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Leukoreduction makes a difference: A pair proteomics study of extracellular vesicles in red blood cell units

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ABSTRACT

Prestorage filtration of blood to remove contaminating donor leukocytes and platelets has substantially increased the safety level of transfusion therapy. We have previously shown that leukoreduction has a mitigating effect on the storage lesion profile by lowering the extent of hemolysis and of RBC aging and removal phenotypes, including surface signaling and microvesiculation. Even though protein composition may determine the fate of EVs in the recipient, the probable effect of leukoreduction on the EV proteome has been scarcely investigated. In the present paired study, we characterized the proteome of EVs released in prestorage leukoreduced (L) and nonleukoreduced (N) RBC units prepared from the same donors, by immunoblotting and qualitative proteomics analyses at two storage intervals. Apart from common proteofrms typically associated with the established EV biogenesis mechanisms, the comparative proteomics analyses revealed that both leukoreduction and storage duration affect the complexity of the EV proteome. Membrane and cytoskeleton-related proteins and regulators, metabolic enzymes and plasma proteins exhibited storage duration dependent variation in L- and N-EVs. Specific proteoforms prevailed in each EV group, such as transferrin in L-units or platelet glycoproteins, leukocyte surface molecules, MHC HLA, histones and tetraspanin CD9 in N-units. Of note, several unique proteins have been associated with immunomodulatory, vasoregulatory, coagulatory and anti-bacterial activities or cell adhesion events. The substantial differences between EV composition under the two RBC preparation methods shed light in the underlying EV biogenesis mechanisms and stimuli and may lead to different EV interactions and effects to target cells post transfusion.

1. Introduction

Extracellular vesicles (EVs) are in the spotlight of the scientific community in the last years. Increasing evidence points to unique EV functions in cellular homeostasis, stress response, intercellular communication and other events that take place under a variety of physiological and pathological conditions [1]. EVs are released by almost all cells *in vivo* and, also, by red blood cells (RBCs) following cell aging and cold storage-related lesions, as an effective means of disposing of damaged, oxidized or potentially harmful signaling molecules and membrane patches [2]. In non-leukodepleted blood units, storage-induced vesiculation concerns the white blood cells (WBCs) and platelets (PLTs) as well [3].

EVs released by stored RBCs are enriched in oxidized/denatured hemoglobin (Hb), raft-associated proteins, metabolic enzymes and chaperones [4,5]. Their concentration, size distribution and biophysical features change during storage. In fact, the EV proteome becomes continuously more complex, presenting additional proteasome components, RBC aging markers and Fas signaling related molecules [1]. The time-dependent diversity in EV features mirrors the progression of the storage lesion in the parent cells. Storage conditions may trigger EV release by a variety of mechanisms, including oxidative stress and

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membrane disorganization [6]. Just like many aspects of stored RBC biology, the extent of vesiculation is affected by the length of storage, donor characteristics [5,7,8] and the processing methods followed from blood donation to preservation in the cold [1,9,10]. Regarding leukoreduction, substantially higher EV accumulation has been found in non-leukoreduced (N) units when compared to leukoreduced (L) ones [8]. More importantly, it seems that these EVs exhibit proinflammatory activity [7].

The acknowledged competence of EVs as potential biological response modifiers raises concerns about their post-transfusion effects. Concrete evidence of their involvement in immunomodulation, inflammatory responses and coagulatory activities following interactions with several types of recipient cells has been offered by both *in vitro* studies and animal models of transfusion [1,11–13]. Even though the EVs' composition may determine their fate in the recipient, few data are available about the effect of prestorage leukoreduction on the EV proteome. To focus on this target, we conducted a paired proteomics study on RBC units prepared by the same donors and stored from the same period with or without prestorage leukoreduction.

2. Material and methods

2.1. Biological samples

In two sequential donation events, packed RBCs were prepared from 4 different eligible young blood donors of the same blood group and stored in citrate-phosphate-dextrose (CPD)/saline-adenine-glucose-mannitol (SAGM) units for 42 days with (L) or without (N) prestorage leukoreduction, as previously reported [9]. The study was approved by the Ethics Committee of the Department of Biology, School of Science, NKUA. Investigations were carried out upon donor consent, in accordance with the principles of the Declaration of Helsinki.

2.2. Microscopic observation

Confocal laser scanning microscopy (CLSM) was used to detect the externalization of phosphatidylserine (PS). Stored RBCs were labeled with fluorescent annexin V and were thereafter observed through CLSM (Digital Eclipse C1, Nikon, NY). Annexin-V-Fluos solution was from Roche Diagnostics (Burgdorf, Switzerland). The morphological evaluation of RBCs was achieved through scanning electron microscopy (Philips SEM515), post fixing with 2% glutaraldehyde and 1% osmium tetroxide, dehydration in ascending ethanol concentrations and coating with gold-palladium (Tousimis Samsputter-2a, Rockville, Maryland). Both methods have been extensively described before [9].

2.3. Isolation of membranes and extracellular vesicles

To obtain RBC membranes, the cells were lysed with hypotonic sodium phosphate buffer containing protease inhibitors. Extracellular vesicles were isolated from the supernatant of the RBC units by high-speed centrifugation at 37,000xg for 1 h, as previously described [4]. The pellet was resuspended in PBS containing protease inhibitors. The protein concentration of isolated membranes and vesicles was determined by the Bradford assay (Biorad, Hercules, CA). Samples of the same group were pooled to proceed to western blot and proteomics analysis.

2.4. Immunoblotting analysis

Pooled samples of membranes and vesicles of equal (individual and total) protein concentration were loaded in Laemmli gels and then transferred to nitrocellulose membranes. A variety of proteins were identified by chemiluminescence detection using primary antibodies and species-specific secondary antibodies conjugated with HRP. The following antibodies and reagents were used: band 3, actin and human IgGs from Sigma–Aldrich (Munich, Germany); Hb from Europa Bioproducts (UK); peroxiredoxin 2 (Prdx2) from Acris GmbH (Herford, Germany); clusterin and calpain-1 (μ-calpain) from Santa Cruz Biotechnology (CA, USA); flotillin-2 from BD Transduction Laboratories (CA, USA); CD95 and caspase 3 from Cell Signaling Technology (Beverly, MA); HRP-conjugated IgGs from GE Healthcare (Buckinghamshire, UK) and Dako Cytomation (Glostrup, Denmark); ECL Western blot detection kit from Perkin Elmer (CA, USA); mAb against stomatin was kindly provided by Prof. R. Prohaska (Institute of Medical Biochemistry, University of Vienna, Austria).

2.5. Proteomics analysis

EV protein extracts were analyzed by a Ge-LC approach as previously described [14]. Briefly, 20 μg of proteins from each sample were loaded on a 12 % SDS-PAGE. Each gel lane was cut in 7 bands and proteins were in-gel digested. Peptide extracts were analyzed by using a split-free nano-flow liquid chromatography system (EASY-nLC II, Proxeon, Odense, Denmark) coupled with a 3D-ion trap (model AmaZon ETD, Bruker Daltonik, Germany) equipped with an online ESI nanosprayer (the spray capillary was a fused silica capillary, 0.090 mm OD, 0.020 mm ID) in the positive-ion mode. For all experiments, a sample volume of 15 μl was loaded by the autosampler onto a homemade 2-cm fused silica precolumn (100 μm I.D.; 375 μm O.D.; Reprosil C18-AQ, 5 μm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Sequential elution of peptides was accomplished by using a flow rate of 300 nl/min and a linear gradient from Solution A (100 % water; 0.1 % formic acid) to 50 % of Solution B (100 % acetonitrile; 0.1 % formic acid) in 40 min over the precolumn on-line with a homemade 15-cm resolving column (75 μm ID; 375 μm OD; Reprosil C18-AQ, 3 μm, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Acquired MS/MS spectra were processed in DataAnalysis 4.0 and submitted to the Mascot search program (Matrix Science, London, UK). The following parameters were adopted for database searches: database = NCBInr; taxonomy = Homo sapiens; peptide and fragment mass tolerance = ±0.3 Da; missed cleavages = 1; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M).

3. Results

Examination of RBC units by confocal microscopy provided morphological evidence of annexin V positive EVs' release by the surface of annexin V positive RBCs. Closer examination of vesiculated cells and EVs by SEM revealed heterogeneity in EV size and shape that was smaller in budding EVs than in released, free EVs (Fig. 1A). SDS-PAGE of pooled storage EVs, isolated by the supernatant of d21 (early EVs) and d42 (late EVs) units, revealed: a) numerous protein bands in the N-compared to the L-EVs, b) a further increase in the number of electrophoretic bands with storage time, and c) a different band profile in EVs compared to that of RBC membrane (Fig. 1B).

Immunoblotting analysis of samples for the detection of typical EV components showed an enrichment of late L-EVs in Hb-oligomers, Prdx2 and membrane microdomain-associated proteins (stomatin, flotillin-2) compared to late N-EVs. However, the extent of Hb oligomerization and band 3 proteolysis was slightly higher in the N-EVs compared to the L-EVs in early storage. Moreover, the N-EVs contained higher levels of actin, calpain, Fas receptor, clusterin and caspase-3 (including the proteolytic fragments of activated molecule) than L-EVs, at both storage times (Fig. 1C).

Subsequent qualitative proteomics analysis of pooled L- and N-EV samples verified the electrophoretic profiles by identifying more proteoforms in N-EVs compared to L-EVs and in late EVs compared to early ones in both groups. The EVs contained secreted or plasma proteins (immunoglobulins, albumin etc) and cell-derived components. The percentage of different plasma proteoforms was slightly higher in the L-EVs vs. N-EVs (Fig. 2A).

Regarding the cell-derived components, all EV samples share in

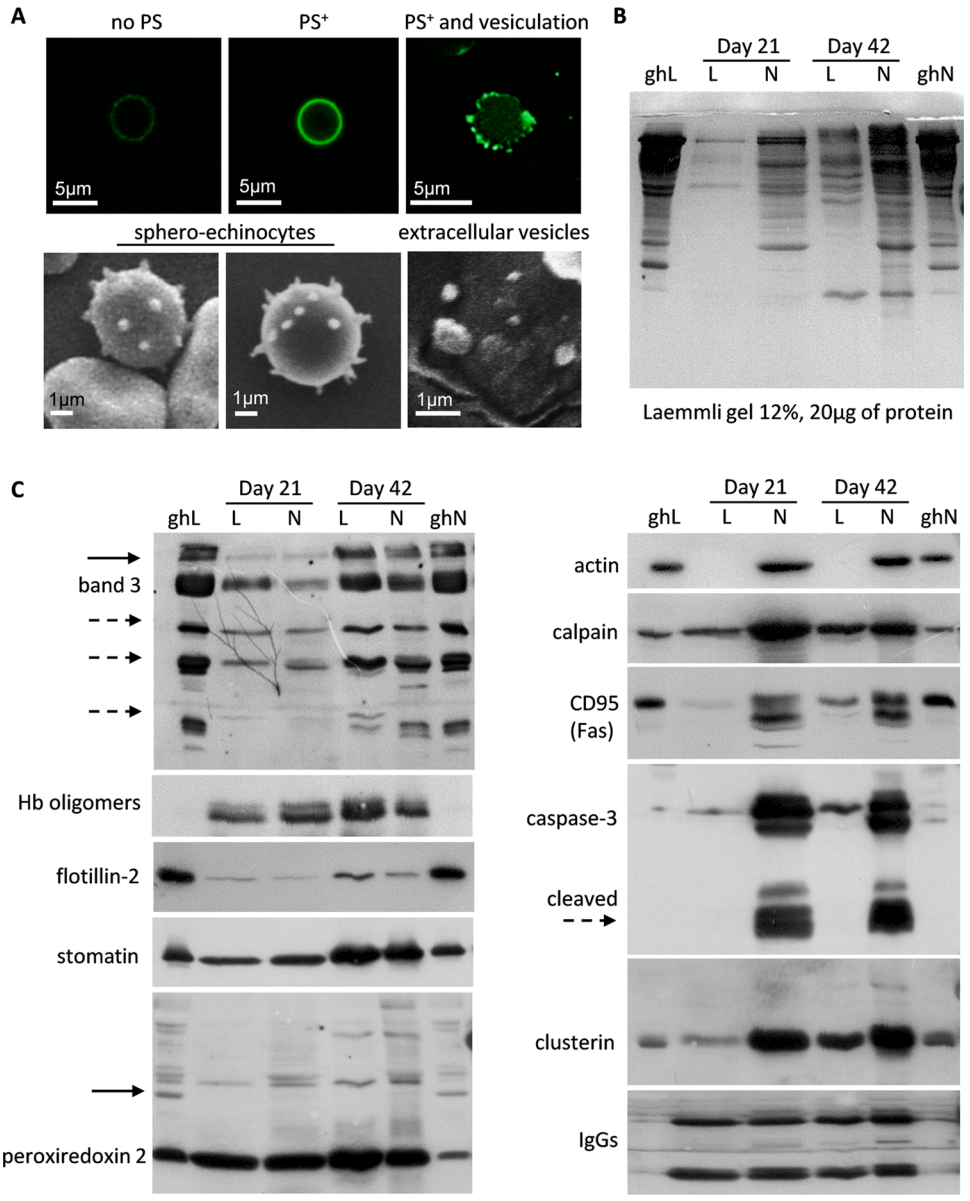

Fig. 1. Immunoblotting and morphological analysis of EVs and RBCs from leukoreduced (L) and non-leukoreduced (N) blood units. (A) Observation of stored RBCs and storage EVs by confocal laser scanning microscopy and scanning electron microscopy. PS: phosphatidylserine. (B) Separation of ghost (gh) and EV samples by SDS-PAGE. (C) Representative western blots for selected EV proteins at middle and late storage. Samples of isolated RBC membranes (ghL and ghN) are shown in order to facilitate comparison between RBC membranes and EVs. -Solid arrows: oligomers; dashed arrows: Band 3 fragments or cleaved (activated) caspase-3.

common membane (band 3) and membrane-linked (stomatin) as well as cytosolic (Hb, carbonic anhydrase-1, Prdx2) proteins that are conditionally associated with the membrane. Though the Mascot score is only an approximate indicator for protein abundance in the analyzed sample, most common EV components predominated in late L-EVs (Fig. 2B).

Late storage EVs contained a great variety of membrane and cytoskeleton proteoforms (eg. CD59, ankyrin and erythrocyte membrane protein band 4.2) compared to EVs released at mid storage (Table 1). Moreover, GLIPR2 (a protein with amyloidogenic properties related to functional aggregation events) and the Ras-protein ARF1 appeared only in later storage EVs of both groups (Table 1). As mentioned above, several typical EV components accumulated predominantly in late vs. early L-EVs (Fig. 2C).

Finally, an array of proteoforms were specifically detected in the L-EV or N-EV proteoms. For instance, L-EVs were characterized by the presence of the transmembrane protein DING (involved in phosphate ion transport; all storage EVs), transferrin, flavin reductase (NADPH) and VCP-ATPase (transitional endoplasmic reticulum ATPase), in agreement with previous proteomics analyses [5]. As mentioned above, additional components of the RBC membrane (glycophorin C, protein 4.1R) appeared only in late compared to middle storage EVs. A small minority of L-EV proteoforms was of non-RBC origin, as exemplified by the CD58 protein, a cell adhesion and signaling molecule expressed mainly in macrophages [5].

On the contrary, the N-EVs, were enriched in PLT- and WBC-derived proteins, although a few proteoforms of potentially mixed cellular origin might be derived from RBCs according to previously reported studies [5, 7]. Among these, heat shock proteins, glucose transporter, small GTPases (RAP1B, GNAI), cytoskeleton-associated proteins/modulators (moesin, cofilin, 14-3-3), catalase, and metabolic enzymes (aldolase A, enolase, TPI1 triosephosphate isomerase) are included (Table 2). However, the majority of N-EV proteoforms have not been detected (at least more than in traces) in L-EV proteoms, as with the case of histones, tetraspanin CD9, platelet glycoproteins, calelectrin, medullasin, MHC HLA, and other leukocyte surface molecules. Most N-unique components present throughout storage were cytoskeleton components, partners, modulators and regulators: actin, talin, filamin, fermitin, vinculin, gelsolin, WDR1, cofilin-1, CAP-1 (adenyl cyclase-associated protein-1) and the Ca^{2+}-dependent annexin A2 (Table 2). Such proteins prevailed in early EVs (Fig. 2D). Annexin A2 and actin equally participated in all

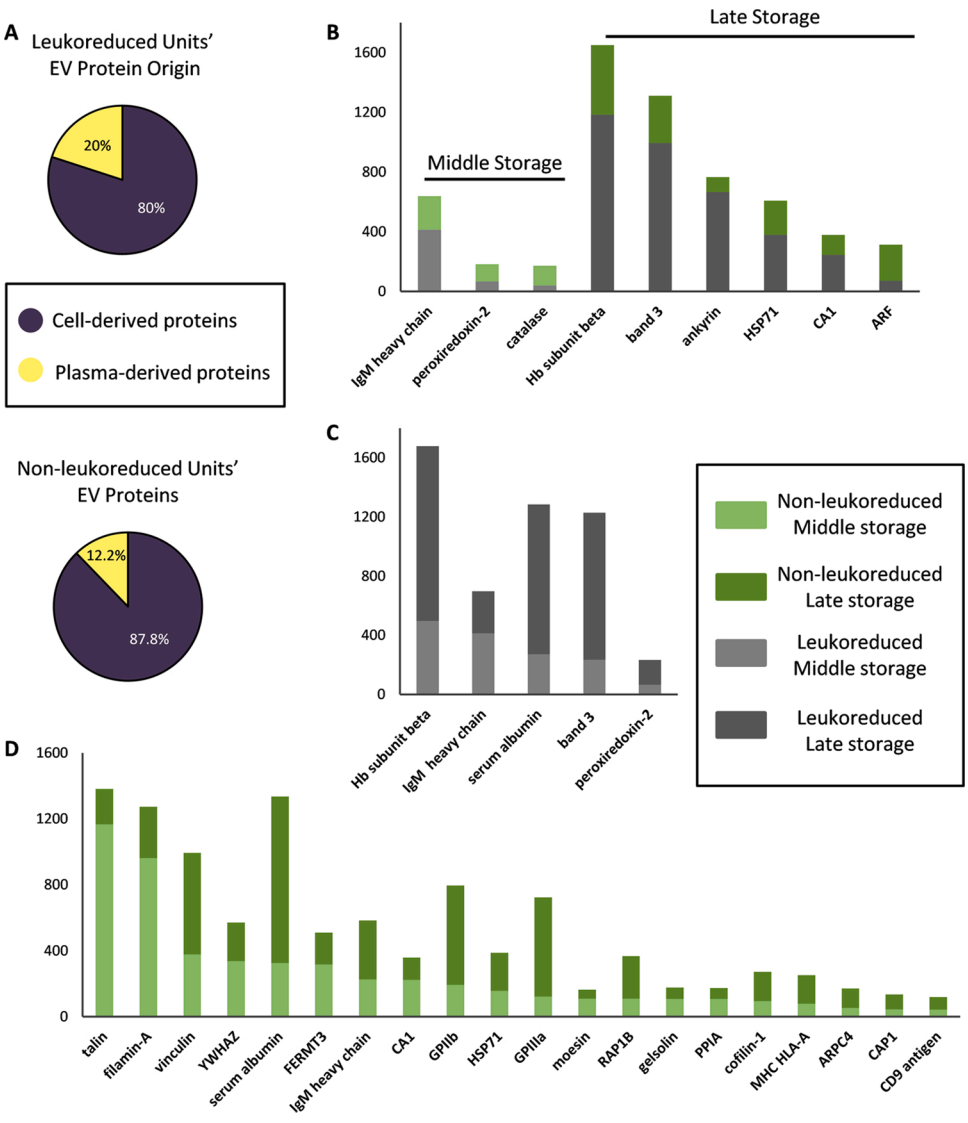

Fig. 2. Proteomics analysis of EVs isolated from leukoreduced (L) and non-leukoreduced (N) blood units. (A) Origin of detected proteins. Proteoforms with highly different Mascot scores between (B) N-EVs and L-EVs, (C) L-EVs of middle and late storage, and (D) N-EVs of middle and late storage. CA1: carbonic anhydrase 1; ARF: ADP-ribosylation factor; YWHAZ: 14-3-3 protein zeta/delta; FERMT3: fermitin family homolog 3; GPIIb: platelet membrane glycoprotein IIb; GPIIIa: platelet glycoprotein IIIa; RAP1B: ras-related protein Rap-1b; PPIA: peptidyl-prolyl cis-trans isomerase A; ARPC4: actin-related protein 2/3 complex subunit 4; CAP1: adenylyl cyclase-associated protein 1.

Table 1
Proteins detected selectively in late storage EVs.

	L-EVs	N-EVs
ankyrin	665	100
erythrocyte membrane protein band 4.2	277	254
immunoglobulin kappa light chain	93	277
CD59 glycoprotein preproprotein	61	59
Golgi-associated plant pathogenesis-related protein 1 (GLIPR2)	169	123
ADP-ribosylation factor (ARF1)	70	243

Data are shown as mascot scores.

storage N-EVs, as verified by immunoblotting analysis (Fig. 1C).

The early storage N-EVs were characterized by the presence of several metabolic enzymes (e.g. pyruvate kinase and L-lactate dehydrogenase) not detected in later storage EVs (Fig. 2D). Moreover, some GTPases (EHD3), skeleton-related proteins (beta-parvin), the cytokine and alarmin S100-A8 (subunit of S100 A8/A9 heterodimer) and the plasma fibronectin-1 were also exclusively detected in those samples (Table 2B).

On the other hand, a greater variety of membrane proteins (platelet glycoproteins, leukocyte surface protein, CD109, claudin), integrins, MHC antigens, membrane-associated proteins (calelectrin), RNA-binding proteins (QKI), histones and several serum or secreted proteoforms (lactoferrin, vitronectin, fibrinogen, clusterin) was found in later storage N-EVs (Table 2B). Cytoskeleton components or regulators (e.g., ARPC5 protein) were less evident in this EV subgroup (Table 2B). Regarding the relative abundanc of N-EV proteins, serum albumin, MHC HLA antigens, PLT glycopoteins, heat shock proteins and small GTPases were predominantly detected in late vs. early storage N-EVs.

4. Discussion

The current study focuses on the effects of prestorage leukoreduction on the protein composition of storage EVs. While growing evidence supports the beneficial role of prestorage leukoreduction on the RBC storage lesion profile and the potential adverse effects of transfusion [9, 15–17], it is not a uniformly applied preparation strategy in RBC transfusions worldwide. In addition, despite lower accumulation of storage EVs in leukoreduced RBC units compared to nonleukoreduced ones (due to the additional vesiculation of PLTs and WBCs as well as to the milder storage lesion of RBCs *per se* [3,9]), the protein content of L- vs. N-EVs is not widely studied. Finally, the substantial individual variability among healthy donors in both the number and the features (including the proteome) of plasma EVs [18,19], favors studying filtration effects in pairs of L- and N-RBC units prepared by the same donors. According to our results, leukoreduction and storage duration independently and in synergy affect the proteome of the EVs accumulated in

Table 2
Proteins detected only in EVs from (A) leukoreduced or (B) non-leukoreduced blood units.

A. Leukoreduced Units Protein	Middle Storage	Late Storage
DING protein	78	90
transitional endoplasmic reticulum ATPase (VCP)	ND	139
transferrin (TF)	ND	137
p48	ND	118
CD58 protein	ND	53
glycophorin C	ND	48
EPB41 protein	ND	40
flavin reductase (NADPH)	ND	48

B. Non-leukoreduced Units Protein	Middle Storage	Late Storage
talin	1166	216
filamin-A	962	312
beta actin	608	622
fermitin family homolog 3 (kindlin-3)	316	194
vinculin	377	616
platelet membrane glycoprotein IIb	192	604
platelet glycoprotein IIIa	121	603
moesin (MSN)	109	54
gelsolin	107	70
platelet glycoprotein Ib alpha	51	92
WDR1 protein	129	72
adenylyl cyclase-associated protein 1 (CAP-1)	44	91
MHC HLA-A	78	174
LIM and senescent cell antigen-like-containing domain protein 1 (LIMS1)	83	115
annexin A2	58	41
14−3-3 protein epsilon (YWHAE)	54	95
14−3-3 protein gamma (YWHAG)	150	155
glycoprotein Ib beta	131	124
ras-related protein Rap-1b (RAP1B)	109	258
CD9 antigen	43	76
ras suppressor protein 1 (RSU1)	41	61
cofilin-1 (CFL1)	94	178
actin-related protein 2/3 complex subunit 4	53	118
histone H3	53	54
peptidyl-prolyl cis-trans isomerase A (cyclophilin A)	107	67
guanine nucleotide-binding regulatory pro-tein alpha-inhibitory subunit	68	ND
platelet glycoprotein IIb	47	ND
fibronectin 1 (FN1)	46	ND
pyruvate kinase	52	ND
EH domain containing 3 (EHD3)	50	ND
p58 NK cell inhibitory receptor NKR-K6	45	ND
aldolase A (ALDOA)	139	ND
CGI-56 protein (beta parvin, PARVB)	115	ND
actin-related protein 3 (ARP3)	88	ND
protein S100-A8	73	ND
alpha enolase (ENO1)	66	ND
L-lactate dehydrogenase A chain (LDHA)	61	ND
p64 CLCP	43	ND
triosephosphate isomerase 1 (TPI1)	119	ND
rho GDP-dissociation inhibitor 2 (ARHGDIB)	67	ND
platelet glycoprotein 4 (CD36)	ND	246
lactoferrin	ND	89
leukocyte surface protein	ND	52
CD109	ND	47
integrin alpha6 subunit	ND	42
calelectrin	ND	113
vitronectin (VTN)	ND	62
MHC class I antigen, partial	ND	162
fibrinogen gamma chain (FGG)	ND	51
apolipoprotein J precursor	ND	46
QKI (STAR)	ND	42
14−3-3 protein eta (YWHAH)	ND	120
RAS-related protein MEL	ND	72
Hpast	ND	70
medullasin	ND	52
beta integrin-linked protein kinase	ND	83
interferon-inducible protein 9−27 (IFITM1, CD225)	ND	184
histone H1b	ND	104

Table 2 (*continued*)

B. Non-leukoreduced Units Protein	Middle Storage	Late Storage
platelet glycoprotein IX	ND	103
actin-related protein 2/3 complex subunit 5 (ARPC5)	ND	78
histone H4	ND	67
histone H2B	ND	55
proteolipid protein 2	ND	54
YPT3	ND	53
ras-related protein Rap-2 (RAP2A)	ND	43
claudin-3 (CLDN3)	ND	42

Data are shown as mascot scores. ND: not detected.

the RBC units and probably, the molecular mechanisms of the underlying vesiculation events. Though both preparations contained typical EV proteins in high adundance [5], the proteome complexity increased with storage time but decreased following leukoreduction.

Deep investigation of storage-derived EVs is equally challenging (mainly because of pre-analytical and analytical issues [1,20]) and significant (for scientific and, probably, clinical reasons). At first, EVs' biogenesis is the result of storage-related cell degradation and as such, it mirrors storage lesion profiles. To support, the rate of vesiculation shows correlations with cell deformability, hemolysis and ATP content [21]. Moreover, as a cellular response to storage-related stress and *in vitro* aging, storage EVs may shed light into the mechanisms of membrane vesiculation. Finally, the biochemical features of storage EVs may modify the risk of post-transfusion complications following potential interactions with soluble and cellular factors in the recipient. EV composition, in particular, is indicative of biogenesis stimuli and mechanisms and may determine the EVs' fate post transfusion [18].

Vesiculation may generally occur by several mechanisms that drive modifications in the organization, composition and topological parameters of the membrane and its interactions with the cytoskeleton [22]. Our resuts verified the intrinsic relation of EV release with the abolishment of membrane phospholipid asymmetry [23–25], the band-3-centered aging pathway and the rearangements of the cytoskeletal network [23,26,27]. Indeed, we detected adundant EV components critically associated with those biogenesis mechanisms, such as stomatin, oxidized/denatured Hb and Prdx2. Consistent with other reports, the EV proteome differed at various storage periods (eg. higher prevalance of metabolic enzymes in early EVs) and, as a general rule, its heterogeneity increased with storage time [21,23,26]. Based on the fact that more cytoskeleton-related proteoforms (eg. ankyrin and protein 4.2) appeared in late storage EVs in both groups, one may suggest that cytoskeleton rearrangements and defects that underlie vesiculation is an ongoing process, substantially exacerbated with storage duration. To support, (a) progressive accumulation of Hb to the cytoskeleton, (b) increase in the oxidative index of the cytoskeletal proteins [28], and (c) a specific kinetic of changes in the cytoskeletal mesh that contained both disruption of filaments and protein clustering [29] have been detected in stored cells. Of note, irreversible changes to the cytoskeleton structure first appear at mid storage [29] when the vesiculation rate substantially increases over baseline.

In both EV groups, we observed prevalence of metabolic and redox proteoforms (e.g., pyruvate kinase, aldolase A, catalase) in early phase EVs and of membrane-/cytoskeleton-related proteoforms (e.g., band 3, ankyrin) in late phase EVs. This finding reveals a sequential order of the storage lesion (including vesiculation) events, namely deregulation of energy and redox metabolism that precedes the severe disruption of normal cytoskeletal protein structure observed at late storage. Indeed, an interesting interplay between several storage-induced vesiculation triggers is taking place during storage [26], such as lipid modifications, oxidative insults and calcium accumulation [6,24]. The metabolic rate is decreased in the RBCs after two weeks in the cold [30,31], and this is reflected in a time-course decrease in the concentration of certain

metabolic intermediates (e.g., phosphoenolopyruvate) in the released EVs [24]. On the other hand, shape modifications, especially towards non-reversible cellular forms, prevail at late storage [9]. Consequently, it's tempting to suggest that metabolic stress accompanies or underlies vesiculation events at an earlier storage time while membrane and cytoskeleton defects (mainly due to accumulated oxidative lesions [32]) consist the influential EV biogenesis event at late storage.

In the same context, the majority of unique proteoforms in N-EVs were cytoskeleton components and partners, such as regulators and molecular motors/switchers (eg. small GTPases). Among these, beta actin, that was substantially underpresented in L-EVs in consistency with previous reports [7]. This finding suggested involvement of actin networks in the release of exosomes and microvesicles by non-erythroid cells and less probably, by the stored N-RBCs as well. To support, N-EVs further contained the GPI-anchored protein CD109 [33] and the tetraspanin CD9 involved in exosome biogenesis [34] in PLTs and other cells [35]. Another exosome-related protein (Alix) was recently detected by nano ultra-HPLC-tandem MS proteomics in L-EVs collected under the same conditions (37,000xg) [5]. Finally, transmission electron microscopy studies have shown that the average size of storage EVs is <200 nm, with many of them ranging in the typical exosome size [4,21]. These observations are of particular importance in the light of studies showing that these small EVs represent the predominantly bioactive fraction of stored EVs, at least with respect to immunomodulation [36].

The mixed cellular population of N-RBC units resulted in greater proteome complexity in N-EVs that further increased by storage time. Apart from actin, they were enriched in calcium dependent membrane-binding proteoforms (calpain, calelectrin etc) compared to L-EVs, suggesting a more influential role of calcium stress in those vesiculation events [37]. Overloading with Fas and caspase-3 (Fig. 1) is not indicative of apoptotic bodies, since the EV isolation protocol followed minimizes the collection of particles >0.8 μm. After all, both proteins are RBC components that following activation by storage [38] accumulate in the EVs at least after the first week of it [4]. In either case, overpresentation of activated caspase-3 along with denatured Hb and Prdx2 in EVs confirms the hypothesis of their homeostatic role in the removal of damaged or dangerous cellular components [39]. Of note, Hb oligomerization and band 3 proteolysis seemed to be higher in the young N-EVs compared to the L-EVs, verifying the aggravating effect of residual WBCs and PLTs on stored RBC functional phenotypes related to aging, redox homeostasis, membrane proteostasis and extent of vesiculation *per se* [9].

According to previous reports, prestorage leukoreduction alleviates not only the accumulation of EVs [9] but also their proinflammatory activity, as revealed by the induction of lung injury in mouse models [10]. Transfusion can trigger immune responses dominated by inflammation, especially in the case of N-RBC units [40]; thus, detection of several proinflammatory molecules in N-EVs came as no surprise. Medullasin, for example, is an inflammatory proteinase that enhances the activity of natural killer cells [41], while peptidyl-prolyl cis-trans isomerase A (or cyclophilin A) functions as a leukocyte chemotactic factor [42] and a proinflammatory cytokine involved in the inflammatory responses observed in several diseases. In the same context, S100-A8 is an immunocyte-derived cytokine and alarmin, that also participates – along with S100-A9 – in inflammatory processes. These members of the S100 family are secreted under several pathological conditions (infections, inflammatory diseases) to mediate the inflammatory response by acting like chemokines. It is interesting though, that the same molecules exhibit anti-inflammatory properties in overwhelming inflammation [43].

Another group of EV proteins found in our N samples is potentially related to immune suppression/modulation effects. CD95, for instance, a member of the TNF family of death factors, when expressed on EVs can enhance cytotoxicity in an antibody-mediated, FcR-dependent way [44]. On the other hand, several N-EV components have been related to tumor biology or could underlie the linkage of blood transfusion with the risk of cancer recurrence and mortality in surgical cancer patients [45,46]. Vitronectin, a fibrin-associated molecule with important roles in thrombogenesis and tissue repair [47], has been related to neuroblastoma tumor aggressiveness [48], while platelet CD109 and annexin A2 to poor prognosis in patients with malignant tumors [49–51]. Moreover, the presence of the immunosuppressive Ras-related protein Rap-2 and interferon-inducible protein 9−27 in N-EVs raises concerns for their probable reactivity in a recipient context related to a multitude of human cancers [52,53]. Indeed, it has been reported that transfusion related immune modulation (TRIM) might be triggered by EV-specific molecules via crosstalk with cancer cells [45].

Finally, several EV (mostly N-EV) components have been related to coagulatory and cell adhesion activities. Among these, PLT-derived EVs that can start a coagulation cascade through fibrinogen binding [54] and PS exposure; $CD9^{+}$ EVs that are capable of triggering PLT activation and aggregation via the Fc receptor pathway [55]; and FERMT3 (or kindlin-3) that mediates adhesion of PLTs and WBCs to endothelium. It is worth mentioning that proteins with antibacterial or antiviral activity, including the iron binding lactoferrin [56] and the immunomodulatory interferon-inducible protein 9−27 (IFITM1) [57] were also detected in N-EVs.

In conclusion, the performed comparative, paired study reveals that the proteome of EVs released in standard RBC units is a function of storage age and can substantially change following prestorage leukoreduction. While several membrane components, cytoskeleton regulators and metabolic enzymes participate at variable extent in EVs of different storage age, a wide array of proteins are specific components of L- or (mostly of) N-EVs. The proteome variation with storage time points to an ordered sequence of storage lesion events that accompany or underly the release of storage EVs, while the putative biological response modifying activities of EVs may determine a differential profile of potential interactions and effects to receptor cells post transfusion, especially in transfusion susceptible recipients, such as surgical cancer patients. Even though a small group of blood donors was examined, and thus, our findings cannot be extrapolated as uniform prestorage leukoreduction-related outcomes, the exclusion of donor variation effects enhances their reliability.

Author contributions

Conceptualization: AGK, KES and MHA; Methodology: SR and MHA; Investigation: SR and VLT; Resources: AGK, KES, ISP, SR and MHA; Data Curation: MHA, VLT and ATA; Writing – Original Draft Preparation: MHA, VLT and ATA; Writing – review & editing: SR, AGK, KES and ISP; Visualization: VLT and ATA; Supervision: MHA and AGK.

Declaration of Competing Interest

The authors report no declarations of interest.

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