ORIGINAL PAPER

Lipid ratios as a marker for red blood cell storage quality and as a possible explanation for donor gender differences in storage quality

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Vox Sanguinis

Background and objectives Red blood cells that are stored for transfusions as red cell concentrates (RCCs) undergo changes during the storage period, culminating in the lysis of the cells. The goal of this work is to find markers that are linked to high haemolysis, in order to explain the inter-donor variability that is known to occur in storage quality, and also the known differences between RCCs from male and female donors.

Materials and methods The relative amounts of lipids at the end of the storage period were compared for one group of low haemolysis samples (24 units, all ≤0·15% haemolysis), and one group of high haemolysis samples (26 units, all ≥0·5% haemolysis). Representative lipids were analysed from different lipid classes, including cholesterol, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and ceramide. Whole membrane preparations were analysed with one mass spectrometry technique, and lipid extracts were analysed with a second mass spectrometry technique.

Results The ratio of palmitoyl-oleoyl phosphatidylcholine (POPC) to sphingomyelin was different for the high and low haemolysis groups ($P = 0·0001$) and for the RCCs from male and female donors ($P = 0·0009$). The ratio of cholesterol to phospholipids showed only minimal links to haemolysis. Higher relative amounts of sphingomyelin were associated with lower haemolysis, and higher relative amounts of ceramides were associated with increased haemolysis.

Conclusion The level of sphingomyelinase activity and the resulting ratio of sphingomyelin to POPC is proposed as a possible marker for RCC storage quality.

Key words: storage, erythrocytes, lipid bilayers, mass spectrometry, sphingomyelin, ceramide.

Introduction

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When red blood cells (RBCs) are stored for transfusions, additives in the bags extend the shelf life of the cells, but there are still changes that take place over the course of the storage period. One of the significant changes is that cells can lyse, a change that is clearly irreversible and that constitutes one of the primary indicators of the storage quality [1]. The extent of the haemolysis increases with time and renders the unit unsuitable for transfusion if it reaches beyond a threshold level (0·8%, Europe/Canada; 1%, USA).

One of the challenges with measurements of storage quality is that RBCs from different donors can lyse at different rates. It is therefore of interest to find markers that are linked to the haemolysis; this could aid in prediction of the storage quality at the beginning of the storage period and potentially provide a foundation to build on for storage quality improvements. The levels of ATP and other metabolites in stored RBCs have been found to have an inheritable component [2], but a large-scale study on the metabolites from RBCs collected from donors previously shown to have either low or high haemolysis did not establish donor-specific clustering of the measured metabolite levels [3].

The RBCs that are collected from female donors have, on average, better storage quality [4-6]. There is a distribution of haemolysis values for RBCs from both male and female donors at the end of the storage period, but a large-scale study has shown that this distribution is shifted towards lower haemolysis values for RBCs from female donors [4]. The reason for the sex-related difference in the haemolysis of stored RBCs has been attributed variously to differences in the levels of progesterone [6] and testosterone [4], but these publications addressed the biological cause of the difference, rather than specific structural variations in the RBC membrane that could be directly linked to haemolysis. The RBC membrane lipids are primarily cholesterol and phospholipids, so that it is unlikely that hormones play a direct structural role in the lipid bilayer. It therefore remains to identify the structural basis of the variable haemolysis.

Haemolysis can involve both the release of free haemoglobin or else the release of small vesicles that can bleb off from the cell surface [7,8], but all forms of haemolysis will necessarily involve disruption of the red blood cell membrane. In this work, we therefore take the simple and direct approach: we assume that variation in the lysis of the RBC membrane is associated with variation in the membrane structure, and therefore investigate the membrane lipids. The lipid composition of the RBC membrane is assessed at the end of the storage period and is compared for RBCs from low and high haemolysis samples and for RBCs from male and female donors.

The lipid bilayer portion of the RBC membrane is approximately 45 mol% cholesterol and 55 mol% phospholipids; the phospholipids are about 29% phosphatidylcholines (PC), 27% phosphatidylethanolamines (PE), 25% sphingomyelins (SM), 15% phosphatidylserines (PS), and a small percentage of other lipids classes [9]. The category of lipid is determined by the hydrophilic head group in the case of PC, PE and PS, and by the presence of sphingosine in the case of SM. For PC, PE and PS, the lipids also have two acyl chains that can vary in length and in number or position of the double bonds; for SM, the sphingosine can vary, as can the one acyl chain present. For the work presented here, representative lipids were chosen from each category based on the distribution of acyl chains known to be present in RBC membrane lipids [9].

Materials and methods

Blood samples

The RCCs were prepared by the German Red Cross Blood Service of Baden-Württemberg – Hessen. Whole blood is collected by weight with an allowed range of 450–505 g. The blood is then anticoagulated with ACD (acid citrate dextrose solution) and processed by buffy coat removal and leucocyte filtration to reduce leucocytes and platelets. The haematocrit of the initially collected whole blood is not measured, but the volume of the final RCCs, which have a haematocrit (volume fraction) of 0·60 ± 0·03 l/l, is recorded; the RCC volume is an indirect measure of the initial whole blood haematocrit. The RCCs were stored in PAGGSM for 42 days at 2–6°C, after which the haemolysis was measured by the quality control (QC) laboratory that monitors 1% of the units collected in the Baden-Württemberg—Hessen region. Free haemoglobin (Hb) was measured following the three wavelength method [10], using a kit from Bioanalytic (Umkirch/Freiburg, Germany); the haemolysis was then calculated using the free Hb values together with haematocrit and total Hb determined using a CELL-DYN Ruby haematology analyser (Abbott Diagnostics, Abbott Park, IL, USA).

Groups of high and low haemolysis samples were obtained from the QC laboratory as described for a previous work that analysed RBC morphology [11]. The QC laboratory identified low and high haemolysis samples each week for five weeks of its routine analysis. The lipids were prepared from these samples as described below within 2 days of the haemolysis measurements and stored frozen. In total, we collected lipids from 26 units of high haemolysis samples, all with haemolysis ≥0·5%, and from 24 units of low haemolysis samples, all with haemolysis ≤0·15%. Of these samples, 22 came from female donors and 28 came from male donors. Donors self-identified as male or female and we therefore use the term gender when referring to our samples. A total of 636 units was assessed by the QC laboratory. Characteristics including blood type and storage bag are given in the Tables S1-S3. The distribution of haemolysis values for the low and high haemolysis groups is given in the results section. Ethical approval for the experiments described here was granted by the Medical Ethics Committee of the Medical Faculty Mannheim, Heidelberg University.

Quality control laboratory data

The quality control laboratory data for one year (1 year and three weeks) for the Baden-Württemberg—Hessen region was used for comparison of the 50 high–low haemolysis samples with the general population and also for additional analysis.

Preparation of RBC ghosts for mass spectrometry

The RCCs from the high and low haemolysis groups were centrifuged briefly so that a fixed volume of packed cells could be removed for preparation of washed membranes. The packed RBCs (300 µl) were washed 4× in water, with centrifugation of the lysed cell membranes for 10 min at 2500 g. After the final wash, 100 µl of the pellet was collected. The RBC ghosts were prepared each week that the samples were obtained from the QC laboratory and then frozen. Samples were then thawed for the matrix-assisted laser desorption–ionization-time-of-flight mass spectrometry (MALDI-ToF), described below and in the supporting information.

Preparation of lipid extracts for HPLC coupled to mass spectrometry

Lipid extracts were prepared for analysis by HPLC coupled to electrospray ionization quadrupole–time-of-flight mass spectrometry (ESI-Q-ToF). The RCCs from the high and low haemolysis groups were washed 4× in phosphate-buffered saline prepared from tablets (VWR), and 20 µl of packed RBCs was collected for the lipid extracts. Lipids were extracted as described elsewhere [9], with minor modifications. Methanol (100 µl, Merck) was added to the packed RBCs in microcentrifuge tubes, mixed and left for 30 min; at the end of the 30 min, 100 µl of chloroform was added, and the tubes were mixed by vortexing and then left for another 30 min. The liquid phase was then collected, and the solvents were added again to the precipitate for a second extraction, but with the methanol and chloroform added together and then left for 30 min. The collected supernatants were then centrifuged a second time to remove unwanted fragments of precipitate. Additional chloroform was then added (320 µl) along with 80 µl of 50 mM KCl; tubes were mixed and then allowed to sit overnight at 6°C; the tubes were then centrifuged; 320 µl was collected from the bottom phase, dried under N_2 and stored dry at −20°C.

Analysis of RBC ghosts by MALDI-ToF

The RBC ghost preparations were collected and then analysed at the same time by matrix-assisted laser desorption–ionization time of flight mass spectrometry, or MALDI-ToF, as described in more detail in the supporting information. The MALDI-ToF analysis provides high-resolution m/z values but without the potential for further fragmentation of the parent ion. Lipids known to be present in high quantities such as cholesterol and POPC can be identified, but it is not possible to distinguish between structures that have the same molecular weight.

Analysis of lipid extracts by ESI-Q-ToF

The ESI-Q-ToF is a tandem mass spectrometer that can fragment the initially obtained parent ions, thus providing robust identification of the observed peaks. One part of the ESI-Q-ToF analysis was to confirm the identity of the peaks at m/z values of 703·56 and 725·56 as determined by MALDI-ToF, while a second part was to obtain relative concentrations of representative lipids from the different phospholipid groups. Experiments were done in positive ion mode and were therefore unsuitable for analysis of phosphatidylserines or phosphatidylinositols. For phosphatidylcholines, POPC was analysed; for phosphatidylethanolamine, PE(16:0/18:1); for sphingomyelins, SM(d18:1/16:0). The selection of the PE and the SM was based on the known frequency of the fatty acyl chains. The SM(d18:1/16:0) was identified as the peaks at 703·56 (the [M + H]$^+$ peak) and at 725·56 (the sodium adduct), based on elution times and fragmentation patterns. The PE was identified based on the presence of the diacylglycerol-like fragment at the m/z value corresponding to the parent ion (m/z of 717·53) minus the PE head group. A ceramide (Cer(d18:1/20:1), from Merck/Avanti) was used as a reference standard.

Statistical analysis

Results are presented as comparisons between pairs of defined sample groups. The Wilcoxon–Mann–Whitney U-test was used to check for systematic differences between the pairs of sample groups, using the calculations on the Vassar website (http://vassarstats.net/utest.html). The two-sided P values are reported with no correction for multiple tests. The values are reported in the captions of the associated figures and are also summarised in tables in the supporting information. The increased probability of having a false positive due to multiple tests is discussed briefly.

Changes in the lipid ratio as a function of time

A small-scale study with 12 units of RCCs stored in SAGM was done to assess the changes in the lipid composition as a function of time. Lipids were extracted at weeks 1 and 5. Additional details are provided in the Supporting Information.

Results

Haemolysis values in the samples and in the overall population

The first requirement of this work is to identify sample sets with good and poor storage qualities, which is to say low and high haemolysis. The distribution of the haemolysis values in the low and high haemolysis groups is shown in Fig. 1a. When the haemolysis values are grouped according to whether the donor was male or female, as shown in Fig. 1b, it can be seen that the low haemolysis group is predominantly from female donors, and the high haemolysis group is predominantly from male donors. The exaggerated difference between RBCs from male and female donors is due to the collection of the samples from the extreme ends of the range of haemolysis values: when the comparison is made for the general population, as in Fig. 1c, it can be seen that the difference is less pronounced. The difference in the distribution of haemolysis values between male and female donors is shown in Fig. S1 of the supporting information and corresponds well with the difference that has been reported for RBCs stored in SAGM [4].

Analysis of lipids with mass spectrometry

The analysis and identification of the lipids is described in detail in the supporting information. The MALDI-ToF peaks of interest were at m/z 369·35 for cholesterol (from the ion $[M + H-H_2O]^+$, due to cholesterol after loss of water), m/z 760·57 (POPC), and a pair at m/z 703·56 and m/z 725·56, attributed to SM(d18:1/16:0) and the sodium

adduct, $[M + Na]^+$. An additional unidentified peak at m/z 616·18 is discussed in the supporting information. The lipids identified and analysed by ESI-Q-ToF were POPC (parent ion m/z 760·58), PE(16:0/18:1) (m/z 718·54), SM (d18:1/16:0) (m/z 703·57), cer(d18:1/24:0) (m/z 648·63) and cer(d18:1/20:0) (sodium adduct, at m/z 616·56). Figures in the results section show changes in lipid ratios; separate values are shown in Figs S2 and S3 of the Supporting Information.

The POPC:sphingomyelin ratio

Figure 2 shows the differences in the ratio of POPC to sphingomyelin for the high and low haemolysis groups, and for comparisons of male and female donors. The differences are significant in all cases and are consistent for the two different sample preparations and measurements techniques (lipid extracts measured by ESI-Q-ToF, and whole membrane preparations measured by MALDI-ToF).

The ceramide:sphingomyelin ratio

The ratio of ceramide content to sphingomyelin showed some difference between the high and low haemolysis groups, as shown in Fig. 3, with a significant difference seen for the cer(d18:1/24:1), but not for the cer(d18:1/20:0) sodium adduct. No differences were seen between male and female donors (Fig. 3, bottom row).

Ratios of cholesterol to PC, PE and SM

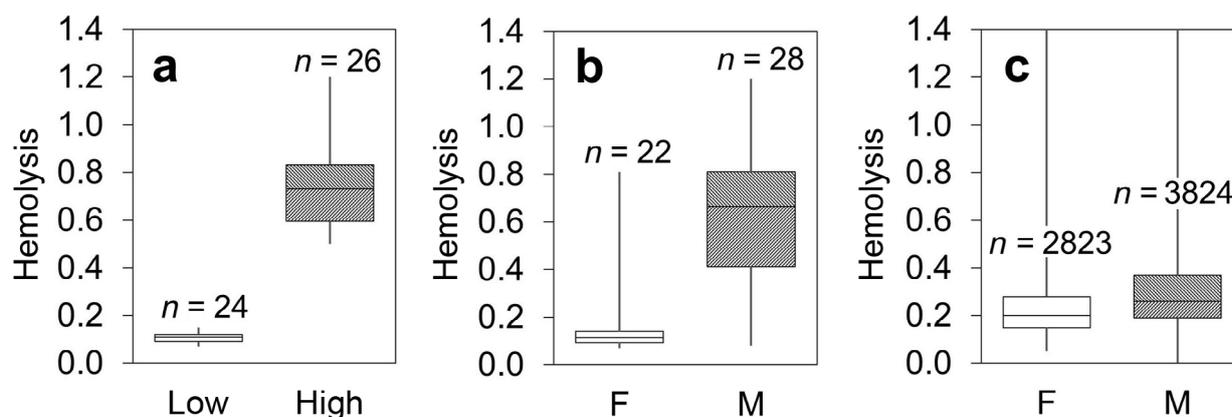The measurements with the ESI-Q-ToF showed that the ratio of cholesterol to representative lipids from three

Fig. 1 (a) Comparison of haemolysis values for the high and low haemolysis groups of the high–low haemolysis sample set, (b) comparison of haemolysis for male and female donors from the high–low haemolysis sample set and (c) comparison of haemolysis for male and female donors for the overall population. The midline of the boxes indicates the median and the top and bottom ends indicate the quartiles. For graphs a and b, the whiskers show the maximum and minimum values. Graph c is shown with the same axes, for comparison; the maximum and minimum reported haemolysis values were, respectively, 6·1% and 0·05% for the female donors and 8·24% and 0% for the male donors. For the comparisons in graphs a and c, the Mann–Whitney *U*-test showed $P < 0·0001$; for graph b, $P = 0·0004$.
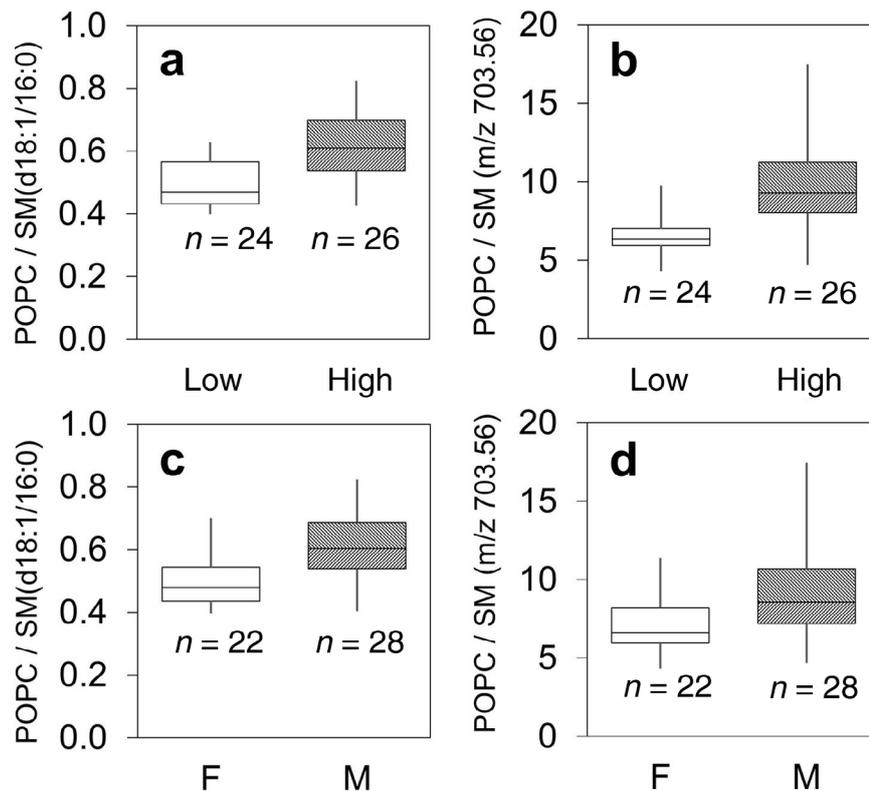
Fig. 2 Ratio of the mass spectrometry peak signals for POPC and a sample SM, showing the link between the POPC: SM ratio and the RBC storage quality. Ratios are of peak areas for ESI-Q-ToF (a, c) and peak heights for MALDI-ToF (b, d). The ratios are different for the low and high haemolysis groups of the high–low haemolysis sample set, for both ESI-Q-ToF analysis of the lipid extracts (a, $P = 0.0001$) and the MALDI-ToF analysis of the whole membrane preparation (b, $P < 0.0001$). When the POPC:SM ratio is compared for female and male donors, the values are also different, in a manner consistent with the known differences in storage quality that are illustrated in Fig. 1 (c, ESI-Q-ToF analysis of the lipid extracts, $P = 0.0009$, d, MALDI-ToF analysis of the whole membrane preparation, $P = 0.0045$).

classes of phospholipids had no variation with the high and low haemolysis groups, or with donor gender, as illustrated in Fig. 4 (all P values ≥ 0.07, see figure caption). The measurements made with MALDI-ToF showed small differences in lipid ratios for the low and high haemolysis groups ($P = 0.021$ for cholesterol/POPC and $P = 0.066$ for cholesterol/SM), and in the comparisons for male and female donors ($P = 0.050$ for cholesterol/POPC and $P = 0.441$ for cholesterol/SM), as shown in Fig. S4 of the supporting information. The differences in the cholesterol:POPC ratio for the MALDI result would be significant if considered as a single experiment ($P < 0.05$).

Storage quality of RCCs from female donors not due to low haematocrit

One possibility is that the improved storage of RBCs from female donors is due to the lower haematocrit of the initially collected blood, an effect that could be associated with a higher turnover of the RBCs and hence a younger average age of individual cells. If this were the case, it

should be possible to find a link between lower haematocrit and improved storage quality. Although haematocrit of the collected blood is not measured directly, it can be inferred from the volume of the processed RCCs, together with the RCC haematocrit. If the haematocrit of the initially collected blood is low, then the RCCs, which are processed to produce a defined haematocrit range, will have a lower volume. Fig. 5 uses the quality control laboratory data for the year and compares the product of the RCC volume and RCC haematocrit for low and high haemolysis groups. The division into low and high haemolysis is arbitrary, and has been done here to match the values used for the group of 50 samples, with the low haemolysis group being all the samples with haemolysis $\leq 0.15\%$, and the high haemolysis group being all samples with $\geq 0.5\%$.

When the low and high haemolysis groups are compared for the whole population, it can be seen from Fig. 5a that the low haemolysis group does indeed have a lower initial haematocrit ($P < 0.0001$) (more precisely, a lower total volume of initially collected RBCs). When the
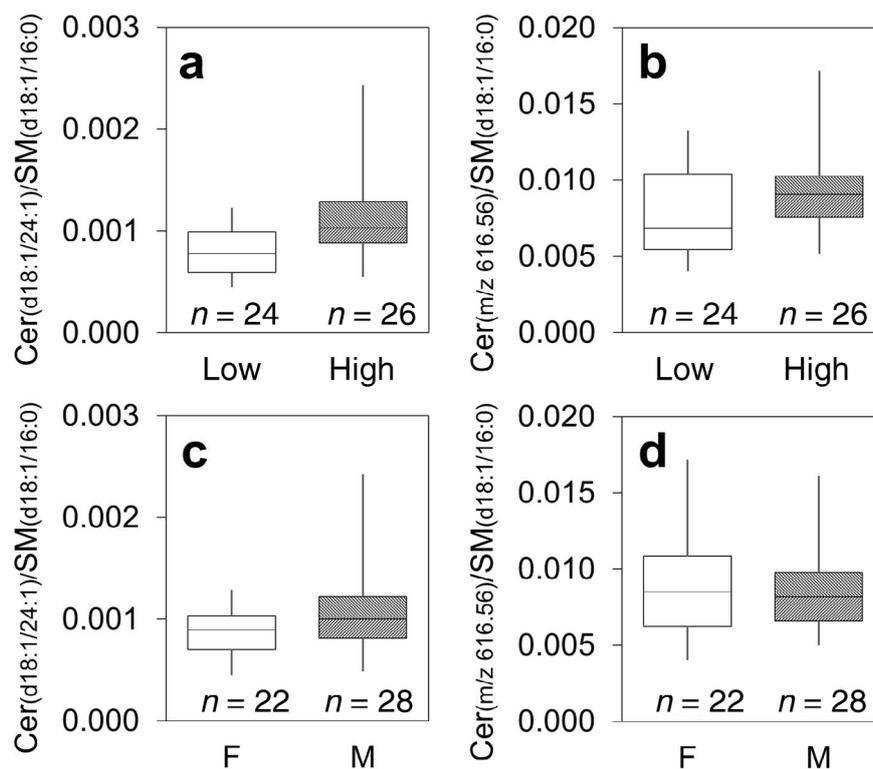
Fig. 3 Ratio of the mass spectrometry peak areas for ceramides and a sample sphingomyelin. Graphs a and b, top row, show ratios indicated on the y-axes comparing the high and low haemolysis groups, for ESI-Q-ToF of lipid extracts; *P* values were, respectively 0·0013 and 0·114. Graphs c and d, bottom row, show comparisons for male and female donors, for ESI-Q-ToF of lipid extracts; *P* values were, respectively, 0·126 and 0·795. The ceramide at m/z 616·56 was identified as the sodium adduct of cer(d18:1/20:0).

results are graphed separately for male and female donors Fig. 5, however, this effect diminishes for RBCs from male donors ($P = 0.018$) and vanishes for RBCs from female donors ($P = 0.596$). It therefore appears that haematocrit is not linked directly to storage quality.

Changes in the lipid ratio as a function of time

A small-scale study with 12 units of RCCs assessed at weeks 1 and 5 of the storage period, as described in the supporting information, showed that the extracted POPC remained constant but that the extracted SM decreased, thus increasing the POPC/SM ratio (Fig. S5).

Discussion

The POPC to SM ratio shown in Fig. 2 was different for the high and low haemolysis groups, for both the lipid extract ($P = 0.0001$) and the whole membrane analysis ($P < 0.0001$). The POPC to SM ratio also differs for RBCs from male and female donors, in a manner that reflects the observed differences in haemolysis. The ratio of cholesterol to phospholipids from three different

categories showed very little association with the haemolysis level or with donor gender as shown in Fig. 4, and the link between haematocrit of the initially collected blood appears to be indirect and diminishes or disappears when the results are separated by donor gender as shown in Fig. 5. The ratio of POPC to sphingomyelin therefore appears to be the most significant factor identified here for both the differences in haemolysis and the donor gender differences in storage quality.

It has been noted previously that the sphingolipid content of RBCs from male and female donors differs, with RBCs from females having higher levels of some sphingomyelins [12], a result that is consistent with the ratios presented in Fig. 2. One possibility is that the increased sphingomyelin content makes the membrane stronger and therefore less prone to haemolysis. Sphingomyelins increase the order in model lipid bilayers [13] and are also associated with the formation of the more ordered structures of lipid rafts in vivo [14]. Vesicles prepared with sphingomyelin and cholesterol are less prone to rupture than vesicles of phosphatidylcholine and cholesterol [15].

A second possibility is that association between sphingomyelin and haemolysis is less direct and relates to their
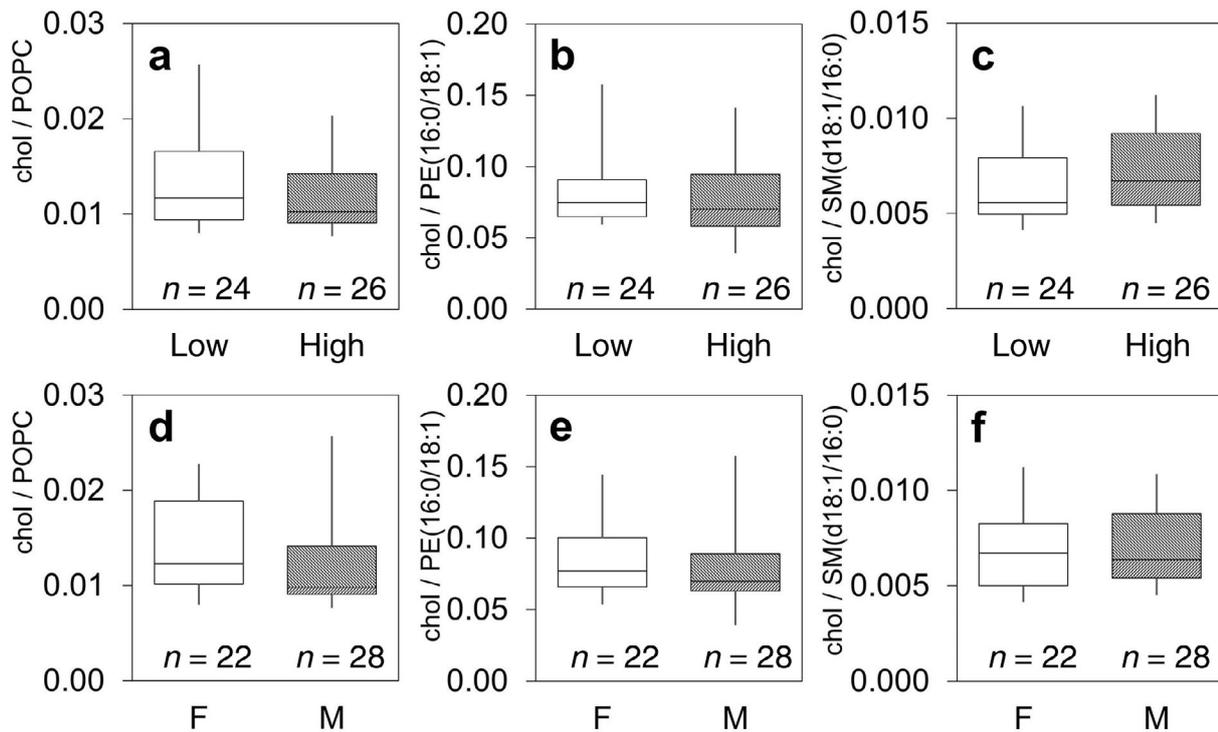
Fig. 4 Cholesterol to phospholipid ratios for the high and low haemolysis groups and for RBCs from male and female donors, showing that there is no apparent link between RBC storage quality and the ratio of cholesterol and the three phospholipids tested, and also no link between donor gender and the cholesterol to phospholipid ratios. All lipid measurements were made with the ESI-Q-ToF mass spectrometer. Graphs a–c, top row, show comparisons of high and low haemolysis groups for (a) cholesterol:POPC, (b) cholesterol:PE and (c) cholesterol:SM; P values were, respectively, 0·298, 0·211 and 0·114. Graphs d-f, bottom row, show comparisons of male and female donors, for (d) cholesterol:POPC, (e) cholesterol:PE and (f) cholesterol:SM; P values were, respectively, 0·070, 0·144 and 0·589.
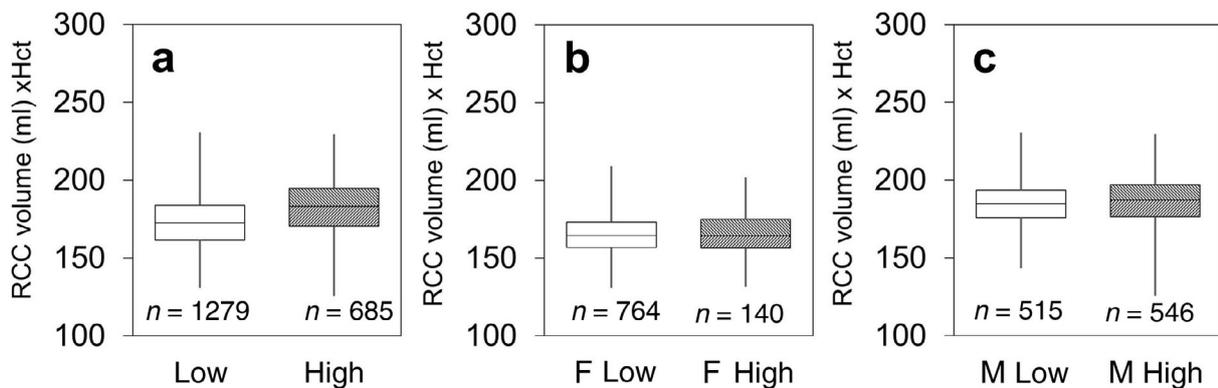

Fig. 5 Product of RCC volume and RCC haematocrit (a measure of haematocrit of initially collected blood) for low haemolysis (≤0·.15%) and high haemolysis (≥0·.5%) samples, for a, the whole population (P < 0·0001); b, female donors (P = 0·596); c, male donors (P = 0·018).

role in the production of bioactive species. Sphingomyelins are hydrolysed by the enzyme sphingomyelinase to produce ceramides, which have been linked to a form of RBC death that is analogous to apoptosis [16,17]. Fig. 3 shows differences in the ratio of one ceramide to sphingomyelin but not a second ceramide and no differences for male and female donors. Ceramides cause lipid flip-flop and may cause the coalescence of lipid domains, leading to lysis [18]. The lower levels of sphingomyelin in RBCs from male donors could be due to higher levels of sphingomyelinase activity, which could then also be associated with increased ceramide formation

and the associated haemolysis. We note in passing that if the formation of ceramides does cause relatively rapid haemolysis in cells, then the presence of ceramide would have been under-reported due to the sample preparation used here: cells that had already lysed would have been lost during the initial washing step. The measurements made at weeks 1 and 5 in the smaller study (Fig. S5) imply that the differences in the sphingomyelin levels are generated over the course of the storage period, implying that the sphingomyelinase activity has a significant effect on the end of storage haemolysis.

If the ratio of phosphocholines to sphingomyelins is associated with the haemolysis of RBCs, then it remains to be seen what use can be made of this information. Analysis of the lipid ratios would not be sufficient to identify all of the high haemolysis donors and would not be helpful if the characteristic ratios only appear later on in the storage period. The other reason for identifying causes of high haemolysis is, of course, to use the information to make general improvements to the RBC storage quality; it is possible that information about sphingomyelins and sphingomyelin metabolism could be employed in this regard. The amounts of some sphingolipids present can be increased if they are collected from fasting subjects [12]; sphingomyelinase activity is higher in the RBCs of people who drink more alcohol [19,20]; the activity of sphingomyelinase has been characterised [21] and could possibly be inhibited; the formation of ceramides and ceramide-related RBC death has also be characterised, along with possible inhibitors [17]; ceramide domains have been associated with haemolysis occurring after cooling [22]. A ceramide-mediated haemolysis could also account for the improved RBC storage in isotonic PAGGSM relative to the storage in the hypertonic SAGM [23]: the formation of ceramides is linked to the osmotically induced cell shrinkages [17],

and so the lower osmolarity of the PAGGSM could therefore account for its improvement of the RBC storage. Future research could therefore focus on the role of sphingomyelinases in RBC storage quality, or on the incorporation of the known aspects of sphingomyelin metabolism into the search for methods to improve the storage quality of RBCs.

Acknowledgements

Funding

Conflict of interest

The authors have no conflict of interest.

Author contributions

KAM designed experiments, prepared samples, analysed data and wrote the paper; MM, FK and GBW prepared samples and collected and interpreted mass spectrometry data; KB arranged sample acquisition and represents the QC laboratory for this work. All authors reviewed the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article: