Terminal maturation of human reticulocytes to red blood cells by extensive remodelling and progressive liquid ordering of membrane lipids

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ABSTRACT

The terminal differentiation of reticulocytes to mature red blood cells (RBCs) in mammals is a topic of intense investigation. Although much has been learned about the early phase of reticulocyte maturation in the bone marrow of the adult mammal, after enucleation of the orthochromatic erythroblast, the final maturation of peripheral circulating reticulocytes into RBCs is still poorly understood. . Research on reticulocyte maturation was predominantly carried out on stress reticulocytes, or mixed populations of RBCs and normal reticulocytes, strongly limiting the possibility of discriminating different maturational phases and the relative underlying mechanisms. Another limitation was a somewhat "proteinocentric" approach, whereby possible qualitative and quantitative changes in the most important structural component of the membrane, the lipids, were not investigated.

In the marrow, the reticulocyte matures through processes intrinsic to the cell (autophagy, ubiquitin/proteasome-dependent proteolysis, exosome release, regulated volume decrease) to reduce its size and simplify its composition. In the circulation, the task is taken over by systemic intervention that is necessary for the removal of extra surface area and to stimulate further volume decrease. The last two processes are preliminary to and go along with the completion of the assembly of the membrane skeleton and its anchoring to the bilayer into a structurally and functionally stable plasma membrane. The selective membrane removal in the marrow phase of maturation leads to the loss of most of the transferrin receptor through release of exosomes enriched in membrane rafts. In the circulatory phase, membrane area decrease is obtained by (a) different mechanism(s), as indicated by several pieces of evidence, including the results of the present study

We have isolated here two populations of circulating reticulocytes at different levels of maturation in vivo, and three subpopulations of RBCs of different age from normal human donors, and characterized the evolution of their lipidome. Sphingomyelin, cholesterol and in part phosphatidylethanolamine increase in relative terms, whereas phosphatidylcholine and phosphatidylserine decrease from immature reticulocytes to mature RBCs, at the same time as the surface area per cell decreases. Moreover, the relative amounts of the more than 70 phospholipid subclasses evaluated in the study, based on the number of carbon atoms (12-24) and of double bonds (0-6) in the fatty acids linked to the phospholipid, also change in the process. This complex remodeling suggests the presence of an underlying blueprint, whereby changes in each individual phospholipid subclass all seem to converge in producing a state of higher liquid order, and thus of higher stability, in the lipid bilayer. Results also strongly hint at the lipid remodeling as the driving force, together with cell shrinkage, for the organization of spectrin in an extended meshwork and its anchoring to the bilayer through vertical interactions with intrinsic membrane proteins. A model is proposed, based on concepts of lipid interdigitation and protein “pinning”.

INTRODUCTION

The terminal differentiation of reticulocytes to mature red blood cells (RBCs) in mammals is a topic of intense investigation since the beginning of RBC research[1]. It is generating renewed interest in recent years[2-7] in connection with the possibility of culturing human RBCs in vitro for transfusion purposes[8-13], also aided by "omics" approaches, aiming at re-evaluating these cells' properties in health and disease[14-21]. Much has been learned about the early phase of reticulocyte maturation in the bone marrow of the adult mammal, after enucleation of the orthochromatic erythroblast, which is largely sustained by processes intrinsic to the cell. However, not much is known about the terminal development of a circulating reticulocyte into a mature RBC. Two main problems have curbed advancements in this field: on the one hand the limited availability of reticulocytes at a synchronized stage of differentiation. Studies have dealt most often with "shift" reticulocytes[22], obtained from humans with natural or induced reticulocytosis, or with "stress" reticulocytes[23], by boosting reticulocyte counts in animals up to 90% of total red cells by phlebotomy or pharmacological treatment[24]. These cells may be synchronized, in a sense, but far from representative of circulating reticulocytes[25] or of "normal" reticulocytes at all[26]. Reticulocyte enrichment by gradient centrifugation, although yielding "normal" reticulocytes, only affords limited purification, thus affecting accurate biochemical characterization of the true circulating reticulocyte[27]. Cord blood also provides significant amounts of reticulocytes, that are, however, again different from the normal adult counterpart[28,29]. Finally, anucleated cells obtained with variable efficiencies by culturing erythroid precursors in vitro are but immature reticulocytes, probably akin to bone marrow reticulocytes at a very early stage of maturation. As a result, the issue as to whether peripheral circulating reticulocytes could mature into "normal" RBCs in vitro is controversial[30].

The second problem lies in a relatively "proteinocentric" approach focused on the characterization of RBC protein expression during erythropoiesis with ever increasing accuracy to the present day[2,31,32], while overlooking, with a few exceptions[33], the lipids as the other essential constituents of cell membranes. This occurred despite the availability of an extensive literature on RBC lipids, whose origin well predates the description of even the earliest functionally characterized RBC membrane protein[34], such as Band 3[35] or spectrin[36], and also despite the fact that the RBC membrane served as a model for the discovery of the asymmetric transverse localization of glycerophospholipids and sphingolipids in the two leaflets of the bilayer[37-39] that was later recognized as a feature of the plasma membrane in all

eukaryotic cells[40]. Furthermore, the current view of the RBC membrane structure and biogenesis is the result of a vast number of studies where single proteins were extracted and purified to ensure absence of any bound lipid. The proteins were then tried for their reciprocal binding, mostly in binary or tertiary combinations. The lipid component was absent, or served as a mere scaffold for protein-protein re-binding assays, as with stripped vesicles or ghosts[41]. As RBCs age in the circulation, they lose membrane surface area and volume in a coordinated manner, so as to retain a remarkably constant surface-to-volume ratio, and avoid premature clearance because of loss of deformability due to sphericization[42]. It is thought that the cell-age related membrane loss occurs through the spontaneous release of vesicles. By analogy with the vesicles that can be obtained in vitro by stimulating RBCs with various manoeuvres, and that are devoid of membrane skeleton[43,44], it is common belief that RBCs release spectrin-free vesicles also in vivo. On the other hand, loss of membrane surface by early reticulocytes maturing in the bone marrow was described to occur via the release of exosomes[45] enriched in transferrin receptor (TfR, CD71)[46,47], and virtually free of Band 3, glycophorin A and, very likely, also spectrin (although this was not directly evaluated,[48,49]). However, we have recently shown[50], and confirm it here, that circulating reticulocytes lose Band 3 and spectrin, as do mature RBCs for the rest of their 120-days circulatory life[51,52]. Membrane rafts appear to be selectively lost with the exosomes released by (stress) reticulocytes in rats, as their membrane is enriched in specific raft proteins[53] and is in the sphingomyelin-rich liquid-ordered state typical of rafts[54]. As reticulocytes mature, therefore, their membrane should become more and more depleted in membrane rafts. However, sphingolipids and cholesterol are largely retained in the membrane of circulating reticulocytes (at the same time that total surface area decreases) to end up enriched in mature RBCs[55]. This evidence points to a significant difference in the mechanisms that regulate the two phases of reticulocyte maturation, in the bone marrow first and then in the circulation, that was not acknowledged earlier because the cell population under study was almost invariably comprised of very immature reticulocytes, more similar to those found normally in the bone marrow than in the circulation. To circumvents these problems, and complete the analysis of membrane lipids over the entire 120 days of the cell life span, we have isolated here two populations of circulating reticulocytes at different levels of maturation in vivo, and three subpopulations of RBCs of different age from normal human donors, and characterized their lipid composition. All the major lipids classes (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine and cholesterol) change in their relative amounts as the cell develops from an immature reticulocyte to a mature RBC and further on to an old RBC. Moreover, the

relative amounts of the more than 70 phospholipid subclasses evaluated in the study, based on the number of carbon atoms (12-24) and of double bonds (0-6) in the fatty acids linked to the phospholipid, also change in the process, at the same time that membrane surface area decreases. This complex remodelling suggests the presence of an underlying blueprint, whereby changes in each individual phospholipid subclass all seem to converge in producing a state of higher liquid order, and thus of higher stability, in the lipid bilayer. Results also strongly hint at the lipid remodelling as the driving force, together with cell shrinkage, for the organization of spectrin in an extended meshwork and its anchoring to the bilayer though vertical interactions with intrinsic membrane proteins. A model is proposed based on concepts of lipid interdigitation and protein “pinning”.

RESULTS

CELL TYPES

We have isolated, starting from a single sample of blood, two populations of $CD71^+$ reticulocytes, Retic 15 and Retic 45, representative of ≈0.1-0.2% more immature and ≈0.4-0.5% more mature circulating reticulocytes. Additionally, from the reticulocyte-depleted population of RBCs we have separated three subpopulations of young (≈5%), middle-age (93.5%) and old (≈1.5) RBCs, and confirmed their different age by analysing their protein 4.1a/4.1b ratio (See Materials and Methods). The young RBCs may be considered in their first week of life, during which they have matured from reticulocytes, whereas old RBCs in their last few days of life, after 17 weeks in the circulation. See also Discussion.

SIZE AND SHAPE

When glutaraldehyde-fixed cells[56] are examined, without staining, by light microscopy, a small but statistically significant ≈4.0% decrease in cell diameter from Retic 15 to Retic 45 and of ≈7.7% from Retic 15 to mature RBCs is measured. By scanning electron microscopy, almost all biconcave discocytes are visible, with a few misshaped cells mostly present in the Retic 15 samples (**Figures 1 and 2**). The reticulocytes are almost indistinguishable from mature RBCs from the morphological point of view.

SPECTRIN AND BAND 3

Spectrin and Band 3 are representative of, respectively, peripheral and integral proteins of the RBC membrane. Band 3, together with glycophorin A, the two most abundant integral membrane proteins, appear to be completely retained in the reticulocyte that matures in the bone marrow, at the same time as the cell releases exosomes enriched in TfR[46]. Whether additional mechanisms could be at play in the bone marrow that contribute to the reduction in surface area of the reticulocyte is not known. If the same conservative mechanism that saves Band 3, glycophorin A and membrane-skeletal proteins, at the expense of TfR, were at the basis of the maturation of the circulating reticulocyte, the levels of, i.a., Band 3 and spectrin should be the same when comparing the same numbers of reticulocytes and young or mature RBCs. **Figure 3** shows that this is not the case, as both proteins decrease, on a per cell basis, from the reticulocyte to the RBC.

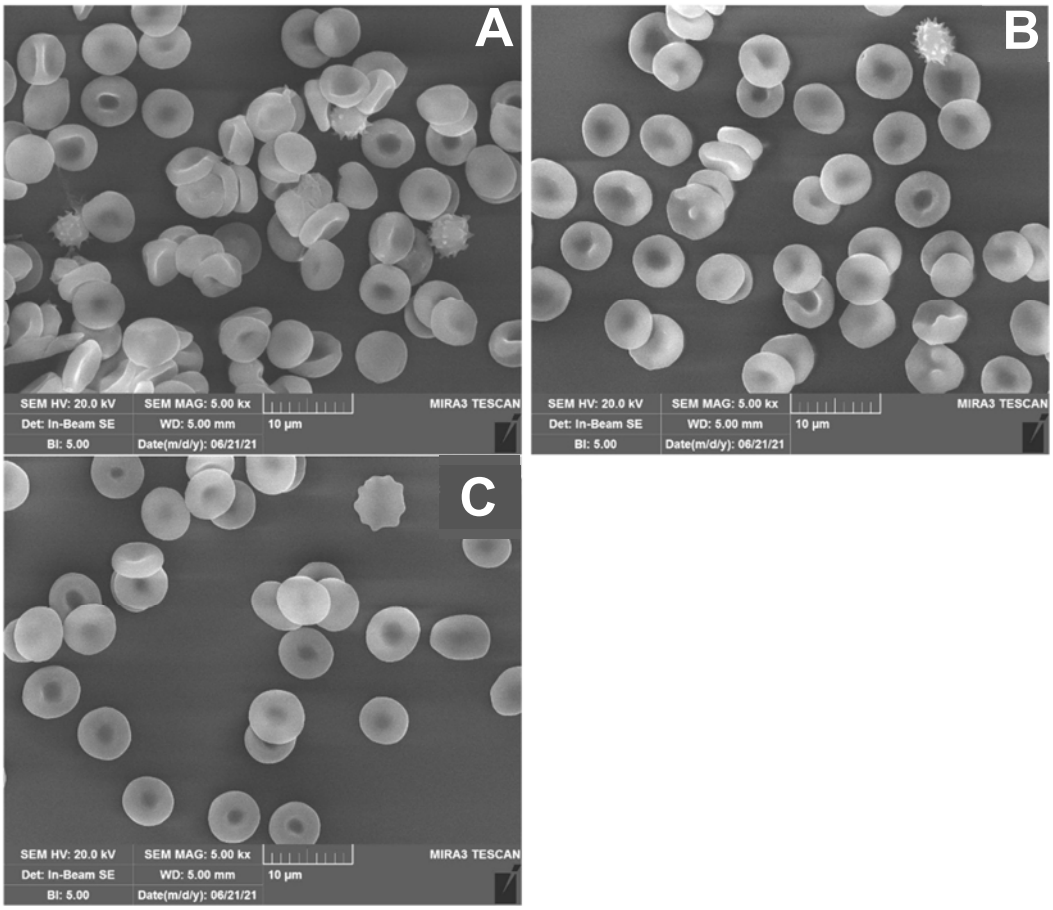


Figure 1. Scanning electron microscopy of reticulocytes and RBCs. (A) Immature circulating reticulocytes (Retic 15); (B) mature reticulocytes (Retic 45) and (C) RBCs from the total population of reticulocyte-depleted cell sample of the same donor. Images are representative of four independent experiments with similar results.

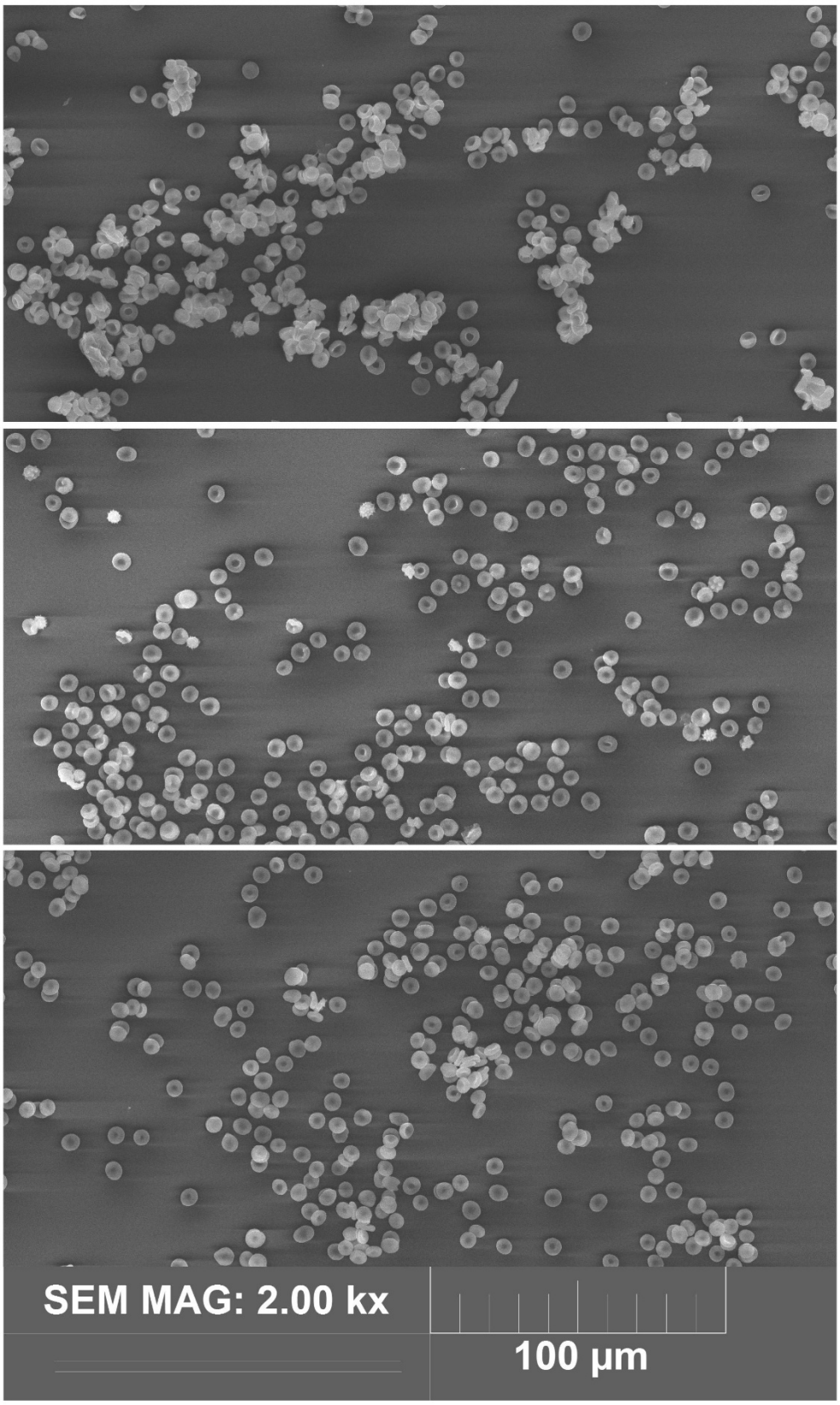


Figure 2. Scanning electron microscopy of reticulocytes and RBCs. A lower magnification field from reticulocyte samples described in the previous figure. From top to bottom, Retic 15, Retic 45 and RBCs. Immature reticulocytes did not separate from the CD71-Microbeads as easily as mature reticulocytes when suspended by pipetting, due to the higher amounts of CD71 in their membrane, so they appear aggregated in clumps.

Quantification by densitometry reveals that the ratio between Band 3 levels in Retic 15 and RBCs is ≈2.5, pointing to a loss of more than half the total Band 3 content in the process. As the decrease in surface area from circulating reticulocytes to RBCs is expected to be ≈20% (see Discussion), this result is clearly absurd, and it is related to the non-linear relationship between chemiluminescence emission and protein levels typical of the Western blotting procedure adopted (see also Materials and Methods). However, this artefact proved to be useful here to amplify differences in Band 3 and spectrin levels that would have been otherwise too small to be detected with a linear method.

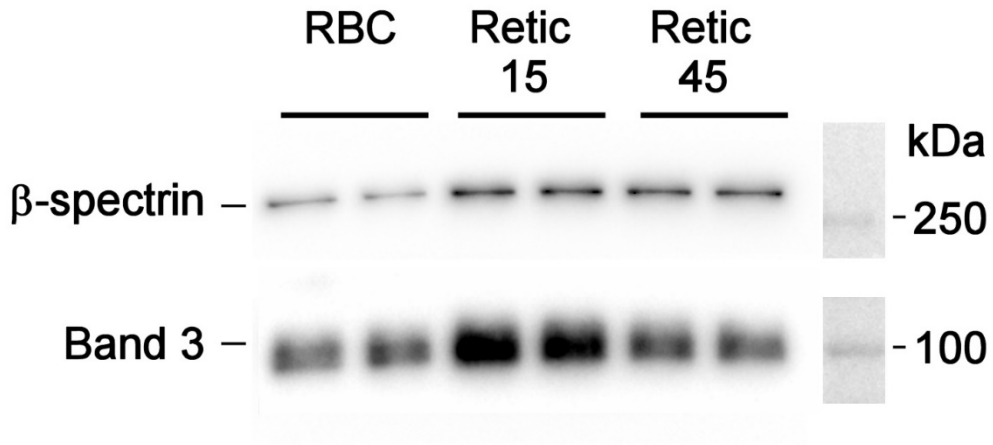


Figure 3. Band 3 and β-spectrin levels in reticulocytes and RBCs. The same number of cells were loaded for each sample, in duplicate: $2x10^5$. The blot shown is representative of four independent experiments with similar results. See text for details.

LIPIDS

Figure 4 shows the content of seven membrane lipids, as mol % of each species over total sample lipids, in the five cell populations under study. A relative increase in cholesterol, sphingomyelin (SM) and phosphatidylethanolamine (PE) (**Figure 4A, D, G**), and a decrease in phosphatidylcholine (PC) and phosphatidylserine (PS) (**Figure 4B, E**) from reticulocytes to RBCs is observed, confirming what previously reported by us in a comparison of a single population of reticulocytes with the total population of RBCs[55]. Starting from the more immature reticulocytes, changes appear to plateau at the mature RBC stage. From mature RBC to old RBC there seems to be an inversion in the trend for SM and PC. PS (**Figure 4E**) displays a continuous decrease all along maturation and ageing to the stage of old RBC, although from Retic 15 to Retic 45 an initial increase is observed. Interestingly, this trend seems to be specular to that of PE (**Figure 4D**), which, after an initial decrease from Retic 15

to Retic 45, displays then a relative increase, which continues to the stage of old RBCs (statistically significant from Retic 45 to middle-age RBCs). Although the availability of only two samples (with minimal variation between the two) prevents statistical elaborations involving Retic 15, the inversions in the trends for PS and PE are nonetheless worth investigating further. PS is the only phospholipid that displays a continuous and statistically significant decrease all along maturation from Retic 45 to old RBCs (**Figure 4E**), whereas PC, the other phospholipid that together with PS displays an overall relative decrease, tends to increase again from mature to old RBCs. This is also worth consideration, as it could reveal interesting features of RBC senescence. When cholesterol and SM are together related to total glycerophospholipids (PC+LPC+PE+PS+PI), a statistically significant increase in "raft lipids" (SM+cholesterol) with respect to "non-raft lipids" is observed (**Figure 4I**). LPC ad PI (**Figure 4C, F**) show a continuous decrease although not statistically significant, due to the high variability in the analysis of these minor components (≈1% of total phospholipids). They will not be considered further here (but see also **Figures S8 and S9)**.

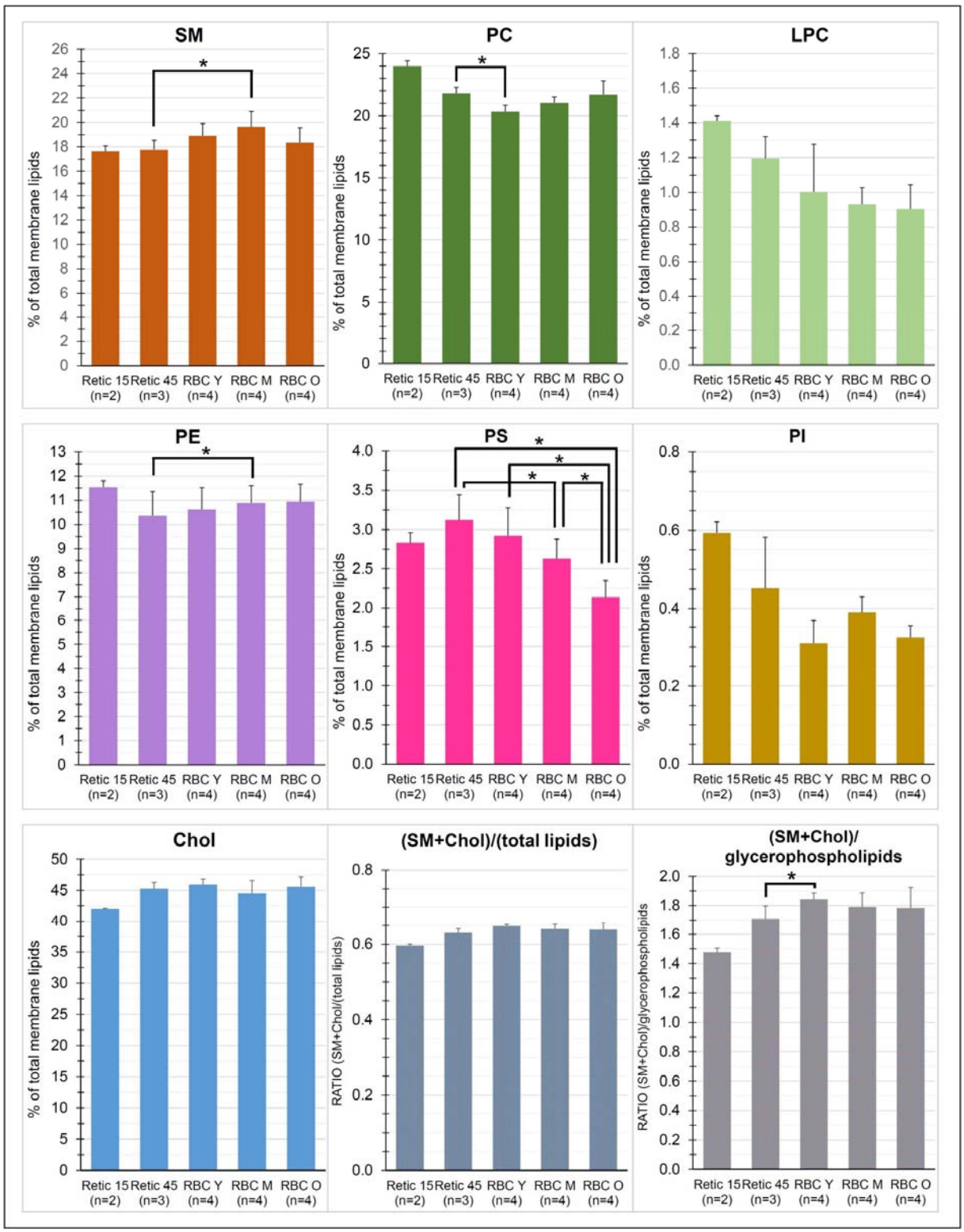


Figure 4. Analysis of the seven main lipid classes of the reticulocyte and RBC membrane. Results are expressed as mol % of each lipid with respect to total lipids in each sample. Error bars represent the standard deviation. For Retic 15, where only two samples were available for analysis, the error bars represent the difference between the mean and the individual values. The tabulated data from which the graphs were derived are available in the Supplementary material, **Figure S1**.

PHOSPHOLIPID SUBCLASSES: PHOSPHATIDYLCHOLINE

When the different subclasses of a given phospholipid are analysed, they are seen to change, to variable extents, in relative abundance in the cell populations examined. Their changes should always be read in context with the underlying change in the relative amounts of that same phospholipid with respect to total lipids, be it a decrease or an increase (**Figure 4**). **Figure 5** shows such changes for PC subclasses (for the corresponding tabulated values see **Figure S2 and S3** in the Supplementary material). Within PC, which shows an overall relative decrease from reticulocytes to RBCs, a bimodal behaviour is observed. One group of PC subclasses contains saturated species that display a relative decrease over total PC [dipalmitoyl-PC (16:0/16:0), 1-palmitoyl,2-stearoyl-PC (16:0/18:0) and 1-palmitoyl,2-oleoyl-PC (16:0/18:1)] and a second group containing all the other subclasses that show a relative increase. In **Figure 5**, the composition in PC subclasses of plasma lipoproteins, "PLASMA" and total RBCs "RBC" is also shown, taken from a literature dataset[57,58]. The PC composition of "RBC" is practically superimposable to that of our sample of middle-age RBCs. During maturation from reticulocyte to middle-age RBC, the relative amounts of a given subclass (with a few exceptions) appear to approach the content of that particular subclass in plasma, as if the change occurred along a concentration gradient (but see also Discussion). Notably, the PC subclasses composition of plasma and RBCs, with the respective differences in relative amounts, was already described in earlier studies[59], and it has been reported in a recent one[60] with virtually identical results (see **Figure S3**).

p values for the paired t test (all samples n=4 except Retic 45: n=3 and Retic 15: n=2)

	32:0	32:1	34:0	34:1	36:1	34:2	36:2	36:3	36:4	38:3	38:4	38:5	38:6	40:6	(32:0 to 34:1)
Retic 45 vs. RBC Y	0.004	0.083	0.106	0.010	0.100	0.009	0.011	0.003	0.875	0.015	0.197	0.010	0.112	0.140	0.007
Retic 45 vs. RBC M	0.000	0.034	0.054	0.005	0.052	0.004	0.001	0.001	0.180	0.006	0.144	0.038	0.111	0.090	0.002
Retic 45 vs. RBC O	0.001	0.038	0.020	0.006	0.033	0.005	0.001	0.005	0.905	0.002	0.167	0.009	0.081	0.072	0.002
RBC Y vs. RBC M	0.006	0.049	0.009	0.007	0.022	0.082	0.001	0.006	0.590	0.001	0.059	0.396	0.051	0.030	0.005
RBC Y vs. RBC O	0.004	0.160	0.007	0.013	0.001	0.055	0.002	0.011	0.876	0.003	0.178	0.347	0.992	0.037	0.007
RBC M vs. RBC O	0.001	0.199	0.054	0.291	0.521	0.709	0.039	0.050	0.486	0.032	0.836	0.838	0.273	0.391	0.029

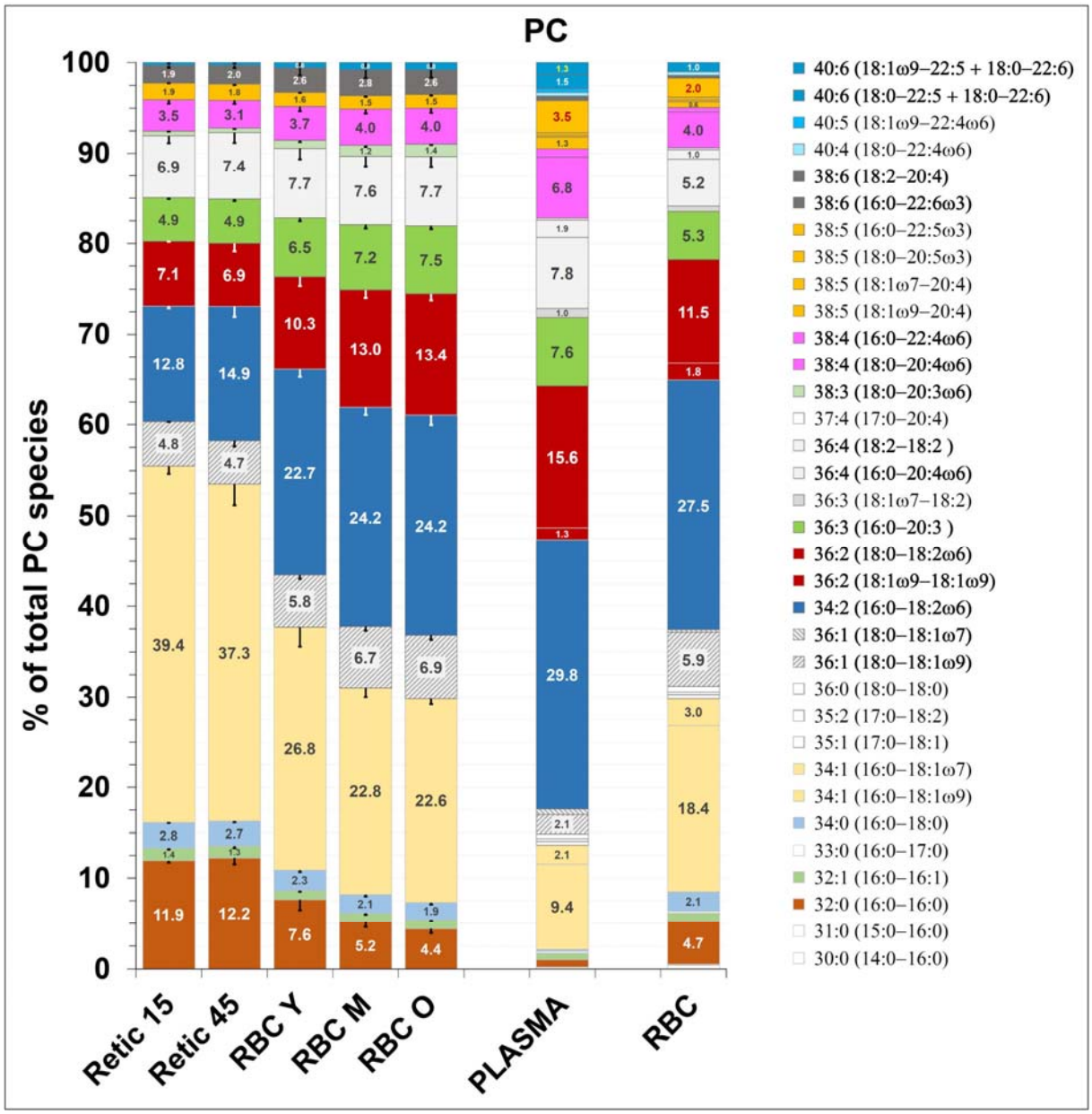


Figure 5. Diacyl PC subclasses in reticulocytes and RBCs. PC subclasses, as mol % of total PC in a given sample (as indicated by numbers inside the histogram bars) are presented with arbitrary colour that will be used consistently for the corresponding phospholipid subclass of diacyl PE, when present (see **Figure 8**). The PC composition of samples "PLASMA" and "RBC" is taken from[57,58], which was also used as a source for annotating the lipid subclasses obtained in our analysis (see Materials and Methods). PC is ≈70% by weight of total lipids in plasma lipoproteins and ≈25 mole % of total phospholipids in RBCs[57,61]. The single saturated species (18:0/18:1) that displays a relative increase, instead of a decrease like all other saturated species (in bold in the legend to the right), is filled with dashed diagonal lines. Error bars represent the standard deviation except for Retic 15, where only two samples were available for analysis; here error bars represent the difference between the mean and the individual values. The table on top of the graph reports the results of the statistical analysis conducted by paired Student's t test of comparisons among reticulocyte and RBC samples. Highlighted in yellow are the p values when ≤0.05.

SPHINGOMYELIN

Figure 6 displays the changes in SM subclasses during maturation of reticulocytes and ageing of RBCs. Additionally, the molecular composition, in terms of SM subspecies, of individual classes of plasma lipoproteins is shown, taken from a literature dataset[57,58]. Changes in relative abundance of various SM subclasses occur with reticulocyte maturation, although to a much lesser extent than for PC, and continue to the stage of old RBCs instead of stabilizing with middle-age RBCs. Like with PC, most of the SM subclasses give the impression of approaching the SM composition of plasma lipoproteins (except for the HDL family). Furthermore, as with PC, a certain regularity of variation can be singled out. SM species carrying amidated fatty acids with less than 20 carbon atoms (from d32:1 to d36:2 included) display a relative increase in abundance, whereas those carrying a fatty acid with 20 or more carbon atoms decrease (see Discussion).

PHOSPHATIDYLETHANOLAMINE

Diacyl PE subclasses also appear to change according to a pattern during the maturation of reticulocytes to RBCs. Again, this phospholipid subspecies can be divided in two groups. Those belonging to the first group display a relative increase with maturation, and contain relatively shorter and more saturated fatty acids (from 26:0 to 36:4). Those belonging to the second group contain longer and more unsaturated fatty acyl chains (from 38:4 to 40:7) (**Figure 7**). Changes are strikingly specular to those undergone by PC subclasses but, unlike for PC, PE subclasses do not give the impression of a tendency to equilibrate with the corresponding plasma diacyl PE species, as changes go in the opposite direction (with a few exceptions) with respect to the PE plasma composition. This and other peculiar aspects will be covered in more detail in the Discussion section.

p values for the paired t test (all samples n=4 except Retic 45: n=3 and Retic 15: n=2)

	d32:1	d33:1	d34:0	d34:1	d34:2	d36:1	d36:2	d38:1	d40:1	d40:2	d41:1	d41:2	d42:1	d42:2	d42:3	d44:1	d44:2	(d32:1 to d36:2)
Retic 45 *vs.* RBC Y	0.138	0.140	0.017	0.392	0.028	0.009	0.005	0.649	0.313	0.100	0.156	0.077	0.803	0.263	0.001	0.232	0.223	0.139
Retic 45 *vs.* RBC M	0.098	0.089	0.041	0.220	0.060	0.000	0.001	0.272	0.066	0.042	0.151	0.175	0.951	0.161	0.036	0.507	0.449	0.054
Retic 45 *vs.* RBC O	0.007	0.065	0.048	0.273	0.012	0.001	0.001	0.137	0.020	0.160	0.133	0.160	0.922	0.015	0.059	0.971	0.111	0.058
RBC Y *vs.* RBC M	0.243	0.005	0.037	0.183	0.020	0.001	0.008	0.001	0.142	0.715	0.194	0.113	0.193	0.021	0.140	0.033	0.449	0.007
RBC Y *vs.* RBC O	0.044	0.038	0.097	0.029	0.007	0.000	0.008	0.009	0.060	0.303	0.066	0.016	0.461	0.042	0.203	0.008	0.285	0.001
RBC M *vs.* RBC O	0.081	0.117	0.863	0.242	0.354	0.003	0.030	0.036	0.058	0.127	0.035	0.172	0.597	0.361	0.807	0.063	0.004	0.024

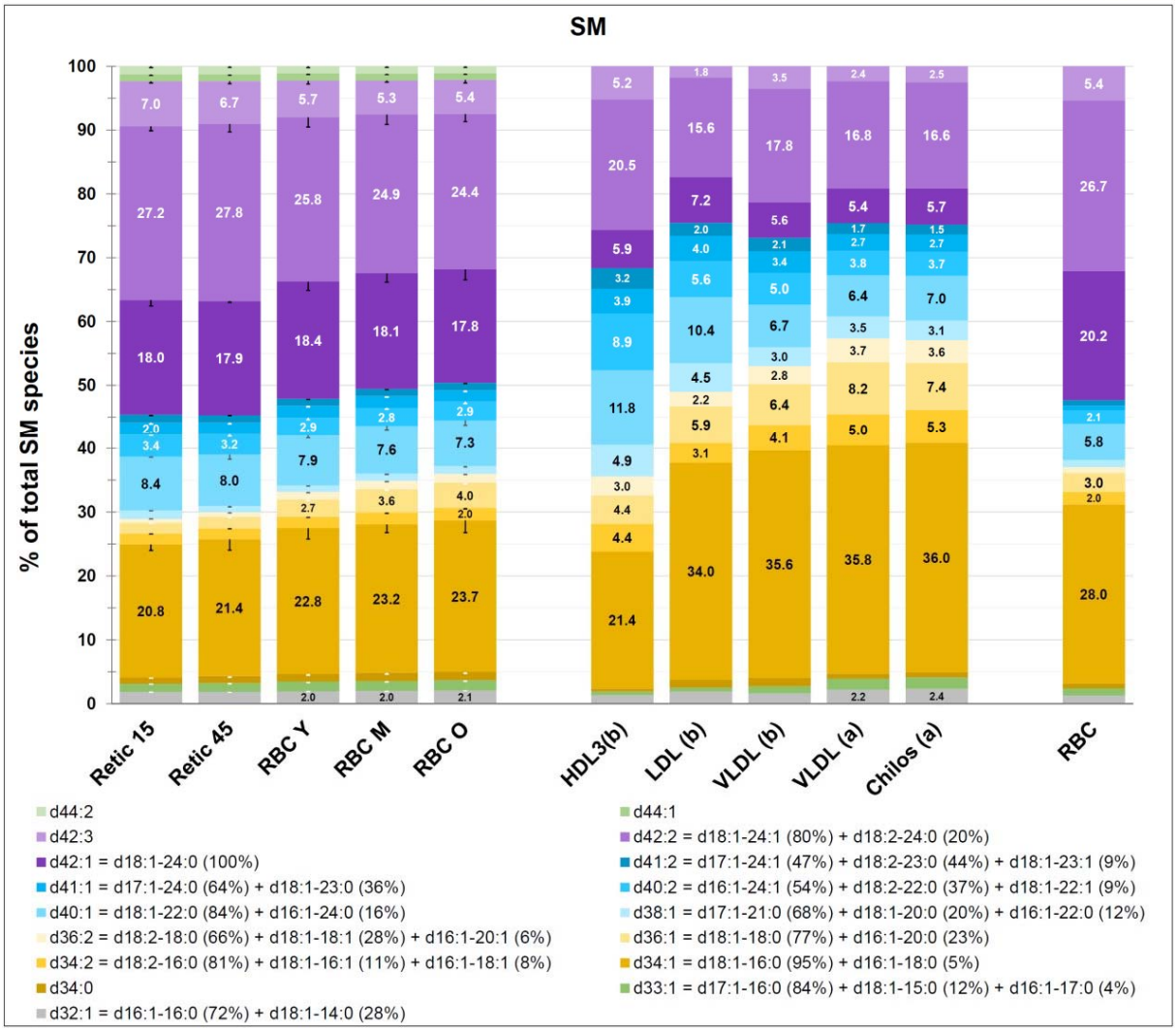


Figure 6. SM subclasses in reticulocytes and RBCs. Each subclass is given as mol % of total SM in a given sample, as indicated by numbers inside the histogram bars. The SM composition of "RBC" is taken from[57], that of plasma lipoproteins from[58]. These two articles have been also used as a source for annotating the SM subclasses obtained in our lipidomics analysis (see Materials and Methods). SM is ≈18% by weight of total lipids in plasma lipoproteins and ≈25% of total phospholipids in RBCs. (a) Postprandial plasma of a normolipemic subject; (b) fasting plasma from three different normolipemic subjects[58]. For error bars and statistics, see previous figure legend. See also **Figures S4** and **S5**.

p values for the paired t test (all samples n=4 except Retic 45: n=3 and Retic 15: n=2)

	26:0	32:1	34:1	36:1	34:2	36:2	36:3	36:4	38:4	38:5	38:6	40:4	40:5	40:6	40:7	26:0 to 36:4
Retic45 vs. RBC Y	0.960	0.858	0.046	0.028	0.966	0.055	0.195	0.199	0.052	0.256	0.305	0.077	0.026	0.237	0.135	0.079
Retic45 vs. RBC M	0.074	0.366	0.010	0.043	0.912	0.015	0.018	0.569	0.001	0.088	0.182	0.054	0.001	0.034	0.089	0.009
Retic45 vs. RBC O	0.616	0.361	0.006	0.022	0.732	0.006	0.017	0.737	0.001	0.043	0.176	0.045	0.002	0.055	0.117	0.007
RBC Y vs. RBC M	0.131	0.078	0.014	0.027	0.600	0.025	0.012	0.031	0.007	0.005	0.007	0.008	0.010	0.014	0.071	0.004
RBC Y vs. RBC O	0.028	0.077	0.004	0.005	0.180	0.006	0.003	0.044	0.002	0.000	0.020	0.005	0.000	0.016	0.061	0.001
RBC M vs. RBC O	0.072	0.411	0.004	0.441	0.018	0.001	0.005	0.230	0.000	0.000	0.048	0.005	0.004	0.034	0.182	0.000

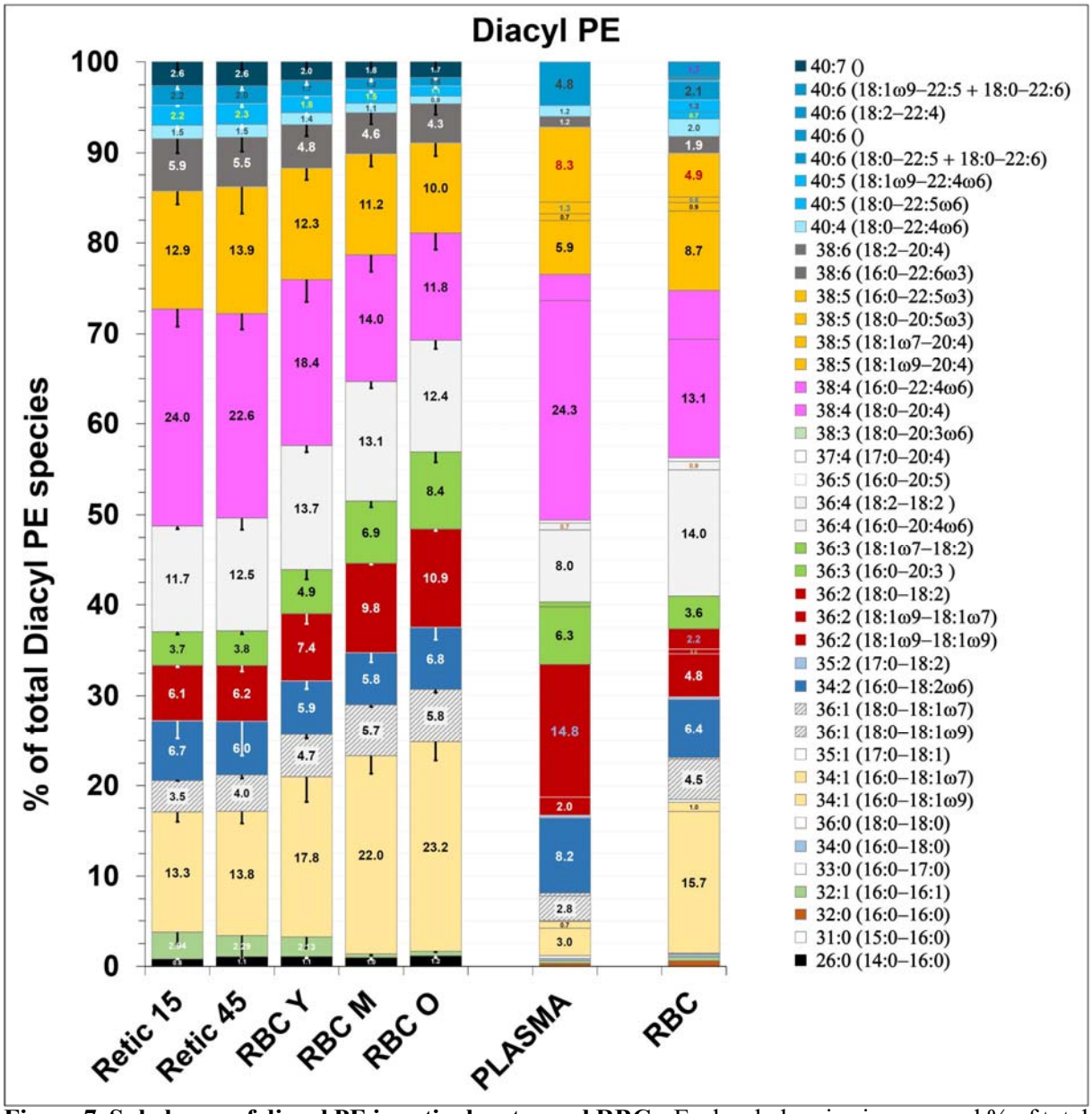
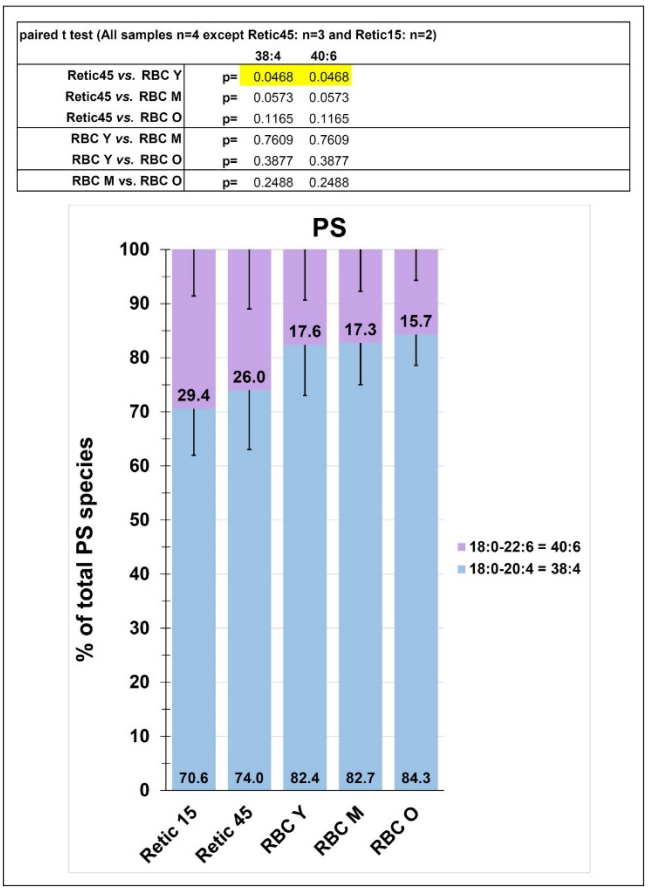


Figure 7. Subclasses of diacyl PE in reticulocytes and RBCs. Each subclass is given as mol % of total diacyl PE in a given sample. The diacyl PE composition of samples "PLASMA" and "RBC" is taken from[57,58], which was also used as a source for annotating the diacyl PE lipid subclasses obtained in our lipidomics analysis (see Materials and Methods). Diacyl PE subclasses of corresponding fatty acid composition as PC subclasses shown in **Figure 5** are shown in the same colour. Tabulated values can be found in **Figure S7**. For error bars and statistical analysis, see previous figure legends.

PHOSPHATIDYLSERINE

Figure 8 displays the changes in PS subclasses that occur during reticulocyte maturation. PS subclasses with the longest and more unsaturated fatty acids decrease more relative to total PS species (see also Discussion).



paired t test (All samples n=4 except Retic45: n=3 and Retic15: n=2)		38:4	40:6
Retic45 *vs.* RBC Y	p=	0.0468	0.0468
Retic45 *vs.* RBC M	p=	0.0573	0.0573
Retic45 *vs.* RBC O	p=	0.1165	0.1165
RBC Y *vs.* RBC M	p=	0.7609	0.7609
RBC Y *vs.* RBC O	p=	0.3877	0.3877
RBC M vs. RBC O	p=	0.2488	0.2488

Figure 8. Subclasses of PS in reticulocytes and RBC samples. See also text. For error bars and statistical analysis, see previous figure legends. For tabulated values, see **Figure S7**.

DISCUSSION

RETICULOCYTE MATURATION AND RBC AGEING

The aim of this work was to have available as many subpopulations as possible of circulating red cells for studying the evolution of some of their biochemical properties from the reticulocyte stage to the stage of 120-days old RBC. We focused on normal human circulating reticulocytes, not contaminated by "stress" or "shift" reticulocytes[23], nor by mature RBCs. We have isolated two populations of $CD71^+$ reticulocytes, Retic 15 and Retic 45, amounting approximatively to 0.15% and 0.45% of total circulating red cells, respectively. The overall reticulocyte recovery was ≈60%, assuming a conservative, average reticulocyte count of ≈1.0% in normal human subjects[62]. Of the ≈40% reticulocytes that could not be recovered, some (≈10%) could have been lost during leukodepletion[55], or because they presented too low levels of CD71 in the membrane for binding to the microbeads or because, after binding, they detached due to shearing during sample manipulation. A certain percentage of circulating CD71-negative reticulocytes could not in principle be isolated with the method adopted. In the worst case, ≈30% of total reticulocytes (≈0.3% of total blood cells), could have remained mixed with the rest of the RBCs in the sample, and could have been collected together with the population of young RBCs isolated by density gradient centrifugation, being the reticulocytes of low buoyant density[27,63]. The young RBCs recovered were ≈5.0% of total cells subjected to density fractionation. If the residual 0.3% "missing" reticulocytes all ended up mixed with the young RBC population, 6% of the cells in this fraction would have been reticulocytes, the rest young RBCs. This should not have had a major impact on the characterization of the "true" population of young RBCs, first because of the limited level of contamination, second because of the advanced degree of maturation of $CD71^-$ reticulocytes. In this respect, and from what has emerged from this work, drawing boundaries in classifying the immature stages of circulating red cells is rather captious, as they are continuously evolving toward a state of maximum stability and functionality that can be identified only in the mature RBC. After further analysis, the Retic 15 cells turned out to be significantly more immature than Retic 45, as indicated by the presence of 20-30 times more CD71 in the membrane of the former with respect to the latter (see Material and Methods). It is difficult to translate differences in CD71 contents into age differences, because the kinetics of TfR decay in normal circulating reticulocytes is not known. Furthermore, absolute quantification of TfR is complicated, and it has produced conflicting results[64]. Because circulating reticulocytes

mature to young RBCs in 1-2 days, it is reasonable to conclude that Retic 15 are in their first 12-24 h after egress from the bone marrow, whereas Retic 45 in their 24-48 h of circulation. Human RBCs have a potential life span of 120 days, i.e. ≈17 weeks[65]. Under physiological conditions, ≈0.83% of new reticulocytes enter the circulation, and the same amounts of old RBCs are cleared every day. Therefore, as ≈5% RBCs turn over in ≈6 days, our ≈5% population of youngest RBCs corresponds to cells in their first week of life. Conversely, the old population (≈2%) of RBCs is representative of cells in their 17th week of life. The dramatic changes in membrane properties that characterize the transition, in the vasculature, from the two stages of reticulocytes to young RBCs take place in ≈6 days, a period that is 5% of the entire expected life span of the cell (100x6/120=5). Furthermore, our young RBCs are still changing significantly. It is not possible in this case to estimate the time required for their composition to reach that of a mature cell because we have compared the young RBCs with the bulk of circulating RBCs (>90% of total RBCs). It is conceivable, however that it may take several more days for a young RBC membrane to reach the state of compositional and mechanical stability typical of the mature RBC, which will remain almost constant to the stage of old RBCs (with some exceptions that are worth consideration, see below). It should be kept in mind, however, that net membrane loss occurs during the entire life of the cell.

SIZE AND SHAPE

The best available quantification of the changes in membrane surface area occurring during reticulocyte maturation in vivo was carried out in baboons[66]. Results showed that reticulocytes lose ≈20% of surface area and ≈15% of cell volume in the first 24-48 h of being reinjected in the circulation after labelling. Erythropoiesis in the animals was boosted with erythropoietin before the experiment; therefore, a fraction of the circulating reticulocytes might have been more immature, "shift reticulocytes"[22] than under resting conditions, producing correspondingly slightly higher cell size values than for normal reticulocytes. For these reasons, and assuming that those results could be extended to human reticulocytes, in our case the decrease in surface area might have been less than 20% from immature, peripheral reticulocytes to middle-age RBCs This is consistent with determinations carried out by others with a different method[67]. Membrane surface area was thought to be lost in the form of exosomes[66], although peripheral reticulocytes are most likely unable to produce and release exosomes. As shown here, a subset of spectrin and Band 3 molecules are lost from maturing circulating reticulocytes, whereas this decrease does not appear to occur for

reticulocytes that mature in the bone marrow. It would seem as if the membrane was also lost selectively by peripheral reticulocytes, although by mechanism(s) that produce specular results to those obtained in the exosome-based process. Regions of the membrane that are in a liquid disordered state and contain mostly PC and PS, are lost preferentially, and with them the residual TfR that is known to partition in non-raft membrane domains[55].

RBCs and circulating reticulocytes may share a common mechanism for reducing the extension of their membrane. Losses of surface area and cell volume from young to oldest 1% RBCs amount to ≈17% and ≈25%, respectively[68]. In all, the circulating cells will lose ≈45% of surface area and ≈45% of cell volume from reticulocyte to 120-days old RBC. The decreases in surface area and volume must occur through separate, yet highly coordinated mechanisms. Separate, because the observed decrease in cell volume largely exceeds that obtainable with the release of membrane in the form of small vesicles, thus requiring that cell volume decrease by a different mechanism[50,51,66]. Highly coordinated, in order to maintain a remarkably constant surface-to-volume ratio of the discocyte throughout its circulatory life[68-71]. The common volume regulatory mechanism for circulating reticulocytes and RBCs may involve mechanosensitive channels and loss of potassium through the Gardos channel[72]. In reticulocytes, both in the marrow and in the circulation, additional mechanisms may contribute, such as the K^+/Cl^--cotransport, whose activity could still be triggered in young RBCs, to later disappear in mature cells[27,73,74].

More focus is usually given to the decrease in surface area that converts a spheroidal cell to a biconcave one. As said, surface area decrease by itself contributes by an insignificant factor to the decrease in cell volume, so that the process should be viewed from the opposite perspective: the decrease in cell volume would drive the transformation of a more spherical cell into a biconcave discocyte. One of the poorly characterized aspects is how volume regulatory mechanisms are modulated during erythroid development[73] to produce the required cell shrinkage. The assembly of the membrane skeleton as a fully extended network in the sub-membranous space should be considered the result of the process rather than the beginning and the cause of discocytic transformation. A discoid shape does not necessarily imply an assembled membrane skeleton[6,75,76]. At the stages of enucleated orthochromatic erythroblast and reticulocyte, the full complement of membrane skeletal components may be already synthesized and available, even in excess of demand, although the skeletal network may be still “threadbare”, to justify the relative instability of the membrane. The cell could become progressively more discoid only thanks to the above-mentioned driving force of

volume decrease (see below for additional considerations about the assembly of the membrane skeleton).

As regards cell morphology, it goes perhaps against common belief that the vast majority of circulating reticulocytes appear, as in our hands, as normal biconcave discocytes, with a few misshaped cells of the knizocyte and codocyte (target-cell) type[77], more abundant among the younger reticulocytes (**Figure 1**). Although these morphologies could be artefacts generated by staining and/or fixation[78,79], we avoided the unnecessary staining with New Methylene Blue, because our reticulocyte populations were by definition ≈100% pure. The observed abnormal shapes are commonly found among RBCs with excess membrane surface area[80]. Previous characterizations of reticulocytes as roundish, irregularly shaped, multi-lobular cells were referring to samples containing significant amounts, perhaps 100%, of marrow reticulocytes. Whenever some cells displayed cup-shaped or discocytic morphology, they could have been mistaken for mature RBCs, while still being reticulocytes. More careful approaches[25,30,78] reveal, as in the present study, that circulating reticulocytes are indeed morphologically indistinguishable from mature RBCs.

This topic brings to the attention the controversial issue of reticulocyte maturation in vitro, a possibility that has seen a few supporters. In most of the studies, "stress" reticulocytes were used (i.a.[30,81,82]), and in a fewer cases peripheral reticulocytes isolated by various means[83-85]. The results depend on which cell parameter was monitored to judge maturation: RNA in the cytoplasm or TfR in the membrane, whose declines follow parallel but independent pathways and different kinetics. In vivo, systemic processing of the cell surface removes TfR faster than endogenous nucleases[1] degrade RNA. Therefore, we can have circulating reticulocytes that are CD71-negative and stain positive for RNA. In vitro, however, the TfR does not decline spontaneously, whereas the RNA does, leading to the erroneous conclusion that peripheral reticulocytes can mature in vitro, when only RNA content is considered. When TfR decline was adopted as a marker for in vitro maturation, reticulocytes were either not directly followed during in vitro incubation[83], or results were obtained with stress reticulocytes or with reticulocytes from Friend leukaemia-infected mice[30,82]. It is therefore safe to conclude that peripheral, circulating reticulocytes cannot be induced to develop in vitro into a mature RBC.

Perhaps even more confused is the issue as to whether circulating reticulocytes can still synthesize Hb. Experiments showing that up to 20% Hb can be produced by these cells are probably flawed by analytical artefacts, possibly due to running automatic haematology analysers out of specification[85]. Even among the most immature stress reticulocytes from phenylhydrazine-treated rabbits, only one third of the cells is active in synthesizing proteins[86].

Therefore, a more conservative view is that no changes in Hb content occur, neither because of synthesis, nor because of loss in vesicles, during the maturation of peripheral reticulocytes[62].

CHANGES IN LIPID SUBCLASSES AND DECREASE IN CELL SIZE

The relatively low amounts of reticulocytes that could be isolated in this study restricted the lipidomics characterization to direct MS analysis without intermediate chemical steps, providing only limited information on the chemical structure of each phospholipid species[87]. Yet, some of the missing information was recovered by annotating most possible phospholipid subclasses based on highly valuable, previously published characterizations of the human RBC lipidome[57,88]. For instance, the C36:2 subclass of PC is ≈7% of total PC in our RBCs and it was annotated as 18:0/18:2 in the figures presented here, because it amounts to ≈90% of all C36:2 PC in RBCs, the rest being 18:1/18:1. Similarly, C36:1, that could be either 18:0/18:1 or 16:0/20:1, among others, was annotated as 18:0/18:1 because it is the only form of C36:1 in human RBCs, although it appears to be present as a mixture of 18:0/18:1ω7 and 18:0/18:1ω9, with the latter prevailing[57]. Related figures in the Supplementary material give additional details on the issue.

The striking complexity of the lipid remodelling that occurs with the maturation of reticulocytes, which involves changes in the relative amounts of at least 70 different phospholipid subclasses as shown here, makes it difficult to find a unifying model. We will examine the behaviour of each phospholipid and its subclasses in detail first and present a comprehensive discussion of the results next. PC species undergo selective substitution during the maturation of reticulocytes and from young RBC to mature RBC. The saturated species dipalmitoyl-PC (16:0/16:0), 1-palmitoyl,2-stearoyl-PC (16:0/16:18) and 1-palmitoyl,2-oleoyl-PC (16:0/18:1) decrease relative to total PC in a statistically significant manner from each of the cell subpopulations to the next more mature. In the youngest reticulocyte fraction (Retic 15), these PC subclasses represent together ≈55% of total PC, whereas they are reduced to ≈30% in the mature RBCs (**Figure 5**). The steepest decline occurs between mature reticulocytes (Retic 45) and young RBCs. There is a slight decrease, still statistically significant, between mature and old RBCs. The decrease in the above-mentioned PC species is mirrored by the relative increase of the species with longer and unsaturated acyl chains. Until now, mature RBCs have been defined based on the absence of any detectable remnants of TfR in the membrane and of RNA in the cytoplasm with respect to reticulocytes. A more

accurate definition should be now based on the inversion of the ratio between the relative amounts of palmitoyl-oleoyl (16:0/18:1) and of palmitoyl-linoleoyl (16:0/18:2) species of PC. The ratio changes from > 2 in reticulocytes to <1 in mature RBCs (**see Table 1**). Notably, young RBCs are still undergoing significant rearrangements in their membrane lipid composition, making them more similar to reticulocytes than to mature RBCs.

Table 1. Ratios of palmitoyl-oleoyl to palmitoyl-linoleoyl phosphatidylcholine in the various populations of cells analysed.

(PC16:0/18:1)/(PC16:0/18:2)						
	Mean		SD	C.V.(%)	paired t test (n=4; n=3 for Retic 45)	
Retic 45	**2.53**	±	0.34	13.3	Retic 45 *vs.* RBC Y, p=	0.0176
RBC Y	**1.19**	±	0.13	11.1	Retic 45 *vs.* RBC M, p=	0.0120
RBC M	**0.95**	±	0.05	5.3	Retic 45 *vs.* RBC O, p=	0.0115
RBC O	**0.93**	±	0.06	6.1	RBC Y *vs.* RBC M, p=	0.0206
					RBC Y *vs.* RBC O, p=	0.0210
					RBC M *vs.* RBC O, p=	0.0898

The above mentioned relative changes in composition for individual PC are to be viewed in a context of general decrease in membrane surface area that appears to be selective as to the lipid class that is preferentially lost (PC and PS) or retained (SM, PE and Chol). Changes in composition within a given phospholipid class could be the result of a selective process that recognizes the lipid and lipid subclasses to be removed, or of a non-selective process of homogeneous membrane removal, coupled with another mechanism responsible for generating the inhomogeneity, or both. The lipid composition of the reticulocyte as it enters the circulation is the result of i) lipid metabolism taking place during erythroid development via de novo synthesis, that continues for some time in the immature reticulocyte[89], and ii) exchange with extracellular compartment(s). The topological asymmetry of phospholipids in the plasma membrane is encoded biosynthetically in the internal membranes, endoplasmic reticulum and Golgi apparatus, and appears to be generated and modulated through mechanisms that vary depending on the phospholipid. In human RBCs, the outer leaflet contains ≈70% of total PC, ≈85% of SM, ≈20% of PE and less than 4% of total PS, to name only the most abundant phospholipids[38,40]. De novo synthesis ends at the more mature reticulocyte stage. From then on, the RBC depends only, mostly for the purpose of repairing damaged fatty acids, on the Lands cycle for reacylation of phospholipids of the inner leaflet[90]

by one or more acyl-transferases[91] that access a cytoplasmic pool of acyl-carnitine[92], and on the exchange of lipids between the two leaflets and between the membrane and plasma[93-95]. Exchange with plasma was originally described as involving mainly PC, consistent with the phospholipid composition of plasma lipoproteins: ≈70% PC by weight, ≈20% SM and less than 5% for the other phospholipids[57,61]. Differences in the molecular species of PC between plasma and the cell membrane were early discovered in rabbit and human RBCs[59], and later confirmed[57,58,60]. Differences were also found in the PC composition of plasma and the RBC membrane between full term infants and their mothers[96] or in acanthocytes of congenital abetalipoproteinemia and advanced liver disease[97-99]. Although the possibility of exchange with plasma had been documented[100,101], the residual differences were perceived as the absence of a significant mass equilibration with plasma[58]. The issue remained unsolved, because the plasma was compared only with the population of mature RBCs. In the present study, the PC composition was analysed in five different populations of cells, and compared with that of RBCs from two literature datasets[57,60]. The PC composition of our middle-age RBC is virtually superimposable to that of RBCs from both cited articles (**Figure 5** and **Figure S3**), a striking feature for studies conducted thirty years apart from each other. This is not surprising, as appreciable changes in composition of RBC phospholipids can be detected only in association with defects in the supply of essential fatty acids, as with low-fat/high-carb diets, or with deranged lipoprotein metabolism, such as, again, in abetalipoproteinemia, or liver disease [97,102,103], hinting to an important role for the liver in maintaining homeostasis of lipids, not only in the plasma but also in the (red) cell membrane. At first sight, our data seem to indicate that an exchange of PC could be driven by the concentration gradient existing between cells and plasma for each of the PC subspecies. Thus, the relatively higher percent of polyunsaturated PC subspecies in plasma would drive an increase of these species in the reticulocyte, where they are at lower levels, so that they will become the most abundant species in the RBC. The saturated species, more abundant in the reticulocyte and low in plasma, would move in the opposite direction (**Figure 5**). Implicit in this model is a one-to-one type of exchange for the phospholipid molecule as a whole, with no net loss of membrane surface area, and a high selectivity in the recognition of various substrates for the (protein mediated?) exchange. Because the contents of each PC subclass are given as relative abundance over total PC for plasma lipoproteins and for the cell membrane, it is difficult to verify whether differences in the absolute amounts of a given phospholipid, i.e. gradients, exist between donor and acceptor compartment. In addition, the striking exception of PC18:0/18:1, which increases relative to total PC from reticulocyte to RBC while being

present at low levels in plasma, would go against exchange with plasma as the main cause of membrane lipid remodelling (see also discussion on PE). Incidentally, altered molecular composition of PC species as found in RBCs from respiratory distress syndrome patients was described as a feature of the disease, involving altered exchange with plasma, although, in the light of results presented here, it can be as well explained by the shift towards a younger population of circulating RBCs in these subjects[60].

The relative amounts of total SM over total phospholipids increase significantly from immature reticulocytes to middle-aged RBCs, counterbalanced by the relative decrease in PC + PS (**Figure 4**). In human RBCs, around 80% of SM is located in the outer leaflet of the bilayer. SM sub-species are differently distributed between the two leaflets, with ≈70% of those bearing amide-linked acyl chains of 20 or more carbon atoms located in the outer leaflet and ≈70% of those with less than 20 carbon atoms in the inner leaflet, independently from their unsaturation state[104]. Interestingly, a significant relative increase of the "shorter" SM species over total SM occurs in the maturation from reticulocytes to RBCs (**Figure 6**), although it is not possible now to tell if the increase involves the SM subclasses of the inner leaflet. Whether the observed differences are due to exchange of SM species with plasma is not clear although, as with PC, they seem compatible with an exchange along concentration gradients. However, the same caveat made for PC holds true here. Furthermore, lower amounts of SM (17.7% by weight) than of PC (67.0%) are present in lipoproteins[58]. Smaller changes than those seen for PC affect SM subclasses and occur throughout the life of the cell rather than being confined to the stage of reticulocyte maturation, like for PC. SM asymmetry originates at the erythroblast level[105] and does not appear to undergo major rearrangements thereafter[104,106], and only 30% of SM seems to be exchangeable with plasma[95]. All this would rule out an important exchange of SM subspecies between cells and plasma.

PE and PS are the two phospholipids predominantly oriented in the inner leaflet. We have examined in detail the diacyl species of PE, which amount to ≈49% of all PE, the rest being plasmalogens (alkylacyl ≈3%; alkenylacyl ≈48%)[57,58]. From the literature, a correlation between the amounts of a given plasmenyl PE subspecies and the corresponding diacyl PE subspecies exists, indicating the common metabolic origin and excluding major reorganization due to exchange with plasma PE, also because of the extremely low levels of PE in plasma lipoproteins[58,61], and, most importantly, because the observed changes go against a hypothetical equilibration with plasma (with a few exceptions) (**Figure 7**). Unfortunately, information about the transbilayer disposition of PE plasmalogens in RBCs is

not available in the literature, and is also scarce for other cell types[107]. Although detailed characterization of PE plasmalogens in reticulocytes and RBCs is in progress, it can be for the moment assumed that diacyl and alkenylacyl PE species change in unison during reticulocyte maturation.

Unlike in other cell types, PS in RBCs is peculiar, being composed mostly of subspecies carrying long and polyunsaturated fatty acids[107]. During reticulocyte maturation, PS displays a decrease relative to total phospholipids, together with PC (**Figure 4**). The decrease in PS actually continues all along RBC ageing. Within this general decrease, PS subclasses with the longest and more unsaturated fatty acids decrease more relative to total PS species (**Figure 8**) (but see further on).

We have described a scenario of progressive increase, with reticulocyte maturation, in the relative amounts of sphingomyelin, cholesterol and PE that, together with the relative decrease in PC and PS, confers an overall higher liquid-ordered state to the membrane.

The main issue is to account for a net loss of membrane at the same time that the membrane changes its composition. One possibility is that of an extraction from the membrane of selected single phospholipid molecules without exchange with another phospholipid class or subclass. The situation may be similar to what proposed for explaining the peculiar lipid composition of bovine RBCs, whose low PC levels (counterbalanced by correspondingly increased SM contents) may be produced by net loss of PC[108]. Strikingly, bovine erythroid precursors display a normal PC content, indicating that the differences with mature RBCs are the result of a process occurring late in maturation, perhaps in the circulation. According to the proposed explanation, PC would be degraded by an endogenous phospholipase A2 (Plase A2) located in the outer leaflet, and the product, LPC, would be lost to the plasma. PE would also be deacylated at the outer surface, but the resulting LPE would be translocated to the inner leaflet by the aminophospholipid translocase, where it would be reacylated to PE using the fatty acid released from PC. The overall result is a relative decrease in PC and increase in PE in the membrane This possibility for the maturation of human reticulocytes remains open, because an endogenous PlaseA2 activity of the type described for bovine RBCs could not be detected in human RBCs[108].

To account for net phospholipid loss, another, not necessarily mutually exclusive explanation is that changes in subclass composition of individual phospholipids is produced autonomously by the cell through metabolism, generating subdomains of the plasma membrane that can be more easily subjected to spontaneous release, or actively removed by an external factor, in association with portions of the membrane skeleton.

The bulk of a given phospholipid that is preferentially located in the outer leaflet must be coupled to the same amounts of another, or a mixture of other, phospholipids in the inner leaflet, not to generate surface area mismatch between the two leaflets (only small inter-leaflet differences in surface area are tolerated and seem to determine the curvature of the membrane, according to the bilayer couple hypothesis [109-111]). By what combinations and assortments of different phospholipid this balance is achieved could only be a matter of speculation now, as we are entering here the largely uncharted territory of lipid interdigitation, interleaflet coupling and the role of sphingolipids and ether phopsholipids[107,112-114]. Yet, current models focus on the possibility that SM species carrying long amidated fatty acids project the last carbon groups of their acyl chains in the inner leaflet and interdigitate with acyl chains of inner layer phospholipids, with cholesterol playing an important role in the interaction. This would make sense, given the presence of higher amounts of the long chain SM species in the outer (70%) with respect to the inner leaflet in RBCs[104]. Depending on the identity of the partner phospholipid in the inner layer, registered or unregistered liquid-ordered domains may form[113]. PS, as the typical inner leaflet phospholipid, is often considered the most likely inner leaflet partner for interdigitating with outer leaflet phospholipids[107]. However, even if all PS were engaged in this coupling, and only with SM, there would remain significant amounts of outer SM species without a partner in the inner layer. PE is more abundant in the inner leaflet than PS (≈18% of total phospholipids *vs.* PS ≈15%), and it should deserve more attention than it has attracted. The difficulties come from the presence of a large percent of plasmalogen PE in RBCs (see above)[57]. Nothing is known about the transversal orientation of the ether PE species in the RBC membrane. However, because 80% of total PE is located in the inner leaflet, a minimum 30% of total PE should be present as the vinyl-ether form in the inner leaflet, and it would not be surprising to find it interacting significantly with SM in a registered liquid-ordered phase. It is interesting that in the making of mammalian RBCs, PC appears to be dispensable, whereas SM must be present, as an essential lipid for the very survival and growth of individual eukaryotic animal cells[106]. Thus, in ruminant RBCs PC can be absent altogether, as in ovines (sheep and goat), or present in very low amounts, as in bovines and camelids[115-117]. Actually, in some mammals, such as rat, dog, horse, and guinea pig, RBCs have higher levels of PC than of SM[118]. In this case, it could be that only selected subclasses of PC (more saturated?) compensate for the missing SM, although, to the best of our knowledge, no information is available on this point. Notably, in front of considerable variability, in RBCs from 11 mammals the relative amounts of PC are the ones that vary more (coefficient of variation=70%), followed by SM (C.V.=57%). However, the variability of the

total choline-containing phospholipids (PC+SM) drops to ≈9%. PE is the single phospholipid that displays the lowest variability of all, with a C.V. of 13%[115]. Perhaps even more remarkably, also the coefficient of variation of (PE+PS) is lower than the coefficient of variation of either of the two phospholipids considered separately (see **Table 2**).

Table 2. Phospholipid distribution in RBCs from different mammalian species

	SM	PC	SM+PC	PE	PS	PE+PS	OTHER
RAT	12.8	47.5	60.3	21.5	10.8	32.3	7.4
DOG	10.8	46.9	57.7	22.4	15.4	37.8	4.5
HORSE	13.5	42.4	55.9	24.3	18.0	42.3	1.8
GUINEA PIG	11.1	41.1	52.2	24.6	16.8	41.4	6.4
RABBIT	19.0	33.9	52.9	31.9	12.2	44.1	3.0
CAT	26.1	30.5	56.6	22.2	13.2	35.4	8.0
MAN	25.8	28.3	54.1	26.7	12.7	39.4	3.9
PIG	26.5	23.3	49.8	29.7	17.8	47.5	2.7
GOAT	45.9	0.0	45.9	27.9	20.8	48.7	5.4
COW	46.2	0.0	46.2	29.1	19.3	48.4	5.4
SHEEP	51.0	0.0	51.0	26.2	14.1	40.3	8.7
MEAN	26.2	26.7	53.0	26.0	15.6	41.6	5.2
SD	15.0	18.7	4.6	3.4	3.2	5.3	2.3
C.V.%	**57.3**	**70.2**	**8.6**	**13.0**	**20.7**	**12.8**	**43.5**

Data elaborated from[115].

When PC is low or nil, such as in sheep RBCs, SM is the most abundant choline phospholipid in the membrane, and prevalently located in the outer leaflet. Correspondingly, increased amounts of PS and PE are observed in the inner layer, and, in bovine RBCs, PE was found to be located almost 100% in the inner leaflet[108]. Additional cues to the importance of PE are the derangements in PE metabolism that have been described in spur cell anaemia, in connection with altered RBC morphology and maturation[119].

As said before, the observed changes in phospholipid subclasses during reticulocyte maturation could be produced autonomously by the cell through metabolism of glycerophospholipid (i.e. not involving the relatively more stable SM) although exchange of PC subclasses with plasma cannot be ruled out. Hinting to a possible explanation is the modality of change in the relative amounts of subclasses of PC and PE, as the main glycerophospholipids of, respectively, the outer and the inner leaflet of the bilayer. The relative decrease in the more saturated species among PC subclasses is mirrored by an increase in the same species among PE subclasses (**Figures 5 and 7**). It would seem as if the reacylation mechanism[90] that works at the cytoplasmic side of the bilayer, transfers the more saturated fatty acids from the PC subspecies that are oriented intracellularly at any given moment, to the PE species in the inner leaflet that carry unsaturated fatty acids. Although PC

is mostly inserted in the outer leaflet, it is more mobile and capable of transbilayer movement than PE[120], thus the transacylation in the direction described above could be favoured by the internalization of saturated PC species from the outer leaflet to support further transacylation of PE with more saturated fatty acids. The latter mechanism may not even be necessary if all the required saturated PC species were already available in the inner leaflet for the exchange. The net result would be a progressive increase in the ordered state of the PE pool in the inner leaflet, which could promote the generation of registered liquid-ordered rafts by interdigitation of PE with SM in the outer leaflet, as mentioned above. At the same time, the PC pool would become more populated with unsaturated species, extending the size of the membrane in a liquid disordered state[121] that would be more prone to spontaneous shedding[122] and/or active removal, together with portions of the spectrin skeleton, through, for instance, splenic "conditioning"[123]. The progressive relative decrease of PS in the membrane (**Figure 4**) would be the result of its preferential interdigitation with PC species in the outer leaflet, with which it would remain coupled while portions of the lipid bilayer are lost/removed. The overall outcome, i.e. a membrane in higher liquid-ordered state than in the reticulocyte, would be produced by internal metabolism alone, without the need to invoke major exchange of phospholipids with plasma lipoproteins, although the possibility of such an exchange cannot be excluded for selected lipid species[58].

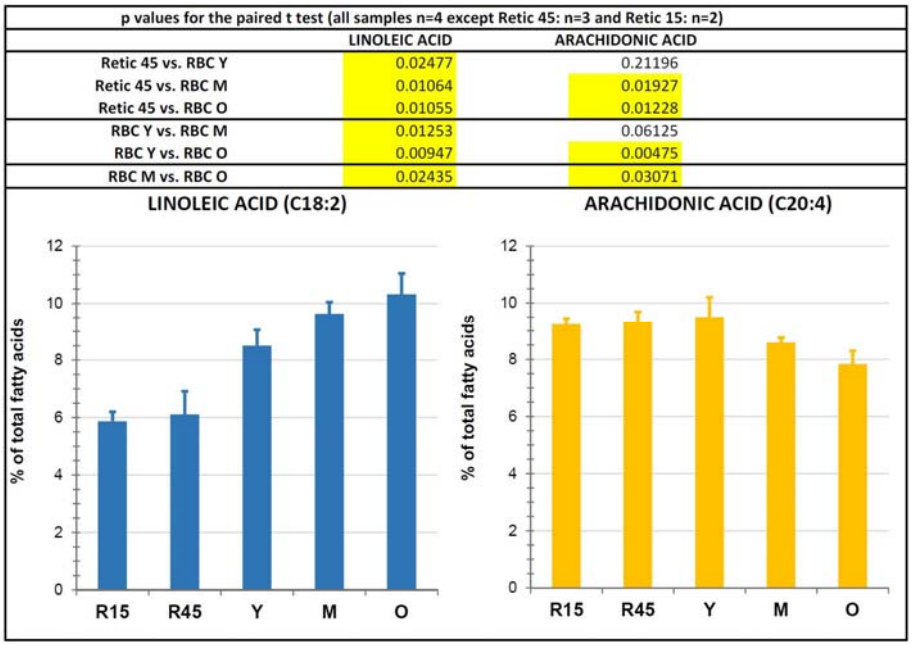
The basic question as to how and where the net parallel decreases in lipid bilayer and membrane skeleton take place is still open. Does it occur in the vascular system or is it localized into a specific tissue/organ? Confinement into a specialized district would account for the high accuracy of the process. Does it involve extracellular lipid-transfer-proteins and phospholipases to provide whole phospholipid molecules or intermediates (lysophospholipids and fatty acids) for the cell to use in remodelling selected membrane phospholipids? Could this compartment be the hepatic sinusoid/peri-sinusoidal space (space of Disse), where the proximity with the site of production and secretion of lipoprotein would ensure a steady supply of exchangeable PC species? Do Kupffer cells play a role? Although the spleen is recognized as a major site for the maturation, conditioning and repairing of normal, and the clearance of diseased RBCs[124], the liver may also play an important role. Old evidence ascribed to the liver the role of repairing RBC and reticulocyte membrane damage in the form of desialylated membrane proteins[125-127]. Could "homing" to the liver be the mandatory checkpoint for a major restyling of reticulocyte membrane lipids? The morphological and compositional anomalies of RBCs observed in liver disease would lend support to this view.

LIPIDS AND MEMBRANE TRANSPORTS

An even less investigated field is the role of lipid remodelling on membrane transport systems in general[128] and during terminal erythroid development in particular. As a general concept, several transport systems appear to decrease with the maturation of the reticulocyte, when evaluated by immunochemical technique (Western blotting, flow cytometry), among which there are the glucose transporter, the Na-K-ATPase, the sodium/hydrogen exchange (NHE1)[2], the K-Cl cotransporter[73], and, as shown here, the anion exchanger 1 (AE1, Band 3). Such decreases in molecule copy number per cell is expected for a cell that is losing membrane area. Nonetheless, a number of transports, both passive and active, have been shown to be sensitive to the lipid composition of the membrane. Classical comparative studies carried out in mammals showed that the membrane permeability to various electrolytes and non-electrolytes positively correlated with the levels of poly-unsaturated fatty acids in the membrane[115]. More specific studies revealed that the activity of the red cell Na-K-ATPase requires physiological levels of PS in the membrane[129] and is sensitive to the amounts of arachidonic acid, especially in PE plasmalogens[130]. The levels of arachidonic acid and linoleic acid were found to positively and negatively correlate, respectively, with the activity of the Na^+-Li^+-countertransport in human RBCs[131]. Comparative studies carried out on RBCs of different mammals have correlated the phospholipid and fatty acid composition of the plasma membrane with the permeability coefficients for several transports. The transport rate for the Band 3-mediated HCO_3^-/Cl^- exchange was found to positively correlate with the percentage of PC over total membrane phospholipids, and negatively with the percentage of SM[132]. Also the fatty acid composition seems to affect the HCO_3^-/Cl^- permeability, which correlates positively with the percentage of arachidonic acid (20:4) and negatively with that of linoleic acid (18:2) in total fatty acids[132]. Interestingly, also active K^+ transport was found to negatively correlate with the SM content and positively with the PC content and the percentage of poly-unsaturated fatty acids [133]. Furthermore, passive K^+ transport, that was originally considered as the residual "leak" transport although it was later attributed mostly to the $K^+(Na^+)/H^+$ exchange[134], was also found to positively correlate with the total content of arachidonic acid in the membrane phospholipids[134-136].

To the best of our knowledge, no functional studies have been performed on possible modulation of membrane transport systems during the maturation of reticulocytes to RBCs in connection with the intervening changes in membrane lipid composition. As we have described here, a relative increase in SM and a decrease in PC accompany the maturation of reticulocytes to RBCs (**Figure 4**). Moreover, a relative increase in the percentage of linoleic

acid with respect to total fatty acids takes place during the reticulocyte maturation and RBC ageing (**Figure 9**). Interestingly, and a decrease in arachidonic acid is also evident, although only with RBC senescence, not during reticulocyte maturation (**Figure 9**). It would seem that changes in lipid composition that take place during the maturation of reticulocytes and young RBCs produce a general decrease in membrane permeability by affecting multiple transport systems although perhaps to a different extent depending on the transport.



p values for the paired t test (all samples n=4 except Retic 45: n=3 and Retic 15: n=2)		
	LINOLEIC ACID	ARACHIDONIC ACID
Retic 45 vs. RBC Y	0.02477	0.21196
Retic 45 vs. RBC M	0.01064	0.01927
Retic 45 vs. RBC O	0.01055	0.01228
RBC Y vs. RBC M	0.01253	0.06125
RBC Y vs. RBC O	0.00947	0.00475
RBC M vs. RBC O	0.02435	0.03071

Figure 9. Total linoleic acid and arachidonic acid in reticulocytes and RBC samples. The total linoleic acid and arachidonic acid was the sum of pmoles of the respective fatty acid in PC, PE and PS for a given sample, and was expressed as percent of total fatty acids of the same sample. Error bars represent the standard deviation except for Retic 15, where only two samples were available for analysis; here error bars represent the difference between the mean and the individual values. The table on top of the graph reports the results of the statistical analysis conducted by paired Student's t test of comparisons among reticulocyte and RBC samples. Highlighted in yellow are the p values when ≤0.05.

SPECTRIN AND BAND 3

One of the most investigated topics in RBC studies is the biogenesis of the membrane-skeleton[137]. It is an extended pseudo-hexagonal network of spectrin tetramers anchored to the lipid bilayer, to which it provides mechanical stability while keeping it highly deformable[138]. Spectrin is a fibrous protein made of multiple modular repeats of 106 amino acids. Each repeat is formed by a triple coiled coil of antiparallel α-helices. In the folded conformation of α-spectrin there are 21 repeats and in β-spectrin 17[139,140]. One α-spectrin and one β-spectrin

monomer associate longitudinally in an antiparallel way to form stable heterodimers, which, in turn, assemble head-to-head, generating a tetramer. The tetramer is considered as the structural and functional unit of the membrane-skeleton, although it appears to be in a dynamic equilibrium with the dimers in vivo. Atomic-level structural information is available only for isolated repeats. However, repeats differ from one another within each of the spectrin monomers, therefore it is unknown whether the features described for isolated repeats[141] are retained in the spectrin dimer. The overall surface properties of the dimer, other than the sites of protein-protein interaction with components of the actin-based junctional complex and with ankyrin[142], are still largely unknown. Despite many studies, spectrin resists characterization also for what it concerns its native conformation and its interaction with membrane lipids in vivo[41,143]. That spectrin associates with lipids has been known since the observation that a variety of membrane phospholipids remain associated with spectrin when the protein is purified. Analysis of the composition of the spectrin-associated lipids revealed that, with respect to the RBC plasma membrane "phosphatidylethanolamine is increased by more than 70%, while the contribution of the other phospholipid species is decreased: phosphatidylserine (-33%), phosphatidylcholine (-25%), lysophosphatidylethanolamine and sphingomyelin (-22%)."[144]. In bovine RBCs, the ratio of phospholipids bound per spectrin molecule is even higher[145]. Evidence from erythroid development studies led to the proposal that the attachment of newly synthesized spectrin to the plasma membrane is mediated by interactions other than spectrin-ankyrin and spectrin-protein 4.1, and therefore precedes the oligomerization and assembly into an extended meshwork[146-148]. We have previously shown that when membrane rafts are isolated from RBCs under conditions of proteolysis from contaminating neutrophils, when ankyrin and protein 4.1 are completely degraded and the membrane skeleton dissociates from the bilayer[149,150], spectrin is recovered in the raft fraction, with β-spectrin only partly affected by proteolytic removal of two short fragments at both the N- and the C-terminal ends[151] (manuscript in preparation). This strongly supports the concept that spectrin can associate with the bilayer independently from ankyrin and protein 4.1. Furthermore, the isolation of membrane rafts as detergent-resistant-membrane from RBCs is not possible if the membrane is treated with the detergent alone at physiological pH, but an increase in pH and ionic strength of the medium is required. This suggests that for the detergent-insoluble domains to be released, electrostatic interactions between the membrane skeleton and the bilayer must be broken[152]. The affinity of spectrin for individual phospholipid classes, or mixtures of them, has been characterized in a large number of studies[153,154]. The view that anionic phospholipids, most notably PS, bind preferentially to spectrin has prevailed from a certain time onwards[155], largely based on the

proposed role for spectrin in maintaining the asymmetric orientation of PS in the inner leaflet of the bilayer[156]. Although such a role was later disproved[157], subsequent studies kept focusing on PS as the elected membrane phospholipid whose interaction with spectrin could be meaningful in vivo, possibly also because of the involvement of PS exposure on the extracellular leaflet in triggering cell phagocytosis by macrophages. Thus, recombinantly-expressed fragments of variable lengths of both α- and β-spectrin bind to artificial, PS-containing liposomes[158]. Yet, PE was earlier found to be equally, if not more, affine to spectrin than PS[159-161]. Moreover, PE seems to compete, when present in artificial monolayers or liposomes, with ankyrin for the binding to a recombinant C-terminal fragment of bovine β-spectrin[162], the same region where the binding site for ankyrin had been previously mapped[140]. We have already highlighted the remarkably constant levels of PE in the membrane of mammalian RBCs, where the relative amounts of other phospholipids vary considerably among different animal species (**Table 2**). All this circumstantial evidence strongly draws the attention on PE as a player in directing membrane skeleton assembly. Its affinity for PE may pull spectrin to a sub-membranous location, ready to assemble with the protein-based junctional complexes that will anchor it stably to the bilayer. Most of the spectrin appears to be synthesized already at the nucleated stages of erythroid development, before full amounts of protein 4.1 and ankyrin are available, and would rapidly associate with the bilayer thanks to its affinity for lipids. Remarkably, α-spectrin appears to be synthesized in large excess and localized preferentially in the cytoplasm, whereas αβ-dimers were found in association with the membrane[146], suggesting that the attachment of spectrin dimers to the membrane may be driven by the previous association of β-spectrin, the only one between the monomers that seems to carry the binding site(s) for PE. The excess α-spectrin would then be targeted for ubiquitylation/proteolytic degradation. Spectrin fragments are present in RBCs of all ages, as possible leftovers of incomplete proteolysis that ended with maturation of the reticulocyte and the dismantling of the proteolytic machinery. These fragments are always found in association with the membrane, not in the cytoplasm[52]. A self-ubiquitylating activity of α-spectrin has even been described[163] and related to a possible self-tagging for proteolytic degradation. However, delivery to the proteasome requires a poly-ubiquitin tag, whereas mono-ubiquitylation is used in other contexts such as endocytosis. If spectrin were only mono-ubiquitylated, could it participate in processes such as coat protein assembly, cargo recruitment and endocytosis?

Oligomerization of spectrin in the extended and compacted sub-membranous network, anchored through “canonical” vertical interactions to the bilayer, would be a relatively late and long

lasting process that could be considered completed only in the mature RBC[164]. Additional binding of spectrin to PS may occur at a later stage, as an additional stabilizing factor with the contribution of PS-binding sites provided by α-spectrin. Whatever the case, the immature reticulocyte in the marrow must still perform a complex trafficking of membrane that involves extensive endocytosis of clathrin-coated vesicles to ferry TfR and other membrane components to the endosomal compartment, which, in turn, re-selects them by endovesiculation to produce intraluminal vesicles. The relatively large multivesicular body will have to be able to reach the lipid bilayer, fuse with it and release its vesicular cargo in the extracellular space as exosomes. Studies describing this trafficking[146] were carried out on nucleated erythroblasts and "stress" reticulocytes from phenylhydrazine-treated rats, with the reticulocyte still in that early phase of maturation that would normally take place in the marrow. Here, the cell's machinery for exosome production, autophagy and proteolysis is operative and it coexists with ongoing synthesis of selected proteins, among which there are Band 3, protein 4.1 and ankyrin. Intuitively, this complex membrane traffic would be obstructed by the presence of a highly organized membrane-skeleton adhered to the bilayer[50]. It would make sense, therefore, if the spectrin dimers and tetramers, apparently already assembled before enucleation[165], remained as such and did not polymerize in an extended network. The appearance of relaxed spectrin tetramers in situ is that of convoluted ruffles[166,167] rather than of linear rods as when skeletons are artificially extended[168,169]. The relatively low density of spectrin in the vast area of the plasma membrane that characterizes the reticulocyte would slow down polymerization of "ruffles" of spectrin dimers/tetramers into higher oligomers. The sub-membranous positioning of spectrin in association with lipids would result in a "reduction of dimensionality"[170] with the consequent enhancement of the rate of membrane skeleton assembly by the establishment of horizontal interactions, and its anchoring to the bilayer, through protein-protein vertical interactions. It is true that the membrane skeleton, when uniformly extended, could cover a surface 7 times larger than the total membrane area of a mature RBC[166], and it could therefore support the membrane extension of even the most immature reticulocyte that is, at most, two times that of the RBC. However, the membrane skeleton is not yet formed at this stage. Its assembly requires that first, spectrin dimers diffuse laterally along the cytoplasmic surface of the membrane and associate into tetramers, and second, that tetramers bind to actin protofilaments and protein 4.1 in a junctional complex to form higher oligomers[171]. The latter process may also be influenced by the availability of protein 4.1, a late-synthesized[146] and extremely proteolytically unstable protein[150]. Ankyrin, another protein of relatively tardive synthesis and as unstable as protein 4.1[150], must also be available to mediate the binding

between spectrin and Band 3, and generate the second main anchoring complex of the membrane skeleton to the bilayer, thus strengthening their interaction[6]. All along this process, partially assembled portion of the membrane skeleton may be disturbed by the ongoing vesicle trafficking to and from the plasma membrane. This view seems not reconcilable with the previous description of the establishment of a functional membrane skeleton in murine erythroblasts[75,76]. However, primitive murine erythroblasts were used for the study. Because these cells develop in the circulatory system, they have probably evolved a different program whereby the generation of a mechanically stable plasma membrane is anticipated. On the other hand, when a normal adult reticulocyte is released in the circulation, the assembly and strengthening of intra and inter-skeletal interactions is still in progress, as evidenced by the fragile nature of the circulating reticulocyte, and of the young RBC. The results presented here indicate even more dramatically how far is the membrane of the reticulocyte and of the young RBC from the stable and resilient structure typical of the mature cell, which is reached only when the lipid component has been properly remodelled to a state of higher liquid order. Furthermore, compelling evidence, although circumstantial, reveals that a finely tuned process of selection exists, for retention in the membrane of a major subset of SM and PE species. These species would become coupled between the two leaflets already at the level of their biosynthesis in internal membranes. When this membrane is trafficked to the plasma membrane, the interleaflet-coupled species would recruit spectrin, possibly starting with β-spectrin, which would from then on be "pinned" to the cytoplasmic leaflet by interaction with PE[113,114]. In an earlier phase, β-spectrin might even travel to the plasma membrane already associated with the Golgi membrane and with the anterograde flux of exocytic vesicles[172], in this case working as the organizer of inner leaflet lipids interdigitated with the outer (luminal) SM species in a pre-raft structure[173]. It may be speculated that early, co-translational association of β-spectrin with the cytoplasmic side of internal membranes would sequester it and slow down its association with α-spectrin, possibly explaining why α-spectrin is overexpressed with respect to its partner monomer.

As for PS, which is entirely confined in the inner leaflet, the situation is more complex. Although interaction with spectrin has been demonstrated in vitro, its importance in vivo has been questioned[157]. In RBCs, highly unsaturated and long fatty acids are abundant in PS subspecies that may be therefore expected to partition in liquid disordered membrane subdomains where increasing amounts of unsaturated PC species are also delivered, perhaps due to the metabolic exchange of fatty acids with PE, as described above. Their possible

association with a subset of spectrin molecules would explain the loss of spectrin that is observed to occur, together with PS, all along reticulocyte maturation[55] as shown also here, and RBC ageing[51,52]. The implications of the results and the proposed model are manifold and could impact on several aspects of RBC physiology and disease, from membrane biogenesis to RBC ageing and clearance.

Although the observations presented here do not show the exact sequence of events by which differences may be produces, they do suggest several possible future lines of inquiry in this regard.

MATERIALS AND METHODS

A detailed version of Materials and Methods is available in the Supplementary material. Human blood collected in lithium heparin as the anticoagulant was leukodepleted and RBCs suspended at ≈20 haematocrit (Ht) in PBSG (5 mM sodium phosphate pH 7.4, 154 mM NaCl, 4.5 mM KCl, 305-310 mosmol/kg H_2O). Aliquots were used for cell count, Ht and Hb determination (Drabkin's reagent), SDS-PAGE and Western blotting, fixation for SEM, and lipid extraction for lipidomics analysis. The RBC suspension was then used for the immunomagnetic isolation of $CD71^+$ reticulocytes by using the "CD71 MicroBeads" (Miltenyi Biotec, Germany). By playing with the flow rate of the RBC suspension through the magnetized separation columns, two different populations of reticulocytes (Retic 15 and Retic 45) were isolated in four independent experiments. In a typical experiment, ≈60% of all circulating reticulocytes were isolated (see Discussion), of which ≈20% as Retic 15 and ≈80% as Retic 45. That reticulocytes in the two populations differed for their degree of maturation was shown as follows. After cell counting and solubilisation with SDS-PAGE sample buffer and Western blotting for CD71 of the same number of cells, the result of the separation of Retic 15 and Retic 45 in four different experiments is shown in **Figure 10**.

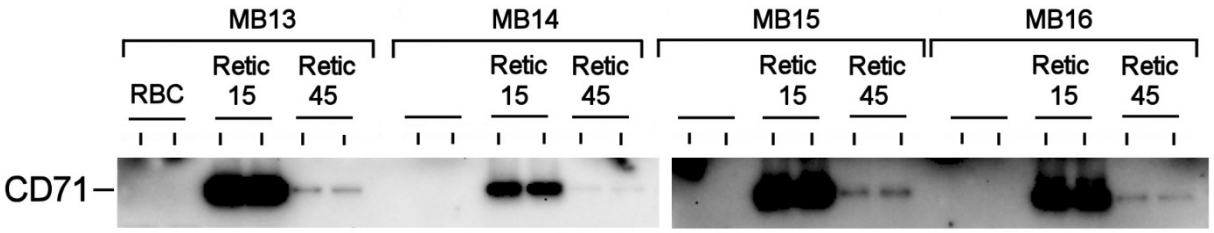


Figure 10. CD71 levels in RBCs and two populations of reticulocytes. Western blotting with anti CD71 of two populations of reticulocytes and one of RBCs from the corresponding donor in 4 independent experiments. For each sample, 2.0 x 10^5 cells were loaded. See text for details.

The CD71 levels appear to be strikingly different in the two populations of reticulocytes, indicating a different degree of maturation in vivo. The non-linearity of the chemiluminescence response to protein levels, and the large signal difference between the two samples, prevented quantification of relative CD71 contents by densitometry. To overcome the problem a different approach was used. Increasing amounts of Retic 15 sample were loaded together with a single amount of Retic 45 in the same gel (**Figure 11, left**). After detection of the signal and

densitometry of the bands, the sample of Retic 15 whose CD71 intensity was more similar to that of Retic 45 was selected. Only when two bands have identical density, the relative CD71 levels in the two samples can be obtained the ratio between the number of cells loaded for one sample to that of the other. For instance, in the example shown, assuming that the CD71 signal produced by $0.6x10^5$ Retic 15 is the same as that of $6.0x10^5$ Retic 45, then the ratio of CD71 between Retic 15 and Retic 45 is 10. To account for remaining differences in band intensity, a correction factor was applied after densitometry of the bands, under the assumption of linearity for small intensity differences. The same procedure was applied to all four experiments.

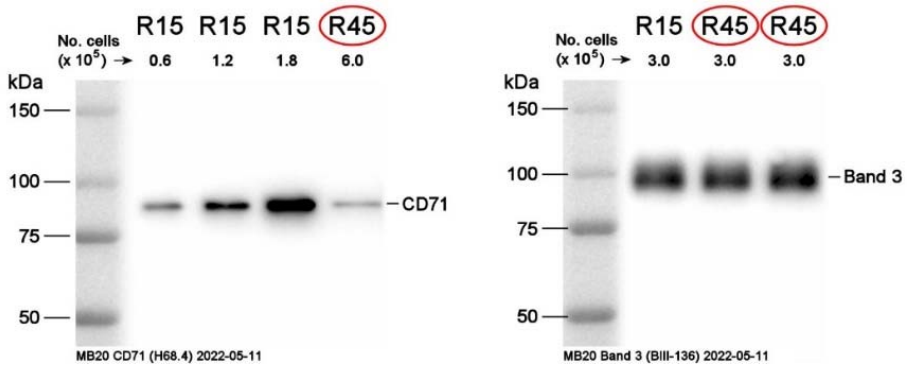


Figure 11. Procedure for determining the ratio of CD71 levels between two samples. (Left) CD71 Western blotting of different amounts of Retic 15 (R15) and one single quantity of Retic 45 (R45). (Right) Band 3 Western blotting of identical numbers ($3.0x10^5$) of reticulocytes from the Retic 15 and Retic 45 populations. Equal amounts of Band 3 confirm the validity of cell count. See text for details.

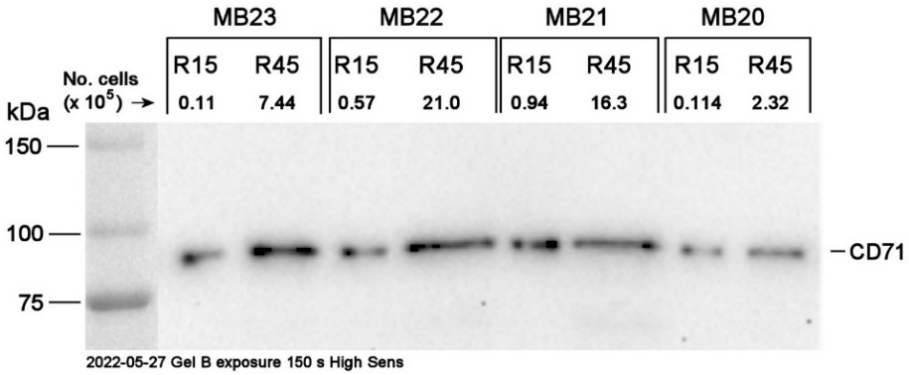


Figure 12. CD71 Western blotting of all the reticulocyte samples obtained in the four experiments. CD71 Western blotting of different amounts of Retic 15 (R15) and Retic 45 (R45) from all the experiments. Cell numbers were chosen in order to have the closest possible band intensity for all samples. See text for details.

Figure 12 shows the result of the loading in a single gel of all the samples obtained in four independent experiments. Because bands were not exactly of the same intensity, an additional correction factor was applied to obtain the results shown in **Table 3**.

Table 3. Ratio of CD71 between reticulocytes Retic 15 and Retic 45.

EXP.		Cell N. ($x10^5$)	Band density (arbitrary units)	Band density (%)	Cell N. after correction ($x10^5$)	(CD71 in Retic 15)/ (CD71 in Retic 45)
MB20	Retic 15	0.114	492	107.5	0.12	**19**
	Retic 45	2.32	529	100.0	2.32	
MB21	Retic 15	0.94	1165	73.9	0.69	**23**
	Retic 45	16.26	861	100.0	16.26	
MB22	Retic 15	0.57	712	172.8	0.99	**21**
	Retic 45	21.00	1230	100.0	21.00	
MB23	Retic 15	0.108	627	206.5	0.22	**33**
	Retic 45	7.44	1295	100.0	7.44	

The levels of CD71 in the more immature reticulocytes are 20 to 30 times the levels in the more mature reticulocytes, confirming that the procedure yielded two populations of circulating reticulocytes at different stages of maturation in vivo.

It was also possible to compare the CD71 levels in samples from different donors/experiments, by arbitrarily setting at 100% the CD71 levels of the reticulocyte population from one donor (highlighted in yellow in **Table 4**) to which the CD levels of the other cell populations could be referred.

Table 4. Ratio of CD71 between reticulocytes Retic 15 and Retic 45 from different donors.

Retic 15						Retic 45					
EXP.	Cell N. ($x10^5$)	Band density (arbitrary units)	Band density (%)	Cell N. after correction ($x10^5$)	**CD71 levels (% of MB23)**	EXP.	Cell N. ($x10^5$)	Band density (arbitrary units)	Band density (%)	Cell N. after correction ($x10^5$)	**CD71 levels (% of MB23)**
MB20	0.114	492	78	0.145	**74**	MB20	2.320	529	41	5.682	**131**
MB21	0.940	1165	186	0.506	**21**	MB21	16.260	861	66	24.463	**30**
MB22	0.570	712	114	0.502	**22**	MB22	21.000	1230	95	22.109	**34**
MB23	0.108	627	100	0.108	**100**	MB23	7.440	1295	100	7.440	**100**

Verification of the purity of the isolated reticulocytes

At some point, concerns were raised for the possible contamination of the reticulocyte samples by RBCs. Direct evaluation by flow cytometry was not feasible due to the small sample size. Also impractical was the manual procedure of staining and evaluation by microscopy, due to

the expected low number of unstained cells (RBCs) to be unequivocally identified among a vast majority of stained cells. We therefore resorted to analysis by Western blotting of protein 4.1R in reticulocyte samples. Protein 4.1R is synthesized in the erythroid precursors as protein 4.1b, which undergoes a time-dependent, non-enzymatic, post translational deamidation of two asparagine residues that converts it to protein 4.1a[174]. Therefore, protein 4.1a is only present in RBCs, in variable amounts depending on the age of the cell. It is expected that possible contaminant RBCs are from the total population of cells, of intermediate age, where protein 4.1a is present in approximately the same amounts as protein 4.1b. Contamination by RBCs could therefore be revealed by the presence of protein 4.1a in reticulocyte samples. As shown in **Figure 13**, representative of all the performed experiments, no protein 4.1a is visible in the sample of Retic 45 reticulocytes, whereas it is clearly detected in the population of young RBCs and progressively increases until becoming the most abundant form in old RBCs (RBCs of different age were obtained from the same donor as described in the next paragraph.)

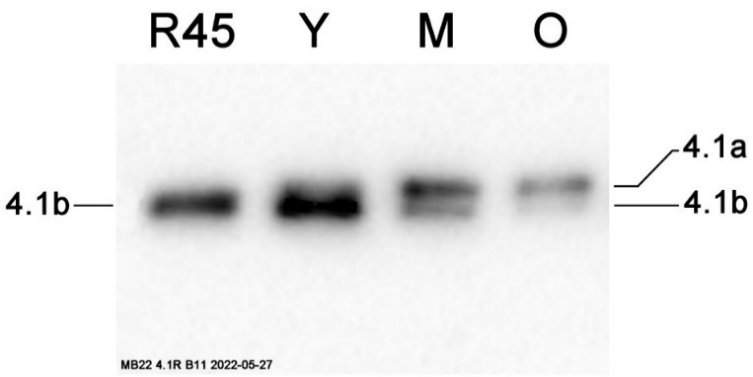


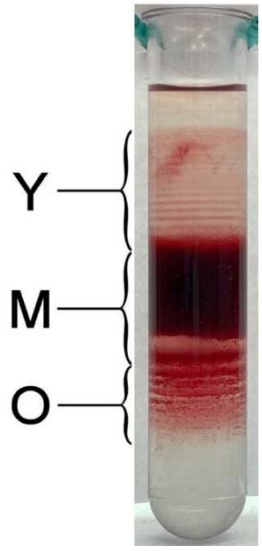
Figure 13. Protein 4.1R Western blotting in reticulocytes and RBCs of different age. The blotting is representative of four independent experiments with identical results. Reticulocytes (R45) and RBCs of different age (Y=young, M=middle-age, O=old) were from the same donor. In each lane, 2.0×10^5 cells were loaded. See text for details.

Separation of RBCs into subpopulations of different age

After isolation of $CD71^+$ reticulocytes, the reticulocyte-depleted RBC sample was subjected to density fractionation to isolate three subpopulations of RBCs of different age: young ("RBC Y"), middle-age ("RBC M") and old ("RBC O"). Cells were centrifuged in self-forming Percoll® Plus (GE Healthcare, Milan, Italy) gradients according to a previously published method[175], with some modifications. Care was taken to isolate less than 5% of the total RBCs in the subpopulations of young and old cells. Results obtained in four independent experiments are shown in **Figure 14.**

Figure 14. **Separation of RBCs according to density in self-forming Percoll® gradients.** One tube only is shown of the two that were typically run in each experiment, as representative of the four performed experiments. The table gives the average cell recovery in the three RBC subpopulations, evaluated as % Hb (or number of cells) recovered in each fraction with respect to total Hb (or number of cells) loaded in the gradient.

		EXPERIMENT			
	Mean ± SD	MB20	MB21	MB22	MB23
RBC Y	5.2 ± 2.6	7.86	6.87	2.72	3.30
RBC M	93.3 ± 2.4	90.70	91.80	95.80	94.70
RBC O	1.6 ± 0.3	1.42	1.30	1.53	1.96



Fractionation according to cell age, not only cell density, was verified by evaluating, by Western blotting, the protein 4.1a/4.1b ratio in the different subpopulations. From the results shown in **Figure 15**, it could be confirmed that there is a progressive increase in the 4.1a/4.1b ratio from young to old RBCs.

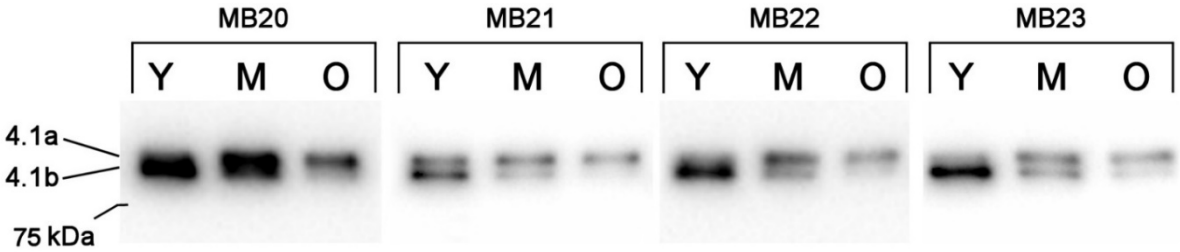


Figure 15. Western blotting of protein 4.1 in density-separated RBC subpopulations. The relative increase in protein 4.1a and decrease of protein 4.1b from samples of young to old RBCs confirmed that density separation resulted in a separation of RBCs of diffent age. Each sample contained 2.0 x 10^5 cells.

Preparation of cells for Scanning Electron Microscopy (SEM)

Before preparation for SEM, RBCs and reticulocytes were fixed with glutaraldehyde (GA) according to a two-step protocol[56]. Fifty µl of a suspension in water of GA-fixed cells were mixed with 50 µl of 2% osmium tetroxide in water (Cod. 75632-5ML, Merck Life Science S.r.l., Milan, Italy). After 30 min at room temperature, cells were washed with deionized water and were subjected to graded alcohol treatment, starting from 200 µl 70% ethanol, followed

after 30 min by 80% ethanol and, after another 30 min, by absolute ethanol, in which cells could be stored at 4 °C. For SEM, a few µl of these suspensions were poured on the surface of a 5x7 mm silicon chip (Ted Pella Inc, Redding, CA, USA). After evaporation of the solvent, the chip was laid on one side of a bi-adhesive plastic disc coated with conductive material (Ted Pella Inc.) which was in turn attached to the surface of a standard pin stub mount for SEM. The surface of the samples was made electrically conductive by a coating of Pt using a Cressington 208HR Sputter Coater. SEM images were obtained through a FEG-SEM TESCAN Mira3 XMU, Variable Pressure Field Emission Scanning Electron Microscope (TESCAN, Brno, Czech Republic) located at the Arvedi Laboratory, CISRiC, Pavia, Italy. Observations were made, at different magnifications, in secondary electrons mode at 20 kV with an In-Beam SE detector at a working distance of 5 mm.

Lipid analysis

Aliquots of packed reticulocytes or RBCs, containing $2x10^7$ cells, were extracted with 0.5 ml+0.5 ml of ice-cold, HPLC-grade methanol and transferred to 10 ml Pyrex® glass tubes with polyphenolic screw cap (1636/26MP, Italtrade S.r.l., Genoa, Italy). The quantitative lipid measurement was done by high resolution mass spectrometry (LTQ-Orbitrap, Thermo Scientific)[176]. Lipids were extracted with an established MTBE (Methyl-tert-butylether) protocol[177]. The Orbitrap Velos Pro hybrid mass spectrometer was operated in Data Dependent Acquisition mode using a HESI II ion source. Full scan profile spectra from m/z 450 to 1050 for positive ion mode and from m/z 400 to 1000 for negative ion mode were acquired in the Orbitrap mass analyser at a resolution of 100k at m/z 400 and < 2 ppm mass accuracy. Samples were measured once in positive polarity and once in negative polarity. For MS/MS experiments, the 10 most abundant ions of the full scan spectrum were sequentially fragmented in the ion trap using He as collision gas (CID, Normalized Collision Energy: 50; Isolation Width: 1.5; Activation Q: 0.2; and Activation Time: 10). Centroided product spectra at a normal scan rate (33 kDa/s) were collected. The custom developed software tool Lipid Data Analyzer was used for data analysis[178,179]. Seven classes of lipids were quantified, together with their subclasses according to the length and number of double bonds of the acyl chains linked to the glycerol or sphingosine moiety: phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyeline (SM), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and cholesterol (Chol).

SDS-PAGE and Western blotting

Standard protocols were adopted for SDS-PAGE in 10% isocratic or 5%-15% gradient polyacrylamide mini-gels (Bio-Rad Laboratories S.r.l., Segrate, Italy), and for Western blotting by electro-transferring proteins from gels to PVDF membranes (0.2 μm pores) using a Trans Blot Turbo system (Bio-Rad Laboratories S.r.l., Segrate, Italy) according to manufacturer's instructions. After incubation with the primary antibody, and the appropriate secondary horseradish-peroxidase-(HRP)-conjugated antibody, membranes were developed with the chemiluminescence kit Prime Western Blotting Detection Reagent (GE Healthcare, Milan, Italy) and the signal was acquired with a Molecular Imager ChemiDoc XRS+ (Bio-Rad Laboratories S.r.l., Segrate, Italy). Densitometry of the bands was performed using the software Scion Image (Scion Corporation, USA). Antibodies usedwere: mouse monoclonal (BIII-136) anti human Band 3 (B9277, Merck Life Science S.r.l., Milan, Italy). Mouse monoclonal (H68.4) anti human CD71, sc-51829; mouse monoclonal anti human β-spectrin (VD4), sc-53901 (Santa Cruz Biotechnology, Dallas, Texas, USA). HRP-conjugated goat anti-mouse IgGs (170-6516, Bio-Rad Laboratories S.r.l., Segrate, Italy).

LIST OF ABBREVIATIONS

DRM, detergent-resistant-membrane; ILV, intraluminal vesicle; MVB, multivesicular body; GPA, glycophorin A; Tfr, transferrin receptor; CD71, transferrin receptor; Chol, cholesterol; GP, glycerophospholipid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyeline; SP, sphingolipid; ST, sterol.

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