TRANSFUSION MEDICINE AND BLOOD

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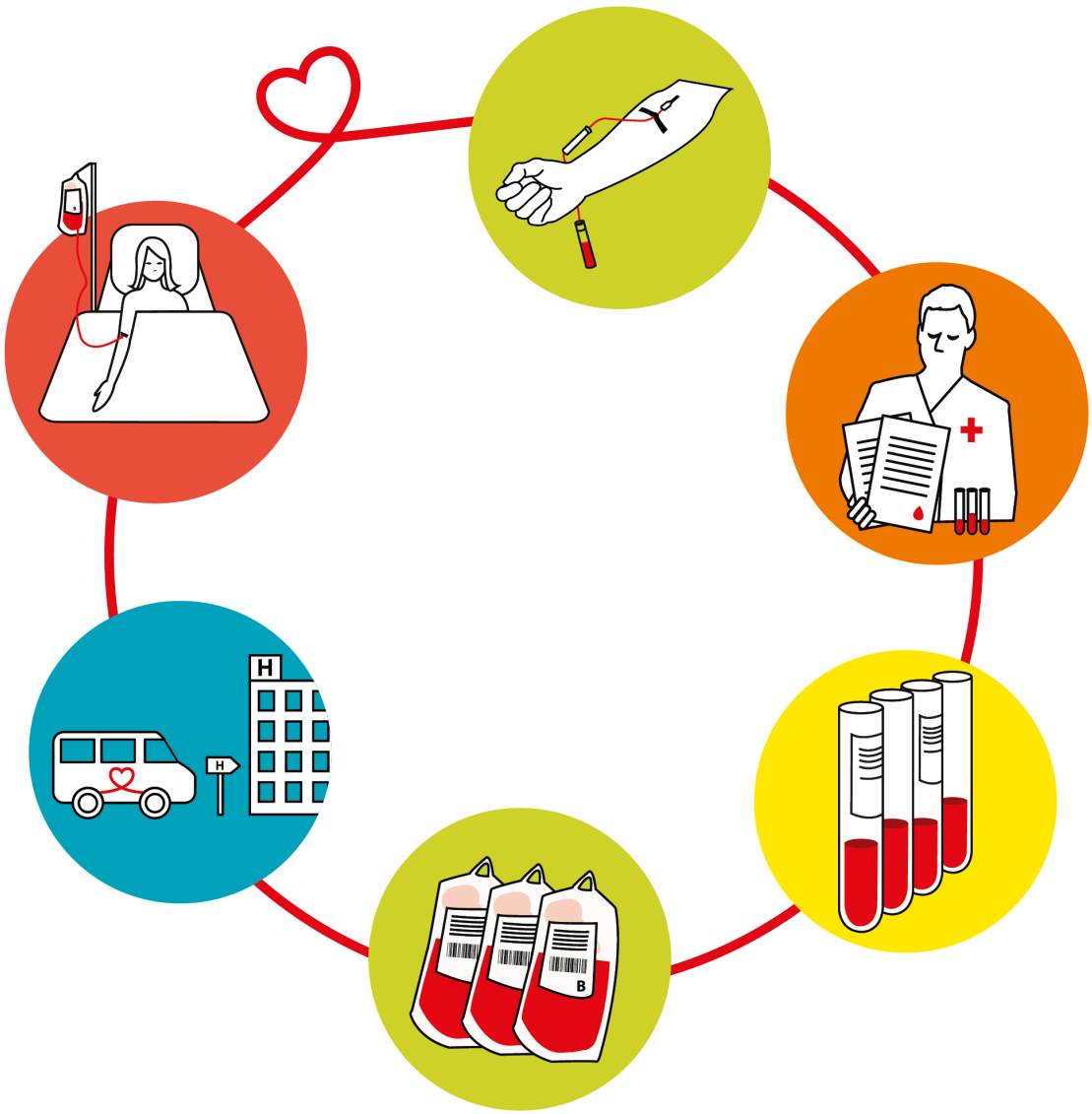

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TRANSFUSION MEDICINE AND BLOOD

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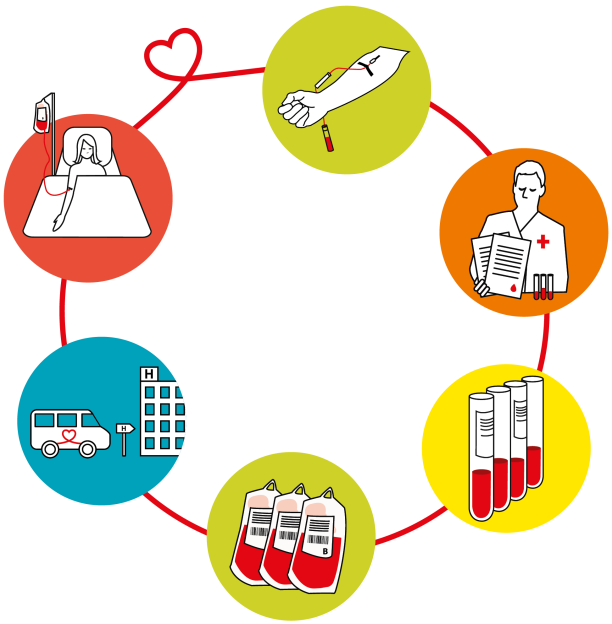

Transfusion chain: from donor to patient. © Agence-NOW.ch

Research in transfusion medicine is diverse and interdisciplinary, involving scientists and physicians in hematology, basic sciences, biology, biotechnology and so forth. It regularly proposes innovation from the donors to the patients along the whole transfusion chain in terms of blood screening, processing and transfusion praxis.

The present Research Topic covers recent advances in transfusion medicine and blood, and provides an overview of the current knowledge. It includes original articles, reviews and perspectives for the future challenges.

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EDITORIAL
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Editorial: Transfusion Medicine and Blood

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Editorial on the Research Topic

Transfusion Medicine and Blood

Transfusion medicine is in perpetual evolution and has faced several challenges since the seventeenth century. Nevertheless, it is a relatively new specialty based on an old and simple concept that is transferring blood from an individual to another one. The history of the discipline is quite rich, the first steps being associated with killing patients (1) (due to numerous reasons sometimes not necessarily related to transfusion) notably because of acute hemolytic reactions related to ABO incompatibilities (beyond xenotransfusion). Indeed, before 1900, the blood groups were unknown (2). Over the years, transfusion medicine evolved from a dangerous concept to a reality, and was really the first example of personalized medicine when transfusion was performed arm to arm: the donor and the recipient were in the same room, with a single doctor caring both individuals as well as the process (3). Furthermore, the blood transfused was a non-denaturized product: it was "true" blood, "pure" blood, without any process and storage lesions.

Nowadays, blood transfusion is the paradigm of both precision and personalized medicine: (i) blood or blood components are collected from donors with well characterized blood groups and/or HLA geno-phenotypes and or sometimes for particular (non)-reactivity with specific pathogens such as hepatitis E or cytomegalovirus, (ii) blood is separated in its different components that are red blood cells (RBCs), platelets, plasma, and other therapeutics. Whatever the "philosophic consideration," more than 100 million of donations per year are performed worldwide and, even if at risk, transfused blood products save lives. Nevertheless, the pressure in terms of efficacy, safety, blood management, and cost constrains currently pushes a further evolution of transfusion medicine. Research in transfusion medicine is diverse, interdisciplinary, and regularly proposes innovation along the whole transfusion chain, involving every stage from the donors to the recipients. Each step (donors and blood collection, screening, preparation, storage, transfusion as well as post-transfusion survey) has been considered in order to improve safety, quality, and benefit to the patients. Some of these aspects are highlighted by the diversity of the published articles in this research topic and introduced below.

GLOBAL AND INDIVIDUAL DIVERSITY

The diversity in transfusion medicine is real (beyond the blood groups), and this diversity is present at different levels. Socio-political and economical aspects of transfusion medicine are of primary importance when western countries participate to the development of blood collection and processing in lower to upper middle-income countries. It is the case in Eastern/Southern Mediterranean countries where surveys are currently ongoing to address diverse issues in transfusion medicine, as reviewed by Haddad et al. The diversity is also dependent on the donors

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(genetically or not), on the patients, and on the processing and storage, where differences in blood component quality were reported. Garraud and Tissot discussed these differences and similarities, and the potential consequences in transfusion.

SAFETY AND INFECTIOUS DISEASE AGENTS

Safety of blood transfusion is achieved at different steps of the chain. The first link is the donor questionnaire based on compliance where both donor and patient health are considered. The questionnaire and the interview are the results of several adjustments and efficient analyzes (as deeply investigated by Gillet and Neijens), where nurses and other health professional play a central role.

The next link is blood testing for infectious disease markers. The first blood donor screening test for an infectious disease agent to be routinely introduced was implemented in the early 1940s to detect a *Treponema pallidum* infection. Until the discovery and characterization of the Australia antigen (HBsAg) in 1968, no further tests were implemented. Up to the early 1990s many blood products were contaminated with HIV, HBV, or HCV, which consequently resulted in many transfusion-related infections. Following the AIDS scandal during which thousands of blood product recipients were infected, there has been a vast increase in specific and elaborated measures to prevent such dramatic infectious disease transmissions. These include the introduction of a sophisticated donor questionnaire, a rigorous disinfection of the skin associated with the elimination of the first few milliliters of the collection in order to decrease to load of skin-related bacterial contamination, the leucodepletion as a toll to eliminate intracellular microbes—beside the reduction of the immunogenicity and of the storage lesions of blood components, highly sensitive and specific serological tests and finally new test technologies such as nucleic acid testing (NAT) and pathogen reduction (PR). All these measures have greatly increased the safety of blood products in relation to the transmission of pathogens. In the last decade there has been a new focus on the so-called (re)-emerging infectious agents, such as West-Nile virus, Chikungunya virus, Dengue virus, and Zika virus. The interest in these diseases led to the introduction of new measures to prepare the blood donation community for the potential emergence of these threats. The current situation in the Latin American continent is described by Levi. Particularly in Europe the viral agents Parvovirus B19, Cytomegalovirus (CMV) and more recently Hepatitis E virus (HEV) have become an important issue. Juhl and Hennig and Dreier et al. show how Parvovirus B19 and HEV, respectively, are relevant in the safety of blood components. Despite the measures described above, there are still several issues which need to be addressed. For instance, it is often not clear whether a pathogen is really transmitted by blood products. If the agent is shown to be transmitted, then the question of the infectious dose needs to be addressed. Both, the transmission and the infectious dose, are essential issues which must be evaluated prior to the design of a specified test strategy. Furthermore, the importance of the virus load, as exemplified for HBV, is addressed by Candotti and Laperche. PR has recently become standard practices in many countries either for platelet concentrates and/or plasma resulting in safer blood products. It remains to be seen whether PR of whole blood and/or the automation of the procedure might, in the future, increase the efficiency. With the progressive introduction of various PR technologies, there is place for discussions how these technologies will either complement or replace the existing screening strategies. Seltsam reviewed in a very informative manner the recent developments in the PR of blood products completed by a comprehensive description of the molecular impact in platelets by Schubert et al. Particularly the possible implementation of an automated PR system of whole blood will lead to debates whether the current highly sensitive NAT systems required or whether less sensitive pool NAT systems will be sufficient. It is also possible to envisage an approach by using multiplexed viruses detection in one NAT reaction in order to save costs.

Ultimately it has to be clarified how, when and why new measures should be introduced. Appropriate decisions must be made together with all the various stakeholders involved, based not only on safety points but also on clear cost-benefit considerations. A well-adjusted safety policy will not be easy to define and certainly will not satisfy everyone involved in the global process. The balance between maximal or optimal safety and the high costs are relevant topics: the subject is elegantly discussed by Zaaijer in his open minded review.

UNDERSTANDING THE BLOOD PRODUCTS QUALITY

PR systems (as stated above) have recently become standard in many countries for platelet concentrates and plasma resulting in safer blood products (Seltsam; Schubert et al.). This innovation is a typical example where the processing impacts several biological functions of blood products. In-depth investigations of RBCs, platelet and plasma components have been carried out during the last decades. For instance, the impact of PR on platelets (reviewed by Schubert et al.) illustrates the role and the advantage of these investigations in understanding the preparation of blood for transfusion. The platelets thus produced exhibit different properties (we meet once again the diversity) that might be particularly relevant for recipients. Another interesting aspect of platelets physiology was pointed out by Handtke et al. They reviewed the relevance of cell subpopulations for transfusion which is of importance knowing that the method of collection and processing might enrich or deplete specific phenotypes. The next cutting-edge innovation is clearly the *in vitro* production of platelets. This field of investigation [as it has been achieved a few years ago for RBCs (4)] is clearly moving forward and Strassel et al. reviewed the advancements and challenges in this promising facet of transfusion medicine that could deeply transform the whole blood chain supply.

RBCs have been also deeply investigated during the last decades and the related articles illustrate here the achievements

reached so far. Nemkov et al. have recently focused on metabolism of citrate in mature RBCs. Their interesting metabolomic investigation reports the consumption of citrate and other carboxylates in function of hypoxia. Using a similar approach, the same group in collaboration with Kriebardis and colleagues (Reisz et al.) highlighted the consequences on storage parameters when blood was collected from glucose 6-phosphate dehydrogenase (G6PD)-deficient donors. These investigations are not only relevant for better understanding of pathophysiological behaviors of RBCs but also for the investigation of storage lesions, which are the consequences of a cascade of events (5). At the other end of metabolism rerouting, cell morphology shifts from a discocyte to a spherocyte with an impact on deformability. Several tools are available to quantify these modifications and Roussel et al. described a simple and elegant method to assess volume and morphological parameters using a microfluidic chamber coupled to fluorescence exclusion without RBC pre-treatment. These approaches will be useful for as well the future developments of preservation strategies as the physiological understanding of RBC biology. The assessment of transfused RBC quality (knowing the deleterious effects of processing and storage) in clinical trials is required. The gold standard is obviously the 24-h post-transfusion recovery that is one of the specifications that has to be met in order to validate red cell concentrates (RCCs) for transfusion. Nevertheless, the different methods reviewed by Roussel et al. exhibited weaknesses and other markers of storability would be a plus in validation procedures and clinical trials.

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Last but not least, we should never forget that blood products are designed and processed for transfusion purposes. The role of RBCs in neurodegeneration was explored in a perspective paper by Bosman. The pathological context of the disease (here neuroacanthocytosis) and the RBC abnormality can affect brain oxygenation; the question on treatments that could reduce RBC defects is thus opened. The previously mentioned metabolomic approach on G6PD-deficient donors was also applied to assess the status of fresh frozen plasma (FFP) derived from these donors by Tzounakas et al. Their results highlighted different properties compared to standard FFP which might deserve the transfusion of plasma taking advantage of antioxidant and procoagulant activities. The transfusion of FFP has his pro and cons clinicians and recent studies plaid for beneficial effects of plasma. In this sense, Barelli and Alberio interestingly discussed the role of FFP in massive bleeding and its ability to preserve the endothelial glycocalyx structure and function. As for the platelet transfusion where their hemostatic role is obvious, Sut et al. pointed out in a detailed review the inflammatory potential of stored platelets and their involvement in adverse reactions.

Another important adverse transfusion event is alloimmunization resulting from the phenotypic disparities between donor and recipient. Repeated blood transfusions can result in the production of alloantibodies against one or more RBC antigens, which in turn complicate subsequent transfusions. Such events often lead to difficulties in providing a compatible RCC for a future transfusion, which can lead to an increased morbidity and mortality in transfusion-dependent patients (as in sickle cell anemia). Furthermore, due to the increasing heterogeneity of populations, the matching of the labile blood components for patients of different ethnicities is becoming increasingly awkward. van Sambeeck et al. reviewed the future approaches in blood screening and patient compatibility of the blood products to improve targeted donor recruitment strategies.

The thematic covered in this research topic on "Transfusion Medicine and Blood" is all but exhaustive: however, it represents recent advances and approaches along the transfusion chain, including specific preparations of blood-derived products such as the serum eye drop reviewed here by Drew et al. to treat dry eye syndrome. The clinical trials, the future of universal blood and all the therapeutics aspects of blood collection and cellular therapies were not included here but are part of our everyday life. The aims of all these researches are always to provide safe and adapted blood products for patients in a sustainable financial context, while keeping a suitable pool of health donors. It is interesting to point out that several papers in this collection consider the costs in their discussion since the margins are quite narrow to improve the whole chain.

In this context, research and development strategies are of primary importance to assist and shape the future of transfusion, and to convince stakeholders and other decision-makers. As in other scientific and medical fields, the economic pressure is important and it is difficult to grant research program. Moreover, the public funds are not always available for transfusion medicine and blood banks have to contribute to finance R&D by themself. Different organizations are in place worldwide partly supported by the academia (around two third of the authors here are employed in academia or university hospital) or 100% by blood banks. It is noteworthy that the investments are compulsory to shape the future and we cannot ignore the vast developments and innovation the R&D can initiate and support. Transfusion medicine has definitively been moving (even fighting against dogma since the 17th, dogma that still exist in different forms) and the science of blood transfusion is at the heart of medicine. Because, as already mentioned, transfusion medicine is both a personalized as well as a precision medicine.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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How Can Eastern/Southern Mediterranean Countries Resolve Quality and Safety Issues in Transfusion Medicine?

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Unlike their Western counterparts, some of the Eastern/Southern Mediterranean countries lack centralized coordinated blood transfusion services leading to an unequal blood safety level. This was recently highlighted by a recent World Health Organization (WHO) regional committee report in which WHO urges these countries to establish and implement a national blood system with well-coordinated blood transfusion activities and to make attempts to reach 100% voluntary non-remunerated blood donation. The objective is thus to meet the same levels or standards as Western countries in term of self-sufficiency and blood safety. This raises the question whether these countries can either comply with Western countries' guidelines and experiences or develop their own safety scheme based on proper sociopolitical and economic features. Another option is to identify efficient and cost-effective strategies setup successfully in neighbor countries sharing cultural and economic features. To address this issue—and make an attempt to achieve this goal—we designed a number of surveys specifically addressed to Mediterranean countries, which were sent out to the national authorities; so far, five surveys aim at covering all aspects in blood collection, processing, testing, inventory and distribution, as well as patient immune-hematological testing and follow-up (including surveillance and vigilances). It is anticipated that such practice can help identifying and then sharing the more successful and cost-effective experiences, and be really focused on Mediterranean areas while not necessarily copying and pasting experiences designed for Western/Northern areas with significantly distinct situations.

Keywords: transfusion, Southern Mediterranean, Eastern Mediterranean, quality, safety, VNRD, blood supply

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INTRODUCTION

Despite having lower to upper middle-income economies, Eastern/Southern Mediterranean countries, compared with high-income countries (often referred to as either Western or Northern countries), provide similar transfusion therapies for a wide range of diseases and conditions. This comprises, among others, care for thalassemia major, and to a lesser extent, sickle cell disease patients, and assistance to transplantation programs, including stem cells (1, 2). However, unlike their Western counterparts, some of these countries lack centralized or coordinated blood transfusion services resulting in

different levels of blood safety in terms of quantity and quality: insufficient blood supply, unequal availability of blood components (BCs) and nationwide health-care coverage, inadequate financial and human resources, etc. (3, 4). All of which were regularly highlighted by World Health Organization (WHO) reports, urging these countries to establish and implement a national blood system with well-coordinated blood transfusion activities. In parallel to its recommendation on blood use and surveillance, WHO also advised all countries to achieve a 100% voluntary non-remunerated blood donation (VNRBD) objective by the year 2020 (recently postponed to 2025 for Eastern Mediterranean countries) (5, 6). In stating this, WHO considers that the fundamental strategy to ensure timely access to safe and sufficient supplies of blood and blood products is the development of a nationally coordinated blood transfusion service based on VNRBDs without any other alternative (7).

CURRENT QUESTIONS ADDRESSED BY DEVELOPING COUNTRIES REGARDING THEIR BLOOD COLLECTION AND TRANSFUSION PROGRAMS

Almost all countries in the process of implementing or strengthening their own national blood transfusion program must address seven key questions:

- (ai) How can we meet the clinical demand and become self-sufficient in procuring BCs?
- (aii) How can we guarantee donors' safety during blood collection?
- (aiii) How can we ensure BCs are issued at the safest level (at least regarding transfusion-transmitted infections and immunohematological compatibility)?
- (aiv) How can we ensure a quality management-driven organization?
- (av) How can we set up a surveillance system to follow-up donors, the BC chain process and recipients?
- (avi) How can we guarantee optimal clinical use of blood and ensure Patient Blood Management (PBM) while avoiding inadequate transfusions and/or loss of expired blood products?
- (avii) How can we educate all staff categories (and/or other stakeholders)?

It is evident that not all countries are at the same level of progress toward these goals. In fact, the majority face a number of obstacles such as (8):

- (bi) Available financial and human resources (including educational level).
- (bii) Cultural habits, traditions, and experiences (including perception of blood donation or infusion and quality management).
- (biii) Unfavorable epidemiological conditions (active circulation of vectors, viruses, and other pathogens that may be transmitted by blood).
- (biv) Multiethnic population with antigenic diversity that makes immunohematological matching difficult to achieve for some patients.

These goals cannot be achieved without a strong commitment from the public authorities and the backing of the Ministry of Health in the country concerned.

Bearing in mind that quality, safety, education, surveillance, and vigilance program apply to all three aspects, the blood transfusion process can commonly be reported as a three-legged stool, i.e., the "A, B, C" of the process:

A. Donors, donations, and/or blood collection.
B. BCs (i.e., blood outside the donor and not yet transfused to the recipient).
C. Recipients.

THE "A, B, C" OF THE BLOOD COLLECTION AND TRANSFUSION PROCESS WITH SPECIAL REFERENCE TO DEVELOPING COUNTRIES

Donors/Donations/Blood Collection

It would be tempting to start with the "A" leg of the stool; however, it must be borne in mind that blood donation and/or collection only exist because there is a demand. There are three major approaches for analyzing this demand: (i) a passive analysis, which is the easiest and consists in retrospectively reviewing all BCs issued over a defined period of time for predefined clinical situations (obstetrical bleeding, trauma, malaria, etc.); (ii) an active or prospective analysis, more difficult to address, which consists in predicting the needs in a given population to fulfill certain clinical indications such as cancer therapy, transplantation, and internal medicine; (iii) a combined (active and passive) analysis, which simultaneously reviews historical data and prospects the needs based on the development of the patient recruitment processes. Therefore, it is instrumental to stratify the actual needs and anticipate any increase in blood demand based on the available hospital strategies to recruit patients and on the consensuses regarding transfusion strategies for every patient category. All of the above is aimed at defining whether a system is self-sufficient or not. Indeed, can anyone consider a system self-sufficient when it only ensures BCs on a daily basis (emergency or/and bleeding situations) as regularly seen in developing countries? Consequently, it can be deduced that self-sufficiency is difficult to define and should be driven by audits assessing both short (or daily basis) and long-term needs.

Once the demand is defined, blood collection programs can be launched to build up inventories. Here again, there are essentially two main pathways: the first applies to small Hospital Blood Banks (HBBs) and consists in fulfilling the arbitrary need on a daily basis. The second—usually seen in larger settings—is based on a program, adjusted according to the statistical consumption of blood. The latter is more suitable with the VNRD-based blood supply system where mobile drives in partnership with non-governmental organizations (NGOs) such as the Red Cross/Red Crescent, can eventually be planned in advance.

The respective values of VNRD and replacement donation can now be discussed.

Replacement donation consists in donating blood voluntarily in case a relative is in need, therefore contributing to blood bank replenishment. This donation mode is predominant in almost all Mediterranean countries. In fact, a recent Greek study found that donors seem to be more sensitized by the need in BCs rather than altruism, contrary to what is seen in VNRD systems (9). The donation is addressed specifically to the bank and not to the patient, since the donor and recipient are not required to have an identical blood group. The recipient will be transfused with already processed BCs, bearing in mind that the anonymity process is always guaranteed. No direct benefits are provided by the patient, donor or blood bank. However, indirect benefits cannot be ruled out for each party. Based on the literature, replacement donation should not be abandoned since it is more efficient in small facilities where the collection of blood and inventory replenishment occurs at all times simultaneously with the BC delivery activity (10).

However, both systems have their own advantages and disadvantages (11). The VNRD system clearly favors intergroup solidarity (region and nation), while the replacement system favors intragroup solidarity (village or neighborhood, family, and work station). Complying with WHO recommendations and abandoning replacement donation should, if adopted, be scheduled progressively and strongly encouraged and supported by the national authorities.

The following two examples appear to illustrate this statement: (a) the first is the Lebanese experience (12). In this country, blood banking does indeed largely depend on replacement donors for several reasons: decentralized system, predominance of the private health-care sector over the public (i.e., a fragmented system) and the cultural habits of its inhabitants who are used to react in emergency situations (13). Recently, NGOs started taking initiatives to promote national solidarity and VNRD and have made considerable progress (14). However, this should be further encouraged by the national authorities who lack complete involvement (12) due to political issues. Meanwhile, should Lebanon encourage family replacement donors who meet all the classical criteria of VNRD to donate regularly? In fact, some authors consider these donors legitimate and indispensable (10, 15). (b) The second experience is the Moroccan one where VNRD is highly valued and where national authorities are fully involved under the blessing of the Royal Family. In fact, the Royal Family has been photographed while donating blood and their pictures are displayed in blood centers clearly to motivate donors and promote voluntary blood donation. Despite cultural similarities with Lebanon, Morocco, which is a centralized state, seems to be on the right track toward achieving 100% VNRD (the WHO target).

VNRD is recognized as the universal goal for all countries since it fully respects the ethical issues of donation; however, replacement donation might be regarded as ethically valuable and efficient in some cultures. In addition, some authors estimate that replacement donors are as safe as VNRD and less costly to health-care systems (16, 17).

In our opinion, an interesting but highly debatable strategy that can significantly alleviate the burden of transfusion-transmitted infections in endemic areas (i.e., Africa) is to establish financial contracts with "safe" donors (committed to safe behavior). However, such compensation would fall into the for-profit category according to the Nuffield Council on Bioethics (18), which raises two points: (a) The first is that this strategy is beneficial to patients in terms of supply and safety, as these donors ought to be at least as safe as or most probably safer than ordinary donors. (b) The second is related to ethical issues: are ethical values so inflexible, universal, and really independent from culture? This is perhaps debatable.

Emerging transfusion systems should also consider donor hemovigilance—set up over a decade after patient hemovigilance—especially when populations' iron stores are threatened by many local reasons such as ethnicity, nutritional aspects, and digestive parasitosis. Furthermore, the frequency of donations also has a strong impact on donor safety and the depletion of iron stores. Finally, the ethics of donation or collection is now regarded as a strong pillar of safety (19).

Thus, it would be an interesting option for each country to consider the establishment of a Blood Supply Committee to discuss the organization and ethical issues of blood supply according to local characteristics and constraints. This committee should comprise not only of professionals and authorities but also representatives from various branches of human and social sciences (economy, ethics, sociology, etc.), and other stakeholders.

BC Processing and Quality Management

The "B" leg of the transfusion stool encompasses the BCs and the quality management system. Nowadays, blood is collected, anticoagulated—which is a manipulation—and processed. Any BCs that are made available need to be defined (whole blood, red blood cell concentrates, plasma for therapeutic use, platelet components, etc.), along with their characteristics (volumes, active compounds with their minimum therapeutic levels, quality indicators, etc.). Furthermore, BCs can be either leukoreduced or not, and, if so, certain indicators must be defined such as date and time, pre- or post-storage (at bedside) leukoreduction and its efficacy, storage conditions (temperature), storage period, and expiry date. BC modifications such as irradiation, pathogen inactivation where available, volume reduction, washing, and splitting or pooling must be considered to define what is accepted by the system and what is not, and in which conditions. Quality indicators at all stages may be set up to monitor each part of the entire process (20).

Finally, testing of either the donor or the donated blood product is mandatory and not optional, but its extent and the decision tree upon biological findings may vary between systems. Indeed, no transfusion system can consider not testing donors for HIV, but not all HIV tests are equal nor are they confirmatory. Furthermore, some systems now retest previously donated BCs (using frozen plasma samples) in the event of positivity on the current donation.

However, not all systems allow the retrieval of historical samples. All of this depends on the organization, policy, and resources allocated to blood testing. An issue which is likely addressed in well-established systems is the definition of acceptance or rejection criteria for a given product (volume, quality, infectious safety level, extended blood grouping/phenotype, and residual leukocyte count to control or reduce inflammation). In fact, defining the infectious safety of a given BC (e.g., for a

given virus) is a very difficult task. For example, is the residual risk of HIV-1 infection defined by less than 1 in 10^6 donations, considered within the acceptable range? This may be unacceptable in countries that control HIV transmission well (i.e., in Europe or the USA), but unachievable in countries with a high prevalence of HIV in their population such as Middle Africa (21). It is clearly not the responsibility of HBB or Blood Establishment (BE) professionals to define such criteria, but rather the national public health authorities.

Recipients (the Beneficiaries of BC Transfusions)

The "C" leg of the transfusion stool represents the recipients. Once the BC is qualified for issuing (and subsequently labeled), which is the main responsibility of the BE alongside the procurement of BCs that meet the demand, there remain a large number of tasks that the HBBs still have to perform, as they have to match the immunogenic and immunophenotypic characteristics of donor and recipient bloods. This is never a simple process in high-income countries and can be extremely difficult in low-income countries due to scarce resources: BCs in the inventory, resources to type and match bloods, etc. The financial burden of transfusions in high-income countries, where BC access was not restricted, has been extensive for decades with probable over-transfusion (22, 23). Those countries have now started to reduce unnecessary transfusions, implementing recommendations on Optimal Blood Use (OBU) and operating the so-called PBM programs (24, 25). Should low-income countries, where over-transfusion does not likely exist, start implementing PBM programs? And should they also apply OBU programs? We would be tempted to answer yes to both questions. In fact, both programs are aimed at improving the quality of medical services delivered to patients in need and reducing complications. Issuing recommendations that follow the general (universal) standards and are adapted to the actual situation of the country/system would be beneficial to both patients and the transfusion systems.

The major issue that developing countries face is the scarcity of surveillance and hemovigilance programs, with limited resources to recognize and report adverse reactions to implement improvement programs based on quality management. Another obstacle is the lack of evaluation of applicable or newly developed practices. Finally, hospital transfusion committees should be encouraged since they have proved to support transfusion safety in many places (26).

CONCLUDING REMARKS

All of the foregoing raises the question of whether Eastern/Southern Mediterranean countries can either comply with Western countries' guidelines and experiences or develop their own safety scheme based on proper sociopolitical and economic features. Another option (which does not necessarily contradict the previous ones) is to identify efficient and cost-effective strategies in neighboring countries that have had successful experiences and share similar cultural and economic features. To address this issue, and attempt to achieve this goal, we designed a number of surveys addressed specifically to Southern/Eastern Mediterranean countries that were sent out to national authorities when they existed, or to pre-identified blood banking specialists. So far, five surveys (comprising of 45 pages in total) have been produced and disseminated aimed at covering all aspects of blood activities [1—organization of the national transfusion service related to donors and staff; 2—prevention of infectious risks and prevalence of infectious diseases; 3—type, quantity, and specifications of produced blood products; 4—quality management system and the specifications of the environment of transfusion practices (education, vigilance, and invoicing); and 5—conditions of release and the use of blood and blood products] to collect and analyze data and standards. The surveys include series of questions tracking carefully all transfusion procedures that can be answered with a YES or NO and sometimes a box exists for some questions to place comments. All Southern/Eastern Mediterranean countries were targeted but only eight (Southern: Egypt, Morocco, Tunisia, Mauritania, and Algeria; Eastern: Lebanon, Jordan, and Palestine) responded to these surveys, which are currently being analyzed and validated before their communication. The preliminary results indicate that the organization of blood service in these countries is heterogeneous, as some countries have national systems and some others a decentralized organization; all countries nevertheless face similar challenges; to cite some: the blood supply relies mainly on replacement male donors; there is no clear strategy to secure the infectious safety of blood (and for instance no nuclear acid testing); there is not always an adequate quality management system; there is an evident lack of proper education; hemovigilance, when existing, is stammering, alongside all vigilances and surveillance processes. Some interesting experiences (e.g., universal leukoreduction in Lebanon, production of derived plasma products in Morocco, etc.) deserve to be highlighted and discussed regarding their outcomes and cost-effectiveness. It is anticipated that such a practice can help identify and then share the more successful and cost-effective experiences, and really focus on Mediterranean areas while not necessarily copying and pasting experiences designed for Western/Northern areas with significantly distinct situations in terms of donors, recipients, politics, economics, and even ethical and philosophical baseline.

AUTHOR CONTRIBUTIONS

AH and OG designed and wrote the paper. TA assisted with the writing and critical revision of the paper.

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Blood and Blood Components: From Similarities to Differences

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Blood transfusion is made possible because, in most countries and organizations, altruistic individuals voluntarily, anonymously, and generously donate (without compensation) either whole blood or separated components that are then processed and distributed by professionals, prior to being allocated to recipients in need. Being part of modern medicine, blood transfusion uses so-called standard blood components when relative to cellular fractions and fresh plasma. However, as will be discussed in this paper, strictly speaking, such so-called labile blood components are not completely standard. Furthermore, the prevalent system based on voluntary, non-remunerated blood donation is not yet universal and, despite claims by the World Health Organization that 100% of blood collection will be derived from altruistic donations by 2020 (postponed to 2025), many obstacles may hinder this ambition, especially when relative to the collection of the enormous amount of plasma destined for fractionation into plasma derivative or drugs. Finally, country organizations also vary due to the economy, sociology, politics, and epidemiology. This paper then, discusses the particulars (of which ethical considerations) of blood transfusion diversity and the consequences for donors, patients, and society.

Keywords: transfusion, blood donation, blood processing, blood components, ethics

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INTRODUCTION

Blood and blood components (BCs) for transfusion chiefly originate from donations made by altruistic individuals. However, although 100% voluntary non-remunerated blood donation (VNRD) is the goal that has been set (by 2020 as publicized by the World Health Organization WHO—later revised to 2025 in some areas) (1, 2), it is far from being achieved at the present time for various reasons linked to contingency (3), and the emergence in low/middle-income countries of modern health-care services requiring more transfusion prescriptions. Blood for therapeutic use comes into two sets: (1) the one consists in labile blood components (LBCs) comprising essentially the cellular components [red blood cells concentrates (RBCCs) and platelet concentrates, PCs as well as part of therapeutic plasma, principally fresh frozen plasma FFP]; and (2) the other one consists in plasma derived or fractionated drugs, and occasionally in FFP obtained from large pools and subjected to stringent pathogen reduction. While the former is principally handled by blood establishments (BEs), many of them overruled by the public sector or Non Governmental, non-for-profit, organizations such as the Red-Cross/Red-Crescent, the latter is largely handled by the plasma fraction industry within the private, for-profit sector.

Labile blood components are usually labeled as "standard blood products," and thus refer to guidelines such as the Council of Europe's "Guide to the preparation, use and quality assurance of blood components" (4). However, all LBCs referring to the same label are, in fact, significantly

different from each other: they depend on the donor, the process and the storage characteristics which are not consistent, as each consists either in single donor originating units or small pools (5 in mean, and >12 by all means); minipools apply to PCs and therapeutic plasma. Indeed, as each LBC reflects both the genetic and non-genetic-based characteristics of the donors (referred to as "storage lesions"), they all differ from one another despite considerable efforts made to minimize deviations in their process.

This paper will briefly outline the most visible community forms of and differences in LBCs, and the way in which differences can affect outcomes in: (1) recipients; (2) health policies and economics (as essential additional safety procedures can result in even more complex disparities between LBC inventories). The circumstances of blood collection and how ethics are concerned when blood supply does not meet demand or when marketing creates new markets will also be discussed.

AS THE ESSENTIAL DETERMINANT IN TRANSFUSION, BLOOD REPRESENTS A COMMON MATTER FOR HUMANS

Every human depends on nearly normal blood functions to survive. Moderate alterations may result in sub-physiological functioning, while more severe defects can be corrected by either drugs or blood derivatives (when available), or both, pending anticipated gene correction. Blood characterizes species and cannot be safely exchanged between species, even close ones such as humans and apes. Human blood transfusion can nevertheless be processed within the human species provided major compatibility rules are respected. This property first allowed blood transfusion to be performed in emergency situations, and later, with technological advances, to be applied almost routinely, despite the fact that "routine" is a word that never applies to blood transfusion from either the donor's or the recipient's perspective (5, 6).

Donated blood is humanity (7, 8) and ideally, VNRD blood is "pure" humanity, yet replacement donation shares the values of assistance and cannot be considered non-altruistic, even if a certain degree of social pressure cannot be ruled out (3). Maybe the lowest common denominator is the root symbol of blood, its sacred characteristic, whatever sacred may mean to people, ranging from religious to atheistic sentiments (9). Thanks to initiatives like Blood Donor Day on the 14th of June, it can be acknowledged on the highest scale that many people worldwide share the idealistic human view of blood donation for transfusion purposes (10). Ideally, this standpoint should make sure that such a process expands and infuses in areas where VNRD is not yet achievable. However, the situation is far from universal or exemplary. Three taints can be outlined as follows: (1) as the demand for BCs expands in many countries, BEs start applying marketing methods to attain prospective new blood donors, potentially altering the voluntary aspect of donation, or the benevolence (or the absence of profit) (11). The same holds true for plasma collectors within the industry (12–16). (2) The benefits of blood have been distorted to serve non-life-threatening medical conditions, and BCs have entered the for-profit market and business where blood is being increasingly used in welfare clinics for doping or cosmetic applications (17, 18). This merchandized blood may, therefore, no longer be available for therapeutic and medical indications, potentially worsening the shortage problem and ultimately leading BEs to use enhanced marketing tools and enter a vicious circle. (3) Blood and BCs for research now represent an expanding market, they can either be "conventional" as BEs trade left-overs from test tubes or residues from blood processing, or BCs purposefully processed, i.e., BEs collect blood from VNRDs not eligible for the therapeutic pathway or presenting characteristics required for specific research. In those cases, VNRDs are usually informed that all or part of their donation will be used for research programmes and they may object to this use. Moreover, some donors sell blood for specific research programmes, with the blood generally being collected outside of BEs; this is possible in some countries but is strictly forbidden in others. Research ethics committees would prevent these possibilities.

SIMILARITIES OF HUMAN BLOOD

As has already been outlined, one common platform for humans considering blood is the general anthropological consideration of this "fluid" which is both material (the blood tissue) and spiritual or the like (altruism, attraction/repulsion, life and death, genetics and lineages, war and peace, and so forth) (9). Regarding its materiality, human blood is unique to the species, and there is by no means any significant difference between ethnic groups, which refutes previous racist theories on the purity of blood. There are indeed certain variations that vary in their frequency in ethnic groups as will be presented in the next section of this paper, but none of them prevent donated blood from being issued to any other human, apart from major (ABH) blood (in fact, tissue) antigen group compatibility. These fundamental characteristics allow the principle of blood transfusion and, moreover, of universal blood transfusion, once the major restriction of the ABH system is taken care of.

DIVERSITIES IN HUMAN BLOOD AND BCs FOR TRANSFUSION

In contrast to what has been presented above, no siblings have identical blood. Indeed, even monozygotic twins display genetic and epigenetic differences. Whereas the Mendelian distribution of HLA antigens is transmitted in blocks (haplotypes), HLA diversity between humans ranges from between 10^6 and 10^7 (19). HLA polymorphism limits, to some extent, platelet and leukocyte availability and may also create complications such as immunization (20). Platelet antigens add diversity by bringing more than 30 additional groups often with two alleles each (about 5 matter essentially in transfusion) (21, 22). The diversity of erythrocyte antigenic alleles is not only huge (by the thousands) but is also non-haplotypic, offering a tremendous assortment of individual cell markers which is fascinating if we consider the uniqueness of each human, but limits the possibility of matching blood groups for transfusion (23). In fact, half a dozen such antigens are considered routine, allowing for wide-scale transfusion. It should be noted that not all individuals are equal and some individuals

can be easily transfused (the vast majority) while others with rare blood groups cannot, requiring special programs with technical, ethical, and organizational problems. Similarly, there is diversity in people's propensity to become alloimmunized after blood transfusion, because HLA loci make people good or bad responders to RBC, platelet, or leukocyte antigens (a common law in innate and adaptive immune responses that varies in individuals) (24–26).

With respect to LBCs, despite being called "standard," and their largely similar appearance category by category, they are not, in fact, all the same. They are defined as standard because they fall within a range of maximum and minimum levels of either desired and undesired constituents, and only a few parameters are evaluated among the thousands of variables that make individuals' blood differ from another (4). Furthermore, for each category of LBC, besides the genetic variations between donors that transfer characteristics to the donated component (gender, tissue and HLA genotype, erythrocyte antigen genotype, platelet antigen genotype, protein variants, etc.), there are additional variation parameters that depend on the donors: the time of donation (morning, afternoon, after a meal or fasting, drug and dietary supplement intake, menstrual cycle for non-menopaused women, diet, hygiene habits, and many other parameters) (27–29). All of these parameters may influence the final characteristics of the LBC, though there is no evidence as yet of the effect apart from the presence of soluble antibodies against blood cell antigens (iso-anti-A or B; anti-HLA in females who have been pregnant). BEs extend the variety since the addition and diversification of devices, machines and automats multiply heterogeneity (**Table 1** aims to illustrate this). Finally, shelf-life duration multiplies LBC delivery diversity by 41 for RBCCs (in theory: 42 minus 1 day, as 1 day is needed to obtain all the parameters allowing the labeling and issue of the RBCC) (30) and 4 (5 minus 1 day) for PCs. It has further been noticed near two decades ago that RBCs do not recirculate the same depending on storage conditions of the component (one ¼ of the infused RBCs even never recirculate) (31).

What then is the clinical relevance of such diversity? The case of storage lesions has been extensively studied (32–34), having basically identified two sets of lesions: reversible and irreversible lesions (35). Conflicting data have been obtained from both experimental and clinical data, sparking disputes over the rationale for using fresh as opposed to old blood (36–38). In simple terms, it appears that neither fresh nor old blood is clearly defined, nor is the readout (patient outcome) providing conclusive information, and relevant clinical trials are still needed to resolve this issue (39–41). This issue is of utmost importance as it may revoke the current blood-banking paradigm (meaning that conflicts of interest when blood banks happen to be research principal investigators are non-negligible).

TABLE 1 | Parameters having proven or theoretical influence on the quality of the processed blood component (BC).

Main categories	Main items adding diversity	Level of diversity
Donor dependent parameters (genetically controlled)	• Sex/gender • Immunogenetic characteristics (blood groups) • Natural iso-antibodies • …	• Two • By the thousands (millions if applied also to HLA antigens) • Variable
Donor dependent parameters (only partly genetically controlled)	• Immunization status • Nutrition, metabolism • Hygiene and intoxications (therapeutic and recreational drugs, supplements, alcohol, tobacco) • Meal; or fast • Nycthemeral cycle • Genital cycle and periods • Outside temperature condition • …	Hundreds of influential parameters
Donor independent parameters (BC processing)	• Shipping time and temperature • Needles, plastics and bags, rotators, automats for collection and intermediate storage • Devices for cell separation • Working temperature • Additives (anticoagulant, solutions, pathogen inactivation, etc.) • Filtration steps (meshes, temperature, timing, etc.) • Pooling steps • Preservation conditions • Physical interactions in shelf-life conditions (stacking, shocks, thermic differences, shipping, etc.)	• Variable • Dozens influential parameters
Patient (recipient, beneficiary) dependent parameters	• Blood group • Immunization status • Matching conditions	• By the thousands

Each parameter being independent from the preceding one, diversity is created by the multiplication as opposed to the addition of all. The final diversity goes by the million or more. Not all parameters are equally influential but it clearly appears from the table that one given BC collected by one individual, despite being "standardized" to a norm, is unlikely to be "standard."

DISPARITIES IN PUBLIC HEALTH POLICIES

Labile blood components are not considered univocal in various countries and systems, though they are not considered to be drugs according to Directive 2002/98/EC (42), in contrast to plasma fractions or derivatives [Directive 2001/83/EC (43)], or industrially prepared and solvent-detergent secured therapeutic plasma originating from large pools. Some national regulations do, however, consider labile LBCs to be drugs, as is the case in Switzerland; and WHO recently (2015) added blood and blood derivatives to its list of essential medicines (44). Semantics between medicines and drugs are imprecise, however, and this decision has displeased a number of blood donor associations who are batting against confusion with LBCs and plasma derivatives (45). Furthermore, the logistics by which certified LBCs can be delivered to patients varies considerably between countries: according to the European directive, there are basically two main actors: BEs which collect, process, test, and distribute BCs, and Hospital Blood Banks or HBBs which acquire LBCs from BEs, build up an inventory and deliver selected LBCs to patients upon *ad hoc* immunohaematological matching (4). On some occasions, BE and HBB functions can be held by the same entity (as, e.g., in France).

In addition, Council of Europe directorate EDQM requests that BEs apply a minimum platform for blood testing, with additional options for BEs willing to raise the LBC safety level with, for example, the implementation of nucleic acid testing (NAT) (4). Some BEs perform NAT on each donation, even on frequent donors' donations, while others restrict it to new donations. NAT is applied to both pools and individuals, and some BEs that used NAT have reverted back to non-NAT because of its relatively low (and debatable) cost-effectiveness (46).

In short, there is significant heterogeneity in stances on the availability of LBCs to patients through organizations and countries' public health policies. Consistent efforts have been made by large bodies such as EDQM, the American Blood Bank Association or the International Society for Blood Transfusion ISBT to define safety and quality parameters that should apply to each issued LBC; however, this is still a range above and beyond which the LBC should not conform to standards; if testing is individual for most infectious markers (and even some are tested in pools), hematological markers are frequently tested by sampling to define quality (4). This is the reason why we call attention here on the false homogeneity of LBCs despite they go by the noun of "standard." This is in sharp contrast to the actual standardization of plasma derivatives obtained from 100 of 1000s of collections and subjected to an industrial processing (47).

DISPARITIES WITHIN ETHICAL CONSIDERATIONS

Ethics are largely cultural and it is difficult to establish a platform that fits all situations worldwide. Nevertheless, as early as 1975, WHO declared that sustainable efforts should be made by all countries to collect blood from VNRDs. WHO later set the objective of 100% VNRD by 2020 (the Melbourne Declaration, 2009) (1). The EDQM has also declared itself in favor of a generalized objective of 100% VNRD where possible (4). The Oviedo Convention (1997), set up under the auspices of the Council of Europe, has established standards for ethics in the field of human rights and dignity, with regard to the use of tissues of human origin (including blood) (48). Nonetheless, many states have been late in signing this convention and others have not yet ratified it. WHO, considering that many countries could not achieve the 2020 VNRD objective, postponed it to 2025 for certain Middle-Eastern countries. State WHO and ISBT in its revised code of Ethics, paid and compensated donations should no longer be acceptable; replacement donations remain questionable (49). The issue of paid versus unpaid plasma collection has been challenged by advocates for the plasma fractionation industry (13, 14, 16) (the case of paid cell donations is still existing in certain countries including Europe but apparently on the decline worldwide). It is worth noting that a very interesting study was published in 2012 questioning the ban on financial contracts with blood donors in Africa, with the declared objective to reduce the infectious burden on "donated" blood (50). This last example questions the universality of ethical values of blood donation when coming to strengthening safety in LBC beneficiaries, opening an additional ethical dilemma. Next, when this comes to plasma for fractionation, there have been requests that collection and fractionation are more equally dispatched in countries that prescribe most to do justice to patients in need in case there is shortage and to alleviate the burden of (paid) collection in countries hosting the majority of plasma collectors such as the USA (51).

We offer an opinion here: as opposed to normative ethics (the Oviedo Convention, the ISBT Code of Ethics), reflective ethics (anthropological and philosophical) are preferred in order to move forward with the safe use (for donors, recipients, stakeholders and society members, i.e. people, civilians and tax payers) of substances of human origin (41). In the meantime, there is clear evidence that ethics are extremely cultural and embedded with acknowledged or hidden religious sentiments with the populations concerned.

In short, it should be noticed that blood within the transfusion process offers an additional paradox: while the LBCs are relatively inhomogeneous relative to active constituents though relatively homogenous relative to the so-called ethicality of their collection, this is the other way around for plasma derivatives that are remarkably homogenous and standardized relative to active constituents but may be obtained both within the profit and the non-for-profit sectors. The latter case has been recently (2016) the subject of important debated in France and Switzerland and journalists reported both in the paper and the TV press on some potential malpractice with respect to donors' own safety and vulnerability.

CONCLUDING REMARKS

The transfusion process is at the crossroads of a multitude of paradoxes (42). It is one of the only medicines that rely on material outside the control of the pharmaceutical industry if we exclude plasma for fractionation. It professes to be precision medicine, yet the issued LBC comprises ±20% (or more) of the expected therapeutic constituent. It professes to be personalized medicine, but blood groups between donors and recipients match for the vast minority. It is prescribed for the most fragile patients, yet practitioners prove to have very limited knowledge on it (according to recent surveys, they usually know which is the target of a biosimilar drug or a therapeutic monoclonal antibody, but only largely relative to LBCs) (43). We believe it is important to advocate for prescribing doctors to receive further training on LBCs and transfusion medicine–setting apart the case of standardized plasma derivative or drugs—education and training are essential to understanding and respecting blood donors and donated LBCs, to making wise choices, and to the optimal management of patients' blood and donated LBCs, within the limitations of prevailing uncertainty (Garraud et al., in preparation). Being knowledgeable in the face of uncertainty would prevent a comparison of apples and oranges in published works, even in high-quality journals. It would be practical for prescribing doctors to forward their everyday questions to blood transfusion specialists; this would certainly be useful in establishing the most appropriate training programmes.

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Both authors contributed equally to this manuscript and its revision.

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An Original Approach to Evaluating the Quality of Blood Donor Selection: Checking Donor Questionnaires and Analyzing Donor Deferral Rate

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Blood donor selection is a cornerstone for blood transfusion safety, designed to safeguard the health of both donors and recipients. In the Service du Sang, Belgian Red Cross, French and German-speaking part of Belgium (SFS), health professionals (HPs) are allowed to interview donors on their own after formal qualification. This qualification is afterward evaluated by means of two complementary quality indicators: monitoring of donor health questionnaires (DHQs) and analysis of donor deferral rate. The study aims to evaluate the degree to which both quality indicators may be useful and appropriate tools to evaluate the quality of blood donor selection. An analysis performed on 2016 data showed that noncompliance detected by means of DHQ monitoring seems to be more frequent in HPs who conduct a low number of interviews compared to all HPs as a group (5.67 vs. 3.23%; $p < 0.001$). Deferral rates are also higher in HPs with a lower activity compared to HPs who interview more donors (14.80 vs. 13.00%, $p < 0.001$). Furthermore, statistically differences are observed between the type of blood donation venue in terms of the global deferral rate (for instance fixed site vs. schools: 11.9 vs. 19.5%; $p < 0.001$), and specific reasons for deferral (such as sexual risk behavior and travel in at-risk areas, the differences being highly significant between each category of blood donation venue; $p < 0.001$). Providing the HPs with feedback on these findings was an opportunity to draw their attention to some aspects of the selection process in order to improve it.

Keywords: blood donor selection, transfusion safety, evaluation, donor health questionnaire, donor deferral rate

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INTRODUCTION

Blood donor selection is a cornerstone for blood transfusion safety, designed to safeguard the health of both donors and recipients. Donor safety (1, 2) is targeted by reducing the risk of complications associated with blood donation (rare but not absent) (3) and, in order to improve recipient safety, blood donor selection attempts to reduce the risk of transfusion-transmitted infections (TTI). A recent study conducted in Senegal confirmed what is globally accepted based on epidemiological data, i.e., the efficacy of blood donor selection in reducing the prevalence of HIV seropositive donors (4).

Abbreviations: DHQ, Donor Health Questionnaire; HP, health professional; QCF, Questionnaire Control Form; SFS, Service du Sang, Belgian Red Cross, French and German-speaking part of Belgium; TTI, transfusion-transmitted infections.

The European Directive 2005/62/EC (5) recommends entrusting the responsibility for blood donor selection to qualified health professionals (HPs). Training in blood donor selection encompasses both a theoretical and a practical approach: the HP has to be able to take the right decision (knowledge of blood donor selection criteria) and to communicate this decision to donors in an appropriately understanding manner (professional relationship with each donor based on trust).

The blood donor selection process usually encompasses four main steps (6):

- Pre-donation information and advice: this is usually provided in a leaflet, especially about transfusion-transmitted infections (and the associated risk factors) and the potential risks of donation.
- Donor Health Questionnaire (DHQ): filled out by the donor alone (before the pre-donation interview) or with the HP (during the pre-donation interview).
- Donor interview: conducted by a qualified HP.
- Donor health assessment: at the end of the interview, the donor is declared eligible to give blood or deferred temporarily or permanently; this decision also takes into account physical and biological parameters, such as hemoglobin level, blood pressure, heart rate, and weight.

Before an HP is allowed to conduct donor interviews and selection alone, qualification must be acquired.

In the Service du Sang, Belgian Red Cross, French and German-speaking part of Belgium (SFS), the following qualification criteria are used and as recommended in terms of training objectives (7), communicated to the HPs at the start of the training session:

- Theoretical training is provided (possibly *via* e-learning) focusing on blood donor selection criteria: a predefined grade must be achieved in order to continue the training,
- the ability to take the right decision is assessed in simulated cases by a senior HP/supervisor,
- a certain number of donor interviews is overseen by a senior HP/supervisor in order to assess the trainee's ability to take the right decision and to communicate properly with donor, especially when the donor is deferred,
- the supervisor monitors the first interviews performed alone by checking the donor health questionnaires (DHQs) (see below).

Each step must be successfully completed before the next step is taken. When all steps are completed, the HP is qualified for donor selection.

After this initial qualification, HPs are allowed to interview donors alone, but their qualification is not infinite. It is nevertheless difficult to evaluate the quality of this activity. As confidentiality is a prerequisite for the pre-donation interview, regular attendance by a third person (supervisor) is not recommended even with a view to evaluating the competency of each HP. Furthermore, the presence of a third person could influence the behavior of both the donor, who might hesitate to disclose all the relevant data [donors' compliance is not always achieved (8, 9)] and the HP, who might be more inclined to apply all the procedures than when conducting the interviews alone.

Blood donor selection by qualified HPs can be evaluated in various ways. For example, the quality of the decision taken by a trainee (nurse, technician, or physician) can be assessed by comparison with the decision taken by a senior physician, the latter being considered as the reference (10). In the SFS, the qualification of each HP is formally monitored through two main quality indicators, the monitoring of DHQs and the donor deferral rate:

- A sample of DHQs filled in by donors and analyzed by the HPs during the pre-donation interview are monitored yearly for each HP in order to assess whether the HP in question collected the relevant data and used them properly.
- The donor deferral rate observed for each individual HP is compared to the overall rate for all HPs within the SFS in order to detect differences that might reflect different ways of selecting donors.

Both quality indicators are used routinely by the SFS. The study aims to analyze their adequacy to evaluate the quality of blood donor selection and to possibly improve blood transfusion safety.

Results of both indicators are provided to each individual HP to improve their blood donor selection, and, as a consequence, to improve blood transfusion safety.

Evaluations performed on blood donor selections, which took place in 2016 in the SFS are analyzed and the results reported in the study that provides some examples of issues detected and thoughts about the further development of these tools.

MATERIALS AND METHODS

The study was conducted as an audit of blood donor medical selection analyzing two indicators: DHQs monitoring and blood donor deferral rate. This audit was submitted to the Erasme-ULB Ethics Committee (Brussels, Belgium), which determined that full review and approval was not required.

DHQs Monitoring

In the SFS, each HP ideally undergoes one annual monitoring by a senior HP (supervisor). The paper version of the DHQ filled in by the donor and checked by the HP with the donor during the face-to-face interview, the data entry in the data-base (eProgesa® software, MAK systems, Paris, France) and the decision taken are checked for a series of DHQs, typically all the DHQs for one blood collection session for this HP.

The DHQ used by the SFS is made up of 43 questions, of which 42 are to be answered by ticking a yes/no box. One text answer is mandatory, i.e., country of birth. The DHQ must mandatorily be dated and signed by both the donor and the HP. In addition, HPs are asked to document the interview and their reading and checking of the DHQ with the donor by writing a comment and/or additional info and/or "ok" or at least by adding a checkmark next to the answer provided by the donor, if this answer could be a potential reason for deferral or when the "yes" answer generates a specific laboratory analysis request when entered into the database. This is the case for example for the question "Have you ever

had a malaria attack?" which induces the detection of malaria antibodies.

Blood Donor Deferral Rate

Data collected in eProgesa from January to December 2016 involving all donations and including HPs identification, decision taken by the HP (donor acceptance or deferral), reason for deferral (when the donor is deferred), and type of blood donation venue (fixed site or mobile site, i.e., village, school, office, or special) were analyzed.

Statistical Analysis

In order to evaluate HPs' compliance in donor selection, the following items are checked, according to the DHQ used by the SFS. These items are listed in a standardized questionnaire control form (QCF) used by supervisors in order to document the control:

- Donor signature
- HP signature
- Donor's "Yes" answers documented
- Answers to all questions
- Coherent answer on DHQ and entry into data-base
- Donor selection.

Data from all QCFs collected in 2016 were recorded in an Excel data-base and analyzed by descriptive statistics (mean, median, and percentiles), including the identification of HPs, the blood donation venue, the category of donor (first-time vs. regular or repeat donor), and the six types of noncompliance listed above.

Blood donor deferral rates were calculated globally and for each specific deferral category for the whole group of HPs, for HPs who conducted fewer than 500 interviews during the research period and for those who conducted 500 interviews or more. Deferral rates in the various blood donation venues were compared. The statistical analysis was performed by using the chi square test in order to evaluate the degree to which observed differences were statistically significant.

RESULTS

During the study period, 209,617 pre-donation interviews were conducted by 125 HPs (**Table 1**).

Out of these 125 HPs, 57 (46%) were assessed with a total of 2,063 DHQs. More HPs may have been monitored but were not included in the study because the QCFs were not provided on time.

Deferral rates were calculated and analyzed for all donations.

Donor Health Questionnaires

This study reports data from 2,063 DHQ checks carried out within the SFS, i.e., 0.98% of all DHQs for 2016.

Table 2 provides data analyzed from the 57 monitored HPs. The proportion of DHQs assessed for first-time donors is 12.4% (256 out of 2,063).

Noncompliances for the various parameters checked by means of the QCF, as described above, are displayed in absolute numbers and in percentages of the total number of DHQs checked for all HPs as a group. The distribution according to median and P10, 75 and 90 shows great inter-individual variability.

Table 3 provides data analyzed from a total of 1,610 DHQs from HPs who interviewed more than 500 donors during the study period ($N = 47$), i.e., a selection of HPs who performed donor selection most regularly.

For the whole group of HPs (**Table 2**), 5.67% of the DHQs were rejected by the supervisor.

The most frequent noncompliance issue was a missing answer, i.e., no box ticked by the donor and no comment provided by the HCP: 3.01% of the checked DHQs.

The second most frequent noncompliance was missing HP signature (2.76%).

The least frequently reported noncompliance was missing donor signature: 5 times out of 2,063, i.e., 0.24%.

Overall, the quality of the decision (donor acceptance or deferral) was considered as being wrong in 0.92% of the cases.

TABLE 1 | Activity of health professionals (HPs).

2016	SFS	Fewer than 500 pre-donation interviews	500 or more pre-donation interviews
HP	125	34	91
Pre-donation interviews	209,617	10,861	198,756
Interview/HP	1,677	319	2,184

TABLE 2 | Monitoring of donor health questionnaires.

	No. of health professionals (HPs)	No. of questionnaires	No. of first-time donors	Questionnaires not OK	Donor's signature missing	HP's signature missing	Response not documented	No response to at least one question	Discrepancy between questionnaire and decision	Quality of decision
SFS	57	2,063	256	117	5	57	22	62	10	19
%				5.67%	0.24%	2.76%	1.07%	3.01%	0.48%	0.92%
Median				0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P10				0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P75				4.76%	0.00%	2.94%	0.00%	1.69%	0.00%	0.00%
P90				8.09%	0.00%	5.91%	4.46%	4.86%	0.00%	3.02%

Data were analyzed from 57 HPs whose questionnaires were monitored in 2016.

TABLE 3 | Monitoring of donor health questionnaires (more than 500 pre-donation interviews).

	No. of health professionals (HPs)	No. of questionnaires	No. of first-time donors	Questionnaires not OK	Donor's signature missing	HPs signature missing	Response not documented	No response to at least one question	Discrepancy between questionnaire and decision	Quality of decision
SFS	47	1,610	193	52	3	34	14	22	2	9
%				3.23%	0.19%	2.11%	0.87%	1.37%	0.12%	0.56%
Median				0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P10				0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P75				4.27%	0.00%	1.14%	0.00%	0.00%	0.00%	0.00%
P90				8.09%	0.00%	5.08%	3.02%	4.63%	0.00%	2.56%

Data were analyzed from HPs who have interviewed more than 500 donors during the study period.

The HP subgroup who interviewed most donors on an annual basis (more than 500) had lower percentages of noncompliance than the whole group for all parameters checked (**Table 3**).

A great variability was observed for the various criteria in both the whole group of HPs and in the group of HPs who interviewed more than 500 donors during the study period.

Blood Donor Deferral Rate

A total of 13.09% of the 209,617 donors interviewed by the 125 HPs were deferred for blood donation (**Table 4**).

The deferral reason was recorded by the HPs in the data-base during the pre-donation interview for 85.12% of donors.

The main reasons were:

- Exposure to potential transfusion-transmitted infectious agents within the deferral period, due to surgery, endoscopy, tattoo, piercing, potentially contaminating accident, acupuncture, mesotherapy, or blood transfusion: 1.60% (traveling and sexual risk behavior are not included in this item).
- Traveling in a region where transfusion-transmitted infectious agents are present, mainly malaria, Chagas disease, and West Nile Virus infection: 1.57%.
- Sexual risk behavior, i.e., new sexual partner within the deferral period, men having sex with men, or sexual partner infected with HIV, HBV, or HCV: 1.09%.

Other deferral reasons, such as current infection or serious medical condition were less frequent.

Quite a number of donors (4.5%) were not allowed to give blood, but blood analyses were performed in order to check previous results. The main reason is probably low hemoglobin level, but this information is not available for analysis.

Table 4 provides data on deferral rates for the group of HPs as a whole, for HPs who interviewed fewer than 500 donors and for HPs who interviewed at least 500 donors during the same study period. SDs suggest major inter-individual differences.

In HPs who interview more donors (at least 500/year) overall deferral rates were lower and, more interestingly, variability tended to decrease, particularly for the global deferral rate (SD: 9.84% compared to 3.28%).

Furthermore, statistically significant differences are observed between the type of blood donation venue, i.e., fixed or the various types of mobile sites (**Table 5**). For instance, there is a significant difference in global deferral rates (**Table 5**) between fixed and mobile sites, such as schools, offices, and special campaigns (for instance, during periods requiring a call to potential donors by media). Differences between each category (fixed sites, villages, schools, offices, and special drives) are highly significant with respect to sexual risk behavior and travel in at-risk areas (**Table 5**).

TABLE 4 | Comparison of deferral rates between health professionals (HPs) who conducted fewer than or at least 500 pre-donation interviews during 2016.

2016		SFS (%)	Fewer than 500 pre-donation interviews (%)	500 or more pre-donation interviews (%)	*p*
Deferral rate	μ	13.09	14.80	13.00	<0.001
	σ	5.95	9.84	3.28	
Recording rate	μ	85.12	78.47	85.54	<0.001
	σ	12.72	14.33	11.50	
Sexual risk behavior deferral rate	μ	1.09	1.25	1.08	NS
	σ	0.68	0.80	0.64	
Travel deferral rate	μ	1.57	1.25	1.59	<0.01
	σ	1.01	0.89	1.05	
Exposure to potential TTI	μ	1.60	1.58	1.60	NS
	σ	0.56	0.73	0.48	

TTI, transfusion-transmitted infections; NS, not significant.

Data for each individual HP are compared with those of the group as a whole and to those of HPs working in the same type of venue as the monitored HPs. This comparison allowed to identify differences that were discussed with the HP, an opportunity to assess their blood donor selection activity and, when needed, to provide additional training.

DISCUSSION

The analysis of the data suggests that checking DHQs and analyzing blood donor deferral rate on an individual basis may be used as quality indicators of blood donor selection and as a basis for improvement of this activity.

Donor Health Questionnaires

The completion of DHQs is a useful tool to provide HPs with adequate data to take the right decision. Using a DHQ reduces the risk of transmission of blood-borne infectious agents by transfusion. A study showed a significant reduction in Gabonese seropositive donors (hepatitis B, hepatitis C, and syphilis) who

TABLE 5 | Comparison of donors' deferral rate between fixed and mobile sites.

2016	Fixed sites (%)	Villages (%)	Schools (%)	Offices (%)	Special (%)	Comments
Overall deferral rate	11.9	12.3	19.5	16.0	19.5	No difference between schools and special drives $p < 0.05$ between fixed sites and villages $p < 0.001$ between all other categories
Sexual risk behavior	0.92	0.66	4.03	1.39	2.32	$p < 0.001$ between all categories
Travel	1.40	0.98	1.79	3.91	2.82	$p < 0.001$ between all categories
Exposure to potential transfusion-transmitted infections	1.19	1.70	2.78	1.83	2.82	$p < 0.001$ between all categories (except between villages and offices, and between schools and special: not significant)

completed a DHQ (11), although this encouraging finding was not observed in all studies (12), probably partly because of differences in the prevalence of pathogens.

One limitation of the DHQ is its understanding by all donors; its form has to be regularly analyzed and reviewed (13, 14), in particular, to ensure donors' compliance with respect to questions about sexual risk behaviors (15, 16); nevertheless, direct questions pinpointing sexual behavior may reduce donor's intention to return for a further donation (especially in the case of experienced blood donors) (16).

Another limitation of the DHQ is the noncompliance on the part of some donors when it comes to responding properly and honestly to all questions. To improve donors satisfaction and probably to make donors more compliant when it comes to filling out the DHQ honestly, an abbreviated questionnaire may be an alternative at least for repeat and regular donors (17).

A standardized DHQ is used by most blood services to collect relevant data in order to evaluate the donor's eligibility to give blood. Each blood service uses its own standardized DHQ. Exceptions are the USA and Canada, where uniform DHQs have been used since 2005. In Germany, a national DHQ has been recently tested (16).

An automated computer-assisted pre-donation interview could improve the collection of relevant data (14, 18) but in most countries, the pre-donation interview is conducted without the assistance of a computer and remains a human activity. An evaluation of this activity could, therefore, be useful to improve donor selection.

These critical findings highlight the importance of analyzing DHQs as a means to evaluate the quality of the blood donor selection, confidentiality required by the donor interview conflicting with the attendance of a supervisor.

In this study, monitoring DHQs by means of the QCF allowed to identify a number of issues which otherwise would have gone unnoticed and adapted feedback was provided to the HPs concerned.

Some issues occured only once, as for any human activity: one HP for example, forgot to make sure that an answer was given to one question in the set of DHQs checked. This HP was then reminded of the importance of staying focused. If several questions remained unanswered, the HP received different feedback and was possibly monitored for a certain period until they improved.

An issue such as a missing HP signature is major, but was mostly due to simple forgetfulness on the part of the HP who made an adequate donor selection. In fact, as HPs are asked to comment/sign off on all "yes" answers that might be a reason for deferral, tracking their comments made it possible to confirm whether the DHQ has been checked during donor selection. Missing signatures could, therefore, be noticed by the supervisor in most cases.

The same type of noncompliance issues detected during the DHQ checks may have a very different impact on transfusion safety. The impact may be possible transmission of a TTI, a health consequence for the donor, or rather noncompliance with the legal framework which may have no immediate consequences for safety in a specific case, but should of course be avoided.

Therefore, it is important that the supervisor who performs the checks have thorough knowledge of the consequences of all issues, and give appropriate feedback to the HP involved.

The supervisor adapts their evaluation of the HP when performing DHQ controls accordingly. The examples below illustrate this individualized approach:

- A not documented "yes" answer or no answer to the question: When the question involves antecedents of allergy or asthma, the consequence is rather minor, whereas when it concerns contact with a person suffering from hepatitis or another infectious disease, this may have major consequences. In fact, the HP may have missed a reason for deferral or for requesting additional laboratory analyses, with potential transmission of a TTI.
- Discrepancies between DHQ completion and data entry:
  For instance, it is more important to enter the actual "yes" answer in the data-base for the question "Have you ever had a malaria attack?" in the case of a first-time donor than for "Have you ever been operated on since you were born?" Indeed, a positive answer to antecedents of malaria automatically generates an analysis request and possibly deferral of the donor. Therefore, if the appropriate answer is not entered, the opportunity for an analysis request and/or donor deferral will be missed.
- Donor selection:
  Missing deferral of a donor who is at risk of having been infected with a blood-borne agent, such as a donor who has a new sexual partner within the deferral period, can have a safety impact. On the other hand, accepting a donor whose hemoglobin level is 0.1 g/dL below the legal threshold for giving blood is wrong, but has no safety consequences for either the recipient or the donor.

The least frequent noncompliance issue observed in this study, the missing donor signature, is a major one as without this confirmation from the donor, the blood bag cannot be used.

Sometimes systematic or very frequent issues were detected in some or all of the HPs in a group, or in an individual HP. For the supervisor, this was an opportunity to remind the HPs of the rules to be followed in order to improve blood collection quality and safety. In some cases, the issue was a subject for a continued education session.

Examples:

- The majority of HPs in a group understood the question involving a "donor born in a malaria region, who has lived in this region for the 5 first years of their life?" as living in a malaria region for the *full* first 5 years. Therefore, if the donor has only lived in this region for 1 year, the answer entered was "no," resulting in the malaria analysis request not being automatically generated on the first blood donation. Not testing for malaria could miss a reason for deferral and transmission of malaria. How to answer this question correctly was explained to the HP group twice by the supervisor, first by email when it became obvious that this was a recurrent error, and then included in the next continued education session. After these two steps, there was a marked improvement.
- One HP did not check properly in which countries Chagas disease is present and forgot to record the relevant journey. As a result, the analysis request was not generated.

The reminder to this HP to check whether a country is affected by Chagas disease had an obvious impact on their data entry.

In some situations, incorrect documentation has no impact on blood quality or safety, but can give the wrong impression of noncompliance. This may induce the presence of findings during an audit or inspection.

For instance, one HP always entered the date of the interview as the start date for a temporary contra-indication (e.g., following a tattoo) instead of the actual date; the end date, however, was correctly calculated. The donor was thus deferred for the correct duration and there was no safety issue whatsoever. However, a *post hoc* administrative check would identify the duration of the deferral as being too short and consider it to be a failure to comply with legal requirements. The HP was thus reminded to enter the actual start date and subsequent checks confirmed that this was being done correctly.

Noncompliance seems to be more frequent in HPs who conduct fewer interviews (5.67 vs. 3.23%; $p < 0.001$). This information is interesting and should be analyzed further in order to assess whether there is a threshold in the number of interviews to be conducted over a specific period.

This finding is also of use for adapting the frequency of DHQ controls to the number of interviews performed, HPs with a high number of interviews needing less frequent controls than those with a low number of interviews.

The variability between all of the HPs as a group and those who interviewed more than 500 donors during the study period encourages individual analysis of the data and the use of the analysis to set up specific strategies for each HP. Even if an HP is well-graded they can potentially improve their competency toward a better grade and continue to reduce the transfusion risk, even it will never be zero.

Blood Donor Deferral Rate

Analysis of deferral rates is another efficient way of evaluating HP donor interviews and provides a different type of information than DHQ monitoring.

Deferral affects the supply of blood components because a blood bag is not collected and because the return rate for a further donation is reduced (19, 20), in particular, for first-time donors.

The comparison of individual deferral rates of an HP with the whole group allowed to identify discrepancies that were discussed with the monitored HP.

Examples:

- An HP had a much higher overall deferral rate than the group as a whole. Discussion with the supervisor revealed that this HP was very anxious about making mistakes, and preferred to defer donors if there was any doubt whatsoever.
- An HP had a much lower deferral rate for new sexual partners than the group as a whole. It appeared that this HP did not feel comfortable asking the question on this topic and relied entirely on the answer provided by the donor on the DHQ. Nevertheless, it is a well-known fact that quite a number of donors misunderstand or ignore the question and answer "no," when in fact they should answer "yes." Therefore, asking the question orally is important. Detection of this deviation was an opportunity to discuss this particular topic with the HP and provide additional training.

These two issues would not have been detected by means of DHQ monitoring; they illustrate the complementarity of the two blood donor selection evaluation tools used by the SFS.

These great inter-individual differences emphasize the importance of providing each HP with personal data in order to give individual advice and plan specific training.

It is important to take into account the various parameters, which may have an effect, such as the type of blood donation venue (**Table 5**). For instance, a frequent observation is that the number of deferrals due to sexual risk behavior is higher in blood collections organized in schools than in other types of venues, most probably due to the younger donor population (students). When an HP performs more donor selections in schools than average, it is logical for them to have a higher deferral rate than the HP group as a whole. On the other hand, the number of deferrals due to sexual risk behavior in blood collections organized in villages is below average. An HP who works mainly in village venues will, therefore, show a lower deferral rate for this risk factor. Donors' deferral rates being statistically different among the types of donation venues, individual data have to be given to each HP with global data as a reference and with the distribution of their interviews in terms of donation venue. When these are considered, unexpected deviations can be selected and discussed with each individual HP, where applicable.

No hard data are available about the impact of the feedback provided by the supervisor to the HPs, but clear improvements

were reported. An objective analysis of this impact may be valuable to validate the monitoring tools.

CONCLUSION

In the SFS as in most blood transfusion centers, donor selection is the result of a pre-donation interview performed confidentially by an HP who has had full *ad hoc* training and has been qualified accordingly. The quality of the selection process is difficult to assess, but is important in the context of blood transfusion safety.

Having a supervisor present during the interview introduces a bias in itself, as both the donor and the HP may act differently compared to a plain face-to-face interview. Therefore, two complementary methods have been developed in the SFS to assess the quality of the selection process, i.e., *post hoc* control of the DHQs and analysis and comparison of deferral rates. Even if the number of DHQs checked was low compared to the total amount of donors selected, it made it possible to identify a number of mistakes made in donor selection, both at individual HP level as well as in a group of HPs. Deferral rates analyses also pinpointed the difficulties experienced by HPs in specific selection situations.

Providing the HPs with feedback on these findings is an opportunity to talk to them and draw their attention to some aspects of the selection process in order to improve it. In certain situations, the topic was included in a continued education session. Clear improvements were reported when further controls and/or deferral analyses were performed.

A comparison of the results for HPs according to the number of interviews conducted on an annual basis showed that for both the control of DHQs and the deferral rates, HPs with a greater number of interviews made fewer mistakes.

In conclusion, controls of DHQs and analyses of donor deferral rate seem to be useful tools to improve the quality of donor selection. It may be interesting to assess whether there is a threshold number of interviews, a HP should conduct per year in order to achieve optimal donor selection quality.

AUTHOR CONTRIBUTIONS

PG collected data recorded by HPs and performed a preliminary analysis of these data in order to develop figures and tables. EN and PG enhanced a deeper analysis of the data, and EN exploited the data with a view to planning individual and overall actions. EN and PG wrote the article.

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Emerging Infectious Agents and Blood Safety in Latin America

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Historically, emerging infectious agents have been an important driving force toward the enhancement of blood safety, illustrated by the sharp reduction in the transmission of infectious agents by blood transfusion after human immunodeficiency virus (HIV) epidemics. In general, Latin American (LATAM) countries have introduced screening for microorganisms with proven blood transmission with some delay in comparison to developed countries, but, nowadays, all LATAM countries comply with a minimum standard of screening which includes Hepatitis B, C, HIV, *Treponema pallidum*, and *Trypanosoma cruzi*. Noticeably, all those agents, in addition to HTLV, cause chronic infections. By contrast, in the last decade, the region has witnessed explosive outbreaks of arboviral diseases, representing a new challenge to the blood system, threatening not only blood safety but also availability. So far, the clinical impact of transfusion-transmitted Dengue, Chikungunya, or Zika has not been evident, precluding immediate reaction from the authorities. A number of other arboviruses are endemic in the region and may, unpredictably, originate new epidemics. Several measures must be taken in preparedness for the potential emergence of another arbodisease.

Keywords: blood transfusion, arboviruses, Latin America, Zika, Dengue, Chikungunya

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INTRODUCTION

The first agent verified to be transmitted by blood transfusion was the malaria protozoa (1) followed by Syphilis, the latter leading to the introduction of predonation testing in the first decades of the previous century (2). During the next 70 years, the safety of blood transfusions was gradually increased, covering a growing range of agents, more importantly the hepatitis viruses and human immunodeficiency virus (HIV). In common, all these pathologies have a short symptomatic acute phase and a variable rate of progression to a chronic asymptomatic period that may last through life. Blood units collected from unaware infected donors are averted from being transfused by detecting specific antibodies against those agents. With the exception of the hepatitis B virus (HBV) surface antigen (HBsAg) detection, the hallmark of prevention by laboratorial analysis has been the development and implementation of imunoassays, including, in some countries, the anti-hepatitis B core antigen (anti-HBc), that detects occult B carriers lacking, by definition, detectable HBsAg.

By the beginning of the millennia, the evolution of nucleic acid testing (NAT) allowed their incorporation to the blood screening routine, in order to interdict window-period donations, pursuing for an unattainable zero risk. A better selection of candidate donors with more stringent epidemiological and behavioral restrictions, summed to the arsenal of screening tests, have dropped the risk of transfusion transmission of infectious agents to a very low level, being nowadays a rare event (3).

Latin American (LATAM) countries are in general still struggling to motivate the population to donate blood voluntarily and regularly. Donations in the region are commonly insufficient for sustaining the transfusion demand, thereby resulting in a permanent blood shortage. In many countries, most of the donations come from replacement, often familiar donors (4) and emphasis is given to transform those into altruistic regular donors. Surprisingly, it has been observed that in some centers, repeat donors pose a risk that is similar or even higher than first-time donors concerning HIV transmission (5). This observation is justified by a fraction of repeat donors being indeed composed of HIV test seekers (6).

With some delay in comparison to developed countries, the four most important screening targets, HBV, HCV, HIV, and Syphillis, in addition to *Trypanosoma cruzi*, of local uttermost importance, were fully implemented in the 1990s (4). Seroprevalence of these agents were and are still much higher when compared to those verified in blood donors from Europe, Japan, Canada, and the US (3, 4, 7).

In this scenario, the explosive outbreaks of arboviruses with a high rate of asymptomatic subjects with a short-term viremia is new to the LATAM blood transfusion community; thus, there is a lot of uncertainty in how to deal with it. There are several proposed measures to mitigate this situation: averting blood collection in affected regions, applying pathogen reduction methods for plasma and platelet concentrates, adopting NAT and quarantine while waiting for post-donation information of donor's health. Some countries may implement all them and others one or none. Certainly, this variability is not only due to scientific gaps in our knowledge but also to the resources available and political determination in each country or region. **Table 1** summarizes selected features of the arboviruses representing today potential threats to the blood supply in LATAM.

EMERGING VIRUSES

Dengue

Viruses transmitted by arthropods (arboviruses) have always been of concern to human health but never much in the radar of blood banks. Dengue was the first arbovirus to cause epidemics in a global scale in the twenty-first century, globally affecting millions, from the Far East to the Americas. In LATAM, Brazil has been the country with the largest number of cases, experiencing yearly outbreaks from moderate to high intensity. Moreover, all four serotypes are now endemic, but there is still a large number of subjects naïve to at least one of the four serotypes, meaning that outbreaks will continue to occur.

TABLE 1 | Selected features of emerging viruses representing a potential threat to the blood supply in Latin America, 2018.

	Arbovirus		
	Chikungunya	**Dengue**	**Zika**
Family/genus	Togaviridae/ alphavirus	Flaviviridae/ flavivirus	Flaviviridae/ flavivirus
Enveloped	Yes	Yes	Yes
Viremic blood donors	Yes	Yes	Yes
Proven TT	No	Yes	Yes
NAT screening commercially available	No	No	Yes
Inactivated by PIT[a]	Yes	Yes	Yes
Vaccine available	No	Yes	No

[a]*Pathogen reduction/inactivation technologies.*

The main impact of dengue outbreaks to the blood system is the fall in the number of candidate donors, thus shortening the supply of blood products, aggravated by the universal practice of transfusing dengue patients with low platelet counts (8). As most dengue infections are asymptomatic, it is likely that such infected subjects are be able to donate and thus eventually transmit the dengue virus to recipients. This possibility was demonstrated by several studies detecting viremic donors in Brazil (9–11), Honduras (9), Mexico (12), and Puerto Rico (13) among others.

However, there is an obvious discrepancy in between the dengue incidence and rates of viremia verified among blood donors during outbreaks and the paucity of reports of dengue cases by the transfusional route (TT-DENV). The most comprehensive study on TT-DENV showed that recipients have an approximately 36% risk to get the virus from a viremic donor, but was unable to depict dengue-specific symptoms on those infected (11). Several reasons have been presented to explain this (un)finding, discussed in detail elsewhere (14), but may be summarized as follows: it seems that dengue viruses are well adapted to the mosquito to human cycle, and, passage through the invertebrate host and inoculation by its bite are required to cause disease on us. So, although TT-DENV is a recognized risk in many endemic countries in LATAM, preventive measures were never taken, since it did not convince clinicians and authorities of its morbidity for recipients. However, it is necessary to emphasize that, in rare instances, severe dengue-associated symptoms were observed on recipients of viremic donations (13, 15), thereby leading to the implementation of laboratorial screening in Puerto Rico, first by using NS1 antigen testing further replaced by NAT (16) but in nowhere else in LATAM.

West Nile Virus (WNV)

The diverse outcome of the different Flaviviruses causing human diseases requires that each viral species to be studied in depth, making analogies and generic Flavivirus models of little practical utility. This was well illustrated when the WNV arrived to the US by the end of the previous century. It took about 2 years to get enough evidence of its aggressiveness when acquired by blood transfusion or organ transplantation (17), since there was no previous recognition of any arbovirus transmission by these modes. When this link was unquestionably proven, it triggered a fast response from the transfusion medicine community, culminating in the introduction of screening by NAT (18). As WNV moved so fast from East to West US, it seemed inevitable that it would spread further South to LATAM, since susceptible vectors are abundant in the region and there is a huge migration of bird species that may harbor high titer viremias, from US and Canada to South America. Contrary to expectations, WNV was never able to cause human outbreaks in LATAM (19).

Zika

The trajectory of Zika virus (ZKV) from an obscure agent to a global health emergence has been comprehensively described (20). The well-studied outbreak in French Polynesia revealed the important association of ZKV to Guillain-Barré syndrome (21), while Brazil was the country to raise and prove the hypothesis of a shocking causal association of this Flavivirus to microcephaly and other fetal neural abnormalities (22). Concerns about blood safety were raised by Musso and co-workers in French Polynesia where NAT, pathogen inactivation and quarantine were deployed to protect the blood supply (23). So far, there are only two published clusters of TT-ZKV, both from Brazil (24, 25). Similar to TT-DENV, on those reports it was shown that ZKV was indeed transmitted to transfusion recipients but they did not develop any symptom associated with Zika disease. In French Polynesia, look-back of 12 recipients transfused with red blood cells from ZKV-RNA$^+$ donors has not also identified any post-transfusion symptoms (23). Even though solid evidence for a severe clinical outcome of TT-ZKV is still missing, the precautionary principle led Fundação Pró-Sangue/Hemocentro de São Paulo, Brazil, to develop a validated in-house NAT (26) and adopt it, from February 2016 on, to provide Zika-RNA-free blood units to approximately 20 pregnant women per month. In Martinique, Guadaloupe, and the French Guyana, pregnant women received blood collected in mainland France (Xavier de Lamballerie, personal communication) while donations were screened by individual NAT in Marseille, France (27). This policy of prioritizing groups at higher risk such as pregnant and highly transfused fertile women, and fetuses was further advocated by experts and organizations in the field (28) and WHO (29). In the US, in observation of FDA recommendations, blood collection was halted in Puerto Rico in between March and April 2016, resumed when NAT screening was introduced in late April. Approximately 0.5% of the donors were found viremic, peaking to 1.8% in July 2016 (30). Mosquito-borne and travel-associated cases in the continental US led the FDA to extend the recommendation of NAT screening to the whole country, being implemented by September/October 2016 and indeed depicting some infected donors, the majority with a recent travel history to an endemic area (31, 32). No country in LATAM so far followed this policy. The sharp decline in Zika incidence in LATAM in 2017 reduced the pressure and debate over this issue. High seroprevalence rates are observed today in the most affected areas of Brazil, what may prevent new large outbreaks in the near future (22, 33).

Chikungunya

Chikungunya (CHKV), in common with Zika and Dengue, is also transmitted by mosquitos from the *Aedes* genus, causing similar symptoms, making difficult to perform a diagnosis relying solely on clinical manifestations. Noticeably, arthralgia is much more pronounced and may last for months in some patients.

Its transmission by blood transfusion remains theoretical since no single case of TT-CHKV has ever been published. CHKV was introduced to LATAM in 2013, hitting first the Saint Martin Island in the Caribbean, brought probably from the South Pacific. The implicated CHKV Asian strain rapidly spread over the Caribbean and to South, Central, and North America (34). In the French West Indies, concerns about blood safety led to early implementation of a lab-developed NAT, in addition to pathogen reduction and quarantine (35). They were able to detect four viremic donors, two of them developed fever after donation and the other two remained asymptomatic. A large outbreak occurred a few months later in Puerto Rico with up to 2.1% of donors testing CHKV-RNA$^+$ (36). In contrast to the fast adoption of NAT for WNV, upon solid evidence of the clinical impact of TT-WNV, and for Zika, even lacking such parallel data, NAT for CHKV was never implemented in Puerto Rico.

From the Caribbean, the Asian strain spread first to the Northern countries of South America; Colombia, Venezuela, Suriname, Guyana, and the French overseas territory of French Guyana (37). The Brazilian Amazon state of Amapá, contiguous to the French Guyana, was the first to report autochthonous cases in September 2014. Curiously, at about the same time, an infected individual brought the East–South–Central Africa (ECSA) strain to the Northeastern state of Bahia, resulting in hundreds of cases and the establishment of this lineage as endemic in the region (38). In the following years, growing number of presumed CHKV cases were verified in Brazil, 38,499 in 2015, 271,824 in 2016, and approximately 200,000 in 2017, with dozens of deaths (39, 40). It is suspected that another arbovirus, Mayaro (MAYV), belonging to the same Alphavirus genus from the *Togaviridae* family, may be hidden among cases attributed to CHKV (41) and DENV (42).

There is a fear in LATAM in general, that huge outbreaks of CHKV will take place in the next years, since the majority of the population is still naïve to this virus. Strategies to mitigate the risk of TT-CHKV are not being actively discussed, since the risk of getting infected by mosquitoes is much higher and the associated clinical picture absolutely clear. In LATAM countries, with several social and health demands, it is debatable whether the resources to prevent a few TT-CHKV should not be invested in vector control, in order to benefit a larger number of inhabitants, including blood recipients that are off course also susceptible to mosquitoes' bites in daily life outside blood transfusion settings. However, availability of, in development, arboviral multiplex NATs allowing for simultaneous detection of Dengue, Chikungunya, and Zika and/or pathogen reduction technologies acting on whole blood (43) or components, necessarily including red cells (44) may perhaps result in cost-effective measures to be implemented in endemic areas, home to the majority of the LATAM population.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Parvovirus B19: What Is the Relevance in Transfusion Medicine?

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Parvovirus B19 (B19V) has been discovered in 1975. The association with a disease was unclear in the first time after the discovery of B19V, but meanwhile, the usually droplet transmitted B19V is known as the infectious agent of the "fifth disease," a rather harmless children's illness. But B19V infects erythrocyte progenitor cells and thus, acute B19V infection in patients with a high erythrocyte turnover may lead to a life-threatening aplastic crisis, and acutely infected pregnant women can transmit B19V to their unborn child, resulting in a hydrops fetalis and fetal death. However, in many adults, B19V infection goes unnoticed and thus many blood donors donate blood despite the infection. The B19V infection does not impair the blood cell counts in healthy blood donors, but after the acute infection with extremely high DNA concentrations exceeding 10^{10} IU B19V DNA/ml plasma is resolved, B19V DNA persists in the plasma of blood donors at low levels for several years. That way, many consecutive donations that contain B19V DNA can be taken from a single donor, but the majority of blood products from donors with detectable B19V DNA seem not to be infectious for the recipients from several reasons: first, many recipients had undergone a B19V infection in the past and have formed protective antibodies. Second, B19V DNA concentration in the blood product is often too low to infect the recipient. Third, after the acute infection, the presence of B19V DNA in the donor is accompanied by presumably neutralizing antibodies which are protective also for the recipient of his blood products. Thus, transfusion-transmitted (TT-) B19V infections are very rarely reported. Moreover, in most blood donors, B19V DNA concentration is below 1,000 IU/ml plasma, and no TT-B19V infections have been found by such low-viremic donations. Cutoff for an assay for B19V DNA blood donor screening should, therefore, be approximately 1,000 IU/ml plasma, if a general screening of blood donors for single donation blood components is considered at all: for the overwhelming majority of transfusion recipients, B19V infection is not relevant as well as for the blood donors. B19V DNA screening of vulnerable patients after transfusion seems to be a more reasonable approach than general blood donor screening.

Keywords: parvovirus B19 infection, B19V, blood donors, transfusion-transmitted infection, blood donor screening

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INTRODUCTION

Parvovirus B19 (B19V) has been described first in 1975: Cossard and colleagues (1) found "parvovirus-like particles" in the sera of nine blood donors and two patients. Meanwhile, these "parvovirus-like particles" are known to be B19V, a small, single-stranded DNA virus of approximately 5,500 nucleotides of which three different genotypes worldwide are existent. Genotype 1 is predominant worldwide,

while genotype 2 is found only sporadically in Europe and the Americas. Genotype 3 seems to be widespread predominantly in north- and west-Africa (2). The diameter of B19V is between 19 and 25 nm, and it is a "bare" virus without any envelope. The icosahedral capsid consists of two structure proteins: VP1 and the smaller VP2 one. VP2 is the main protein in the capsid with a percentage of approximately 95% of the capsid while 5% of the capsid consists of VP1 (2, 3). The genome also encodes, besides for VP1 and VP2, for the non-structure protein NS1 which is essential for the replication of the viral DNA and responsible for the host cell apoptosis (4).

The tropism of B19V is very specific: B19V infects only cells with the blood group antigen P (globosid) on their surface (5). The antigen P is expressed on erythrocyte precursor cells and megakaryocytes as well as on endothelia cells, placental cells, and fetal myocardium.

Crucial for virus entry into the host cell is besides the P-antigen on the cell surface a distinct part of VP1, the unique amino-terminal region VP1u (6). The role of antibodies against epitopes on VP1 in the protection of erythrocyte progenitor cell and in the termination of the acute B19V infection underlines the importance of VP1 for the establishment of the infection by host cell entry (7, 8). Although B19V enters all cells with the antigen P, a productive infection with virus replication and the formation of progeny viruses happens only in erythrocyte progenitor cells, leading to the apoptosis of the infected cell.

However, many infections with B19V are asymptomatic or manifests only with mild of unspecific symptoms, flu-like symptoms, or arthralgia. The association of B19V with a disease was initially unclear (1) but only several years later, B19V could be linked to an exanthematous children's disease, the erythema infectiosum or fifth disease (9).

The ability to enter P-antigen expressing tissues, may explain the distinct clinical picture of B19V infection in some patient at risk for a more severe course of the infection: acute infection causes an affection of a large number of erythrocyte precursors with consecutive apoptosis. Mass apoptosis of these cells may lead to a short-time arrest of hematopoiesis and slight drop of the hemoglobin value (10) or the hematocrit, respectively, but due to the life span of red cells of approximately 140 days, severe anemia due B19V infection in patients without high erythrocyte turnover are rare and only single cases have been reported (11–13). However, in patients with increased red blood cell destruction resulting in high erythrocyte turnover and a shorter half-life of red cells from other reasons, e.g., hemolytic anemia or hemoglobinopathies, already the short-time hematopoetic arrest due to B19V infection can cause a life-threatening aplastic crisis (4).

Penetration of other cells with the P-antigen on their surface can induce some other clinical pictures of B19V infection: the typical rash during the erythema infectiosum may be a sign of the B19V infection of cutaneous endothelial cells, although a deposit of immune complexes, formed by antigen-bound antibodies is also a possible explanation.

In non-immune, antibody-negative pregnant women, B19V-infection can be transmitted *via* the placenta to the unborn child. In first-trimester pregnancies, transplacental infection of the fetus can lead to miscarriage, in the second or third trimester, infection of fetal erythrocyte precursor cells in the liver, which is the site of fetal haematopoesis, and infection of myocardial cells causes a severe fetal illness with anemia and myocardial failure, a clinical picture that is called hydrops fetalis.

After the experimental infection of otherwise healthy volunteer subjects, the course of the B19V infection was characterized in detail first in 1985 (14): B19V is naturally droplet transmitted by aerosol *via* the upper respiratory tract, and the infection of the volunteers in this study was thus performed by intranasal inoculation with B19V. Already few days after infection, B19V was detectable in the plasma of the infected volunteers, and virus levels reached a peak 6–10 days after infection was induced. In this acute stage of infection, viral DNA at a concentration of more than 10^{10} IU/ml (15) plasma is detectable, followed by IgM antibody formation which precedes the appearance of IgG antibodies about some days (14, 16). Individuals, in whom IgG antibodies against B19V are present, are considered to be immune against a new infection with any B19V genotype.

Due to the infection path *via* droplet transmission, many infections occur during childhood: while in infants at an age of below 5 years, in only 2% antibodies against B19V are detectable as a marker of a past infection, the percentage of antibody-positive infants increases with the age: between an age of 5 and 9, in 21% of infants antibodies against B19V are detectable and 36% in adolescents between 10 and 19 years. In 49% of adults, between an age of 20 and 39 years, antibodies against B19V were detectable in this study (17). In Germany, in 66.9% of the adolescents at an age of 18–19 years, antibodies against B19V are detectable, also indicating that many infections occur already during childhood. Overall 72.1% of the adults between 18 and 79 years in Germany tested positive for anti-B19V-IgG as a marker for an infection anytime in the past (18).

B19V INFECTION IN BLOOD DONORS

Seroprevalence of B19V in Blood Donors

As shown by Anderson et al. (14) and known for other viral infections, also infection with B19V is accompanied by the formation of B19V-specific IgG antibodies, which are detectable for many years or even lifelong. The rate of B19V IgG-positive blood donors, the seroprevalence, thus serves for the assessment of the rate of donors who have had a B19V infection at any time in the past. Data about the prevalence of antibodies against B19V are available from several countries (**Table 1**). The seroprevalence differed between 9.78 and 79.1% in the different countries, but not only geographical differences might led to the differing seroprevalence rates but also differences in the numbers of investigated donors as well as different sensitivities in the antibody tests that were used.

Data about the seroprevalence of B19V in German blood donors are lacking. However, the seroprevalence of B19V in blood donors is likely similar to that in the general adult population and can probably be transformed to the blood donor population: the seroprevalence of B19V in adults from the age of 18 years in Germany has been assessed to 72.1% and these individuals can be considered as probably immune against a B19V infection (18).

TABLE 1 | Seroprevalence, measured by anti-parvovirus B19 IgG determination, in blood donors.

Country	Seroprevalence (%)	No of investigated donors	Reference
Spain	9.78	92	(19)
India	27.96	1,633	(20)
Russia	29.70	1,000	(21)
Chile	55	400	(22)
Brazil	55.30	47	(23)
China	55.43	184	(24)
Brazil	60	100	(25)
Spain	64.70	136	(26)
Tunisia	65	378	(27)
Belgium	74	441	(27)
Italy	79.10	446	(28)

Prevalence of B19V DNA in Blood Donors

Provided that 72% (or slightly less) of all blood donors are immune against B19V due to the presence of antibodies formed by a B19V infection in the past, the remaining blood donors without detectable antibodies are susceptible for a B19V infection. As the acute infection is often asymptomatic, especially in adults, affected blood donors are often not apparently ill and hence are allowed to donate blood. That way, viremic donations can be taken and B19V DNA can be detected in the plasma by nucleic acid testing (NAT). In the last years, with the availability of international standards (29, 30), it became common, to express the viral load of a blood sample by the quantity of B19V DNA in "International Units per milliliter (IU/ml)" rather than genome equivalents with one international unit being approximately equivalent to 0.6–0.8 genome equivalents (29).

Several reports about the prevalence of B19V DNA in blood donors or blood donations, respectively, have been published in the past 15 years. Investigation of blood donations for B19V DNA showed that detection of B19V DNA in blood donations is not a rare event: Already in studies performed in the 1990s, in 0.03–0.6% of all blood donations, detection of B19V DNA was reported (31–33). An overview about the prevalence of B19V DNA in blood donations as well as the number of investigated donations and the pool size is provided in **Table 2**.

The early prevalence data from the 1990s could be confirmed by more recently published reports: B19V DNA was present in one out of 625 (or 0.16%) donations in Belgium between 1999 and 2000 (34). As in many studies dealing with the issue of B19V DNA in blood donations, the screening for B19V DNA in this study was performed by minipool-testing: That means, not each single donation is tested for B19V DNA, but several donations are brought and tested together by NAT.

In another study from the USA, 5,020 archived samples collected in the years 2000 and 2003, were investigated for B19V DNA. The prevalence was 0.88% (37) and considerably higher compared to a further study that was performed in the Netherlands (38) between 2003 and 2009: 6.5 million blood donations have been tested for B19V DNA. However, donations with a concentration of B19V DNA below 1×10^6 IU/ml plasma were not considered in the study. With the limitation that only donations with a high DNA concentration were considered, 411 out of the 6.5 million donations (or 0.006%) tested positive for B19V DNA.

TABLE 2 | Prevalence of parvovirus B19 (B19V) DNA in blood donations.

Country	Prevalence of B19V DNA (%)	No of investigated donations	Pool size	Reference
UK	0.03	20,000	500	(31)
Japan	0.6	1,000	10	(32)
USA	0.1	9,568	50	(33)
Belgium	0.16	16,859	480	(34)
Portugal	0.12	5,025	10	(35)
Germany/ Austria	0.013[a] and 0.26[a]	2.8 million	96	(36)
USA	0.88	5,020	Single donation	(37)
The Netherlands	0.006	6.5 million	480	(38)
Korea	0.1	10,032	24	(39)
China	0.58	3,957	Single donation	(40)
Brazil	1	100	Single donation	(25)
Germany	0.61	53,789	96	(41)
China	0.06[b]	5,040	96	(42)
	0.079[b]	5,030	96	
Brazil	1	91	Single donation	(43)

[a]*Prevalence of B19V DNA was assessed for high (>10^6 IU B19V DNA/ml plasma) and low (<10^6 IU B19V DNA/ml plasma) viremic donations.*
[b]*Prevalence data were assessed for source plasma and for whole blood donors separately.*

In a Chinese study (40), 3,957 donor samples were screened for B19V DNA, 23 of those samples (0.58%) tested positive. In this study as well as in the study from the USA, only samples with lower DNA concentration had been detected (2.48×10^2–6.38×10^4 IU/ml and <20–1,869 IU/ml plasma), in contrast to the Dutch study. Acute B19V infections with high DNA concentrations seemed to be only shortly detectable, and thus, a large number of blood donations have to be screened for B19V DNA in order to detect a high viremic donation.

In another study from Austria and Germany (36), 2.8 million donations were screened for B19V DNA by minipools within 4 years. Minipools with a B19V DNA concentration >10^5 IU/ml were resolved to identify the high viremic donation and the positive donation was discarded, but minipools with a lower B19V DNA concentration were not resolved. That way, 12.7 positive donations per 100,000 donations (0.13%) with a high viral load (>10^5 IU/ml) were found and, presumably, 261.5 positive donations per 100,000 (0.26%) donations with a low (<10^5 IU/ml) viral load.

In a recent study (41) concerning the issue of B19V DNA in blood donations was performed also in Germany: 53,789 donations from overall 23,889 donors (first time and repeat blood donors) were screened for B19V DNA within 1 year. In 326 donations (0.61%), B19V DNA was detectable, in most cases at a low concentration. Only in eight donations, more than 10^5 IU B19V DNA/ml plasma were detectable.

Also the incidence as well as the prevalence of B19V DNA in blood donors were assessed in this study: 77/17,231 repeat blood

donors were B19V DNA positive when tested first time during the study period and from 34/17,231 repeat blood donors, at least one negative sample were drawn, before they became infected during the study period. By these data, the prevalence of B19V DNA was calculated to be 0.45% and the annual incidence to be 0.20%.

The results of these studies are compatible with each other although a comparison of these studies is difficult due to differences in the size of the study population, the pool size [e.g., 480 donations in the Belgium study (34) vs 96 in the German/Austrian study (36) or vs single donation testing in the US study (37)] and use of different tests with a different analytical sensitivity for B19V DNA screening. Moreover, it is known that the occurrence of acute B19V infections follows a seasonal pattern, with many infections in spring and less or no infections in autumn (38). The point in time when a study was performed might, therefore, influence the prevalence of B19V DNA, especially if the study did not comprise the period of an entire year. It is also known, that in some years B19V epidemics occur, with a higher number of acute B19V infections in 1 year, while in other years only few B19V infections happen (38, 44).

COURSE OF THE B19V INFECTION IN BLOOD DONORS

Clinical Course

At present, there are no studies available which investigated systematically the clinical picture of the B19V infection in blood donors. However, in many otherwise healthy individuals like blood donors, the infection often goes unnoticed or presents with only mild or unspecific symptoms. One can assume that B19V infections in blood donors demonstrate a likewise course and from that reason, many B19V-infected blood donors appear to donate and are allowed to donate in the absence of any signs of disease.

Hematological Course

As B19V affects erythrocyte precursor cells and megakaryocytes, a change in the hematological parameter (hemoglobin and platelets) could be expected in blood donors and was already described after the experimental infection of a low number of otherwise healthy individuals (14). The relation between B19V infection and blood count in blood donors has been investigated in one study in Germany (41): blood counts of 345 samples with detectable B19V DNA were compared to 100 B19V DNA-negative controls. While no differences in the quantity of leukocytes, erythrocytes, and platelets were observed, the mean hemoglobin value, the hematocrit, the mean corpuscular volume, the mean cellular hemoglobin value, and the mean hemoglobine concentration of the 345 B19V DNA-positive samples were statistical significant lower than the 100 controls. However, although statistical significant, the differences were only moderate and without any clinical relevance. Also, in the context of acute B19V infection with high DNA concentration, no major differences in the blood count could be observed in comparison to controls or to the B19V DNA-negative samples of the same donors which were drawn before the B19V infection.

Course of the Viremia

The course of the viremia during B19V infection in blood donors is well investigated. The results of the first study, which investigated the course of the viremia after the infection, led to the conclusion that viremia is rapidly cleared in acutely infected, not immune compromised individuals (14). However, the method used to detect B19V DNA (dot-plot hybridization) was less sensitive compared to the methods that are currently used in the blood donor screening, but such a sensitive method was not available at that time. Especially in recent studies, the concentration of B19V DNA, measured in the plasma by NAT, serves as parameter for the level of the viremia of blood donors. And despite the use of more sensitive methods for the detection of B19V DNA, until the beginning of the last decade, the viremia in the acute B19V infection was considered as a rather short phenomenon, lasting only for several weeks (45, 46). The duration of viremia in blood donors has been estimated to be 17.5 days (95% CI 11.0–53.0) (47). The persistence of the virus or chronic infections were considered as extremely rare (48) and a longlasting viremia as a phenomenon that is limited to more severely ill and immunocompromised individuals rather than blood donors, although the data of Jordan and colleagues (33) already suggested that a longer period of viremia might be possible: at least one of the 11 B19V DNA-positive donors in this study was tested positive again during a follow-up investigation performed over 5 months later.

Compatible with this observation were the results of a longitudinal study from France (49): 76 patients who suffered from hemoglobinopathies, and were thus, like blood donors, not immunocompromised, have been followed up for several years. In six of these patients, persistence of B19V DNA at low levels (10–100 IU B19V DNA/ml plasma) for several years (up to 60 months) was determined. Also in a Japanese study, 20 B19V DNA-positive blood donors were followed up for several years and investigated for B19V DNA biannual. A decline of the B19V DNA concentration was found to be below 10^4 IU B19V DNA/ml plasma after 1 year and 10^3 IU/ml plasma after 2 years, subsequently, B19V DNA concentration persisted between 10^3 and 10^1 IU B19V DNA/ml plasma in the third and fourth year (50). In a German-Austrian Study (36), a rapid decline in the median B19V DNA concentration from 4.85×10^7 to 4.6×10^2 IU/ml within approximately 12 weeks was observed. Another study (51) investigated the duration of B19 viremia in 75 blood donors, in whom several consecutive samples could be investigated during 5.5 years. In this period, only in a minority of blood donors, the entire duration of viremia could be assessed, as the last B19V DNA-negative sample before infection *and* a B19V DNA-negative sample after the infection became available, indicating a long duration of viremia. However, the mean interval between drawing the first positive sample and the last positive samples in the study period was 21.5 months (range: 2.3–52.4 months; 95% CI: 19.1–23.9 months). Compatible with the rapid decline of B19V DNA concentration in the former studies (36, 41), a rapid decline of the B19V DNA concentration from a mean value of 2.23×10^8 IU B19V DNA/ml plasma (95% CI: 0–6.48×10^8 IU/ml plasma) to a mean value of 1,598 IU B19V DNA/ml plasma (95% CI: 1,157–2,039 IU/ml plasma) was assessed between the first B19V DNA-positive sample and the second sample, which has

been taken after a mean time of 135.8 days later. B19V DNA then persisted in the donors who could be investigated for a longer period, for several years at low (10^2–10^3 IU/ml plasma) or very low levels (<10^2 IU/ml plasma).

Persistence of B19V DNA has been proven (52–54) in several tissues (e.g., liver, heart, tonsils, synovia) and it has been suggested that, after acute infection, possibly bare B19V DNA, and *not* mature virions, are released from these tissues into the plasma (53, 55). A recent study provided evidence that the positive detection of B19V DNA in the plasma of blood donors approximately 6 months after acute infection is based on bare DNA strands and not on mature, infectious virions. These DNA strands may persist for years after the acute infection (56). Based on this assumption, most of the B19V DNA-positive donations, namely those with low DNA concentrations, might not be infectious for the recipients and the persistence of B19V DNA in blood donors after the decrease of the peak B19V DNA concentration might be irrelevant.

Humoral Immune Response in B19V-Infected Donors

The humoral immune response in the B19V infection in blood donors has already been studied thoroughly and might influence the assessment of the importance in Transfusion Medicine. Already Anderson and colleagues (14) observed the typical antibody response in experimentally infected individuals with formation of anti-B19V IgM in the second week after infection, followed by the formation of anti-B19V IgG few days later. However, the epitope specificity (anti-NS1, anti-VP1, anti-VP2) of these antibodies was not reported.

Early studies on blood donors reported the prevalence of IgG and IgM antibodies in B19V DNA-positive blood donors: in the Netherlands (38), a subgroup of 67 out 411 B19V DNA-positive blood donors was investigated for antibodies of both classes. In 47 (70%) of the B19V DNA-positive donors, no antibodies against B19V were detected, neither of the IgM nor of the IgG class. 16 (24%) donors tested positive for anti-B19 IgM, and 4 (6%) tested positive for IgG *and* IgM. A further characterization of these antibodies was not performed and the findings of this study (no antibodies or predominant IgM detectable) are compatible with acute B19V infections in the investigated blood donors and not with longlasting infections. A German-Austrian study (36) was the first, which investigated the course of the humoral immune response in relation to the B19V DNA concentration by investigation of follow-up samples from 50 B19V-infected donors. IgG antibodies against epitopes on the viral capsids (VP1, VP2) were detectable in approximately one-third of the donors in the first sample, which has been taken during the acute infection. Already in the first follow-up sample, taken 12 weeks thereafter, in all of the donors, IgG antibodies against epitopes on the viral capsid, were detectable. These antibodies were also detectable in a second follow-up sample. As these IgG antibodies were directed against epitopes on the virus surface (VP1 and VP2), it can be hypothesized that these antibodies are able to neutralize the virions by hindering their binding to the cellular receptor on the target cells for B19V. This assumption was corroborated by another experiment in this study: samples with high B19V DNA concentrations and with low B19V DNA concentrations were filtered through a protein G column. Afterward, the DNA concentration was assessed again, and the reduction of B19V DNA after protein G filtration was significantly higher in samples with low DNA concentration compared to those samples with high DNA concentration. This finding indicated the presence of strong binding, high avide IgG antibodies on the surface of B19V, leading to clearance of B19V in the sample by adsorption of IgG-binded virions to the protein G *via* the Fc-fragment of the IgG.

In another study from Germany (51), the humoral immune response in 75 B19V-infected blood donors was investigated, 29 of them had an acute B19V infection during the study period, in the remaining donors, the infection occurred before the study period. Overall 410 samples with detectable B19V DNA have been provided by these donors within 5.5 years and could be considered in the study. That way, the course of B19V infection in blood donors could be studied over a longer period. Besides B19V DNA, samples were investigated for anti-B19V IgM, quantitative for anti-B19V IgG, and the avidity of anti-B19V IgG antibodies was determined. In only six samples with high B19V DNA concentrations, no anti-B19V IgG was detectable. The decrease of B19V DNA concentration was accompanied by an increase of the anti-B19V IgG titer, compatible with the findings of another study (41). Out of 29 donors with an acute, recently acquired B19V infection, in 24 (82.8%) already IgG antibodies, directed against epitopes on VP1 and VP2 with high avidity, were detectable. In five donors, no IgG antibodies against B19V were detectable in the first B19V DNA-positive donation, but at the point in time, when the next follow-up samples was drawn from these donors, also anti-B19V IgG with high avidity, directed against viral capsid proteins, were detectable. The study could also demonstrate that detection of anti-B19V IgM is not a suitable marker for the acute B19V infection as B19V IgM could not be detected in many donors during the acute infection.

TRANSFUSION-TRANSMITTED (TT-) B19V INFECTION BY BLOOD PRODUCTS, IN PARTICULAR BY SINGLE DONATION BLOOD PRODUCTS

Data suggesting a B19V transmission by plasma derivates are available since the 1990s (57, 58). Due to its physicochemical properties, B19V is hard to inactivate or to remove by processes used for other viruses during the manufacturing process of plasma derivatives. In addition, because of the prevalence of B19V DNA among blood donors, the entering of a donation with high DNA concentration in a plasma pool for fractionation is not a rare event, leading to the contamination of the plasma pool from several thousand donations. Thus, it is not astonishing that in a large number of plasma pools for fractionation, B19V DNA is detectable, when untested donations are entering in a pool (59) and that many TT-B19V infections by plasma derivatives have been reported in the past (57). To avoid further manufacturing of such plasma pools, B19V DNA testing of plasma pools or plasma units for fractionation is recommended by the Food and

Drug Administration. The B19V DNA concentration should not exceed 10^4 IU B19V DNA/ml plasma,[1] a level that is considered as the maximum acceptable in plasma pools for fractionation. Nevertheless, there is further evidence for a B19V transmission by plasma derivatives despite the exclusion of plasma donations or plasma pools with high B19V DNA concentration for fractionation (60).

Measures to avoid potential B19V transmission by single donation blood products have not been established so far in many countries, although the first cases of TT-B19V infection were reported also in 1990s (61, 62). Despite the frequent detection of B19V DNA in blood donors, suspected TT-B19V infections are rarely reported in Germany, a country in which several millions of transfusions are carried out annually.[2] If a case of viral transmission by transfusions is suspected in Germany, the German authority has to be informed according to the German transfusion act. However, the publications of this authority in which suspected cases of TT infection were reported during the period from 1997 to 2012, included no cases of suspected TT-B19V infection.[3,4] In 2013, seven B19 infections in a donor and two in 2014 were reported, but infection of these donors never led to a transmission to the recipients of their blood components and no suspected cases of a TT-B19V infection due to an overt infection in a recipient of single-donor blood components was reported to the German authority by a treating physician.[5] TT-B19V infections hence seem to be either a rare event or with a low clinical relevance, so that many infections are overlooked by the treating physicians. Also data reported to the Serious Hazard Of Transfusion (SHOT-) registry in the UK support this assumption: only one case of major morbidity due to a TT-B19V infection has been reported to SHOT between 1996 and 2016 (63).

Initially, only blood products provided from donors with high B19V DNA concentrations seemed to be infectious for their recipients. In a study from the USA, a fourfold increase in the IgG antibody titer in an already anti-IgG antibody-positive recipient of a red blood cell concentrate from a donor with 2.9×10^{10} IU B19V DNA/ml plasma was detected. The authors interpreted their finding as an anamnestic immune response, triggered by a TT-B19V infection through the red blood cell concentrate (15), but they detected no TT-B19V infection through blood products with a B19V DNA concentration of less than 10^6 IU/ml plasma and concluded, that transmission with blood components from donors with a lower B19V DNA concentration do either not occur or are at least a rare event. Shortly later, a TT-B19V infection in a susceptible, antibody-negative recipient by a red blood cell concentrate from an antibody-negative donor with an acute B19V infection was reported. The B19V DNA concentration in the donor's plasma was 5×10^9 IU/ml, the genome of the B19V of the donor and the recipient shared 100% of their sequences, making the red blood cell concentrate as the origin of infection very probable (64).

However, the threshold DNA concentration in blood donors that was considered as being infectious for the recipients of their single donation blood products decreased more and more in the following years. Already in the year 2011, it could be demonstrated that red blood cell concentrates from B19V-infected donors with DNA concentration of 10^5 IU/ml plasma are probably able to transmit B19V by their donation: after transfusion of red blood cell concentrates from nine out of 18 donors with a DNA concentration of 10^5 IU B19V DNA/ml plasma or more, an infection in the recipients occurred, but no TT-B19V infection was observed after transfusion of red blood cell concentrates from donors with less than 10^5 IU B19V DNA/ml plasma. In this study, phylogenetic analysis of B19V in the donors and the infected recipients yielded the blood products as the probable origin of infection (65). In donors with lower B19V DNA concentration ($<10^5$ IU/ml plasma) without transmission of B19V infections to the recipients, a higher proportion of probably neutralizing antibodies was detectable. Besides the lower DNA concentration, the presence of such antibodies might also be protective for the recipients of the blood products. But already in the same years, transmissions of B19V infections through blood donations from donors with still lower DNA concentrations were reported: In a Japanese study (66), a TT-B19V infection through a red blood cell concentrate provided by a donor with a B19V DNA concentration of 5.1×10^3 IU B19V DNA/ml plasma and proved by genome sequence analysis, occurred. Also IgG and IgM antibodies were detectable in the donor's plasma, demonstrating that antibodies in the donor are not always protective for the recipient of his blood products. Although a further probable TT-B19V infection after transfusion of a red blood cell concentrate from a donor with low ($10^{3,2}$ IU B19V DNA/ml plasma) DNA concentration and *with* detectable B19V IgG antibodies was reported (67), single donation blood products with such a low B19V DNA concentration, seem not to be infective in either case: no TT-B19V infections have been observed after transfusion of 15 single donation blood products (eight red blood cell concentrates, four pooled platelet concentrates, and three fresh frozen plasma) from donors with a B19V DNA concentration between 10^3 and 10^4 IU B19V DNA/ml plasma (68).

In the latest report of a TT-B19V infection, a red blood cell concentrate taken from a donor with a B19V DNA concentration of 1.1×10^4 B19V DNA/ml plasma was the probable source of the infection. The B19V infection of the recipient has been accompanied by an immune thrombocytopenia (69).

It is noteworthy that, in only three of the recent studies, the symptomatology of the transfusion recipient finally led to the investigation of the donor: in one Japanese study (66), the infections in the recipients became evident due to more or less serious, miscellaneous symptoms like reticulocytopenia, delayed recovery of red blood cells after chemotherapy or pure red cell aplasia, and also rash or febrile disease. In France (67), a recipient suffered from erythroblastopenia after transfusion of a red blood

[1] https://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm078510.pdf.

[2] http://www.pei.de/DE/infos/meldepflichtige/meldung-blutprodukte-21-transfusionsgesetz/berichte/berichte-21tfg-node.html.

[3] http://www.pei.de/SharedDocs/Downloads/vigilanz/haemovigilanz/publikationen/haemovigillanz-bericht-1997-2008.pdf;jsessionid=734FDB0D86C7B79C8E95E0E9AE86EF2F.1_cid329?__blob=publicationFile&v=1.

[4] http://www.pei.de/SharedDocs/Downloads/vigilanz/haemovigilanz/publikationen/haemovigilanz-bericht-2011.pdf?__blob=publicationFile&v=6.

[5] http://www.pei.de/SharedDocs/Downloads/vigilanz/haemovigilanz/publikationen/haemovigilanz-bericht-2013-2014.pdf?__blob=publicationFile&v=4.

cell concentrate due to sickle cell disease, and in Japan again, fever and thrombocytopenia led to the diagnosis of B19V infection (69). In the other studies (15, 64, 65, 68), positive screening of blood donors for B19V DNA induced the retrospective investigation of the recipients of their blood products, in whom no B19V infection had been suspected so far. Retrospective analysis of the red blood cell counts in one study (68) revealed a slight drop of the hemoglobin values in the two infected recipients after transfusion of red blood cell concentrate. However, the slight decrease of the hemoglobin level might also be attributed to other reasons like allogeneic blood stem cell transplantation, or iatrogen due to excessive blood sample withdrawal. No specific symptoms were specified later by the affected recipients in another study (66) and no symptoms in the recipients have been reported in two further studies (64, 65).

CONCLUSION: WHAT IS THE RELEVANCE OF B19V IN TRANSFUSION MEDICINE AND WHAT CAN BE DONE?

Approximately 30% of the potential blood donors starting at an age of 18 years have no antibodies against B19V and are thus susceptible for new B19V infections. And as B19V infection often go unnoticed especially in adults, B19V-infected donors cannot be recognized by clinical symptoms or evident aberrances in the blood count and are, therefore, allowed to donate. From that reasons, detection of B19V DNA in blood donations is not a rare finding, at least in comparison to other transfusion-transmissible viral agents like HIV, HCV, and HBV. Unlike suggested in the past, B19 viremia after the acute infection is not a short phenomenon limited to several days or weeks, but persistence of B19V DNA for months to years, also in otherwise healthy blood donors, seems to be the norm rather than the exception and that way, multiple consecutive B19V DNA-positive donations can be taken even from a single donor. However, high DNA concentrations indicates peak viremia in the acute infection, but shortly after the acute infection DNA concentration decreases rapidly, accompanied by formation of potentially neutralizing IgG antibodies.

That TT-B19V infections are seldom reported by treating physicians may serve as an indication of the minor relevance of B19V for the transfusion of single donation blood components, either because they do not occur at all or because of their missing clinical consequences. In many reports, dealing with the issue of TT-B19V infection, only the detection of B19V DNA in the donor induced the investigation of the usually asymptomatic recipient of the blood products, and it was not a symptomatic transfusion recipient who was the cause for the investigation of a donor for a B19V infection. This could indicate that the overwhelming majority of TT-B19V infections (if they occur at all) are overlooked by the treating physicians either due to missing or only mild and unspecific symptoms and only single cases of TT-B19V infections with severe consequences were reported (66, 67, 69).

To avoid TT-B19V infections, several measures have been proposed: In Japan, screening for B19V is performed by hemagglutination assay to avoid donations with high viral load entering a plasma pool for fractionation (66, 70). However, the method is less sensitive, detects only donations with a very high viral load and, therefore, more suitable to detect plasma donation that should not enter a plasma pool for fractionation due to their high B19 viral load. As many single donations with lower viral loads and thus the potential to transmit B19V to recipient are overlooked by this assay, the performance in blood donor screening for single donation blood products is not reasonable.

The most efficient method to avoid TT-B19V infections is a general blood donor screening for B19V DNA by NAT. This can be also done by minipools, in which multiple donations are brought together for NAT screening. Screening assays nowadays have a sufficient sensitivity (36, 71, 72) to reliably detect donations even with minimal amounts of B19V DNA. On the basis of the current knowledge, maximal security in terms of avoiding a TT-B19V infection is provided, if the sensitivity of the assay is sufficient to detect a single donation with a B19V DNA concentration of 10^3 IU B19V DNA/ml plasma.

In contrast to NAT screening of blood donations, anti-B19V IgM screening is not suitable to detect blood donations at risk for the transmission of a B19V infection: although IgM is generally regarded as formed during the acute B19V infection, IgM antibodies are not always detectable during the peak viremia in the acute infection stage (51). Reasons might be a pre-seroconversion acute infection when B19V DNA precedes the antibody formation or the disappearance of IgM antibodies during the Ig-class switch.

Another, antibody testing-based strategy for providing "B19V safe" single donation blood components has been proposed in the Netherlands (73): single-donor blood components can be considered as "B19V safe" if they are donated from donors, in whom anti-B19V IgG has been detected in two separate samples, taken after an interval of at least 6 months. And although B19V DNA is usually detectable over a longer period than 6 months after seroconversion (unlike believed at that time), the measured concentration is low, the detection of B19V DNA is probably based on bare DNA strands and, moreover, B19V DNA accompanied by the presence of protective antibodies in at least all donors with ongoing B19V infection. This approach warrants more protection against TT-B19V infection by single-donor blood products. However, anti-B19V IgG testing, preferably fully automated, is required just as an efficient algorithm, which warrants anti-B19V IgG testing after 6 months and then the declaration of single donation blood products as "B19V safe."

Besides donor screening for B19V DNA, inactivation or removal by the virions is applied during the manufacturing process for plasma derivates. Measures like pasteurization or treatment with low pH were effective in the elimination of B19V (74) as well as nanofiltration (75) and an additional gain of security concerning the transmission B19V by plasma derivatives can be expected.

Some data are currently available concerning the effectiveness of pathogen reduction technologies like the amotosalen/UVA treatment or the riboflavin/UV-light treatment of cellular blood products in the inactivation of B19V. By such treatment, a considerably reduction of human or porcine B19V has been reported (76). However, a possible transmission of B19V by an

amotosalen/UVA treated pooled platelet concentrate has been also reported recently (77), making a final conclusion about the effectiveness of pathogen inactivation of B19V in cellular blood products currently difficult.

Whether a general screening for B19V DNA or implementation of other measures for providing "B19V safe" single donation blood components is meaningful should be debated thoroughly. According to current knowledge, B19V seems to be of minor relevance in the administration of single-donor blood components. Symptomatic TT-B19V infection are very rarely reported by clinicians. This may be because they rarely occur for donor reasons: the viral load, if mature virions are present at all in the donor, is too low and/or circulating virions are neutralized by coexisting anti-B19V IgG antibodies with specificity for VP1, protecting the recipient against the infection. Also patient reasons are possible: the seroprevalence in the patient population is comparable to that of the general population. This means that approximately 70% of the transfusion recipients have antibodies against B19V and are probably immune. Another explanation is that TT-B19V infections occur, but are overlooked by the treating physician because they do not have any clinical relevance.

Unlike other transfusion-transmissible viruses (e.g., HBV, HCV, HIV), B19V infections can be well treated, e.g., by transfusion of further red blood cells or by administration of i.v. IgG, and thereby major consequences of TT-B19V infections can be prevented. Moreover, HBV, HCV, and HIV pose a threat for a majority or almost all transfusion recipients and cannot or hardly be community acquired, in contrast to B19V, which is droplet-transmissible but against which already 70% of transfusion recipients are immune.

Hence, another approach than a general blood donor screening to protect recipients for TT-B19V infection is to generate awareness in clinicians for the possibility of TT-B19V infections. Distinct susceptible patient groups for a more severe course of a potential B19V infection (e.g., pregnant women, immunosuppressed patients, patients with high erythrocyte turnover) should be investigated thoroughly for symptoms of a B19V infection after transfusion and also performance of a B19V NAT screening in the patient can be considered. In this way, not only rarely occurring TT-B19V infections but also the presumably more frequently occurring community-acquired B19V infections can be detected.

AUTHOR CONTRIBUTIONS

DJ and HH reviewed the literature and wrote the manuscript.

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Transfusion-Transmitted Hepatitis E: NAT Screening of Blood Donations and Infectious Dose

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The risk and importance of transfusion-transmitted hepatitis E virus (TT-HEV) infections by contaminated blood products is currently a controversial discussed topic in transfusion medicine. The infectious dose, in particular, remains an unknown quantity. In the present study, we illuminate and review this aspect seen from the viewpoint of a blood donation service with more than 2 years of experience in routine HEV blood donor screening. We systematically review the actual status of presently known cases of TT-HEV infections and available routine NAT-screening assays. The review of the literature revealed a significant variation regarding the infectious dose causing hepatitis E. We also present the outcome of six cases confronted with HEV-contaminated blood products, identified by routine HEV RNA screening of minipools using the highly sensitive RealStar HEV RT-PCR Kit (95% LOD: 4.7 IU/mL). Finally, the distribution of viral RNA in different blood components [plasma, red blood cell concentrate (RBC), platelet concentrates (PC)] was quantified using the first WHO international standard for HEV RNA for NAT-based assays. None of the six patients receiving an HEV-contaminated blood product from five different donors (donor 1: RBC, donor 2–5: APC) developed an acute hepatitis E infection, most likely due to low viral load in donor plasma (<100 IU/mL). Of note, the distribution of viral RNA in blood components depends on the plasma content of the component; nonetheless, HEV RNA could be detected in RBCs even when low viral plasma loads of 100–1,000 IU/mL are present. Comprehensive retrospective studies of TT-HEV infection offered further insights into the infectivity of HEV RNA-positive blood products. Minipool HEV NAT screening (96 samples) of blood donations should be adequate as a routine screening assay to identify high viremic donors and will cover at least a large part of viremic phases.

Keywords: hepatitis E virus, blood donor, blood safety, NAT testing, transfusion–transmission

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INTRODUCTION

Hepatitis E virus (HEV) is an emerging infectious threat to blood safety. In the recent decade, there have been several reports of transfusion-transmitted hepatitis E virus (TT-HEV) infection [for review, see Ref. (1)], although the risk of infection through consumption of raw or undercooked pork and wild boar is even greater (2). In industrialized countries, HEV infection, mainly with genotype 3, usually causes an acute, self-limiting, asymptomatic, or mild hepatitis. However, the significance of HEV genotype 3 infections has changed because chronic hepatitis with rapidly

progressive cirrhosis in organ transplant recipients and patients with hematological malignancy, as well as fulminant hepatitis in patients with chronic liver disease, have been observed (1, 3). HEV-infected immunocompromised patients develop chronic hepatitis E in approximately 60% of cases (4).

Since 2004, HEV has gained importance as a transfusion transmissible infectious agent, although earlier reports pointed to the risk of infection (5, 6). HEV has been transmitted in samples of red blood cells, platelet preparations, pooled granulocytes, and fresh frozen plasma (FFP) (solvent-detergent treated, amotosalen-treated, secured by quarantine). As a result, the public health implications of HEV in Europe have gained greater momentum due to an increasing number of hepatitis E cases and recent reports of chronic, persistent HEV infections associated with progression to cirrhosis in immunosuppressed patients. The question of hepatitis E and contribution of NAT screening on blood safety is currently extensively discussed, not only by several European committees and local blood authorities but also internally by a large number of blood transfusion facilities. Domanovic questioned the situation as "a shift to screening" and summarized the epidemiology of HEV infections among blood donors and outlined strategies to prevent TT-HEV in 11 European countries (7). A nationwide HEV RNA screening of blood donations was introduced in Ireland, the UK, and recently the Netherlands. Several blood establishments in Germany, France, and recently Switzerland perform selective screening intended for use in high-risk patients or universal screening for HEV RNA. Blood authorities in Greece, Portugal, Italy, and Spain are evaluating the situation (7). Regardless of the risk of HEV transmission *via* blood products, most authorities have recommended HEV monitoring of immunosuppressed patients. The implementation of a HEV run control for screening human plasma pools requested by the Ph. Eur. 1646 (8) is another indication toward a transfusion relevance of HEV. So far, there have been only specific case reports of HEV transmission by SD-treated plasma (SDP) but not by other plasma-derived medicinal products. However, it is not unlikely that cases might have been overlooked due to diagnostic failure (9). Past serologic investigations in Japan implicated coagulation factors in the transmission of HEV. The conclusion was based on the significantly higher prevalence of HEV antibody in hemophilia patients receiving coagulation factors that were not subjected to virus inactivation or removal, compared with patients who received virus-inactivated coagulation factors (10). Cost-effectiveness analyses were carried out in the Netherlands to assess whether an appropriate measure should be implemented for blood donor screening (11). The analysis led to the conclusion that the prevention of HEV transmission through the screening of blood donations is not markedly expensive compared with other blood-screening measures. However, the key issue of these cost-effectiveness analyses is the minimum viral load required to be detected in the donor blood. Thus, attention is now focused on the limit of detection of NAT (ID versus pool NAT), which is primary influenced by the minimum infectious dose of a blood product triggering an infection in the recipient. The German Advisory Committee on Blood (Arbeitskreis Blut) recommended a NAT sensitivity of 100 IU HEV RNA/mL [per single donation (12)], which is difficult to achieve with the currently available NAT assays using minipool NAT. For these reasons, the ongoing discussions address the question of the most appropriate and effective strategy to minimize the risk of TT-HEV infection, taking into account the costs, the logistics of testing, and the infection risk and outcome of HEV-infected blood recipients. The present review provides a comprehensive view of the various aspects of TT-HEV infection and a discussion on the current status on the issue of screening for this virus.

MATERIALS AND METHODS

HEV RNA Screening, Serological Testing, and Measurement of Liver-Specific Parameters in Blood Donors and Transfusion Recipients

Routine HEV RNA screening of therapeutic blood products was introduced in our blood donation service in January 2015. From January 2015 to July 2017, a total of 235,524 donations from 86,933 donors were screened for the presence of HEV RNA revealing 182 HEV RNA-positive donors. For four of these HEV RNA-positive donors, a lookback procedure need to be initiated, and a total of nine viremic previous donations of these donors were identified, which were transfused to six different recipients (**Table 1**).

Hepatitis E virus RNA-positive blood donors were identified using the RealStar HEV RT-PCR Kit (Altona Diagnostics, Hamburg, Germany), as described previously (13). Total nucleic acid (RNA/DNA) was extracted from 500 µL of donor and recipient samples using the NucliSens easyMAG (bioMerieux, Nürtingen, Germany) automated RNA/DNA extraction system followed by HEV RNA detection using the RealStar HEV RT-PCR Kit (13). HEV titer of positive samples was quantified using the first WHO international standard for HEV RNA for NAT-based assays (Paul Ehrlich Institute, Langen, Germany). The linear range of quantification was from 25 to 10E+07 IU/mL.

Screening for the presence of HEV-specific IgM and IgG antibodies was performed using the Anti-HEV ELISA (IgM and IgG, Euroimmun, Lübeck, Germany) according to the manufacturer's instructions. Serum concentrations of glutamate dehydrogenase (GLDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin were measured in plasma samples using the respective enzymatic assays on the Architect system (Abbott Diagnostics Europe, Wiesbaden, Germany). All HEV-infected donors underwent pre-donation medical examination and negated current diseases or any known risk factors for viral infection. The study protocol followed the ethical guidelines of the Ruhr University, Bochum, and was approved by the institutional review board; donors provided informed consent.

Processing of Blood Products and Quantification of the Viral Load

Apheresis-derived single-donor PCs (APCs) were prepared after standard processing with the Haemonetics MCS + (Haemonetics GmbH, München, Germany). The final product consisted

TABLE 1 | Cases of transfusion of blood products containing hepatitis E virus (HEV) RNA from this study.

	Donor				Recipient					Outcome		
Donor	**Blood product**	**Viral load (IU/mL), genotype**	**Infectious dose (IU)**	**Anti-HEV IgM/IgG**	**Recipient, sex and age**	**Anti-HEV IgG[a]**	**Primary disease**	**Immuno-compromised**	**Follow-up period (days)**	**Outcome**	**HEV-PCR**	**Anti-HEV IgG**
1	RBC (314 mL)	<25 GT 3	<2.50E+02	Negative	1 M, 23 years	Negative	Heart transplantation	Yes	134 days	No HEV infection	Negative	Negative
2	PC 1 (234 mL)	<25 GT 3	<4.68E+03	Negative	2 M, 76 years	Negative	Heart valve failure, atrial fibrillation	No	35 days	No HEV infection, died sepsis	Negative	Negative
	PC 2 (243 mL)		<4.86E+03		3 M, 54 years		Left ventricular heart failure	No	50 days	No HEV infection	Negative	Negative
3 donation 1	PC 1 (230 mL) PC 2 (254 mL) Total	27.8 GT 3	5.12E+03 5.65E+03 1.08E+04	Negative	4 F, 26 years	Negative	Hypertrophic cardiomyopathy	No	16 days	No HEV infection[b]	Negative	Negative
3 donation 2	PC 1 (244 mL) PC 2 (244 mL) Total	69.4 GT 3	1.35E+04 1.35E+04 2.71E+04	Negative	5 M, 72 years	Negative	Arrhythmia	No	NA	Died arrhythmia	NA	NA
4	PC 1 (242 mL) PC 2 (244 mL) Total	<25 GT 3	<3.97E+03 <4.00E+03 7.97E+03	Negative	6 F, 79 years	Negative	Leukemia	Yes	NA	Died leukemia	NA	NA

[a] *At the date of transfusion.*

[b] *Patient no longer available.*

of 2.0–4.0 × 10E+11 platelets/unit (205–295 mL) containing 0.76–0.84 mL/mL human plasma and 0.16–0.24 mL/mL ACD-A stabilizer. For the preparation of RBC, whole blood donations were collected into a multiple bag system with inline filtration for leukoreduction (CompoFlow quadruple 4F, 70-mL CPD/100-mL PAGGS-M—WB + PDS-V, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany), followed by centrifugation of the filtered whole blood unit at 4,182 × *g* and 22°C for 45 min. Automated fractionation was carried out using the CompoMat G5 separator (Fresenius Kabi), and RBCs were stored directly at 4 ± 2°C. The final product volume averaged from 200 to 400 mL. The residual plasma volume was estimated to be 10 mL per RBC. The corresponding plasma products (FFP) contained a total volume of 180–380 mL with 0.75–0.82 mL/mL human plasma.

Viral RNA in the different blood components (FFP, RBC, RBC supernatant, PC) was extracted from 4.8-mL sample with the Chemagic Viral DNA/RNA reagent kit (Viral 5k, PerkinElmer Chemagen Technology GmbH, Baesweiler, Germany) combined with the automated Chemagic magnetic separation module MSMI (PerkinElmer Chemagen Technology GmbH) according to the manufacturer's instructions. For the recovery of RBC supernatant, 50 mL of RBC were transferred to EDTA-containing monovettes followed by centrifugation for 10 min at 5,000 rpm. Therefore, the RBC supernatant contained CPD/PAGGS-M stabilizator and a minimal proportion of residual plasma. For RNA extraction of RBCs, the alternative lysis buffer CMG-825 (lysis buffer blood, PerkinElmer Chemagen Technology GmbH) was used. The 95% lower limit of detection was calculated by Probit analysis to 4.7 IU/mL [confidence interval: 3.6–7.5 IU/mL (13)] for FFP, PC, and RBC supernatant and to 8.9 IU/mL (confidence interval: 6.5–21.1 IU/mL) for RBCs.

Searching Criteria

For the systematic review of HEV cases, the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/), a public search engine maintained by the United States National Library of Medicine (NLM) at the National Institutes of Health (NIH) that provides access to over 24 million citations in all fields of life sciences, mostly from the MEDLINE (Medical Literature Analysis and retrieval System Online), was searched for publications between 2004 and 2017 (publications dates) using specific search strings including "hepatitis E/HEV infection," "transfusion transmitted hepatitis E/HEV infection," and "hepatitis E/HEV blood donor screening."

Statistical Analysis

All values are given as mean ± SD. Median values and SD were calculated and Spearman's rank correlation analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Statistical analysis to assess differences between values was performed using the Mann–Whitney *U* test.

RESULTS

A systematic review of individual case reports regarding the transfusion of HEV-contaminated blood products from 2004 to

2017 is summarized in chronological order of occurrence in Table S1 in Supplementary Material. Cases including those patients who died shortly after transfusion for reasons other than HEV infection were excluded. We further describe six new cases of patients transfused with HEV-contaminated blood products, and none of the recipients developed HEV infection.

Case Description

Table 1 summarizes the donor and recipient information of all six cases. Anti-HEV IgM and IgG antibodies were not detected in any HEV RNA-positive donor and serum concentrations of GLDH, AST, ALT, and total bilirubin were all within normal range (data not shown). The presence of HEV RNA was confirmed in a secondary sample. Additionally, the corresponding plasma product of the RBC of donor 1 was available and the presence of HEV RNA was further confirmed. Donors 1 and 4 did not return for blood donation after HEV infection. For the other two donors, anti-HEV IgG seroconversion was observed after 149 days (donor 2) and 116 days (donor 3) after the first HEV RNA-positive donation. For HEV genotyping of all donor samples, HEV-nucleotide sequence, corresponding to a 242-bp fragment of the ORF1 region, was amplified and sequenced. Phylogenetic analysis showed that the samples clustered together and were related to HEV genotype 3, which is prevalent in Germany.

All recipients were anti-HEV IgM and anti-HEV IgG negative at the time of transfusion of HEV RNA-positive blood products. The viral load in plasma samples of donors 1, 2, and 4 was determined to be 17, 12, and 20 IU/mL, respectively. These values were below the linear range of quantification (<25 IU/mL), and therefore a maximum infectious dose was calculated assuming a viral load of 25 IU/mL. Recipient 1, an immunocompromised man after heart transplantation, received one RBC. Assuming a residual plasma volume of 10 mL per RBC, the maximum corresponding infectious dose was calculated as 250 IU. This patient did not develop HEV infection within the follow-up period of 134 days, and neither HEV RNA nor anti-HEV antibodies were detectable.

Each apheresis platelet donation resulted in two APCs. The two immunocompetent recipients R2 and R3 received APCs from donor 2 with infectious doses of 4.68E+03 IU (APC1) and 4.86E+03 IU (APC2), assuming an average residual plasma volume of 0.8 mL per APC. Neither patients developed an HEV infection within the follow-up period of 35 days (recipient R2) or 50 days (recipient R3); moreover, no HEV RNA or anti-HEV antibodies were detectable. Accordingly, recipient R6 received two apheresis platelets (APC1: 242 mL, APC2: 244 mL) from donor 4 with a total maximum infectious dose of 7.97E+03 IU (total volume transfused: 486 mL), but she died shortly after transfusion for reasons other than HEV infection.

Donor 3 donated platelets regularly approximately every 14 days and showed the highest viral load compared with the other donors. The first viremic donation (donation 1) contained 27.8 IU/mL HEV RNA, and the second donation, 18 days later (donation 2), contained 69.4 IU/mL HEV RNA. The double APCs were transfused to two immunocompetent recipients (recipients R4 and R5). Recipient 4 received a total infectious dose of 1.08E+04 IU HEV RNA (donation 1, total volume of 484 mL, APC1: 230 mL, APC2: 254 mL). This patient did not develop an acute HEV infection within 16 days after transfusion, and neither HEV RNA nor anti-HEV antibodies were detectable. However, the observation period was short and the possibility that HEV infection may have occurred later could not be ruled out. This patient was released from hospital and unfortunately no follow-up samples were sent to our laboratory for further follow-up. The second HEV-positive donation of donor 3 had a total infectious dose corresponding to 2.71E+04 IU HEV RNA (donation 2, transfusion of a total volume of 488-mL APC, APC1, and APC2: 244 mL). The recipient (R5) of these two apheresis platelets died shortly after transfusion for reasons other than HEV infection.

The cases of recipients 4–6 were excluded from the subsequent overview due to the short follow-up period.

Distribution of Viral RNA in Different Blood Products

In order to determine if a reduction of the viral load occurs during the manufacturing process of blood products, e.g., by centrifugation or by adsorption to components of the blood bag system, including the filter used for leukoreduction, viral loads were quantified in the plasma of HEV RNA-positive donors and additionally quantified in the corresponding blood products, FFP and RBC. Results from the respective blood products were correlated with the expected viral loads calculated with quantified results for plasma of HEV RNA-positive donors, assuming no removal during the production process. Calculation of virus titer assumed a mean plasma content of 0.80 mL/mL human plasma (80%) for FFP. For RBCs, the remaining plasma volume of 10 mL per RBC was assumed for consideration of the total volume of each individual RBC after processing. A total of 73 value pairs were available for correlation analysis of FFPs (**Figure 1A**), of which three were excluded due to low viral load (<25 IU/mL). Likewise, a total of 73 value pairs were available for RBC (**Figure 1B**), of which 31 with low viral load (<25 IU/mL) were excluded. Spearman's correlation analysis revealed a good correlation of $r = 0.9418$ (95% CI: 0.9065–0.9641) for FFP and a good correlation of $r = 0.9290$ (95% CI: 0.8538–0.9663) for RBC. The wider distribution between the measured and calculated HEV titer in RBC is based on the considerably lower plasma amount of only 10 mL per RBC (mean RBC volume 268 mL, mean plasma proportion 3.7%) and the resultant higher method-specific quantification error.

In order to determine if HEV RNA or virus particles are bound to the surface of red blood cells, HEV RNA was quantified in 20 different RBCs as well as in the cell-free supernatants of RBCs (**Figure 1C**). Spearman's correlation analysis again revealed a good correlation of $r = 0.9390$ (95% CI: 0.8492–0.9760), indicating that no specific binding to red blood cell surfaces had occurred.

Figure 2 displays the distribution of viral RNA in FFP and RBC depending on the viral load, quantified in the plasma of HEV RNA-positive donors. HEV RNA was detected in the RBCs of all donations where the viral load in plasma was quantified as >1,000 IU/mL (**Figure 2A**). Quantified FFPs contained a total mean volume of 287 mL, corresponding to a total plasma volume of 230 mL. Comparison of the quantified mean values for FFP

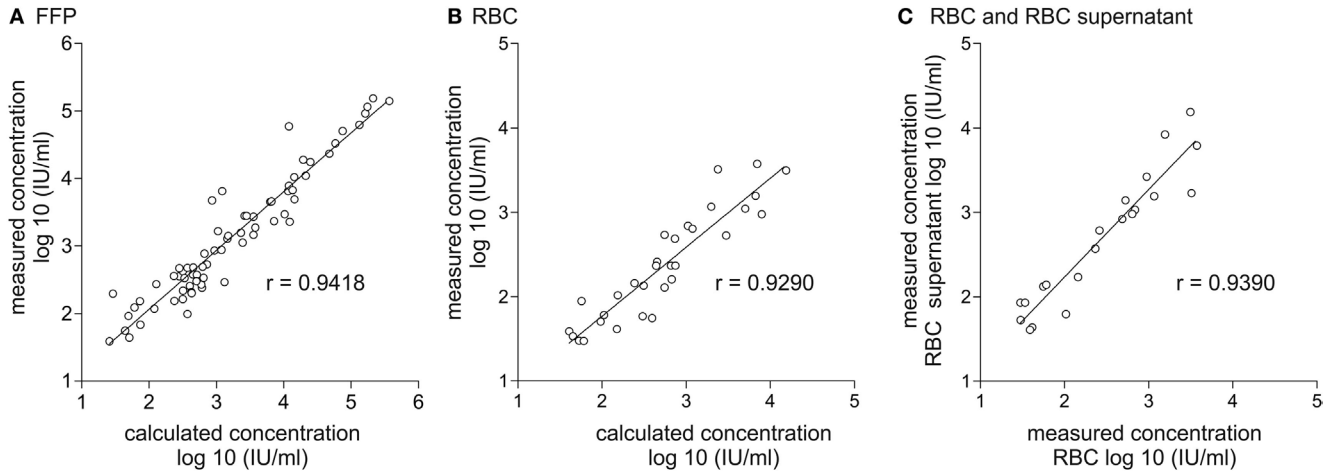

FIGURE 1 | Correlation of calculated and effectively quantified viral load in fresh frozen plasma (FFP) and red blood cell concentrate (RBC) and correlation of viral load in RBC and RBC supernatant. Displayed is the correlation between the effectively quantified hepatitis E virus (HEV) titer and the expected viral load in FFP **(A)** and RBC **(B)**. Calculation of the expected viral load in FFP is based on quantification results of HEV viral load in plasma of donors assuming a mean plasma content of 0.8 mL/mL plasma in the corresponding plasma product. Calculation of the expected viral load in RBC is based on quantification results of HEV viral load in plasma of donors assuming a residual plasma content of 10 mL/RBC. **(C)** Correlation of the effectively quantified HEV titer in RBC and RBC supernatant. The linear range of quantification was from 25 to 10E+07 IU/mL. Therefore, all values <25 IU/mL were excluded.

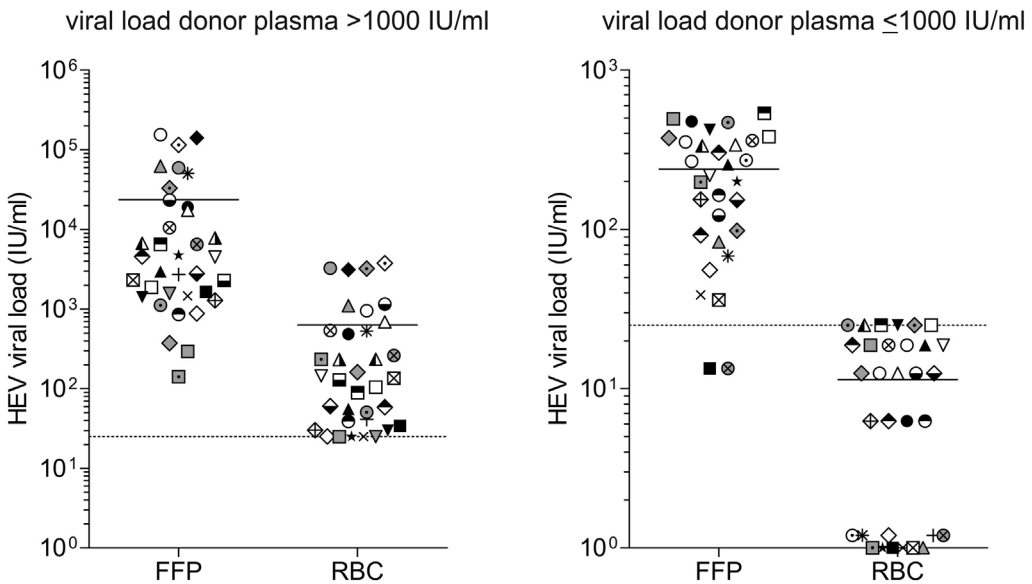

FIGURE 2 | Distribution of viral load in different blood products. The hepatitis E virus (HEV) titer in plasma of donors and the corresponding blood products fresh frozen plasma (FFP) and red blood cell concentrates (RBCs) was quantified using the first WHO international standard for HEV RNA for NAT-based assays. The distinction of viral loads >1,000 IU/mL **(A)** and ≤1,000 IU/mL **(B)** is based on quantification results of HEV viral load in plasma of donors, not in the corresponding blood products. Equal symbols present quantification results in different blood products from the same donor, quantification was performed in quadruplicate. The linear range of quantification was from 25 to 10E+07 IU/mL. Values <25 IU/mL were displayed as 25 IU/mL. For RBCs with low viral loads, not all replicates were positive for HEV RNA. Results were displayed as follows: 0 IU/mL: 0/4 positive replicates; 6.25 IU/mL: 1/4 positive replicates; 12.5 IU/mL: 2/4 positive replicates; 18.75 IU/mL: 3/4 positive replicates; and 25 IU/mL: 4/4 positive replicates (quantification results <25 IU/mL). The solid horizontal line represents mean values, and the dotted horizontal line is representative for the value 25 IU/mL.

(2.34E+04 ± 4.08E+04 IU/mL) with those obtained for RBCs (6.29E+02 ± 1.05E+03 IU/mL) revealed the percentage proportion of 2.7% for RBCs, essentially corresponding to the calculated mean plasma proportion of 3.7%.

For RBCs, where the viral load in plasma was quantified >1,000 IU/mL (**Figure 2B**), a maximum viral load of 25 IU/mL was detected and often not all replicates were positive for HEV RNA. Negative results might either indicate that RBC

contains no viral RNA or the viral load is below the detection limit of the assay (8.9 IU/mL).

DISCUSSION

German public health authorities have recognized an increasing number of acute HEV infections, which is probably due to higher clinical awareness but more likely due to detection of HEV-infected asymptomatic blood donors identified through NAT screening by blood donation establishments. A high frequency of HEV viremic donors have been reported in recent large screening studies in several European countries (13–17). The asymptomatic infection is mostly characterized by a period of asymptomatic viremia, with an estimated duration of 68 days (18). The typical serological course of an acute HEV infection showed detectable IgM antibodies following an incubation period of 2–6 weeks, decline to baseline levels within three to 6 month, followed by longer lasting IgG antibodies which remains detectable for up to 15 years (19–21). The progression of anti-HEV immunoglobulins in asymptomatic cases is comparable with symptomatic cases (22). However, the factual incidence of TT-HEV infection and the real clinical importance is currently unknown. The rate of reported TT-HEV infections is very small, probably due to underreporting, failure to recognize or misinterpretation of symptoms (23), or development of HEV infection long after transfusion, hampering any association with an earlier transfusion event (24). Moreover, the occurrence of primary asymptomatic infection in recipients is certainly an option. Besides a recent large study in England (24), only a small number of individual cases of TT-HEV infection (Table S1 in Supplementary Material) and cases of transfusion of HEV-containing blood without TT-HEV infection (Table S2 in Supplementary Material) have been described (14, 23, 25–46). The large 2012–2013 study in England retrospectively screened 225,000 English blood donors for HEV by NAT. Follow-up of the recipients who had received HEV genotype 3-contaminated blood components indicated that 42% had evidence of TT-HEV infection, with transmission possibly linked to the absence of HEV-specific antibodies (24). A high virus load in the donor, corresponding with the volume of plasma transfused with the final blood component, rendered infection more likely. Moreover, multiple different kinds of blood products were involved, but the transmission rates varied. Of all transfused RBC, only 25% caused HEV infection, whereas 40% of transfused PPCs, 50% of transfused APCs, and 100% of transfused FFPs or pooled granulocytes caused HEV infection (24).

Analysis of 19 Japanese cases of TT-HEV infection by Satake et al. found a comparable rate of infection of 50% (29). All TT-HEV cases present in the Satake's study were included in our case analysis (Table S1 in Supplementary Material). The studies by Satake et al. and Hewitt et al. [18 patients (24)] also identified several patients transfused with HEV-contaminated components without the development of HEV infection [5 patients (29), 18 patients (24)], but these cases were not considered in Table S2 in Supplementary Material because no detailed case descriptions are available.

Unfortunately, some cases only revealed poor data sets, missing important facts for both, the recipients of contaminated blood products or the respective donors. For example, the pretransfusion serostatus in recipients is often only assumable based on the posttransfusion status, or the serostatus is entirely absent. The serological status of the blood donors is also often missing. Additionally, the duration between transfusion, determination of infection, and follow-up of patients including the accompanying therapy and laboratory parameters is often incomplete or untraceable. Most important, the viral HEV load and the resulting infectious dose is not determined. Taken into consideration only the individual cases included in Tables S1 and S2 where the viral load infused is available, 39 patients received blood products containing HEV, of whom 28 patients develop TT-HEV infection.

Tedder and colleagues performed an estimation of the infectious dose of the individual blood product types involved in the UK study in a subsequent analysis, demonstrating that components causing TT-HEV infection had a considerably higher median infectious dose of 1.44E+06 IU than components not causing TT-HEV infection [median total viral load transfused: 2.40E+04 IU (2, 24)]. Accordingly to this study, our systematic case review analysis showed a significant difference in the median viral load transfused between HEV-infected (median: 5.20E+05 IU, this study) and non-infected patients (median: 1.91E+03 IU, $p < 0.0001$, **Figure 3A**). Statistical significant differences in the median viral load transfused were also observed between HEV-infected (median: 4.40E+05 IU) and non-infected non-immunocompromised patients (median: 1.91E+03 IU, $p = 0.0002$), whereas no differences were observed between HEV-infected (median: 4.80E+05 IU) and non-infected immunocompromised patients (median: 1.55+04 IU, $p = 0.1006$). When the immune status of the recipient, which was mentioned to have a major impact on the actual risk of TT-HEV infection, was also taken into account, no differences were observed in the median viral load transfused between immunocompromised and non-immunocompromised patients, independently from the infection outcome (HEV-infected, $p = 0.6286$; non-HEV-infected, $p = 0.5044$). The lowest infectious dose resulting in TT-HEV infection observed in general was 7.05E+03 IU. When the type of blood product was considered, the lowest infectious dose transfused was 7.05E+03 IU for PCs without subdivision of APC and PPCs, 3.16E+04 IU for RBCs, and 3.60E+04 IU for FFPs (**Figure 3B**). Tedder et al. demonstrated that the lowest dose of virus resulting in an infection was 2.00E+04 IU, whereby only 55% of the components containing this dose transmitted an infection (2). Among non-transmitting components, 60% contained or exceeded this infectious dose. Satake et al. summarized that infusion of total viral loads between 2.00E+04 IU and 2.60E+05 IU can occur without HEV transmission (29). In our systematic case review analysis, all components with a viral load >5.00E+04 IU caused infection (**Figure 3A**), independently from the immune status of the recipient; however, only one of the non-transmitting components exceeded this value. Furthermore, the pretransfusion serostatus of recipients receiving HEV-contaminated blood products might have an impact on the development of HEV infection. With the exception of three cases in the study by Hewitt et al. (24) and two additional cases (29, 38, 39, 46), all described cases had a seronegative pretransfusion status. Future studies including IgG positive pretransfusion cases might contribute to

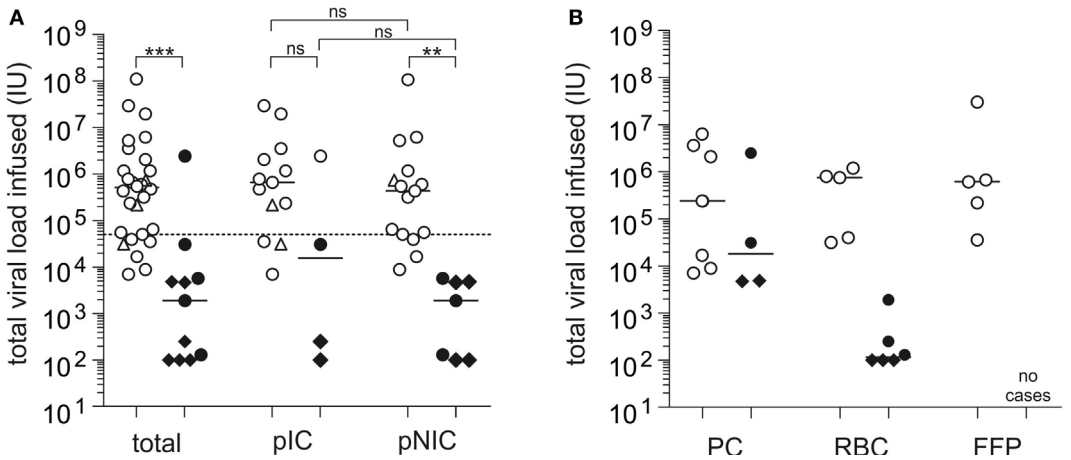

FIGURE 3 | Systematic case review analysis of the total viral load transfused observed in individual case studies (Tables S1 and S2). **(A)** Displayed is the total viral load transfused resulting in posttransfusion hepatitis E virus (HEV) infection or no posttransfusion HEV infection, independently from and depending on the immune status of the recipients ($n = 39$). pIC, possibly immunocompromised; pNIC, possibly not immuno-compromised. **(B)** Displayed is the total viral load transfused resulting in posttransfusion HEV infection or no posttransfusion HEV infection depending on the transfused blood product ($n = 25$). RBC, red blood cell concentrates; PC, apheresis or pooled PCs; FFP, fresh frozen plasma. ◊: values specified with <IU/mL, viral loads for these cases are placed at the maximum possible value, Δ: estimated infectious dose, solid bars indicate median viral load. The solid horizontal line represents median values, and the dotted horizontal line represents the minimum infectious dose. White symbols: HEV infection and black symbols: no HEV infection. ***$p < 0.0001$, **$p = 0.0002$, and *$p < 0.05$, ns, not significant.

the assessment of a protective effect of previous experienced HEV infection or the effectiveness of future available vaccination of donors and/or at risk recipients.

In addition to the cases mentioned in Table S1 in Supplementary Material, Arankalle et al. described two cases of putative, but in our opinion unlikely, posttransfusion hepatitis. HEV infection was assumed due to seroconversion of both patients within weeks after transfusion, but no HEV RNA was detected in patients for comparative sequence analysis. Additionally, donor screening of five of the six involved donations revealed no HEV RNA (6). In Japan, seven further posttransfusion hepatitis E cases (six cases of RBC transfusion, one case of PC transfusion) were detected, according to the official announcement of the Japanese Red Cross Society, but no information on either donor or patient antibody status or the infectious dose were available (26). The German authorities also announced four further posttransfusion hepatitis E cases (two RBC, two PC) in their actual hemovigilance report so far, without detailed case information (47).

The lack of a small animal model and efficient cell culture system has hampered the study of HEV replication, pathogenesis, and infectious dose determination. HEV isolates of genotypes 3 and 4 have been adapted to grow *in vitro*, but HEV cell culture is inefficient and limited, and requires genetic modifications of the HEV isolates (48–51). Experimental HEV infection in the rhesus monkey model led to acute hepatitis E after transfusion of 10 mL plasma from a HEV-infected donor (52). Immune-deficient human-liver chimeric mouse also serves as an appropriate model to study HEV genotype 1 and 3 infection, virus–host interactions, and drug efficacy (53, 54). For example, chronic HEV infection was observed after intrasplenic injection of HEV-GT1-containing preparation with an infectious dose of 2.5E+05 IU (53). These models could serve as a starting point to determine the infectivity and pathogenicity of HEV. However, it is currently questionable whether these models are faithful representation of human infection and could answer the question of infectious dose in humans.

The plasma proportion of the transfused blood product seemed to affect the risk for TT-HEV at most, but so far no information is available on the partitioning of HEV into the different components from a single blood donation (9). It is conceivable that manufacturing processes during the fractionation of whole blood might result in lower viral loads than what is expected on the basis of viral plasma load of blood donors and the assumed residual plasma content. We have shown that the fractionation process in our blood transfusion facility does not considerably reduce the concentration of viral RNA, but this may not generally valid for other production processes.

The question remains as to which screening strategy is necessary and practicable. Screening may constitute a universal approach to include all blood products or a selective screening can be performed for only the products that would be used in at-risk patients. This issue is primarily influenced by two sides: the hospital-sided clear definition of at-risk patients and the logistic implications for the order of blood products, and supply and availability, managed by the blood establishment. The second question is whether minipool screening of up to 96 samples or ID testing is necessary. We would submit that the required detection limit, which need to be derived from the infectious dose, plays an important role for the second issue. Thus, the decision for ID or pool NAT depends on logistic and costs, which are in part dictated by the required sensitivity. **Table 2** summarizes the currently available commercially HEV NAT-screening assays including the analytical sensitivities. The analytical sensitivity (95% LOD) of HEV NAT assays ranges from 4.7 to 18.6 IU/mL, and all assays used for blood screening detected positive donations of all genotypes 1 to 4 and demonstrated a good

TABLE 2 | Overview of currently commercially available hepatitis E virus (HEV) NAT-screening methods.

Kit name	RealStar HEV RT-PCR Kit 1.0	Cobas HEV test	Procleix HEV assay	HEV NAT kit
Manufacturer	**Altona Diagnostics**	**Roche Diagnostics**	**Grifols**	**GFE Blut**
Automation	No	Full automation cobas® 6800/8800 Systems	Full automation Procleix® Panther® System	Full automation PoET System
FDA/CE-IVD	No/yes	No/yes	No/yes	No/yes
Sample preparation/nucleic-acid extraction				
Virus enrichment pre-extraction	No	No	No	No
Maximal MP size	96	Depending on regional regulation	12	Up to 96
Nucleic-acid extraction procedure	Chemagic Viral RNA/DNA Kit on MSM-I (4.8-mL protocol)	Magnetic glass particles for fully automated NA-extraction	Target-specific extraction—magnetic microparticles capture viral nucleic acids with viral-specific capture oligonucleotides	Fully automated magnetic bead extraction
Processed sample volume (plasma mL)	4.8	0.85	0.525	1.3
Elution volume (µL)	100	50	n.a., single-tube format	90
Plasma-equivalents (mL)/PCR (%)	1.2 (100)	0.425 (35)	0.525 (44)	0.433 (36)
NAT/detection				
Principle of NAT detection	RT-PCR, TaqMan probes	RT-PCR, TaqMan probes	Transcription-mediated amplification	RT-PCR, TaqMan probes
NAT instrument	Rotorgene Q	cobas® 6800/8800 Systems	Procleix® Panther® System	PoET System
Target (gene region)	ORF3	5′UTR	n.a.	n.a.
Eluat/PCR volume (µL/µL)	25/50	25/50	100% of the sample is processed and used in the amplification reaction	30/75 or 10/25
Test specifications				
Analytical sensitivity (95% LOD IU/mL)	4.7 (451.2–96 pool)	18.6	7,88	8.2 (787.2–96 pool, 75-µL PCR)
Specificity	100% Genotype 1–4	100% Genotype 1–4	99,98% Genotype 1–4	100% Genotype 1–4
Accomplishment				
Hands on time	30 min	15 min	15 min	n.a.
Time to result	4 h	3 h	3.5 h	5 h
Throughput	960 results (10 pools of 96 samples) in 4 h	96 results (94 pools plus 2 controls) in 3 h 384 results (376 pools plus 8 controls) in 8 h shift (cobas® 6800 System)	5,775 results (275 pools of 16 samples) in 8 h 10,500 results (500 pools of 16 samples) in 12 h	Depending on configuration. e.g., 8,448 in 5 h, 16,896 in 9 h (176 pools of 96 samples)
Remarks	Automation on AltoStar system for ID NAT pending		Intended use includes cadaveric (non-heart beating) donors	Preliminary data; IVD certification pending.

performance in routine testing (**Table 2**). In most settings, the Procleix HEV (Grifols) is used in individual testing (ID NAT), where the 95% LOD was determined to be 5.5–12.78 IU/mL, which is slightly different than that of the manufacturer's value (15, 55). The disadvantage of current commercial HEV NAT assays is their requirement of special screening platforms that are fully integrated and automated, and not as flexible as open NAT platforms. For this reason, we introduced routine minipool HEV NAT screening (96 donations) using an in-house testing regime in our transfusion facility in January 2015. The setting of HEV NAT using RealStar HEV RT-PCR Kit 1.0 [Altona Diagnostics, 95% LOD 4.7 IU/mL (CI: 3.6–7.6, 452 IU/mL) per single donation] is compatible to the virus NAT screening used in our blood transfusion service (13, 56). Our HEV NAT is comparable with commercial HEV NAT-screening methods (**Table 2**) in spite of a lower level of automation and throughput. The novel automation platform AltoStar allows ID NAT testing or alternatively a higher automation grade for pool NAT. It is to be noted that the sensitivity of the RealStar HEV RT-PCR Kit strongly depends on the nucleic-acid extraction method used, ranging from 4.7 to 37.8 IU/mL (13, 55, 56). In our screening setting, we use the fully automated nucleic-acid extraction method Chemagic Viral DNA/RNA Kit that allows the processing of large plasma volumes (4.8 mL). Compared with the other commercial HEV NAT-screening methods from GFE Blut, Roche, and Grifols, the processed sample volume of our method is 3.7-fold, 5.6-fold, and 9.1-fold higher, respectively, resulting in a considerably higher number of HEV plasma-equivalents per PCR reaction. Therefore, this combination is, to the best of our knowledge, the most sensitive HEV NAT. It does not fully meet the sensitivity of 100 IU HEV RNA/mL recommended by the German authorities, but at present, it is not clear whether this sensitivity is absolutely necessary. Our minipool screening strategy aims to identify high viremic donors and will cover at least a large part of viremic phases (22, 57). The European medicine agency so far has also recommended minipool screening in their reflection paper on

hepatitis E (9). However, it remains to be seen in the future whether all relevant viremic phases that could result in TT-HEV infections will be detected.

AUTHOR CONTRIBUTIONS

JD and TV designed the study, analyzed and interpreted the data, and drafted the manuscript. CK designed the study and revised the manuscript critically. All authors contributed to drafting the text and approved the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fmed.2018.00005/full#supplementary-material.

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REVIEW
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Hepatitis B Virus Blood Screening: Need for Reappraisal of Blood Safety Measures?

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Over the past decades, the risk of HBV transfusion–transmission has been steadily reduced through the recruitment of volunteer donors, the selection of donors based on risk-behavior evaluation, the development of increasingly more sensitive hepatitis B antigen (HBsAg) assays, the use of hepatitis B core antibody (anti-HBc) screening in some low-endemic countries, and the recent implementation of HBV nucleic acid testing (NAT). Despite this accumulation of blood safety measures, the desirable zero risk goal has yet to be achieved. The residual risk of HBV transfusion–transmission appears associated with the preseroconversion window period and occult HBV infection characterized by the absence of detectable HBsAg and extremely low levels of HBV DNA. Infected donations tested false-negative with serology and/or NAT still persist and derived blood components were shown to transmit the virus, although rarely. Questions regarding the apparent redundancy of some safety measures prompted debates on how to reduce the cost of HBV blood screening. In particular, accumulating data strongly suggests that HBsAg testing may add little, if any HBV risk reduction value when HBV NAT and anti-HBc screening also apply. Absence or minimal acceptable infectious risk needs to be assessed before considering discontinuing HBsAg. Nevertheless, HBsAg remains essential in high-endemic settings where anti-HBc testing cannot be implemented without compromising blood availability. HBV screening strategy should be decided according to local epidemiology, estimate of the infectious risk, and resources.

Keywords: hepatitis B virus, transfusion, blood safety, nucleic acid testing, HBsAg, anti-HBc, residual risk

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INTRODUCTION

Despite a vaccine and antiviral treatments being available, hepatitis B infection remains a global serious public health issue that affects more than two billion people worldwide. Hepatitis B virus belongs to the Hepadnaviridae family, which genome is a ~3.2-kb partially double-stranded circular DNA enclosed in an icosahedral capsid composed of HBV core (HBc) protein and an outer lipid envelope constituting the 30–42 nm in diameter viral particle. Three viral glycosylated surface proteins (large, middle, and small) embedded in the lipid envelop and are involved in virus binding of and entry into susceptible hepatocytes. During the viral life cycle, non-infectious subviral particles, designed HBV surface antigen (HBsAg), that lack the nucleocapsid and are composed of lipids and small surface proteins are produced in 1,000–10,000 excess compared with infectious virions (1). Due to its limited size, the HBV genome has a highly compact structure consisting in four overlapping reading frames for P, S, C, and X genes, which code for the reverse transcriptase/DNA polymerase, surface, core, and X proteins, respectively. The reverse transcription of a pre-genomic RNA intermediate during HBV replication contributes to a significant natural genetic diversity among viral

strains. According to this genetic heterogeneity, HBV variants are classified currently into nine genotypes (A–I), some of them being further subdivided in subgenotypes (2). HBV genotypes and subgenotypes have different geographical distributions and are increasingly associated with differences in the natural history, clinical outcome of the infection, and detection. HBV chronic carriage prevalence varies according to geographical regions. Sub-Saharan Africa, South East Asia, China, and the Amazon Basin are highly endemic (≥8% HBsAg seroprevalence) or of higher intermediate endemicity (5–7.99%). Countries from the Mediterranean area, Eastern Europe, the Middle East, and North-West of South America are of lower intermediate endemicity (2–4.99%). Western and Northern Europe, North America, part of South America, India, and Australia have mostly low endemicity levels (<2%) (3).

HBV is transmitted through direct exposure to infected blood or organic fluids. The main routes of infection are sexual, vertical from an infected mother to her child during birth or shortly after, and parenteral including blood transfusion. Before 1970, approximately 6% of multi-transfused patients acquired HBV infection through transfusion. Over the past decades, the risk of HBV transfusion–transmission has been steadily reduced by the successive implementation of various safety measures that include donor selection based on risk-behavior evaluation, serological screening for HBsAg and antibodies against the core protein (anti-HBc), and nucleic acid testing (NAT) for HBV DNA. Nevertheless, hepatitis B remains a viral infection transmissible by transfusion with a residual risk varying according to HBV epidemiology, donor populations, and screening strategies (4). The HBV calculated residual risk estimate ranged between <1 and 1.4 per million donations in low-endemic countries and 16 and >100 in high-endemic countries (5–11). These estimates depend on the mathematical models used and are limited by the lack of recent published reports especially from sub-Saharan Africa. Nevertheless, the residual risk of HBV transfusion–transmission is associated mainly with blood donations tested negative for HBsAg and/or HBV DNA and collected during the early phase of primary infection or during the late stages of infection. Success or failure to intercept such potentially infectious donations may depend on the screening strategy and the performance of both serological and molecular assays used. Despite the existence of this residual risk, questions regarding the apparent redundancy of some of the safety measures implemented over the years (i.e., testing for two direct markers HBsAg and viral DNA) prompted debates on how to reduce the cost of HBV blood screening. However, it appears essential to consider carefully the potential negative impact on blood safety before considering removing any safety procedure, especially in high HBV prevalence settings.

The aim of this review is to examine the intrinsic limits and complementarity of HBV screening strategies of blood donations according to the epidemiologic situation.

BLOOD DONOR SELECTIVE RECRUITMENT

In recent years, careful selection of blood donors became an essential and pragmatic element of blood safety management. In that respect, WHO actively promotes the recruitment of voluntary non-remunerated donors (VNRDs) (12). The generally high prevalence of bloodborne pathogens observed in paid donors supported this strategy. Blood safety is improved further by encouraging VNRDs to become regular donors who show considerably lower prevalence of viral markers (13). This policy was successfully implemented in most of high-income countries but might have negative consequences by excluding traditional family/replacement donors (FRDs) that constitute 4–100% of the blood supply in middle- and low-income countries (mainly in Latin America, Africa, and Central Asia), and therefore perpetuating blood shortage and increasing the cost of blood transfusion (14). Exclusion of FRDs relied mainly on the assumption that these donors could not be differentiated from unsafe paid donors. However, during the past few years, a wealth of evidence has been collected that showed no epidemiological and social difference between FRDs and first-time VNRDs (13, 15–17).

A second level of donor selection based on risk-behavior evaluation and at-risk exposure is used by most blood services worldwide to refuse high-risk individuals to donate blood temporarily or permanently. This procedure generally involves pre-donation risk assessment that requires first-time and regular donors to self-declare or self-complete a questionnaire every time before donation followed by a confidential interview with a medical counselor. However, the effectiveness of this donor self-deferral system strongly depends on donor education and accurate and truthful risk disclosure. Despite limited comprehensive data, the prevalence of overall non-compliance with transfusion-transmitted infection (TTI) risk-related deferral criteria was estimated between 1.65 and 13% in general donor populations, irrespective of blood screening results (18, 19). Studies exploring the rate of non-compliance reported substantially higher rates (~25%) among donors tested positive for viral infection(s) post-donation (20, 21). Recently, an overall 10% non-compliance rate was reported in HBV-infected blood donors from the Netherlands (21). Multiple and complex factors were found associated with non-compliance varying from deliberate (e.g., test seeking, social discomfort, disagreement with deferral criteria, and misunderstanding of the pre-donation screening purpose since donations are tested further) to genuine (e.g., misinterpretation of questions, failure of recall, and erroneous no-risk belief associated with temporally remote exposure) non-disclosures. Furthermore, a main risk factor associated with HBV infection in donors is to originate from an endemic region, and this cannot constitute selection criteria for obvious ethical and practical reasons. It would be unethical to consider this criterion for selection. Albeit the efficacy of donor risk-behavior selection is reflected by the significant lower prevalence of TTIs commonly reported among eligible donors compared with general populations, donor non-compliance may compromise transfusion safety and still needs to be minimized (22).

SERUM ALANINE AMINOTRANSFERASE LEVEL TESTING

Serum alanine aminotransferase (ALT) level testing was initially introduced in blood services as a surrogate marker for what was

then called "non-A non-B" hepatitis and was later identified as hepatitis C. Elevated ALT level in an asymptomatic donor may constitute an unspecific marker for a wide range of active and potentially transmissible viral hepatitis infections (i.e., HBV, HAV, HCV, and HEV) (23). Therefore, exclusion of donors with elevated ALT is still used in several middle- and low-income countries, particularly where alternative molecular screening remains not affordable due to cost and technical constraints. However, ALT elevation could be mainly caused by various heterogeneous life style factors that are not related to viral infections and do not constitute a direct threat to blood safety. Unnecessary deferral of donors with elevated ALT might exacerbate the problem of blood shortage as debated in Japan and China where the ALT exclusion threshold was raised to 60 and 50 IU/L, respectively, in an attempt to mitigate the problem (24, 25). Following the implementation of effective serological and NAT for HCV and HBV, most of Western countries discontinued ALT routine donor screening as it was reported to have no significant added value in preventing HBV or HCV TTI (26, 27).

HBsAg TESTING

HBsAg is the first serological marker to appear during the course of HBV infection and remains the first line of HBV screening in blood donors. However, HBsAg screening required an optimal analytical sensitivity to limit the so-called "window period" (WP) phase, commonly defined as the time between infection and detection of the viral antigen, and to enhance the ability to detect the smallest amount of HBsAg during the asymptomatic late stage of chronic infection. Since the first assay available in 1970, the sensitivity and specificity of HBsAg testing has been steadily improving with the development of enzyme immunoassays (EIAs) including enzyme-linked immunoabsorbent assays that use chemoluminescence and polyclonal antibodies. A comparative evaluation of 70 HBsAg assays (51 EIAs and 19 rapid tests) from around the world indicated sensitivities ranging between 0.013 and 1 IU/mL for 84% of the EIAs tested (28). The pre-HBsAg WP was estimated to 32.5 days when using assays with <0.13 IU/mL sensitivity (**Figure 1**). Recently, an

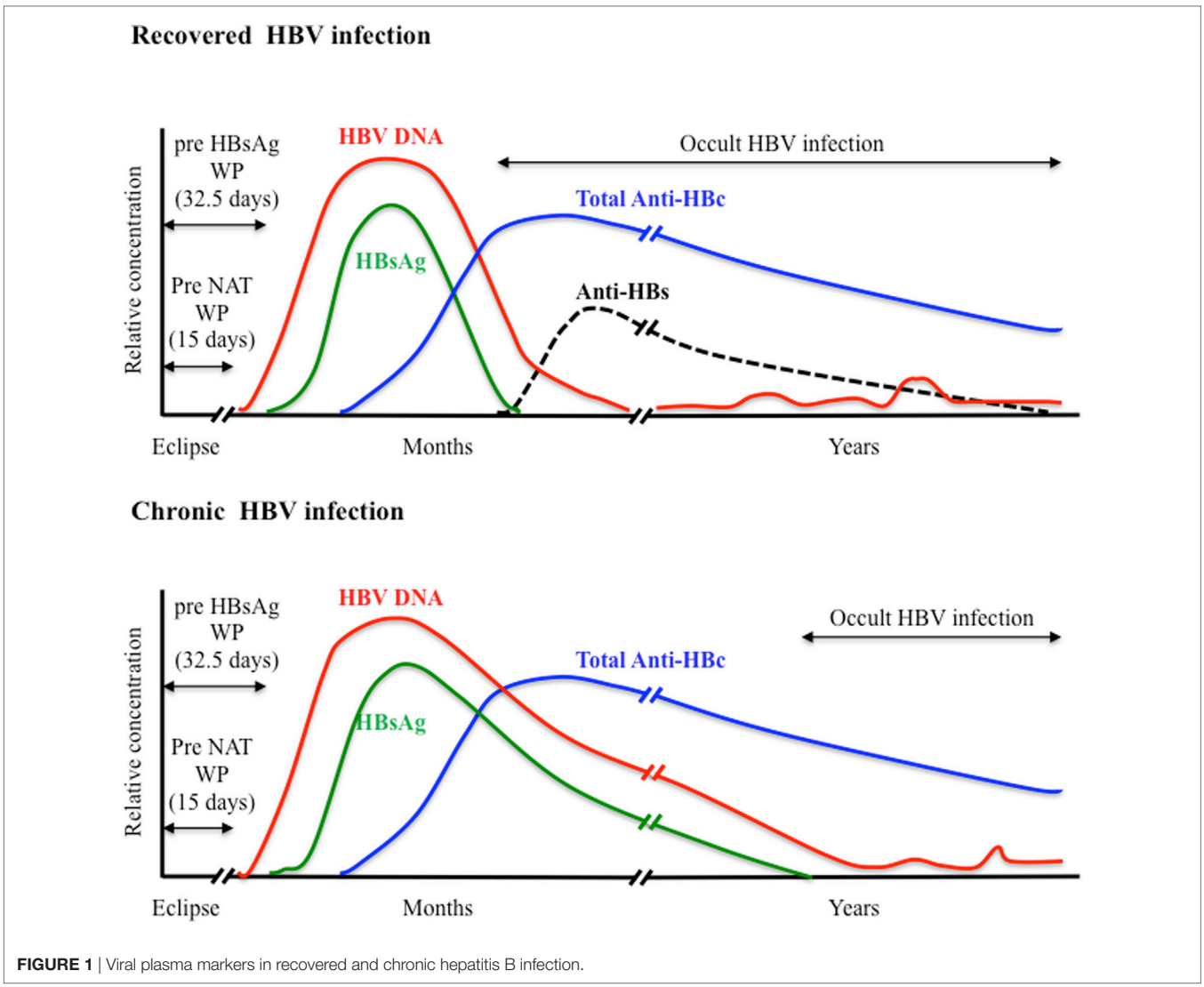

FIGURE 1 | Viral plasma markers in recovered and chronic hepatitis B infection.

enhanced HBsAg chemiluminescent EIA (HBsAg-HQ) and an ultra-high sensitive HBsAg assay employing a semi-automated immune complex transfer chemiluminescence enzyme technique (ICT-CLEIA) were developed that showed 5 and 0.5 mIU/mL sensitivities, respectively (29, 30). These highly sensitive assays were reported to detect HBsAg before HBV DNA in few cases and to possibly reduce the WP to ~14 days (30, 31). However, they were developed mainly to monitor HBV reactivation in treated patients, and their suitability regarding blood donor screening has not been evaluated so far.

Although HBsAg EIAs proved to be effective in blood donor screening, they have many limitations in endemic low/middle-income countries that include high cost, need for sophisticated equipment and trained technicians, continuous supply of electricity, and long turnaround times. Despite showing reduced sensitivity ranging between 1.5 and >4 IU/mL compared with EIAs, rapid tests offer the advantage of low cost and rapid delivery of results and may constitute the only available HBV screening alternative in some resource-limited regions (28, 32–34).

Aside from the WP, HBsAg screening may fail to identify donors infected with HBV variants (35). Mutations within and outside the immunodominant regions of the S protein have been functionally associated with HBsAg structural changes that may lead to impaired detection by the current immunoassays (36, 37). These mutations may arise from escaping the host immune response during infection, vaccine, or HBV immunoglobulin treatment (36, 38). Because of the overlap of P and S ORFs, drug-selected changes in the reverse transcriptase/polymerase may also influence HBsAg detection (39). Recently, chronic HBV infection with antigen levels below the detection threshold of HBsAg assays was increasingly identified in donors and was defined as occult HBV infection (OBI) (40). Studies suggested that undetected HBsAg levels might be associated with mutations in the surface promoter impairing S gene expression or to mutations in the S protein and deletions in the pre-S1/S2 region that reduced HBsAg production and secretion from infected hepatocytes (41–44). In addition, the impact of HBV genotypes on the efficiency of HBsAg detection remains unclear. Albeit the most sensitive and commonly used HBsAg assays showed similar sensitivity in detecting all genotypes, some others had impaired sensitivity for genotypes D–F (28, 45). To overcome the risk of HBsAg false-negative results related to HBV variants, monoclonal antibodies were replaced by polyclonal antibodies against both "wild-type" and variant viruses. HBsAg assays using multiple monoclonal antibodies for capture together with a polyclonal conjugate for detection appear to be the most efficient in detecting a wide range of HBsAg epitopes. Another cause of HBsAg detection failure may be the formation of immune complexes in the presence of HBV surface antibodies (anti-HBs) (46). Furthermore, few studies described unusual cases of acute asymptomatic infections in blood donors detected by HBV NAT that, in contrast to overt acute HBV infection, never showed detectable HBsAg despite seroconverting to anti-HBc overtime and therefore were so-called acute primary OBI (47).

Generally, blood donor samples that initially reacted on a primary screening are retested either in duplicate with the same assay or with an alternative immunoassay. Despite a ≥99.5% specificity level estimated for the majority of HBsAg assays, repeat reactive samples not confirmed by further testing may represent either biological false-reactive or true positive with indeterminate testing results raising issues for donor management and unnecessary loss of blood components (28, 48). An Australian study reported similar HBsAg false-reactive rates of 0.02 and 0.03% in first-time and repeat donors, respectively (49). The causes of HBsAg false reactivity remain unclear but there were reports that HBV vaccination could result in a transient antigenemia in vaccinees (50). False reactivity appeared to be specific for an assay, mostly transient with ~85% of these donors found consistently negative at subsequent donations, and partially associated with low sample-to-cutoff (s/co) ratios (51). This predictive value of s/co ratios should be considered with caution as the s/co ratio distributions for false-reactive and confirmed-positive HBsAg results showed some overlap. Therefore, it is advisable that donors initially testing HBsAg repeat reactive are subject to serologic confirmation using a second immunoassay and a neutralization assay.

ANTI-HBc TESTING

Anti-HBc antibodies usually appear 6–12 weeks after infection are considered non-protective and remain detectable lifelong in immunocompetent subjects constituting the most sensitive marker for exposure to HBV irrespective of the current infection state (**Figure 1**). Anti-HBc may be the only serological marker of HBV infection at the end of a resolving infection when anti-HBs decline to undetectable levels or in OBI where HBsAg may be undetectable and HBV DNA only intermittently detectable (52–55). Recently, increasing evidence of HBV transmission by anti-HBc-reactive donors who repeatedly tested HBsAg and HBV individual donation (ID)-NAT negative with the most sensitive assays available has been reported (56–59).

Since it was first introduced in the late 1980s as a surrogate marker for non-A non-B hepatitis, anti-HBc screening for blood donors remains controversial. It is generally admitted that deferring anti-HBc reactive units would too severely affect blood supply and at a non-affordable cost in medium- and high-endemic areas where anti-HBc prevalence in blood donors ranges between 8 and >50% (i.e., Mediterranean area, East Asia, and sub-Saharan Africa). By contrast, the donor loss caused by universal anti-HBc screening was considered sustainable in some medium/low-endemic countries including Canada, France, Germany, Ireland, the Netherlands, Lebanon, and USA (60, 61). To limit potential donor loss associated with a ~5% anti-HBc prevalence, Japan implemented a complex screening algorithm that includes anti-HBs testing of anti-HBc only donations (56). Donations anti-HBc-reactive only that contain anti-HBs levels >100–500 IU/L are considered eligible for apheresis plasma donation for fractionation while red blood cells and platelets are discarded, and donations with low anti-HBc and anti-HBs levels are rejected. Plasmas from recovered anti-HBc-reactive individuals containing high levels of anti-HBs (e.g., >8,000 IU/L in France) still are needed to supply human hepatitis B immunoglobulin (HIBG) essential to prevent infection in immunosuppressed transplant patients and newborns from HBV-infected mothers. Setting of

a minimum limit in anti-HBs titer (usually 500 IU/L) by plasma fractionators and/or national regulatory bodies and implementation of virus reduction procedures assure viral safety of products produced from anti-HBc positive plasmas (http://www.who.int/bloodproducts/publications/en/).

Blood products containing low levels of HBV DNA were found poorly infectious when transfused in the presence of anti-HBs (54). However, the protective level of anti-HBs remains a matter of debates as cases of HBV transfusion–transmission despite concomitant detectable anti-HBs were documented (56, 62, 63). Furthermore, the frequency of anti-HBs carriers among anti-HBc only donors may vary according to HBV epidemiology and vaccine coverage. Studies conducted in Europe, Japan, and North America reported that approximately 90% of anti-HBc-reactive donors carried also anti-HBs and 63–70% of them had titers >100 IU/L (56, 61, 64–66). By contrast, in Ghana, a country with high HBV endemicity, anti-HBs was detected in 24.5% of anti-HBc-reactive donors (67). Caution is required when comparing seroprevalences between studies due to differences in screening algorithms and methodology.

There are still limitations to anti-HBc screening even in low-endemic countries. Albeit recently improved, the specificity of anti-HBc testing is not optimal with reported false-reactivity rates of 16–75% according to assays and screening algorithms (60, 65, 66, 68–70). Recombinant/peptide antigen-based confirmatory assays being not available, secondary testing with an alternative EIA is needed to distinguish between true- and false-positivity and to confirm borderline reactive results that might be associated with low avidity or low titer of antibodies (65, 71). Additional testing for anti-HBs, anti-HBe, and/or HBeAg was considered to have confirmatory value for anti-HBc (56, 65, 66, 68). These complex confirmatory algorithms add economic and organizational constraints to blood services, but it is beneficial for the donor not to be permanently deferred due to false-positive outcome. Another limitation is that anti-HBc screening does not identify WP infections. In addition, simultaneous detection of HBV DNA and anti-HBs in the absence of detectable anti-HBc has been described. These cases were mostly associated with various degree of immunosuppression in patients, core regions deletion, and immunotolerance to HBc antigen in children born from HBeAg-positive mothers (45, 72, 73). However, rare anti-HBc negative/HBV DNA positive cases were also identified in immunocompetent blood donors irrespective of the presence of anti-HBs (53, 74, 75). The frequency of this unusual serological profile seems to vary according to the geographical origin of the donors and possibly vaccine coverage as it was detected in approximately 2 and 13% of OBI donors from Europe and Southeast Asia, respectively (53, 75).

HBV NAT

Nucleic acid testing for HBV DNA was introduced initially in Austria, Germany, and Japan in the late 1900s. After 2004, its implementation for routine blood donation screening was extended worldwide when high-throughput commercial multiplexed NAT assays that included HBV DNA detection in addition to HIV and HCV RNAs were developed and licensed (76). The fully automated commercial multiplex (HBV/HCV/HIV) NAT assays mainly used in transfusion laboratories are the PCR-based cobas TaqScreen MPX version 1 or 2 assays (Roche Diagnostics), and the Procleix Ultrio or Ultrio Plus/Elite assays (Grifols Ltd.) that employ transcription-mediated amplification. The most recent cobas TaqScreen MPX v2 and Procleix Ultrio Plus assays showed specificity of 99.9% and similar 95% limit of detection (LOD) of 2–4 IU/mL for HBV DNA when applied to ID testing (77, 78). This high sensitivity allowed HBV NAT to reduce significantly the WP left by HBsAg testing to an estimated eclipse phase of ~15 days following infection (**Figure 1**) (79). In addition, HBV NAT uncovered a relatively large number of HBsAg-negative occult HBV infection (OBI) among blood donors who tested anti-HBc and/or anti-HBs positive (40). The majority of OBI donors are characterized by a viral load <50 IU/mL and, in some cases, the presence of a high amino acid variability within the S protein that might impair recognition by HBsAg assays (37, 53, 80). The sensitivity of HBV NAT not only depends on the efficiency of the amplification and detections methods used but also on the input plasma volume and the efficiency of the nucleic acid extraction (81). Moreover, the NAT analytical sensitivity may vary considerably between HBV genotypes and between strains of the same genotype, especially genotype D that is the most polymorphic types of HBV (82).

HBV NAT implementation may be limited by the considerable cost of high-throughput fully automated commercial platforms and reagents, especially in low- or medium-income countries of Africa, Asia, and South America. In high-income countries with usually low HBV prevalence, the clinical risk reduction benefit of NAT was associated with an extremely low cost-effectiveness (83). In addition to multiplexing, testing for viral genomes in plasma pools of various sizes was implemented to reduce the cost of NAT. However, there has been a constant progression toward screening smaller pools of six to eight plasmas and to ID. Indeed, the dilution factor introduced by the pooling process reduces the sensitivity of HBV NAT and its ability to detect the low levels of HBV DNA observed in the majority of OBI donors (9, 76, 78). Nevertheless, even ID-NAT may not be sensitive enough to detect potentially infectious blood products with extremely low levels of HBV DNA (56–58).

Discrepancies between serological and molecular testing and the increasing sensitivity of NAT assays make difficult to distinguish between true- and false-positive HBV DNA results. While the commercial multiplex cobas TaqScreen MPX v2 assay allows the simultaneous detection and direct identification of HBV, HCV, and HIV by using virus-specific probes labeled with different dyes, the cobas TaqScreen MPX v1 and Procleix Ultrio Plus assays indicate the presence of viral genomes with a single consensual signal that does not discriminate between these viruses. Therefore, three additional separate virus-specific discriminatory NAT assays are necessary to identify the virus in the originally reactive sample. Discriminatory assays do not fully qualify for confirmation since they are using the same technology and reagents as the initial screening assay. Furthermore, 0.09–0.29% of tested donations reactive in the initial multiplex assay might be non-reactive in the discriminatory assays and/or in the multiplex assay when repeated and were designed non-repeat-reactive

(NRR) (55, 84–87). The reasons of these discrepancies remained unclear but probably reflect Poisson distribution statistics of HBV DNA levels around the assay's LOD, especially in OBI donors, since multiplex and discriminatory assays showed no significant difference in sensitivity according to manufacturers (81, 85). Therefore, ID-NAT screened NRR donations are not released for transfusion in most countries, but donors may remain eligible to donate again as false-positive results cannot be totally excluded.

In the absence of serological investigations or detectable serological markers (i.e., WP), false-positive NAT results due to cross-contamination may be ruled out by retesting a clean sample from the initial plasma bag and by donor follow-up. However, caution should apply when considering the intermittently detectable HBV DNA levels observed in some OBI donors over time (53). NRR donations might be tested for anti-HBc to identify occult HBV carriers (88). NRR donations were reported more frequently reactive for anti-HBc than HBV DNA-negative donations (57 versus 7%, respectively) (85). However, this is not applicable in high-endemic countries such as China that showed an anti-HBc detection rate of 48% in HBV DNA non-reactive donations implicated in reactive minipools of 6 and 68% in ID-NAT NRR donations (86, 87). Alternatively, most ID-NAT users have adopted a serology-like algorithm to discriminate true from false initial reactive results. Multiple repeat tests are performed to identify NRR donations with low viral load using either the multiplex assay or a second independent commercial or in-house assay preferentially targeting a different region of the viral genome. This approach has its drawbacks as it is costly and NAT assays show different levels of sensitivity. Even the most sensitive assays may fail to detect extremely low levels of HBV DNA consistently (81). NAT sensitivity can be enhanced by several non-exclusive changes in the standard procedures aiming to increase the number of HBV DNA templates in the amplification reaction. This can be achieved by purifying viral DNA from larger volumes of plasma and/or by concentrating viral particles with high-speed centrifugation (84). Nevertheless, these approaches are not suitable for large-scale blood donation screening.

HBV SCREENING STRATEGY: ARE ALL VIRAL MARKERS OF VALUE?

Blood donation screening for multiple HBV markers showed discrepant results. The frequency of these discrepancies is difficult to evaluate as they largely depend on the performance of the assays used. Nevertheless, a recent large-scale multiregional study using a comparable HBV screening algorithm showed that among 9,455 confirmed HBV-infected donors, 84.8% were consistently reactive for the three markers, 5.9% were anti-HBc and HBV ID-NAT reactive (OBI), and 2.65% were HBV DNA reactive only [WP (2.25%), primary OBI (0.13%), and anti-HBs only OBI (2.27%)] (89). In addition, 6.45% of donors were HBsAg and anti-HBc reactive but ID-NAT non-reactive. Previous studies reported absence of detectable HBV DNA in 2–20% of HBsAg reactive/anti-HBc reactive donors depending on the LOD of the molecular assays used (67, 76, 78, 82, 90). No confirmed HBV-infected donation testing HBsAg only has been identified so far.

In low-endemic affluent countries, the implementation of both HBsAg, anti-HBc, and HBV NAT provides the optimal level of blood safety by allowing detection of both the early phase of acute infection, persistent occult infection with potential transient detectable viremia, and genetic and/or antigenic viral variants. In addition, ID-NAT should be preferred, as it appeared more efficient in reducing the transmission risk by both WP and occult infections compared with MP-NAT (91). A residual risk would be left by the remaining early infection eclipse phase before HBV DNA becomes detectable. However, questions regarding the apparent redundancy of testing, especially for the two direct markers HBsAg and HBV DNA, prompted debates on how to reduce the cost of HBV blood screening without compromising blood safety. Accumulating data suggests that the apparently efficient combination of NAT and anti-HBc to detect both WP donations and low viremic chronic carriers precludes the need for HBsAg testing. There is increasing evidence that anti-HBc screening, if applied, would have interdicted infectious donations containing extremely low HBV DNA level undetectable with the most sensitive NAT (56–58). Despite being recommended by WHO and included in the European directive, the question of maintaining HBsAg testing might be raised but the absence of potential negative impact on blood safety needs to be assessed before considering discontinuing HBsAg. Therefore, the infectivity of such donations needs to be investigated. However, HBV infectivity studies are limited by the lack of physiologically reliable *in vitro* cell culture and susceptible animal models that generally require high doses of virus for infection (92). An alternative approach might be to isolate and amplify the viral genome present in HBsAg positive/HBV DNA negative donations and to use it in *in vitro* transfection experiments to study the virus replicative properties as a surrogate of infectivity. Dropping a screening test is highly challenging because it is politically sensitive and must not be perceived by the public as exposing recipients to higher risk. Solid scientific evidence about absence or minimal acceptable infectious risk should be provided to regulatory agencies and decision-makers who have the final decision.

In moderate- and high-endemic countries, anti-HBc testing cannot be implemented without compromising blood availability. Therefore, HBsAg testing in combination with NAT would be preferable when resource is available. Highly sensitive ID-NAT only might be considered, as it appears more efficient in detecting HBV chronic carriers than even enhanced sensitivity HBsAg assays. However, the existence of HBsAg reactive/HBV DNA non-reactive donations comforts maintaining HBsAg screening. In high-endemic countries with limited resource, HBV blood safety still relies essentially on HBsAg testing with inexpensive rapid tests as mentioned earlier. Pre-donation viral screening of blood donors using such rapid tests was shown effective and cost-effective, particularly in high-endemic areas (i.e., sub-Saharan Africa and China) where their use reduced wastage of collecting infected blood (93, 94). Additional testing of rapid test-negative donations with a different and more sensitive serological assay and/or expensive NAT still is needed to ensure an acceptable level of safety. The cost limitation of NAT may be addressed by developing in-house multiplex assays and/or by adapting assays using less expensive technologies that have been recently developed

for monitoring viral infection at the point-of-care (93, 95, 96). In addition, quality assurance (QA) issues may persist in some resource-limited settings even with relatively simple serological assays such as HBsAg EIAs (33). Possible implementation of sophisticated but non-standardized in-house NAT assays may be prone to even bigger QA problems. Cheaper and well-validated commercial NAT assays may still be preferable to avoid false sense of biosecurity. However, the suitability of these new molecular methods for high-throughput blood screening remains to be evaluated. Discussions on the cost of NAT implementation must also take into account the multiplex format of the currently available systems that include HCV and HIV testing.

Decisions on screening strategy face the dilemma between cost-effectiveness and clinical benefit in terms of HBV TTI risk reduction. The HBV residual transmission risk depends essentially on the infectivity of the blood products from undetected HBV-infected donations. The minimum 50% infectious dose by transfusion was estimated between 20 and 200 IU (100–1,000 virions) in the absence of anti-HBs antibodies (54, 58). The HBV residual TTI risk may also vary according to the donation testing algorithms, the sensitivity of the serological and NAT assays used, and the HBV epidemiology. A recently developed mathematical model estimated this residual risk based on the probability distribution of the HBV DNA load in randomly selected OBI donors, the probability that a given DNA load remains undetected by NAT, and the probability that this DNA load causes infection in the recipient (4). According to this model, 3 and 14% of ID-NAT undetected OBI donations might cause infection by red blood cell concentrates and fresh-frozen plasmas, respectively. Another model based on lookback data reported similar 2–3% residual estimates of OBI transmission (58). When HBsAg and anti-HBc serology in combination with ID-NAT are used, the residual risk may be associated essentially with the remaining DNA-negative eclipse phase in early acute infection and the rare cases of anti-HBs only OBI with intermittent detectable DNA, albeit the infectivity of corresponding blood products is still unknown (75, 79).

Pathogen reduction technologies (PRTs) might represent an attractive strategy. Although PRTs are currently used to complement current testing, there are still limitations to overcome before considering it as a full alternative to testing. Indeed, PRTs were reported not 100% effective against infectious agents present in high loads (97). Efficacy of 2 to >5 log reduction in HBV infectivity has been reported using different PRTs (98). Therefore, the HBV infectious risk may be diminished but not eliminated since HBV viral loads ranging between undetectable to $>10^9$ IU/mL are observed in blood donors (99, 100). In developed countries, PRTs are applied currently to fresh-frozen plasmas and platelet concentrates but remain unavailable for red cell concentrates. Some controversies also persist regarding their impact on the functional aspects of the treated components, albeit the clinical efficacy of treated products is generally satisfactory [see Ref. (98) for review]. Recently, the ability of pathogen reduction of whole blood to provide safer products at an affordable cost for low- and middle-income countries while retaining the ability to prepare functional components was raised (98, 101). The benefits of PRTs might be amplified in low-resource and high-risk countries due to the efficacy against different types of local bloodborne pathogens, including major TTIs (e.g., HBV, HCV, and HIV) and others widely endemic but yet unaddressed (e.g., malaria and bacterial infections). Few reports demonstrated that implementation of PRTs in resource-limited settings was feasible (98, 101). More studies are needed to assess the practical sustainability in terms of infrastructures, supplies, and cost-utility of PRTs implementation in settings where serology and NAT are already limited.

Finally, effective HBV vaccines have been available since the early 1980s, and vaccination has led to a 70–90% decrease in chronic HBV carrier rates in the countries where it has been implemented (102). Therefore, the extension of HBV vaccine coverage in both donor and recipient populations has the potential to reduce significantly the residual risk of HBV transfusion–transmission. However, 5–10% of healthy vaccinees failed to mount an adequate antibody response, vaccination alone failed to protect 10–30% of newborns from HBsAg/HBeAg-positive mothers, and occult HBV infection was frequently reported in individuals with protective anti-HBs levels. Suboptimal protection might be due to heterologous HBsAg (sub)genotypes or to the decline of anti-HBs level over time in vaccinees (63, 102). Nevertheless, a recently developed new generation of recombinant HBV vaccines that contain correctly folded HBsAg and additional neutralizing epitopes of the preS antigens was shown to be highly immunogenic, inducing faster and higher seroprotection rates against HBV compared with conventional vaccines. With optimal vaccines and vaccination coverage, eradication of HBV might be possible but that is another story.

AUTHOR CONTRIBUTIONS

DC and SL contributed equally to the conception and writing of the work and approved it for publication.

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MINI REVIEW
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Pathogen Inactivation of Cellular Blood Products—An Additional Safety Layer in Transfusion Medicine

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In line with current microbial risk reduction efforts, pathogen inactivation (PI) technologies for blood components promise to reduce the residual risk of known and emerging infectious agents. The implementation of PI of labile blood components is slowly but steadily increasing. This review discusses the relevance of PI for the field of transfusion medicine and describes the available and emerging PI technologies that can be used to treat cellular blood products such as platelet and red blood cell units. In collaboration with the French medical device manufacturer Macopharma, the German Red Cross Blood Services developed a new UVC light-based PI method for platelet units, which is currently being investigated in clinical trials.

Keywords: transfusion, platelets, pathogen inactivation, ultraviolet light, red blood cells

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INTRODUCTION

From the late 1970s to the mid-1980s, contaminated hemophilia blood products were a serious public health problem, resulting in the infection of large numbers of hemophiliacs with the human immunodeficiency virus (HIV). If safety measures had been implemented in a timely and consistent manner after identification of the acquired immune deficiency syndrome (AIDS) epidemic in 1981 and isolation of the HIV in 1983, the transmission of HIV infection by these blood products could have been prevented in most cases. This contaminated blood scandal made the community aware that new pathogens may emerge and threaten blood safety at any time. However, there was a significant delay in the introduction of HIV detection systems in some countries and in some cases, the detection tests that were implemented proved to be unreliable. In addition, the plasma products used for therapy were not even treated by heat inactivation—a pathogen inactivation (PI) method that was readily available and approved at that time. Consequently, blood and blood components became subject to drug law in some countries (1, 2).

Increasingly stringent donor eligibility criteria and more sensitive virus detection methods have reduced the risk of transfusion-transmitted infection (TTI) by blood products significantly, but a residual risk of TTI with viruses, bacteria, protozoa, and prions remains. False-negative test results due to test failures, very low-pathogen concentrations in the peripheral blood or escaped mutants can result in TTI in spite of negative screening tests (e.g., for *Treponema pallidum*, hepatitis B, hepatitis C, and HIV). In addition, transfusion recipients may be infected by pathogens not targeted in regular blood donor screening programs (e.g., hepatitis A and bacteria). Transfusion safety is particularly susceptible to pathogens that enter regions in which they are not yet endemic. The fact that viruses that are usually endemic in tropical regions have recently caused outbreaks in Western countries demonstrates that these pathogens can arise and threaten transfusion safety at any time (3, 4).

Blood safety is still mainly based on the reactive principle of introducing new test systems or new donor election criteria after a threat to transfusion recipients has been identified. In other words, infections by contaminated blood products must first occur before appropriate counter-measures are established. At the beginning of the last decade, a number of cases of West Nile virus occurred in the USA through the transmission of blood components before the first detection system for donor testing was implemented (3). The recent Zika virus outbreak on the American continent has heightened concerns over this reactive approach to blood supply safety (5, 6).

During an international consensus conference, transfusion experts and other stakeholders in the field of transfusion medicine recommended a change from the hitherto reactive strategy toward a proactive, preventive approach to blood safety (7). Recently, developed and approved PI technologies for cellular blood products, such as red blood cell (RBC) and platelet units, are considered key measures for closing or at least reducing the safety gap caused by emerging pathogens. While virus reduction procedures are an integral part of the process of manufacturing plasma derivatives from plasma pools, and although the methylene blue system has been used for PI of single donor plasma units for nearly two decades (8), a new generation of PI methods for platelet units have recently become available (9, 10). PI technologies for the treatment of RBC units are still in development and have not received market authorization yet.

TECHNOLOGIES

The use of PI technologies for blood products has a number of advantages. Because they inactivate most clinically relevant viruses, bacteria, and protozoa, they can help to eliminate the residual risk of infection during the "window period" when transfusion-relevant pathogens (e.g., HIV) cannot be detected by donor screening tests. Their broad activity against pathogens also helps to reduce the risk of recognizable infectious agents (e.g., bacteria), which still cannot be prevented completely. In contrast to screening tests for transfusion-borne pathogens, PI proactively protects against emerging infectious agents entering the blood supply in a given community.

All PI methods used to treat cellular blood products work by impairing the target pathogen's ability to replicate. When used alone or in combination, ultraviolet (UV) light and alkylating agents cause irreversible damage to the nucleic acids of pathogens. Therefore, they effectively eliminate classical pathogens such as viruses, bacteria, fungi, and protozoa, but are ineffective against prions. The latter protein-based pathogenic agents can cause sporadic and variant Creutzfeldt–Jakob disease in humans.

The following PI technologies for cellular products are currently available or in the pipeline.

INTERCEPT Blood System for Platelets and Plasma

The INTERCEPT Blood System for platelets and plasma is manufactured by Cerus Corporation (Concord, CA, USA). The mechanism of action of this PI technology is based on the properties of amotosalen HCl (S-59), a photoactive compound which penetrates cellular and nuclear membranes and binds to the double-stranded regions of DNA and RNA. When activated by low-energy UVA light (320–400 nm), amatosalen cross-links nucleic acids and thus irreversibly blocks the replication of DNA and RNA (11). After illumination, residual amotosalen and its photoproducts must be removed during an incubation step lasting up to 16 h. The amatosalen/UVA procedure is not suitable for RBCs because of UVA light absorption by hemoglobin.

MIRASOL PRT System for Platelets and Plasma

The MIRASOL system was developed by TerumoBCT (Lakewood, CO, USA). This photodynamic procedure employs riboflavin (vitamin B2) and broad spectrum UV light (mainly UVA und UVB, 285–365 nm). On exposure to UVA and UVB light, riboflavin associates with nucleic acids and mediates oxygen-independent electron transfer, causing irreversible damage to the nucleic acids (12). Because naturally occurring vitamin B2 and its photodegradation products are non-toxic and non-mutagenic, they do not need to be removed prior to transfusion. In addition to plasma and platelets, protocols for extension of the MIRASOL system to whole blood are now in development.

THERAFLEX System for Platelets

THERAFLEX UV-Platelets is a novel UVC-based PI technology that works without photoactive substances. It is the product of a joint venture between Macopharma (Mouvaux, France) and the German Red Cross Blood Service NSTOB in Springe, Germany. Shortwave UVC light (254 nm) directly interacts with nucleic acids to form pyrimidine dimers that block the elongation of nucleic acid transcripts (13). UVC irradiation mainly affects the nucleic acids of pathogens and leukocytes and does not impair plasma and platelet quality. As no photoactive substances are involved, UVC treatment is just as simple but faster (takes less than 1 min) than gamma irradiation, and can easily be integrated into the manufacturing processes at blood banks (**Figure 1**). The THERAFLEX system was originally developed for platelets but is also suitable for plasma and RBC units.

S-303 PI System for RBCs

The S-303 PI system (INTERCEPT RBC system, Cerus Corporation, Concord, CA, USA) was specifically developed for RBC units. S-303 is a modular compound that prevents nucleic acid replication by targeting and cross-linking nucleic acids. Once added to the RBC unit, this amphipathic compound rapidly passes through cell and viral envelope membranes and intercalates into the helical regions of nucleic acids. S-300, the non-reactive byproduct of this reaction, is subsequently removed by incubation and centrifugation, which can take up to 20 h (14). In contrast to the other PI technologies described here, the S-303 system does not require UV light. However, glutathione (GSH), a naturally occurring antioxidant, must be used to prevent non-specific reactions between S-303 and other nucleophiles present in the RBC unit. These may include small

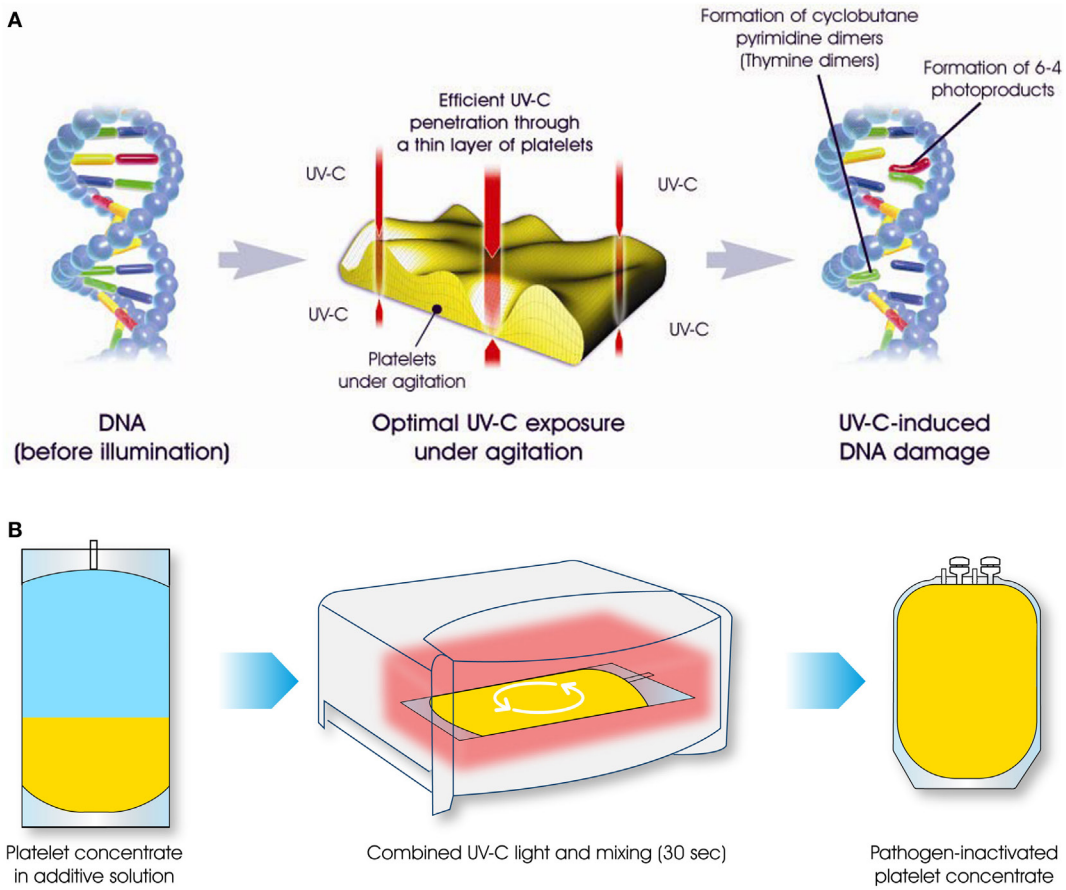

FIGURE 1 | The THERAFLEX ultraviolet (UV)-Platelets pathogen inactivation system uses UVC light to induce irreversible damage to the nucleic acids of viruses, bacteria, fungi, protozoa, and leukocytes. Intense agitation of the platelet bag during UVC illumination results in efficient mixing, ensuring the uniform treatment of all blood compartments **(A)**. For the illumination step of this simple and rapid procedure, platelet units are placed in the irradiation device for a period of less than 1 min **(B)**. Afterward, the pathogen-reduced platelet product can be used for transfusion.

TABLE 1 | Pathogen inactivation technologies.

	Technology			
	INTERCEPT blood system	**MIRASOL PRT system**	**THERAFLEX UV-Platelets**	**S-303 system**
Mechanism of action	UVA plus amotosalen (alkylating agent)	UV plus riboflavin (vitamin B2 = photosensitizer)	UVC alone	Alkylating agent
Blood products	Plasma and platelets	Plasma and platelets (in development for whole blood)	Plasma and platelets (in development for RBCs)	RBCs
Status	Approved in some countries	Approved in some countries	In clinical development	In clinical development

UV, ultraviolet light; UVA, wavelength A; UVC, wavelength C; RBC, red blood cell.

molecules, such as phosphate and water, and macromolecules, such as proteins.

The INTERCEPT and MIRASOL systems for platelets and plasma have already been approved in the USA and some European and Asian countries, while both the THERAFLEX system and the S-303 system are still in clinical development. The UVC-based THERAFLEX system is expected to receive marketing authorization within the next few years (**Table 1**).

CLINICAL STUDIES

Platelets

Clinical studies show that platelets retain their hemostatic efficacy after PI treatment. Following prophylactic transfusion, there was no difference in the ability of pathogen-reduced and untreated platelet units to prevent severe bleeding (15). However, almost all clinical trials demonstrated that post-transfusion survival

and recovery rates were consistently lower in patients receiving platelets treated with PI technology than in those transfused with untreated platelets (16–19). Accordingly, the transfusion of pathogen-reduced platelets resulted in lower platelet count increments (CIs), lower corrected count increments, shorter intervals between platelet transfusions, and a higher number of platelet transfusions per patient. However, observational studies showed no evidence of increased product consumption rates when pathogen-reduced platelet units were used in a routine setting (20).

Interestingly, the rate of acute transfusion reactions may be lower after the transfusion of pathogen-reduced versus untreated platelets. However, there have been concerns over acute respiratory distress associated with amatosalen/UVA-treated platelets (15). While the results of animal studies suggest that UV light-treated platelets mediated a higher risk of pulmonary toxicity (21), an analysis of clinical data by an expert panel does not confirm significant differences in the rates of acute lung disorders between PI-treated and untreated platelets (22). The results of ongoing large-scale phase III and hemovigilance studies will help to further clarify open questions with respect to therapeutic efficacy and potential side effects of pathogen-reduced platelets (23).

Red Blood Cells

The S-303 system, which is in clinical development, is the only PI technology available for RBCs. Current studies are investigating the second-generation S-303 PI process. The first-generation S-303 procedure only marginally affected RBC quality and function, but after reports of immunization against pathogen-inactivated RBCs in transfused patients emerged, a new generation of the S-303 system had to be developed. In the second-generation S-303 system, the quencher concentration of GSH was increased from 2 to 20 mmol/l in order to decrease the affinity of S-303 for proteins and thus to avoid the formation of neoantigens on the surface of erythrocytes (24). However, recent studies show that immunization against S-303-coated RBCs still occurs after modification of the S-303 system (25). In particular, the fact that antibodies against S-303-treated cells were also detected in healthy blood donors who had never been transfused with pathogen-reduced RBCs suggests that some individuals may be immunized by S-303-like substances in the environment (e.g., food or air) or may have naturally occurring antibodies against epitopes on the S-303 molecule. These data clearly show that the use of chemical agents for PI of cellular products increases the risk of immune responses against blood components in transfusion recipients. Various phase III clinical trials to test the second-generation S-303 PI system for RBCs in acute and chronic anemia patients are currently ongoing or planned.

IMPLEMENTATION IN ROUTINE USE

The INTERCEPT and MIRASOL PI systems for platelets and plasma are used in some parts of Asia, Europe, and the USA. In Europe, the willingness to use pathogen-reduced platelet units varies between countries and regions. PI technologies are implemented nationwide in some countries (e.g., Switzerland and Belgium), but only regionally in others (e.g., Poland). Evaluation of PI technologies for platelets is under way at some blood centers in Germany. In 2011, the Swiss national authority (Swissmedic) ordered the nationwide implementation of PI of platelet units. This measure was mainly aimed at preventing or at least minimizing the risk of fatal transfusion reactions caused by bacterially contaminated platelet units. Analysis of Swiss hemovigilance data revealed that without PI, one fatal case of transfusion-transmitted sepsis by contaminated platelet units would occur in Switzerland every 2 years. The US Food and Drug Administration (FDA) recently recommended the use of approved PI technologies as an alternative to bacterial detection methods in order to adequately control the risk of bacterial contamination of platelets (26, 27).

The preventive potential of PI of cellular blood components first became apparent during a chikungunya virus epidemic on the French island of La Reunion in the Indian Ocean in 2006 (28). Because more than 30% of the inhabitants were infected, local blood donation was suspended to prevent TTI. To sustain the availability of platelet components, the French national blood service (Etablissement Français du Sang) implemented universal PI of platelet components on the island. The success of this measure demonstrated that PI can effectively support the availability of safe labile blood components during an epidemic.

The West Nile virus epidemic in the USA was the first example of a large-scale arboviral threat to the blood supply of a Western country that required an urgent response across government agencies and non-governmental organizations. The dramatic spread of Zika virus in the Americas since 2015 has generated a sense of public health urgency akin to AIDS, along with immediate concerns over blood safety. In areas of active transmission, "FDA guidance recommends that blood be outsourced from unaffected areas, unless there are measures to screen donations using a laboratory test, or unless the blood components are subjected to PI technology" with an approved method (29). The INTERCEPT system was approved by the FDA in 2014 and has already been implemented at a number of US blood centers.

OUTLOOK

Despite the increasing and profound safety and efficacy record of pathogen-reduced blood cellular products, there are still concerns that impede the introduction of PI technology in hemotherapy. The INTERCEPT protocol includes incubation and adsorption steps that result in a significant loss of platelets (up to 15%) during preparation and PI treatment. However, this loss could be offset by performing PI with higher platelet counts in the starting products. The platelet yields could be increased by using more buffy coats (e.g., five instead of four) to manufacture pooled platelets, or by collecting higher numbers of platelets during the apheresis process. Moreover, this measure could compensate for reduced platelet CIs in transfusion recipients and thus lower the possible need for increased platelet unit utilization.

All PI technologies mentioned in this review exhibit gaps in their PI efficacy. The amatosalen/UVA-based system (INTERCEPT) is ineffective for non-enveloped viruses such as hepatitis A, hepatitis E, and parvovirus B19 (30). The riboflavin/UV-based system (MIRASOL) has only weakly effects against

bacteria and some viruses (31). The UVC light-based system (THERAFLEX) is highly effective against bacteria and most transfusion-relevant viruses, but only moderately effective against HIV (32). However, when highly sensitive screening tests for HIV are performed, UVC-based PI could further reduce the risk of virus transmission during the "window period" in which the pre-nucleic acid testing can be negative and in patients with occult infections. Despite these weaknesses, PI systems generally have the potential to significantly add an additional layer of safety to blood transfusion.

Major concerns surrounding the implementation of PI have to do with its impact on the integrity of blood components and the toxicity of the chemicals used in these systems. In particular, acute and chronic toxicities may be caused by PI technologies that use active chemicals. Although only small quantities of photochemical compounds are used in PI technologies and they appear to provide sufficient safety margins, it cannot be excluded that alkylating agents such as amatosalen may be carcinogenic in the long term in a subset of transfused patients. A major advantage of the THERAFLEX system is that it works without photoactive substances, thus eliminating the risk of photoreagent-related adverse events (10, 13).

According to various stakeholders in the field of transfusion medicine, it is crucial to inactivate pathogens in all blood components in order to increase the safety margin of the entire blood supply. As long as PI is not routinely implemented in the production of RBC units (the most commonly used blood components), PI cannot achieve its full potential to enhance blood safety. Experts and health authorities are increasingly recommending the implementation of PI systems for platelets and plasma as an important step toward improving blood safety. A Canadian risk-benefit analysis suggests that if a new pathogen entered the blood supply, the use of pathogen-reduced plasma and platelets would reduce the risk of TTI by 40% (33).

The additional costs of PI implementation may be responsible for the hesitant acceptance of this technology by hospitals and funding agencies. Although based on assumptions and simplifications, the available cost-effectiveness analyses suggest that PI implementation, like other measures for the improvement of blood safety, has an acceptable cost–benefit ratio in this specific application (34, 35). The potential cost savings from PI implementation could offset some costs associated with the technology (e.g., production costs); however, the amount of potential offsetting cost reductions may vary considerably between different countries and regions and must be evaluated on an individual basis for blood centers and hospitals (36). Finally, the available resources influence how politicians and health authorities decide on how to meet public concerns for safety in transfusion medicine. If emerging evidence continues to demonstrate the efficacy of PI, it will be difficult to explain to individuals with severe transfusion-associated infections why this readily available risk mitigation and safety measure was not implemented.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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PERSPECTIVE
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Prevention of Transfusion-Transmitted Infections: Dilemmas

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To make blood components and blood products safe, many safety measures are applied to avoid transfusion-transmitted infections. Defining a balanced safety policy is not easy, we face several dilemmas: How safe should blood be? Should we opt for maximal or optimal safety? Are perceived threats real and relevant? Should blood be clean while food, air, or mosquitoes are not? Is vCJD still a threat? It seems wise to discuss these issues more in the open.

Keywords: blood safety, donor screening, preventive measures, precautionary principle, transfusion-transmitted infection, blood transfusion

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In developed countries, a variety of safety measures ensures a low risk of transfusion-transmitted infections. These safety measures include donor selection (limiting imported and window infections); skin disinfection and diversion bags (limiting bacterial contamination during blood donation); the screening of donations (enabling timely detection of HBV, HCV, HIV, and *Treponema pallidum*); specific processing (such as leukodepletion, pathogen reduction, and inactivation, which remove or kill certain pathogens); quarantine of plasma (preventing window infections); bacterial culturing (detecting contaminated platelets); and post-donation and post-transfusion notification (respectively, enabling donor- and recipient-triggered look-back procedures, which identify infected recipients). This impressive preventive machinery does not mean that the problem of transfusion-transmitted infections has been solved. New infectious threats arise or are being recognized. Threats may arise locally, such as hepatitis E virus genotype 3 in parts of Europe and Babesia in parts of the USA; or abroad, causing temporary deferral of traveling donors, returning from an ever changing set of affected areas. This dynamic situation necessitates ongoing monitoring, and discussion of safety measures that could be introduced.

Ongoing discussion on another level concerns the notion that existing safety measures may be discriminatory (e.g., asking men-who-have-sex-with-men not to donate) or cost-ineffective (e.g., screening for HTLV after implementation of leukodepletion; or screening for HBsAg after implementation of screening for HBV-DNA and anti-core antibodies). At the same time an entirely new, additional layer of safety is becoming available: pathogen reduction based on photo-chemical treatment of plasma, platelets, or whole blood. Should this technique be implemented on top of existing safety measures, or should pathogen reduction replace (some) existing safety measures? In either scenario: what would be the cost per prevented transmission? Considering the complex mixture of old and new infectious threats, and of old and new safety measures, it is hard to determine at which stage the blood supply is safe enough. Discussing the safety of the blood supply, several dilemmas can be recognized.

HOW SAFE SHOULD BLOOD BE?

Sometimes clear-cut economic guidelines are applied regarding preventive measures in public health. For example, recently universal vaccination of Dutch children against rotavirus was considered cost-ineffective as long as costs per quality adjusted life year (QALY) gained are more than € 20,000 (1). At the same time, in Dutch blood banking very high costs per QALY occur for HCV and HIV PCR

donor screening, which since their start, respectively, in 1999 and 2000, only detected one HCV window infection and no cases of HIV window infection (2). Without thinking, new safety measures may automatically be stacked upon existing safety measures. It must be realized that spending money in blood banking on cost-ineffective preventive measures may be unethical, because the money involved could safe more lives elsewhere in health care. (This line of reasoning must be applied carefully because, for example, any money spent in rich countries would safe more lives when spent in very poor countries.) Advanced pathogen reduction techniques are becoming available. Before these techniques are introduced, it must be analyzed whether and which existing safety procedures can be abolished (3). In addition, the costs of one QALY, gained by pathogen reduction, must be estimated and evaluated using local parameters. Unless these prerequisites are met, it seems wise to withstand the pressure from companies to introduce their pathogen reduction techniques "because they are available." Complicating an estimation of the cost/benefits ratio is the possibility that pathogen inactivation procedures could harm the functionality of the therapeutic cells or proteins involved.

MAXIMUM OR OPTIMAL BLOOD SAFETY?

Among the dilemmas surrounding the prevention of transfusion-transmitted infections, the dilemma whether one can accept a certain residual risk (optimal safety), or whether one should cover each threat (maximum safety), is an easy one. Maximum safety does not exist. Even if all available safety tests and procedures for all agents would have been implemented, one could perform each screening test not once, but twice on each donation, thus increasing the sensitivity of detection. Input volumes for PCR extractions and reactions could be increased, etcetera. Hence, implicitly we always implement a reasonable set of safety measures (optimal safety), not a maximum set of safety measures.

IS THE THREAT A TRANSFUSION-TRANSMITTED THREAT?

Transfusion-transmitted West Nile virus (WNV) infection can cause serious disease in affected recipients (4). WNV and other arboviruses are on the rise. The Americas were, respectively, invaded by WNV in 1999, by chikungunyavirus in 2013, and by zikavirus in 2015. Worldwide, different serotypes of dengue virus spread into each others areas. Secondary outbreaks of chikungunya and dengue occur in Mediterranean Europe. Recently, in Germany and the Netherlands widespread death occurred in blackbirds and owls, caused by usutuvirus, another arbovirus. Subsequently, usutuvirus viremia has been detected in a German blood donor and in Austrian blood donors. Considering that disease is caused both by mosquito-borne and by transfusion-transmitted WNV, one may assume that all arboviruses cause "their" diseases, whether transmitted by mosquito or *via* transfusion. This assumption may be wrong. The apparent lack of significant disease caused by transfusion-transmitted dengue-, zika-, and chikungunyavirus, even in immunosuppressed patients, suggests that these viruses need transmission *via* a mosquito bite to cause disease. Of course, one must be careful because sound epidemiological data on this topic is scarce. On the other hand, we must realize that tick-bore encephalitis historically seems assumed to be irrelevant for blood safety. Before automatically assuming that zika-, dengue-, chikungunya-, and usutuvirus necessitate blood safety measures like WNV does, we must study the actual threat they pose to blood safety. We must consider to give these viruses the "benefit of the doubt": as long as significant post-transfusion pathology (in the number of cases and in the nature of pathology) seems absent, blood safety measures may be avoided. In the "post-post-HIV era," it is too easy to automatically apply the precautionary principle again, which demands that preventive measures should be taken even if cause and effect relationships are not established scientifically (5).

SHOULD BLOOD BE CLEAN WHILE FOOD/AIR/MOSQUITOES ARE NOT?

In 2009, the third year of the large Q-fever epidemic in the Netherlands, 3 of 1,004 local blood donations were found to be confirmed positive for *Coxiella burnetii* DNA by PCR (6). Should blood donors indeed be screened for *C. burnetti* infection, while nothing was done to decrease the massive exposure of the population (including transfused patients) *via* air? History repeats itself: since July 2017 Dutch blood donations are being screened for the presence of hepatitis E virus, another zoonotic agent. During the first 11 weeks of screening, 48 of 85,023 (1:1,771) donations were found confirmed positive for HEV. We calculated that in Holland only 1 of 700 HEV infections is caused by blood products. In multitransfused at-risk patients, one of 3.5 cases of chronic hepatitis E is caused by blood products (7). Considering the Q-fever and hepatitis E example, one could argue that when "society" accepts large infection pressure *via* common routes such as air and food, it is not necessary to make transfusion safe. On the other hand, it seems that blood transfusion services have the responsibility to provide safe blood to vulnerable patients, even when other significant transmission routes are not eradicated.

IS vCJD STILL A THREAT?

Since the outbreak of BSE in Great Britain with subsequent cases of vCJD in humans, many blood banks maintain safety measures to prevent transmission of vCJD *via* blood transfusion and blood products. Examples of safety measures are the exclusion of blood donors who stayed at least 6 months in the UK during 1980–1996, and the exclusion of donors who themselves were transfused. Is it time to lift these restrictions? Until recently, it seemed that the outbreak of vCJD had ended. Unfortunately, in 2016 a new vCJD patient was reported. This patient was found to be heterozygous (methionine/valine) for codon 129 of the human prion gene. This is alarming, because so far all tested vCJD patients were methionine homozygous. Possibly, this first heterozygous patients reflects the start of a second wave of vCJD cases, with longer incubation times than the former homozygous cases. Apparently, people may harbor the infection during many years, and, regarding blood banking, could be seen as asymptomatic, but possibly infectious

carriers. On the other hand, no more cases of blood-transmitted vCJD have surfaced. Studies of archived appendices suggest that roughly 1 in 2,500 British appendices tests positive for the vCJD agent. The first and second appendix study involved 12,674, respectively, 32,441 appendices, from persons born between 1961 and 1985, respectively, 1941–1985 (8, 9). These persons experienced dietary exposure to the vCJD agent around 1990; and their appendices were removed in 1995–1999, respectively, 2000–2012; in which 3, respectively, 16 vCJD-prion-positive appendices were found. Is this finding really linked to dietary exposure to the vCJD agent in the 1980s and 1990s, or is there an unrelated, harmless, and natural background presence of the protein in appendices? The Appendix-III study included appendices and persons who did not live in the late 1980s and early 1990s (10). The findings of the Appendix-III study are confusing: possibly the period of dietary exposure is larger than assumed. Alternatively, there may be no connection between appendices testing positive and the BSE/vCJD outbreak. Currently, it seems wise not to abandon vCJD blood safety measures yet.

In summary, several dilemmas exist concerning the desired level of safety of blood transfusion. Perhaps it is good to discuss these issues more in the open, and keep the struggle with difficult and expensive decisions not confined to blood transfusion services and ministries of health.

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REVIEW
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Ultraviolet-Based Pathogen Inactivation Systems: Untangling the Molecular Targets Activated in Platelets

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Transfusions of platelets are an important cornerstone of medicine; however, recipients may be subject to risk of adverse events associated with the potential transmission of pathogens, especially bacteria. Pathogen inactivation (PI) technologies based on ultraviolet illumination have been developed in the last decades to mitigate this risk. This review discusses studies of platelet concentrates treated with the current generation of PI technologies to assess their impact on quality, PI capacity, safety, and clinical efficacy. Improved safety seems to come with the cost of reduced platelet functionality, and hence transfusion efficacy. In order to understand these negative impacts in more detail, several molecular analyses have identified signaling pathways linked to platelet function that are altered by PI. Because some of these biochemical alterations are similar to those seen arising in the context of routine platelet storage lesion development occurring during blood bank storage, we lack a complete picture of the contribution of PI treatment to impaired platelet functionality. A model generated using data from currently available publications places the signaling protein kinase p38 as a central player regulating a variety of mechanisms triggered in platelets by PI systems.

Keywords: platelets, pathogen inactivation, transfusion, mechanisms, signaling

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THE CHALLENGES OF PLATELET TRANSFUSIONS

Platelets play an essential role in hemostasis, fibrinolysis, and vascular integrity, which are critical physiological processes to prevent and control bleeding (1–3). Platelet concentrates (PCs) are transfused to treat bleeding in thrombocytopenic, trauma, or surgery patients (4–6) as well as for prophylactic treatment of patients with hypoproliferative thrombocytopenia (7, 8). Over the last decades, development of improved therapies and the subsequent introduction of new transfusion guidelines have changed the practice of platelet transfusion (9, 10) which has, in turn, influenced the management of platelet inventories in the blood bank.

Additionally, the integrity and safety of platelet preparations could be compromised by the presence of pathogens, such as viruses, bacteria, and parasites (11). Serious complications or death due to bacterially contaminated units have been well documented, leading to several changes in the collection procedures, including stricter donor screening, improved skin disinfection methods and diversion of the first few milliliters of collected blood, and bacterial culture of PCs (12–16). However, the risk still exists, not only for undetected bacterial contamination but for the increasing number

of emerging and re-emerging pathogens, particularly viruses for which screening tests may not be in place.

Finally, even with the use of pre-storage leukoreduction, the transfer of residual allogeneic donor leukocytes in PCs still occurs and can potentially cause adverse reactions in certain platelet recipients (17). All pathogen inactivation (PI) systems show inactivation capacity of these residual leukocytes (18, 19).

These challenges of platelet storage have led to the development and increasing implementation of PI technologies which are based on ultraviolet (UV) light-mediated damage of nucleic acids and subsequent inactivation of most pathogens as well as passenger white blood cells.

A BRIEF OVERVIEW OF CURRENT PI SYSTEMS

Currently, three PI systems to produce pathogen-reduced PCs are commercially available, utilizing UV in the presence or absence of a photosenzitizer. These technologies are extensively reviewed in the literature (20–29); therefore, only key points necessary for the context of this review are provided.

The INTERCEPT system (Cerus Corporation, Concord, CA, USA) uses amotosalen as photosensitzer in combination with UVA light (320–400 nm). Amotosalen penetrates the cellular membrane forming non-covalent links between pyrimidine residues in DNA and RNA. UV illumination induces a photochemical reaction that transforms the preexisting link into an irreversible covalent bond, preventing DNA replication and RNA transcription. Excess amotosalen and its photoproducts need to be removed by an in-line compound absorption device (30, 31).

The MIRASOL system (Terumo BCT, Lakewood, CO, USA) uses vitamin B2 (riboflavin) as the photosensitizer and UVA/UVB light (270–360 nm) (32, 33). In the presence of riboflavin, illumination generates free oxygen radicals causing irreversible damage to guanidine nucleotide bases. Riboflavin does not need to be removed following illumination as it is a common dietary element and generally considered to be safe.

The THERAFLEX-UV Platelets system (MacoPharma, Tourcoing, France) uses UVC light in combination with strong agitation which facilitates light penetration and does not require a photosensitizer. UVC acts directly on nucleic acids to induce pyrimidine dimers to block DNA replication (34, 35).

PATHOGEN-REDUCED PLATELET PRODUCTS

Pathogen-reduced PCs can be obtained by direct treatment of platelet components using a PI system, or they can be derived by treating whole blood with the MIRASOL (36, 37) or potentially the INTERCEPT system once a current trial turns out to be successful followed by processing into the (platelet) components (**Table 1**).

It is noteworthy to point out that the THERAFLEX system require PCs produced in platelet additive solution (PAS) while the MIRASOL and INTERCEPT systems can treat PCs in plasma or PAS.

TABLE 1 | Overview of pathogen inactivation (PI) treatment options to obtain pathogen-reduced platelet products.

Product treatment	Storage solution	PI system		
		INTERCEPT	MIRASOL	THERAFLEX
AP/PC	Plasma	+	+	–
	PAS	+	+	+
PRPC or BC/PC	Plasma	+	+	–
	PAS	+	+	+
WB (prior to PRPC or BC/PC production)	Plasma	–	+	–
	PAS	–	+	–

AP/PC, apheresis platelet concentrates; PRPC, platelet-rich plasma concentrate; BC/PC, buffy-coat-derived platelet concentrate; WB, whole blood; PAS, platelet additive solution.

ONGOING DEBATE: SAFETY VS EFFICACY OF PI

More than a decade ago, the interest in PI prompted many large-scale discussions (38–40). The outcome of these deliberations included the provision of information required for implementation of PI systems such as implementation criteria, component specifications, licensing requirements, and the impact in blood product inventories, as well as clinical issues such as transfusion efficacy, risk management issues, and cost–benefit assessments. Since then, numerous studies have been conducted to provide answers to questions on product safety, clinical efficacy, and quality.

In order to assess inactivation efficacy, studies spiking pathogens relevant to blood transfusion into PCs prior to illumination have been performed (34, 41–44). All PI systems currently on the market have demonstrated effectiveness in inactivating most tested pathogens with moderate to highly effective inactivation capacities for several emerging viruses including West Nile virus (45), chikungunyah virus (46), Zika virus (47, 48), dengue virus (49), and hepatitis-E-virus (50). Additionally, a comparative study (51) revealed that HIV-1 can be similarly inactivated by MIRASOL and INTERCEPT, however, less efficient compared to other viruses due to its resistance to UV light. Furthermore, INTERCEPT demonstrated a higher inactivation capacity for bovine viral diarrhea virus and pseudorabies virus compared to MIRASOL while both technologies showed similar log reductions for hepatitis-A-virus and porcine parvovirus. However, due to their chemistry, PI systems are only able to target pathogens that contain nucleic acids and consequently they are ineffective against prions and transmission of variant Creutzfeldt–Jakob disease (52).

In order to demonstrate clinical efficacy, several large clinical trials using these PI systems have been conducted or are underway (22, 53) and extensive hemovigilance studies have also been undertaken. The main message is that PI treatment damages the platelets in many ways including alterations in membrane integrity, signaling pathways and in some capacity functionality of miRNAs, which results in reduced recovery and survival in healthy volunteers (54, 55). Similarly, shorter transfusion intervals have been observed in patients receiving PI-treated platelets, but these observations for the most

part have not been associated with increased World Health Organization grade 2 or greater bleeding in patients receiving pathogen-reduced platelets, as hemostatic efficacy seems to be maintained (22, 26). Furthermore, some evidence points toward the fact that transfusion of PI-treated platelets does not affect mortality, the risk of clinically significant or severe bleeding, or the risk of a serious adverse event (AE) (56). However, as pointed out by Kaiser-Guignard and colleagues, the results of the published clinical studies should be interpreted with caution, and their characteristics and possible biases should be taken into account (22), such that interpretation of clinical outcome data cannot be generalized across different PI systems (22). A recent systematic review presented strong evidence that transfusion of PI-treated platelets appears to increase the risk of platelet refractoriness and the frequency of platelet transfusions (56).

The majority of contributions to investigations of PIs are *in vitro* quality studies. Multiple analyses have been conducted to monitor potential changes in the platelet quality following illumination with the three different PI systems in combination with products of different characteristics (see **Table 1**). These studies have typically measured common blood banking parameters, including metabolic activities, platelet activation, and platelet function to evaluate product quality, and to determine whether quality control requirements of the individual jurisdictions were met. Comparisons of different studies; however, are hampered by the fact that these measures are influenced by the type and proportion of the platelet storage medium. PI treatment of platelets in different PAS differentially alters platelet quality features (57). Recent studies with the riboflavin/UV system (MIRASOL) revealed that the quality of platelets is similar whether stored in plasma or PAS; however, transfusion of treated PCs in PAS led to fewer transfusion reactions (58). This observation is corroborated by the finding that PAS seems to have a protective effect on platelets upon illumination (59).

Based on these diverse studies, in recent years, many (individual) opinions have been published outlining the pros and cons of PI in light of safety and efficacy (20, 60–64). Ongoing discussions are guided by experiences from blood centers that have implemented PI (65–67).

PLATELET STORAGE LESION (PSL): A GENERAL OVERVIEW

Many studies measuring changes to platelet *in vitro* quality indicate that PI treatment accelerates the progression of the PSL. This term describes the sum of all the deleterious changes in platelet structure and function that arise from the time the blood is withdrawn from the donor to the time the platelets are transfused to the recipient (68–73). It is mainly explained by triggering platelet activation during preparation and handling of PCs, especially the heightened metabolic activity and activation-specific changes to surface glycoproteins observed in stored platelets (74). Transient derangement of platelet metabolism can be rescued by plasma replacement, resulting in improved morphology scores, stabilized osmotic recovery, and completely restored platelet secretory responses (75).

THE IMPACT OF PI ON PLATELET FUNCTIONS

PLT Activation, Degranulation, and Protein Release

As mentioned above, the main feature of PSL seems to be platelet activation, which is commonly determined by the expression of P-selectin (CD62P) on the platelet surface, as a consequence of the release of the alpha-granule content. Many studies have shown that PI increases the surface expression of CD62P (58, 76–78).

Additional features of storage-mediated platelet activation are the increased phosphatidylserine (PS) externalization (79) and changes in the protein profile of platelet surface receptors (80, 81) which are further altered upon PI treatment (82).

Among other changes, the level of cytokines and chemokines also increases in the supernatant of the storage solution during platelet storage (83–85). Although some controversy continues in the literature (86), PI treatment appears to induce platelet degranulation, hence further increasing the levels of immune factors under some treatment conditions (86–91). The altered releasate composition may affect the immunomodulatory capacity of platelets. As a consequence of this accumulation, supernatants of MIRASOL PI-treated platelets can suppress LPS (lipopolysaccharide)-induced monocyte IL-12 production (92), as well as increase LPS-induced mononuclear cell production of IL-8 (93). A recent study has demonstrated that increased supernatant levels of pro-inflammatory molecules resulting from platelet granule release are associated with reactive oxygen species generation during storage (94). This finding is corroborated by an observed increased ROS production in MIRASOL PI-treated PCs (77, 95).

A brief summary is provided in **Table 2** highlighting the changes of platelet storage features by the individual PI systems.

Development of Platelet Apoptosis

There is an ongoing debate regarding the extent to which platelet activation and programmed cell death (apoptosis) in platelets overlap at the molecular level (113). Platelets contain most of the apoptotic machinery, including pro- and anti-apoptotic Bcl-protein family members as well as caspases (114). Activation of these pathways leads to microvesiculation with expression of PS in the outer layer of the platelet membrane (115). As PS exposure is believed to contribute to the development of inflammatory or immunomodulatory processes and ultimately regulates clearance of platelet from circulation, PS exposure monitored by annexin-V binding is commonly used to measure the development of platelet apoptosis.

Pathogen inactivation treatment also results in the externalization of PS (59, 116, 117). MIRASOL PI-treated PLTs exhibit an increased expression of proapoptotic proteins Bak and Bax, but not anti-apoptotic proteins Bcl-XL (109, 116). Additionally, MIRASOL PI-triggered activation of caspase cleavage leads to proteolytic cleavage of their respective substrate proteins (116). Similar results have recently been shown in INTERCEPT PI-treated platelets (118). However, these features are not prominent until later in storage (typically 5–7 days) and may only need to be considered in the context of extended platelet storage.

TABLE 2 | Summary of impact of pathogen inactivation (PI) treatment on platelet features compared to untreated control.

Platelet storage feature	PI system		
	INTERCEPT	MIRASOL	THERAFLEX
Metabolic activity	± (96); ↑ (97)	↑ (98)	↑ (99)
Platelet activation (CD62P expression)	↑ (96, 100)	↑ (98)	↑ (99)
Platelet adhesion (under flow)	± (101); ↑ (102)[a]	↓ (102); ±(103)	n.d.
Clot formation (thrombo-elastography)	↓ (104)	↑[b], ↓[c] (105)	↓ (99)
Responsiveness (to agonists)	↓ (102); ±↓[d] (106)[c]	↓ (98)	± (99)
Platelet apoptosis (PS exposure)	± (107); ↑ (108)[a]	↑ (109)	↑ (99)
Platelet microparticle release	↑ (110)	↑ (111)	↑ (112)
Free mitochondria release	n.d.	↑ (95)	n.d.

↓ = decrease; ± = similar; ↑ = increase; n.d. = not determined. The references are only examples of published studies, but are not comprehensive. Differences in some study outcomes could be due to variations in production methods used (platelet-rich plasma vs BC/PCs or apheresis PCs), composition in storage solution—plasma vs platelet additive solution (in different concentration)—and assay procedures.

[a]*At end of storage.*

[b]*Thrombus stability.*

[c]*Aggregation.*

[d]*Agonist-dependent.*

Microvesicle (MR) Release

Platelets are known to generate heterogeneous populations of cell-derived MVs (119). Platelet MVs have a bilayered phospholipid structure exposing procoagulant PS and expressing various membrane receptors, and they serve as cell-to-cell shuttles for bioactive molecules such as lipids, growth factors, microRNAs (miRNAs), and mitochondria (120). Further, the presence and quantity of MVs has been associated with the clinical severity of the atherosclerotic disease, diabetes, and cancer (6, 121). These features along with the observation that the number, function, and content of MVs in the components varies with age, gender, lipid, and hormone profiles of the blood donor (122) makes them one of the most discussed, controversial, and interesting topics in current blood banking and transfusion medicine (123). Different studies have demonstrated that all UV-based PI treatments increase the release of MVs from platelets compared to untreated controls (36, 95, 112, 124). To our knowledge, no study has directly addressed the impact of INTERCEPT on the release of MVs during PC storage; however, Kanzler et al. found a reduction of MVs in the platelet product immediate after INTERCEPT treatment (125).

Role of Platelets in Inflammation

Although once primarily recognized for their role in hemostasis and thrombosis, platelets have been increasingly recognized as a multipurpose cell. There is growing recognition of the critical role of platelets in inflammation and immune responses. Platelets release numerous inflammatory mediators such as RANTES or CD40L, modifying leukocyte and endothelial responses to a range of different inflammatory stimuli (88). Additionally, platelets form aggregates with leukocytes and form bridges between leukocytes and endothelium, largely mediated by platelet P-selectin. Through their interactions with monocytes, neutrophils, lymphocytes, and the endothelium, platelets are, therefore, important coordinators of inflammation and both innate and adaptive immune responses. As mentioned above, studies have shown that MIRASOL-treated platelets release such mediators (92, 93) and, therefore, might modulate inflammatory responses.

Mitochondria and Mitochondrial DNA (mtDNA) Release

Mitochondria are known as the powerhouse of cells and play a crucial role in maintaining platelet function throughout platelet storage (126). Mitochondria are released from activated platelets and upon hydrolysis of the mitochondrial membrane release mtDNA (127). MIRASOL-PI treatment also causes release of free mitochondria, mainly at the later stages of storage (95). Potentially associated with the mitochondria release, free mtDNA has been associated with AEs following platelet transfusion, and may be predictive of some types of AEs (128). mtDNA is a highly potent inflammatory trigger (128) that can be released from platelets during storage (129). Illumination of platelets with PI systems modifies mtDNA (129–131). Detection of PI-modified mtDNA using PCR assays can be used to monitor and confirm PI treatment (131). Furthermore, the relationship of mtDNA levels and AEs related to immunomodulation should also be considered; with a recent study showing an association between mtDNA and the incidence of respiratory distress posttransfusion (132).

MicroRNA

MicroRNAs are small (~20–24 nucleotides) RNA sequences generated by ribonucleases in the nucleus (by Drosha) and cytosol (by Dicer 1) through sequential enzymatic trimming of double stranded miRNA precursors. miRNAs are thought to fine tune gene expression through degradation of their mRNA targets (133). Although platelets are anucleate, high-throughput sequencing has revealed that human platelets harbor a complex array of miRNAs, which are key regulators of mRNA translation in different cell types (134). Activated platelets can deliver mRNA regulatory Argonaute-2 miRNA complexes to endothelial cells *via* MVs leading to modulation of cell function (135).

INTERCEPT, but not MIRASOL PI treatment has been shown to affect the platelet mRNA transcriptome (27, 136). However, miRNA synthesis and function were not affected and no cross-linking of miRNA-sized endogenous platelet RNA species was observed; rather miRNA levels were reduced (136, 137). Further, the reduction in the platelet miRNA levels induced by INTERCEPT correlated with platelet activation and an impaired platelet aggregation response to ADP (136). In contrast, a recent study presented by Arnason et al. (138) demonstrated that INTERCEPT treatment did not change the quality or significantly altered the miRNA profile of PCs. These controversial results prompted further investigations and as the clinical significance of MV-associated miRNAs is unknown, and speculation of a negative effect of PI-treated platelets including long-term consequences for recipients is as yet unwarranted. This is a relatively

new area of research, and additional studies are required to fully understand the impact of PI treatment on miRNA synthesis and the resulting impact on platelet quality.

mRNA Levels and Protein Synthesis

Although anucleate, platelets have the capacity to synthesize biologically relevant proteins that are regulated *via* gene expression programs at the translational level in response to physiological stimuli (139–141). Recent studies have demonstrated that levels of specific mRNA species are reduced following MIRASOL PI treatment while others are less affected (142). Subsequent studies have revealed that this observation is mirrored in the platelet translatome, demonstrating that platelets are still capable of synthesizing proteins following PI treatment, suggesting that they may possess mechanism(s) to protect their mRNA from damage by the PI treatment (143). The clinical relevance of this finding, however, is still unknown.

Impact of PI Treatment on Platelet Lipidomics

Although the application of lipidomics to platelet biology is still in its infancy, seminal studies have shaped our knowledge of how lipids regulate key aspects of platelet biology, including aggregation, shape change, coagulation, and degranulation, as well as how lipids generated by platelets influence other cells, such as leukocytes and the vascular wall, and thus how they regulate hemostasis, vascular integrity, and inflammation, as well as contribute to pathologies, including arterial/deep vein thrombosis, and atherosclerosis (144). Mapping the human platelet lipidome revealed cytosolic phospholipase A2 as a regulator of mitochondrial bioenergetics during activation (145). A recent study has demonstrated that psoralen and UV light increased the order of lipid phases by covalent modification of phospholipids, thereby inhibiting membrane recruitment of effector kinases such as BTK and Akt and consequently affecting GPVI- and PAR1-mediated signal transduction (99).

FURTHER INVESTIGATIONS TOWARD UNDERSTANDING THE MOLECULAR MECHANISMS OF PI-INDUCED PLATELET ALTERATION: FROM PROTEOMICS TO SIGNALING

A variety of untargeted proteomic approaches have been used to assess the impact of PI systems on platelets (146–148). The effect of the PI treatment on the proteome appears to be different according to the particular technology. A comparative analysis of proteomic data revealed that MIRASOL seems to impact proteins involved mainly in platelet adhesion and shape change while INTERCEPT affects proteins of intracellular platelet activation pathways and THERAFLEX influences proteins linked to platelet shape change and aggregation (149). These conclusions are based on a relatively small number of studies and further analyses are required for verification.

A more targeted approach using a phospho-kinase antibody-based array demonstrated that a variety of kinases were activated by MIRASOL PI treatment (150). p38MAPK plays a central role in MIRASOL PI-mediated signaling by regulating a variety of platelet features, such as apoptosis (109), mitochondrial function, and release of free and MV-encapsulated mitochondria (95). The INTERCEPT system also triggers p38MAPK activation in platelets, and the phosphorylation of the p38MAPK substrate Tace is directly linked to GPIb cleavage possibly explaining the reduced adhesion of those platelets under flow conditions (118). The role of p38MAPK in mediating PI-triggered signaling linked to features

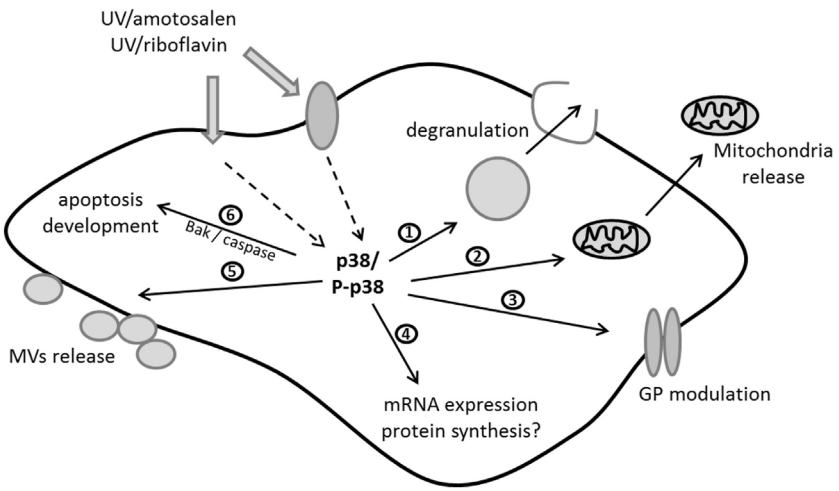

FIGURE 1 | Current molecular model of signaling triggered by ultraviolet (UV)/riboflavin (MIRASOL) and UV/amotosalen (INTERCEPT) in platelets: UV can penetrate either directly or *via* surface/receptor proteins to activate p38MAPK kinase as one of the central players in the signaling cascade. Thus far it has been shown that p38 activation/phosphorylation (P-p38) is involved in regulating (1) degranulation, (2) release of free mitochondria, (3) the modulation of glycoproteins (GPs), (4) the expression levels of mRNAs and potentially protein synthesis, (5) microvesicle (MVs) release, and (6) the development of apoptosis *via* proapoptotic protein expression and caspase activation. This figure was modified from Ref. (150).

of PSL is supported by studies demonstrating a regulatory role of p38MAPK in regulating PSL development (151) and platelet *in vivo* recovery and survival in mouse models (152). This body of work suggests that similar signaling pathways are activated by both of these PI systems as modeled in **Figure 1**. Although only a few studies to date have investigated the signaling aspect in platelets, it could be hypothesized that p38MAPK activation in response to the stress associated with the PI treatment may have a regulatory role in platelet life span (153) as inhibition of this protein leads to decreased apoptosis (109, 118).

CONCLUSION AND FUTURE DIRECTIONS

Although there are numerous studies in the literature assessing the impact of UV-based PI systems on platelet *in vitro* and *in vivo* function, only a few conclusions can be drawn. All technologies seem to accelerate the development of some form of the PSL but this likely results through different modes of action; therefore, it is likely that many divergent, as well as overlapping molecular mechanisms are triggered. Most of the functional studies conducted to decipher the role of signaling pathways in PI-treated platelets have been carried out using the INTERCEPT and MIRASOL system and thus the effects of the THERAFLEX system remain relatively unknown. However, it is clear that PI-treated platelets are different to untreated platelets, and the differences may go some way toward explaining some of the clinical observations following transfusion of PI-treated platelets. Proteomic analyses and in future other -omics approaches such as metabolomics (154) will likely shed more light into the specific effects of PI treatment. Additional targeted approaches will guide the formulation of signaling models, which may ultimately identify pathways known to impact platelet function upon illumination, and provide potential (protein) markers to assist with the fine-tuning of these technologies. We need to keep in mind, however, that the PI treatment does not only affect platelets *per se*, these procedures trigger the release of MVs, proteins, and nucleic acids in to the storage medium which also gets transfused. Whether any of these components will have deleterious effects on the recipients remains to be determined even though the initial clinical studies do not show significant clinical effects from PI treatment of PCs.

AUTHOR CONTRIBUTIONS

All authors contributed to this manuscript and approved the final version for submission.

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Toward the Relevance of Platelet Subpopulations for Transfusion Medicine

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Circulating platelets consist of subpopulations with different age, maturation state and size. In this review, we address the association between platelet size and platelet function and summarize the current knowledge on platelet subpopulations including reticulated platelets, procoagulant platelets and platelets exposing signals to mediate their clearance. Thereby, we emphasize the impact of platelet turnover as an important condition for platelet production *in vivo*. Understanding of the features that characterize platelet subpopulations is very relevant for the methods of platelet concentrate production, which may enrich or deplete particular platelet subpopulations. Moreover, the concept of platelet size being associated with platelet function may be attractive for transfusion medicine as it holds the perspective to separate platelet subpopulations with specific functional capabilities.

Keywords: platelet subpopulation, platelet size, platelet turnover, platelet clearance, platelet maturation

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INTRODUCTION

Platelets recognize vessel damage, trigger coagulation and enhance clot formation at the site of injury (1). Beyond hemostasis, platelets also act as mediators in immunity and inflammation (2–5).

Circulating platelets differ in age, maturation state, or density. An obvious physical feature of platelets is their size, which can vary substantially among platelets of one individual. It was an early concept, that large platelets represent a rather young and reactive platelet subpopulation (6). Later, this concept was abandoned when consecutive experiments demonstrated no clear correlation between platelet size and age (7, 8).

The observation that some platelets have particular procoagulant capabilities led to the concept of platelet subpopulations with different biological functions (9). Other examples for platelet subpopulations are reticulated (rather young) platelets and platelets exposing signals mediating their clearance from the circulation (rather old platelets). It is conceivable albeit unclear, whether other platelet subpopulations exist which play a more pronounced role in immunological or inflammatory processes, e.g. by expression of CD40 or release of CD40L (10, 11).

Epidemiological studies found an association between an increased platelet size and thrombotic outcomes in patients with cardiovascular disease (12) resulting in a revival of the "old" hypothesis of an association between a larger platelet size and enhanced platelet function in hemostasis.

Clarifying the hypothesis of different biological features of platelet subpopulations is potentially relevant for transfusion medicine. Enrichment of distinct platelet subpopulations in platelet concentrates (PCs) during production may modulate the biological effects of PCs.

In this review, we summarize the current knowledge on platelet subpopulations with a special emphasis on platelet size, its association with platelet function and the impact of platelet turnover on platelet production.

SIZE AS A PLATELET CHARACTERISTIC

Platelet Formation, Turnover, and their Role for Platelet Size

Platelet size is genetically determined and relatively stable over the lifetime in healthy individuals. Genome wide association studies in healthy subjects identified several genes associated with platelet size (13–18).

Under steady-state conditions, platelets are generated from megakaryocytes in the bone marrow after stimulation with thrombopoietin. The amount of circulating thrombopoietin is regulated by the mass of circulating platelets. They bind thrombopoietin, providing a negative feedback mechanism to control thrombopoiesis (19). In mice, thrombopoietin administration increases platelet size (20) whereas in humans the opposite seems to be the case (21).

In the bone marrow, preplatelet intermediates are formed as extensions of elongated megakaryocyte-pseudopodia and released into the sinusoidal blood vessels (22, 23). Glycoprotein Ib mediates transmigration of megakaryocytes into the sinusoids *via* the small GTPases Cdc42 and RhoA (24). Preplatelets convert into barbell-shaped proplatelets that form platelets (23, 25) mediated by integrin αIIbβIII signaling (26). Platelet size is established during the formation of barbell proplatelets from circular preplatelets and limited by microtubule bundling, elastic bending, and actin-myosin-spectrin cortex forces (27).

Thrombopoiesis in the bone marrow is spatially regulated (28) but platelet maturation does not end in the bone marrow. Preplatelets are also formed from proplatelets in the circulation (29) and can maturate in the lungs (30).

In vivo, the mechanisms of proplatelet formation are very dynamic and influenced by platelet turnover (31). In case of inflammation an alternative pathway of platelet production can occur. Nishimura et al. found that increased serum levels of the inflammatory cytokine IL-1α induce platelet release by the rupture of megakaryocytes as a distinct mechanism in the absence of elevated thrombopoietin (32). *Via* this mechanism, larger platelets are produced than in thrombopoietin-stimulated megakaryocytes in mice.

We have established a model of enhanced platelet production in healthy volunteers using platelet apheresis showing that platelet apheresis stimulated platelet production leads to reversible changes in the platelet proteome (33). This further indicates an impact of platelet turnover on the phenotype of circulating human platelets.

Platelet Size and Function during Steady-State Platelet Production

Most studies identified large platelets as a subpopulation with a higher prohemostatic capacity, if generated under steady state. However, it is still debated whether a larger size alone contributes to this higher capacity (34), or if there are specific features in large platelets which over-proportionally increase their prohemostatic potential. **Table 1** provides an overview of functional comparisons between large and small human platelets. The majority of experiments included adjustments for cell size, suggesting a hyperproportional prohemostatic capacity of large platelets.

Steady-state large platelets have a higher capacity for glucose metabolism, resistance to osmotic shock (36), and lipid peroxidation (38). They aggregate faster and release more ATP and alpha granule proteins (34, 37), contain more fibrinogen, and serotonin (40), and express more human leukocyte antigen-I molecules (41) and membrane glycoproteins (43).

Platelets synthesize proteins (50) and large platelets have more ribosomes and incorporate more amino acids (35). Probably, large platelets have a higher capacity to translate mRNA. This needs to be demonstrated by future studies, which adequately control for residual leukocytes in the large platelet fraction.

Opper et al. found different patterns of cGMP synthesis and protein phosphorylation patterns after stimulating platelets of different size (44, 46), suggesting differences in signal transduction between large and small platelets.

The ability to mobilize Ca^{2+} in the cytosol is pivotal for platelet activation. Li et al. showed that the cytosolic Ca^{2+}-concentration is similar in resting large and small platelets, whereas higher amounts of Ca^{2+} are mobilized by large platelets (45).

Large platelets express more surface-bound fibrinogen, bind more von Willebrand factor, and metabolize more arachidonic acid (39), express more P-selectin, activate more integrin αIIbβ3 after ADP-stimulation (42, 47, 51), and release more thromboxane after collagen- and thrombin-induced aggregation in proportion to platelet size (39).

A recent study indicates that large-size platelets are functionally different compared to small platelets. Brambilla et al. found that large platelets express not only significantly higher amounts of tissue factor and tissue factor mRNA compared to small platelets. Large platelets also expose functionally active tissue factor on their cell membranes whereas the activity of tissue factor in small platelets is almost completely quenched by tissue factor pathway inhibitor (48). These results extent previous findings showing that platelets translate tissue factor (52) and point toward specific roles of large and small platelets in hemostasis.

Platelet Size during Increased Platelet Turnover

If platelet production is enhanced in healthy humans by application of thrombopoietin, the peripheral platelet concentration increases whereas platelet size measured by the mean platelet volume (MPV) slightly decreases without changes in platelet viability, platelet responsiveness to physiologic agonists, or expression of platelet activation markers (21).

In contrast, disease-related increased platelet turnover is often associated with an increase in platelet size (6, 53), e.g., in case of enhanced destruction of platelets by autoantibodies (54–56), during recovery after bone marrow suppression by chemotherapy (49), or in situations with increased consumption in patients with severe arterial disease (57, 58). These

TABLE 1 | Functional characterization of human large and small platelets.

Reference	Results	Size adjustment	Evidence for a hyperproportional difference between large and small platelets
Steady-state platelet production			
Booyse et al. (35)	Only large platelets contain ribosomes	Not performed	Yes
Karpatkin (36)	Large platelets: higher glycogen, higher orthophosphate, higher total adenine nucleotide, higher glucogenolysis capacity, higher glycolysis activity, higher protein synthesis, higher glycogen synthesis, higher resistance to osmotic shock	Ratios of analytes compared to ratios of platelet volumes	Yes
Karpatkin (37)	Large platelets: lag time to aggregation shorter; higher ATP release; following aggregation higher ADP release; higher release of platelet factor 4	Not performed	Not applicable
Karpatkin and Strick (38)	Large platelets: higher activity of glycolysis enzymes, less lipid peroxidation product, more resistant to lipid peroxidation	Equal amount of protein extract taken from large and small platelets	Yes
Thompson et al. (34)	Large platelets: maximal aggregation after activation by collagen or thrombin increased; contain larger amounts of ATP and beta-thromboglobulin	Relative change within each size fraction (aggregometry); relative comparison of ATP and beta-thromboglobulin before and after stimulation	Yes
Jakubowski et al. (39)	Large platelets: release more thromboxane after collagen or thrombin stimulation Platelet size correlates with the amount of metabolized arachidonic acid	Correlation to MPV	No
Mezzano et al. (40)	Large platelets: more fibrinogen, more serotonin and more absolute protein	Not performed	Not applicable
Pereira et al. (41)	Large platelets: more $P1^{a1}$ molecules; small platelets: more HLA-A2 molecules, more total HLA class I-molecules	Not performed	Not applicable
Frojmovic et al. (42)	Large platelets: more fibrinogen receptor expressed on membrane when activated; faster aggregation rate	Correlation of ratios large/small with size ratio large/small	No
Polanowska-Grabowska et al. (43)	Large platelets: faster adhesion to collagen, less sensitive to inhibition by prostacyclin, increased content of glycoprotein Ia/iia complex	Not performed	Not applicable
Opper et al. (44)	Large platelets: higher basal level of cgmp, higher cgmp synthesis rate after stimulation with sodium nitroprusside, lower activity of camp-dependent phosphodiesterases	Adjustment of protein content and platelet size	Yes
Li et al. (45)	Large platelets: higher maximal aggregation after stimulation with thrombin, increased ATP secretion, higher degree of calcium mobilization	Relative change within each size fraction (aggregometry)	Yes
Opper et al. (46)	Large platelets: higher degree of protein phosphorylation after thrombin stimulation, higher rate of ADP-ribosylation by cholera toxin; small platelets: higher basal phosphorylation levels of several proteins, higher ADP-Ribosylation by pertussis toxin and C3 exoenzyme, higher basal Ca^{2+}-level	Equal amount of protein extract taken from large and small platelets	Yes
Mangalpally et al. (47)	Large platelets: express more surface-bound fibrinogen, bind more von Willebrand factor after arachidonic acid- or ADP-stimulation, express more P-selectin, more activated glycoprotein iib/iiia after ADP stimulation; higher proportion of reticulated platelets	Adjustment to the platelet surface area	Yes
Brambilla et al. (48)	Large platelets: contain higher amounts of tissue factor and tissue factor mrna; mainly large platelets expose functionally active tissue factor	Not performed	Not applicable

(*Continued*)

TABLE 1 | Continued

Reference	Results	Size adjustment	Evidence for a hyperproportional difference between large and small platelets
Increased platelet turnover			
Balduini et al. (49)	Old platelets: MPV and P-LCR reduced; young platelets: MPV and P-LCR higher compared to old and to control; aggregation response faster in young platelets	Relative change within each size fraction (aggregometry)	Yes

MPV, mean platelet volume; P-LCR, platelet large cell ratio; HLA, human leukocyte antigen.

studies suggest that platelet size in disease is regulated by other mechanisms than the ones regulating platelet size during thrombopoietin-mediated megakaryocytopoiesis in healthy volunteers. Severe thrombocytopenia induced by disseminated intravascular coagulation in children is also associated with an increase in platelet size (59). However, in view of the findings of Nishimura et al. (32), this likely results from platelet production by the alternative pathway involving IL-1α induced fragmentation of megakaryocytes.

Platelet Size and Platelet Age

The first attempts to characterize young platelets were driven by the hypothesis, that large platelets are considerably younger than small platelets because they are more functionally active (37, 38). However, later studies did not reveal a direct relationship between platelet size and age. This was convincingly underscored by an experiment in baboons under conditions of steady-state platelet production. The animals received radioactively labeled methionine being incorporated by megakaryocytes (8). Radioactively labeled platelets were afterwards present in each assessed size fraction of platelets indicating that size and age of platelets do not correlate under steady-state conditions. Also in humans platelet size is likely not strongly associated with platelet age (7). After transfusion of radioactively labeled autologous platelets, the mean survival of a high-density platelet population was shorter than that of platelets with low density. The mean volumes of high- and low-density platelets were not different suggesting that platelet size is unrelated to platelet age under normal conditions, but implicating a role of platelet density for the age of circulating platelets.

Platelet Size as Risk Factor for Adverse Clinical Outcomes

Epidemiological studies in patients with cardiovascular disease found an association between an increased MPV and a higher prevalence of thromboembolic complications (12, 60–62). An increased platelet size due to increased platelet turnover also correlates with refractoriness to antiplatelet therapy (58) and predicts a higher incidence of adverse outcomes after coronary intervention (63).

It is unclear, whether the increased MPV is the cause or the consequence of an increased risk for thromboembolic outcomes (60, 64). An alternative explanation is that individuals with large platelets have *per se* an increased risk for thrombotic complications because genetic traits have been identified, which are at the same time associated with an increased MPV and an increased risk for cardiovascular disease (65).

An increased MPV also characterizes inherited bleeding disorders with dysfunctional large platelets (66).

PLATELET SUBPOPULATIONS

Reticulated Platelets

Ingram et al. first observed a unique population of newly formed platelets soon after the induction of acute blood loss in beagle dogs. They stained platelets with methylene blue and noticed coarse and punctate condensations in platelets similar to those seen in reticulocytes of red cells. Therefore, this platelet fraction was named "reticulated platelets" (67). Later, reticulated platelets were shown to contain more RNA, staining with nucleic acid-specific fluorescent dyes, such as thiazole orange (68).

Reticulated platelets likely represent the youngest platelet fraction. After *in vivo* biotinylation, freshly formed platelets carrying reduced levels of biotin were shown to be reticulated (69). These platelets are younger than 24 h (70) and decay their RNA during aging (71).

In healthy humans with steady-state platelet production around 8% of circulating platelets are reticulated (72). Furthermore, the proportion of reticulated platelets is enriched in the fraction of large platelets compared to the fraction of small platelets (47), suggesting a relationship between platelet size and age.

A limitation of studies applying thiazole orange to stain reticulated platelets is, however, the tendency of this dye to bind unspecifically to alpha-granule contents (73). Therefore, a higher proportion of thiazole orange positive platelets observed in larger platelets could in part result from unspecific binding and may not represent young platelets (74). This limitation may be overcome by more RNA-specific dyes (75), which may finally elucidate the relationship between the size and age of platelets under steady-state platelet production.

In patients with high platelet turnover, the MPV is increased and likewise the proportion of reticulated platelets (72, 76, 77). One example that these changes may have biological relevance is their response to antiplatelet therapy. Despite dual antiplatelet therapy, large platelets with a higher proportion of reticulated platelets show increased *in vitro* reactivity compared to small platelets (78). Moreover, newly formed reticulated platelets show increased thrombogenicity after stopping prasugrel (75). Both observations suggest consequences for individualized antiplatelet therapy.

Procoagulant Platelets

About 30% of circulating platelets (range of 15–55%) can exhibit a procoagulant phenotype after stimulation with the agonists *co*llagen *a*nd *t*hrombin (79, 80). They were named COAT-platelets, which was later changed to coated platelets. Coated platelets express high levels of functional α-granule derived Factor V (FV) (79) and other α-granule proteins on their surface, including fibrinogen, von Willebrand factor, thrombospondin, fibronectin, and α_2-antiplasmin (81). Furthermore, coated platelets expose procoagulant phosphatidylserine (PS) on their surfaces (79, 82). PS exposure on the outer platelet membrane is closely related to disruption of inner mitochondrial membranes in the cells (83). In platelets, this process is controlled by calpain and not by caspases as in other cells (84). Therefore, PS exposure on procoagulant platelets is not necessarily related to apoptosis (85, 86). As not all PS-exposing platelets show the typical features of coated platelets, coated platelets seem to represent a procoagulant subgroup of PS-exposing platelets (82, 87).

Activation of the protease activated receptor 1 with thrombin, SFLLRN, and AYPGKF had strong additional effect (80) on the collagen-induced calcium peak and induced a sustained cytoplasmatic elevation of Ca^{2+} which is crucial for the formation of procoagulant platelets (88). Differential phosphorylation of PKCalpha and p38MAPK may drive the different calcium fluxes in coated compared to non-coated platelets (89). Increased cytosolic Ca^{2+} levels result in the inactivation of adenylatecyclase and activation of phosphatidylinositol 3-kinase and Src tyrosine kinase which further promotes procoagulant platelet segregation (90). On the other hand, elevated cytosolic Ca^{2+} levels can reverse integrin αIIbβ3 activation by stimulating intracellular cleavage of the β3-chain *via* calpain (91). PAC-1 binding is reduced in coated platelets although surface expression of αIIbβ3 is not diminished (89). The underlying mechanism is displacement of PAC-1 a stronger bond rather than inactivation of αIIbβ3 (92). This may explain why coated platelets do not take part in the formation of aggregates mediated by αIIbβ3.

Thus, platelet subpopulations arrange differently in a thrombus (93). Within a thrombus platelets with activated αIIbβ3 integrins assemble to aggregates. Those with inactive αIIbβ3 integrins remain solitary and form blebs and shed microparticles (93–95), the typical features of coated platelets. Independently of αIIbβ3, coated platelets attach to aggregates by forming caps of colocalized fibrinogen and thrombospondin on the PS-positive platelet surface (96). This allows coated platelets to become incorporated into thrombi independently of activated αIIbβ3 integrins.

Interestingly, platelet size has not yet been directly investigated as a feature of procoagulant human platelets. In rabbits, young platelets showed a similar size and the same ability to form procoagulant platelets under steady state compared to older platelets (74). If size is associated with the procoagulant capability of human platelets, it could be applied to enrich or deplete procoagulant platelets in PCs.

Platelets Exposing Signals for Clearance

Platelets survive for up to 10 days under normal conditions (97, 98). Platelets exposing signals to induce their clearance may be seen as another subpopulation with a limited life span. It would be desirable to reduce the amount of these platelets in PCs to prolong survival of transfused platelets.

Three main mechanisms have been identified by which platelets mediate their clearance (99). First, degraded glycans appear as a signal on platelet membranes which are recognized by the hepatic Ashwell Morrel Receptor (100). This has been demonstrated for cold stored platelets (101) and for platelets in sepsis (102, 103). Concomitantly, the removal of glycan deprived platelets *via* the Ashwell Morell Receptor in the liver induces hepatic thrombopoietin-mRNA expression and leads to increased megakaryocyte numbers and *de novo* platelet production (100).

The second mechanism is platelet apoptosis. Platelet survival is extended if the proapoptotic proteins Bak and Bax are lacking and reduced if the prosurvival proteins Bcl-2, Bcl-xL, and Mcl-1 are absent (104). Recently, protein kinase A was identified as a mediator of platelet life span by regulating apoptosis (105). However, the exact signals on the platelet surface and the corresponding receptor recognizing apoptotic platelets for platelet clearance are not yet identified. It is also unknown whether apoptotic signals appear differently in platelets of different size.

Finally, platelets are cleared after being opsonized with antibodies, which can be autoantibodies in diseases such as autoimmune thrombocytopenia, or alloantibodies in case of feto-maternal incompatibility, or after platelet transfusion (106). This mechanism is likely independent of platelet size.

Of note, P-selectin is an adhesion receptor for leukocytes expressed by activated platelets and was suggested to mediate platelet clearance. Berger et al. demonstrated that P-selectin does not mediate platelet clearance but may modulate leukocyte recruitment or thrombus growth (107).

CONCLUSION AND PERSPECTIVES

Understanding features differentiating platelet subpopulations has greatly improved. For example, platelet size correlates with platelet reactivity and mRNA content, which may classify large platelets as a prohemostatic subpopulation. These large platelets could be enriched in blood centers by differential or density gradient centrifugation, or special apheresis techniques in order to produce more potent PCs, e.g., for trauma patients.

It remains unclear, if large platelet fractions also include more procoagulant platelets. To gain further insight, PS-exposure, Ca^{2+}-mobilization and the ability to form coated platelets should be assessed in large and small platelets. Additionally, no data exist whether immunological functions of platelets correlate with platelet size.

Highly relevant for the interpretation of any study on the association of platelet size and platelet function is the fact that platelet turnover is important for platelet formation. Large platelets under steady state are likely different from large platelets generated under conditions of increased platelet turnover. This difference may explain some of the conflicting results on large and small platelets reported in the literature. It will be mandatory for future studies to exactly define the conditions of platelet turnover under which the investigated platelet population is generated as well as the agonists mediating thrombopoiesis in health and disease (108).

Platelet turnover may also be relevant for the production of PCs. Platelets derived from whole blood donation are collected under steady-state conditions because the donation procedure routinely lasts ~5–15 min and will unlikely result in changes in the collected platelets. In contrast, repeated platelet apheresis procedures may stimulate platelet generation because it lowers the platelet content more rapidly over a period of 60–90 min (109) and can be performed up to 3 times a week. This may have an effect on the collected platelet population, as shown for the platelet proteome after repeated apheresis (33). Moreover, platelets collected from hypertensive donors may differ in phenotype and functionality compared to those from normotensive donors (110).

Finally, PCs are produced by differential centrifugation leading to a loss of very large and very small platelets. Recently it was shown that the preparation procedure of red cell concentrates is associated with mortality (111). Enrichment of a specific platelet subpopulation in PCs by different preparation methods might also be relevant for the outcomes of transfused patients.

In summary, there is increasing evidence on platelet subpopulations with different biological functions, which are particularly interesting for transfusion medicine. Better understanding of the characteristics and functions of platelet subpopulations may be applied to develop new or improved platelet products.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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On the Way to *in vitro* Platelet Production

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The severely decreased platelet counts (10–30. 10^3 platelets/μL) frequently observed in patients undergoing chemotherapy, radiation treatment, or organ transplantation are associated with life-threatening increased bleeding risks. To circumvent these risks, platelet transfusion remains the treatment of choice, despite some limitations which include a limited shelf-life, storage-related deterioration, the development of alloantibodies in recipients and the transmission of infectious diseases. A sustained demand has evolved in recent years for controlled blood products, free of infectious, inflammatory, and immune risks. As a consequence, the challenge for blood centers in the near future will be to ensure an adequate supply of blood platelets, which calls for a reassessment of our transfusion models. To meet this challenge, many laboratories are now turning their research efforts toward the *in vitro* and customized production of blood platelets. In recent years, there has been a major enthusiasm for the cultured platelet production, as illustrated by the number of reviews that have appeared in recent years. The focus of the present review is to critically asses the arguments put forward in support of the culture of platelets for transfusion purposes. In light of this, we will recapitulate the main advances in this quickly evolving field, while noting the technical limitations to overcome to make cultured platelet a transfusional alternative.

Keywords: platelets, *in vitro* production, megakaryocytes, biomanufacturing, hematopoietic stem cells

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INTRODUCTION

Blood platelets are small anucleate cells (2–4 μm in diameter) derived from the cytoplasmic fragmentation of their MK precursor (1). MKs are produced in the bone marrow through a highly orchestrated process (2). Hematopoietic stem cells (HSCs) lie at the apex of this process and give rise to progenitors which progressively commit to the megakaryocytic lineage to produce immature MKs (3). MK maturation involves an increase in DNA content (up to 64N) through endomitosis accompanied by massive enlargement of the cytoplasm, the emergence of numerous alpha and dense granules and the development of an extensive membrane network, the demarcation membrane system (DMS) (4–6). Terminally differentiated MKs are intimately associated with the sinusoidal endothelium of the bone marrow. Following extensive cytoskeletal remodeling, fully mature MKs extend cytoplasmic projections called proplatelets into the vessel lumen, where platelets are released under shear forces produced by the circulating blood (7, 8). The entire sequence is strongly influenced by cytokines, extracellular matrix components, surface topography, matrix stiffness, and blood flow (9). This efficient procedure generates 10^{11} functional platelets per day to sustain an average count of 3.10^{11} platelets/L in man (10).

THE CULTURED PLATELETS IN THE TRANSFUSIONAL CONTEXT

More than 100 million blood donations are collected each year, but the transfusion situation varies greatly in different parts of the world. Nearly half of the donations are made in high-income countries, where < 20% of the world's population lives (WHO). In industrialized countries, blood banks operate on a just-in-time basis. Maintaining an adequate platelet supply, ensuring their appropriate use and guaranteeing transfusion safety, together with the prevention of the transmission of infectious diseases, are the main concerns of these blood banks.

In this context, the field of platelet and transfusion research has witnessed an increasing interest in producing platelets *in vitro*. A number of arguments are frequently put forward to justify this research on the grounds of three main threats: i) a risk of shortage, ii) the contamination hazard, and iii) the immunological risk.

i) The shortage threat: Maintaining appropriate stocks of platelet concentrates is becoming a major concern worldwide, due to the ever increasing number of patients experiencing long periods of severe thrombocytopenia related to bone marrow failure, anti-cancer therapy, bone marrow grafts, or immune-related or drug-induced thrombocytopenia (11). The short *in vivo* half-life of human platelets imposes regular platelet transfusions for these patients, while a maximum shelf-life of 5 days further increases the demand for platelets. In the USA, platelet transfusion rose by 7.3% from 2008 to 2011 and the market for platelets is expected to grow at a rate of 5.3% per annum over the next decade (12). This enhanced need has been cited to advocate the development of *in vitro* platelet production, although these figures might not apply equally to all countries. In France, for example, platelet transfusion increased by only 0.5% from 2012 to 2016 and has remained stable since, principally due to new guidelines allowing a reduction in the number of transfused platelets per unit body weight (13). Whereas this has shelved the prospect of a short term shortage, the long term trend merits surveillance. In any event, all countries are facing situations with peak demands and/or periods of low blood donation (vacations, public holidays…) where cultured platelets could represent a real alternative to maintain optimal stocks of platelet concentrates.
ii) The contamination hazard: Platelet transfusion has been routine practice for over five decades (14) but is however not devoid of potential risks. A bacterial contamination remains the major cause of platelet transfusion-related morbidity and mortality (15). Fortunately, the introduction of pathogen inactivation systems and bacterial detection tests, together with careful donor screening and rigorous skin disinfection, has raised transfusion safety to levels never achieved before (16). Nevertheless, the risks of biological hazards and contamination of blood products cannot be totally eliminated and also vary widely between countries. Platelets can capture emergent pathogens which remain undetectable or possibly resistant to inactivation, leading to a residual risk of infection (17). To circumvent these drawbacks and reach conditions of absolute safety, cultured platelets could be an attractive alternative.
iii) The immunological risk: Alloimmunization and platelet refractoriness remain major complications associated with platelet transfusion, despite the introduction of leukodepletion methods (18). The selection of HLA-compatible platelets and/or crossmatch-negative donors can solve these problems but is often difficult to achieve (19). In addition to alloimmunization, ABO-incompatibility can result in weaker transfusion efficacy (20). These problems could be resolved by the generation of universal cultured platelets lacking HLA class I and expressing preferably 0 antigens to improve their compatibility (21).

In summary, although platelet transfusion remains self-sustainable and safe, transfusion practices are destined to evolve, justifying as a precautionary measure research focusing on the efficient culture of platelets. The availability of cultured platelets, free of infectious, inflammatory, and immune risks, would undoubtedly be a real step forward for patients requiring frequent blood transfusions or lacking suitable compatible donors.

OVERVIEW OF THE CHALLENGE

Platelet culture for transfusion will be quite a challenging task. It will require their production in amounts equivalent to one unit ($2–5.10^{11}$) of apheresis- or buffy coat-derived platelets and with the quality and functionality of native platelets. In essence, the *in vitro* conditions need to reproduce as closely as possible the *in vivo* environment. Assuming that each bone marrow megakaryocyte (MK) generates 2000–3000 platelets, 250.10^6 mature MKs will be needed to produce one unit of platelet concentrate. However, despite an increasing knowledge of the molecular and cellular mechanisms governing platelet production and the development of innovative bioreactor technologies, the current yields have remained limited to 100 to 150 platelets/MK over the past several years (22, 23). To meet the challenge still ahead, there is a need to develop further knowledge (**Figure 1**).

1. To reach sufficient MK progenitor amplification efficiencies to obtain the equivalent of one unit of platelets ($\sim 5.10^{11}$ platelets);
2. To obtain a level of MK maturation closely matching that of the bone marrow;
3. To efficiently release platelets from mature MKs;
4. To demonstrate native hemostatic properties and functionality following transfusion.

IMPROVING MK AMPLIFICATION EFFICIENCIES

The source of hematopoietic progenitors/stem cells is of paramount importance and conditions the strategies and expansion capacities. Two main sources have been used i) pluripotent stem cells, including human embryonic stem cells

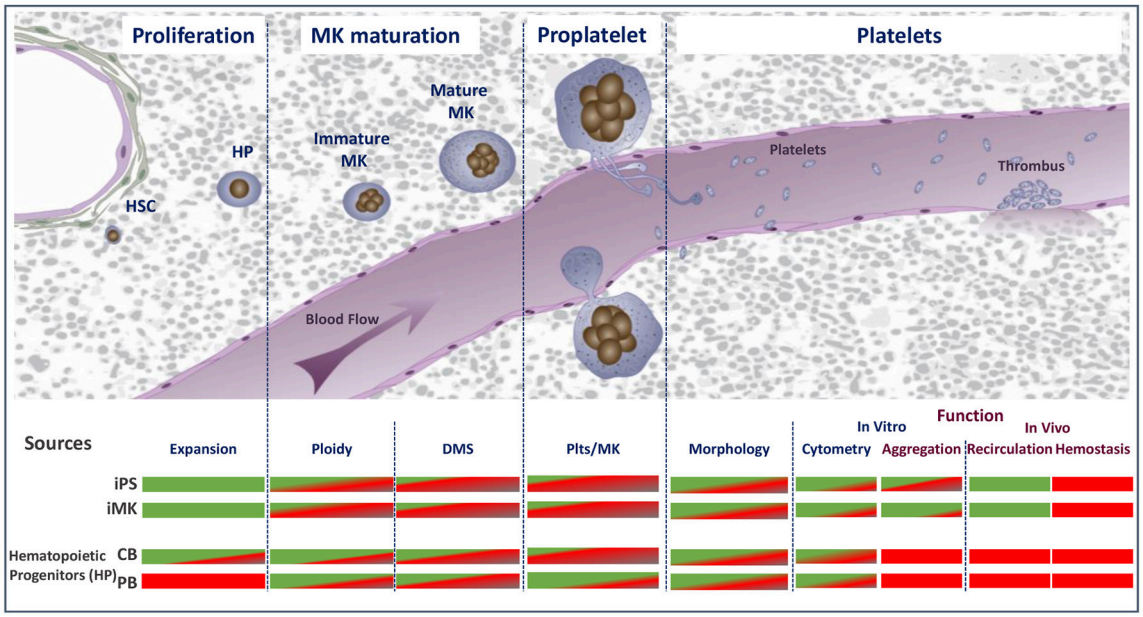

FIGURE 1 | Schematic representation of the major stages of platelet biogenesis coupled with an overview of the main technical or biological hurdles that have either been overcome (green) or need to be met (red) to consider cultured platelet as a clinical alternative. HSC, hematopoietic stem cells; HP, hematopoietic progenitors; MK, megakaryocytes; DMS, demarcation membrane system; Plts, platelets; iPS, induced pluripotent stem cells; iMK, immortalized megakaryocytes.

(hESCs) and induced pluripotent stem cells (iPSCs), and ii) hematopoietic progenitors derived from bone marrow (BM), cord blood (CB) and peripheral blood (PB) (CD34+ cells). Each of these offers advantages and disadvantages for the development of a transfusion product.

i) Pluripotent stem cells: hESCs and iPSCs both possess the significant advantage of a self-renewal capacity. iPSCs offer the additional benefit of avoiding the ethical concerns raised by hESCs and therefore constitute the most attractive pluripotent stem cells (24). The reader can refer to two previous excellent reviews on this subject (25, 26). Significant progress has been made in iPSC engineering to enhance platelet production. A promising development has undoubtedly been the generation of two types of expandable MK line (25). One type was obtained following several optimization steps resulting in the sequential introduction of c-MYC, BMI1, and BCL-XL (27). The second was developed by overexpressing the transcription factors GATA-1, FLI1, and TAL1 under chemically defined conditions (28). Both cell lines tolerate cryopreservation and can be expanded upon demand to generate platelets with higher efficiency and in shorter times as compared to iPSC-derived MKs. In the objective of avoiding platelet transfusion refractoriness, another remarkable achievement has been the generation of iPSC-derived HLA class I-silenced MKs and platelets using RNA-interference TALEN or *CRISPR/Cas9* editing strategies (29–31).

   Although the above arguments speak in favor of iPSCs, this source still faces a number of drawbacks. The yield of platelets remains low with < 10 platelets/MK, possibly due to some immaturity of iPSC-derived MKs (low ploidy and a less well developed DMS) (25, 27, 28, 32). Another drawback for clinical applications is the potential tumorigenicity of these cells. This risk is considered to be minor on the grounds that platelets lack replication and can be irradiated before transfusion (22, 25). However, caution might prevail and impede their acceptance by regulatory authorities. In any case, careful separation of the *bona fide* platelets from other cellular elements, such as nucleated cells including immature MK, large fragments with remnant nuclear material (DNA, RNA), in the final culture suspension will be required to minimize gene-related risks.

ii) Hematopoietic progenitors: As compared to hESCs and iPSCs, hematopoietic progenitors, conventionally isolated through their CD34 positivity, are technologically easier to manage for platelet generation. They can be derived from cord blood (CB), bone marrow (BM) or peripheral blood (PB) and their harvest is easy and rapid with no ethical concerns and low cost (33). The major advantage of CD34+ cells is their platelet yield, usually around 100–150 platelets/MK, while several studies have mentioned that platelets derived from these progenitors share ultrastructural and functional characteristics with circulating platelets (34, 35).

One limitation often evoked to oppose the use of CD34+ cells is their finite expansion. However, large numbers of safe CB-derived HSCs are stored around the world and could be used for the bio-manufacture of platelets. Similarly, PB-derived CD34+ cells eluted from leukoreduction filters (LRFs) represent a source with strong potential. Around $0.4.10^6$ PB-derived CD34+ cells

can be eluted from one LRF and the French Blood Bank, for example, destroys more than 3.10^6 LRFs/year (36). LRFs are an easily available and safe source of cells submitted to a stringent quality control process. Automation and standardization of the process would allow the constitution of large homogeneous and safe CD34+ cell pools. Even if these cells do not possess the theoretical unlimited expansion potential of iPSCs, recent studies indicate that this could be partly overcome by the use of novel agents like StemRegenin 1 (SR1) (37), nicotinamide (NAM) (38), mesenchymal stromal cell (MSC) coculture (39), or notch ligands (40). Moreover, CD34+ cells harbor the same potential as iPSCs to produce HLA-deficient platelets, since both CB- and PB-derived HSCs could be selected and pooled according to their HLA/ABO phenotype to generate compatible platelets for transfusion. Altogether, the availability of safe, HLA/ABO-pooled, CB-, and LRF-derived CD34+ cells combined with new strategies favoring their proliferation could lead to a regain of the use of CD34+ cells for platelet production.

Choosing between iPSCs and hematopoietic progenitors will be a matter of compromise taking into account the proliferation and maturation potentials of the cells when planning large-scale cultures. Despite undeniable technological advances, platelet production from iPSCs requires relatively complex and sophisticated methods, which might complicate the industrial-scale generation of cultured platelets. We postulate that CD34+ cells derived from PB, which are presently underexploited, could represent an interesting trade-off in terms of their availability, cost, and MK maturation and platelet yields (35).

OBTAINING A LEVEL OF MK MATURATION CLOSELY MATCHING THAT OF THE BM

Efficient platelet production requires a high degree of MK maturation, which is itself dependent on i) efficient endomitosis and ii) DMS expansion (2). Endomitosis contributes to the production of large amounts of proteins and to organelle development within a single MK (6). DMS expansion provides the reservoir of membranes required to feed the extension of numerous proplatelets and *in fine* the release of individual platelets. To reach an optimal degree of MK maturation *in vitro* we have to faithfully mimic these processes, which are influenced by specific microenvironments in the bone marrow (cytokines, stiffness) and depend on efficient lipid biosynthesis.

i) Ploidization. So far, even under optimal conditions, MKs derived from adult progenitors or iPSCs present lower ploidy levels than MKs resident in the bone marrow, indicating a certain lack of maturity. Consistent with this finding, it has been observed that smaller and less polyploid MKs produce fewer platelets than larger MKs (41). This has also been documented in fetal and neonatal MKs, which are significantly smaller and of lower ploidy than adult MKs and produce fewer platelets than their adult counterparts (42). Whereas the mechanisms underlying the small size and low ploidy of neonatal MKs remain unclear, Elagib et al. recently identified an RNA-binding protein, IGF2BP3, regulating the human fetal-adult MK transition (43). These authors demonstrated that downregulation of IGF2BP3 using a lentiviral strategy enhanced neonatal MK enlargement, growth arrest, and polyploidization. In addition, use of a pharmacological inhibitor of IGF2BP3 expression elicited adult features in neonatal MKs. This could open the way to the development of new strategies to enhance MK maturation and platelet production. Other molecules favoring endomitosis have been identified, such as actin polymerization inhibitors and Rho kinase inhibitors (44–46), but these agents did not significantly improve platelet yields.

ii) DMS expansion. The production by a single MK of thousands of platelets requires considerable membrane synthesis and its folding into a well-organized DMS. The DMS is fueled by invagination from the outer membrane with further contributions from internal golgi-derived membranes and contacts with the endoplasmic reticulum, which together provide a continuous membrane supply (4). It has been reported that immature MKs have a high capacity for cholesterol and phospholipid synthesis and are also able to capture fatty acids (47). The importance of cholesterol uptake is further suggested by studies showing that hypercholesterolemia positively influences platelet production (48). A better knowledge of the lipid pathways involved in MK maturation could help us to devise culture media supplements favoring platelet production.

Bone marrow is a complex and dynamic cellular tissue where MKs interact with other cells and protein matrices in a 3-dimensional (3D) configuration (49). Recent findings have highlighted the environmental stiffness of the bone marrow as a key regulator of MK maturation, whereby adaptation of the cells to the surrounding physical constraints favors higher ploidy and proplatelet formation (34, 50). These observations can be applied directly to *in vitro* platelet production. Thus, Aguilar et al. recently showed that MKs grown in 2% methylcellulose (30–60 Pa) exhibited enhanced DMS expansion leading to increased platelet production. Mechanistically, these authors demonstrated the increased nuclear translocation of an important regulator of MK maturation, megakaryoblastic leukemia factor-1 (MKL1), which was triggered by the physical constraints (51). Identifying the stiffness-mediated factors involved in MK maturation should provide an important means of improving the production of platelets in culture.

EFFICIENTLY RELEASING PLATELETS FROM MATURE MKS

In vivo, under physiological conditions, efficient platelet release requires i) the transmigration of proplatelets/MK fragments through the endothelial barrier and ii) their exposure to the blood flow (8).

i) Endothelial cells. Upon reaching the sinusoids, mature MKs come into contact with endothelial cells. A seminal study conducted by Rafii et al. demonstrated that human BM microvascular endothelial cells (BMECs) specifically supported the MK differentiation of CD34+ progenitors

(52). More recently, the interplay between MKs and the sinusoidal barrier has been examined in more detail. It could be shown that MKs form podosomes which are able to degrade the extracellular matrix, allowing elongation of proplatelets into the lumen (53). The importance of podosomes in thrombopoiesis is further suggested by the occurrence of thrombocytopenia in primary genetic deficiencies affecting podosome formation (WASP, CDc42, α-actinin, or CD44) (54, 55). Work by Antkowiack et al. also indicates that EC contacts triggering podosome formation could participate in the DMS polarization preceding proplatelet extension (56).

Current efforts in bioreactor development will require additional research to reveal the specific mechanisms involved in the transmigration of mature MKs into the lumen. Endothelial cells have already been introduced into 3D flow systems but did not appear to positively affect proplatelet elongation or platelet numbers (34, 57). The positive impact of EC might depend on the additional presence of soluble factors such as Il1b, an inflammatory cytokine reported to enhance MK and EC interactions (58). Stimulating the endothelium through the VEGFR1-mediated pathway also increased platelet production (59). Finally, a signaling lipid circulating in the blood, S1P, has been proposed as a new key regulator of platelet release, *in vitro* and under flow conditions (60).

ii) Blood flow. When left under static conditions, MKs extending proplatelets liberate individual platelets with a very low efficiency (61). Thus, to mimic *in vivo* conditions where platelet release depends heavily on shear forces (8, 60), a number of laboratories have integrated flow into newly developed scalable microfluidic platelet bioreactors. Baruch et al. have designed a bioreactor comprising a multitude of staggered pillars covered with von Willebrand factor. MKs adhering to the pillars and subjected to hydrodynamic forces stretch out long extensions and release platelet-like elements (62). Another microchamber developed by Thon et al. consists of one channel separated by a series of 2 μm diameter gaps from another channel where flow is applied. Trapped MKs extend proplatelets and release platelets into the second channel more efficiently than in the absence of flow (63). Nakagawa et al. have designed original gaps where trapped MKs are submitted to a bidirectional flow, applied at a 60° angle reported to be 3.6 times more effective than a 90° platelet release angle (64). Although this is an attractive variant, incorporation of this geometry into future devices will require thorough morphological and functional analysis of the platelets released. The development of these bioreactors has established proof of the feasibility of *ex vivo* platelet production. However, despite taking into account extracellular matrix proteins, stiffness, and flow, the platelet yields obtained are only of the order of 30–50 platelets/MK.

DEMONSTRATING NATIVE FUNCTIONALITY AND HEMOSTATIC PROPERTIES FOLLOWING TRANSFUSION

If cultured platelets are to be considered as a clinical alternative, they must equal the quality of donor-derived platelets in terms of i) morphology and ultrastructure and ii) *in vitro,* and iii) *in vivo* functionality (65). So far, studies of the quality and functionality of cultured platelets are still fragmentary.

i) Morphology and ultrastructure. Native platelets are typically anucleate, exhibit a characteristic discoid shape and are filled with secretory granules (α, δ, lysosomes) which contain endogenously synthesized or endocytosed molecules.

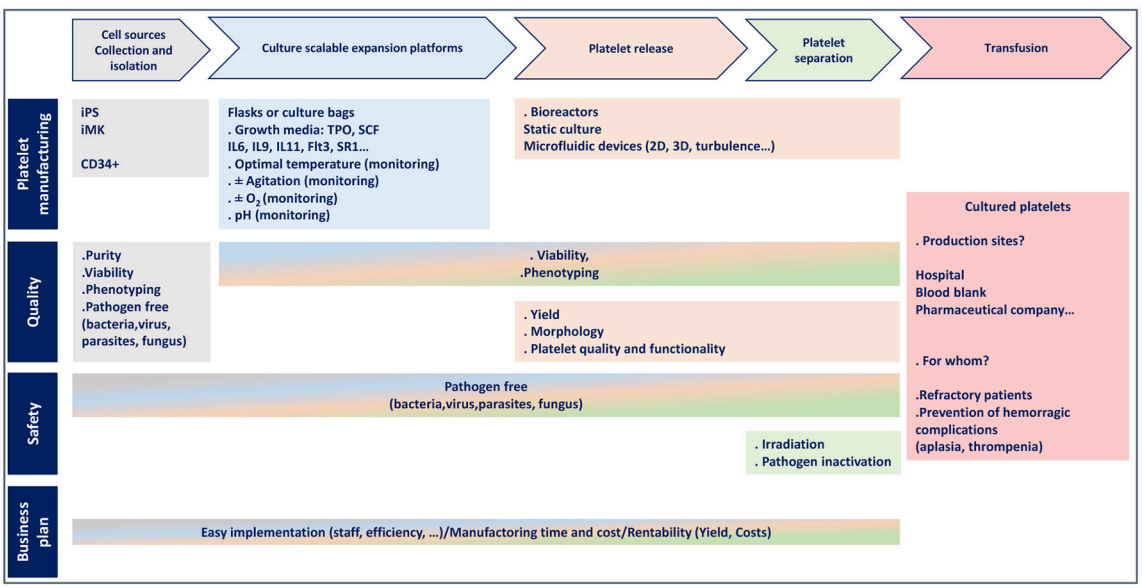

FIGURE 2 | Flowchart for platelet manufacturing.

Microscopic analyses have revealed that cultured platelets are typically larger than native ones and have an increased RNA content, two characteristic features of "young" platelets (27, 32, 35, 57). This raises the question of whether such immaturity is useful or detrimental for platelet recirculation after transfusion. In addition, platelet functions are largely dependent on the molecules stored in their granules. Culture media do not usually contain certain components required for platelet function such as fibrinogen or serotonin. Studies will be required to determine whether we need to load platelets with proteins they lack during culture, or whether they are capable of filling their granules during recirculation to ensure their normal function.

ii) *In vitro* evaluation of platelet function. In the majority of studies, the functionality of the platelets generated *in vitro* has only been incompletely addressed. The tests have mostly relied on flow cytometric measurement of P-selectin exposure and PAC-1 binding to detect GPIIb-IIIa activation. Usually, a large proportion of cultured platelets express these activation markers upon stimulation by agonists such as ADP or thrombin (27, 32, 34). One may note that a state of pre-activation, visualized by P-selectin expression, is often observed in the absence of any agonist (66, 67). In itself, this positivity does not inevitably predict poor transfusion properties. Indeed, in one study it was reported that circulating degranulated platelets rapidly lose surface P-selectin to the plasma pool but continue to circulate and function *in vivo* (68). The demonstration of platelet aggregation has often been restricted to a flow cytometric approach (2 color assays), due to the limited numbers of purified platelets obtained in culture (27, 34). However, as shown by Feng et al., this should not routinely exempt us from standard aggregometry testing as a quality control for transfusion applications (32).

iii) *In vivo* evaluation of platelet function. The *in vivo* functionality of cultured platelets has mainly been attested on the basis of their ability to participate in a developing thrombus after vessel injury in the mouse (27, 32, 35). Concerning their capacity to recirculate, this has only been demonstrated in a few studies. A quite similar half-life to that of native platelets was observed after transfusion into immune-deficient mice (27, 28, 32). Although these results are encouraging, they do not provide a definitive answer to the question of the true ability of cultured platelets to fulfill their functions. Finally, there are to date no available data concerning the *in vivo* hemostatic properties of the cells, i.e., their capacity to correct a bleeding tendency in thrombocytopenic individuals. With the declared ambition of being a transfusion substitute, it is now time to move on in the area of the functionality of cultured platelets with an accurate evaluation of their hemostatic potential in thrombocytopenic mice.

CONCLUSION AND PERSPECTIVES

Over the past 5 years, considerable efforts have been made to improve the production of platelets *in vitro,* mainly in relation to iPSC generation and the availability of universal platelets, together with the design of original and scalable bioreactors. A dozen dedicated teams around the world are competing to achieve *ex vivo* platelet production. However, in addition to their important research efforts, it is also important to consider production costs which have to be greatly reduced to make cultured platelet an economic reality (**Figure 2**). In this respect, optimization of the crucial steps of platelet generation (MK maturation and platelet release) and a better understanding of the molecular and cellular mechanisms governing platelet production will be required to make cultured platelets a clinical alternative in certain situations. In any case, one must recall that despite the technical advances and enthusiasm underlying this challenge, nothing will ever replace the voluntary, free and anonymous donation of blood.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Metabolism of Citrate and Other Carboxylic Acids in Erythrocytes As a Function of Oxygen Saturation and Refrigerated Storage

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State-of-the-art proteomics technologies have recently helped to elucidate the unanticipated complexity of red blood cell metabolism. One recent example is citrate metabolism, which is catalyzed by cytosolic isoforms of Krebs cycle enzymes that are present and active in mature erythrocytes and was determined using quantitative metabolic flux analysis. In previous studies, we reported significant increases in glycolytic fluxes in red blood cells exposed to hypoxia *in vitro* or *in vivo*, an observation relevant to transfusion medicine owing to the potential benefits associated with hypoxic storage of packed red blood cells. Here, using a combination of steady state and quantitative tracing metabolomics experiments with $^{13}C_{1,2,3}$-glucose, $^{13}C_6$-citrate, $^{13}C_5{}^{15}N_2$-glutamine, and $^{13}C_1$-aspartate *via* ultra-high performance liquid chromatography coupled on line with mass spectrometry, we observed that hypoxia *in vivo* and *in vitro* promotes consumption of citrate and other carboxylates. These metabolic reactions are theoretically explained by the activity of cytosolic malate dehydrogenase 1 and isocitrate dehydrogenase 1 (abundantly represented in the red blood cell proteome), though moonlighting functions of additional enzymes cannot be ruled out. These observations enhance understanding of red blood cell metabolic responses to hypoxia, which could be relevant to understand systemic physiological and pathological responses to high altitude, ischemia, hemorrhage, sepsis, pulmonary hypertension, or hemoglobinopathies. Results from this study will also inform the design and testing of novel additive solutions that optimize red blood cell storage under oxygen-controlled conditions.

Keywords: hypoxia, metabolomics, mass spectrometry, tracing experiments, flux analysis

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INTRODUCTION

Approximately 31,000 packed red blood cell (RBC) units are transfused every day in the US alone (1), thus illustrating the importance of RBC transfusion as a life-saving procedure for millions of people around the world. One hundred years of advancements in the field of transfusion medicine [as reviewed here (2, 3)] have tackled many of the issues associated with making ~110 million units/year

available for transfusion all over the world. Though logistically inevitable, refrigerated storage of packed RBCs in the blood bank results in the progressive accumulation of a series of biochemical and morphological alterations, collectively termed the "storage lesion" (4–6). Hallmarks of the storage lesion include the early onset of an impaired energy and redox metabolism (7), which in turn affects redox homeostasis of proteins (8–10), lipids (11–13), and various small molecule metabolites (13–15). Reassuringly, evidence from randomized clinical trials [RCTs—extensively reviewed by Belpulsi and colleagues (16)] suggests that the general standard of care would not be improved by exclusively issuing fresh RBCs, at least for the clinical indications addressed by, and within the statistical power of, the completed RCTs. One tentative explanation reconciling the lack of correlation between the well-established storage lesion and the RCT results could involve the underappreciated role that donor and recipient biology plays in mediating transfusion safety and efficacy (17). In the last 7 years, such large-scale studies as the Recipient Epidemiology and Donor Evaluation Study-III have addressed the issue of biological variability and found that biological variability across donors (i.e., donor ethnicity, gender, and age) affects RBC storability and stress hemolysis (18). Such observations have been supported by smaller scale laboratory studies in humans (19, 20) that demonstrated heritability of the metabolic storage lesion (21–23), as well as studies performed in mice (24, 25) showing that post-transfusion recoveries are greatly variable across donors (26). Of note, Yoshida and colleagues have recently provided preliminary evidence suggesting that hemoglobin oxygen saturation (SO_2) at 8 h from donation and routine processing varies significantly across donors (27), potentially contributing to the donor-dependent development of the storage lesion. This is relevant in light of accumulating evidence suggesting that SO_2 significantly impacts RBC metabolism, as is the case in exposure to high-altitude hypoxia or hemorrhagic hypoxia (28, 29), as well as hypoxic storage in the blood bank (30–32). Hypoxic storage boosts energy metabolism and limits oxidative challenge to stored RBC proteins (10, 33), a phenomenon in part explained by the intracellular alkalinization accompanying the simultaneous removal of oxygen and carbon dioxide from the unit (34), as well as by the oxygen-dependent metabolic modulation of glycolytic enzyme activity (10, 35–37). Some of the benefits of anaerobic storage can indeed be phenocopied by alkaline additives (38, 39), which have been shown to boost glycolysis, Rapoport-Luebering shunt and pentose phosphate pathway activation (40) through a positive pH-dependent regulation of phosphofructokinase, bisphosphoglycerate mutase, and glucose 6-phosphate dehydrogenase (2). Because beneficial effects of metabolic interventions to attenuate the storage lesion have been demonstrated by washing and/or rejuvenating end-of-storage erythrocytes (41), boosting RBC metabolism through a combination of SO_2 control and novel additive solutions may represent a viable strategy to tackle the storability issue and further improve RBC storage quality in the future. Understanding how erythrocyte metabolism is affected by normoxia and hypoxia *in vivo* and *ex vivo* under refrigerated conditions is key to the development of novel additive solutions tailored to packed RBCs stored under oxygen-controlled conditions. In this view, it is worth considering how recent advancements in proteomics have expanded our understanding of the RBC proteome complexity, which was thought to include ~750 proteins just a decade ago (42) and is now known to enlist ~2,800 (43) and counting (44). While identification of trace levels of an enzyme in RBCs does not necessarily imply that the enzyme is functionally active, it has been recently demonstrated through flux experiments using stable isotope tracers that cytosolic isoforms of Krebs cycle enzymes are present and active in mitochondria-devoid human erythrocytes (44), an observation that is relevant for the RBC metabolism of citrate when stored in the most common additives in Europe [SAGM (45, 46)] and in the US [e.g., AS-3 (13)]. In these studies, it was shown that citrate metabolism can contribute to a varying percentage of lactate generation during storage progression (13, 45, 46). Since hypoxia promotes glycolysis and lactate generation in a SO_2-dependent fashion (10), we hypothesized that carboxylic acid metabolism (including citrate metabolism) in mature RBCs may be affected by hypoxia *in vivo* and *ex vivo* during short term (24 h) and prolonged refrigerated storage (up to 42 days) under SO_2-controlled conditions. To test this hypothesis, we re-analyzed RBCs from individuals exposed to high-altitude hypoxia to specifically look for carboxylates, as an expansion of the AltitudeOmics study (28). Moreover, we performed integrated metabolic tracing experiments in the presence of different stable isotope-labeled substrates (citrate, glucose, aspartate, and glutamine) in order to determine how hypoxia affected RBC metabolism of these substrates under normoxic and hypoxic conditions.

MATERIALS AND METHODS

Blood samples were collected from healthy donor volunteers upon receiving written informed consent and in conformity with the Declarations of Helsinki under protocol approved by the University of Texas Houston and University of Colorado Denver institutional review boards (no. AWC-14-0127 and 11-1581, respectively). Commercial reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA) unless otherwise noted.

Human RBCs, Stored under Normoxic or Hypoxic Conditions

Blood was collected from healthy donors at the Bonfils Blood Center (Denver, CO, USA) according to the Declaration of Helsinki. Filter leukocyte-reduced (>99.95% WBC depleted—Pall Medical, Braintree, MA, USA) packed RBCs were stored in CP2D-AS-3 ($n = 4$; Haemonetics Corp., Braintree, MA, USA). Units were sterilely sampled (0.1 mL per time point) on a weekly basis until storage day 42, and cells and supernatants were separated by centrifugation at 2,000 × *g* for 10 min at 4°C.

High-Altitude Studies

Whole blood was collected from 12 male and 9 female healthy human volunteers at sea level or after 3 h (ALT1 am), >8 h (ALT1 pm), or 7 days (ALT7) of exposure to high-altitude hypoxia (5,260 m) in Mt. Chacaltaya, Bolivia, within the framework of the AltitudeOmics study (28). RBCs were separated from whole

blood through gentle centrifugation (~99% WBC depleted), as described (28).

Labeling Experiments

$^{13}C_{1,2,3}$-Glucose and RBC Storage under Controlled Oxygen Saturation Conditions

Filter leukocyte-reduced (>99.95% WBC depleted—Pall Medical, Braintree, MA, USA) packed red blood cells ($n = 4$) were collected, processed, and stored in CP2D-AS-3, as described above, supplemented with additional 11 mM $^{13}C_{1,2,3}$-glucose (no. CLM-4673-PK—Cambridge Isotope Laboratories Inc.—Tewksbury, MA, USA) prior to storage at six different oxygen saturation conditions, monitored throughout storage duration—including controls (untreated—averaging $SO_2 = 47 \pm 20$), hyperoxic ($SO_2 > 95\%$), and hypoxic ($SO_2 = 20\%$, 10%, 5%, or <3%), as previously described (10, 27).

Tracing Experiments from Heavy Citrate, Glutamine Aspartate, and Glucose in Hypoxia and Normoxia for 24 h

Filter leukocyte-reduced (>99.95% WBC depleted—Pall Medical, Braintree, MA, USA) RBCs ($n = 3$) were stored for up to 24 h under normoxia ($PO_2 = 21\%$) or hypoxia ($PO_2 = 8\%$) in CP2D-AS-3 prepared in house (four independent experiments) in the presence of U-^{13}C-glucose (55 mM—Sigma-Aldrich Catalog no. 389374), $^{13}C_6$-citric acid (Sigma-Aldrich Catalog no. 606081—2.2 mM), $^{13}C_1$-aspartate (Sigma-Aldrich Catalog no. 489972—1 mM), or $^{13}C_5{}^{15}N_2$-glutamine (Sigma-Aldrich Catalog no. 607983—4 mM).

Sample Processing

Packed RBCs and supernatants were extracted in ice cold extraction solution (Optima LC-MS grade methanol:acetonitrile:water 5:3:2 v/v) at 1:10 or 1:25 dilutions, prior to vortexing for 30 min at 4°C. Insoluble proteins were pelleted by centrifugation at 4°C for 10 min at 10,000 × *g* and supernatants were collected and stored at −80°C until subsequent analysis.

UHPLC-MS Metabolomics Analysis

Sample extracts were analyzed by UHPLC-MS, as previously reported (47). Briefly, analyses were performed on a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were resolved over a Kinetex C18 column, 2.1 mm × 150 mm, 1.7 µm particle size (Phenomenex, Torrance, CA, USA) at 25°C using an isocratic runs with 5% B for 3 min at 250 µl/min or a 9 min method from 5 to 95% B flowed at 450 µl/min and 30°C, where mobile phase A consisted of water + 0.1% formic acid (for positive mode) or 5 mM ammonium acetate (for negative mode) and mobile phase B consisted of acetonitrile water + 0.1% formic acid (for positive mode) or 5 mM ammonium acetate (for negative mode). The mass spectrometer was operated independently in positive or negative ion mode scanning in Full MS mode (2 µscans) at 70,000 resolution from 60 to 900 *m/z*, with electrospray ionization operating at 4 kV spray voltage, 15 shealth gas, 5 auxiliary gas. Calibration was performed prior to analysis using the Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data was converted from .raw to .mzXML file format using Mass Matrix (Cleveland, OH, USA). Metabolite assignments, isotopologue distributions and correction for expected natural abundance of 13C and 15N isotopes were performed using MAVEN (Princeton, NJ, USA) (48).

Graphs were plotted and statistical analyses (either *T*-test or repeated measures ANOVA) performed with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was assessed through repeated measure ANOVA (time course), two way-ANOVA (SO_2 conditions), and *T*-test (% isotopologue enrichment)—threshold being $p < 0.05$.

RESULTS

High-Altitude Hypoxia Affects Steady-State Levels of Carboxylates in Human RBCs

Red blood cells were collected from 21 healthy volunteers (12 male and 9 female) at sea level (SL—Oregon) or within <3 h (ALT1 noon), 8–12 h (ALT1 pm), 7, or 16 days (ALT7 and ALT16, respectively) of exposure to high-altitude hypoxia in Bolivia (Mt. Chacaltaya, >5,260 m) (**Figure 1A**), within the framework of the AltitudeOmics study (28, 29). Even though previous metabolomics analyses of these RBCs did not cover carboxylic acids (28), new analyses were performed in light of the recent appreciation of carboxylic acid metabolism in mitochondria-deficient mature erythrocytes (13, 45, 46). Exposure to high-altitude hypoxia resulted in a progressive decrease in the RBC levels of carboxylic acids citrate, alpha-ketoglutarate, and 2-hydroxyglutarate from baseline levels at SL, and proportionally to the duration of stay at high altitude (**Figure 1B**). Transient decreases within hours after exposure to high altitude and progressive increases after 8–12 h during altitude acclimatization were observed for RBC fumarate and malate (**Figure 1B**). In parallel, elevated ratios of pyruvate/lactate [a proxy for NADH/NAD + ratios according to the mass action law (49)] and reduced/oxidized glutathione (GSH/GSSG) (**Figure 1B**) were observed, representing markers of a progressively increased reducing environment in the cytosol of RBCs from individuals acclimatizing to high-altitude hypoxia.

Ex Vivo Preservation of Packed RBCs under Controlled SO_2 Conditions Promotes Citrate Consumption and Accumulation of Fumarate, Malate, and Alpha-Ketoglutarate

To determine whether the observations in RBCs from individuals exposed to high-altitude hypoxia would be translatable to RBCs stored under oxygen-controlled conditions, we stored RBCs under normoxia (untreated—$SO_2 = 47\% \pm 21$, mean ± SD), hyperoxia ($SO_2 > 95\%$), or four hypoxic conditions ($SO_2 = 20$, 10, 5, or <3%—**Figure 2**). Citrate consumption proportional to the degree of hypoxia was observed in supernatants and, most

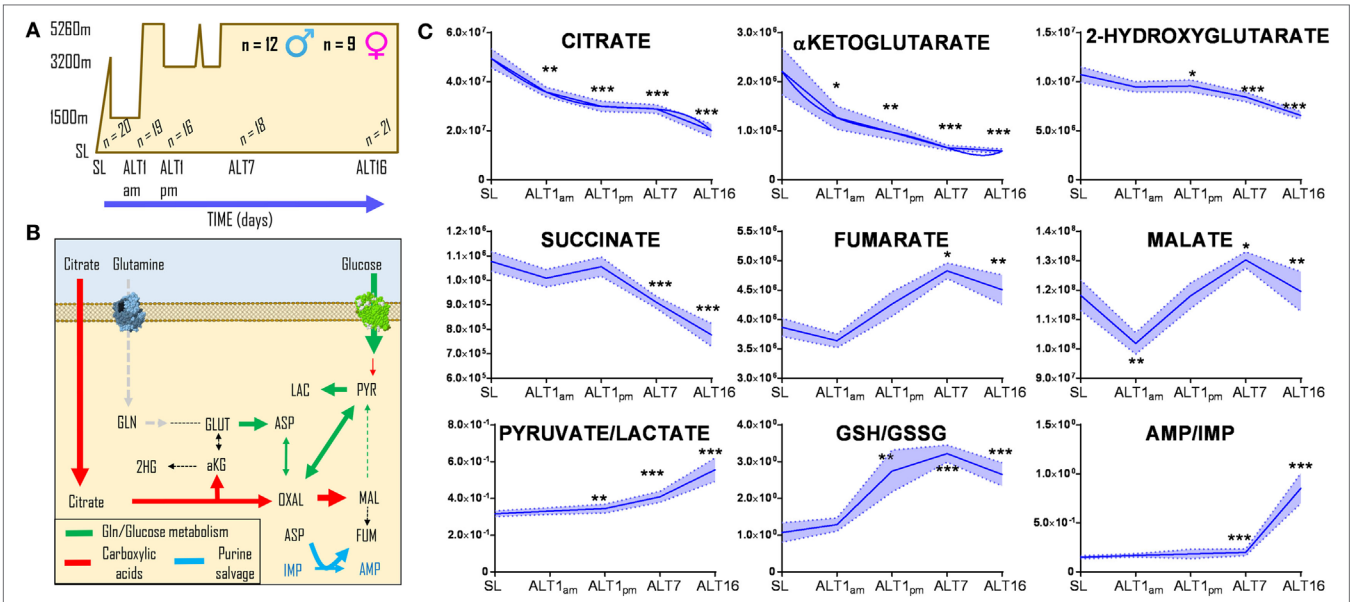

FIGURE 1 | Acclimatization to high-altitude hypoxia decreases steady-state levels of carboxylic acids in human red blood cells. Twenty-one healthy volunteers (12 male and 9 female) were flown from sea level (Oregon) to Bolivia (>5,260 m) for up to 16 days **(A)**, within the framework of the AltitudeOmics study (28, 29). While all of them successfully acclimatized to high-altitude hypoxia (28, 29), red blood cell (RBC) levels **(B)** of citrate, alpha-ketoglutarate, hydroxyglutarate, and succinate decrease from sea level to high altitude, proportionally to the duration of stay at over 5,000 m. Transient decrease and progressive increases in fumarate and malate were observed, paralleled by increases in the pyruvate/lactate ratios and reduced/oxidized glutathione (GSH/GSSG) ratios, suggestive of a progressively more reducing environment in the cytosol of RBCs from individuals acclimatizing to high-altitude hypoxia. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (repeated measures ANOVA). All data points on *x* axis were tested [*n* for each data point is reported in panel **(C)**].

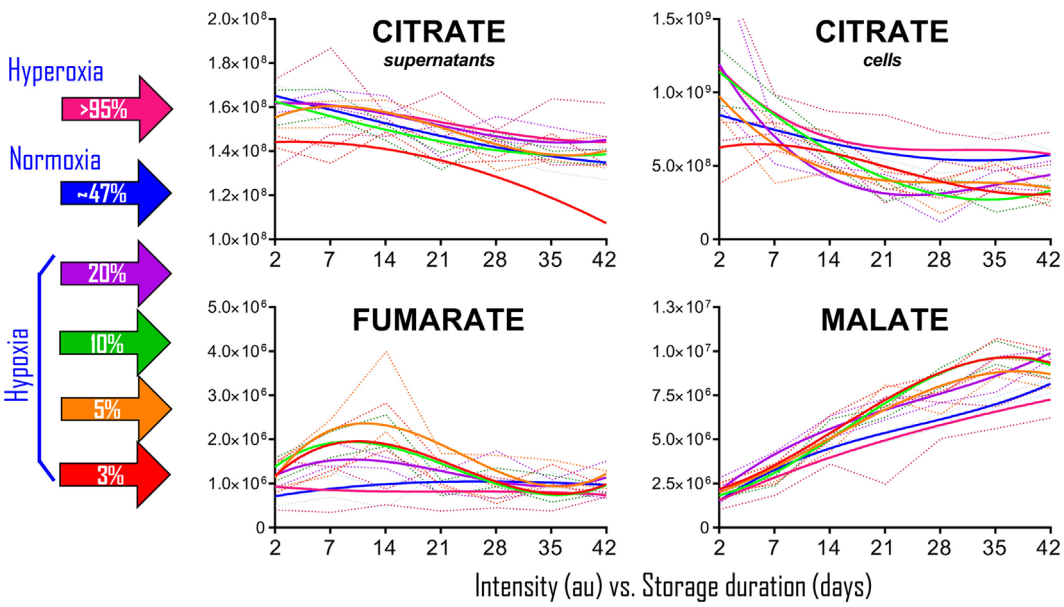

FIGURE 2 | Packed red blood cell (RBC) storage under controlled hemoglobin oxygen saturation conditions recapitulates high-altitude hypoxia-induced decreases in citrate and accumulation of fumarate/malate. RBCs were stored under normoxic conditions (untreated–SO_2 = 47 ± 21, mean ± SD—solid blue line), hyperoxia (SO_2 > 95%—solid purple line), or four hypoxic conditions (SO_2 = 20, 10, 5, or <3%—solid purple, green, orange, and red lines, respectively). Supernatant citrate was significantly lower than controls ($p < 0.05$) in SO_2 < 3% hypoxic RBCs at all tested time points. Fumarate was significantly higher than controls ($p < 0.05$) at storage day 7 and 14, while malate at day 14 onward in all hypoxic RBCs when compared to controls and hyperoxic counterparts. Dotted lines indicate ranges (same color-code—lighter tone). All data points on *x* axis were tested ($n = 4$).

notably, in cells during storage in AS-3, therefore suggesting increased consumption of citrate in hypoxic RBCs (**Figure 2**). In parallel, hypoxic RBCs generated more fumarate for the first 3 weeks of storage, and malate through the whole storage period (**Figure 2**). Recent proteomics (43, 44), metabolomics (13, 45), and computational evidence (46) has suggested that carboxylate metabolism in mature RBCs can be regulated by enzymatic reactions that are downstream to glucose-derived pyruvate by cytosolic isoforms of Krebs cycle enzymes such as acteyl-coA ligase, phosphoenolpyruvate carboxylase—PEPCK [or PEPCK-like activity of hemoglobin (50)], fumarate hydratase, isocitrate dehydrogenase 1, and malate dehydrogenase 1. To determine whether such reactions were affected by the degree of hypoxia, we incubated RBCs with $^{13}C_{1,2,3}$-glucose under varying SO_2 conditions (from <3% to >95%) and monitored ^{13}C distribution in downstream metabolites according to the reactions summarized in **Figure 3**. While generation of ^{13}C-fumarate from $^{13}C_{1,2,3}$-glucose was not observed, accumulation of $^{13}C_3$-malate and $^{13}C_3$-alpha-ketoglutarate isotopologues was observed during storage and followed a trend that was inversely proportional to SO_2 (i.e., higher generation of these compounds from heavy glucose was observed with hypoxia—**Figure 3**).

Determination of Isotopologue Distributions upon RBC Exposure to Hypoxia *Ex Vivo* in Presence of Stable Isotope-Labeled Citrate, Glutamine, and Aspartate

Our previous results showed encouraging evidence suggesting that the generation of malate and alpha-ketoglutarate from glucose could indeed occur in mature erythrocytes proportional to hypoxia. However, the amount of isotope-contribution was not sufficient to explain the observed increases in steady-state levels of these compounds during hypoxic refrigerated storage (<10% of which were derived from glucose oxidation in both cases of malate and alpha-ketoglutarate). Therefore, we hypothesized that hypoxia-induced catabolism of substrates other than glucose could more completely explain the observed increase in malate and altered metabolism of RBC carboxylic acids. To test this hypothesis, we incubated RBCs for 24 h under normoxic and hypoxic conditions using an in-house generated AS-3 supplemented with U-^{13}C-glucose or $^{13}C_6$-citratic acid (thereby replacing the unlabeled components in the formulation), $^{13}C_1$-aspartate, or $^{13}C_5{}^{15}N_2$-glutamine in four independent experiments ($n = 3$ for each). Heavy isotopologues derived from the catabolism of these substrates were quantified as a percentage of the total levels of the compound of interest, and included carboxylic acids (citrate, malate, and alpha-ketoglutarate), amino acids derived from transmination/oxidation of alpha-ketoglutarate (glutamate, 5-oxoproline), and lactate (**Figure 4**). In **Figures 4** and **5**, we provide a bar graph representation of the percent contribution to the generation of the aforementioned compounds from each of the heavy tracers in normoxia and hypoxia. Of note, >60% of RBC citrate was labeled independently from hypoxia, suggesting that the majority of this metabolite is uptaken from the media (**Figures 4** and **5**). Notably, citrate catabolism to malate was significant under normoxic conditions (~40% of the total) and reduced by hypoxia (<15%), which in turn promoted oxidative citrate metabolism to glutamate and 5-oxoproline (**Figure 4**). Minimal contribution of citrate catabolism to lactate generation (**Figures 4** and **5**) was observed under either normoxic or hypoxic

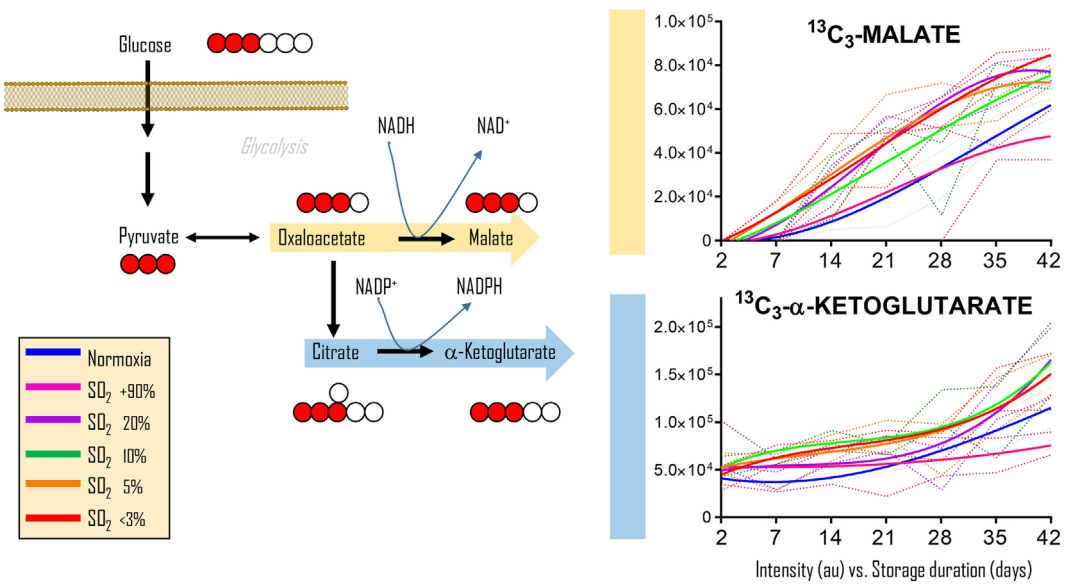

FIGURE 3 | Glucose tracing experiments indicate hypoxia-induced increases in carboxylic acids deriving from glucose. Cytosolic isoforms of Krebs cycle enzymes are present in mature red blood cells (RBCs) and can theoretically catalyze the reactions graphed here, reactions that could contribute to RBC reducing equivalent homeostasis. Heavy fumarate and malate accumulation in hypoxic RBCs was significantly higher ($p < 0.05$) than controls at all tested storage days after day 7. Hyperoxic RBCs had significantly ($p < 0.05$) lower heavy fumarate than control RBCs only at storage day 42. All data points on *x* axis were tested ($n = 4$).

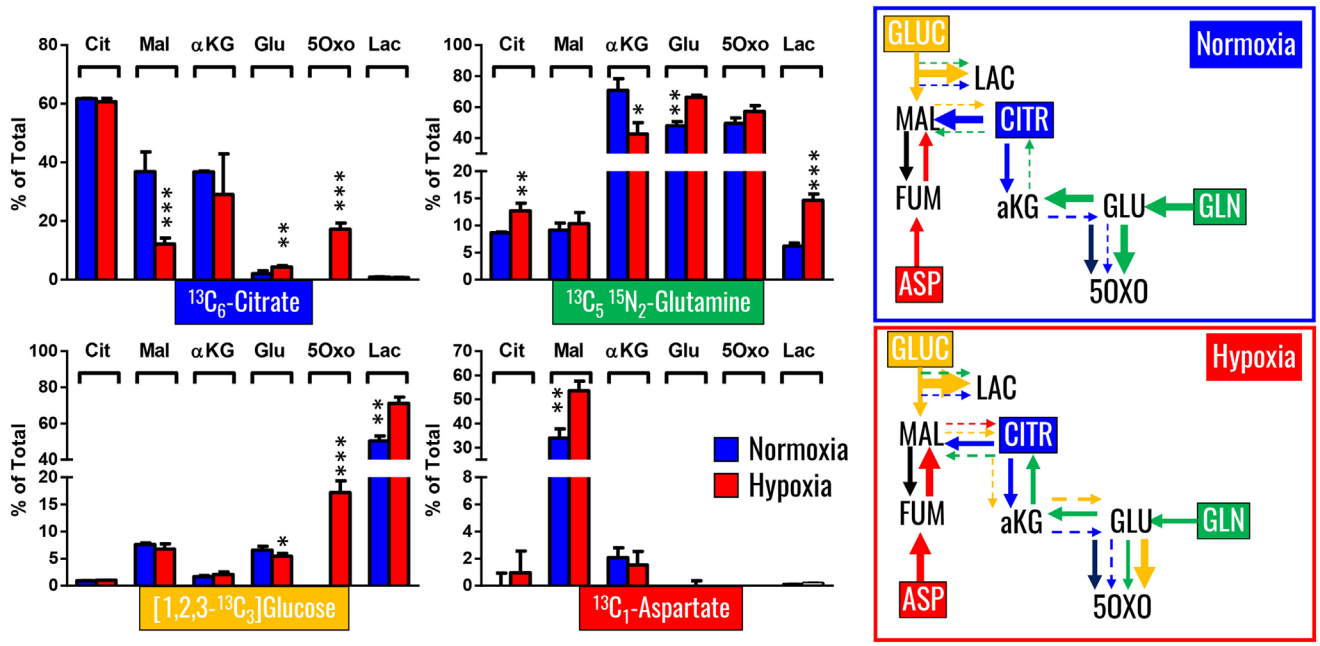

FIGURE 4 | Isotopologue distribution of heavy carbon atoms from heavy citrate, glutamine, glucose, and aspartate indicate a complex rewiring of red blood cell carboxylic acid metabolism in response to hypoxia, as summarized in the panels to the right. Bars indicate median (± SD)% accumulation of heavy isotopologues vs the total levels of the compound, as measured in three independent experiments per each condition (normoxia vs 24 h hypoxia—blue and red bars, respectively). Arrows in the panels to the right indicate metabolic rewiring in normoxia and hypoxia and color-code are consistent with the colors used to identify stable isotope tracers indicated in the four panels to the left. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (*T*-test to normoxic control).

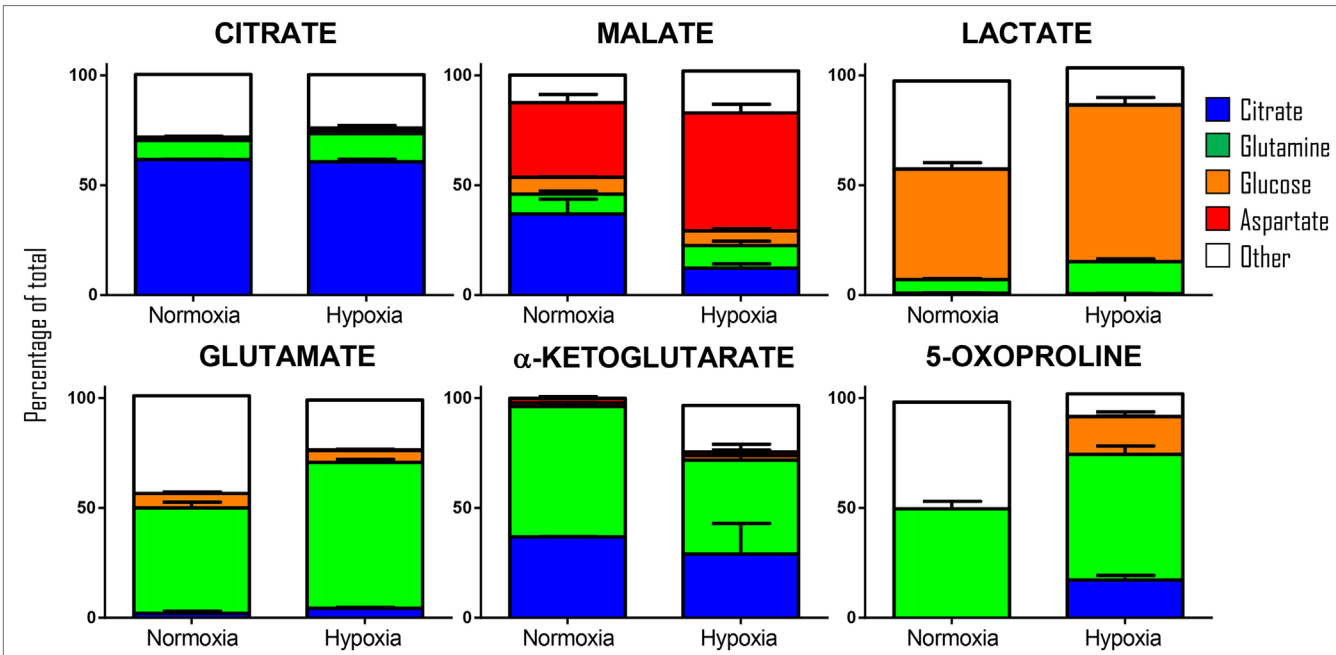

FIGURE 5 | Relative contribution of metabolic substrates (citrate, glutamine, glucoose, aspartate, other) to the generation of citrate, malate, lactate, glutamate, alpha-ketoglutarate, and 5-oxoproline under normoxic or hypoxic conditions (24 h). Mean ± SD are shown from three independent experiments per condition. Other here indicates either endogenous levels of the metabolite or derivation from other sources than the stable isotope tracers used here. Significant increases in glucose-derived lactate and glutamine-derived glutamate, but not ketoglutarate were observed under hypoxic conditions. Citrate and glucose-derived 5-oxoproline increased significantly ($p < 0.05$) under hypoxic conditions.

conditions for 24 h (<2.5%), suggesting that previous observations in AS-3 (13) may be explained by a metabolic switch only occurring later on during storage. Glutaminolysis mostly fueled the generation of alpha-ketoglutarate and its transamination byproducts glutamate and 5-oxoproline, a phenomenon that was exacerbated by exposure to hypoxia for 24 h (**Figure 4**). Metabolism of heavy glutamine contributed in part (<10%) to lactate generation under normoxia, and increased under hypoxia (up to 15%) where the contribution of glutamine to citrate reservoirs increased to ~13% of the total (**Figures 4** and **5**). Glucose catabolism mostly fueled lactate generation (55 to >70% of total lactate after 24 h in normoxia and hypoxia, respectively) and ~18% generation of 5-oxoproline under hypoxic conditions (**Figures 4** and **5**). Limited glucose incorporation into malate is consistent with tracing experiments with glucose during storage (**Figure 3**), though hypoxia-triggered increases in glucose metabolism to malate only became apparent after 1 week of storage rather than 24 h (**Figures 3** and **4**). This is important because we have previously shown that hypoxic RBCs may use glucose-derived carbons to synthesize amino acid moieties necessary for the synthesis of the tripeptide glutathione during hypoxic storage (27). Finally, aspartate catabolism was identified to influence malate generation (<40% under normoxia and up to 60% under hypoxia—**Figure 4**), making it the main source of hypoxic malate in human RBCs in this study (**Figure 5**).

DISCUSSION

Red blood cells are by far the most abundant host cell in the human body, accounting for nearly 80% of the 30 trillion host cells that make up the body of a 175 cm tall 70 kg man (44). Although loaded with hemoglobin (98% of the cytosolic proteome) and devoid of nuclei and organelles, RBCs are far more complex than previously believed (until the last decade or so). Appreciation through proteomics of the presence of cytosolic isoforms of Krebs cycle enzymes in mature erythrocytes has prompted the field to reconsider whether these enzymes are actually active and, if so, whether they actually influence RBC metabolism during routine storage in the blood bank. Indeed, tracing experiments in packed RBCs have suggested that citrate can be metabolized into lactate when stored in SAGM (45) and AS-3 (13); the latter being more directly relevant due to its elevated concentration of citrate (>20 mM) that compensates for the removal of the osmolite mannitol from its formulation. In light of these tracing experiments, it has been suggested that reactions catalyzed by cytosolic isoforms of Krebs cycle enzymes may contribute to the homeostasis of RBC reducing equivalents NADH and NADPH through reactions alternative to glycolysis, pentose phosphate pathway, and methemoglobin reductase, thereby expanding well-established understanding of RBC metabolic networks (51). Refinement of such networks is indeed important for the development of new storage additives, as *in silico* elaboration of quantitative metabolic information of metabolic markers of the storage lesion (52) would help in predicting the metabolic state of RBCs exposed to novel additives (53). In this study, we provide additional information to refine such models by determining the metabolic effect of RBC SO_2 modulation on carboxylate metabolism. Decreased RBC levels of 2-hydroxyglutarate and succinate in response to high-altitude acclimatization are relevant in that these metabolites are well-established markers of tissue hypoxia [e.g., ischemic (54) and hemorrhagic hypoxia (55)]. In nucleated cells, succinate accumulation is interesting given that it promotes the stabilization of hypoxia inducible factor 1α by inhibiting prolyl hydroxylase, therefore promoting acclimatization responses to hypoxia (56). Since all the subjects enrolled in the AltitudeOmics study effectively acclimatized to high-altitude hypoxia (57), it is interesting to note that declining levels of RBC succinate may be a marker of decreased tissue hypoxia as the subjects acclimatized.

For the first time, we provide evidence that exposure to hypoxia *in vivo* or *ex vivo* affects RBC capacity to metabolize (consume or generate) carboxylic acids. Through a combination of metabolic flux experiments using different stable isotope tracers, we confirm that RBCs can uptake carboxylic acids such as citrate and metabolize them into di-carboxylates (e.g., malate) or transamination intermediates (e.g., alpha-ketoglutarate, glutamate, 5-oxoproline) in an SO_2-dependent fashion. Most notably, we show that malate accumulation during storage and the exacerbation of this phenomenon under hypoxia are potentially explained by varying metabolic mechanisms, in that aspartate catabolism predominantly contributes to malate generation under hypoxia, rather than glucose or citrate catabolism. In this view, it is interesting to speculate that purine catabolism [deamination of purines to hypoxanthine and xanthine, a well-documented phenomenon in stored erythrocytes (7, 14, 15, 25, 52, 58)] may be influenced by hypoxia. Indeed, aspartate consumption *via* purine salvage reactions would explain increased fumarate accumulation, which in turn would become a substrate for fumarate hydratase [present and active in mature RBCs (46)] for the generation of malate. Future studies will investigate this interesting corollary to the observations reported here. Alternatively, aspartate may represent an eligible substrate (amino group donor) for transamination reactions. This hypothesis is consistent with the observed decrease in the level of alpha-ketoglutarate and increased glutamate isotopologues (both M + 5 and M + 5 + 1). Such observation can only be explained by combined glutamine metabolism to alpha-ketoglutarate (carbon backbone + 5), which is turn transaminated back to glutamate *via* glutamate oxaloacetate transaminases, previously identified in mature RBC proteomics datasets (43, 44).

Finally, though merely observational, the present study provides interesting hypothesis-generating evidence to investigate why carboxylic acid metabolism may be affected by hypoxia in an enucleated cell incapable of *de novo* protein synthesis, as is the case with RBCs. It is fascinating to speculate that, in similar fashion to the oxygen-dependent metabolic modulation model (28, 29, 35–37), post-translational modifications such as phosphorylation mediated by adenosine/AMPK-dependent signaling (59)—recently identified to contribute to hypoxic adaptations in eukaryotes as simple as *S. cervisiae* (60)—may influence enzyme sub-cellular compartmentalization, formation of multi-protein complexes, and activity. RBC multi-enzyme protein complexes have been preliminarily described in mature RBCs and reported

to be susceptible to the storage lesion (61). Therefore, it remains to be assessed whether some of the observations reported here could be attributed to factors other than hypoxia-driven intracellular alkalinization that affects the activities of many RBC cytosolic enzymes, such as sub-cellular compartmentalization (e.g., membrane vs cytosol) or oligomerization of Krebs cycle enzymes into alternative multi-protein complexes under hypoxic conditions. Last but not least, the results presented here may be also interpreted as a result of as of yet uncharacterized reactions involving alternative to Krebs cycle cytosolic isoforms. A paradigmatic example of this notion is the conversion of late glycolytic trioses to oxaloacetate, an intermediate in malate/citrate generation/consumption in mature erythrocytes and a reaction that could be catalyzed by hemoglobin (50) through moonlighting functions (62). Similar considerations could be made for other carboxylates such as 2-hydroxyglutarate, which could be generated by lactate dehydrogenase under hypoxic conditions (63). Therefore, future studies will be necessary to disentangle and possibly identify new metabolic networks that are modulated by oxygen level in RBCs.

ETHICS STATEMENT

The AltitudeOmics study has been approved by the University of Colorado Institutional Review Board, Protocol no. 11-1581.

AUTHOR CONTRIBUTIONS

TN, JR, KH, and ADa performed metabolomics analyses and plotted the results. KS, EW, AW, and YX generated samples for *ex vivo* tracing experiments. RR designed, performed, and provided samples for high-altitude studies. TY and ADu generated technology and samples for *ex vivo* oxygen-controlled preservation of packed RBCs. ADa wrote the first draft of the manuscript, and all the authors critically contributed to its finalization.

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Metabolic Linkage and Correlations to Storage Capacity in Erythrocytes from Glucose 6-Phosphate Dehydrogenase-Deficient Donors

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Objective: In glucose 6-phosphate dehydrogenase (G6PD) deficiency, decreased NADPH regeneration in the pentose phosphate pathway and subnormal levels of reduced glutathione result in insufficient antioxidant defense, increased susceptibility of red blood cells (RBCs) to oxidative stress, and acute hemolysis following exposure to pro-oxidant drugs and infections. Despite the fact that redox disequilibrium is a prominent feature of RBC storage lesion, it has been reported that the G6PD-deficient RBCs store well, at least in respect to energy metabolism, but their overall metabolic phenotypes and molecular linkages to the storability profile are scarcely investigated.

Methods: We performed UHPLC-MS metabolomics analyses of weekly sampled RBC concentrates from G6PD sufficient and deficient donors, stored in citrate phosphate dextrose/saline adenine glucose mannitol from day 0 to storage day 42, followed by statistical and bioinformatics integration of the data.

Results: Other than previously reported alterations in glycolysis, metabolomics analyses revealed bioactive lipids, free fatty acids, bile acids, amino acids, and purines as top variables discriminating RBC concentrates for G6PD-deficient donors. Two-way ANOVA showed significant changes in the storage-dependent variation in fumarate, one-carbon, and sulfur metabolism, glutathione homeostasis, and antioxidant defense (including urate) components in G6PD-deficient vs. sufficient donors. The levels of free fatty acids and their oxidized derivatives, as well as those of membrane-associated plasticizers were significantly lower in G6PD-deficient units in comparison to controls. By using the strongest correlations between *in vivo* and *ex vivo* metabolic and physiological parameters, consecutively present throughout the storage period, several interactomes were produced that revealed an interesting interplay between redox, energy, and hemolysis variables, which may be further associated with donor-specific differences in the post-transfusion performance of G6PD-deficient RBCs.

Conclusion: The metabolic phenotypes of G6PD-deficient donors recapitulate the basic storage lesion profile that leads to loss of metabolic linkage and rewiring. Donor-related issues affect the storability of RBCs even in the narrow context of this donor subgroup in a way likely relevant to transfusion medicine.

Keywords: glucose 6-phosphate dehydrogenase deficiency, transfusion medicine, red blood cell storage lesion, donor variation, mass spectrometry, metabolomics, interactome

INTRODUCTION

Routine storage of packed red blood cells (RBCs) in the blood bank is a logistic necessity that makes ~110 millions of units available for life-saving transfusions to millions of recipients worldwide every year. Storage in the blood bank is associated with the progressive accumulation of a series of biochemical and morphological alterations to RBCs collectively referred to as the storage lesion (1, 2). Deranged metabolic homeostasis of stored RBCs is a heritable trait, i.e., it is affected—like hemolysis—by the donor's genetic background (3–5). The metabolic storage lesion can be cursorily summarized in two main components, i.e., decreased energy metabolism and increased oxidative stress (6–10). Despite laboratory observations suggesting that old blood may be associated with poorer transfusion outcomes, reassuring evidence from randomized clinical trials has been generated to support the overall safety and efficacy of current transfusion practices (11). The apparent disconnect on the age of blood issue between laboratory observations and randomized clinical trials is in part reconciled by the appreciation of the many confounding factors affecting the interpretation of prospective clinical trials and limited size of cohorts tested in most basic science/laboratory studies. Introduction of high-throughput omics technologies and combination of omics results with functional outcomes (12) has fostered a new era in the field of transfusion medicine where the focus has been shifted from the final product to the donor (13–15) and the intrinsic variability across the donor population. Animal studies further strengthened this conclusion, reporting that while not all (mouse strain) donor RBCs store similarly (16), transfusion of RBCs from different (mouse strain) donors may result in a "good apple/bad apple" effect (17), further increasing the complexity of the donor/recipient system and increasing the noise of clinical studies where exclusively and consistently young or old blood is hardly ever transfused to the same recipient (18). In humans, factors such as donor age, ethnicity, and gender ultimately affect RBC storability (influencing parameters such as hemolysis or oxidative stress-induced hemolysis) (12). Gender in particular may be an underestimated confounder (19).

Glucose 6-phosphate dehydrogenase (G6PD) deficiency is an X-linked (20) recessive inborn error of metabolism that affects ~400 million individuals worldwide and results in impaired antioxidant capacity. Indeed, carriers of G6PD-deficient traits are characterized by a reduced capacity to generate antioxidant equivalents (i.e., NADPH) through the pentose phosphate pathway (PPP), which in turn results in an increased susceptibility to hemolysis. As oxidative stress has been considered an etiological contributor to the RBC storage lesion, it has been anticipated that RBCs from G6PD-deficient donors may suffer from exacerbated alterations during storage in the blood bank (21). While clinical evidence on the issue is still missing, preliminary omics studies have revealed that RBCs from G6PD-deficient donors unexpectedly better preserve energy homeostasis and morphology during storage in the blood bank though they are increasingly more susceptible to temperature and oxidative stress-induced hemolysis than stored RBCs from G6PD sufficient donors (22, 23). In that preliminary study, we focused specifically on glycolysis and the PPP. However, Tang and colleagues (24) recently showed that RBCs from G6PD-deficient donors challenged with pro-oxidant stimuli such as diamide are characterized by a wide series of alterations, including alterations of purine homeostasis which in turn result in activation of AMP protein kinase. Other pathways, such as fatty acid metabolism, are significant correlates to post-transfusion recoveries in mouse models (25, 26). In this study, we expand on our previous observation on the metabolic phenotypes of RBCs from G6PD-deficient vs. sufficient donors. Results are correlated to physiological measurements of potential clinical impact, such as extracellular potassium, oxidative lesions, RBC fragility, and susceptibility to hemolysis *in situ* or at post-storage mimicking conditions (e.g., incubation at 37°C).

MATERIALS AND METHODS

Subjects, Blood Collection, and Processing

Six male, 22–30 years old G6PD-deficient ($G6PD^{-}$, Mediterranean variant, <10% residual activity of the enzyme) and three gender- and age-matched G6PD-normal ($G6PD^{+}$) regular blood donors were recruited. Venous blood was collected into EDTA or citrate vacutainers just before blood donation and preparation of packed RBCs. RBC storage quality was evaluated in citrate phosphate dextrose (CPD)/saline adenine glucose mannitol (SAGM) log4 leukofiltered units (Haemonetics Corp., MA, USA) stored for 42 days at 4–6°C. Samples were collected aseptically at weekly intervals of the storage period (days 7, 14, 21, 28, 35, and 42). The study was approved by the Ethics Committee of the Department of Biology, School of Science, NKUA. Investigations were carried out upon signing of written consent, in accordance with the principles of the Declaration of Helsinki.

Hematological, Biochemical, and Physiological Measurements

Pre-donation blood and RBC concentrates of G6PD-deficient donors were further evaluated for almost 45 hematological,

biochemical, and physiological parameters before and throughout the storage period in CPD/SAGM, as described in the previously published study (22, 23). Shortly, Hb concentration and RBC indexes (RBC and reticulocyte counts, hematocrit, mean corpuscular volume, mean corpuscular Hb, mean corpuscular Hb concentration, and RBC distribution width) were measured using the Sysmex K-4500 automatic blood cell counter (Roche), while serum biochemical analysis (triglycerides, cholesterol, low density lipoproteins, high density lipoproteins, iron, ferritin, total billirubin, uric acid, aspartate transaminase, alanine aminotransferase, potassium, and sodium) was performed using the analyzers Hitachi 902, 9180 and Elecsys Systems Analyzer (Roche). Levels of glycated Hb (HbA1c) and G6PD activity were measured in fresh blood and in packed RBCs on the last day of storage. Levels of extracellular (free) Hb, total or uric acid-dependent/independent antioxidant activities, total and RBC-derived microparticles (MPs), and MP-associated pro-coagulant activity were evaluated in plasma/supernatant by standard biochemical assays, flow cytometry, or ELISA approaches. Fresh and stored RBCs were finally evaluated for shape modifications (scanning electron microscopy), osmotic and mechanical fragility, membrane protein carbonylation, and accumulation of intracellular reactive oxygen species (ROS) and calcium. Measurements of RBC fragilities and ROS accumulation were performed before and after 24 h incubation at 37°C, while ROS accumulation was estimated before and after treatment with the oxidative agents diamide (dROS) and *tert*-butyl hydroperoxide (tBHP, tROS). All measurements were run in triplicate.

Metabolomics Analyses

Metabolomics analyses were performed as previously reported (22). Briefly, 100 μL of stored RBCs were collected on a weekly basis and extracted at 1:6 dilutions in methanol:acetonitrile:water (5:3:2), vortexed, and centrifuged to pellet proteins, prior to analysis by UHPLC-MS (Ultimate 3000 RSLC-Q Exactive, Thermo Fisher). Sample extracts (10 μL) were loaded onto a Kinetex XB-C18 column (150 mm × 2.1 mm × 1.7 μm—Phenomenex, Torrance, CA, USA). A 9-min gradient from 5 to 95% B (phase A: water + 0.1% formic acid and B: acetonitrile + 0.1% formic acid) eluted metabolites into a Q Exactive system (Thermo, Bremen, Germany), scanning in full MS mode (3 min method) or performing acquisition independent fragmentation (MS/MS analysis—9 min method) 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 assignment was performed against an in house standard library, as reported (27), through the freely available software Maven (Princeton University, USA) (28). No data pre-processing (neither normalization nor log-transformation) was performed. In our previous study (22, 23), only glycolysis, ribose phosphate, glutathione, and NADH/NAD+ ratios were reported. Here, we expanded the analysis to amino acids, lipids, purines, and other metabolites, as extensively reported in Table S1 in Supplementary Material.

Statistics

For statistical analysis, the Statistical Package for Social Sciences (SPSS, IBM) was used. Correlations between parameters were evaluated by the Pearson's and Spearman's tests after checking out the variables for normal distribution profile (by using the Shapiro–Wilk test) and presence of outliers. Briefly, in the absence of normal distribution, Spearman test was performed. In addition, and since Pearson's test is sensitive to extreme outliers, in the presence of such an outlier the value was excluded and the analysis was performed again, to minimize the possibility of false results likely associated with the small size of the cohort. If the outcome of the subsequent Pearson analysis was not modified compared to the first one, the outlier was included back to the cohort. If not, Spearman analysis was preferred. Outliers (any measurement outside the range of mean $\pm 2 \times$ SD) were identified by using both the Shapiro–Wilk test and detrended normal Q–Q plots. Significance was accepted at a p value of less than 0.01.

Network Analysis

All hematological, biochemical, omics, and physiological parameters collected from G6PD-deficient donors (for abbreviations, see Table S2 in Supplementary Material) were used for the construction of biological networks connecting variables of fresh donor's blood (*in vivo* state) with those of packed RBCs (*ex vivo* state) by significant and repeated correlations that existed throughout (namely, at every time point of) the storage period (with the exception of the G6PD activity and percentage of HbA1c, for which only end-of-storage measurements were available). The reasoning behind selection of correlations that were repeatedly evident at all time points of storage (namely, fresh blood vs. 7th and 14th and so on until the 42nd day) was to find out sound links between variables regardless of storage duration and in parallel, to minimize the false discovery rate that is intrinsically connected to any small sized sample. To increase the confidence level, the outputs of that analysis were further analyzed by a Bonferroni-like correction for multiple comparisons. The multiply checked and thus, most probably true, correlations were topologically represented in undirected biological networks by using Cytoscape version 3.2.0 application, as previously described (15). The length of each edge was inversely proportional to the r value (the shortest the edge, the higher r value).

RESULTS AND DISCUSSION

Metabolic Phenotypes of G6PD-Deficient Donors Recapitulate the Storage Lesion Observed in G6PD Sufficient Donors

Overall, a total of 293 metabolites were monitored in this study, as extensively reported in Table S1 in Supplementary Material. Recently, Palsson's group (29, 30) recognized three stages identifying the metabolic age of blood (18). Multivariate analysis of metabolomics data from RBC concentrates stored in different additives (29–31) results in a U-shaped graph which is indicative of three time-dependent metabolic phases as RBCs age during storage. Consistently, Paglia et al. have reported that transition from phase 2 to phase 3 occurs after storage day 18 (29). Here, multivariate analysis of metabolomics data from SAGM-stored RBCs from G6PD-deficient donors suggests that such transition may occur earlier in this population (storage day 14—**Figure 1A**),

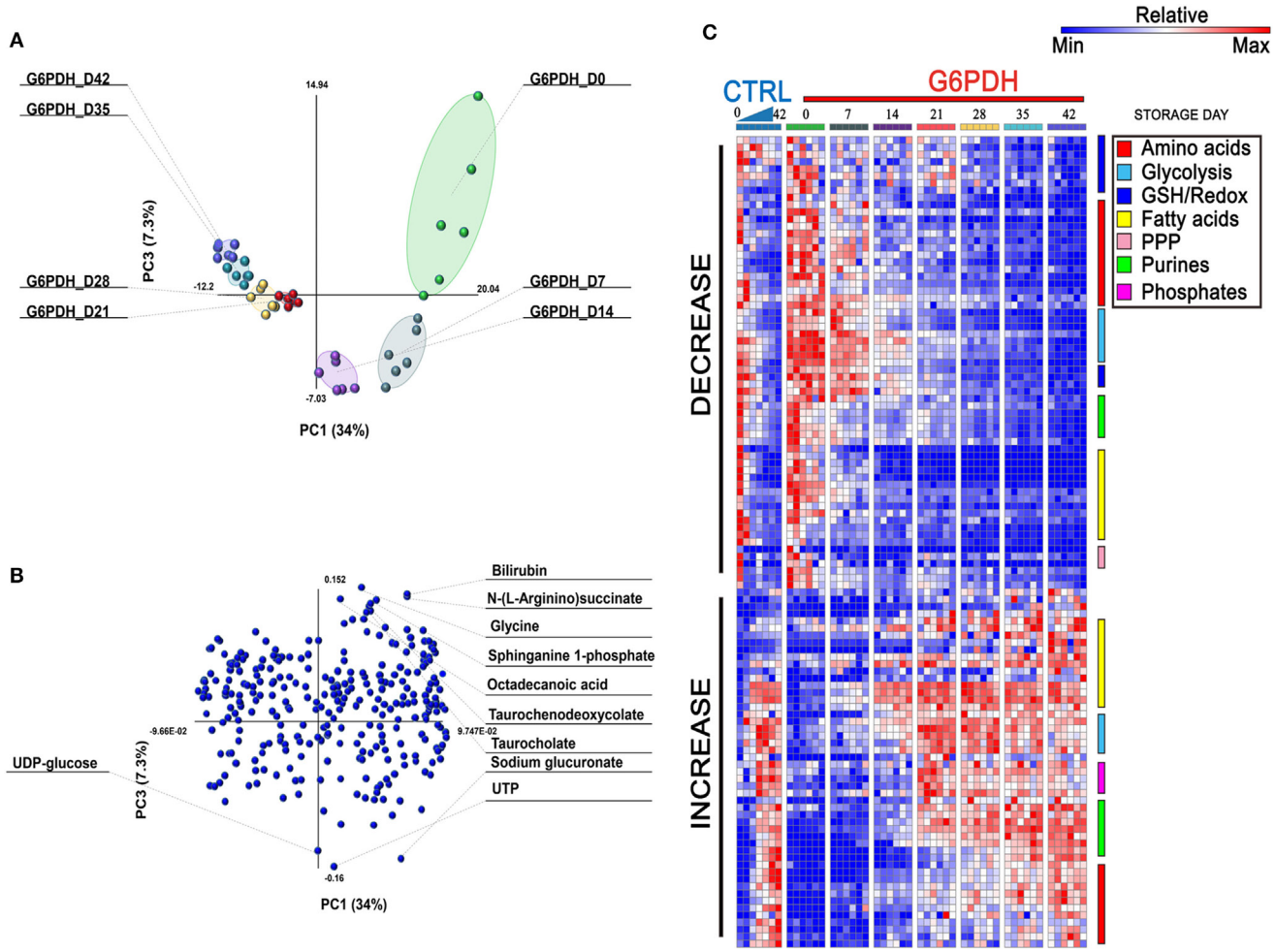

FIGURE 1 | Multivariate analysis of metabolic phenotypes of stored red blood cells (RBCs) from glucose 6-phosphate dehydrogenase (G6PD)-deficient donors. Partial least square discriminant analysis [PLS-DA—panel **(A)**] revealed a U-shaped distribution of packed RBC samples from G6PD-deficient donors over storage in the blood bank, consistent with previous reports from Palsson's lab (29, 30) and our group (9, 31). A loading plot for most significant variables informing the PLS-DA discrimination is shown in panel **(B)**, and includes bioactive lipids and bile acids. In panel **(C)**, a heat map of time course metabolic changes in RBCs from G6PD sufficient (median) or deficient (n = 6) donors, divided by pathway as specified by color codes in the top right corner of the panel.

though greater temporal resolution in the 10–18 storage day range would be necessary to further support this conclusion. Overall, the top variables discriminating RBC concentrates for G6PD-deficient donors include bioactive lipids and free fatty acids, bile acids, glycolytic metabolites, purines, and amino acids, as reported in the loading plot and heat maps in **Figures 1B,C**, respectively. A vectorial version of the heat map with hierarchical clustering is provided as Figure S1 in Supplementary Material.

G6PD-Deficient Donors Are Characterized by Alterations in One-Carbon Metabolism, Glutathione/Urate Homeostasis, and Fatty Acid Metabolism Compared to G6PD Sufficient Donors

Two-way ANOVA comparing storage-dependent trends in G6PD-deficient vs. sufficient donors revealed significant changes in metabolites involved in one-carbon and sulfur metabolism (including cystathionine, methionine, S-adenosyl-L-methionine, methylenetetrahydrofolate, and homocysteine—**Figures 2A–E**), metabolites involved in glutathione homeostasis and antioxidant defenses (urate, glutamine, glutathionylcysteine—**Figures 2F–H**), and the carboxylic acid fumarate (**Figure 2I**), all significantly lower in the G6PD-deficient group except for cystathionine and homocysteine.

Consistent with a better preserved morphology (22) and a trend of decreased storage vesiculation degree (23), the levels of free fatty acids and oxidized derivatives (e.g., HPETE/LTB4 or isobaric isomers) were significantly lower in G6PD-deficient donors in comparison to control RBCs, with the exception of oleate and linoleate (**Figure 3**). Previous proteomics analyses revealed increased oxidation and stress markers accumulation but also increased levels of antioxidant enzymes in the plasma membrane and the extracellular vesicles released by the stored G6PD$^-$ RBCs (22). Of note, the levels of several polyunsaturated fatty acids, including the linoleate, were found to be both

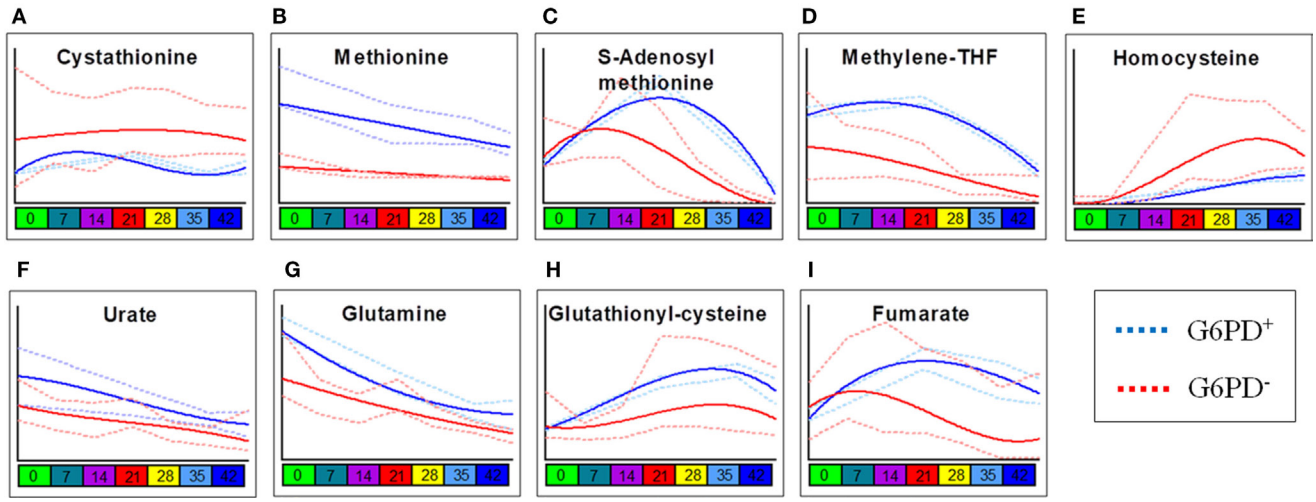

FIGURE 2 | Most significant metabolic differences between glucose 6-phosphate dehydrogenase (G6PD) sufficient and deficient red blood cells (RBCs). Panels indicate alterations to sulfur/one-carbon metabolism **(A–E)**, purine oxidation **(F)**, glutathione homeostasis **(G,H)**, and carboxylates **(I)** in G6PD sufficient (blue line) and deficient (red line) RBCs during storage in the blood bank (median + ranges are shown in light blue or red for both G6PD sufficient and deficient groups, respectively).

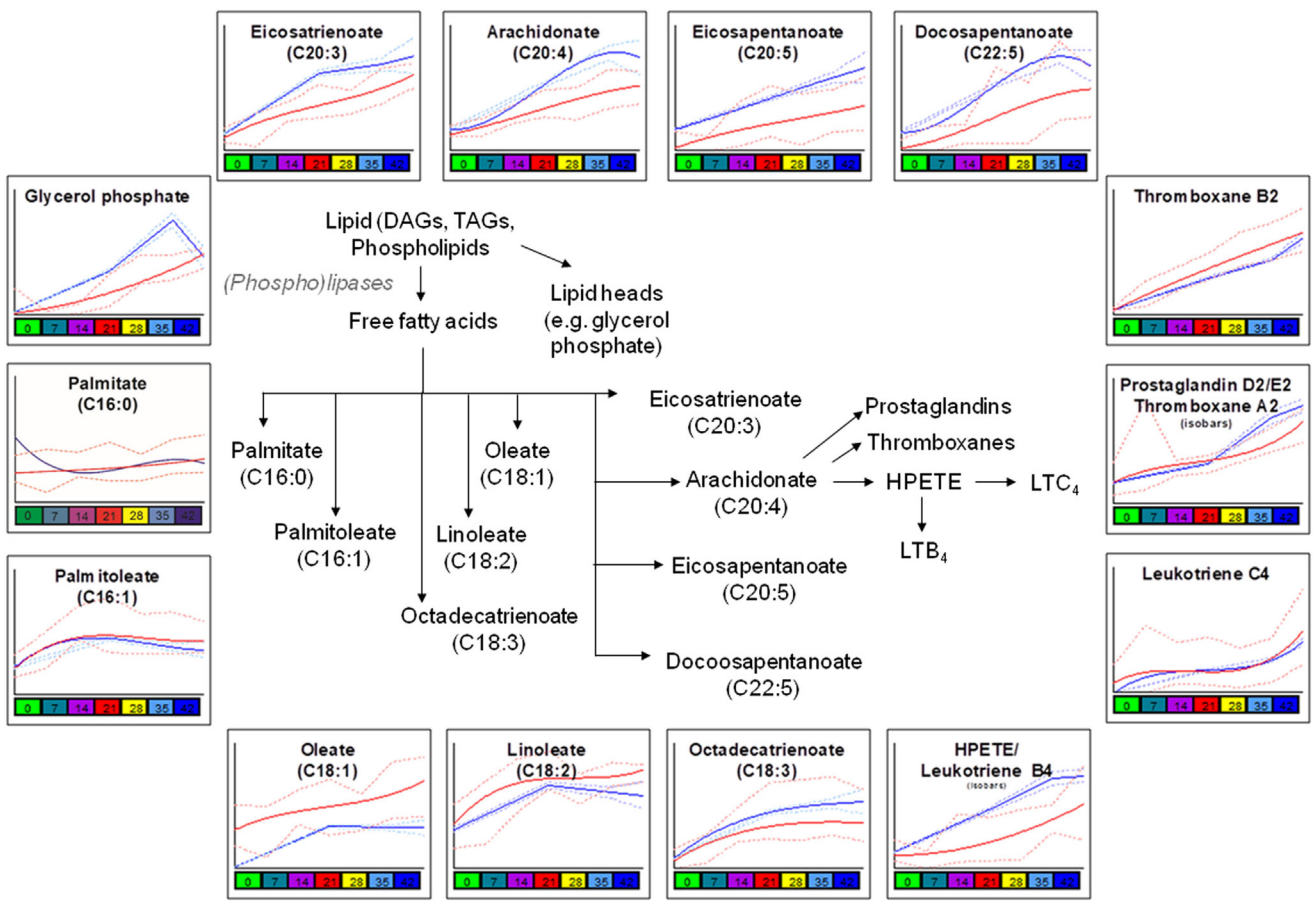

FIGURE 3 | Significant differences in fatty acid metabolism and oxidation between glucose 6-phosphate dehydrogenase (G6PD) sufficient (blue line) and deficient (red line) red blood cells during storage in the blood bank (median + ranges are shown in light blue or red for both G6PD sufficient and deficient groups, respectively).

heritable and associated with ATP levels in AS-3 stored RBCs (4), while in this study, the linoleate concentration in G6PD$^-$ donors at donation time had a strong negative correlation with the 2,3-biphosphoglycerate (2,3-BPG) levels in stored RBCs (see below). The different concentration of oleate in fresh and stored G6PD$^-$ blood compared to control blood may be associated with a different rate of incorporation into phosphatidylcholine that significantly decreases during storage (32).

Similarly, lower levels of plasticizers monoethyl-hexylphthalate and phthalate were detected in RBC concentrates from G6PD-deficient donors (**Figure 4**). Approximately 28% of the available bis(2-ethylhexyl) phthalate (DEHP) is taken up by stored RBCs where it exerts a protective effect on membrane stability and flexibility similar to that of mannitol (33). Since NADPH is indispensable for the synthesis of fatty acids and cholesterol, the RBC membrane in G6PD deficiency of Mediterranean type is characterized by increased fluidity and decreased cholesterol-to-phospholipid ratio (34). Considering that RBCs from both donor cohorts were processed and stored through comparable manufacturing processes, and that the DEHP levels in G6PD$^-$ units were equal to the control levels, it is plausible to speculate that the lipid remodeling in G6PD deficiency may favor incorporation of DEHP in the membrane, preventing thus its hydrolysis to MEHP and phthalate. This kind of protective effect is consistent with the previously reported trend of stored G6PD$^-$ RBCs to reduced mechanical fragility compared to that of control RBCs at body temperature (23).

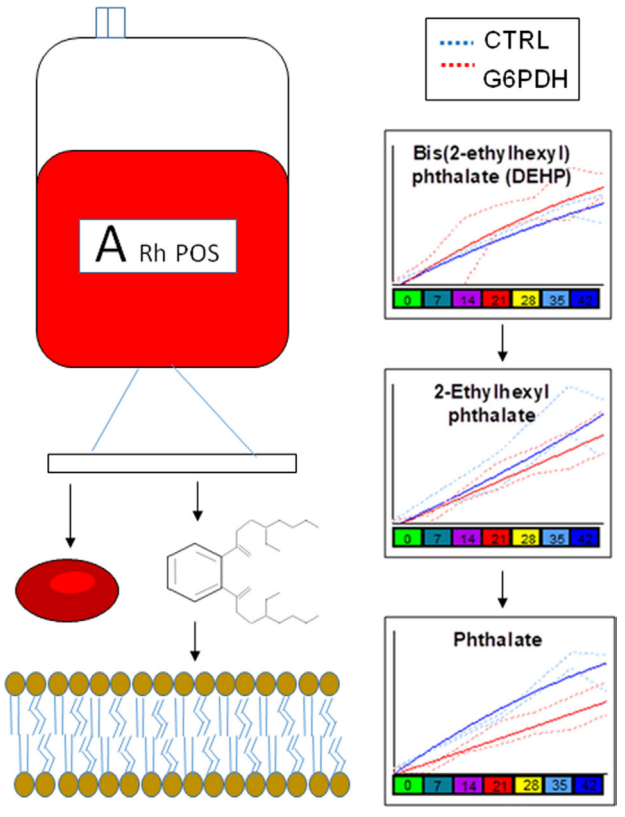

FIGURE 4 | Different levels of phthalate plasticizers and breakdown products are observed in red blood cells from glucose 6-phosphate dehydrogenase (G6PD) sufficient (blue line) and deficient (red line) during storage in the blood bank (median + ranges are shown in light blue or red for both G6PD sufficient and deficient groups, respectively).

Loss of Metabolic Linkage and Metabolic Rewiring in G6PD-Deficient Donors

Though correlations do not necessarily imply causation, in the field of metabolomics, a high degree of correlation is observed among the levels of metabolites from pathways that are linked by biochemical constraints of enzymatic reactions (35). The identification of such correlates under physiological conditions and the disruption of such correlations under pathological conditions (e.g., here G6PD deficiency, **Figure 5**) are indicative of metabolic rewiring. Here, for example, we identify alterations between the correlates of pyruvate/lactate ratios, suggestive of disrupted NADH/NAD+ homeostasis in G6PD-deficient subjects. This observation confirms and expands upon our previous report about increased levels (and potentially increased activity) of methemoglobin reductase in RBCs from G6DP$^-$ donors (22). On the other hand, we also noted a disruption in the correlation between acetylcarnitine and fructose. Of note, RBC levels of carnitine and acetylcarnitine are, respectively, comparable and higher to the levels observed in plasma (36). Indeed, RBCs are equipped with a functional ATP-citrate lyase, as we (31, 37) and others (38) have shown with tracing experiments with ^{13}C-glucose and other stable isotope tracers. Disruption of correlation between acetylcarnitine and fructose levels in G6DP$^-$ donors is suggestive that, in normal RBCs, at least part of the acetylcarnitine pool is derived from fructose sugar and that this metabolic route is dysregulated in G6DP$^-$ donors. Follow-up tracing experiments with ^{13}C-fructose will be necessary to expand on this observation.

In addition, apart from the intrametabolic correlates, several couples of correlations involving metabolites and physiological RBC/plasma characteristics can be identified in G6PD$^-$ donors, physically linked to each other by exhibiting the same variation profile in fresh blood (non-stored, NS) and throughout storage in CPD/SAGM (**Figure 6**). Results are indicative of a correlation between the energy state of the stored RBC (as gleaned by 2,3-DPG, glucose, and lactate levels) and the preservation of a discocytic phenotype, while MPs release and phosphatidylserine exposure correlated with markers of impaired glutathione homeostasis and (maybe merely spuriously) with the total levels of phthalates measured at any given time point. Of note, correlations between AMP levels and gamma-glutamyl-cycle end-product 5-oxoproline (in oxoprolinase-deficient mature RBCs) is suggestive of an intertwinement between energy and redox metabolism, further confirming our recent reports on the role of oxidative stress in stored RBC energy impairment secondary to AMP deaminase activation (39).

The Metabolic and Biopreservation Profiles of G6PD$^-$ Packed RBCs Were Closely Related to the Biological Profile of the Donor: Intra-Parameter Relationships

Several metabolites and physiological characteristics of G6PD$^-$ packed RBCs fluctuate throughout the storage period

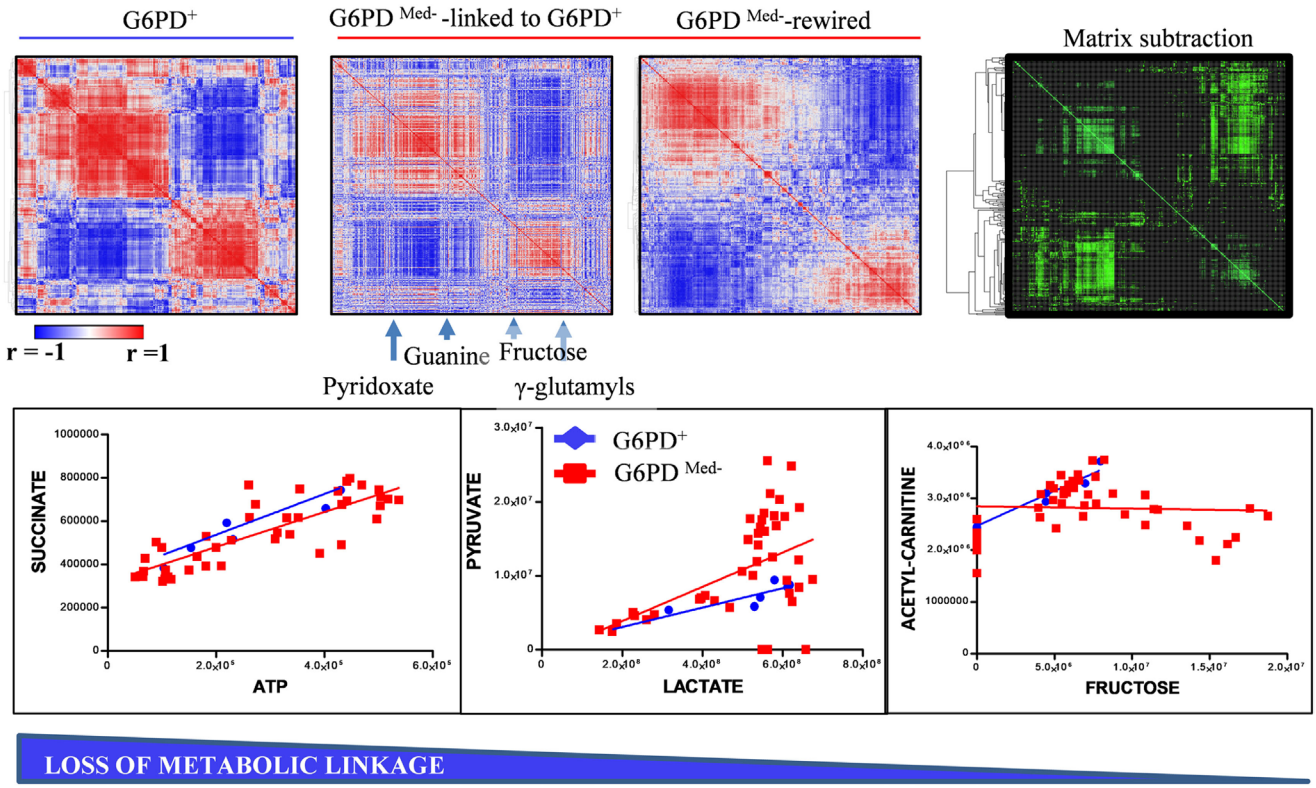

FIGURE 5 | Metabolic linkage (35) analyses in glucose 6-phosphate dehydrogenase (G6PD)-deficient and sufficient red blood cells (RBCs). Metabolite levels were correlated to each other in both groups (independently of storage age), to identify variations ($\Delta|r| > 30\%$) in metabolite levels secondary to metabolic rewiring in G6PD-deficient donors when compared to G6PD sufficient controls. Phenotypic alterations that disrupted this fine tuning of the kinetics of specific metabolic pathways would be highlighted by a differential analysis of correlation of metabolites across conditions. Blue to red = $-1 < r < +1$. A highlight of correlations varying >30% between these two conditions is shown in green. For example, significant alteration of the linkage between lactate and pyruvate or acetyl carnitines and fructose, but not succinate and ATP, was observed in G6PD-deficient RBCs (red line and squares) vs. G6PD sufficient counterparts (blue lines and rhomboids).

proportionally to their own baseline levels *in vivo*, as shown in Table S3 in Supplementary Material (whole storage) and in the representative scatter plots of **Figure 7** (end-of-storage). Among these, the decreasing over storage (22, 40) G6PD activity, reducing power (NADPH) and antioxidant capacity (23), along with the increasing glycated Hb (HbA1c) (41) and osmotic hemolysis levels (23) were included. Similar findings regarding the donor-dependent resistance of RBCs to osmotic lysis and the antioxidant capacity of the supernatant have been previously detected in packed RBCs from G6PD$^+$ donors (14, 15, 42, 43), signifying a "donor-signature effect" on storage. Indeed, while their absolute values vary following variations in storage duration, mediums, and strategies, or in the genetic background (current study), their overall storage profile is steadily a function of donor levels. In other words, the blood banking affects them equally and by a "stable factor" of effect.

Inter-Parameter Relationships and Networking

In addition to those "intra-parameter" relationships, more than 900 repeatable correlations observed between variables in fresh and stored RBCs, as shown in the G6PD$^-$ specific *in vivo*-vs. -*ex vivo* biological network of Figure S2 in Supplementary Material. This complex structure is a correlation-based, strict assessment of distinct and multifarious experimental metrics, designed to minimize the unreliable interrelations, as each connection is statistically significant at 6 different time points of the storage period (a total of 36 replicates in the 6 G6PD$^-$ units). Certain hub nodes represented specific storability variables that correlated to a large number of donor entities (and *vice versa*). The higher density of connections is observed at the upper right area of the network (I), where in-bag hemolysis and susceptibility to hemolysis (I-A) clustered with the 2,3-BPG (I-B) and dehydroascorbate (DHA, I-C) hub nodes. In a clockwise way, the areas of bile acids (II), extracellular K^+ (III), G6PD activity (IV), redox (including ROS, urate, and antioxidant capacity, V), hematological (MCV, MCH, HbA_{1c}, VI), and fatty/bile acids (VII) can be observed (Figure S2 in Supplementary Material). To focus on entities intrinsically related to the G6PD deficiency, the progress of storage lesion (e.g., redox status) and the quality of RBC concentrates (e.g., hemolysis-related variables) fragmentary analyses of sub-networks were subsequently performed.

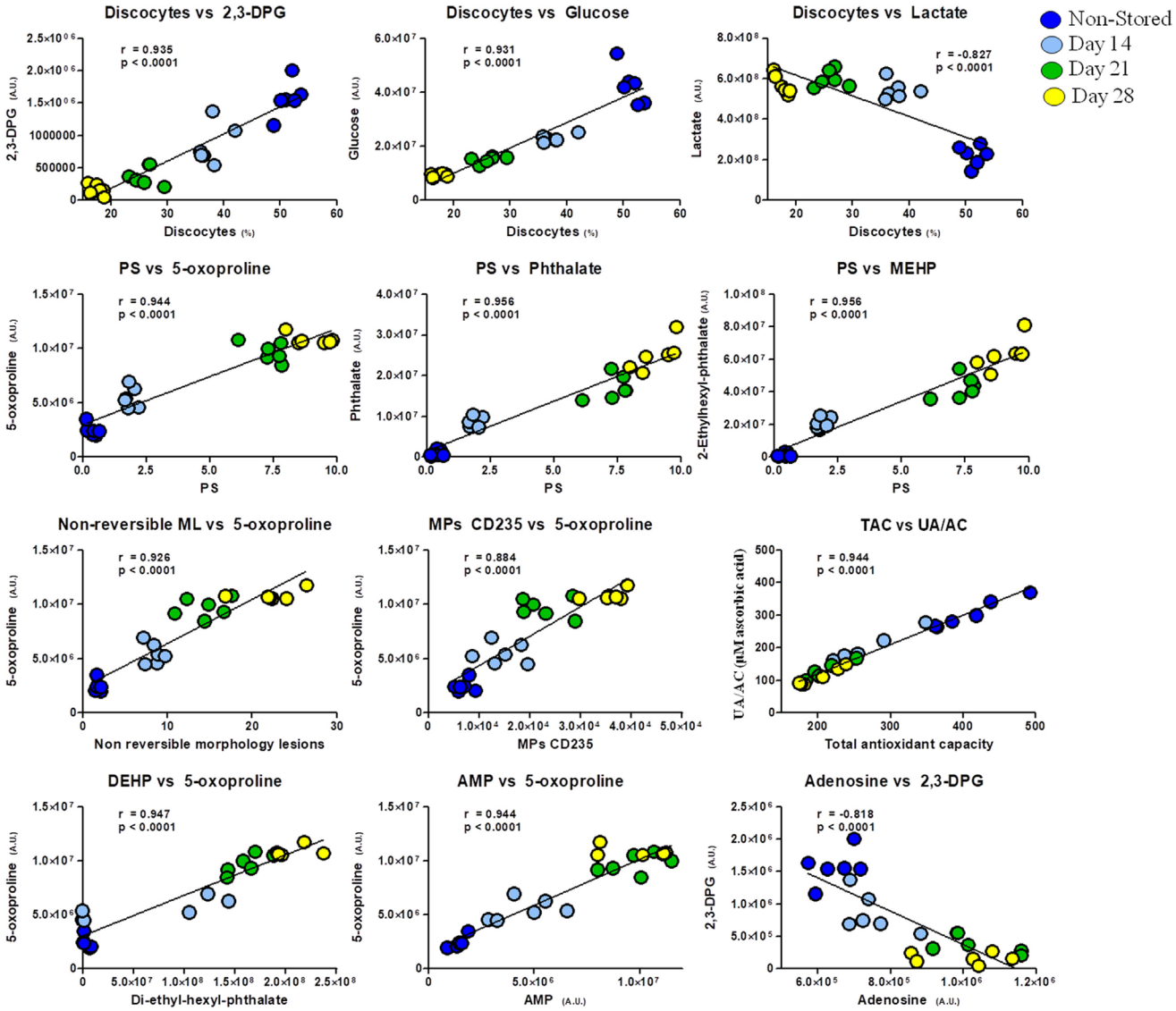

FIGURE 6 | Correlation analysis of metabolites with physiological features and morphological outcomes in glucose 6-phosphate dehydrogenase-deficient red blood cells at different storage days (non-stored/fresh blood: dark blue; day 14: light blue; day 21: green; day 28: yellow dots).

G6PD and Metabolic Networking Revealed that RBC Storage Biology Is Physically Related to Donor Biology, though at a Broader Level of Interwoven Underlying Pathways

In the G6PD activity network (**Figure 8**), the baseline levels had positive correlations with the in-bag levels of amino acids and 2-OH-glutarate. On the other side, in-bag G6PD activity had inverse correlations with metabolites of the PPP cycle, monounsaturated fatty acids, bile acids, and oxidized lipids in fresh blood.

Regarding the main metabolic pathways, the end-products of glycolysis (Figure S3 in Supplementary Material) *in vivo* had negative correlations with the levels of 2,3-BPG but opposite correlations with numerous hemolysis/fragility parameters of the RBC concentrates and supernatant K^+. In the same way, in the PPP and one-carbon metabolism pathways (Figure S4 in Supplementary Material), sedoheptulose-1-phosphate, and folate levels in fresh RBCs had positive correlations with in-bag levels of 2,3-BPG and redox variables (DHA) but inverse correlations with hemolysis-related metrics. An interesting link between *in vivo* levels of protein carbonylation and osmotic fragility with the reducing power of the packed RBCs was also noticed. In reverse, *in vivo* levels of glucono-1,5-lactone-6P had strong correlations with those of the UA-dependent antioxidant capacity of the supernatant and of the G6PD activity. In the glutathione cycle, transaminases, and malate-aspartate shuttle, the majority of connections concerned *in vivo* GSH/GSSG content, fumarate, and malate toward stored RBCs' amino acid and fatty acids metabolism, DHA, malate, 2,3-BPG, hemolysis, and extracellular K^+.

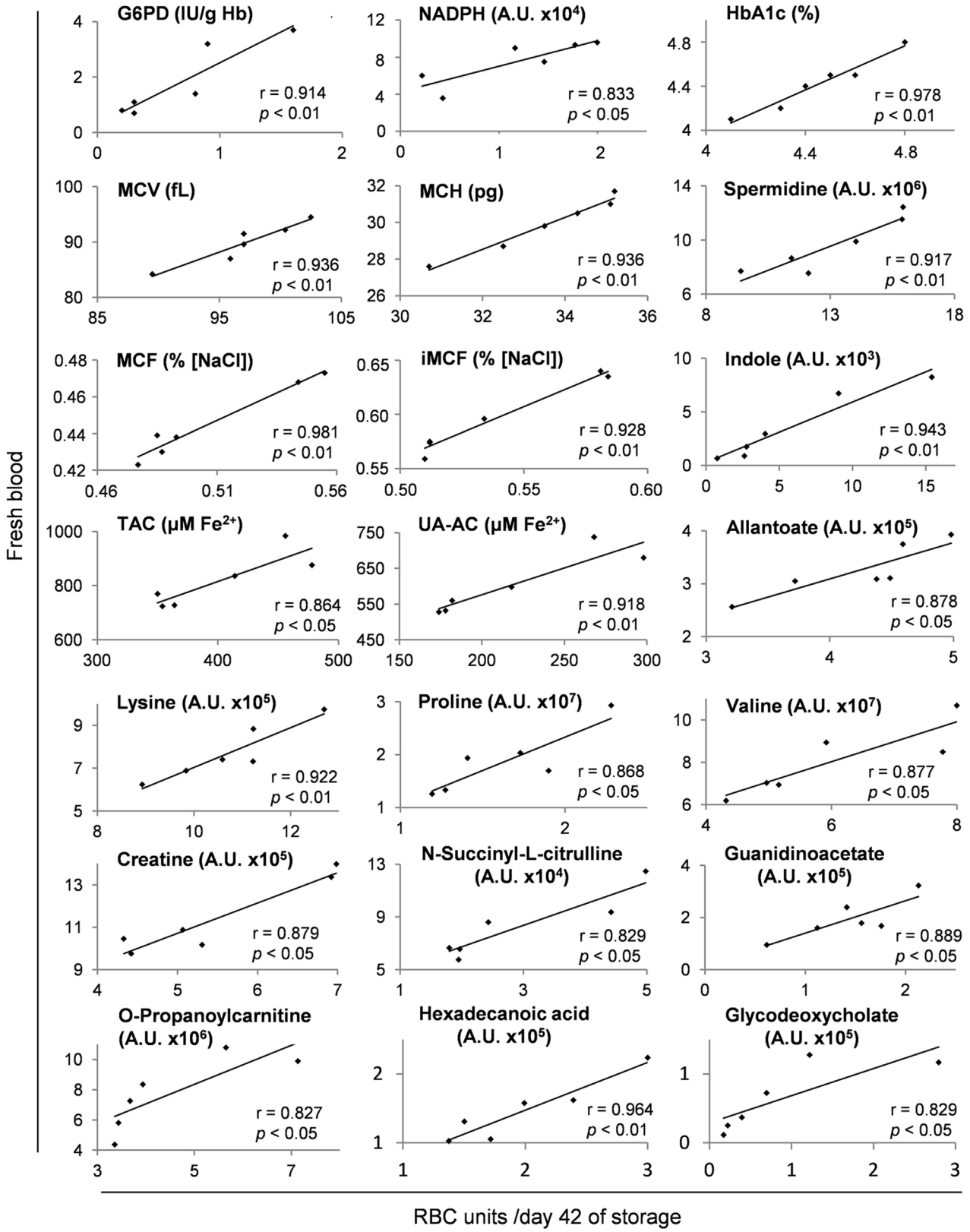

FIGURE 7 | Representative scatter plots of Pearson's correlations between hematological, physiological, and metabolism parameters in fresh donors' blood and packed red blood cells (RBCs) of the same donors after 42 days of storage. Apart from day 42 shown here, these fresh blood variables had statistically significant correlations (see Materials and Methods) with those of stored RBC units at every time point of the storage period, as shown in Table S3 in Supplementary Material.

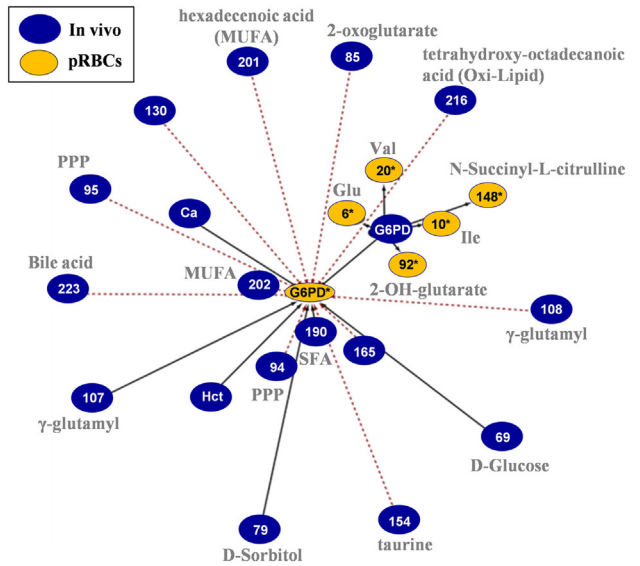

FIGURE 8 | *In vivo* (fresh blood) vs. *ex vivo* [packed red blood cells (RBCs)] network analysis of glucose 6-phosphate dehydrogenase (G6PD) activity in G6PD⁻ donors. The G6PD activity sub-network consists of 23 statistically significant and repeatable at any storage duration correlations (see Materials and Methods). Same as all networks currently reported, only the correlations of Day 42 with fresh blood measurements are shown; however, those pairs of statistically significant connections (with slightly different *r*) applied to all possible storage durations (Days 7, 14, 21, 28, 35, and 42). The length of each line is inversely proportional to the *r* value of the correlation (the shorter the edge, the higher *r* value). Continuous black lines: positive correlations; dashed red lines: negative correlations.

Consistent with the involvement of G6PD (through the production of NADPH) in the reductive biosynthesis of fatty acids and cholesterol (44), two-way ANOVA processing of the metabolomics analyses revealed clear differences in lipid content, biosynthesis, and metabolism between G6PD⁻ and control RBCs both *in vivo* (e.g., palmitate, oleate) and during storage (e.g., glycerol phosphate, eiosatrienoate, arachidonate) (**Figure 3**), in a way likely affecting recoveries, as shown in multiple mouse strains (16, 25). In those studies, lipid metabolism, degradation and oxidation emerged as the strongest correlates with poor 24-h recoveries. Moreover, in both animal and human transfusion contexts, bioactive lipid components of the transfusate are considered clinically relevant for the transfused recipients (45). Subsequent network analysis (Figure S5 in Supplementary Material) further verified the central role of lipids in defining the demanding membrane properties and thus, the quality of stored G6PD⁻ RBCs (5, 25). The levels of linoleate, thromboxane B2, and leukotriene C4 had inverse correlations with those of 2,3-BPG and DHA in the RBC unit, while those of eicosapentaenoic/eicosatetraenoic and hexadecanoic acids at donation correlated well (negatively) with the fragility of stored RBCs and the concentration of extracellular potassium in the supernatant.

The interesting interplay among the G6PD-affected metabolic pathways (e.g., NAD+/glucose-6-phosphate, GSH/2,3-BPG) before and during refrigerated storage, suggests that RBC storage biology reflects a part of donor biology, in a way hardly revealed by individual factor metrics. Lactate levels in the G6PD⁻ donors, for example, were not proportional to lactate levels in stored RBC units, but rather with those of the pathway-interconnected 2,3-BPG.

The Quality of the Biopreservation of the G6PD⁻ RBC Unit Is Linked by Biochemical and Cellular Pathways with the *In Vivo* State

The network analyses made clear that the redox and hemolysis-related parameters of the G6PD⁻ RBC unit are among those most correlated with the *in vivo* state. These variables characterize not only the storability of G6PD⁻ RBCs but also a part of their post-transfusion performance. The sub-interactome shown in **Figure 9** contains the sum of the *in vivo* physiological and omics variables having correlations with the in-bag levels of oxidant/antioxidant variables. According to this map, donor levels of RBC ROS had no correlation with those of stored RBCs, but they did have a strong inverse correlation with DHA levels, throughout the storage period. Reversely, susceptibility of stored RBCs to thermal- or oxidant-induced ROS generation had strong correlations with the levels of serum UA in fresh blood. In addition, the antioxidant capacity of the supernatant had positive correlations with the *in vivo* levels of UA/allantoin, PPP components, GSH, arachidonate, and fatty acid metabolism. It seemed that high levels of GSSG in fresh blood predispose the RBC unit to low levels of UA-dependent antioxidant activity throughout the storage period. Storage DHA was the most important hub nod in the redox network. It showed positive correlations with amino acids, nucleotides, GPLs such as sphingosine-1-phosphate, prostaglandin D3, leukotriene A4, and folate, but opposite correlations with the levels of intracellular ROS, fumarate, malate, and leukotriene C4 at donation.

Regarding the hemolytic variables of the RBC units in G6PD deficiency, an impressive polyparametric sub-network of 173 connections links them to the *in vivo* state (**Figure 10**). The network refers to in-bag levels of hemolysis and to the osmotic/mechanical fragility of stored RBCs *in situ* or in post-storage-simulating conditions. A few donor variables had individual correlations with in-bag hemolysis (e.g., AMP, 5-oxoproline, GSH), or osmotic hemolysis (e.g., NADPH, xanthine, citrulline), or mechanical hemolysis (e.g., homocysteine); however, the vast majority of them correlated (positively or negatively) with most of, or all, the hemolysis-related variables of packed RBCs (*r*-independent circular network in the upper panel of **Figure 10**). Thus, increased levels of amino acids, lipids, and lipid metabolism factors (including sphingosine-1-phosphate and prostaglandin D3), serine biosynthesis metabolites, NADPH, and GSH, among others *in vivo* predispose RBCs to better storability profiles (*r*-driven sub-network in the down panel of **Figure 10**). A clear opposite trend was seen for the protein carbonylation, osmotic fragility, AMP (see also purines involving network in Figure S6 in Supplementary Material), lactate, malate, 2-OH-glutarate (that is a marker of hypoxia), fumarate, and oxoproline. In the hemolysis network, certain donor metabolites are components of the one-carbon and sulfur metabolism (see also Figure S7

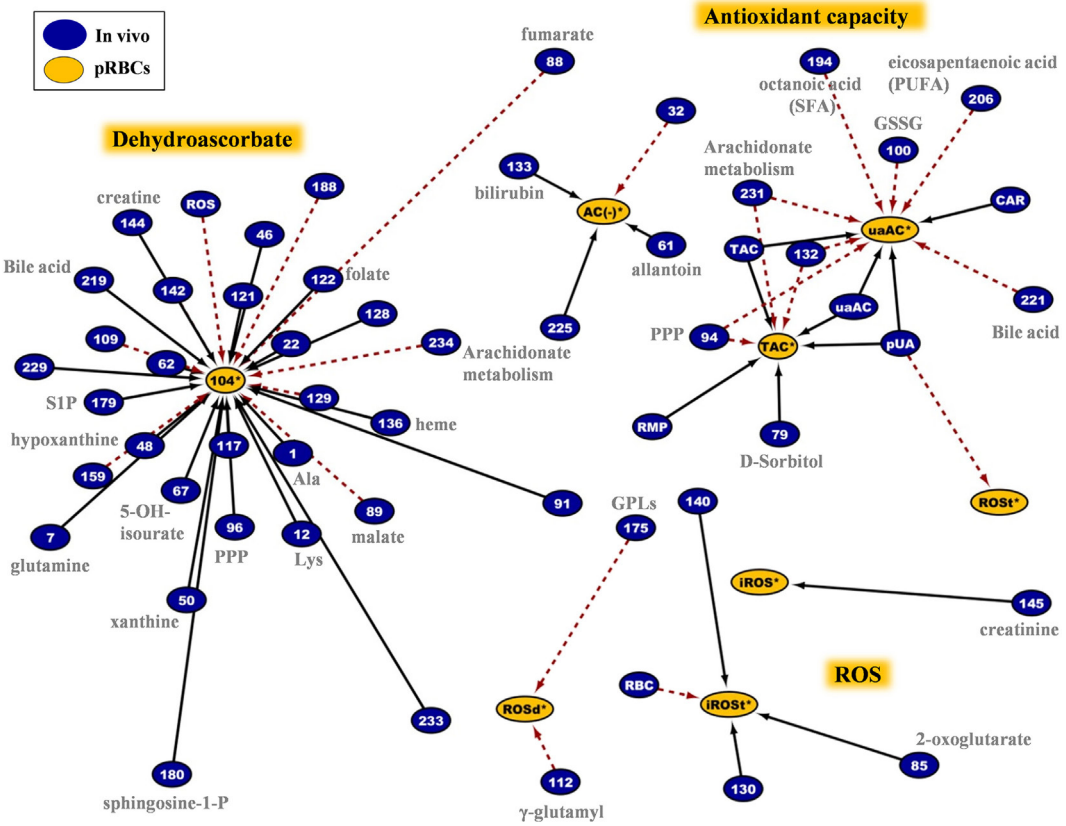

FIGURE 9 | Network analysis of donors' variables at time of donation that had significant correlations with the redox status of the packed red blood cells (RBCs) regardless of the storage duration. The sub-network consists of 62 connections involving intracellular reactive oxygen species (ROS) accumulation, antioxidant capacity of the supernatant, DHA, and other variables. In-bag DHA and ROS levels at post-storage-mimicking conditions represented the most important hub nods. Plasma uric acid and its attributed antioxidant capacity revealed their "intra-parameter" dynamics (see also **Figure 7**). Continuous black lines: positive correlations; dashed red lines: negative correlations.

in Supplementary Material) that were found at lower (e.g., methionine) or higher (e.g., homocysteine) levels in the G6PD$^-$ donors compared to controls (**Figure 2**). It is worth noting that while G6PD activity *in vivo* had no correlation itself with in-bag hemolysis or cellular fragilities, several G6PD-related metabolites (GSH, NADPH, etc.) had strong correlations with at least one hemolysis-related node, verifying the significantly higher analytical power of metabolomics compared to one-molecule-targeted biochemical approaches for probing cellular physiology (46).

The G6PD$^-$ RBCs are more susceptible than the G6PD$^+$ RBCs to K^+ leak (22), which potentially increases the risk of hyperkalemia-induced arrhythmia in susceptible recipients (47). End-of-storage extracellular potassium had numerous negative correlations with donor metabolites in fresh RBCs, most of which in the categories of fatty acids and lipid metabolism or metabolites potentially involved in lipid and protein oxidation (e.g., the highly expressed L-homocysteine) (**Figure 11**, *left panel*). As in the case of in-bag hemolysis and mechanical fragility, high *in vivo* levels of fumarate, phosphoenolopyruvate, and hydroxybutyrate correlated with high in-bag potassium concentration. The progressive degradation of RBC membrane with storage apparently leads to increasing levels of extracellular Hb (as both free Hb and extracellular vesicles) and potassium. Of note, donor levels of AMP were negatively associated with both variables. Finally, the phthalate-specific network support the above mentioned hypothesis that the distinct lipid composition and mechanical properties of the membrane in G6PD deficiency may drive the differential incorporation of DEHP in the bilayer of stored RBCs (**Figure 11**, *right panel*).

Hemolysis Is a Multivariate "Phenotype" of the RBC Storage Lesion, Functionally Connected to Donor Biology by More than One Tethers

Strikingly enough, donor levels of free Hb and RBC mechanical fragility did not correlate with any of the hemolysis-related variables of the RBC concentrates (**Figure 10**). According to a number of metabolomic studies, genetic factors contribute substantially to the degree of storage hemolysis (5), while several donor-specific "metabotypes" have been described in stored RBCs (48). Hemolysis, however, is a multivariate phenotype of the stored

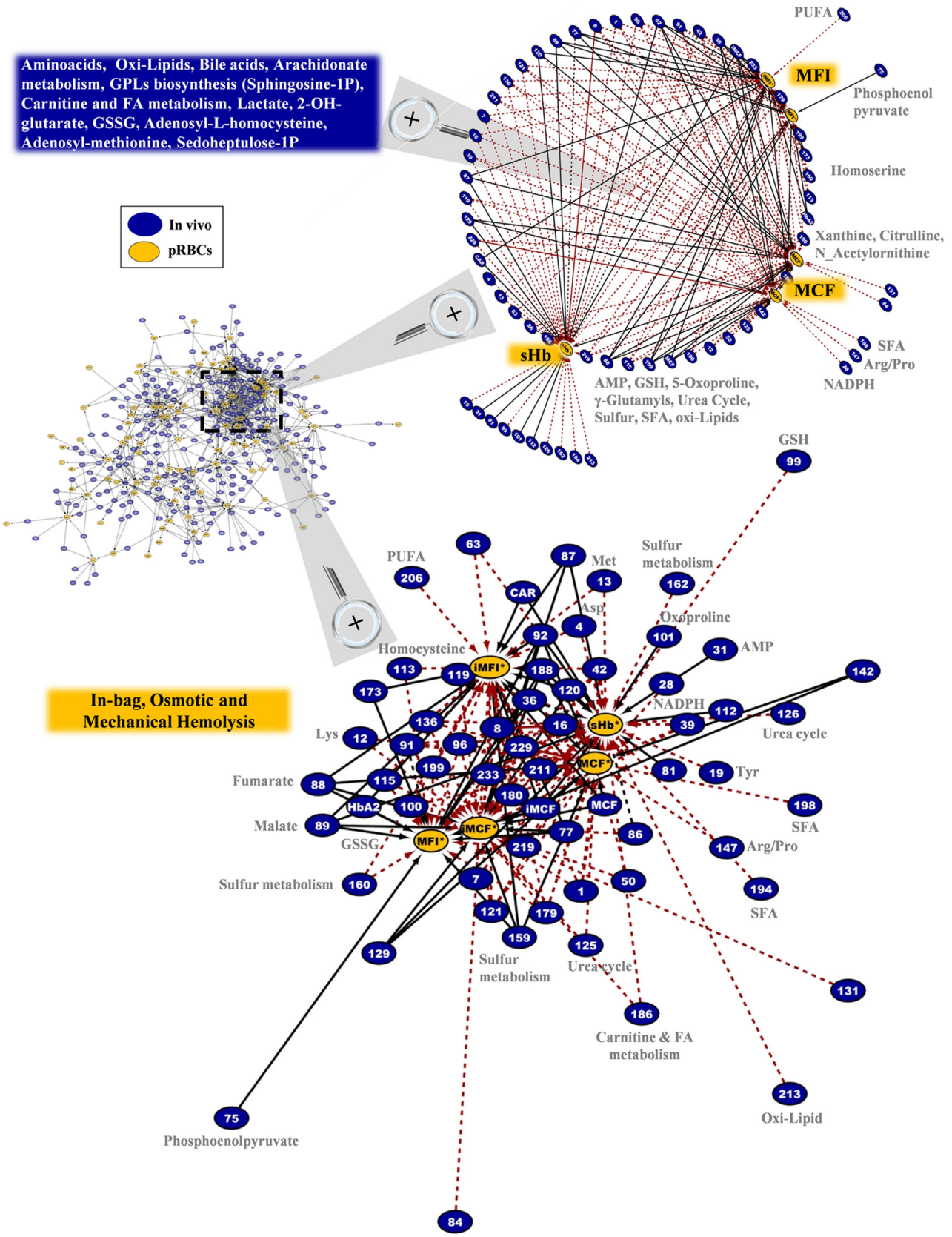

FIGURE 10 | Continued

FIGURE 10 | In-bag hemolysis (sHb) and the resistance of stored G6PD⁻ red blood cells (RBCs) to osmotic or mechanical lysis had several correlations with the *in vivo* state. Magnification of the most dense network cluster (dashed frame) that contains the hemolysis-related nodes of the packed RBCs. *Upper panel*: circle layout of the donor variables reveals that only few of them had correlations with individual hemolysis metrics in packed RBCs (right side of the map), as the majority are connected with at least two of them (blue box in the left side). In this layout, the length of the connections is unrelated to the correlation coefficient *r* value; however, the positive/negative correlations follow the color code shown in **Figure 8** (black-solid/red-dashed, respectively). *Down panel*: the interactome connecting hemolysis-related nodes by 173 repeatable correlations captures a part of the polyparametricity of the "hemolysis" phenotype and the "intra-parameter" dynamics of RBC osmotic fragility both *in situ* (MCF) and following incubation for 24 h at 37°C (iMCF). Continuous black lines: positive correlations; dashed red lines: negative correlations.

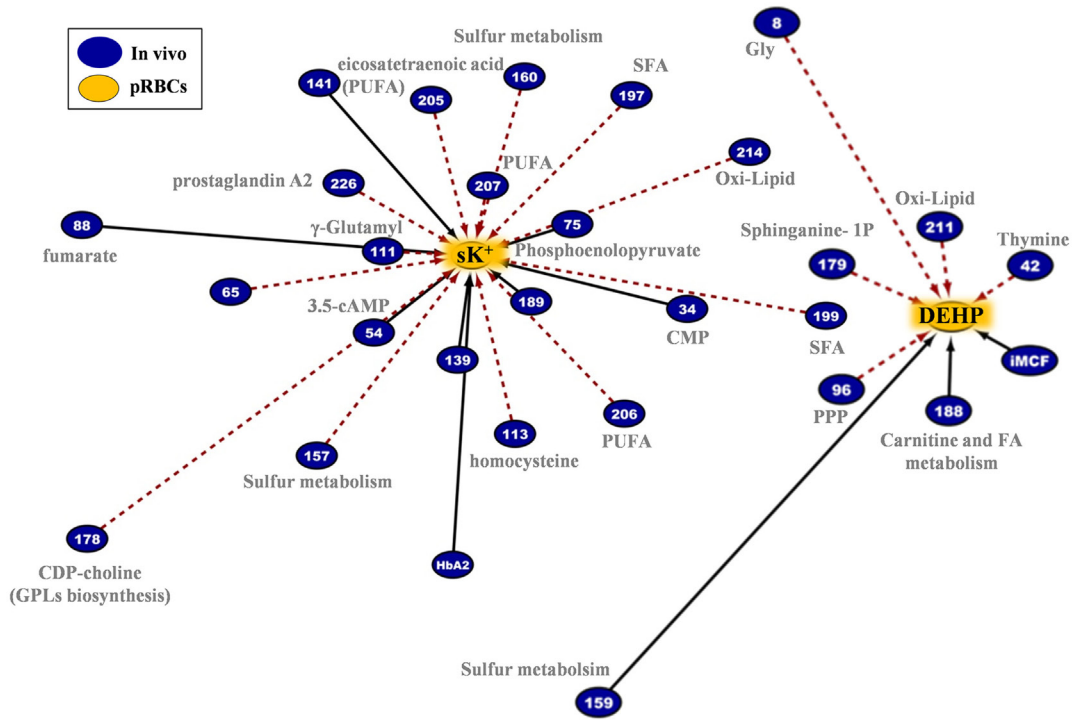

FIGURE 11 | Extracellular potassium and bis(2-ethylhexyl) phthalate (DEHP) connections in pRBCs donated by G6PD⁻ donors. Lipids and lipid metabolism constitute significant contributors in both networks. Continuous black lines: positive correlations; dashed red lines: negative correlations.

RBCs and our study revealed only a fraction of the multitude and the complexity of the donor factors that likely affect it. Without doubt, additional omics analyses (proteomics, lipidomics, etc.) would further elucidate the phenomenon. The biological complexity is too high and the *in vitro* system substantially different compared to the *in vivo* state where the homeostatic mechanisms of healthy RBCs were evolved to meet the cell integrity needs. In-bag hemolysis and post-transfusion recovery represent the overall effect of storage- and recipient-related stresses on distinct physiological characteristics of RBCs that might be donor related. For instance, the osmotic fragility of CPDA-stored RBCs has a correlation with the levels of in-bag hemolysis, but, in quantity terms, fragility can reveal no more than 11% of its variation (42). Moreover, stored RBCs from G6PD⁺ donors that repeatedly exhibit high in-bag hemolysis at outdate are also characterized by reduced ability to resist osmotic stress compared to those exhibiting normal hemolysis (49). In a similar way, in-bag hemolysis in G6PD⁻ units had a positive correlation with the baseline levels of osmotic hemolysis at body temperature (**Figure 10**).

Interplay of Redox, Energy, and Hemolysis-Related Factors before and during Storage

The hemolytic phenotype is likely based on changes in redox and energy metabolism that affect the deformability and stability of RBCs under physiological or pathologically levels of stress (50). The redox activity of RBCs, which contain a strongly oxidizing cytoplasm, governs their lifetime in circulation (51, 52), and thus, changes in the antioxidant activity during storage (53) may have a substantial effect on pre- and post-storage viability. Quantitative proteomics analyses have identified a number of proteins in the supernatant of RBC units showing linear correlations with the absolute levels of extracellular Hb (54), while the donor-related susceptibility of stored RBCs to hemolysis was associated with modifications in RBC membrane proteins involved in oxidative response pathways and decreased storage levels of 2,3-BPG (49). The present study in G6PD⁻ RBCs, which are more susceptible to metabolic changes and protein oxidation compared to normal

cells (22, 24), further revealed the strong interplay of in-bag hemolysis and donor biology, since, for instance, RBC protein oxidation at baseline had positive correlations with both in-bag and mechanical hemolysis at body temperature (**Figure 10**).

Moreover, the levels of a relatively homogeneous panel of metabolites in fresh blood (including amino acids, carboxylic acids, GPLs, fatty acids, and purine metabolism components) strongly predispose RBCs to either good or poor storage. Previous studies in stored RBCs have identified some of them (e.g., amino acids) as having similar trend of correlations (negative) with the same-day hemolysis levels (5). In our study, sphingosine-1-phosphate showed positive correlation with the quality of the RBC unit. This bioactive signaling lysophospholipid, is functionally related to RBC (55, 56) and transfusion biology (57) as having critical roles in blood homeostasis and vascular permeability (58). The interconnections of redox, energy, and hemolysis parameters before and during storage (e.g., lactate/hemolysis, GSH/hemolysis, NADPH/osmotic fragility, ROS/2,3-BPG in fresh/stored samples, respectively), further highlight the usefulness of omics/bioinformatics analyses in revealing the complexity of the RBC storage lesion as a function of inter-donor variability and its underlying mechanisms.

Some Pieces of the Interactomes Might Be Related with the Post-Transfusion Performance of G6PD-Deficient RBCs

According to previous reports, the G6PD$^-$ RBCs exhibited normal levels of ROS and in-bag hemolysis; however, exposure to post-transfusion mimicking conditions, including recipient plasma, body temperature (37°C), and oxidants (24), promote hemolysis and ROS accumulation compared to control RBCs (22). The deformability of stored RBCs may determine their quality and post-transfusion performance (59), while *in vitro* testing of RBC responses in recipient-mimicking contexts would reveal clinically relevant sublethal injuries of transfused RBCs (2, 60, 61). In this context, it was interesting that the redox network of **Figure 9** includes several responses of stored RBCs to oxidants (tBHP, diamide) that may indeed interfere with the function of stored RBCs in recipients characterized by (medication- or infection-induced) redox disequilibrium. Moreover, variation in the RBC osmotic fragility throughout the storage period was found proportional to its baseline levels in G6PD$^-$ donors (**Figure 10**). In a similar way, numerous biochemical components *in vivo* (including S-adenosyl methionine that provides cysteine to support GSH synthesis) had significant correlations with the fragilities of stored G6PD$^-$ RBCs after 24 h staying at body temperature (iMCF, iMFI). Of note, alpha-tocopherol levels in fresh mouse RBCs were reported to correlate with recovery in animal studies (25) and its levels in G6PD$^-$ donors correlated not only with in-bag hemolysis but also with the pro-hemolytic features of the stored RBCs. Our findings may be functionally linked to the performance of G6PD$^-$ RBCs, which has been questioned at both laboratory (22) and clinical levels (21). To support, *in vivo* levels of aspartate, glutamine and thymine were indeed reported to correlate with recovery in mice (25). Despite the fact that these correlations should be confirmed by *in vivo* studies in human, they provide an insight into the pathophysiology of post-transfusion complications in therapies involving G6PD$^-$ donors.

CONCLUSION

This study showed for the first time that the metabolic phenotypes of G6PD-deficient donors recapitulate the basic storage lesion profile observed in G6PD sufficient donors, which is characterized by loss of metabolic linkage and rewiring, in spite of certain differences observed in one-carbon metabolism, glutathione/urate homeostasis, and fatty acid pathways. Moreover, it revealed that donor variability issues affect the storage quality even in the narrow context of this small donor subgroup characterized by an enzymatic genetic defect. We reported an interesting and informative interplay between redox, energy, and hemolysis parameters before and during storage, namely, between factors which differ among G6PD$^-$ donors at the time blood was harvested, and the storability of donated RBCs, a part of which may be related to their performance in the transfused patient. Our data provide mechanistic insight into the biology of RBC storage in G6PD deficiency and could guide future studies focusing on donor biology-related factors involved in the regulation of storage-induced hemolysis that is a multivariate phenotype of the RBC storage lesion. Development of reliable physiological and metabolic biomarkers of storage quality and post-transfusion performance (e.g., post-transfusion recovery, iron metabolism) in fresh or stored blood from G6PD sufficient or deficient donors would allow donor screening and thus improved management of both donor and RBC inventory at time of collection or prior to release of the RBC concentrates from the Blood Bank.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Department of Biology, School of Science, NKUA. Investigations were carried out upon signing of written consent, in accordance with the principles of the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

Each author has contributed to the submitted work as follows: AK, AD, and MA designed the study. JR, TN, and AD performed the UHPLC-MS analyses. VT, AK, and MA prepared the RBC units and performed the hematological and physiological analyses. AV performed the biological networks. VT, AD and MA analyzed the results, prepared the figures, and wrote the first draft of the manuscript. IP critically commented on the interpretation of data and drafting of the manuscript, and all the authors contributed to the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fmed.2017.00248/full#supplementary-material.

Table S1 | Metabolomic analysis of G6PD sufficient (control) and G6PD deficient (G6PD def.) leukoreduced red blood cell concentrates throughout the storage period in CPD-SAGM.

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Fluorescence Exclusion: A Simple Method to Assess Projected Surface, Volume and Morphology of Red Blood Cells Stored in Blood Bank

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Red blood cells (RBC) ability to circulate is closely related to their surface area-to-volume ratio. A decrease in this ratio induces a decrease in RBC deformability that can lead to their retention and elimination in the spleen. We recently showed that a subpopulation of "small RBC" with reduced projected surface area accumulated upon storage in blood bank concentrates, but data on the volume of these altered RBC are lacking. So far, single cell measurement of RBC volume has remained a challenging task achieved by a few sophisticated methods some being subject to potential artifacts. We aimed to develop a reproducible and ergonomic method to assess simultaneously RBC volume and morphology at the single cell level. We adapted the fluorescence exclusion measurement of volume in nucleated cells to the measurement of RBC volume. This method requires no pre-treatment of the cell and can be performed in physiological or experimental buffer. In addition to RBC volume assessment, brightfield images enabling a precise definition of the morphology and the measurement of projected surface area can be generated simultaneously. We first verified that fluorescence exclusion is precise, reproducible and can quantify volume modifications following morphological changes induced by heating or incubation in non-physiological medium. We then used the method to characterize RBC stored for 42 days in SAG-M in blood bank conditions. Simultaneous determination of the volume, projected surface area and morphology allowed to evaluate the surface area-to-volume ratio of individual RBC upon storage. We observed a similar surface area-to-volume ratio in discocytes (D) and echinocytes I (EI), which decreased in EII (7%) and EIII (24%), sphero-echinocytes (SE; 41%) and spherocytes (S; 47%). If RBC

dimensions determine indeed the ability of RBC to cross the spleen, these modifications are expected to induce the rapid splenic entrapment of the most morphologically altered RBC (EIII, SE, and S) and further support the hypothesis of a rapid clearance of the "small RBC" subpopulation by the spleen following transfusion.

Keywords: red blood cell volume, red blood cells, transfusion, red blood cell storage, fluorescence exclusion, red blood cell morphology

INTRODUCTION

Surface area-to-volume ratio is a major determinant of red blood cell (RBC) deformability and ability to circulate (1). Normal discocytes (8 μm in diameter) must indeed withstand stringent deformation as they navigate along 4- to 6-μm-wide microvessels and across 1- to 2-μm-wide inter-endothelial slits in the spleen. Modifications of surface area-to-volume ratio have consequences in physiology (removal of senescent RBC) (2, 3) and pathology (anemia in hereditary spherocytosis and other RBC hemoglobin and membrane disorders) (4–6). Furthermore, RBC display a tight relationship between morphology and deformability, and these parameters may impact transfusion yield. Previous studies have shown that RBC stored in blood bank exhibit morphological alterations that become significant after 3 or 4 weeks of storage depending on the technique and the cell classification (7–10). It has been also suggested that these alterations are associated with surface and volume modifications (11). A decrease in surface area-to-volume ratio induces a decrease in RBC deformability that can lead to their retention and elimination in the spleen (12–14). We recently showed that alterations in the morphology of stored RBC was accompanied by a decrease in the projected surface area that only impacts a subpopulation of RBC (10). The proportion of this subpopulation increases upon storage but is highly variable between donors. Determination of the projected surface area of these stored RBC was conducted using imaging flow cytometry, a high throughput tool allowing a detailed and objective quantification of cell morphology and dimensions (15). Imaging flow cytometry however cannot determine cell volume hence misses the key pathophysiological feature of RBC: surface area-to-volume ratio.

So far, single cell measurement of RBC volume has remained a challenging task achieved by a few sophisticated methods, such as micropipette aspiration (16, 17), quantitative phase microscopy (18) including digital holographic microscopy (19), some of these methods are flawed by potential artifacts due to labeling (confocal laser scanning microscopy) (20) or sphering (optical scattering methods) (21). A reproducible and ergonomic method enabling the assessment of the volume and morphology of RBC at the single cell level would be a useful tool to study the storage lesion and more generally RBC physiology and pathology.

The method developed here is based on the dye exclusion principle first proposed by Gray et al. (22) and adapted recently to mammalian cells (23–25). RBC were suspended in a medium supplemented with a fluorescent dye coupled to a dextran molecule and inserted in a microfluidic chamber of fixed height. Dextran is a biocompatible polysaccharide that does not cross cell membranes; fluorescence is thus excluded from the cells which allows volume calculation as the drop in fluorescence intensity is directly related to the thickness of the object (22). RBC volume is obtained by integrating the fluorescence signal over its projected area (see Material and Methods).

This technique also generates brightfield images enabling a simultaneous precise determination of their morphology.

Abbreviations: D, Discocyte; E, Echinocytes; SE, spheroechinocytes; S, spherocytes; RBC, red blood cell; MCV, Mean Corpuscular Volume; SAG-M, Saline-Adenine-Glucose-Mannitol; CPDA, Citrate-Phosphate-Dextrose-Adenine; RPMI, Roswell Park Memorial Institute medium.

MATERIALS AND METHODS

Chamber Design and Fabrication

Molds were fabricated using classic soft lithography methods or micromachining (Minimill3, Minitech Machinery) (24). Pillars were evenly positioned (interpillar distance 200 μm) in the observation chamber to set a very stable height in the chamber. Their value of fluorescence provides a stable signal useful for calibration (see Volume and projected area calculation in Material and Methods). Chips were made using a mixture 1:10 of PDMS (polydimethylsiloxane) and its cross linker (Sylgard 184, Dow Corning) cured at 66°C for 2 h. Inlets and outlets were created with 2 or 3 mm punchers before bonding. Chambers were bond on glass-bottomed petri dishes (Fluorodish) using air (Harrick) plasma cleaner or corona SB (Elveflow). Chamber surface was passivated with Poly(L-lysine) grafted poly(ethylene glycol) (PLL-g-PEG, Surface Solutions) for 30 min to 1 h after bonding. This pre-treatment induces the formation of a polymeric brush that prevents RBC from adhering to the surface. Chambers can be used immediately or stored at 4°C in PBS solution for a few days before use. Prior to RBC injection, PBS was changed to Krebs-Albumin 0.5% solution (Krebs-Henseleit Buffer modified with 2 g glucose, 2.1 g sodium bicarbonate, 0.175 g calcium chloride dehydrate and 5 g AlbuMAX II Lipid-Rich BSA for 1 L sterile water, pH 7.4) supplemented with FITC-dextran. According to its composition, Krebs-Albumin medium should have a refractive index similar to classical cell culture medium and thus be estimated at 1.337 (26).

Sample Preparations

Leukoreduced RBC in Saline-Adenine-Glucose-Mannitol (SAG-M) from healthy donors were supplied by the Etablissement Français du Sang (French Blood Service) 3 days after blood collection. All units were stored in optimal blood bank conditions between 2 and 6°C and for 42 days, according to regulations. Samples were aseptically collected

to perform experiments. Just before analyses, RBC were diluted (1/50) in a Krebs-Albumin 0.5% solution. pH-related morphological alterations were induced by suspension of RBC in a Krebs-Albumin 0.5% solution after adding either HCl or NaOH up to the desired pH. Heated-RBC (HRBC) were produced by incubation of a RBC suspension at 1% hematocrit in RPMI at 50°C in a glass tube for 20 min. We used HRBC and RBC exposed to low and high pH as well-known examples of clear-cut volume and surface modifications RBC. In addition, HRBC have been repeatedly used to measure the biomechanical retention of stiff RBC *in vivo*, including in human subject (27, 28).

Fluorescence Exclusion Sample Preparation

RBC were suspended in the medium supplemented with 1 mg/mL FITC-Dextran (10 kDa) from Sigma-Aldrich. The hydrodynamic radius of the FITC-Dextran used in this study has been determined previously to be 1.86 nm (29) and thus far exceeded the maximum pore radius of the erythrocytes membrane (0.4 nm) (22, 30). RBC concentration was adjusted by modifying the dilution from 1:30 to 1:50 in order to obtain an optimal number of RBC in the chamber and avoid superimposition of RBC or contacts between them that would hamper measurements.

Imaging

Imaging was performed with an automated Nikon Eclipse-Ti microscope equipped with a 20x objective NA.0.75, or a Zeiss AxioObserver microscope equipped with a 20x objective NA.0.8. Compatibility and precision of such objectives have already been shown (25). Fluorescence and brightfield images were acquired sequentially within <0.5 s according to the microscopes manufacturers.

Volume and Projected Area Calculation

Volume calculation was performed as described (24). Briefly, image analysis was performed using homemade MatLab program (The Math Works Inc., Natick, MA, USA). Calibration of the relationship between fluorescence intensity and height was performed for each field using values of fluorescence around each RBC and over the pillars as described (25). Briefly, α is extracted using a robust fit from $I_B = \alpha \cdot h + I_0$, where I_B and I_0 are the background and pillar fluorescence respectively and h the height of the chamber. To correct for the inhomogeneity of the fluorescent lamp, background is locally subtracted and the volume is then calculated by integrating over an area S larger than cell ($V_{Cell} = \iint_S \frac{I_B(x,y)-I(x,y)}{\alpha} dS$). This procedure also integrates the dye deposition on the chamber walls that can occur after several hours. After background removal, the fluorescence value around the RBC is close to zero, the area of integration does not play a crucial role and thus limits errors that could be introduced by a precise segmentation of the cell area. Projected area was extracted from brightfield images after background removal by a basic thresholding and filling procedure using built-in MatLab functions. Briefly, a square region of interest (ROI) was defined around the cell, then a binary gradient image was obtained using the edge function, the outlines of the binary image were dilated (imdilate) and the open area were filled (imfill). Eventually, objects too small or on the edge of the ROI were discarded (imclearborder, imerode). Parameters used during this procedure were adapted by user accordingly to images quality and exposure properties.

Morphological Analysis of RBC

After acquisition, brightfield images where anonymized and randomized for blind evaluation and RBC were classified in 6 morphological categories according to Bessis et al. (31). adapted for DIC microscopy (10). namely discocytes, echinocytes (I, II and III), sphero-echinocytes and spherocytes. Small RBC were defined as previously described (10) as a morphologically altered RBC population that exhibited a decrease in projected surface area and included 3 subpopulations, namely echinocytes III, sphero-echinocytes and spherocytes.

RESULTS

Determination of RBC Volume by Fluorescence Exclusion Is Simple and Requires Limited Specific Instruments

RBC were injected in a microfluidic chamber higher than the maximum size of the cells to prevent mechanical stress (chamber height = 6.8 μm). Its height was however limited to enable the capture of images with excellent contrast. The microfluidic chamber was designed to determine the volume of large numbers of RBC within a single chamber (typically from 500 up to 1,500 RBC depending on the working concentration). Regularly spaced pillars (interpillar distance 200 μm) ensure a constant height across the whole chamber and enable the calibration of the relationship between height and fluorescence (see Material and Methods). The inlet and the outlet were in immediate proximity and a fluid bridge was established between them to easily stop the flow. This prevented any fluid flow circulation in the chamber thereby enabling measurements on still RBC (**Figure 1A**).

Brightfield (**Figure 1B**) and fluorescence (**Figure 1C**) images were acquired using a 20x objective and each acquired fluorescence image enabled the volume determination of 10 to 50 RBC, depending on the concentration. Determination of RBC morphology can be evaluated simultaneously by acquiring and analyzing the corresponding brightfield image and was achieved according to Bessis et al. (see Methods).

Training to prepare chips required a few hours to a few days. The air plasma cleaner was replaced by a cheaper corona SB (Elveflow) that efficiently activated the surfaces before bonding (see Methods). Manufacturing steps are shown in **Figure 2**.

The RBC Volume Quantification by Fluorescence Exclusion Is Precise and Reproducible

To explore the reproducibility of the chip preparation and technique, the RBC volume from a RBC concentrate at day 10 of storage was assessed, on the same day, in two chips (namely A and B), manufactured from the same mold. Volume measurement showed a normal gaussian distribution (**Figure 3A**). Comparison

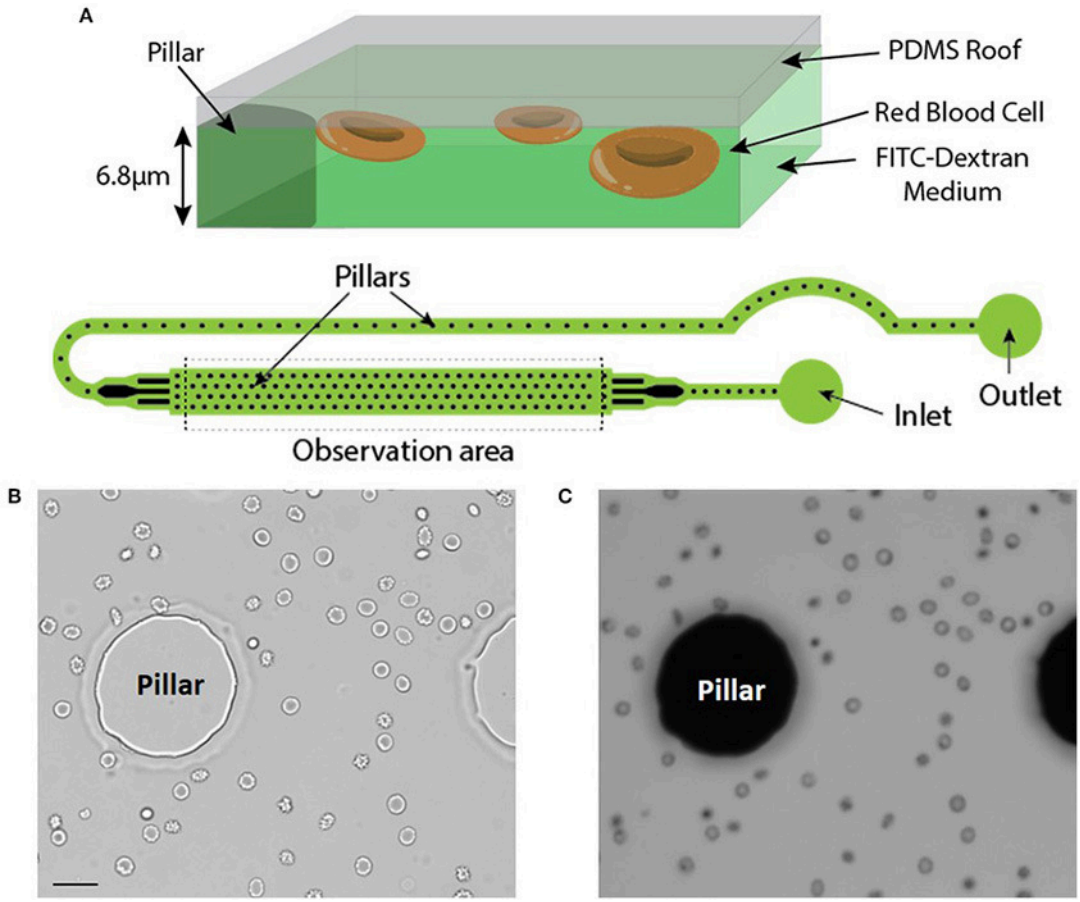

FIGURE 1 | (A) Top: Principle of the fluorescence exclusion method, bottom: design of the microfluidic chamber. **(B,C)** Brightfield and fluorescence images of the same field (x20) showing RBC stored for 42 days in SAG-M (healthy donor) scale bar = 20 µm.

of RBC volume between the two chips revealed a difference in the median volume of 3% (97 μm^3 in chip A vs 100 μm^3 in chip B) and a similar distribution (**Figure 3B**). The repetition of this experiment showed a variation of the median volume of 1.6% (97.4 vs. 99 μm^3) (data not shown). This difference is very likely due to variations in chip height that originate from the master mold itself (one mold contains several chip designs for multiplexing the PDMS chip fabrication) as the error on the height is also 3%. Other sources of error would include, the chip bonding to glass, local variations from height variation within one chip. The background cleaning step during the image processing would also contribute, but only to width distribution and not to the inter chip variations.

Precision of the technique was next assessed using samples containing an increasing concentration of RBC of reduced volume (**Figure 3C**). HRBC were used since they are known to display a loss of volume. Normalized frequency histogram of RBC volume distribution showed a progressive shift to the left when the proportion of heated RBC increased in the sample (0, 25, 50, and 100%) (**Figure 3D**). Mean volume (± SEM) of each sample was 98.4 (±0.54) μm^3, 95.0 (±0.44) μm^3, 92.4 (±0.54) μm^3, and 84.4 (±0.48) μm^3 respectively (**Figure 3E**).

Fluorescence Exclusion Measures Volume While Defining RBC Morphology

We next incubated RBC in media of acidic or basic pH to assess the RBC volume modifications associated with the pH-induced morphological modifications (**Figure 4**). Acidic pH (4.2) generated stomatocytes and sphero-stomatocytes (**Figure 4B**) which exhibited a mean 3% volume loss compared to physiological conditions (pH 7.4) (**Figure 4C**) while basic pH (9.4) generated echinocytes III, sphero-echinocytes and spherocytes (**Figure 4D**) that had lost a mean 11.4% of their volume. Differences in RBC volume were significantly different when measured either at low or normal pH ($p < 0.01$, ** Mann Whitney non-parametric test) and between high and low pH (****$p < 0.0001$).

Fluorescence Exclusion Shows a Decrease in Surface Area-to-Volume Ratio in Red Blood Cell Stored in Blood Bank Conditions

We determined the volume and the projected surface area of RBC stored for 42 days in 3 blood bank concentrates from healthy donors and correlated this measure with their

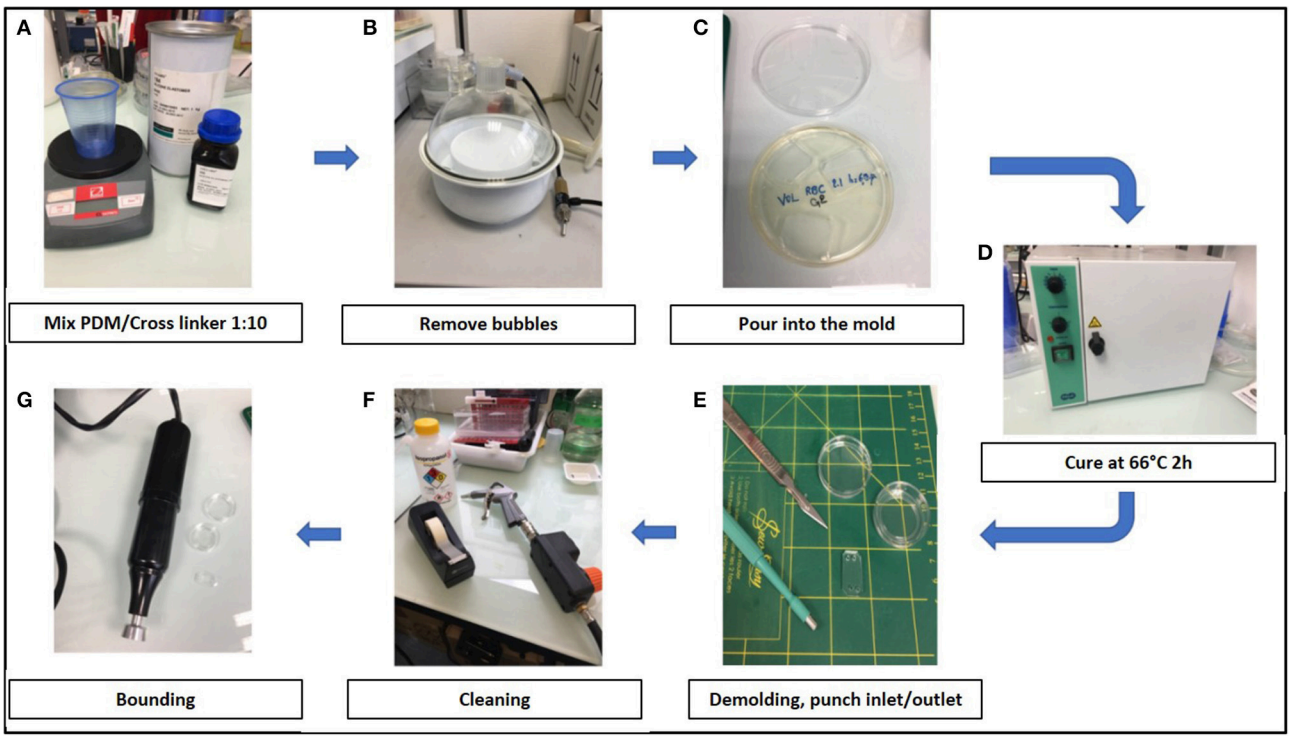

FIGURE 2 | Stepwise preparation of chips: **(A)** Chips were made using a mixture 1:10 of PDMS (polydimethylsiloxane) and its cross linker (Sylgard 184) **(B)** The mixture was degased to remove air bubbles, **(C)** poured into the mold and **(D)** cured at 66°C for 2 h. **(E)** After demolding, inlets and outlets were created with 2 or 3 mm punchers and **(F)** the surface was cleaned using isopropanol, air gun and tape before bonding. **(G)** Chambers were bond on glass-bottomed petri dishes (Fluorodish) using air plasma cleaner (Harrick) or a corona SB (Elveflow).

storage-induced morphological alterations (**Figure 5A**). For the 3 donors, surface distribution on normalized frequency plots was bimodal and confirmed the existence of a subpopulation of "small RBC" with a reduced mean projected surface area (<58 μm^2) (**Figure 5B**). Mean volume (±SD) was 86.0 ± 14.4 μm^3 for donor 1, 85.2 ±14.1 μm^3 for donor 2 and 93.9 ± 13.2 μm^3 for donor 3 (**Figure 5C**). We then determined the projected surface area, volume and surface area-to-volume ratio of RBC for each morphological category (**Figures 5D–F**).

We observed a projected surface area loss of altered RBC when compared to discocytes (D): Echinocytes (E) I, II and III exhibited a mean projected surface area loss of 2, 14, and 32% respectively while the most intensely altered RBC, namely spheroechinocytes (SE) and spherocytes (S) had lost 49 and 51% of their projected surface area, respectively. Altered RBC exhibited also a decrease in their volume although to a lesser extent. When compared to D, the mean volume loss of storage-damaged RBC was 2% (EI), 6% (EII), 10% (EIII), 14% (SE) and 6% (S). This resulted in a surface area-to-volume ratio not modified for EI and decreased of 7 and 24% for EII and EIII respectively, and of 41 and 47% for SE and S. Small RBC (EIII, SE and S) exhibited a mean decrease in surface area and volume of 40 and 11% respectively resulting in a decrease of surface area-to-volume ratio of 32%.

DISCUSSION

We adapted the fluorescence exclusion method to determine RBC volume. This method is reproducible and sensitive and allows the simultaneous measurement of volume and projected surface area together with a detailed determination of RBC morphology at a single cell level.

Several methods such as automated complete blood count and impedance-based Coulter-Counter have been described to measure cell volume, but while of major clinical impact, they have disadvantages regarding mechanistic exploration of RBC. These high-throughput methods indeed provide reproducible data but volume can only be measured on global populations of RBC and not at a single cell level (32). Light scattering in flow cytometry is also a high-throughput method which is frequently used because of the wide availability of flow cytometers. It measures individual RBC volume together with hemoglobin concentration but requires pre-treatment of the cell (sphering). Furthermore, flow cytometry does not enable fine morphological observation of RBC (32–34). Morphology, volume and surface of single RBC can be accurately assessed by micropipette aspiration (16, 17) which also provides biomechanical information such as membrane viscosity and multiple elastic moduli. Micropipette measures are however of low throughput, technically challenging, and mastered by a very few specialized teams. Moreover, mechanical constraints on the RBC during micropipette aspiration may

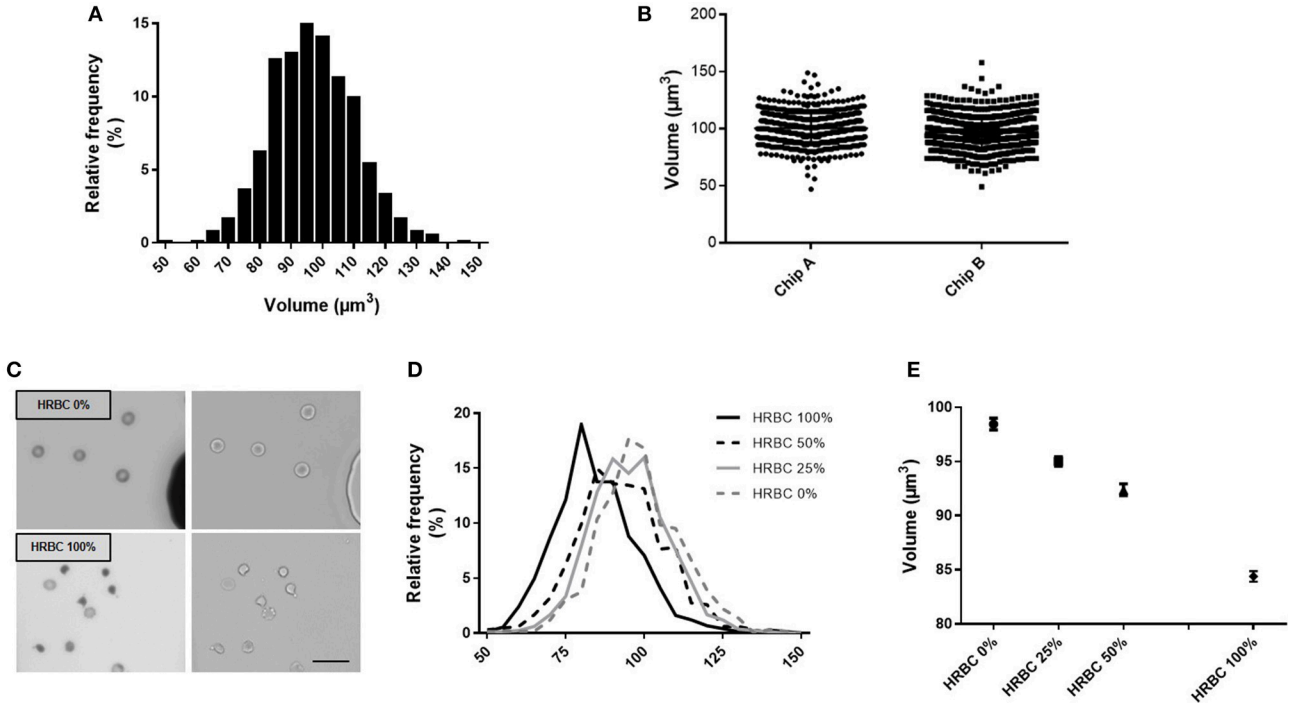

FIGURE 3 | Fluorescence exclusion to measure RBC volume: a precise and reproducible method. **(A)** Normalized frequency histogram of RBC volume distribution from a healthy donor at day 10 of storage in SAG-M. **(B)** Individual volume of RBC from a healthy donor measured using 2 differents chips manufactured from the same mold (chip A and chip B). **(C)** Fluorescence (left) and brightfield (right) images (20x) of control RBC (top) or 100% heated RBC (bottom) scale bar = 20 µm; **(D)** Normalized frequency histogram of RBC volume distribution, and (E) Mean volume (+/−SEM) of samples containing increasing concentrations of heated-RBC.

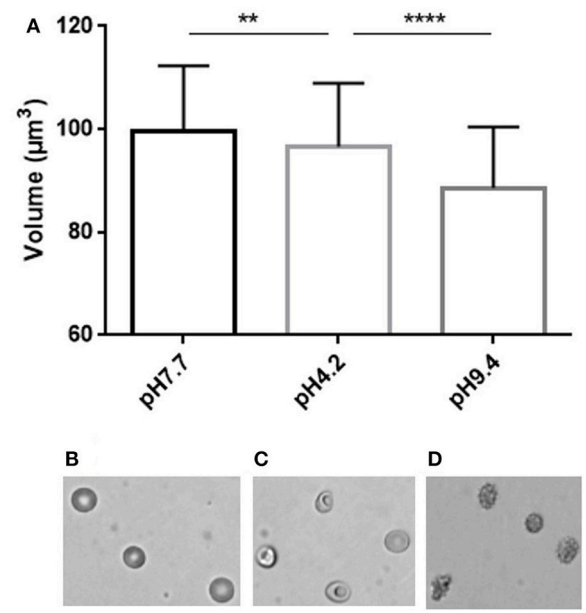

FIGURE 4 | Simultaneous determination of RBC volume and morphology using fluorescence exclusion and microscopy. **(A)** Mean (standard deviation) volume of RBC samples exposed to physiological (left), low (center) and high (right) pH. Stars represent the significance level of the Mann-Whitney statistic test, ** when $p < 0.01$, **** when $p < 0.0001$ **(B–D)** Representative brightfield images of RBC in the same samples.

generate artifactual changes in morphology or volume. Lately, new techniques to obtain RBC volume have been proposed, based on sophisticated microscopical methods and microfluidics. Guo et al. have generated a microfluidic device to measure RBC volume based on electric current modification as cells pass through a gate (MOFSET-based detection) (35). Confocal microscopy can also be used but, in addition to requiring a specific instrumentation, this technique requires membrane labeling that can modify RBC morphology (19). Quantitative phase imaging, including digital holographic microscopy can provide RBC volume and RBC refractive index, but these two parameters are measured separately in isotonic liquids of different refractive indexes and thus requires RBC adhesion to the surface or complex microfluidic setup in order to perfuse solutions (19, 36). Scanning electron microscopy has also been proposed, but its usefulness is limited by a very low throughput and the limited availability of the instrument. Not least cell fixation is required and known to induce both morphological and volume changes.

The principle of dye exclusion has been recently adapted to RBC by Schonbrun et al. (26). RBC are suspended in an index-matching absorbing solution and volume can be measured from the modification in light absorbance between the cells and the background (37). Measures with this technique are independent from cell refractive index and provide microscopic spatial resolution of single cells as well as medium throughput, but microfluidic controllers are needed.

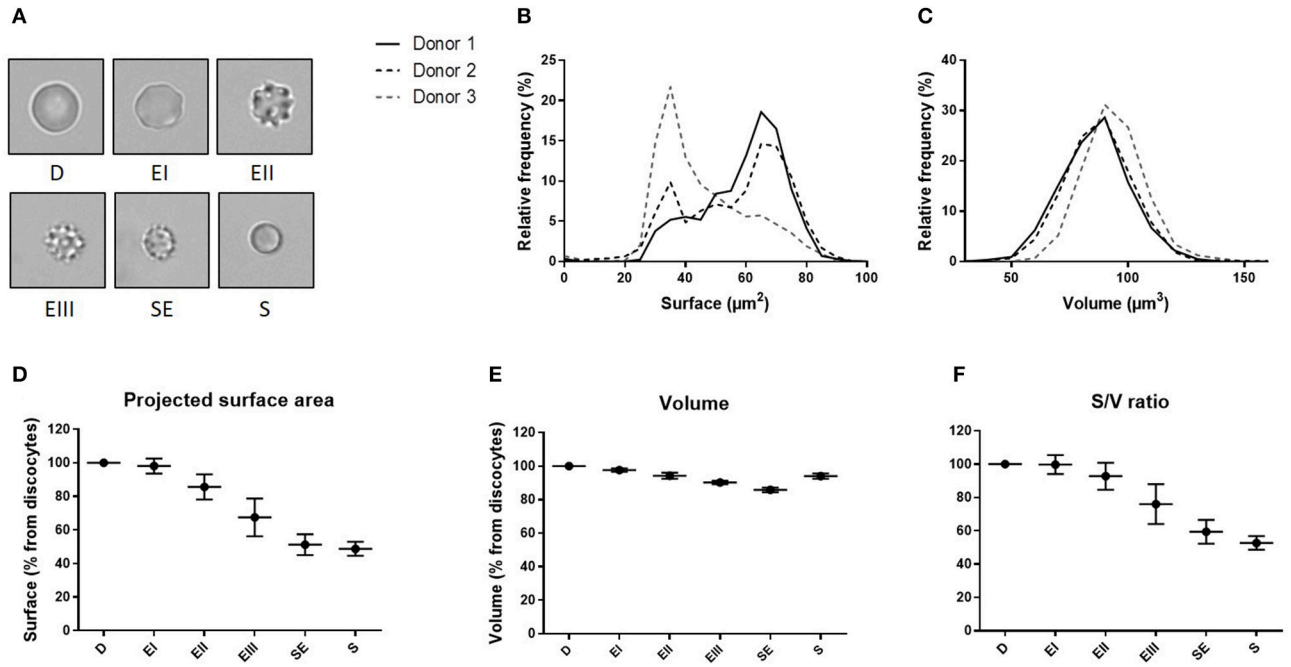

FIGURE 5 | Fluorescence exclusion shows a decrease in surface to volume ratio during storage of red blood cell concentrates. **(A)** Morphological categories of RBC as defined in the Material and Methods section, namely discocytes (D), echinocytes I (EI), echinocytes II (EII), echinocytes III (EIII), spheroechinocytes (SE) and spherocytes (S). **(B–C)** Normalized frequency of RBC projected surface area (μm^2) and RBC volume (μm^3) of RBC concentrates after 42 days of storage in SAG-M ($n = 3$). **(D–F)** Mean (SD) projected surface area, volume and projected surface area-to-volume ratio normalized to the value of discocytes (D).

Unlike most aforementioned methods, the fluorescence exclusion technique described here does not require any pre-treatment of RBC and can be performed in physiological buffer. and could then be used for long term experiments with multiple cell types [see Cadart et al. (25)]. These 2 experimental features are real assets when studying RBC. Morphology and dimensions of RBC (especially those of altered RBC) are indeed exquisitely labile and sensitive to any fluctuation in the pH of the medium, its composition and most labeling procedures. Also, the chips are loaded without requiring pressure control or any specific infusion device. RBC adherence to the surface is not warranted since the immobility of the RBC is obtained by creating a bridge of fluid between the inlet and the outlet. Not least, measuring fluorescence exclusion only requires a fluorescence microscope.

We showed that fluorescence exclusion can detects variations of volume of a RBC population as small as 3%. Mean volume of RBC populations containing increasing proportions of small heated RBC were very close to theoretical predictions. RBC heated at 50°C during 20 min exhibited a 14% volume loss. Samples containing 25 and 50% heated-RBC should have exhibited 3.5 and 7% volume loss while measured values were 3.5 and 6.1%, respectively. Reproducibility was robust when measuring the mean RBC volume in the same samples using 2 different chips. The method still has weaknesses however. In its current version it displays a relatively low throughput. Improvement is envisioned by combining the analysis program to an algorithm for automated classification of RBC morphology, as described by Piety et al. (38). Also, like imaging flow cytometry, our method measures the projected surface area of RBC, a proxy for the total RBC surface generally considered accurate but that may be suboptimal for echinocytes that exhibit membrane spicules.

Using this new method, we confirmed and expanded recent findings. We measured the projected surface area, volume and surface area-to-volume ratio of RBC stored 42 days in blood bank conditions.

Measures of the volume of the subpopulation of "small RBC" (EIII, SE and S) by fluorescence exclusion provided direct confirmation that this subpopulation exhibits an overall volume loss of 11%. Because surface loss was proportionally greater than volume loss, the result was a reduced surface area-to-volume ratio of 32%. Previous observations had shown that a reduction in surface area-to-volume ratio was correlated to splenic retention of RBC. A reduction> 21% had led to a rapid entrapment of 79% of RBC in normal human spleens perfused *ex-vivo* (14). If RBC dimensions determines indeed the ability of RBC to cross the spleen, EIII, SE and S induced by storage (i.e small RBC), that exhibit a decrease in surface area-to-volume ratio of 24, 41, and 47% respectively (resulting in an overall decrease of 32%), are expected to undergo splenic retention. Not least, the marked difference in projected surface area-to-volume ratio observed between EII and EIII, as well as between EIII and SE is consistent with the similarly marked difference in the capacity of these RBC subsets to circulate in a microfluidic device (39). These data further support the hypothesis of a rapid clearance of "small RBC" by the spleen following transfusion. Recent publications

showed that approaches consisting in reducing the appearance (anaerobic storage) or selectively remove this sub-population (washing in hypotonic solution) improved the ability of stored RBC to perfuse in an artificial microvascular network (40, 41).

Little is known about volume modification of stored RBC at the single cell level. Several studies showed an increase in RBC mean corpuscular volume (MCV) upon storage (42, 43) but a recent studies using quantitative phase imaging observed no significant volume modification after 6 weeks of storage (36, 44). We observed that the volume of RBC stored in SAG-M for 42 days was smaller in EI, EII, EIII, and SE than in D (with an almost linear decrease from EI to SE). The volume of S, although reduced, was greater than that of EIII and SE. In the context of transfusion, we found no previous direct observation on the volume of the different morphological subpopulations of RBC. We are thus currently unable to address the external consistency of this somewhat unexpected observation. In physiology, RBC with irregular shape in pre-term and term neonates show a similar decline in volume from D to SE but the volume of S is lower than that of SE, when assessed by micropipette aspiration (26). The higher-than-expected volume of S that we observed using fluorescence extinction may be artefactual or correspond to alterations of transmembrane flow of ions and water, as S are the most altered RBC subpopulation in red blood cell concentrates. This may have escaped observations with micropipettes because mechanical constraints linked to the aspiration may artificially modify RBC volume. When submitted to mechanical forces, RBC can indeed undergo dehydration via the activation of the mechanosensitive cation channel, Piezo 1 (45). By contrast, our method does not require manipulation of RBC and the height of the chips (6.8 μm) protects RBC from stringent mechanical forces. These hypotheses require experimental testing. Which method is the most accurate to quantify the volume of S will be determined by future work, for example by direct comparisons using all available methods with an array of altered RBC.

The method described here has the potential to bring important insight into the ability of RBC to circulate, in the context of RBC transfusion but also in RBC membrane or volume disorders.

AUTHOR CONTRIBUTIONS

CR, SM, OH, CL, YC, MP, PA, and PB: designed the research; CR, SM, MD, and EF: performed the experiments; CR, SM, MD, and EF: analyzed the data; SM, CR, PA, and PB: wrote the paper.

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Measuring Post-transfusion Recovery and Survival of Red Blood Cells: Strengths and Weaknesses of Chromium-51 Labeling and Alternative Methods

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The proportion of transfused red blood cells (RBCs) that remain in circulation is an important surrogate marker of transfusion efficacy and contributes to predict the potential benefit of a transfusion process. Over the last 50 years, most of the transfusion recovery data were generated by chromium-51 (^{51}Cr)-labeling studies and were predominantly performed to validate new storage systems and new processes to prepare RBC concentrates. As a consequence, our understanding of transfusion efficacy is strongly dependent on the strengths and weaknesses of ^{51}Cr labeling in particular. Other methods such as antigen mismatch or biotin-based labeling can bring relevant information, for example, on the long-term survival of transfused RBC. These radioactivity-free methods can be used in patients including from vulnerable groups. We provide an overview of the methods used to measure transfusion recovery in humans, compare their strengths and weaknesses, and discuss their potential limitations. Also, based on our understanding of the spleen-specific filtration of damaged RBC and historical transfusion recovery data, we propose that RBC deformability and morphology are storage lesion markers that could become useful predictors of transfusion recovery. Transfusion recovery can and should be accurately explored by more than one method. Technical optimization and clarification of concepts is still needed in this important field of transfusion and physiology.

Keywords: transfusion recovery, red blood cell, spleen, red blood cell morphology, red blood cell deformability, storage lesion

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INTRODUCTION

Each year, more than 85 million red blood cells (RBCs) units are transfused worldwide. This demanding human and organizational task is conducted by national or local organizations. Collection, transformation, storage (for a maximum of 35–49 days), and distribution of blood products are tightly quality controlled, most commonly at the national level. In industrialized countries, most of the transfused RBCs are stored as red cell concentrates (RCC), from which plasma, platelets, and leukocytes have been almost entirely removed, usually using centrifugation and/or leukoreduction filters.

The objective of an RCC transfusion is to increase the oxygenation capacity of the recipient by increasing the number of functional RBC in circulation. Improvement in tissue oxygenation following transfusion is arguably the most relevant marker of transfusion efficacy but measuring it is technically and logistically challenging in patients and impossible in healthy volunteers in whom tissue oxygenation is not altered. Measuring the proportion of RBCs that remain in circulation after transfusion thus appears as a suitable surrogate marker to evaluate the efficacy of a transfusion. That a reasonable proportion of transfused RBC stays in circulation for long enough to operate the expected correction is indeed a prerequisite for transfusion efficacy.

Early studies have identified that, after storage, a variable proportion of transfused RBC is removed from the circulation in the first 24 h following transfusion (1). Then, the remaining transfused RBCs have a normal survival. Although the long-term survival of RBC is an important parameter to evaluate transfusion efficacy, most studies have focused on the measure of the 24 h transfusion recovery.

Several techniques have been developed and used in the last 100 years to measure transfusion recovery. Transfusion recovery using chromium-51 (^{51}Cr) labeling is now a regulation criterion to license new storage systems or RCC preparation processes by the Food and Drug Administration (FDA). The FDA threshold to approve a preparation and storage process of RBC is a maximum 1% *in vitro* hemolysis and a 24 h *in vivo* recovery of at least 75% after reinfusion of autologous ^{51}Cr-labeled RBC in healthy volunteers, at the limit of storage (2). The ^{51}Cr-labeling technique was first used in the early 1950s and became the gold standard in the 1970s when the International Committee for Standardization in Hematology (3) proposed it as the reference technique. The use of a standardized protocol is essential to compare studies distant in space or time. Over the last 50 years, most of the transfusion recovery data were generated by ^{51}Cr-labeling studies, mostly to validate new storage systems and RCC preparation processes. As a consequence, our understanding of transfusion efficacy strongly depends on the strengths and weaknesses of ^{51}Cr labeling. However, other methods have been developed and validated, the advantages and limitations of which deserve careful analysis.

We will provide a brief overview of the methods used to measure transfusion recovery in humans. We will compare their strengths and weaknesses and critically analyze their potential limitations. Also, based on our understanding of the spleen-specific filtration of damaged RBC, we will discuss the relevance of storage lesion markers to predict transfusion recovery. We will finally discuss the current state of knowledge in the RBC transfusion field and propose future directions. Animal studies published in the recent years on this topic are beyond the scope of this analysis.

METHODS USED FOR TRANSFUSION RECOVERY STUDIES

Differential Agglutination (DA)

Differential agglutination was the first method used to measure transfusion recovery of a complete RCC (1, 4). In the 24 h post-transfusion blood sample, RBCs from the donor or the recipient are agglutinated with an appropriate antiserum, and the remaining RBCs are counted (5, 6). Similarly, an automated DA technique was developed where agglutinates are removed automatically and the remaining hemoglobin is quantified colorimetrically (7–13). The "100%" initial point is calculated from the prediction of the recipient's normal blood volume (using its height and weight). Alternatively, the initial point can be obtained from an estimation of the recipient's red cell volume using radioactive labeling of "fresh" RBC.

Radioactive Labeling Methods With ^{51}Cr and Other Isotopes

Here, 15–30 ml of RBCs from a donor is labeled with ^{51}Cr (14) and injected to the recipient (most of the time the donor himself) (15–31). RBC recovery is quantified after transfusion by taking a blood sample at early time points (5, 7.5, 10, 12.5, and 15 min) and 24 h after injection. In each sample, a radioactivity count number is acquired, and the initial point is extrapolated by linear regression. Alternatively, transfusion recovery can be evaluated using the technetium-99 (^{99m}Tc)/^{51}Cr double-labeling technique (32–43). In this method, the recipient's RBC volume is first evaluated using a known amount of "fresh" RBC labeled with ^{99m}Tc (^{32}P and ^{52}Cr were also used in older studies) (44–46), which is then used to calculate transfusion recovery. Similarly, ^{51}Cr labeling can be associated with ^{125}I-labeled albumin to evaluate the recipient's plasma volume, which is then used in the calculation of transfusion recovery (35, 46–48). A reduced transfusion recovery was observed in some studies (34, 46, 48) that compared the double labeling with the single-labeling method. This is probably due to an undervaluation of the very short-term component of survival when using the single-label method (since some RBCs are rapidly removed from the circulation in the very first minutes following injection) and suggests that the double-label method is worth the extra complexity.

Biotinylation

Red blood cells from a donor (5–30 ml) are labeled with biotin and injected to the recipient. RBC recovery is usually quantified after transfusion by taking a blood sample at an early time point (10 min) and 24 h after injection (49, 50). Fluorescent labeling of biotinylated RBC and flow cytometry detection quantify the proportion of transfused RBC. By labeling RBC with different densities of biotin, it is possible to evaluate the recovery of up to three RBC populations in the same recipient. Care must be taken to avoid too high concentrations of biotin since it has been correlated with increase of transfused RBC clearance and anti-biotin antibodies in the recipient (51). A GMP grade biotin is now available and could be used in countries where radioactive labeling procedures are not authorized (52). This method theoretically allows the determination of transfused RBC characteristics.

Minor Antigen Mismatch

In the minor antigen mismatch method, an antibody directed against a minor antigen (e.g., Fy), which differs between the donor and the recipient, is used to determine, by flow cytometry, the proportion of transfused RBC in the 24 h post-transfusion

sample (53, 54). No manipulation of the transfused RBC is necessary, and recovery is evaluated after the transfusion of a complete RCC, but the measure is dependent on the prediction of the recipient blood volume (from its height and weight). Theoretically, the characteristics of transfused RBC can be observed after transfusion.

Increase in Blood Counts

A simple method to evaluate transfusion recovery is to measure the increase in blood count (hemoglobin level or hematocrit) between a pretransfusion sample and post-transfusion samples (55–58). Limitations from such a method include the limited accuracy of the blood count measure and the unknown recipient's blood volume (and its variable response to transfusion) that both contribute to the inaccuracy of the measure.

LIMITATIONS OF EXISTING RECOVERY STUDIES

Strengths and weaknesses of the different methods are summarized in **Table 1**. One of the potential bias of the chromium or biotinylation techniques stems from the labeling protocol necessary to perform these studies. In the normal transfusion setup, RBCs are transfused to the recipient directly from the bag while in these two methods, RBCs are manipulated, centrifuged, and incubated in PBS or saline solution. It is conceivable that these steps modify labeled RBC in a way that affects their ability to stay in circulation, although a recent study showed that some RBC properties are only slightly modified by the biotinylation protocol (59). The situation is different with DA and minor antigen mismatch, as these techniques do not require any RBC manipulation before transfusion, thereby eliminating this potential source of artifact.

Another potential limitation of the accuracy of the ^{51}Cr or biotinylation techniques is the infusion of a relatively small volume (5–30 ml) of RBC rather than a complete RCC. Transfusion recovery may indeed be influenced by the volume of transfused RBC. To explore this possibility and mimic more closely a complete RCC transfusion, ^{51}Cr-labeled or biotinylated RBC could be co-transfused with the rest of the RCC. One study (60) reported a lower transfusion recovery of an entire unit (using automated DA) when compared with a 10–30 ml transfusion volume (using ^{51}Cr labeling). However, it is not possible to ascertain that the "volume of transfusion" was responsible for this difference since other potential factors related to the method used to quantify transfusion recovery method (automated DA vs ^{51}Cr) may have impacted the observation. The exact clearance mechanism(s) of potentially damaged RBC stored for many weeks are not well known. To what extent transfusion recovery data using a small amount of RBC accurately predict the outcome of a complete—or massive—RCC transfusion remains therefore an open question.

To reduce the risk of adverse events including transmission of infectious diseases, most of the transfusion recovery studies are conducted using autologous transfusion of stored RBC to healthy volunteers. In this setup, conditions of transfusion are probably appropriate to evaluate storage and donor effects. However, they do not take into consideration the possible complex interaction between damaged-stored RBC and potential recipient specificities related to its physiopathological condition. Along this line, it has been shown that survival of transfused RBC is abnormally low in some thalassemia patients with splenomegaly (61). Normal survival was restored following splenectomy suggesting that the spleen is where most RBCs that were no longer present in the circulation 24 h after transfusion had been retained. This is an example of how the medical condition of the recipient can impact transfusion recovery.

STORAGE LESION, TRANSFUSION RECOVERY, AND SPLEEN FILTRATION

Storage Lesion and Transfusion Recovery

Recently, a number of prospective clinical studies have been conducted to evaluate the potential benefit of transfusing RCC stored for a short period (61–66). These studies have shown that transfusion of RCC stored for a short period does not reduce in-hospital morbidity or mortality in adult and children transfused for acute anemia. The "standard of care" collection and storage processes thus appear to be currently adequate when (accurately) assessed on clinical endpoints. However, these complex prospective clinical studies assessing predominantly safety may be difficult to conduct in some cohorts, such as chronically transfused patients, where the long-term impact of transfusions may be even more relevant. In addition, clinical studies of safety did not specifically examine the effect of transfusing RCC stored for a long period (more than 35 days) and did not directly address the efficacy of the procedure. This evaluation could be important in light of the well-documented RBC alterations that accumulate during hypothermic storage (67). The clinical relevance of this storage "lesion" to predict the efficacy and safety of the transfusion for the recipient is still a matter of controversy but suggests that RBC quality does not remain stable during storage.

It has been assumed that the decrease in transfusion recovery related to storage is due to RBC damages that accumulate after several weeks of storage. Studies performed more than 50 years ago have shown indeed that the extent of the storage lesion increases with storage duration while transfusion recovery decreases accordingly (5, 8, 10, 45). Few studies have directly explored the correlation between *in vitro* markers of storage lesion and *in vivo* recovery. In these studies, three markers (intracellular ATP, deformability, and morphology) have been shown to correlate with transfusion recovery (**Box 1**). The proportion of RBC removed from circulation (calculated from transfusion recovery) could correspond to the proportion of RBC, damaged during the storage process, which are over a recipient "clearance threshold." If this assumption is correct, an optimal marker of storage lesion should identify and quantify the subpopulation of RBC that undergoes early premature clearance.

The spleen has a specific filtering function that operates the clearance of damaged or senescent RBC from the circulation. Knowledge on the spleen filtration process is therefore relevant to understand transfusion recovery.

TABLE 1 | Strengths and weaknesses of the different methods to measure transfusion recovery.

Method	Principle	Strengths	Weaknesses
Differential agglutination (DA)	Red blood cells (RBCs) from the donor or recipient are agglutinated, and the remaining RBCs are counted	• Transfusion recovery of a normal transfusion volume can be determined • One or more RBC populations can be quantified in parallel • The persistence of transfused RBC in circulation may be followed for several weeks • The method can be used in patients, in infants, pregnant women, and any vulnerable group • RBCs from the donor or the recipient are not manipulated before transfusion	• Quantification is inaccurate when/if agglutination is incomplete • Only allogeneic RBC transfusion can be studied with this method • The method is dependent on the prediction of the recipient's blood volume (from its height and weight) or the calculation of the recipient RBC volume using radioactivity
Automated DA	RBCs from the donor or recipient are agglutinated and the remaining hemoglobin is quantified	• Variability is reduced when an automated procedure is used	
Chromium-51 (^{51}Cr)	Donor RBCs are labeled with ^{51}Cr and then injected to the recipient Recovery is quantified in serial samples	• This is a reference Food and Drug Administration-approved method to test new devices/procedures for transfusion/storage • The procedure is standardized which allows comparison between different studies • Autologous RBC transfusion can be studied with this method	• Only relatively small volumes (15–30 ml) of labeled RBC can be transfused • Elution of ^{51}Cr from RBC limits the evaluation of long-term persistence in circulation (less than 30 days) • There are regulatory, logistical, and technical constraints related to the use of radioactivity • Protected populations cannot be studied because recipients are exposed to radioactivity • RBCs from the donor are manipulated before transfusion
Technetium-99 (^{99m}Tc) /^{51}Cr	Blood volume in the recipient is first evaluated using a known amount of tracer "fresh" RBC labeled with ^{99m}Tc	• Quantification is expected to be more robust because recipient's RBC volume is measured with ^{99m}Tc-labeled RBC	
Biotin	One or more donor RBC populations are labeled with different concentrations of biotin then quantified in serial samples by flow cytometry	• The persistence of transfused RBC in circulation may be followed for several weeks • Up to 3 RBC populations can be quantified in parallel • Autologous RBC transfusion can be studied with this method • The method can be used in patients, in infants, pregnant women, and any vulnerable group • The characteristics of transfused RBC can be observed after transfusion	• Only relatively small volumes (15–30 ml) of labeled RBC can be transfused • The recipient is at risk of developing anti-biotin antibodies • RBCs from the donor are manipulated before transfusion
Antigen mismatch	Following transfusion of compatible RBC, minor antigen differences (e.g., Fy) are used to quantify RBC from the donor by flow cytometry	• Transfusion recovery of a normal transfusion volume can be determined • The persistence of transfused RBC in circulation may be followed for several weeks • One or more RBC populations can be quantified in parallel • The characteristics of transfused RBC can be observed after transfusion • The method can be used in patients, in infants, pregnant women, and any vulnerable group • RBCs from the donor or the recipient are not manipulated before transfusion	• Only compatible transfusions with at least 1 minor antigen difference can be studied with this method • The method is dependent on the prediction of the recipient's blood volume (from its height and weight)
Increase in blood counts	Blood hemoglobin levels (or hematocrit) are measured before and after transfusion	• Transfusion recovery of a normal transfusion volume can be determined • The method can be used in patients, in infants, pregnant women, and any vulnerable group • RBCs from the donor or the recipient are not manipulated before transfusion	• The quantification is inaccurate when the blood volume of the recipient is abnormal • Processes or interventions other than transfusion can impact on hemoglobin blood level (or hematocrit)

Spleen Filtration Capacity

In the splenic circulation, RBCs engage into two parallel pathways, the fast or slow microcirculations (71). In the fast and "closed" microcirculation, RBCs remain in endothelialized pathways and transit from arterioles to the venous sinus lumen through pathways in the perifollicular zone (72). In the slow and "open" microcirculation, RBCs navigate in tortuous microcirculatory beds of the red pulp, devoid of endothelium, before returning to the venous circulation by squeezing through 1- to 2-μm-wide slits between endothelial cells in the wall of sinuses (71, 73). Macrophages account for approximately half the volume of the cords, and their abundance facilitates direct RBC–macrophage interactions (71). The spleen likely contributes through one or more of these mechanisms to the clearance of transfused RBC.

BOX 1 | Connecting storage lesion with transfusion recovery.

Intracellular ATP

An inverse correlation between the intracellular content in ATP in the RCC and transfusion recovery has been reported in a number of studies (1, 7, 9, 44). ATP content declines during long-term hypothermic incubation in a non-physiological solution. This is probably at the root of most RBC alterations that accumulate during storage. Intracellular ATP quantification remains, however, difficult to standardize and allows evaluation of the RCC quality at a cell population rather than a single-cell level.

Morphology

At least two studies using the ^{51}Cr technique have shown that morphological modifications of RBC in a RCC do correlate with transfusion recovery. In the first study, the proportion of RBC with a discoid shape was positively correlated with transfusion recovery (16), while in the second, the morphology index after rejuvenation correlated with recovery (20). An evaluation of RBC morphology thus seems a good potential predictor of transfusion recovery provided that individual RBC shape can be categorized reliably. However, morphology analyses are low-throughput and operator-dependent making them difficult to standardize and implement. New technologies such as imaging flow cytometry may help circumvent this problem. We have recently identified a subpopulation of small spherocytic RBC that appears and expands during storage with wide variations between donors (68). This spherocytic shift could be a relevant marker as it readily identifies a subpopulation of RBC expected to be cleared rapidly after transfusion. However, direct evidence is lacking that small spherocytic RBC are prematurely cleared following transfusion, hence account for all or part of a suboptimal recovery.

Deformability

A recent study in patients with thalassemia showed that the increase in hemoglobin following transfusion was inversely correlated to the proportion of "less deformable" RBC in the RCC (57). In this study, a cell flow analyzer (69) was used to measure the elongation index of individual RBC that adheres to a polystyrene slide. Deformability can also be evaluated by measuring an RBC elongation index using ektacytometry (70). Both technologies have shown a decrease in RBC deformability during storage but the cell flow analyzer, although not commercially available has the advantage of measuring individual RBC elongation. In principle, the automated rheoscope and cell analyzer would provide interesting individual cell data on the evolution of RBC during storage.

Intensity and kinetics of this clearance depend on the proportion of altered RBC in an RCC and on the intensity of the alterations. In physiologic conditions, the spleen can process at least 20 ml of RBC per day. It is conceivable that its filtration capacity might be overwhelmed by the amount of damaged RBC transfused, potentially leaving in circulation RBC that should normally be removed.

Spleen Filtration Threshold

The spleen-specific filtration process can trigger the clearance of senescent or altered RBC based on the sensing of surface modifications, mechanical alterations, or a combination of both. Inter-endothelial slits in the spleen exert a stringent challenge on RBC and retain least deformable ones (74, 75). Macrophages sense the shape and altered deformability of RBC and phagocytize them (76). In hereditary spherocytosis, morphology and deformability of RBC are linked (77), surface area-to-volume ratio being the main major determinant of RBC ability to cross narrow inter-endothelial slits in the spleen (74, 78). *Ex vivo* experiments with human spleens have confirmed the correlation between RBC retention in the spleen and the loss in projected surface area (79). Retention was almost complete when more than 17.5% of surface area had been lost. There is therefore a "splenic clearance threshold" that senses biomechanical and morphological changes of RBC which has also been determined by modeling *in silico* (75). Deformability and morphology of transfused RBC are expected to be very important determinants of transfusion recovery.

CONCLUSION

Recovery of autologous RBC in healthy non-anemic recipients using ^{51}Cr labeling, 24 h after transfusion, is the method usually performed to determine the validity of the RCC preparation/storage processes. When examining transfusion recovery studies, we identified three parameters, namely transfusion volume, labeling protocol and the recipient pathophysiological state that have been under-evaluated and may impact the determination of transfusion recovery. For example, monocytes and macrophages, that possess a limited clearance capacity (80), could be saturated and leave in circulation damaged RBC when a large volume of RBC is transfused. Such an assumption is supported by data showing that transfusion of more than five RCC leads to a decreased deformability of circulating RBC (81). In the case of the labeling protocol, it has been shown that RBC stored for a long period are "primed" and more sensitive to an incubation in medium at 37°C (82) and may react differently when incubated in non-physiological solutions used in certain labeling protocol. Also, the observation that transfusion recovery is reduced in recipients with a splenomegaly (4, 61, 83) strongly suggests that individual characteristics or a pathological condition in the recipient impacts the recovery and survival of transfused RBC, even in absence of alloantibodies. These technical differences between transfusion recovery studies in healthy volunteers and transfusion of an anemic patient in a medical context suggest that available transfusion recovery data may not reflect transfusion efficacy in anemic recipients in some physiopathological conditions. A better understanding of RBC clearance mechanisms is warranted and could be explored by conducting transfusion recovery studies. In doing so, an appropriate experimental design, considering the strengths, weaknesses of the available methods, should be selected. As such, antigen mismatch method appears to offer a number of theoretical advantages over the other methods but would ideally be coupled with a non-radioactive labeling method to evaluate the RBC volume in the recipient.

That some storage lesion markers correlate with transfusion recovery reinforces the potential relevance of these *in vitro* studies which may deliver clinically relevant information. However, in the current conditions of blood collection and processing, this correlation between transfusion recovery and storage lesion remains poorly explored. Identification of a marker that could predict transfusion recovery would be a valuable tool for transfusion medicine and help to bridge the gap between storage lesion and the morbi-mortality studies. Future studies that evaluate transfusion recovery should be designed to include selected storage lesion markers to verify potential correlations. Deformability

and morphology, preferably at the individual RBC level, appear as key potential markers since both spleen physiology and historical transfusion recovery data identify them as potentially predictive of transfusion recovery.

In vitro studies have shown that marked RBC alterations appear and worsen during storage, but a paradox remains since clinical studies have not found correlations between using RCC stored for a short time (generally less than 7–10 days) and improved clinical outcome. This apparent discrepancy is a source of interrogation in the transfusion community. Clinical studies were appropriately designed to guide transfusion policy. The current conclusion is that there would be no benefit at keeping "fresh blood" for specific situations. The impact of studying storage "lesion" has been questioned as well as the medical relevance of cellular alterations that do not translate into any negative outcome. Is storage "lesion" merely a misnomer, to be replaced advantageously by storage "changes"? This is not so sure yet. Many have argued that clinical studies did not assess the effect of transfusing RCC stored for more than 28 days, while storage lesion studies indicate that the extent of damage rapidly increases after 4 weeks of storage (84). Furthermore, clinical studies were not designed to assess transfusion efficacy and particularly the influence of storage duration on transfusion recovery. On the other hand, *in vitro* studies of the storage lesion are not often correlated with *in vivo* recovery. *In vitro* studies of the storage lesion and clinical studies deliver complementary information while addressing different questions. Studies that explore both dimensions of knowledge are difficult to implement since their designs differ. Large safety studies collect simple data from many patients while transfusion recovery collects complex repetitive samples, which are analyzed using relatively sophisticated methods. The way forward is probably to set-up ancillary recovery studies in the context of large safety trials.

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All the authors listed have made a contribution to the work and approved it for publication.

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Disturbed Red Blood Cell Structure and Function: An Exploration of the Role of Red Blood Cells in Neurodegeneration

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The structure of red blood cells is affected by many inborn and acquired factors, but in most cases this does not seem to affect their function or survival in physiological conditions. Often, functional deficits become apparent only when they are subjected to biochemical or mechanical stress *in vitro*, or to pathological conditions *in vivo*. Our data on the misshapen red blood cells of patients with neuroacanthocytosis illustrate this general mechanism: an abnormal morphology is associated with an increase in the susceptibility of red blood cells to osmotic and mechanical stress, and alters their rheological properties. The underlying mutations may not only affect red cell function, but also render neurons in specific brain areas more susceptible to a concomitant reduction in oxygen supply. Through this mechanism, an increased susceptibility of already compromised red blood cells to physiological stress conditions may constitute an additional risk factor in vulnerable individuals. Also, susceptibility may be induced or enhanced by systemic pathological conditions such as inflammation. An exploration of the literature suggests that disturbed red blood cell function may play a role in the pathophysiology of various neurodegenerative diseases. Therefore, interventions that reduce the susceptibility of red blood cells to physiological and pathological stress may reduce the extent or progress of neurodegeneration.

Keywords: aging, deformability, neuroacanthocytosis, neurodegeneration, red blood cell

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INTRODUCTION

The statement that a healthy red blood cell is essential for organismal homeostasis may sound as a truism, but this depends on the functional definition of a healthy red blood cell. There are many genetically determined, structural abnormalities in the hemoglobin chains that, in most circumstances, do not affect red blood cell integrity and do not seem to affect transport of oxygen binding and release in lungs and tissues, respectively (1). Also, many obvious deviations of the classical discoid red blood cell shape, due to inborn errors in integral membrane proteins and cytoskeletal components, have no obvious clinical implications (2). In addition, there are hardly any data indicating that physiological aging *in vivo* or *in vitro* during storage in the blood bank has a notable effect on oxygen supply of the tissues and carbon dioxide removal (3).

The gas transport capacity of red blood cells is not only determined by the characteristics of hemoglobin, but also by the capacity to regulate intracellular pH, deformability, ATP production, redox status, resistance to osmotic and mechanical stress, and recognition and removal by the

immune system. The role of most of these processes emerges mainly upon recognition of their putative involvement in pathophysiological mechanisms, and in most cases their molecular details become clear only after detailed study *in vitro*.

The absence of conspicuous clinical consequences, such as hemolysis and anemia, of many structural and functional flaws under physiological circumstances indicates that the red blood cell has considerable reserves to maintain structure and function. The limits of these reserves, in addition to the resilience provided by the erythropoietic system, may be reached when red blood cells are exposed to pathological processes, such as inflammation (4). Errors that are inborn or flaws that are acquired in the circulation in critical structural, functional, or metabolic red blood cell components are likely to increase the rate at which the weakest links in these defenses are breached. For example, a decrease in the capacity to maintain phospholipid asymmetry increases the likelihood of recognition by macrophages, that is mediated by the exposure of phosphatidylserine (PS) in the outer leaflet of the red blood cell membrane. Aging renders red blood cells more susceptible to PS exposure after osmotic stress (5, 6).

Here we explore the boundaries of these reserves, how they may be breached, and their pathological implications. The starting point of this exploration is the complex of structural and functional characteristics of the aging red blood cell, that was the foundation of our study of the misshapen red blood cells that accompany the neurological problems of patients with neuroacanthocytosis.

RED BLOOD CELL AGING

Physiological aging *in vivo*, as well as aging *in vitro* during storage in the blood bank, induces changes in the red cell membrane (7), in the activity of the main metabolic pathways (8, 9), and in hemoglobin (10). These changes not only affect function by decreasing deformability (11, 12), but also lead to the appearance of signals that trigger recognition and removal by the immune system. Especially the latter process is induced by the conditions that the cells normally encounter in their journey through the circulation, such as mechanical stress, oxidation and hyperosmotic conditions (5, 13, 14). A number of pathological conditions may trigger the same changes, as exemplified by the detrimental effects of inflammatory lipases on red blood cell structure and the association between inflammation and anemia (4, 15). Thus, the biophysical, biochemical, immunological, and functional characteristics of the healthy, aging red blood cell provide us with the tools to study the red blood cell structure-function relationship in a clinically relevant context.

NEUROACANTHOCYTOSIS

Neuroacanthocytosis (NA) is a family of rare neurodegenerative disorders, that includes chorea-acanthocytosis, McLeod syndrome, Huntington's disease-like 2, and panthothenate kinase-associated neurodegeneration. Patients with NA suffer from devastating movement disorders, caused by degeneration of spinal neurons in the basal ganglia. One hallmark of NA is the presence of acanthocytes, red blood cells with thorny protrusions, in the blood, but detailed morphological analysis shows the presence of many other misshapen red blood cells as well (16, 17). The presence of acanthocytes is mostly considered as an indication that the pathways that lead to the red blood cell abnormalities are the same as those involved in neuronal degeneration. The molecular similarities between the putative mechanisms inducing acanthocytosis in red blood cell membrane organization and in neurodegeneration in patients with NA have been discussed extensively (18, 19).

In patients with NA, the degree of acanthocytosis may vary over time. There are no clues for the identity of the processes that might cause a transition of mature discocytes to acanthocytes. A recent inventory of the available data has led us to the hypothesis that red blood cells with an acanthocyte shape may already be present in the final stages of erythropoiesis, and appear into the circulation as such (20). This is supported by the observation that an artificially induced, long-term disturbance of red blood cell membrane architecture had a lasting effect on erythropoiesis and caused the appearance of acanthocytes in the circulation (21).

Recent applications of various combinations of immunochemical, (phospho) proteomic, lipidomic and metabolomic approaches have provided indications for the mechanisms responsible for the acanthocyte shape. In acanthocytes, Lyn kinase-mediated phosphorylation and phosphatidylinositol-involving signaling pathways show altered activities. These pathways regulate the interaction between the main cytoskeletal and integral membrane proteins, and may be involved in autophagy during erythropoiesis (19, 20, 22, 23). As a band 3 plays a central role in multiprotein complex formation during erythropoiesis (24), disturbance of this process is likely to affect the stability of the binding of the cytoskeleton to the band 3-based ankyrin-complex and/or the junctional complex. A band 3-centered disturbance of this binding leads to various abnormal cell shapes, varying from spherocytosis to ovalocytosis and acanthocytosis (2, 25). Therefore, the processes that are affected in NA must have very specific, but a yet unknown characteristics in order to induce the characteristic acanthocyte shape. Band 3 does not only provide high-affinity binding sites for the actin-spectrin cytoskeleton, but also for deoxyhemoglobin and for key enzymes of the glycolytic enzyme complex. This interaction plays a regulatory role in red blood cell metabolism and function (26). Metabolomic analyses indicate that NA-associated alterations in band 3-centered protein-protein interactions may also affect the metabolism of red cells (16). The effect of the latter changes on red blood cell survival or function are presently unclear.

Clinical descriptions of patients with NA focus on the neurological symptoms, and in general do not provide clear indications for NA-specific red blood cell dysfunction. Measurement of deformability and relaxation *in vitro* shows

that acanthocytes from NA-patients assume a normal bullet-like shape when passing through a microfluidic., capillary-mimicking system, and relax toward their original shape as quickly as cells with a normal morphology. However, acanthocytes have difficulties when passing through a spleen-mimicking device *in vitro* (16). Also, the misshapen red blood cells of NA patients show a decreased deformability as well as an abnormal aggregation behavior (**Figure 1**). Together, these data constitute strong indications for an altered rheology and decrease in deformability, that may not only be responsible for the splenomegaly and hemolysis described in patients with McLeod disease as well as in a patient with acanthocyte-associated band 3 mutations (18, 28), but may also contribute to the neurological problems (see below).

The abnormal cytoskelon/membrane associations that underly genetically determined alterations in red blood cell morphology are, in general, associated with a decreased deformability *in vitro* (12, 29). Decreased deformability is, in most cases, associated with a decrease in hematocrit and in hemoglobin concentration *in vivo*. Even at subclinical levels, these may not only induce an increased susceptibility to red blood cell-centered pathology, as exemplified by the anemia of aging (30), but also hypoperfusion and thereby hamper oxygen delivery. In the brain, deprivation of oxygen leads to excessive glutamate release and NMDA-receptor activation-induced neuronal cell death. The latter is stimulated by Lyn-related kinases, that are also implied in acanthocyte formation during erythropoiesis and neuronal dysfunction *in vitro* (23, 31). These data, together with sporadic clinical observations, led us to the hypothesis that, in patients with neuroacanthocytosis, the compromised function of acanthocytes and otherwise misshapen red blood cells contributes to the neuronal degeneration in the striatum (20). The most likely underlying mechanism would be a decrease in red blood cell rheology, resulting in a restricted perfusion of sensitive brain areas. More subtle metabolic effects of alterations in cell morphology on oxygen binding or release by hemoglobin may play a role as well. The former mechanism may primarily be caused by defective cytoskeleton-membrane interactions, the latter by defective, membrane-centered regulation of pH, ATP production, and/or redox status.

An etiological role of acanthocytosis has been postulated in the damage to the globus pallidus and development of choreoathetosis as rare complications of cardiopulmonary bypass during open-heart surgery, especially in young children (32). In this hypothesis, the mechanical stress exerted by the extracorporeal circulation system constitutes a mechanical

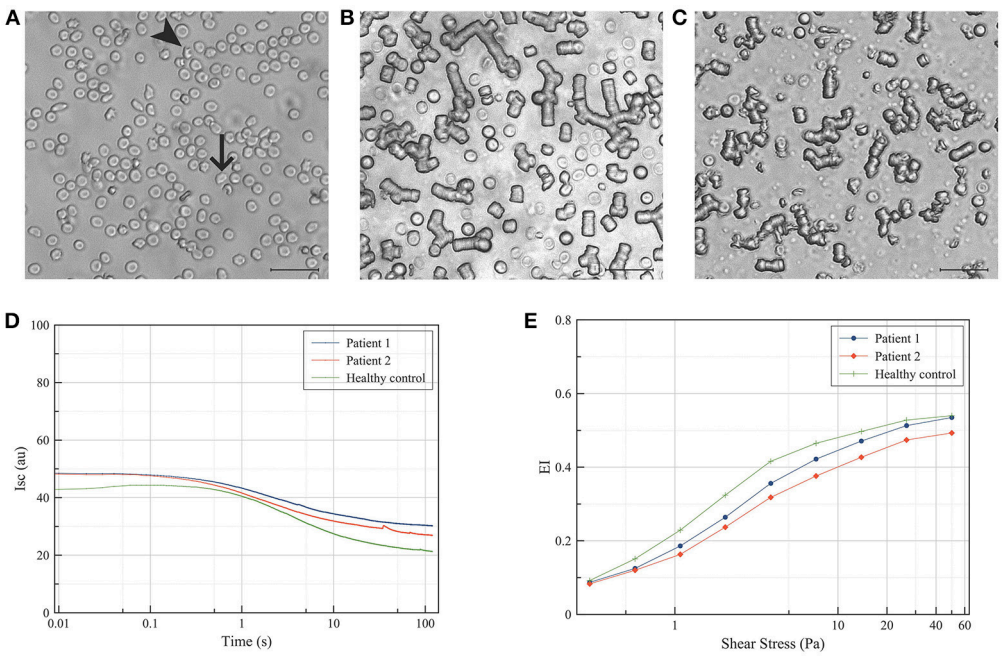

FIGURE 1 | Deformability and aggregation of red blood cells from patients with neuroacanthocytosis. Red blood cells were isolated from patients with neuroacanthocytosis as described before (16), and their morphology, aggregation and deformability were compared with those of a healthy control donor. (**A)** Bright-field microscopy of the red blood cells of one patient (0.1% hematocrit in phosphate-buffered saline), showing acanthocytes (arrowhead) and otherwise misshapen red blood cells (arrow); (**B)** Bright-field microscopy of the red blood cells of a healthy control donor (1% hematocrit in plasma), showing aggregates mostly as rouleaux after 2 to 3 min of incubation at room temperature; (**C)** Bright-field microscopy of red blood cells of an acanthocytosis patient showing smaller rouleaux and much more disordered aggregates; (**D)** Syllectograms of the red blood cells of a healthy control donor and two neuroacanthocytosis patients obtained in 40% hematocrit in plasma, showing altered aggregation characteristics of the patients' red blood cells; (**E)** Deformability curves of the red blood cells of one healthy control and two neuroacanthocytosis patients, showing a lower maximum elongation index (EI) in the patients' red blood cells. Aggregation and deformability were measured using a Lorrca (RR Mechatronics, Hoorn, The Netherlands) as described before (12, 27).

trigger that, in combination with hypothermia, spleen dysfunction, and/or altered pH regulation, may lead to the formation of misshapen red blood cells with a decreased deformability and to a hampered oxygen supply to the brain. A similar phenomenon may underlie the neurological problems following coronary-artery bypass surgery (33), and the higher risk of postoperative cognitive dysfunction in patients with diabetes (34). In most cases, the "postpump" chorea is transient (32). However, in NA patients a chronic acanthocytosis might lead to a chronic deficit in oxygen supply and thereby to a more severe and progressive neurodegeneration.

RED BLOOD CELLS AND NEURODEGENERATION

This hypothesis provided an additional trigger to explore the literature for indications that abnormal red blood cell function may be an etiological factor in neurodegeneration.

Acanthocytosis

Acanthocytes are present in patients with disorders of lipid metabolism such as abetalipoproteinemia and hypolipoproteinemia. However, these patients do not have any signs of NA-like neurodegeneration, and their red blood cells have a different molecular phenotype (25, 35). Acanthocytosis has been described in patients with aceruloplasminemia, and anemia has been reported to precede neurological symptoms in almost all patiens with this defect in copper transport and iron metabolism (36, 37). These data indicate that acanthocyte generation may be due to various causes, and that the functional properties of at least some types of acanthocytic red blood cells may contribute to the development of specific neurological deficits.

Anisocytosis

Abnormally shaped red blood cells display an increased heterogeneity in cell volume, due to impaired erythropoiesis or to excessive fragmentation or destruction. This heterogeneity, expressed as an increase in red blood cell distribution width (RDW), is associated with ischemic cerebrovascular disease (38), with increased odds of having dementia (39), with Alzheimer disease (40), and with the severity of leukoaraiosis (41). In related studies, we found indications for disturbed red blood cell aging, which is associated with changes in cell morphology, in patients with beginning demantia (42). Also, abnormal red blood cells were reported to be associated with cognitive performance in a large longitudinal aging study (43). Such associations may reflect the expression at different organs of a common pathological process. Alternatively, the abnormally shape of red blood cells in individuals with an increased RDW is likely to affect not only cellular deformability and thereby oxygen delivery (29), but may also be an indication for impaired red blood cell signaling-mediated vasodilation by NO, ATP and adenosine (44). In addition, correlations between RDW and sedentary behavior, and between RDW and muscle strength suggest that RDW may be a component of frailty in the elderly (45).

A closer look at red blood cell abnormalities in patients with various neurodegenerative diseases yields indications for abnormal cell morphology and/or red cell function in patients with Huntington's disease (46–48), Parkinson's disease (49), and Alzheimer's disease (50). These abnormalities may reflect peripheral phenomena of the major neurodegenerative mechanism, as indicated by the increased concentration of the *PARK7*-coded protein DJ-1 in red blood cells of early-stage Parkinson's disease patients (51) or by the alfa-synuclein levels in red blood cells with Parkinson's disease (52). Independent of the underlying mechanisms, the effects of these abnormalities on red blood cell function may constitute a risk factor, as has recently be argued for Alzheimer's disease (53).

Red Blood Cell-Centered Diseases and Neurological Problems

Various red blood cell-centered diseases have been reported to be associated with neurological problems. In patients with sickle cell disease and thalassemia, impaired cognitive and neuropsychological functioning are likely due to inadequate oxygen supply in the frontal, parietal and temporal lobes (54–56). In these hemoglobinopathies, decreased deformability and increased aggregation are likely to be the primary causes of the neurological problems. Also, some hereditary red blood cell enzymopathies that are accompanied by hemolytic anemia are associated with neurological problems (57). The latter may be due to the expression of the same mutated genes in the brain and in hematopoietic stem cells, but also to a functional impairment of the mature red blood cells.

In addition, treatment of anemia with red blood cell concentrates, especially in transfusion-dependent patients, may pose its own problems due to its effect on perturbed iron homeostasis, also in the brain [e.g., (58)]. The molecular interplay between red blood cell homeostasis, chronic transfusion and brain pathology remains to be established.

CONCLUSIONS

The data presented here indicate that physiological and pathological circumstances may affect red blood cell function, especially by diminishing their capacity to withstand pathophysiological stress conditions. In other words, in normal conditions, the characteristics of aging, stored, and genetically affected red blood cells may have only subclinical consequences. However, during periods of stress, for example during inflammation, already compromised cells may become less deformable, more fragile, or more prone to recognition by the immune system.

Our data on acanthocytosis illustrate that an abnormal red cell structure increases the susceptibility of the misshapen red cells to mechanical stress and alters their rheological properties. The underlying mutations may not only affect red cell shape

and function, but also render neurons in vulnerable brain areas more susceptible to a concomitant reduction in oxygen supply.

Thus, interventions that reduce the susceptibility of red blood cells to pathological as well as physiological stress conditions may reduce the extent and/or progression of neurodegeneration.

ETHICS STATEMENT

The data shown here were obtained in a study that was approved by the Medical Ethical Committee of the Radboud University Medical Center and in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Redox Status, Procoagulant Activity, and Metabolome of Fresh Frozen Plasma in Glucose 6-Phosphate Dehydrogenase Deficiency

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Objective: Transfusion of fresh frozen plasma (FFP) helps in maintaining the coagulation parameters in patients with acquired multiple coagulation factor deficiencies and severe bleeding. However, along with coagulation factors and procoagulant extracellular vesicles (EVs), numerous bioactive and probably donor-related factors (metabolites, oxidized components, etc.) are also carried to the recipient. The X-linked glucose 6-phosphate dehydrogenase deficiency (G6PD$^-$), the most common human enzyme genetic defect, mainly affects males. By undermining the redox metabolism, the G6PD$^-$ cells are susceptible to the deleterious effects of oxidants. Considering the preferential transfusion of FFP from male donors, this study aimed at the assessment of FFP units derived from G6PD$^-$ males compared with control, to show whether they are comparable at physiological, metabolic and redox homeostasis levels.

Methods: The quality of $n = 12$ G6PD$^-$ and control FFP units was tested after 12 months of storage, by using hemolysis, redox, and procoagulant activity-targeted biochemical assays, flow cytometry for EV enumeration and phenotyping, untargeted metabolomics, in addition to statistical and bioinformatics tools.

Results: Higher procoagulant activity, phosphatidylserine positive EVs, RBC-vesiculation, and antioxidant capacity but lower oxidative modifications in lipids and proteins were detected in G6PD$^-$ FFP compared with controls. The FFP EVs varied in number, cell origin, and lipid/protein composition. Pathway analysis highlighted the riboflavin, purine, and glycerolipid/glycerophospholipid metabolisms as the most altered pathways with high impact in G6PD$^-$. Multivariate and univariate analysis of FFP metabolomes showed excess of diacylglycerols, glycerophosphoinositol, aconitate, and ornithine but a deficiency in riboflavin, flavin mononucleotide, adenine, and arginine, among others, levels in G6PD$^-$ FFPs compared with control.

Conclusion: Our results point toward a different redox, lipid metabolism, and EV profile in the G6PD$^-$ FFP units. Certain FFP-needed patients may be at greatest benefit of

receiving FFP intrinsically endowed by both procoagulant and antioxidant activities. However, the clinical outcome of G6PD⁻ FFP transfusion would likely be affected by various other factors, including the signaling potential of the differentially expressed metabolites and EVs, the degree of G6PD⁻, the redox status in the recipient, the amount of FFP units transfused, and probably, the storage interval of the FFP, which deserve further investigation by future studies.

Keywords: transfusion medicine, fresh frozen plasma, G6PD⁻ donors, donor variation, metabolomics, extracellular vesicles, antioxidant capacity, interactome

INTRODUCTION

Fresh frozen plasma (FFP) is commonly used in transfusion therapy to maintain the coagulation status in patients with acquired multiple coagulation factor deficiencies and severe bleeding after injury (1). In addition, FFP units can be used as a pool for biopharmaceutical fractionation in order to manufacture medicinal products (2). Practically, FFP is used for its ability to generate thrombin and form a clot as a result of intrinsic components, including coagulation factors, calcium and procoagulant phospholipid surfaces, involved in the assembly of coagulation complexes, and coagulation activation (3). However, apart from coagulation factors and procoagulant extracellular vesicles (EVs), numerous bioactive signaling factors and oxidized lipids and proteins (4) pass to the FFP recipient.

The extent of this risk is partly related to inter-individual donor characteristics. Indeed, several studies have recently reported significant donor-to-donor variation in numerous blood properties *in vivo* and in labile blood products. In the case of red-cell concentrates, apart from in-bag hemolysis and 24-h posttransfusion recovery, units from different donors might have substantial variation in antioxidant capacity (5, 6), cellular fragility (6, 7), or surface removal signals (8). Extracellularly, the uric-acid-dependent antioxidant capacity of the supernatant (that influence the storage lesion, and thus, the quality of the blood component) significantly varies among donors (9–11). In the same context, and since the plasma reflects the physiological state of donor's cells and tissues (12), significant variation has been observed among FFP units used for transfusion, in terms of EV characteristics and lipid peroxidation (4, 13).

Certain aspects of the so-called "donor variation effect" are attributed to genetic factors that dictate subclinical inter-donor differences in blood physiology as clearly exemplified by the distinct blood profile of beta thalassemia trait and glucose-6-phosphate dehydrogenase (G6PD)-deficient donors (14). In the last case, the subjects are characterized by extremely low levels of G6PD activity that catalyzes the first reaction in the pentose phosphate pathway converting glucose 6-phosphate to gluconolactone-6-phosphate. Pentose phosphate pathway feeds cells with reducing equivalents (like nicotinamide dinucleotide hydrogen phosphate, NADPH) needed for the maintenance of redox equilibrium. In cases of oxidative stress, NADPH helps in the regeneration of reduced glutathione, in the detoxification of hydrogen peroxide and in the prevention of oxidative damage in membrane lipids and proteins. G6PD deficiency (G6PD⁻) affects the energy and redox status of cells and consequently, a range of energy-dependent cellular activities, including the transport properties of cell membrane, a feature that might link changes in cell metabolomes to those of plasma (15).

Genetic factors may determine the quality of stored blood and probably, its posttransfusion performance and effects. Thus, a study of donors carrying the most common human enzyme genetic defect might be highly relevant to blood transfusion. Moreover, since G6PD⁻ is an X-linked defect, males are more commonly affected than females. Considering that G6PD activity influences both the cellular and plasma homeostases and that a typical transfusion practice is the use of FFP units donated exclusively by male donors, the study of G6PD⁻ male donors is especially relevant to FFP transfusion. However, and despite this intrinsic clinical interest, little is known about the physiological properties and the metabolome of FFP donated by eligible, G6PD⁻ donors. This study aimed at the comparative assessment of FFP units produced by whole blood donations from G6PD-deficient and -sufficient male donors, by using a number of biochemical measurements, flow cytometry and mass spectrometry, in addition to statistical and bioinformatics tools.

MATERIALS AND METHODS

Blood Donors and Fresh Frozen Plasma (FFP) Preparation

Blood from 12 eligible male regular donors was used for the production of FFP units. G6PD⁻ donors under study ($n = 6$) carried the common Mediterranean variant of G6PD⁻ (16). After donation of approximately 465 mL of blood and addition of 63 mL of CPDA-1 (citrate–phosphate–dextrose–adenine) anticoagulant, clinical-grade FFP was prepared according to the standard blood banking procedures (17), directly from whole blood units at 4°C. Briefly, after centrifugation at 4,500× *g* for 15 min, the supernatant plasma was squeezed off by a plasma expressor (Fenwall Laboratories, Deerfield, IL, USA) and frozen for 12 months at −20°C. For analysis, FFP samples were rapidly thawed for 15–20 min at 30–37°C to avoid precipitation of cold-precipitating proteins, consistent with the blood banking procedure for the thawing of clinical FFP for transfusion and the standard AABB operating procedures. The study was approved by the Ethics Committee of the Department of Biology, School of Science, NKUA. Investigations were carried out upon signing of written consent, in accordance with the principles of the Declaration of Helsinki.

Free Hemoglobin, Redox Parameters, and Protein Analysis

Free hemoglobin was calculated by using the Harboe method as previously described (10). Total (TAC) and uric-acid-dependent antioxidant capacity (UA/AC) of FFP samples were determined in the absence or presence of uricase (Sigma-Aldrich, Munich, Germany) treatment, respectively (18), by using the ferric reducing antioxidant power assay (19). Lipid peroxidation of FFP units was assessed by measuring the levels of malondialdehyde (MDA), a natural by-product of lipid peroxidation. Briefly, after deproteinization of each sample with 15% trichloroacetic acid, thiobarbituric acid was added (all chemicals by Sigma-Aldrich, Munich, Germany). After heating of the samples for 50 min at 95°C, the absorption of the produced chromogenic MDA–thiobarbituric acid complex was measured at 532 nm. Measurements were plotted against a standard curve of known MDA concentration.

For the FFP protein characterization, 20 μg of FFP samples were separated in homogeneous 10% sodium dodecyl sulfate polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with primary antibodies against advanced glycated end products (AGEs, 1:1,000 Millipore AB9890), soluble clusterin (sCLU, 1:1,000 Santa Cruz Biotechnology), human hemoglobin (Hb, 1:15,000, Europa Bioproducts), IgGs (1:1,000; Sigma I-2011), and horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed using a standard enhanced chemiluminescence reagent kit and the relative amount of each protein was quantified by scanning densitometry (Gel Analyzer v.1.0 image-processing program, Athens, Greece). In addition, FFP samples were processed for the detection of protein carbonylation using the Oxyblot detection kit as per manufacturer's specifications (20).

Extracellular Vesicles (EV) Profiling

Extracellular vesicle-associated procoagulant activity was estimated by using a functional Elisa assay kit (Zymuphen MP-activity, Hyphen BioMed, Neuville-sur-Oise, France) as per manufacturer's instructions. All FFP samples were supplemented with calcium, Factor Xa, and thrombin inhibitors before addition into microplate wells precoated with streptavidin and biotinylated Annexin V (AnnV). Subsequently, samples were incubated at 37°C before introduction of factor Xa–Va and prothrombin. After addition of the chromogenic substrate, thrombin activation induced by the AnnV positivity ($AnnV^+$) EVs was detected at 405 nm and expressed as nM of phosphatidylserine (PS) equivalents.

Enumeration and phenotyping of FFP EVs was performed by flow cytometry within 15 min from units' thawing, as previously described (13). Briefly, EVs were identified by size (<1 μm), exposure of cell-specific markers, and $AnnV^+$. All samples were double stained with AnnV-phycoerythrin (PE Annexin V Apoptosis Detection Kit I, 559763) and CD235a-fluorescein isothiocyanate (clone GA-R2, HIR2, 559943) or integrin-α2b-FITC (CD41a, clone HIP8, 555466) or CD45-fluorescein isothiocyanate (clone HI30, 555482) from BD Biosciences to identify $AnnV^+$ red-cell-derived, platelet-derived, or leukocyte-derived vesicles, respectively. After addition of phycoerythrin-AnnV and a cell-specific and fluorescein isothiocyanate-conjugated monoclonal antibody in AnnV buffer environment, samples were incubated in the dark for 15 min at room temperature. The samples run within 30 min in a FACScan flow cytometer (Beckton Dickinson) using CELL Quest Software (Becton Dickinson, San Jose, CA, USA). TruCount™ tubes (340334, BD Pharmingen) were used to calculate the absolute EVs count/μL.

The protein composition of EVs isolated by FFP units was estimated by immunoblotting analysis. To this purpose, EVs were precipitated with high-speed centrifugation of 1-mL FFP at 30,000× *g* for 1 h at 4°C. The produced pellet was resuspended in saline buffer and washed twice under the same conditions. The EV proteins were separated in homogeneous 10% sodium dodecyl sulfate polyacrylamide gels, and transferred onto nitrocellulose membranes. Membranes were probed with primary antibodies against vesicular proteins [anti-Hb 1:15,000, Europa Bioproducts; anti-IgGs 1:1,000 Sigma; anti-Hsp70 (K-20) 1:300 Santa Cruz Biotechnology; anti-sCLU 1:1,000 Santa Cruz Biotechnology; anti-Alix 1:1,000 Cell Signaling Technology; and monoclonal antibody against stomatin, kindly provided by Prof. R. Prohaska, Institute of Medical Biochemistry, University of Vienna, Austria] diluted in 5% non-fat milk for 1 h at room temperature. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:8,000–1:14,000), the immunoreactivity was visualized by enhanced chemiluminescence.

Statistical and Biological Network Analyses

For statistical analysis, the Statistical Package for Social Sciences (SPSS, IBM) was used. After checking all variables for normal distribution profile and presence of outliers (by using the Shapiro–Wilk test and detrended normal $Q - Q$ plots), inter-groups differences were evaluated through independent *t*-test or Mann–Whitney test as appropriate. In addition, and according to the outcome of the normal distribution and outliers' analyses, correlations between parameters that subsequently used for the construction of biological networks were evaluated by the Pearson's and Spearman's tests. The statistically significant correlations between biochemical, metabolomics, and physiological parameters collected from $G6PD^-$ and control FFP samples were used for the construction of undirected biological networks. The topological representation was processed by the Cytoscape version 3.2.0 application, as previously described (6). The length of each edge was inversely proportional to the *r* value (the shortest the edge, the higher the *r* value). Significance for both network and inter-group analyses was accepted at $p < 0.05$.

Metabolite Extraction and LC–MS Analysis

Metabolites were extracted by adding 200 μL of plasma sample (control: $n = 5$; G6PD: $n = 6$) to 200 μL of chloroform/methanol/water (1:3:1 ratio) solvent mixture stored at −20°C. Samples were vortexed for 1 min and left on ice for

2 h for complete protein precipitation. The solutions were then centrifuged for 15 min at 15,000× *g*. Twenty microliters of supernatants (two technical replicates) were injected into an ultra high-performance liquid chromatography (UHPLC) system (Ultimate 3000, Thermo) and run in positive ion mode. A Reprosil C18 column (2.0 mm × 150 mm, 2.5 μm—Dr Maisch, Germany) was used for metabolite separation. Chromatographic separations were achieved at a column temperature of 30°C and flow rate of 0.2 mL/min. A 0–100% linear gradient of solvent A (ddH2O, 0.1% formic acid) to B (acetonitrile, 0.1% formic acid) was employed over 20 min, returning to 100% A in 2 min and a 6-min post-time solvent A hold. The UHPLC system was coupled online with a mass spectrometer Q Exactive (Thermo) scanning in full MS mode (2 μscans) at 70,000 resolution in the 67–1,000 m/z range, target of 1×10^6 ions, and a maximum ion injection time (IT) of 35 ms. Source ionization parameters were as follows: spray voltage, 3.8 kV; capillary temperature, 300°C; sheath gas, 40; auxiliary gas, 25; S-Lens level, 45. Calibration was performed before each analysis against positive ion mode calibration mixes (Piercenet, Thermo Fisher, Rockford, IL, USA) to ensure subppm error of the intact mass.

Metabolomic Data Processing and Statistical Analysis

Raw files of replicates were exported and converted into mzXML format through MassMatrix (Cleveland, OH, USA), then processed by MAVEN software[1] (21). Mass spectrometry chromatograms were elaborated for peak alignment, matching and comparison of parent and fragment ions, and tentative metabolite identification (within a 2-ppm mass-deviation range between observed and expected results against the imported KEGG database). Univariate (two-sample *t*-test, Volcano plot) and multivariate (PCA, PLS-DA) statistical analyses were performed on the entire metabolomics data set using the MetaboAnalyst 3.0 software[2]. Before the analysis, raw data were normalized by sum and pareto scaled in order to increase the importance of low-abundance ions without significant amplification of noise. False discovery rate (FDR) and Holm–Bonferroni method were used for controlling multiple testing. The web-based tools MSEA (metabolite set enrichment analysis) and MetPA (metabolic pathway analysis), which are incorporated into MetaboAnalyst platform, were used to perform metabolite enrichment and pathway analyses, respectively. Data for identified metabolites detected in all samples were submitted into MSEA and MetPA with annotation based on common chemical names. Verification of accepted metabolites was conducted manually using HMDB, KEGG, and PubChem DBs. *Homo sapiens* pathway library was used for pathway analysis. Global test was the selected pathway enrichment analysis method, whereas the node importance measure for topological analysis was the relative betweenness centrality.

[1]http://maven.princeton.edu/.

[2]http://metpa.metabolomics.ca/.

RESULTS

High-Antioxidant Capacity and Low-Oxidative Defects to Plasma Lipids and Proteins in FFP Units from G6PD$^-$ Donors

Oxidative stress and antioxidant capacity are donor-related factors that may contribute to the quality of FFP products. Biochemical analysis of FFP units stored for 12 months at −20°C revealed substantial differences in the oxidant/antioxidant equilibrium and the extent of oxidative defects between the two groups. Both TAC and UA/AC were significantly higher in G6PD$^-$ FFP units vs. control (TAC: 698 ± 92 vs. 574 ± 52 μM Fe^{2+}, UA/AC: 466 ± 89 vs. 360 ± 46 μM Fe^{2+}, respectively, mean ± SD, $p < 0.05$, $n = 12$) (**Figures 1A,B**). In addition, the levels of lipid peroxidation, as measured by the production of malonyldialdehyde (MDA), formed during the breakdown of peroxidized fatty-acid side chains of the phospholipids (TBARS assay), was lower in the G6PD$^-$ units as compared with control FFPs (0.175 ± 0.113 vs. 0.597 ± 0.219-μM MDA, $p < 0.01$, respectively, mean ± SD, $n = 12$), as shown in **Figure 1C**. To analyze the FFP proteins for probable oxidative defects, we performed immunoblotting analysis of an equal quantity of plasma proteins (20 μg per sample, **Figure 1D**). Substantially lower levels of albumin carbonylation ($p < 0.05$, $n = 12$) along with a trend for decreased levels of advanced glycation end-products (AGEs) and sCLU were detected in G6PD$^-$ FFP units compared with controls. Only traces of soluble Hb were detected in some units, signifying low pre-donation levels of autohemolysis and high-quality level of the FFP preparation procedure followed, which resulted in minimal lysis of RBCs. IgG immunodetection was used for loading control.

The EV Component of the FFP Units Differed between the Two Groups of Donors

In large consistence with the results of the immunoblotting analysis of FFP proteins (**Figure 1D**), extremely low levels of free hemoglobin (<5 mg/dL) were detected by the Harboe method in all the FFP samples ($n = 12$) under study, without any intergroup difference (**Figure 2A**). On the contrary, the EV-associated procoagulant activity of the FFP (**Figure 2B**), which stands for the total concentration of PS exposed on EVs' surface, was significantly higher in G6PD$^-$ units vs. control units (33.93 ± 8.98 vs. 19.03 ± 11.76 nM PS, respectively, $p < 0.05$, mean ± SD).

To further characterize the EV part of the FFP units, we proceeded to enumeration and phenotyping analysis by flow cytometry. There was no statistically significant difference in the concentration of total EV populations between the two groups under examination; however, the concentration of AnnV$^+$ EVs was higher in G6PD$^-$ FFPs (**Figure 2C**), verifying the finding of high procoagulant activity of G6PD$^-$ FFP. The platelet-derived EVs (CD41$^+$) were the most abundant, followed by the red-cell-derived (CD235$^+$) and the leukocyte-derived (CD45$^+$) vesicles. The G6PD$^-$ FFP units contained more red-cell EVs and more AnnV$^+$ red-cell EVs ($p < 0.05$) compared with controls. Moreover, while similar concentrations of platelet EVs and leukocyte EVs

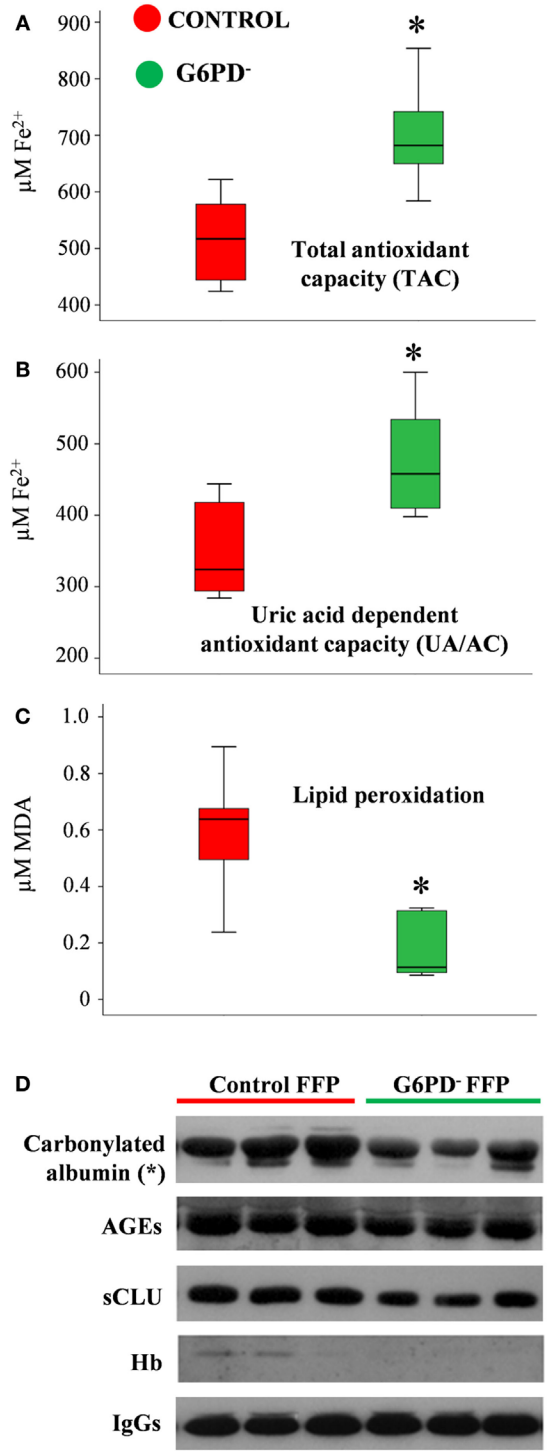

FIGURE 1 | Redox status of fresh frozen plasma (FFP) units prepared from G6PD deficient (G6PD⁻) and control donors. Levels of total antioxidant capacity **(A)**, uric-acid-dependent antioxidant capacity **(B)**, and lipid peroxidation **(C)** in FFP units from G6PD⁻ donors ($n = 6$) compared with control ($n = 6$). MDA: malondialdehyde. **(D)** Representative immunoblots showing variation in the expression of stress protein markers in the two groups of FFP. AGEs: advanced glycation end-products. sCLU, soluble clusterin (apolipoprotein J). *$p < 0.05$, G6PD⁻ vs. control FFPs.

were measured in the two FFP groups, the platelet EVs from G6PD⁻ FFP units demonstrated a higher percentage of AnnV⁺ as compared with the control group (96.5 ± 4.1 vs. 88.6 ± 5.4%, respectively, $p < 0.05$, mean ± SD).

The above-mentioned differences in the PS exposure and cell origin between the otherwise similar EV pools of G6PD⁻ and control FFPs prompted us to a rough examination of their protein composition by immunoprobing of selected components typically associated with the microvesicles or the exosomes (**Figure 2D**). Indeed, the vesicles precipitated by high-speed centrifugation of an equal volume (1 mL) of G6PD⁻ and control FFPs differed significantly between them in protein expression, by showing lower levels of oxidized Hb, IgGs, Hsp70, sCLU, and the red-cell lipid raft marker stomatin in the G6PD⁻ samples. As a component of the late endosomal machinery, the Alix protein has been considered a marker of endosome-derived EVs, namely of exosomes (22). Of note, traces of Alix were detected in some control samples, but not in G6PD⁻ FFP EVs.

Metabolome FFP

Metabolites were extracted from plasma samples of five healthy and six G6PD⁻ donors and were analyzed by LC–MS (two technical replicates). More than 2,000 peaks per sample were obtained referring to the KEGG database; among them, 195 metabolites were analyzed more precisely and identified. To compare the metabolomes between control and G6PD⁻ donors, both multi- and univariate statistical analyses were performed. For unsupervised multivariate analysis, principal component analysis (PCA) showed that the 70.6% of variance was captured by the first three principal components and sample groups could be clearly distinguished in the 3D-PCA score plot (**Figure 3A**). However, to maximize the separation achieved by PCA, partial least square discriminant analysis (PLS-DA) was subsequently performed and the obtained 3D score plots are shown in **Figure 3B**. The prediction accuracies were assessed by cross-validation and the best performance was obtained with three PCs (accuracy 1, R2 > 0.94, Q2 > 0.87; Figure S1 in Supplementary Material). As a supervised method, PLS-DA also enables the identification of the metabolites most contributing to the segregation of the diagnostic groups, thus variable importance in the projection (VIP) scores were calculated to rank the significance of these metabolites as potential biomarkers. Considering that variables having a VIP score of ≥1 are interpreted as being highly influential (23), 15 metabolites were considered as significant important features to differentiate control from G6PD⁻ FFP (**Figure 4A**). These changed plasma metabolites were mainly lipids (including 1,2-diacylglycerol, DAG), amino acids, nucleotides, and organic acids. To further confirm the specificity and significance of potentially discriminating metabolites identified from PLS-DA, univariate analysis of each metabolite was performed by combining statistical significance (Student's *t*-test) with fold-change (FC) variations. The generated Volcano plot (FDR adjusted $p < 0.05$; FC > 2) is displayed in **Figure 4B** where additional metabolites included riboflavin, FMN, ornithine, D-glucono-lattone-6-phosphate, and acyl-glycerophosphoinositol, among others. Quantitative variations

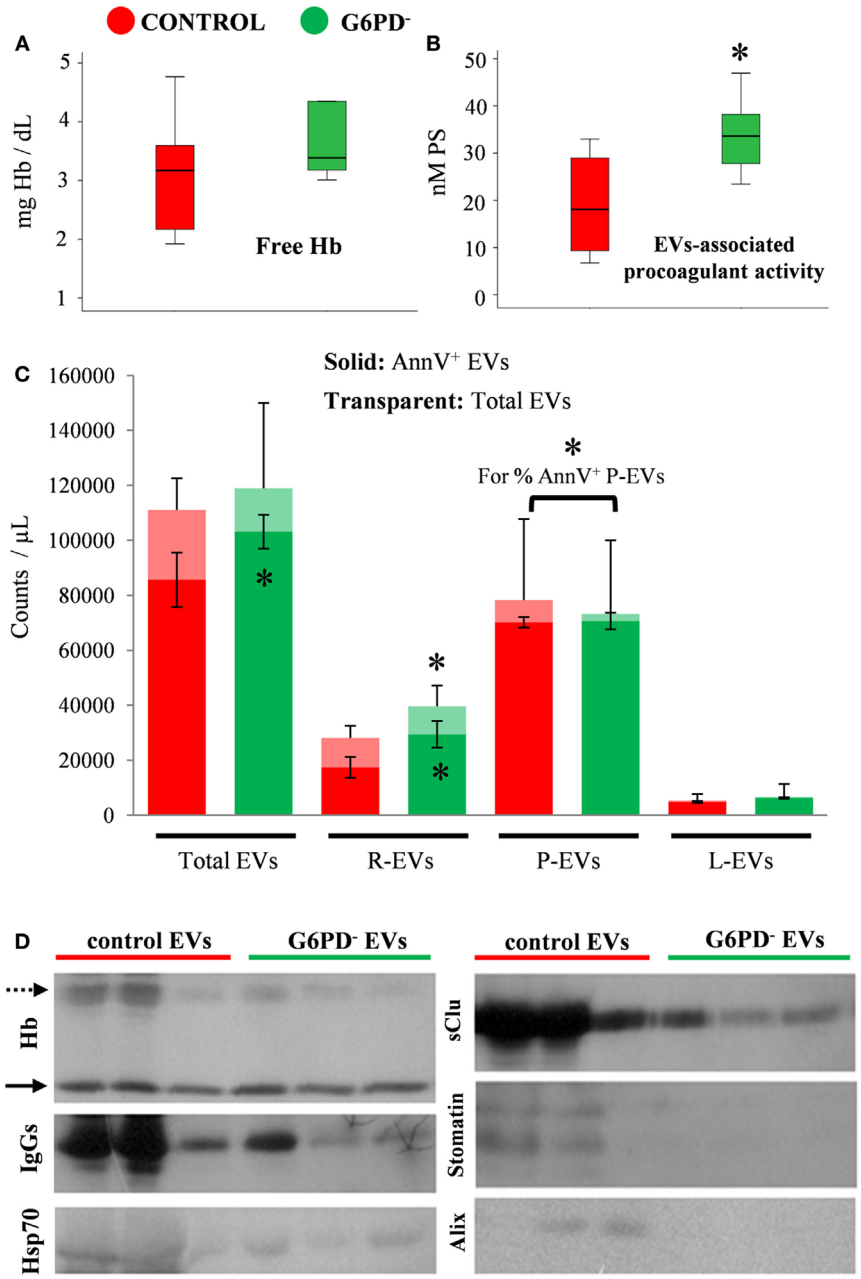

FIGURE 2 | Hemolysis and extracellular vesicles (EV) analyses in fresh frozen plasma (FFP) units prepared from G6PD-deficient (G6PD⁻) and control donors. Free hemoglobin (Hb). **(A)** and EV-associated procoagulant activity **(B)** levels in the G6PD⁻ and control FFP units. **(C)** Enumeration and phenotyping of total and annexin V positive (AnnV⁺) EVs by flow cytometry. R-, P-, L-EVs stand for red cell-, platelet-, and leukocyte-derived EVs, respectively. *$p < 0.05$, G6PD⁻ vs. control FFPs; error bars: mean ± SD. **(D)** Representative immunoblots showing similar Hb levels (solid arrows) but variable expression of other protein components in EVs precipitated by high-speed centrifugation of equal volumes of G6PD⁻ and control FFP. Dashed arrow: oxidized Hb bands.

for a number of important metabolites are shown in **Figure 5**. Nevertheless, by performing PLS-DA or Volcano-plot analysis, the potential to identify subtle but substantial changes among a group of related compounds could be weakened. To overcome this obstacle, an MSEA was performed on plasma metabolites along with their relative concentrations by using the web-based platform MetaboAnalyst (**Figure 6A**). Metabolomic data from control and G6PD⁻ FFP showed that the pathways significantly enriched (FDR < 0.05) were as follows: (i) riboflavin metabolism, (ii) phospholipid biosynthesis, (iii) purine metabolism, (iv) tricarboxylic acid cycle, and (v) histidine metabolism. In parallel, we also utilized the MetPA module of MetaboAnalyst, which combines results from the pathway enrichment analysis with the pathway topology analysis. A graphical list of the pathways

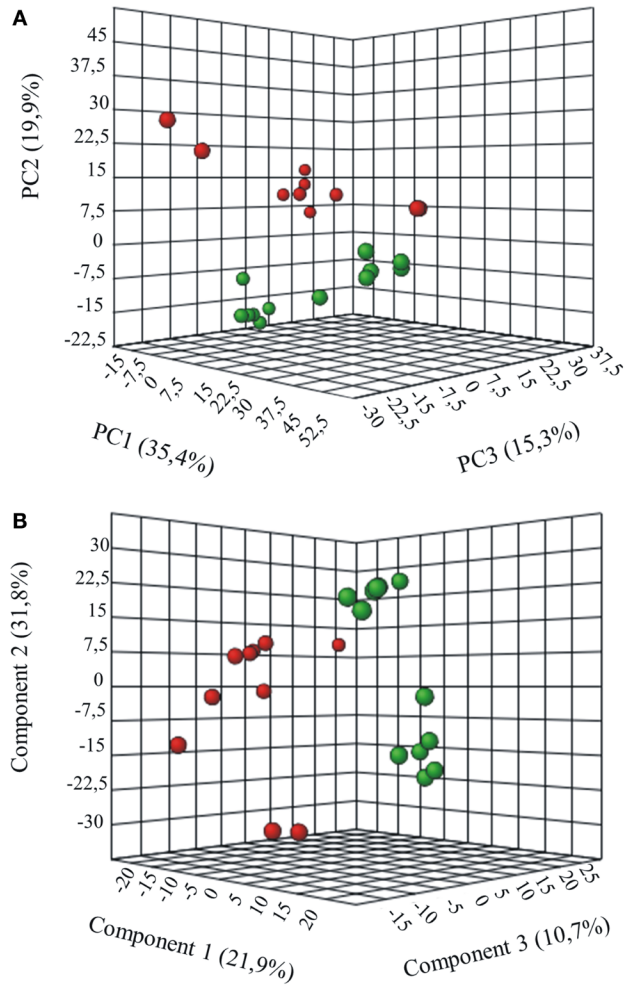

FIGURE 3 | Multivariate statistical analysis of metabolomics data from control and G6PD⁻ donors. Three-dimensional principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) score plots are shown in **(A)** and **(B)**, respectively. Control sample groups are in red; G6PD⁻ sample groups are in green.

identified and their relative impact is shown in **Figure 6B**. The most important ones (FDR < 0.05; impact values > 0.1) included tricarboxylic acid cycle and the metabolism of the following compounds: (i) glycerolipids, (ii) glycerophospholipids, (iii) purines, (iv) riboflavin, (v) glyoxylate/dicarboxylates, and (vi) inositol phosphate. Taken together, these results point out that both analyses concurred on most of the pathways, with MetPA being slightly more sensitive.

The Biological Networks of G6PD⁻ and Control FFP Were Different

More than 1,000 statistically significant correlations ($p < 0.05$) were detected between the biochemical, physiological, and metabolic variables in control FFP units. They were topologically arranged in an untargeted biological network according to the power of the correlation coefficient r (the shorter the edge, the higher the r value, small magnification network in **Figure 7**). A significant part of that network ($n = 266$ pairs) referred to connections between redox, EVs, and metabolic parameters (see Table S1 in Supplementary Material for code numbering and abbreviations). Focusing on that part of the control FFP network resulted in the interactome shown in **Figure 7**. In that subnetwork, uric acid and uric-acid-related physiological features and metabolites (antioxidant capacity, allantoin) exhibited the higher degree of connectivity, followed by the hub nodes of EVs, homocysteine, aconitate, and riboflavin. Half of the connections involved at least one of those variables. Worth to mention here, allantoin, the precursor of allantoate in serum, is a biomarker of oxidant generation *in vivo* (24). Uric acid may be oxidized non-enzymatically to allantoin by various ROS, leading to hydrogen peroxide generation. Uric-acid-related correlations included lactate, numerous amino acids, phosphoinositol, carnitine, creatinine, and NADP/NADPH. The concentration of the AnnV⁺ EVs in the control FFP was strongly interconnected with the levels of lipid peroxidation, uric acid, adenosine, glycerophospholipids, lactate, ornithine, and, again, with several amino acids. AnnV⁺ red-cell EVs and platelet EVs had a similar degree of interconnection, while both of them, in addition to the EV-associated procoagulant activity of the FFP, strongly correlated with the levels of amino acids, lipid metabolism, and redox state components (ascorbic, uric acid, lipid peroxidation). In fact, the procoagulant activity had negative correlations with several amino acids but positive correlations with acetylcarnitine. PS exposure on platelet-derived EVs seemed to be more influenced by lipid metabolites, and thus the relevant node was arranged out of the main core of the network. Homocysteine showed significant connections with adenine and citrulline, while aconitate with citrulline, lactate, purines, choline, and ascorbic acid, among others. Riboflavin and the correlated ascorbic acid localized to the center of the network. Riboflavin had correlations with adenine, acetylcarnitine, and lipid peroxidation, while ascorbic acid with several components (adenosine-monophosphate, glycerophosphocholine, ornithine) and the uric-acid-independent antioxidant capacity of the control FFP, along with vitamin B6 metabolites.

Regarding the biological network of G6PD⁻ FFP, it was substantially bigger than that of control FFP, with more than 2,000 statistically significant connections at total, and 434 connections in the relevant subnetwork shown in **Figure 8**. It was also different compared with the control network, in terms of pairing, topography, and hub nodes. While the uric-acid-related box and aconitate represented main hub nodes here too, they constructed along with the hubs of DAG, lipid peroxidation, and glutathione the extremely dense core of the network that included strongly interconnected variables. The degree of PS exposure on EVs was strongly interconnected with the levels of pyruvate, allantoin, adenosine, and inosine and, again, with several amino acids, similarly with the control network. In contrast, however, to the control FFP, the PS-exposing red-cell EVs had more connections compared with the other EV subtypes in the G6PD⁻ FFP, mostly with adenosine, amino acids, and purine metabolism variables. The platelet-EV node was again located away from the network's core, being linked, though, with it by the pyruvate–glutathione connection. The procoagulant activity of the G6PD⁻ FFP had positive

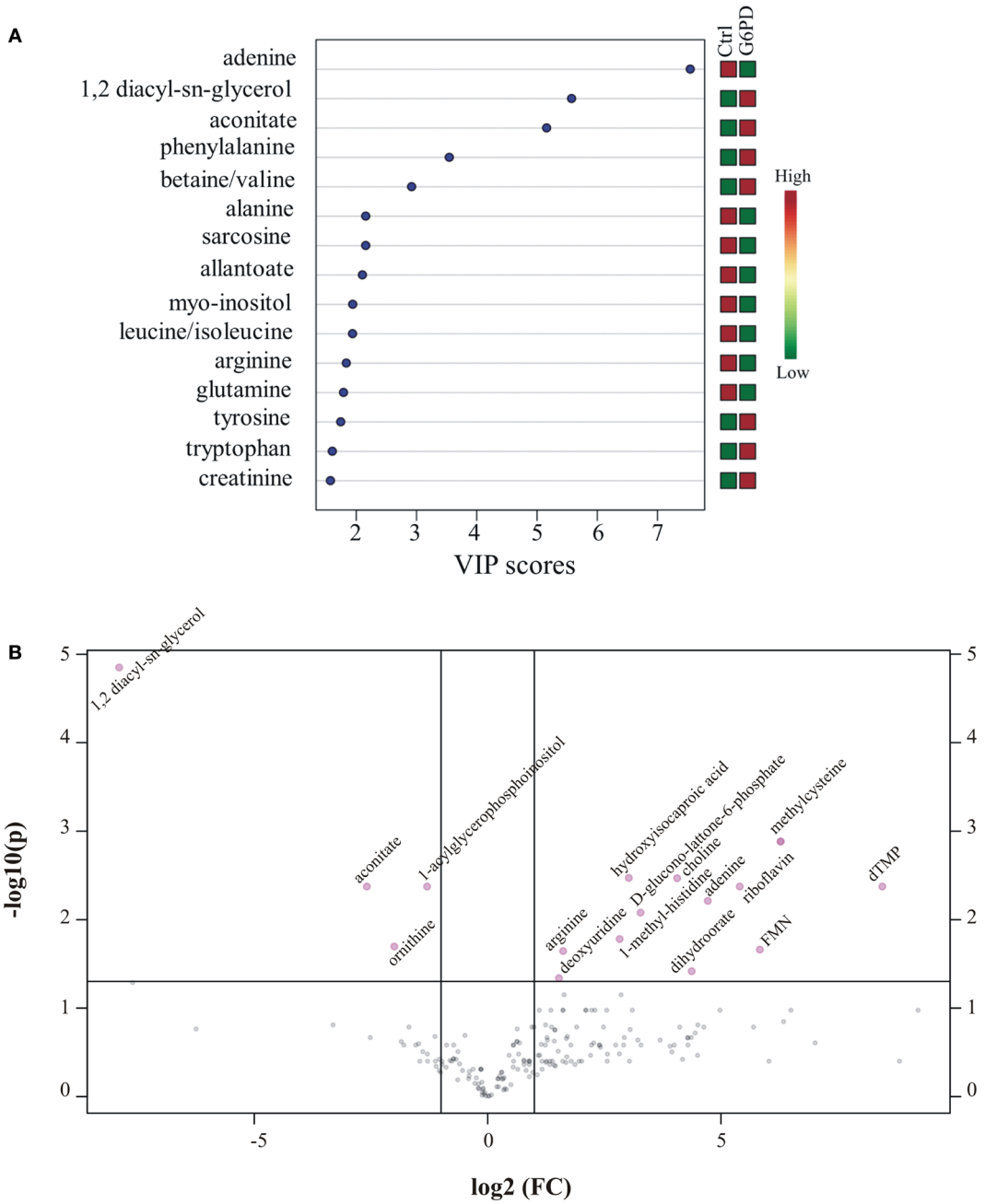

FIGURE 4 | Important features identified by uni- and multivariate analyses. **(A)** G6PD⁻ vs. control variable importance in projection (VIP) plot; colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under current study. **(B)** Volcano plot showing the distribution of the fold changes in metabolite concentrations. Metabolites with absolute fold change >2 and adjusted *p*-value (FDR < 0.05) are indicated in pink. Comparisons were analyzed using Student's *t*-test.

correlations with pentose phosphate pathway intermediates and allantoin. Riboflavin and ascorbic acid were not connected with the core of the network, while the uric-acid-independent antioxidant capacity had no correlation with the levels of ascorbic acid, as occurred in the control FFP. Uric acid and related variables were connected with lipid biosynthesis, transfer, and metabolism, in addition to amino acids and arginine metabolism/urea cycle components. The newly appearing hub node of DAG was strongly connected to the other hub nodes of the network, namely, the lipid peroxidation (MDA), aconitate, glutathione, and allantoate, in addition to lactate and many lipid-related, amino acids, purine, and arginine metabolism components. The hub of MDA was further connected to those of glutathione and aconitate, in addition to adenine, purine metabolism components, glycerophospholipids,

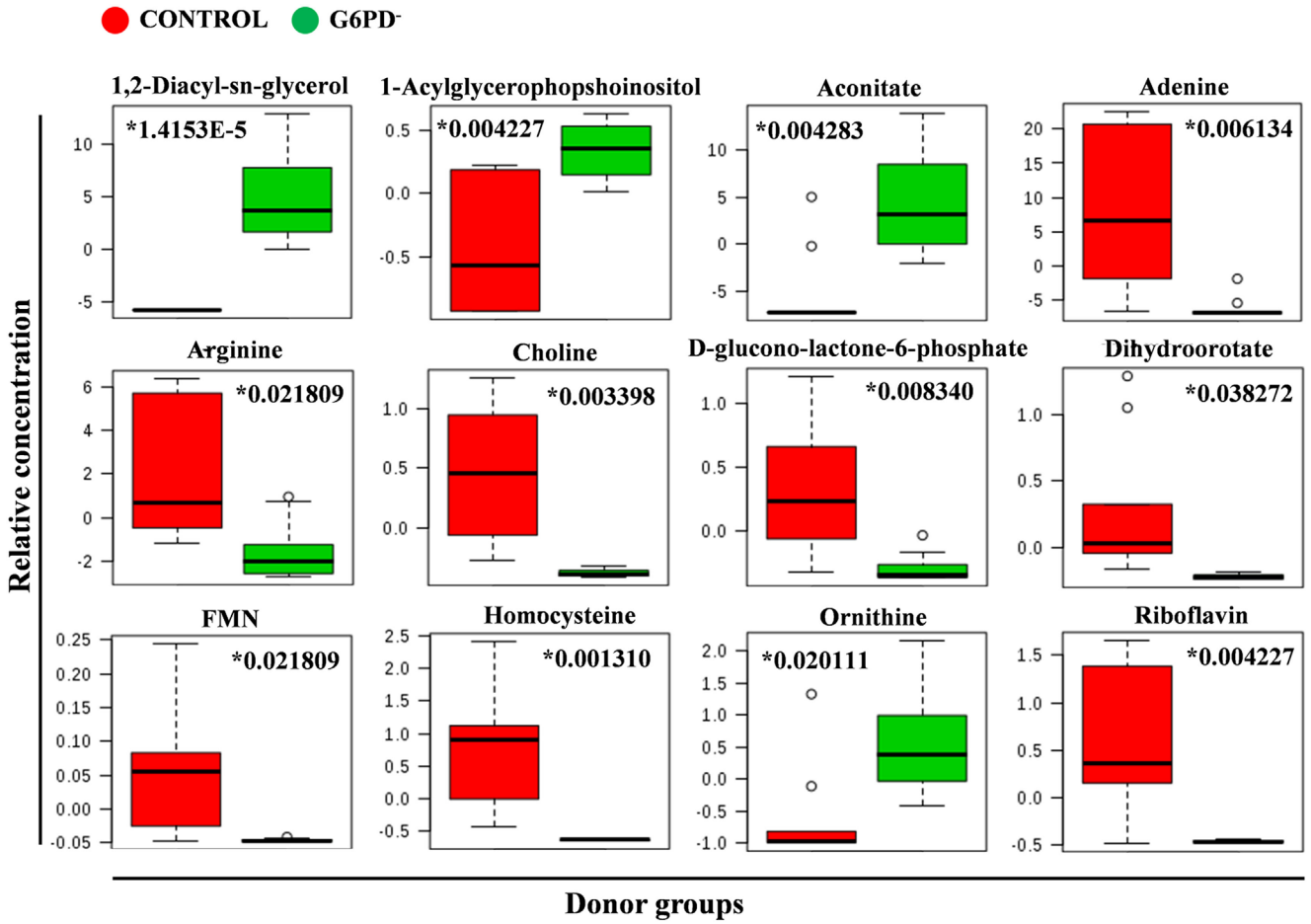

FIGURE 5 | Quantitative assessment of selected metabolites. Box and whisker plots for selected significantly altered metabolites in G6PD⁻ FFP compared with control (units in normalized and scaled concentrations). The *x*-axis shows the specific metabolite and the *y*-axis is the relative concentration. Medians are indicated by horizontal lines within each box. Outliers are plotted as individual points. Numbers with asterisks indicate adjusted *p*-values.

and aminoacids. Glutathione hub was related to glycolysis and arginine metabolism, glycerophospholipids, and aminoacids and finally, aconitate had connections with purine and arginine metabolism, lipids, and amino acids.

DISCUSSION

Human plasma has been often utilized in biomarker discovery studies because its molecular composition reflects the physiological state of donor's cells and tissues (12, 25). According to recent reports, inheritable omics variation among labile blood products, including FFP, may be associated with inter-donor differences observed in their quality, before and following transfusion (26). In similarity with the storage effect on blood components (27), G6PD⁻ affects the glycolysis and the pentose phosphate pathways, and thus, the energy and redox status of cells. Since the "fluxome," namely, the transport properties of cell membrane, in G6PD⁻ would interconnect the intracellular and plasma metabolomes (15), we used untargeted mass spectrometry-based metabolomics strategies to study the systemic metabolic effects of G6PD⁻ on FFP used for transfusion in association with other physiological assessments, including the antioxidant capacity and the EV component, compared with G6PD⁺ controls. To the best of our knowledge, this is the first study to show FFP metabolome and physiological changes related to G6PD⁻.

Metabolomics and Physiological Analyses Supported the Low Level of Oxidative Defects Seen in G6PD⁻ FFP Based on Uric Acid

G6PD⁻ cells are extremely sensitive to the deleterious effects of oxidants. As a probable adaptation to this inherent danger, FFP from G6PD⁻ donors was characterized by higher antioxidant capacity, which was mostly uric-acid-dependent. In fact, uric acid and its metabolic (allantoin, hypaxanthine) and physiological (antioxidant capacity) relatives constituted as high as 24% or 30% of the statistically significant correlations between the currently measured plasma variables in control and G6PD⁻ FFPs, respectively, signifying the central role of purine

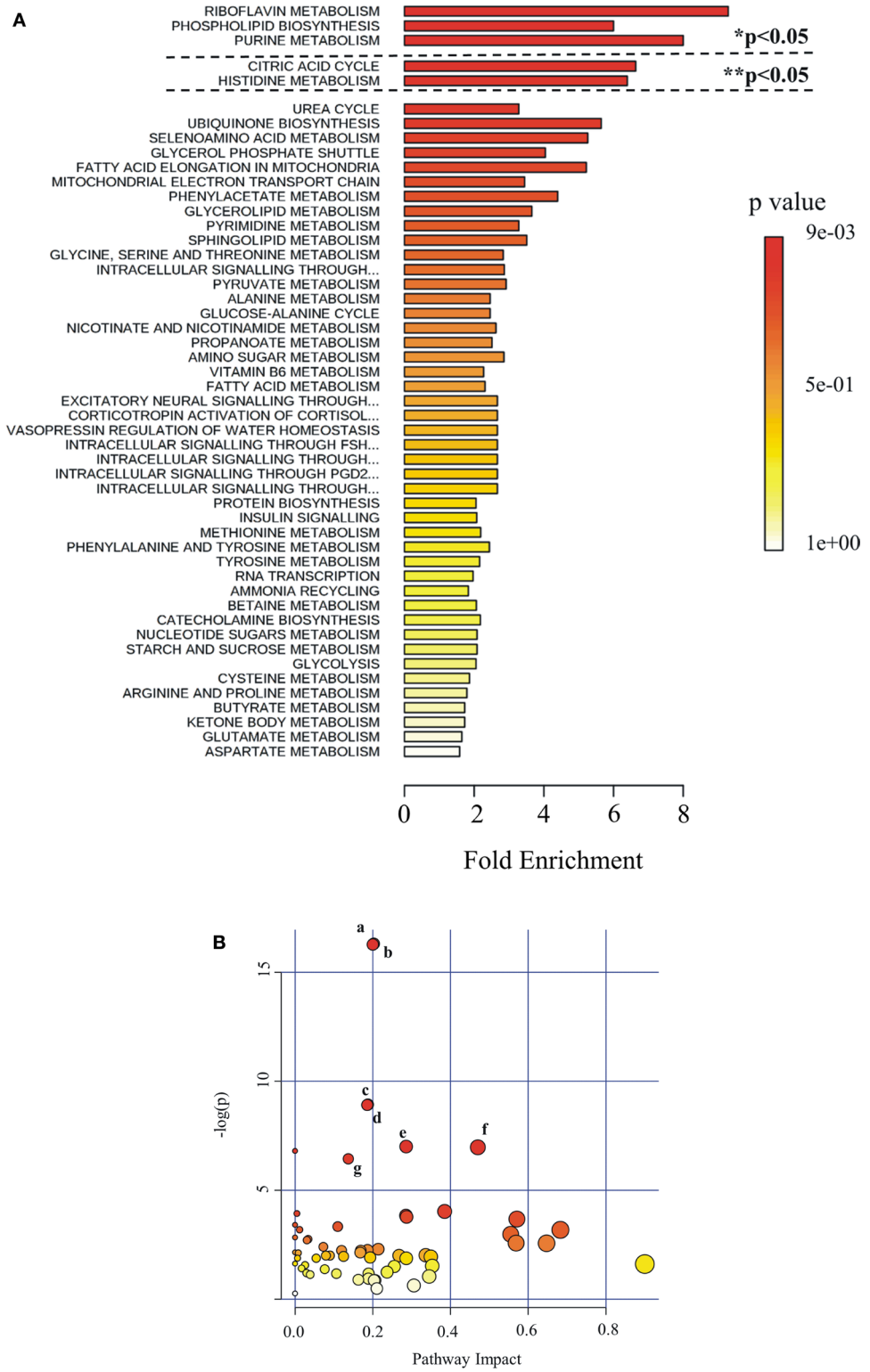

FIGURE 6 | Pathway analysis as generated by MetaboAnalyst software package. Identified metabolites and their relative quantity were used to calculate the enrichment and statistical significance. **(A)** Metabolite set enrichment analysis (MSEA). Top 50 perturbed pathways are shown. The dashed lines indicate the cutoff of the adjusted *p*-value (*Holm; ** FDR). **(B)** Metabolic pathway analysis (MetPA). All the matched pathways are displayed as circles. The color and size of each circle are based on *p*-value and pathway impact value, respectively. The most impacted pathways having high statistical significance scores are indicated with letters: a, glycerolipid metabolism; b, glycerophospholipid metabolism; c, purine metabolism; d, riboflavin metabolism; e, glyoxylate and dicarboxylate metabolism; f, tricarboxylic acid cycle; g, inositol phosphate metabolism.

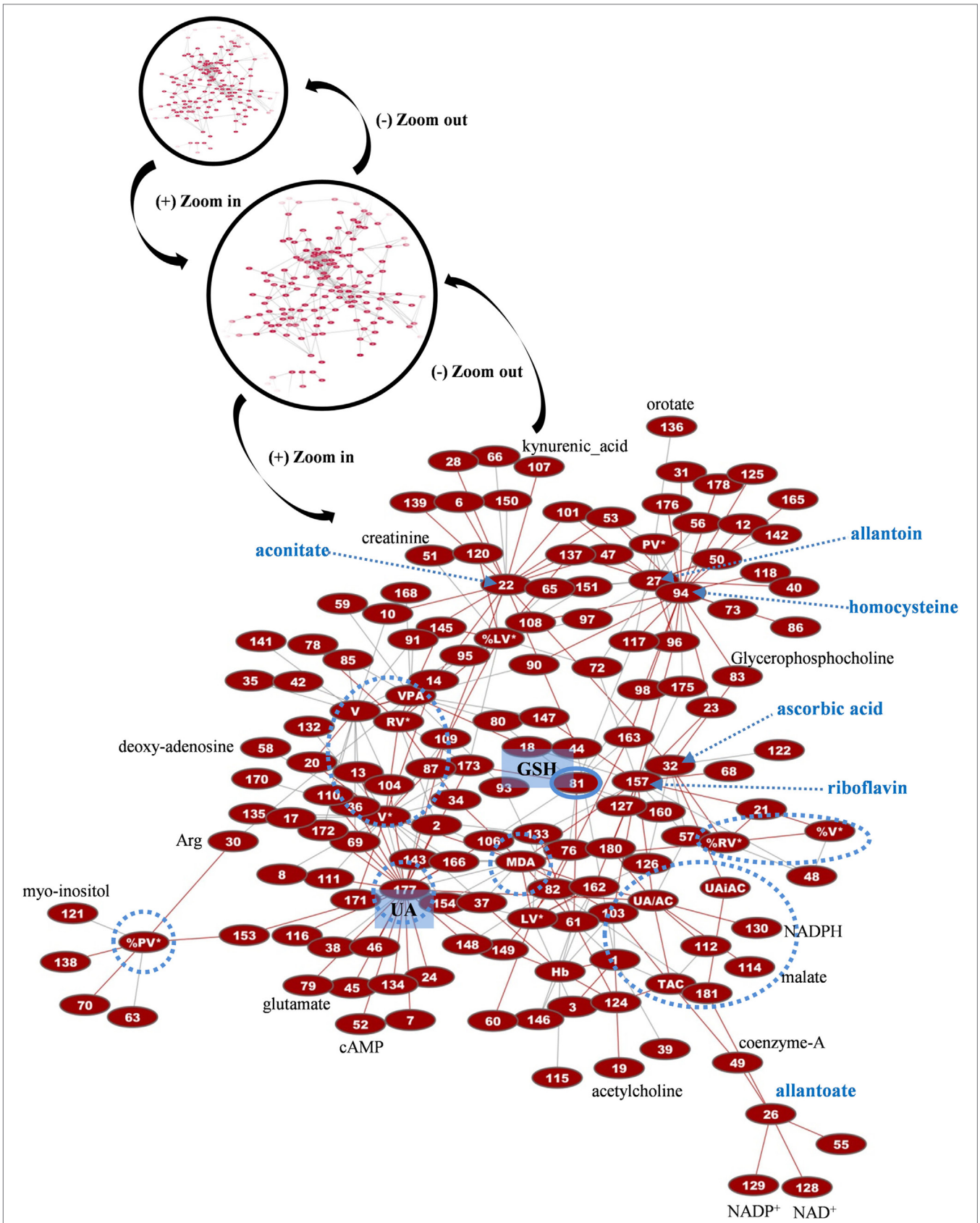

FIGURE 7 | Continued

FIGURE 7 | Network presentation of correlations among biochemical, physiological, and metabolomic variables in control FFP units. "Magnification" of a part of the total interactome of control fresh frozen plasma (FFP) units (networks in black circles) allowed focusing on the main connections between redox, EVs, and metabolic parameters. In the subnetwork of 266 connections (see Table S1 in Supplementary Material for code numbering and abbreviations), uric acid (UA), homocysteine, and reduced glutathione (GSH) constituted significant hub nodes, while ascorbic acid had correlations with the uric-acid-independent antioxidamt capacity of the FFP unit. Light blue dashed arrows indicate metabolites of high connectivity, and light blue circles stand for distinct groups of highly interconnected variables, or "boxes," corresponding to FFP EVs and antioxidant capacity. Only the statistical significant correlations at $p < 0.05$ are shown. The length of each line is inversely proportional to the *r* value of the correlation (the shorter the edge, the higher the *r* value). Red lines: positive correlations; gray lines: negative correlations.

metabolism in plasma homeostasis. The high-antioxidant capacity of FFP in G6PD$^-$ is very reminiscent of the high small molecule antioxidant capacity found in the umbilical cord blood from G6PD$^-$ newborns (28). Notably, the antioxidant capacity of fresh plasma in G6PD$^-$ was found similar to that of G6PD$^+$ plasma (29). Thus, our data suggest either a G6PD$^-$ donor variation effect or an effect of preparation and storage manipulations on the antioxidant capacity, and likely on other features of the donated plasma, which renders it only in part analogous to the *in vivo* state.

G6PD$^-$ FFP under investigation had normal levels of free hemoglobin (which otherwise might trigger oxidative reactions), and substantially lower oxidative modifications to both lipids and proteins. MDA units increase as a result of oxidative stress and according to previous studies (4), their levels are influenced by pre-analytical and donor-related factors. According to our results, G6PD$^-$ represents a donor-related variable with significant effects on lipid peroxidation, protein carbonylation, membrane vesiculation, and metabolome of FFP, in addition to their "wiring" in biological networks. Lipid peroxidation had significant correlations with uric acid and riboflavin in control FFP samples and with glutathione, xanthine/hypoxanthine, adenine/adenosine, and DAG in G6PD$^-$ samples.

The metabolomic assessment is in harmony with the substantially low lipid peroxidation in G6PD$^-$ units. Underrepresentation of riboflavin in G6PD$^-$ FFP, for instance, compared with the control FFP is likely the effect of its consumption in the context of a homeostatic antioxidant activity. This water-soluble vitamin is very effective in ameliorating oxidative stress, especially lipid peroxidation, through many molecular pathways, including reduction–oxidation reactions of the molecule itself and participation in the glutathione redox cycle (30). Riboflavin is a hub node in the network of control FFP, having strong correlation with lipid peroxidation, while in G6PD$^-$ FFP it correlated with glycolysis metabolites and biotin, another vitamin involved in fatty-acid metabolism and amino-acid catabolism. In addition, riboflavin exhibits anti-inflammatory and neuroprotective effects and seems to be involved in immune-mediated clinical conditions like sepsis and multiple-organ failure (30). Apart from riboflavin, the high levels of aconitate may be associated with the redox homeostasis of G6PD$^-$ FFP, since in both mouse model of human G6PD$^-$ (31) and in Alzheimer's disease subjects (32) the activity of aconitase had correlations with the oxidative stress and the antioxidant protection. In our samples, aconitate was a hub node in both control and G6PD$^-$ FFP networks, with significant correlations with the redox state of FFP (ascorbic acid, uric acid, lipid peroxidation), amino acids, lipids, and several metabolic pathways (glycolysis, purines, arginine). The increased antioxidant capacity but low riboflavin levels of G6PD$^-$ FFP compared with control, which are reported for the first time, may be important for FFP recipients characterized by increased systemic oxidative stress, but this finding deserves further examination at clinical level.

Increased Red-Cell EVs and EV-Associated Procoagulant Activity in G6PD$^-$ FFP

By affecting the redox potential, G6PD has a critical role in the development, cell survival, and apoptotic cell death (33). PS exposure and increased vesiculation rates characterize the surface of stressed, activated, or apoptotic cells (34). Indeed, higher concentration of circulating PS$^+$ microparticles (both red-cell- and platelet-derived) was reported in G6PD$^-$ subjects in close association with the severity of G6PD$^-$ (35). In FFP, the population of EVs is a mixture of (ex-) circulating EVs and those produced during the preparation, storage, and thawing of the unit. In our study, their cellular origin was similar to that found in previous reports (4, 13). Despite the expected (13) profound donor-dependent variation in EV enumeration among FFP samples in both groups, the G6PD$^-$ samples were characterized by invariably increased procoagulant activity and percentage of PS exposure mainly on red-cell- and platelet EVs, in consistence with previous studies showing enhanced PS exposure on circulating G6PD$^-$ red cells (36). Moreover, these EVs were characterized by different protein composition compared with those isolated from control FFP, and probably by a different origin, as shown by the different pattern of Alix staining. The lower expression of stress protein markers in G6PD$^-$ EVs, including oxidized Hb, heat shock protein 70, and clusterin (37), was in line with the higher antioxidant capacity of the G6PD$^-$ FFP.

The PS- or tissue factor-exposing EVs are likely to have procoagulant activities (38) and of note, higher levels of coagulation cascade components have been detected by proteomics analyses in EVs released by G6PD$^-$ stored RBCs in comparison to controls (36). Consequently, the FFP prepared by G6PD$^-$ donors probably represents a unique case, where its EV-based hemostatic activity (38–40) is combined with a high-antioxidant capacity and low-oxidative defects. The baseline rate of EV generation is strongly modulated by the endogenous or exogenous oxidative stress levels and the capacity of the antioxidant machinery, under a wide variety of physiological and pathological conditions (41). Indeed, in both FFP groups, the extent of PS exposure on EVs had significant correlations with the levels of redox state components including lipid peroxidation, uric acid/allantoin, and ascorbic acid. Moreover, the concentration of PS$^+$

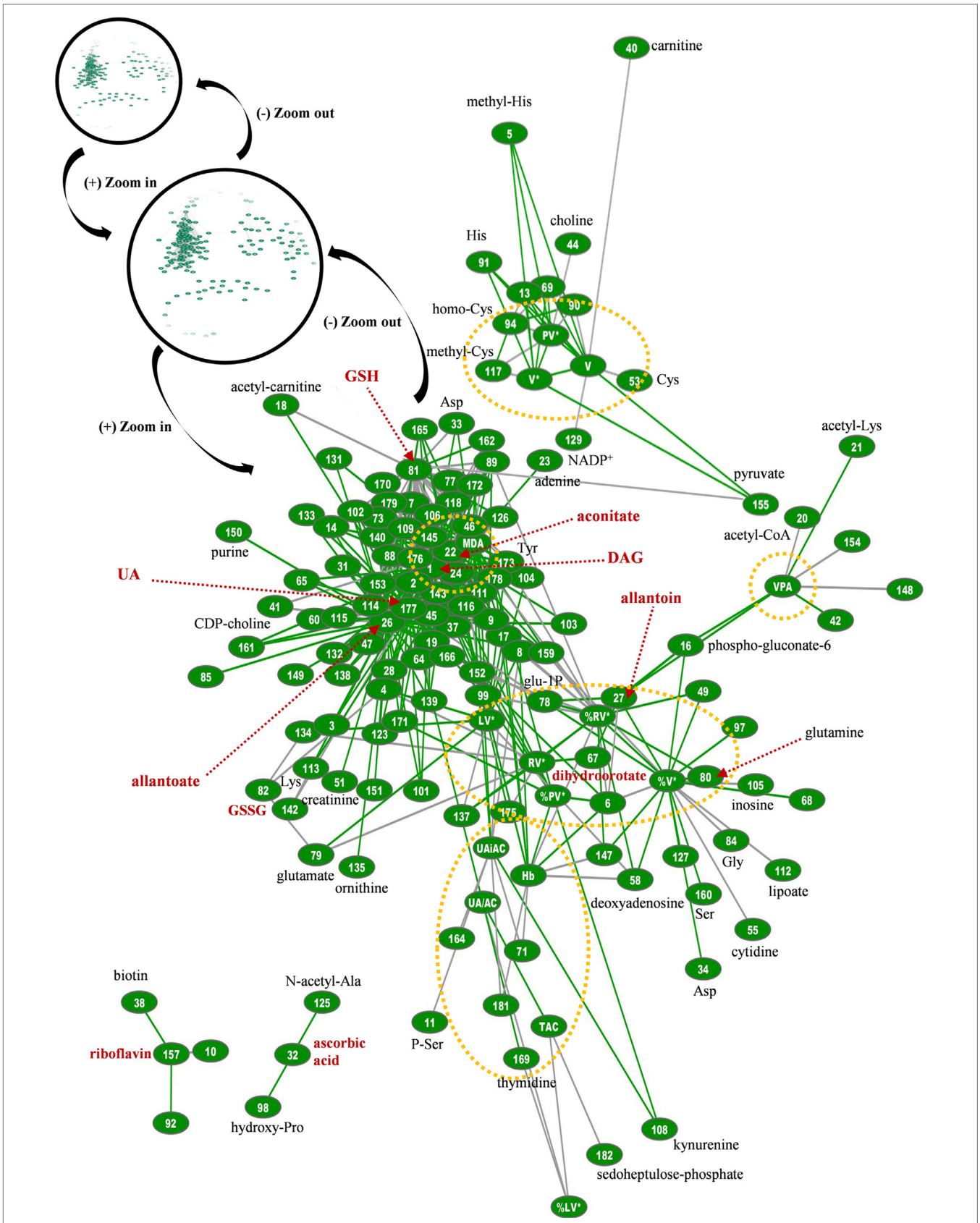

FIGURE 8 | Continued

FIGURE 8 | Topological presentation of correlations between biochemical, physiological and metabolomic variables in G6PD⁻ fresh frozen plasma (FFP) units. Starting from the total G6PD⁻ network (shown in black circles) and by using continuous "zoom in" tools we studied the main connections between redox, EVs, and metabolic parameters. This subnetwork was bigger and quite different compared with that of control FFP. It consisted of 434 connections (see Table S1 in Supplementary Material for code numbering and abbreviations), and while uric acid is also a hub node, diacylglycerol (DAG), lipid peroxidation (MDA), allantoate, aconitate, and reduced glutathione (GSH) have substantially more connections. The red dashed arrows indicate metabolites of high connectivity, and the yellow dashed circles stand for "boxes" of distinct groups of interconnected variables that correspond to the core of the network, FFP EVs, and antioxidant capacity. Only the statistical significant correlations at $p < 0.05$ are shown. The length of each line is inversely proportional to the r value of the correlation (the shorter the edge, the higher the r value). Green lines: positive correlations; Gray lines: negative correlations.

EVs seemed to have correlations with the levels of adenosine, amino acids, and energy metabolism, despite the fact that the individual components differed between the two groups (e.g., lactate instead of pyruvate). RBC vesiculation and EV-related procoagulant activity, which was assessed by thrombin generation *in vitro*, were found especially increased in G6PD⁻, in close association with adenosine, pentose phosphate pathway, and purine metabolism variants, showing a diverse pattern of wiring compared with the control FFP, in which ascorbic acid, lipids, and lipid modifications were more influential.

Main Metabolic Changes in G6PD⁻ FFP with Probable Impact on Signaling

The main metabolic profile of G6PD⁻ FFP reflects to some extent a systemic cellular response to G6PD⁻, as revealed, for example, by the deficiency in gluconolactone-6-phosphate. Favic response in G6PD⁻ mice includes alterations in a similar group of plasma (e.g., ornithine) and liver (e.g., phosphoglycerols and adenine) metabolites (42). Apart from riboflavin metabolism, purine metabolism, arginine metabolism/urea cycle components, and phospholipid biosynthesis constituted the most significant differences between the G6PD⁻ and G6PD⁺ FFP units. Indeed, increased levels of ornithine were found in G6PD⁻ FFP at the expense of L-arginine, suggesting increased energy consumption/waste by the G6PD⁻ cells (43).

The significantly low extracellular levels of the purine derivative adenine in the G6PD⁻ FFP verify the previously suggested strong positive correlation of plasma adenosine (adenine nucleoside) levels with glycolysis (44, 45). RBCs, in particular, use extracellular purines to maintain their intracellular nucleotide pool and to exploit the pentose moiety for energy production. More importantly, this finding suggested a different dynamics in purinergic signaling, since purinergic receptors are widely expressed in almost every cell type, including erythrocytes (46). Adenosine arising by the metabolism of extracellular nucleotides can transmit signals through G-protein-coupled receptors and anti-inflammatory adenosine (P1 purinergic) receptors (47). Extracellular adenosine signaling serves regulatory functions in inflammation, in acute lung injury (48) and in the O_2 delivery ability of red cell targets through boosting the production of 2,3-BPG (46). Nucleoside transporters assist in the control of plasma adenosine levels, and notably, decreased nucleoside transporter hENT1 [that also mediates hypoxanthine transport (49)] expression and activity was detected in G6PD⁻ RBCs (50). In G6PD⁻ FFP, adenosine was correlated with red-cell vesiculation, lipid peroxidation, hemolysis, DAG, oxidized glutathione, and uric acid/allantoate levels, suggesting more influential effects compared with the control FFP and a second level of intercellular signaling potential.

Aberrations in glycerolipid biosynthesis have been associated with G6PD⁻ from nematodes to humans (51). Increased activity of phospholipase A2 is observed during eryptosis which is enhanced in G6PD⁻ subjects (52). As a probable result of lower NAPDH, which is used in the reductive biosynthesis of fatty acids and cholesterol (53), lower cholesterol content (54), and clear differences in the lipid repertoire, biosynthesis and metabolism were detected between the Mediterranean type G6PD⁻ and control red cells both *in vivo* and during refrigerated storage (55). Notably, the levels of free fatty acids and oxidized derivatives were found significantly lower in stored G6PD⁻ compared with control red cells.

The presence of the highly hydrophobic DAG extracellularly was not expected. DAG has significant signaling potential that is exerted, however, at cell and subcellular membranes loci. Despite that, targeted lipidomics analysis revealed increased levels of DAG in the plasma of Alzheimer's disease patients (56), a clinical condition with indirect, however strong, pathophysiological connections with the G6PD activity. In fact, G6PD⁻ predisposes to a variety of chronic neurological diseases by undermining the defenses against the endogenous ROS-mediated neurodegeneration during aging (57). The high oxygen concentration and fatty acids levels but low antioxidant activity in brain tissue render it highly susceptible to peroxidation and oxidative damage (58). While DAG was a minor component of the control FFP network, it was the central hub node in the G6PD⁻ network, showing numerous direct connections with glycerophospholipid biosynthesis, amino acids, and components of glycolysis, glutathione, purine, glutamate, and arginine pathways, and indirect connections with the EVs through choline, purine, and glycolysis pathways metabolites.

Partitioning of DAG into the circulating vesicles may account for its detection in biological fluids in primary G6PD⁻ and in G6PD-related clinical conditions, principally characterized by enhanced release of EVs. While exosomes are not enriched in DAG compared with the cell membrane of origin (59), DAG is a component of urinary exosomes (60, 61), and of mesenchymal stem-cell-derived EVs (62). Of note, DAG and DAG-kinase have critical role in the polarized secretion of exosomes in T and B (and probably in other kinds of) cells (63). The putative DAG-bearing exosomes in G6PD⁻ FFP may transfer powerful signaling hits to target cells through fusion or endocytosis, especially in neuronal and immune tissues that principally express the targeting molecules (namely, the C1 domain-containing proteins) (64). FFP DAG may be arisen from the hydrolysis of phosphatidic acid produced by the activity of exosomal phospholipase D (65) on

extracellular phosphatidylcholine, which is quite probable, since choline—the second product of phosphatidylcholine hydrolysis by phospholipase D—was extremely low in G6PD$^-$ FFP. In the same context, the levels of 1-acylglycerophosphoinositol (that is formed *via* cytidine diphosphate-DAG by reaction with inositol) were found substantially increased compared with control samples.

CONCLUSION

The metabolome and several physiological features of FFP units prepared from donors with G6PD$^-$ differ compared with control FFP. Higher EV-related procoagulant activity, PS concentration, red-cell vesiculation, and antioxidant capacity, along with lower oxidative modifications in lipids and proteins were detected in G6PD$^-$ FFP than in G6PD$^+$ units. The EVs of G6PD$^-$ FFP further varied in number, cell origin, lipid and protein composition, and probably, in generation pathway. Metabolomics analysis revealed that riboflavin metabolism, purine metabolism, and glycerolipid/glycerophospholipid biosynthesis constitute the most significant variances between the two groups of FFP. Units prepared from G6PD$^-$ donors had excess of DAGs, glycerophosphoinositol, aconitate, and ornithine but they were deficient in riboflavin, flavin mononucleotide, adenine, and arginine, among others. Certain FFP-needed patients may be at greatest benefit of receiving units from G6PD$^-$ donors, intrinsically endowed by both procoagulant and antioxidant activities and low oxidative defects. However, the clinical outcome is likely affected by various other preparation and donor/recipient-related factors (4), including the signaling potential of the differentially expressed metabolites and EVs, the degree of G6PD$^-$, the redox status in the recipient, the amount of FFP units transfused, the leukoreduction (66), and probably, the storage interval of the FFP (13). All these parameters deserve further investigation by large-scale laboratory and clinical studies.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Department of Biology, School of Science, NKUA. Investigations were carried out upon signing of written consent in accordance with the principles of the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SR and MA designed the study. VT performed the biochemical and ELISA assays. HG performed the immunoblots. AK performed the flow cytometry analysis. FG and SR performed the UHPLC-MS analyses. VT, FG, SR, and MA analyzed the results, prepared the figures, and wrote the manuscript. LZ and IP critically commented on the interpretation of data and drafting of the manuscript. All the authors contributed to the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fmed.2018.00016/full#supplementary-material.

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MINI REVIEW
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The Role of Plasma Transfusion in Massive Bleeding: Protecting the Endothelial Glycocalyx?

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Massive hemorrhage is a leading cause of death worldwide. During the last decade several retrospective and some prospective clinical studies have suggested a beneficial effect of early plasma-based resuscitation on survival in trauma patients. The underlying mechanisms are unknown but appear to involve the ability of plasma to preserve the endothelial glycocalyx. In this mini-review, we summarize current knowledge on glycocalyx structure and function, and present data describing the impact of hemorrhagic shock and resuscitation fluids on glycocalyx. Animal studies show that hemorrhagic shock leads to glycocalyx shedding, endothelial inflammatory changes, and vascular hyper-permeability. In these animal models, plasma administration preserves glycocalyx integrity and functions better than resuscitation with crystalloids or colloids. In addition, we briefly present data on the possible plasma components responsible for these effects. The endothelial glycocalyx is increasingly recognized as a critical component for the physiological vasculo-endothelial function, which is destroyed in hemorrhagic shock. Interventions for preserving an intact glycocalyx shall improve survival of trauma patients.

Keywords: massive hemorrhage, shock, resuscitation, fresh frozen plasma, endothelium, glycocalyx

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The aim of this mini-review is to give an overview on plasma treatment in massive bleeding. We will briefly describe current pathophysiological concepts of vascular damage in hemorrhagic shock, summarize data on the use of plasma as a resuscitation fluid, and report experimental data suggesting a protective role of plasma on endothelial integrity.

TRAUMA, MASSIVE HEMORRHAGE, AND TRAUMA-INDUCED COAGULOPATHY (TIC)

Epidemiology and Definition of Massive Hemorrhage

The World Health Organization estimates that in the year 2000, 5 million people died of injuries, accounting for 9% of global annual mortality (1). After central nervous injury, massive hemorrhage represents the second-leading cause of death, being responsible for 30–40% of trauma-related mortality (1). Death can occur within 3–6 h by exsanguination from uncontrolled hemorrhage and one-third to half of the deaths occur before reaching the hospital (1, 2). Modern transfusion practices and blood supply make massive hemorrhage a potentially preventable cause of death in different settings (e.g., civilian or military trauma, surgery, post-partum). The benefit of blood component transfusion in the context of trauma has been discussed for many years but it is only since the retrospective study of Borgmann published in 2007 (3) that plasma transfusion has been recognized

as a probable positive factor for survival. However, "survival bias" remains an unsolved pitfall of retrospective studies, not only for interpreting potentially causative factors related to survival (i.e., did the patient "survive because she received plasma transfusion" *or* did she "get plasma transfusion because survived long enough to receive it"?) but also for defining massive hemorrhage. In fact, the classical definition of massive hemorrhage is based on the number of packed red blood cells (PRBC) units transfused during the first 24 h after admission. High mortality rates during the first 24 h and rapid time course of massive hemorrhage make transfusion rate (e.g., ≥3 PRBC units/60 min) a more appropriate definition (4). In addition, data analysis from the PROMMT study enabled Rahbar et al. to identify those patients most likely to develop massive hemorrhage based on emergency admission variables, such as systolic blood pressure, heart rate, pH, and hemoglobin (5). This prospective observational study showed that transfusion with higher ratio of plasma to PRBC early in resuscitation is associated with an improved survival at 24 h (6). Specifically, adult trauma patients surviving beyond 30 min from admission and transfused with ≥1 unit of PRBC in the first 6 h and ≥3 units of PRBC during the first 24 h showed a significantly higher survival at 6 and 24 h, and 30 days when receiving plasma units and PRBC at a ratio of at least 1:1 (6, 7). Of note, such high plasma to PRBC ratios beyond the first 24 h was not associated with survival by day 30.

Pathophysiological Concepts

The so-called acute trauma coagulopathy (ATC) (8) and TIC (9) have been conceptualized through different models, all converging to the key concept of «endothelial stress» (10–20), also named «endotheliopathy of trauma» (21, 22) or «shock-induced endotheliopathy» (23). The endothelium covering an area of about 5,000 m^2 is one of the frailest and initial victims of massive hemorrhage (24). For instance, severe hypo-perfusion is associated with increased levels of circulating heparan sulfate, a component of the endothelial surface with anticoagulatory properties similar to heparin (25). Moreover, the co-existence of severe tissue injury, leading to high *in vivo* thrombin generation, and severe hypo-perfusion, leading to endothelial sufferance and thrombomodulin shedding, is complicated by circulating thrombin–thrombomodulin complexes culminating in systemic protein C activation and fibrinolysis (8, 9).

Several factors drive the system into a vicious circle: (1) on the one side, endothelial injury with enhanced vascular permeability leads to further loss of intravascular volume, hypovolemia, tissue hypoxia, and exacerbated shock and (2) on the other side, resuscitation-related blood dilution with acidosis and hypothermia (the classical iatrogenic triad) further impair vasculo-endothelial functions. In sum, massive hemorrhage means perfusion, oxygenation, coagulation, and metabolic failures.

PLASMA AS A RESUSCITATION FLUID

Plasma Type, Delivery, and Supply

Plasma sources and plasma processing have been developed during these last decades (18). Each preparation addresses and mitigates particular risks related to transfusion hazards: single donor fresh frozen plasma (FFP) vs pooled plasma or quarantine FFP vs pathogen-inactivated FFP to diminish the risk of transfusion-transmitted infections; FP24 (frozen within 24 h after donation) instead of standard FFP (frozen within 8 h after donation) to enable HLA testing and remove high risk units for TRALI; frozen plasma vs liquid or thawed plasma to extend storage duration; lyophilization formulas for rapid reconstitution. Study of the variability of coagulation factors and natural anticoagulants levels in different plasma preparations are summarized elsewhere (26). Of note, the factor V and factor VIII, known to be «labile» and critical in the evaluation of manufacture practice, show heterogeneous decrease during storage, depending on formulas. Several studies reveal how processing conditions (whole blood hold-time, storage duration/temperature before freezing, freezing mode, leucodepletion, pathogen inactivation, lyophilization) specifically influence coagulation factor levels, microparticles content, clot generation capacity and protein composition in plasma (27–30) and clotting factor stability after thawing (31). In massive hemorrhage management, logistical concerns, besides biological aspects such as type of plasma, FFP to PRBC ratio or functional monitoring of clot generation, matters as well. Time between trauma and transfusion, transport of plasma from blood bank to the clinical unit, mode of checking plasma unit before transfusion and provision of thawed/liquid plasma are most critical aspects in massive transfusion protocols (32–34).

Hence, one logistical challenge for blood bankers is plasma supply. Benefit from plasma transfusion in massive hemorrhage lead to growing use of «universal» but scarce AB plasma (35). At the same time, implementation of the «male policy» (plasma donation from male donors only) to improve the transfusion safety regarding risk of TRALI significantly restricts plasma availability. This demand/supply imbalance led the American Red Cross to consider the use of group A plasma to adult trauma patients (36). Novak et al. reported the experience of 12 trauma centers participating in the PROPPR study in managing plasma inventory to meet new guidelines issued by the American College of Surgeons in 2013 for trauma resuscitation (37). Rapid delivery is made possible by the selection of group A plasma with low titer anti-B, in addition to plasma formulations and thawing systems with short turnaround time.

Benefit of Plasma Transfusion: Do Coagulation Factors Tell the Whole Story?

Since the time Borgman et al. demonstrated in 2007 that FFP transfusion in massive hemorrhage resulted in increased survival (3), researcher started to wonder which mechanisms may be responsible for this effect. The first hypothesis at hand would have been the correction of coagulopthy. However, plasma transfusion in the form of FFP cannot replace coagulation factor loss (38). Therefore, several publications aimed to investigate the benefit of plasma resuscitation on other pathophysiological variables, such as endothelial restoration (39–45).

ENDOTHELIAL GLYCOCALYX

The Endothelial Glycocalyx Structure and Function

The endothelial glycocalyx is a thick (about 0.2–3.0 μm *in vivo*) (46, 47), negatively charged carbohydrate-rich layer coating the vascular endothelium (48–52). The glycocalyx *sensu stricto* is formed by cell membrane-bound sulfated proteoglycans, consisting of a core protein (e.g., transmembrane syndecan, membrane-bound glypican, or basement matrix-associated perlecan) with glycosaminoglycans side chains (e.g., heparan sulfate, hyaluronic acid, and chondroitin sulfate) (53), and cell membrane glycoproteins bearing sialoproteins (50, 53). Syndecan-1 (CD 138), a heparan sulfate containing proteoglycan, is one of the major constituents ensuring endothelial integrity (51). Under physiological conditions, positively charged soluble components (such as plasma proteins, enzymes, growth factors, cytokines, amino acids, and cations) and water are trapped in the glycocalyx forming an extended endothelial surface layer. The mesh formed by the gylcocalyx contains ~1 to 1.5 l plasma, which are in dynamic equilibrium with the flowing blood (48, 54, 55).

The glycocalyx has several recognized functions (**Table 1**) (49–52). In particular, it forms a physical barrier between blood and vessel wall (48, 56–60); it maintains blood fluidity by modulating the interactions of the endothelium with blood cells and proteins (50, 61–63); it regulates cell adhesion and vascular permeability (64); it creates a high intravascular colloid-osmotic gradient (65, 66); and it acts as a mechano-transducer, e.g., by sensing shear stress and inducing endothelial release of nitric oxide (60, 63, 67, 68). As it may be expected from its many functions, the disruption of the glycocalyx leads to several clinically relevant pathologies (48, 52, 61). In the following paragraph, we will discuss the effect of hemorrhagic shock and type of resuscitation fluid on the glycocalyx.

TABLE 1 | Some recognized functions of the endothelial glycocalyx (48–50, 52, 69, 70).

Functions	Mechanisms	Reference
Barrier and filter	Protection from shear	(71)
	Exchange of water and solutes	(48)
	Sieve for plasma proteins	(57, 59)
	Uptake of low density lipoproteins	(63, 72)
	Repels red blood cells	(50)
Cell adhesion regulation	Prevents leukocyte adhesion	(56–58, 60, 73)
	Prevents platelet adhesion	(74)
Anticoagulation	Tissue factor pathway inhibitor	(48)
	Antithrombin	(50, 75)
	Thrombomodulin	(50)
Complement regulation	Complement factor H binding	(76)
Colloid-osmotic gradient	Absorption of albumin and smaller solutes	(65, 71)
Mechano-transducer	Nitric oxyde production	(50, 68, 77)
	Prostacyclin production	(48)
Inter-endothelial communication	Regulation of endothelial gap junctions	(78, 79)

Hemorrhagic Shock, Endothelial Glycocalyx, and Resuscitation Fluid

Shedding of endothelial glycocalyx components has been shown to occur in response to, e.g., ischemia and hypoxia (80), reactive oxygen species (81), inflammation and sepsis (82), and trauma-related sympatho-adrenal activation (83). As recently reviewed by Becker et al., loss of glycocalyx appears to be mediated by "sheddases," such as matrix metalloproteases, heparanases, hyaluronidases, and proteases (60) and to be responsible for endothelial inflammatory changes and vascular hyperpermeability (51, 64). In hemorrhagic shock, loss of the endothelial glycocalyx correlates with a dismal outcome. For instance, human studies indicate that in trauma patients with severe bleeding, high levels of syndecan-1 on admission (≥40 ng/ml) correlate with the extent of tissue damage, laboratory indicators of ATC and, in particular, mortality (84–86). Rahbar et al. showed that high circulating syndecan-1 levels correlate with increased vascular permeability (87). Since plasma-based resuscitation appears to exert a beneficial effect on survival (6, 7, 88), the question is whether plasma as a resuscitation fluid may have an impact on the endothelial glycocalyx and, therefore, potentially on vascular integrity and function.

Investigations in animal models may help framing a working concept (**Table 2**). Kozar et al. (40) employed a pressure-controlled model of hemorrhagic shock. Rats were bled to a mean arterial pressure of 30 mmHg for 90 min then resuscitated with either lactated Ringer's solution (LR) or fresh plasma to a mean arterial pressure of 80 mmHg. These animals were compared to shams (all procedures without bleeding) and positive controls (hemorrhagic shock without resuscitation). The authors found that (1) hemorrhagic shock is associated with a significant shedding of the endothelial glycocalyx, as indicated by circulating syndecan-1 levels, cell surface expression of syndecan-1, and electron microscopy imaging; (2) loss of the endothelial syndecan-1 correlates with the extent of lung injury, as assessed by alveolar wall thickness, capillary congestion, and cellularity; (3) resuscitation with plasma partially restores the endothelial glycocalyx while LR cannot, as assessed by electron microscopy on post-capillary venules obtained from the small bowel mesentery and by syndecan-1 expression in the lung; (4) the endothelial glycocalyx appears to be restored within 3 h after plasma resuscitation; (5) a clinically potential beneficial effect of plasma is suggested by the observations that plasma resuscitation required significantly less volume to maintain the mean arterial pressure at 80 mmHg compared to LR, and by the fact that plasma reduced lung injury while LR resuscitation increased it (40). These observations were expanded by the work of Torres et al. (42). In their model, a 40% blood volume hemorrhage was induced in rats. After 30 min of shock, animals were resuscitated with LR, hydroxyethyl starch (HES) or FFP, and compared to sham and hemorrhage without resuscitation. First, the authors confirmed that the endothelial glycocalyx is significantly damaged by the hemorrhagic shock and can be restored only with FFP, as assessed by circulating syndecan-1 levels and glycocalyx thickness. Second, a clinically beneficial effect of plasma-based resuscitation was

TABLE 2 | Studies[a] investigating endothelial integrity through plasma exposition in HS conditions.

Reference	Experimental models	Types of plasma	Main results
Pati et al. (39)	Studies on HUPECs monolayers (hypoxia-induced permeability) with assessment of EC permeability (FITC-Dextran) after FFP treatment, comparing FFP stored for 0 vs FFP stored for 5 days *In vivo* studies on rat model of HS for testing capacity of FFP (comparing FFP stored for 0 vs for 5 days) to restore MAP	Human FFP (ABO blood types, same donor or pooled from three donors, thawed-aliquoted-stored at 4°C for 0 or 5 days before use)	Day 0 FFP inhibits EC permeability; day 5 FFP demonstrated a diminished capacity to inhibit EC permeability Day 0 FFP, but not day 5 FFP, restores blood pressure to baseline
Kozar et al. (40)	Studies on a rat model of HS, comparing effect of LR vs fresh plasma resuscitation with assessment of endothelial glycocalyx on mesenteric vessels (electronic microscopy), relative expression level of syndecan-1 (QRT RT PCR) and cell surface expression of syndecan-1 (immunostaining) in lung tissue	Fresh plasma (not otherwise specified)	Glycocalyx is partially restored by plasma resuscitation Syndecan-1 expression in lung is enhanced by plasma Lung injury is lessened by plasma resuscitation
Haywood-Watson et al. (86)	Studies on HUVECs monolayers (hypoxia-induced permeability) with assessment of VE-cadherin and syndecan-1 expression (immunofluorescence), topographical properties (AFM), permeability (FITC-Dextran) after LR vs FFP treatment Patients admitted to ICU for shock, resuscitation with plasma, syndecan-1 and cytokines measurements	FFP (not otherwise specified)	Vascular integrity is disrupted by shock but mitigated by FFP FFP hastens syndecan-1 restoration compared to LR Injured patients in shock shed syndecan-1; syndecan-1 correlates with specific inflammatory cytokines
Torres et al. (42)	Studies on a rat HS models comparing effect of LR/HS vs fresh plasma resuscitation with studies on blood samples (including thromboelastometry) and on endothelium (glycocalyx thickness measurements by fluorescent dye-exclusion method)	FFP defined as plasma frozen within 6–8 h of collection and stored at −20°C, prepared by separation form whole blood collected on donor rats	Restoration of coagulation function by a small-volume resuscitation with FFP in contrast to resuscitation with LR/HS groups
Peng et al. (41)	Studies on HUPECs monolayers (VEGF-A165-induced permeability) with assessment of EC permeability (TEER/ECIS and FITC-Dextran) and leukocyte-endothelial binding Mouse model of HS and trauma comparing effect of LR vs FFP resuscitation with *in vivo* studies (MAP monitoring, measurement of syndecan-1 in plasma) and *in vitro* studies on harvested lungs: vascular permeability (intravenous fluorescent dye extravasation), infiltration of neutrophils (MPO immunofluorescence staining and activity), syndecan-1 detection (anti-syndecan-1 antibody)	Human FFP used in both *in vitro* and *in vivo* studies (frozen within 8 h after donation, kept frozen until the day of experiment and used within 1–2 h of thaw)	In HUPECs monolayers, FFP compared with LR reduces pulmonary endothelial hyper-permaeability and leukocyte binding In mouse HS models, FFP and LR similarly restore MAP. FFP mitigates lung hyper-permeability, reduces lung inflammation, increases lung syndecan-1, and reduces syndecan-1 shedding compared with LR resuscitation
Wataha et al. (44)	Studies on HUVECs and PECs monolayers (VEGF-A165-induced permeability) comparing effect of FFP, SD-FFP, SDP (controls: LR/HS) with assessment of EC permeability (FITC-Dextran), WBC binding assay (fluorescent labeling), surface adhesion molecules/integrin expression (flow cytometry) and VE-cadherin/β-catenin mobilization to cell surface (staining)	Human FFP (frozen at −20°C, thawed at 37°C and used on day 0–1 of thaw) SDP defined as pooled liquid plasma that has been dehydrated by means of spray drying and reconstituted citric acid and monobasic sodium phosphate (SD-FFP being the starting material)	FFP, SD-FFP, and SPD equivalently inhibit vascular permeability, ensures EC adherens junctions integrity and endothelial WBC binding Lack of difference between FFP and SD-FFP and between SD-FFP and SDP indicating that solvent-detergent treatment and spray drying do not affect the ability of plasma product to modulate endothelial function
Potter et al. (45)	Studies on HUVECs monolayers (VEGF-A165-induced permeability), comparing FFP and SDP (controls: LR) by testing endothelial permeability (TEER/ECIS), cytokine production in EC and gene expression Mouse model of HS comparing FFP and SDP (controls: LR) with *in vivo* studies (MAP and BE monitoring) and measurement of EC adherent junctions stability (immunofluorescence and histological staining) on harvested lungs	FFP obtained from human donors plasma by apheresis collection, used freshly thawed (same day of thaw) SDP from multidonor plasma (more than 150 type AB donors)	On HUVECs monolayers, FFP and SDP decrease endothelial permeability, induce similar patterns of gene expression and cytokines production in EC In mouse HS models, SDP and FFP equivalently correct MAP and BE, reduce pulmonary vascular leak, equivalently inhibit leukocyte infiltration and breakdown of endothelial adherens and tight junctions
Torres Filho et al. (90)	Rat model of HS for studying quantitatively the relationship between plasma biomarkers and changes in microvascular parameters, including glycocalyx thickness after resuscitation with FWB, PRBC, FFP, 5% albumin, or crystalloids (RL, NS, and HTS)	FWB (3.2% citrate, stored at 4°C, used with 24 h), PRBC (used within 48 h), and FFP (frozen within 6–8 h of collection, stored at −80°C for up to 1 year) all from donor rats	Changes in glycocalyx thickness (and microvascular permeability) negatively (positively) correlated with changes in plasma levels of syndecan-1 and heparane sulfate FWB and FFP, but neither colloid or crystalloid resuscitation, support vascular stabilization by reconstitution of the endothelia glycocalyx after HS

(*Continued*)

TABLE 2 | Continued

Reference	Experimental models	Types of plasma	Main results
Diebel et al. (91)	HUVEC lined microfluidics model for studying endothelial cell activation/injury and glycocalyx barrier function after simulation of HS by treatment with epinephrine and hypoxia reoxygenation	5% human plasma perfused immediately following treatment or after a 3 h delay	"Early" plasma mitigates glycocalyx degradation and inflammatory prothrombotic endothelial response
Pati et al. (92)	Studies on HUVECs monolayers (VEGF-A165-induced permeability), comparing FFP and LP (controls: LR or no treatment) by testing EC permeability (FITC-Dextran), EC resistance (TEER/ECIS), VE-cadherin/β-catenin mobilization to cell surface (staining), leukocyte-binding (fluorescent labeling) Mouse model of HS comparing FFP and LP (controls: LR or no treatment) with assessment of inflammation (MPO staining), vascular permeability (dye extravasation) and tissue edema (wet-to-dry weight ratio)	Human FFP (male donors O+) thawed and used freshly (day 0 of thaw) LP defined as lyophilized plasma (male O+) reconstituted in buffer	On HUVECs monolayers, FFP and LP decrease endothelial permeability, preserve EC adherens junctions, attenuate EC-leukocyte-binding In mouse HS models, LP and FFP reduce pulmonary vascular permeability, edema, and inflammation

AFM, atomic force microscopy; BE, base excess; EC, endothelial cell; ECIS, electric cell-substrate impedance system; FFP, fresh frozen plasma; FITC, fluorescein isothiocyanate-conjugated; FWB, fresh whole blood; HES, hydroxyethyl starch; HS, hemorrhagic shock; HTS, hypertonic (3%) sodium chloride; HUPEC, human pulmonary endothelial cell; HUVEC, human umbilical vein endothelial cell; LP, lyophilized plasma; LR, lactated ringers; MAP, mean arterial pressure; NS, normal saline; PEC, pulmonary endothelial cells; PRBC, packed red blood cells; QRT RT PCR, quantitative real-time reverse-transcription polymerase chain reaction; SD, solvent detergent; SDP, spray-dried plasma; TEER, trans-endothelial electrical resistance; VE-cadherin, vascular endothelial cadherin; WBC, white blood cell.

[a]*Studies identified by searching the terms "glycocalyx, haemorrhagic shock, plasma" on PubMed and secondary references.*

indicated by the fact that FFP corrected metabolic acidosis significantly better than LR and HES, as assessed by pH, base excess, and lactate. This was associated with an improved microcirculation and a lesser degree of hemodilution by FFP compared to LR and HES (42). This latter point was also observed by a recent publication of Nelson et al. (89), who demonstrated that resuscitation with FFP resulted in a circulating volume expansion equaling the volume of blood loss, while circulating volume expansion by Ringer's acetate was less effective.

The pulmonary effects of hemorrhagic shock and resuscitation fluids were addressed by Peng et al. (41). They investigated pulmonary endothelial inflammation and hyper-permeability employing a coagulopathic mouse model of hemorrhagic shock and trauma. Mice were bled to a mean arterial pressure of 35 ± 5 mmHg for 90 min (93) and subsequently resuscitated over 15 min with either LR (at 3× shed blood volume) or FFP (at 1× shed blood volume). Resuscitated animals were compared to shams (all procedures without shock) and positive controls (hemorrhagic shock without resuscitation). Major findings were as follows: (1) lung permeability, assessed *in vivo* by the extravasation of a fluorescent dextrane or Evan's blue, was significantly increased after hemorrhagic shock compared to shams, and FFP resuscitation was significantly more effective than LR in preventing/correcting shock-induced pulmonary hyper-permeability; (2) similarly, lung inflammation, assessed by detecting myeloperoxidase which reflects neutrophils infiltration, significantly increased after hemorrhagic shock and was lessened by FFP resuscitation; (3) shock-induced loss of pulmonary syndecan-1 was most efficiently prevented by resuscitation with FFP. Of note, similar results on pulmonary inflammation and permeability were reported by Potter et al. employing FFP and spray-dried plasma (SDP) (45).

A recent publication by Torres Filho et al. (90) employing a rat model of hemorrhagic shock showed that (1) syndecan-1 and heparan sulfate represent valuable biomarkers of glycocalyx shedding and (2) fresh whole blood and FFP support vascular stabilization by reconstitution of the endothelial glycocalyx (see **Table 2**).

Syndecan-1 as a Key Mediator of Plasma's Effect

A key question is which plasma component may exert a beneficial effect on the glycocalyx. *In vitro* experiments have shown that FFP enhances pulmonary endothelial syndecan-1 expression in a time- and dose-dependent manner (94). A key role for syndecan-1 is supported by *in vivo* experiments as well. Utilizing the model of trauma-hemorragic shock described by Peng (41), Wu et al. investigated the pulmonary response to the type of resuscitation fluid (FFP vs LR) in wild-type and *Syndecan* gene knock-out ($Sdc1^{-/-}$) mice (94). They found that the inability to synthesize syndecan-1 abrogated the protective effect observed with plasma. In particular, they demonstrated that in absence of syndecan-1 synthesis: (1) the ability of FFP to mitigate the increase in lung permeability induced by hemorrhagic shock was abrogated; (2) FFP lost its ability to dampen the shock-induced increase of pulmonary neutrophil infiltration; and (3) FFP lost its protective effect on histopathologic signs of lung injury. Similar results have been reported by Ban et al. with an animal model of gut injury and inflammation after hemorrhagic shock (95).

Plasma: Coagulation Factors or Other Components?

Intriguingly, a major plasma component that may play a role in preserving endothelial integrity appears to be albumin. While loss of circulating albumin correlated with loss of the glycocalyx and increased fluid extravasation (96), albumin supplementation attenuated glycocalyx shedding and reduced interstitial edema in a guinea pig heart model of cold ischemia (97). Kheirabadi et al. (98) studied the role of albumin in a model of uncontrolled hemorrhage. Rabbits were subjected to a splenic injury. Ten minutes after injury, at a mean arterial pressure less than 40 mmHg, the rabbits received equal volumes (15 ml/kg) of rabbit plasma, HES, or 5% human albumin, targeting a mean arterial pressure of 65 mmHg. The authors observed that: (1) onset of resuscitation initiated additional

bleeding and total blood loss did not differ among the three groups; (2) thromboelastography revealed a faster and stronger clot formation in the plasma and albumin groups compared to HES; (3) shock indices were increased in all three groups but less in the albumin one; (4) the albumin group had the highest survival rate (8 out of 9 rabbits) compared to plasma and HES (both 4/10), and positive controls (1/9). This apparent beneficial role of albumin, if confirmed in further studies, may be related to its ability to attenuate neutrophil adhesion to the endothelium and other anti-inflammatory properties, its scavenging and buffering capacity, its potential to enhance nitric oxide production and stabilize glycocalyx (50, 60, 99). However, a recent publication showed that a four-factor prothrombin complex concentrate (containing vitamin K-dependent coagulation factors and several other plasma proteins) and FFP but not albumin inhibit vascular permeability in an *in vivo* mice model (100). Thus far, it is not known which soluble factor present in the factor concentrate might be responsible for its beneficial effect (100).

As of coagulation factors, despite a current of thought supporting the use of fibrinogen in massive bleeding, we are not aware of publications investigating its impact on glycocalyx and endothelial functions. A recent work observed a U-shaped association between initial fibrinogen concentration in major bleeding and in-hospital mortality, with similar rates of increased mortality for fibrinogen levels <1 g/l and >4 g/l (101). A possible explanation for the negative effect of higher fibrinogen levels is offered by *in vitro* data, suggesting that fibrin promotes endothelial transmigration of neutrophils and inflammation (102).

As of other plasma proteins, adiponectin is an interesting candidate (103). Adiponectin is produced in adipocytes and has been shown to have anti-inflammatory properties and to prevent cytokine-induced endothelial cell hyper-permeability (104–106). Employing a mouse model, Deng et al. demonstrated that (1) hemorrhagic shock leads to a significant decrease of adiponectin levels and a disruption of the lung vascular barrier function; (2) plasma resuscitation improves adiponectin levels and reverses lung injury; (3) the beneficial effect of plasma-based resuscitation is abolished by immunodepletion of adiponectin; and (4) it is restored when plasma was replenished with adiponectin (103). These findings suggest that adiponectin may be an important component contributing to a vasoprotective effect of plasma-based resuscitation.

In sum, several animal studies suggest that early use of plasma in hemorrhagic shock may exert a clinically significant beneficial effect by preserving or even restoring the glycocalyx layer and, therefore, maintaining critical endothelial functions. This appears to be due to the ability of a plasma component to lessen endothelial inflammatory response, possibly by limiting neutrophil adhesion. As of today, it is not known which plasma components are responsible for these effects, which impact plasma processing may exert on them, and which might be the dose–effect relationship.

Human Studies

From a clinical point of view, the key question is whether early resuscitation of hemorrhagic shock with plasma is truly able to improve vasculo-endothelial function and survival. As a proof of principle, a small study in non-bleeding critically ill patients demonstrated that plasma transfusion decreased syndecan-1 and factor VIII levels, suggesting an endothelial stabilizing effect (107). To our knowledge, the only human study prospectively investigating the effect of early plasma-based resuscitation in humans is the COMBAT study (108). In this prospective randomized trial, casualties are treated with 2 units of FFP (thawed in the ambulance) vs conventional crystalloids as initial pre-hospital resuscitation. The study aims to verify whether a "plasma first" resuscitation strategy might be able to (1) attenuate acute traumatic coagulopathy; (2) improve metabolic recovery; (3) decrease blood component transfusion; (4) reduce the incidence of acute lung injury and multiple organ failure; (5) decrease mortality at 24 h or 28 days. According to www.clinicaltrials.gov, the study has been closed after having enrolled 144 patients as per protocol. Results are eagerly awaited.

In conclusion, plasma as early resuscitation fluid for massive hemorrhage appears to exert beneficial effects improving patient survival. Experimental data suggest that this may be related to its ability to preserve endothelial glycocalyx structure and function. We think that these fascinating data shall be confirmed in prospective randomized clinical trials and the mechanisms underlying these effects shall be revealed in order to develop more targeted treatments.

AUTHOR CONTRIBUTIONS

Both authors discussed the literature, wrote the manuscript, and approved the final version.

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REVIEW
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The Non-Hemostatic Aspects of Transfused Platelets

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Platelets transfusion is a safe process, but during or after the process, the recipient may experience an adverse reaction and occasionally a serious adverse reaction (SAR). In this review, we focus on the inflammatory potential of platelet components (PCs) and their involvement in SARs. Recent evidence has highlighted a central role for platelets in the host inflammatory and immune responses. Blood platelets are involved in inflammation and various other aspects of innate immunity through the release of a plethora of immunomodulatory cytokines, chemokines, and associated molecules, collectively termed biological response modifiers that behave like ligands for endothelial and leukocyte receptors and for platelets themselves. The involvement of PCs in SARs—particularly on a critically ill patient's context—could be related, at least in part, to the inflammatory functions of platelets, acquired during storage lesions. Moreover, we focus on causal link between platelet activation and immune-mediated disorders (transfusion-associated immunomodulation, platelets, polyanions, and bacterial defense and alloimmunization). This is linked to the platelets' propensity to be activated even in the absence of deliberate stimuli and to the occurrence of time-dependent storage lesions.

Keywords: platelets, transfusion, CD40L, serious adverse reaction, inflammation, innate immunity

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INTRODUCTION

Blood platelets are small anucleate cells essentially originating from megakaryocyte (MK) fragmentation. These cells have a dense cytoskeleton that maintains their discoid shape in normal state and changes the platelets to a spherical form after their activation (1). Platelets play a key role in vascular repair and maintenance of homeostasis, particularly in primary hemostasis. The platelet membrane glycoproteins can interact with the elements of the injured endothelium, mediating their adhesion, followed by activation and finally aggregation, resulting in the formation of a thrombus formed by aggregation of interconnected platelets by fibrinogen to close the vascular gap (1, 2). Platelets also play an important role in innate and adaptive immunity by interacting directly or indirectly with other immune cells to trigger or maintain the inflammatory response (1–3). Several factors are involved in the platelet inflammatory process, in particular, by membrane expression of several immune receptors, such as cytokines (CKs), chemokines (CHs), and a large number of soluble factors contained in their granules (in α-granules, this includes CKs/CHs, immunomodulatory factors, and growth factors, etc.) (4, 5) (**Figure 1**). Moreover, platelets also release other factors: (i) growth

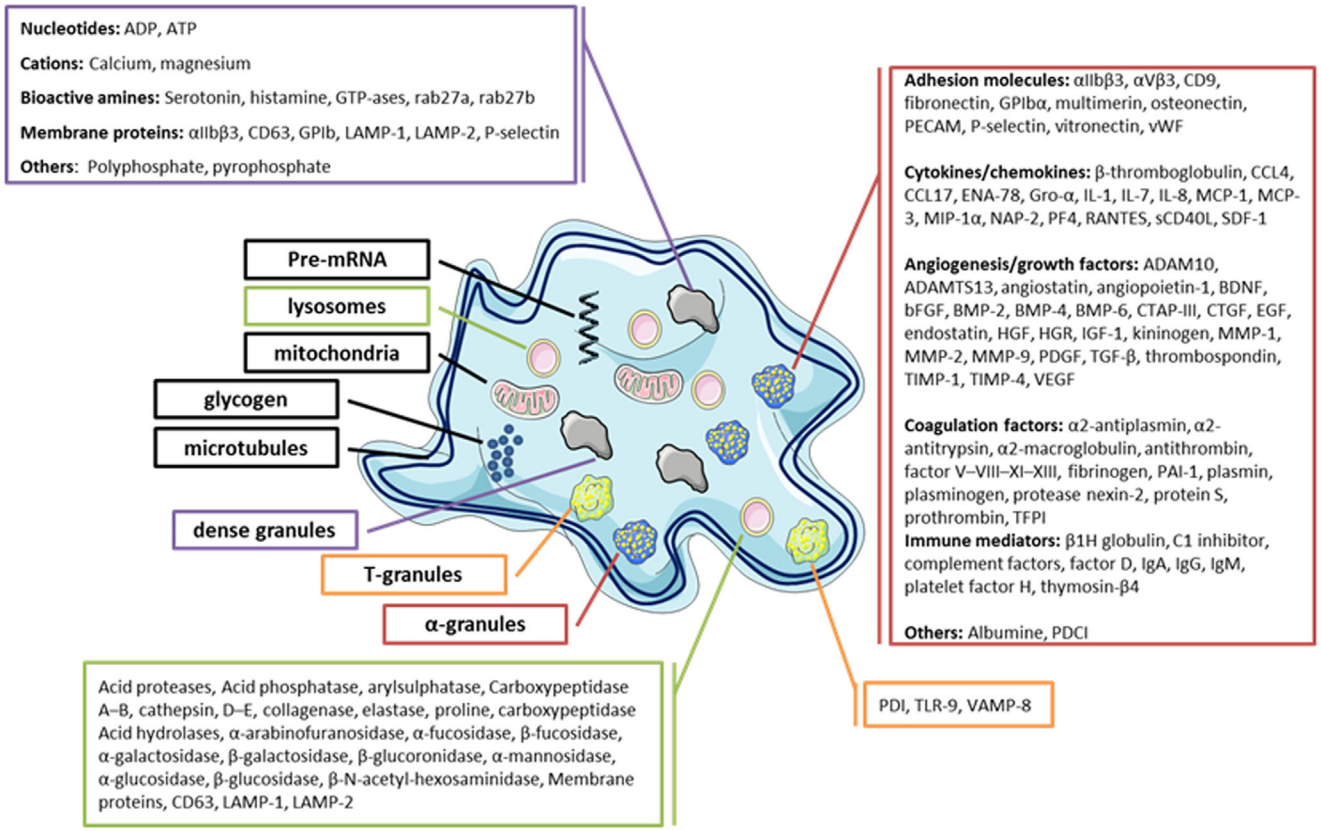

FIGURE 1 | Diagram of the platelets' main granules and their secretory content. Most products that can be released by platelets are listed.

factors promoting angiogenesis, which are also required to repair damage to inflammatory sites (6–8), (ii) clotting factors required for platelet hemostatic functions (9, 10), (iii) antibacterial peptides (1, 11), (iv) adhesion factors (12), and (v) inflammatory mediators, such as serotonin and histamine (13–15).

Platelet-derived soluble CD40L (sCD40L) is a key mediator of the immune system (16–19). Platelet receptor and signaling is important and drive the stimulated platelet granule secretion in these differential profiles—a completely new concept with regard to an anucleate cell—also appear to be strictly regulated by intraplatelet signaling pathways, depending on the stimuli (20–23). This review summarizes current information surrounding the association between inflammation and transfused platelets.

A BRIEF OVERVIEW OF PLATELET FUNCTIONS

Blood platelets are important reservoirs of soluble, preformed mediators (CKs/CHs, hemostatic factors, and immunomodulators) that are present in secretory granules, in particular, α-granules, δ-granules, and lysosomes, which are released upon their activation (1, 15, 24–28). Platelets contain a large variety of CKs/CHs, which are mainly synthesized in the MK and stored in α-granules in most cases (**Figure 1**). CKs/CHs may directly interact with cells of the innate and adaptive immune system or indirectly through immune or non-immune relay cells, such as endothelial cells. CK/CH platelets help regulate the surrounding cells, including their proliferation, differentiation, and activation (1). Interestingly, platelets also express receptors for several CKs/CHs that they secrete, showing their potential to establish autocrine and paracrine bidirectional loops. Platelet immunomodulatory factors include growth factors and CKs/CHs, but also molecules sharing the main characteristics of CHs and CKs, such as sCD40L/CD40L, soluble P-selectin/CD62P, platelet-derived growth factor AB (PDGF-AB), transforming growth factor β, interleukine 1β, regulated on activation normal T cell expressed and secreted (RANTES), and platelet factor 4 (PF4 ou CXCL4).

Although platelets have generally not been considered central to innate immunity and inflammation, this paper provides evidence that platelets may play a key role. Recent data report evidence that platelets can also recycle a number of CKs/CHs and regulatory products called biological response modifiers (BRMs) for which they also express the pairing ligand, which is the case for sCD40L (platelets are the major purveyors of this molecule in the circulation) (17, 29). Platelets also express membrane CD40 and, unlike CD40L, CD40 is detectable on the surface of resting as well as activated platelets (1).

Cytokines and other platelet products can be readily detectable at the onset of acute inflammation (8). In this regard,

transfusion is an excellent model of the pathological process, as here, the mediators of inflammation are transfused with consecutively rare, but then, severe serious adverse reaction (SARs). It is currently and widely admitted that sCD40L is the master platelet-associated CKs (17, 30). When it was first described in 2001 in association with platelets, this was in the context of platelet component (PC) transfusion hazards. Subsequently, platelet-sCD40L and the CD40/CD40L pair have been described in many pathologies. The conclusion that, e.g., febrile non hemolytic transfusion reaction (FNHTRs), where sCD40L appears to be chiefly responsible for pathological symptoms, was indeed inflammatory. This conclusion seems similar to their role in diabetes, cardiovascular disease, atheromatous plaques, and inflammatory bowel disease, where CD40/CD40L have now been acknowledged as being influential (1, 31).

PLATELET INTERACTIONS WITH OTHER BLOOD CELL ELEMENTS

Interactions of sCD40L with its CD40 receptor (expressed on immune cells or other cells, such as endothelial cells) can modulate the responses of each of the different cell partners (5, 32). Indeed, platelet sCD40L, interacting with CD40 on endothelial cells, induces inflammatory responses characterized by the expression of adhesion receptors (E-selectin, P-selectin, intercellular adhesion molecule 1, vascular cell adhesion molecule 1) for the release of proinflammatory CKs/CHs (CCL2, IL-6, IL-8) and the recruitment of leukocytes to the inflammatory sites (16). The *in vitro* engagement of neutrophil CD40 by sCD40L induces the generation of reactive oxygen species (ROS) and the destruction of lung endothelial cells suggesting this factor's role in transfusion-related acute lung injury (TRALI) (33). Moreover, platelet sCD40L creates a link between innate and adaptive immunity in promoting maturation (19, 34), activation (35), secretion (36), and presentation of antigen by dendritic cells (DCs), which are cells capable of activating naive T cells to induce an adaptive immune response (5). Elzey et al. have demonstrated that platelet sCD40L can, both *in vitro* and *in vivo*, amplify the activity of pathogen-specific CD8+ T lymphocytes (Lyt), which results in the production and function of IFNγ, and in the enhancement of their lytic function (37). Iannacone has further shown that the number of cytotoxic Lyt during infection with lymphocytic choriomeningitis virus was dramatically reduced in the absence of platelets, involving CD40/CD40L: thrombocytopenia is estimated to result almost exclusively from the antiplatelet antibodies (38). CD40/CD40L also plays a major role in the interaction of $CD4^+$ T lymphocytes (and CD8+) and B lymphocytes, which supports proliferation, differentiation, and production of immunoglobulin by plasma cells. Platelet or MK-derived sCD40L, which is continuously released into the circulation in large quantities (37, 39, 40), is a key molecule regulating the immune system and increased release of sCD40L plays a major role in the pathogenesis of the immune-mediated disease.

Platelets are also essential for the formation of neutrophil extracellular traps (NETs) by neutrophils, The NET formation is an apoptotic process, most important to release of neutrophil DNA, which entraps bacteria resulting in bacterial clearance and concentrating antibacterial factors but in enhancing thrombosis. Toll-like receptor 4-activated platelets bind to neutrophils and initiate NET formation. Platelets facilitate NETosis *via* several protein interaction as CD62P-PSGL-1, involving of platelet GPIbα or neutrophil lymphocyte-function-associated-antigen-1. Moreover, platelet release several soluble factor initiate NET formation and increase bacterial clearance [CXCL4, von Willebrand factor, high-mobility group box 1 protein, thromboxane A2, and β-defensin (41)]. Platelet–leukocyte interactions has focused on platelet interactions with monocytes and neutrophils, as described above, but platelets present a role in T cell responses. Chapman et al. show elegantly that platelets express T cell costimulatory molecules, process, and present Ag in MHC class I and directly activate naive T cells in a platelet MHC class I-dependent manner. The group of Craig N. Morrell define new concept that platelets not only support and promote acquired immune responses but platelets may also directly participate in the initiation of acquired immune responses (42). While for the role in primary hemostasis, platelets primarily interact with endothelial cells, they also interact directly or indirectly *via* their released CK/CH with many of cell types, hereby, strongly influence their function. Platelets can, indeed, activate (and be mutually activated by) almost all types of leukocytes (monocytes, T-lymphocytes, B-lymphocytes, and neutrophils) and DCs (1, 30, 43). When allogeneic (donor) platelets are transfused to patients, the recipients' circulating cells make foreign encounters [e.g., by human leukocyte antigen (HLA) class I molecule expressed on platelets] and can potentially be activated by those encounters, and vice versa. This led to a recent re-examination of the concept of pathogens defense mechanisms, extending it to non-infectious "dangers" such as foreign (transfused) cells (15, 26, 27, 44, 45). PCs are stored for a maximum of 5 days (most countries) before being issued to a patient in need; prior to that, during their shelf life, platelets "spontaneously," i.e., with no acknowledged exogeneous stimulus, release a number of CKs, particularly sCD40L (17, 30) in high enough quantities to exert functional activities on target cells possessing the *ad hoc* receptors. sCD40L was found to be consistently and significantly elevated in PCs that had led to SARs comprising various syndromes, including (antibody independent) TRALI (although this is disputed in such particular case) (30, 33).

A BRIEF OVERVIEW OF PC TRANSFUSION BENEFITS AND COMPLICATIONS

Platelet component transfusions have two main indications, aimed at being either curative or prophylactic (46). Curative transfusions are given to patients presenting with active bleeding and low to very low platelet counts (in exceptional circumstances, the platelet count can be normal, but platelets are non-functional), or massive blood loss. Curative transfusions are not under debate, unlike the protocols and timing of other blood component transfusions [red blood cell concentrates (RBCCs) and fresh plasma] and/or blood derivatives, such as prothrombin complex concentrate or fibrinogen. There is no consensus on prophylactic transfusions, however, although many practitioners still recommend not exposing at-risk patients to bleed. Thresholds for transfusion and quantities of transfused platelets vary consistently

in different countries and with different systems. In short, PC transfusion provides a benefit to patients and prevents bleeding and deterioration of otherwise serious clinical conditions. PC transfusion is supportive in many chemotherapy protocols and stem cell transplantation.

Platelet component transfusions can lead to adverse inflammatory reactions. The majority of adverse inflammatory reactions in patients receiving blood (recipients) appear either FNHTRs or allergy, both being clearly inflammatory conditions. FNHTR is characterized and associated with fever (≥38°C or ≥1°C above baseline, if baseline ≥37°C), or chills and rigors, but not directly with hemolysis, caused by cytokines that accumulate in the product during storage. FNHTR is also initiated by the presence of recipient antibodies reacting to donor HLA or other antigens. Allergic reactions (e.g., urticaria) occur within minutes after the start of the transfusion. Allergic reactions may be associated with mild upper respiratory symptoms, nausea, vomiting, abdominal cramps, or diarrhea. Allergic reactions could be severe (e.g., anaphylaxis). Patients can present a severe hypotension, cough, bronchospasm (respiratory distress and wheezing), laryngospasm, angioedema, urticaria, nausea, abdominal cramps, vomiting, diarrhea, shock, and/or loss of consciousness. This may be a fatal reaction. Severe allergic reactions could be dependent of (i) IgA-deficient patients who have anti-IgA antibodies, (ii) patient antibodies to plasma proteins (such as IgG, albumin, haptoglobin, transferrin, C3, C4, or cytokines), (iii) transfusing an allergen to a sensitized patient (for example, penicillin or nuts consumed by a donor), or (iv) rarely the transfusion of IgE antibodies (to drugs, food, etc.) from a donor to an allergen present in the recipient (47).

On rare occasions, PC transfusion can lead to immediate to short-delayed inflammatory adverse reactions (grades 1–3: 0.24%); however, in some cases (0.006%), grade 3 reactions can be life-threatening (48). The rationale for the relatively high number of SARs with PC transfusion [from 1/4 to 1/2 of all reported SARs, while PCs represent only about 10% of transfused blood components (BCs)] may be deduced from their propensity to secrete copious amounts of pro-inflammatory BRMs as outlined in the previous section. In addition, PC transfusion can be associated with volume overload, as PCs frequently come into large volumes and elevated levels of proteins and lipids exerting a surfactant effect (49). The latter can be prevented by close patient monitoring and by replacing 2/3 of plasma with platelet additive solutions (50). The case of TRALI and the responsibility of platelets have been presented elsewhere (45). Finally, PC transfusion carries a greater risk of bacterial contamination, which can be life threatening especially in severely immuno-compromised patients (51). The introduction of Pathogen Reduction Technologies has abrogated much of the adverse effects associated with pathogen contamination of platelet products (52, 53). Pre-storage leukoreduction proved to significantly reduce inflammatory reactions as well as viral infections (54). In brief, PC transfusions can induce unwanted effects, e.g., volume, plasma, inflammatory reactions, pathogen transmission, etc., in addition to their therapeutically intended effect, i.e., improving hemostasis. However, since PC-transfused patients are particularly fragile patients, close monitoring and careful dosing can prevent many complications such as volume overload.

PLATELET STORAGE AND OUTCOMES OF CRITICALLY ILL PATIENTS

Over the platelet storage period, certain biochemical and functional changes occur in the platelets and their storage medium. These changes, called storage lesions (**Figure 2**), include acidification of the storage medium secondary to anaerobic platelet metabolism, platelet activation (55), and an increase in CKs and lipids level in PCs (56, 57). Several authors applied a metabolomics approach to the issue of donor variability in poststorage platelet viability. Metabolomic analysis of the stored platelets identified multiple specific metabolites that correlated with either PLT recoveries or survivals after transfusion (Lipid metabolism components, caffeine, and its metabolites) (58). Interestingly, platelet storage lesion is not associated with a linear decay of metabolism, but rather with successive metabolic shifts (59). Prudent et al. review the key findings of the proteomic analyses of platelet concentrates (PCs) treated by the Mirasol Pathogen Reduction Technology, the Intercept Blood System, and the Theraflex UV-C system, respectively, and discuss the potential impact on the biological functions of platelets. The impact of the Pathogen inactivation treatment on the proteome appears to be different among the Pathogen inactivation systems (53, 60).

These storage lesions may compromise the platelets' viability and functionality, and, therefore, the transfusion's efficacy (61). They may also lead to adverse reactions in the recipients.

Critically ill patients are the second largest patient group to receive platelet products after oncology–hematology patients. Around 15% of critically ill patients require a platelet transfusion during their intensive care unit stay for treatment or prophylaxis of bleeding (62). Critically ill patients are characterized by a coexisting inflammatory state, making them theoretically more susceptible to blood product adverse reactions. A "two-hit" hypothesis has largely been used to explain the pathophysiology of transfusion adverse events including TRALI, the first hit being a pro-inflammatory condition and the second hit being the administration of antibodies or BRMs through blood component transfusion (63). Results of *in vitro* and animal studies suggest platelets storage lesions have a key effect on the occurrence of non-antibody mediated TRALI (33, 63). Khan et al. have observed an increase in sCD40L level over the PC storage period, and higher levels of sCD40L in platelet products implicated in TRALI, suggesting that the accumulation of sCD40L during platelet storage induces TRALI (33). Consistent with these findings, Vlaar et al. have found that stored platelet supernatant compared with fresh platelet supernatant led to an increase in systemic and pulmonary coagulopathy in lipopolysaccharide pretreated rats (63).

CD40/CD40L complex could be a major target in a TRALI prevention strategy. Improving the conditions in which the PCs are prepared and stored would contribute to controlling partly the risks of non-immune TRALI.

Prolonged platelet storage has been associated with a decrease in posttransfusion platelet increment and a shorter

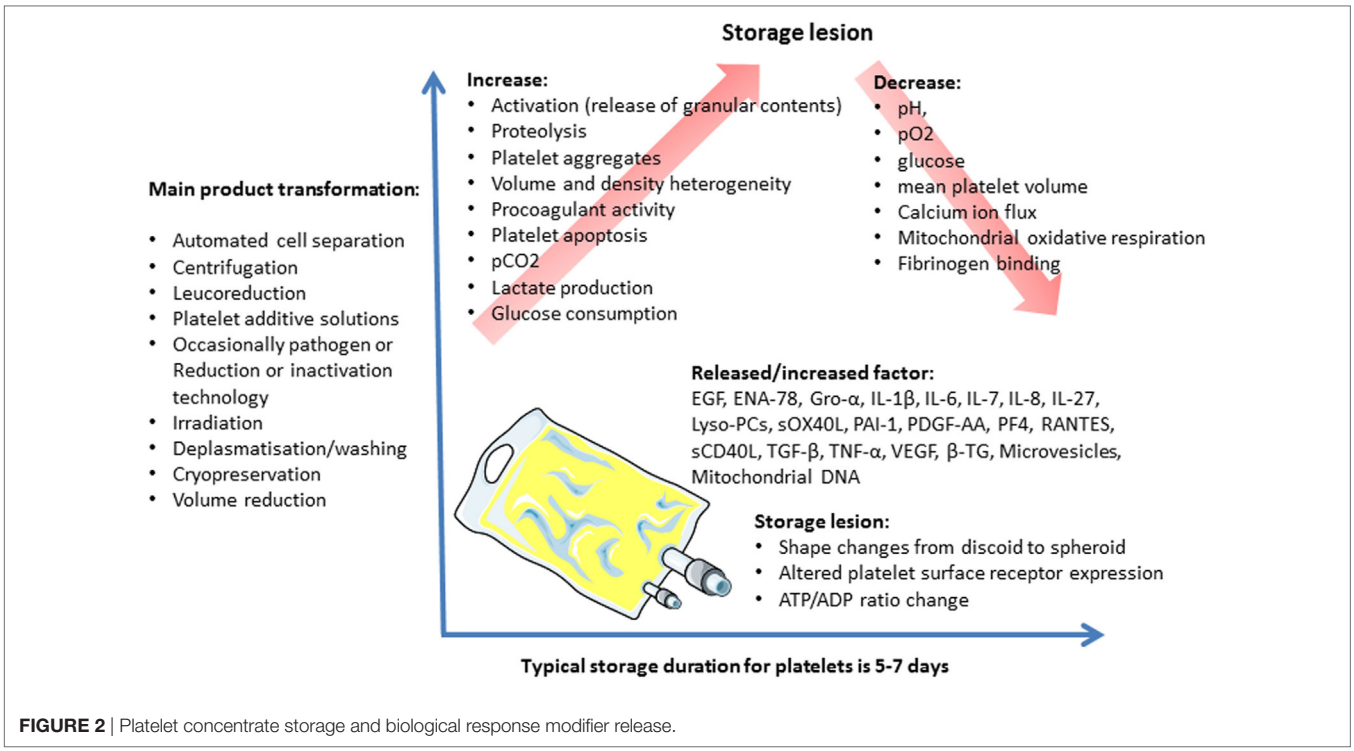

FIGURE 2 | Platelet concentrate storage and biological response modifier release.

time to next platelet transfusion in oncology–hematology patients (64–66), but the clinical consequences of the platelets storage lesions remain uncertain (66–68). To our knowledge, no study has investigated the association between transfusion efficacy and platelet storage duration in critically ill patients. Five observational studies have investigated the association between PC storage duration and critically ill patient outcomes; one included post-cardiac surgery patients only, two studies included trauma patients only, and two all critically ill patients (69–73). There was no association between mortality and storage duration in the three studies evaluating this outcome (69, 70, 73). In a study of 381 trauma patients, those receiving platelets stored for 5 days developed more complications, including sepsis, than patients transfused with platelets stored for less than 5 days (5.5% sepsis in patients receiving platelets stored for 3 days or less, versus 16.7% in patients receiving platelets stored for 5 days, $p = 0.03$) (70). After adjustment for confounders, patients receiving PCs stored for 5 days had a 2.4-fold higher risk of developing complications, including acute renal failure, acute respiratory distress syndrome, and sepsis, than patients transfused with fresher platelets (70). All these studies are retrospective and have numerous limitations in their methods making it impossible to draw any definitive conclusion on the impact of platelet storage duration on clinical-centered outcomes. Prospective research is warranted to determine whether prolonged platelet storage has an impact on the prognosis of critically ill patients. In the meantime, better understanding of platelet transfusion-related immunomodulation may help us to understand the reported association between platelet transfusion and an increased risk of hospital-acquired infections (74, 75).

TRANSFUSION-ASSOCIATED IMMUNOMODULATION

Transfusion-related immunomodulation or TRIM is a complex event with dual effects that are potentially beneficial, but in general, mostly considered harmful (76). The long-term effect of transfusions is suspected to modulate (dampen) immune responses and consequently favor the emergence of secondary malignancies and infections. It is, however, extremely difficult to decipher the respective roles of causal pathologies in severely sick intensive care patients or patients receiving chemotherapy and immunosuppressants, monoclonals and biosimilars, and BCs. TRIM induced by PCs would be best understood in patients having received PCs only, but it is almost impossible to delineate the immunosuppressive role of platelets relative to red blood cells as only very few patients receive PCs and no RBCCs. Furthermore, in case of plasma-rich BCs, plasma polyreactive immunoglobulins (Igs) may counterbalance certain immunosuppressive effects. In short, whether PC transfusions may be immunomodulatory remains elusive and difficult to assess, though it would be of interest to investigate this in order to provide patients with optimized care.

PLATELETS, POLYANIONS, AND BACTERIAL DEFENSE

While the above-described mechanisms clearly indicate that platelets interfere with the immune system, only a few studies clearly show a causal link between platelet activation and immune-mediated disorders. One well-investigated example of

the role of platelets in mediating immune reactions is the interaction of platelets with heparin.

The adverse drug effect of heparin-induced thrombocytopenia (HIT) will, therefore, be used to exemplify the interaction of platelets and the immune system. HIT is a prothrombotic adverse drug reaction caused by the transient production of IgG-class platelet-activating antibodies that recognize multimolecular complexes of the positively charged PF4 and the polyanion drug heparin. These antibodies activate platelets and also monocytes *via* their FcγRIIa receptors. This causes transformation of an immune reaction into a prothrombotic reaction, resulting in massive thrombin generation and paradox thrombotic complications. If unrecognized, the risk for new thrombosis in affected patients is 5% per day and the risk of mortality is 25–30% (77). There is no doubt that with HIT, platelets mediate an extremely powerful reaction, which results from concomitant activation of the immune system and the coagulation system.

The reason for this massive response is that HIT is likely a misdirected bacterial host defense (78). PF4 binds charge-related to Gram-negative and Gram-positive bacteria. On Gram-negative bacteria, lipid-A is the binding site for PF4 (79). The binding site of PF4 on Gram-positive bacteria has not yet been identified. The question raised is how and why PF4 induces such a potentially dangerous immune response.

The following section summarizes our recently proposed working model (80, 81). All bacteria expose strong negative charges on their surface. This negative charge is likely a mechanism by which bacteria are kept apart from each other, and by which bacteria are protected from phagocytosis. The zeta potential-mediated repulsive forces generated by the negative charges push bacteria apart from each other and away from their "predators" (the reader is invited to watch the following YouTube video demonstrating this principle https://www.youtube.com/watch?v=Kb-m1uDoWfU). Eukaryotic cells, however, must not have this strong negative charge as the repulsive forces would be incompatible with a complex multicellular organism. In view of this consideration, we propose that a strong negative charge is a fundamental feature of prokaryotes. In line with this concept, basic mechanisms of the innate immune system, like the alternative and classic complement pathway, the intrinsic clotting system with factor XII and factor XI, as well as the kininogen–bradykinin pathway are strongly activated by negative charges (82). However, the adaptive immune system (T cell receptors, B cell receptors, antibodies) do not recognize charge, they recognize structures. The platelet-derived CH PF4 has the role of translating charge into structure. After binding to negative charges, PF4 undergoes complex structural changes [for review, see Ref. (83, 84)]. These structural changes expose a neoepitope, which is recognized by anti-PF4/polyanion antibodies, the same antibodies that induce HIT. After binding of anti-PF4/P antibodies to PF4-labeled bacteria, these opsonized bacteria mediate very efficient phagocytosis by granulocytes (78). The evolutionary advantage of using such a mechanism is that it enables an early IgG response toward bacteria the organism has not seen before. The newly encountered bacteria also bind PF4; PF4 undergoes its conformational change due to the negative charge on the bacteria surface and is then recognized by the preformed anti-PF4/P antibodies. In line with this concept, most likely a secondary immune reaction, natural anti-PF4/P antibodies are found in the general population where their presence is highly correlated with the presence of chronic infections like chronic periodontal disease (85). On the basis of this concept, these antibodies must be very common. Indeed, the adverse drug reaction HIT has helped to prove this. In HIT, anti-PF4/P IgG are formed in high titer between day 5 and day 10 (86). As B cells cannot produce IgG antibodies during a primary immune response within 5–10 days, HIT is always a secondary immune reaction, even in patients who have never received heparin before. As 65% of patients develop these antibodies after cardiac surgery, a plausible explanation for such frequent primary immunization is the above-outlined concept of bacterial infection-related priming of the immune system.

However, the above-outlined concept of the role of PF4 and platelets as mediators between innate and specific immunity places platelets in a very special position, bridging two major parts of our immune defense system. Platelets secrete or expose many molecules with a specific role in immunity. Little information exists on how platelet storage modifies the structure of these molecules or their spatial presentation within platelet compartments or on the platelet surface. As exemplified by the structural changes of CH PF4 induced by polyanions like heparin, conformational changes in these proteins may transmit a danger signal to the transfusion recipient's immune system, which erroneously triggers potent pathogen defense mechanisms, resulting in adverse transfusion reactions. Although this has not been shown yet, it is conceivable that other platelet-derived mediators such as sCD40L intensify and probably orchestrate the interaction of platelets and other immune cells with pathogens. If misdirected, this can cause SARs. The adverse drug reaction of HIT provides one of the most prominent examples of the potentially deleterious consequences for patients.

The risk that our immune system develops autoimmune-like reactions toward platelet proteins when they are modified during storage is probably quite low, although such autoimmune reactions may occur. Again, this has been demonstrated for the immune reaction toward conformationally changed PF4. In the past decade, it has become recognized that certain patients present with clinical symptoms and laboratory features of HIT despite not having previously received heparin either in the recent past or at all. Sera from these patients contain antibodies that strongly activate platelets even in the absence of heparin. To date, ≈20 cases of spontaneous HIT syndrome have been reported (87–96). In the plasma of these patients, antibodies are found, which bind to PF4 with such high avidity that they cluster two PF4 molecules, thereby inducing the same conformational change as polyanions. These clusters of conformationally changed PF4 attach to platelets and endothelial cells, giving the immune system a false signal of the presence of strong negative charges, which prompts the above-described bacterial defense mechanism.

As the negative charge is a danger signal for the human defense system, bacteria have naturally developed counteracting methods to hide this danger signal. One of which is long lipopolysaccharide (LPS) chains covering and "hiding" the negative charges or the Fc-part of the anti-PF4/P antibodies bound to conformational-changed PF4 on the bacteria surface. Lipid A is the basis of LPS.

PF4 has a diameter of 5 nm; when an IgG molecule (which is about 10 nm long) binds to conformationally changed PF4 bound to lipid A, the entire complex has a height of about 15–18 nm. The LPS chain, however, can reach lengths of up to 25 nm. This covers the Fc part of the antibody and thereby recognition of opsonized bacteria by the immune system's Fc receptors. However, platelets, in addition to PF4, secrete polyphosphates from their δ-granules. Polyphosphates are also negatively charged and bind to the PF4 molecule on the bacteria surface, attracting other PF4 molecules and finally forming large multimolecular PF4/polyphosphate complexes, which extend well out of the bacteria's LPS shield (97). This has two effects: conformationally changed PF4 is now exposed for antibody recognition, and consequently several anti-PF4/P antibodies can bind to these complexes on the bacterial surface, forming immune complexes, which are then readily recognized by the Fc receptors of human defense cells.

Platelets also found a new way in which platelets defend against bacteria. When platelets are incubated with *Escherchia coli* in the presence of PF4 and anti-PF4/P antibodies, platelets kill up to 75% of *E. coli* by direct platelet bacteria interaction. Upon investigating this mechanism in more detail, we found a new way in which platelets defend bacteria. It is well established that platelets can internalize IgG-coated targets (98–100); however, it is debated whether phagocytosis of bacteria (i.e., *Staphylococcus aureus*) (101) is really a major mechanism for bacterial host defense (102, 103). Although platelets store bactericidal substances in their α-granules (12), α-granules are designed to be released and it is unlikely that phagocytosed bacteria are transported within the platelet into the α-granule. Such a mechanism would be incompatible with platelet shape change during activation where platelets are spread thinly over a large area with the α-granules concentrated within the immediate granulomere zone (104). We propose an alternative mechanism, where platelets cover bacteria by widely extending their membranes and then actively contracting them, thereby centralizing bacteria until they are very close to the granulomere of the platelets, where the substances with antibacterial potency are stored (105). When a threshold concentration of platelet-activating signals is reached due to platelet interaction with the opsonized bacteria, the activated platelets release their α-granules preferentially at the site of the bacteria, thereby locally reaching high concentrations of antibacterial substances. This phenomenon is similar to the pore-forming perforin released from the granules at the immunological synapse potentiated by cytotoxic T lymphocytes (106).

The above-outlined mechanisms are not the only ways in which platelets interfere with bacteria and other pathogens (107–110). Through complex mechanisms involving the platelet Fc-receptor FcγRIIA (111–113), glycoprotein αIIbβ3, GPIbα, complement receptors (e.g., gC1q-R), and toll-like receptors (e.g., TLR-2 and TLR-4), platelets interact with bacteria and become activated by bacteria (27, 114). Upon activation, platelets release antimicrobial substances such as ROS, antimicrobial peptides, defensins, kinocidins, and proteases (11, 115–117). Taken together, there is ample evidence that platelets play an important role in the defense against pathogens.

Recognition of pathogens by platelets is at least partly mediated by conformationally changed endogenous, platelet-derived proteins. The challenge for transfusion medicine and immunohematology is to identify whether platelet proteins with an important role in danger signaling are also conformationally changed during platelet processing and storage, thereby presenting a danger signal with an increased risk of triggering misdirected host defense mechanisms.

PLATELETS, POLYMORPHISMS, AND ALLOIMMUNIZATION

Platelet component transfusions are extremely difficult to match for surface antigens between donors and recipients, apart from the ABO groups (A and/or B antigens can be variably expressed on platelets) (118). Moreover, platelets exhibit numerous copies of highly polymorphic HLA class I antigens. The functions associated with HLA class I molecules on platelets are currently under debate, as platelets are not consensually considered capable of presenting antigens. HLA transfer to other cells has recently been evidenced experimentally in mice, opening up novel avenues on the subject. HLA immunization of patients is not uncommon, but pre-storage leukoreduction has proven to be tremendously efficacious in limiting it, since leukocytes—10-times more loaded with HLA moieties than platelets—seem to potentiate immunization against platelet antigens, HLA, and human platelet antigens (HPA) (119). HPA are actually polymorphic variants of platelet glycoproteins, representing "platelet-specific blood groups." Almost 20 such molecules are recognized as being immunogenic, with less than five being implicated in the most frequent immunization, while the others stand for rare antigens. Those HPA antigens usually come in two antithetical moieties termed "a" and "b," "a" being the frequent allele and "b" the rarest. In certain circumstances, HLA or HPA testing and matching is the only option available to efficiently transfuse refractory patients; indeed, patients presenting with allogeneous anti-HLA and/or HPA Abs may destroy transfused platelets especially if Abs are directed at frequent Ags, leading to refractory states and imposing cross-matching of donor PCs against recipients' plasma whenever possible (outside emergency situations) (120); rarely, transfer of allogenous Ags onto recipient's platelets may create aggravated thrombocytopenic states with posttransfusion purpura (121). Transfusion of pooled platelets may be an option to saturate allo-Abs and give a chance to increase the patient's platelet count during the critical phase (at the expense of creating further immunization, however). Regarding female patients having been alloimmunized during pregnancies, it is preferable to transfuse them using either HPA (HLA) typed, or cross-matched, PCs, to avoid the rebound of allo-Abs and refractoriness (122). It should be noted that as residual red blood cells exist even in very small numbers in PCs, patients transfused with PCs can be immunized against red blood cell antigens, especially when these are highly immunogenic such as Rhesus-D; this occurrence is nevertheless infrequent. Whereas it is strongly advised not to transfuse a Rhesus D negative female recipient in child-bearing age with platelets obtained from a Rhesus D positive donor unless prophylaxis is available if needed; Rhesus D negative men and females with no longer child-bearing potential are assumed to be

safely transfused by Rh D positive donor's PCs (especially pooled PCs according to recently published studies) (123); the case of Rhesus negative men (such as HSC transplanted) undergoing repeated PC transfusion is debated but should be discussed for Rh-D prophylaxis (124). Finally, it has recently been hypothesized that ABO mismatched platelets favor alloimmunization (125), although this hypothesis has yet to be ascertained with respect to its clinical impact.

CONCLUSION

In conclusion, transfusion of platelets is generally safe and largely beneficial to patients. On rare occasions, SARs (which cannot be prevented by current measures), occur with clinical presentation of acute inflammation. In all cases investigated to date, either based on clinical observations or tested experimentally, BRMs (comprising chiefly of CKs and CHs and related molecules such as sCD40L) are found to be in close association. Potentially, these SARs are misdirected physiological defense mechanisms. This we have exemplified by the complex pathogenesis of HIT, which, however, involves just one of the many immunomodulatory CHs released by platelets. Additional safety measures to prevent those SARs would be beneficial to patients; however, it is likely they would be extremely difficult to establish and would not be cost effective. Again, transfusion-linked inflammation is likely the result of a combination of factors related to the donor, the BC, and the recipient. The only factor that can be targeted at present is the BC and measures to improve BC quality are being implemented when identified within the industry, in partnership with blood establishments. The identification of parameters that may be related to patients (recipients) would be desirable to identify at-risk patients and apply measures to prevent the severity of the hazards. If parameters are linked to donors, the situation becomes much more difficult, because further medical investigations in donors would scarcely be acceptable, and would have the potential to jeopardize BC stocks. How can one explain to a generous blood donor that he or she is perfectly safe and healthy, but "at risk" of inflicting harm on "certain" recipients? This problem is medically, ethically, and psychologically difficult to address. Alternatively, transfusion medicine may become one of the first medical specialties where personalized medicine comes into effect: "How can a given patient be given the BC most suited to his or her condition"?

AUTHOR CONTRIBUTIONS

CS, CA, HH-C, AG, OG, and FC: wrote the paper. CS, ST, CA, ChA, HH-C, PB, SL, AG, OG, and FC: participated in all steps of the process and reviewed the manuscript.

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A Conceptual Framework for Optimizing Blood Matching Strategies: Balancing Patient Complications Against Total Costs Incurred

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Alloimmunization is currently the most frequent adverse blood transfusion event. Whilst completely matched donor blood would nullify the alloimmunization risk, this is practically infeasible. Current matching strategies therefore aim at matching a limited number of blood groups only, and have evolved over time by systematically including matching strategies for those blood groups for which (serious) alloimmunization complications most frequently occurred. An optimal matching strategy for controlling the risk of alloimmunization however, would balance alloimmunization complications and costs within the entire blood supply chain, whilst fulfilling all practical requirements and limitations. In this article the outline of an integrated blood management model is described and various potential challenges and prospects foreseen with the development of such a model are discussed.

Keywords: blood supply chain, alloimmunization, cost-effectiveness, optimization, modeling

1. INTRODUCTION

In a utopian world every blood transfusion would be handled like an organ transplant, which means that one would try to find a perfect match between donor and recipient. The reality however is that completely matched donor blood is impossible in practice due to the abundance of blood group antigens, costs associated with blood typing, and complications the logistics for such a scheme would impose. As a consequence only a handful of blood group antigens are matched, posing transfusion recipients at risk for alloimmunization and associated transfusion complications. An ideal matching strategy would be one that minimizes the risk of alloimmunization, is cost-effective, and fits within the practical limitations of the blood supply chain. In the past, matching strategies

have been guided by the frequency of alloimmunization incidents, without systematically considering all consequences such strategies impose on the blood supply. Since a selected matching strategy will either directly or indirectly affect the entire blood supply chain (**Figure 1**), an integrated approach is required. Such an approach would, for any particular blood matching strategy, allow balancing the costs of donor recruitment, donor typing, inventory management, blood product logistics, patient blood typing, and alloimmunization complications in transfusion recipients. Besides costs also the effects of transfusion complications on patients health should be taken into account. This article describes the outline of a generic integrated blood management model, its components, their interaction and potential complicating factors and limitations currently foreseen for such a model.

We will first provide a description of all elements within the blood transfusion chain that are relevant to such a blood management model. Next we will describe how various elements are combined into an integrated model. Finally, we will discuss which challenges are foreseen with the implementation of the model and potential prospects. Challenges will concern knowledge required for shaping the modeling structure and the availability of data for various model parameters. Not only will the model guide the search for a rational choice of an optimal matching strategy, it will create transparency for the decision arena: the balance between costs and patient outcomes will become explicit for whatever optimal decision is selected. Secondly, by developing an integrated model, any blind spots in knowledge regarding any of the elements of the decision model will become visible and will have to be filled in.

The elements identified for the integrated blood management model are: the patient population, transfusion practice, predisposition of transfusion complications, typing and matching strategies, and the donor population. Note that as the patient is the primary concern, it is the patient that should be the starting point of the analysis. From there we will work our way back through the blood transfusion chain toward the donor population.

2. TRANSFUSED PATIENTS, EXPOSURE AND TRANSFUSION COMPLICATIONS

Blood transfusion is one of the most common medical procedures performed in hospitals. Despite its benefits, patients exposed to red blood cell (RBC) alloantigens may produce antibodies, which can cause acute or delayed hemolytic transfusion reactions (HTR). In addition, upon pregnancy in alloimmunized women, hemolytic disease of the fetus and newborn (HDFN) may occur. Not all patients form antibodies after RBC transfusion. According to current views, most are so-called non-responders and will never form antibodies despite numerous transfusions. Others seem to have an increased immunization risk and develop multiple antibodies after a few antigenic exposures, these are referred to as the (hyper)responders (1). It is currently not possible to prospectively identify patients that will form antibodies. In the absence of phenotypic matching, RBC alloimmunization risks vary between patient groups; it occurs in less than 5% of all transfusion recipients, increases to about 10–30% in patients with thalassemia, auto-immune hemolytic anemia or myelodysplastic syndromes, and can be

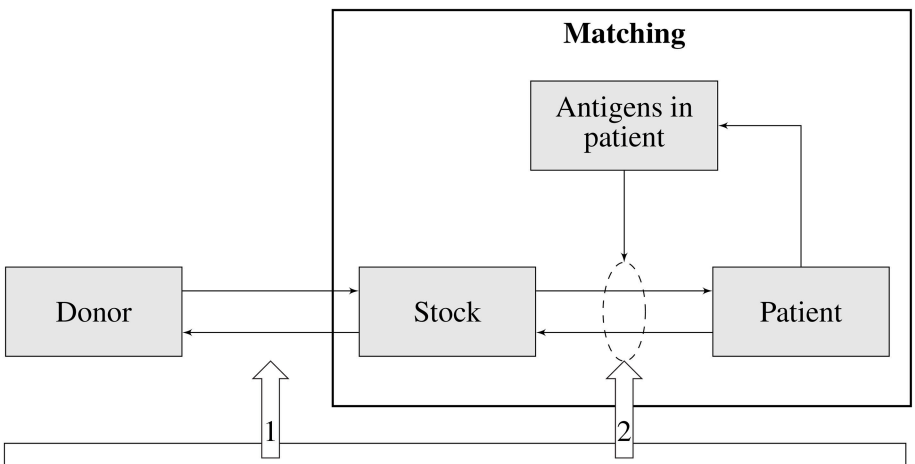

FIGURE 1 | Schematic overview of blood type matching and its impact on the blood supply chain.

more than 50% in sickle cell anemia patients (2, 3). In addition, patients with antibodies are at increased risk for additional antibody development upon subsequent transfusions (4, 5). During pregnancy, maternal RBC antibodies against paternal inherited antigens can pose the child at risk for HDFN. Besides anti-D, anti-E, anti-K, and anti-c are the most frequently encountered antibodies with the potential to seriously complicate pregnancy if the fetus carries the cognate antigen. The risk for severe HDFN in these fetuses, requiring intra-uterine or postnatal (exchange) transfusion, is estimated to be 12% for anti-K, 8.5% for anti-c and about 1% for anti-E. While for anti-D, administration of anti-D immunoglobulin (besides preventive D-matching) has reduced the risk of D immunization from 15% to 0.3%, such measures are not available or not always applied for other antigens, which are in the majority of cases elicited by previous transfusions (6).

The impact of transfusion reactions may vary widely, ranging from serologic observations or mild symptomatic anemia only, to life-threatening complications and death. It is obvious that with increasing severity, costs of treatment will also increase, although studies reporting on such associations and associated costs are currently limited or completely lacking (7). Maximum benefits of alloimmunization prevention can be obtained by administering extended antigen matched blood to patients who have an a priori high risk for alloimmunization. Therefore, unraveling genetic and environmental conditions enhancing RBC immunization would support preventive strategies. Although most studies on this subject have been performed in sickle cell disease (SCD) patients, factors such as age, sex, inflammatory status, MHC class-II genotype, polymorphisms associated with immune modulation and altered immune (regulatory) cells and disease or therapy associated immunosuppression seem to influence the immune response toward transfusion exposed alloantigens (1, 8–13). Due to logistic constraints, elaborate preventive matching based on a responder-profile is expected to be only feasible for a small proportion of patients. Targeting patients with (chronic) elective transfusions is likely to be feasible. Also, two recent prospective studies showed that less than 50% of surgery patients, who according to the local hospital pre-operative blood-ordering schedule had a high transfusion risk, were actually transfused. Extensive preventive matching as a routine policy is therefore expected to require a substantial amount of additional work and costs. Moreover, about 25% of patients required more than the anticipated number of RBC units during surgery and extended matched units were not readily available (14, 15).

As the blood management model is aiming to optimize strategies for preventing HTRs, the risk of alloimmunization in patients, its associated cost and health impact needs to be explicated. The ongoing Dutch R-fact study in which the predisposition for formation of antibodies is studied will allow modeling the likelihood of antibody formation. This information, combined with data on blood use for various patient groups, which will be obtained from the Dutch PROTON study (in which detailed transfusion data from a large number of hospitals are combined in a Dutch Transfusion Datawarehouse), will provide the information required to model the likelihood of HTRs in various patient groups. Research on the cost and health impact associated with HTRs will also be required to complete the model for patient and health outcome of transfusion complications.

3. CURRENT MATCHING STRATEGIES IN THE NETHERLANDS

In the Netherlands all RBC transfusions are compatible for ABO and D antigens. Since 2011 the guideline for selection of RBC units prescribes preventive matching for specific blood group antigens for different patient subgroups. Since 2004 it has been policy to select K-negative RBCs for women aged under 45, which in 2011 was extended with matching for c and E. These measures aim to prevent HDFN. In the updated guideline four patients groups with a putative increased risk of alloimmunization were defined, on grounds of either underlying disease, transfusion frequency, or potential (hyper-)respondership. The four patient groups concern (1) patients with autoimmune hemolytic disease; (2) patients with myelodysplastic syndrome and (3) patients with an immediate early antibody (IEA) against a clinically relevant RBC antigen. For these three patient subgroups Rh phenotype (CcDEe) and K compatible RBCs are selected. Finally, the fourth group consists of patients with hemoglobinopathies (SCD or thalassemia) for whom Rh phenotype, K and Fy(a) compatible RBCs are selected, and whenever available, Jk(b), S or s compatible RBCs. The recommended matching strategies formulated in Dutch transfusion guidelines are summarized in **Table 1** (16).

Apart from these specific patient groups, patients in the Netherlands are routinely tested for the presence of IEAs prior to RBC transfusions. When IEAs are detected, both their specificity and clinical importance are investigated. In case of a clinical important IEAs it is essential to select donor erythrocytes that are negative for corresponding antigens to prevent HTRs. Furthermore, dependent on the matching strategy, it may be required that donor erythrocytes are compatible with other antigens of the patient (extended matched), to prevent the formation of additional IEAs. Because antibodies may lose detectability over time, accurate recording and accessibility of patient antibody formation is of the utmost importance (17–19). Besides in-hospital records, a national database is available in the Netherlands (TRIX, Transfusion Register Irregular antibodies and X(cross)-matching), in which hospitals register patients with RBC antibodies and cross-match problems (20). This system is accessed for the evanesced antibodies in all patients

TABLE 1 | Matching strategies for various patient groups as recommended in the 2011 Dutch Transfusion guideline.

Patient group	Matching strategy
Sickle cell anemia and thalassemia	Rh phenotype, K and Fy(a) (and if available, Jk(b), S and s)
Autoimmune hemolytic anemia	Rh phenotype and K
Myelodysplastic syndrome	Rh phenotype and K
Alloimmunized with clinical important antibodies	Rh phenotype and K
Woman of childbearing age	c, E and K

with a transfusion request to prevent re-exposure to the cognate antigen. However, these registrations will not prevent re-exposure due to an inadequate antibody follow-up after transfusion.

The blood management model will have to accommodate matching strategies currently implemented as well as various extended matching strategies. The model should incorporate all costs involved for various matching strategies considered (e.g., costs of personnel and materials used).

4. TYPING THE DONOR POPULATION

Different matching strategies will pose different requirements on the availability of typed blood products. The required number of typed blood products, the variation in its demand, and the required service level (the probability of not being able to deliver a requested typed blood product) will determine the number of typed blood products that will have to be available in stock at any time, and hence the level of typed donors. A large typed donor population has the advantage that in most cases donor erythrocytes can be selected directly from inventory, even when blood products need to be typed negative for combinations of antigens. However, there will always be a balance between the additional efforts required to fulfill requirements for typed blood products and extending the pool of elaborately typed donors.

5. DONOR RECRUITMENT

Transfusing matched blood is only feasible if there are enough donors that are typed negative for specific (combinations of) blood group antigens. For instance, many Blood Services in Western countries have a structural shortage of Fy(a)-neg, Fy(b)-neg, e-neg donors. This blood type is most common in populations from Sub-Saharan Africa, of which relatively few individuals are enrolled as blood donors (21). In addition, in many countries a broad variety of ethnic minority populations exist. Shifting immigration patterns and mixing of these populations will increase the demand for rare blood type combinations. A valuable side effect of recruiting among minority groups is a potentially increase of donors for HLA-matched substances of human origin, such as stem cells. Blood Services therefore need to identify which specific ethnic minority populations to focus on in terms of rare blood type prevalence.

6. INTEGRATION

In the previous sections various elements of the blood transfusion chain and their interdependencies were discussed (see **Figure 1**). Each of these elements and their interactions need to be modeled in order to allow evaluation of the impact of a particular matching strategy on the transfusion risk of patients (i.e., acute and delayed HTRs) and on other parts of the blood supply chain (e.g., the availability of matched blood products, costs of type and screen, storage, outdating, and targeted donor recruitment). The main elements of the blood supply chain and the associated sub-models describing various interactions required for an integrated blood management model is depicted in **Figure 2**.

The starting point for any evaluation is the blood matching strategy, as this, in combination with the patient mix, will determine the demand for particular blood products. Depending on the matching strategy and patient mix (patient subgroups) there will be a risk of antibody formation and subsequent risk for adverse transfusion complications. Moreover, the combination of patient mix and associated matching strategy will determine the demand for typed blood products in the inventory. The availability of typed blood products in the inventory is dependent on the availability of typed blood donors, which again is dependent on the efforts and requirements of targeted donor recruitment.

The assessment of the transfusion complication risk requires estimates of the likelihood of antibody formation and subsequent transfusion reactions in patients given a particular matching strategy. Such estimates should incorporate the transfusion pattern and the ethnic (blood type) composition of various patient sub-groups. Also, antigen specific estimates for the likelihood of developing antibodies as well as for transfusion complications are required. The likelihood of transfusion complications in combination with cost and the health impact

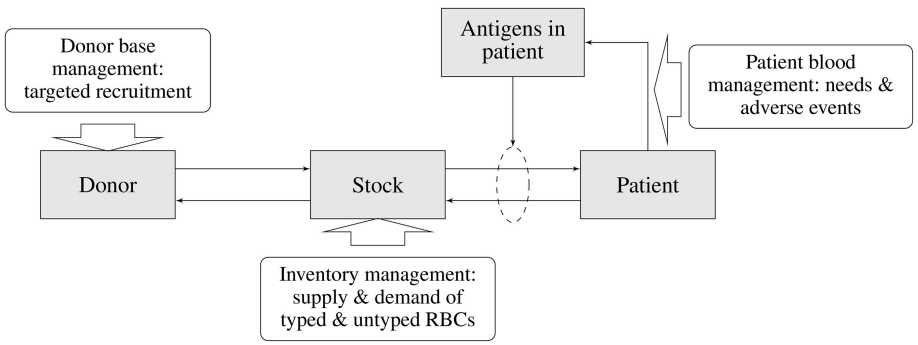

FIGURE 2 | Main elements of the blood supply chain and associated sub-models of the blood management system.

will allow estimation and subsequent balancing of the costs and benefits from the matching strategy applied.

To enable matching blood for transfusion recipients antigen and antibody profiles of patient subgroups have to be determined. Next, compatible RBC units have to be selected from inventory. Detailed information on blood use and the antigen profiles per patient group allows assessment of the blood inventory required to meet patient needs. This will be a description of the required inventory both in terms of amount and composition of RBCs in various stocks along the blood transfusion chain. Blood product demand will show a stochastic behavior and a realistic blood management model will therefore have to be able to accommodate such random variations. Given the patient mix, matching strategy and associated transfusion characteristics, for any pre-specified acceptability rate for the unavailability of (matched) blood products and inventory management strategy, the required blood inventory size and composition can be determined. The resulting costs and effects for the complete blood transfusion chain (outdating, size of the inventory, logistics, and material handling costs) can now be estimated. Note that the unavailability of matched blood products will impact the likelihood of transfusion complications in patients. Therefore, optimization of the overall blood transfusion chain will require a separate sub-optimization for the inventory management strategy.

The availability of compatible RBC units required in the inventory is directly linked to the availability of typed donors and hence guides the typing strategy and targeted donor recruitment efforts. The typing strategy will be aiming at fulfilling the requirements for maintaining sufficient inventory levels, but this will be dependent on the availability of specific antigen profiles in the (typed) donor population. Whenever these are insufficient, targeted donor recruitment efforts will have to ensure adequacy of the desired antigen profiles in the un-typed donor population, and ultimately those in the typed donor population. Estimates for the costs of recruiting specific donor subgroups in order to ensure a sufficient level of typed blood groups in the donor population are required to estimate the costs for maintaining the required inventory levels. Other than in the inventory management, which is an in-line process, it is presumed that the required levels of typed donors will be met by increasing donor recruitment efforts.

7. DISCUSSION

In this article we discussed a conceptual framework for a blood management model which allows optimization of blood matching strategies. The model links various elements from the blood transfusion chain to allow an assessment of the full impact of any particular matching strategy. The approach is unique in the sense that in the past matching strategies were guided by the prevention of transfusions complications observed with the administration of blood products, without consideration its impact on the underlying blood supply process. In theory this new approach seems sensible, however, in practice there will be a number of complicating factors.

First of all, except for some specific patient subgroups there is only limited evidence available on the effectiveness of matching strategies for the prevention of transfusion complications. Despite the fact that transfusion complications are accurately analyzed, patient exposure is far more difficult to ascertain. More evidence however has been gained for the risks of alloimmunization in various patient cohorts in the Netherlands in the ongoing Risk-Factors for alloimmunization after red blood Cell Transfusion (R-FACT) study (22). This concerted collaboration of several large hospitals will provide the information required to model risk factors for some patient subgroups. Also, looking back at the reduction of transfusion complications after implementation of altered matching strategies may support inference on its effectiveness. However, this effect may also be confounded by transfusion practice.

Another complicating factor is the impact of transfusion complications on patients, as this may vary from serologic observations or mild symptomatic anemia to life-threatening complications and death. Not only are predictors for predisposing factors lacking, but the impact of various levels of transfusion complications on patient health (apart from death) are not readily available, and neither are the associated costs. Assessing costs of complications is complex as it requires separation of the costs of patient treatment from costs of complications which are confounded by definition. Similar complications occur when estimating the impact on patient health. Nonetheless, an increasing number of publications on the impact of transfusion complications are becoming available (23–25).

In most settings detailed information on transfusion practice (number of transfused blood products for specific patient subgroups and the variation herein) is lacking. In the PROTON II study for a large number of Dutch hospitals detailed information on blood transfusions administered to patients is collected in one central datawarehouse (26). These data consist not only of transfused products, but also patient diagnosis and lab results. These data are indispensable when modeling the logistics of the blood supply in general, and for specific patient groups. Optimized inventory and dispatching strategies can be developed for both hospital and regional distribution centers and may be tailored to specified matching strategies. Note that with data on blood use the requirements and constraints for such models are available.

For the assessment of the risk of transfusion reactions (depending on the matching strategy) information on historical exposure of patients to blood products is required in order to assess the likelihood of antibody development. Such data is at present only available at a large scale for Denmark and Sweden where long term follow-up data on transfused patients is recorded in the SCANDAT database (27, 28). Such information may be used to estimate an approximate risk of exposure to red blood cells in other settings.

The development of an integrated blood management model will increase transparency in costs and effects of selected

matching strategies and is therefore -if applied- expected to contribute to an improved efficiency in blood transfusion practice.

AUTHOR CONTRIBUTIONS

JvS, PdW, JL, BV, KvdH, AvD, MK, MvK, CvdS, HS, WdK, and MJ design of the framework. JvS, PdW, BV, KvdH, AvD, HS, and MJ initial draft of the paper. All authors review and update of the final paper.

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Reflections on Dry Eye Syndrome Treatment: Therapeutic Role of Blood Products

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Dry eye syndrome (DES) is a multifactorial, frequent, pathology characterized by deficient tear production or increased evaporation of tears and associated with ocular surface alteration and inflammation. It mostly affects, but not exclusively, older individuals and leads to varying degrees of discomfort and decreased quality of life. Although the typical treatments of DES rely on using artificial tears, polyunsaturated fatty acids, integrin antagonists, anti-inflammatory agents, or on performing punctal occlusion, recently, standardized blood-derived serum eye drops (SED) are generating much interest as a new physiological treatment option. The scientific rationale in using SED for treating or releasing the symptoms of DES is thought to lie in its composition in multiple factors that resembles that of tears and contributes to the healing and protection of the ocular surface. This manuscript seeks to provide relevant background information on the management of DES, and on the increasing role that various types of SED or platelet lysates, from autologous or allogeneic origins, are playing in the improved therapeutic management of this pathology. The increasing role played by blood establishments in producing better-standardized SED is also addressed.

Keywords: dry eye syndrome, keratoconjunctivitis, artificial tears, serum eye drop, platelet lysate, blood

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DRY EYE SYNDROME (DES): EPIDEMIOLOGY, PATHOLOGY, AND SOCIO-ECONOMIC IMPACTS

Epidemiology

Dry eye syndrome, also known as dry eye disease or keratoconjunctivitis sicca (KCS), is among the most common ocular complaints that older patients seek eye care for (1). Often under-recognized, DES is a multifactorial disease associated with varying degrees of discomfort and decreased quality of life (2). Awareness of DES varies much among populations, largely influenced by criteria used for self-diagnosis. For instance, in a survey conducted in Japan, 33% of participants estimated to be affected by DES (3). DES prevalence increases with age. One study found that there are no significant differences in DES prevalence in men of differing races or regions in the United States (1). Females of all age groups have a greater likelihood of developing DES than males, with DES prevalence increasing with age (4). Schaumberg et al. estimate that, in the United States, 1.68 million men over the age of 50 years experience DES and this number is expected to grow to 2.79 million by 2030

as life expectancy increases (1), whereas a previous health study found that over 3.23 million women are currently suffering from DES (4).

In another study, it was extrapolated that 4.3 million people over 65 years in the United States suffer from ocular irritation at least occasionally (5).

Pathology

Dry eye syndrome pathology is typically divided into two types: deficient tear production or the evaporation of tears. Deficiency in tear production can be further divided into two more categories: Sjögren's syndrome (SS), which is an autoimmune disease, or non-Sjögren's syndrome (non-SS) (2, 6–8). Evaporation of tears refers to the loss of water from the ocular surface and is often the result of a meibomian gland dysfunction leading to a lipid bilayer deficiency in the tear film. The meibomian gland loses its function with age, leading to tear film instability and evaporation of tears; the quality and function of the meibomian gland has been linked, at least in part, to androgen levels (9). As males have higher androgen levels than females, this is consistent with the higher frequency of DES in females, especially after menopause.

Deficiency of tears caused by the decrease of aqueous tear production or excessive tear evaporation could increase osmolarity, with deleterious effects on the ocular surface. DES is associated with inflammation, and tear hyperosmolarity is an important mediator of this inflammation (2). Hyperosmolarity is associated with a key pathogenic mechanism of DES with negative effects on epithelial cells, including decreased cell volume, damage to DNA repair systems, increased apoptosis, and increased oxidative stress (2). It also stimulates multiple inflammatory events involving metalloproteinase-9 (MMP-9), tumor necrosis factor-alpha (TNF-α), and mitogen-activated protein kinase (MAPK) (2). Indeed, overexpression of proinflammatory cytokines/chemokines on the ocular surface has been found to be associated with the symptoms of dry eye (10–12), including interleukin (IL)-1β, IL-6, IL-17, IL-22, interferon-γ, tumor necrosis factor α (TNF-α), chemokine (C-Cmotif) ligand 2 (CCL2), and matrix metalloproteinases (13, 14).

In addition to dryness, symptoms associated with DES include pain, burning sensations, eye fatigue, redness, blurred vision, discharge, contact lens intolerance, sensitivity to light, and the feeling of foreign bodies present in the ocular region (2). Depending on the severity of DES, some patients experience problems carrying out basic daily activities such as reading, watching television, using a computer, driving a vehicle, and working (15). The discomfort caused by DES has also been tied to depression and decreased quality of life (2, 16–18). Furthermore, one study conducted a battery of tests, including tear function and ocular surface evaluations, and questionnaires on DES patients and determined a correlation between lower DES symptoms and patient happiness, suggesting that DES may influence a patient's psychiatric well-being (19). A study of depression in DES subjects identified that these patients experience poor sleep quality (16). DES patients tend to sleep later, less, and use more sleep medications and antidepressants than non-affected subjects. However, antidepressants are being investigated as a potential contributor to DES (20), and patients with severe DES that progressively worsens over time suffer from increased anxiety and other mood disorders (2, 16).

Socio-economic Impacts

Dry eye syndrome places a substantial economic burden on society due to hospital visits, medical costs, surgeries, and drugs, in addition to indirect costs such as loss of productivity (21). In the United States, the average DES patient makes approximately 6 hospital visits annually at a total cost of nearly $800 USD, adding up to a national cost of nearly $4 billion USD. These costs have risen over the years. When taking loss of productivity into account, annual societal costs are estimated to exceed $55 billion in the United States (22). In Europe, the estimated annual cost for ophthalmologist-managed care ranged from approximately $270 USD in France to $1,100 USD in the United Kingdom (21). In Japan, DES patient annual medical costs amounted to roughly $470 USD, mostly for drugs (21). Additionally, loss of work productivity in Japan was calculated to be approximately $536 USD per patient (21, 23). Surprisingly, the economic burden of DES due to loss of productivity drastically outweighed the direct expenses from receiving care from healthcare professionals or prescription drugs (22). Although the apparent costs vary among countries, the real costs of DES in each country are likely higher than data shows when taking into account that the purchase of over-the-counter artificial tears is not always incorporated into cost calculations (24) and data are incomplete, in particular in some parts of Asia (21).

CURRENT THERAPEUTIC STRATEGIES

The past 5 years have witnessed substantial developments in DES treatment options. Current treatment strategies that are not based on blood products include artificial tears, lubricants, steroids, immunosuppressant eye drops, dietary supplements associated with eyelid cleansing, and in more extreme case, anti-inflammatory drugs or punctal occlusion, a procedure consisting of inserting a plug into the tear drainage area to maintain tears in the eyes. Generally, such treatments, which can be combined, are selected based on disease severity and medical history of the patient. For the majority of DES cases, treatments focus on alleviating symptoms rather than addressing the causes of DES (6–8). Treatment effectiveness on symptoms must be regularly assessed (25). Regular use of artificial tears, anti-inflammatory drops, or punctal plugs provides only transient release and can often induce ocular side effects.

Artificial Tears

The main functions of artificial tears are to increase moisture and provide lubrication of the ocular surface (26). There is a variety of artificial tear formulations available, differing in osmolarity, viscosity, electrolyte content, preservative content, and solute combinations (27). Artificial tears are currently formulated as osmoprotectants, with the purpose of restoring cell volume, decreasing cell stress, and reducing inflammatory reactions that occur under hyperosmotic conditions (28). One

eye drop product uses propylene glycol (PG), polyethylene glycol (PEG), and hydroxypropyl guar (HP-Guar) with polyquaternium-1 preservative, which decreased ocular surface inflammation and DES symptom severity (29). Similarly, another eyedrop formulated using hyaluronic acid (HA) and trehalose stabilizes the bilipid membranes and protects labile proteins from desiccation, as well as prevents oxidative damage (30, 31).

A recent Cochrane analysis (27) could not identify whether different over-the-counter artificial tears provide "similar relief of signs and symptoms when compared with each other or placebo." However, 0.2% polyacrylic acid-based artificial tears were found to be more effective than 1.4% polyvinyl alcohol-based artificial tears. In addition, artificial tears are not free of inducing some adverse events.

One limitation of artificial tears is the lack of some of the components of natural tears such as lipids, salts, proteins, and hydrocarbons, as well as growth factors, immunoglobulins, albumin, and vitamins present in serum, as discussed later (28, 32–34). Additional possible drawbacks of artificial tears include the presence of preservatives and other potentially toxic and allergenic compounds (35). Benzalkonium chloride (BAK), the most frequently used preservative compound in eye drops, may contribute to hyperosmolarity by disrupting tear films. BAK-induced damage extends to destruction of goblet cells, the corneal epithelium barrier, and deeper ocular tissues including release of proinflammatory cytokines, oxidative stress, and apoptosis (35). These factors should be taken into consideration when prescribing DES treatments.

Polyunsaturated Fatty Acids (PUFAs)

Omega 3 and 6 fatty acids are essential fatty acids that cannot be synthesized in the human body. Their improper balance can lead to an omega 6 proinflammatory effect (8). Dietary supplementation of polyunsaturated fatty acids (PUFAs) may help manage DES (8, 36). In a randomized, double-blind study, omega-3 supplementation promoted tear film stabilization, reducing tear evaporation and DES symptoms as a result of increased goblet cell counts and improved epithelial cell morphology (8, 36). Balanced combination of omega-3 and omega-6 was recently found to attenuate contact lens-related DES (37).

Integrin Antagonist

Lymphocyte function-associated antigen-1 (LFA-1), an integrin expressed on T-cells, is upregulated in the conjunctiva of DES patients (38). The interaction between LFA-1 and intercellular adhesion molecule-1 (ICAM-1) is key in T-cell adhesion with endothelial cells, as well as for T-cell interaction with antigen presenting cells (38). One approach to treat DES aimed to block the interaction between LFA-1 and ICAM-1. A small LFA-1 antagonist called Lifitegrast (SAR 1118) demonstrated in phase III clinical trials to significantly and safely relieve DES symptoms (39). Lifitegrast acts as an antagonist to LFA-1, resulting in the inhibition of T-cell activation, migration, and proliferation (40). However, other parameters to assess ocular function, such as Schirmer's test results, tear breakup time, and inferior corneal staining, did not improve significantly (41). In July 2016, Xiidra® was the first United States Food and Drug Administration (US-FDA)-approved LFA-1 agonist for treating DES (40).

Anti-Inflammatory Therapies and Immunomodulators

Corticosteroids are one among several anti-inflammatory drugs to treat DES. In addition to reducing cellular infiltration, restoring vascular permeability and inhibiting chemotaxis, corticosteroids decrease fibroblast proliferation, reduce capillary dilation and suppress collagen deposition (40). They are considered highly effective toward the treatment of immune-mediated inflammatory diseases (40). However, their efficacy is limited to short-term usage (4 weeks or less) (41) as long term use leads to intraocular pressure and the formation of cataracts (40, 42). A combination with anti-inflammation agent (epigallocatechin gallate, EGCG) and mucoadhesive component, hyaluronic acid (HA) was used for the treatment of DES in a rabbit experimental model (43). Its therapeutic effect was evidenced via increased tear production, inflammation relief, and corneal epithelium recovery providing an alternative inflammatory inhibition agent for clinical DES treatment.

Cyclosporine is preferred over corticosteroids as a long-term treatment for DES. Cyclosporine A is a topical immunomodulator, first approved by the FDA in 2002 (Restasis®) for treating dry eye by increasing tear production (44) and by the European Union in 2015 (Ikervis®) (45). When administered topically, cyclosporine A acts as an immunomodulator, and when administered systemically, it acts as an immunosuppressant (8). This drug elicits anti-inflammatory properties by inhibiting cell-mediated reactions and preventing the release of proinflammatory cytokines, while upregulating the production of anti-inflammatory cytokines (44). Multiple studies have reported minimal side effects associated with topical application of cyclosporine A, under conditions increasing tear production and conjunctival goblet cell density (8, 45–47).

Punctal Occlusion

Lacrimal punctal occlusion by plug is the most common non-pharmacological therapy for DES (48, 49). Although many authors recommend temporary occlusion by plugs as a trial treatment, permanent occlusion can be achieved through surgical obstruction of the lacrimal punctum. It has been described as being like "blocking the drain in a tub and collecting the water dripping from the tap" (50), which in other words means preventing tear drainage toward the nasal cavity by physically blocking the lacrimal punctum/canaliculus. Punctal occlusion is typically recommended for patients suffering from DES symptoms after failed attempts of using traditional aqueous treatment options (49). Although punctal occlusion may improve DES symptoms, there is a concern that it could retain unhealthy tears on the ocular surface causing irritation (51) and does not decrease tear cytokines and MMP-9 levels (52). An international panel of dry eye specialists recommended that factors associated with inflammation be handled prior to performing punctal occlusion (53). A study comparing the effects of administering punctal

occlusion alone versus a punctal plug regime in combination with cyclosporine treatment demonstrated that for the near term, punctal occlusion, alone or with cyclosporine, yielded swift improvement in moisture. However, for the long term, treatment regimes involving punctal occlusion in combination with cyclosporine produced equal or superior results to treatment regimes using occlusion plugs only (50). A recent Cochrane study has identified a "very low-certainty evidence on symptomatic improvement" of punctal occlusion, commonly associated with epiphora and inflammatory conditions (54).

BLOOD PRODUCT-BASED DES TREATMENT OPTIONS

Scientific Rationale

Human blood has been for many decades the source of a wide range of cell-based or protein-based therapeutic products. Cellular products include red blood cell (erythrocyte) concentrates, buffy coats/granulocytes concentrates, and platelet (thrombocyte) concentrates. Therapeutic proteins encompass coagulation factors, albumin, and immunoglobulins. More recently, new platelet-derived preparations, rich in growth factors, have been increasingly used for therapeutic applications in wound healing, tissue repair and regeneration (55), and *in vitro* clinical-grade cell propagation and tissue engineering (56).

There is now great interest in the application of human blood derived products as eye drops for DES. The most common blood product used as eye drops is serum, which is obtained by a physiological clotting process of blood collected without anticoagulant, as described in details below. The therapeutic benefits of blood-derived serum eye drops (SED) are probably multifactorial and may be explained by a composition that, in part, shares similarities with that of tears (32–34, 57). Like tears, SED contains carbohydrates, lipids, and various electrolytes, but 10 times more proteins including albumin, fibronectin, and transferrin (33). SED contains natural antimicrobial components, like complement component (58), and IgG, but less lysozyme than tears (32). Tears and SED provide vitamins and both share a similar osmolality (close to 300 mosm/l) as they contain comparable sodium and anion levels, and a similar pH (close to pH 7.4) (33, 59, 60). Potassium ion levels are about five times higher in tears than in SED, but calcium ions and phosphate levels are less in tears than in SED (33). However, the total protein content of tears is only about 10% that of SED (33). IgA is the major immunoglobulin in tears, playing a role in protecting against infections. Vitamin A is less in tears than in serum. Vitamin C and glutathione antioxidants are present at higher levels in tears than in serum. Most importantly, SED, like tears (61), also contain a mixture of cell growth promoting agents (62, 63), since blood clotting is associated with a degranulation of the platelets and a release of a plethora of growth factors from their alpha-granules (56, 64, 65). Growth factor composition is said to be qualitatively equivalent in tears and serum, but concentrations may be higher in serum, as is the case for transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF). **Table 1** presents some of the known similarities existing between tears and SED.

Serum Eye Drop

Preparation

Serum refers to the fluid portion of blood, devoid of cellular components that is obtained by letting blood collected without an anticoagulant to clot. It is typically prepared by collecting blood from patients (autologous source) or donors (allogeneic source), allowing the blood to clot for several hours prior to a centrifugation step at ca. 3,000 × *g* for approximately 10 min at 20–25°C to recover a supernatant serum. Serum may be passed through a 0.22-μm pore-sized filter for bacterial sterilization and clarification (34, 57, 66). In such a preparation, the platelets are not concentrated compared to the level found in the blood circulation, by contrast to newer SED formulation made from platelet concentrates where platelets are threefold to fivefold enriched compared to blood. When SED are made from platelet concentrates for transfusion, the content of serum plasma protein depends upon whether the platelets are suspended in 100% plasma or a mixture of plasma and platelet additive solution (PAS).

An informative survey of methods used at international levels to prepare SED has recently been conducted by the Biomedical Excellence for Safer Transfusion (BEST) Collaborative (67). A summary of the preparation methods of SED is illustrated in **Figure 1**. Briefly, this survey indicates that SED for clinical use are prepared by national or regional blood establishments (also known as blood centers), as well as by hospitals or medical centers. Although most centers are manufacturing SED of autologous origin, an increasing number is now producing SED from allogeneic blood donors (68–70). When the SED are from allogeneic origins, procedures are in place, e.g., by preparing SED from AB group donors to hold a single blood group inventory or by donation screening to match all blood groups to ensure hemato-immunological matching between donors and recipients. It is, however, still unknown whether presence of anti-B agglutinins affect corneal healing (67).

A small majority of centers (most likely the blood establishments familiar with the production of blood components for transfusion) prepare SED from blood collected into blood bags rather than into tubes and use larger volumes of 200 mL or more. While the clotting time to get serum may be less than 6 h, it can be up to 24 h and (somewhat surprisingly) up to 3 days in some places. Most often, the serum is centrifuged to clarify the supernatant. Most centers do not perform a bacterial filtration step, whereas others do, implying that they apply the standard close-system manufacturing practices familiar to blood establishments. A small majority of the centers dilute the serum twofold to fivefold in saline or phosphate-buffered saline solution, before immediate dispensing in 0.5–5 mL aliquots into vials/eye dropper bottles or tubing segments before freezing (67).

Formulation

To date, the optimal formulation and dilution factor of SED for DES treatment remains uncertain. This is not unexpected considering the biological complexity of the serum material compared to artificial tears. Sometimes, the serum is diluted to approach

TABLE 1 | Comparison of tears and serum composition (individual variations may affect the values).

	Tears	Serum	Physiological function possibly relevant in ocular defect treatment
Physico-chemical parameters (33, 57)			
Osmolality, mosm/l	302	300	Maintains physiological osmolality and pH
pH	7.2–7.4	7.2–7.4	
Proteins (33, 55, 56, 74)			
Total proteins, mg/mL	7.37	60–70	Support tear surface tension, physiological hydration of the ocular surface, and ocular homeostasis
Albumin, mg/mL	0.05	35–40	Anti-apoptotic activity, detoxification
Fibronectin, µg/mL	21	200–300	Adhesion protein supporting wound healing
IgG, mg/mL	0.032	8–12	Anti-microbial
IgA, mg/mL	0.41		Anti-microbial
IgM, mg/mL	–	0.5	Endotoxin binding
IgD, µg/mL	–	3–300	
IgE, µg/mL	–	0.25–0.7	
Alpha 2-macroglobulin		2.6	Anti-collagenase
Complement system			Anti-microbial; bacteriostatic
Lactoferrin, mg/mL	1.51	–	Anti-microbial and anti-inflammatory
Transferrin, mg/mL	–	2–3	Iron-carrier; anti-microbial
Lysozyme, mg/mL	1.4	6	Iron carrier; anti-microbial
Growth factors (33, 55–57, 61)			
TGF-β1, ng/mL	2–10	6–50	Epithelial and stromal repair processes
PDGF, ng/mL	0.09–1.7	30–100	Enhances mitosis and scarring
EGF, ng/mL	0.2–3	0.5–1	Accelerates the migration of epithelial cells; anti-apoptotic
HGF, ng/mL	0.2–0.5	0.1–1	Supports corneal epithelial cells
VEGF, ng/mL	0.019	1–5	Supports conjunctival endothelial permeability
Vitamins (33)			
A, ng/mL	16–20	800–1000	Prevents squamous metaplasia and helps maintain the normal histology in the conjunctiva
C, µg/mL	117	7–20	Antioxidant
Antioxidants (33)			
Tyrosine, µM	45	77	
Glutathione, µM	107	ND	
Electrolytes (33)			
Na+, mEq/L	145	135–146	
K+, mEq/L	24.1	3.5–5.0	
Ca^{2+}, mM	1.5	1.1	
Cl^{-}, mM	128	96–108	
HCO_3^- mM	26	21–29	
NO_3^- mM	0.14	0.19	
PO_4^{3-} mM	0.22	1.42	
SO_4^{2-} mM	0.39	0.53	

the composition of tears and to decrease the concentration of TGF-β, which may exert an anti-proliferative activity and impair the healing of epithelial cells (33). There is, nevertheless, no real consensus yet nor evidence-based information on the optimal formulation (71). One cannot exclude that formulation may have to be adjusted to the disease treated or its extent (dryness or epithelium defect). Lower dilution factors (50%), or even no dilution at all, have been used (57, 72), while other authors have proposed to dilute SED to 20% in a sodium hyaluronate solution in particular to improve retention time and decrease the frequency of the administration (73). Higher SED concentrations have been reported to increase the speed of epithelial healing and closure in a patient recovering from laser *in situ* keratomileusis (LASIK) eye surgery (66).

Safety Aspects

Autologous SED do not essentially present risks of extraneous virus contamination when produced under GMP restricting the risks of cross-contamination or mislabeling with SED from another patient. Release testing focusing on microbial sterility of the final batch is carried out by about half of the producers that were recently surveyed (67). Preservative solutions are not added in SED; preparation procedures should therefore be carefully controlled and monitored to prevent bacterial contaminations.

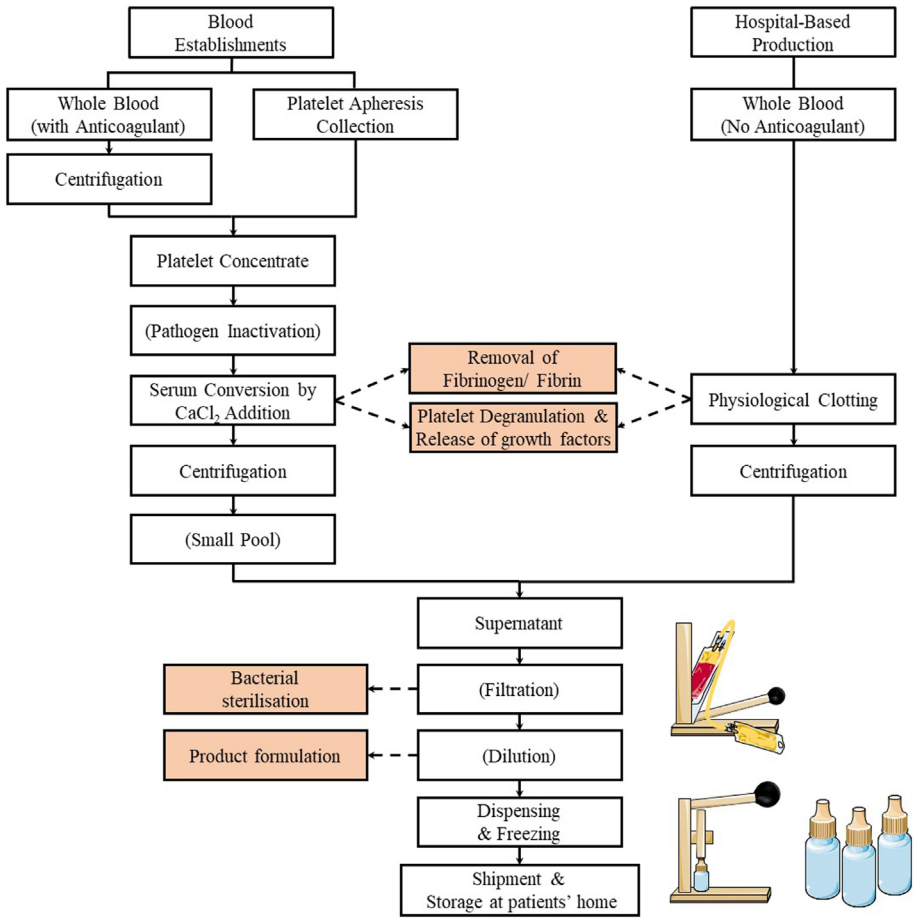

FIGURE 1 | Flowchart of the preparation methods of serum eye drop by blood establishments and hospitals. Typically, the process involves the release of the platelet growth factors by physiological or $CaCl_2$ stimulated clotting of blood (or platelet concentrates), followed by centrifugation, filtration (optional), dilution (optional), dispensing, and freezing.

Allogeneic blood donors donating blood for the production of SED should be screened for virus markers using the same standards that are applied to donations devoted to the manufacturer of transfused blood products (67, 74, 75). The main transfusion transmitted infections associated with allogeneic serum are viruses, most notably human immunodeficiency virus, and hepatitis B and C viruses (76). Emerging viruses, like West Nile virus, Dengue virus, Chikungunya virus, Ebola virus, and Zika virus, may also be a potential threat (77). However, efficient safety measures in place in blood establishments, namely donors' screening and donation testing, dramatically restrict the risks of viral transmissions in a regulated blood collection jurisdiction (78). Particular future attention may need to address the pathological consequences of risks of transmission of other blood-borne viruses, such as the Herpes simplex virus, that may lead to ocular complications and affect vision (79).

Photochemical pathogen inactivation methods are in use for transfused plasma and platelet concentrates (80, 81), but they are not a current option as no dedicated or licensed pathogen inactivation treatment has been approved for application to therapeutic serum, although experimental studies have shown applicability to serum for cell expansion (82). As therapeutic platelet concentrates can be pathogen-inactivated using licensed treatment, this may speed-up the development of allogeneic pathogen-inactivated SED for clinical use (67). The well-established solvent-detergent (S/D) treatment, already applied to a wide range of biopharmaceutical preparations and plasma products (76), was experimentally proven applicable to rabbit SED (83). This S/D-treated rabbit serum was used as allogeneic SED equivalent to treat DES-rabbits, showing promising results. The safety and efficacy of such S/D-treated SED was demonstrated through the restoration of a corneal epithelium in a DES rabbit model. This preclinical study supports the possibility of using S/D virally inactivated SED to treat DES for the application of allogeneic human SED (83).

Shipment and Storage

Most often, patients themselves collect the SED from the production site and store the bottles at home in a domestic freezer. The typical specified shelf life set by producers of SED ranges from 3 to 12 months until thawing and up to 24 h to 1 week after thawing. Currently, SED storage at patients' home is not specifically controlled and is under patients' responsibility

(33, 67). Studies have suggested that SED can be stored liquid at 4°C for up to 1 month, or frozen at −20°C or −80°C for up to 3 to 6 months, and in the dark to limit the decay in vitamin A (62, 71, 72). The stability of factors in serum, such as vitamin A, EGF, and TGF-β, was shown over up to 9 months. However, stability evaluations based on functional or biological activity (e.g., using cell cultures or animal models), rather than immunological tests (e.g., ELISA measurement of growth factors), should be conducted to determine the shelf-life. Furthermore, variations in the preparation methods of SED may impact its quality and properties (57, 72, 84) and, potentially, influence its long-term stability. Topical application of SED, which do not contain preservatives in order to prevent toxicity, requires careful handling to avoid microbial contamination.

Regulations

The regulatory status of current SED varies, but these preparations are typically regulated as blood products with variations from country to country depending upon jurisdictions (85). The increasing number of blood centers producing SED should eventually lead to the recognition and regulation of SED as a blood product, and to the establishments of international guidelines underlying their manufacture, and efforts towards implementing guidelines for standardization and product specifications. Clinical trials are expected to provide more rigorous information of clinical efficacy in various ocular pathologies and guidance for optimal products' performance and clinical outcomes (67).

Clinical Rationale and Experience

The clinical strategy behind administering autologous serum is to take a comprehensive approach to treating dry eye, rather than just serve as a lubricant. Recent studies and review papers generally confirm the benefit of SED, from autologous or allogeneic sources, providing improved tear film stability, ocular surface health, and subjective comfort in refractory DES (57, 59, 71, 86–93). According to a Cochrane review based on a limited number of randomized clinical trials, autologous SED alleviate dry eye symptoms better than artificial eye drops for the first couple of weeks, but data still remain inconclusive at determining clinical efficacy over long-term periods (72). Therefore, randomized clinical trials involving larger cohorts of various patient groups should be conducted to better delineate the short-term and long-term benefit of SED in the treatment of DES and other ocular diseases (26, 72, 87).

Cost Consideration and Reimbursement Policy

Cost is a major limitation of using autologous SED. In the United States, most health insurance providers do not cover this form of dry eye treatment, resulting in out-of-pocket costs between $175 and $250 for a 2-month supply. The cost of this treatment may therefore makes it an option to consider for patients who have already exhausted more conventional forms of dry eye treatment.

Pending Issues

Autologous versus Allogeneic Products

Currently, there is no universal consensus of criteria on suitable patient selection for autologous blood donation (72). Another disadvantage of using autologous serum is that occasionally the frequent drawing of blood can be inconvenient to patients with prolonged treatment (59). For the elderly and for newborns with serious infections, autologous serum products may be unavailable or contraindicated (94). Cultural considerations are also playing some role. Patients of many Asian cultures, especially the elderly Chinese and Taiwanese, hold the belief that frequent venipuncture causes weakness and makes them more prone to bacterial infection (94). Also some people fear phlebotomy. Additionally, some patients may be too old to donate due to poor venous access or do not possess blood suitable for conversion into autologous SED due to clinical conditions such as previous cerebrovascular accidents, cardiovascular disease, anemia, use of anticoagulant medications or coagulation factor deficiency, or presence of inflammatory mediators (59, 70, 92). Allogeneic serum consists of the same general substances as those in autologous serum, but from a different source and provides a potential alternative treatment for these patients (59). Allogeneic SED are thus being researched for their efficacy in treating a variety of eye disorders associated with DES including persistent corneal epithelial defect (PED), KCS, chronic graft-versus-host disease (cGVHD), and many more (69, 88, 94). In other words, some ocular pathologies may actually benefit from SED made from allogeneic source, rather than autologous.

The use of allogeneic SED poses some risks of its own including the transmission of blood-borne pathogens, hypersensitivity and immune reactions, and potential legal or ethical concerns (59). To overcome some of the risks associated with allogeneic serum, some researchers have limited their investigations to SED obtained from family members (69). It has been reported that these eye drops are clinically comparable to autologous serum (94), but obtaining blood from family members does not imply the absence of risks, including infectious one's. A ready-made, ABO-specific allogeneic eye drop study involving 34 patients (20 patients with KCS and 14 with PED) observed no side effects in any of the subjects and recorded objective improvement in 59% of the subjects. Of patients with KCS, relief was reported in 80% of the patients after allogeneic eye drop treatment (69). In a separate study investigating allogeneic serum in 36 PED patients, the epithelial defect of 16 subjects had healed in 2 weeks time (94). These results were confirmed with the observation of partial or full corneal changes in 16 of the 20 patients. This particular study supports the clinical potential for, and safety of, allogeneic eye drops. However, several immunological and physiological concerns still need to be given due consideration, namely ABO and HLA antibodies that may initiate inflammation (69). As such the virus safety and immune-hematological screening criteria of blood donations used to make allogeneic SED should be in line with those used for blood components for transfusion.

Due to the risk of transfusion-transmitted infections, it is highly recommended that manufacturers and documenters of allogeneic blood products implement good manufacturing practice as is recommended for the collection of blood components by blood establishments (67, 74, 75, 85).

Newer and Emerging Strategies Using Other Blood Products

Various other blood-derived preparations can be considered as therapeutic options to relieve DES symptoms and improve

patients' quality of life. It was identified, using a dry eye rat model, that plasma albumin provides a therapeutic benefit that was attributed to suppression of apoptosis (95). Albumin added to an eye drop formulation also helps to relieve DES symptoms in a rabbit model (96). Recent trends in development of blood products to treat DES focus on using blood fractions enriched in platelets (therefore equivalent to therefore somewhat equivalent to what is typically known as platelet-rich-plasma or PRP) as source material as the combination of platelet growth factors is believed to provide a scientific rationale to support its healing potential of DES (69). In the above-mentioned international survey (67), four centers manufacture eye drops either from (a) platelet-rich plasma (PRP) from human cords, (b) autologous platelet rich plasma donations, or (c) plasma. There is great interest in producing SED from PRP or platelet concentrate, as this blood fraction contains a threefold to fivefold higher platelet count than does whole blood.

A product termed Eye-PRP ("E-PRP") is prepared by collecting whole blood in the presence of a 3.2% sodium citrate anticoagulant solution (97) in order to avoid serum formation. Anticoagulated whole blood is centrifuged to sediment red blood cells and to recover a platelet-enriched supernatant plasma. This PRP is then directly divided into aliquots of 3–4 mL and stored in 4°C refrigeration for 1 week or stored in −20°C freezer for extended periods (97, 98). Growth factors in E-PRP act to stimulate angiogenesis, promote cell repair, and activate macrophages (97). These essential molecules are actually commonly used in ophthalmology to promote epithelial wound healing of the cornea (98). 89% of patients using E-PRP eye drops four to six times per day reported subjective absence of DES symptoms. Benefits extended to include increased visual acuity, increased tear production, and improvements in ocular surface condition (98). A similar conclusion was reached by a study investigating the effect of this PRP on human lacrimal function (99).

An alternative to autologous serum and E-PRP is "plasma rich in growth factors" (PRGF). PRGF contains, like serum and E-PRP, a number of platelet growth factors, including platelet-derived growth factor, angiopoietin-1 (ANG-1), epidermal growth factor (EGF), VEGF, and many more (100, 101). PRGF can be prepared by collecting 30 mL of whole blood in tubes containing 3.8% sodium citrate and centrifuging the tubes, using soft spin, at 460 × *g* at room temperature for 8 min. The plasma supernatant portion is recovered, and the platelets are activated using 22.8 mM calcium chloride (102). Addition of calcium chloride induces a process of serum-conversion where a fibrin clot is generated, and growth factors are released due to platelet activation and degranulation. Afterward, the growth factor-rich supernatant serum is collected and filtered. It can be diluted with 0.9% sodium chloride down to 20%. All of these steps are performed under sterile conditions. The final product is distributed into eye drop dispensers, ready for use. For immediate usage, the eye drops could be stored in 4°C refrigeration up to 1 week, and for long-term storage, at −20°C for no longer than 3 months. Patients administer eye drop solution four times per day. Treatment cycles last approximately 3 months, but treatment can be extended several more months to include more cycles if symptoms do not improve. A study investigating the efficacy of PRGF to treat DES reported that out of 16 patients, 75% experienced moderate to substantial improvements. Use of PRGF has demonstrated an ability to reduce symptoms of squamous metaplasia in patients suffering from DES (101).

As mentioned above, the development of human platelet lysates (HPL) manufactured from platelet concentrates collected following the licensed procedures in place to prepare blood products for transfusion is very likely and opens the roadmap for the development of more standardized SED (103).

Finally, recently, a limited case study was conducted in the UK where patients applied a drop of whole blood to the affected eye(s) four times daily for 8 weeks. Significant improvements were noted in several parameters, such as visual acuity, corneal staining, tear break-up time (TBUT), and ocular comfort index (OCI), but not Schirmer's test (104).

CONCLUSION AND FUTURE PROSPECTS

Dry eye syndrome is a common eye condition with a range of causes and degrees of severity and tremendous socioeconomic implications in addition to reductions in quality of life. There is a wide variety of medical products and procedures currently available or under development for the treatment of DES, each with their own advantages and disadvantages. Emerging treatment options include products derived from whole blood, such as autologous or allogeneic SED, E-PRP, PRGF, and HPL.

Relevant questions regarding the production method, quality, efficacy and safety of blood products used to treat DES remain, as already identified in particular with regards to standardization and formulation (87, 103). Similar to most claimed applications of platelet-derived preparations used in regenerative medicine, work is needed to design and standardize SED production methods to yield formulations with optimized blood proteins and growth factors composition to best address various DES and ocular pathologies. Reliable *in vitro* tests should be identified and validated as predictor of clinical outcomes (87). Furthermore, pre-clinical studies using valid animal models (105) to delineate the respective contribution of the plasma and platelet proteomes in the benefits of blood-derived eye drops in releasing DES symptoms should be performed. As is the case in other fields of regenerative medicine (106), dedicated platelet lysates may be needed to tackle the specific micro-environment of the diseased tissues and promote optimal repair strategies. An increasing involvement of blood establishments in producing SED is expected to contribute to improve standardization, quality, and safety (67).

In summary, blood products are well known for their benefits in relieving a variety of symptoms associated with DES. Many new and emerging blood products are currently being assessed for the presence of key growth factors and their overall effects weighed against their potential risks. Ultimately, as more evidence-based knowledge is obtained on the specific growth factors and their direct impact, patients with ocular defects should be able to receive personalized treatments, better customized to

their individual needs and pathology, which are becoming the buzzword of all clinical interventions.

AUTHOR CONTRIBUTIONS

VJD and TB wrote the first draft. CLT made additions. JS reviewed and modified the final draft. All authors approved the final version.

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