Joint narrative of RBC storage lesion: Use of advanced technologies for better understanding of variability associated with process, storage media/ time, donor/ donation and recipient issues

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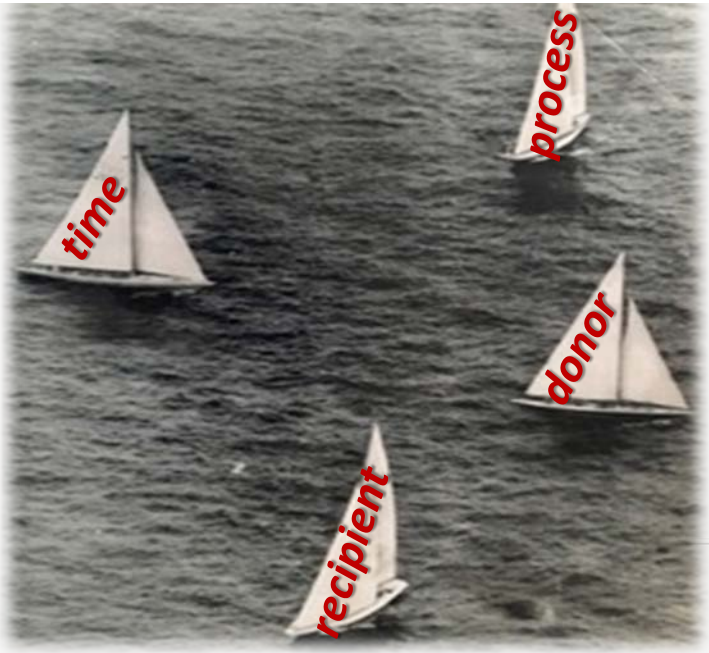
RBC STORAGE LESION (RSL) ALLEGORY:
Smart sailing, in the open sea of RSL, closer to the new wind of technical advances, in 4 interrelated areas, that optimize RBC quality with consistency: The outward looking ride will not be smooth but Coordinated Training & Team Working with Holistic Haemovigilance will help!



Red Cell Special Interest Group

"Clinical benefits vs potential harmful side effects of RSL-Derived EV/MP"

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BBTS Annual Conference 2016
21st - 23rd September

I . The Unresolved CHALLENGES of Red Blood Cell Storage Lesion (RSL)

BBTS Annual Conference 2016

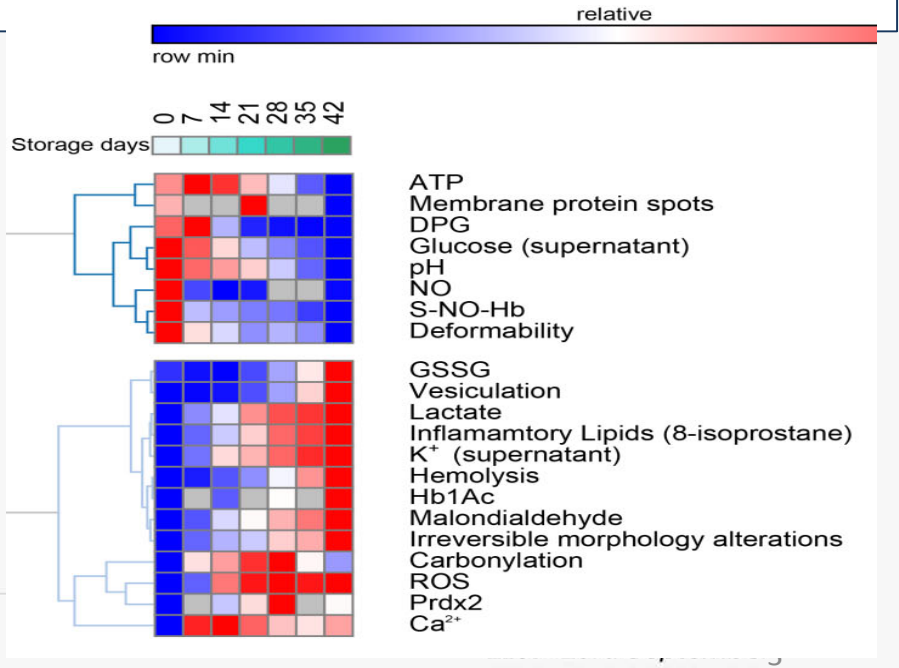
During storage at cold [not a natural state] packed red blood cells (pRBCs) undergo considerable changes at 3 levels of cellular homeostasis:

- ✓**Energy metabolism**
- ✓**Redox metabolism**
- ✓**Shape changes and secretion of membrane enclosed vesicles, with multifaceted functions.**

These changes are collectively referred as Red Cell Storage Lesion, with some yet unclear clinical outcomes

1. Major RSL concerns:

- ✓**Quality/safety of aged RBCs!**
- ✓**Levels of residual cells/ debris**
- ✓**Levels of** BRMs in **Supernatant**
- ✓**Risk of infection & Immune/Non Immune side effects in recipients**



D'Alessandro et al., Transfusion 55:205; 2015

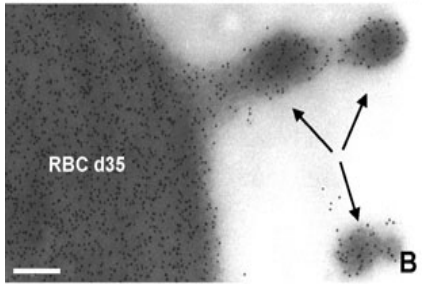
2. Quality Assessment of aged RBC units:

RSL is conventionally measured by **changes in main physiological parameters:**

II. Dynamics of the main lesions during RBC storage

***1 Variable changes in RBCs viability, functionality & signaling*:**

- ✓**shape changes, rheology**
- ✓**defected RBC deformability, fragility, adhesion potential**
- ✓**RBC senescence/removal signaling** (surface markers, erythrophagocytosis)
- ✓**RBC death (hemolysis, eryptosis)**
- ✓**Sub-lethal lesions (susceptibility to physiological levels of stress)**



Kriebardis et al., Transfusion 48:1943, 2008

2.Variable changes in soluble bio-active factors in supernatant:

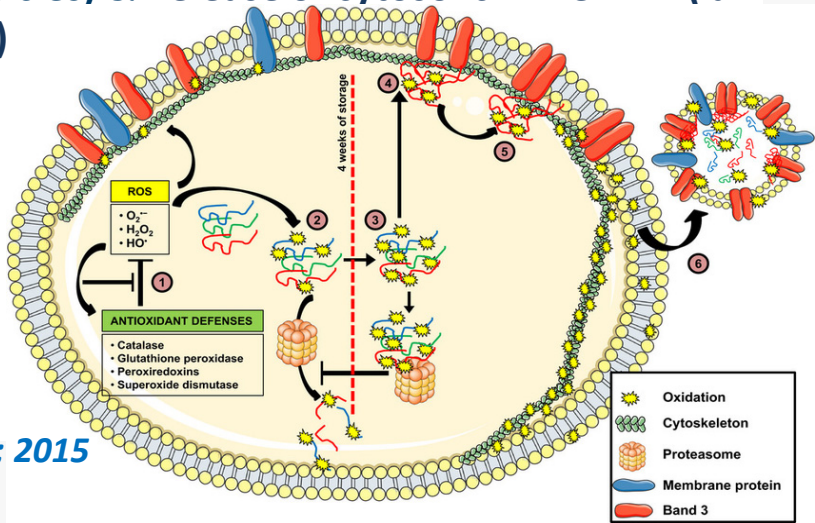
- ✓ **Release of extracellular vesicles (MVs +Exs+ Apoptotic vesicles) & Release of cytosolic Annexin V (a new ELISA quality indicator of the overall cellular damage)**
- ✓ **Bioactive lipids**

(that prime neutrophils and may contribute to TRALI)

- ✓ **Free and non-transferrin bound iron**

(that may promote growth of siderophilic bacteria)

- ✓ **Cytokines and chemokines** (especially in units that are not filter leukoreduced or Pathogen inactivated)
- ✓ **Extracellular K+**



Delobel et al., WJH 4(4):54-68; 2015

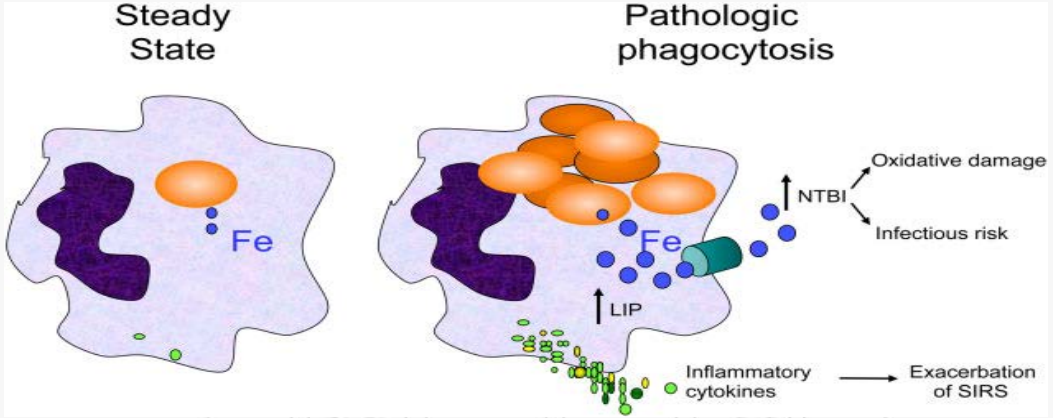
III. Main Physiological impacts of Hemolysis and release of highly heterogenious EVs:

Each unit of RBCs contain variable levels of modified RBCs, EVs, free Hb, hemin and Iron

* **Free Hb &MP-Hb are Scavengers of nitric oxide (NO)** and **inhibitors of NO generation**

** Older **RBCs-inhibit NO synthase** (↓NO bioavailability, vasoconstriction, decreased blood flow, tissue hypoxia)

Roback JD, Hematology 2011 &
Owusu et al., Antioxid Redox Signal 19 :1198; 2013



➢Metabolism of the Hb in the **large amount of ingested RBCs:**
increases the **intracellular free iron levels**, which, through a signal transduction pathway, enhances the production and secretion of **proinflammatory cytokines**

➢The latter can enhance the severity of the **systemic inflammatory response syndrome** (SIRS). In addition, excess free iron is exported from the cell through **ferroportin (green cylinder).** If the amount of exported iron exceeds the binding capacity of transferrin, then **NTBI** is produced, which can induce oxidative damage and **enhance pathogen proliferation**

Phagocytosis of damaged stored RBCs
THE IRON HYPOTHESIS

Spitalnik SL. Transfusion 54:2365; 2014
EMILY COOLEY AWARDS LECTURE

IV. Narrative Challange : " Precision Transfusion Medicine" based on donor/ recipient needs

From clinical standpoint RSL reflects *the totality of changes that RBC undergo from collection, through processing, storage and transport to transfusion, requiring* " **Continual Quality Improvement**" , at all levels to ensure that **all STORED pRBC units** are clinically equivalent **irrespectively of the storage duration**.

"Quality, safety and adequate management of transfusion practices" involved:

- ✓**recruitment** and **retention** of right donors;
- ✓timely **collection** of right blood donation in right **bags;**
- ✓**Separation** in right time using right **processes and storage media**; and
- ✓**Transfusion** to the right patients in right time

We need better understanding of:

- **pathophysiological mechanisms underlying RSL AND clinical relevance of RSL**

We need to focus on:

1) Minimizing of RSL based on clinical outcome data (new ASs, new processing and storage strategies)

2) Establishment of newly agreed quality criteria for pRBC units, to predict RSL profile based on simple haematological/biochemical baseline factors

3) Clarifying the donor-related variation effects

4) Managing of blood supplies based on paired donor and recipient issues,

so called "Precision Transfusion Medicine"

V. Methodological Aspects: Traditional tools for RSL assessment

- **In-bag hemolysis**/RBC cellular integrity
- **24-h recovery**: number of **RBCs that remain in circulation 24h post-transfusion**

V. 1. Advanced metrics tools for measuring RSL

- ✓**Hb** outside of intact RBCs (eg, either free or in the form of **MPs**)
- ✓Amount of **S-nitrosylation of Hb** itself (SNO-Hb)
- ✓Adhesion of RBCs to **endothelial beds**
- ✓Effects of stored RBCs on **models of vascular tone** (eg, isolated aortic rings), in animals and directly in humans
- ✓Membrane **vesiculation** & Release of Evs and soluble/ MV-bond Annexin –V, the cytosolic component of all blood cells
- ✓**Sublethal lesions of stored RBCs** affecting post-transfusion recovery and reactivity

Zimring JC. Established and theoretical factors to consider in assessing the red cell storage lesion. Blood 2015

V.2. Classical analytical approaches to better define RBC properties

Method	Information
Biochemical tests	pH, ATP, 2,3-DPG, hemolysis etc
Microscopy (CLSM, TEM, SEM)	structure, morphology, size, phenotyping
Fluorometry	intracellular ROS, Ca^{2+} etc
Flow cytometry	changes in surface molecules, ROS, Ca^{2+}
Physiological assays	cellular fragility, deformaility, erythrophagocytosis, rheology, etc

V.3. Novel analytical approaches to better define RBC morphology

Method	Information
Optical tweezers, Cell Transit Analyzer , Multiplexed fluidic plunger (MFP) mechanism	deformability of individual RBCs
Atomic force microscopy (AFM)	3D nanoscale topographical map of RBC surface
Digital holographic microscopy	quantitative information about 3D morphology of RBCs and characteristic properties such as MCH
Spatial Light Interference Microscopy (SLIM)	thickness, dry mass area density, refractive index , stiffness, deformability
RBC and supernatant miRNAs	novel in vitro biomarkers of RSL (their changes correlate with RSL)

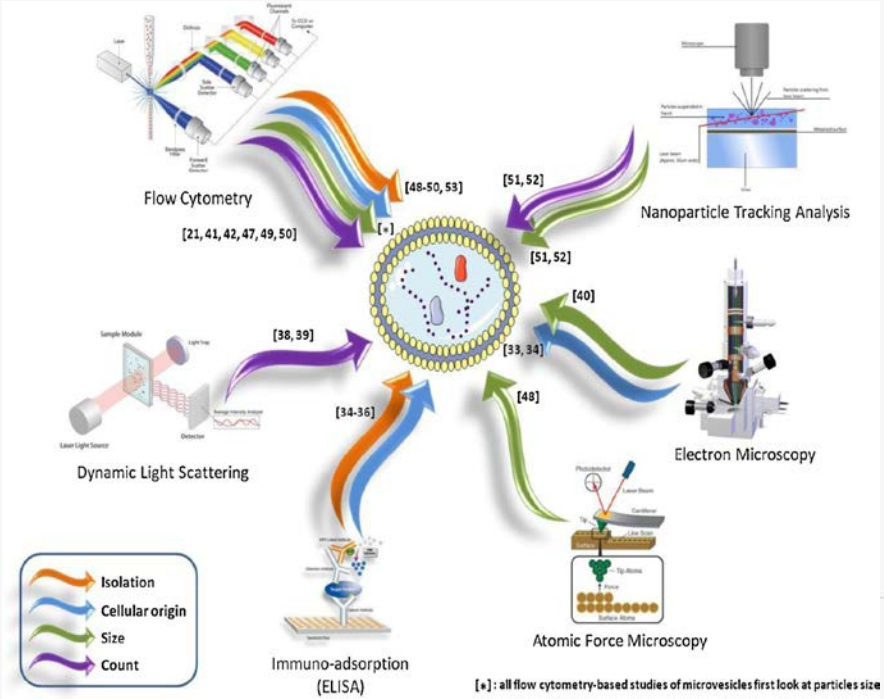
V. 4. Current approaches used to Characterize EVs' heterogeneity

Method	Information
Electron microscopy (EM) Cryo-EM, TEM, Immunogold EM, High-resolution transmission EM	Characterization of EVs <300-400nm size, enumeration, composition , «phenotyping" (cellular origin) of thousands of individual EVs in suspension in a short time
Atomic Force Microscopy (AFM)	3D nanoscale topographical maps of EVs surface with an imaging resolution of 1–10,000nm- Preservation of natural structure, size, antigenic properties and numbers
Conventional and dedicated, high-resolution Flow Cytometry	Size, structure, composition
Nanoparticle tracking analysis (NTA) (optical particle tracking method)	Concentration, size distribution (and potentially phenotype) of each EV through image tracking analysis
Dynamic light scattering (DLS)	average particle size (and potentially count)
Tunable resistive pulse sensing (TRPS, commercialized as the IZON qNano technique)	A novel alternative to NTA for concentration, size distribution, surface charge and zeta potential measurements of EVs present in a complex and polydispersed samples
Ultrasound standing waves technique	size and density of <200nm EVs

V. 5. The values of current practices to define EVs heterogeneity, the hallmark of RSL

EVs' characteristics: small **size** (below the detection range of conventional detection methods)

- low **refractive index**
- large **heterogeneity**



- **Most EVs <100nm** ⇒ significant numbers of particles may potentially **be missed**

⇒"**tip of the iceberg**" (probably only **1 to 2%**) of all vesicles present in biofluids

RBC-derived MVs/EVs: Small things with big multifaceted beneficial/ harmful effects

Seghatchian & Petrik , Trasci 2016

(Tissot et al., Translational Proteomics 1(2013) 38-52)

VI. Utility of Omics analysis (for RBCs, EVs, Supernatant)

"Big data platforms on single classes of biomolecules"

Genomics, ***proteomics***, ***metabolomics***, ***lipidomics***, ***phosphoproteomics***, ***carbonylomics***…

To explore small molecule composition

VI.1. Omics technologies

Traditional systems of analysis: **measure one** (or at best several) analyte at a time and require substantial **sample volume** to do so

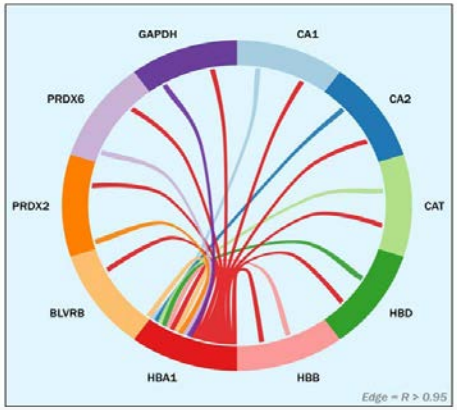
Linkage of chromatography to tandem mass spectrometers: has provided the capability to simultaneously elucidate **thousands of analytes** on **small specimens**

unimaginable panoply of new data in RSL

what happens "in the bag" during storage → **Optimizing bio-preservation**

1. Confirm **historical observations on RSL**
2. **New molecular changes** that correlate with **RBC storage age (Biomarkers of RSL)**

VI.2. Other benefits of Omics technologies in RSL



Some **proteins and metabolites have significant correlations with storage duration and Hb levels**

New markers of hemolysis: metabolic enzymes , antioxidant enzymes and stress-related chaperone proteins

D'Alessandro et al., Transfusion 56(6):1329; 2016

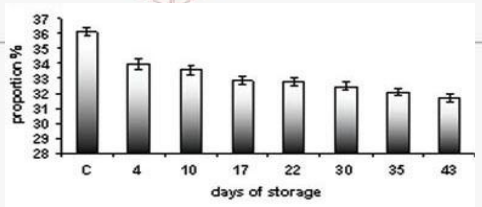
1) to identify the **donor-specific factors** that influence the quality of stored RBCs (biological mechanisms)
2) to better understand the influence of **storage conditions /storage time** on RBC **metabolic** pathways, **function**, **quality** and **survival**

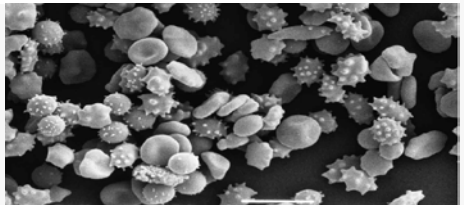
3) to speed up the design of **innovative storage strategies and solutions** developed to improve the quality, safety, and effectiveness of blood components

Omics highlight the main variables that change across different conditions:

- ✓Inter-donor differences
- ✓storage duration and media
- ✓Leukoreduction
- ✓Gamma irradiation
- ✓pathogen inactivation

VII. Influence of storage -related changes In RBC & Studies on RSL time profile, measured with traditional and newer technologies

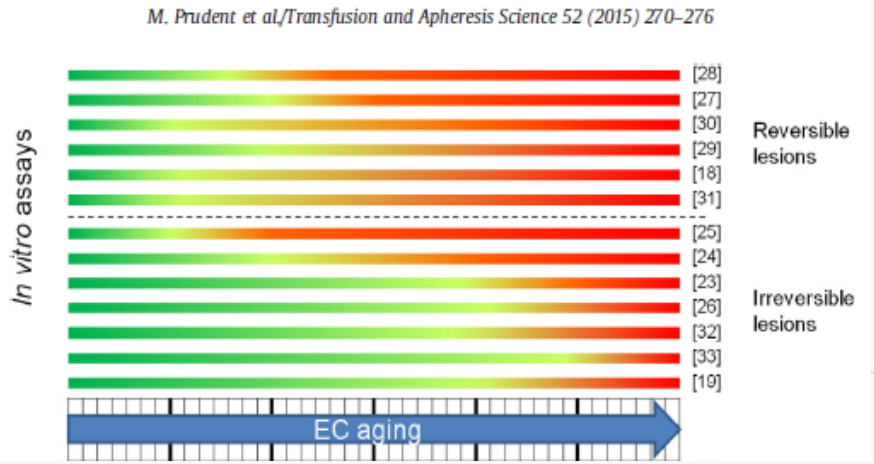




- ✓Loss of membrane proteins
- ✓accumulation of oxidative lesions in lipids and proteins (including Hb)
- ✓RBC transformation and EVs release
- ✓Biochemical changes
- ✓Physiological changes
- ✓RBC ageing-related modifications

D'Alessandro et al., Haematologica 97(1) : 107, 2012

Kriebardis et al., J Cell Mol Med, 11:148; 2007

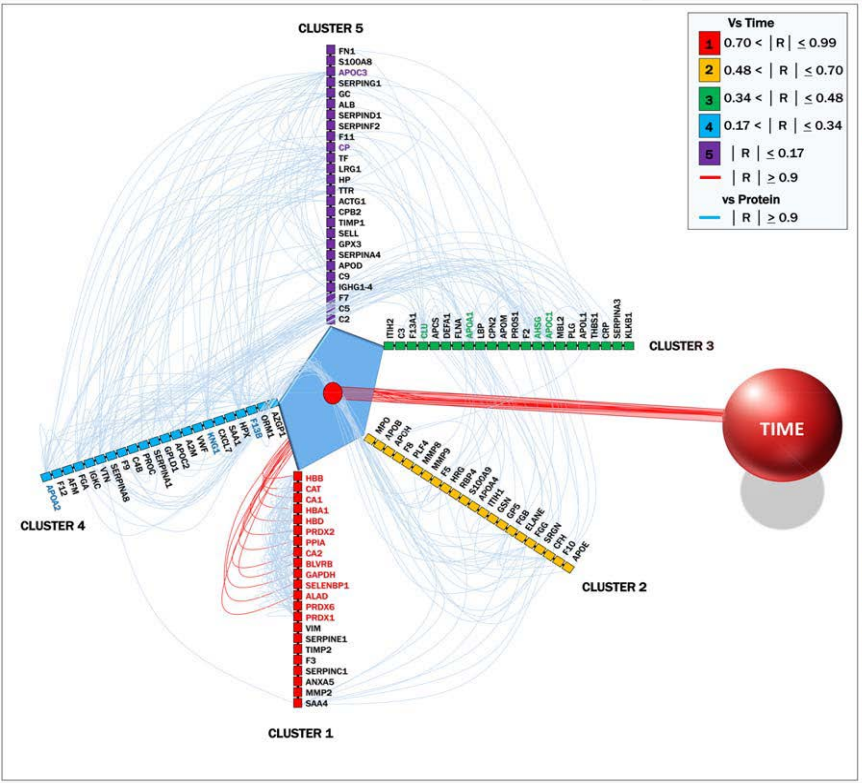


VII. 1. In depth study of RSL Time profile

Reversible lesions (mainly related to **metabolism** activity)**:** around the **2nd week of storage**

Irreversible lesions (mainly related to proteins, shape, EVs): around the **4th week of storage**

VII.2. TIME IS NOT the ONLY DETERMINANT of RSL



Of the **114 proteins** monitored in supernatants of AS-3 RBCs during routine storage:

5 clusters ranged from having **very high** (Cluster 1) to very **poor correlation with storage duration**

Poor correlations with storage duration: (probably owing to the initial depletion by **leukoreduction** and delayed increase only after **the second week of storage**), when the rates of **vesiculation** events are known to increase

D'Alessandro et al., Transfusion 56(6):1329; 2016

VIII. Shortcomings of some of the current RSL' Research studies Plan

❖⇒the field of **transfusion biology** is very much in need of **new measures of RBC storage quality** that **predict how the RBC units will perform *in vivo:***

❖Unfortunately **none** of the *in vitro (***traditional and novel)** metrics RSL has a strong correlation to **high 24h recovery** (performance):

➢**Extremely bad values** (e.g., very low pH, extreme morphologic changes): can **predict poor** 24-h recoveries; however,

➢**Good laboratory values**: in **no way guarantee** acceptable 24h recovery

Fortunately data from animal model of transfusion (murine model) support the view that ***lipid peroxidation*** *and* ***metabolites*** *that differed in fresh blood* were found associated with **poor 24h recovery**, allowing screening of good donors at time of collection

de Wolski et al., Metabolic pathways that correlate with post-transfusion circulation of stored murine red blood cells. Haematologica 2016

IX. Influence of collection / Processing strategies: intrinsic variability in RSL that make it "a moving target"

1. **Intrinsically there is a great chance for variations in all collection techniques used**

2. **Buffy-coat method** (Top/Bottom): significantly lower **hemolysis** compared to units processed using the **whole-blood filtration method (Top/Top)**

3. **Leukoreduced vs. Non-leukoreduced units**: **NL supernatants** accumulate proteins with likely immunogenic and **pro-inflammatory** potential (***Dzieciatkowska et al., Vox Sanguinis 2013).***

4. ***While validated leukofiltration showed little changes in generation/ retention of MV and prion protein But the c***ombined leukofiltration , S303 treatment & newly designed **Prion removal filter** reduces by 5 fold RBC-EVs (MPs) ***(Krailadsiri et al, transfusion 2006. & CDL report, BBTS newsletter]***

5. **Gamma irradiation:** at the biochemical level **accelerates metabolic aging of stored RBCs:** Four pathways significantly affected by irradiation [***Patel et al Transfusion 2015***]

6. **Anaerobic storage of RBCs- Metabolomics**: confirmed that **energy metabolism is** improved **at the expense of the PPP** and general **antioxidant potential (**RBCs stored anaerobically **in SAGM).** Focus on the **optimal hypoxic conditions** that preserved redox homeostasis

(D'Alessandro et al., Mol BioSyst; 2013)

X. Influence of additive solutions

- **CPD/SAGM**-stored RBCs are characterized by slower stimulation of common **recognition signaling** pathways and **EVs release** compared to **CPDA-**stored cells probably because **mannitol** works as a free radical scavenger and membrane stabilizer.
- **Alkaline ASs (Cl^--free)** have been designed to promote glycolytic enzyme activities by mitigating pH-dependent negative feedbacks on glycolytic fluxes during routine storage in the blood bank
- **Energy and redox homeostasis have been found to be better preserved in low-chloride, high-bicarbonate ($HCO3^-$), alkaline solutions, such as PAGGGM, AS-3, AS-7.**

- There is a clear underlying signature of RSL in **RBC metabolic age** where certain extra-cellular compounds [i.e . lactic acid, xanthine, glucose, adenine etc.] can be used as **biomarkers** of it **across storage media**

Paglia et al BLOOD 2016

X I. Influence of Donor-dependent variability in RSL and post-transfusion recovery

1. Baseline hematologic characteristics of each donors are useful candidate **biomarkers of storage quality in terms of " storability"**.

2. Certain donors have **lower initial quality** and lower storage capacity ("storability") and an "**old**" unit from a **good "storer"** might be better than a **"fresh"** one from a donor with **poor storability.**

3. Some **characteristics of RSL** are **heritable traits** in humans (**animal models** and **human twin** studies) and the percentage of **PS-exposing circulating RBCs** correlate to **in-bag hemolysis.** (***Dinkla et al., Blood Transf 12:204; 2014***).

4. Clinically silent **familial pseudohyperkalemia may be proved harmful**: increase in **K+** levels in the supernatant of the pRBC units, during the **first period of storage**

5. RBCs from donors with higher uric acid levels, a plasma antioxidant, appears to fare better in storage compared to RBCs from donors with lower plasma uric acid levels, probably due to the antioxidant capacity of uric acid.

Tzounakas et al., Transfusion 55:2659, 2015 & Bawazir et al., Transfusion 54:3043; 2014

6. Human and transgenic mice **HbAS trait RBCs** demonstrate **accelerated storage time-dependent hemolysis** and **reduced post-transfusion RBC recovery**. The rapid post-transfusion clearance of stored HbAS RBC is unrelated to macrophage-mediated uptake or intravascular hemolysis, but by enhanced sequestration in the spleen, kidney and liver.

7. Glucose 6-phosphate dehydrogenase deficient subjects are probably better "storers" than donors of red blood cells. G6PD-deficient RBCs store well in relation to energy, calcium and morphology related parameters, though at the expenses of a compromised anti-oxidant system. Additional stimuli, mimicking post-transfusion conditions promote hemolysis and oxidative lesions in stored G6PD- cells compared to controls

Tzounakas et al., Free Radic Biol Med 96:152:2016

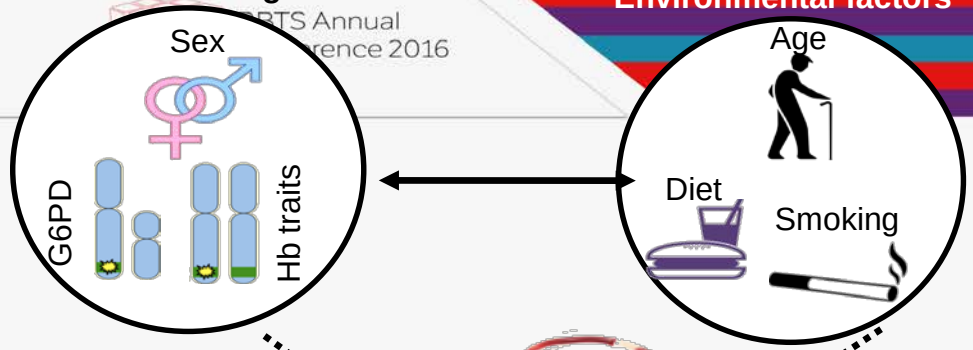
8. Murine model: RBCs form **iron-deficient donors** exhibit low 24h **recovery** *in vivo*

Bandyopadhyay et al., Transfusion 2015;5A(S3)

9. Donor Related Variations: Summary

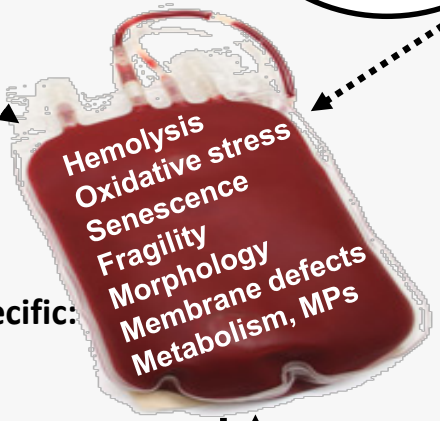
Genetic background

Environmental factors



RBC age/senescence
energy metabolism
Sensitivity to oxidative stress

Donor-specific:



Post-transfusion RBC recovery

Recipient variation

RBC fragility, PS exposure, Hb concentration
GSH and GSH/GSSG ratio, MCV
Plasma/supernatant **nitrate/nitrite**
antioxidant capacity

fluctuated throughout the storage **period in proportion** to their values in fresh blood and thus, can be safely **predicted**

Development of **predictive model(s) of RSL** for particular **groups of donors**

Tzounakas et al., Transfusion 56(6):1274; 2016
& Tzounakas et al., Proteom Clin Applic, 2016

XII . Donor- Recipient-Related Issues

❖Transfusion is the **synergistic result** of donated blood and recipient's pathological characteristics

Kanias et al., Transfusion 52: 1388; 2012

❖**Recipient's clinical status** is a major determinant of **RBCs performance** post-transfusion.

"double hit" hypothesis

❖**Inter-donor** differences in **RBC storability** might become more evident **post-transfusion** when cellular and soluble components of high physiological variability **cross-talk** with **pathophysiologic factors** exhibiting considerable diversity in the **recipient**

❖Adverse clinical outcomes have been reported in **neonates**, hemolytic G6PD patients, **multi-transfused** patients and patients **on oxidative medication** after transfusion of pRBCs from eligible **G6PD-deficient donors** but not in other adult recipients.

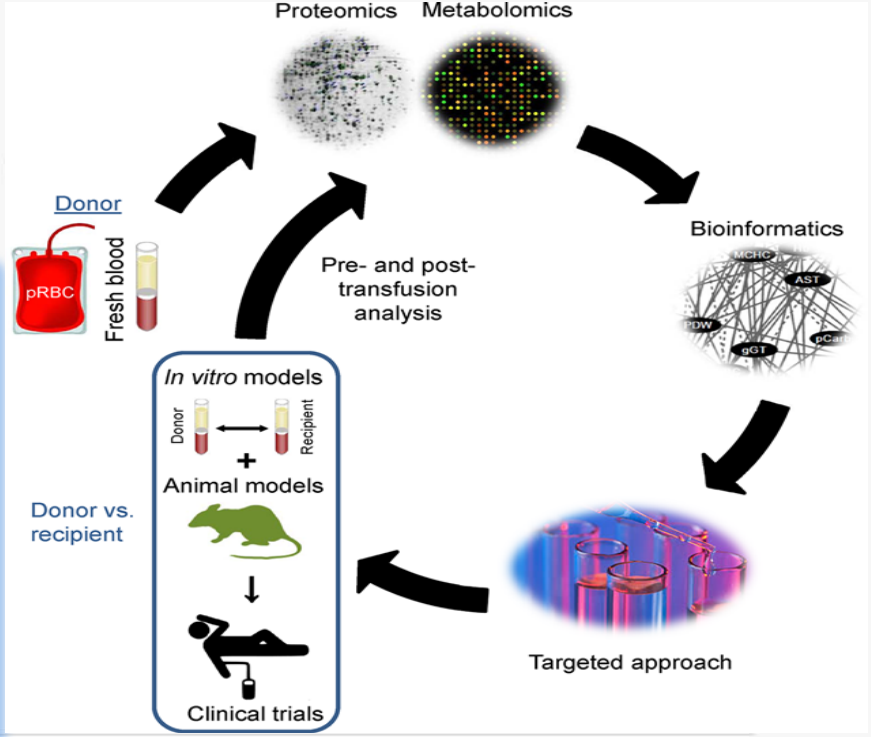
Shalev et al., Vox. Sang. 64:94–98; 1993
Nabavizadeh et al., Hematology 12:85–88; 2007

❖**Diabetic mice** (who had more severe endothelial dysfunction) were more sensitive to free Hb (or Hb-EVs) Hb **NO-scavenging abilities** than their nondiabetic counterparts.

XIII. So, what can we do in terms of future DDR strategies???

Tzounakas et al., Proteom Clin Applic, 2016

1. Focusing on relevant **clinical studies** and **experimental models (human and animal; *in vitro* and *in vivo*)** to assess the correlation between main characteristic **RSL** profiles and the clinical outcomes of transfusion in specific group of **recipients**.



❖**Autologous** transfusions

❖**pair studies** of well characterized (in terms of RSL) RBC units **(in vitro)** from **different donors and *in vivo*** recovery in healthy recipients or certain disease contexts.

Identifying this **crosstalk** would backwards lead to the development of **storage strategies** that **mitigate or change the RSL profile**

2. Focusing on the donor -reciepient integrated approach of RSL:

1) Donor/ Donation (before/during blood collection)
2) pRBCs unit during the storage
3) Recipient (post transfusion)

Cooperation between collection teams, laboratory experts and clinicians to find out correlations and mechanistic relationships of Biological / hematological factors (*in vivo/in vitro*) **WITH clinical outcomes**

In particular focussing on:

✓ Substantial **harmful effects confined to the latest peirod of storage, especially in patients receiving multiple units of microbiologically safe blood at nearest to outdate, occurring with** some frequency, in patients in real practice.

✓ **Narrow and discrete recipient groups**, such as trauma patients .The **clinical susceptibilities** of the patients in the existing RCTs **may not reflect susceptibilities in other patient populations**.

✓ **Donor and processing-associated variation, where** substantial intra-unit differences exist, as a result of donor and processing variations

✓ **Multifaceted role of EVs** on RBC quality, safety and efficacy, **using additional tools to assess the clinical outcome**: tissue oxygenation, adequate removal of CO2, NO bioavailability & Performing new **Randomized Clinical Trials** (RCTs)

3. Finaly focusing on the impact of heterogenieous levels of multifunctional Evs, present in aged red cell to recipients' RBC biology and clinical outcome:

* Not all pRBC units are equivalent products clinically

** Each unit of RBC is **unique** – contains variable levels of modified **RBCs**, carrying removal signals (senescent markers) and sublethal lesions & containing bio-reactive **Evs** / **MPs** with PS on the surface (hence are potentially procoagulant) though some appearing as inside out vesicles, or lost their adhesive proteins(hence can be highly immunogenic.

Currently ongoing RBC-omic study being conducted in the **REDS-III program (Recipient Epidemiology and Donor Evaluation Study-III** is evaluating **donor genetic and metabolic characteristics** that regulate differential rates of RBC **oxidative**, **osmotic**, and spontaneous **hemolysis** during storage. This study will also investigate the **genetic characteristics** of donor **Hb production** and **iron metabolism** that influence **RBC recovery post-transfusion**.

Kleinman et al., The National Heart, Lung, and Blood Institute Recipient Epidemiology and Donor Evaluation Study (REDS-III): a research program striving to improve blood donor and transfusion recipient outcomes. Transfusion 2014;54:942-55

Current Opinions on Clinical Outcomes: Fresh vs. Aged Blood in Transfusion Practice

Significant RSL-associated changes are those that **impact patient transfusion outcomes**.

The multicenter randomized and blinded **ABLE trial** compares RBCs stored for <8 days vs. standard issue cells with respect to 90-day mortality. [***Lacroix J et al. NEJM*, 2015**].

Conclusion: **fresh RBCs do not confer a 90-day survival benefit compared to standard issue blood in critically ill patients**.

These large scale RCTs raise the provocative question: what's the relevance of laboratory study of RSL in the absence of demonstrable evidence of adverse medical outcomes correlated to prolonged storage?

❖It has been, nevertheless, suggested that a **larger total volume of older blood** being transfused can **increase patient mortality by a dosage effect.** [*Aubron et al. Age of red blood cells and transfusion in critically ill patients, Ann Intensive Care, 2013.*

Acute medical conditions with accompanying massive haemorrhage (polytrauma, acute variceal gastrointestinal bleeding etc) are potential candidates for this sub-group analysis. [O'Brien KL et al & *How do we manage blood component support in the massively hemorrhaging obstetric patient? Transfusion*, 2016].

XIV. WHERE ARE WE NOW : THE CURRENT CLNICAL & DDR STRATEGIES: { "NHLBI Project /2015" }

To improve storage strategies -effectiveness – safety:

*Definition of **Optimal storage conditions** at two levels of importance:

1) minimising **in-bag** hemolysis
2) Optimising **post-transfusion survival**, function, effectiveness, reactivity

** **Individualized Transfusion Medicine**

Taking into consideration specific features of each contributing part :

1) **Prognosis of RSL profile for each donor**
2) **RSL profile-associated clinical effects in specific groups of recipients**

***Better "Blood management"** by blood donation and transfusion services: ⇒ Take the best from each donor to the benefit of patients:

1. **Low storability units**: storage under optimum conditions
2. **High storability units**: in susceptible recipients (critically ill, transfusion-dependent patients, infants)

RSL Allegory:" The End of the Beginning"

" Rapidly adjust sails to the winds of newer developments, in 4 interrelated areas of RSL with the holistic **training / team working & haemovigilance** "

" TO AGE, OR NOT TO AGE" THAT IS THE QUESTION!!!

"The pessimist complains about the wind;
The optimist expects it to change;
The realist adjusts the sails".

William A. Ward

- Unlike the good wine, RBC does not improve with age.
- Our Narrative: RSL is a multifaceted function of variability in **collection, processing,** storage **media**, storage **time**, **donor/donation** and **recipient** issues, as four "circles of dependence of Quality and Safety".
- Best transfusion practice, even with the use of validated aged RBC, is a real balancing act between **safety/efficacy** vs. immune/non immune **side effects.**
- As realist believing on the optimistic word of "possible", based on **evidence** provided here, more **collaborative, integrated works,** employing **contemporary technologies are needed** to achieve more **efficient** and **safer transfusions in patients –targeted manner**:
- The ride will not be easy but is achievable with perseverance & surveillance.

II. LIST OF SOME RELEVANT READINGS: Current opinions & Future perspectives

1) *The use of* **advanced methods in the field of RBC research (**such as AFM etc) *"**in a patient-orientated precision medicine approach**" (**Pretorius et al., Blood Reviews 2016***

2) **Extending the use Omics approaches: need small volumes** for analysis, **provide big data** platforms of clinical relevance. (***Antonelou & Seghatchian].*** Update on EVs inside red blood cell storage units: Adjust the sails closer to the new wind . *Transfusion and Apheresis Science 55 (2016).*

3) Circulating **EVs, are now considered as useful biomarkers of health/disease,**

(with some beneficial role in hemostasis and some harmful effects such as carrying blood-born infection[including TSE], -viruses might use EVs as a mode of trafficking to avoid recognition and stimulation of immune response).

4) Measurement of EVs as Biomarkers of Consequences or Cause Complications of Pathological States, and Prognosis of both Evolution and Therapeutic Safety/Efficacy. ***Amiral & Seghatchian*** . *Transfusion and Apheresis Science 55 (2016)*

5) The multifaceted roles of **EVs** in the components **quality**, **therapy**, **immunomodulation** and **infection** highlighted recently[Editorial]. ***Seghatchian & Petrick***."***Big things in small heterogeneous packages***" - With enormous clinical significance. *Transfusion and Apheresis Science 55 (2016).*

6) The **vesicles of pRBCs units** have not been characterized in depth, but have been used for **delivering drugs in cancer patients**, as immunocompatible biomimetic nanocarriers, to avoid the host immune system while delivering their cargo safely to the site of action with extended blood circulatory time.

(***Burnouf et al Transfusion Apheresis Science 54 2016)***.