Label-free quantitation of phosphopeptide changes in erythrocyte membranes: towards molecular mechanisms underlying deformability alterations in stored red blood cells

While the probability of troublesome adverse effects related to the transfusion of older red blood cell (RBC) units is still a matter of debate and of clinical investigation, what is now known for certain is that blood storage affects the biochemical and biological properties of RBCs. The accumulating changes, collectively known as "storage lesions", include alterations to either functionality (essentially metabolism and oxygen delivery capacity), or morphology (transition from a discoid to a spherocytic phenotype). These latter are mostly irreversible and result in a more rigid cell structure, with cytoskeleton disorders and perturbation of membrane protein interactions; therefore, they are likely the most responsible for reducing transfusion efficacy. Protein phosphorylation is known to be one of the most important and better-studied posttranslational modifications that affect protein-protein binding interfaces. Interestingly, all components of the red cell membrane skeleton (except actin) are phosphoproteins.[1] Past research has demonstrated that their phosphorylation is involved in the mechanical properties of the erythrocyte membrane.[2-4] However, there have been no studies at all on the phosphorylation events occurring during *in vitro* RBC aging. Based on these considerations, we aimed to investigate the phosphorylation status of erythrocyte membranes while undergoing blood storage for transfusion purposes by means of phosphoproteomics technologies. To this end, we decided to apply a gel-free shotgun proteomics approach to obtain qualitative phosphorylation site mapping of RBC ghosts. Specifically, packed RBCs were lysed with 9 vol of cold 5 mM phosphate buffer pH 8.0 containing 1 mM EDTA, 1mM phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitor cocktails (P5726, P0044 Sigma-Aldrich). Two hundred micrograms of red cell membrane proteins were subjected to in-solution tryptic digestion[5] followed by selective pre-enrichment of phosphorylated peptides through TiO_2 affinity chromatography microcolumns.[6] Eluted phosphopeptides were then analyzed by LC-MS/MS with both electron transfer dissociation (ETD) and neutral-loss triggered MS^3 in collision-induced dissociation (CID), as previously reported.[7] Experiments were performed in triplicate at 0-day storage time with five leuko-reduced CPD-SAGM RBC units (biological replicates) collected from different donors. Results are shown in Table 1. As expected, pTyr occurrence was very low, but in agreement with the estimated percentage,[8] while numerous Ser/Thr phosphopeptides were identified. Five of these were chosen to be quantitatively monitored during storage (at 0, 21 and 35 days); selection criteria are described in Table 1. The analytical strategy adopted for the quantitation consisted in a targeted approach because we only quantified (by LC-MS) those individual peptide ions of interest that had been detected and identified previously in data-dependent LC-MS/MS experiments. In detail, targeted quantification was performed by adopting a conventional label-free MS-based workflow relying on the calculation of the extracted ion chromatogram (EIC) peak height from LC-MS runs.[9,10] To minimize technical variability, each sample (i.e. 200 µg of digested erythrocyte membrane proteins) were spiked with 25 fmol/µg of bovine α-casein digest prior to TiO_2 enrichment. Among the α-casein phosphopeptides detected, the one showing the lower coefficient of variation (CV) was chosen as internal standard for data normalization (i.e. YKVPQLEIVPNSpAEER, m/z 976.40, CV = 14%). Figure 1 shows graphs obtained by plotting the normalized ion intensities of each phosphopeptide *versus* storage time. Measurements of phosphopeptide intensity variation

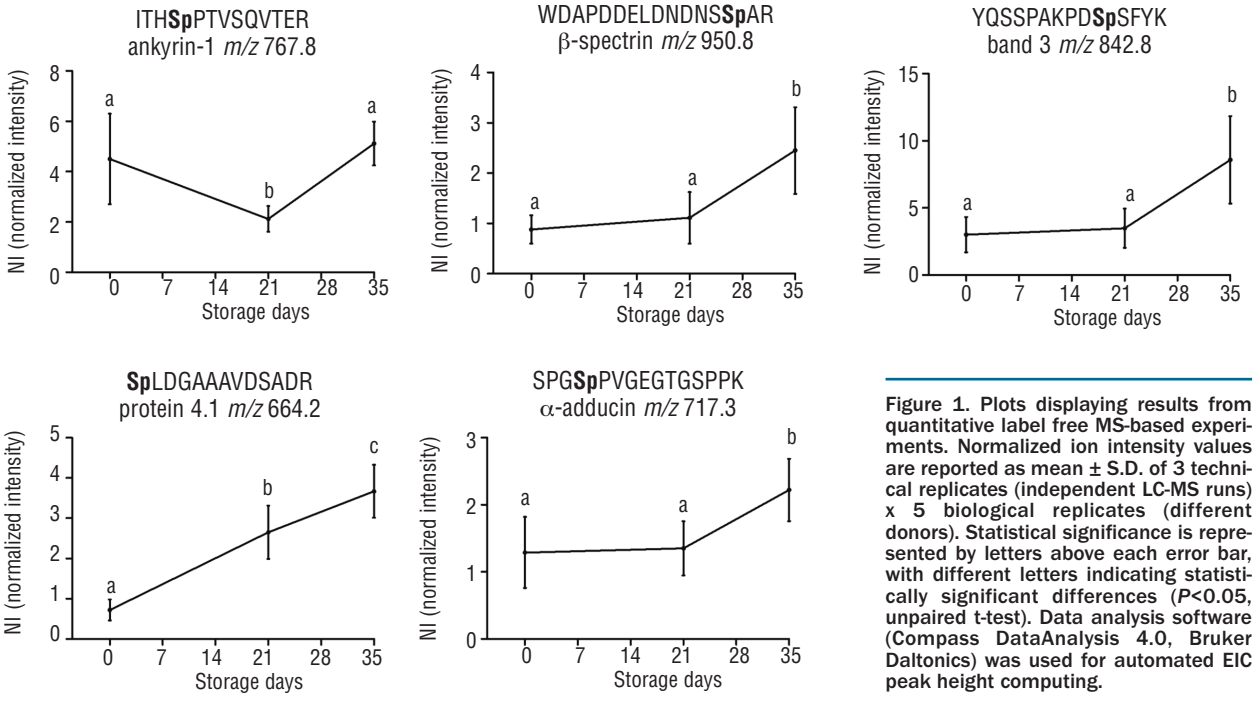

Figure 1. Plots displaying results from quantitative label free MS-based experiments. Normalized ion intensity values are reported as mean ± S.D. of 3 technical replicates (independent LC-MS runs) x 5 biological replicates (different donors). Statistical significance is represented by letters above each error bar, with different letters indicating statistically significant differences ($P<0.05$, unpaired t-test). Data analysis software (Compass DataAnalysis 4.0, Bruker Daltonics) was used for automated EIC peak height computing.

Table 1. Qualitative phosphopeptide mapping by LC-MS/MS in red cell membranes at 0-day storage.[a]

Protein ID (NCBInr Accession N.)	m/z	Charge state	Phosphopeptide sequence	Mascot score	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
ankyrin-1 (gi|70780359)									
	474.75	2+	**Tp**PTPLALR	40 (ETD); 47 (NL)	X		X	X	
	526.73	2+	GA**Sp**PNVSNVK	36 (ETD); 67 (NL)			X	X	
	644.37	2+	LGYI**Sp**VTDVLK	56 (ETD); 75 (NL)	X	X			
	512.25	3+	ITH**Sp**PTVSQVTER	90 (ETD); 82 (NL)		X	X	X	X
	767.86	2+	ITH**Sp**PTVSQVTER	102 (ETD); 80 (NL)	**X**	**X**	**X**	**X**	**X**
	571.60	3+	GEIVNMLEGSGRQ**Sp**R	70 (ETD); 50 (NL)	X			X	X
	1004.97	2+	NGA**Sp**PNEVSSDGTTPLAIAK	87 (ETD)		X			
	659.77	4+	RQDDATGAGQD**Sp**ENEVSLVSGHQR	133 (ETD); 68 (NL)		X	X		
	582.93	3+	LS**Tp**PPPLAEEEGLASR	79 (ETD); 58 (NL)		X	X		
	827.35	3+	QDDATGAGQD**Sp**ENEVSLVSGHQR	45 (NL)				X	X
spectrin beta chain (gi|67782319)									
	962.40	2+	LSS**Sp**WESLQPEPSHPY	76 (ETD); 64 (NL)	X		X	X	X
	668.60	3+	L**Sp**S**Sp**WESLQPEPSHPY LS**Sp**S**Sp**WESLQPEPSHPY	70 (ETD); 56 (NL) 66 (ETD); 70 (NL)		X	X	X	X
	1042.38	2+	L**Sp**S**Sp**WE**Sp**LQPEPSHPY	37 (ETD)	X	X	X		X
	922.45	5+	QIAERPAEETGPQEEEGE**Tp**AGEAPVSHHAATERTSPVSLWSR	61 (ETD)		X			
	950.81	2+	WDAPDDELDNDNS**Sp**AR	104 (ETD)	**X**	**X**	**X**	**X**	**X**
	633.87	3+	WDAPDDELDNDNS**Sp**AR	98 (ETD); 92 (NL)	X	X		X	X
	556.77	2+	T**Sp**PVSLWSR	55 (ETD)			X	X	X
	822.13	4+	DASVAEAWLIAQEPYLASGDFGHTVD**Sp**VEK	57 (NL)				X	
	652.29	4+	AWLQDAHRLL**Sp**GEDVGQDEGATR	42 (ETD)					X
band 3 anion transport protein (gi|4507021)									
	842.89	2+	YQSSPAKPD**Sp**SFYK	91 (ETD); 44 (NL)	**X**	**X**	**X**	**X**	**X**
	562.25	3+	YQSSPAKPDS**Sp**F**Yp**K	47 (ETD)			X	X	X
	614.32	3+	RYQSSPAKPD**Sp**SFYK RYQSSPAKPDS**Sp**FYK	82 (ETD); 42 (NL) 72 (ETD); 40 (NL)		X	X		
	827.59	5+	YQSSPAKPDS**Sp**FYKGLDLNGGPDDPLQQTGQLFGGLVR	49 (ETD)		X			
alpha-adducin (gi|559044)									
	717.30	2+	SPG**Sp**PVGEGTGSPPK	76 (ETD); 110 (NL)	**X**	**X**	**X**	**X**	**X**
	728.28	2+	QKG**Sp**EENLDEAR	100 (ETD)	X		X	X	
	613.65	3+	AAVVT**Sp**PPPTTAPHKER	90 (ETD); 56 (NL)			X	X	
	777.40	2+	AAVVT**Sp**PPPTTAPHK	50 (ETD)		X	X		
	786.80	2+	GDEASEEGQNGS**Sp**PK	69 (ETD); 32 (NL)				X	
	600.24	2+	G**Sp**EENLDEAR	53 (ETD)			X		
	762.63	3+	WLNSGRGDEASEEGQNGS**Sp**PK	59 (ETD); 39 (NL)	X		X		
	763.87	4+	SPG**Sp**PVGEGTGSPPKWQIGEQEFEALMR	50 (ETD)	X			X	
	908.64	4+	YSDVEVPASVTGYSFASDGDSGTC**Sp**PLRHSFQK	54 (NL)				X	
dematin (gi|62089016; gi|166706883)									
	523.01	4+	**Sp**LPDRTPFHTSLHQGTSK	114 (ETD); 63 (NL)	X	X		X	
	770.25	3+	RGAEEEEEEEDDD**Sp**GEEMoxK	90 (ETD)	X	X		X	X
	571.28	2+	HL**Sp**AEDFSR	53 (ETD); 55 (NL)		X		X	
	677.32	2+	QRE**Sp**VGGSPQTK	55 (ETD)	X	X	X		
	662.84	2+	GN**Sp**LPCVLEQK	41 (NL)	X		X		X
	864.67	4+	LQS**Tp**EFSPSGSETGSPGLQIYPYEMoxLVVTNK LQ**Sp**TEFSPSGSETGSPGLQIYPYEMoxLVVTNK	40 (ETD); 70 (NL) 55 (ETD)	X				X
	509.27	2+	SS**Sp**LPAYGR	46 (NL)			X	X	
	507.59	3+	TPFHT**Sp**LHQGTSK	91 (ETD)	X	X			
	718.25	3+	GAEEEEEEEDDD**Sp**GEEMoxK	86 (ETD); 73 (NL)			X		X

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protein 4.1 (gi42716291)								
664.28	2+	**Sp**LDGAAAVDSADR	64 (ETD); 108 (NL)	**X**	**X**	**X**	**X**	**X**
410.55	3+	HHA**Sp**ISELKK	76 (NL)		X		X	
954.94	4+	SLDGAAAVD**Sp**ADRSPRPTSAPAITQGQVAEGGVLDASAK	56 (NL)					X
394.22	3+	RL**Sp**THSPFR	51 (ETD)		X	X		
596.64	3+	QA**Sp**ALIDRPAPHFER	64 (ETD)	X	X			
glycophorin alpha (gi225711)								
1129.62	3+	KSPSDVKPLPSPDTDVPLS**Sp**VEIENPETSDQ	137 (NL)	X	X	X		
815.60	4+	SPSDVKPLP**Sp**PDTDVPLSSVEIENPETSDQ	68 (ETD); 84 (NL)					
		SPSDVKPLPSPD**Tp**DVPLSSVEIENPETSDQ	80 (ETD)			X	X	X
spectrin alpha chain (gi115298659)								
563.77	2+	KE**Sp**LNEAQK	42 (ETD); 60 (NL)	X	X			
413.70	2+	WG**Sp**LQR	37 (ETD)		X	X		
769.86	2+	WG**Sp**LQRLADEQR	63 (NL)				X	
hCG2041529 protein (gi119591886)								
862.84	2+	DGV**Sp**LGAV**Sp**STEEASR	93 (ETD); 70 (NL)		X		X	
902.81	2+	DGV**Sp**LGAV**Sp****Sp**TEEASR	50 (NL)		X			X
glycophorin C (gi183327)								
929.74	3+	GTEFAESADAALQGDPALQDAGDS**Sp**RK	73 (NL)		X	X	X	X
aquaporin (gi688358)								
798.00	3+	VWT**Sp**GQVEEYDLDADDINSR	48 (ETD)					
		VWTSGQVEEYDLDADDIN**Sp**R	66 (ETD); 42 (NL)	X	X	X	X	
chain D, Structure And Control Of The Actin Regulatory Wave Complex (gi313103963)								
478.60	3+	GGSGGSGGSGGSGG**Sp**GGSK	54 (ETD)	X			X	
KIAA0233 protein (gi1510143)								
707.30	2+	SG**Sp**EEAVTDPGER	58 (NL)			X		X
glucose transporter glycoprotein (gi183303)								
860.37	2+	**Tp**PEELFHPLGADSQV	52 (ETD); 51 (NL)					
		TPEELFHPLGAD**Sp**QV	61 (NL)		X	X		X
erythroid membrane-associated protein (gi15808373)								
704.34	2+	**Sp**EESIVPRPEGK	57 (ETD)	X	X			
HSP 90 (gi33987931)								
787.52	2+	IEDV**Sp**DEEDDSGK	51 (ETD); 79 (NL)			X	X	X

[a]*Whole blood (450 mL ± 10%) was collected from 5 healthy volunteer donors [male = 3, female = 2, age 40±11.2 (mean ± SD)] into CPD (citrate-phosphate-dextrose) anticoagulant (63 mL). After separation of plasma and buffy coat by centrifugation, leukocyte-filtered RBCs were suspended in 100 mL of SAG-M (saline, adenine, glucose, mannitol) additive solution. RBC units were stored for up to 42 days under standard blood bank conditions (4 ± 2°C) and samples were removed aseptically and used for the subsequent analyses. Only phosphopeptides where the phosphorylation site was confidently determined (Mascot delta score greater than 5) are listed. The presence of each phosphopeptide has been verified in all the 5 donors and annoted in the table with an X. Bold annotations highlight the phosphopeptides chosen for quantitation. Selection was firstly based on the obtained results by Western blotting with anti phospho Ser/Thr antibodies where bands corresponding to spectrin, ankyrin, adducin, band 3 and band 4.1 proteins were the most reactive ones (data not shown). Then, for each of these proteins we selected one representative peptide by considering the maximal confidence in the phosphosite localization (in terms of tandem mass spectral quality) and we combined this parameter with the presence of such a peptide in all the biological replicates. The higher Mascot scores obtained for ETD and/or neutral loss (NL)-triggered product ion spectra are shown in brackets (ion score cut off = 30). The detected pSer:pThr:pTyr ratio was 87.5:10.7:1.8.*

across all replicates for each condition resulted in CV values ranging from 16% to 40%. Quantitative data have been validated by multiple reaction monitoring (MRM)-based experiments (*data not shown*). Although each phosphopeptide showed a distinctive trend, it seems that 21-day storage represents a crucial point for the erythrocyte, when either a decrease or a progressive increase in the phosphorylation status occurs. This may correspond to the intensification of mechanisms inducing reduction of deformability observed during blood storage.[11] However, further investigations are needed to explore the functional effects of these phosphorylative changes on the quality of stored RBCs.

All the phosphopeptides quantitatively monitored in this study map in protein regions essential for the functional and structural organization of the red cell membrane architecture, suggesting that they may be involved in the molecular processes that control shape, flexibility and aggregability in stored RBCs. For example, the phosphopeptide ^{333}SpLDGAAAVDSADR^{345} belonging to band 4.1

falls in the 16 kDa protein domain. Curiously, this region seems to affect band 4.1 interactions with spectrin and actin proteins, in turn influencing membrane stability.[2,12] On the other hand, band 3 ^{347}YQSSPAKPDSpSFYK^{360} maps in proximity of one of the contact sites (represented by the sequence ^{343}LRRRY^{347}) between CDB3 and the protein 4.1,[13] thus phosphorylation of band 3 Ser-356 may affect the band 3-band 4.1 interactions. Interestingly, the β-spectrin N-terminal region, where our monitored phosphopeptide ^{25}WDAPDDELDNDNSSpAR^{38} maps, is known to serve as the counterpart for the 4.1R binding.[14] As far as α-adducin is concerned, it is known that the its 'neck domain' contains crucial amino acids (Ser-408, Ser-436, Ser-481) whose phosphorylation has previously been seen to favor the detachment of adducin from the spectrin/actin network,[3] but the same region is also clearly involved in association of adducin monomers to heterodimers, which constitute the functionally active form of the protein.[15] The α-adducin phosphopeptide monitored in our study (^{355}SPGSpPVGEGTGSPPK^{369}) maps within the neck domain, very close to the sites cited before, thus Ser-358 may either represent a new phosphorylation site with regulatory properties in the adducin oligomerization process, or may be involved in the control of vertical interactions anchoring the junctional complex to the lipid bilayer. On the other hand, still little information is available for the region of ankyrin where the ^{1683}ITHSpPTVSQVTER^{1695} phosphopeptide is located (downstream a 'death domain' at the C-terminus), thus any hypothesis about its involvement in maintaining membrane stability would, for the moment, be purely speculative given our present knowledge.

In conclusion, our MS-based approach allowed, for the first time, to follow peptide-specific quantitative changes in the phosphorylation status of ghost proteins in preserved RBCs, clearly demonstrating an increase in the Ser/Thr phosphorylation levels of RBC membrane skeletal proteins during blood storage. In our opinion, the investigation of the phosphorylation-triggered molecular events occurring during *in vitro* erythrocyte aging will be of great benefit in elucidating the causes of the reduced survival of transfused RBCs. In this direction, future large-scale phosphoproteomics analyses can provide new biological insights.

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