comparison of PS data in human RBCs with those of 37 different human cell and tissue samples^a

PS species	37 cell/tissue samples [22]	Human RBCs [19]	Human RBCs [20]	Interaction with CHOL
PS 18:0/18:1	48 ± 14%	6%	7%	Strong interaction
Sum PUFAs	21 ± 16%	81%	88%	Expel CHOL

^aThe amount of PS 18:0/18:1 and the sum of all PUFA PS species are shown as the percent (mean ± sd, *n* = 37) of all PS species [22]. PS 18:0/18:1 interacts strongly with CHOL [23,24], whereas PUFAs expel CHOL [25,26].

the headgroup is important for interactions between PLs and CHOL. Thus, PS 18:0/18:1 interacted more strongly with CHOL than PC, PE, and PA with similar hydrophobic chains, such that PS 18:0/18:1 gave by far the strongest interaction with CHOL among all the PL species they tested. Their observation that the headgroup is also important for interaction with CHOL was later verified by Nyholm *et al.* [25]. Together, these data demonstrate that the various PS species interact very differently with CHOL, and that the general statement that CHOL has a higher affinity for saturated than for unsaturated PLs is not always correct [23–25].

To sum up the comparison of lipid content in RBCs and other cells and the interaction of CHOL with various lipid species, RBCs contain very little PS 18:0/18:1 and very high levels of PS with PUFA groups. Areas with high levels of PUFA groups have been described as expelling CHOL [25,26], and the very low levels of PS 18:0/18:1 in human RBCs cannot be expected to contribute significantly to interacting with CHOL and keeping it in the inner leaflet. Thus, the very different composition of PS species in RBCs and other cells may contribute to less CHOL in the cytoplasmic leaflet of RBCs than has so far been believed to be the case for most other cells.

There are also large differences between the lipid composition of RBCs from various animals. They all have a sum of SM and PC that is approximately the same as the sum of the other PLs. However, human RBCs contain similar amounts of SM and PC [19], whereas, for example, rats and dogs have approximately four times more PC than SM and bovine and sheep have only SM and lack PC [10,32]. Thus, it would be interesting to see if treatment with SMase and PLA2 of RBCs from these other species would fit the model proposed for human RBCs.

Molecular dynamic simulation studies of RBCs

Doktorova *et al.* [2] performed molecular dynamic simulation studies to support their view on the membrane structure, the enrichment of CHOL in the exoplasmic leaflet, and the overabundance of other lipids in the cytoplasmic leaflet. In these simulation studies, PS 16:0/20:4 was set to constitute 22–40% of the PLs in the inner leaflet; in their earlier study, this species was reported to constitute 33% of the lipids in the inner leaflet [1]. In the 37 datasets discussed earlier [22], PS 36:4 was not reported in 20 of the datasets and was reported to be less than 1% in the remaining 17 datasets. It should be mentioned that the PS species reported for the five blood cells by Leidl *et al.* [19] included high levels of PS 18:0/20:4 in human RBCs (38%) but no PS 16:0/20:4; similar data have been published by two other groups [11,20]. However, for samples other than blood cells in the 37 datasets discussed earlier, PS 38:4 was not reported or reported to be less than 1% in 10 of the datasets. In the remaining datasets (not including blood cells), PS 38:4 constituted $4.8 \pm 3.5\%$ ($n = 20$) of total PS species. Thus, the recent molecular dynamic simulations by Doktorova *et al.* [2], based on their lipidomic data and those published earlier by the same group [1], are based on calculations using a lipid composition very different from that found in the 37 samples discussed earlier [22]. This raises the question of how representative molecular dynamic simulation studies based on the lipid data for RBCs are for the structure of the PM in other cells. The lipid data used for simulation studies by the Levental group have also been used by others for similar studies [33], and their data are also discussed in recent reviews [34,35].

We should also note that more than 10 years ago, Ingólfsson *et al.* [36] performed molecular dynamic simulation studies of what they called a ‘general cell PM’, consisting of 63 lipid species with 14 types of headgroups and 11 different FA chains. They reported a small enrichment of CHOL in the outer leaflet (54% of the total). They also reported lipid order domains forming and disappearing on a microsecond timescale and noted that these domains were coupled across the two leaflets.

Concluding remarks and future perspectives

There has, for many years, been a focus on studies of RBCs to learn about membrane structure, as the RBCs have lost all intracellular organelles and have only one lipid bilayer. The publications discussed in this article, suggesting a new model for PMs, use an enzymatic strategy based on the assumption that SMase will hydrolyze all SMs in the outer leaflet to Cer, and PLA2 should remove all FA groups from the *sn*-2 position of all PLs in the outer leaflet. As discussed in the present article, there are several challenges when performing such studies, including the uncertainty of whether the enzymes are 100% effective or if lipids may redistribute to the other leaflet during the experiments. We have discussed such issues in this article, and we have listed several key questions and control experiments that should be addressed to prove the new model (see [Outstanding questions](#)).

Even if data obtained by performing the control experiments discussed indicate that the enzymatic strategy gives a correct picture of the lipids present in the two leaflets of human RBCs, more studies are necessary to determine whether a similar conclusion can be reached for the PM of other cells. We have discussed the large differences in the lipidome between RBCs and 37 other cells and tissue samples. We believe that the very large differences in amounts of PS species showing strong interaction with CHOL (PS 18:0/18:1) and expelling CHOL (PUFA-containing PS) will contribute significantly to the distribution of CHOL between the two membrane leaflets, such that it would not be very surprising if PMs in other cells have a structure very different from that now described for human RBCs. Obviously, it is much more challenging to perform such studies in cells with intracellular organelles. We propose, however, that it may be a useful strategy not only to study the PM from other cells but also to extend investigations to exosomes from different cell types, as well as to RBCs from species known to contain a lipidome very different from that of human RBCs. Such studies should be based on quantitative lipidomic data and combined with molecular dynamic simulation studies.

Declaration of interests

The authors declare no competing interests.

References

- Lorent, J.H. *et al.* (2020) Plasma membranes are asymmetric in lipid unsaturation, packing and protein shape. *Nat. Chem. Biol.* 16, 644–652
- Doktorova, M. *et al.* (2025) Cell membranes sustain phospholipid imbalance via cholesterol asymmetry. *Cell* 188, 2586–2602.e24
- Bogdanova, A. *et al.* (2013) Calcium in red blood cells—a perilous balance. *Int. J. Mol. Sci.* 14, 9848–9872
- Singer, S.J. and Nicolson, G.L. (1972) The fluid mosaic model of the structure of cell membranes. *Science* 175, 720–731
- Bretscher, M.S. (1972) Asymmetrical lipid bilayer structure for biological membranes. *Nat. New Biol.* 236, 11–12
- Balla, T. (2013) Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol. Rev.* 93, 1019–1137
- Merrill, A.H., Jr. (2011) Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics. *Chem. Rev.* 111, 6387–6422
- Steck, T.L. and Lange, Y. (2018) Transverse distribution of plasma membrane bilayer cholesterol: picking sides. *Traffic* 19, 750–760
- Harayama, T. and Riezman, H. (2018) Understanding the diversity of membrane lipid composition. *Nat. Rev. Mol. Cell Biol.* 19, 281–296
- Minetti, G. (2026) Reticulocytes and their maturation. In *Handbook of Red Blood Cells* (Kaestner, L.B., ed.), pp. 1–40, Springer Nature
- Minetti, G. *et al.* (2025) Insights from lipidomics into the terminal maturation of circulating human reticulocytes. *Cell Death Discov.* 11, 79
- Verkley, A.J. *et al.* (1973) The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim. Biophys. Acta* 323, 178–193
- Zwaal, R.F. *et al.* (1975) Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. *Biochim. Biophys. Acta* 406, 83–96
- Murate, M. *et al.* (2015) Transbilayer distribution of lipids at nano scale. *J. Cell Sci.* 128, 1627–1638
- Allan, D. and Walklin, C.M. (1988) Endovesiculation of human erythrocytes exposed to sphingomyelinase C: a possible explanation for the enzyme-resistant pool of sphingomyelin. *Biochim. Biophys. Acta* 938, 403–410
- Zha, X. *et al.* (1998) Sphingomyelinase treatment induces ATP-independent endocytosis. *J. Cell Biol.* 140, 39–47
- Trajkovic, K. *et al.* (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319, 1244–1247
- Heiner, A.L. *et al.* (2008) Effects of cholesterol on physical properties of human erythrocyte membranes: impact on susceptibility to hydrolysis by secretory phospholipase A2. *Biophys. J.* 94, 3084–3093
- Leidl, K. *et al.* (2008) Mass spectrometric analysis of lipid species of human circulating blood cells. *Biochim. Biophys. Acta* 1781, 655–664
- Lauren, E. *et al.* (2018) Phospholipid composition of packed red blood cells and that of extracellular vesicles show a high resemblance and stability during storage. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1863, 1–8

Outstanding questions

Do lipids flip between the two leaflets as a result of using phospholipase A2 to remove the *sn*-2 chain of phospholipids in the outer leaflet?

Is phospholipase A2 100% effective for all phospholipids, even when they are present in highly ordered (tightly packed) membrane areas in the studies discussed?

Is there an increase in the cytosolic Ca²⁺ concentration during the experiments with phospholipase A2, which may affect enzymes important for maintaining the lipid asymmetry of the membrane ([Box 1](#))?

Is the large difference in lipid composition between red blood cells and other cells important for creating differences in their plasma membranes?

Keeping in mind that phosphatidylserine 18:0/18:1 interacts strongly with cholesterol, whereas polyunsaturated phospholipids expel cholesterol one may ask: Will the large differences in lipid composition of phosphatidylserine species between red blood cells and other cells be important for the outcome of the molecular dynamics simulation studies used to estimate the distribution of cholesterol in the two leaflets?

Will the proposed model for human red blood cells be representative of red blood cells from other species, such as rats or dogs, which have four times more phosphatidylcholine than sphingomyelin, or for species such as bovines or sheep, which have only sphingomyelin and lack phosphatidylcholine in their red blood cells?

Will sphingomyelinase treatment, by removing the phosphocholine headgroup of sphingomyelin (transferring sphingomyelin to ceramide), result in invagination of the red blood cell membrane and possibly lead to endocytosis? This could result in sphingomyelinase not being able to reach all sphingomyelin.

Will the various control experiments described earlier support or disprove the newly proposed model for leaflet composition of human red blood cells?

21. Skotland, T. and Sandvig, K. (2019) The role of PS 18:0/18:1 in membrane function. *Nat. Commun.* 10, 2752
22. Skotland, T. and Sandvig, K. (2022) Need for more focus on lipid species in studies of biological and model membranes. *Prog. Lipid Res.* 86, 101160
23. Rog, T. *et al.* (2016) Interdigitation of long-chain sphingomyelin induces coupling of membrane leaflets in a cholesterol dependent manner. *Biochim. Biophys. Acta* 1858, 281–288
24. Maekawa, M. and Fairn, G.D. (2015) Complementary probes reveal that phosphatidylserine is required for the proper transbilayer distribution of cholesterol. *J. Cell Sci.* 128, 1422–1433
25. Nyholm, T.K.M. *et al.* (2019) The affinity of sterols for different phospholipid classes and its impact on lateral segregation. *Biophys. J.* 116, 296–307
26. Wassall, S.R. and Stillwell, W. (2009) Polyunsaturated fatty acid–cholesterol interactions: domain formation in membranes. *Biochim. Biophys. Acta* 1788, 24–32
27. Llorente, A. *et al.* (2013) Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. *Biochim. Biophys. Acta* 1831, 1302–1309
28. Skotland, T. *et al.* (2017) Lipids in exosomes: current knowledge and the way forward. *Prog. Lipid Res.* 66, 30–41
29. Skotland, T. *et al.* (2023) Lipids in extracellular vesicles: what can be learnt about membrane structure and function? *Cold Spring Harb. Perspect. Biol.* 15, a041415
30. Jin, F. *et al.* (2025) Phosphatidylserine-positive extracellular vesicles for detecting multiple operable cancers. *Cell Rep.* 44, 116259
31. Seo, S. *et al.* (2020) Pivotal role of interdigitation in interleaflet interactions: implications from molecular dynamics simulations. *J. Phys. Chem. Lett.* 11, 5171–5176
32. Bernhardt, I. and Kaestner, L. (2025) Historical view and some unsolved problems in red blood cell membrane research. *Front. Biosci. (Landmark Ed)* 30, 25331
33. Varma, M. and Deserno, M. (2022) Distribution of cholesterol in asymmetric membranes driven by composition and differential stress. *Biophys. J.* 121, 4001–4018
34. Pabst, G. and Keller, S. (2024) Exploring membrane asymmetry and its effects on membrane proteins. *Trends Biochem. Sci.* 49, 333–345
35. Caputo, M. *et al.* (2025) Lipid asymmetry and membrane trafficking: transbilayer distribution of structural phospholipids as regulators of exocytosis and endocytosis. *J. Biol. Chem.* 301, 110441
36. Ingolfsson, H.I. *et al.* (2014) Lipid organization of the plasma membrane. *J. Am. Chem. Soc.* 136, 14554–14559