since 1956

BLOOD TRANSFUSION

Official journal of
Società Italiana di Medicina Trasfusionale e Immunoematologia - SIMTI
Associazione Italiana dei Centri Emofilia - AICE
Hrvatsko Društvo za Transfuzijsku Medicinu - HDTM
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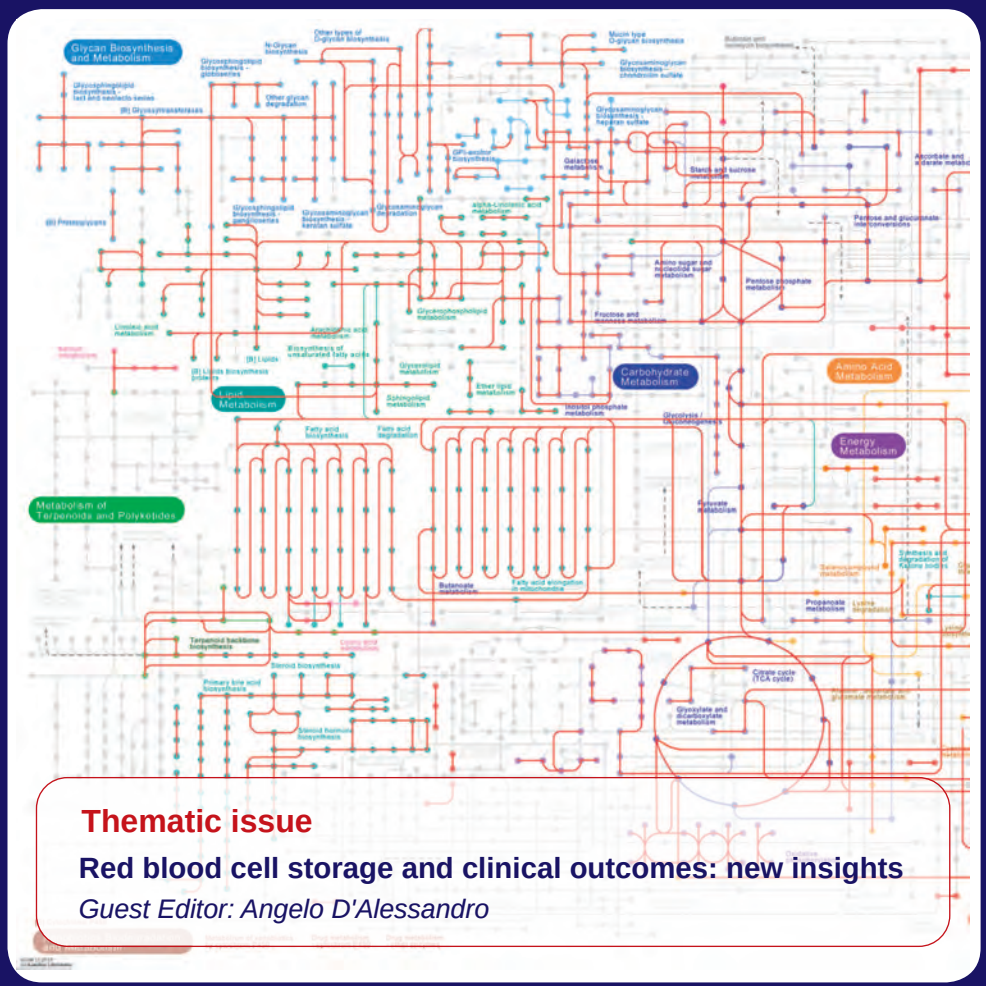

Thematic issue

Red blood cell storage and clinical outcomes: new insights

Guest Editor: Angelo D'Alessandro

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Contents

Cover Picture

Adapted from "Red blood cell proteomics update: is there more to discover?" by D'Alessandro et al. *(page 183).*

Information and Instructions to Authors

Aims and Scopes

Blood Transfusion is a bimonthly Journal founded in 1956 by the Italian Society of Transfusion Medicine and Immunohaematology (Società Italiana di Medicina Trasfusionale e Immunoematologia, SIMTI).

It is the official journal of SIMTI, Italian Association of Haemophilia Centres (Associazione Italiana dei Centri Emofilia, AICE), Croatian Society of Transfusion Medicine (Hrvatsko Društvo za Transfuzijsku Medicinu, HDTM), Spanish Society of Blood Transfusion and Cellular Therapy (Sociedad Española de Transfusión Sanguinea y Terapia Celular, SETS), and Italian Society for the Study of Haemostasis and Thrombosis (Società Italiana Studio per lo Studio dell'Emostasi e della Trombosi, SISET).

The journal also has two Affiliated Societies: Portuguese Association of Immuno-Haemotherapy (Associação Portuguesa de Imuno-Hemoterapia, APIH), and Hellenic Society of Blood Transfusion (HSBT).

It publishes Editorials, Reviews, State-of-the-Art Reports, Original Articles, Brief Reports, Case Reports, Letters to the Editor, Book Reviews, Forums and Debates on all topics related to Transfusion Medicine, e.g.: transfusion clinical practice, immunohaematology, blood component collection and production, transfusion-transmissible diseases, immunogenetics, histocompatibility, transplantation, haemostasis, medical-legal correlations, biotechnology and connected molecular biology.

Blood Transfusion is published in English only. Proceedings of Congresses and Meeetings may be published in the original language. Authors' opinions may not necessarily reflect those of SIMTI and the other Affiliated Societies or Editorial Board of Blood Transfusion.

Blood Transfusion is an **Open Access Publication**, as the Editorial Board and all the Affiliated Scientific Societies believe that works reporting the results of scientific research should be openly accessible and freely usable by the entire scientific community.

Manuscript submission

All manuscripts must be submitted online. Manuscripts submitted via fax or by e-mail will not be considered. We inform that Blood Transfusion website is optimised for IExplorer and FireFox. In any case, if you cannot submit online please contact the Editorial Office (francesca.fermi@bloodtransfusion.it).

For any information about manuscript composition, Authors should refer to papers previously published in Blood Transfusion or contact the Editorial Office. Useful guidelines are given at the site www.icmje.org and you can refer to our Review "How to write a scientific manuscript for publication" by Liumbruno GM *et al.* (http://www.bloodtransfusion.it/articolo.aspx?idart=002471).

Once an article has been submitted, the corresponding author will receive an e-mail indicating the submission number.

Please make sure that you cite your submission number in all correspondence with the Editor or Editorial Office. If the editing style does not comply with the instructions published in Blood Transfusion, the Author will be notified and asked to revise the paper before it is sent out for peer-review.

Editorials, Reviews and State-of-Art Reports are usually solicited by the Journal Board and are subject to peer-review. Unsolicited Review Articles may be submitted. Authors who wish to submit reviews to the journal are requested to submit a short synopsis of their chosen subject to the Editor-in-Chief, and to indicate the deadline by which they expect to submit their final manuscript, which, however, will be peer-reviewed after submission.

Copyright

Authors will grant copyright of their manuscript to SIMTI Servizi Srl. No formal permission will be required to reproduce parts (tables or illustrations) of published papers, provided the source is quoted appropriately and reproduction has no commercial intent. Reproductions with commercial intent will require written permission and payment of royalties. Please contact the office for requests: francesca.fermi@bloodtransfusion.it.

Peer review

All submissions are subject to peer-review by at least two experts selected by the Editor-in-Chief and the Associate Editors.

The review process takes about 6 weeks, and most manuscripts require revision before final acceptance.

Each paper is first evaluated by one or more editors, who will assess the overall quality and novelty of the work and the article's appropriateness for the scope of Blood Transfusion. Articles that are not found to be relevant or doesn't reach sufficient priority for Blood Transfusion will not be sent out for external review. The remaining articles are reviewed by external referees (second step of classical peer-review).

The referees' comments are returned to the Authors, who may be asked to revise their submission. Authors who resubmit a revised version must log in again into the Blood Transfusion website and replace the file of the manuscript according to the referees' criticism and the e-mail received. Authors who do not agree with the referees' criticism must clearly state their reasons. It is up to the Editors to accept such reasons.

A copy of the manuscript with highlighted changes in the text is required in order to help speed the review process. Revised papers are reviewed by the original referees, unless the Editor-in-Chief or the Associate Editor opt for an immediate decision. Acceptance of a revised paper is not granted but contingent on the acceptability of the changes made in compliance with the referees' criticisms.

The final decision on a paper's acceptability for publication is made by the Editor-in-Chief on the basis of the comments of an Associate Editor and the referees.

Clinical trials, systematic reviews and observational studies

Blood Transfusion prioritises reports of original research that are likely to change clinical practice or thinking about a disease.

Obligation to register clinical trials. Blood Transfusion, according to the International Committee of Medical Journal Editors (ICMJE), believes that it is important to foster a comprehensive, publicly available database of clinical trials. As ICMJE, Blood Transfusion defines a clinical trial as any research project that prospectively assigns human subjects to intervention or concurrent comparison or control groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Medical interventions include drugs, surgical procedures, devices, behavioral treatments, process-of-care changes, and the like.

Blood Transfusion requires, as a condition of consideration for publication, registration in a public trials registry. The journal does not advocate one particular registry, but requires authors to register their trial in a registry that meets several criteria. The registry must be accessible to the public at no charge. It must be open to all prospective registrants and managed by a not-for-profit organisation. There must be a mechanism to ensure the validity of the registration data, and the registry should be electronically searchable. An acceptable registry must include a minimum of data elements. Blood Transfusion accepts registration in the following registries: www.anzctr.org.au, www.clinicaltrials.gov, www.ISRCTN.org, www.umin.ac.jp/ctr/index/htm, www.trialregister.nl, https://eudract.ema.europa.eu/. In addition to these registries, Blood Transfusion also accepts registration in any of the primary registries that participate in the WHO International Clinical Trials Portal (see http://www.who.int/ictrp/network/primary/en/index.html). Because it is critical that trial registries are independent of for-profit interests, Blood Transfusion policy requires registration in a WHO primary registry rather than solely in an associate registry, since for-profit entities manage some associate registries. The details of the ICMJE policy are reported at http://www.icmje.org/faq_clinical.html.

Randomised clinical trials. Reports of randomised trials must conform to CONSORT 2010 guidelines, and should be submitted with their protocols. All reports of randomised trials should include a section entitled Randomisation and masking, within the Methods section. Meta-analyses of randomised controlled trials, should refer to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA statement, http://www.prisma-statement.org) or to the guidance document published by the Cochrane Collaboration

(http://www.cochrane-handbook.org/). Documented review and approval from a formally constituted review board (Institutional Review Board -IRB- or Ethics committee) is required for all studies (prospective or retrospective) involving people, medical records, and human tissues. Blood Transfusion requires that the authors provide this information on the manuscript's website, and also that they report it explicitly under design and methods. When reporting experiments on animals, authors should be asked to indicate whether the institutional and national guide for the care and use of laboratory animals was followed.

Observational studies. Observational studies (cohort, case-control, or cross-sectional designs) must be reported according to the STROBE statement, and should be submitted with their protocols (http://www.strobe-statement.org/index.php?id=strobe-home).

Ethical concerns

In case of experimental/clinical reports involving patients or volunteers, the Authors must provide information about institutional, regulatory and ethical Committee authorisation, informed consent from patients and volunteers, and the observance of the latest release of the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/index.html).

How to prepare a manuscript for submission

Each manuscript must be original work never published before in any substantial part; it must be submitted solely to Blood Transfusion and should not be under consideration for publication elsewhere. Meeting or Congress Abstracts and Lectures are not considered as prior publications.

If a manuscript is accepted, all copyrights, including the right to reproduce all or part of the manuscript, will be assigned exclusively to the Publisher, i.e. SIMTI Servizi Srl. The terms for copyright, authorship and financial disclosure must be accepted when submitting an article. Once accepted, at the Editor's discretion, your contribution may be published in one of the Journal issues or in a special issue supplement.

Language

All manuscripts must be written in English (British English).

Authorship declaration, Copyright release and Conflict of interest disclosure

Please accept online the terms for:
- Privacy;
- Copyright;
- Authorship;
- Financial Disclosure.

If there is any possible conflict of interest, the Author(s) should describe it fully; the text will be automatically printed in the Authorship Declaration to be signed and returned to the Editorial Office.

Examples: "NAME is collaborating with XXX industry for research in the field" or "NAME will speak about this issue in a sponsored symposium of XXX industry".

Authorship declaration, Copyright release and Conflict of Interest disclosure (declaration.pdf) are automatically e-mailed to the corresponding author upon manuscript submission and should be signed and returned to the Editorial Office by fax (+39 02 23.95.16.21) or e-mail to luisa.stea@bloodtransfusion.it. The Author who submits a paper for publication is responsible that all other Authors agree to its submission and to its content.

Funding and resources

Institutional, research, private or any other funds and resources, totally or partially used to carry out the original work, must be declared.

Types of article and manuscript requirements

Original article

Be up to 4,000 words (excluding the abstract, references, tables, and illustrations).

The text must be ordered as follows:
- Abstract
  Structured in four paragraphs (Background, Material and methods, Results and Discussion), not exceeding 2,000 characters (including spaces). The abstract should be sufficiently explanatory of the whole paper and should only include text, without any citations and, possibly, any abbreviations.
- Introduction
- Materials and methods
- Results
- Discussion
- Conclusions
- Acknowledgements (if any)
- Funding and resources (if any)
- Authorship contributions
- Disclosure of conflicts of interest
- References
- Tables
- Figure legends.
  No more than 6 tables/figures are allowed.

Long articles may need subheadings to clarify some sections, such as Materials and methods, Results and Discussion.

Supplemental data - to be published online only - may include additional information regarding methodology, supplemental figures or tables, or primary data sets; it must be submitted with the original manuscript submission so it can be peer-reviewed.

Brief communication

Be up to 2,000 words (excluding references).
The text must be ordered as follows:
- Introduction
- Materials and methods
- Results
- Discussion
- Conclusions
- Acknowledgements (if any)
- Authorship contributions
- Disclosure of conflicts of interest
- References (a maximum of 20 references is allowed)
- Tables
- Figure legends.
  No more than 3 tables/figures are allowed.

Case report

Be up to 1,500 words (excluding references).
The text must be ordered as follows:
- Introduction
- Case report with results
- Discussion
- Acknowledgements (if any)
- Authorship contributions
- Disclosure of conflicts of interest
- References (a maximum of 20 references is allowed)
- Tables
- Figure legends.
  No more than 3 tables/figures are allowed.

Review article

Review articles are welcomed by the journal and are generally solicited by the Editor-in-Chief or the Associate Editors. Authors who wish to submit reviews to the journal are requested to submit a short synopsis of their chosen subject to the Editor-in-Chief, and to indicate the deadline by which they expect to submit their final manuscript. Review articles should focus on recent scientific or clinical advances in an area of broad interest to those in the field of Transfusion Medicine. These articles should not simply go over or summarise general information which is already known, but also discuss the importance of the data and provide a critical view on

how the findings of the papers reviewed have transformed or will transform the Transfusion Medicine field. Manuscripts are not to exceed 5,000 words excluding references, tables, and illustrations. The paper must include an abstract of maximum 2,000 characters (spacing included); it should be sufficiently explanatory of the whole manuscript and include text only, without any citations and, possibly, any abbreviations. A maximum of 100 references is recommended.

All Reviews, including those solicited by the Editors, are rigorously peer reviewed before a final publication decision is made.

Any involvement of medical writers, editors, or researchers supported by the pharmaceutical industry is not acceptable for the preparation of Review Articles published in Blood Transfusion.

Guideline/Recommendation

These articles do not have a specific structure.

Editorial/Commentary

Editorials are commentaries or opinions on specific articles or on general concepts in Transfusion Medicine. These articles are usually invited articles, but proposals are welcome and should be addressed to the editorial office. These articles have a free structure, contain about 1,500 words (excluding references). A maximum of 20 references is recommended.

Letter to the Editor

Be up to 1,000 words (excluding references).
Are allowed a maximum of:
- 2 tables and/or figures
- 5 essential references.

For all manuscripts

The first page must contain:
- title of the article;
- names of Authors (full first name and surname);
- institutions;
- a running title of no more than 60 characters (including spaces);
- keywords: Original Articles, Case Reports and Reviews require 3-5 keywords to be entered into the field. Please use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/MBrowser.html);
- corresponding author (full address and e-mail).

Files

All text elements (tables included) should be submitted in one file only.

The following file types are accepted:
- Microsoft Word (.doc and .docx);
- generic Rich Text Format (RTF).

Measurements

All measurements should be expressed in the International System of Units (SI); please see also www.bloodtransfusion.it for further and more complete information. Abbreviations should be spelled out on their first use in the text (unless it is a standard abbreviation or a unit of measurement) and thereafter applied consistently throughout the manuscript. The first time any equipment, apparatus or reagent is used, the name of the manufacturer(s) (and identifying address) must be provided in parentheses.

Terminology

Nomenclature of red cell antigens and genes should follow the conventions outlined in Garratty, G. Dzik W, Issitt PD, et. al. Terminology for blood group antigens and genes--historical origins and guidelines in the new millennium. Transfusion 2000; **40**: 477-89.

Nomenclature for factors of the HLA system should follow the convention outlined in the report by Bodmer JG et al. (Vox Sang 1999; **77**: 164-91).

Granulocyte antigens should be named according to the convention described in the publication by ISBT Granulocyte Antigen Working Party (Vox Sang 1999; **77**: 251, with the clarification of Lalezari in Transfusion 2002; **42**: 1396-7).

Nomenclature of platelet-specific antigens should follow the conventions outlined in Metcalfe P, Watkins NA, Ouwehand WH et al. Nomenclature of human platelet antigens. Vox Sang 2003; **85**: 240-5.

Additional instructions for manuscripts dealing with the molecular biology of RBC's, WBC's, and platelets.

1. Use accepted nucleotide, amino acid, and allele notation and symbols as defined by the ISBT and available on the ISBT Working Party on Red Cell Immunogenetics and Terminology website. Use c. to indicate the position of the nucleotide change in the mRNA, and p. to modify the amino acid change in the protein. For example, c.574C>T, p.Arg192Trp.
2. cDNA sequencing or full gene sequencing is required to associated a specific polymorphism with an observed phenotypic change and to define a new allele.
3. Nucleotide(s) change(s) identified by cDNA analysis should be confirmed in genomic DNA (by sequencing, PCR-RFLP, SS-PCR).
4. Accession numbers of new or novel sequences should be provided by depositing in an appropriate database (GenBank/EMBL).
5. Web-based resources used (database, online computer programme, etc.) should be given along with the URL in a separate section entitled "Web resources", following the Acknowledgments. URLs should not to be included in the text as these can change. The date the URL was accessed must also be included.

Tables

Collected at the end of the manuscript, must have a brief title and a legend and must be numbered consecutively in the same order as they are first cited in the text, using upper case Roman numbers. All explanatory information should be placed in a legend at the bottom of the table and not in the heading. Each Table must be cited in the text.

Illustrations

Include figures, photographs and graphics. Each figure must be supplied as a separate file from the text file; only .jpg, .bmp, .gif and .tif formats, with a resolution of at least 300 dpi (dots per inch), maximum size 10 MB, can be used. Artwork in other format is not acceptable. Please note that images downloaded from the Internet generally have a resolution of 72 dpi only and are therefore unsuitable for conventional printing purposes. Please do not increase their resolution manually. Illustrations must be numbered progressively according to their order of appearance in the text, using Arabic numbers. Each Figure must be cited in the text. The illustration's title and a short legend, if necessary, must be entered into the field "Title/legend" (ex. "Figure 1 - Algorithm for the diagnosis of thrombocytopenias"). Please be aware that illustrations will be printed in black and white. Please do not use Microsoft Organization Chart application to create figures, graphics or tables.

References

Only strictly relevant references must be quoted in the reference list at the end of the manuscript. They should be numbered consecutively in the order in which they appear in the text. A reference in the text is identified as a superscripted number (without parentheses) after the text that quotes the corresponding paper listed in the reference list (e.g. blood transfusion[1]).

If more than one citation is required, the reference numbers in the text are separated by a comma with no space in between (e.g. blood transfusion[1,3,7]).

If reference numbers in the text are more than two and consecutive, only the first and the last numbers separated by a hyphen should be cited (e.g. blood transfusion[1-4]). In the reference list, Journal names should be abbreviated as described in Index Medicus, Medline and PubMed. Abbreviations can also be obtained from http://www.nlm.nih.gov/tsd/serials/lsiou.html website.

The names of up to four authors should be listed. If a reference has five or more authors, only the first three should be listed, followed by "et al".

Personal communications, unpublished observations, and manuscripts that have been "submitted" or are "in preparation" must be cited in the text, but not included in the reference list.

Only papers accepted for publication can be included in the reference list, even if they are not published, yet. In this case, add the DOI number.

References must be written in compliance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals published by the International Committee of Medical Journal Editors (www.icmje.org). Some examples are given below:

- *Citation from a Journal article*

Hess JR [without punctuation marks after the initial(s) of the first name], Hill HR, Oliver CK, et al. Alkaline CPD and the preservation of RBC 2,3-DPG. Transfusion 2002; **42** [bold-type]: 747-52.

- *Citation from a Journal article published online ahead of print.*

Hess JR [without punctuation marks after the initial(s) of the first name], Hill HR, Oliver CK, et al. Alkaline CPD and the preservation of RBC 2,3-DPG. Transfusion 2002; DOI xx2001-xxx10001.x.

- *Citation from a Book*

Issitt PD, Anstee DJ. (punctuation mark between authors and title) *Applied Blood Group Serology*. [title of the book in italics with the first letter of all words in capitals, except prepositions such as "the", "of", "about" etc.) in capitals] 4th ed. Durham, NC: Montgomery Scientific Publications; 1998.

- *Citation of a Chapter in a book*

Beatty PG. The molecular revolution in histocompatibility testing: relevance to blood and marrow transplantation. In: Garratty G, editor. *Application of Molecular Biology to Blood Transfusion*. Bethesda, MD: American Association of Blood Banks (or AABB); 1997. p. 51-72 [If there are five or more Editors, list only the first three and add "et al."].

- *Citation of an Editorial*

Garratty G, Petz LD. Approaches to selecting blood for transfusion to patients with autoimmune hemolytic anemia [editorial]. Transfusion 2002; **42**: 1390-2.

- *Citation of an Abstract*

Kleinman SH, Stramer SL, Mimms L, et al. Comparison of preliminary observed yield of HCV and HIV minipool (MP) nucleic acid testing (NAT) with prediction from incidence/window period (inc/wp) model [abstract]. Transfusion 2000; **40** (Suppl 1): ABS010.

- *Citation of a Paper Conference*

Anderson JC. Current status of chorion villus biopsy. In: Tudenhope D, Chenoweth J, editors. Proceedings of the 4th Congress of the Australian Perinatal Society; 1986: Brisbane, Queensland: Australian Perinatal Society; 1987. p. 190-6.

- *Citation of a Letter*

Jimenez M, Sanz C, Alvarez A, Pereira A. Massive intravascular haemolysis in a patient with Clostridium perfrigens sepsis [letter]. Vox Sang 2002; **82**, 214.

- *Citation of Electronic material (paper/document)*

A concept paper on prestorage leukocyte reduction of transfusion blood components. Food and Drug Administration. Available at: http//www.fda-gov/ohrms/dockets/ac/00/backgrd/3620b2.doc., followed by "Accessed on dd/mm/yyyy" (the date when the paper was read/downloaded from the website).

- *Citation of Electronic material (general content)*

Eurordis.org [Internet]. Brussels. EURORDIS Rare Diseases Europe. Available at: www.eurordis.org. Accessed on dd/mm/yyyy.

Online pre-publication

As soon as a paper is accepted, edited and corrected, it is posted online in its definitive version (.pdf format), at www.bloodtransfusion.it, before being assigned to a print issue.

Online pre-publication allows accepted articles to be visible and indexed about 6 months earlier than the print publication date. The early online date counts as the official publication date. To this purpose a Digital Object Identifier (DOI) number is assigned to the online article to allow the pre-published article to be cited well before its appearance in print.

Galley proofs

Galley proofs are sent to the Corresponding Author for approval and corrections. They must be returned to the Editorial Office within one week. Major alterations to the text are not accepted at a proof stage. Please keep a copy of the corrected galley pages. Reprints may be requested by the Corresponding author.

Address

Blood Transfusion publishes the corresponding Author's e-mail and full postal address in the Journal.

Alert service

Please note that the e-mail addresses of Author and Co-authors will be included in the Blood Transfusion free alert service, to receive e-mail notification when new articles are published.

Units of measurement

The International System (IS) of units of measurement must be adopted, using prefixes and symbols as follows:

Prefix	Factor	Symbol
xa	10^{18}	E
peta	10^{15}	P
tera	10^{12}	T
giga	10^{9}	G
mega	10^{6}	M
kilo	10^{3}	k
hecto	10^{2}	h
deka	10^{1}	da
deci	10^{-1}	d
centi	10^{-2}	c
milli	10^{-3}	m
micro	10^{-6}	µ
nano	10^{-9}	n
pico	10^{-12}	p
femto	10^{-15}	f
atto	10^{-18}	a

Concentration units			
molar	M	normal	N
mole	mol	molality	molal
Length units			
micrometre	µm	metre	m
ångström	Å	nanometre	nm
Volume units			
litre	L	decilitre	dL
millilitre	mL	microlitre	µL
Mass units			
kilogram	kg	gram	g
decigram	dg	microgram	µg
Time units			
second	s	week	week
day	day	hour	h
minute	min	month	month
Electric current units			
ampere	A	ohm	Ω
volt	V	watt	W
Statistical terms			
Analysis of variance	ANOVA	probability	p
not tested	NT	correlation coefficient	*r*
coefficient of variation	CV	standard error	SE
not significant	NS	degrees of freedom	df
confidence interval	CI	Student's *t* test	*t*-test

Principal abbreviations

2,3 BPG	2,3 biphosphoglycerate acid
2-ME	2-mercaptoethanol
5-Ht	5-hydroxytryptamine (not serotonin)
ACD-2	acid citrate dextrose-2
ADP	adenosine diphosphate
AIDS	acquired immune deficiency syndrome
ALT	alanine aminotransferase
AST	aspartase aminotransferase
BFU-E	burst-forming unit-erythroid
BMT	bone marrow transplantation
bp	base pair
BSE	bovine spongiform encephalopathy
BSA	bovine serum albumin
C	Complement
°C	degrees Celsius
CCI	correct count increment
CD	cluster designation
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CFU	colony-forming unit
CFU-G	colony-forming unit-granulocyte
Ci	Curie
CJD	Creutzfeldt-Jakob disease
CMV	cytomegalovirus
CO	cut-off
CPD	citrate phosphate dextrose
CPD-A	citrate phosphate dextrose-adenine
cpm	counts per minute
cps	counts per second
CSF	colony-stimulating factor
Da	Daltons
DAT	direct antiglobulin test
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetracetate
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Eq	equivalent
F(ab)	antigen-binding fragment
FBS	foetal bovine serum
FCS	foetal calf serum
FDA	Food and Drug Administration
FFP	fresh-frozen plasma
FITC	fluorescein isothiocyanate
G-6-PD	glucose-6-phosphate dehydrogenase
g	force of gravity
G-CSF	granulocyte-colony stimulating factor
GP	glycoprotein
GPA	glycophorin A
GPB	glycophorin B
GPC	glycophorin C
GPD	glycophorin D
GvHD	Graft-versus-Host disease
Gy	Gray radiation(s)
HAV	hepatitis A virus
Hb	haemoglobin
HBc	hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HBSS	Hanks balanced salt solution
HBV	hepatitis B virus
Hct	haematocrit
HCV	hepatitis C virus
HDN	haemolytic disease of the newborn
HES	hydroxyethyl starch
HGV	hepatitis G virus
HHV	human herpes virus
HIV-1	human immunodeficiency virus, type 1
HIV-2	human immunodeficiency virus, type 2
HLA	human leucocyte antigen(s)
HNA	human granulocyte antigen(s)
HPA	human platelet antigen(s)
HSA	human serum albumin
HPC(s)	haematopoietic progenitor cell(s)
HTLV-I	human T-lymphotropic virus, type I
HTLV-II	human T-lymphotropic virus, type II
LDH	lactate dehydrogenase
LISS	low-ionic-strength solution
MCHC	mean cell (RBC) Hb concentration
M-CSF	macrophage colony-stimulating factor
MCV	mean cell (RBC) volume
MHC	major histocompatibility complex
MNC(s)	mononuclear cell(s)
MoAb	monoclonal antibody
mRNA	messenger RNA
NA	neutrophil antigen(s)
NAT	nucleic acid amplification testing
NIH	National Institute(s) of Health
NK	natural killer (cells)
OD	optical density
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBSC(s)	peripheral blood stem cell(s)
pCO_2	partial pressure of CO_2
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	negative logarithm of hydrogen ion concentration
PHA	phytohaemagglutinin A
PMN(s)	polymorphonuclear leucocyte(s)
pO_2	partial pressure of O_2
PrP(sc)	prion protein (scrapie)
PrP(c)	prion protein (cellular)
PVC	polyvinylchloride
QC	quality control
R	Röntgen radiation(s)
Rad	radiation absorbed dose
RBC(s)	red blood cell(s)
RFLP	restriction fragment length polymorphism
RHu	recombinant human
RIA	radioimmunoassay
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
rpm	revolutions per minute
s	sedimentation coefficient
S	Svedberg unit of sedimentation (10-13 s)
SCID	severe combined immunodeficiency
SDS	sodium dodecylsulphate
S/D	solvent/detergent
TNFα	tumour necrosis factor α
TRALI	transfusion-related acute lung injury
tRNA	transfer RNA
UTR	untranslated region
UV	ultraviolet
vCJD	variant Creutzfeldt-Jacob disease
VNTR	variable number of tandem repeats
vWD	von Willebrand's disease
vWF	von Willebrand factor
WBC(s)	white blood cell(s)

Red blood cell storage and clinical outcomes: new insights

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The Good

Transfusion of packed red blood cells (RBCs) is a life-saving intervention for millions of chronically or massively transfused recipients worldwide every year. After over a century of improvements, ten years ago a highly-debated retrospective clinical paper[1] suggested the potential negative association between storage "age of blood" and transfusion outcomes. This controversial observation fuelled the debate about the potential clinical relevance of the so-called storage lesion(s), a wide series of biochemical and morphological alterations RBCs undergo during storage in the blood bank (as extensively reviewed[2-5]). Ten years later, a series of comprehensive randomised clinical trials (RCTs) have come to an end, providing reassuring evidence about the lack of a detectable difference between fresh blood and standard of care at the limits of the statistical power of these studies[6-10]. This translates into the appreciation of the fact that the general standard of care will not be improved by preferentially issuing fresh blood[11], at least to the specific categories of recipients enrolled in those RCTs. Many have noted the limitations of the RCTs (including several contributors to this thematic issue of Blood Transfusion). Limitations relate, for example, to the lack of comparison of fresh blood products *vs* products close to the end of their shelf-life (35 days or older) owing to ethical concerns hampering the design and feasibility of such studies. It may be provocatively argued that "if we do not deem ethical to design a study where half of the recipients will only receive >35 day old blood, then we should not transfuse the oldest blood to actual patients as well", as recent studies seem to suggest[12]. Still, it is undeniable that RCTs reassured the field to such an extent that it became reasonable to conclude that, quoting American Association of Blood Banks (AABB) guidelines, "a restrictive transfusion threshold is safe in most clinical settings and the current blood banking practices of using standard-issue blood should be continued"[11]. In other terms, current practices are for the most part as safe and effective as they have ever been in the history of Transfusion Medicine. Nevertheless, it ought to be noted that, as Zimring and Spitalnik suggest in this issue[13], "when approximately 80 million RBC units are transfused annually worldwide, even vanishingly small (transfusion-associated negative) events, if they are real, can affect actual human lives; it then becomes a question of ethics and economics whether it is *worthwhile* to study and attempt to prevent them".

It is a matter of pride for all the members of the international Transfusion Medicine community to note that, despite the reassuring evidence coming from RCTs, the field still fancies the opportunity to define an international agenda to pursue the amelioration of blood storage strategies. An example of this critical commitment by decision makers in the United States and Italy is represented by the recent 2016 meetings at the National Heart, Lung, Blood Institutes, Food and Drug Administration and Italian National Blood Center, where some of the leading experts in the field gathered to identify current issues associated with blood storage, and shared strategies to address such issues.

The Bad

While clinical trials have informed us about the substantial safety of current transfusion practices at large, laboratory sciences, especially omics technologies, have contributed insights into the reason why the transfusion therapy may mediate, in a minimum but not negligible number of cases, untoward transfusion-related events (e.g. transfusion-related acute lung injury [TRALI], transfusion-related immunomodulation [TRIM]) or aggravate underlying conditions (e.g. sepsis[14]). Improving our understanding of the storage lesion at a molecular level is a critical step towards the introduction of improved blood processing and storage guidelines. Many groups have contributed to document the energy and oxidative lesions targeting stored RBCs (as extensively reviewed by several authors in this issue). RBC energy and redox metabolic reprogramming during storage in the blood bank has been associated with the processes of vesiculation, impaired morphology and functionality (e.g. gas transport and off-loading), as well as *in vivo* survival in animal models and humans (as extensively reviewed in this issue and elsewhere)[2-5]. Protein[15] and metabolic markers[16] of the RBC storage lesion have been proposed by us and others. The metabolic phenotype of stored RBCs follows a specific 3-stage sequence, as gleaned through multivariate analysis of metabolomics data

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from different storage additives (as detailed by Prudent and Colleagues, Bordar, and us in this issue). We now understand that RBC metabolic reprogramming during storage in the blood bank is a biochemical necessity driven by refrigeration and excess oxidative stress, hence the necessity to restore reducing equivalents in order to counteract oxidative stress to functional proteins, such as haemoglobins and anti-oxidant enzymes (e.g. peroxiredoxin 2). Energy and redox homeostasis in stored RBCs are intertwined to such an extent that storage additives may be designed to boost either or both metabolic necessities, such as in the case of alkaline additives or hypoxic storage of erythrocyte concentrates (as discussed in this issue). For the interested reader, this thematic issue offers the opportunity to get a glimpse of the recent advancements in this field, as well as to get a general overview of the main technologies that contributed to our making the most significant strides forward in this research endeavour (i.e. omics technologies*)*.

The Ugly

The apparent disconnection between laboratory science and clinical trials has been increasingly explained in the past 12 months by the small scale of laboratory omics studies performed to date and by the necessity to investigate the biology of the donor and the recipient along with the evolution of the storage lesion *per se*; a "Copernican revolution" we had anticipated in 2009[17-19]. As pointed out by some of the contributors to this issue, until recently donor and recipient biology had often been overlooked in laboratory and clinical studies of the RBC storage lesion. While the clinical relevance of the storage lesion(s) remains a matter of debate, large-scale studies such as the REDS III Omics initiative will tackle this relevant issue in the coming years.

To further support the statements above, it is worthwhile recalling the 2008 study by Dumont and Aubuchon in which the results were published from a large retrospective study of radiolabelled RBC recoveries in autologous healthy volunteers (n=641)[20]. Results indicated that end of storage RBCs had recoveries averaging around 82.4±6.7%, with some donors showing 24-h *in vivo* survival as low as 35-40%[20]. These numbers are also suggestive that, on average, approximately 17% of the RBCs in a transfused unit are lost during storage and transfusion to healthy volunteers[20] (as pointed out by Mays and Hess in this issue). These numbers would theoretically be even worse if the biology of actual recipients were taken into account, in that heterologous chronically or massively transfused recipients would respond differently to blood transfusion than autologous healthy volunteer recipients owing to their repeated exposure to allogeneic cells or the underlying pro-inflammatory/metabolically-deranged physiology, respectively (as pointed out in this issue by several groups).

The Bad, the Good and the Ugly: old blood, new blood or better stored blood all over again

You may have noticed that paragraph subtitles in this editorial are a tribute to Sergio Leone's masterpiece "The Good, the Bad and the Ugly" which celebrates its 50th anniversary. The title of the film has entered the English language as an idiomatic expression, one that is typically used to describe something by referring to its upsides (the Good), downsides (the Bad), and the parts that could, or should have been done better, but were not (the Ugly). Besides the poetic license of the comparison to Sergio Leone's title, the whole field seems to have lost interest in the "new blood - old blood" diatribe, and rather agrees on the necessity to welcome the opportunity omics/laboratory studies have provided us with to further improve storage quality[21]. For the foreseeable future, small molecule/protein pre-storage markers of the lesion may inform us about the possibility of designing specific storage strategies for a given blood product for which the biology of the donor is already known, before matching it to the biology and specific clinical indications for the recipient. Alternatively, as suggested by Yoshida and Colleagues[22] in this issue, strategies such as hypoxic storage may exploit biochemical constraints to normalise inter-donor variability and provide more homogeneous blood products to the community.

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Large retrospective effects, clear differences in animals, and multiple negative randomised controlled trials: this is exactly how it is supposed to work

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A series of randomised controlled trials (RCTs) have recently been completed regarding the clinical relevance, or lack thereof, of the "age of blood" in red blood cell (RBC) transfusion[1-5]. The results of the most recently completed study, the INFORM trial ("Informing Fresh versus Old Red cell Management"), were presented during the Plenary Session of the 2016 American Association of Blood Banks (AABB) meeting, and the paper is now available in the New England Journal of Medicine[3]. To date, none of these RCTs have detected any significant differences in the medical outcomes measured following transfusion of fresh RBCs *vs* standard issue (i.e. "older") RBCs. Although no study has purposefully transfused the oldest possible RBCs (i.e. 42 days) to any patient population, analyses of those who did receive the oldest units by chance likewise failed to detect any significant differences in any evaluated clinical outcome, although, the latter analyses did not have a great deal of statistical power. These results have led to some concern regarding the discrepancy between the new results with RCTs and prior results from observational studies in human patients and prospective studies with healthy human volunteers and animal models. For example, why did the study by Koch *et al.*[6], and many other studies, observe increased morbidity and mortality associated with transfusions of longer-stored RBC units? In addition, why has so much animal model data in multiple species (mice, guinea pigs, dogs, sheep, etc.) similarly observed multiple adverse outcomes, by multiple pathophysiological mechanisms, following transfusions of longer-stored RBCs? Such a clear failure of outcomes must reflect some fundamental error; in short, where did so many of us go so wrong to mislead the field and waste significant resources on misguided science?

This question, while seemingly complex, is actually very easy to answer; that is, nothing went wrong. Indeed, this is exactly how scientific inquiry is supposed to work. Thus, although RCTs remain our best tool to mitigate bias in observing natural phenomena in human populations (in accordance with methodological constraints consistent with our ethical values), RCTs are resource intensive efforts. Therefore, it is not cost effective to perform RCTs as a first-line approach to observing clinical events, adverse or otherwise, especially those that occur at a low frequency. Rather, retrospective and observational studies, driven by analysis of captured data, or even inspired by anecdotal observations, remain our most powerful surveillance method for phenomena that would otherwise go undetected. Indeed, the history of medicine is rife with such examples, encompassing successful ones (e.g. digitalis, smallpox vaccination, artemisinin) as well as many failures. Such critical analysis is ongoing in essentially all fields by our front-line observers; for example, were it not for real-time observational reports, then the association between Zika virus infection and microcephaly would not have been detected. Not to perform such studies and publish such observations would be, in our view, an abdication of our responsibility to monitor and assess human (patho) biology and medicine. It is exactly because such studies can result in type I errors, due to the unavoidable bias intrinsic to such methods, or due to just chance alone, that it is wise and appropriate that resources then be expended on subsequent RCTs, when justified by the size and/or implications of the detected effects. However, the failure of subsequent RCTs to observe the phenomena detected by the antecedent retrospective studies does not demonstrate that the retrospective approach is wasteful. Rather, as vanguards of what human diseases or sequelae that we can detect, a substantial false determination rate is an expected and inevitable quality of approaches with sufficient sensitivity to allow detection of important medical issues. In addition, RCTs will not detect (nor can they confirm or refute) very low frequency occurrences, due to the practical limit on statistical power that RCTs carry as a function of the costs required for their performance. Indeed, the suspicion that longer-stored RBCs may produce adverse effects is reasonable based upon general notions of metabolic ageing, *in vitro* analysis of stored human RBCs (i.e. the RBC "storage lesion"), and animal models that demonstrate such sequelae, in general, and also in specific settings. Thus, we would have been remiss as a field not to

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study this issue and address this question in the most rigorous way possible. That the recent RCTs showed no detectable differences at the limits of their statistical power provides great comfort that the general standard of care will not be improved by issuing fresh blood, at least for the clinical indications that were studied.

Of course, we cannot rule out very small effects that may be detected by large retrospective or observational studies, but for which RCTs cannot be powered in any practical way. However, if we accept that science consists of a body of knowledge, the final adjudicator of which is observation of the natural world, and if we accept that our observations are limited by the resources available, then there is no practical difference between phenomena that we cannot observe and those that do not exist ("For whereof we cannot speak, thereof we must be silent")[7]. Still, when approximately 80 million RBC units are transfused annually worldwide, even vanishingly small events, if they are real, can affect actual human lives; it then becomes a question of ethics and economics whether it is "worthwhile" to study and attempt to prevent them.

What are we then to make of all the animal data generated in modelling a human effect that may not even exist? How many resources were "wasted" on such investigations and how many animals were needlessly subjected to "meaningless" studies? The key redeeming fact about such studies is a counterintuitive utility even to that which does not translate. If transfusions of longer stored RBCs have no clinically-important effects in humans, then studies of their effects in animals cannot translate to the human setting. Accordingly, what is the meaning of modelling something that does not exist? At first consideration, it seems that this notion is not possible; if the phenomenon being "modelled" does not exist, then is one modelling anything at all? Of course, the answer is "yes", just because scientists may not know what they are modelling does not mean they are not modelling something useful.

In one of the best descriptions of how research really works, Peter Medawar, the immunologist and Nobel laureate, detailed how experiments in tumour transplantation in mice examined a biology that does not occur in humans[8]. However, far from modelling nothing, they were inadvertently modelling tissue transplantation, and, eventually, the biology of histocompatibility and immune recognition, in general. When Bruce Beutler, another immunologist and Nobel laureate, and his colleagues became interested in the effects of endotoxin in mice, it was already well understood that "septic mice" have a drop in temperature instead of a fever, which seems to be a poor model for human biology. Even worse, Dr. Beutler focused on the very few mouse strains in which endotoxin had no effect; this was clearly the single worst model for sepsis! However, it was precisely this "broken" response that allowed him to identify a specific toll-like receptor as the murine, and then human, response element to endotoxin[9]. Like Medawar's description, the greatest utility of this finding was in enhancing our understanding of innate immunity in all of its manifestations.

So, now, what about all the animal data generated regarding transfusion of longer-stored RBCs and its various sequelae? Do they model effects that really occur in humans in general, but too infrequently to be observed? Or do they model effects in particular patient populations that have not yet been studied in the RCTs? Alternatively, are we modelling some other biology that we are, as yet, unaware of that is linked to the biology of longer-stored RBC transfusions? Perhaps we are modelling something unrelated to human biology at all, although this seems unlikely, given the close evolutionary relationships among mammals. Still, it remains possible that we are studying biology relevant only to our livestock, our companion animals, and the vermin in our sewers. Nevertheless, although we do not yet understand the utility of this knowledge, the history of science teaches that the effort is not wasted effort as long as the experiments are properly designed, rigorously performed, and accurately reported. The data are what the data are, but their interpretation, meaning, and importance will evolve over time in the context of our ever-expanding base of scientific knowledge.

At the cost of sounding self-congratulatory (as the authors are a small part of the modelling efforts of longer-stored RBC transfusions), it seems appropriate to congratulate the field of Transfusion Medicine and the various funding agencies for rising to the occasion and keeping an open mind regarding potential issues affecting patient care, for asking whether such effects might be present, and for acting in a rigorously scholarly and scientific fashion. The detailed investigations of humans, and the simultaneous modelling studies *in vitro*, in animals, and in human volunteers, have generated a new body of knowledge, the greatest utility of which is likely not yet clear. However, what is clear is that we not only kept a watchful eye on potential problems, but also did not rush impetuously into making reflexive changes, without careful scrutiny and study. The field is also indebted to the support from government, public sector, and industry sources to allow such studies to be performed. At the end of the day, we understand much more now than we did when we started, and we are more knowledgeable about what is best for our patients, and how best to save human lives, while doing the least harm. This is exactly how it is supposed to work. The lack of any detectable effect of RBC storage on clinical outcome in the published RCTs is a highly pertinent

negative, and the last decade has been fruitful, even if the end point of the journey has not been what, at least some of us, anticipated at the beginning. In the words of George Harrison, paraphrasing a conversation between Alice and the Cheshire Cat[10], "If you don't know where you're going, any road will take you there"[11]. What has just occurred in the field of blood storage biology is exactly how the scientific process is supposed to work.

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The red cell storage lesion(s): of dogs and men

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Abstract

The advent of preservative solutions permitted refrigerated storage of red blood cells. However, the convenience of having red blood cell inventories was accompanied by a disadvantage. Red cells undergo numerous physical and metabolic changes during cold storage, the "storage lesion(s)". Whereas controlled clinical trials have not confirmed the clinical importance of such changes, ethical and operational issues have prevented careful study of the oldest stored red blood cells. Suggestions of toxicity from meta-analyses motivated us to develop pre-clinical canine models to compare the freshest *vs* the oldest red blood cells. Our model of canine pneumonia with red blood cell transfusion indicated that the oldest red blood cells increased mortality, that the severity of pneumonia is important, but that the dose of transfused red blood cells is not. Washing the oldest red blood cells reduces mortality by removing senescent cells and remnants, whereas washing fresher cells increases mortality by damaging the red blood cell membrane. An opposite effect was found in a model of haemorrhagic shock with reperfusion injury. Physiological studies indicate that release of iron from old cells is a primary mechanism of toxicity during infection, whereas scavenging of cell-free haemoglobin may be beneficial during reperfusion injury. Intravenous iron appears to have toxicity equivalent to old red blood cells in the pneumonia model, suggesting that intravenous iron and old red blood cells should be administered with caution to infected patients.

Keywords: transfusion, storage lesion, red blood cells, canine model.

The earliest modern transfusions of human blood were either performed at the bedside immediately after removal of blood from the donor or even simultaneously *via* arterio-venous anastomoses[1]. The advent of solutions containing citrate anticoagulant and dextrose permitted blood collected for transfusion to be separated from the blood donor in both space and time. Refrigerated storage of whole blood or red cells (RBCs) permitted the establishment of blood depots to support the troops in World War I and eventually the modern blood bank[2,3]. However, the convenience of having inventories of stored refrigerated blood was accompanied by a drawback. Red cells clearly changed during cold storage as documented by a number of *in vitro* measurements of size, shape, enzyme content, rheology, and filterability[4]. Furthermore, modern tools can now detect thousands of changes in red cell metabolomics that occur within a few weeks of refrigerated storage[5]. The critical question, however, concerns the clinical importance of these changes.

For much of the 20th century, a major challenge in RBC transfusion involved efforts to extend component shelf life without sacrificing quality and efficacy. Early investigators settled on RBC recovery and survival *in vivo* at the end of storage as the key measure of RBC quality. The US Food and Drug Administration, which licenses RBCs, containers and storage solutions, relies primarily on two surrogate measures of efficacy and safety: 24-hour recovery and survival of >75% of radiochromium-labelled RBCs, and haemolysis of <1% at the end of storage. No clinical studies have ever been required. These were clearly minimal standards and although a variety of other analytical measurements were routinely provided (ATP, 2,3, DPG, lactate, whole blood oxygen binding curves, potassium concentration), surrogate measures had not been correlated with patient outcomes. Nevertheless, these surrogate measures seem to have served us well.

My research interest in this area was stimulated in 2003 when I was asked to write an editorial about a retrospective study that had concluded that stored RBCs posed a risk of toxicity to patients[6]. It occurred to me that whereas small compromises in erythrocyte quality and quantity, commonly referred to as the "storage lesion", are tolerated as the price of increased blood availability, clinicians do not expect this price to include harm to their patients. A few years later, an influential and controversial paper published in the New England Journal of Medicine provided a retrospective analysis of several thousand cardiac surgery procedures and concluded that transfusion of older red cells was accompanied by increased mortality[7]. This was not the first such study, but it was the largest and most widely publicised. In response, a collaborative group from the Department of Transfusion Medicine and the Critical Care Medicine Department in the intramural program

of the National Institutes of Health undertook a series of studies to determine whether "old blood" is associated with more clinical adverse events than "fresher" units. We determined that studying the oldest and freshest RBCs transfused to patients in specific clinical states would pose logistical and ethical problems, so we determined to adapt a well-validated canine model of severe pneumonia with a mortality end point to the transfusion setting. We believed such a model might detect a risk of transfusing stored RBCs if such a risk existed, and if so, could explore the mechanism(s), dose responses, critical length of storage, and potential interventions.

Before developing the pre-clinical study model, we undertook a literature review and meta-analysis of all studies published in English that reported the age of the RBCs transfused and included mortality as an outcome. Twenty-one such studies met the pre-determined criteria and our analysis revealed a highly statistically significant difference in mortality [odds ratio 1.16, 95% confidence interval (CI) 1.07-1.24, p=0.0001] favoring transfusion of fresher cells[8] (Figure 1). Despite the obvious limitations of such meta-analyses, a series of sensitivity analyses of these data [adult intensive care unit (ICU) studies, paediatric studies, surgery studies, non-surgery studies, studies in different decades] all yielded consistent findings, further supporting the hypothesis of the storage lesion.

The pre-clinical model we developed examined the extremes of fresh and old RBC storage as well as massive amounts of RBC transfusion in critically ill animals. We reasoned that if such a model showed no differences in mortality, then it would seem fruitless to pursue further studies of RBC storage. We infused a known concentration of bacteria into the lungs of purpose-bred beagles, and between hours 4-16 after bacterial challenge, performed four 20 mL/kg exchange transfusions with commercially prepared, leucoreduced RBCs, either 5 days old (fresh) or 42 days old for a 70% RBC exchange. Animals were supported with antibiotics, mechanical ventilation and titrated pressor therapy similar to management of patients in an ICU. At 96 hours, any surviving animals were sacrificed for necropsy. Older blood transfusion increased mortality (p=0.0005), the arterial-alveolar oxygen gradient (p<0.01), and histological lung damage (p=0.03). Older blood resulted in increased *in vivo* haemolysis, releasing free iron in the form of non-transferrin bound iron (NTBI) and cell-free haemoglobin (CFH) (p<0.03) and decreasing plasma haptoglobin levels[9]. Consistent with the vasoconstrictive effect of CFH, older blood increased both systemic and pulmonary pressures (all p<0.02). Mortality was associated with extensive pulmonary necrosis; other organ toxicity was not observed. This was the first randomised blinded animal trial showing blood transfused at end of storage period can increase mortality during infection (Figure 2).

Based on our studies in canines, we have proposed two main mechanisms as underlying the reported adverse effects of older stored blood. Briefly, we found that older RBCs are more fragile and prone to *in vivo* haemolysis when transfused, resulting in increased release of plasma haemoglobin and iron. This is also the case with human red cells. Both cell-free haemoglobin (CFH) and free iron have recognised toxicities and can potentially worsen outcomes in transfusion settings[10,11]. CFH is well known to scavenge nitric oxide (NO), an endogenous potent vasodilator, resulting in vasoconstriction. The vasoconstrictive effects of CFH can induce ischaemia and vascular endothelial injury. Haptoglobin, the plasma protein known to bind CFH, may become saturated during haemolysis and unable to promote its clearance by the reticuloendothelial system (RES). In addition, haemolysis releases free and protein-bound iron. Iron is a critical nutrient for bacterial growth. Iron metabolism

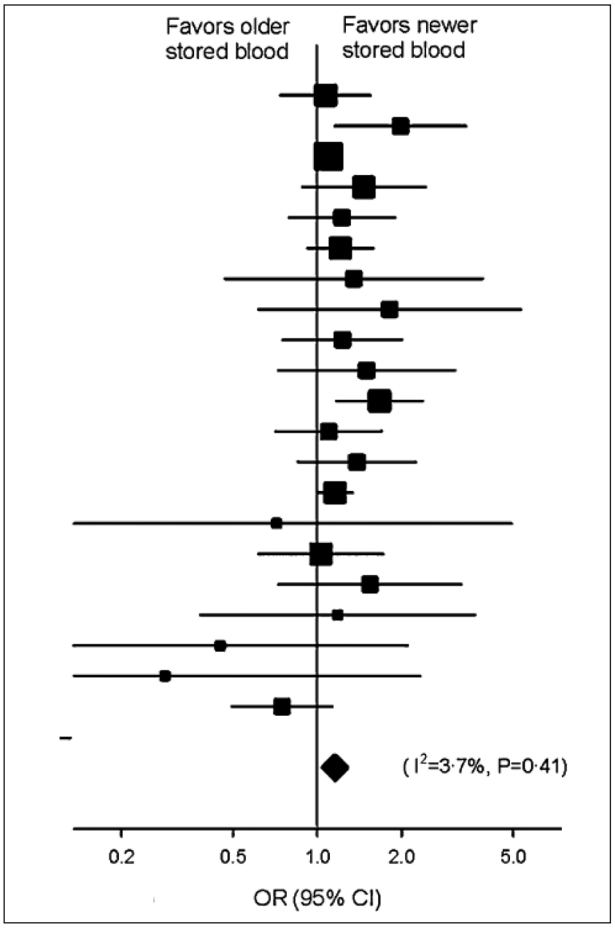

Figure 1 - Mortality.
The size of the data markers is proportional to the inverse variance of each point estimate.

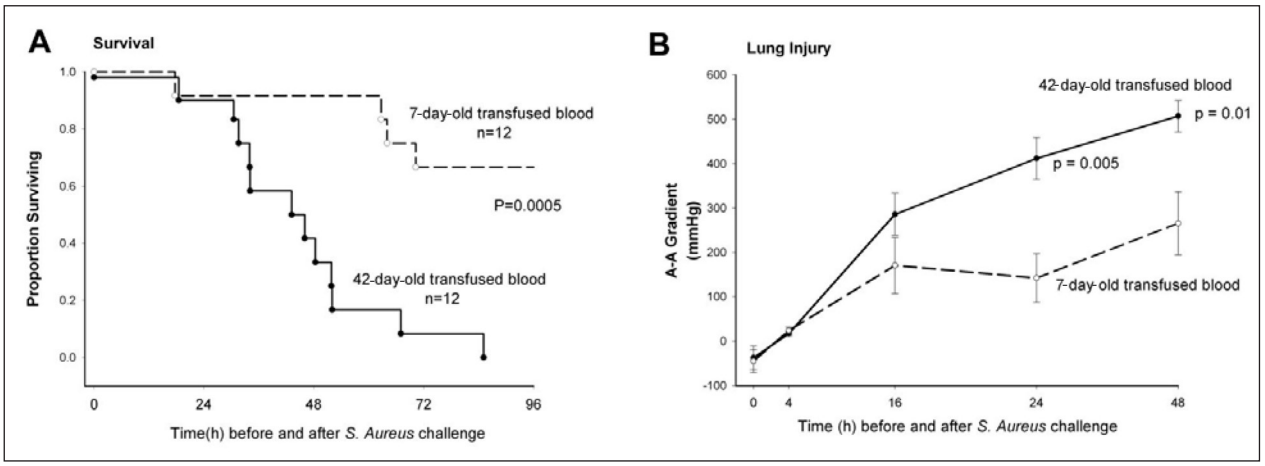

Figure 2 - Survival curves.
(A) Kaplan-Meier plot over the 96 hours of study comparing animals challenged with intrapulmonary *S. aureus* and exchange transfused with 42-day-old (solid circle, solid line) or 7-day-old (open circle, dashed line) stored blood. (B) Serial measures of lung injury. Arterial-alveolar oxygen gradient shows lung damage was significantly worse (higher) in the animals with pneumonia receiving 42-day-old (solid circle, solid line) *vs* 7-day-old (open circle, dashed line) stored blood at 24 hours (p=0.005) and 48 hours (p=0.01) after bacterial challenge.

is ordinarily carefully controlled to prevent direct oxidative toxicity and to limit access by pathological microorganisms. Human physiology has evolved multiple mechanisms to remove free iron available for micro-organisms from the circulation. In conditions of iron overload (haemochromatosis, transfusional haemosiderosis) the binding capacities of such proteins as transferrin and ferritin are saturated resulting in increases in plasma non-transferrin bound iron (NTBI). However, bacteria have evolved sophisticated strategies to scavenge iron directly from binding proteins with the help of high-affinity siderophores.

Next, we investigated the effect of increasing bacterial doses and severity of infection on the risks associated with age of blood transfused in this canine model[12]. Forty-eight animals were challenged intrabronchially with either 0 (n=8), 1.0×10^9 (n=8), 1.25×10^9 (n=24), or 1.5×10^9 (n=8) *S. aureus* colony-forming units/kg and then exchange-transfused with either 7- or 42-day-old canine universal donor blood (80 mL/kg in four divided doses). Without bacterial challenge, levels of CFH and NTBI were significantly higher with older *vs* fresher RBC transfusion but there were no significant differences in measurable injury. With higher-dose bacterial challenge, the elevated NTBI levels declined more rapidly and to a greater extent after transfusion with older blood, and older blood was associated with significantly worse shock, lung injury, and mortality. The CFH levels were markedly elevated over days regardless of severity of infection. The augmented *in vivo* haemolysis of transfused older RBCs, resulting in excess plasma CFH and iron release, appears to require the presence of established infection to worsen outcomes. These data suggest that transfused older RBC increase the risks from infection in septic subjects and define an infection dose-response.

During canine bacterial pneumonia with septic shock, but not in controls, older stored RBC were associated with significantly increased lung injury and mortality. We wondered whether transfusion of older RBCs would cause similar adverse effects during shock and inflammatory injury without infection. Therefore, 2-year-old purpose-bred beagles (n=12) were transfused similar quantities of either older (42-day) or fresher (7-day) stored universal donor canine RBC 2.5 hours after undergoing controlled haemorrhage producing shock[13]. With older transfused RBCs, CFH ($p<0.0001$) and NTBI (p=0.004) levels increased, but lung injury (p=0.01) declined and there was a trend toward lower mortality (18% *vs* 50%). The increased levels of CFH with older RBC transfusion were associated with an improved haemodynamic response to haemorrhage-reperfusion, with lowered exogenous norepinephrine requirements ($p<0.05$) and cardiac outputs ($p<0.05$). This haemodynamic effect is consistent with the ability of CFH to scavenge NO causing vasoconstriction. Thus, in haemorrhagic shock, older RBCs altered resuscitation physiology but did not worsen clinical outcomes. Elevated CFH lowers norepinephrine requirements and cardiac output, ameliorating reperfusion injury. In our infection model, we had previously shown that older blood increases NTBI levels transiently during transfusion and the rapid clearance of iron is associated with increased lung injury and mortality. In contrast, during haemorrhagic shock, NTBI levels persist longer after transfusion, and increased levels of iron are not

associated with worsened outcomes. These pre-clinical data suggest that whereas iron derived from older RBCs promotes bacterial growth, worsening septic shock mortality during infection, release of CFH and NTBI during haemorrhagic shock is not necessarily harmful.

We next conducted a blinded randomised controlled study (RCT) of RBC washing in this canine infection model of transfusion injury[14]. We hypothesised that washing older units of blood before transfusion would improve clinical outcomes by removing older fragile RBC and prevent increases of CFH and iron, whereas washing fresher units would have no effect on outcome. Twenty-four 2-year-old purpose-bred beagles (n=24) with *S. aureus* pneumonia were exchange-transfused with either 7- or 42-day-old washed (commercially available Haemonetics blood cell processor with standard washing procedure, Haemonetics Corp., Braintree, MA, USA) or unwashed canine universal donor blood (80 mL/kg in 4 divided doses). Washing RBCs before transfusion had a significantly different effect on canine survival, multiple organ injury, plasma iron, and CFH levels depending on the age of stored blood (all $p<0.05$ for interactions). Washing older units of blood improved survival rates, shock score, lung injury, cardiac performance and liver function, and reduced levels of NTBI, possibly by lysing and washing away old cells and supernatant. In contrast, washing fresh blood worsened these clinical parameters and increased CFH levels. Our data suggest that fresh blood should not be washed routinely because washing induces sublethal membrane damage to the RBC, and in a setting of established infection, washed RBC are prone to lyse, release CFH and iron, and result in worsened clinical outcomes. These findings, along with our previous studies, indicate that transfusion of fresh blood in infected subjects results in less haemolysis, CFH and iron release, and is less toxic than transfusion of older blood in critically ill infected subjects. However, if older blood must be used during established infection, washing prevents elevations in plasma circulating iron and improves survival and lessens multiple organ injury.

We next examined the results of altering volume, washing, and age of RBCs. Two-year-old purpose-bred infected beagles were transfused with increasing volumes (5-10, 20-40, or 60-80 mL/kg) of either 42- or 7-day-old RBCs (n=36) or 80 mL/kg of either unwashed or washed RBCs with increasing storage age (14, 21, 28, or 35 days) (n=40)[15]. All volumes transfused (5-80 mL/kg) of 42-day-old RBCs resulted in like increases in iron, CFH, lung injury, and mortality rates after transfusion. Transfusion of 80 mL/kg of RBCs stored for 14, 21, 28 and 35 days resulted in increased CFH and NTBI in between levels found at 7 and 42 days of storage. However, washing RBCs of intermediate ages (14-35 days) does not alter NTBI and CFH levels or mortality rates. Thus, our pre-clinical data suggest that any volume of 42-day-old blood potentially increases risks during established infection. In contrast, even massive volumes of 7-day-old blood result in minimal CFH and NTBI levels and risks. In contrast to the extremes of storage, washing blood stored for intermediate ages does not alter risks of transfusion or NTBI and CFH clearance.

Our most recent as yet unpublished studies examined the effect of intravenous iron infusion on mortality in the canine pneumonia model and compared it with the effects of RBC transfusion. Intravenous iron has been aggressively marketed as a safer alternative to RBC transfusion and is used widely in intensive care unit (ICUs)[16,17]. We reasoned that if iron released from old, stored RBC enhanced bacterial growth, a similar effect might result from administration of intravenous iron. We compared two forms of commercially available intravenous iron, iron sucrose, a "2nd-generation" preparation, and ferumoxytol, a novel iron oxide nanoparticle with a polyglucose sorbitol carboxymethylester coating designed to minimise immunological sensitivity, with fresh RBC transfusion. In the canine model of mild anaemia and pneumonia, both iron preparations resulted in similar mortality and pulmonary toxicity, both statistically increased compared to transfusion of 7-day-old RBC. These findings suggest that both old stored RBC and intravenous iron should be used with caution in the setting of established infection.

Because the number of clinical studies investigating the effects of age of blood on mortality has recently surged, and several large, RCTs have been completed, we up-dated our meta-analysis[18]. Among 31 observational studies, we found an overall significantly increased risk of death with older blood, confirming our original findings. In our up-dated meta-analysis, 6 RCTs now account for more than 4,000 patients, sufficient to perform a separate analysis from the observational studies. A significantly different overall effect on outcome was found in RCTs (p=0.02), with no differences in survival between older and fresher blood (OR: 0.91, 95% CI: 0.77-1.07). We attribute the different results found in observational studies and RCTs to methodological differences. While observational studies divide current transfusion practice into two groups of older and fresher blood transfusion, and "old" is up to 42 days, RCTs compare current practice (with an average storage age of 2-3 weeks) to transfusion of the freshest available RBCs (1-10 days old). Consequently, the median age for both the fresher and older blood arms was significantly higher in observational studies compared to RCTs (p=0.01). Our analysis of RCTs confirms that the freshest RBCs are not superior to the 2-3-week-old stored RBCs transfused during current practice. However, the RCTs cannot exclude the possibility,

which observational studies suggest, that older stored units available for transfusion, namely 4-6 week old RBCs, increase mortality risk. Since publication of our analysis, a pragmatic, controlled trial conducted at six hospitals in four countries randomised 31,497 patients to receive either fresher or older RBC; the findings are consistent with our conclusions[19].

Randomised controlled trials remain the "gold standard" for determining the risk of stored red cells, but studies in man have both ethical and practical limitations. One cannot ethically "store RBCs" in order to give the oldest cells to patients in a toxicity study. In addition, all clinical conditions cannot be studied. Pre-clinical studies can provide important clues to risk, pathophysiology, and therapy. Our studies suggest that the oldest RBC should not be administered to patients with established infection. We have, therefore, limited the age of stored RBC at NIH to 35 days. Our studies further suggest a potential benefit to washing the oldest RBC, but an increased risk to washing fresh cells. Finally, in a canine model, IV iron increases the mortality risk in the setting of established infection. We believe that until appropriate studies are conducted in patients, IV iron should be used with caution in infected patients.

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The controversy over the age of blood: what do the clinical trials really teach us?

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Abstract

Red blood cell transfusions have been used in clinical practice for decades and represent the most common therapeutic procedure performed in hospitalised patients. Depending on the storage solution and national regulatory requirements, red blood cells can be stored in the refrigerator up to 42 days before transfusion. We reviewed five of the most recent randomised clinical trials that examined clinical outcomes in specific patient populations. Although these studies provide some comfort regarding our current standard of care, they do not address whether the oldest blood is associated with harm in certain patient populations.

Keywords: blood transfusion, storage lesion, red blood cell, haemolysis, non-transferrin-bound iron.

Introduction

All medications have side effects. Although red blood cell (RBC) transfusions have been used in clinical practice for decades and represent the most common therapeutic procedure performed on hospitalised patients[1], one might expect them to have unintended side effects as well. We are more familiar with the risks posed from infectious disease transmission (e.g., HIV, hepatitis) and transfusion reactions (e.g., haemolytic allergic, febrile, acute lung injury, circulatory overload); however, those resulting from the "RBC storage lesion" are less well characterised. Depending on the storage solution and national regulatory requirements, RBCs can be refrigerator stored for up to 42 days before transfusion[2]. During refrigeration, RBCs undergo multiple metabolic and structural changes, collectively termed the "RBC storage lesion"[3]. Although the clinical outcomes resulting from the RBC storage lesion are debatable, everyone agrees that refrigerator damage *in vitro* does induce haemolysis *in vivo* of some of the storage-damaged RBCs[4]. In various animal models (e.g., mice, guinea pigs, dogs, sheep), under certain clinical circumstances, the haemolysis of transfused, storage-damaged RBCs produces adverse effects[5-7]. In addition, in healthy human volunteers, there is increasing haemolysis *in vivo* of transfused RBCs in proportion to increasing storage time before transfusion[4]; nonetheless, there was no definitive evidence of clinical harm in these research subjects.

A provocative observational study[8] concluded that transfusions of RBCs stored for more than two weeks were associated with significantly increased risks of post-operative complications after cardiac surgery, accompanied by increased short-term and long-term mortality. Although not the first such observational study examining the effect of prolonged RBC storage on clinical outcomes, this one initiated a flurry of well-designed clinical trials to examine this question more closely. Based on the conclusions from the five major clinical trials published since then, which primarily addressed the potential benefit of "fresher" RBCs, some have argued that "additional trials do not appear warranted at this time"[9], because no benefit of a fresher blood policy was demonstrated. However, we believe that one must examine these clinical trials more closely before concluding that the current standards do not have unintended consequences in certain clinical settings. To this end, we herein review the five major clinical trials published to date and discuss the strengths and limitations of each.

The Age of Red Blood Cells in Premature Infants (ARIPI) randomised trial

The ARIPI trial of fresh red blood cell transfusions in premature infants[10] was a double-blind randomised controlled trial in 377 premature infants with birth weights less than 1,250 grams admitted to six Canadian tertiary-care neonatal intensive care units (ICUs) between May 2006 and June 2011. The objective was to determine if RBCs stored for seven days or less, as compared with the standard of care, decreased rates of major nosocomial infection and organ dysfunction in neonatal ICU patients requiring at least one RBC transfusion. The overall conclusion was that a fresher RBC transfusion policy did not improve outcomes in premature, very low-birth-weight infants. However, we caution against concluding that the use of older RBCs is safe in this population, because this was not tested in the ARIPI trial; this is of special interest given the typical practice of reserving one unit for transfusion to the same neonate over the course of their hospitalisation, providing older and older aliquots as time goes on. In particular, the median age of "standard of care" RBCs in the Canadian sites participating in the ARIPI trial

was 14.6 days, whereas other studies in neonates[11,12] suggest that there is very little evidence of haemolysis from transfusing RBCs stored for less than two weeks. Because the standard of care arm in the ARIPI trial represented reasonably fresh RBCs, it is perhaps more justifiable, based on this study, to conclude that a "really fresh" RBC policy is not superior to a "fresh" RBC policy in neonates.

The Age of Blood Evaluation (ABLE) trial

The ABLE trial on the age of transfused blood in critically ill adults[13] was a multicentre randomised controlled trial comparing RBCs stored for less than eight days with standard-issue RBCs. Critically ill adults, 18 years of age or older, from tertiary-care ICUs at 64 centres were enrolled. Patients were eligible if a first RBC transfusion was prescribed within seven days after admission to the ICU and if they were expected to require invasive or non-invasive mechanical ventilation for at least 48 hours. The study interventions aimed to compare RBCs stored for less than eight days (mean 6.1±4.9 days) to standard-issue RBCs (mean 22.0±8.4 days). This study did not find any benefit attributable to the transfusion of fresh RBCs in critically ill patients. Although the primary outcome was clinically relevant and important for both patients and clinical decision makers, the study was not designed to answer the question of "Is old blood bad?" but, rather, "Is fresh blood better than the standard of care?"[13]. We recently demonstrated that circulating non-transferrin-bound iron (i.e., circulating iron not bound to the physiological iron transport protein, transferrin), a marker of excessive acute haemolysis, is not produced in transfused healthy volunteers until after 35 days of RBC storage[14,15]. Furthermore, evidence suggests that non-transferrin-bound iron can potentially mediate at least some of the adverse effects of transfusion[5-7,16,17]. Taken together, if non-transferrin-bound iron is a mediator of adverse effects resulting from the RBC storage lesion, it would be difficult to observe a clinical difference between fresh and standard-issue RBCs, unless the standard-issue RBCs were particularly old (e.g., 35-42 days). Thus, although this study provides some comfort regarding the current standard of care, it does not address the question as to whether RBCs stored up until the last week allowable result in harm in this vulnerable population.

The Informing Fresh *vs* Old Red Cell Management (INFORM) trial

The INFORM trial on the effect of short-term *vs* long-term blood storage on mortality after transfusion[18], conducted from April 2012 to October 2015, was a large, multicentre, pragmatic, randomised trial involving a general population of hospitalised patients aged 18 years and older who required a red cell transfusion. The 31,497 patients were randomised in a 1:2 ratio to receive the freshest or the oldest RBC units available. The median storage duration in the "old" transfusion storage group was 23.6 days. There was no difference observed in the primary outcome, comparing the rate of death between the patients transfused with the freshest or oldest available RBCs. Although this was the largest study performed to date, like the ABLE study[13], it was not designed to answer the question of whether the oldest RBCs (e.g., 35-42 days) are harmful in specific vulnerable patient populations. To confound this study further, by including multiple clinical conditions, an adverse effect in one sub-population may mask a beneficial effect of older RBCs in another. For example, if older RBCs do, in fact, function in a pro-coagulant fashion, this aspect may benefit a bleeding patient, but harm a patient with a thrombotic diathesis[3]. Based on this study, on average, fresher blood was not superior to older blood (although the median age of the older blood was approximately 24 days); however, in a given patient, it does not address whether a 42-day old unit will result in harm if transfused. In contrast, multiple animal studies suggest longer-stored RBCs have the potential to be harmful in certain circumstances[5-7,17,19]. Furthermore, a recent, albeit observational, study suggests that RBCs stored for 35-42 days are, indeed, associated with increased morbidity and mortality[20]. Although the INFORM trial is a large study, one must be careful in concluding that "old blood is safe" given these limitations.

The Red Cell Storage Duration Study (RECESS) trial

The RECESS trial on the effects of red-cell storage duration on patients undergoing cardiac surgery[21] was a multicentre, prospective, randomised clinical trial that compared clinical outcomes after cardiac surgery in patients who received transfused RBCs stored for ten days or less ("fresh" blood) or for 21 days or more ("old" blood). Running from 2010 to 2014, participants were 12 years of age or older undergoing complex cardiac surgery and likely to receive transfused blood. The primary outcome was the change in Multiple Organ Dysfunction Score (MODS; range 0-24, with higher scores indicating more severe organ dysfunction) from the pre-operative score to the highest composite score through Day 7 or the time of death or discharge. There was no difference in MODS observed between transfusions of fresh or old RBCs. It is very important to note that the cardiac surgery patients in this study were on bypass for a median time of 140 minutes. Cardiac bypass is associated with a significant amount of haemolysis from RBC damage caused by the pump[22,23].

The incremental haemolysis from the transfusion of one or two units of stored RBCs is expected to be small compared to the effects of the underlying haemolysis due to the bypass pump. Thus, to the extent that haemolysis of transfused storage-damaged RBCs is responsible for the adverse effects of transfusion, the results of this study are not generalisable to other patient populations without baseline haemolysis from bypass. Although this study may not be generalisable to other patient populations, it does answer an important clinical question raised by Koch *et al.*[8]. Based on that study[8], cardiac surgeons had an argument for demanding fresher blood for their cardiac surgery patients. The RECESS trial suggests that in patients on bypass, with significant pump-induced haemolysis, the additional effect of haemolysis from transfusion of older RBCs does not pose a significant clinical risk and the current standard of care is acceptable.

The Tissue Oxygenation by Transfusion in severe anaemia with Lactic Acidosis (TOTAL) trial

The TOTAL trial on the effect of transfusion of red blood cells with longer *vs* shorter storage duration on elevated blood lactate levels in children with severe anaemia[24] was a randomised non-inferiority trial of 290 children (aged 6-60 months) with severe anaemia, most with malaria or sickle cell disease, presenting between February 2013 through May 2015 in Uganda. The objective was to determine if older RBC units (25-35 days) were non-inferior to fresher RBC units (1-10 days) with regards to tissue oxygenation, as measured by reduction in blood lactate levels and improvement in cerebral tissue oxygen saturation. The primary outcome was the proportion of patients with a lactate level of 3 mmol/L or lower by eight hours after transfusion using a margin of non-inferiority equal to an absolute difference of 25%. Among children with lactic acidosis due to severe anaemia, transfusion of older RBCs did not result in inferior reduction of elevated blood lactate levels. Again, it must be noted that, analogous to the RECESS trial, virtually all the patients in the TOTAL trial had significant ongoing baseline haemolysis from their underlying illness (almost all had malaria and/or sickle cell disease, with a haemoglobin level on hospital admission of 5 g/dL or lower). Thus, to the extent that haemolysis is responsible for the adverse effects of transfusing older RBCs, this baseline haemolysis would make it very difficult to see a clinical difference in transfusion efficacy between fresh and older blood. Furthermore, this study could not test the hypothesis that the oldest blood, that is blood stored for more than 35 days, is responsible for adverse effects, because the standard of care in Uganda limits the outdate to 35 days. This policy is similar to other countries and institutions, which have reduced the maximum outdate due to the precautionary principle, such as the United Kingdom, the Republic of Ireland, the Netherlands, and the Blood Bank of the National Institutes of Health[25,26]. Given the difficulty of performing randomised clinical trials to test RBCs stored exclusively for 35-42 days, and the successful experience of some countries in limiting the allowable outdate, some would invoke the precautionary principle to argue that the maximum outdate should simply be changed to 35 days universally[15].

Conclusions

Although the clinical trials completed to date provide answers to clinically-relevant questions, they have yet to answer the question of whether transfusing RBCs after 35-42 days of storage is safe. One must be careful to generalise from patient populations with baseline hemolysis from an underlying disease to other patient populations. This raises the issue that, in some patients, older RBCs are not expected to be harmful; furthermore, one can even imagine patient populations in which older RBCs would be beneficial. Thus, a more nuanced approach is necessary before concluding that no further clinical studies are necessary[9]. In addition, none of the completed trials were designed on the basis of a mechanistic understanding of why transfusions of older RBCs might produce adverse outcomes (e.g., production of non-transferrin-bound iron can enhance biofilm formation of "ferrophilic" pathogens)[27]. Without a mechanistic basis, there remains the concern that the clinical trial design might include too much "noise" to reveal the "signal" of concern (e.g., a primary outcome of all infections may result in a different conclusion than using a primary outcome of biofilm-related infections from "ferrophilic" pathogens). Finally, although the clinical studies published to date provide some comfort regarding our current standard of care, the specific patient populations that would benefit from a fresher RBC strategy, or from avoidance of the oldest RBCs, remain to be fully explored.

The Authors declare no conflicts of interest.

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Red blood cell components: time to revisit the sources of variability

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Abstract

Quality and safety of red blood cell (RBC) components is managed by screening of donors and strict regulatory controls of blood collection, processing and storage procedures. Despite these efforts, variations in RBC component quality exist as exemplified by the wide range in storage-induced haemolysis. This article provides a brief overview of the variables that contribute or potentially contribute to the quality of stored RBC components, including blood collection, processing, and donor-related variables. Particular focus is made on donor health and lifestyle factors that are not specifically screened and may impact on the physicobiochemical properties of RBCs and their storability. Inflammatory and oxidative stress states may be especially relevant as RBCs are susceptible to oxidative injury. Few studies have investigated the effect of specific donor-related variables on the quality of stored RBC components. Donor-related variables may be unaccounted confounders in the "age of blood" clinical studies that compared outcomes following transfusion of fresher or longer-stored RBC components. The conclusion is drawn that the blood donor is the greatest source of RBC component variability and the least "regulated" aspect of blood component production. It is proposed that more research is needed to better understand the connection between donor-related variables and quality consistency of stored RBC components. This could be very important given the impact of modern lifestyles that sees escalating rates of non-communicable health conditions that are associated with increased oxidative stress, such as hypertension, obesity and diabetes in children and adults, as well as an ageing population in many countries. The effect of these changes to global health and population demographics will impact on blood donor panels, and without significant new research, the consequences on the quality of stored blood components and transfusion outcomes are unknown.

Keywords: blood donor, component processing, red blood cells, transfusion.

Introduction

In many jurisdictions throughout the world, red blood cells (RBCs) for transfusion are regulated as a medicine using similar codes of practice that are applied to the pharmaceutical industry for the manufacture of chemical-based drugs. Unlike a chemical pharmaceutical, in which all raw ingredients and formulations are precisely defined, it is not possible to achieve this same level of precision for complex biological medicines such as RBC components for transfusion. Whereas batch-to-batch variation is minimal for a chemical pharmaceutical, each blood donor and blood donation is biologically unique, and therefore each RBC component is a single batch. Furthermore, unlike the pharmaceutical industry, in which each batch of drug is tested prior to release, this does not occur for RBC components. Nevertheless, with the peace of mind provided by strict regulatory control of blood component production, there has been a tendency to overlook the possible impact of RBC component variability on transfusion outcomes[1]. The common view held by medical personnel is that all RBC components are essentially equivalent.

An exception to the notion of equivalence is the concern about storage duration of RBC components and the associated "storage lesion" that occurs during the permissible shelf-life of up to 42 days[2-5]. Whether shorter-stored RBCs provide improved transfusion outcomes compared to longer-stored RBCs has been a subject of active debate. Recent findings from several large "age of blood" randomised clinical trials (RCTs)[6-9], as well as large observational clinical studies[10], have reported a lack of effect of RBC component storage duration. These findings are somewhat at odds with those expected based on the numerous *in vitro* studies that have documented a progressive decline in RBC component quality caused by the storage lesion[2-5], and results reported by many retrospective "age of blood" clinical studies[11].

Storage duration alone is just one of many factors that impact on RBC component properties and quality. It has long been known that RBCs from some donors store well while others store poorly[12-15]. Evidence of donor-related variability is seen by the haemolysis profiles obtained from large datasets of RBC component quality control information[14,16,17], *in vivo* 24-hour post-transfusion RBC recovery data[12,15], as well as studies in twins[18] and different strains of inbred mice[17]. Donor-related variability may be an unaccounted for confounder in the "age of blood" clinical studies reported to date[5,19]. It is

timely to revisit the vast list of variables that influence RBC component properties and consider the donor-related factors more closely. The following commentary provides a brief overview of the different sources and types of variables, including donor and processing variables, along with the role of regulatory oversight.

Regulatory oversight

Regulatory oversight of donor health screening, blood collection, component processing, testing, quarantine, quality control and storage is intended to ensure blood component purity, potency and safety for the transfusion recipient[20]. Over the past few decades, the implementation of strict regulatory oversight, stream-lining of procedures, and rigorous training of front-line personnel have dramatically reduced technical variabilities in the collection and processing of blood components. Many national blood services and large blood centres have standardised their procedures to increase operational efficiency and improve the consistency of the blood components produced[21,22].

Donor selection

For blood collection centres, the objective of donor selection is equally focused to provide a safe and efficacious product for the recipient of the donation as well as to avoid any harm to the health of the donor[23]. Donor acceptance criteria depend heavily on the donor reporting to be well at the time of blood donation and identifying certain risks that could harm the recipient, such as transmission of infectious disease or allo-antibody reactivity.

Donation acceptance

Physical criteria for acceptable donations rely on the donor's haemoglobin (Hb) level measured at donation and the volume of blood collected, both of which must be within defined limits that may vary between jurisdictions[23,24]. For example, the prescribed volume of blood collected for a whole blood (WB) donation is typically 450 mL with a ±10% margin; in other words the acceptable volume range is 405-495 mL, which equates to a 90 mL difference between the smallest and largest acceptable donations[25]. In many jurisdictions, the minimum Hb limit is lower for female donors than male donors[24], and is inherently variable between donors and donations. Thus, the total Hb content of individual RBC components can differ markedly simply based on the variability of the volume of blood collected and the donor Hb level[26].

Routine quality control of RBC components

Only a small proportion of randomly selected RBC components are checked for quality control purposes. The intention of quality control is to provide assurance that the manufacturing system is performing within specification and looks for shifting trends rather than deviations of individual products. Some RBC components may be checked prior to storage to verify acceptable leucoreduction, while other tests are conducted at product expiry. The panel of expiry quality control measurements may simply include product volume, cell count, haematocrit and Hb content. Some blood centres perform additional quality measurements, such as percent haemolysis[14]. An array of other tests, such as pH, levels of RBC metabolites and membrane properties may be used for validation or research studies; however, the cost implications of a more expansive quality control testing regime would be prohibitive. Regardless of this, it is still unclear which, if any, of the available tests predict efficacy of the component when transfused[27,28]. Thus, for the vast majority of RBC components, specific information about RBC content and quality is not known.

Collection and processing variables

There are numerous sources of "allowable" variables within the collection and processing stages of blood component production that can influence the biological properties of the finished product (Table I).

In addition to the variables of blood volume and total Hb content mentioned above, other examples of variables include the elapsed time between WB collection and processing, temperature and handling conditions during the hold period. In many countries, including Europe, the United Kingdom, Canada and Australia, WB can be held for up to 24 hours at room temperature prior to processing, whilst other countries, such as the United States of America (USA), require that if WB is not processed within 8 hours of collection it must be refrigerated. Longer hold time is known to affect RBC biochemical properties; however, the RBC components meet accepted quality criteria[29]. Differences in the processing procedures used, such as the buffy coat method, plasma-reduction or apheresis, and types of leucocyte-reduction filter (WB-filter or RBC component filter) influence the characteristics of the final product[30,31]. A recent Canadian clinical study has highlighted that differences in processing methods of RBC components may have a greater impact on recipient outcome than previously appreciated[32]. This Canadian study is noteworthy. However, a caveat regarding its conclusions is necessary because the handling and processing of donations in Canada may be different to those used in other jurisdictions, and the study did not attempt to account for donor-related variables.

Donor-related variables

Some of the donor variables that potentially contribute to RBC component composition and quality are listed in

Table I - Blood collection and processing variables that contribute or potentially contribute to red blood cell component composition and quality.

Collection/processing step	Variables
Collection	
Type of donation	Whole blood, RBC apheresis
Venepuncture patency	Phlebotomy; donor venous accessibility
Blood volume collected	Set target volume±10% margin, e.g. 450±45 mL
Blood collection/storage pack	Different manufacturers, configurations, plastics, plasticisers, etc.
Anti-coagulant	Different formulations and manufacturers (CPD, CP2D, CPDA-1, ACD, etc.)
Hold time and temperature prior to processing	Varies within and between jurisdictions, e.g. up to 24 hours hold at room temperature or cooled
Handling and transportation prior to processing	Varies within and between jurisdictions
Processing	
Processing method	Packed RBCs, buffy coat method, apheresis
Processing conditions (centrifugation settings, temperature, etc.)	Varies within and between jurisdictions
Additive solution	Different formulations and manufacturers (SAGM, AS-1, AS-3, AS-5, AS-7, PAGGSM, MAP, CPDA-1, etc.)
Pre-storage leucoreduction	Universal leucoreduction is not mandated in all countries, e.g. USA
Type of pre-storage leucoreduction filter	Different manufacturers, filter chemistries, specifications for use, etc.
Final component volume, RBC and Hb content	Dependent on donation and processing variables
Post-processing manipulations (irradiation, washing, cryopreservation/thaw, etc.)	Procedures and revised component out-date varies between jurisdictions
Maximum storage time and inventory management	Varies between jurisdictions
Storage conditions, handling, transport prior to transfusion	Varies within and between jurisdictions

RBC: red blood cell; Hb: haemoglobin.

Table II. Only a few of the donor characteristics listed are used as selection criteria, including donor age, weight and Hb level. Typically, donors are between 18 and 70 years old. Some jurisdictions allow donors as young as 16 years to donate providing parental or medical consent is obtained. Donors older than 70 years may donate providing they are well and/or obtain medical consent. Acceptable donor weight is usually at least 50 kg to donate a full WB unit (i.e. 450 mL) or over 70 kg for double RBC apheresis. An upper weight limit is not usually defined. Blood pressure is recorded in some jurisdictions, several of which accept donors with moderately abnormal readings if the donor is otherwise well[23]. Selection limits for Hb levels may be sex specific and vary in different jurisdictions[24]. The minimum inter-donation interval varies between jurisdictions; for example, 56 days in Canada, the Netherlands and the USA, compared to around 120 days in Croatia, Israel, Luxembourg, Malta and Slovenia[24,25]. Higher donation frequency tends to correlate with increased rate of donor deferral due to unacceptably low Hb levels, particularly in female donors[24]. Certain diseases, such as previous leukaemia, trigger permanent deferral. Acceptance of donors with other diagnosed diseases, medical conditions or traits varies between jurisdictions, but the donor must report to be in good health in the weeks prior to donation, and where applicable, their condition controlled by prescribed medication[23]. As indicated in Table II, there are a myriad of other variables that contribute to donor health, and could potentially influence RBC properties and their storability positively or negatively, which are not considered in the selection process. It is reasonable to hypothesise that the blood donor is the greatest source of variability of RBC component quality rather than collection or processing variables.

Donor factors and transfusion outcomes: recent research

To date, very few studies have specifically addressed the influence of donor variables on RBC transfusion outcomes[33]. New research is beginning to emerge that will fill this void, but already, seemingly contradictory results are being reported. For example, RBC components from young female donors have been reported to be more resilient to mechanical or osmotic stresses and have lower *in vitro* haemolysis[17,34]. Such attributes could be expected to favour improved function and survival of stored RBCs following transfusion. However, a large longitudinal cohort study of 30,503 transfusion recipients in Canada reported that RBC components from female donors or young donors less than 30 years of age were associated with increased mortality[35]. In

Table II - Donor variables that contribute or potentially contribute to red blood cell component composition and quality.

Donor variable	Used as selection criteria	Acceptance limits or information documented by blood centre
Sex	No	Documented
Age	Yes	Minimum-maximum limits apply; limits vary between jurisdictions
Body weight	Yes	Minimum weight limit; varies between jurisdictions
Body mass index	No	Not systematically documented
Blood pressure	Yes/No	May be documented; varies between jurisdictions
Blood group antigens	No	ABO, Rhesus D are documented; other alloantigens are documented if extended typing performed
Hb level	Yes	Different acceptance limits for female and male donors; varies between jurisdictions
Other RBC indices (cell concentration, cell size, haematocrit, etc.)	No	Not routinely measured for whole blood donations
Iron deficiency	Yes*	*Applies only to centres that have implemented routine screening
Diagnosed RBC pathologies or carrier traits (G6PD deficiency, haemochromatosis, sickle cell trait, thalassaemia carriers, etc.)	Yes/No	Acceptance of donors varies between jurisdictions
Diagnosed medical conditions (diabetes; obesity; metabolic syndrome; inflammatory conditions; immunosuppressive conditions; allergy; respiratory conditions; auto-immune diseases; hormonal imbalance; hypertension; hyperlipidaemia; periodontitis; previous non-haematologic cancer; intestinal, kidney, liver conditions)	No**	Not systematically documented **Donor acceptance dependent on the donor reporting to be well at the time of donation
Health and lifestyle choices (diet; vitamins and supplements; contraceptive/ hormonal therapies; tobacco intake; alcohol consumption; physical activity)	No	Not systematically documented
Immediately prior to donation (food, drink consumed; high physical activity; vitamins, supplements, medication taken; tobacco intake; anxiety, stress level)	No	Not systematically documented
Inter-donation interval	Yes	Minimum time period since previous donation varies between jurisdictions
Seasonality of donation	No	Documented
Unknown or undisclosed variables (undiagnosed conditions or diseases, e.g. cardiovascular disease, diabetes, RBC or haematologic pathologies, inflammatory or immunosuppressive disorders, auto-immune diseases, lung, intestinal, kidney or liver conditions, etc.; pre-clinical conditions, e.g. pre-diabetes; metabolic variants; genetic variants)	Not possible	Unknown variable; cannot be documented

Hb: haemoglobin; RBC: red blood cell.

contrast, a large retrospective study of 136,639 RBC transfusion recipients in Sweden and Denmark did not find an effect of donor age on 30-day or 1-year mortality[36]. Similarly a smaller French retrospective cohort study of 2,715 cardiac surgery patients did not find an effect of donor age, sex or component storage duration on 1-year mortality[37].

The effect of sex mismatch between donor RBCs and patient-recipient has also been investigated. In the French study[37], a trend towards increased risk of death was noted for female recipients (n=247) of male-only RBCs compared to male recipients (n=237) of female-only RBCs (hazard ratio [HR] 2.03, 95% CI: 0.87-4.73 *vs* 0.96, 95% CI: 0.57-1.61, respectively), but the effect did not reach statistical significance in this study cohort. In a Swedish retrospective study of 5041 cardiac surgery patients followed for up to 12 years after transfusion, there was a significant association of

increased long-term mortality in patients who received only sex-mismatched RBC components (HR 1.08, 95% CI: 1.03-1.14; p=0.003)[38]. This study did not differentiate the direction of sex mismatch (i.e. female RBCs-male recipient or male RBCs-female recipient). These recent studies add to an earlier Dutch study that reported significantly increased mortality at 90 days and 6 months after transfusion in male recipients less than 55 years old who received female RBCs[39]. The association was not significant at 5 years after transfusion, nor was it significant at any of the time points for female recipients who received only male RBCs.

Based on findings to date, the influence of donor age or donor sex on transfusion outcomes remains unclear. It may be too simplistic to select only one or two basic donor-related variables when there are many more donor variables that could influence the properties of RBC components, such as indicated in Table II.

Changing donor demographics and potential impact on RBC component quality

With the exception of donor age and donor sex noted above, very few studies have specifically investigated the effect of donor-variables on the quality of stored RBC components. The significantly changing demographics of the global population, including an increasing proportion of the elderly age group[40,41], and escalating rates of non-communicable health conditions, such as hypertension, obesity and type-2 diabetes in children and adults[42], raises questions about the potential impact of these evolving changes on the demographic profile of blood donor panels and the possible implications for the quality of blood components for the future.

For example, in many countries, the obesity rates have doubled since 1980; as of 2012, the average rate of adult obesity of 34 countries of the OECD (Organisation for Economic Co-operation and Development) was 18%, with countries such as Canada, Australia and the United States having much higher rates of 25%, 28% and 35%, respectively[43]. Likewise, the rate of metabolic syndrome, which is defined as having three or more risk characteristics that include excess abdominal adiposity, hypertension, raised blood lipids, raised fasting plasma glucose or diabetes, is also rapidly rising, particularly in countries with higher incidence of obesity, such as the United States and Australia[44-46]. Based on 2012 data, the prevalence of metabolic syndrome in the United States is reported to be 35% of the adult population and 50% in adults aged 60 years or older[44].

Cardiovascular disease, obesity and diabetes individually and combined are associated with numerous biochemical changes, including increased oxidative stress and inflammatory markers, that affect blood characteristics, together with RBC properties[47-50]. The extent of the changes to RBCs caused by these health conditions is further influenced by genetic and inherited metabolic differences between individuals[51-57], and even seasonal effects[58]. It is very likely that the numbers of blood donors with undiagnosed or pre-clinical stages of these and related health conditions is increasing. The direct effect of these health conditions on the quality of stored RBC components has not been specifically investigated.

Donor lifestyle variables and RBC component quality

Blood donors are not routinely asked about lifestyle variables such as diet, physical activity, alcohol and tobacco intake, although all of these variables can significantly influence RBC physicochemical properties[19]. Decreased RBC anti-oxidant capacity and increased oxidative stress markers are consistent characteristics of unhealthy lifestyle choices, such as poor or unbalanced diet, excess alcohol, tobacco intake or physical inactivity.

Hyperlipidaemia

Red blood cell components prepared from donors with hyperlipidaemia, particularly raised triglycerides, have been found to have increased haemolysis early in storage that can reach unacceptably high levels before component out-date[59]. The lipid content of RBC membranes is known to vary according to the relative concentrations of lipid species in the extracellular milieu[60,61], and this in turn can alter RBC membrane fluidity. Furthermore, hypercholesterolaemia is associated with increased oxidative stress and inflammatory mediators that can damage RBCs and increase RBC membrane rigidity[47]. Plasma lipid levels can be transiently raised by the consumption of a high fat meal[62,63], which is relevant in the context of blood donation and variability in the quality of RBC components[59,64]. Although very turbid plasma products are discarded by some blood centres, the RBC components prepared from the same donations are not usually discarded.

Diet and alcohol

The physicobiochemical properties of RBCs can be significantly affected by diet. A high fat diet may not necessarily result in raised plasma lipids, but can still contribute to significant RBC dysfunction *via* increased inflammatory and oxidative stress mechanisms[65]. On the other hand, a healthy diet and one that is rich in natural antioxidants may enhance the capacity of RBCs to counter the damage inflicted by oxidative stresses[66-69]. However, dietary antioxidants, preservatives and colourants included in the "Western" diet may

influence immune regulation, including the development of allergic, inflammatory or immunosuppressed responses[70,71].

Moderate consumption of red wine can improve RBC membrane fluidity[72], but excess ethanol negatively affects RBC rheology by inducing macrocytic morphological changes with reduced RBC deformability and aggregation, reduced RBC anti-oxidant capacity, increased oxidative markers, and increased blood viscosity[73-75].

Physical activity

Insufficient physical activity and extreme exercise are each associated with raised oxidative stress markers, which negatively impact on RBCs[76,77]. Moderate and regular exercise and physical activity has significant benefits on RBC redox homeostasis[78,79].

Tobacco smoking

Tobacco smoking causes marked changes to RBC rheological properties due to the consequences of inhaled toxins that increase the levels of oxidants and plasma lipids[80,81]. The levels of induced oxidants has been shown to vary with the age and sex of the smoker, with young female smokers being the most adversely affected[82]. Oxidant levels are further exacerbated in smokers after ingestion of a meal, which can be alleviated by moderate exercise[83]. Furthermore, consumption of red wine prior to smoking can reduce the negative haematologic changes associated with smoking[84].

Donor medical conditions and RBC component quality

In addition to the medical conditions mentioned above (i.e. hypertension, obesity, diabetes, etc.), there is a plethora of other ailments that can affect RBC physicochemical properties (Table II). Furthermore, an unknown proportion of donors will have undiagnosed or pre-clinical forms of these medical conditions. Given that progressively increased oxidative stress is a feature of the RBC storage lesion[4,85], it is reasonable to hypothesise that RBCs collected from donors with inflammatory conditions, including autoimmune and hyperallergic predisposition, may be more susceptible to storage-induced injury. Detailed studies of stored RBC components prepared from donors with inflammatory profiles have not been reported. A few studies have been reported concerning RBCs collected from donors with certain inherited disorders that affect RBCs and these are briefly discussed below.

Genetic pathologies

Donors who disclose genetic pathologies that affect RBCs, such as hereditary haemochromatosis (HH), sickle cell trait (SCT), and glucose-6-phosphate dehydrogenase (G6PD) deficiency, are accepted in many jurisdictions providing the donor feels well at the time of donation, and where applicable their condition is controlled[19,24]. Many more donors may be unaware of their conditions as clinical symptoms are often not apparent.

Iron overload is the hallmark of HH and exposes RBCs to increased oxidative challenge[86,87]. HH is one of the most common genetic diseases that affect RBCs, with a prevalence of around 0.6% in individuals of European ethnicity. As it is an autosomal recessive disorder, HH is significantly more frequent in males. Irreversible organ damage may have already occurred before clinically significant symptoms become apparent, which tend to emerge in mid-life or later. HH is associated with a heightened risk of other diseases, including type-2 diabetes[88]. However, individuals homozygous for the common HH-related *HFE* gene C282Y mutation have been reported to have lower total lipid and low-density cholesterol levels[89], which may be advantageous. Phlebotomy is standard therapy for the treatment of HH. Patients with HH are often referred to blood centres for their phlebotomy therapy. In many jurisdictions, blood collected from patients with controlled HH is deemed acceptable and is processed as a routine blood transfusion component. Thus, the frequency of HH in blood donor panels is likely to be higher than the frequency in the general population. Although RBC components from HH donors appear to display acceptable quality following storage[90,91], extensive investigations have not been reported.

Sickle cell trait, or sickle cell carrier, is the heterozygous form of sickle cell disease, which is one of several types of haemoglobinopathies. Due to the presence of the abnormal sickle haemoglobin gene (HbS), RBCs from SCT individuals tend to be more rigid[92]. SCT is particularly prevalent in individuals of African black ethnicity; the incidence of SCT in the African-American population is estimated to be around 7-9%[93,94]. For RBC components prepared from SCT donors, the increased rigidity of the RBCs can be problematic during pre-storage leucoreduction filtration, with increased risk of filter blockages and damage to the RBCs[95,96]. Recent studies using a mouse SCT model have demonstrated increased haemolysis and post-transfusion clearance of stored SCT RBCs[97]. Transfusion of SCT-RBC components to sickle cell disease recipients is avoided, particularly during a sickling crisis, due to the risk of exacerbation of vascular occlusion. The world-wide incidence of other haemoglobinopathies, including the thalassaemias, is increasing and becoming more diversified due to immigration, mixed-ethnic births, and improved medical standards in developing

countries where many of the haemoglobinopathies have a higher frequency[98]. The effect on the quality of RBC components prepared from donors with genetic variants of Hb has not been investigated in detail.

Glucose-6-phosphate dehydrogenase deficiency is the most common genetic enzyme defect in humans. It is an X-linked recessive inheritance and consequently affects males, while females are more likely to be heterozygous carriers. G6PD is part of the pentose phosphate pathway (PPP) that is active in all cells. RBCs rely on the PPP to generate large amounts of NADPH required to produce the anti-oxidant, glutathione. Consequently, G6PD-deficient RBCs are vulnerable to oxidative stress and ultimately, haemolysis[99]. Individuals with G6PD-deficiency are usually asymptomatic, and for this reason are accepted as blood donors in most jurisdictions. However, recent studies have questioned the safety and efficacy of stored G6PD-deficient RBC components[100-102].

Future focus

It is clear that the blood donor is likely to be the greatest source of variability of RBC component quality and arguably is the least "regulated" step in the production line of blood components. Research focused to better understand the effect of donor-related variables is needed.

To obtain meaningful results will require collection of additional health, medical and lifestyle information from blood donors as well as certain biochemistry tests, such as plasma lipids and oxidative markers. More detailed studies could utilise the power of "omics" technologies (proteomics, metabolomics, lipidomics and transcriptomics) to reveal biological patterns associated with good or poor "storers". Some research is already being undertaken in this direction, such as the large USA-based "RBC-Omics" project within the REDS III programme that will use a genome-wide association study (GWAS) approach to investigate the genetic basis of RBC storage variability and haemolysis of 14,000 US blood donors[103].

These research approaches could lead to the identification of markers in donor blood that predict the storability of blood components. To be fully informative, these endeavours will need to gather together the expertise of a range of disciplines, including medical, scientific, public health, sociology and bioinformatics. It is a major exercise that will require much planning and investment of resources. However, it will be worth the effort. Not only might this research be of great benefit to Transfusion Medicine, but will potentially be of enormous value to public health research and programmes to deal with the implications of the sizeable shifts that are occurring in the health demographic profiles of the global population.

Conclusions

There are a multitude of variables that contribute or potentially contribute to the quality of stored RBC components. Many variables related to collection and processing of blood are managed by strict regulatory controls and standardised procedures, and, to some extent, by donor selection criteria. However, the blood donor remains the greatest source of variability of RBC component quality. Donor selection criteria are principally focused on avoiding the transmission of infectious diseases to the transfusion recipient as well as avoiding harm to the health of the blood donor in the context of blood depletion. Few studies have investigated the effect of specific donor-related variables on the quality of stored RBC components. Donor-related variables have not been accounted for in the 'age of blood' RCTs, or in many of the observational studies.

With the changing demographics of the global population, that is seeing more aged people, and rapidly rising rates of non-communicable medical conditions such as hypertension, obesity and diabetes, in children and adults, more research is needed to understand how these changes in population health impact on the quality of stored RBC components and transfusion outcomes. Identification of donor-related markers that are predictive of RBC storability could be invaluable in achieving improved consistency of RBC component quality and recipient outcomes. The challenge is there for us to grasp.

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Unraveling the Gordian knot: red blood cell storage lesion and transfusion outcomes

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Abstract

What is following the impressive progress that has been made? During the last couple of years several tremors have shaken the field of Transfusion Medicine. The epicentres of those tremors were located on novel insights into the RBC storage lesion, on emerging connections between storage lesion and post-transfusion performance and effects, and on acknowledging that storage time is only one (rather than the most prominent) of the parameters which contribute to the progression of storage lesion in any given unit of blood. The optimisation of bio-preservation conditions emerged at the same time with all-new scientific knowledge gained by advances in research tools, implementation of technological innovations, and application of elegant *in vitro* and *in vivo* models of transfusion. Simultaneously, one after another, all the reported randomised clinical trials concluded, with spectacular consensus, that there is no significant difference in the rate of adverse clinical events (including death) among patients who underwent transfusion with fresh (and presumably good) or standard of care (and presumably bad) blood. The comparative analysis and comprehension of the aforementioned data would set the context for the next generation of research in blood transfusion science, since the need for safer and more efficient transfusions remains.

Keywords: red blood cell storage lesion, donor/recipient variation, transfusion outcome, clinical relevance, research opportunities.

Back to basics

When Dern and his colleagues revealed a significant donor to donor variability in erythrocyte storage characteristics in 1966[1], they probably did not imagine that studying the impact of a donor's intrinsic biological "signature" would not only be relevant but truly revived almost 50 years later. Nowadays, increasingly more studies focus on the impact of donor characteristics on red blood cell storage lesion profile and transfusion outcomes[2]. The rationale behind those donor-oriented studies was similar to that widely adopted in solid organ or bone marrow transplantation research[3]. Moreover, metabolites crucial for red blood cell (RBC) physiology, such as glutathione, might be considered inherited characteristics that retain their donor-dependent pre-storage dynamics[4]. In the same context, several RBC and plasma characteristics, including osmotic fragility and plasma antioxidant capacity, share the ability to characterise in part the blood unit already at the time of donation, since they fluctuate during storage in close relation to their pre-storage and donor-dependent levels[5]. In addition, factors apparently irrelevant to storage quality, such as serum uric acid, can also affect storage lesion metrics in blood units[6]. Despite being based on low-quality evidence from 59 studies, female donor sex, positive white blood cell antibodies, HLA-DR and RBC antigen selection were identified as unique donor characteristics with potential impact on RBC transfusion recipient outcomes[2]. The realisation that distinct groups of donors demonstrate different degrees of storage lesion progression, or post-transfusion performance, offers the opportunity to design a blood logistics model for optimal selection of blood donors and donor-recipient matching. In fact, the donor's genetic background is not neutral in respect to storage lesion progression, as RBC units from donors with clinically silent familial pseudohyperkalaemia, for instance, exhibit increased potassium accumulation in the supernatant at the early storage period[7], while RBC units from G6PDH deficient donors demonstrate poor post-transfusion performance *in vitro*[8]. Further proteomic analyses that produce large amounts of data have demonstrated donor-associated, storage induced changes that can be attributed to intrinsic variation in specific oxidative markers[9]. Apart from the genetic context, lifestyle factors, dietary habits and, probably, the frequency of blood donation are also strong contributing factors to RBC physiology, and probably to storage quality[10]. To sum up, the study of donor variation effects offers a golden opportunity to clarify those aspects of the storage lesion that are not related to the duration of storage and the conventional

ageing of stored RBCs. Although seemingly opposed to the recently reported conclusions of the randomised clinical trials showing that the age of blood is not related to post-transfusion mortality and morbidity in those specific settings[11], donor variation considerations stand side by side with those trials by appreciating time as only one among the many parameters that affect the quality of blood labile product. As a matter of fact, any study focused on donor properties has the ability to detect storage- and/or transfusion-associated differences between blood units of exactly the same age, although the evidence collected so far is insufficient to draw definite conclusions about the clinical relevance of those differences for any donor characteristics. Assessment of the relativity of time and of the cell-to-organism complexity of the biological systems would inevitably lead to the acknowledgement that donor variability stands hierarchically above the age of stored blood.

Gazing ahead to the future: (re)searching in the new era

Speaking of time and relativism, recent studies from pioneer groups in the field re-defined the meaning of time in respect to RBC ageing and storage lesion by using state-of-the-art metabolomic analyses[12]. Their work means we are now on the verge of establishing a new way of measuring stored RBCs' age, besides the conventional approach, by reading the signature of metabolic ageing in stored RBCs. After all, measuring storage time in μM/mM of equivalent metabolites instead of days seems extremely interesting. Extracting this kind of information would not be possible at all without the development of omics technologies and their application to blood storage. New generation proteomics platforms have provided the opportunity for absolute quantification that resulted in the introduction of novel candidate protein biomarkers of RBC haemolysis and vesiculation and, thus, of RBC quality during storage[13]. At the same time, metabolomics analyses have revealed an impairment of energy and redox metabolism in RBCs, like the storage-dependent reversible oxidation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which promotes metabolic reprogramming during storage[14], while several metabolic features of the storage lesion are found to be heritable (thus highly donor-related) in human or mouse models[15,16]. In addition, contemporary advances in optical and non-optical high-resolution technologies allow for the thorough study of extracellular vesicles generated in the bag in order to assess their possible clinical relevance[17]. This panoply of data acquired by the application of innovative techniques to blood units has offered challenging opportunities in Transfusion Medicine research and identified numerous parameters with a storage and/or transfusion-outcome biomarker potential. Even after sorting the data using bioinformatics tools, the need to link storage lesion variables with transfusion performance remains vital. Thus, the introduction of *in vitro* models of transfusion has been proved very helpful for a first-line evaluation of the post-transfusion phenotype[8,18]. In a second step, transition to the *in vivo* state, by using animal models of transfusion, provides further insight into the correlation between storage quality and transfusion effects[19] and, eventually, both types of models fuel extended clinical trials in humans. Bearing in mind that all of the above-mentioned approaches for studying post-transfusion efficacy and effects have their own pros and cons, it would be really informative to combine them, focusing on what each of them can provide instead of what each of them may conceal based on their intrinsic limitations[20]. Therefore, future clinical trials designed on the basis of more reliable and upward tested/checked input (and output) parameters would help in clarifying current uncertainties and controversial issues. Advances in omics and small particle biology technologies might permit the establishment of a large donor-to-recipient data infrastructure to achieve a robust assessment of the clinical relevance of various blood donor characteristics. In this context, the Recipient Epidemiology and Donor Evaluation Study-III (REDS-III) premier research of the National Health, Lung and Blood Institute (NHLBI) programme[21], which involves basic, translational and clinical research, has committed to the innovative development of comprehensive databases which will link information on donor/donation/components to that of transfused recipients (compared to untransfused controls) at all participating hospitals. These cumulative databases, will contribute to address key research questions in blood banking and Transfusion Medicine, and inform blood policy decisions.

The story of a dog chasing his own tail: the transfusion paradox

Both assessment and interpretation of clinical trials are of high importance for the evolution of Transfusion Medicine services. Despite research opportunities offered by the strictly controlled system of a blood unit to biomedical sciences, donated blood and its components represent precious therapeutic substances of human origin that are limited by their very nature. Consequently, it makes sense that the primary outcome measured by almost all of the recent randomised clinical trials was the ultimate human good, namely survival[22,23]. On the other hand, owing to the numerous systemic factors implicated, the outcome of a specific transfusion is by default a highly complex, multifaceted phenomenon. When evaluating the effects of a given transfusion therapy,

one must take into account not only the variability of the blood components used (donor, processing and storage strategy variations), but also the specific biomedical context of the treated recipient in need (recipient variation), similar to the strategic planning of the REDS-III programme. As a result, the paradox lies in the heart of the approach chosen. Although *in vivo* studies aim to overcome the limitations of *in vitro* human models in evaluating post-transfusion effects, instead of this, they unintentionally feed and multiply the complexity of the findings and their interpretation. In other words, the combination of storage lesion variables (probably related to post-transfusion efficacy) with the infinite systemic factors of the recipient, results in an exponential output of possible conditions rather than a cumulative one. To support this concept, although lower 24-hour post-transfusion recovery of stored G6PDH-deficient RBCs was reported about fifty years ago, studies on post-transfusion haemolysis have provided contradictory data, highlighting the presence of an uncharted universe of interactions and crosstalk (between storage, processing, donor and recipient) that take place during or soon after transfusion therapy[24]. In that case, retrospective studies regarding the efficacy of transfusion or its adverse effects for distinct groups of recipients treated with standard practice or (as much as possible) "equal" blood units might be of great value. Moreover, in terms of transfusion, it is clear that "what you see (or measure) is not always what you get", as several aspects of an RBC storage lesion remain hidden. A set of sub-lethal lesions and defects are only evident under physiological or near-physiological levels of stress (osmotic, mechanical, biochemical, etc.) encountered in the recipient[25]. Nevertheless, it is really interesting that in our own studies, *ex vivo* haemolysis, the gold standard for blood bag quality assessment, and other haemolysis-related factors are found to be linked to donor-specific variation in almost 200 RBC or plasma parameters (*Tzounakas M, unpublished data; 2017*). These examples give only a glimpse of the complexity of transfusion-related research, pointing towards a more systemic approach to answer outstanding issues.

The end is the beginning is the end is…

Every end represents a new beginning and *vice versa*[26]. Novel means and new findings offer the chance for a new dawn in Transfusion Medicine research. Storage lesion and post-transfusion performance and effects represent two different worlds that are connected (?) by a still obscure link. Something has been lost in translation, since, to date, it still has to be proved that really important storage lesion parameters are crucial for post-transfusion metrics. This second paradox is partly owing to the swarm of pragmatic and multifaceted difficulties in the design and implementation of relevant studies. However, it is also well-fed by the strict, one-dimensional target orientation of the majority of the clinical trials performed. By representing the ultimate "gold" checkpoint for transfusion effects, they focus solely on the age of the blood component to measure obviously important things (such as mortality and other hospital metrics) but not on other metrics of patient status (tissue oxygenation, NO-biology issues, etc.) and established storage lesion metrics (e.g. extracellular vesicle levels and signalling) with probable severe effects on safety and efficacy issues[27]. Moreover, they did not take into account donor- and recipient-associated variables. As a result, such clinical trials cannot come to definitive conclusions on the impact of the storage lesion, even on the conventional age of stored blood, on transfusion outcomes, especially in massive transfusion and traumatic haemorrhagic shock contexts. For instance, what is the impact of transfusing a bolus of donor-specific communicable extracellular vesicles along with a set of vesiculation triggers in specific groups of patients well-characterised by overproduction of bio-active, pro-inflammatory/pro-coagulant vesicles or by a vesiculation-prone endothelium? A recent longitudinal cohort study focused on donor variation parameters such as age and sex showed that RBCs transfusions from younger or female donors were associated with increased mortality[28]. Moreover, another prospective, observational study in critically ill children found that post-transfusion haemolysis was independent of RBC storage duration; in contrast, most storage duration effects on haemolysis were overwhelmed by recipient and/or donor factors[29].

Given the influence and impact of donor characteristics on numerous bio-medical settings, it is probably time to re-evaluate research priorities. The novel technologies in combination with established post-transfusion research tools (including *in vitro* and *in vivo* models of transfusion), pave the way for a better understanding of the storage lesion and effects. The field is in constant evolution, from evidence-based, cohort Transfusion Medicine, to knowledge-based, personalised Transfusion Medicine. The path to the core of transfusion research resembles a labyrinth, since there are many ways of entry, but only one way out (Figure 1). This particular journey might prove to be more difficult than we anticipate, since several pieces of the labyrinth are in fact, a mirror maze. We have all entered through different doorways and we follow distinct scientific paths that (sometimes) cross each other. Nevertheless, we all seek the same exit. The odds are favourable for the members of the transfusion research community to find their way out of the labyrinth.

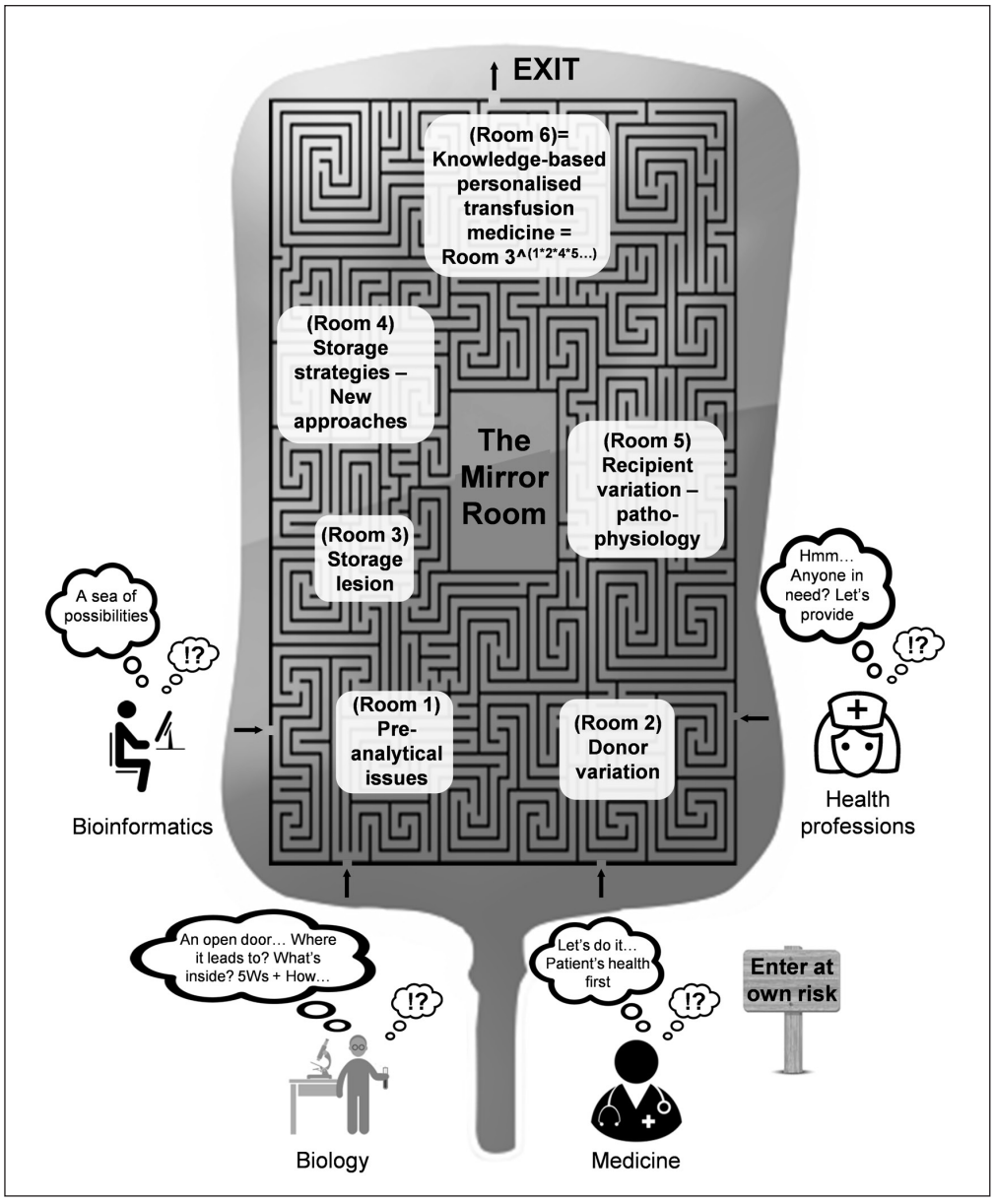

Figure 1 - The labyrinth of research in transfusion biology and medicine and its aspects.

Let us enjoy this fascinating, mind-opening journey. Let us be prepared for what we cannot see and expect the unexpected.

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The accumulation of lipids and proteins during red blood cell storage: the roles of leucoreduction and experimental filtration

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Abstract

Pre-storage leucoreduction has been universally adopted in most developed countries in Asia, Europe and the Americas. It decreases febrile transfusion reactions, alloimmunisation to HLA antigens, cytomegalovirus exposure, the accumulation of a number of pro-inflammatory mediators in the supernatant, including the accumulation of platelet- and leucocyte-derived proteins and metabolites during routine storage. This review will highlight the lipids and proteins, biological response modifiers (BRMs) that accumulate, their clinical effects in transfused hosts, and methods of mitigation.

Keywords: red blood cells, transfusion, transfusion-related acute lung injury (TRALI), leucoreduction, neutrophils.

Introduction

Transfusion of red blood cells (RBCs) has saved numerous lives, far outnumbering any adverse events induced by their infusion. RBC transfusions allow for lengthy and complicated surgeries, survival from life-threatening injuries in both military and civilian settings, organ, bone marrow, and stem cell transplantation, treatment of malignancies with myelotoxic chemotherapy, survival from haemorrhagic diatheses, and haematologic disorders in which RBC production is significantly decreased or destroyed. While the benefits of transfusions far outweigh the risks of a reaction, these reactions still occur, and therefore efforts have been made to improve haemotherapy in order to further decrease clinical morbidity and mortality.

Pre-storage leucoreduction of RBCs (LR-RBCs) by buffy coat depletion, simple filtration, or a combination of the two removes leucocytes and platelets from the RBC units. Buffy coat depletion causes a one log depletion of both leucocytes and platelets while filtration decreases leucocytes by more than 3 logs and platelets by 2 logs[1]. Universal pre-storage leucoreduction significantly decreases febrile non-haemolytic transfusion reactions and decreases exposure to HLA antigens, HLA alloimmunisation, and decreases the accumulation of platelet and leucocyte derived pro-inflammatory mediators, biological response modifiers (BRMs), including: interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), soluble CD40 ligand (sCD40L), and lysophosphatidylcholines (lyso-PCs)[2-8]. Despite these decreases, pro-inflammatory mediators still accumulate during routine storage of LR-RBCs, a number of which have been implicated in the pathogenesis of transfusion-related acute lung injury (TRALI) and post-injury multiple organ failure (MOF), which includes acute lung injury (ALI)[5,9]. This review will detail the mediators/BRMs in question, their clinical effects and possible mitigation, as well as proposing novel strategies to inhibit their production during routine storage.

Transfusion-related acute lung injury

TRALI, which is a rare, adverse event, has been linked to the infusion of bioactive lipids which accumulate during the routine storage of RBCs, and are released into and accumulate in the supernatant of the RBC units[5,10-12]. In unmodified RBCs, there are two classes of lipids, based upon their retention time *via* normal phase with further characterisation by reverse phase high pressure liquid chromatography (HPLC) and identification by mass spectrometry: a mixture of lyso-PCs and non-polar lipids consisting of arachidonic acid, 5-hydroxyeicosotetraenoic acid (HETE), 12-HETE and 15-HETE. These data have been reported by a number of other groups[5,8,13-20]. These lipids were increased in patients at the time TRALI was recognised, and both the supernatants and the lipids from stored RBCs, both day (d)28 and d42 of storage, induced TRALI as the second event in a 2-event animal model[5,12,21,22]. Pre-storage leucoreduction by filtration, specifically the Haemonetics BPF4 filter, removes two logs of platelets and the lyso-PCs from LR-RBC units[1,5,12]. The neutral lipids are not affected and may still serve as the second event in a 2-event animal model of TRALI[5,12,22]. In addition, this removal of platelets also decreases the accumulation of sCD40L, a reported co-factor in TRALI, which has the capacity to alter PMN physiology, e.g. prime the PMNs through the CD40 receptor on the cellular membrane[3]. Importantly, animal models are employed to mimic human disease and to give relevance to suspected mediators; however, just because each and

every rodent experiences TRALI, for example, does not mean that each and every human will also manifest this adverse event[23].

The accumulation of bioactive lipids has been questioned; however, these studies measured lyso-PCs in LR-RBCs, by both buffy coat removal and filtration[24]. Pre-storage leucoreduction nullifies the accumulation of these lipids because of effective platelet removal (approximately 2 logs). Moreover, flow-based measurement of oxidase activity is a qualitative test, and because of time constraints, it is not amenable to quantification since the actual assays are not done simultaneously like the 96-well plate assays that measure superoxide dismutase-inhibitable reduction of cytochrome c[1,24]. Lastly, in a prospective clinical study of TRALI, bioactive lipids (lyso-PCs) were risk factors for TRALI in the univariate but not the multivariate analyses[25]. In addition, the bioactivity measurement on PMNs, increased surface expression of CD11b/CD66, did not demonstrate significant pro-inflammatory activity. However, the details of these assays are important because: 1) the bioactive lipids present: AA and 5-, 12-, and 15-HETEs affect the surface expression of CD11b in five minutes and with longer incubations the surface expression disappears; and 2) if fixed with paraformaldhyde prior to incubation with the antibodies to CD11b/CD66, the antigens are changed for CD11b such that the increased surface expression may be diminished by more than 30%[25]. As stated above, lyso-PCs and sCD40L do not accumulate in LR-RBCs due to platelet removal by the filter and the analyses looked at lipids that should not be present in the RBCs but would be in the platelet concentrates[3,5,25].

TRALI mitigation and experimental filtration

Transfusion-related acute lung injury mitigation has centred on the male-only plasma donors to obviate female plasma which may contain antibodies to human lymphocyte antigens (HLA) or human neutrophil antigens (HNA) due to pregnancy. These efforts have significantly decreased TRALI secondary to plasma transfusion but have not eliminated it[26-28]. Nevertheless, there are few formal mitigation strategies for RBC transfusions and reported clinical series have shown that 20% of TRALI follows RBC transfusions, with this percentage likely to increase because of the decrease in TRALI to plasma[26,27,29]. RBCs contain 5-10 mL of plasma so the relative amount of antibodies to HLA or HNA antigens is relatively sparse compared to plasma or even apheresis platelets, although only 10-20 mL of antibody-containing plasma may elicit TRALI[30,31]. To this end, an experimental filter was developed that removes virtually two logs of IgG. Filtered plasma samples from multiparous females known to have antibodies to HLA or to HNA-3a were deemed negative *via* measurements with Luminex™ beads and flow cytometry at two HLA reference laboratories or for HNA-3a at the Granulocyte Laboratory, Blood Center of Southeastern Wisconsin, USA, employing standard techniques in a blinded fashion[1]. Lastly, these experimental filters also removed neutral lipid priming activity which accumulates during routine storage. (This will be discussed under the proteomics section)[1].

TRALI modelling

The 2-event model of TRALI has been recently criticised because humans given endotoxin (LPS) from *E. coli* followed by stored LR-RBCs or the lipids from LR-RBCs did not manifest TRALI[32,33]. Unfortunately, these studies are marred by a number of factors, most of which appeared in the literature many years ago. In rats, LPS from *E. coli* may not be an effective first event; activation of the pulmonary endothelium did not result in PMN sequestration, which is to be expected because rats are known to live successfully in sewers, which have high levels of *E. coli* and *E. coli* LPS from human waste. Thus, for all rodent experiments, the first event was LPS from *S. enteritides* given *via* an intraperitoneal injection[11,34,35]. This first event caused the animals to become: 1) febrile with rigors and shaking; 2) tachypneic; and 3) despondent, although they respond to pain, with all rats having copious diarrhoea[1,11,22,34-37]. On the cellular level, IP *S. enteritides* LPS in rats causes activation of the pulmonary endothelium and sequestration of PMNs to the capillaries as evidenced by increased pulmonary myeloperoxidase and the lung histology without ALI[1,11,22,37]. The *S. enteritides* LPS concentration administered is 2 mg/kg with 99% animal survival[1,11,22,37]. Although critics of this model have deemed this dose to be supra-physiological, 2 individuals were injected with 2 μg-1 mg of either *E. coli* or *S. enteritides* and both became acutely ill with fevers, hypotension, gastroenteritis, increased respiratory rate, somnolence, and malaise, with one admitted to the intensive care unit with mild ALI and multi-organ dysfunction; both survived[38-40]. Additionally, the treatment of human neurosyphilus was LPS infusion that reached 1 mg intravenous (IV) with the overwhelming majority of the patients surviving[38]. Recent human TRALI models gave *E. coli* LPS IV at a concentration of 2 ng/kg which corresponds to 40 pg/mL of plasma for males and 48 pg/mL of plasma for females and resulted in fever over 38 °C, pulse rates of over 90 beats/min, and mild tachypnea with respiratory rates over 20 breathes/min[32,33]. There was no evidence that any of the human subjects had pulmonary endothelial activation or PMN sequestration in the lung, prerequisites for the 2-event model of TRALI[32,33]. *In*

vitro LPS, whether from *E. coli* or *S. enteritides* did not cause significant activation of human pulmonary microvascular endothelial cells (HMVECs), as measured by increased surface expression of intercellular adhesion molecule-1 (ICAM-1) or chemokine release, until a concentration of 20 ng/mL was reached[9,41,42]. In addition, LPS primes PMNs; however, *E. coli* LPS did not prime fMLF activation of the respiratory burst of human PMNs at concentrations of 2 ng/mL and did induce priming of the oxidase at 20 ng/mL but to a lesser extent compared to *S. enteritides* LPS, which was reported to have an almost identical concentration curve for PMN priming of the fMLF-activated respiratory burst and lyso-PC activation of the oxidase[42]. Unlike intact animals, there is no way to process or excrete the LPS, and the human modelling used concentrations much less than the concentrations needed to cause physiological changes in human cells; thus, clinical TRALI from the human modelling is unlikely because of an insufficient first event[43-45]. Lastly, the administration of LPS (intravenous *vs* intraperitoneal) may also have ramifications for its suitability as the first event of a 2-event model of TRALI in humans.

The proteome of the RBC supernatant

To determine the role of pre-storage leucoreduction on the release of proteins during routine storage, 5 units of red blood cells were drawn; 50% (by weight) were left unmodified and the other 50% was pre-storage leucoreduced by filtration. Both were stored in AS-5[46]. The protein concentration increased 2-3-fold in both the unmodified- and LR-RBCs from day (d)1 to d42 of storage[46]. Leucoreduction decreased the total number of proteins in the supernatant from 401 to 231, and of these, 84 proteins increased (>2-fold increase) with 42 being unique to d42, 30 decreased (<2-fold decrease) with 7 being unique to d1, and 117 remained unchanged[46]. Preliminary data with 3 RBC/LR-RBC units from female donors compared to 3 RBC/LR-RBC units from male donors only demonstrated an increase in pregnancy zone protein, which is increased in the female sex[46]. As expected, the leucocyte and platelet-derived proteins, present in the unmodified RBCs, were not present in the LR-RBC supernatant. However, the glycolytic enzymes were more pronounced in LR-RBC supernatant, including: transaldolase, fructose-bisphosphate aldolase, phosphoglycerate kinase, and α-enolase[46]. Other proteins of interest that increased in the LR-RBC supernatant included: latexin (also known as endogenous carboxypeptidase inhibitor and implicated as a mediator of the haematopoietic stem cell compartment), Prdx1, Prdx2, and Prdx6. These all increased during storage in the LR-RBC supernatant likely due to protease activity. Importantly, Prdx6 contains a phospholipase domain which requires either acidic pH or T-phosphorylation for activity; immunoblotting of the Prdx6 in LR-RBCs showed T-phosphorylation indicating an active enzyme[38,47-50]. There was also significant accumulation of MMP-8 and MMP-9, which display extracellular protease activity, most proteosome subunits, and a drastic decrease in cystatin C[46,51,52]. The presence of an active phospholipase in LR-RBCs may explain the accumulation of AA and 5-, 12-, and 15-HETEs, which have been implicated in TRALI[5]. In addition, these lipids can be used as not only the second event, but also the first event in a 2-event animal model of ALI.

RBC supernatant lipids and proteins and the injured patient

Massive RBC transfusion, more than 6 units in the first 12 hours, was an independent risk factor for the development of post-injury MOF[53-56]. With a more conservative transfusion target, haemoglobin of 7.0 g/dL, the transfusion of fewer RBCs has resulted in less MOF, despite increasing patient age and increased injury severity scores, both risk factors for MOF[57]. In these early studies that controlled for the number of RBC units transfused, older, stored RBCs were implicated in MOF[56]. As stated, MOF has decreased; however, post-injury ALI still plagues more than 12.5% of severely injured patients ISS more than 17[58]. In older LR-RBCs, neutral lipids accumulate, notably AA and 5-, 12-, and 15-HETEs, and pilot data have demonstrated that they induce activation of HMVECs and human liver sinusoidal endothelial cells (LSECs) at concentrations that would be reached by 2, 4 and 6 units of LR-RBCs transfused[5].

The proteome of LR-RBCs and that of the injured patients may provide some insight into the development of trauma-induced coagulopathy (TIC). Recent work on TIC has subdivided trauma patients based on their thrombolytic phenotype: systemic hyperfibrinolysis, physiological fibrinolysis and fibrinolysis shutdown[59,60]. A number of proteins in the LR-RBC supernatants have an affinity for plasminogen, especially α-enolase which is the plasminogen cellular receptor, and may be involved in the prolongation of TIC with respect to fibrinolysis: shutdown, physiological or hyperfibrinolysis[9].

Preliminary data have implicated a role for α-enolase in injured patients at risk for ALI (based on the number of transfusions) who also have evidence of fibrinolysis shutdown. These patients are also prone to organ injury, as well as venous thromboembolism (VTE). *In vitro*, α-enolase significantly increased ICAM-1 surface expression on HMVECs and induced the adherence of PMNs to these activated endothelial cells[9]. This HMVEC activation was inhibited by anti-proteases, required human plasma, and served as the first event in a 2-event

model of PMN cytotoxicity[9]. α-enolase was shown to also co-precipitate with PAR-2 and plasminogen/plasmin in HMVECs and enzymatic activity was not required[9]. Thus, proteins that accumulate during RBC storage as a risk factor for ALI, such as α-enolase, may also elicit previously unrecognised adverse clinical events, both TIC and ALI.

Possible mitigation

Experimental filtration of RBC units, as discussed above, not only removes 2 logs of IgG but it also significantly decreases the priming activity and obviated stored RBCs as the second event in a 2-event animal model of TRALI[1]. In addition, the measured concentrations of AA and 5-HETE were also decreased in the units that underwent experimental filtration *vs* those that were just leucoreduced using the Haemonetics BPF4 filter[1]. As stated previously, active Prdx6 accumulates during RBC storage. When inhibitors of phospholipase activity were added (aristocholic acid and MJ33, a specific inhibitor of the Prdx6 phospholipase), the generation of lipid priming activity was significantly decreased by 25±3% and 26±2%. In addition, when the structure of 5-lipoxygenase activating protein (FLAP) and 5-lipoxygenase were investigated, they demonstrated more than 10% homology with IgG and thus may be removed by the experimental filters. To investigate this removal, immunoblots from pre-filtration supernatants, and supernatants from both the leucoreduced (control) or experimentally filtered units, demonstrated that the FLAP and 5-LO immunoreactivity, present in pre-filtration and in leucoreduced supernatants, was removed by the experimental filters (Figure 1). These data demonstrate that these experimental filters not only remove the immunoglobulins implicated in TRALI, but also the enzymes required to generate the neutral lipids during storage, which have been implicated in both TRALI and post-injury ALI[1].

Conclusions

Pre-storage leucoreduction of RBCs results in fewer febrile transfusion reactions, decreased HLA alloimmunisations, decreased exposure to CMV, and decreased amounts of pro-inflammatory molecules including leucotrienes, lyso-PCs, and sCD40L. It also decreases the release of proteins from contaminating leucocytes and platelets. The non-polar lipids which do accumulate may be obviated by the use of a new leucoreduction filtration system, and possibly by the use of additive solution-3 (AS-3) and other novel storage methods. While transfusions of LR-RBCs has saved countless lives, further work is needed to continue to improve efficacy.

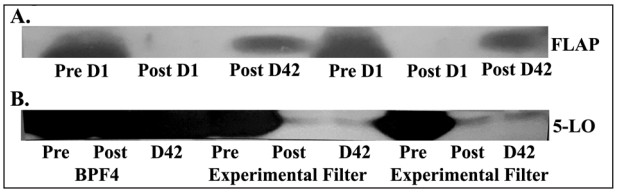

Figure 1 - Pre-storage experimental filtration removes 5-lipoxygenase activating protein (FLAP) and 5-lipoxygenase (5-LO) from red blood cell (RBC) units.
(A) There is significant FLAP immunoreactivity prior to filtration in day 1 (Pre D1) supernatants that is removed by filtration (Post D1) which re-accumulates during routine storage by day 42, the end of storage (Post D42). The supernatants from two different RBC units were used. (B) There is significant reactivity for 5-lipoxygenase (5-LO) both before (Pre) and after (Post) leucoreduction and at the end of storage (D42) with the Haemonetics BPF4 filter. The 5-LO immunoreactivity is present prior to experimental filtration (Pre) that is removed from the supernatant of 2 separate RBC units that does not re-accumulate during routine storage D42. Figure representative of 3 separate experiments which demonstrated similar results.

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Omics markers of the red cell storage lesion and metabolic linkage

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Abstract

The introduction of omics technologies in the field of Transfusion Medicine has significantly advanced our understanding of the red cell storage lesion. While the clinical relevance of such a lesion is still a matter of debate, quantitative and redox proteomics approaches, as well quantitative metabolic flux analysis and metabolic tracing experiments promise to revolutionise our understanding of the role of blood processing strategies, inform the design and testing of novel additives or technologies (such as pathogen reduction), and evaluate the clinical relevance of donor and recipient biological variability with respect to red cell storability and transfusion outcomes. By reviewing existing literature in this rapidly expanding research endeavour, we highlight for the first time a correlation between metabolic markers of the red cell storage age and protein markers of haemolysis. Finally, we introduce the concept of metabolic linkage, i.e. the appreciation of a network of highly correlated small molecule metabolites which results from biochemical constraints of erythrocyte metabolic enzyme activities. For the foreseeable future, red cell studies will advance Transfusion Medicine and haematology by addressing the alteration of metabolic linkage phenotypes in response to stimuli, including, but not limited to, storage additives, enzymopathies (e.g. glucose 6-phosphate dehydrogenase deficiency), hypoxia, sepsis or haemorrhage.

Keywords: mass spectrometry, advanced omics, Transfusion Medicine, blood, storage.

The red blood cell storage lesion(s) and clinical trials

Over the past ten years, concerns have been raised upon the publication of retrospective clinical evidence[1] suggesting the potential negative association between storage "age of blood" and transfusion outcomes. Following these controversial observations, literature has burgeoned around the description of the so-called storage lesion(s), a wide series of biochemical and morphological alterations red blood cells (RBCs)[2] undergo during storage in the blood bank. Many groups (as extensively reviewed[3-6]), including ours[7-20], have contributed to document the energy and oxidative lesions targeting stored RBCs. Compelling biochemical rationale and laboratory evidence[14-16,21-24] *in vitro* and *in vivo* (animal models[25-27]) have been produced to support the hypothesis that prolonged storage does not only negatively affect RBC physiology and functionality (e.g. gas transport[18,22,28]), but it also influences RBC survival in animal models *in vivo*[29]. These observations have led to question whether the storage lesion could thus impair the effectiveness of the transfusion therapy and likely mediate untoward transfusion-related events (e.g. transfusion-related acute lung injury [TRALI], transfusion-related immune modulation [TRIM][30-33]) or aggravate underlying conditions (e.g. sepsis[26,33]). However, reassuring evidence from independent randomised clinical trials (RCTs)[34-38] showed no detectable difference between fresh blood and standard of care at the limits of the statistical power of these studies, which prompted the field to conclude (as summarised in the most recent American Association of Blood Banks [AABB] guidelines[39]) that the general standard of care will not be improved by preferentially issuing fresh blood, at least to the specific categories of recipients enrolled in those RCTs. Many have noted the limitations of the RCTs[40], as elegantly described in several of the papers appearing in this issue of *Blood Transfusion*. For example, none of the RCTs performed to date has actually compared the transfusion of fresh blood products *vs* products close to the end of their shelf-life owing to ethical concerns[40]. However, the general take home message from the RCTs is overall comforting and suggests that, and to quote AABB guidelines, "a restrictive transfusion threshold is safe in most clinical settings and the current blood banking practices of using standard-issue blood should be continued"[39]. Still, quoting Zimring and Spitalnik in this issue[41], "when approximately 80 million RBC units are transfused annually worldwide, even vanishingly small (transfusion-associated negative) events, if they are real, can affect actual human lives; it then becomes a question of ethics and economics whether it is 'worthwhile' to study and attempt to prevent them".

In the light of these considerations, welcoming the challenge to further advance the field of Transfusion Medicine, in 2016 the National Heart, Lung, Blood Institutes and Food and Drug Administration gathered leading experts in the field to identify current issues associated with blood storage and define an agenda to

pursue the amelioration of blood storage strategies[42,43]. Discussions in these meetings highlighted the lack of consensus in the definition of parameters of transfusion efficacy, while classic parameters necessary to obtain FDA clearance for new packed RBC storage additives (haemolysis and 24-hour *in vivo* survival in autologous healthy volunteers) have been unanimously regarded as necessary, but not sufficient, to predict transfusion efficacy in the clinical setting. Several studies suggest that useful, often orthogonal, parameters can be obtained through modern omics technologies.

Omics markers of the RBC storage lesion

Over the last ten years[44], the introduction of omics technologies in the field of Transfusion Medicine has brought about significant advancements in the understanding of the RBC storage lesion[2,4,6,45]. We can now perform state-of-the-art quantitative proteomics approaches (see Figure 1A for the QconCAT approach[10,15,46,47]) or redox proteomics analyses (e.g. switch-tag redox proteomics[15,18]; Figure 1B) to highlight the proteomics storage lesion with unprecedented specificity and sensitivity. In addition, recent advancements in the field of metabolomics enabled us not just to expand on our understanding of the metabolic storage lesion[2,7,8,11,12,14,17,19,21,45,48-53], but also to perform ultra-high throughput[54] quantitative tracing experiments with heavy labelled substrates[14,15,55] to inform biomarker[16] and metabolic flux analyses[14,15,21,55] (Figure 1C). However, we[17] and others[21] have recently acknowledged that molecular appreciation of the storage lesion is but the first step in defining novel strategies to improve storage quality. Protein[8,10,15,24] and metabolic markers[13,14,16,21,51] of the RBC storage lesion have been

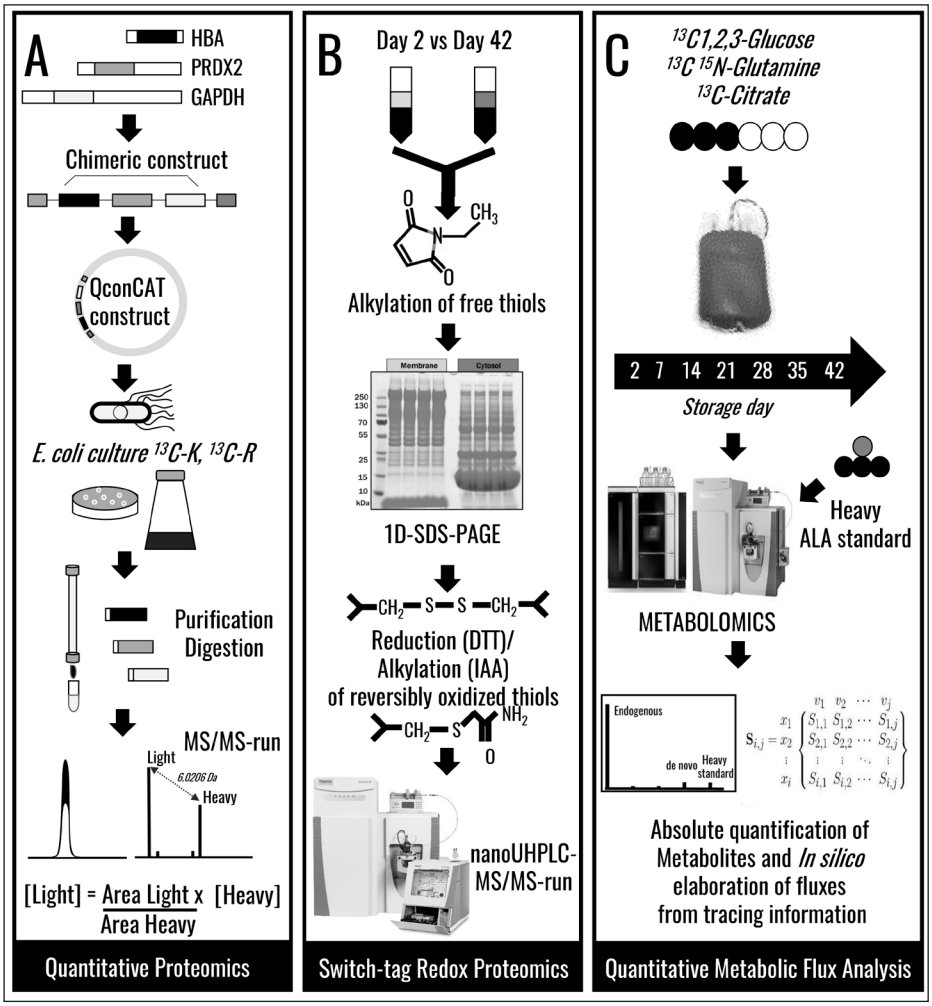

Figure 1 - Advancements in omics technologies for Transfusion Medicine applications. (A) An overview of the QconCAT approach for quantitative proteomics[10] and (B) switch-tag approach for redox proteomics applications[15,18]. (C) An overview of a tracing quantitative metabolic experiment, a workflow that can be exploited to inform quantitative metabolic flux analysis elaboration with systems biology tools.

proposed by us and others. The metabolic phenotype of stored RBCs follows a specific 3-stage sequence, as gleaned through multivariate analysis of metabolomics data of SAGM and AS-3 RBCs (Figure 2A)[16]. Metabolic reprogramming of stored RBCs has been associated with the necessity to restore reducing equivalents in order to counteract oxidative stress to functional proteins, such as haemoglobins[18] and anti-oxidant enzymes (e.g. peroxiredoxin 2[8,24]). Of note, these redox systems appear to be linked, in that irreversible thiol oxidation

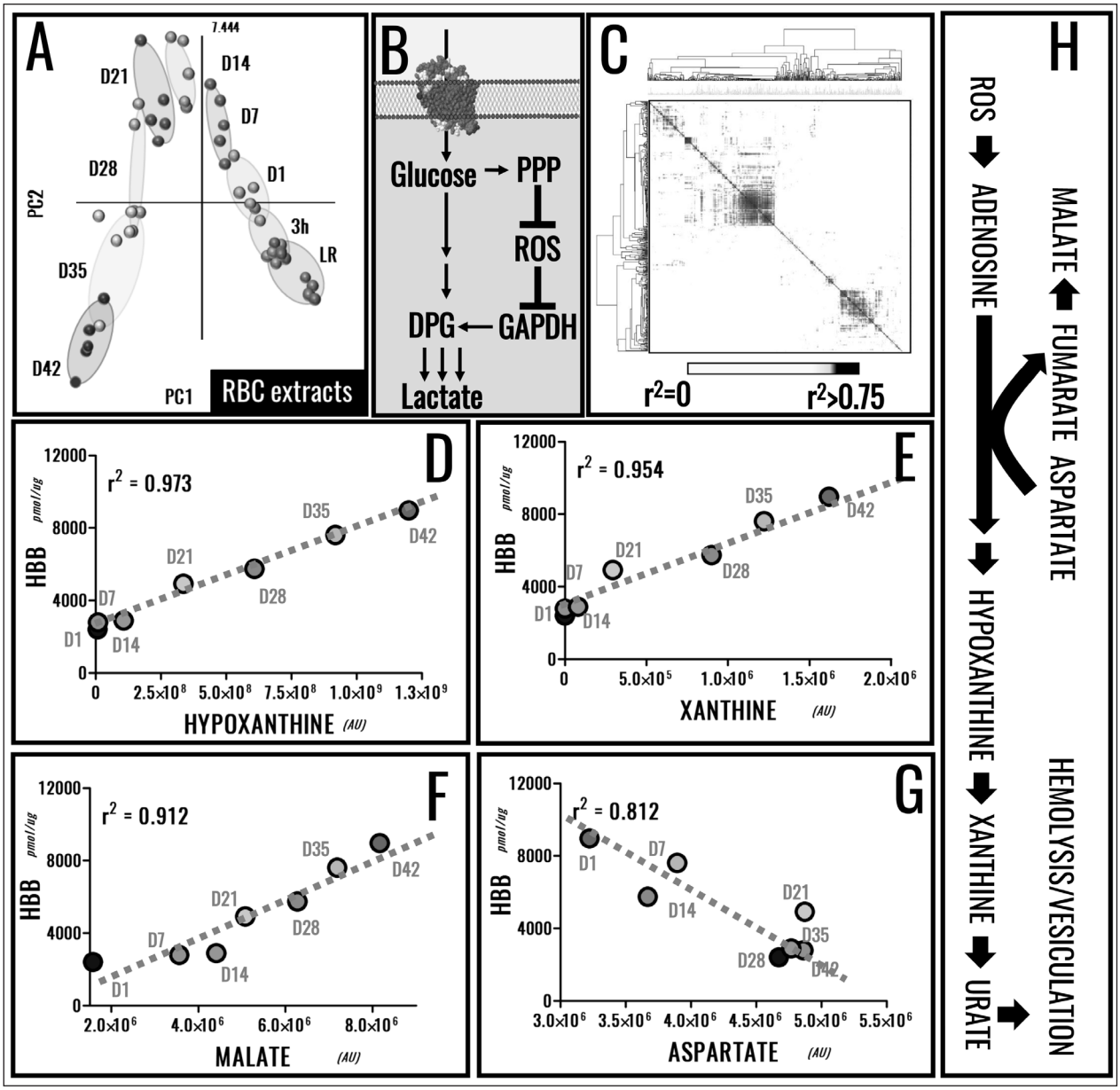

Figure 2 - Metabolic markers of the red blood cell (RBC) storage lesion have been identified through statistical analysis, such as Partial least-square discriminant analysis (A) and receiver operating characteristic curves (ROC)[16,21].
A combination of redox proteomics, quantitative proteomics and metabolic flux analyses has revealed a role for the oxidative stress-dependent regulation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity in mediating the activation of the pentose phosphate pathway (PPP) to generate reducing equivalents in the attempt to counteract oxidative stress over storage[15,23]. (B). Correlative analysis of metabolic[13] and protein markers[10] of the storage lesion was here performed and plotted as a heat map (black = R>0.75) (C). Of note, metabolic markers of the RBC storage age correlated with the absolute concentration of supernatant haemoglobin over storage, a marker of RBC vesiculation and haemolysis (D-G). Though only correlative analyses are here provided, it is interesting to note that all the metabolites showing the highest positive correlations with supernatant haemoglobin were part of the purine catabolism/salvage pathway, a pathway that is activated by oxidative lesion to the purine nucleoside pool and is in part counteracted by salvage reactions fueled by aspartate consumption and resulting in fumarate-malate accumulation (H).

of cysteine 93 of haemoglobin beta (a residue necessary for haemoglobin S-nitrosylation and thus haemoglobin-mediated nitric oxide signalling) impairs recycling of the oxidised active site cysteine of peroxiredoxin 2, inhibiting catalysis[22]. Oxidative lesion(s) to stored RBCs also affect redox sensitive amino acid residues in active site pocket of glyceraldheyde 3-phosphate dehydrogenase (GAPDH)[15,23], a biochemical adaptation that promotes RBC metabolic reprogramming from glycolysis to NADPH-generating pentose phosphate pathway (PPP) in response to oxidative stress (Figure 2B). However, these salvage mechanisms appear to be insufficient to fully counteract the oxidative lesion, resulting in the progressive release of irreversibly oxidised/functionally impaired proteins and small molecule metabolites (including oxidised lipids) into packed RBC supernatants[8,15,18,23,24,31,48]. Of note, protein and metabolite markers of the RBC storage lesion show significant correlations among each other (Figure 2C-G), resulting from the elaboration of data from our previous publications[10,13,16]. Importantly, there is a significant correlation between the levels of metabolic markers of the storage age and the absolute concentrations of supernatant haemoglobin (Figure 2D-G), a marker of RBC storage haemolysis/vesiculation, as we recently proposed[10]. Though merely correlative, these observations are relevant in that they suggest that monitoring RBC markers of the metabolic age could provide information about the quality of stored RBCs and potentially predict the effectiveness of the transfusion therapy, in addition to guiding the design and development of novel storage strategies/additives to decrease RBC storage haemolysis.

From omics markers of the storage lesion to *in vivo* survival and haemolysis

Some of the metabolic markers of the storage lesion are not just related to energy metabolism, but also to purine homeostasis and oxidation, such as the adenosine triphosphate catabolites hypoxanthine and xanthine[16,51]. Oxidised lipids and purines that accumulate in packed RBC supernatants during refrigerated storage correlate with *in vivo* performances of transfused cells in animal models[29]. These observations are suggestive of the likely clinical relevance of the storage lesion, in that it is a safe statement to conclude that, to function, RBCs must at least circulate. Similarly, the metabolic phenotypes (especially in terms of energy and redox homeostasis) correlate with RBC storage haemolysis, both parameters being affected by the genetic phenotype of the donor[56-58]. Studies on RBC storage haemolysis and 24-hour *in vivo* survival suggest that there is room for improvement of the current processing and storage strategies. A large retrospective study of radiolabelled RBC recoveries in autologous healthy volunteers (n=641) by Dumont and Aubuchon reported that end-of-storage RBCs have recoveries of 82.4±6.7%[59]. These numbers indicate that approximately 17% of the RBCs in a transfused unit are lost during storage and transfusion to healthy volunteers[59]. In the light of these data, it is reasonable to assume that heterologous chronically or massively transfused recipients would respond to blood transfusion differently to autologous healthy volunteer recipients owing to their repeated exposure to allogeneic cells or the underlying pro-inflammatory/metabolically-deranged physiology, respectively. Donor and recipient biological variability have often been overlooked in laboratory and clinical studies of the RBC storage lesion, a trend that has been rapidly changing in recent years[60-64]. It is still not fully understood whether omics phenotypes of stored RBCs are affected by donor variability and whether that relates to RBC *in vivo* performances and resistance to the storage lesion, such as in particular haemolysis. Studies such as the REDS III Omics initiative will tackle this important issue in the coming years[65].

Biological variability, metabolic poise and enzymatic constraints: introducing the concept of metabolic linkage

Despite biological variability, RBC metabolism has evolved to preserve the metabolic poise. To achieve this task, RBC metabolism is tightly regulated by biochemical constraints as a result of evolutionarily conserved enzymatic activities, a phenomenon that can be appreciated through systems biology *in silico* elaboration of RBC metabolism[66]. Owing to such biochemical constraints, small molecule metabolites do not just show extremely high correlations with storage age[16], but also among each other (Figure 3A-J), a phenomenon we refer to as metabolic linkage. We believe that future advancements in the field of Transfusion Medicine[17], personalised medicine and clinical biochemistry[67] will be driven by the appreciation of the metabolic linkage and how such linkage is affected by various stimuli; e.g. metabolic responses to hypoxia under physiological (high altitude[68-70]) or pathological (haemorrhagic shock[71] or sepsis[72]) conditions. Similar considerations may also apply with respect to enzymopathies and the role they may play within the framework of the RBC storage lesion. One paradigmatic example is the case of glucose 6-phosphate dehydrogenase deficiency, the most common human enzymopathy that, depending on the variant and thus specific enzymatic mutation, results in different metabolic reprogramming that can affect RBC capacity to cope with stresses upon storage and transfusion into recipients[73,74]. Elaboration of data available in the literature reveals that glucose 6-phosphate dehydrogenase deficient donors are characterised by a re-arranged metabolic linkage phenotype (Figure 4A

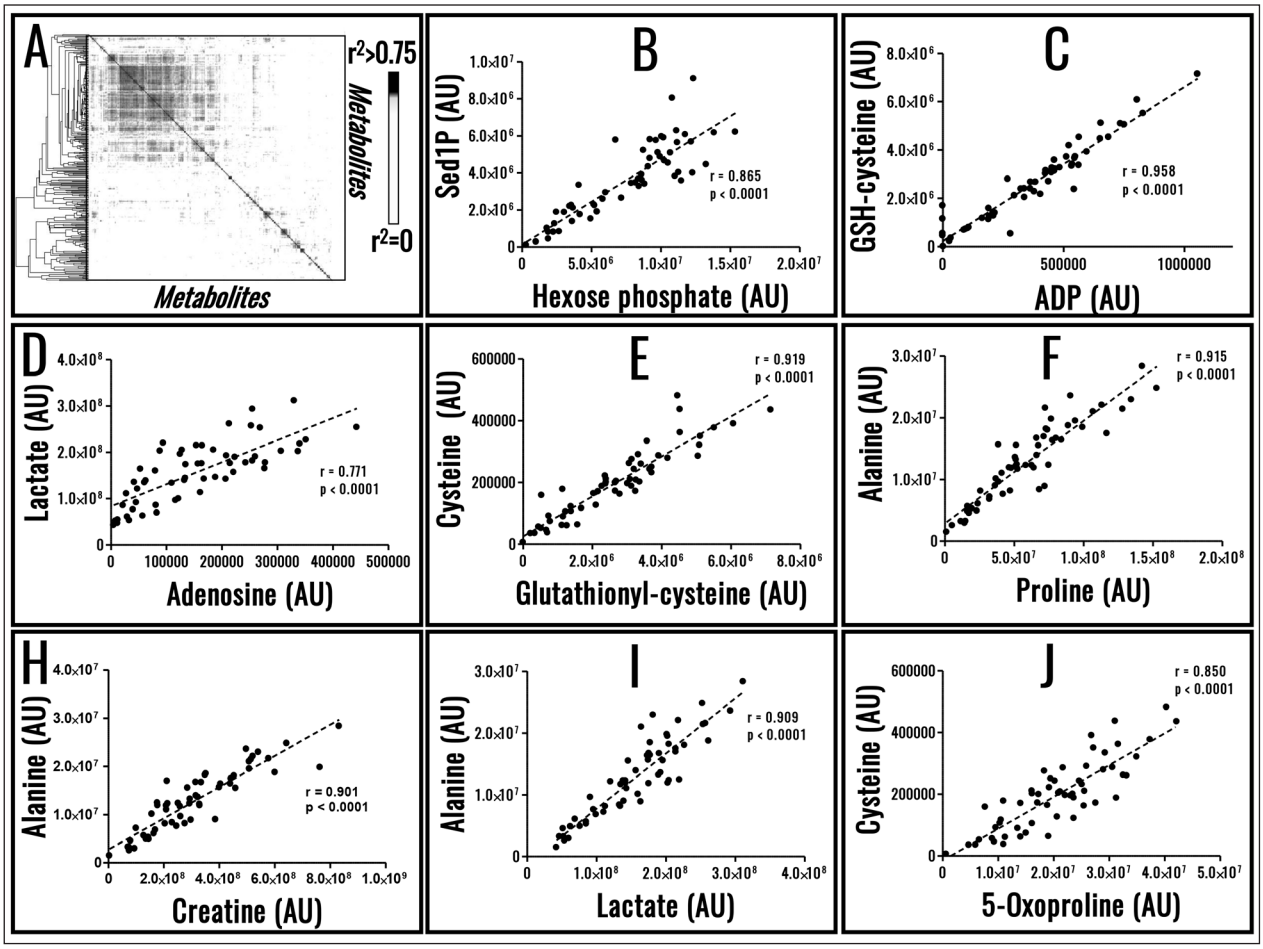

Figure 3 - Metabolic linkage. Metabolite levels in stored red blood cells (RBCs) are significantly correlated, a phenomenon that is explained by enzymatic biochemical constraints and here defined as metabolic linkage (A).
A few examples of metabolites showing significant correlations among each other in 60 packed RBC extracts sampled at random storage time points is shown in (B-J). Figures and panels are the result of the elaborations of metabolomics analyses of samples kindly provided by Dr. Eldad Hod at Columbia University, NY, USA.

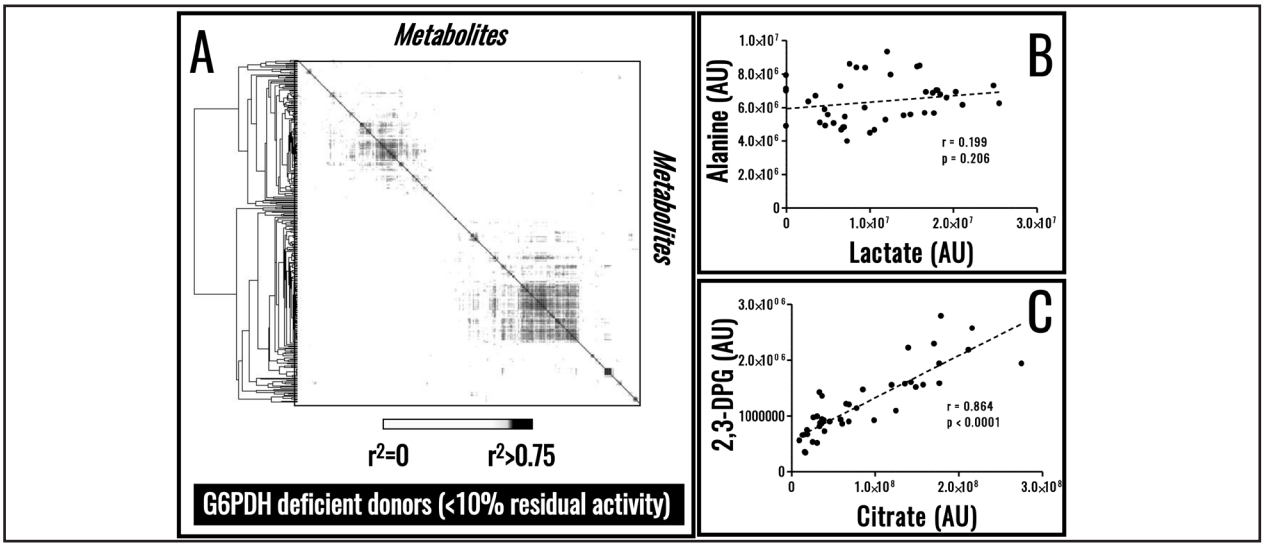

Figure 4 - Enzymopathies affect the metabolic linkage.
Determination of the metabolic linkage in stored red blood cells (RBCs) from glucose 6-phosphate dehydrogenase deficient donors reveals a re-wiring of RBC metabolism (A). As a result, significant correlations observed in healthy volunteers are lost (B), while novel ones appear (C). Figures and panels are the result of the elaborations of freely available data from Tzounakas *et al.*[74].

in comparison to Figure 3A), resulting in the loss of significant correlations between some of the metabolites whose level are linked in healthy donors (e.g. lactate and alanine; Figure 4B in comparison to Figure 3I). On the other hand, as a result of a metabolic re-wiring as previously suggested[74], novel significant correlations emerge (e.g. citrate and 2,3-diphosphoglycerate; Figure 4C), suggesting that recently appreciated metabolic pathways in stored RBCs, such as cytosolic metabolism of citrate in mitochondria-deficient RBCs[14], may play a key role in preserving reducing equivalent homeostasis through alternate pathways in RBCs from glucose 6-phosphate dehydrogenase individuals.

Thesis, antithesis and synthesis

As for the three moments of Hegelian philosophy, the transfusion community at first hypothesised that storage age affected the safety and effectiveness of the transfusion therapy (*thesis*). Despite laboratory and retrospective clinical evidence, reassuring reports from the recent randomised clinical trials have suggested that the current standard of care is not inferior to the transfusion of the freshest units available (*antithesis*). For the foreseeable future, the combination of omics technologies and clinical evidence, through ambitious studies like the REDS III Omics trial[65], will enhance our understanding of the effects of handling processes (e.g. leucoreduction, storage additives) and donor/recipient biological variability, and likely reconcile (*synthesis*) the apparent inconsistencies of the past ten years of Transfusion Medicine research.

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Disclosure of conflicts of interest

Though unrelated to the contents of the manuscript, the Authors disclose that ADA, TN and KCH are part of Endura LLC. ADA is a consultant for New Health Sciences Inc. The other Authors declare no conflicts of interest.

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Duration of red blood cell storage and inflammatory marker generation

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Abstract

Red blood cell (RBC) transfusion is a life-saving treatment for several pathologies. RBCs for transfusion are stored refrigerated in a preservative solution, which extends their shelf-life for up to 42 days. During storage, the RBCs endure abundant physicochemical changes, named RBC storage lesions, which affect the overall quality standard, the functional integrity and *in vivo* survival of the transfused RBCs. Some of the changes occurring in the early stages of the storage period (for approximately two weeks) are reversible but become irreversible later on as the storage is extended. In this review, we aim to decipher the duration of RBC storage and inflammatory marker generation. This phenomenon is included as one of the causes of transfusion-related immunomodulation (TRIM), an emerging concept developed to potentially elucidate numerous clinical observations that suggest that RBC transfusion is associated with increased inflammatory events or effects with clinical consequence.

Keywords: red blood cell, inflammation, storage.

Introduction

An emerging transfusion community interest concerns the ability for blood transfusion to modulate the immune system of recipients[1-5]. Transfusion-related immunomodulation (TRIM) has been implicated in adverse clinical outcomes[6,7]. The present "gold standard" for maximum shelf-life of red blood cells (RBCs) is six weeks (42 days)[8]. Even if storage of blood at 4 °C is proposed to slow down RBC metabolism and the accumulation of soluble factors, amongst other main pointers of quality and safety of stored blood, it does not stop the overall process often referred to as storage lesions. Storage lesions have been extensively researched[9-11]. Classically, storage lesions in RBCs are categorised as either biochemical or rheology changes. However, inflammatory markers are poorly evaluated in the literature. Even if the rise in inflammatory markers observed in transfusion-related immunomodulation[12] may be improved through leucocyte reduction, several immunomodulatory factors stored in RBCs participate in inflammation[13-16]. The potential of other modes of processing for creating storage lesions, e.g. through degradation of nucleic acids to enhance pathogen safety of red cell concentrates, deserves to be fully established. Therefore, a goal of the current review is to summarise the biochemical or rheology changes occurring in relation to the duration and processing of RBC storage, focusing on the generation of inflammatory markers.

Biomechanical changes in stored RBCs

During the typical storage conditions of blood, abundant biochemical alterations take place[17-21]. Such changes primarily refer to the generation of aggregates and biochemical debris that accumulate in the supernatant during prolonged storage of RBCs (Figure 1). Biomechanical storage lesions occur in the cytoskeleton and cellular membranes, defined as membrane and cytoskeleton protein oxidation, membrane phospholipid loss, abnormal rearrangement of membrane phospholipids, and morphological changes[22,23]. As an example, increased storage induces an increased level of extracellular potassium, lactate, and a decrease of sodium and glucose which leads to acidosis, particularly obvious by the end of the second week of storage (approximately after day [d]14). Extended RBC storage is also identified, resulting in reduced levels of ATP and 2,3-diphosphoglycerate (2,3-DPG) (Figure 1). Taken together, the above events are useful markers that could be indicative of a storage lesion in a given stored unit of blood[17,18,24-28]. Moreover, stored RBCs reveal functional changes to RBCs during storage, and particularly reduced deformability and increased rigidity, which may affect the flow of transfused RBCs through micro-capillaries, cell-to-cell aggregation, and adhesion to endothelial cells. The decreased levels of 2,3-DPG

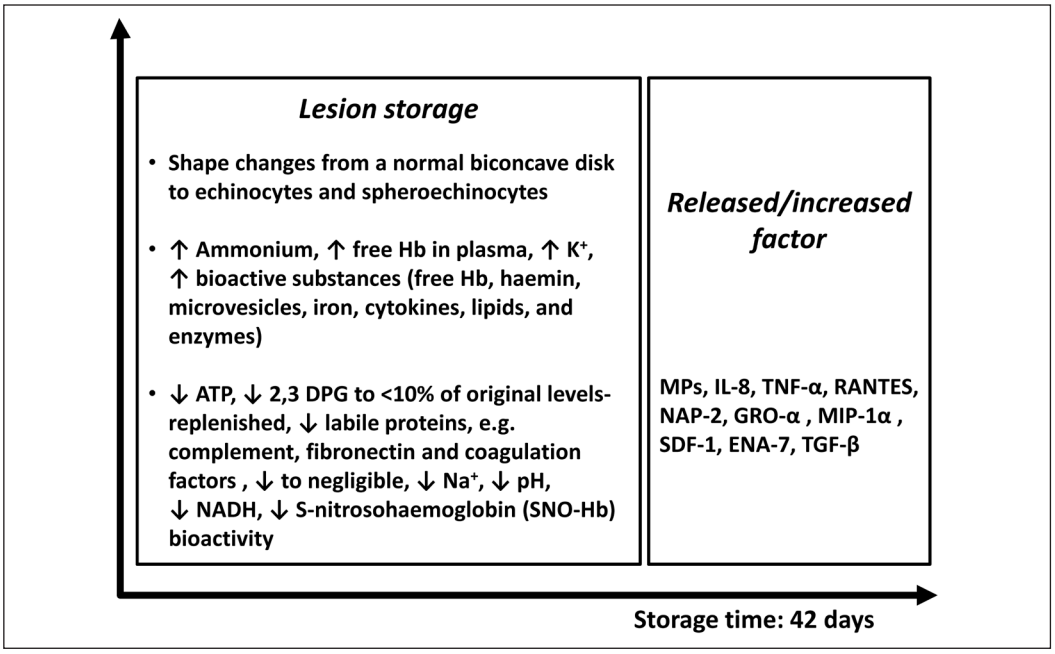

Figure 1 - Red blood cell product storage: lesion storage and biological response modifier release. Hb: haemoglobin; ATP: adenosine triphosphate; 2,3-DPG: 2,3-diphosphoglycerate; NADH: nicotinamide adenine dinucleotide; MPs: microparticles; IL-8: interleukine 8; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T cell expressed and secreted; NAP: neutrophil-activating peptide; GRO: growth-related oncogene; MIP: macrophage inflammatory proteins; SDF: stromal cell-derived factor; ENA-7: epithelial neutrophil-activating protein 7; TGF: transforming growth factor.

increase the affinity of haemoglobin to oxygen, which results in reduced oxygen delivery[26,29-32]. D'Alessandro *et al*. performed comprehensive metabolomics and quantitative tracing metabolic experiments that revealed that mature RBCs can metabolise substrates other than glucose, such as citrate. This observation was highly relevant to Transfusion Medicine, influencing particularly the process of RBC preparation and the formulation of novel additives[33,34].

Red blood cell-derived lipids during storage

During RBC storage, the implications of RBC membrane breakdown and release of potentially harmful bioactive lipids could be quantified, and contributed to the quality assessment of RBC[18,22,26,28,35,36]. The damaging oxidative storage effects on the RBC lipid membrane have numerous functional implications. As an example, increasing oxidative stress on stored RBC is a determining primer to increase phosphatidylserine translocation to the RBC surface membrane[13,37,38]. This phenomenon could mediate adhesion of transfused RBC to endothelial cells and induce the shedding and accumulation of bioactive microvesicles (Mvs)[26,35,39,40]. These bioactive lipids have been implicated in transfusion-related acute lung injury (TRALI) pathogenesis due, mainly, to their[26] polymorphonuclear neutrophil (PMN) priming abilities. In 2011, Silliman *et al*. explored the effect of infusing d1 or d42 lipids that were isolated from healthy human donor into lipopolysaccharide (LPS) or saline-treated male rats[41]. The study evaluated the PMN-priming capacity of the lipids as well as the effect of their infusion on acute lung injury as part of the "two-hit" TRALI model[41-44].

Red blood cell-derived microvesicles during storage

One implication of RBC membrane failure could be the release of potentially injurious bioactive microvesicles[26,35,39,40]. Phospholipids of the membrane are released during the microvesiculation process, which was first defined by Rumsby *et al*. in 1977[45]. Microvesiculation is a cellular process that leads to intracellular communication and cell apoptosis. Membrane lipid oxidation and cytoskeletal protein oxidation can dislocate the plasma membrane and cytoskeleton[10,22,46-48]. This disturbance could be a key phenomenon contributing to the increased release, during the RBC storage, and accumulation of bioactive microvesicles. Recently, several studies investigating the composition of the RBC membrane, particularly during microvesiculation, revealed a significant increase of RBC-derived microparticles as storage exceeded day 42[22,40,49-53]. Moreover, these RBC membrane-derived microvesicles present a significant

physiological and inflammatory pathophysiological process, principally involving vascular dysfunction. However, there is little clinical and *in vivo* evidence linking the effects of microvesicles during transfusion. As defined and summarised elegantly by Michel Prudent *et al*.[54], analysis of *in vitro* data highlights the presence of reversible and irreversible storage lesions demonstrating that RBCs exhibit two limits during storage: one around two weeks and another one around four weeks of storage. Microvesiculations could be considered irreversible storage lesions as degradation/oxidation of proteins, protein aggregations, protein activation, such as the proteasome 20S, shape change and deformability[54].

Reisz *et al*.[55] hypothesised that routine storage of erythrocyte concentrates promotes metabolic modulation of stored RBCs by targeting functional thiol residues of GAPDH and identified ex vivo functionally relevant reversible and irreversible (sulfinic acid; Cys to dehydroalanine) oxidations of GAPDH without exogenous supplementation of excess pro-oxidant compounds in clinically relevant blood products. Palia *et al*. propose that 8 compounds (lactic acid, nicotinamide, 5-oxoproline, xanthine, hypoxanthine, glucose, malic acid, and adenine) strongly correlate with the metabolic age of packed RBCs, and can be prospectively validated as biomarkers of the RBC metabolic lesion[56]. In the same way, Wither *et al*. show that several of the oxidised residues identified play well-established roles in haeme iron co-ordination, 2,3-diphosphoglycerate binding, and nitric oxide homeostasis[57].

Recently, Straat *et al*. hypothesised that extracellular vesicles in RBC products during storage contribute to a pro-inflammatory host response in recipients, which is related both to their amount as well as to the storage duration[58]. The authors clearly demonstrate that incubation of whole blood with both fresh and stored supernatant containing extracellular vesicles induced a strong host response with production of tumour necrosis factor (TNF), interleukin(IL)-6 and IL-8. Moreover, once supernatant was depleted from extracellular vesicles, this host response was completely abolished.

Immunomodulatoty factors in stored red blood cell concentrates

The accumulation of immunomodulatoty factors in stored RBC concentrates has been implicated as a potential cause of transfusion reactions associated with the use of such products[8,9,25,26,29,32,59-61]. Data suggest high concentrations of TNF, IL-1 and IL-6 in random donor RBC concentrates[62-64], and an association between the period of storage of blood components and the risk of developing acute transfusion reactions to platelet concentrates (PCs) and RBCs[3,22,53,65,66].

Cytokine could orchestrate a systemic inflammatory response. Kristiansson *et al*. report that plasma-soluble immunomodulatory factor concentration increases on the first post-operative day after major surgical trauma, the Author observing a relationship between the amount of RBC concentrates transfused perioperatively and post-operative systemic plasma IL-6 concentration[67]. The interaction between these cytokines is complex, each being able to up-regulate and down-regulate their own expression as well as that of the other cytokines. Nevertheless, the cytokine content may reflect the presence of leucocytes, in other words, an association with the initial amounts of leucocytes in RBC concentrates. Leucoreduction may significantly decrease febrile non-haemolytic transfusion reactions and may decrease cardiopulmonary transfusion reactions (TRALI and transfusion-associated circulatory overload)[3,8,41,68,69]. Presumably, this ensues through reduced levels of bioactive lipids and soluble CD40L in leucoreduced RBCs, which would have been produced by leucocytes, had they not been removed from the blood product. Donor leucocytes release cytokines and lipid factors able to activate neutrophils in a time-dependent course during RBC storage[70]. Pre-storage leucoreduction decreases the release of metabolites and cellular components into the RBC product. TNF-α, a multipotent cytokine, perfoms several immunological functions and is involved in maintaining the homeostasis of the immune system. It is known, that TNF-α, like IL-1 and IL-6, suppresses erythropoiesis by direct inhibitory effects on bone marrow RBC production[71,72]. Moreover, TNF-α, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumourigenesis and viral replication, and respond to sepsis *via* IL-1 and IL-6 producing cells. IL-6 is an important mediator of fever and of the acute phase response. It is capable of crossing the blood-brain barrier and initiating synthesis of prostaglandin E2 (PGE2) in the hypothalamus, thereby changing the body's temperature set point. In muscle and fatty tissue, IL-6 stimulates energy mobilisation that leads to increased body temperature[73,74].

Therefore, Muylle *et al*. demonstrated a relationship between TNF-α and IL-6 levels and febrile transfusion reactions[75]. IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils, but also other granulocytes, causing them to migrate toward the site of infection. IL-8 is also known to be a potent promoter of angiogenesis[76,77]. Cases of TRALI have been consistently associated with high levels of cytokines/chemokines, specifically IL-8[78], which has been shown to promote assembly of cholesterol-enriched microdomains or so-called lipid rafts on human neutrophils[79]. Moreover,

McKenzie *et al.*, proposed that antibodies bind monocytes (instead of neutrophils), leading to increased IL-8, which results in neutrophilic pulmonary infiltrate with subsequent TRALI[80]. IL-1 is intensely produced by tissue macrophages, monocytes, fibroblasts, and dendritic cells, but is also expressed by B lymphocytes, natural killer (NK) cells and epithelial cells[81]. They form an important part of the inflammatory response of the body against infection. These cytokines increase the expression of adhesion factors on endothelial cells to enable transmigration (also called diapedesis) of immunocompetent cells, such as phagocytes, lymphocytes and others, to sites of infection[82,83]. They also affect the activity of the hypothalamus, the thermoregulatory centre, which leads to fever. IL-1, appears to be associated with the occurrence of febrile non-haemolytic transfusion reaction (FNHTR) and other transfusion reactions, such as urticaria, hypotension, anaphylaxis, or TRALI[62,84,85]. The main critical factors in determining the accumulation of cytokines are considered to be the WBC content and the age of the blood component; moreover, accumulation is heterogeneous and there is a large inter-individual variation related to donors' hereditary and social habits[86]. Interestingly, the cytokines/chemokines in RBCs might be caused by haemolysis of the cells. This could be a comparable phenomenon to that detected in haemolytic transfusion reactions *in vitro* where there are high concentrations of cytokines/chemokines[87,88].

In the majority of the cases, antibodies against HLAs and/or human neutrophil antigen (HNA) present in the transfused product are thought to be responsible for initiating TRALI. TRALI is assumed to result from two hits, the first hit being caused by the underlying clinical condition of the patient, whereas the second occurs when the antibodies or factors are transferred to the recipient during transfusion[89]. Peters *et al*. investigated 18 healthy male volunteers (aged 18-35 years) infused with LPS to induce systemic inflammatory response syndrome. Two hours later, each participant received either one unit of 2-day stored autologous RBCs, 35-day stored autologous RBCs, or an equal volume of saline. Every 2 hours up to 8 hours after LPS infusion, haemoglobin, haemolysis parameters, and iron parameters, including non-transferrin bound iron (NTBI), were measured. The author concluded that 35-day stored autologous RBCs do not cause haemolysis or increased levels of NTBI during human endotoxemia[90,91]. Production of cytokines/chemokines could originate from an activation of RBC contact with the storage bag system during the storage period, indicating that these storage lesions should also be considered for future evaluations. Foreign material may stimulate cytokine synthesis and release, though this may be less likely during storage at a temperature of 4 °C.

Recently[13], our group focused on the characterisation of stored RBC with regard to cytokine/chemokine content, and investigated the possible influence of storage time (Figure 1). Individual RBC concentrate (RBCC) supernatants were processed by double centrifugation at 2,600 *g* for ten minutes. Samples were kept frozen at −80 °C and shipped on dry ice to the sample-processing laboratory. Levels of soluble cytokines growth-related oncogene (GRO)-α, IL-16, epithelial-derived neutrophil-activating protein 78 (ENA-78), macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), stromal cell-derived factor 1 (SDF-1) and transforming growth factor (TGF) β1, 2, and 3 were measured in triplicate from aliquots using Luminex technology[92], and amounts were expressed in ng/RBC unit. Supernatants from RBCCs were collected over time and tested for the presence of a variety of soluble chemokines and cytokines. GRO-α, IL-16, ENA-78, MIP-1α, MCP-1, SDF-1 and TGF β1, 2, and 3 were selected on the basis of previous reports[25,59,93]. There were no differences in ENA, GROα, MIP1α, MCP1, SDF1, IL-16 or TGF β3, either between the groups or over time[13]. However, TGF β1 and TGF β2 decreased over time in both RBCC groups, with a significant difference at d0 *vs* d42[13]. The biological activities of TGF-β are not species-specific. TGF-β isotypes share many biological activities and their actions on cells tend to be qualitatively similar, though there are a few examples of distinct activities. In some systems, TGF-β3 appears to be more active than the other isotypes. TGF-β2 is the only variant that does not inhibit the growth of endothelial cells. TGF-β2 and TGF-β3, but not TGF-β1, inhibit the survival of cultured embryonic chick ciliary ganglionic neurons. TGF-β is the most potent known growth inhibitor of normal and transformed epithelial cells, endothelial cells, fibroblasts, neuronal cells, lymphoid cells and other haematopoietic cell types, hepatocytes, and keratinocytes. Although TGF-β inhibits endothelial cell growth, it promotes angiogenesis in several bioassays, though TGF-β may also inhibit angiogenesis under certain circumstances[94,95]. At higher concentrations, TGF-β stimulates the growth of these cells. TGF-β has mainly suppressive effects on the immune system by inhibiting the IL-2 dependent proliferation of T cells and B lymphocytes. TGF-β inhibits the proliferation of B lymphocytes and thymocytes induced by IL-2 and IL-1, respectively, and inhibits the maturation of B cells[96]. It also suppresses interferon-induced cytotoxic activity of NK cells, cytotoxic T-lymphocyte activity, and the proliferation of lymphokine-activated killer cell precursors. TGF-β also inhibits the synthesis of immunoglobulin (Ig)G and IgM by B lymphocytes and

stimulates the synthesis of IgA. TGF-β1 is the most potent known chemoattractant for neutrophils[97,98].

In this same report[13], we performed a functional assay of RBCC supernatant on EA.hy926 endothelial cells. The human endothelial hybrid cell line EA.hy926 was obtained by fusion of primary umbilical vein endothelial cells with the human lung carcinoma cell line A459/8 (ATCC #CRL-2922). EA.hy926 cells were cultured in Dulbecco's modified MEM medium supplemented with 10% foetal calf serum and 1% penicillin-streptomycin and then incubated at 37 °C in a humidified atmosphere in 5% CO_2 until the cell monolayer reached confluence. The cells were then exposed to stored RBCC supernatants. IL-6, sCD141 and sCD62E levels were measured by enzyme-linked immunosorbent assay. In this *in vitro* model, we investigated the bioactivity of soluble immunomodulatory factors in endothelial cells *in vitro*. We tested the potential bioactivities of the soluble immunomodulatory factors from RBCs over time using EA.hy926. There was a difference in the expression of marker molecules generally associated with EA.hy926 cell activation (CD141) during storage (d1-d42) and similar results were observed for the expression of soluble markers generally associated with EA.hy926 cell activation (sCD141, sCD62E and IL-6). This result, revealing the bioactivities of soluble immunomodulatory factors in the supernatant of RBCCs on endothelial cells *in vitro*, suggests a potential generation of inflammatory markers during RBC storage. Further investigation could be carried out to determine the nature of these inflammatory markers.

Conclusions and perspectives

The increase of inflammatory soluble markers observed in transfusion-related immunomodulation[12,99] is reduced by leucocyte reduction[60,63,100-103]. However, stored RBCs deliver large quantities of iron to the monocyte/macrophage system and could thus induce inflammation, and transfusion of older, stored RBCs, therefore, produces a proinflammatory response associated with increased iron levels in the liver, spleen, and kidney, and increased circulating levels of non-transferrin bound iron[3,104].

However, it is currently unclear whether the storage lesions simply reflect an accelerated ageing of the RBCs, or something else, and the consequences *in vivo* (after transfusion) remain largely unknown. In addition, RBC preparation and storage processes (cryopreserved for extended periods of time, cryoprotectant, plastic bag, etc.) could be investigated to quantify the RBC inflammatory soluble markers observed in transfusion-related immunomodulation. In this context, we note that several current concepts of intervention (reduction of biological response modifiers) focus on methods to attenuate the cytokine response[105-108]. Another characteristic could be considered concerning the transfusion-related immunomodulation, as for platelet component[2,109-112]. The variability in cytokine/chemokine concentration in RBCs could reveal a biological variation in donors with regard to their capacity to synthesise and release mediators. Moreover, differences in measured cytokine/chemokine concentrations associated with various commercial immunoassay kits should be considered and standardised in the future.

The clinical implications of transfusing RBCs containing cytokines/chemokines to critically-ill patients have not been clarified[113]. It might be that the cytokine content of transfused RBCs may fuel the systemic inflammatory reaction in conditions of trauma and infection, and simulate non-haemolytic transfusion reactions. Investigations have confirmed that stored RBC transfusions seem to up-regulate proinflammatory gene expression in the leucocytes of the transfusion recipient[114]. Moreover, McFaul *et al.*[115] observed an *in vivo* inflammatory effect of transfusion with an increasingly proinflammatory RBC function of storage.

Numerous studies have evaluated a wide variety of photosensitisers and alkylating agents as candidates for a pathogen inactivation process of RBC suspensions, but few with a focus on the inflammatory role of RBC. Consequently, future questions could probably investigate:

i) how this blood component differs from classical RBC components in use;
ii) what are the benefits to the patients of possible pathogen inactivation processes to be used for RBC suspensions;
iii) whether there a reduction in acute transfusion reactions in patients receiving future pathogen-reduced RBC (febrile non-haemolytic and/or allergic transfusion reactions, TRALI).

Future animal and clinical studies could probably increase knowledge of the effect of RBC storage on post-transfusion outcomes and TRALI, with a specific focus on the inflammatory soluble markers observed in TRIM. Moreover, knowledge of TRIM could help answer questions concerning a possible difference between fresh and old blood, and, more interestingly, the medical effects of transfusing stored RBCs. As elegantly defined in animal models by James C. Zimring[16], the question now is to understand the "induction of cytokine storm" on RBCs during storage, and the potential promotion of acute transfusion reactions.

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Modelling the effects of blood component storage lesions on the quality of haemostatic resuscitation in massive transfusion for trauma

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Background. All blood components undergo loss of potency during storage. These loss-of-potency storage lesions are important in trauma resuscitation because they reduce the haemostatic capacity of mixtures of components that attempt to reconstitute whole blood. Even red cell storage-related loss of potency, which averages 17% with modern additive solutions, is important because 6 units of red cells must be given to achieve the effect of 5 fully potent units.

Materials and methods. Loss of potency of stored units of red blood cells, plasma, platelets, and cryoprecipitate were summed for dilutional, storage-related, pathogen reduction-related, and splenic sequestration-related causes and expressed as fractional plasma coagulation factor concentrations and platelet counts.

Results. Production of reconstituted whole blood from 1:1:1 unit ratios of red cells:plasma:platelets is associated with a 38% loss of plasma coagulation factor concentration and 56% loss of platelets. Storage losses of 17% for red cells, 10% for coagulation factors, and 30% for platelets are additive to pathogen reduction-related losses of 18% for coagulation factors and 30% for platelets.

Discussion. Component preparation and storage-related losses of potency for all blood components are serious problems for trauma resuscitation. Even red cell storage contributes to this problem and this can be made better in ways that can save many lives each year.

Keywords: red cell recovery, red cell loss of function, haemostatic resuscitation, damage control resuscitation.

Introduction

Patients suffering severe injury frequently have an accompanying coagulopathy characterised by reduced concentrations of coagulation factors and platelets that arises though haemorrhagic loss, dilution of remaining blood elements with asanguineous fluids, and consumption on damaged vascular surfaces. Acidosis and hypothermia can further impair the function of the remaining coagulation factors and platelets. Treatment is needed to address the underlying causes. Surgical control of all bleeding is desirable but not always fully achievable, especially in damaged tissues. Volume resuscitation to correct haemodynamics and oxygen transport can simultaneously address acidosis and hypothermia, while timely repletion of haemostatic components minimises further blood loss. In this setting, replacement of lost blood volume with crystalloid fluids and red blood cells (RBCs) can lead to dilution of platelets and coagulation factors. This insight provides the basis for balanced resuscitation with an approximate 1:1:1 ratio of RBCs, platelets, and fresh frozen plasma (FFP).

Blood that is stored as separate components and then given to a patient will not attain the original concentration of the separate blood elements because of losses during processing and dilution of components with anticoagulant and additive solutions. With the effects of storage lesions on these products, the *in vivo* recovery of RBCs, platelets, and plasma coagulation factors can be substantially lower. For patients undergoing operative management, general triggers for transfusion are to maintain a haematocrit greater than 21%, a platelet count above 50×10^9/L, and an International Normalised Ratio (INR) below 1.5 or 2.0[1]. The recovered portion of transfused blood products is sufficiently low to be barely above these traditional transfusion triggers. Patients with severe trauma are among those who can least afford to have a borderline haemostatic profile; therefore, the minimisation of storage lesions is critical for adequate resuscitation in this patient population.

Haemodilution as a product of storage

The act of collecting and storing blood as components dilutes the original donation. A typical whole blood donation of 450 mL will start with an average haematocrit of 42%, coagulation factor concentrations of 100%, and a platelet count of 250×10^9/L. This

original whole blood volume is collected into 63 mL of anticoagulant solution and the whole blood unit is split into its components. The cellular components are leucoreduced in filters that retain some of the initial components and volume. Finally, 100 mL of an additive solution is added to the RBCs.

The resulting RBC concentrate contains about 180 mL of RBCs in 9 mL of anticoagulant, 40 mL of plasma, and 100 mL of additive solution for a total volume of about 330 mL and a storage haematocrit of 55%. After collection and processing, a single whole-blood-derived platelet contains approximately 55×10^9/L platelets in 9 mL of anticoagulant and 40 mL of plasma. The platelets occupy about 0.5 mL of a 50 mL total volume. The plasma unit typically contains about 200 mL of plasma and 45 mL of anticoagulant, giving a total volume of 245 mL of 80% plasma in anticoagulant. Due to this loss and dilution, an idealised pool of the three components in a 1:1:1 unit ratio of plasma, platelets, and RBCs will have a haematocrit of 29%, a factor concentration of 65%, and a platelet concentration of 88×10^9/L[2].

The effect of storage lesions on recoverable haematocrit

The above calculations represent the upper limit of attainable haematocrit in a trauma patient receiving nothing but balanced transfusions of units with average sizes and concentrations of fresh products. Barring the removal of storage solutions, the use of unusually concentrated products, or the transfusion of additional amounts of one product that displace the other blood components, a further increase is not possible. Adding a storage lesion to any of the components decreases the recovery even further. In a retrospective study of radiolabelled RBC recoveries, Dumont *et al*. reported that mean recoveries for radiolabelled RBCs that were transfused autologously after 42 days of refrigerated storage averaged 82.4±6.7% for 641 units[3]. The unrecovered fraction of 17.6% represents the best estimate of storage-related loss among current US Food and Drug Administration-approved RBC storage systems.

It appears that it is possible to make better RBC storage solutions. A second-generation additive solution (AS-7) evaluated by Cancelas *et al.* found a 24-hour recovery at 42 days storage of 88±5%[4]. Incremental improvements in storage solutions can thus improve recovery and allow for successful balanced resuscitation while allowing more "room" for intravenous fluids for drug delivery.

Table I demonstrates the effect of applying storage lesions of varying size to blood components transfused in a 1:1:1 ratio. These values were calculated with the included equations (Figure 1) after allowing for a storage lesion of the indicated size. According to these estimates, transfused units with a recovery of about 70% produce a haematocrit approximately equal to suggested transfusion triggers.

The effect of storage lesions on coagulation factor concentration

Recovery of plasma coagulation factors is also highly dependent on the conditions of processing and storage. Factors V and VIII are heat labile, and these factors will quickly degrade if plasma is stored in a non-frozen state. Because Factor VIII is synthesised in endothelial cells and it is an acute phase reactant, it is likely less clinically relevant within the context of severe trauma. In contrast, insufficient repletion of Factor V is likely to be clinically relevant, particularly in severely injured patients with activation of the Protein C system.

A unit of FFP contains about 70% of the plasma from one unit of whole blood. This 200 mL of plasma is diluted with 50 mL of anticoagulant solution to obtain a typical unit volume of 250 mL. The dilution produces a product with 80% of the original factor concentration of the donated plasma. This FFP is added to the remaining plasma present in RBCs and platelets that has been diluted with anticoagulant and red cell storage solution; the combined factor concentration in this reconstituted whole blood (RWB) is about 65%. In a previous study of RWB wherein these products were combined in a 1:1:1 fashion, the pooled products were found to have an average INR of 1.31 and PTT of 42[5]. The clotting factor concentrations measured in these products were

Table I - Calculated haematological indices for whole blood, reconstituted whole blood (RWB), and RWB at varying degrees of recovery after applying reductions due to a storage lesion of a given size.

Recovery	HCT	Platelet count (10^9/L)	Coagulation factors
Whole blood	0.420	250	100%
100% RWB	0.288	88.282	64.67%
90%	0.259	79.365	58.13%
80%	0.230	70.447	51.60%
70%	0.201	61.530	45.07%

Indices were calculated with the equations in Figure 1, but the pathogen reduction and splenic sequestration terms were not applied.
HCT: haematocrit.

$$haematocrit = \frac{Vol_{RBC}}{Vol_D + Vol_{AC} + Vol_{AS}} \times (1 - s)$$

$$factor\ concentration_i = factor\ concentration_o \times \frac{Vol_P}{Vol_P + Vol_{AC} + Vol_{AS}} \times (1 - s) \times (1 - PR_{FFP})$$

$$circulating\ platelets = \frac{PLT_o}{Vol_{RBC} + Vol_P + Vol_{AC} + Vol_{AS}} \times (1 - s) \times (1 - PR_{PLT}) \times (1 - spleen)$$

Figure 1 - Equations estimating recovered indices after transfusion in a 1:1:1 ratio.
Values in parentheses are the values used for calculations in this paper. Vol_{RBC}: volume of red blood cells (RBCs) in a stored unit (180 mL); Vol_D: volume of the original donation (450 mL); Vol_{AC}: volume of anticoagulant (63 mL); Vol_{AS}: volume of additive solution (100 mL); s: percentage of storage lesion; factor $concentration_i$: concentration of clotting factors after product manipulation; factor $concentration_o$: original clotting factor concentration (100%); Vol_p: total plasma volume (280 mL); PR_{FFP}: percentage of clotting factors lost due to pathogen reduction technology (10%); PLT_o: platelet count in a unit of whole blood platelets (55×10^9/L); PR_{PLT}: percentage of platelets lost due to pathogen reduction technology (30%); spleen: percentage of platelets sequestered in the spleen (0.33).

similar to the expected concentrations calculated for RWB in this present report.

This concentration, however, assumes perfect storage and recovery of coagulation factors, and it assumes that the donor has factor concentrations at the population median. While clotting factor levels actually vary widely and reference ranges generally fall from 50 to 150%, this detail likely has a limited impact on average factor levels in massive transfusions where many units of plasma are given. In contrast, storage conditions do decrease factor levels across all units. Cardigan *et al.* found that room temperature storage of whole blood for 24 hours caused a 23% decrease of Factor VIII and small but statistically significant decreases of Factors II, IX, and X. These units also had PTT prolongation of about two seconds when compared to plasma frozen within 8 hours of collection[6].

In addition to loss of factors during the component production process, prolonged storage of plasma in the thawed state also leads to degradation. Downes *et al.* measured the factor content of refrigerated thawed plasma over a period of five days. As confirmed by prior studies, Factor VIII was the most labile factor, losing 40% of its concentration from day 1 to day 5. Factors V and VII saw decreases of 16 and 20% respectively, and other measured factors had negligible decreases[7]. Because pre-thawed plasma is disproportionately used in massive transfusions, degradation during storage for these factors disproportionately affects patients with severe trauma and it likely reduces the effectiveness of the replacement product for some patients.

This degradation is compounded by the fact that 30% of the plasma being given to the patient, that which is present in RBCs and platelets, is not stored in a way meant to preserve coagulation factors. While the amount of degradation varies by factor, it is reasonable to estimate that Factor V and VIII levels are low in RBCs stored for six weeks, and that more stable factors also suffer some degradation. Similarly, the approximately 40 mL of plasma present in a unit of whole blood platelets has been stored at room temperature; it is reasonable to estimate that the coagulation factors in this plasma have degraded at least as quickly as indicated by the data of Downes *et al.*, and it is likely that further losses have been sustained because platelets are stored at room temperature. Pathogen reduction adds further degradation of factors: plasma treated with methylene blue contains approximately 20% less fibrinogen, and solvent/detergent treated plasma has been shown to have 10% reductions of all factor levels[8]. A graph of the coagulation factor content and platelet count of RWB at varying recovery levels can be seen in Figure 2. The figure also includes estimates for pathogen-reduced products.

The effect of storage lesions on platelet recovery

Among blood components, platelets have the greatest discrepancy between the amount of product recovered after transfusion and its theoretical expectation. An apheresis collection or a 6 unit pool of whole blood-derived platelets containing over 300×10^9 platelets/L would be expected to produce an increase of circulating platelets of over 60×10^9/L. However, as about one-third of these transfused platelets become sequestered in the spleen and 30% of the remainder are lost to storage lesions, the average 4-hour increment in patients transfused with platelets in the PLADO trial was about 28×10^9/L[9]. While part of this reflects losses due to patient disease states, most is degeneration during storage, as further analysis found that only about 1% of

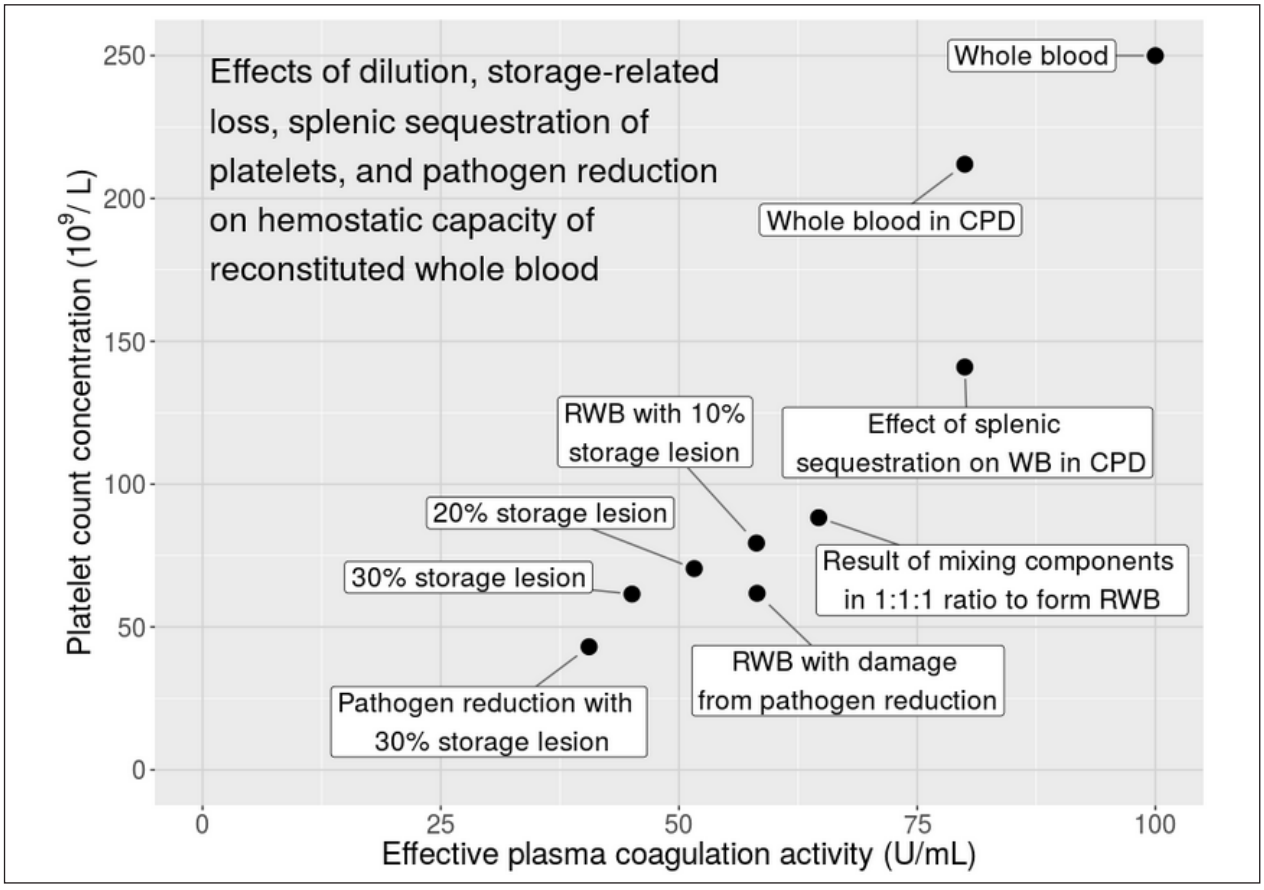

Figure 2 - The effects of dilution, storage-related loss, splenic sequestration of platelets, and pathogen reduction on haemostatic capacity of reconstituted whole blood.
Plot of clotting factor concentrations and platelet counts for whole blood (WB), WB in anticoagulant, and reconstituted whole blood (RWB) after mixing in a whole blood-derived stored components in a 1:1:1 ratio. These values were calculated with the equations and values specified in Figure 1. Percentages refer to RWB at varying degrees of recovery after applying reductions due to a storage lesion of a given size. Pathogen reduction: the effects of subsequent storage lesions on products treated with pathogen reduction technology, with an assumed 30% reduction in platelet counts and 10% reduction in clotting factors. With the exception of "Effect of Splenic Sequestration on WB in CPD", the effect from splenic sequestration was not applied, and *in vivo* recoveries would be expected to be correspondingly decreased.

those patients in the trial had alloimmunisation-based refractoriness[10]. With an average increment of 28×10^9/L, attributing half of the unrealised gain to consumption and half to a storage lesion implies that storage losses are nearly the same magnitude as the total increase in circulating platelets after transfusion.

As with pathogen-reduced FFP, pathogen-reduced (PR) platelets have been shown to improve product safety at the cost of decreased platelet recovery and activity. Prior studies have shown a reduction in total platelet recovery to the amount of approximately 25-44%[11]. One representative study found autologous recoveries in PR platelets of 50.0±18.9%, compared to 66.5±13.4% for control platelets[12]. Applying a 30% reduction to an expected platelet count in RWB would reduce the platelet count from a maximum of 88×10^9/L platelets to a maximum of 62×10^9/L for pathogen-reduced platelets. The cumulative effect of both pathogen reduction and storage lesions is demonstrated in Figure 2, using the equations in Figure 1. This, if transfused, is further reduced by splenic sequestration of one-third to a platelet count of 41×10^9/L before taking into account any storage lesions on the product. An additional storage lesion of 25% would reduce the platelet count of that RWB to, at best, about 30×10^9/L. In a patient with severe trauma, pathogen reduction reduces platelet counts from what is already a tenuously low replacement product.

Conclusions

For patients with severe trauma, correction of coagulopathy is accomplished by surgical control of bleeding and balanced resuscitation with stored blood products in ratios that attempt to restore haemostasis. This reconstituted whole blood is more diluted than whole blood because of the addition of storage solution and anticoagulant. The end product is a mixture that is, at

best, only slightly better than traditional haematological indices associated with unfavourable outcomes; storage lesions exacerbate this problem. Red cells, FFP, and platelets all have storage lesions of a clinically significant magnitude to a patient with severe trauma because reconstituted whole blood is already limited in its haemostatic profile by the dilution of stored components. Minimisation of storage lesions, therefore, represents an excellent opportunity for improving resuscitation in these patients.

Authorship contributions

JAM wrote the article, performed the calculations, prepared the illustrations, and reviewed the final product. JRH provided the basic idea, gave suggestions on citations and design of graphics, edited the article, and reviewed the final product.

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Iron-deficient erythropoiesis in blood donors and red blood cell recovery after transfusion: initial studies with a mouse model

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Background. Most frequent red cell (RBC) donors and many first-time donors are iron deficient, but meet haemoglobin standards. However, the effects of donation-induced iron deficiency on RBC storage quality are unknown. Thus, we used a mouse model to determine if donor iron deficiency reduced post-transfusion RBC recovery.

Methods. Weanling mice received a control diet or an iron-deficient diet. A third group receiving the iron-deficient diet was also phlebotomised weekly. This provided 3 groups of mice with different iron status: (1) iron replete, (2) mild iron deficiency with iron-deficient erythropoiesis, and (3) iron-deficiency anaemia. At ten weeks of age, blood was collected, leucoreduced, and stored at 4 ºC. After 12 days of storage, 24-hour (h) post-transfusion RBC recovery was quantified in recipients by flow cytometry.

Results. Before blood collection, mean haemoglobin concentrations in the iron-replete, iron-deficient, and iron-deficiency anaemia donor mice were 16.5±0.4, 11.5±0.4, and 7.0±1.4 [g/dL± 1 standard deviation (SD)], respectively ($p<0.01$ for all comparisons between groups). The 24-h post-transfusion RBC recoveries in recipients receiving transfusions from these three cohorts were 77.1±13.2, 66.5±10.9, and 46.7±15.9 (% ±1 SD), respectively ($p<0.05$ for all comparisons between groups).

Discussion. In summary, donor iron deficiency significantly reduced 24-h post-transfusion RBC recovery in recipient mice. RBCs from mice with mild iron deficiency and iron-deficient erythropoiesis, with haemoglobin levels similar to those used for human autologous blood donation, had intermediate post-transfusion RBC recovery, as compared to iron-replete donors and those with iron-deficiency anaemia. This suggests that, in addition to the effects of iron deficiency on donor health, frequent blood donation, leading to iron-deficient erythropoiesis, may also have adverse effects for transfusion recipients.

Keywords: red blood cells, iron deficiency, blood donation, mouse model.

Introduction

Iron deficiency is common among frequent blood donors, but red blood cell (RBC) storage quality and post-transfusion RBC recovery of RBC units from iron-deficient donors have not been rigorously examined. Evidence from animal and human studies indicates that, when the iron supply for erythropoiesis is inadequate, the RBCs produced have multiple defects that could impair their ability to tolerate refrigerated storage.

Iron deficiency, a decrease in the amount of body iron, is detected clinically by measuring indicators of iron storage (e.g., serum ferritin) and of the adequacy of iron supply to erythropoietic precursors (e.g., RBC zinc protoporphyrin). In the absence of complicating factors, as iron stores decrease, serum ferritin levels decline. In addition, RBC zinc protoporphyrin monitors the supply of iron available for RBC production. In the developing RBC, the insertion of iron into protoporphyrin IX is the final step in producing haeme for incorporation into haemoglobin. If iron is unavailable, divalent zinc is incorporated instead, producing zinc protoporphyrin, which binds to haemoglobin, persists for the life of the RBC, and is a biochemical indicator of a deficient supply of iron for RBC production[1,2].

Using these biochemical markers, and other means, four successive stages of iron deficiency can be distinguished (Figure 1):

1) Reduced iron stores *(data not shown)*: as iron stores decrease, serum ferritin levels decline proportionally.
2) Iron depletion: iron stores are absent (e.g., serum ferritin <12 μg/L), but the combination of iron recycled from senescent RBCs and that derived from gastrointestinal iron absorption maintains delivery of iron to the erythroid marrow and other tissues for producing haemoglobin and other functional iron compounds. Because the iron supply for RBC

production is maintained, RBC zinc protoporphyrin levels remain in the reference range.

3) Iron-deficient erythropoiesis: with further reductions in total body iron, the lack of iron limits production of haemoglobin and other iron-requiring compounds, resulting in iron-deficient erythropoiesis. The RBC zinc protoporphyrin concentration in newly formed erythrocytes increases, but any further falls in the nominal serum ferritin levels have no physiological meaning. The effect on the circulating haemoglobin concentration is insufficient to be detected by the standards used to screen blood donors. As recently produced RBCs replace senescent RBCs, the circulating RBC zinc protoporphyrin concentration progressively increases, providing an index reflecting the severity and duration of the inadequate supply of iron for erythropoiesis[1,2].
4) Iron-deficiency anaemia: a further diminution in total body iron produces frank iron-deficiency anaemia with haemoglobin levels falling below the standards used to screen blood donors and leads to their deferral from donation.

Iron deficiency is commonly seen in volunteer blood donors. Although surprisingly prevalent in first-time donors[3,4], the prevalence is even higher in the particularly altruistic, frequent, repeat donors, especially among women of childbearing age[5,6]. For example, in the United States in 2011, of the donors who provided the approximately 15.7 million units of RBCs that were collected, 69% were repeat donors[7]. In addition, in Canada, approximately 90% of RBC units collected for transfusion are provided by repeat donors[8]. In the United States, the REDS-II Donor Iron Status Evaluation (RISE) study[9] found that up to 49 and 66% of male and female frequent donors, respectively, exhibited either iron depletion (i.e., absent iron stores) or iron-deficient erythropoiesis. Similar frequencies of iron deficiency were reported in Canadian[8], Austrian[10], Danish[11], and Dutch[12] populations.

Red blood cells from iron-deficient donors may have impaired tolerance for refrigerated storage and decreased post-transfusion RBC recovery. As examples, RBCs from individuals with iron-deficiency anaemia have decreased levels of endogenous antioxidants[13,14],

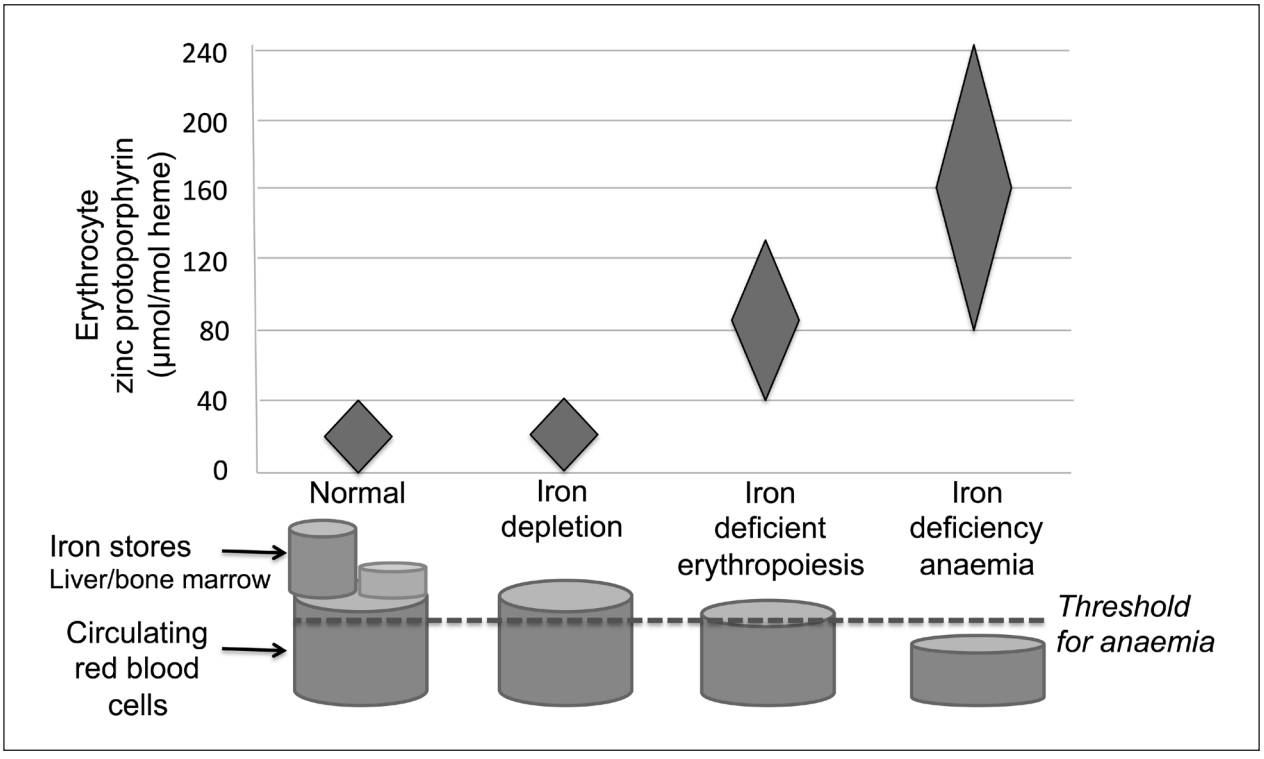

Figure 1 - Red blood cell (RBC) zinc protoporphyrin progressively increases with increasing severity of iron deficiency. Iron deficiency progresses from reduced iron stores to iron depletion, in which there is normal erythropoiesis with normal zinc protoporphyrin levels, but decreased ferritin due to absent iron stores. The next stage is iron-deficient erythropoiesis, in which increased RBC levels of zinc protoporphyrin and very low serum ferritin levels reflect both absent iron stores and the lack of sufficient iron for normal haemoglobin production. As iron deficiency progresses further, frank iron deficiency anaemia develops with insufficient iron for maintaining adequate haemoglobin levels. *Adapted from Hastka J* et al. *and Brittenham GM*[1,2].

have evidence of oxidative damage[15,16], and are more sensitive to oxidative stress[14,16] and low pH[14]; the latter, in particular, decreases progressively during RBC storage[17]. Furthermore, refrigerated storage induces oxidative stress in donor RBCs and inhibits their oxidative stress defence mechanisms[15,18-24]. Oxidative damage *per se* also impairs RBC deformability[25], and RBCs from humans, rats, and rabbits with iron-deficiency anaemia have impaired deformability[16,26]. In addition, the deformability of RBCs from healthy human donors is impaired following refrigerated storage[27]. Indeed, circulatory RBC lifespan is decreased in humans[14,28-30] and in relevant animal models[26,31] with iron-deficiency anaemia. In humans, this decreased circulatory lifespan is most likely due to extravascular haemolysis in the spleen[28-30] and is corrected by iron repletion[14,29]. Remarkably, in several older studies[14,28,32], RBCs obtained from donors with iron-deficiency anaemia were transfused into healthy recipients, albeit without prior refrigerated storage. In each study, the transfused iron-deficient RBCs had a decreased circulatory lifespan/recovery, most likely due to splenic clearance. In addition, when RBCs obtained from healthy donors were transfused into recipients with iron-deficiency anaemia, the transfused RBCs had a normal lifespan, suggesting that the iron-deficiency-induced defect was intrinsic to the RBC and not due to enhanced clearance mechanisms[32,33].

The RBCs transfused in these earlier studies (from the 1940s-1970s) were from donors with iron-deficiency anaemia, not from donors with iron-deficient erythropoiesis. Repeating these studies with modern storage systems and deliberately transfusing healthy human volunteer recipients with allogeneic RBCs obtained from donors with iron-deficiency anaemia are no longer ethically feasible. Nonetheless, if RBCs collected from donors with iron-deficient erythropoiesis do exhibit poor storage quality, this would have particular importance for patients dependent on chronic transfusion therapy, including those with sickle-cell disease and β-thalassaemia. Therefore, we developed a mouse model to determine whether iron-deficient erythropoiesis in donor mice decreased the 24-h post-transfusion recovery of their RBCs in healthy murine recipients.

Materials and methods

Mice

Three murine donor cohorts of different iron status were prepared using wild-type C57BL/6 mice. To this end, weanling male mice were placed on a defined iron control diet of 45 ppm iron (TD.110593, Harlan Laboratories) or an iron-deficient diet of 0-4 ppm iron (TD.110592). A third group was fed the iron-deficient diet and was also phlebotomised weekly (50-100 μL per bleed). This approach generated three groups of mice, respectively, with different iron status: (1) iron replete, (2) mild iron deficiency with iron-deficient erythropoiesis, and (3) iron-deficiency anaemia. At approximately ten weeks of age, blood was collected from each cohort, leucoreduced, packed, and stored at 4 ºC in CPDA-1 for 12 days, as described previously[34,35].

Transfusion-recipient mice were fed an iron-replete diet and were transgenic for expression of the enhanced Green Fluorescent Protein (eGFP) under the control of the human ubiquitin C promoter and were on the C57BL/6 background. Thus, these mice are syngeneic with the wild-type C57BL/6 RBC donors, except for expression of eGFP, which is expressed in all of their cells, including RBCs.

Laboratory measurements of iron status

Within a week prior to preparing the mouse blood banks, small blood samples were collected from all donor mice by submandibular or saphenous vein bleed and pooled by cage, with 5 mice per cage. Haemoglobin was measured by a modified cyanomethemoglobin assay[36]. Complete blood counts were performed for the third replicate experiment using an automated haematology analyser (Forcyte Veterinary Hematology Analyzer; Oxford Science Inc., Oxford, CT, USA). To measure zinc protoporphyrin, RBCs were washed with saline and analysed using a haematofluorometer (Model 208; Aviv Biomedical Inc., Lakewood, NJ, USA).

Non-haeme liver iron was determined, as described previously[37]. This approach was used, rather than determining serum ferritin levels, because measuring the hepatic non-haeme iron concentration is a reference method for evaluating body iron stores. Briefly, the wet weight of each liver obtained at necropsy was quantified, and a weighed, approximately 100 mg portion was dried at 65 °C for 24 h and digested in an acid digestion mixture at 65 °C for 24 h. The iron content of the centrifuged, acidified sample was determined using a bathophenanthroline-based colorometric method. The absorbance at 535 nm of samples and iron standards was measured spectrophotometrically in duplicate and mean values used for calculating total liver iron.

Post-transfusion RBC recovery studies

After 12 days of storage in CPDA-1, 24-h post-transfusion RBC recovery was determined in recipient transgenic mice using flow cytometric detection of transfused RBCs, as described previously[34,35]. In this approach, the non-fluorescent donor RBCs are detected and quantified against the background of fluorescent, eGFP-expressing recipient RBCs. The experiment was repeated three times in its entirety (n=24 per group total).

Statistical analysis

All statistical analyses were conducted using Prism 6 (Graph Pad Software, La Jolla, CA, USA). All column graphs in figures represent mean and standard deviation. Differences among means were analysed by one-way ANOVA with Tukey's post-test.

Results

Generation of cohorts of blood donor mice with different iron status by dietary manipulation

Three groups of weanling mice (n=15-20 per group in each experiment) were fed either an iron-replete or an iron-deficient diet for six weeks. A third group was fed the iron-deficient diet and was phlebotomised weekly to induce severe iron-deficiency anaemia. After six weeks on the diet, the mice were euthanised and blood collected by exsanguination. The average haemoglobin per group (in g/dL ±1 SD) decreased significantly from normal (16.5±0.4) to iron-deficient erythropoiesis (11.5±0.4) to frank iron-deficiency anaemia (7.0±1.4) (Figure 2A). In addition, liver iron measurements (in ng iron/mg tissue ±1 SD) (Figure 2B) demonstrated that iron stores were virtually absent in mice with either iron-deficient erythropoiesis or iron-deficiency anaemia (26.6±2.7 and 28.2±3.0, respectively); in contrast iron-replete mice had normal iron stores (84.6±12.4)[38]. Finally, zinc protoporphyrin levels (in µmol/mol haeme ±1 SD) were quantified in the pooled blood from each group (Figure 2C); these levels increased in proportion to decreasing iron status, measuring 59.0±9.9, 158.8±4.6, and 230.0±33.9, respectively, in iron-replete mice, mice with iron-deficient erythropoiesis, and mice with severe iron-deficiency anaemia.

Other aspects of the complete blood count further confirmed the validity of this mouse model of iron deficiency. Thus, the decreasing trend in the number of circulating RBCs with decreasing body iron stores was significant ($p<0.05$) (Figure 3A). In addition, the mean corpuscular volume was normal in iron-replete mice, but equivalently decreased in mice with mild iron deficiency and iron-deficient erythropoiesis and in those with iron-deficiency anaemia (Figure 3B); as expected, the mean corpuscular haemoglobin concentration was virtually the same across all three groups (Figure 3C). As a marker of dysregulated erythropoiesis, the RBC distribution width increased progressively in proportion to decreasing iron status (Figure 3D). Finally, similar to what has been observed in humans[39], murine platelet counts also increased progressively in proportion to decreasing iron status ($p<0.01$ for linear trend in data) (Figure 3E); nonetheless, as expected, there were no significant changes in the white blood cell counts (WBC) among these three groups of mice (Figure 3F).

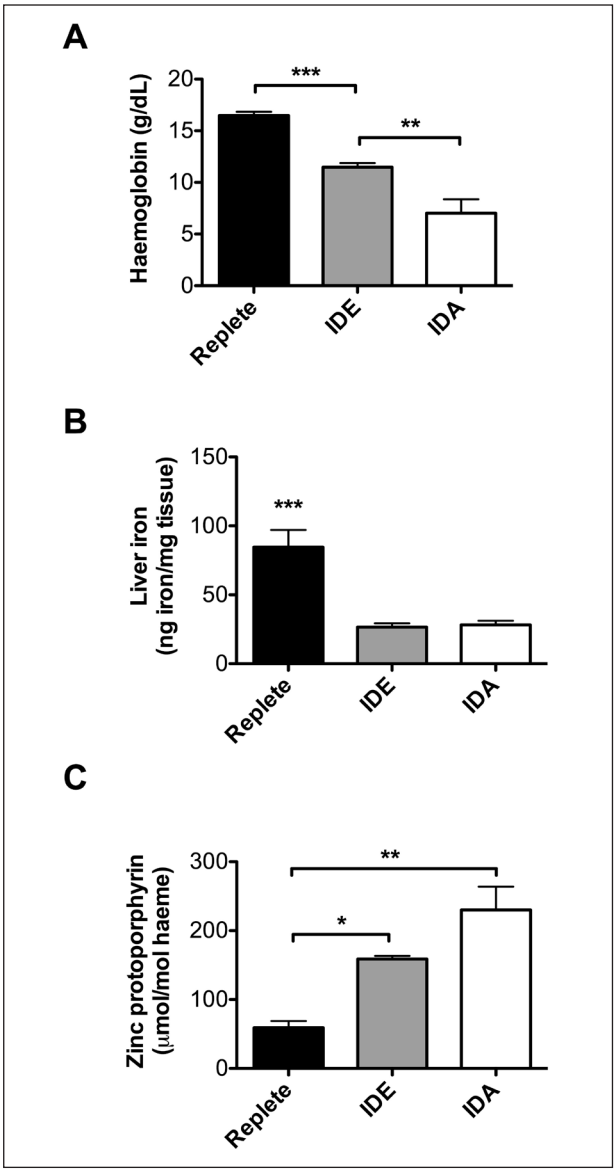

Figure 2 - Blood donor mice with varying iron status were generated by dietary manipulation.
Three groups of weanling mice (N=15-20 per group) were fed either an iron-replete or iron-deficient diet (IDE) for 6 weeks. A third group was fed the iron deficient diet and was phlebotomised weekly to induce iron deficiency anaemia (IDA). After 6 weeks on the diet, the mice were euthanised and blood collected by exsanguination. (A) The average haemoglobin per group across 3 replicate experiments. (B) Liver iron, as a measure of total body iron, quantified in 5 representative mice per group from a single experiment. (C) Zinc protoporphyrin levels in the pooled blood from each group across two representative experiments (these measurements were not performed in one of the replicate experiments). *p<0.05, **p<0.01, ***p<0.001 by One-way ANOVA with Tukey's multiple comparison test. ***Without brackets in panel B represents significance compared to both other groups.

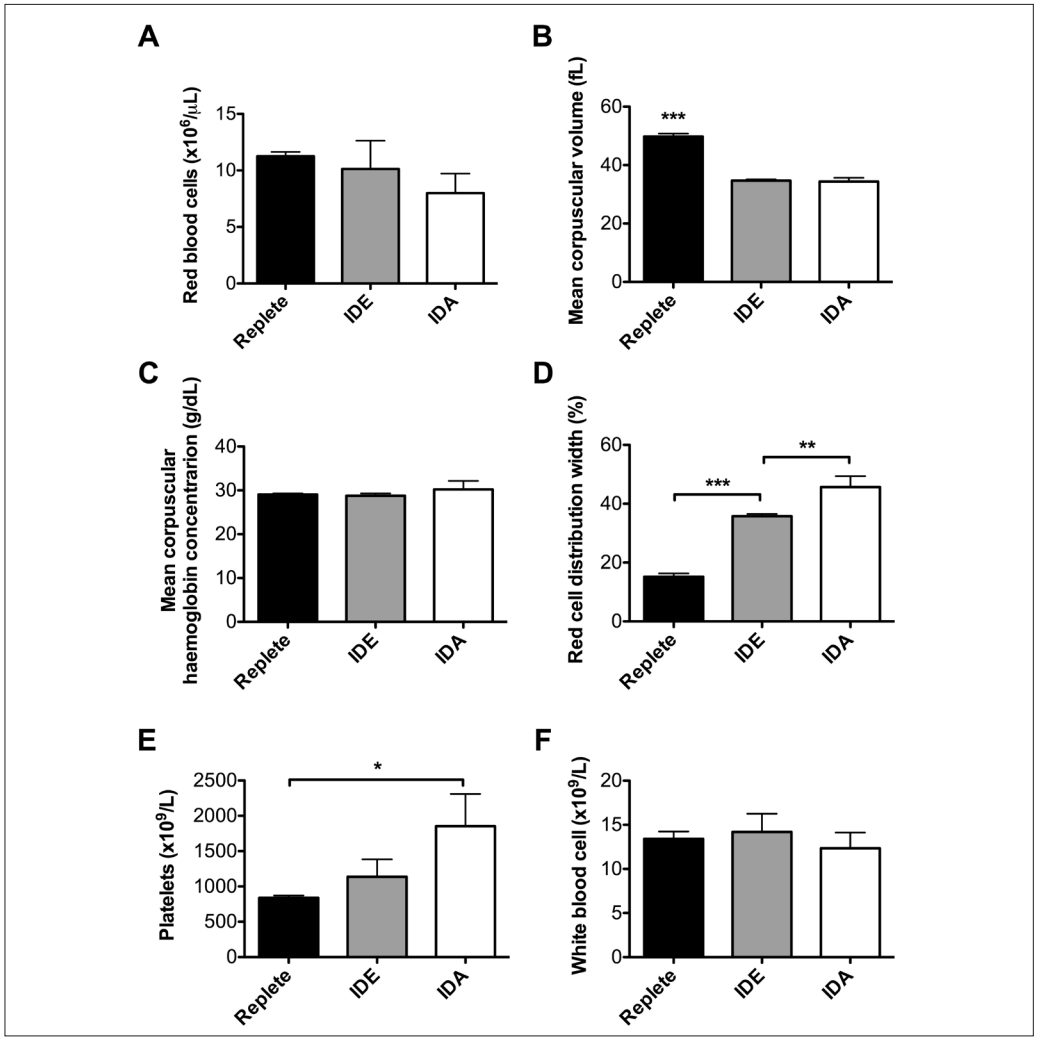

Figure 3 - Additional laboratory parameters demonstrating the validity of the mouse model of variable iron status.

Additional parameters were measured on the animals described in Figure 2. Three groups of weanling mice (N=15-20 per group) were fed either an iron-replete or iron-deficient diet (IDE) for 6 weeks. A third group was fed the iron deficient diet and was phlebotomised weekly to induce iron deficiency anaemia (IDA). After 6 weeks on the diet, blood was collected from the saphenous vein and pooled per cage in each group. The measurements are as labeled in the figure panels. *$p<0.05$, **$p<0.01$, ***$p<0.001$ by One-way ANOVA with Tukey's multiple comparison test. ***Without brackets in panel B represents significance compared to both other groups.

Post-transfusion RBC recovery studies

Similar to prior results[40], refrigerator-stored, transfused RBCs from iron-replete donors into eGFP congenic, healthy recipients had a mean 24-h post-transfusion recovery of 77.1% ±13.2%, as expected (Figure 4). In addition, as expected from human studies and various animal models[14,28,32], the 24-h post-transfusion recovery was decreased using RBCs from donors with severe iron-deficiency anaemia (mean 46.7% ±15.9%; $p<0.001$) whereas the results using donors with mild iron deficiency and iron-deficient erythropoiesis were intermediate: 66.5% ±10.9% ($p<0.05$ compared to iron-replete mice).

Discussion

These results show that murine donor iron deficiency significantly reduced 24-h post-transfusion RBC recovery in healthy recipient mice. RBCs obtained from mice with haemoglobin levels similar to the cut-off used for autologous blood donation in humans, but with mild iron deficiency and iron-deficient erythropoiesis, exhibited intermediate post-transfusion RBC recovery, as compared to iron-replete donors and those with iron-deficiency anaemia. The data in Figures 2 and 3 demonstrate the development of a mouse model of iron deficiency, producing three mouse cohorts with different iron status: (1) iron replete, (2) mild iron deficiency

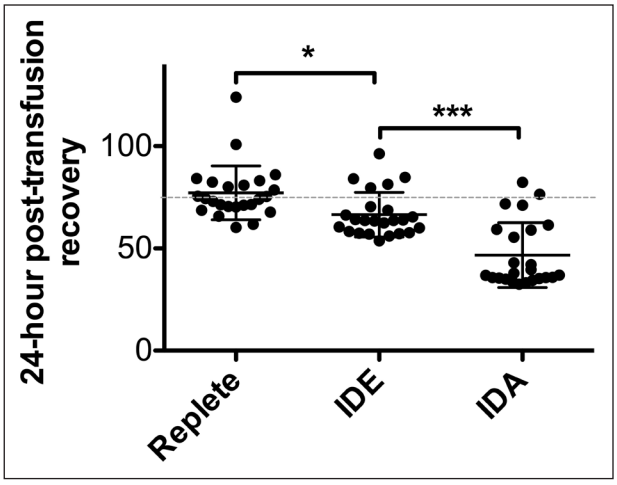

Figure 4 - Donor RBCs from mice with iron deficiency exhibit decreased 24-hour post-transfusion recovery.
Three groups of adult mice with varying iron status: iron replete, iron-deficient erythropoiesis with mild iron deficiency (IDE), and severe iron deficiency anaemia (IDA) were generated by modifying the iron-content of their diets with or without additional phlebotomy. At ~10 weeks of age, blood was collected from each cohort, pooled, leucoreduced, packed, and refrigerator-stored in CPDA-1 for 12 days. The 24-hour post-transfusion recoveries of the stored RBCs were quantified using flow cytometry in enhanced Green Fluorescent Protein-transgenic recipient mice. The experiment was repeated three times in its entirety (n=8 recipient mice per group per experiment; 24 mice per group total). Shown are the individual 24-hour post-transfusion recovery results for each recipient mouse, combined across all three replicate experiments. *p<0.05, ***p<0.001 by One-way ANOVA with Tukey's multiple comparison test.

with iron-deficient erythropoiesis, and (3) severe iron-deficiency anaemia. The storage quality of the RBCs obtained from murine donors with mild iron deficiency and iron-deficient erythropoiesis was suboptimal, with a mean 24-h RBC recovery of only 66.5%. These units did not average a 75% recovery and, thus, would not pass the United States Food and Drug Administration regulatory criteria for adequacy. Therefore, these results in a mouse model provide evidence of the need for clinical evaluation of the effects of donor iron deficiency in humans. For patients with urgent transfusion requirements, RBC units with diminished RBC recovery may, or may not, increase the risk of harm[41]. However, in the chronic transfusion setting (e.g., for transfusion-dependent patients with sickle cell disease, ß-thalassaemia major, or other forms of refractory anaemia), transfusing the best quality RBC units, with the highest post-transfusion recoveries, would reduce the number of transfusions required over a lifetime and, thus, reduce the lifetime burden of iron overload[42].

One limitation of this study is that the murine cohort with iron-deficient erythropoiesis is slightly more affected than that found in humans who meet typical RBC donation standards. Thus, these mice with mild iron deficiency and iron-deficient erythropoiesis have decreased RBC mean corpuscular volumes and haemoglobin concentrations and a greater elevation in zinc protoporphyrin levels than typically seen in humans with iron-deficient erythropoiesis. Nonetheless, the trend in our results suggests a strong correlation between body iron stores and post-transfusion RBC recovery after refrigerated storage, with decreased recovery expected in donors with iron-deficient erythropoiesis. We acknowledge the truism that "mice aren't human" and, in the current case, that our mouse model of iron-deficient erythropoiesis does not exactly correspond to the human counterpart in every possible way (Figures 2 and 3)[43].

Conclusions

In conclusion, it will be important to determine whether or not similar findings are observed in human volunteers. Such a randomised clinical trial is now beginning and will study autologous RBC transfusions in otherwise healthy volunteers with documented iron-deficient erythropoiesis (i.e., the Donor Iron-Deficiency Study. Registered at: clinicaltrial.gov NCT02889133). The results of this human trial will determine whether the adverse effects of iron deficiency are limited to donors or also compromise the quality of RBC units from iron-deficient donors who meet current standards for RBC donation.

Disclaimer

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Authorship contributions

EAH and SLS designed the studies. SB performed the experiments with the assistance of EAH. SB, GMB, EAH, and SLS evaluated and interpreted the data. EAH and SLS prepared the first draft of the manuscript. All Authors read, edited, and approved the final manuscript. EAH and SLS contributed equally to this work.

The Authors declare no conflicts of interest.

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The purified vepoloxamer prevents haemolysis in 42-day stored, DEHP/PVC-free red blood cell units

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Background. Use of the plasticiser di(2-ethylhexyl) phthalate (DEHP) in polyvinyl chloride (PVC) blood bags poses a potential dilemma. The presence of DEHP in blood bags has been shown to be beneficial to red blood cells during storage by diminishing haemolysis. However, DEHP use in PVC may be carcinogenic or estrogenising. Vepoloxamer is a poloxamer with rheological and cytoprotective rheological properties and a favourable toxicity profile in clinical trials. We hypothesised that vepoloxamer may be sufficient to replace the plasticiser DEHP to prevent elevated haemolysis while conserving the biochemical and redox potential++ in RBCs stored for up to 42 days.

Materials and methods. Paired analyses of aliquots from pooled RBC suspensions of ABO identical donors were aseptically split into test storage containers (DEHP/PVC or DEHP-free/ethylene vinyl acetate [EVA]) supplemented with or without vepoloxamer (at concentrations of 0.1, 1, 5 or 7.89 mg/mL) and cold stored for up to 42 days.

Results. Vepoloxamer significantly prevented the increased haemolysis induced by the absence of DEHP in EVA bags in a dose-dependent manner by days 28 and 42 of storage (approx. 50% reduction of the maximum concentration of vepoloxamer; $p<0.001$). There was an inverse correlation between the concentration of vepoloxamer used and the haemolysis rate ($r^2=0.27$, $p<0.001$) and a direct correlation between haemolysis and phosphatidylserine (PS) exposure ($r^2=0.42$; $p<0.01$). Increased osmotic fragility and shear induced deformability of 42-day stored RBC in EVA bags was significantly corrected by the addition of vepoloxamer.

Discussion. Vepoloxamer, in a concentration-dependent fashion, is able to partly rescue the increased haemolysis and PS exposure induced by the absence of the commonly used plasticiser DEHP. These results provide initial but strong evidence to support vepoloxamer use to replace DEHP in long-term storage of RBC.

Keywords: blood, red blood cell, virus, pathogen, inactivation.

Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is a plasticiser produced commercially to provide flexibility to an otherwise rigid polyvinyl chloride (PVC)[1] with concentrations in PVC of approximately 30%, although these can be up to 80%[2,3]. Since DEHP is non-covalently bound to the PVC polymer, its lipophilic nature means it can leach into the storage content of a container, especially when the surface comes into contact with lipid-containing fluids. The concentration of DEHP in whole blood (WB) or blood components can be efficiently measured[4] and increases upon storage[5,6], although absolute amounts depend on component composition and storage temperature. While most DEHP is found in fluid phase, especially bound to lipoproteins[7], a small proportion can be associated with red blood cells (RBCs)[8], resulting in intravenous exposure and circulating DEHP in blood[9,10]. The value of DEHP in RBC bags is not only related to the increased flexibility of the bag but also because it improves RBC survival during storage and after transfusion[11]. DEHP affects RBC integrity by inhibiting the deterioration of the RBC membrane, which prevents the haemolysis, microvesicle formation, and morphological changes that occur during refrigerated storage[12]. DEHP is associated with the RBC membrane and cytosol, and this is a potential mechanism for the increased RBC stability[13]. While the molecular mechanism of this protective role is not completely understood, some studies have demonstrated that DEHP protects against membrane damage by altering the interaction between phospholipids and pro-oxidant adenine nucleotide translocators in the cytoplasmic or mitochondrial membrane of cells[14].

Although human urinary excretion levels are usually below the exposure limits of the US Environmental Protection Agency (EPA) and other worldwide environmental agencies, extensive laboratory studies have been done into the long-term effect on health due to the cumulative exposure in massive transfusion protocols and/or chronic exposure to DEHP and possible synergistic endocrine effects. DEHP undergoes metabolic degradation resulting in the formation of the monoester, mono(2-ethylhexyl)phthalate (MEHP), and further oxidative forms[15]. MEHP has been shown to be carcinogenic *via* the activation of two nuclear transcription factors, PPARα and PPARγ, important to cell differentiation[16]. In addition, DEHP is a developmental and reproductive toxicant suspected of having endocrine disrupting or modulating effects[17] and promoting inflammation[18].

As a consequence of the harmful effects of DEHP, alternative methods to replace the protective effect of DEHP on haemolysis are warranted. A possible alternative is the use of a rheological agent with a favourable toxicity profile that mimics the beneficial effects of DEHP. Vepoloxamer is a highly purified form of the linear non-ionic amphiphilic copolymer poloxamer 188 (Figure 1). It is comprised of an internal hydrophobic polyoxypropylene chain flanked at either end by hydrophilic polyoxyethylene blocks. It exhibits rheologic, anti-thrombotic and cyto-protective properties *in vitro* and *in vivo*[19-31]. Its basic structure confers surface-active properties that enable the modulation of the biophysical properties of the cell membrane, including stability, hydration repair, flexibility, and adhesive properties, all of which serve crucial roles in biological responses. Vepoloxamer inhibits polymer-induced RBC aggregation and adhesion to endothelial cells[32,33]. Substantial research has demonstrated that vepoloxamer has cytoprotective and haemorheologic properties, and inhibits inflammatory processes and thrombosis[19,22,29,34]. The drug has been used in clinical trials of sickle cell patients with vaso-occlusive disease including a Phase III, double blind, placebo-controlled trial (registered as NCT01737814). In this last trial, although vepoloxamer was found not to reduce the duration of the vaso-occlusive disease in sickle cell patients when compared with a placebo control, the drug was found to be well tolerated and safe when administered in healthy and seriously ill humans.

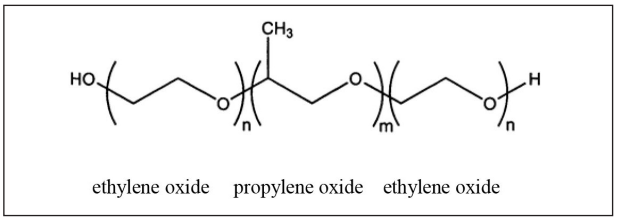

Figure 1 - Chemical formula for vepoloxamer. Vepoloxamer consists of repeated ethylene and propylene oxide groups. With n=80 and m=27, Vepoloxamer has a calculated molecular weight of 8,624 Daltons.

This study was designed to determine whether vepoloxamer could be used in RBC storage additive solutions to replace the effect of DEHP in reducing the haemolysis rate after long-term storage, and, if it proved to be protective, to explore the mechanisms involved in improving RBC viability *in vitro*.

Materials and methods

Blood collection and processing

Whole blood (500 mL ±10%) from 30 consenting donors aged 18-70 years old who fulfilled American Association of Blood Banks (AABB) and US Food and Drug Administration (FDA) criteria for donation (except for travel) was collected into a collection set with CPD anticoagulant (Code 4R3329, Fenwal Inc., Lake Zurich, IL, USA). Units were leucoreduced using the integral RS-2000 filter and stored in additive solution AS-1 within 8 hours of collection. Pools of two RBC suspensions from ABO identical donors aseptically split into five aliquots containing 95 mL of RBC suspension, and stored either DEHP/PVC or DEHP-free/ethylene vinyl acetate (EVA) bags (Evolve EV-12+F-M12, Origen Biomedical, Austin, TX, USA), supplemented with or without 5 mL of GMP-grade vepoloxamer (Figure 1) or saline control and mixed before storage. Groups of paired analysis consisted of DEHP/PVC with no vepoloxamer (with added physiological saline solution, n=15), EVA with no vepoloxamer (with added physiological saline solution, n=15) and EVA with vepoloxamer at concentrations of 0.1 (n=9), 1 (n=15), 5 (n=15) or 7.89 (n=6) mg/mL in physiological saline solutions. RBC units were cold stored (1-6 ºC) for 42 days.

Methods

For all aliquots, a complete blood count was analysed (Coulter Ac.T5 Diff CP analyzer, Coulter Corp., Miami, FL, USA). Spun haematocrit was determined using a microhaematocrit centrifuge, as previously described[35]. Supernatant haemoglobin and haemolysis rates were calculated as previously described[36]. pH, pO_2, pCO_2, extracellular glucose, sodium, potassium and lactate concentrations were measured using ABL805 FLEX Analyzer (Radiometer, Copenhagen, Denmark) following the manufacturer's instructions. Intracellular adenosine-5'-triphospate (ATP) levels were determined as previously described[37]. Osmotic fragility was analysed as previously described[38] and deformability under shear

stress conditions was assessed by ektacytometry at a maximum pressure of 60 Pa[39]. The above listed analyses were performed on days 28 and 42 of storage.

Also on day 42 of storage, oxidative stress was assessed by determination of the oxidised glutathione/reduced glutathione (GSSG/GSH) ratio[40]. Oxidised peroxiredoxin-2 (PRX2) levels were analysed by Western blot as previously described[41], and band density analysed by the Image J software[42] in relation to the loading control developed with anti-β-actin (Sigma, St. Louis, MO, USA) and normalised to the values of the DEHP group. Eryptosis was analysed by determination of the percentage of cells with exposed extracellular phosphatidylserine (PS) residues as assessed by annexin-V binding and flow cytometry analysis. Briefly, RBCs were washed in annexin-V binding buffer containing (in mM) 125 NaCl, 10 HEPES, pH 7.4, and 5 $CaCl_2$ (BD Biosciences, San Jose, CA, USA). Erythrocytes were stained with annexin-FITC (BD Biosciences) at a 1:10 dilution. After 15 min, samples were washed with annexin-V binding buffer and measured by flow cytometry (FACSCalibur; Becton Dickinson). Annexin-V binding was analysed on a gate of appropriate forward and sideward scatter (logarithmic transformation) and annexin-V fluorescence intensity was measured in FL1.

Data are presented as average ±1 standard deviation (SD). Comparative statistical analysis was performed by ANOVA test with Bonferroni correction. $p<0.05$ was considered statistically significant.

Results

As expected, the absence of DEHP in EVA bags results in increased haemolysis by days 28 and 42 of storage (Figure 2A and B). Addition of vepoloxamer significantly reverses the increased haemolysis induced by removal of DEHP in EVA bags in a dose-dependent manner by day 28 and day 42 of storage (Figure 2A and B). Addition of vepoloxamer at the highest concentration tested (7.89 mg/mL) resulted in reduction of haemolysis at levels similar to the levels observed in the PVC/DEHP control. The haemolysis rate was inversely correlated with increasing concentrations of vepoloxamer ($r^2=0.27$, $p<0.001$) indicating an association between both parameters. Similarly, the lack of a plateau phase suggests that the highest concentration of vepoloxamer may have not reached the peak of its biological effect on haemolysis prevention. A similar effect was observed by day 28 and especially by day 42 of storage on RBC osmotic fragility (Figure 2C and D) and deformability under shear stress at 60 Pa (Figure 2E and F), with a significant dose-dependent effect of vepoloxamer on the reversal of the increased osmotic fragility of day 42 stored RBC (Figure 2D) and shear-stress deformability of RBC stored in EVA bags (Figure 2E and F). These changes may have resulted in a reversal of the eryptosis of RBC when stored in EVA, as assessed by analysis of PS exposure through the determination of the percentage of RBC with the ability to bind annexin-V in units containing increasing concentrations of vepoloxamer by day 42 of storage (Figure 3A), with a direct correlation between haemolysis and PS exposure ($r^2=0.42$; $p<0.001$). These results indicate that vepoloxamer prevents the RBC storage lesion associated with replacement of PVC/DEHP for EVA as constituent of the storage bags and are consistent with the hypothesised role of vepoloxamer as an RBC membrane intercalator that interferes with the process of eryptosis and *in vitro* haemolysis.

Interestingly, the amelioration of eryptosis and *in vitro* haemolysis is not associated with modifications in the concentrations of different biochemical parameters associated with metabolic, transmembrane potential or redox stress. There were no significant changes in ATP (Figure 3B), glycolytic flux as assessed by changes in the extracellular concentrations of glucose (Figure 3C) or lactate (Figure 3D), or potassium leakage (Figure 3E). Similarly, despite a modest reduction in oxidative stress of EVA-stored RBC compared with DEHP/EVA stored bags (Figure 3F), vepoloxamer addition did not result in any changes in the intracellular levels of oxidised peroxiredoxin-2 (PRX2) (Figure 3F) or of the intracellular levels of GSSG (Figure 3G) or in the overall intracellular concentration of the GSH (Figure 3H).

Discussion

In this study, we analysed for the first time the possibility of replacing the effect of the DEHP plasticiser used in PVC bags by adding vepoloxamer into malleable EVA bags by adding vepoloxamer at increasing concentrations. Our data support the concept that vepoloxamer does prevent the RBC storage lesion *in vitro* as it reduces the haemolysis, eryptosis and osmotic fragility, and increases the erythrocyte deformability under significant shear stress of up to 60 Pa. This effect seems to be unrelated to modifications in the energy production, glycolytic flux, cat ion exchange activity or redox potential, suggesting that its actions occur by direct interactions of the polymer with lipids and lipoproteins on the red cell membrane leaflets.

Toth *et al.*[32] demonstrated that unpurified vepoloxamer, at concentrations of 0.5-5 mg/mL, inhibited both the extent and strength of RBC aggregation in a dose-dependent manner in the presence of aggregating concentrations of Dextran-70. Vepoloxamer at a concentration of 5 mg/mL was more effective in improving the rheology of younger, less dense cells.

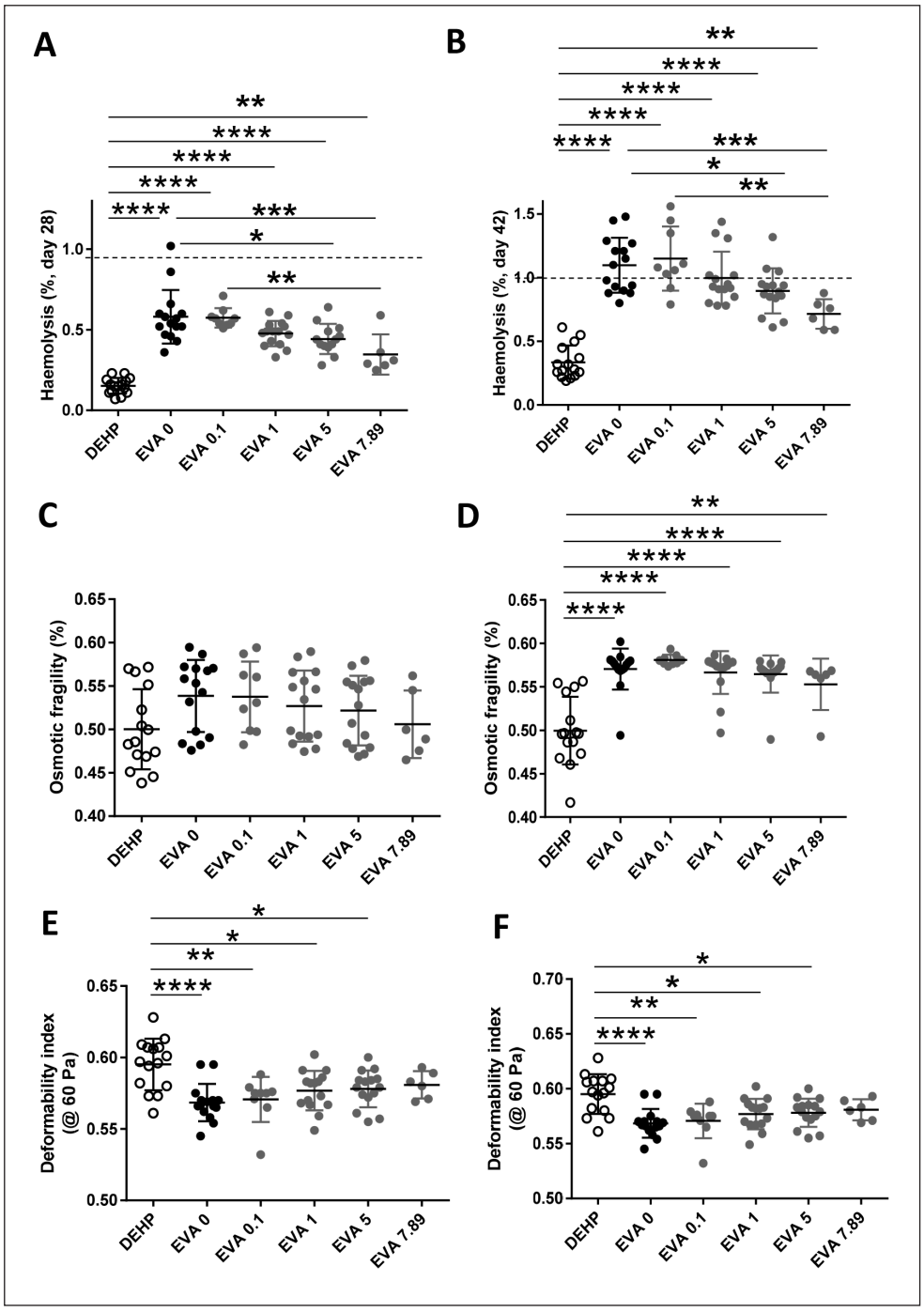

Figure 2 - Addition of vepoloxamer results in a dose-dependent reduction in haemolysis and fragility of red blood cells (RBC) stored in ethylene vinyl acetate (EVA) bags.
(A and B) Haemolysis. (C and D) Osmotic fragility. (E and F) Deformability index (ektacytometry at 60 Pa). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (ANOVA test with Bonferroni correction). DEHP: polyvinyl chloride bag with di(2-ethylhexyl) phthalate (DEHP) plasticiser.

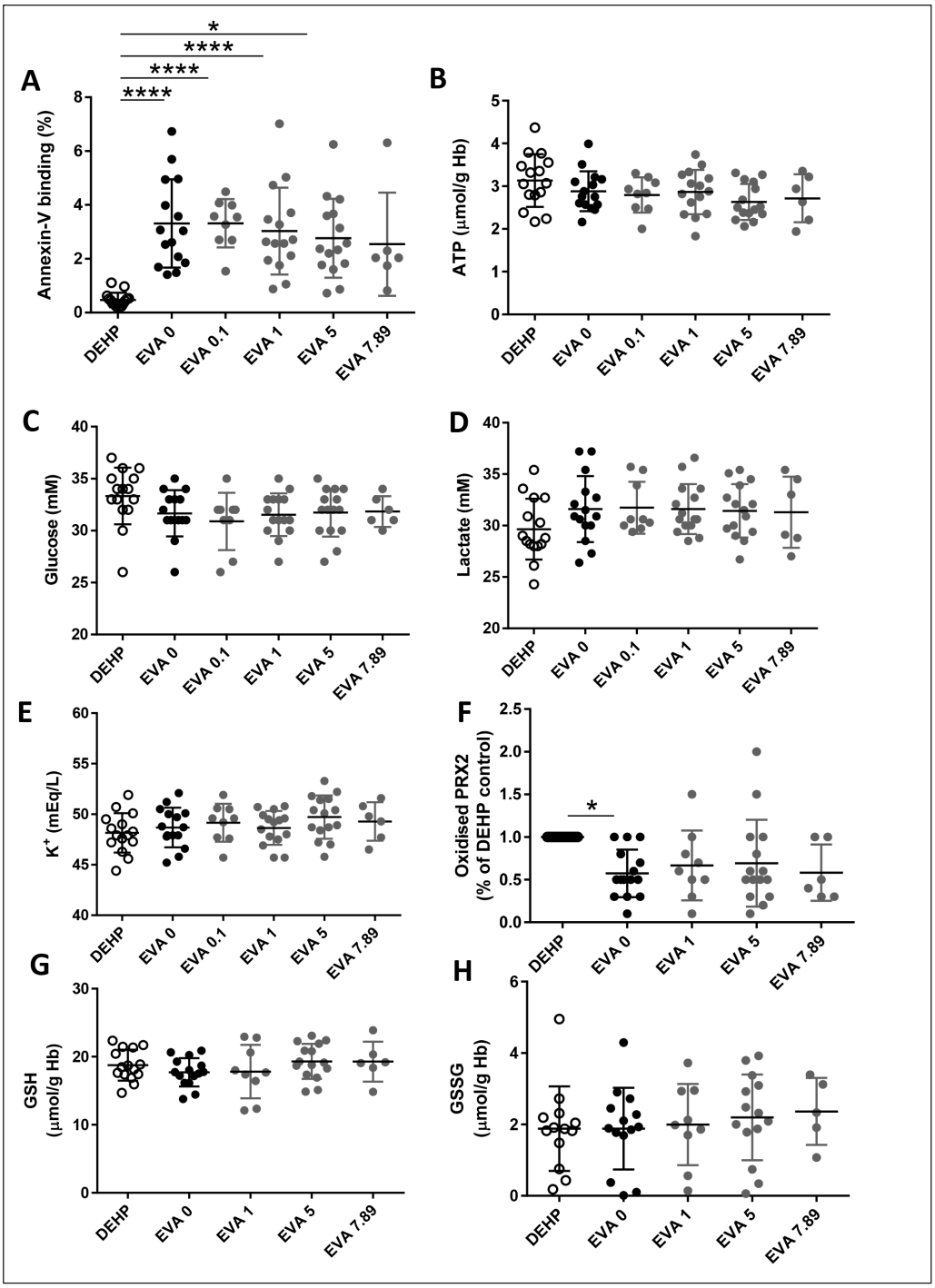

Figure 3 - Addition of vepoloxamer results in a dose-dependent reduction of eryptosis with no changes in glycolytic flux, potassium leakage or redox potential by day 42 of storage.
(A) Percentage of red blood cell (RBC) binding annexin-V. (B) Intracellular adenosine-5'-triphospate (ATP) concentration levels. Extracellular concentration of (C) glucose and (D) lactate. (E) Extracellular potassium concentration. (F) Intracellular level of oxidised PRX2. Intracellular levels of (G) reduced glutathione (GSH) and (H) oxidised glutathione (GSSG). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ (ANOVA test with Bonferroni correction). DEHP: polyvinyl chloride bag with di(2-ethylhexyl) phthalate (DEHP) plasticiser.

Based upon the depletion model for polymer-induced aggregation, these authors suggest that vepoloxamer acts by penetrating the depletion layer near the glycocalyx, thereby reducing the osmotic gradient between the intercellular gap and the suspending medium. Sandor *et al*.[33] also demonstrated that unpurified poloxamer 188 significantly reduces blood viscosity, and RBC aggregation and adhesion to endothelial cells, possibly by acting as an intercalating agent in cell membranes. Our results further support the effect of vepoloxamer on modifications of the RBC membrane that result in stabilisation as assessed by significant reduction of their osmotic fragility and increase in their deformability upon shear stress.

Conclusions

Vepoloxamer is able to significantly rescue the increased haemolysis induced by the absence of the commonly used plasticiser DEHP in a concentration-dependent fashion. While the biochemical/biophysical mechanism of this restoration remains unclear, the improved osmotic fragility and shear-stress deformability index strongly suggests that vepoloxamer may act as an intercalating agent with the ability to increase the RBC membranes flexibility and fitness to challenging storage-dependent rheological conditions. These results provide evidence that the rheological agent vepoloxamer may provide an alternative to the current use of potentially harmful plasticisers like DEHP for long-term RBC storage.

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Authorship contributions

JAC designed experiments, analysed data and wrote the manuscript. NR analysed data and supervised the study. SN and SEH performed experiments. RME and DSMc-K designed experiments. All Authors read the manuscript and contributed to the finalisation of the manuscript.

Disclosure of conflicts of interest

RME and DSMc-K are employees of Mast Therapeutics Inc. Mast Therapeutics Inc. provided study materials and funds to conduct this study. The other Authors declare no conflicts of interest.

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Enhancing uniformity and overall quality of red cell concentrate with anaerobic storage

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Background. Recent research focused on understanding stored red blood cell (RBC) quality has demonstrated high variability in measures of RBC function and health across units. Studies have historically linked this high variability to variations in processing, storage method, and age. More recently, a large number of studies have focused on differences in donor demographics, donor iron sufficiency, and genetic predisposition of the donor to poor storage, particularly through mechanisms of accelerated oxidative damage. A study was undertaken to evaluate a potential additional source of unit to unit variation in stored RBC: the role of variable percent oxygen saturation ($\%SO_2$) levels on blood quality parameters during storage.

Materials and methods. $\%SO_2$ data from 492 LR-RBC/AS-3 units used for internal and external collaborative research was included in the analysis. Whole blood units were processed into red blood cells, AS-3 added, leucocyte reduced, in compliance with American Association of Blood Banks guidelines. LR-RBC/AS-3 products were subsequently analysed for $\%SO_2$ levels within 3-24 hours of phlebotomy using a co-oximeter. Separately, to evaluate the impact of pre-storage as well as increasing levels of $\%SO_2$ during storage, a pool-and-split study was performed. Four units of LR-RBC/AS-3 were split 6 ways; "as is" (control), hyperoxygenated to more than 90%, and four levels of pre-storage $\%SO_2$. The units were periodically sampled up to 42 days and analysed for $\%SO_2$, pCO_2, methaemoglobin, ATP, 2,3-BPG as well as with the metabolomics workflow.

Results. The measured mean $\%SO_2$ in LR-RBC/AS-3 within 24 hours of collection was 45.9±17.5% with (32.7-61.0 IQR). $\%SO_2$ in all products increased to approximately 95-100% in three weeks. Measured blood quality parameters including ATP, % haemolysis, methaemoglobin, oxidised lipids, and GSH/GSSG indicated suppressed cellular metabolism and increased red cell degradation in response to higher $\%SO_2$ levels.

Discussion. The surprisingly high variability in starting $\%SO_2$ levels, coupled with negative impacts of high oxygen saturation on red blood cell quality indicates that oxygen levels may be an important and under-appreciated source of unit-to-unit variability in RBC quality.

Keywords: blood quality, red blood cell storage, oxygen saturation, oxidative stress, storage lesion.

Introduction

Blood transfusions save and/or sustain thousands of lives every day in the United States and across the globe. Improvements in pathogen screening, storage solutions, and leucocyte reduction have continued to improve the clinical benefits of blood transfusion and reduce the risks. Nevertheless, the biochemical and biomechanical degradation that occurs over the course of six weeks of storage has been well established. More than 80 million units of red blood cell (RBC) are collected and stored globally. Once RBCs are removed from circulation in donors and separated as red cell concentrate (RCC), they experience progressive damage during refrigerated storage (hypothermic storage lesions). Even when transfused within the current 6-week limit, stored RBC tend to exhibit lower quality (e.g. increased fraction of RBC removed after transfusion, compromised oxygen delivery capacity, reduced deformability) and increased toxicity, often manifested as the clinical sequelae of transfusion therapy[1-15]. This view is supported by a large and growing number of articles in the literature[4,6,7,9-14,16-24]. Oxidative damage is considered the major cause of various storage lesions[25-34]. Owing to the high concentration of haemoglobin and molecular oxygen in stored RBC, chemical oxidation of haemoglobin to form methaemoglobin and subsequent denaturation products are the major culprits of oxidative stress in

stored RBC[33]. Furthermore, oxygen is the substrate for both non-enzymatic oxidation of lipids in the membrane catalysed by the products of oxidised haemoglobin, as well as for the production of biologically active lipid oxidation products during storage. Of two main reactants for methaemoglobin formation during hypothermic storage, haemoglobin and oxygen, the former is regulated by maintaining a minimum donor haemoglobin requirement, while the latter is not specifically controlled or scrutinised. The accumulation of oxidative storage lesions is reduced by storing RBC under hypoxic conditions[35,36], as reducing O_2 during storage removes the primary initiating factor for oxidative stress.

In order to design and develop an optimal commercial disposable device to reduce oxygen content of RCC prior to refrigerated storage, we purchased a large quantity of fresh RCC from several sources over the past two years. All of the units were fresh (less than 24 hours from collection) and key parameters such as complete blood count (CBC), blood gas and haemoglobin oxygen saturation (%SO_2) values were recorded. This relatively large data set allowed us to examine for the first time the oxygen content, as measured by %SO_2, of a large number of leucoreduced RCC shortly after component separation. In this report, we present the %SO_2 distribution from 492 units of post-production RCC and show the kinetics of gain in %SO_2 during 42-day storage. We also describe the effect of oxygen content during six weeks of storage on several oxidative stress markers and discuss the potential implications of uncontrolled %SO_2 distribution on the blood supply.

Materials and methods

Blood products

Blood products, whole blood in CP2D anticoagulant (WB), packed RBC (component-separated RBC without additive solution) and/or leucoreduced red cell concentrate (LR-RCC in AS-3 additive solution) were purchased from collection facilities: BSC (Biological Specialty Corporation, Colmar, PA, USA); ResBC (Research Blood Components, Boston, MA, USA); Bonfils Blood Center, Denver, CO, USA; IBR/MBC (Innovative Blood Resources/Memorial Blood Center, St. Paul, MN, USA); RIBC (Rhode Island Blood Center, Providence, RI, USA). Blood was collected from healthy donors. Blood products from ResBC and BSC are collected from remunerated donors specifically for research use. At the time of donation, all blood donors signed an institutional review board approved informed consent form.

Methods for collection, leucoreduction and shipping of red cell concentrate products from each source

ResBC: WB (CP2D) and pRBC (without AS)

pRBC (no additive solution added) was either placed in 1-6 °C storage or held at ambient temperature until it was transported by courier to the New Health Sciences, Inc. (NHSi) Blood Service Lab (BSL), Cambridge, MA, USA, within 2-6 hours of collection. AS-3 additive solution was added and leucofiltered by Leukotrap SC RC System (Haemonetics, Braintree, MA, USA), 40 units/case (PN: 430-40) at ambient temperature as per the manufacturer's instructions. WB products were collected into Haemonetics CP2D/AS-3 sets and transported by courier at ambient temperature to NHSi BSL. WB units were sampled then further processed by centrifugation followed by the Compomat G5 (Fresenius-Kabi, Bad Homburg, Germany) to express the plasma and add AS-3 additive solution to the pRBC to make RCC in AS-3 additive. The RBC/AS-3 product was leucofiltered as per the manufacturer's instructions and RCC parameters were measured as below.

Whole blood products in CP2D: BSC, IBR/MBC and Bonfils BC

WB products were collected into Haemonetics CP2D/AS-3 set, then shipped overnight on wet ice (IBR/MBC), 1-10 °C (Bonfils) or at ambient temperature (BSC) to the NHSi BSL where it was processed to RCC as described above. Products from BSC were less than 18-hours old upon arrival at the NHSi BSL.

Leucoreduced RCC in AS-3: Bonfils BC

LR-RCC (AS-3) products were prepared from WB (CP2D) or by apheresis, packed at ambient temperature then shipped overnight to NHSi BSL.

RIBC: LR-RCC (AS-3)

LR-RCC (AS-3) products were prepared from WB (CP2D), packed in wet ice and transported *via* courier for delivery by or before 8:00 am next day to the NHSi BSL.

Extended storage of undisturbed RBC

For the oxygen uptake study, 10 full units of AS-3 LR-RCC in standard PVC RBC storage bag (HAE PN 126-92 or 126-93) were sampled at day 0, then stored upright in blood shoes (acrylic blood unit holders) at 1-6 °C in a blood bank refrigerator. At day 21, the bags were mixed thoroughly and sampled for blood quality testing, then placed back into undisturbed refrigerated storage until the last sampling at day 42.

Preparation of samples for %SO_2 dose response study

Each leucoreduced double red blood cell unit in AS-3 additive solution was split 6 ways: unprocessed control, and five levels of pre-storage %SO_2: hyper-oxygenated (90-98%), 20±2%, 10±2%, 5±2% and <3%. Hyperoxygenated unit was made by adding calculated volume of O_2 gas in the bag; for O_2-reduced subunits, RCC was circulated through neonatal membrane oxygenator (Sorin D100, Sorin, Arvada, CO, USA) as

per the manufacturer's instruction using N_2 and CO_2 sweep gas adjusted to achieve desired levels of $\%SO_2$ and pCO_2 (25±5mmHg, 37 °C). pCO_2 levels were unaltered for unprocessed control and hyperoxygenated units. Oxygen-reduced units were stored inside gas-tight canisters filled with N_2, with two packs of oxygen sorbent (SS-200, Mitsubishi Gas Chemical America, New York, NY, USA). Units were sampled bi-weekly for metabolomics workflow and blood gas/co-oximetry. Oxygen-reduced units were sampled inside a glove-box filled with N_2, separated into RBC pellet and supernatant fractions, snap frozen and stored at −86 °C, then shipped in dry ice to University of Colorado, Denver, USA, for metabolomics workflow[25]. Adenosine triphosphate (ATP), 2,3-bisphosphoglycerate (BPG) and haemolysis were measured at days 1, 21 and 42, as described below. RCC from 4 volunteer subjects were processed and analysed.

Laboratory measurements

Cell counts and RBC indices were measured by a blood cell analyser (Sysmex XE2100-D, Kobe, Japan), blood gas, total haemoglobin concentration and $\%SO_2$ were measured by ABL90 Flex (Radiometer, Copenhagen, Denmark) and supernatant Hb was measured by spectrophotometry (Hemocue PLS LOW HB, Brea, CA, USA) for haemolysis calculation. ATP and 2,3-BPG samples were deproteinised with TCA (DiaSys; Deutschland Vertriebs-GmbH, Holzheim, Germany; cat# G10784) stored frozen at −86 °C until measurement using a CE-marked ATP Hexokinase kit (DiaSysDeutschland Vertriebs-GmbH; reagent cat# 1 6201 99 10 021; standard cat# 1 6200 99 10 065) and the Roche IVD 2,3-BPG kit (Sigma-Aldrich, St. Louis, MO, USA; cat# 10148334001), a standard was prepared from 2,3-BPG pentacyclohexylammonium (Sigma-Aldrich; cat# D9134).

Results

A total of 492 units RCC (including 209 units component-separated from WB received after overnight shipment) from five different sources were examined for oxygen content as represented by $\%SO_2$. The data set is separated according to type of process; handling history and other pertinent characteristics such as duration of RBC exposed to leucocytes/platelets prior to component separation along with mean $\%SO_2$ values are summarised in Table IA and IB.

Table IA - $\%SO_2$ of leucoreduced red cell concentrate (RCC) obtained as is or manufactured into RCC from various suppliers.

Supplier	N	Mean (95% CI)	SD	Minimum	Median	Maximum	Skewness	Kurtosis	ANOVA
All	492	46.1 (43.5, 47.6)	17.3	11.4	43.8	96.5	0.54	−0.41	
Bonfils BC, Denver, CO, USA	22	35.6 (29.9, 41.3)	12.9	18.7	34.4	68.9	1.33	1.99	
BSC (Biological Specialty Co.), Colmar, PA, USA	83	39.8 (36.1, 43.4)	16.5	11.4	35.6	82.8	0.8	−0.12	
Memorial BC, St. Paul, MN, USA	9	41.2 (28.4, 54.0)	16.3	21.5	40.7	74.1	0.79	0.59	Note 1
Research Blood Components, Boston, MA, USA	136	58 (55.0, 61.0)	17.5	19.1	59.2	94.6	-0.17	−0.53	
Rhode Island BC, Providence, RI, USA	243*	42.7 (40.9, 44.5)	14.4	16	39.7	96.5	0.78	0.51	
All suppliers except research blood components	367	41.6 (40.0, 43.1)	15	11.4	38.8	96.5	0.77	0.26	Note 2

Note 1: there is a statistically significant effect for $\%SO_2$ by supplier ($p<0.001$). Note 2: No significant statistical difference between suppliers for $\%SO_2$ if Research Blood Components (ResBC) is excluded ($p=0.105$). *Includes n=15 with modified saline-adenine-glucose additive in place of AS-3, with $\%SO_2$=44.3±12.5. CI: confidence interval; SD standard deviation.

Table IB - $\%SO_2$ from various blood product types as received.

Category of blood product	N	Mean (95% CI)	SD	Minimum	Median	Maximum	Skewness	Kurtosis	ANOVA
Received as whole blood	209	34.4 [32.0, 36.8]	17.6	4.8	29	88.2	0.73	−0.31	
Received whole blood leucoreduced	105	36.5 [33.1, 39.8]	18.5	8.4	32.5	82.0	0.66	−0.4	Note 3
Received as leucoreduced red cell concentrate#	243	42.7 [40.9, 44.5]	14.4	16.0	39.7	96.5	0.78	0.51	

Note 3: there is a statistically significant effect for $\%SO_2$ by product type ($p<0.001$). #Leucoreduced within 8 hours and shipped to NHSi laboratory in Cambridge, MA, USA. CI: confidence interval; SD standard deviation.

Distribution of collected whole blood and processed RCC within 42 hours of blood collection

The $\%SO_2$ of 492 units of RCC, all measured within 24 hours of phlebotomy, is shown in Figure 1. Based on a probability plot to determine normality of the distribution, it was not normal according to the Anderson-Darling test ($p<0.005$; *data not shown*). The mean was 46.1% with a standard deviation of 17.3% with a surprisingly large range of 11.4-96.5%. The mean was significantly lower than expected when compared to the normal value for $\%SvO_2$ as measured from a central venous line (approximately 74%)[37]. In order to further examine the source of the wide pre-storage $\%SO_2$ distribution, the blood sources, handling, and product types of all measured RCC units were further investigated (Table I). From five different suppliers who provided whole blood, leucoreduced whole blood, non-leucoreduced and leucoreduced pRBC, all units were processed to leucoreduced RCC within 24 hours of collection (Table IA). Comparing five suppliers, LR-RCC from ResBC showed 16.4% higher $\%SO_2$ than the other four. Whole blood shipped overnight showed lower oxygen levels compared to leucoreduced RCC made at the collection site and shipped overnight to our laboratory, while leucoreduction processed at our laboratory caused a small increase in $\%SO_2$ (Table IB).

Estimating oxygen absorbed by RCC during storage

Unless stored in an oxygen-free environment or wrapped in an oxygen barrier film, RBC gradually absorb oxygen through the PVC storage bag. We examined this rate by storing 10 whole units of RCC (leucoreduced in AS-3 additive) in a standard PVC bag that was a part of the blood collection kit. Units were sampled on days 0 or 1, 21 and 42. During storage, units were undisturbed, except for mixing to obtain samples at day 21. The results from individual RCC units are shown in Figure 2A. During the 42-day shelf life of these RCC, the rate of $\%SO_2$ can be empirically fitted with a power function.

The oxygen absorption rate depends on the frequency and extent of mixing, as well as how much of the bag surface is exposed to ambient air during storage. To provide a rough estimate of how this oxygen absorption through the PVC bag during RCC storage might affect $\%SO_2$ distribution, Figure 2B was generated by applying an empirical equation obtained from data in Figure 2. For this specific storage configuration, three points in each unit can be empirically fit by a power function with R^2 more than 0.99:

$$\%SO_{2\,(\text{day } x)} = \%SO_{2\,(\text{day 1})} \times t_{(x\,\text{day})}^{\,(k)};$$
$$k = 0.3187 - 0.00356 \times \%SO_{2\,(\text{day 1})} \quad (1)$$

where $\%SO_2$ (day) is simulated $\%SO_2$ at day x with the initial $\%SO_2$ (day 0 or 1) at day t, and the exponent k is a function of the initial value of $\%SO_2$. Using this simulation, the distribution on Figure 1 with a mean $\%SO_2$ increased from 45.9±17.6% (range 11.4-96.5%) at day 0 to 77.1±12.5% (range 32.2-100%) after day 42 of storage.

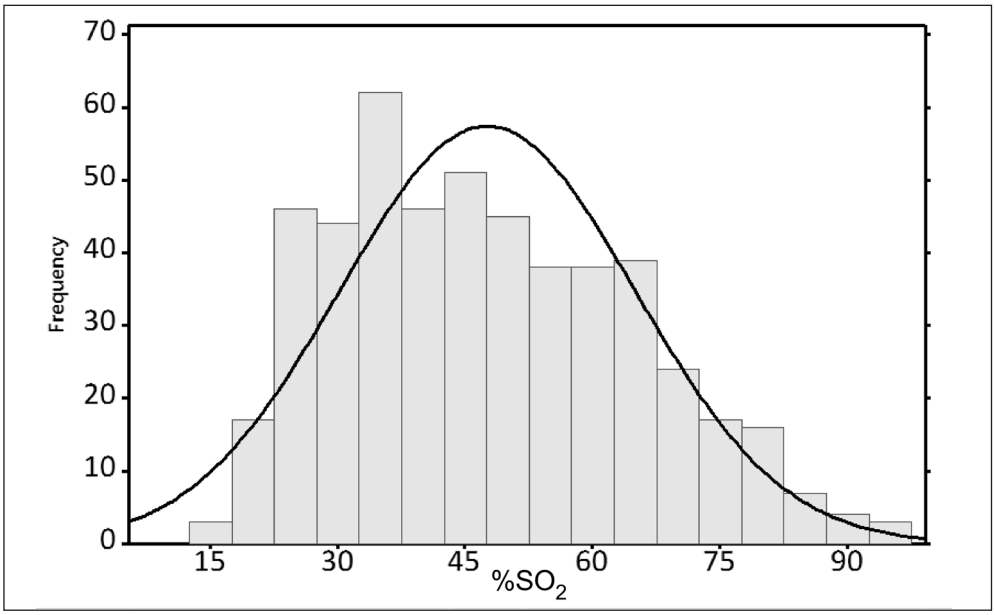

Figure 1 - Histogram of the incoming $\%SO_2$ measured after leucoreduction.
The overall distribution of leucoreduced red cell concentrate failed an Anderson-Darling test for normality when viewed on a probability plot ($p<0.005$). It was skewed right (skewness 0.54, kurtosis −0.041), though the shape of the distribution is generally normal. Likely the mean SO_2 (46.1% 95% CI [43.5, 47.6]) is a valid measure of central tendency, though median SO_2 (43.8%) is also reported. SO_2: oxygen saturation.

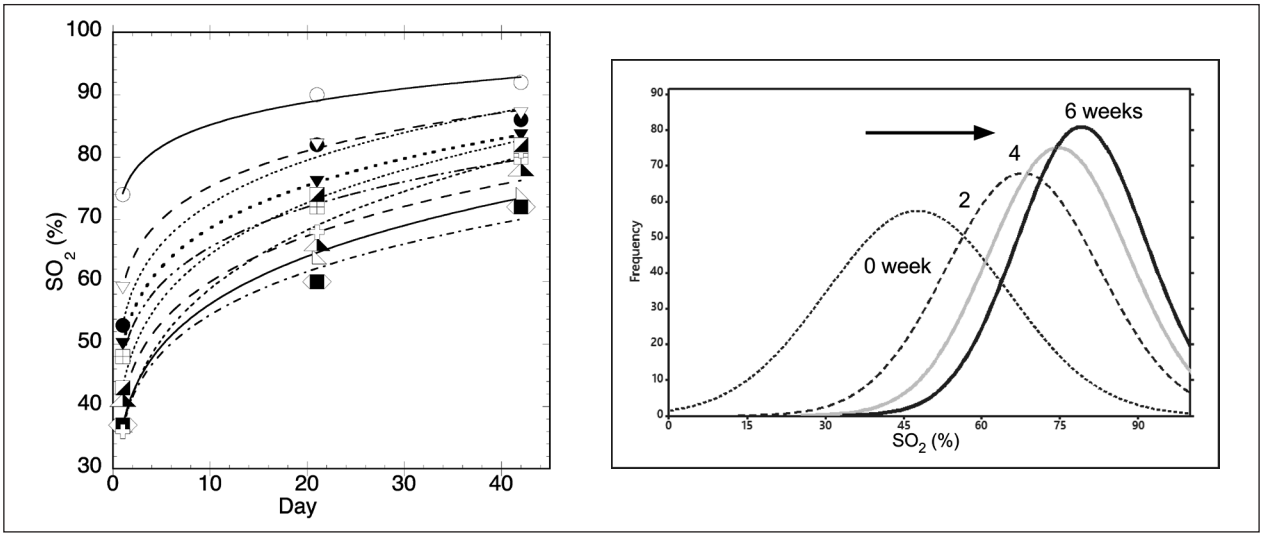

Figure 2 - Oxygen uptake by red cell concentrate (RCC) stored undisturbed during 6-week storage.
(Left) $\%SO_2$ gain measured in 10 full units during six weeks of storage. Except for mixing and sampling at day 21, units were undisturbed at 4 °C. Lines are empirical fit using power function. (Right) Visualisation of oxygen gain during storage. $\%SO_2$ distribution from Figure 1 was transformed for weeks 2, 4, and 6 based on data from the left panel using an empirical simulation algorithm (equation 1 in the Results section). $\%SO_2$ is shown in X-axis as in Figure 1. Three bars on the right represent frequency of units that were calculated to be above 90%. Numbers above curves and bars represent weeks in storage. $\%SO_2$: percent oxygen saturation.

Effects of oxygen content on the quality of stored RBC

Although evidence for accumulation of oxidative storage lesions is abundant in the literature[24,26,31,38], the relationship between the dose of oxygen and the extent of oxidative damage has not been investigated systematically. We undertook a study to examine this relationship with outcome parameters in standard blood quality as well a full, non-specific "omics" workflow (D'Alessandro *et al., 2017; unpublished manuscripts*). From each double RBC unit obtained from a single donor, we prepared 6 split units with differing levels of pre-storage $\%SO_2$. In this study, a smaller volume of RCC (initially 100 mL in a 150 mL transfer bag) was mixed and sampled bi-weekly. This bi-weekly mixing and sampling leads to a higher oxygen gain rate compared to the full unit storage (Figure 2), reaching 100% saturation in three weeks for unprocessed control units stored in ambient air. On the other hand, $\%SO_2$ levels slowly decreased in the O_2-depleted units as they were stored in an oxygen-free atmosphere. Instead of rapidly gaining oxygen, these units lost nearly 50% of haemoglobin oxygen saturation during 42 days (20% to 11%, 10% to 5%, 5% to 3% and 3% to less than 1.3%; well below accurate detection capabilities of the the co-oximeter).

Free intracellular methaemoglobin is an unstable molecule that readily denatures into haemichromes and then to globin and haemin during refrigerated storage of RBC. However, it is apparent from Figure 3B that the steady-state concentration of methaemoglobin is $\%SO_2$-dose dependent and it appears to reflect $\%SO_2$ levels at each sampling time.

Representative biomarkers reflective of the overall redox status or oxidative damage are shown in Figure 4A-C. Glutathione (GSH)/glutathione disulfide (GSSG) levels are an indicator of the overall redox environment in the RBC cytosol and are inversely dose dependent with $\%SO_2$ levels, especially at the end of storage with the exception of the lowest $\%SO_2$ unit (Figure 4A).

In order to examine levels of lipid oxidation during RCC storage, we examined selected oxidation products of polyunsaturated fatty acids using a quantitative lipidomics workflow[39] (Figure 4B and C). 16- and 17-hydroxydocosahexaenoic acid (HDoHE) as well 8-, 9-, and 11-hydroxyeicosatetraenoic acid (HETE) are non-enzymatically oxidised products of docosahexaenoic acid and arachidonic acid, respectively. Significant reductions in accumulated oxidation products of polyunsaturated fatty acids were observed with low $\%SO_2$ storage.

The effects of $\%SO_2$ levels on standard parameters of RBC storage quality are shown in Figure 4D-F. ATP levels are reduced in both the control and high $\%SO_2$ levels compared to less than 20% SO_2 after 21 days (Figure 4D). The hyperoxygenated units showed significantly lower ATP levels compared to the unprocessed control. No apparent oxygen dose response was observed below 20%. 2,3-BPG levels are significantly elevated on day 1 and day 21 for all oxygen-reduced units regardless of $\%SO_2$ level compared to

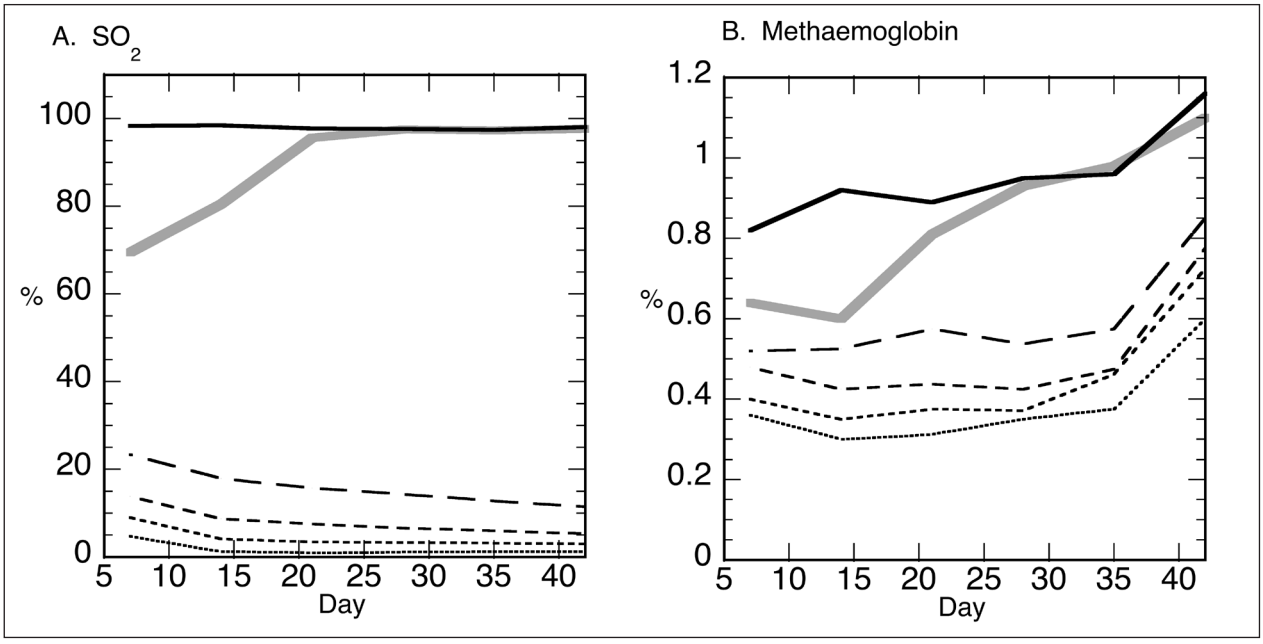

Figure 3 - Changes in $\%SO_2$ levels and concentration of methaemoglobin.
Parameters are measured weekly using co-oximeter/blood gas analyser. (A) $\%SO_2$ levels during storage. (B) Methaemoglobin levels during storage. Set pre-storage $\%SO_2$ values at day 0: from bottom to top lines; < 3% (. . .); 5% (- - -); 10%, unprocessed (– –); 20% (— —); unprocessed (thick gray line); and more than 90% (solid line). $\%SO_2$: percent oxygen saturation.

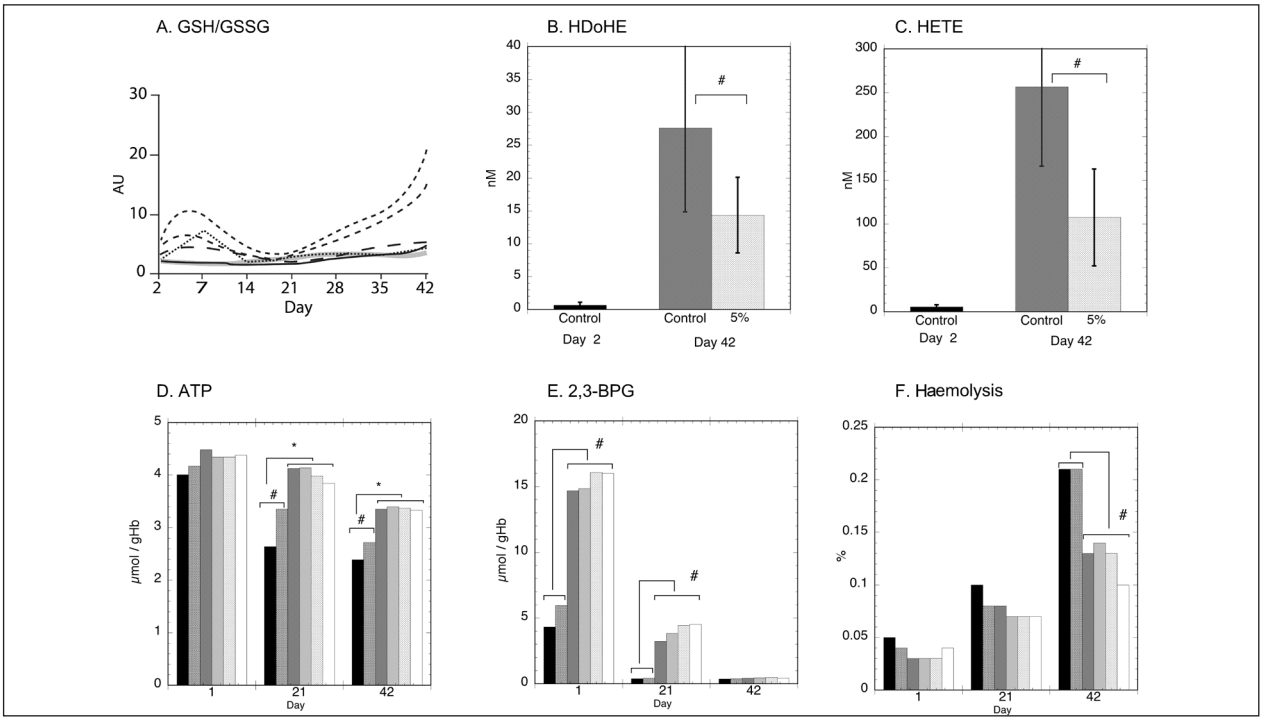

Figure 4 - Effects of $\%SO_2$ levels on metabolic and biochemical parameters.
(A) GSH/GSSG ratio during storage. Set pre-storage $\%SO_2$ values at day 0: from bottom to top lines; < 3% (. . .); 5% (- - -); 10%, unprocessed (– –); 20% (— — —); unprocessed (thick gray line); and more than 90% (solid line). (B) Concentrations of non-enzymatically oxidised PUFA by oxidation of docosahexaenoic acid (16- and 17-HDoHE) at the end of storage. (C) Concentrations of non-enzymatically oxidised PUFA by oxidation of arachidonic acid (8-, 9-, and 11-HETE) at the end of storage. (D) ATP levels at days 1, 21 and 42. (E) 2,3-BPG levels. (F) Haemolysis. Pre-storage $\%SO_2$ levels are indicated in dark to light bars (from left to right). Unprocessed control: more than 90%, 20%, 10%, 5%, and less than 3%. * and #: $p<0.05$. $\%SO_2$: percent oxygen saturation; GSH: glutathione; GSSG: glutathione disulfide; PUFA: polyunsaturated fatty acids; HETE: hydroxyeicosatetraenoic acid; ATP: adenosine triphosphate; BPG: bisphosphoglycerate.

units without any O_2 control (Figure 4E). A significant reduction in haemolysis was observed for all O_2-reduced units compared to the non-reduced units. The lowest level of haemolysis was observed with lowest $\%SO_2$ at day 42, reaching significance ($p<0.049$) compared to the units with 20% SO_2 (Figure 4F).

Discussion

Data shown in Figure 1 resulted from the first detailed study with a large number of units (n=492) on oxygen content of whole blood and prepared RCC. Since more than 95% of the oxygen in a typical unit of blood is bound to haemoglobin, $\%SO_2$ was used as a measure of oxygen content in RCC prepared for blood bank storage.

Measuring $\%SO_2$ using a co-oximeter requires mixing and obtaining a representative sample of RBCs from a unit immediately prior to measurement. To our knowledge, no systematic study to determine freshly collected $\%SO_2$ from blood donors is available. The mean value of 45.9±17.6% appears lower than 61.8±3.7% reported in a reference book for healthy subjects[40], or 73.5±13.3% reported using central venous line[37]. The observed distribution of $\%SO_2$ values was surprisingly wider than expected from those published values.

Peripheral blood $\%SvO_2$ is likely determined from a small volume of blood obtained from the antecubital vein pressurised with a tourniquet prior to venipuncture. On the other hand, the higher values measured in central venous lines were from patients in the intensive care unit. Although it is generally assumed that $\%SvO_2$ of free-flowing blood collected in the bag is similar to reference values in text books, this newly collected data may offer new insights into oxygenation status of healthy individuals. The high end of observed values cannot be explained by trapped air inside the blood collection bag, O_2 dissolved in an anticoagulant solution, or O_2 added with leucofitration and additive solution as evidenced by lack of a large increase in $\%SO_2$ between WB before and after filtration, as well as the final resultant LR-RCC shown in Table IB. Respiring leucocytes and platelets may have caused a decrease in $\%SO_2$ levels from whole blood units held overnight at room temperature as evidenced by approximately 7% higher oxygen saturation of LR-RCC observed produced shortly after phlebotomy at the collection facility and shipped overnight. However, it cannot explain a large number of units with low $\%SO_2$ values that were not held overnight as whole blood. These data might suggest that $\%SvO_2$ values in healthy subjects are not as tightly controlled as blood pH, and that it has a wide range among the diverse donor population, donor age, sex, fitness status, body mass index, as well as genetic predisposition represented by this data. When mean $\%SO_2$ from different suppliers was compared, ResBC showed significantly higher $\%SO_2$ than the other suppliers. It is noteworthy that ResBC is a "for profit" supplier of blood components for research purposes that relies on remunerated blood donors, located near several college campuses. Their donor profile is probably different from typical non-remunerated volunteer blood donors. Data shown here warrant further study to identify various donor characteristics affecting the observed wide distribution of $\%SO_2$ of collected blood.

For leucoreduced red cells, oxygen content increases gradually as the PVC film of the storage bag is permeable to O_2. This phenomenon was previously described with a small study of non-leucoreduced blood[41], but no study has reported on leucoreduced RCC stored undisturbed for a 3-week interval. Because of the relatively low solubility of oxygen in an aqueous salt solution (RBC additive solution), coupled with the moderate permeability of PVC, the rate of $\%SO_2$ increase during storage is dependent on mixing, as well the storage bag surface area-to-RCC volume ratio. Increased surface area-to-volume ratio and mixing substantially increase O_2 uptake rate as compared to the full volume that was mixed only once (Figure 3 *vs* Figure 2A). On the other hand, $\%SO_2$ gain rate was slower than in Figure 3 when full units were undisturbed for the entire 42 days and then measured (*data not shown*).

There is a general consensus among the researchers that oxidative damage is one of the major drivers of RBC storage lesion development[24,26,31,38], and the critical role played by oxidised haemoglobin had been recognised more than two decades ago[29,31,33]. Since oxygen is a substrate for haemoglobin oxidation, as well as a sustaining substrate for lipid peroxidation cycle, the rate of oxidised product formation is expected to be positively correlated with concentration of free oxygen in the cytosol.

Methaemoglobin is unstable and readily denatures to haemichromes, haemin and globin at cold temperature[42], and thus its concentration does not increase significantly during RCC storage. However, steady-state concentration of methaemoglobin is highly dose dependent on oxygen (Figure 3B). The ratio of reduced to oxidised glutathione indicates an extent of oxidative stress in stored RCC, and such higher ratios were observed with lower $\%SO_2$. An exception was found in the lowest $\%SO_2$ unit, which measured less than 1.5% beyond day 28, well below the limit of detection of 3% by co-oximetry. At such an extremely reduced intracellular environment, there might be some unknown chemical reactions that can cause GSH depletion.

In order to examine the effect on oxygen depletion on oxidative stress to membrane lipids, quantitative

metabolomics of selected samples was undertaken to quantify concentrations of non-enzymatically oxidised polyunsaturated fatty acids in supernatant. The results confirm a significant reduction in specifically non-enzymatic polyunsaturated fatty acids (PUFA) oxidation products arising from docosahexanoic acid as well as arachidonic acid (Figure 4B and C).

Dependence of $\%SO_2$ levels on standard RBC quality parameters, ATP, 2,3-BPG and haemolysis were generally better with oxygen reduction but not dose dependent as long as oxygen was reduced to at least 20%. Day 21 and 42 ATP levels of hyperoxygenated units were significantly lower than the control, while $\%SO_2$-reduced units were higher than control. Higher ATP and 2,3-BPG levels achieved by O_2-reduced units is in agreement with enhanced glycolytic flux through metabolic modulation caused by increasing the ratio of deoxy/oxyhaemoglobin after $\%SO_2$ reduction. For control and hyperoxygenated units, by diverting the glycolytic flux away to the pentose phosphate pathway reducing ATP production, it allows NADPH replenishment and subsequent GSH synthesis[43].

A consequence of uncontrolled $\%SO_2$ levels in collected blood at the time of storage is the contribution this variable could make to the inconsistency of red blood cells at the time of use. This study has shown that the starting $\%SO_2$ levels vary widely at the beginning of storage and change at variable rates during storage. The effect oxygen has on various *in vitro* parameters of blood quality has also been shown, including dose-dependent relationships, e.g. methaemoglobin levels. By choosing to not control oxygen at the time of and throughout storage, this variability becomes part of the characteristics of the blood supply. In contrast, applying a strategy to manage oxygen levels below a certain range (ex. 15% in Figure 5) and maintaining this range during storage, two benefits are possible: 1) the improvement of the many parameters measured that are affected by $\%SO_2$; and 2) significantly reduced inconsistency with regard to $\%SO_2$, and by extension the parameters that change based on oxygen saturation. This strategy would be consistent with best practices in biopharmaceutical manufacturing where building quality into a product means establishing identity, strength, purity, and other quality characteristics designed to ensure the required levels of safety and effectiveness, and by designing the product and manufacturing process in such a way as to consistently produce a quality product (e.g., Code of Federal Regulations Part 211, USA). The improvement of *in vitro* characterisation tools has allowed the degradation of red blood cells during storage to be sufficiently elucidated such that the root causes of these changes can be identified and dealt with. Management and control of oxygen levels during storage of RBC is a process that can provide increased quality and consistency of this critical therapy.

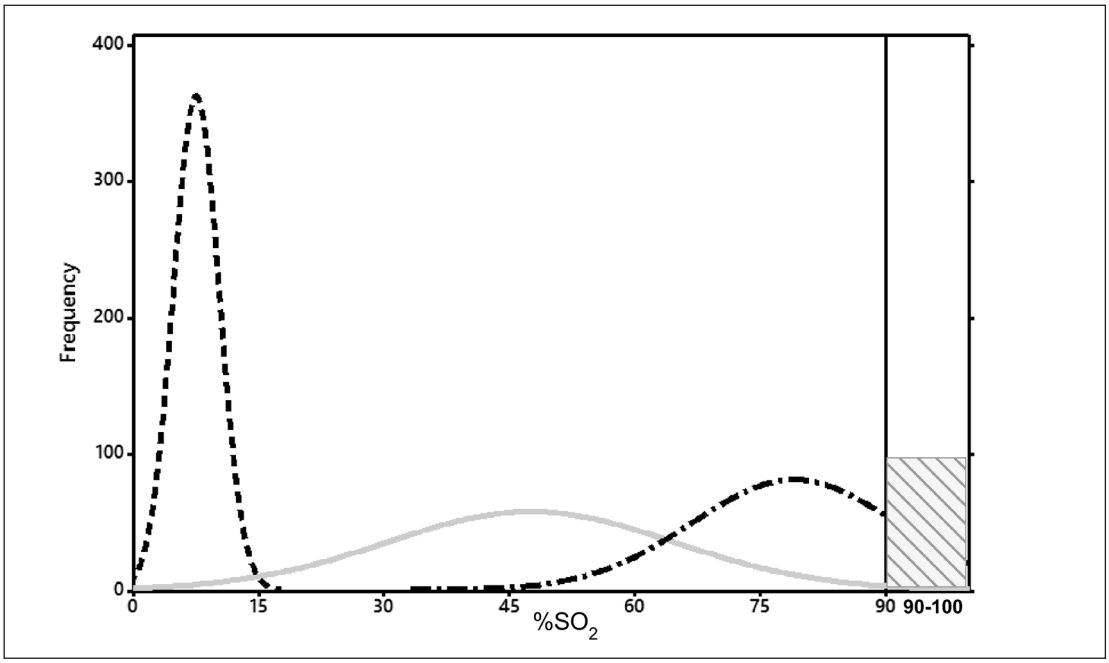

Figure 5 - Controlling $\%SO_2$ levels throughout storage using a disposable device.
Pre-storage $\%SO_2$ distribution (from Figure 1) is shown as a gray curve in centre. Right curve and a bar graph beyond 90% represent $\%SO_2$ gain after 42 days of storage (from Figure 2). The left curve is generated based on transforming the $\%SO_2$ distribution days of storage (from Figure 2). $\%SO_2$: percent oxygen saturation.

Limitations

Quality of RCC varies depending on specific procedures used for blood collection, transportation and component processing that include time-course, temperature history and component separation/leucoreduction methods[44,45]. $\%SO_2$ distribution reported in Figure 1 is for whole blood or RCC that underwent specific processing conditions as shown in Table I, and thus may not be applicable for other processes, such as overnight room temperature hold, buffy coat method as well as immediate cool down (on ice pack) followed by component processing/filtration within 72 hours. The same limitations apply to the kinetics of oxygen gain by stored RCC during 6-week shelf life. Several factors affect the rate of $\%SO_2$ gain independent of factors attributed to donor and blood collection (haemoglobin content, volume, and haematocrit). Such examples include placement during storage (surface area of the bag exposed to air) and transportation, as well as mixing. Ten units used for this study were mixed and sampled only once and were otherwise undisturbed during 6-week storage, which may cause $\%SO_2$ increase rate to be underestimated at blood banks.

Conclusions

Oxidative damage is often cited as an important source of damage to red blood cells during storage and is implicated as a source of adverse events or reduced efficacy of transfusions. In order to improve the understanding of the impact that oxidative damage has on both individual units of blood and the blood supply as a whole, this study evaluated $\%SO_2$ in red blood cell units, including the variability of $\%SO_2$ in fresh blood at the beginning of storage, the changes in the $\%SO_2$ level during storage, and the $\%SO_2$ dose dependence of a subset of salient measures of blood quality. The study reveals that the degradation of important blood quality parameters is clearly $\%SO_2$ dose dependent. Coupled with the findings that $\%SO_2$ levels vary widely at the start of storage and inevitably increase towards full O_2 saturation, these data strongly indicate that $\%SO_2$ levels in blood is an important and underappreciated source of both reduced quality for a given unit of blood during storage and inconsistency across all units of blood in storage. Thus, control of $\%SO_2$ may lead to a reduction of adverse events associated with RBC transfusion while increasing its efficacy.

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Authorship contributions

TY designed the study, analysed the data and wrote the manuscript. AB prepared the samples, measured the parameters, created/managed the database and analysed the data. ADA and TN conducted metabolomics and lipidomics. MD analysed the data. CCS contributed to study design and blood procurement for metabolomics. ADA designed the study and wrote the manuscript.

Disclosure of conflicts of interest

TY, AB, MD and ADA are employees of New Health Sciences Inc. which is developing Hemanext, a device to establish and maintain optimum $\%SO_2$ levels in blood before storage. TY is listed as an inventor on several patents and patents applications describing red blood cell storage technology to control oxygen in stored units. ADA and CCS have received research support from New Health Sciences Inc., and serve as consultant and member of scientific advisor board. TN has received research support from New Health Sciences Inc.

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Red blood cell proteomics update: is there more to discover?

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Introduction

A great deal of interest has been attracted by back-of-the-envelope calculations suggesting that bacterial cells (recently revised to be ~3.9×10^{13}) outnumber the total number of host cells in the human body ($3.0x10^{13}$, estimated for a 70 kg "reference man")[1,2]. Although early estimates of ~2.8×10^{13} red blood cells (RBC) in adult humans[3] seem excessive in comparison to these numbers, it is universally accepted that RBC constitute the most abundant cell type in the body (~2.5×10^{13}) in foetal and post-natal life[3]. With an average lifespan of 120 days in circulation, every day ~2×10^{11} RBC are continuously cleared/released in the blood stream[3]. However, mature RBC are devoid of nuclei and organelles and each mature RBC contains ~270×10^{6} molecules of haemoglobin, accounting for approximately 90% of its dry weight[4]. It is not, therefore, surprising that RBC have been almost exclusively viewed as simple biological envelopes for circulating haemoglobin, in the light of their lack of nuclei and organelles and their clear and unique role in oxygen transport and gas homeostasis functions.

Understanding the complexity of RBC biology beyond gas transport is instrumental to the closely related fields of haematology and Transfusion Medicine. Approximately 108 million units of donated blood are collected globally every year (WHO, 2015). Transfusion of packed RBC is currently the only life-saving intervention for massively or chronically transfused recipients, such as trauma and sickle cell disease/blood cancer patients, respectively. Of note, trauma is currently the leading cause of death under the age of 59 (Center for Disease Control, 2010-2014), further highlighting the clinical relevance of transfusion of packed RBC.

In the last 14 years, proteomics technologies have been used to shed light on the 10% low abundance RBC proteome, revealing an unanticipated level of complexity proportional to the advancements in the analytical technologies and pre-fractionation strategies used to perform the analyses (Figure 1A)[5-13]. Strides in the field of mass spectrometry (MS)-based proteomics technologies have enabled the detection of up to 1,578 unique protein species in a single experiment, following extensive sample pre-fractionation with combinatorial hexapeptide ligand libraries, two-dimensional gel electrophoresis and nano-high performance liquid chromatography (HPLC)-MS/MS analysis[11]. This extensive dataset was integrated with previous -omics results to generate an early *in silico* interaction map of human RBC[9,14], and subsequent expansions to annotate up to 2,289 distinct gene products in mature erythrocytes[13].

Recently, ambitious proteomics projects have expanded our understanding of the human proteome[15,16]. However, none of these massive studies has focused on the RBC proteome, either under the assumption that the majority of the RBC proteome had been substantially unveiled by extant studies or to avoid the technical issues associated with the overwhelming abundance of RBC haemoglobin. The assumption that the RBC proteome had been fully elucidated has been recently challenged by in-depth proteomics studies, which combined advances in the field of proteomics technologies, MS-instrumentation and database searches to enable the identification of up to 2,838 unique proteins in adult and cord erythroid cells prior to or following reticulocyte maturation[12]. Here we highlight how these advances have made it possible to monitor ~2,000 RBC proteins by exploiting a routine laboratory proteomics workflow, using the GeLC-MS approach[17]. Since the presence of specific enzymes in the proteomics dataset does not necessarily indicate residual activity of the given enzyme, we integrated proteomics results with tracing metabolomics experiments by incubating human packed RBC with $^{13}C_{1,2,3}$-glucose-supplemented additives. The findings confirmed the presence and activity of cytosolic versions of Krebs cycle enzymes in mature packed RBC. The metabolomics tracing experiments also suggested that the present proteomics map is incomplete, by highlighting the presence and storage-dependent activation of enzymes that can catalyse pyruvate transamination reactions, an observation that paves the way for future, more in-depth analyses of the RBC proteome and opens new potential scenarios in the formulation of alternative storage additives for transfusion purposes.

Materials and methods

Blood collection and processing

Blood was collected from four healthy donor volunteers, in agreement with the Declaration of Helsinki.

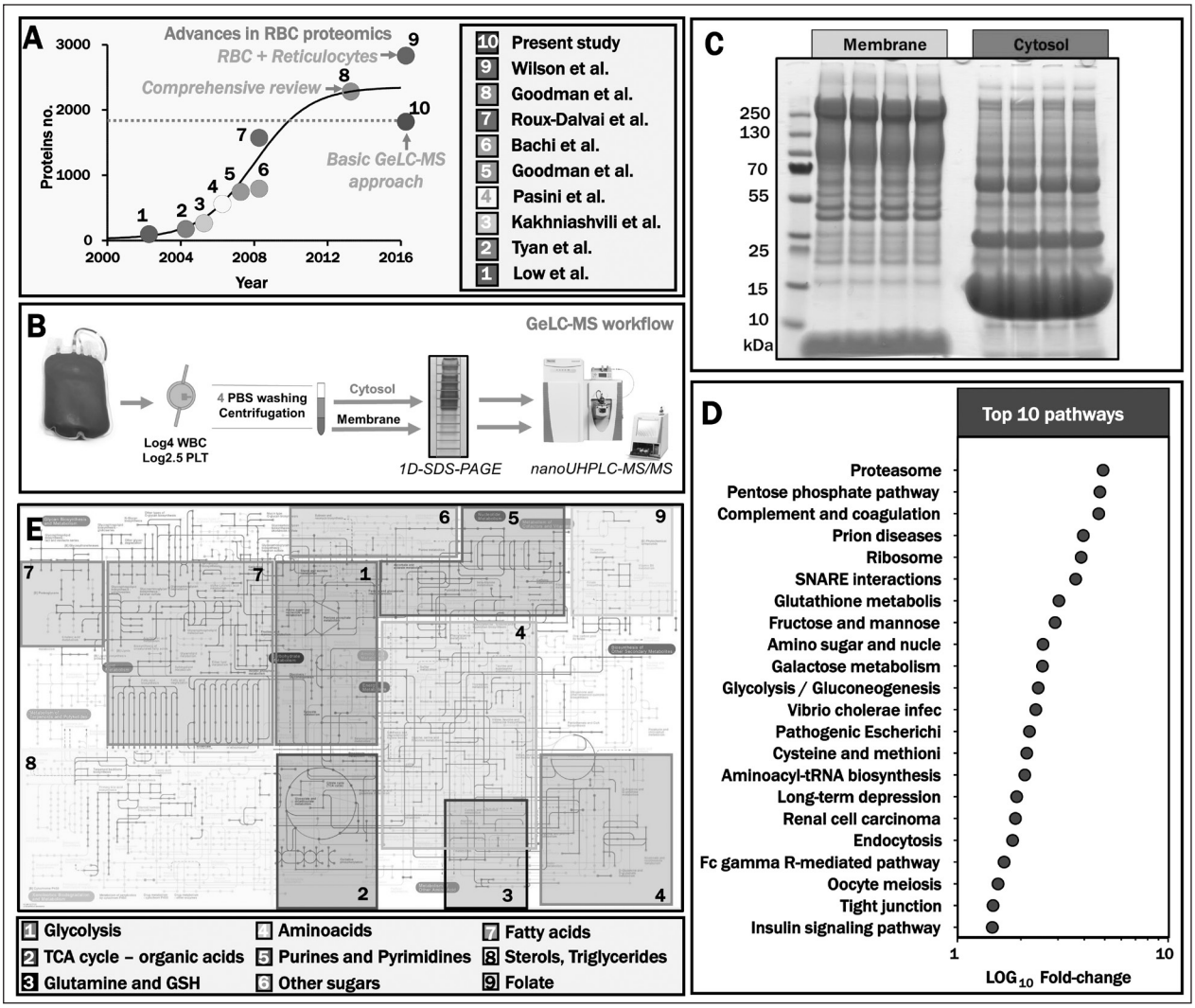

Figure 1 - The RBC proteome: an update.
(A) Proteomics technologies are continuously expanding our understanding of RBC complexity. (B) Here, proteomics analyses were performed through the GeLC-MS proteomics workflow[17]. (C) A representative 1D-SDS-PAGE preparative gel for RBC membrane and cytosol proteomics analyses. (D) Top pathways resulting from gene ontology term enrichment for biological function through DAVID (accessible at https://david.ncifcrf.gov/). (E) Metabolic enzymes identified in the RBC proteome were mapped against the KEGG pathway database (matching nodes are highlighted in dark grey; general pathways are indicated in the legend at the bottom of panel). A high-resolution version of this panel is provided in Online Supplementary Figure S1.

Packed RBC were stored in CP2D-AS-3 (Haemonetics, Braintree, MA, USA), as previously reported[18]. Samples were leucofiltered (RC2D, Haemonetics) to achieve a $\log_4$ reduction in white blood cells (WBC), to $<1\times10^6$ WBC/L or <0.5 WBC/1×10^6 RBC, improving upon previous erythrocyte enrichment protocols[11]. To remove any residual trace of plasma (90% of which is removed by filtration), platelets ($\log_{2.5}$-filtered) and WBC, the RBC were further washed three times with PBS + PMSF (154 mM NaCl, 10 mM phosphate buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride)[11]. At each step supernatant and top RBC layers were removed to eliminate residual contaminating cells/plasma, prior to the final separation of RBC cytosol and membrane fractions[11].

GeLC-MS proteomics analysis

Proteomics analyses were performed using a GeLC-MS approach[17]. Briefly, 30 µg of protein extracts were run on a 4-12% gradient one-dimensional SDS-PAGE gel. Eighteen bands were cut from each lane, trypsin digested upon reduction and alkylation of unmodified cysteine residues, and analysed by nano-UHPLC-MS/MS (Easy-nLC1000, QExactive HF - positive ion mode, Thermo Fisher, Waltham, MA, USA). A volume of 8 µL of sample was injected through a trapping column

for desalting (ZORBAX 300SB-C18, 5×0.3 mm, 5 µm particle size, Agilent Technologies, Santa Clara, CA, USA) and then switched online at 600 nL/min over a home-made 100 µm × 150 mm fused silica capillary packed with Synergi Hydro C18 resin (4 µm, 80 Å from Phenomenex, Torrance, CA, USA). Samples were run on a 350 nL/min 90 min linear gradient of 5-32% ACN with 0.1% formic acid to separate the peptides. The mass spectrometer was operated in the positive ion mode, scan range m/z 400-2,000, followed by collision-induced dissociation MS/MS of the 20 most intense precursor ions at a normalised collision energy of 35 eV. Raw data files were converted into .mgf files which were then merged using an in-house script. Error tolerant searches were performed using Mascot (v. 2.4) against the human UniprotKB database (release date 2015.1.8), including decoy sequences (cysteine carbamidomethylation and methionine oxidation set as fixed and variable modifications, respectively). Mass tolerances for membrane and vesicle data were set to ±15 ppm for precursor ions and ±0.6 Da for fragment ions. For all Mascot search results, peptide spectral matches (PSM) were filtered at a 95% confidence threshold (excluding PSM with an expectation value >0.05).

$^{13}C_{1,2,3}$-glucose tracing metabolomics experiment

CP2D-AS-3 leucofiltered RBC concentrates were spiked with 1.1 mM $^{13}C_{1,2,3}$-glucose (SIGMA Aldrich, St. Louis, MO, USA), resulting in a ~20% addition to the total glucose (unlabeled ^{12}C-glucose monohydrate in AS-3 is ~5.55 mM[18]). Samples were sterilely harvested on storage days 2, 7 and on a weekly basis until the end of the shelf-life of the unit (storage day 42). RBC and supernatants were separated through gentle centrifugation, and measurements of $^{13}C_3$-alanine were performed as previously reported[18,19] using UHPLC-MS (Vanquish, Q Exactive - Thermo Fisher, San Jose, CA, USA). RBC were collected on a weekly basis and immediately extracted in ice-cold lysis/extraction buffer (methanol:acetonitrile:water 5:3:2) at 1:3 dilutions. Samples were then agitated at 4 °C for 30 min followed by centrifugation at 10,000 *g* for 15 min at 4 °C. Protein and lipid pellets were discarded, while supernatants were stored at –80 °C prior to metabolomic analyses. Twenty µL of RBC extracts were injected into an UHPLC system (Vanquish, Thermo Fisher) and run on a Kinetex C18 column (150 x 2.1 mm i.d., 1.7 µm particle size - Phenomenex, Torrance, CA, USA) at 250 µL/min (phase A: Optima H_2O, 0.1% formic acid; phase B: acetonitrile, 0.1% formic acid). The UHPLC system was coupled online with a Q Exactive mass spectrometer (Thermo Fisher), scanning in Full MS mode (2 µscans) at 70,000 resolution in the 60-900 m/z range, 4 kV spray voltage, 15 sheath gas and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs). Metabolite assignments and isotopologue distributions were determined using Maven software (Princeton, NJ, USA), upon conversion of .raw files into .mzXML format through MassMatrix (Cleveland, OH, USA), correction for natural abundance of ^{13}C, and assignments confirmed against a heavy label standard for alanine (^{13}C ^{15}N-alanine and >650 standard compounds - IROATech, Sigma Aldrich, St. Louis, MO, USA). Reproducibility of extraction efficiency, chromatographic and MS technical stability were tested by determining coefficients of variation for the quantitation of spikes in heavy labelled standard mixes (0.25 µM final concentration - Cambridge Isotopes Laboratories, Inc., Tewksbury, MA, USA) and the xenometabolite 5-fluorouracil (2.5 µM final concentration - F6627-1G, Sigma Aldrich).

Absolute quantitation was performed by exporting the values for integrated peak areas of light metabolites and heavy labelled standards (1 µM - Cambridge Isotope Laboratories, Inc.) into GraphPad Prism 5.0.

Results and discussion

Proteomics analyses were performed using a GeLC-MS workflow (Figure 1B). Sample pre-fractionation strategies to remove haemoglobin and the most abundant RBC proteins (e.g. carbonic anhydrase)[11,20,21] had previously been used to make the low abundance RBC proteome amenable to detection through analytical proteomics workflow. Here, preliminary 1D-SDS-PAGE fractionation was adopted as part of a routine proteomics workflow. The resulting gel lanes for membranes and cytosol protein extracts where excised into 18 different bands, trypsin-digested prior to nanoUHPLC-MS peptidomics analysis. This workflow resulted in the identification of a total of 1,826 proteins (Online Supplementary Table SI). Bioinformatic analysis suggests that RBC can engage in many different pathways other than just gas transport (Figure 1D, Online Supplementary Table SII). In particular, mapping of the experimental results into the most comprehensive KEGG metabolic pathway (ko01100) revealed an unanticipated metabolic complexity of human RBC (highlighted in red - Figure 1E; Online Supplementary Figure S1). For example, here we identified cytosolic versions of enzymes involved in tri- and dicarboxylic acid metabolism, such as acetyl-coA citrate lyase, isocitrate dehydrogenase 1, pyruvate carboxylase, malate dehydrogenase 1 and fumarase (Figure 2A). Additionally, enzymes potentially promoting cytosolic reactions of one-carbon metabolism (serine hydroxymethyltransferase 1 and methyltetrahydrofolate reductase 1) have been identified (Figure 2B). Some of the enzymes mentioned above have the capacity to catalyse reactions that can generate

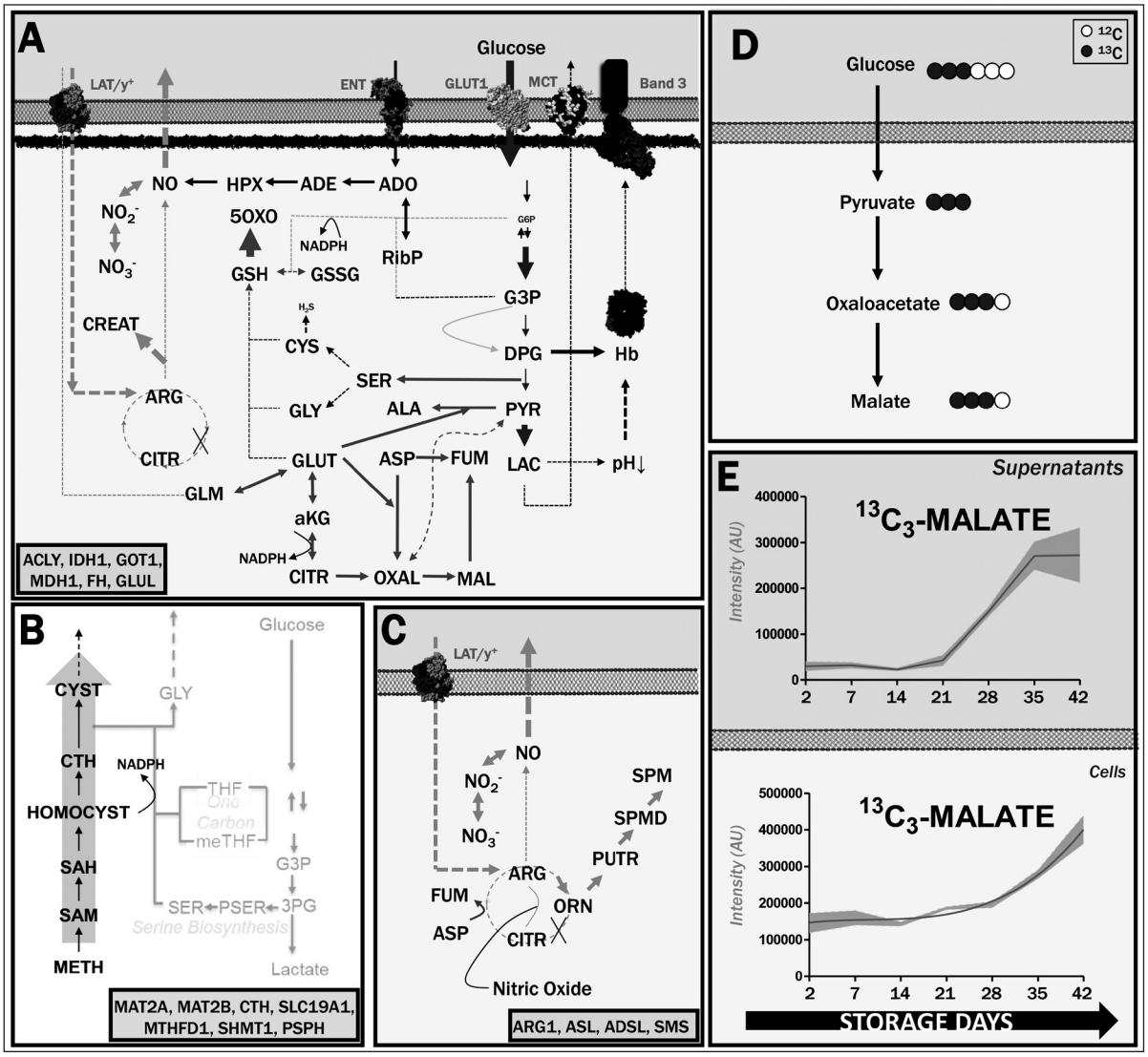

Figure 2 - Proteomics and tracing experiments reveal the presence of previously unappreciated metabolic pathways in mature RBC.
(A) An overview of the central metabolic pathways that are reasonably active in RBC in the light of proteomics evidence. Di- and tri-carboxylates can be metabolised by the enzymes indicated in the bottom left corner of the panel. (B) Enzymes identified in the cytosol of RBC in the present study which may be involved in sulphur/methionine synthesis and serine/folate metabolism are shown. Some reactions catalysed by IDH1 (A) or MTHFD1 (B) can generate NADPH without the activation of the oxidative phase of the pentose phosphate pathway (A). (C) An overview of metabolic reactions in nitrogen/arginine metabolism that are theoretically possible in RBC in the light of the enzymes identified in this study. (D) An overview of the $^{13}C_{1,2,3}$-glucose labelling experiment and expected isotopologue distribution in downstream glucose oxidation, pyruvate carboxylation and malate dehydrogenase 1-dependent generation of malate. (E) Experimental results of $^{13}C_3$-alanine detection in supernatants and cell extracts of stored RBC incubated with $^{13}C_{1,2,3}$-glucose. Median ± interquartile ranges are shown. Enzyme abbreviations are consistent with Uniprot names in Online Supplementary Table SI.

NADPH, a key antioxidant reducing equivalent until now thought to be produced exclusively by reactions from the oxidative phase of the pentose phosphate pathway in RBC. Additionally, we found evidence of the presence of enzymes involved in nitrogen metabolism (incomplete urea cycle) and polyamine synthesis in the RBC cytosol (Figure 2C).

Since identification of an enzyme in a proteomics dataset does not necessarily imply that this protein preserves its catalytic function in the mature erythrocyte, tracing experiments were designed by incubating leucocyte-filtered packed RBC with $^{13}C_{1,2,3}$-glucose prior to refrigerated storage for up to 42 days. Potential time-dependent incorporation of heavy

labelling in oxaloacetate and malate was predicted by the identification of pyruvate carboxylase and malate dehydrogenase 1 (cytosolic) in the proteomics dataset (Online Supplementary Figure S1). An overview of the predicted labelling pattern from $^{13}C_{1,2,3}$-glucose is provided in Figure 2D. MS-based metabolomics tracing experiments confirmed the previously reported storage-dependent accumulation of malate in packed RBC[18], suggesting that a minor percentage (<1%) of the total malate actually derives from heavy glucose catabolism through glycolysis, carboxylation of glucose-derived pyruvate and conversion of the latter substrate into malate (isotopologue M+3 of oxaloacetate and malate, respectively; Figure 2E). This observation also suggests that the majority of malate accumulating in stored RBC derives from sources other than glucose, aspartate or citrate-derived oxaloacetate representing the most likely candidate. While future studies will shed further light on this issue, in the present study the integration of proteomics results with evidence from metabolomics experiments in the presence of stable isotope tracers reveals for the first time that cytosolic versions of Krebs cycle enzymes are present and enzymatically active in mature RBC, an observation with clear potential implications for the future design and development of novel storage additives for improved *ex vivo* preservation of mature erythrocytes.

Extensive, tracing metabolomics experiments suggest that our protein coverage is not comprehensive. Metabolic labelling of packed RBC with $^{13}C_{1,2,3}$-glucose shows a storage duration dependency in pyruvate transamination to alanine (resulting in the accumulation of the $^{13}C_3$-alanine product in stored RBC and supernatants; Figure 3A,B). Even though our labelling data and previous targeted studies[22]but are unable to transport this amino acid across their cell membrane. Consequently, erythrocytes rely on de novo glutamate biosynthesis from α-ketoglutarate and glutamine to maintain intracellular levels of glutamate. Erythrocytic glutamate biosynthesis is catalyzed by three enzymes, alanine aminotransferase (ALT confirmed the presence and activity of glutamate pyruvate transaminase in human RBC, we did not identify this enzyme in our dataset. Similarly, we did not identify other low abundance RBC enzymes that may play adaptive regulatory roles in healthy or pathological RBC, such as sphingosine kinase 1[23].

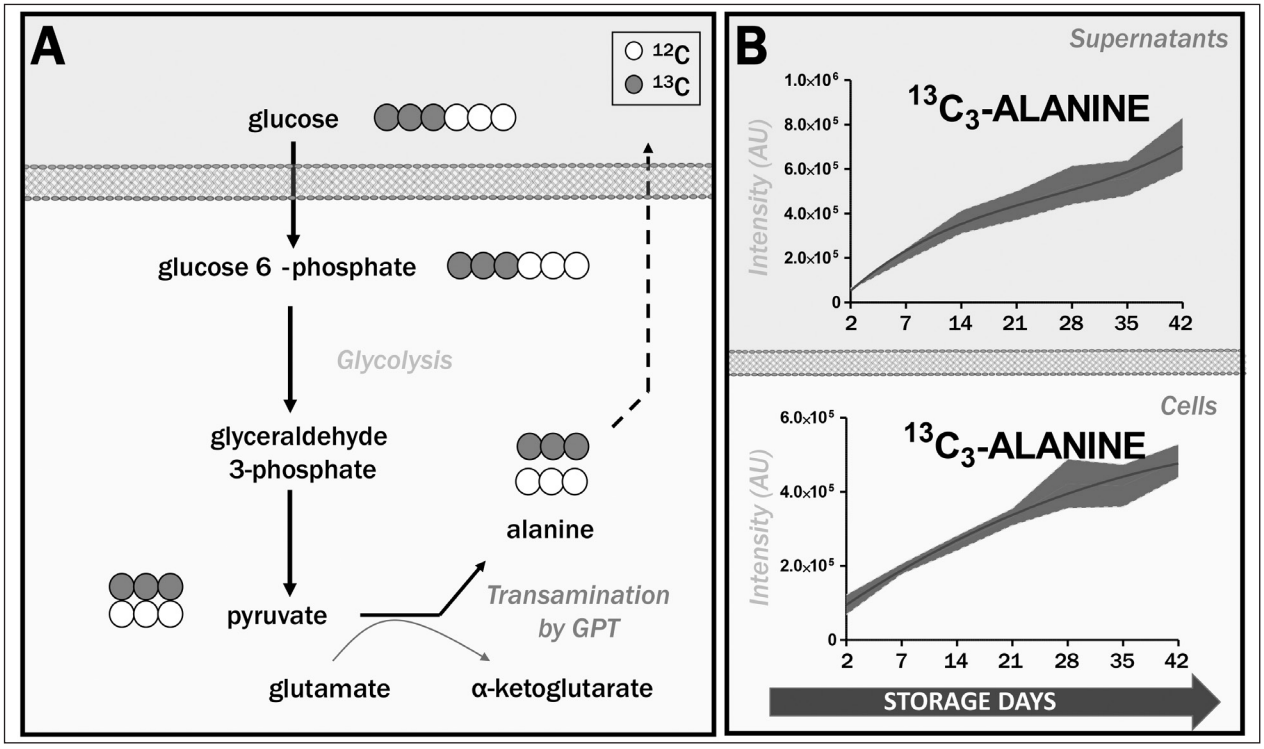

Figure 3 - Tracing experiments suggest that some enzymes that have not been identified in the updated proteomics dataset may actually be expressed and active in mature RBC.
(A) An overview of the $^{13}C_{1,2,3}$-glucose labelling experiment and expected isotopologue distribution in downstream glucose oxidation and pyruvate transamination reactions. (B) The experimental results of 13C3-alanine detection in supernatants and cell extracts of stored RBC incubated with $^{13}C_{1,2,3}$-glucose. Median ± interquartile ranges are shown.

Conclusions

The present dataset, although not definitive, supports the idea that there is still ample opportunity for discovery in one of the most historically studied human cells. Recent comprehensive updates of the RBC proteome complexity -only some of which are referenced here- have been detailed using advanced proteomics technologies and investigating both mature and immature mitochondria-endowed (reticulocyte) RBC fractions[12]. Further extensions of RBC protein complexity and biochemistry are anticipated, stemming from the application of advancing bioanalytical technologies. Meanwhile, this study could serve as a reference for the feasibility of large-scale RBC proteomics studies using routine laboratory analytical strategies while delivering results as comprehensive as those obtainable only with state-of-the-art technologies less than a decade ago. Combined advances in discovery-mode and functional -omics technologies are thus currently available to perform large-scale validation studies and enable the identification of novel modes of therapeutic intervention for the most abundant host cell in the human body.

Authorship contributions

MD, ADA performed the proteomics analyses and organised the resulting data. ADA, TN performed metabolomics analyses. KCH supervised the experiments and provided critical proteomics expertise and key instrumentation. ADA wrote the manuscript and all the Authors critically contributed to its finalisation. ADA and MD share first authorship.

Keywords: mass spectrometry, proteomics, error tolerant, erythrocyte, haematology.

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The 3-phase evolution of stored red blood cells and the clinical trials: an obvious relationship

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Dear Sir,

Recent clinical trials testing the effect of erythrocyte concentrates (ECs) stored for various periods of time on transfused patients arrived at the conclusion that the transfusion of ECs fresher than standard of care is not beneficial for patients[1,2]. However, they do not provide data on long-term stored ECs; nor were they powered to collect such data[2,3]. Whereas the shelf life of ECs has been fixed by gold standards (haemolysis at the end of storage and red blood cell [RBCs] survival rates in the recipient's circulation), a detailed analysis of *ex vivo* RBC ageing has brought new insights beyond a recurrent 14-day limit. First of all, let's focus on the biology and biochemistry of stored RBCs.

During storage in different known additive solutions, RBCs accumulate storage lesions[2-4]. These lesions impact various aspects of the cell ranging from energy metabolite depletion to cell morphology in a cascade of events. They can be classified as either reversible or irreversible, the former term referring to lesions that are reversed either *in vivo* once transfused or *in vitro* after adapted treatments[3]. Reversible lesions include defects in metabolism and protein activity, whereas irreversible lesions include accumulation of metabolites, protein oxidation, shape change, microvesiculation and haemolysis. A detailed review of all these lesions show that they are time dependent and linked together. Reversible lesions (mainly related to metabolism) mostly happen in the first two weeks of storage, and those irreversible lesions only after the fourth week of storage. The 28-35-day stage is the most critical limit in EC storage since irreversible lesions, which are *per se* permanent, start accumulating. Last but not least, two confounding factors should also be taken into account: firstly, ECs contain the whole spectrum of the donor's RBCs, from just-matured cells to close-to-senescence RBCs, and secondly, there are more and more data showing the importance of donor's characteristics on the quality of stored RBCs.

In published clinical studies, the mean age for long-term storage is 26±8 days (including data from Heddle *et al.*[1])[3]. Because of the design of these trials, ECs that contain irreversible lesions are not really taken into account[2,3]. Even though most routine transfusions involve ECs around 17±8 days (in our institution), 35 day-old ECs are still transfused. In critically ill patients, these products stored for more than 35 days were associated with increased morbidity and mortality compared to ECs transfused before 21 days of storage, and the length of stay was increased in both ECs stored for more than 28 and 35 days[5]. These observations are consistent with biochemical data and the accumulation of irreversible lesions.

In summary, three phases are observed during storage. The first transition involving reversible lesions occurs around the second week and the second transition involving irreversible lesions around 4-5 weeks of storage. The clinical trials have focused on the first stage and offer reassurance on RBC transfusion. However, the existence of irreversible lesions should be considered because they might be harmful in some circumstances (in an intensive care unit setting, older patients, etc.). Two issues will have to be considered. First, improve the RBC quality markers (in relation to transfusion efficiency) so as to be able to capture the exact level of RBC storage lesions. Second, find the strategy to postpone or reduce irreversible lesions that might be the reason of adverse clinical outcomes. Biological and biochemical data analyses (including omics and system biology) will contribute to better characterise ECs, to improve storage, and to select the right product for the right patient. Because for our patients, we can still do better.

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Interpreting the deluge of omics data: new approaches offer new possibilities

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Dear Sir,

Developments in assay technologies have transformed biology from a data-poor qualitative field to a data-rich quantitative field. The cost of biological data generation has exponentially decreased in the past two decades, allowing simultaneous measurement of several types of biomolecules, including DNA, RNA, proteins, and metabolites. Particularly for Transfusion Medicine, studies have generated metabolomics and proteomics data of stored red blood cells (RBCs) and platelets detailing time-dependent changes. These high-throughput, or "omics", datasets measure hundreds of metabolites and proteins at tens of time points for each replicate, resulting in studies with over 10,000 data points. As seen in other fields that have generated omics datasets for nearly two decades, it is apparent that data analysis and interpretation, rather than data generation, is becoming the bottleneck of biological discovery[1].

The need for sophisticated data analysis is evident from many of the recently published metabolomics studies on the RBCs and platelet storage legion. Such studies are encyclopedic in nature, often only validating existing biochemical findings previously found using low throughput assays. With so many significant changes, it can be daunting to ipinpoint which metabolite(s) are indicative of a diagnostic biomarker or are important to change for better preservation of red cells. For example, in a recently published metabolomics dataset for RBCs stored in SAGM additive solution, 75% of the over 100 measured metabolites significantly changed over the storage period[2]. How can such high-dimensional data be conceptualised and used for biological discovery and intervention?

Systems biology and bioinformatics are disciplines for deciphering the complex interactions of cells at a global level using high-throughput datasets. In these fields, many statistical and mechanistic methods exist for analysing datasets and modelling cellular processes, each with their own advantages and disadvantages. Researchers should choose techniques based on the cellular process in question and the number of experimental datasets available. Using these considerations, we developed a systems biology platform that: 1) determined RBCs undergo three metabolic states during storage, 2) identified metabolic biomarkers that differentiate between the metabolic states, and 3) pinpointed key regions of cellular metabolism to experimentally perturb that will likely alter and prolong the earlier metabolic states (Figure 1).

First, we measured 100+ intracellular and extracellular metabolites over time for RBCs stored in SAGM additive solution using absolute quantitative metabolomics[2]. Using statistical analyses, we reduced the high dimensional data set. In particular, principal component analysis was used to determine that the cells undergo three distinct metabolic phases during storage. To our surprise, more traditional *in vitro* haematologic parameters currently used for quality assessment, such as haemolysis, ion and gas concentrations, and RBC indices, were less indicative of elapsed storage time. Thus, the biochemical signature we identified indicative of the storage lesion may act as a better quantitative *in vitro* measure of RBC quality, allowing for better benchmarking of novel RBC processing techniques.

Second, the principal component analysis-derived biochemical signature for the storage lesion was further refined by selecting a smaller but "core" subset of the 100+ metabolites. In subsequent work, we showed that tracking just eight metabolites could accurately assess which of the three metabolic states the RBCs are in[3]. Further, the signature was found to be general as the work was experimentally validated in different labs using different mass spectrometry techniques, different RBC processing methods, and different additive solutions (SAGM and AS3).

Third, we have developed comprehensive metabolic networks of RBCs and platelets to assess the changes in metabolic pathway usage during storage. Metabolic network reconstructions are an aggregation of the available primary literature from low- and high-throughput datasets representing the current knowledge of a particular cell's metabolism. These networks are suited for modelling metabolism at the cellular scale[4] and even for analysing blood metabolomics datasets from individuals[5]. For example, the RBC network was constructed from 60+ research articles and textbooks published over the past 50 years. The network contains 292 metabolic reactions catalysed by 281 enzymes. Metabolic networks are analogous to flow networks, in which metabolites "flow" through the network similar to liquids flowing in a pipe. By integrating the time-course measurement of a large portion of metabolites during storage with the underlying "pipeline" of the RBC metabolic network, the status of flow throughout the network can be computed for each of the three metabolic states. Similar to the large number of significant changes seen for the metabolites, we found that the activity of roughly 50% of metabolic reactions significantly change as RBCs shift from one metabolic state to the next. In order to determine important and potentially causal changes to the RBC storage legion, we developed and applied network modelling algorithms that determine

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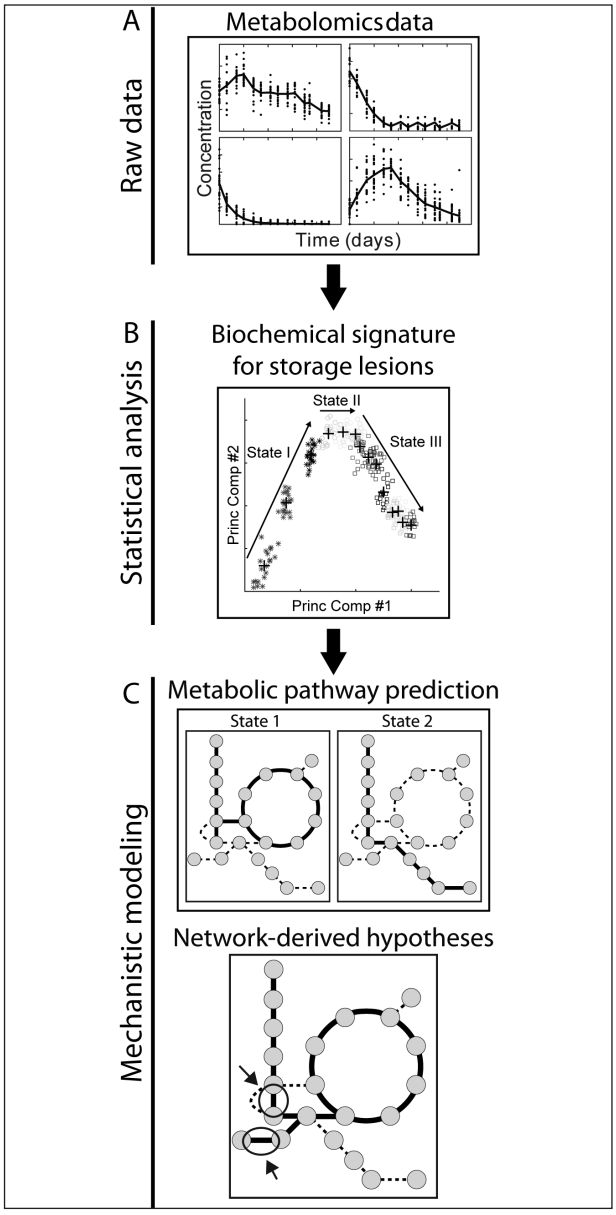

Figure 1 - Workflow of a systems biology platform for analysing high-throughput data in Transfusion Medicine.
(A) Absolute quantitative metabolomics data are generated for stored red blood cells and platelets. (B) Statistical analyses, such as principal component analysis, are used to define a biochemical signature for the storage lesions and for determining the time intervals of each metabolic state during storage. The signature can be used for *in vitro* assessment of blood cell age and a benchmark for new processing methods. (C) Metabolomics data can be integrated with metabolic networks to determine pathway usage for each metabolic state. Network modeling algorithms can then be applied to generate hypotheses on experimental perturbations to help minimise pathway usage changes that occur in subsequent metabolic states. A toy example is shown in which through two perturbations the second metabolic state is reverted to a state similar to the first.

a minimal set of metabolic reactions that if modified would globally alter and prolong the first and second metabolic states.

Using the computationally derived metabolic "lynchpins" of the RBC storage lesion, we have devised perturbation strategies to assess our ability to alter and prolong the first and second metabolic states, thus avoiding the later state of the storage lesion. In collaboration with Drs. D'Alessandro and Hansen at the University of Colorado in Denver, these computationally derived hypotheses will be tested. Metabolomics data will be generated to assess if the interventions affect the metabolic state, a quantitative *in vitro* benchmark for improving red cell quality. Successful interventions will be tested for positive effects on non-metabolic markers of the RBC storage lesion, such as micro-particle generation and cellular rheology. Further, predictions found to be inaccurate allow the reconciliation of the generated high-throughput data with the network structure for discovering new biology not previously captured in the literature.

Systems biology and bioinformatics methodologies provide statistical and mechanistic methods to interpret high-dimensional datasets. A metabolic network's structure represents the compilation of the currently available knowledge of a particular cell's metabolism. Integrating datasets from stored RBCs and platelets will provide a novel opportunity to interpret and harness these unwieldy datasets.

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AB is a co-founder of Sinopia Biosciences.

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