Diversity in a blood bag: application of omics technologies to inform precision Transfusion Medicine

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In recent years, red blood cells (RBCs) have finally received well-deserved attention from scientists in the field of transfusion medicine. These biconcave-shaped oxygen-carrying cells were often ignored by cell biologists for being "too simple", due to their lack of intracellular organelles and DNA, and for being relatively unresponsive to their external environment. They were often regarded as an annoying source of contamination from haemoglobin when processing tissues or isolating white blood cells. Special attention was given to studies of RBC response to cold storage and cryopreservation, which gave rise to the widely used, and perhaps obsolete, term of "the RBC storage lesion"[1]. It all made sense: prolonged cold storage (6 weeks in the USA) of RBC units does not fully suspend animation, as undesirable metabolic reactions may occur that ultimately compromise cell function and their ability to survive in the bag or later in the patient circulation[2].

Among the many stress signals associated with the storage lesion, the amount of spontaneous haemolysis and the release of redox active and toxic free haemoglobin have been shown to steadily increase with storage time[3,4]. Key preclinical studies using transfusion models in small or large mammals strongly suggested that prolonged RBC storage (42 days in most studies) is associated with increased risk of vasoconstriction mediated by haemoglobin consumption of nitric oxide, oxidative injury, sepsis, and accumulation of non-transferrin bound iron in the circulation[4-9]. These observations, along with retrospective studies of age of blood transfusions in humans, led to several international randomised clinical trials that evaluated patient outcomes in response to short-term (1-16 days storage) *vs* long-term (approximately 21-28 days of storage in most studies) RBC storage[10-13]. The majority of these clinical trials failed to demonstrate any difference in the primary outcomes being evaluated (such as mortality) in relation to storage duration. If RBC ageing in the bag does not fully explain variations in the quality of RBC units or adverse clinical outcomes in transfused patients, what else is out there?

The concept of the RBC-Omics Study

In 2011, experts designing the Recipient Epidemiology Donor Evaluation Study (REDS)-III programme[14,15], funded by the National Heart Lung and Blood Institute (NHLBI) of the US National Institutes of Health, were brainstorming new projects that could advance transfusion efficacy and safety. Among the hot topics considered, a few questions received special attention. Does genetic or biological variability in blood donors determine the quality of blood products and resilience of RBCs to withstand cold storage? Are there truly "super donors" whose RBCs store extremely well and "extreme haemolysers" whose RBCs quickly degrade in response to cold storage? If so, which genetic mutations or metabolites predict the fate of RBC storage and post-transfusion outcomes? These questions led to the creation of the RBC-Omics Study, and to a multidisciplinary collaboration among transfusion medicine scientists from RTI International, the University of California San Francisco, the University of Pittsburgh, Versiti Wisconsin (formerly the Blood Center of Wisconsin), Vitalant Research Institute (formerly Blood Systems Research Institute, San Francisco and the Institute for Transfusion Medicine, Pittsburgh), and Yale University (in collaboration with the American Red Cross, CT).

RBC-Omics was one of the largest studies to challenge an old assumption that, except for allogeneic mismatch considerations, the contribution of the blood donor to variability in RBC storage capacity is negligible. In other words, RBC units were similar to mass-produced assembly line products, such as your favourite brand of yogurt. They usually look and taste the same, and have a batch number and expiry date. They may taste funny when they go past their "use by" date, but are unlikely to cause severe side effects unless you are lactose intolerant. But unlike dairy products that are produced by mixing milk from multiple "consenting" donors, RBC units come from an altruistic human being, whose genetic and biological imprints can be quantified and analysed to determine the quality and resilience of their RBCs to survive cold storage. Indeed, pioneering studies in humans and mice have suggested that genetic

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and biological variables (e.g., glucose 6-phosphate dehydrogenase [G6PD] deficiency[16], plasma uric acid[17], donor sex) may impact the resilience of donor RBCs to withstand cold storage[18-21].

After nearly three years of brainstorming, protocol development, and pilot studies[22,23], RBC-Omics was launched in late 2013 with the goal of defining a genetic and metabolomic basis for donor-specific differences in RBC storage stability. In order to achieve this goal, 13,403 blood donors of different race/ethnic backgrounds or donation history gave their consent and enrolled at four blood centres in the United States. These study participants completed study questionnaires and successfully provided whole blood donations from which an aliquot of leucocyte-reduced (LR) RBCs were processed and stored at 4 °C for 42 days in "transfer bags" specifically sourced for the study. Donor differences in RBC storage capacity were defined by performing three high-throughput haemolysis assays on the stored RBC, including the traditional spontaneous/storage haemolysis, and two stress-induced assays that quantified washed RBC responses to osmotic or oxidative stress[24]. Processing laboratories worked around the clock to collect vital specimens from each donor, including LR-filter-derived white blood cells (WBCs) that yielded DNA for subsequent genome-wide association studies (GWAS) of haemolysis, and whole blood for plasma ferritin and haematological indices. Similarly, two testing laboratories faced the daunting challenge of testing thousands of RBC samples for haemolysis assays and creating a biorepository of stored RBCs, WBCs and DNA for future studies.

Donor characteristics are reflected in your average blood bag

The RBC-Omics Study clearly demonstrated the associations between donor characteristics (e.g., sex, age, race/ethnicity, donation frequency, iron intake) and *in vitro* measurements of haemolysis in the blood bag. As these observations have been reported and discussed elsewhere[3,24,25], we chose to highlight some of the major findings. Male sex was associated with enhanced susceptibility to haemolysis in response to cold storage or applied stress that induced osmotic, mechanical and oxidative haemolysis[3,24]. These data confirmed previous reports of differences according to sex in haemolysis in humans and mice that are partially explained by the actions of testosterone[18]. Race/ethnic differences were noted in osmotic fragility, for which RBCs donated by African American donors exhibited enhanced resistance to osmotic haemolysis[24]. Surprisingly, older age (60 years and over) was associated with reduced oxidative haemolysis, and subsequent metabolomic studies revealed age-specific differences in RBC antioxidant capacity[26]. Reduced oxidative haemolysis and ferritin levels were also observed in RBCs from frequent blood donors, particularly in those who had donated nine or more units 24 months prior to the study. Intake of iron supplements was associated with improved RBC resistance to oxidative or osmotic stress[25]. Likewise, use of oral contraceptives or sex hormone therapies was associated with changes in RBC predisposition to haemolysis or with iron deficiency in men[27].

Recalling "extreme" donors

One of the critical questions in RBC-Omics concerned the reproducibility or heritability of haemolysis. To answer this question, 652 RBC-Omics donors whose RBCs exhibited extreme resilience or susceptibility to haemolysis (i.e., subjects above the 95th percentile or below the 5th percentile of spontaneous, osmotic or oxidative haemolysis) were recalled to the study[3]. In addition to investigating intra-donor reproducibility of the haemolysis measurements, recall donor RBCs were processed through the course of LR-RBC component storage into snap frozen aliquots to enable metabolomic profiling that identified biomarkers and metabolic pathways associated with predisposition to haemolysis[23,28,29]. One of the most nerve-racking moments in the RBC-Omics Study was waiting for the first set of analyses that tested the reproducibility of the haemolysis assays between index enrolment and subsequent recall donations. Among the three haemolysis measurements, osmotic haemolysis was proven to be remarkably reproducible (Pearson's r=0.86), followed by oxidative haemolysis (Pearson's r=0.53). Spontaneous storage haemolysis was found to be the least reproducible measurement (Pearson's r=0.40)[3]. These findings suggested that donor genetics largely determine predisposition to osmotic and oxidative haemolysis, whereas spontaneous storage haemolysis, a common tool to evaluate the quality of new RBC products and for quality assurance of routinely produced RBC components, is likely to be determined by non-biological factors, such as blood collection procedures or the type of additive solution; subsequent GWAS of haemolysis in the same donors largely supported these observations[30,31].

RBC-Omics GWAS gives kudos to osmotic fragility

RBC predisposition to osmotic haemolysis has proven to be not only the most reproducible measurement tested in RBC-Omics, but has also provided new insight into genes that modulate erythrocyte osmotic fragility in cold storage and in haemolytic diseases. GWAS of osmotic haemolysis identified multiple genome-wide significant loci across

the entire RBC-Omics donor cohort, and additional loci that were localised in a single racial/ethnic group[31]. These newly identified gene variants may be used in subsequent studies to evaluate their impact on RBC survival and function not only after storage *in vitro*, but also after transfusion. Findings from the RBC-Omics osmotic haemolysis analysis also showed how powerful a simple old test of osmotic fragility can be. A single measurement assay known as the "pink test"[32,33], that was originally developed in the '80s as a diagnostic tool for RBC disorders (e.g., thalassaemia, sickle cell trait, spherocytosis), provided additional GWAS hits that contributed to an outstanding database to investigate modern Omics correlates of haemolysis.

Metabolomic findings

Metabolomic studies of recall donors with extreme levels of oxidative haemolysis yielded preliminary findings of the associations between donor demographics, storage solutions, and spontaneous and stress-induced haemolysis on metabolic pathways[28,34]. Ongoing metabolomics analyses are linking the metabolomics and GWAS findings by identifying biomarkers associated with genetic polymorphisms with known metabolic pathways perturbed by RBC mutations, such as G6PD-deficiency, along with dysregulation in NADPH and glutathione-dependent detoxification pathways of oxidised lipids[1,26]. RBC-Omics metabolomics studies also introduced the concept of storage-induced changes in RBC protein methylation, which provided a novel mechanistic understanding of oxidative stress in stored RBCs[28].

From donor characteristics to patient outcomes

Although RBC-Omics excelled in revealing new biomarkers associated with haemolysis, it was also designed to investigate the impact of donor-specific differences in RBC storage stability and the predictive value of stress haemolysis, genetic and metabolomics biomarkers on clinical outcomes in patients transfused with RBC components[14]. This topic has been much debated among transfusion medicine scientists, largely due to the lack of clinical studies specifically designed to investigate the impact of gene variants or donor characteristics (sex, age, iron status, drugs/hormone consumption) on patient outcomes. Current understanding is based on retrospective analyses that produced conflicting conclusions, partially due to differences in their approaches to statistical analysis or in the selection of transfusion outcomes, for which risk of transfusion-related mortality is commonly used[35,36]. For example, a retrospective study of patient cohorts from Scandinavia and the United States found no associations between donor sex or age and patient survival[37], whereas a comparable study in Canada associated cross-sex transfusions (mainly male donor RBCs transfused into female patients) and younger donor age (<45 years) with increased risk of in-hospital death[38]. In order to resolve such controversies, new studies are needed that use novel approaches to determine transfusion efficacy and recipient outcomes. For example, evaluation of the increase in haemoglobin following blood transfusions may provide information regarding the quality of the transfused RBCs, for which higher increments are associated with better efficacy. So far, pilot studies have suggested that factors such as gamma irradiation of RBC units or increased RBC storage duration may result in lower increases in haemoglobin after transfusion[39,40], whereas transfusion of male RBCs resulted in higher increases in haemoglobin compared with female RBCs[41]. Although these observations require further validation, this approach may be used in future studies aiming to determine transfusion outcomes from highly characterised RBC donors.

The legacy of RBC-Omics

With the closure of the NHLBI REDS-III programmes in 2019, RBC-Omics will soon be establishing databases of the findings of the study, including demographics, haemolysis, genetics and metabolomics, as well as a large biospecimen repository; this is to be made available to the public in 2020. This will provide a goldmine of information for those who seek to advance the knowledge of red blood cell biology and pathology or interactions between donor characteristics and patient outcomes. An equally important legacy is the demonstration of multidisciplinary teamwork in a transfusion medicine project that required outstanding commitment from all RBC-Omics members, including team leaders, administrative staff, the data co-ordinating centre, researchers, technicians, and, of course, the nearly 14,000 blood donors who agreed to take part in this study.

REDS-IV-Paediatric programme

In April 2019, the NHLBI launched the REDS-IV-Paediatric (P) programme, focusing on adult and paediatric transfusion research. This programme will develop and carry out new research projects aimed at advancing transfusion safety and efficacy. It is hoped that with the launch of this programme, and the establishment of aligned collaborative efforts with the United States and international researchers in transfusion medicine, genetics, and RBC biology, further investigations that could expand the RBC-Omics database and biospecimen collections will be carried out to advance the development of precision transfusion medicine.

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