Transfusion Medicine and Proteomics. Alliance or coexistence?

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Introduction

Transfusion of blood components is one of the most frequently performed clinical operations[1,2]. Beginning with the discovery of the AB0-blood group system by Karl Landsteiner[3], the clinical relevance of transfusing blood, blood derived cells or proteins has increased constantly. Today transfusion medicine is a medical discipline and blood transfusion is embedded into one of the most advanced and regularized quality management systems in clinical medicine. In this system collection, production, storage, and administration of blood products are events separated in time and location, thus facilitating the existence of networks of donation sites and blood banks and the treatment of patients with human blood derived therapeutics even in far off areas.

Today blood processing allows the provision of separate blood constituents such as cellular blood products e.g. red blood cell concentrates, platelet concentrates and stem cells, as well as plasma derived therapeutics such as fresh frozen plasma, coagulation factor concentrates, or immunoglobulins. Such blood products are either obtained by fractionation following donation of whole blood or they are selectively obtained from the blood donor using cell separators and apheresis procedures to obtain either cellular subfractions of the blood or plasma. These advances in the production of blood components enable storage of blood products under different conditions e.g. platelets at 20 °C and red blood cells at 4 °C. Blood fractionation also facilitates the targeted selection of specific blood components in order to provide patients with distinct blood functions. Thus, the different blood components are administered according to special indications such as the need for oxygen carriers or the maintenance of blood coagulation.

Moreover, transfusion medicine achieved a remarkable reduction in the risk of pathogen transmission with blood products such as human immunodeficiency virus (HIV), hepatitis C, and hepatitis B[4]. While transmission of these pathogens has become a rare event in the Western world, bacterial contamination of blood products is still an issue, especially for platelet concentrates[5]. Despite the achievements just mentioned, transfusion medicine still faces a number of challenges with potential great impact on medical care systems. First the blood supply to demand ratio will change tremendously in the Western world due to the demographic changes with a growing older population and a declining birth rate[2]. This will require further reduction of waste of blood products e.g. by improvement of storage conditions and prolongation of storage time. Second, new emerging pathogens, which may not necessarily be known today or considered as a relevant transfusion transmissible agent, could easily thwart the blood supply. Prions causing variant Creutzfeld-Jacob-Disease (vCJD) are one recent example illustrating the need for ongoing activities to prevent pathogen transmission via blood products[6].

All approaches to reduce pathogen transmission or to extend storage of blood components have to maintain the integrity and function of blood cells and plasma proteins. In view of the complex composition of blood products and the constantly increasing regulatory requirements in transfusion medicine, monitoring of the effects of different maneuvers and interventions on the integrity of a blood product is becoming an issue of increasing importance. This will likely also require the application of more sensitive technologies.

Technology advancements in the field of proteomics but particularly mass spectrometry have provided an entirely new view on the protein inventory

of cells and cellular compartments by allowing fast and highly sensitive identification and analysis of thousands of proteins, both qualitatively and quantitatively. Such a comprehensive monitoring of dynamic changes of the protein levels but also of post-translational modifications[7] is particularly useful for blood plasma and cells without a nucleus such as red blood cells and platelets. Although de novo protein synthesis has been convincingly demonstrated in platelets[8] it occurs to a limited extend and is confined to pre-existing RNA.

Application of proteomic technologies to different blood cells and plasma proteins has been subject of some excellent review articles[9-12]. Most of these studies have been performed for research questions not directly related to transfusion medicine. Only within the last five years, researchers in transfusion medicine have started to adopt proteomics to a larger extent to analyze blood products for patient care[13]. This review does focus on the potential of proteomics to address some of the current issues in transfusion medicine and provides a current status of proteomics in transfusion medicine.

Technical considerations

For the analysis of therapeutic blood products a wide range of proteomic technologies are available. Based on the specific question and needs a rational decision between currently available proteomic strategies has to be made, which also has to consider cost issues.

Human protein samples such as plasma or blood cell populations are highly complex and thus extensive prefractionation is necessary to reduce the complexity of the protein mixture prior to mass spectrometric analysis. Prefractionation of blood cell derived samples can be accomplished by purifying subcellular protein fractions as shown for erythrocyte membranes[14-16] and subcellular organelles of platelets[17,18]. For plasma-based samples highly abundant proteins such as albumin or immunoglobulins can be removed to increase the coverage of lower abundant plasma proteins[19].

Reduction of the complexity of protein/peptide samples can also be achieved by using the physicochemical properties of proteins/peptides relying either on gel-based strategies like two-dimensional gel electrophoresis (2-DE)[20], or non gel-based strategies, combining different types of chromatography with online mass spectrometric analysis[21]. Yet another approach is the combination of SDS-PAGE with chromatographic prefractionation and online mass spectrometric analysis[21].

Proteomic approaches based on 2-DE such as differential in gel electrophoresis (DIGE) have several advantages for the study of stored blood cells because they offer a convenient approach for comprehensive and, most important, also quantitative monitoring of proteins at different time points[22]. Since they utilize separation at the protein level, gel-based approaches also offer easy detection and assessment of protein degradation[20]. However, this sensitive prefractionation at the protein level is also responsible for limitations in resolution of proteins with either very low (< 10 kDa) or high molecular mass (>> 250 kDa). Probably the biggest disadvantage of gel-based methods is their inability to analyze membrane proteins, which, however, are very important for many functions of blood cells.

Current gel-free proteomic applications also cover membrane proteins and accomplish a deeper coverage of proteins in cell- or plasma preparations. For most precise quantification gel-free methods make use of *in vivo* labeling approaches such as stable isotope labeling with amino acids in cell culture (SILAC), a technique developed by Ong *et al*.[23]. In SILAC labelled protein samples are added as an external standard to the samples of interest immediately after collection, thus eliminating or strongly reducing bias during the process of sample preparation[24-28]. Unfortunately, SILAC is not applicable to cell types which cannot be cultivated for sufficiently long time periods in defined cell culture media, e.g. platelets, which due to the lack of a nucleus are only capable of limited proteins synthesis in culture. Therefore, alternative methods have to be used that introduce a label by chemical or enzymatic incorporation after sample preparation and digestion such as isobaric tags for relative and absolute quantification (iTRAQ)[29].

Recently, labelfree mass spectrometry-centered quantification methods came into fashion, which require dedicated software packages and then rely on quantification of the intensity values recorded in the mass spectrometric measurements. However, gel-free and mass spectrometry-centered proteomics approaches focus on the analysis of peptides obtained from the original proteins by enzymatic digestion and not on the analysis of native proteins[26,29]. Hence,

compared to protein-centric approaches, these peptide-centric technologies have the drawback that they will not capture information on concomitant occurrence of multiple posttranslational protein modifications on different peptides or protein degradation.

However, specific techniques such as Combined Fractional Diagonal Chromatography COFRADIC[30-32] have also been developed which provide insights into protein degradation by specifically analyzing N-termini of proteins. Thus, even if 2-DE based approaches are still of great value particularly for time course experiments, with the further improvement of mass spectrometry-centered gel-free approaches they will likely be replaced in the future mainly because of the superior sensitivity of the latter techniques.

Improving storage of blood products

Ideally, stored blood products should not require cost intensive logistics and should maintain their function over a reasonable amount of time. Storage conditions vary significantly between different blood products, but most products need a rather narrow temperature range for optimal preservation. For example, red blood cell concentrates have to be kept at 4 °C ± 2 °C for a maximum of 49 days, platelet concentrates at 22 °C ± 2 °C for not more than five days and fresh frozen plasma at less than –30 °C not exceeding 36 month of storage. This requires an expensive infrastructure for blood banks which is especially challenging to realize in less industrialized countries.

Improving blood storage and extending storage time of blood products will have major clinical and economical impact. Proteomics has already led to a number of new observations that will help to guide the development of new strategies for blood storage (see below). The major contributions of proteomic studies of stored blood products will be discussed throughout the following chapters.

Proteomics and red blood cell storage

Proteomic studies have evaluated different storage conditions for red blood cells (RBCs). Anniss and coworkers compared supernatants of leucodepleted and non-leucodepleted RBC concentrates (RBCCs) and found potentially bioactive proteins accumulating in supernatants of non-leucodepleted products. In leucodepleted products, however, a higher number of RBC derived proteins accumulated[33].

Storage under aerobic or anaerobic conditions was investigated by D'Amici *et al*.[34]. Oxidative stress was found to be a major cause leading to protein alterations in stored red cells. Hence, protein degradation significantly decreases when RBCs were stored in an oxygen-reduced environment.

Bosman and colleagues found a storage-associated increase in haemoglobin binding to the RBC-membrane and aggregation as well as degradation of the membrane protein band 3[35]. These authors concluded that membranes of erythrocytes show an age-related membrane remodeling, which may affect RBC-clearance after transfusion.

RBC-microparticles were the subject of a study by Rubin *et al*. who showed that microparticles with a changing proteome pattern are released from erythrocytes during storage[36].

Proteomic data of stored RBCCs have revealed, that RBC-storage is connected with a rearrangement of RBC-membranes, an exchange of biologically active proteins between RBCs and the storage media and that different storage conditions such as leucodepletion or an anaerobic environment have an impact on RBC-biology. However, these experiments are only the starting point for more extensive proteomic research in this field. This will most probably result in a more complete definition of optimal storage conditions for RBCCs.

In this context, proteomic studies might add important information to the current controversial discussion on the impact of storage age of RBCs on adverse outcomes[37].

Whereas some studies[38] describe a negative impact on patient outcomes related to the storage age of RBCs, other studies did not find any effect[39]. Identification of RBC-storage lesions on the molecular level by proteomic studies might be instrumental to generate new models of how stored RBCs may cause adverse effects. Furthermore, proteomic studies will be important to define factors influencing RBC storage lesions. This may finally help to reduce storage lesions of RBCs and potentially even increase the storage time.

Proteomics and platelet storage

Platelet concentrates (PCs) are instrumental for maintaining primary haemostasis in severely

thrombocytopenic patients e.g. patients undergoing high dose chemotherapy. Currently, PCs have a rather short storage time of 4-5 days only, primarily because they are stored at room temperature which increases the risk of bacterial growth in these blood products.

Actin was the first protein identified by a proteomic study being changed during PC-storage[40,41]. It was later demonstrated that actin polymerization differs between different preparation methods of PCs and that actin polymerization is a reversible process when platelets were rested after preparation of the PC[42,43].

The possibility to assess the impact of different manipulations on platelet integrity in PCs was one of the first questions for which proteomic methods have been applied in transfusion medicine. This required techniques allowing precise and reproducible quantification of protein changes within a single PC over time. Applying DIGE to analyze protein changes in platelets during storage, we found that more than 99% of the observed protein spots did not change significantly over five days of storage and about 97% still remained stable after nine days[22]. Thon *et al*. combined DIGE with two gel-free quantitative proteomic approaches and showed superior proteome coverage based on the complementary nature of the applied methods but also found the vast majority of platelet proteins being stable during storage[24].

The influence of platelet storage on the PC supernatant was investigated by Glenister and colleagues. They found several cytokines being released from platelets during a storage period of 7 days[44].

More recently, platelet storage was used as a stress model to further investigate changes in platelets of diabetic patients when these platelets were stored *ex vivo* under blood bank conditions. In comparison to platelets of non diabetic blood donors, stored platelets of individuals with type II diabetes showed several changes in their proteome[45]. This study has two implications. One is that this approach can be used to study certain metabolic alterations in patients with diabetes type II using their *ex vivo* stored platelets as a relatively easy obtainable model system. The other implication is that it may allow identification of critical proteins in platelets which deteriorate under stress conditions and therefore require special attention in storage studies as these proteins may serve as markers for "high risk platelets" that render platelet transfusion less effective or even unsafe.

Several interacting platelet proteins were identified to change during PC storage using proteomics, such as zyxin, vinculin, talin, or alpha-actinin[46]. These proteins are players in the GPIIb/IIIa-activation pathway which is essential for platelet aggregation. Thus, proteomics has already identified functionally relevant proteins which change during storage of PCs[47].

Now it needs to be shown whether these markers are suitable indicators of storage lesions. If they do so, the observed platelet storage markers could be implemented into routine-quality testing of platelet concentrates. In more easy to perform assays such as an ELISA it is likely that monitoring of these marker proteins will also have an impact on the development of new methods for PC production and storage. Potentially, these assays may in part substitute current *in vivo* platelet survival studies using radiolabeled platelets in volunteers.

Several concepts of platelet cold storage were developed to maintain platelet integrity during prolonged storage and to reduce bacterial growth. Unfortunately, platelets stored at temperatures below 15 °C perform very poorly *in vivo* which was found to be based on an elevated clearance of transfused cold stored platelets[48]. While galactosylation was shown to prevent this effect *in vitro*[49], *in vivo* galactosylated and cold stored platelets showed only poor survival[50,51]. Proteomic studies might give some new impulses for storage conditions of platelets; however, to date they were not applied for the investigation of PCs stored at temperatures below 15 °C.

Although proteomic methods provide currently the most comprehensive assessment of platelet storage, they reveal only limited information on the functional activity of proteins.

Therefore, proteomics and functional assays are complementary and the correlation between current applied quality testing and proteomics needs further evaluation.

Proteomics in plasma storage

Tremendous progress has been achieved in the field of plasma proteomics[52,53]. To date more than 10,000 distinct proteins have been identified in human plasma[54,55]. This is not surprising as plasma is one of the easiest to obtain clinical specimen and serum or plasma samples are a key for many diagnostic procedures.

Although transfusion of human plasma is a basic treatment for severe coagulopathies or major bleeding and thus a frequently used blood product, there are only few proteomic studies investigating plasma used for therapeutic purposes. Under current blood bank conditions, plasma is stored at –30 °C. Under these conditions it is unlikely that plasma proteins change during storage. However, the required logistics of a cold chain for frozen plasma is challenging for many health care systems in less industrialized countries.

Additionally, thawing of fresh frozen plasma prior to transfusion is time consuming and hinders the release of plasma in emergency situations. We therefore applied quantitative proteomic technologies to study *in vitro* characteristics of lyophilized plasma after 24 months of storage and found that plasma lyophilization has no impact on the plasma proteome[56]. Thus, lyophilized plasma might be an attractive option to provide the most important basic treatment for severe coagulopathies in areas without cold chain and to provide plasma without time delay due to thawing in emergency situations.

Recently, it has been suggested to store plasma in liquid form after thawing at 4 °C for several days even after long time storage at –30 °C[57]. It was demonstrated that functional clotting assays remain remarkably stable over several days of storage of liquid plasma. If proteomics of liquid stored plasma confirms stability of plasma proteins, liquid plasma might become a prosperous option for managing patients with major blood loss.

Improving safety of blood products

Prevention of pathogen transmission by blood products

Prevention of pathogen transmission was the main area of research in transfusion medicine since the early 1980s. Beside the establishment of strict selection criteria for blood donors and standardized skin disinfection procedures before venipuncture, pathogen reduction of blood products is now an emerging topic. Pathogen reduction technologies (PRTs) are already available for plasma-derived therapeutics and for platelet concentrates. The latter are mainly based on irradiation with UV-light and / or pretreatment with a photochemical agent. In this regard three questions are important: i) whether PRTs damage functionally relevant proteins in blood products which could lead to a loss of function; ii) whether there are differences between the different PRT approaches; and iii) whether photochemical treatments have the potential to create neoantigens by modifying proteins in the blood product. The latter bears the risk to induce antibodies as already observed when PRTs were applied to RBCCs[58,59]. However other studies indicate only a low risk for antibody formation after transfusion of pathogen reduced PCs[60,61].

Proteomics of PRT in plasma products

Methylene blue treated plasma was studied by Crettaz *et al*., revealing that the gamma chain of fibrinogen, apolipoprotein A1 and transthyretin were affected by this treatment[62]. However, the concentrations of methylene blue leading to changes in this experiment were higher compared to the dose used under routine conditions and highly abundant proteins were not removed making it difficult to judge changes in low abundant proteins.

Also the effect of solvent/detergent-treatment on pooled plasma has been investigated using gel-based proteomics, which showed the known alterations in α1-antitrypsin, α1-antichymotrypsin and α2-antiplasmin[56].

With the large experience in plasma proteomics, the available quantitative proteomic approaches should be applied to further assess protein changes in plasma induced by PRTs.

Proteomics of PRT in platelet concentrates

Current available PRTs for PCs employ the addition of a photochemical agent, followed by irradiation with UV-light[63,64]. Proteomic analyses of effects on platelets caused by these PRTs, which are clinically approved, have not been reported so far. However, a novel pathogen reduction technology using UVC-light was studied using DIGE[65]. The cytosolic platelet proteome was compared after UVC-irradiation and after treatment with other well established phototreatments, namely UVB- and gamma-irradiation. Gamma-irradiation showed the most pronounced effect on the platelet proteome followed by UVB-irradiation.

The set of protein spots displaying changes in intensity was smallest after UVC-treatment. Compared to UVB- and gamma irradiation, UVC-irradiation induced only two individual changes in

spots containing the protein disulfide isomerase A4 (ERp72), and the Ras GTPaseactivating- like protein (IqGAP-2). The changes in the disulfide isomerase, however, raise the issue, whether these platelets can maintain functionally relevant disulfide bonds. UVC-irradiation in high doses was shown to disrupt S-S bonds in membrane proteins[66]. This needs to be addressed in specific functional studies.

It is likely that preclinical assessment of product changes by PRTs using proteomic technologies will become an important part of the development process for both the manufacturers as well as for the regulatory agencies and PRTs will then become a major focus for further proteomic studies.

Transfusion reactions

Severe transfusion reactions can have a devastating influence on the outcome of transfused patients. A prime example of the relevance of proteomics in transfusion medicine is the characterization of the missing antigen involved in the currently most frequent fatal transfusion reaction, transfusion related acute lung injury (TRALI).

Transfusion related acute lung injury

TRALI is one of the major causes of transfusion-associated mortality. TRALI is defined as acute respiratory distress that occurs within 6 h of blood transfusion, with new bilateral infiltrates in the chest X-ray and signs of hypoxemia in the absence of circulatory overload and other risk factors for acute lung injury[67]. Plasma-rich blood components, such as fresh frozen plasma and platelet concentrates, cause TRALI considerably more often than do plasma-poor packed red cell concentrates[68]. About 80% of reported TRALI cases have been associated with the transfusion of blood products containing unsuspected leucocyte-specific alloantibodies[69]. These alloantibodies arise in individuals lacking the antigen on their own blood cells but whose immune system is exposed to blood cells containing the antigen. While TRALI induced by antibodies is in principle a preventable complication, preventive screening of blood donors was not feasible because the most relevant antigen HNA-3a was not known. In a recent study, we combined immuno-precipitation and proteomic approaches to characterize the human neutrophil antigen 3 (HNA-3) system[70]. It was shown that HNA-3a arises from a nucleotide polymorphism in the choline transporter-like protein-2 gene. The resulting variation at amino acid position 154 determines the reactivity of the protein with HNA-3a–specific antibodies. The molecular characterization of this antigen paved the way for the development of assays for blood donor screening and to lower the risk of TRALI.

Other Transfusion Reactions

Cytokine release from blood cells into storage media is one cause of febrile transfusion reactions. Applying proteomics, the protein composition of supernatants of stored cell based blood products was investigated[33,44]. Thereby it was demonstrated that physiologically active cytokines accumulate in the storage media with ongoing storage. The molecular mechanisms for this cytokine release are not yet fully understood. A deeper insight into cytokine release reactions will help to guide improvements in storage media composition, leading to less cytokine release und improved product safety.

Neoantigen formation and immune response

It is well known that cellular blood products can induce the formation of alloantibodies, with red cells being the most prominent example. However, also plasma-based therapeutics can induce antibody formation. These antibodies seem to be especially triggered if the protein has been structurally altered, as shown for erythropoietin[71] and thrombopoietin[72]. The biggest risk for the patient is that these antibodies, although originally triggered by an altered form of the protein can then cross react with the native protein in this individual causing depletion of the protein by an autoimmune-like process.

Another, although slightly different situation is the induction of antibodies against clotting factor VIII (FVIII) in haemophiliac patients receiving FVIII-concentrates[73]. To date there is a controversy, whether structural alterations of the protein in the blood product increases the risk for antibody formation, although at least one example exists in the literature showing that a change in the production process of a FVIII-concentrate resulted in an increased rate of anti-FVIII-antibodies[74]. Especially plasma derived clotting factor concentrates contain a large number of "contaminating" proteins. It is still not clear,

whether alterations of contaminating proteins may also trigger any immune responses and proteomic approaches will likely add substantial information in this regard.

Proteomics of clotting factor concentrates

Proteomic data are available for concentrated FVIII and FVIII/von Willebrand factor-concentrates[75], FIX-concentrates[76] and prothrombin complex concentrates[77]. These studies showed a large variety of differences between products of different manufacturers but revealed low batch to batch variability of products of the same manufacturer. Furthermore, numerous protein impurities such as complement factors, clusterin or vitronectin were found in these products. Given that clotting factor concentrates are standardized only by distinct clotting factor activities, proteomic methods discovered that these therapeutics contain different amounts of inactive forms of these clotting factors[77]. Whether these inactive molecules are structurally modified or are normal constituents of human plasma should be further investigated.

In a pilot study we used gel-based proteomics to separate a plasma pool derived FVIII-concentrate (Figure 1), demonstrating that proteomic technologies facilitate a more comprehensive view on the constitution of plasma based clotting factor concentrates than currently applied methods for quality control.

Conclusions and future prospects

Proteomics has proven to be a powerful tool for the investigation of blood products and therefore proteomic applications are increasingly applied in transfusion medicine. Proteomic science adds a new perspective here in providing comprehensive overviews on the protein constitution of blood products. However, it is obvious that the biological relevance of the complex changes observed has to be proven in the context of blood transfusion as a next step.

In contrast to blood products obtained from large plasma pools or those produced recombinantly, such as enriched clotting factors, cellular blood constituents will always show certain differences between each other when analyzed by proteomic methods due to the biological diversity of blood donors who are genetically different individuals. Moreover, laboratory-to-laboratory variations in proteomic analyses potentially limit the comparability of proteomic studies. However, these inter-laboratory differences are being recognized as a challenge by the proteomics community and robust reproducibly workflows are being developed[78]. It is now the challenge to identify those parameters and signals within the thousands of proteins within each blood product, which are important for sufficient function, low immunogenic potential, and product stability.

One of the most promising areas for proteomics in transfusion medicine is the use of proteomic

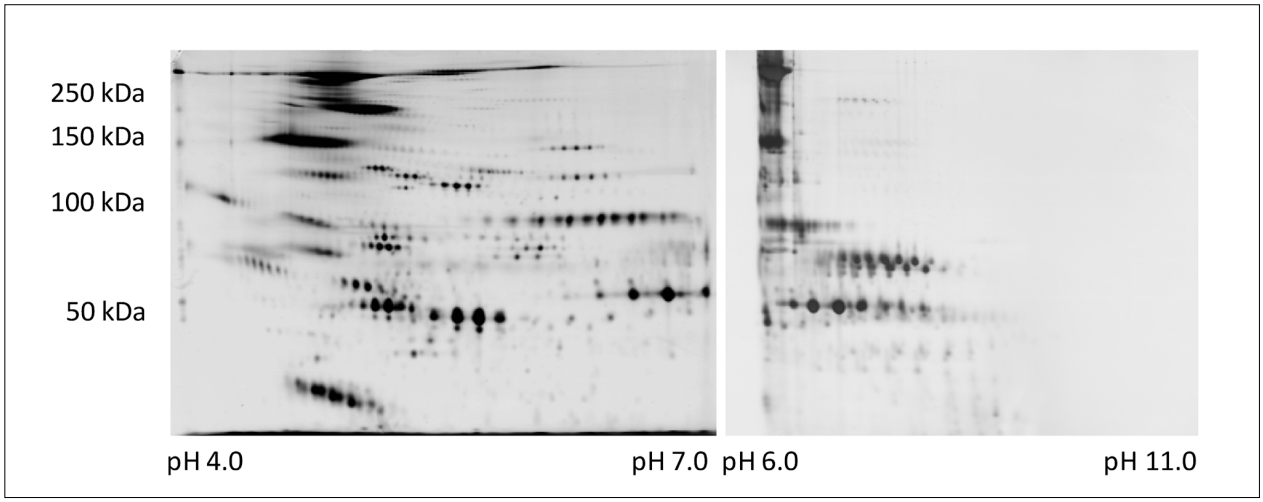

Figure 1 - Example of a two-dimensional gel pattern of a plasma derived FVIII-concentrate. Image of silver nitrate stained proteins in a representative FVIII-concentrate separated by twodimensional electrophoresis using pH gradients 4-7 and 6-11. Polyacrylamide gels contained 7.5 % acrylamide to achieve a better separation of high molecular weight proteins. The image demonstrates that several other proteins are present in a FVIII-concentrate. Due to its high molecular weight FVIII itself was not detected in this study.

technologies to gain preclinical data on newly developed blood products. As a consequence, researchers with knowledge in the field of blood proteomics and transfusion medicine need to find a consensus on how to apply the powerful methods of proteomic technologies to analyze blood products in transfusion medicine. This requires certain standards of how to handle the plasma and cellular products during the preanalytical preparation, the performance of proteomic experiments, and the ways how to interpret proteomic data. A reliable concept of how to perform and interpret proteomic studies in the context of transfusion medicine will be of utmost importance for a meaningful use of proteomic technologies: i) to understand the impact of certain steps in production, processing and storage of blood products; ii) as an instrument for quality control; iii) as a tool to gain a more deeper insight into the molecular characteristics of new production or pathogen reduction technologies before the application of these products to humans. Such a consensus is a requirement for including proteomic data into the preclinical evaluation of new preparation methods for blood products.

Finally, it is important to be aware that ultimately only clinical studies will unfold differences in transfusion outcomes. Thus, proteomic and clinical data will have to be evaluated concomitantly in order to draw biologically relevant conclusions on blood derived therapeutics. Thereby proteomic data will help to formulate hypothesis for clinical trials. Additionally, insights of the mechanisms leading to clinically observed effects of blood products might be provided by proteomic experiments.

Proteomics and transfusion medicine are disciplines complementing each other and researchers of both fields should form an alliance to improve blood products and to solve transfusion related issues in the upcoming years.

Key words: proteomics, transfusion medicine, pathogen reduction, blood supply, immunogenicity.

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