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Alterations of the erythrocyte membrane proteome and cytoskeleton network during storage – a possible tool to identify autologous blood transfusion

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Mature red blood cells (RBCs) are the end-stage of a development process that starts in the bone marrow and continues to differentiate, through reticulocyte stage, entering into the circulation with a four-month lifespan. While stored, RBCs undergo different changes. The aim of this study was to evaluate changes occurring in RBC membranes during storage that could be used as possible markers to detect the misuse of blood transfusion in sports.

Whole blood was collected from two volunteers in blood bags and stored for 42 days at 4°C. At different times (1, 7, 21, and 42 days of storage) whole blood was extracted under sterile conditions and submitted to RBC membrane ghost preparation and further analysis. Proteomic methods were applied using two strategies: protein oriented using 2-DE gels and peptide oriented using isobaric tags for relative and absolute quantitation (iTRAQ).

In both approaches, the goal was to compare detectable changes in RBC membrane proteome before and after standard storage at different times. Some of the changes were confirmed with both methodologies employed, while with others only with one of them. Complementarities of the methods in this case showed to be an advantage.

Changes were observed in two different protein complexes. In one of them, changes consisted of proteins decreasing, while increasing in the other during storage of RBCs. They are mostly located in cytoskeleton - spectrin β, band 4.2, ankyrin-1, tropomodulin-1, β adducin, band 4.9 (dematin), tropomyosin, while some changes were also observed in transmembrane proteins (glycophorin C, aquaporin-1, band 3). Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

One of the greatest challenges in anti-doping control in sports in recent decades is to develop strategies to detect the misuse of methods to increase the number of red blood cells (RBCs) and oxygen delivery capacity. Athletes are using different strategies to increase oxygen delivery capacity, such as erythropoietin (EPO) administration or transfusion of whole blood or RBC concentrates, either autologous or homologous.[1,2] As all of these methods are banned by the World Anti-Doping Agency (WADA),[3] different methodologies of detection are available. An official method for homologous transfusion detection exists, while an official method for autologous transfusion is not yet available. For reasons of convenience and safety, autologous transfusions, in which the cells are the athlete's own, are possibly far more common than homologous transfusions, in which the cells belong to someone else with compatible blood.

The traditional autologous blood transfusion procedure begins by the withdrawal of one to several units of blood (1 unit equals 450 ml of blood) a few weeks before competition. The blood is centrifuged and the corpuscular elements, mainly RBCs, are stored refrigerated at 4°C or frozen (using cryoprotector such as glycerol) at −80°C.[4] Earlier studies have shown that more than a 5% increase in circulating haemoglobin is necessary to improve performance, suggesting that athletes would need to infuse at least one unit of blood to obtain a significant advantage.[5]

While stored, the RBCs undergo changes known as storage lesions.[6–11] RBCs storage lesion includes both metabolic and physical changes. Principal metabolic changes over time while stored include declining pH, reduced ATP, reduced 2,3-diphosphoglycerate (2,3-DPG), and accumulating extracellular potassium. Physical changes include membrane loss and the associated changes in RBCs shape and rheology. Losses of membrane carbohydrates, lipids, and proteins also occur.[10] While stored, RBCs undergo a slow change in shape associated with membrane loss. They evolve from smooth biconcave discs to spheres called spheroechinocytes. After

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reinfusion, RBCs regain normal shape which occurs in parallel with increasing ATP concentrations, regeneration of 2,3-DPG and the restoration of normal sodium, potassium, and calcium gradients.

Beyond the early spheroechinocyte stage, the RBCs lose some membrane as micro-vesicles, and this process is irreversible.[10] Some of the changes in the proteome of RBC membranes during prolonged storage time, such as protein losses or translocations, could be demonstrated with different proteomic methods.

The first step of any proteomic analysis is the purification of proteins from cells or tissues. For complex samples, a subsequent fractionation step is also needed. The proteomics approaches used in this study – protein and/or peptide oriented – are defined by the order of their processing steps.[12–16] Protein-oriented approaches require that the proteins first be separated, isolated, and then digested, whereas peptide-oriented approaches involve the digestion of the protein mixture before separating the resulting peptides.

Two-dimensional gel electrophoresis (2D-GE) is the method of choice in protein-oriented approaches. The first dimension separates the proteins by isoelectric point and the second dimension by molecular weight.[17,18] After separation of the proteins and gel staining, quantitative changes can be detected by comparing intensities of corresponding spots. The main advantage of the 2D-GE approach is that it is protein centric, very visual, and complementary with liquid chromatography-tandem mass spectrometry (LC-MS/MS) approaches. It is also relatively low cost and easy to use. It has the capacity to combine high-resolution power with the ability to quantify and assess protein modifications, such as phosphorylation; and has the possibility of automation with robotic spot pickers, to make the 2D-GE technique the method of choice for certain investigations.[13] On the other hand, the loss of very large, extremely basic, acidic or hydrophobic proteins are drawbacks of this approach and severely limit the capacity of 2D gel-based technologies, including differential gel electrophoresis (DIGE). These disadvantages, and in particular the well-known hydrophobicity for membrane proteins, led to the consideration of alternative proteomics strategies to study changes in RBCs over time.

Peptide-oriented approaches, such as isotope-coding affinity tags (ICAT)[19] or isobaric tags for relative and absolute quantitation (iTRAQ)[20] technologies, provide information not accessible using 2D gel separation, such as the detection of low-abundant or hydrophobic (membrane) proteins, and are at present complementing and even partially replacing 2D-GE. Nevertheless, the use of a single proteomics approach (either protein or peptide oriented) may give only partial information. So, our strategy was to combine the use of complementary proteomic approaches in order to obtain a comprehensive analysis of changes during RBC storage.

In this study, we evaluated protein changes occurring in RBC membranes during storage with the final goal of finding potential markers to detect the misuse of blood transfusion in sports. RBC membrane proteins were chosen for our study because they are readily available and have been well characterized in many studies with different approaches like 2D DIGE,[21] 1D and 2D electrophoresis[22] and LC-MS.[23,24]

Materials and methods

Materials

Blood bags PL146-CPDA-1 were from Baxter (Deerfield, IL, USA). Sodium chloride was from Scharlab (Sentmenat, Barcelona, Spain) sodium di-hydrogen phosphate, di-sodium hydrogen phosphate and formic acid (FA) were purchased at Merck (Whitehouse Station, NJ, USA).

Urea was obtained at Amersham Bioscience (Piscataway, NJ, USA) and thiourea, 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS), amidosulfobetaine-14 (ASB-14), dithiothreitol (DTT), glycerol, iodacetamide (IAM), acetonitrile (ACN), monopotassium phosphate (KH_2PO_4) and potassium chloride (KCl) were from Sigma Aldrich (St Louis, MO, USA). Protease inhibitor cocktail, 2-D Quant Kit, 2-D Clean-Up Kit, NL ampholite carrier, DeStreak reducing agent and IPG strips (24 cm, 3–11 NL IPG DryStrips) were purchased at GE Healthcare (Little Chalfont, Buckinghamshire, UK). Bromphenol blue was from Pharmacia Biotech (Uppsala, Sweden). Sodium dodecyl sulphate (SDS) was obtained from USB Corporation (Cleveland, OH, USA). Tris–HCl, SDS/PAGE and electrophoresis reagents, kaleidoscope prestained protein standard were from Bio-Rad (Hercules, CA, USA). Trypsin was from Promega (Madison, WI, USA). iTRAQ reagents were purchased at Applied Biosystem (Carlsbad, CA, USA). Oasis HLB cartridges were from Waters Corporation (Milford, MA, USA) and trifluoroacetic acid (TFA) was purchased at Thermo Fischer Scientific (Waltham, MA, USA).

Methods

Whole blood storage

Whole blood was collected from two volunteers in blood bags and stored for 42 days at standard transfusiology conditions (4°C). At different times (1, 7, 21, and 42 days of storage) certain quantity of whole blood was extracted under sterile conditions and submitted to RBC membrane ghost preparation and further analysis.

The experimental setup applied to blood samples is described in Figure 1.

RBC membrane ghost and protein extract preparation

Human erythrocyte ghosts were obtained by hypotonic lysis as follows. RBCs were washed twice in isotonic sodium chloride solution (0,9%), at pH 7.4, and sedimented with centrifugation at 300x g for 10 min. The supernatant and buffy coat were removed by aspiration. The soft pellet of the cells was recovered and haemolyzed by resuspension in 30 pellet volumes of 5 mM sodium phosphate (pH 8.5) and maintained on ice for 20 min. Membranes were pelleted by centrifugation (10000x g, 20 min). RBC membrane ghosts were then washed twice (10000x g, 20 min) in 20 mM sodium phosphate (pH 8.5). Pellets of this preparation of erythrocyte ghosts had a pink colour. Nearly white pellets of erythrocyte ghosts were obtained by four consecutive washes (each wash consisting of 20-min incubation in 5 mM sodium phosphate and then pelleting of ghosts). White pellets of erythrocyte membranes ghosts were stored at −80°C until usage. These white pellets of erythrocyte ghosts were used for all the analyses.

The pellet of thawed ghosts were homogenized in buffer containing 7M urea, 2M thiourea, 2% CHAPS, 2% ASB-14, 40mM DTT. Protease inhibitor cocktail was added. The

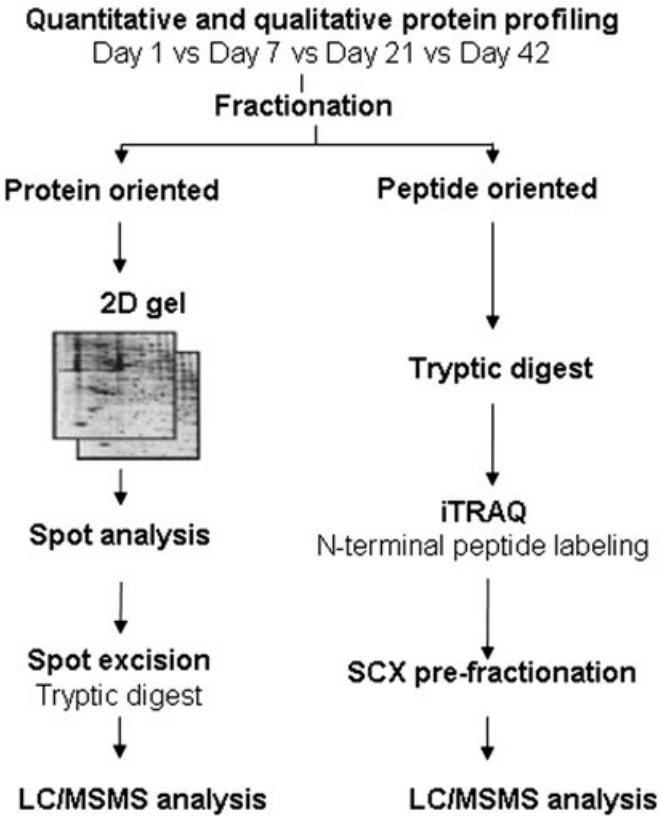

Figure 1. Experimental set-up and workflow for a complementary proteomic assessment of changes occurring in RBC membranes during storage.

extracts were incubated for 30 min on ice and disrupted by sonication. The samples were then centrifuged at 12000x g for 25 min at 4°C and supernatants containing membrane proteins were transferred into new micro-centrifuge tubes and stored at -80°C for further analysis.

Determination of protein concentration

The 2-D Quant kit (Amersham Biosciences, Uppsala, Sweden) was used to quantify protein concentration.

After quantification, samples were precipitated and purified with the 2-D Clean-Up Kit following manufacturer's instructions (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Protein-oriented approach

2-DE. Analytical gels of pooled sample were made in triplicate for each storage time: 150 μg of protein were dissolved in 450 μl of rehydratation buffer (7M urea, 2M thiourea, 2% CHAPS, 0.5% ASB-14, pH 3–11 NL ampholite carrier, bromophenol blue trace and 5 μl of Destreak reducing agent). After mixing and spinning down in centrifuge for 5 min at 12000x g, sample was loaded in prepared strip holders for passive rehydration. After passive rehydratation of IPG strips (24cm, 3–11 NL IPG DryStrips) for at least 16h, isoelectrofocusing was performed on a Multiphor II system as follows: step 1: 500 V step and hold 1 h, step 2: 1000 V Gradient 1 h, step 3: 8000 V Gradient 3 h, step 4: 8000 V step and hold 3 h, step 5: 10000 V Gradient 3 h, step 6: 10000 V step and hold 2 h 30 min, step 7: 500 V step and hold 12 h.

After IEF, the strips were incubated in equilibration buffer I (6M urea, 2% SDS, 0.375 M Tris–HCl pH 8.8 and 20% glycerol), containing 2% *w/v* of DTT (added freshly), for 15 minutes. After this equilibration step, strips were incubated for additional 15 min in equilibration buffer II (containing the same reagents as equilibration buffer I without DTT) supplemented with 4% *w/v* iodoacetamide (added freshly). After the equilibration step, the IPG strips were transferred to the top of a 12% polycrylamide gel and SDS-PAGE was performed overnight in an Ettan Dalt system at permanent power of 2 W per gel. Marker of the proteins (Bio-Rad kaleidoscope marker) was placed adjacent to the samples.

Protein visualization and image analysis. Gels were silver stained. Scanned 2 DE images were uploaded to Ludesi Analysis Center (Sweden, www.ludesi.com) for image analysis using Ludesi's proprietary image analysis software. The protein spots were automatically detected, and the results were manually verified and edited where needed. The gels were automatically matched using 'all to all' matching, avoiding introduction of bias caused by use of a reference gel. The matching was iteratively improved by optimization of matching parameters and manual editing. Integrated intensities were measured for each spot, background-corrected and then normalized mathematically, minimizing the median expression differences between matched spots.

Mathematical analysis. The statistical analysis was performed using Ludesi REDFIN software (Sweden, www.ludesi.com). Normalized spots volumes were used.

First filter applied was a volume filter, which removed spots for further analysis when its volume was smaller than 250 (arbitrary units). Another filter applied was fold change, which removed spots for further analysis when increment change among different spots was less than 2.0. The statistical analysis was performed using ANOVA test.

All spots identified with previously mentioned filters were manually verified to confirm that were correctly detected, quantified and matched in all gels.

In–gel digestion and MS analysis. Spots selected by the mathematical analysis using Ludesi's software were excised and trypsinized following a previously described protocol,[25] leaving out reduction and alkylation. Extracted peptides were analyzed on a LTQ-Orbitrap XL (Thermofisher) fitted with a nanospray source (Proxeon) previous nanoLC separation in an Agilent 1200 nano flow system (Agilent). Peptides were separated in a reverse phase column, 100μm x 150mm (Nikkyo Technos Co., Ltd) with a gradient of 2.4 to 36% ACN with 0.1% FA in 24 min at a flow of 0.5 μl/min.

The LTQ-Orbitrap XL was operated in positive ion mode with nanospray voltage set at 2.2 kV and source temperature at 275 °C. The instrument was externally calibrated using Ultramark 1621 for the FT mass analyzer. An internal calibration was performed using the background polysiloxane ion signal at *m/z* 445.120025 as the calibrant. The instrument was operated in data dependent analysis mode (DDA). In all experiments, full MS scans were acquired over a mass range of *m/z* 350–2000 with detection in the Orbitrap mass analyzer at a resolution setting of 60000.

Fragment ion spectra produced via collision induced dissociation (CID) were acquired in the LTQ mass analyzer. In each cycle of DDA analysis, following each survey, the top five, most intense ions were scanned above a threshold ion count of 5000 and were selected for fragmentation at

normalized collision energy of 35%. All data were acquired with Xcalibur 2.1 software.

Protein identification was performed using MASCOT (Matrix Science) as search engine. Data were processed and searched against IPI human v3.51 database. Oxidation of methionine was set as variable modifications. Peptide tolerance was 7ppm in MS and 0.5Da in MS/MS mode, maximum number of missed cleavages was set at 2.

Peptide-oriented approach

The protein extracts from ghosts were labelled for iTRAQ.

iTRAQ labelling. Erythrocyte membrane-bound proteins were labelled with iTRAQ reagents using a modified protocol. Briefly, the membrane pellet of pooled sample for each storage time was resuspended in a mixture of 8M urea, 2% CHAPS, 2% ABS-14 and 3mM DTT; it was incubated on ice until the suspension clarified. A small amount of undissolved material was usually present and was removed by centrifugation; 100 μg of each sample was reduced with DTT, and cysteine residues were blocked with IAM. Samples were diluted 10-fold and digested with trypsin at 37°C overnight. Peptides generated in the digestion were desalted with a cartridge Oasis HLB cartridge. The desalting process consists of (1) a cleaning step (highly organic, to remove possible contaminants from the reversed phase material), 1x5 ml ACN; (2) a conditioning step that uses the same solvent in which the sample will be loaded, 1x5 ml 5% FA; (3) a loading step, sample dissolved in at least 1 ml 5% FA; (4) a washing step, to remove the salts, 3x5 ml 5% FA; and (5) an elution step, 1x5 ml 70% ACN, 5% FA. The volumes in the different steps are pressed through the column by air pressure using a 10-ml syringe. The eluates were evaporated to dryness, dissolved in 0.5M triethylammonium carbonate, and labelled with iTRAQ reagents at lysines and terminal amine groups.

The four isobaric labels have a nominal mass of 145 Da and consist of a 'reporter' functional group (114, 115, 116, or 117 *m/z*), a 'balance' group (31–28 amu), and a peptide reactive group. Each of the four chemical labels dissociates in the mass spectrometer to produce one of the discrete reporter ions, which is measured in an MS/MS scan and provides the peaks used for peptide quantitation. Samples from membrane preparations were labelled as follows: reporter 114 – sample time 1 day; reporter 115 – sample time 7 days; reporter 116 – sample time 21 days; and reporter 117 – sample time 42 days.

Strong cation exchange chromatography (SCX). The sample containing labelled peptides was diluted ten times with loading buffer (10mM KH2PO4, 25% ACN) and the pH was adjusted to 3. Peptides were separated in 10 fractions by cation exchange cartridge SCX prior to RP-LC-MS/MS; than peptides were manually eluted with loading buffer containing increasing concentrations of KCl (25, 50, 80, 125, 175, 225, 275, 300, 350, and 500 mM). Salts were removed with a cartridge Oasis HLB cartridge following the previously described protocol. Peptides eluted from the cartridges were dried under vacuum and reconstituted in 8 μl 0.1% TFA.

Mass spectrometry analysis. Four μl of the peptide mixture were run on a Q-Star Pulsar (Applied Biosystems) instrument fitted with a nano-ESI source (Proxeon), previous nanoLC separation in an Ultimate II system (LCPackings). Peptides were separated in a reverse phase Atlantis dC18 NanoEase Column, 75μm x 150mm (Waters Corporation), using a gradient of 15 to 60% ACN with 0.1% formic acid in 60 min and 60 to 90% ACN with 0.1% FA in 5 min at a flow of 0.2 μl/min. Equlibration time after 90% ACN lasted 15 min. The mass spectrometer was operated in positive ion mode, with a selected mass range from 350 to 1700 *m/z*. An electrospray voltage of 2400 V was used. Data were acquired using an information-dependent acquisition (IDA) method in which precursor ions of charge state +2, +3, and +4 were considered and the three most abundant ions above 20 counts were selected for MS/MS and dynamically excluded for 60 s with a mass tolerance of 50 mDa. Fragment ion spectra were produced via collision induced dissociation (CID) and data were recorded from 100 to 2000 Da. Collision energy was calculated as a function of mass and charge.

Database search and quantification. Protein identification and quantitation was performed by Protein Pilot software v.2.0.1 (Applied Biosystems) using the Paragon algorithm. Data was searched against IPI Human v. 3.49 database. Carbamidomethylathion for cysteines was set as fixed modification. Other parameters as peptide tolerance in MS or MS/MS mode, trypsin efficiency as well as any other modification are built in functions of Protein Pilot software.

Identification of protein was performed with 95% confidence. For quantitation, the program excluded peptides with confidence $<1\%$, also peptides shared between different proteins or peptides where the spectrum is also claimed by a different protein, but with unrelated peptide sequence (precursor overlap). Also, peptides that do not have an iTRAQ modification were excluded. Protein Pilot software provided on one hand the accuracy of each protein ratio by an error factor and on the other hand a p-value to assess whether the protein is significantly differentially present. Only ratios with p-values lower than 0.05 were considered for evaluation of protein differential appearance.

Results and discussion

We have used two complementary proteomics approaches to monitor storage changes of RBC membranes proteome over time. First, we analyzed the samples through 2D gel electrophoresis, compared spots intensities and performed protein identification on the spots where we detected significant changes. Second, we digested the protein samples, labelled them with isobaric labels (iTRAQ), and performed LC-MS/MS to identify and quantify proteins that change over time.

Protein-oriented approach

Figure 2 shows the silver-stained 2D electrophoresis gels of RBC membrane proteins obtained from blood stored after different times.

The 2D electrophoretic method used allowed us to obtain reproducible 2D maps, comparable to those already reported in the literature.[22,26,27] Ludesi software identified 1664 spots comparing sets of 2D electrophoresis gels from fresh blood samples and samples after 7, 21, and 42 days of storage. As was hypothesized, some spots present in the control showed a decrease in intensity of staining over time, whereas other

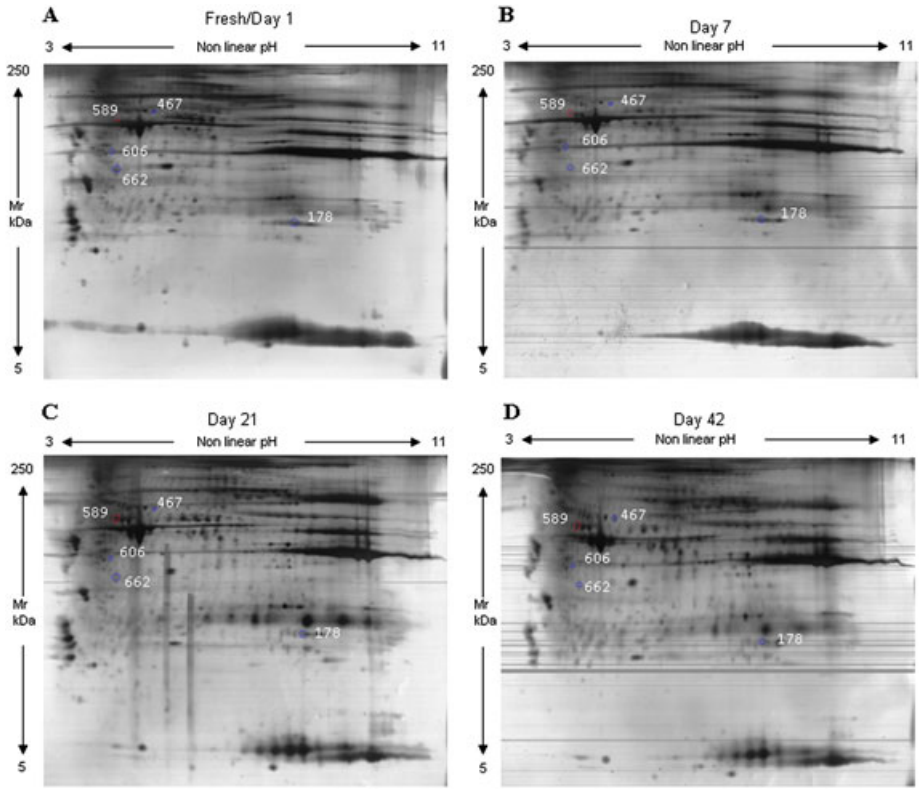

Figure 2. Silver-stained 2DE gels of erythrocyte membrane proteins extracted from fresh blood (A) and blood stored during 7 (B), 21 (C), and 42 days (D) in blood bags under standard transfusiology conditions (4°C). The numbers shown refer to spots identified by mass spectrometry and detailed in Table 1. Total protein sample load: 150 μg.

spots showed an increase of staining. Both phenomena were considered for further analysis.

Figure 3 shows examples of evaluation reports of different gel spots coming up after analysis with Ludesi software.

To verify changes in normalized volumes of the spots, different filters were applied; these filters discarded spots for further analysis.

When no filter was activated, 1664 spots were apparent; when artifacts were excluded, 1589 spots were apparent from specific area for the analysis.

We selected 312 spots with the filters 1.5-fold change (ANOVA $p < 0.05$) and 29 spots with the fold change minimum of 2.0 (ANOVA $p < 0.05$).

At the end, 29 spots were excised and analyzed by LC-MS/MS after trypsin digestion in order to identify the proteins responsible for the differential intensity of the 2D gel spots.

The results from the database search generated a score for each protein as well as the sequence coverage, the fragment distribution, the molecular mass, and the pI.

Out of the 29 spots analyzed by LC-MS/MS for protein identification, only those that were determined with two or more peptides were considered. In spot 589, tropomodulin-1 was detected; in spot 467, β-adducin; in spot 662, band 3; while in spots 606 and 178 β spectrin and ankyrin-1 were detected, respectively. Proteins out of the scope for our study (such as haemoglobin or Ig) were excluded.

The ultimate aim of our study was to achieve a clear distinction between proteins that decrease over time in the membrane content, and proteins that increase or move from the interior of the cell to its surface. Comparing theoretical and experimental values of identified proteins (Table 1), it was possible to verify a mobility shift of intrinsic protein bands. This modified the protein's molecular mass and its pI, which could be due either to aggregate formation or protein fragmentation and degradation generated during storage, as was already reported.[6] In a comprehensive study[6] using 2D SDS-PAGE in combination with MS/MS, it was shown that changes in RBCs after 7 or 14 days of storage produce new spots with lower molecular masses. Furthermore, over time, fragments and high molecular-mass aggregates appeared. Some of the protein changes turned out to be shifts in isoelectric point. Protein identification revealed that most of the modified proteins were located in the cytoskeleton.

Taking into account the study mentioned above, mobility shift and pI shifts were considered very cautiously. Based on the results obtained from MS analysis, it was concluded that tropomodulin-1 and β adducin increased over time while, band 3 and spectrins (α and β) and ankyrin-1 decreased. Also, an increment of proteins Hb and Ig in some spots was detected even though they were out of the scope of this study. All of these results would be confirmed with peptide-oriented approach.

Peptide-oriented approach

The same protein extracts were subjected to the alternative approach of quantification by isobaric labels (iTRAQ) to monitor changes over time during storage. After digestion, labelling, and cation exchange separation, protein identification and quantitation by LC-MS/MS was performed. Samples from membrane preparations were prepared in such a way that every sample representing different storage times were

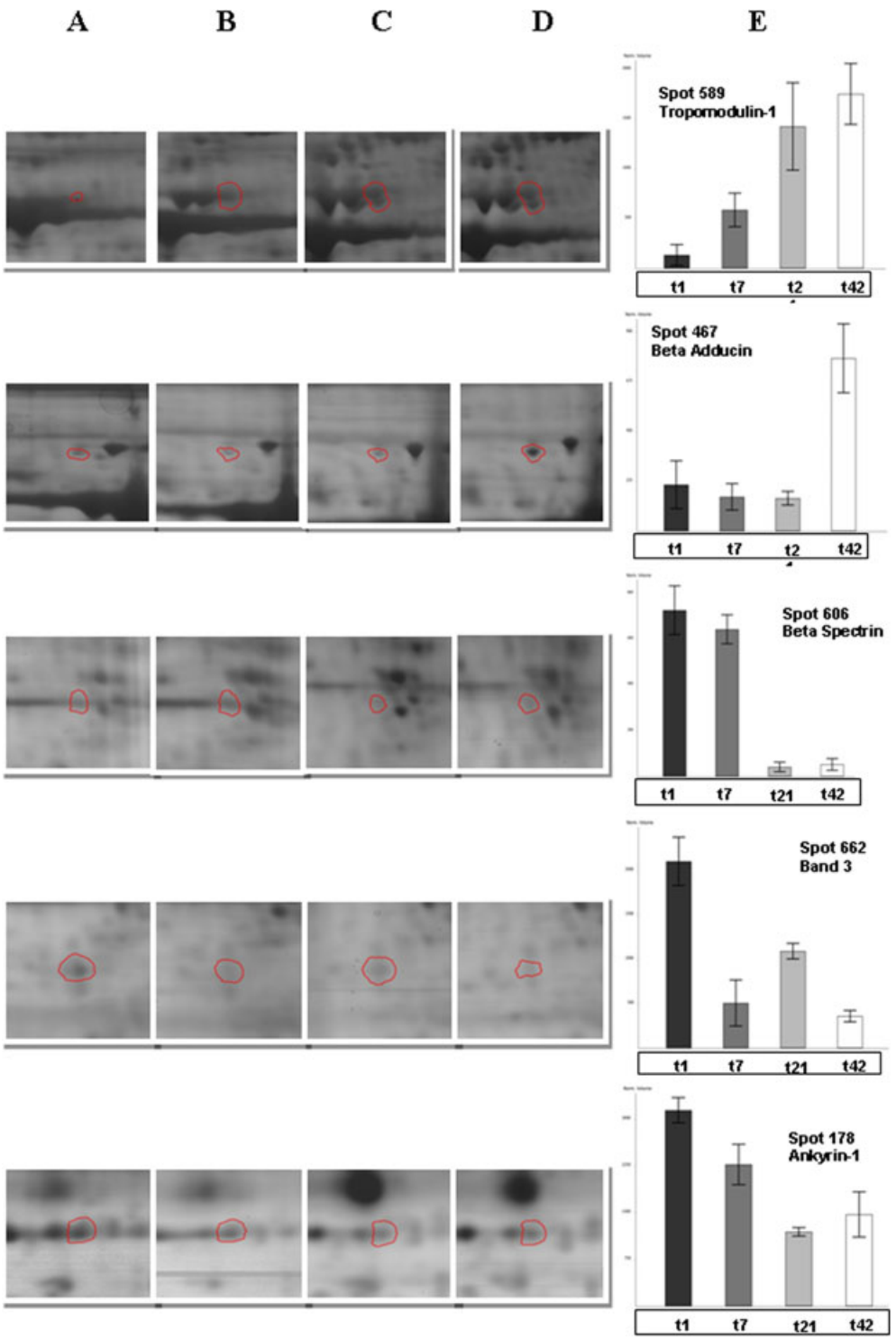

Figure 3. Different gel spots coming after analysis with Ludesi software. A, B, C, and D: amplification of selected spots in 2DE gels corresponding to fresh blood (day 1) and day 7, day 21, and day 42 of storage. E: normalized spot intensities at different storage times (mean value and SD are calculated from three technical replicates of the same time). Each graph corresponds to selected spots on the left. Indicated is spot number and protein identified by mass spectrometry.

labelled with different 'reporter' functional groups and they were quantified by MS/MS as described.

In Figure 4, examples of peptide quantitation by MS using iTRAQ are shown. The intensity of the *m/z* of the reporter functional group at each storage time (*m/z* 114, 115, 116, 117) allows the quantitation of the peptide in the sample. As can be seen in representative examples in Figure 4, some of the peptides increase with long storage time (tropomodulin-1, Figure 4A); others decrease (erythrocyte membrane protein band 4.2 isoform 2, Figure 4B); while others are maintained (55 kDa erythrocyte membrane protein, Figure 4C).

Using this approach, it was possible to identify 46 proteins, but not all of them demonstrated changes over time. Some proteins identified had a valid ratio change over time

Table 1. Proteins that change over time identified in 2DE maps of RBC membranes corresponding to day 1 (fresh blood) and day 7, day 21, and day 42 of storage.

Spot N°	M_r, kDa theor./exp.	p*I* predict./exp.	Mascot score	Protein name
589	40.5/45	5.03/4	3371	Tropomodulin-1
467	80/50	5.7/5	354	Beta adducin
662	100/37	5.2/4.5	866	Band 3
606	270/40	5.3/4.5	461	Beta spectrin
178	206/25	5.7/7	169	Ankyrin-1

Only spots present in the control which showed either decrease or increase in intensity of staining over time and only spots matching across all the replica maps were selected and analyzed (see additional criteria in Material and methods). The theoretical and experimental values of molecular weight and isoelectric point, Mascot score and protein name are reported.

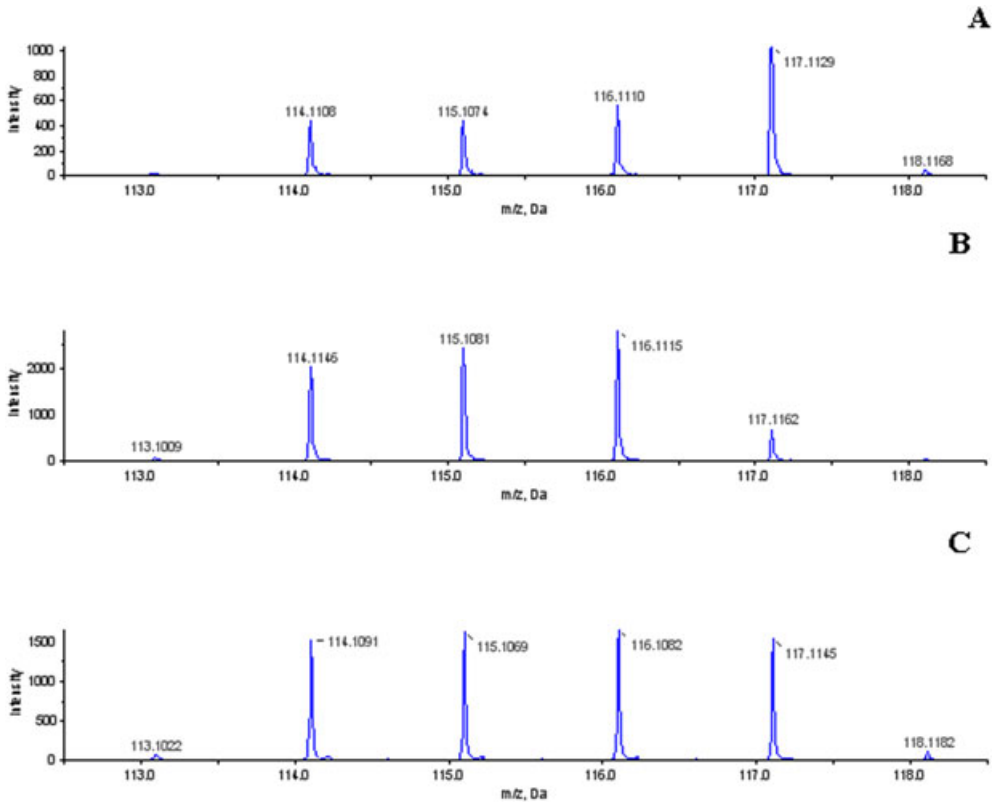

Figure 4. Examples of peptide quantitation information by MS, using iTRAQ for the following proteins: tropomodulin-1 (A), band 4.2 (B) and 55 kDa erythrocyte membrane protein (C). The intensities of the m/z of the reporter functional group for each time (114, 115, 116, and117) is an indication of the abundance of the peptide, verifying changes in quantity of the protein over time of storage of the RBC.

but unsatisfactory p-value; others didn't exhibit sufficient change over time; and others were identified with only one peptide and therefore were not considered.

In Table 2 we have listed the proteins in which significant changes (p-value < 0.05) were found. The ratios between the different reporters are ratios between the day indicated in the table and the reference day which, in this case, is the day of the blood extraction (day 1).

The proteins in which significant changes were detected could be separated into two main groups – one group of proteins increased over time, and another decreased. Some of the proteins appear unrelated to the study as they are either a product of manipulation (proteins such as keratin or filaggrin), or simply they are a product of serum contamination (serum albumin) or hemolysis (haemoglobin). These protein identities are not shown in Table 2.

Proteins that significantly increase over time (Table 2) are band 4.9 (dematin), tropomyosin, tropomodulin-1, β-adducin, ghlycophorin C. Proteins that significantly decrease over time (Table 2) appear to be β spectrin, band 3, aquaporin-1, band 4.2. Very close to the lower limit of change were also two proteins such as ankyrin-1 (which was also detected as discretely decreasing with protein oriented approach) and a protein highly similar to fructose-bisphosphate aldolase; in both cases their p-values are satisfactory. Most of these findings are in a good agreement with a semiquantitative overview of the changes in the RBC membrane/cytoskeleton network[28] where a decrease in the relative content of spectrin, ankyrin-1, and band 3 of RBC membranes especially between 21 and 42 days of storage was shown.

Similarly, a strong storage-associated reduction of the cytoskeletal proteins spectrin, ankyrin-1, protein 4.2, and actin

Table 2. iTRAQ analysis results.

Protein name	Day 7/Day 1	Day 21/Day 1	Day 42/Day 1
Erythrocycte membrane protein band 4.9 isoform 3	0,81	0,37	5,59
Tropomyosin 3	1,00	0,87	2,53
Tropomodulin-1	1,15	1,08	2,27
Isoform 2 of Tropomyosin alpha-3 chain	0,92	0,87	2,04
Isoform 1of Beta-adducin	1,04	0,90	1,89
Glycophorin C isoform 2	1,39	1,22	1,48
Specrtin beta isoform b	0,98	1,02	0,80
Aquaporin-1	0,95	1,11	0,62
Band 3 anion transport protein	1,01	1,01	0,61
Erythrocyte membrane protein band 4.2 isoform 2	1,10	1,19	0,47

All values in the table are ratios between the day indicated in the table and reference which in this case is day of the blood extraction (day 1). Only ratios lower than 0.8 and higher than 1.2 has been considered. All the values for the corresponding proteins have p-value equal or lower than 0.05.

in the membrane had also been observed and it was hypothesized that this decrease was due to their degradation.[29] RBCs lose membrane during the storage of whole blood by releasing vesicles in a process of vesiculation. A study of RBC-derived vesicles[30] suggested that the vesiculation operates as an effective way of eliminating damaged proteins produced by storage.

The cytoskeleton is an organized network of proteins comprising of major (e.g. α- and β-spectrin, actin, protein 4.1, ankyrin-1) and minor components (protein 4.2, dematin - band 4.9, α- and β-adducin, tropomyosin, tropomodulin-1 etc.), many of which interact not only with each other, but also with proteins and lipids of the membrane.[14] All these interactions and associations among different proteins can explain the fact that some of them have similar fate during storage of RBCs.

Most of the affected proteins investigated in our study are located in the cytoskeleton, while some of them are also transmembrane proteins (aquaporin-1, band 3, glycophorin C).

Changes in protein quantity observed were located in two multiprotein complexes.

In complex A (Figure 5), proteins decrease in the quantity during storage and proteins included in this complex could be confirmed either with both methodologies employed (band 3, β spectrin) or only by one (band 4.2, ankyrin-1).

In complex B, proteins increase in quantity during storage and could be confirmed either with both methodologies employed (tropomodulin-1, β adducin), or only by one (band 4.9 (dematin), tropomyosin, glycophorin C).

Figure 5 shows a schematic representation of the interactions between membrane and underlying cytoskeleton of the RBC and also two protein complexes identified and the protein identities as a main finding of this study.

Conclusions

The results of this study provide the first step in finding potential biomarkers for transfusion abuse using proteomics. Here we

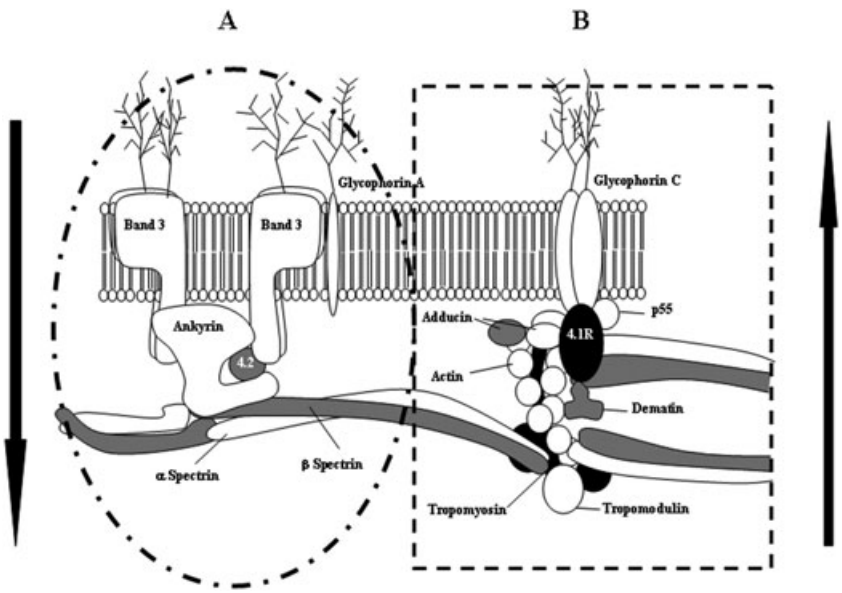

Figure 5. Simplified schematic representation of the interactions between membrane and underlying cytoskeleton of the RBC. To clarify our findings two multiprotein complexes in the red cell membrane (A and B) are represented. Complex A consists of proteins decreasing over time. Proteins detected in our study are band 3, beta spectrin, ankyrin-1 and band 4.2. Complex B consists of proteins increasing over time. Proteins detected in our study in this group are tropomodulin-1, beta adducin, dematin (band 4.9), tropomyosin and glycophorin C.

have shown that there is a differential proteome over storage of RBC and these data can be used eventually to develop a reliable test for autologous/homologous transfusion detection.

In order to verify possible changes in proteome due to storage, we used two complementary approaches: one protein oriented (2 DE gels) and one peptide oriented (iTRAQ labelling). Some of the changes were confirmed with both methodologies, while some others only with one of them. Taking into account the limitations and drawbacks of the methods used, complementarity in this case showed to be an advantage. Changes observed are mostly located in cytoskeleton - spectrin β, band 4.2, ankyrin-1, tropomodulin-1, β adducin, band 4.9 (dematin), tropomyosin, while there are also some changes in transmembrane proteins (glycophorin C, aquaporin-1, band 3). Basically we observed changes at the level of two multiprotein complexes. In complex A (Figure 5), proteins levels decrease during storage and this decrease was also confirmed either with both methodologies employed (band 3, β spectrin) or only by one (band 4.2, ankyrin-1). In complex B (Figure 5), proteins levels increase over time during storage and this was confirmed either with both methodologies employed (tropomodulin-1, β adducin), or only by one (band 4.9 - dematin, tropomyosin, glycophorin C).

Next step would be to verify the candidates described in this study by other appropriate methodologies. Using proteomics, candidates can be validated using a targeted approach, which is an alternative methodology to confirm quantitatively levels of proteins in a large-scale throughput manner. Another option could be the use of flow cytometry using the proteins in which we have detected changes as markers. This technique could be routinely used and help to develop a reliable method for detection of transfusion misuse.

Our results created modest optimism that in the future it would be possible to use the candidates we have selected using differential proteomics as a tool for detection of recent blood transfusion.

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