JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v3i1.123

Red Blood Cell Lipidomics analysis through HPLC-ESI-qTOF: application to red blood cell storage

Anna Maria Timperio[1], Cristiana Mirasole[1], Angelo D'Alessandro[1], Lello Zolla[1,*]

[1]Department of Ecological and Biological Sciences, University of Tuscia, Largo dell'Università, snc, 01100 Viterbo, Italy

Received: 11 January 2013 **Accepted:** 02 February 2013 **Available Online**: 11 March 2013

Abstract

Recent developments in mass spectrometry (MS) have enabled fast and sensitive detection of lipid species in different biological matrices. In the present study we performed an on-line HPLC-microTOF-Q MS approach to the red blood cell (RBC) lipidome. We thus exploited bioinformatic tools for the interrogation of novel databases, such as LIPID MAPS. By means of *ad hoc* software suites for mass spectrometry-based metabolomics analyses, we could address the key biological issue of the RBC lipidome, within the framework of RBC storage for transfusion purposes. Samples were collected from subjects living in the province of Viterbo, where olive oil consumption represents a central aspect of the diet. On this ground, we could postulate a diet specific effect on the accumulation of lipid-specific storage lesions. The analyses yielded the tentative identification of a huge number of lipid molecules on the basis of accurate intact mass values and retention times, and MS/MS validation. This analytical workflow was exploited to consolidate existing knowledge on the RBC lipid composition and individuate statistically significant fluctuations of lipids throughout storage duration of RBC concentrates under blood bank conditions. Our analysis indicated ceramides, glycerophospholipids and sterols as key targets of RBC storage lesions to the lipidome, that will deserve further targeted investigations in the future. It also emerged how compositional analyses of the RBC lipidome might end up yielding different results on the basis of the background of the blood donor (i.e. diet), which might translate into region-specific lipidomic alterations over storage progression of RBC concentrates.

Keywords: Red Blood Cells; Mass Spectrometry; Blood Storage; Lipidomics.

1. Introduction

Lipidomics is the systematic identification of the lipid molecular species of a biological matrix (either a cell, organelle, globule, or whole organism) with emphasis on the relative quantitation of composition changes in response to a perturbation, such as ageing or drug treatments [1].While the term "lipidomics" dates back to a decade ago, investigations of the lipid content of specific biological matrices was an already consolidated field of research over the last fifty years [2]. In particular, this holds true for those matrices that are largely available and display limited biological complexity, such as anucleated cells and, in particular red blood cells (RBCs) [2-14].

Indeed, RBCs are also devoid of organelles and of any *de novo* lipid synthesis capacity, which makes their lipidome rather stable in comparison to other cell types. Indeed, phospholipid synthesis is known to be active in reticulocytes and suppressed in mature RBCs [15]. Nonetheless, alteration of lipid homeostasis is strictly tied to membrane reorganization during RBC ageing *in vivo* and *in vitro* (RBC storage), mainly owing to lipid peroxidation phenomena which promote membrane shape alterations through the progressive loss of lipids (and membrane-associated proteins) via vesiculation [16-18]. Therefore, it is small wonder that the RBC lipidome has long attracted a great deal of interest over the last five

***Corresponding author:** Lello Zolla, E-mail Address: zolla@unitus.it; Phone Number: +39 0761 357 100; Fax Number: +39 0761 357 630

decades.

Yet in 1959, Phillips and Roome provided a preliminary portrait of the human RBC phospholipidome [2]. However, it was only in 1960 that Hanahan and colleagues described a more complex scenario, also by including species-specific differences between human and bovine RBCs [3]. Four years later, Ways and Hanahan reported a detailed lipid class composition of normal human RBCs, indicating the following percentages: cholesterol 25%, choline glycerophosphatides 30%, sphingomyelin 24%, ethanolamine glycerophosphatides 26%, and serine glycerophosphatides 15% [5]. Meanwhile, Farquhar and Ahrens [4] had showed that 67% of the PE, 8% of the PS, and 10% of the lecithin of human RBCs are in the plasmalogen form, with a vinyl ether linkage at the sn-1 and an ester linkage at the sn-2 position. In 1967, Dodge and Philips described a silicic acid thin-layer chromatography strategy to investigate the phospholipid and phospholipid fatty acids and aldehydes in human RBCs [6]. Thirty-three fatty acids and five aldehydes were separated and tentatively classified into lipid classes, including phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), lecithin, and sphingomyelin (SM) 24:0 and 24:1, while fatty acid moieties were tentatively attributed. Of note, the values reported by Dodge and Philips [6] were consistent with those by Ways and Hanahan [5]. Interesting results were obtained also as far as it concerned the composition in fatty acid moieties of the different lipid classes. About 37% of the total fatty acid in PS was 18:0, while only about 3% was 16:0; in PE and lecithin, 16:0 was the major saturated fatty acid, with the level in lecithin being over twice that in PE. The relative amount of 18:1 was also much lower in PS than in PE and lecithin. The fatty acid distribution of sphingomyelin differed markedly from that of the glycerophospholipids (GP), in particular in the greater degree of saturation [6]. Only about 33% of the fatty acids were unsaturated; in addition, less than 6% of the fatty acids appeared to have more than one double bond and less than 3% more than two double bonds. The 16:0, 24:0, and 24:1 made up almost 7% of the total fatty acids. Essentially all of the 24:0 and most of the 24:1 of the human RBC phospholipids appeared to reside in sphingomyelin.

Different instrumentations and techniques have been tested for the improvement of lipid analysis. During the last two decades, big technological strides have prompted the dissemination of chromatography separation and mass spectrometry-based lipidomics studies of RBCs [9-14]. At the dawn of the mass spectrometry-based lipidomics era the complexity of the lipidome did not enable comprehensive studies like the ones performed with thin layer chromatography (TLC) or gas chromatography (GC) described in the previous paragraphs [2-8]. The expensive instrumentation and the lack of bioinformatic tools to handle the high-throughput amount of data collected via the mass spectrometry-based workflow hampered at first its diffusion in the field [1]. More recently, the introduction of highly accurate and less expensive instruments (in comparison to the ones available decades ago) was also paralleled by consistent improvements in the field of bioinformatic elaboration of the raw mass spectra [1]. The acquired expertise have helped laboratories worldwide to cope with the intrinsic difficulties related to lipid mass attribution and fueled new efforts to bring about the systematic classification of lipid species and structures [19, 20]. The current burgeoning of OMICS disciplines has thus given new verve to the field of lipidomics research, while enabling further steps forward.

Regarding RBC lipid homeostasis, as premised by Farquhar and Ahrens [4], lipid composition of human RBCs is largely influenced by the diet. In this view, Dougherty and colleagues performed an extensive investigation to relate region specific diets to the lipid content of plasma, platelets and RBCs [8]. By comparing RBCs of individuals from rural areas in Finland, Italy (province of Viterbo) and the United States, the Authors demonstrated how diets largely relying on fish and olive oil consumption (in Finland and Italy, respectively), resulted in a significant decrease (in comparison to the US counterparts) in the levels of polyunsaturated fatty acids (PUFA), which they relate to the potential production of unhealthy prostaglandins (thromboxane and prostacyclins) byproducts [8]. Finally, the Authors also noted that in all plasma and RBC glycerolphospholipids, the monounsaturated fatty acids (especially oleic acid 16:1 and palmitic acid, 16:0) were highest in the Italian and the saturated fatty acids were highest in the Finnish samples. In this frame, we exploit novel databases such as LIPID MAPS and *ad hoc* software suites for mass spectrometry-based metabolomics analyses (such as MAVEN [21]) to address the key biological issue of the RBC lipidome. Our investigation shares some features with the study by Dougherty and colleagues [8], for it was performed on RBCs collected from subjects living in the province of Viterbo, where olive oil consumption represents a central aspect of the diet. We further address the RBC storage issue (from a lipidomic standpoint) as to conclude that wider transfusion medicine-relevant studies should be carried out to investigate whether inter-regional donor differences might lie upon peculiar RBC lipidomic profiles, which in turn are likely to reflect the heterogeneity of local alimentation regimes across Italy.

2. Materials and Method

2.1. *Sample collection*

Red blood cell units were drawn from healthy donor volunteers according to the policy of the Italian National Blood Centre guidelines (Blood Transfusion Service for donated blood) and upon informed consent in accordance with the declaration of Helsinki. We studied RBC units collected from 10 healthy donor volunteers [male=5, female=5, age 39.4 ± 7.5 (mean ± S.D.)]. RBC units were stored for up to 42 days under standard conditions (CDP-SAGM, 4°C), while samples were removed aseptically for the analysis on a weekly basis (at 0, 7, 14, 21, 28, 35 and 42 days of storage).

2.2. *Untargeted Metabolomics Analyses*

2.2.1. Metabolite extraction

For each sample, 0.5mL from the pooled erythrocyte stock was transferred into a microcentrifuge tube (Eppendorf ® Germany). Erythrocyte samples were then centrifuged at 1000g for 2 minutes at 4°C. Tubes were then placed on ice while supernatants were carefully aspirated, paying attention not to remove any erythrocyte at the interface. Samples were extracted following the protocol by D'Alessandro et al. [22]. The erythrocytes were resuspended in 0.15mL of ice cold ultra-pure water (18 MΩ) to lyse cell, then the tubes were plunged into a water bath at 37°C for 0.5 min. Samples were mixed with 0.6mL of -20°C methanol and then with 0.45mL chloroform. Subsequently, 0.15mL of ice cold ultra-pure water were added to each tube and they were transferred to -20°C freezer for 2-8 h. An equivalent volume of acetonitrile was added to the tube and transferred to refrigerator (4°C) for 20 min. Samples with precipitated proteins were thus centrifuged for 10000 x g for 10 min at 4 °C .

Finally, samples were dried in a rotational vacuum concentrator (RVC 2-18 - Christ Gmbh; Osterode am Harz, Germany) and re-suspended in 200 µl of water, 5% formic acid and transferred to glass auto-sampler vials for LC/MS analysis.

2.3. *Rapid Resolution Reversed-Phase HPLC*

An Ultimate 3000 Rapid Resolution HPLC system (LC Packings, DIONEX, Sunnyvale, USA) was used to perform metabolite separation. The system featured a binary pump and vacuum degasser, well-plate autosampler with a six-port micro-switching valve, a thermostated column compartment. Samples were loaded onto a Reprosil C18 column (2.0mm×150mm, 2.5 µm - Dr Maisch, Germany) for metabolite separation.

For lipids multi-step gradient program was used. It started with 8% solvent A (ddH_20, 20 mmol L^{-1} ammonium formiate; pH 5) to 6% solvent A for 3 min than to 2% solvent A for 35 min and finally to 100% solvent B (methanol) in 30 minutes. At the end of gradient, the column was reconditioned with 8% solvent A for 10 min. The overall run time was 68 min. Column oven was set to 50°C and the flow rate was 0.2 mL/min.

2.4. *Mass spectrometry analysis through microTOF-Q*

Due to the use of linear ion counting for direct comparisons against naturally expected isotopic ratios, time-of-flight instruments are most often the best choice for molecular formula determination. Thus, mass spectrometry analysis was carried out on an electrospray hybrid quadrupole time-of flight mass spectrometer MicroTOF-Q (Bruker-Daltonik, Bremen, Germany) equipped with an ESI-ion source.

MS analysis was carried out in negative ion mode capillary voltage 2800V, nebulizer 45 psi and dry gas of 9 l/min, scan mode 100-1500 m/z. For sample injection, solutions were evaporated to dryness and reconstituted in an adequate volume of methanol:ethanol 1:1. Lipids extracts were prepared by dilution to a concentration of 5 pmol*L^{-1} (where total phospholipids concentration was 2.5 pmol*L^{-1}). Tandem mass spectrometry (MS/MS) is used for glycerophospholipid species structural characterization. Unambiguous species identification is done by analysis of the retention time and fragmentation pattern and through direct comparison against the same parameters, as acquired from chemically defined standards (Avanti Polar Lipids, Inc., Alabaster, Al.), in agreement with Ivanova et al. [23].

Automatic isolation and fragmentation (AutoMS^n mode) was performed on the 4 most intense ions simultaneously throughout the whole scanning period (30 min per run). Calibration of the mass analyzer is essential in order to maintain an high level of mass accuracy. Instrument calibration was performed externally every day with a sodium formate solution consisting of 10 mM sodium hydroxide in 50% isopropanol, water, 0.1 % formic acid. Automated internal mass scale calibration was performed through direct automated injection of the calibration solution at the beginning and at the end of each run by a 6-port divert-valve.

2.5. *Data elaboration and statistical analysis*

In order to reduce the number of possible hits in molecular formula generation, we exploited the in house SmartFormula application of MAVEN [21], which directly calculates molecular formulae based upon the MS spectrum (isotopic patterns) and transition fingerprints (fragmentation patterns). This software generates a confidence-based list of chemical formulae on the basis of the precursor ions and all fragment ions, and the significance of their deviations to the predicted intact mass and fragmentation pattern (within a predefined window range of 5 ppm). Triplicate runs for each one of the 10 biological replicate at day 0 and over storage duration were exported as mzXML files and processed through METLIN/XCMS data analysis software (Scripps Centre for Metabolomics) [24] and MAVEN [21]. Mass spectrometry chromatograms were elaborated for peak alignment (m/zwidth = 0.025, minfrac = 0.5, bw = 5), matching and comparison of parent and fragment ions, and tentative metabolite identification (within a 20 ppm mass-deviation range between observed and expected results against the imported LIPID MAPS database [20] – annotations included adduct ions in positive ion mode). XCMS and MAVEN are open-source software that could be freely used or downloaded from their websites (http://metlin.scripps.edu/download/ and http://genomics-pubs.princeton.edu/mzroll/index.php?show=download).
Quantitative variations were determined against day 0 controls and only statistically significant results were considered (fold change > 2.5 and ANOVA *p-values* < 0.01). Data were further refined and plotted with GraphPad Prism 5.0

(GraphPad Software Inc.).

3. Results and Discussion

HPLC-MS analysis of the RBC lipidome yielded the tentative identification of a huge number of lipid molecules on the basis of accurate intact mass values and retention times (RT) (Supplementary Tables 1-5). Results were further validated against MS/MS feature transitions (fragmentation patterns) for RBC storage time course analyses, where we reported statistically significant variations ($p < 0.01$ ANOVA) of specific lipid molecules over storage duration on a weekly basis in comparison to day 0 controls (Table 1). This helped coping with the difficulties related to the attribution of fatty acid moieties in detected lipids, a problem that hampered major translational applications of early MS-based approaches to the RBC lipidome [9,10].

A 2D map overview of the lipid features identified in a single run is provided in Figure 1, where compound class specific separations are indicated according to the established nomenclature (fatty acids – FA; glycerolipids – GL; glycerophospholipids – GP; sphingolipids – SP; sterols – ST; prenols – PR and polyketides – PK). While FA, GL, GP and SP eluted rather early (within the first six minutes of RT), ST first and PR or PK displayed higher RTs, consistently with their more hydrophobic nature.

In the following paragraphs, we will detail the major findings of the currently proposed investigation through the description of the main distinct lipid classes. Results will be discussed in the light of existing literature in the field.

3.1. *Fatty acids*

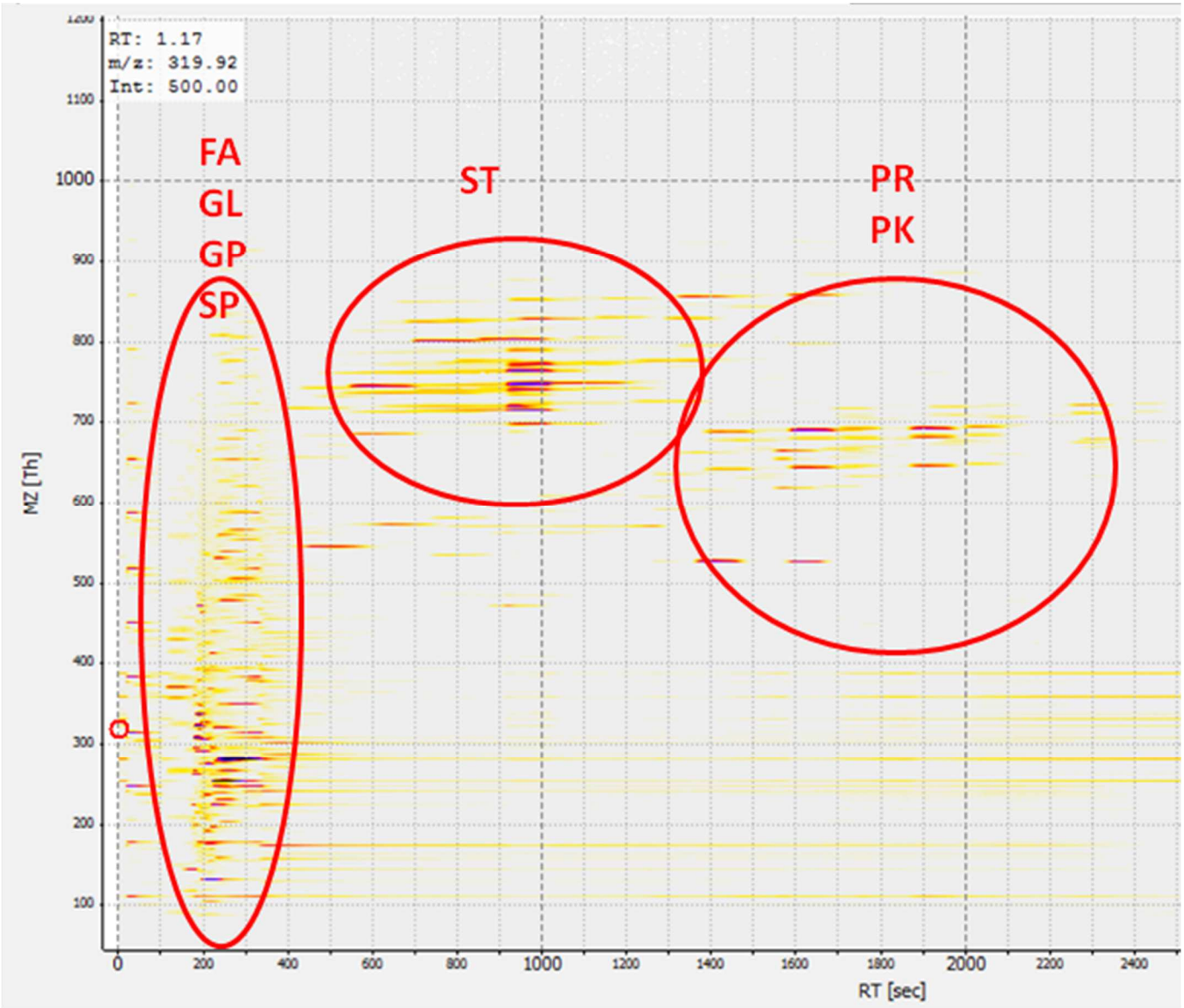

Figure 1. 2D map overview of the lipid features identified in a single run compound. Class specific separations are indicated according to the established nomenclature (fatty acids – FA; glycerolipids – GL; glycerophospholipids – GP; sphingolipids – SP; sterols – ST; prenols – PR and polyketides – PK).

Table 1. Time course alterations of lipids in long stored Red Blood Cells (RBCs). The table reports a weekly comparison of the lipidomes of stored RBCs. Mass spectra chromatograms of day 0, 7, 14, 21, 28, 35 and 42 lipid extract samples were aligned and matched with XCMS software. Statistically significant variations (fold change > 2.5 and p-value < 0.01 ANOVA) against day 0 controls were reported in the table, depending on the specific lipid class (through a direct reference to the LIPIDMAPS database). Red=Increase;

Lipid class		0 vs 7	0 vs 14	0 vs 21	0 vs 28	0 vs 35	0 vs 42
FA		Anandamide (20:l, n-9)	Clupanodonyl carnitine	Cervonyl carnitine		Mayolene-18	11,12-dihydroxy arachidic acid
		4-fumarylacetoacetic acid		Dodecanoylcarnitine		N-(5-hydroxy-pentyl) arachidonoyl amine	omega-hydroxy hendecanoic acid
		3-O-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoic acid		triacontan-1,14-diol		Triacontan-1,14-diol	15-oxo-18Z-tetracosenoic acid
		1-O-[(6'-O-hexadecanoyl)-aD-glucopyranosyl]-(2-hexadecanoyloxy)-eicosan-1-ol		Hentriacontan-16-one		Hentriacontan-16-one	
				15(S)-15-methyl PGF2α isopropyl ester		(Z)-N-(2-hydroxyethyl) hexacos-17-enamide	
				PGF2α dimethyl amine			
		C24:1-OH Sulfatide		Prostaglandin E2		Prostaglandin E2	
				(1R,2R)-3-oxo-2-pentyl-cyclopentanehexanoic acid		3-methyl-tetradecanedioic acid	
				7-oxo-11E-Tetradecenoic acid		5,13-docosadienoic acid	
DG		DG(17:1(9Z)/17:1(9Z)/0:0)					
		54:3 SLBPA					
TG		TG(20:5(5Z,8Z,11Z,14Z,17Z)/22:3(10Z,13Z,16Z)/22:5(7Z,10Z,13Z,16Z,19Z))[iso6]					
		TG(17:0/18:2(9Z,12Z)/20:0)[iso6]					
GP	PC				PC(O-1:0/O-18:0)		PC(10:0/18:0)
	PI	PIP2(16:0/18:0)	D-myo-Inositol-1,2,4,5,6-pentaphosphate			PI(13:0/0:0)	D-myo-Inositol-1,3,4-triphosphate (sodium salt)
		D-myo-Inositol-1,2,4,5,6-pentaphosphate					
		Inosine 5'-tetraphosphate					
	PE			LysoPE(0:0/22:2(13Z,16Z))		LysoPE(0:0/22:2(13Z,16Z))	

Table 1 Continuation) Red=Increase; Blue=decrease

Lipid class	0 vs 7	0 vs 14	0 vs 21	0 vs 28	0 vs 35	0 vs 42
ST	1-Hydroxyvitamin D3 3-D-glucopyranoside	Vitamin D3 / Cholecalciferol (Vitamin D3) skeleton	1 α -hydroxy-25-methoxyvitamin D3 / 1 α -hydroxy-25-methoxycholecalciferol	Cucurbitacin H	Cucurbitacin H	(6R)-vitamin D2 6,19-sulfur dioxide adduct / (6R)-ergocalciferol 6,19-sulfur dioxide adduct
					1-Hydroxyvitamin D3 cellobioside	
					19-norandrosterone	
	1α,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrovitamin D3 / 1α,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrocholecalciferol	5β-Cholestane-3α,7α-diol	Campesterol	Desmosterol(d6)	Campesterol	
					5a-Tetrahydrocorticosterone	
			1 α -hydroxy-23-[3-(1-hydroxy-1-methylethyl) phenyl]-22,22,23,23-tetradehydro-24,25,26,27-tetranorvitamin D3	(22R)-1 α,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrovitamin D3 / (22R)-1 α,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrocholecalciferol	Desmosterol(d6)	
		1α-hydroxy-2β-(5-hydroxypentoxy)vitamin D3 / 1α-hydroxy-2β-(5-hydroxypentoxy) cholecalciferol	Ecalcidene		(2-fluoroethyl)-25-hydroxy-26,27-dimethyl-24a-homo-22-oxavitamin D3 / 1α-(2-fluoroethyl)-25-hydroxy-26,27-dimethyl-24a-homo-22-oxacholecalciferol	
			1 α,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrovitamin D3 / 1α,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrocholecalciferol		3α,7α,12α,24-tetrahydroxy-24-methyl-5α-cholestan-26-oic acid	
		1α-hydroxy-25-methoxyvitamin D3 / 1α-hydroxy-25-methoxycholecalciferol	3α,12α,25-trihydroxy-5β-cholestan-7-one	3 α,12 α,25-trihydroxy-5 α -cholestan-7-one	1α,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrovitamin D3 / 1α,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrocholecalciferol	
					1α -hydroxy-22-(3-hydroxyphenyl)-23,24,25,26,27-pentanorvitamin D3 / 1α-hydroxy-22-(3-hydroxyphenyl)-23,24,25,26,27-pentanorcholecalciferol	
			(22R)-1α,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrovitamin D3 / (22R)-1 α,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrocholecalciferol		Gorgosterol	
					5?-Cholestane-3?,7?-diol	
					Ecalcidene	

Table 1 Continuation) Red=Increase; Blue=decrease

Lipid class	0 vs 7	0 vs 14	0 vs 21	0 vs 28	0 vs 35	0 vs 42
SAC	1-(O-alpha-D-glucopyranosyl)-3-keto-(1,25R,27R)-octacosanetriol			1-O-alpha-D-glucopyranosyl-1,2-nonadecandiol		
PR			30,32-dihydroxy-2b-methyl-bishomohopane	4,4'-Diapo-zeta-carotene	4,4'-Diapo-zeta-carotene	
			(+)-24-Dammarene-3alpha,12beta,20S-triol		2-Hexaprenyl-3-methyl-6-methoxy-1,4 benzoquinone	
			2-Hexaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone		3-Hexaprenyl-4-hydroxy-5-methoxybenzoic acid	
					4,4'-Diapophytoene	
PK			Delphinidin 3-(6-p-coumaroylglucoside)-5-[6-(malonyl)-4-(rhamnosyl)glucoside)]			
SP Cer			C-8 Ceramide		C-8 Ceramide	C-2 Ceramide
			Ceramide (d18:1/12:0)		Ceramide (d18:1/12:0)	
SP SP			N,N,N-trimethyl-sphingosine	Sphingosine	Phytosphingosine	
				Phytosphingosine	D-erythro-Sphingosine C-15	

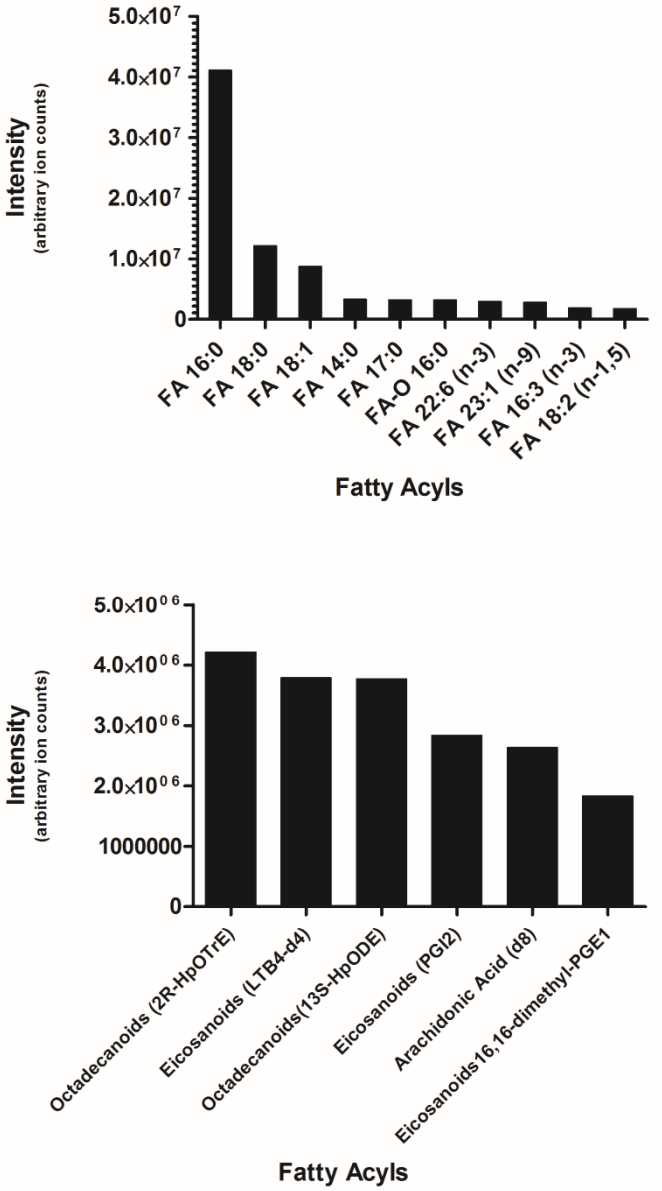

Figure 2. Fatty acid distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database [20].

Fatty acid distribution indicated that palmitic acid (FA C16:0 – Figure 2) was the most abundant free fatty acid (extended results are reported in Supplementary Table 1). This is also consistent with oldest reports on the RBC fatty acid composition available from the literature [5], despite the extreme differences between the TLC and the HPLC-MS analytical approaches. Palmitic acid might be tied to the modulation of calcium signaling in RBCs by mediating Ca^{2+} fluxes via specific membrane pores [25], thereby modulating RBC survival.

Among the most abundant individual fatty acids we could detect 16:0 (palmitic), 18:0 (stearic), 18:1 (oleic), and 22:6 n-3 (docosahexaenoic acid), in agreement with previous studies on fatty acids of erythrocytes obtained from healthy Italian subjects [26]. Furthermore, octadecadienoic acid (18:2 n-1,5) had already been reported among the most abundant ten fatty acids of RBCs [5]. The abundance of oleic acid in particular was an expected result, since olive oil holds a key role in the frame of the Mediterranean diet and, in particular, in the province of Viterbo (Italy) where blood samples were collected from healthy donor volunteers. The intertwinement between oleic acid relative concentrations and high olive oil consumption rates had already been postulated and demonstrated through TLC approaches [8], and hereby confirmed through MS.

On the other hand, no previous investigation indicated myristic acid (14:0) as one of the most abundant fatty acid in RBCs, except for those studies suggesting a role for myristic acid supplementation as a substitute of oleic acid in the diet, which results in the relative increase of α-linolenic and doxosahexaenoic acid levels [27] and alterations of RBC membrane fluidity [28]. Analogously, heptadecanoic acid (17:0) has been proposed as a controversial biomarker for the assessment of energy and macronutrient composition in response to specific diets [29].

Eicosanoids and octadecanoids and their peroxidation products (relative abundances are reported in Figure 1) are thought to play a role in mediating RBC maturation from reticulocytes by promoting the degradation of mitochondrial membranes and thus elimination of these organelles [30]. Also, eicosanoids serve as substrates for cycloxygenase, lipoxygenase and epoxygenase activities, which result in the production of pro-inflammatory factors that are associated with increased cardiovascular risk and cancer [31].

3.2. *Glycerolipids and glycerophospholipds*

Relative abundances of RBCs glycerolipids (GL) and glycerophospholipids (GP) are reported in Figure 3 and 4 (extended results are reported in Supplementary Table 2 and 3), respectively, whereas the latter class has been further subdivided into phosphatidic acid (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserine (PS), in the light of the observed elevated concentrations in RBCs.

Fatty acid incorporation stages into RBC membrane GLs and GPs has been long investigated [32-34], indicating higher rates for reticulocytes in comparison to adult RBCs [35,36]. Indeed, RBC, GL and GP metabolism is a key aspect in RBC survival [37], since during their 120 days approximate lifespan in the circulatory system RBCs shed approximately 1 microvesicle/h, thus continuously remodeling their membrane and its lipid composition. Also, early approaches to GP composition of RBCs have been
purported via TLC [38] indicated a relation of GP composition with RBC membrane anomalies, such as in the case of spheorcytosis [39]. These considerations are relevant in the light of the incomplete long chain fatty acid synthesizing system which characterizes RBCs [40]. The introduction of high-resolution capillary gas chromatography approaches recently shed new light on this delicate issue [41], further evidencing compositional anomalies of GL and GP in cancer

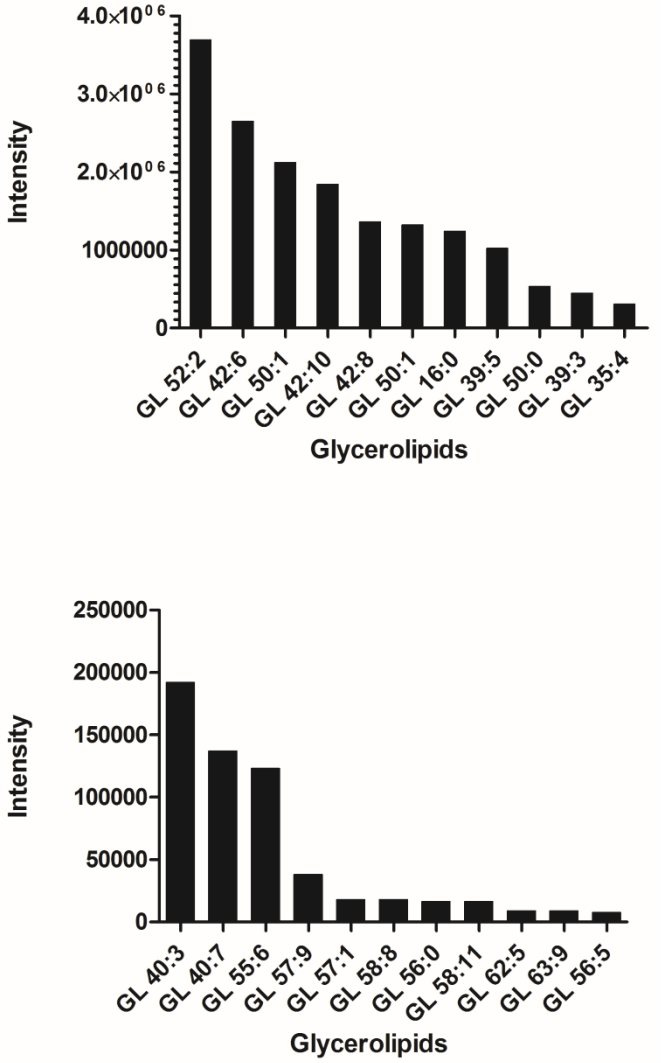

Figure 3. Glycerolipids distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database [20].

evidencing compositional anomalies of GL and GP in cancer patients [42].

Our results provide further supporting evidence about increased levels of elevated levels of C16:0 and C18:0 fatty acids in lyso-PCs from adult RBCs (Figure 4), as previously reported with different approaches [8,41,43]. Analogous considerations can be made for PE 38:4, PE 40:6 and lyso-PE 18:0, as well as for PS 38:4 (Figure 4), in agreement with the literature [8,43]. In particular, PS 38:4 had been recently indicated as the most abundant RBC-specific PS, in comparison to other blood cell types [43]. Distribution of saturated and unsaturated fatty acids in GL and GP was also consistent with the literature [5,8,43].

As expected, compositional differences were observed as well, which are probably attributable to the different diets of the subjects enrolled in the present study in comparison to data available from the literature.

3.3. *Sphingolipids, sterols and prenol lipids*

Sphingolipids (SP), among which ceramides (Cer), have been recently associated with *in vivo* and *in vitro* ageing of RBCs [18]. Though the mechanisms have not yet been fully elucidated, sphinogosines and ceramides seem to affect RBC survival by serving as signaling molecules upon acid sphingomyelinase hydrolysis of sphingomyelin into ceramide [44] or rather they directly affect RBC membrane stability by forming specific pores and thus altering membrane permeability and potential [45]. Ceramide-enriched membrane domains have been indeed associated with hot-cold hemolysis [46]. Besides, SP metabolites, including ceramides, sphingosine and sphingosine 1-phosphate have recently emerged as a new class of lipid biomodulators also in the extracellular space [47].

In Figures 5 and 6 we report the relative abundances of the top SP, with a focus on ceramides, respectively. Of note, C16 sphingosine (Figure 5) has been recently reported to be the most abundant RBC-specific SP [43]. On the other hand, while we expected elevated levels of Cer 24:1 and 24:0, in agreement with Leidl et al. [43], we could instead observe elevated levels of Cer 18:0 and 18:1 in all ceramide subclass (Figure 6), which might reflect the relative composition of free fatty acid, as mentioned in the previous paragraphs.

Recent studies have also demonstrated that sphingolipids dynamically cluster with sterols to form lipid microdomains or rafts, which function as platforms for effective signal transduction and protein sorting [48]. Sterol profiling of RBCs is also a powerful diagnostic tool to investigate the effects of total parental nutrition diet supplementation to the newborn, a lifesaving therapy in children with intestinal failure [49].

An overview of the most abundant sterol lipids is provided in Figure 7, where sterol lipids are reported with their relative name from the Lipid Maps database [20], owing to the impossibility to adapt graphic limitations to the lengthy extended names of each sterol lipid. However, full details are provided in the Supplementary Table 4.

Prenol lipids are often under-investigated class of lipids, which are synthesized from five carbon isoprene units. Recent lipidomics studies focused on plasma levels of dolichols (a group of α-saturated polyprenols characterized by 14 to 24 isoprene subunits) and ubiquinones (a group of 1,4-benzoquinones modified with 9-10 repeated isoprene units) [50]. However, to the best of the Authors' knowledge, little is known about the composition of prenol lipids in adult RBCs.

While in Figure 8 we graphed the relative abundances of prenol lipids on the basis of their relative molecular formula, in Supplementary Table 5 we also provided extended details about their common name and abbreviations, according to the Lipid Maps database nomenclature [20]. However, further dedicated studies are mandatory to shed further lights on the relative concentrations and biological functions of these molecules within the framework of RBC biology.

3.4. *Red blood cell Lipidomics: application to the storage of erythrocyte concentrates*

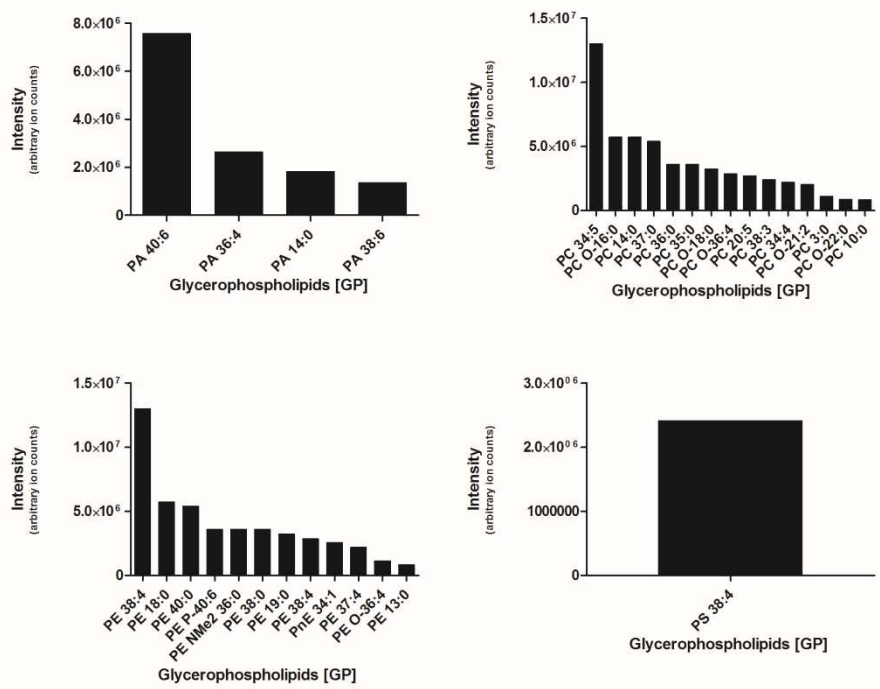

Figure 4. Glycerophospholipids distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database [20].

RBC concentrates for transfusion purposes are routinely stored at 2-6°C for up to 42 days, according to international standard guidelines [51].

Despite decades of substantial improvements in the field of RBC storage [52], concerns still arise and persist about the quality of longer stored RBCs, since it is clearly emerging –at least from a biochemical standpoint– that storage progression corresponds to the accumulation of a wide series of RBCs storage lesions [52], as, among others, we could recently document at the morphologic, metabolomics and proteomics level [53-55]. On the other hand, lipidomic aspects of RBC storage in the blood bank still lie undisclosed. Recently, Bosman's group demonstrated that specific treatment with exogenous sphingomyelinases resulted in the accumulation of ceramides RBC morphological lesions, thereby mimicking the effects of long-stored RBCs [18]. Indeed, accumulation of ceramides and their metabolites (sphingosine and sphingosine 1-phosphate – S1P) might promote intrinsic stimuli leading to the exacerbation of ageing phenomena in RBCs [18], by altering membrane conformation [45] or mediating specific intra- or extra-cellular signaling cascades [44]. In this view, it is worthwhile noting that plasma S1P mainly originates from erythrocytes, since RBCs display alkaline (but not acid or neutral) ceramidase activity on D-e-C(18:1)-ceramide [56]. First of all, we wish to stress that the most abundant ceramides we could detect in control adult RBCs could be catalogued as C18:0 or 18:1 (Figure 6). Moreover, in the present study prolonged storage of RBC was apparently associated with statistically significant decrease (p < 0.01 ANOVA) of ceramides (C-8, Ceramide d18:1/12:0 and ceramide C-2 – Table 1) after three weeks of storage, which is a critical timespan threshold for the accumulation of storage lesions at the biochemical level, as we could previously report at the proteomics and metabolomics level [54,55]. These results are suggestive of a likely ceramidase-mediated digestion of ceramides, or rather of an alteration of the lipid composition of long-stored RBCs probably reflecting the membrane remodeling occurring over RBC storage duration [53]. However, we could also observe a decrease in the levels of several sphingosines (N,N,N-trimethyl sphingosine, sphingosine, phytosphingosine and D-erythro-Sphingosine C-15 – Table 1), which did not help us ruling out any definitive scenario to explain the observed phenomena.

After an initial increase (attributable to phospholipase activities [57]), prolonged storage of RBC concentrates hereby resulted in the progressive statistically significant (p<0.01 ANOVA) decrease of a wide series of fatty acids, prostaglandins (such as PGF2α and prostaglandin E2) and fatty acid oxidation products ((1R,2R)-3-oxo-2-pentyl-cyclopentanehexanoic acid) (Table 1). This is consistent with the reported progressive accumulation of lipid oxidation byproducts in the supernatants of long-stored erythrocyte concentrate units [17,57].

The initial increase in the levels of diacyl-glycerols (DG) and triacyl-glycerols (TG) (Table 1) is difficult to interpret, if not in the light of the need for RBCs to cope with the initial free fatty acid accumulation through their sequestering and accumulation in the form of DGs and TGs. This is consistent with the hypothesis that, whether a Save or Sacrifice mechanism is innate in RBCs, as suggested by *in silico* elaborations [58,59], this mechanism is active within the first two weeks of storage [53-54].

Recently, Bicalho et al. investigated the alterations to the RBCs phospholipidome by performing a direct comparison of fresh RBC phospholipids against the phospholipid composition of RBC-shed microvesicles [60]. As a result, the Authors could point out the alterations of PS 38:4 and PS 38:1 composition in fresh controls and RBC-derived microvesicles [60]. In the present study, while we could confirm previous evidences about PS 38:4 being preponderant in RBCs (Figure 4), also in agreement with Leidl et al. [43], we could not detect any statistically significant variation as far as PS are concerned. On the other hand, we could detect significant decrease in the levels of two PCs (O-1:0/O-18:0 and 10:0/18:0 – Table 1), PEs (lyso-PE(0:0/22:2(13Z,16Z)) and lyso-PE(0:0/22:2(13Z,16Z)) – Table 1), while PIs followed a controversial trend, especially within the first two weeks of storage.

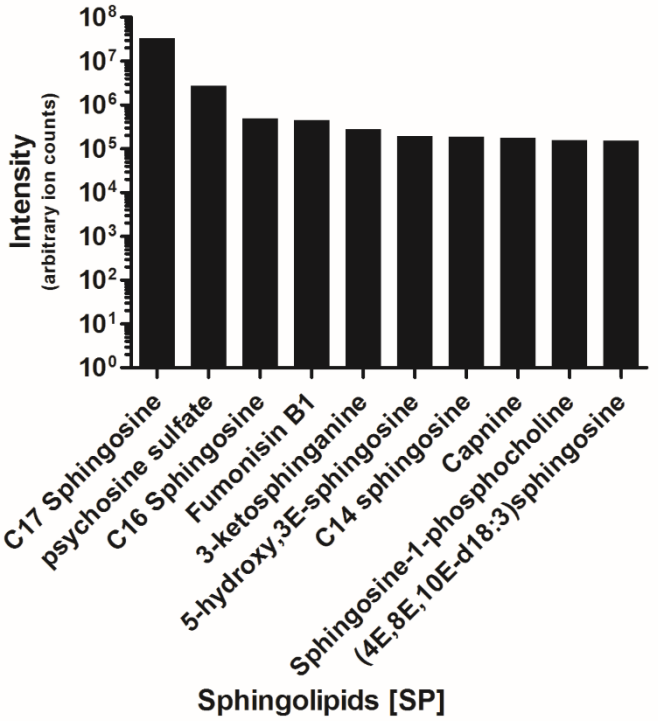

Figure 5. Sphingolipids distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database [20].

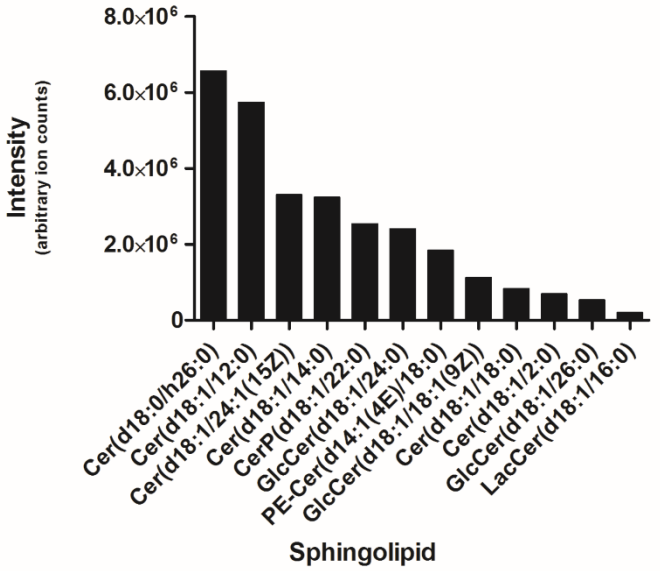

Figure 6. Ceramides distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database [20].

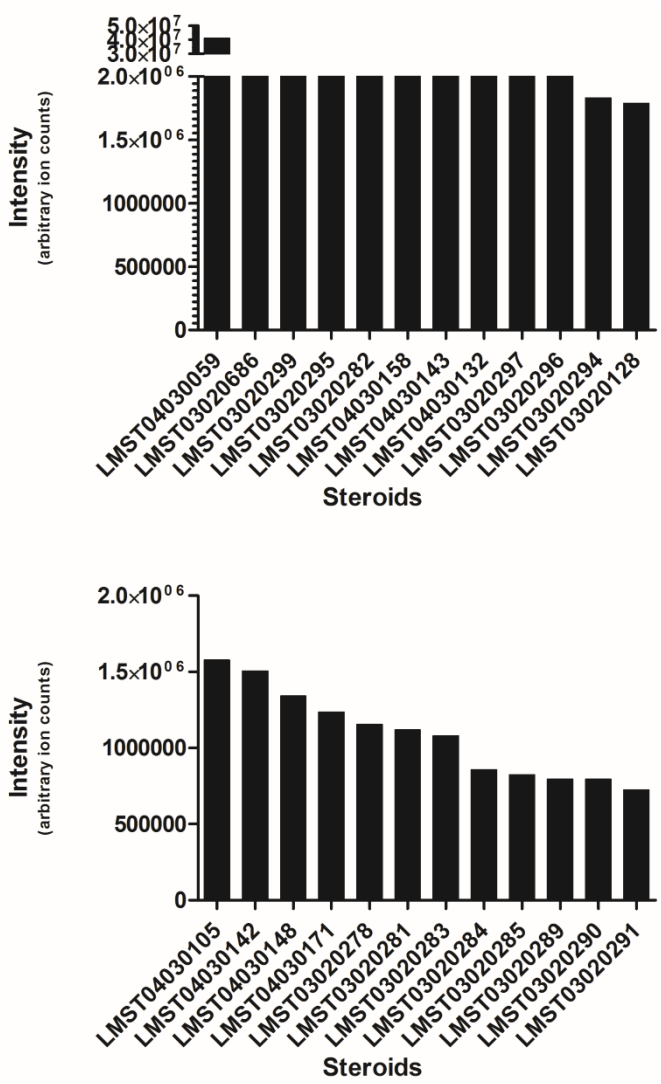

Figure 7. Steroids distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database [20].

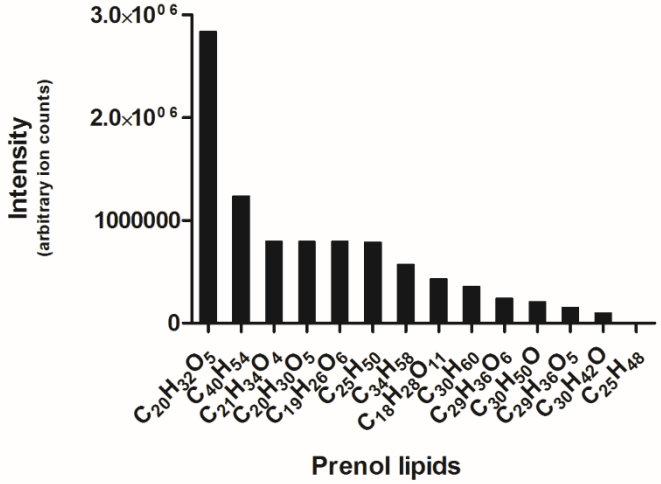

Figure 8. Prenols distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database [20].

Finally, sterols, prenols, saccharolipids and polyketides were hereby investigated for the first time within the framework of RBC storage. Intriguingly, all these classes of lipids statistically significant decreases throughout storage duration (Table 1). Most of the observed decreases account for sterols (e.g. desmosterol, gorgosterol), and in particular for vitamin D3-related metabolites (Table 1). This is relevant in the light of the well-established role for Vitamin D in modulating RBC survival [61], also by influencing anti-oxidant potential and Ca^{2+} permeability [62], a phenomenon which is strictly tied to erythrocyte-specific apoptosis, also known as eryptosis [63].

On the other hand, earliest studies on the likely long term effect of RBC storage on the lipidome suggested that cholesterol loss is limited in comparison to the loss of phospholipids and phosphoinositides [64]. Finally, our results about a generalized decrease in lipid contents of the major lipid classes in long stored RBCs also confirm and expand/complement recent evidences by Acker's group [60,65].

4. Conclusion

Despite decades of investigations, the field of lipidomics recently drained new lymph from the introduction of recent technical innovations. From TLC to gas chromatography and MS, consolidated lipidomics expertise in the field of RBC biology has paved the way for a deeper understanding of the functioning of this pivotal cell and, in parallel, to the accumulation of a wealth of knowledge that will be soon transferred to the clinical setting. Indeed, owing to their relative abundances and widespread biological activities, lipids are well suited to play the role of biological markers and will soon serve this purpose.

In this study, we presented an HPLC-microTOF-Q approach to investigate the RBC lipidome. We could exploit this analytical workflow to consolidate existing knowledge on the RBC lipid composition and individuate statistically significant fluctuations of lipids throughout storage duration of RBC concentrates under blood bank conditions. While this field of research still warrants future investigations, which could be exploiting for example recently introduced imaging mass spectrometry approaches [66], we could indicate ceramides, glycerophospholipids and sterols as key targets of RBC storage lesions to the lipidome, that will deserve further targeted investigations in the future.

Finally, in the light of minor differences with other reports available from the literature, we posited how compositional analyses of the RBC lipidome might end up yielding different results on the basis of the background of the blood donor (above all, the diet), which might translate into region-specific lipidomic alterations over storage progression of RBC concentrates. This is relevant in the light of the constant efforts pursued by transfusion services to improve the quality of blood-derived therapeutics [67], by shifting the focus of attention from the end-product (RBC concentrates) to their providers (the donors).

Acknowledgments

The authors are supported by funds from the Italian National Blood Centre (Centro Nazionale Sangue - CNS - Istituto Superiore Sanita` - Rome, Italy). Angelo D'Alessandro was supported by mobility studentship funds by the Interuniversity Consortium for Biotechnology (CIB). **Funding** TAM, CM, ADA and LZ are supported by funds from the Italian National Blood Centre (Centro Nazionale Sangue - CNS - Istituto Superiore Sanita` - Rome, Italy).

Conflict of interest statement. All the authors disclose any conflict of interest.

Authorship contributions

Timperio Anna Maria – Designed the experiment and performed mass spectrometry analyses.

Cristiana Mirasole – elaborated the results, prepared figures and tables.

Angelo D'Alessandro – wrote the paper.

Lello Zolla – Supervised the experimental design and methods, provided laboratory equipment and contributed with his expertise in the field of biochemistry to the revision of the draft of the manuscript..

References

1. H. Alex Brown, Curr Opin Chem Biol 16 (2012) 221-226. DOI: 10.1016/j.cbpa.2012.02.003.
2. G.B. Philips, N.S. Roome, Proc Soc Exp Biol Med 100 (1959) 489-492. DOI: 10.3181/00379727-100-24670.
3. D.J. Hanahan, R.M. Watts, D. Papajohn, J Lipid Res 1 (1960) 421-432. DOI:jlr.org/content/1/5/421.short.
4. J.W. Farquhar, E.H. Jr Ahrens, J Clin Invest 42 (1963) 675-685. DOI: 10.1172/JCI104759.
5. P. Ways, D.J. Hanahan, Lipid Res 5 (1964) 318-328. DOI:jlr.org/content/5/3/318.short.
6. J.T. Dodge, G.B. Phillips, J Lipid Res 8 (1967) 667-675. DOI:jlr.org/content/8/6/667.short.
7. J.S. Owen, K.R. Bruckdorfer, R.C. Day, N. McIntyre, J Lipid Res. 23 (1982) 124-132. DOI: jlr.org/content/23/1/124.short.
8. R.M. Dougherty, C. Galli, A. Ferro-Luzzi, J.M. Iacono, Am J Clin Nutr 45(1987) 443-455. DOI: http://ajcn.nutrition.org/content/45/2/443.short.
9. X. Han, R.W. Gross, Proc Natl Acad Sci U S A 91 (1994) 10635-10639. DOI: 22/10635/10639.
10. C. Beermann, M. Mobius, N. Winterling, et al. Lipids 40 (2005) 211-218. DOI: 40/L9604/2005.
11. C.M. Skeaff, L. Hodson, J.E. McKenzie, J Nutr 136 (2006) 565-569. DOI: jn.nutrition.org/content/136/3/565.short.
12. P. Rise, S. Eligini, S. Ghezzi, et al. Prostaglandins Leukot Essent Fatty Acids 76 (2007) 363-369. DOI: 10.1016/j.plefa.2007.05.003.
13. E.K. Kabagambe, M.Y. Tsai, P.N. Hopkins, et al. Clin Chem 54 (2008) 154-162. DOI: 10.1373/clinchem.2007.095059.
14. T.P. Novgorodtseva, Y.K. Karaman, N.V. Zhukova, E.G. Lobanova, M.V. Antonyuk, T.A. Kantur, Lipids Health Dis 10 (2011) 82-92. DOI: 10.1186/1476-511X-10-82.

15. A.K. Percy, E. Schmell, B.J. Earles, W.J. Lennarz, Biochemistry 12 (1973) 2456–2461. DOI: 10.1021/bi00737a014.
16. A. D'Alessandro, G.M. D'Amici, S. Vaglio, L. Zolla, Hematologica 97 (2012) 107–115. DOI: 10.3324/haematol.2011.051789.
17. F. Gevi, A. D'Alessandro, S. Rinalducci, L. Zolla, J Proteomics. 76 (2012) 168-180. DOI: 10.1016/j.jprot.2012.03.012.
18. S. Dinkla, K. Wessels, W.P. Verdurmen, C. Tomelleri, J.C. Cluitmans, J. Fransen, B. Fuchs, J. Schiller, I. Joosten, R. Brock, G.J. Bosman, Cell Death Dis 18 (2012) 410-418. DOI: 10.1038/cddis.2012.143.
19. E. Fahy, S. Subramaniam, H.A. Brown, et al. J Lipid Res 46 (2005) 839-861. DOI: 10.1194/jlr.E400004-JLR200.
20. M. Sud, E. Fahy, D. Cotter, A. Brown, E.A. Dennis, C.K. Glass, A.H. Merrill Jr, R.C. Murphy, C.R. Raetz, D.W. Russell, S. Subramaniam. Nucleic Acids Res. 35 (Database issue) (2007) D527-532. DOI: 10.1093/nar/gkl838.
21. M.F. Clasquin, E. Melamud, J.D. Rabinowitz, Current Protocols in Bioinformatics. 14 (2012) 14-11. DOI: 10.1002/0471250953.bi1411s37.
22. A. D'Alessandro, F. Gevi, L. Zolla, Mol Biosyst. 27 (2011) 1024-1032. DOI: 10.1039/c0mb00274g.
23. P.T. Ivanova, S.B. Milne, H.A. Brown, J Lipid Res 51 (2010) 1581-1590. DOI: 10.1194/jlr.D003715.
24. R. Tautenhahn, G.J. Patti, D, Rinehart, G, Siuzdak, Anal Chem 84 (2012) 5035-5039. DOI: 10.1021/ac300698c.
25. K.N. Belosludtsev, A.S. Trudovishnikov, N.V. Belosludtseva, A.V. Agafonov, G.D. Mironova, J Membr Biol 237 (2010) 13-19. DOI: 10.1007/s00232-010-9302-1.
26. V. Pala, V. Krogh, P. Muti, V. Chajès, E. Riboli, A. Micheli, M. Saadatian, S. Sieri, F. Berrino, J Natl Cancer Inst 93 (2001) 1088-1095. jnci.oxfordjournals.org/content/93/14/1088.short.
27. V. Rioux, D. Catheline, E. Beauchamp, J. Le Bloc'h, F. Pédrono, P. Legrand. Animal. 2 (2008) 636-644. DOI: 10.1017/S1751731108001705.
28. H. Dabadie, C. Motta, E. Peuchant, P. LeRuyet, F. Mendy, Brit J Nutr 96 (2006) 283-289. DOI:10.1079/BJN20061813.
29. S.D. Poppitt, P. Kilmartin, P. Butler, G.F. Keogh, Lipids Health Dis 4 (2005) 30-36. DOI: 10.1186/1476-511X-4-30.
30. C. Grüllich, R.M. Duvoisin, M. K. Wiedmann, van Leyen. FEBS Lett. 489 (2001) 51-54. DOI: 10.1016/S0014-5793(01)02080-4.
31. W.L. Smith, R.C. Murphy, Chapter 13 In Biochemistry of lipids, lipoproteins and membranes (4th Edition) 2002; Vance DE and Vance JE Eds. Elsevier. DOI: books.it/booksid=LZprTsjcvIMCpg=PA331d.
32. M.M. Oliveira, M. Vaughn, Incorporation of fatty acids into phospholipids of erythrocyte membranes. J Lipid Res 5 (1964)156-162. DOI: jlr.org/content/5/2/156.short.
33. R.K. Donabedian, A. Karmen, J Clin Invest 46 (1967) 1017-1027. DOI:10.1172/JCI105591.
34. E. Mulder, J. de Gier, L.L.M. Van Deenen. Biochim Biophls Acta 70 (1962) 94-96. DOI:11.1572/BBA133211
35. S.B. Shohet, D.G. Nathan, M.L. Karnovsky, J Clin Invest 47 (1968) 1096-108. DOI: 10.1172/JCI105799.
36. C.Van Gastel, D. Van Den Berg, J. de Gier, L.L.M. Van Deenen, Brit. J Haematol 11 (1965) 193-199. DOI: 10.1111/j.1365-2141.1965.tb06577x.
37. E. Mulder, J.W.O. Van Den Berg, L.L.M. Van Deenen, Biochim. Biophys. Acta. 106 (1965) 118-125. DOI:10.1016/0005-2760(65)90100-1.
38. V.P. Skipski, R.F. Peterson, M. Barclay, Biochem. J 90 (1964) 374-378. DOI: 12.1202627/0003(64)-2.
39. M. Kates, A.C. Allison, A.T. James, 1961. Biochim Biophys Acta 48 (1961) 571-582. DOI: 10.1016/0006-3002(61)90055-5.
40. J.G. Pittman, D.B.J. Martin, Clin Invest 45 (1066) 165-172. DOI: 10.1172/JCI105328
41. V. Jakobik, I. Burus, T. Decsi, Eur J Pediatr 168 (2009) 141-147. DOI: 10.1007/s00431-008-0719-9.
42. N. Mikirova, H.D. Riordan, J.A. Jackson, K. Wong, J.R. Miranda-Massari, M.J. Gonzalez, PR Health. Sci J. 23 (2004) 107-113. DOI: 10.1002/ijc.2910450502.
43. K. Leidl, G. Liebisch, D. Richter, G. Schmitz, Biochim Biophys Acta 1781 (2008) 655-664. DOI:10.1016/j.bbalip.2008.07.008.
44. E.L. Smith, E.H. Schuchman, FASEB J. 22 (2008) 3419-3431.DOI: 10.1096/fj.08-108043
45. L.J. Siskind, S. Fluss, M. Bui, M. Colombini, J Bioenerg Biomembr 37 (2005) 227-236. DOI: 10.1007/s10863-005-6632-2.
46. L.R. Montes, D.J. López, J. Sot, L.A. Bagatolli, M.J. Stonehouse, M.L. Vasil, B.X. Wu, Y.A. Hannun, F.M. Goñi, A. Alonso, Biochemistry. 47 (2008) 11222-11230. DOI: 10.1021/bi801139z.
47. M. Tani, M. Ito, Y. Igarashi. Cell Signal 19 (2007) 229-237. DOI:/10.1016/j.cellsig.2006.07.001.
48. K. Simons, E. Ikonen, Nature 387 (1997) 569-572. DOI: 10.1038/42408.
49. P. Pianese, G. Salvia, A. Campanozzi, O. D'Apolito, A. Dello M. Russo, G. Pettoello-Mantovani, J. Corso, Pediatr Gastroenterol Nutr 47 (2008) 645-651. DOI: 10.1097/MPG.0b013e318170956a.
50. O. Quehenberger, A.M. Armando, A.H. Brown, S.B. Milne, D.S. Myers, A.H. Merrill, S. Bandyopadhyay, K.N. Jones, S. Kelly, et al., J Lipid Res 51 (2010):3299-3305. DOI: 10.1194/jlr.M009449.
51. European Directorate for the Quality of Medicines & HealthCare. Guide to the Preparation,Use and Quality Assurance of Blood Components. Recommendation No. R (95) 15.16th Edition. Council of Europe (2011) DOI:10.1111/j.1423-0410.2002.tb05271.x.
52. A. D'Alessandro. G. Liumbruno, G. Grazzini, L. Zolla, Blood Transfus 8 (2010) 82-88. DOI: 10.2450/2009.0122-09.
53. B. Blasi, A. D'Alessandro, N. Ramundo, L. Zolla, Transfus Med 22 (2012) 90-96. DOI: 10.1111/j.1365-3148.2012.01139.x.
54. G.M. D'Amici, C. Mirasole, A. D'Alessandro, T. Yoshida, L.J. Dumont, L. Zolla. Blood Transfus 10 (2012) 46-54. DOI: 10.2450/2012.008S.
55. R. Xu, W. Sun, J. Jin, L.M. Obeid, C. Mao, FASEB J 24 (2010) 2507-2515. DOI: 10.1096/fj.09-153635.
56. N.A. Neidlinger, S.K. Larkin, A. Bhagat, G.P. Victorino, F.A. Kuypers, J Biol Chem 281 (2006) 775-781 DOI:10.1074/jbc.M50579020.
57. R. Chaudhary, R. Katharia, Blood Transfus 10 (2012) 59-62 . DOI: 10.2450/2011.0107-10.
58. S.R. Goodman, A. Kurdia, L. Ammann, D. Kakhniashvili, O. Daescu, Exp Biol Med (Maywood) 232 (2007) 1391-408. DOI: 10.3181/0706-MR-156.
59. A. D'Alessandro, P.G. Righetti, L. Zolla, J Proteome Res 9 (2010) 144-163. DOI: 10.1021/pr900831f.
60. B. Bicalho, J.L. Holovati, J.P. Acker, Biochim Biophys Acta 1828 (2013) 317-326. DOI: 10.1016/j.bbamem.2012.10.026.
61. M. Alexander, Biomedicine (1977) 108-110. DOI: 10.890015/k.1965-1765.1977.
62. R.P. Holmes, M. Mahfouz, B.D. Travis, N.L. Yoss, M.J.

Keenan, Ann N Y Acad Sci 414 (1983) 44-56. DOI: 10.1111/j.1749-6632.1983.tb31673.

63. G. Pompeo, M. Girasole, A. Cricenti, G. Boumis, A. Bellelli, S. Amiconi, Biochim Biophys Acta 1798 (2010) 1047-1055. DOI: 10.1016/j.bbamem.2010.02.002.
64. T.J. Greenwalt, C. Zehner Sostok, U.J. Dumaswala. Vox Sang 58 (1990) 90-3. DOI: 10.1111/j.1423-0410.1990.tb02068.x.
65. R. Almizraq, J.D. Tchir, J.L. Holovati, J.P. Acker. Transfusion. 2013 DOI: 10.1111/trf.12080.
66. Mainini V, Bovo G, Chinello C, et al. Mol Biosyst. 2013 DOI: 10.1039/C2MB25296A.
67. G. Liumbruno, A. D'Alessandro, G. Grazzini, L. Zolla, J Proteomics 73 (2010) 483-507. DOI: 10.1016/j.jprot.2009.06.010.