

Proteomics: applications in transfusion medicine

Giancarlo Maria Liumbruno

Servizio di Immunoematologia e Medicina Trasfusionale, AUSLN. 6–Livorno, Italy.

Introduction

Since the completion of the mapping of the human genome^{1,2}, which allowed the identification of over 30,000 genes, continuous efforts have been made to associate the data acquired with DNA functions. New tools for analysing these data have been developed and new disciplines of study have been generated to explore the whole range of the potential applications of human genome-related information; the names of all these new fields of study end in *omics*.

Genomics is the comprehensive analysis of DNA structure and function, whereas *transcriptomics* is the study of the mRNA pool found within a cell and describes gene expression³, *proteomics* instead studies the location, structure and function of proteins expressed in a biological system⁴.

The genome is the total chromosome (hereditary) content of a biological system, while the proteome is all the proteins that the genome produces through biological transcription and translation⁵.

The term "proteome" (PROTEins expressed by a genOME) was coined by Wilkins and colleagues in 1996⁶. Initially the word proteomics referred to the techniques used to analyse a large number of proteins at the same time, but, at present, this word covers any approach that yields information on the abundance, properties, interactions, activities, or structures of proteins in a sample⁷.

The name "protein", derived from the Greek term *proteios*, meaning "the first rank", was used for the first time by Berzelius in 1838 to emphasise the importance of these molecules⁸. The number of proteins produced by the 30,000–40,000 genes of the human genome is estimated to be three or four orders of magnitude higher⁹. The reasons for this numerical superiority and complexity are^{4,10}: i) differential splicing of mRNA gene transcripts, which allows

a single gene to produce multiple protein products; ii) the capability many proteins have of associating with other proteins to form complexes; iii) post-translational modifications, which are additional changes that proteins initially translated within a cell may undergo. These are covalent modifications that regulate protein functions, determining their activity state, cellular location and dynamic interactions with other proteins; the most important and best-studied post-translational modifications are phosphorylation and glycosylation, but many others are common (acetylation, methylation, lipid attachment, sulphation of tyrosine, ubiquitination and disulphide bond formation) among over 300 different known types.

The genome, compared to the proteome, is stable^{4,5}; the proteome, on the other hand, is dynamic and changes based on the type and functional state of a cell. The number of proteomes that can be defined within a biological system therefore increases as the complexity of the latter becomes greater⁷; indeed the number of proteomes within a cell is much lower than the number within a tissue and really much lower than the one within an organism. This makes proteomics a challenging field, largely due to the sheer size of the proteome and the volume of data that can be generated by it¹¹.

Transfusion medicine is a clinical discipline characterised by one of the most advanced quality management systems, which is structured so as to assure the production of blood components and *raw materials*, for biopharmaceutical fractionation, which are safe, efficient and effective¹². In Italy, the collection and production procedures performed at blood banks are closely regulated by State laws and/or directives issued by government agencies. At present, proteomics seems to be the most promising tool for global quality assessment of the production process of blood components and blood derivatives.

The potential role of proteomics in transfusion medicine has been addressed in several articles, since 2004^{4,7,12-15}. The objective of this review is to provide a brief overview of contemporary proteomics technologies and published studies on their applications in transfusion medicine, which are mainly the characterisation of blood product proteomes and their modifications caused by production or storage processes.

Proteomics technology

Proteomics in transfusion medicine exploits several technologies such as two-dimensional polyacrylamide gel electrophoresis (2D-GE) and other non-gel based separation techniques, mass spectrometry (MS), and protein microarrays.

The first step of proteomics is the purification of proteins from cell or tissues and their separation; after protein separation using a technique such as 2D-GE, the gel is stained and the single protein "spots" are digested by an enzyme to yield peptides, which are then analysed through MS. The identity of the digested protein is determined based on the pattern of the resulting mass spectrum⁴.

Gel-based separation techniques

The central method for proteomic analysis is 2D-GE, which has been available since 1975 and is still a state-of-the-art research tool. This technique was developed to separate complex protein mixtures into individual components and is based on orthogonal separation of proteins by isoelectric point and molecular weight^{16,17}. After 2D-GE, the separated proteins are visualised by staining with colloidal Coomassie brilliant blue (CBB), or the more sensitive silver nitrate, or by the use of a pre-labelled sample obtained with fluorescent dyes, such as SYPRO Ruby and cyanine dyes (Cy2, Cy3, Cy5)¹⁸⁻³⁰; this last approach offers an improved sensitivity, a linear dynamic range that can exceed three orders of magnitude, and allows for a quantitative comparison of gel-based protein patterns^{7,12}. A summary of the characteristics of dyes currently used for proteome analysis can be found in an article recently published by Thiele *et al*¹².

After separation and staining of the proteins, a gel contains 1,000-2,000 protein spots even if, in theory, it is possible to display up to 10,000 protein species on a 2D gel³¹. Higher resolution separation can be achieved by using several 2D-GE with overlapping narrow pH gradients, which reduce the presence of protein per spot, thus improving the detection of low-abundance proteins^{8,32,33}.

In 2D-GE, after staining, the gels are scanned and

subjected to computer analysis through specific software, which facilitate pattern matching between gels and the relative quantification of proteins^{7,11,12,32}. Spots of interest are then excised and digested, usually with the hydrolytic enzyme trypsin, which generates peptides that, on average, have the right size to be usefully detected and sequenced through MS¹⁰.

An elegant and very attractive modification of the traditional 2D-GE is the technique referred to as difference in-gel electrophoresis (DIGE), invented by Unlü and colleagues, which uses the fluorescent cyanine series of dyes and allows relative quantitative analyses of up to three samples^{7,8,12,28,34}.

The advantages of 2D-GE include its relatively low cost and ease of use, the capacity to combine high-resolution power with the ability to quantify and assess protein modifications, such as the substitution/modification of a single amino acid, and the possibility of automation with robotic spot pickers. Its main limitations are that: i) it is not a high-throughput technique; ii) it is not suitable for the separation of membrane proteins; and iii) its sensitivity is not satisfactory for low-abundance proteins^{7,12,15}. Highly abundant proteins, such as albumin and immunoglobulins in plasma (or actin in cell-derived samples), mask low-abundance proteins by obscuring their signals in 2D-GE. This is an important issue for proteomic analysis in the field of transfusion medicine, as the dynamic range of protein concentrations in human plasma samples extends over ten orders of magnitude, separating albumin, at the high-abundance end (normal concentration range 35-50 mg/mL, or 35-50 x 10⁹ pg/mL) and the rarest proteins now measured clinically at the low-abundance end, such as interleukin-6 (normal range 0-5 pg/mL)³⁵.

Prefractionating samples into cellular components or into water-soluble, peripheral and integral membrane-proteins, is a strategy to increase the sensitivity and the relative quantification of low-abundance proteins^{7,8,32}. Several methods have been proposed over time^{11,36-42} to address this limitation of 2D-GE, but multiply affinity columns are the approach most commonly used^{36,43-52}. The most important limitation of using such columns is the risk of eliminating low-abundance proteins at the same time¹⁵.

Non-gel based separation techniques

Non-gel based separation techniques provide information not accessible using two-dimensional gel separation, such as the detection of low-abundant or hydrophobic (membrane) proteins, and are at present complementing and even partially replacing 2D-GE. These alternative approaches mainly rely on the two-dimensional

separation of peptides, rather than proteins, and are focused on the subsequent characterisation of protein/peptide mixtures by MS^{12,53}. Other significant advantages of these techniques over 2D-GE are: i) the potential high-throughput capabilities; ii) the possibility of full automation; iii) direct integration with MS; iv) higher sensitivity; and v) the smaller amount of starting material needed.

Liquid chromatographic methods are most commonly used to fractionate samples on the base of two or more biophysical characteristics (determining selective binding to a column matrix), such as surface charge (ion exchange chromatography), hydrophobicity (hydrophobic interaction chromatography), or affinity to particular compounds [affinity, dye ligand, reversed-phase liquid chromatography (RPLC)]^{7,12,53-55}. Protein samples are first partially degraded with a protease such as trypsin, which reduces them into smaller components and thus increases the binding of the peptides to the chromatographic column matrices and allows for direct analysis of samples through MS, after elution from the column^{7,10,12}.

A promising application of two-dimensional liquid chromatography (2D-LC) is multidimensional protein identification technology (MUDPIT)^{56,57}. The protein sample is reduced, alkylated, and digested in solution; the resulting peptide mixture is fractionated through 2D-LC using a strong cation exchange column, which separates peptides on the base of their charge, and the derived fractions are further separated, on the base of hydrophobicity, on a reversed-phase (RP) column. MUDPIT is used to fractionate highly complex protein mixtures and, currently, seems to be the best choice for analysing samples that cannot be efficiently resolved on gels because of their physico-chemical properties. The chromatographic column is coupled on-line with a mass spectrometer equipped with an electrospray ionisation (ESI) source, which ionises samples from liquid phase, in the so-called LC-ESI-MS technique, which is fully automated, even in database searching^{7,10,12}.

Another method, typical of the so-called shotgun proteomics which has the aim of decreasing the time required for analyses⁵⁸, is combined fractional diagonal chromatography (COFRADIC)⁵⁹. In COFRADIC sets of representative peptides are isolated out of a complex tryptic digest through a specific modification of their side chains, performed between two identical RP-LCs; these peptides shift away from their original elution position and are collected, so that the complexity of the resulting peptide mixture is reduced⁶⁰.

Another approach, used for the in-depth analysis of the membrane and cytosolic proteome of red blood cells

(RBCs), combines a protein-based pre-fractionation by conventional sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a peptide-based separation of the tryptic digests of SDS-PAGE gel slices, which are analysed by capillary LC coupled on-line with tandem MS⁶¹.

In conclusion, it must be said that none of these methods enable conclusions to be drawn regarding relative protein concentrations⁷.

Mass spectrometry

MS has been used for the analysis of proteins and peptides since 1989, when ESI and matrix assisted laser desorption ionisation (MALDI) were introduced^{10,62,63}. These two techniques, for which the inventors both received the Nobel Prize in Chemistry in 2002⁵³, are used for gas phase ionisation of biomolecules such as peptides, proteins, metabolites and oligonucleotides, and are defined "soft", because they do not break covalent bonds within the sample⁸.

The main components of a mass spectrometer are an ion source (the device that brings the analytes into gas phase and ionises them), one or more mass analysers, which is the central component and measures the mass-to-charge ratio (m/z) of the ionised analytes, and a detector, which registers the number of ions at each m/z value.

In ESI ions are formed from a liquid solution, while in MALDI a laser pulse induces the sample to sublimate out of a dry crystalline matrix. Gaseous ions formed by MALDI and ESI are accelerated into the mass analyser by an electric potential, and the m/z ratios are determined by the motion of the ions through the mass analyser. The detector converts the stream of ions into a voltage that is interpreted by a computer and converted to a mass spectrum^{4,7,8}.

Examples of mass analysers currently used in proteomics are: time-of-flight (TOF), quadrupole (Q), triple quadrupole or linear ion trap (LIT), ion trap (IT), Fourier transform ion cyclotron resonance (FTICR), and Orbitrap⁶⁴. Two analysers can be placed in sequence to perform two-stage MS in the so-called tandem MS, commonly referred to as MS/MS^{4,10}.

The TOF analyser separates ions based on the differences in transit time (time of flight) from the ion source to the detector in tubes under vacuum. The Q analyser transmits only ions with a narrow m/z range and uses the stability of the trajectory to separate them according to their m/z ratio on four parallel cylindrical metal rods. In the triple quadrupole or LIT, ions of a particular m/z are selected in a first section, fragmented in a collision cell, separated and captured in the third section, where they are excited

through resonant electric fields and scanned out creating the tandem mass spectrum. The IT analyser captures or traps the ions, which are then subjected to MS or MS/MS analysis. FTICR mass spectrometer is also a trapping device operating under high vacuum in a high magnetic field. The recently introduced Orbitrap provides high resolution, high-mass accuracy, and good dynamic range; its detection method is similar to that used in FTICR.^{65,66}

MALDI is usually coupled to TOF analysers, which measure the mass of intact peptides⁷; it has also been implemented in TOF-TOF mass spectrometers to provide true MS/MS capabilities. ESI has mostly been coupled to IT and Q instruments, which exhibit high resolution and mass accuracy in MS and MS/MS modes⁶⁴. The ESI-triple/quadrupole instrument is generally used to generate data from peptide fragments⁷.

MS is highly sensitive, requiring only 10-15 femtomoles of peptides as starting materials, and extremely accurate, being capable of resolving ions that differ by only 0.001 atomic mass units in terms of m/z ⁶⁷.

In conclusion, a typical set-up for proteome analysis requires: i) highly reproducible and standardised sample preparation; ii) extensive pre-fractionation of the sample, at either the protein or peptide level, after digestion of protein mixtures with specific proteases; iii) protein-based separation (gel-based techniques) or peptide-based separation (non-gel based techniques) approaches; iv) MS characterisation of enzymatically generated peptide mixtures.

After determination of the peptide masses and their fragmentation patterns, publicly available [e. g. Swiss-Prot (<http://www.ebi.ac.uk/swissprot/>), National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi>), IPI (<http://www.ebi.ac.uk/IPI/Databases.html>)] or in-house databases are then interrogated, through dedicated software, with the peptide mass fingerprints and the fragmentation data, to identify proteins and their modifications¹².

The protein is identified by comparing the peptide mass fingerprint with peptide masses obtained from theoretical digestion in protein sequence databases. If protein identification through database searching is unsuccessful, further MS is possible to improve protein identification. Specific peptide ions are selected and subjected to further fragmentation within the mass spectrometer in the tandem-MS: typical approaches are either MALDI-TOF tandem MS or the on-line combination of LC with ESI-MS/MS. The protein is then identified through specifically developed computer algorithms^{7,8,10,12,32}.

Mass spectrometry-based quantitative analysis

2D-GE can provide quantitative data through densitometry, which can be used to compare spots between different gels or the same gel, if DIGE is performed. One significant limitation of MS is the lack of quantitative information, which is crucial for analysing many properties of the proteome, and the biological effects induced by perturbations of the system^{7,8,11}.

Several MS-based strategies have been developed for quantitative proteomic analysis; these techniques also allow relative quantification of basic, hydrophobic, or large proteins excluded by 2D-GE or DIGE. These methods involve the differential labelling of two or more sets of proteins or peptides, derived from different cell states, with light and heavy isotopes of the same chemical reagent, and subsequent MS. The rationale is that chemically identical analytes of different stable-isotope composition can be distinguished in a MS due to their mass difference; indeed the light- and heavy-labelled peptides appear in the mass spectrum as doublets, and the peak height comparison enables the calculation of the relative abundance of the protein in each cell state^{7,8,11,53,64}. This allows for the measurement of slight changes (< 2-fold) in abundance between samples⁵³.

Current methods for protein quantification mostly involve the use of isotope-coded affinity tags (ICAT); next generation techniques, such as isobaric peptide Tagging system Relative and Absolute Quantitation (iTRAQ), label all primary amines. Another approach involves metabolically labelling peptides, either by synthesis or by growing cells with labelled amino acids [Stable Isotope- Labelling with Amino acids in Cell culture (SILAC) method]; other alternative quantification methods currently in development are attempting to avoid the labelling process entirely^{7,11,53}.

Protein microarrays

Protein-protein interactions play an integral role in every biological system. Two methods can be used to analyse these interactions in a high-throughput fashion: yeast two-hybrid screening and peptide/protein arrays. As the former technique, recently reviewed by Page and Colleagues⁷, has several limits for the study of human proteins, array technology has been suggested as an alternative. The term microarray is often used interchangeably with the term biochip and there is also some confusion about the "probes" and the "samples": in most cases the molecules of known identity, immobilised on the microarray surface, are termed

probes, and the investigated mixtures of molecules, though labelled in many cases, are called samples⁶⁸.

Protein chips differ from previously described methods, which can potentially detect any protein, because they can only provide data on sets of proteins selected by the investigator⁶⁴. The chips consist of a glass or plastic surface spotted with a checkerboard-like grid of molecules (> 100 probe elements/cm²) designed to capture specific proteins at specific sites^{4,69}. Advanced fluid systems have been developed to deliver micro-volumes of reagents and samples to the chip, where bound molecules are detected through a secondary antibody tagged with a fluorescent marker or directly, if the protein sample has been fluorescently labelled. Scanners are required to read the chips, and dedicated software is used to analyse and interpret the data^{4,7}.

The capturing agents on the chip surface, in addition to the classic monoclonal and polyclonal antibodies, include⁷⁰: i) affibodies, which are robust affinity proteins based on the structure of protein A; ii) ankyrin repeat proteins, which are highly thermostable members of large combinatorial libraries, mimicking natural ankyrin repeat proteins in *Escherichia coli* cultures⁷¹; iii) aptamers, which are short strings of single-stranded DNA or RNA molecules, that are chemically synthesised and can form tertiary structures capable of recognising and binding non-nucleic acid structures (proteins) with very high affinity and specificity⁷²; and iv) photoaptamers, which are modified aptamers that bind covalently to the target molecule, resist more stringent washing conditions, and have lower background levels^{4,7,11,73}.

Although microarray sensitivity and reproducibility can possibly be improved, the availability of a wide spectrum of protein-binding molecules has several diagnostic applications and allows mapping of the interactions of proteins with various other molecules, identification of potential disease biomarkers, especially in the area of cancer biology, and screening for potential drug targets^{4,8,11,32,68,69}.

Coupling microarray protein fractionation with surface-enhanced laser desorption/ionisation (SELDI) TOF-MS provides a powerful system for analysing differential protein expression on spot arrays. Unlike other MS-based strategies, the SELDI approach does not require pre-processing (enzymatic digestion) of the sample; however, the key disadvantage is that the mass spectrum obtained does not enable the direct identification of the proteins analysed, and additional work is required^{8,11,32}.

A rapid and cost-effective test for the diagnosis of

thrombotic thrombocytopenic purpura (TTP) using SELDI-TOF-MS was recently been developed; this assay is capable of quantifying ADAMTS-13 activity as low as 2.5% in plasma within 4 hours, and of determining the inhibitory titre of ADAMTS-13 antibody in cases of acquired TTP⁷⁴.

MALDI-TOF-MS has also been applied to microarray technology^{8,11,68}.

Other approaches for global analysis of protein functions are provided by chemical proteomics in the form of activity-based protein profiling (ABPP); this strategy is useful for determining the activity of specific classes of enzymes in complex proteomes, or for the study of membrane proteins that elude conventional proteomic characterisation^{7,8,75,76}.

Proteomics in transfusion medicine

Red blood cells

The first proteomic study of RBCs dates back to 1981 and was performed by Roseblum, who applied 2D-GE to the study of RBC-membrane proteins in normal adults, neonates, and patients with erythrocyte membrane disorders⁷⁷. Later, three studies used different mass spectrometric techniques to analyse the RBC proteome⁷⁸⁻⁸⁰. In 2002, Low and Colleagues, using one-dimensional SDS-PAGE and 2D-GE in combination with MALDI-MS, identified a total of 102 protein spots on 2D gels and 25 additional proteins found exclusively on one-dimensional gels⁷⁸. In 2004, Kakhniashvili analysed the RBC proteome by IT-MS/MS coupled on-line with RP-LC and identified a total of 181 unique protein sequences: 91 in the membrane fractions and 91 in the cytoplasmic fractions⁷⁹. In 2005, Tyan identified 272 proteins by profiling RBC proteins using proteolytic digestion chips followed by two-dimensional ESI-MS/MS⁸⁰. More recently, Pasini and co-workers combined a protein-based pre-fractionation by conventional SDS-PAGE with a peptide-based separation of the tryptic digests of SDS-PAGE gel slices, which were analysed by capillary LC coupled on-line with MS/MS, using Q and FTICR mass spectrometers; in this study they identified and validated 314 membrane and 252 soluble proteins⁶¹.

Proteomics was also successfully employed to profile the erythrocyte membrane proteins from patients with sickle-cell disease. Through DIGE followed by in-gel tryptic digestion and LC coupled with MS/MS, performed with an ESI-IT device, the authors identified 44 forms representing different modifications of 22 original protein sequences⁸¹.

Further proteomics studies analysed: i) the structure-function relationship in the erythropoietin receptor signalling complex, and the protein involved in signalling

pathways, using phosphoproteomics to identify post-translational modifications characterised by phosphorylation, which is the most frequent post-translational modification involved in protein regulation and signal transduction⁸²; ii) the surface proteins of malaria-infected RBCs (identifying two novel surface proteins), and the life cycle of *Plasmodium falciparum*, through MUDPIT^{83,84}; iii) the influence of type 2 diabetes mellitus on RBC membranes, a restricted number of which were shown to be dysregulated⁸⁵; and iv) the dynamic changes in transcription factor complexes throughout erythroid differentiation, employing non-gel-based quantitative ICAT⁸⁶.

At present, only two studies have addressed the changes that RBCs undergo during storage. Annis *et al.* carried out a proteomic analysis of supernatants of stored RBC products⁸⁷, whereas Zolla's group recently published an article on proteomic analysis of RBC membrane protein degradation during blood storage in the *Journal of Proteome Research*⁸⁸.

In the first study, proteomics was used to identify proteins that accumulate in supernatants, as storage time increases, and to determine the effect of pre-storage leucofiltration on the number and types of proteins present in the supernatants of stored RBC units. Supernatant samples of standard non-leucoreduced RBC units (S-RBC) and pre-storage filtered RBC units (F-RBC) were collected at fortnightly intervals until unit expiry, at day 42, and analysed using 2D-GE, with detection by CBB staining, combined with LC-ESI-MS/MS identification. Much lower amounts of proteins were present in the supernatants of F-RBC and, although the majority of proteins identified by MS were common to both types of RBC concentrates, transthyretin (a transport/binding protein), Ig k-light chain (Igk), serum amyloid P (SAP), and connective tissue activating peptide III (CTAP-III) accumulated predominantly in S-RBC, whereas cytosolic enzymes such as carbonic anhydrase I and thioredoxin peroxidase B were found to accumulate in F-RBC. The unexpected increase of serum proteins, such as transthyretin, Igk, and SAP, can be explained by the fact that they are absorbed on the cell surface and then released during storage¹⁵. CTAP-III is a cleaved peptide of platelet basic protein and promotes neutrophil adhesion⁸⁹; its presence is relevant in the context of recently published in-vitro studies on the role of prestorage white blood cell (WBC) reduction in abrogating the pro-inflammatory response elicited by supernatants from stored RBCs⁹⁰, and on the influence of storage duration and WBC burden in increasing the number and strength of

adhesion of RBCs to vascular endothelium⁹¹. The accumulation of cytosolic proteins in the supernatant of F-RBC is, on the other hand, explained by storage-related haemolysis^{15,87}.

The second study was undertaken with the aim of understanding and describing the changes of the RBC cytoskeleton during storage of SAG-M-preserved non-leucodepleted RBC units⁸⁸. WBC reduction was not performed, in order to include any contribution of leucocytes to proteolytic cleavage and the production of active oxygen species. 2D-GE, with staining by CBB or silver, and a reversed phase-high pressure liquid chromatography-ESI-MS/MS were used to identify RBC membrane changes over storage time under atmospheric oxygen or helium, in the presence or absence of protease inhibitors; indeed, the aetiology of lesions in RBC membranes involves both active oxygen species, and proteolytic enzyme activity. A gradual increase in the number of protein spots was observed during the first 14 days of storage, followed by a decline at 42 days. In the presence of oxygen many new protein spots were generated as a result of cytoskeleton protein attack by reactive oxygen species. In contrast, only a small number of changes were related to proteolytic cleavage, which seems to play a minor role in storage lesions in comparison to protein oxidation. During the first 7 days of storage, oxidative damage was observed prevalently in band 4.2, to a minor extent in bands 4.1 and 3, and in spectrin. Protein degradation was greatly reduced in the absence of oxygen, when blood was stored under helium. In line with recent research indicating that anaerobic storage may allow a reduction in the effect of the storage lesion⁹², this study also showed that removal of oxygen is a more effective way of limiting RBC storage lesions than any chemical addition.

The proteomic approach is a valuable way to perform a global screening of storage-related RBC lesions and to study both the role of pre-storage WBC reduction on the pro-inflammatory potential of transfused RBCs and the mechanisms of the potential biological consequences on the transfusion recipient. Moreover, identifying and recognising storage-associated RBC changes may be important for evaluating protective factors against oxidative damage, to be used possibly in future storage protocols aimed at improving the quality and prolonging the shelf-life of RBC units.

Platelets

Platelets are essential cellular components in haemostasis; they are cytoplasmic fragments of

megakaryocytes and excellent candidates for proteomics; indeed, the absence of a nucleus prevents them from being studied through classic molecular biology techniques, and transcriptomics is also difficult, due to the small amount of mRNA left³.

In 1979, Clemetson used 2D-GE to analyse platelets⁹³. Nowadays, different proteomic techniques, including 2D-GE and multidimensional chromatography followed by various systems of MS identification, have been applied to identify platelet proteins, either at different stages of activation, or in different locations inside the cell³. In 2005, standards were published by the International Society on Thrombosis and Haemostasis to combine proteomic information with data from other experimental approaches, to establish a database on protein expression and function in platelets⁹⁴. Platelet proteomic studies can be grouped into two distinct but at the same time overlapping subcategories: proteomic analyses of quiescent platelets (the static platelet proteome) or activated platelets (the functional platelet proteome)⁹⁵. Numerous studies have addressed: i) the global mapping of platelet proteins⁹⁶⁻¹⁰⁰; among these studies, 2D-GE based approaches allowed the detection of nearly 2,300 distinct protein spots and the identification of more than 400 different proteins^{99,100}; this number exceeded 1,000 with the proteins detected through non-gel-based techniques, such as COFRADIC¹²; ii) the investigation of phosphorylated proteins and the impact of protein phosphorylation on signal transduction pathways/cascades¹⁰¹⁻¹⁰⁵; iii) the exploration of N-glycosylation sites on human platelet proteins; indeed glycoprotein isoforms are a prominent and functionally important group of platelet proteins, including secretory proteins and plasma membrane receptors¹⁰⁶; iv) the membrane proteome^{107,108}; v) the platelet releasate of activated platelets: this study led to localisation of novel platelet proteins in human atherosclerotic lesions¹⁰⁹; and vi) the platelet microparticle proteome¹¹⁰. Current advances in platelet proteomic studies and their application to platelet research, also emphasising the importance of parallel transcriptomic studies to optimally dissect platelet function, have been recently addressed in several articles^{95,111-113}. Platelet proteomics shows a great potential for dissecting the mechanisms involved in both thrombotic and haemorrhagic disorders³; many approaches are being used to identify new therapeutic targets for platelet-related diseases, such as coronary artery disease and atherothrombosis in general, though other groups use the proteomics of circulating platelets as a way to study pathologies that have not yet been brought in contact with

platelet physiology or pathophysiology, such as Alzheimer's disease^{67,114}.

Platelet concentrates (PCs) are of major importance for modern transfusion therapy; in Europe, they are currently prepared mainly through two different methods: i) apheresis of platelets from a single donor, using a cell separator; and ii) obtaining platelets from pooled buffy coats of four to six different donors^{12,115}. Platelet concentrates are stored at 22 °C under continuous agitation for optimal gas diffusion through gas-permeable bags¹¹⁶; they are currently stored for up to 5 days to limit the risk of bacterial contamination. Storage causes a variety of changes in platelet function and morphology, which are known as platelet storage lesions^{117,118}. At present, the quality of PC is primarily determined *in vitro* by selective methods, assessing platelet morphology, pH, or platelet function by aggregometry and flow cytometry¹¹⁹, although these techniques provide only limited information on certain features of platelet function or morphology¹¹⁵.

Protein changes occurring during the storage of PCs were initially analysed, using 2D-GE, by Snyder and colleagues, who noted storage-related changes in the pattern of 30 cytosolic and membrane proteins, and identified two actin fragments that grew significantly during 7 days of platelet storage¹²⁰.

Recently, DIGE and MS were used to analyse the time-dependent changes in the platelet proteome occurring during 15 days of PC storage, with the aim of identifying proteins that can be used as sensitive markers for storage-related changes in PCs¹¹⁵; the same study also addressed whether the method used to prepare the PC could affect the platelet proteome. The authors compared 401 cytosolic protein spots present in apheresis and buffy coat PCs, showing that the platelet proteome remained rather stable during the first 5 to 9 days of storage, whereas major alterations occurred between days 9 and 15. During the first 24 hours after donation, the apheresis PCs showed proteome alterations, probably due to the different production procedure that may allow buffy coat PCs a longer time to recover from preparation stress. The protein patterns of septin 2, actin β -chain, and gelsolin changed earlier than those of other platelet proteins, and were identified as suitable, potential surrogate markers of storage-related changes in PCs. Interestingly, septin 2 and gelsolin are altered by apoptosis, and not primarily by platelet activation, indicating that apoptosis in PCs may have an impact on platelet storage and its inhibition might become an additional approach to prolong storage time.

A more recent report describes a new method for

characterising platelet storage lesions as an approach to monitoring the *in vitro* quality of stored PC and predicting their *in vivo* performance¹²¹. The authors combined hydrophobic interaction LC and MALDI-TOF-MS/MS to analyse the supernatants of apheresis and buffy coat PC, in which they identified platelet factor 4 (PF4) and β -thromboglobulin (β -TG). PF4 and β -TG are stored in platelet α -granules and their concentrations (after 24 hours) in the supernatants of buffy coat PCs were lower than those in apheresis PCs; this suggests less activation of the former concentrates. This method is suitable for profiling supernatant peptides and proteins in stored PCs, for subsequent identification by MS, and could be useful for evaluating different platelet collection and storage protocols.

These two studies clearly show the role that PLT proteomic techniques will probably have in the near future in validating and optimising new methods/devices for collecting, processing and storing PCs.

Further validation of PCs should include the platelet membrane proteome and the phosphoproteome, and thus also the application of non-gel-based proteomic approaches¹².

Plasma

Human plasma is a blood component obtained from the fractionation of whole blood, or by apheresis collection. It is produced by blood banks as fresh frozen plasma (FFP) for the treatment of coagulation disorders, or as *raw material* for biopharmaceutical fractionation, to manufacture medicinal products¹²².

The history of human serum and plasma proteomics is closely associated with the history of proteomics itself¹². From 1977, when human plasma proteins were first profiled through 2D-GE¹²³, all the available separation techniques have been used to study human serum and plasma proteome^{35,124-127}.

Blood plasma is an exceptional proteome in many respects³⁵; it is the most complex human-derived proteome, because it contains other tissue proteomes as subsets; moreover it is the most difficult protein-containing sample to characterise, due to the large proportion of albumin (55%), the wide dynamic range in abundance of other proteins, and the enormous heterogeneity of its predominant glycoproteins. The total number of human serum proteins alone is estimated to be around 10,000¹²⁷. The Human Proteome Organisation-Plasma Proteome Project (HUPO-PPP) is attempting to put together a comprehensive analysis of plasma and serum protein constituents, and identify the location of the biological

sources of variation within individuals over time; with 35 collaborating laboratories and many analytical groups, a publicly available database containing 3,020 proteins has been generated and this is the base for further studies¹²⁸.

Various procedures are applied for pathogen inactivation, but it should be emphasised that none of the currently applied methods inactivates all types of pathogens, and all have some effect on plasma quality when this product is compared to FFP¹²⁹. Pooled solvent/detergent (S/D)-plasma is the best-documented clinical product, followed by methylene blue (MB) light treated-plasma. Recently, psoralen light treated-plasma has been introduced (CE-marked product in Europe), while riboflavin light treated-plasma is still under development.

The effect of pathogen inactivation procedures on the integrity of plasma proteins has been addressed by several studies. In 1994 Tissot and co-workers analysed, by high-resolution 2D-GE, untreated and photochemically treated apheresis FFP to verify whether MB, in combination with visible light, induced protein alterations¹³⁰. This study revealed no change in the silver-stained 2D-GE pattern before and after the treatment. To overcome the problems of the potential genotoxicity of MB¹³¹, it has been proposed that this dye should be removed by filtration, and efforts have been made to efficiently remove the dye after photo-treatment^{132,133}. A more recent study, in 2004, examined the effect of MB treatment plus light, followed by removal of different concentrations of MB and various times of light exposure¹³⁴; the proteomic investigative techniques used were 2D-GE and LC-ESI-MS/MS. Modifications were noted in the γ -chain of fibrinogen, concomitantly with functional alterations of this protein; the second change on 2D-GE gels was secondary to a relatively large increase of one of the isoforms of transthyretin, while the last modification was an enlargement of the spot of apolipoprotein A-I. Removal of MB by filtration did not cause additional protein alterations. However, the effect of over-treatment of plasma with a very high concentration of MB (50 μ M) in association with prolonged light exposure (3 hours) led to severe damage to most of the plasma proteins. By contrast, S/D inactivation was, apparently, not associated with modifications of the 2D-GE spot patterns of plasma proteins^{3,12}.

Few investigators have used proteomic strategies to evaluate the effect of pathogen inactivation techniques on blood plasma preparations, but the findings may be important for the quality control of the various plasma preparations available on the market, and may reveal protein modifications otherwise not suspected¹³⁴.

These studies relied on gel-based techniques without the removal of high abundance proteins, but the depletion of the latter could increase the sensitivity of the methods and possibly the plasma protein changes detected¹². Moreover, the impressive and constant progress achieved in the knowledge of the plasma proteome will aid in evaluating the different FFP preparations used in daily clinical practice¹⁵.

Other plasma derivative products

Protein products fractionated from human plasma are an essential class of therapeutics used, often as the only available option, in the prevention, management, and treatment of life-threatening conditions resulting from trauma, congenital deficiencies, immunological disorders, or infections¹³⁵.

Various procedures used for protein purification (and pathogen inactivation) may modify or degrade proteins¹⁵.

Recently, the analysis of prothrombin complex concentrates (PCCs) was used as a model to evaluate to what extent proteomic technologies can detect differences in blood-derived therapeutic products, beyond that of standard quality control, and identify modified proteins¹³⁶. PCC contain the procoagulant zymogens of the prothrombin complex, namely factor II, factor VII, factor IX, and factor X, as well as the inhibitory factors protein C and protein S. They are used in patients with hereditary or acquired deficiencies of vitamin K-dependent clotting factors¹³⁷⁻¹⁴¹, and are standardised by their factor IX activity¹⁴². Three PCCs (two batches each) were analysed for differences in protein content by functional assays, and 2D-GE coupled with MALDI-MS/MS or LC-ESI-MS/MS. The results were compared to those of a pool of 72 normal plasma samples. The authors found major differences between products from different manufacturers, whereas no significant batch-to-batch variability was seen in the same product. Besides the labelled clotting factors, 41 additional proteins were identified, including fibrinogen, complement factors, and several apolipoproteins. Many proteins were present in multiple spots (factor II, factor X, protein C, vitronectin), indicating a high degree of post-translational modifications. Interestingly, protein expression in the 2D-GE pattern did not correlate with the activities of clotting factors, suggesting a loss of biological function during the manufacturing process. In comparison with untreated pooled plasma, PCCs displayed several low-molecular-weight variants of proteins that probably constitute potential degradation products. Thus, proteomic technologies could become a useful tool for transfusion

medicine to assess the impact of processing on the integrity of blood-derived therapeutics, as well as on the difference in recovery and immunogenicity between clotting factor concentrates^{12,136}.

α_1 -antitrypsin (A1AT) deficiency is an autosomal recessive disorder characterised by quantitative and qualitative abnormalities of α_1 -proteinase inhibitor (A1PI)¹⁴³. A1PI is a potent inhibitor of serine protease, especially neutrophil elastase, which degrades connective tissue in the lung; its deficiency, therefore, increases the risk of emphysema. A1AT augmentation therapy is indicated for the treatment of patients with lung emphysema secondary to congenital A1AT deficiency¹³⁵. Since the mid-1980s, A1PI deficiency has been treated with specific plasma-derived concentrates, to increase circulating A1PI to and above theoretically protective levels.

Mature A1PI is a single-chain 52-kDa glycoprotein consisting of 394 amino acids and 12 percent by weight of carbohydrate; its heterogeneity is due to several post-translational modifications. There are now several licensed A1AT plasma-derived concentrates; three commercially available products were recently submitted to biochemical analysis of the protein backbone as well as MS (LC-ESI-MS/MS) to perform a glycoproteomic characterisation of the A1PI protein, in order to identify and characterise potential alterations, caused by the exposure to different physicochemical conditions and various enzymes, during the industrial manufacturing process¹³⁵. The study showed that these commercially available A1PI products differ from A1PI directly analysed in plasma; however, the observed structural variations between products probably do not have a substantial biological role, because all three concentrates have similar half-lives and specific neutralising activities. According to the Food and Drug Administration (USA), there are currently no data suggesting an influence of these differences on the functional activity and immunogenicity of A1PI concentrates.

Intravenous immunoglobulin (IVIG) is used for an increasingly diverse number of therapeutic applications as an immunomodulatory drug¹⁴⁴⁻¹⁴⁹. At present, no data have been published on the proteome composition of therapeutic IVIG¹². Sapan and co-workers did, however, recently evaluate the use of genomics and proteomics for the study of immunomodulation therapeutics; the authors presented a description of DNA and protein microarray technologies, their application to the study of immune system cells, and a review of their uses in the study of gene expression in response to IVIG¹⁵⁰. They concluded that DNA and protein

microarray can be used to: i) provide rational indications for the clinical use of IVIG; ii) analyse the composition of raw material and final IVIG, and iii) to develop new, more focused and/or targeted therapeutic products.

Granulocytes

Neutrophils are the main class of WBCs in human peripheral blood; they have a pivotal role in eliminating extra-cellular pathogens by phagocytosis, and are the major component of the innate immune response³. Infections associated with therapy-related neutropenia continue to be a major cause of morbidity and mortality. Although granulocyte concentrates are currently not a standard therapeutic product, renewed interest in granulocyte transfusion therapy, as treatment for severe bacterial and fungal infections in neutropenic patients undergoing haematopoietic stem cell transplantation and dose-intensive chemotherapy for malignant diseases, has been generated by the observation that large doses of granulocytes can be obtained from donors who have been stimulated with granulocyte colony-stimulating factor (G-CSF)^{151,152}. Granulocyte concentrates collected by centrifugation leucapheresis, from G-CSF-stimulated donors, can be effectively stored for 24 hours with preservation of functional activity^{153,154}.

Human neutrophil proteomic studies have addressed: i) calcium-dependent secretion¹⁵⁵; ii) the substrates of mitogen activated protein kinase - activated protein kinase 2 (MAPK-APK2), a critical downstream kinase for a number of functions dependent on the p38 MAPK pathway, which has a central role in the regulation of neutrophil functional responses¹⁵⁶; iii) the modulation of their proteome, via a tyrosine oxidation pathway induced by pro-inflammatory mediators¹⁵⁷; iv) their granules¹⁵⁸; and v) a methodology for proteomic analysis of isolated neutrophils and its applications¹⁵⁹.

Granulocyte concentrates, probably because of their limited clinical use, in part a consequence of their short storage life, have not been studied through proteomics, although this field of research may in the future provide a useful tool to guarantee the safety, reliability, and possibly wider clinical use of this blood component¹².

CD34⁺ haematopoietic stem/progenitor cells

Haematopoietic stem cells (HSCs) are capable of self-renewal and differentiation into different haematopoietic lineages, including myelomonocytic, megakaryocytic, erythrocytic and lymphocytic cells. HSCs can be collected from bone marrow, from peripheral blood through apheresis,

or from umbilical cord blood; they can be employed to repopulate a bone marrow transplant recipient suffering from various haematological disorders after chemotherapy and/or radiotherapy¹⁶⁰⁻¹⁶².

Functional genomics approaches have been used to study myeloid differentiation, applying different proteomic techniques, such as oligonucleotide microarrays and 2D-GE MS, and DNA microarrays together with quantitative ICAT and DIGE; these genomic and proteomic analyses of the myeloid differentiation programme showed that throughout cellular development there is a strong contribution of post-transcriptional regulatory mechanisms, which can be unveiled only through integrated analysis of both proteins and mRNA^{12,163-165}.

The proteome of primary HSCs is determined by rates of transcription, translation, and protein turnover. Stem cell commitment to differentiation is a critical step in development, during which proteomic changes are observed as a cell undergoes successive development steps; the potential of MS-based protein quantification techniques, such as iTRAQ, for characterising the proteome signature of stem cells and their progeny, was recently demonstrated by Unwin and colleagues, who compared the proteomes of long-term reconstituting HSCs and non-long-term reconstituting progenitor cells¹⁶⁶.

The mechanisms by which mRNA is translated into protein are highly regulated, and it remains unclear how the transcriptome reflects the functional state of the HSC, as defined by its protein output. A comparison between the transcriptome and proteome of primary HSCs can reveal post-translational regulation of the proteome in stem cell populations; the factors which must be considered when comparing proteomic and transcriptomic datasets, and the relative merits of these two approaches in the study of stem cell populations, have been recently reviewed¹⁶⁷.

In 2003, the proteome of human CD34⁺ cord blood cells was explored for the first time, through LC-ESI-MS/MS, in order to identify stem cell-specific proteins; a database search led to the identification of 215 proteins. The authors concluded that to obtain a more complete picture of the human stem cell proteome, and also to access low-abundant proteins, it would be necessary to pool more than one CD34⁺ preparation, thus increasing cell numbers and protein content¹⁶⁸. Two years later, the same group compared the proteome profile of CD34⁺ from five different umbilical cord samples, through 2D-GE and silver staining followed by LC-MS/MS; the resulted extensive heterogeneity of the cytosolic protein pattern, indicating a not fully developed (or instable) proteome of stem cells,

was attributed to their dissimilar differentiation states¹⁶⁹.

A proteomic approach was also used to compare the proteins differentially present in CD34⁺ and in the more terminally differentiated CD15⁺ cells from human cord blood¹⁷⁰. This study presented, for the first time, global cellular protein constituents in both population of cells, and identified changes in cellular protein composition during myeloid differentiation; indeed 2D-GE, CBB staining, and MALDI-MS revealed preferential expression of 112 and 15 cytosolic protein spots in CD34⁺ and in CD15⁺ cells, respectively, thus suggesting that the former cells have a relatively larger proteome than the latter mature CD15⁺ myeloid cells.

The most comprehensive study of human cord blood-derived CD34⁺ cells was recently published by Liu and colleagues in Proteomics¹⁷¹. The study described the proteome and the transcriptome of these cells and identified 370 proteins, using 2D-GE and LC-MS; nerve, gonad, and eye-associated proteins were reliably identified, thus providing new insights into the potential of the stem/progenitor cells. Interestingly, the transcripts of about 36% of these proteins were not found by the prevalent transcriptome approaches. This is the first study to provide an integrated and complementary approach of the '-omics' for human haematopoietic CD34⁺ cells and a unique view of them; the integration of proteomic and transcriptomics data is indeed a necessary step for a better understanding of the processes involved in the differentiation of progenitor cells into mature blood cells, or during long-term culture^{12,15,171,172}. Trends in stem cell proteomics have been reviewed recently⁶⁴.

The analysis and description of the processes that regulate HSC development and the functional activity of mature cells has improved with proteomic approaches to the characterisation of proteins, their interactions, and their relative quantification³; moreover, proteomic techniques will probably facilitate comprehensive screening of a large subset of marker proteins, thus allowing the integrity and functionality of HSC therapeutics to be monitored¹².

Conclusion and perspectives

Proteomics has been used for the analysis of blood constituents since the mid-1970s, but, notwithstanding its potential to provide comprehensive information about changes occurring during the processing and storage of blood products, it has not been widely applied to assess or improve them, or for monitoring their production processes¹².

The study of peptides and peptide pools, the so-called

peptidomics, is useful for a better understanding of cell biology and for an in-depth analysis of various biological fluids, and will also certainly influence all the steps involved in the different methods of plasma preparation used in transfusion medicine; this approach could also be of great interest for the characterisation of small peptides and may open up a new outlet for the field of *degradomics*. Quantitative proteomic techniques could prove useful not only to compare identified peptides in blood components/derivatives, but also to follow the evolution of these therapeutic products over time, through quantitative monitoring of the appearance and disappearance of proteins in supernatants¹⁵.

Moreover, the description of the proteome of crude components will reduce contraindicative impurities and could allow high-value components to be further extracted from blood. Viral inactivation methods could be evaluated by their effect on protein levels and functional activities, or for the possible neoantigens generated during production, thus guiding their improvement^{7,12,14,15}. Proteomics could, therefore, become part of the quality-control process to verify the identity, purity, safety, and potency of various blood products⁴.

Several articles have recently dealt with proteomic analysis of cell membrane microparticles, a heterogeneous population of vesicles that circulate in the blood of healthy donors and are generated during various biological phenomena^{110,173-176}. The study of the generation as well as the composition of both RBC and platelet microparticles, both during the preparation and storage of blood components, is of primary clinical importance, as it could enable an assessment of the role of different treatment and storage protocols on the quality and shelf-life of blood components¹⁵.

In conclusion, proteomics is a rapidly developing science, which offers the potential for significant improvements in transfusion medicine, and allows a much more precise assessment of the quality of the blood products transfused to patients. Although the costs of proteomics are still fairly high^{12,14}, this branch of science undoubtedly has the potential to provide the necessary tools to meet and verify several of the various quality requirements set by regulatory government agencies and/or national laws in the field of plasma-derivative production and pathogen inactivation.

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Correspondence: Dr. Giancarlo Maria Liumbruno,
Viale Italia, 19 – 57126 Livorno, Italy
e-mail: giancarlo@liumbruno.it