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Application of proteomics to hematology: the revolution is starting

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“... proteomics is pervading all fields of blood-related disciplines... and is beginning to be used as a qualification tool for clinical practices in transfusion sciences”

Since the advent of proteomics, a huge effort has been put into the cataloging of proteins from body fluids, mainly because of their ready availability and potential for the discovery of disease biomarkers. Plasma is the best studied among body fluids: from 40 proteins identified in 1977 [1], the numbers have constantly grown through the years. Sennels *et al.* recently reported a list of 1559 identified proteins in a single experiment [2], and the HUPO Plasma Proteome Project now holds a core consensus dataset of 3020 nonredundant identified proteins [3,4]. In contrast, the red blood cell proteome and the platelet proteome, though to some extent more relevant to biologists and clinical practitioners, have received much less attention. In 2002, Low *et al.* identified 84 membrane proteins [5]; a number that grew up to 181 proteins in 2004 [6], and 566 in 2006 [7], for a collectively identified dataset of 751 proteins. In parallel, the platelet proteome started with 120 proteins in the year 2000 [8], up to 264 in 2004 [9–11], 641 in 2005 [12] and for a current consensus dataset of 1200 proteins.

However, to date, this flood of data has resulted in little gain for hematological science and clinical practice. The reasons for this dead valley between basic research (the brute-force acquisition of proteomic data) and translational medicine lie in the difficulty of extracting valuable biological information from the data. First, the way a sample is preprocessed may have a profound influence on the observed profile of peptides and proteins; a difficulty that has now been relatively well documented, especially from the biomarker discovery field. For example, the use of protease inhibitors

results in a dramatically different protein pattern (compared with noninhibited one), as demonstrated by 2D gel electrophoresis (GE): when protease inhibitors are omitted, considerable amounts of low-molecular-weight proteolytic fragments appear during sample processing [13].

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Similarly, Ayache *et al.* have studied the influence of sample processing (delay between blood collection and plasma isolation, and addition of protease inhibitors) on the concentration of 99 soluble factors in plasma (including interleukins, chemokines, matrix metalloproteinases and so on) [14]. They found that there is a clear influence of the delay between sample collection and plasma preparation (2 h in this study) on the level of these 99 soluble factors; the addition of protease inhibitors has relatively little influence on the soluble factors concentration (as measured by immunoassays). Given the relatively poor documentation of protease inhibitor cocktails, and the broad spectrum of their specificities, it is highly probable that they do not suppress all proteolytic activities, and that different cocktails result in different residual proteolytic activities. Similarly, EDTA/citrate or heparin anticoagulation treatments result in quite different low-molecular-weight plasma proteomes [15–17].

A trickier case is the study recently published by Davis *et al.*, who analyzed the influence of sample preprocessing on the

low-molecular-weight profile of plasma [18,19]. They clearly showed that any delay in the isolation of plasma from the serum results in the appearance of *in vitro* degradation fragments (mainly fibrinogen fragments) that are directly due to the exposure of plasma proteins to the clotting system. A more complex case arose when identifying such fragments generated *in vivo* (i.e., when all possible causes of *in vitro* artifacts were controlled) in patients suffering from a wide range of diseases when compared with healthy subjects. Whereas there was some hope that such proteolytic fragments be generated at a tumor site (e.g., by specifically overexpressed matrix metalloproteinases), a more plausible explanation is that these fragments are generated by *in vivo* processing of plasma proteins by altered hemostatic processes, which are known to be present in a broad spectrum of diseases. Although these *in vivo* generated fragments are linked indirectly to the presence of the disease, their potential selectivity for disease diagnosis remains highly questionable.

“Besides the brute-force search for disease biomarkers, proteomics has already delivered some important contributions to the field of blood-related diseases.”

Another relatively neglected artifact in blood proteomics is the presence of circulating microparticles, which are small vesicles of less than 1 μm in size released by red blood cells and platelets. The function of these microparticles is still unknown: they may serve in cell–cell communication, participate in hemostasis (platelet microparticles) or simply aid in protein degradation and clearance from parent cells. However, there is now clear evidence that they contain a large fraction of the parent’s proteome (in terms of number of different proteins). For example, Miguet *et al.* recently analyzed the proteome of T-lymphocytic cell line-released microparticles and found a set of 390 proteins, among

which 34% were plasma membrane proteins [20]. In many cases, these microparticles retain the function of their parent’s cell: for example, platelet microparticles may have up to 50-times more hemostatic activity than whole platelets [21,22]. Unfortunately, unlike red blood cells and platelets, these microparticles are not eliminated from serum by low centrifugation, and thus remain in the isolated plasma if no appropriate measure is taken. Additionally, it is our experience that multiplying sample preparation steps (centrifugation, filtration and pipetting) tends to stimulate the *in vitro* production of microparticles from red blood cells and platelets [RUBIN O, CRETZAZ D, CANNELINI G, TISSOT JD, LION N. BLOOD MICROPARTICLES IN STORED RED BLOOD CELLS. (2008) (IN PREPARATION)]. It is highly dubious that the presence of these microparticles plays no role in the stability of the isolated plasma.

The plethora of possible *in vitro* artifacts makes the extraction of valuable biological information from large-scale body fluid proteomics very difficult beyond protein cataloging, and trying to establish standard procedures for sample collection, handling and preparation can rapidly become extremely costly and difficult [15,23].

Do these difficulties imply that proteomics is only a new toy in the life-science toolbox that is far from delivering results of biomedical interest? The fact is that in blood-related disciplines, proteomics is pervading all fields and is evolving from a brute-force, biomarker discovery approach, to an extensive armamentarium that is available to clinical practitioners and researchers to address focused problems in the fundamental knowledge of blood components (plasma, red and white blood cells and platelets), in disease-related research, and to examine their medical practice at an unprecedented level of comprehensiveness (FIGURE 1).

A typical example of the contribution of proteomics to the fundamental knowledge of blood components can be found in the field of platelet proteomics, which started 20 years ago with 2DGE combined with western blotting for the identification of a set of 20 proteins. The development of mass spectrometry-related techniques for protein identification boosted the field in the mid-1990s, and thus the latest projects identified hundreds of platelet proteins in a single experiment (FIGURE 2).

This global effort not only resulted in a compendium of approximately 1200 proteins identified collectively, but also provided new information about the regulation of resting platelets, for example, Zahedi *et al.* recently identified a new phosphorylation site on GPIb α [24]. GPIb α and GPIb β are covalently linked through a disulfide bridge to form GPIb, a central part of the von Willebrand factor binding GPIb-IX-V receptor complex, which mediates initial platelet adhesion and activation. Although GPIb β was already known to be regulated by protein kinase A and G (PKA/PKG; negative regulation of von Willebrand factor

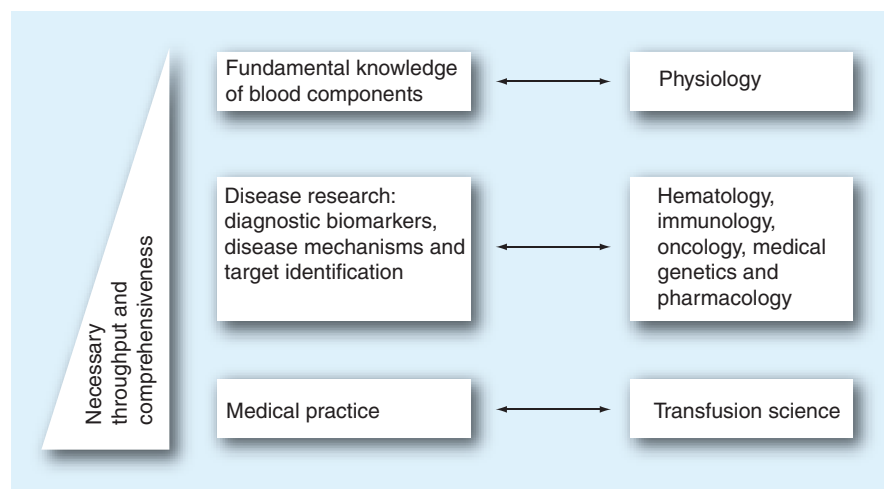


Figure 1. Disciplinary relationships between thematic proteomic projects and traditional medical disciplines.

binding to the GPIb-IX-V receptor complex), it was unknown that GPIb α was also a target for phosphorylation. Moreover, the new phosphorylation site is not a consensus sequence for PKA/PKG, which implies that another (unknown) kinase may play a role in this inhibition process. These kinds of results are canonical to what can be expected from proteomics: it confirms results from decades of biochemical and physiological research (in this case, known inhibition processes of platelet activation through PKA/PKG phosphorylation), and provides new insights into living phenomena (e.g., regulation of GPIb α by an unknown kinase). Furthermore, Albert Sickmann's group has undergone a huge effort to understand platelet function from protein cataloging [12], study of phosphorylation [24] and glycosylation [25,26]. However, as highlighted by several authors, assembling a list of proteins, even together with their list of post-translational modifications, is just the beginning of the search for the knowledge of the mechanisms of red blood cell or platelet physiology [27,28]; the next task is to aggregate these data and build functional models of metabolism, signaling or activation networks.

Besides the brute-force search for disease biomarkers, proteomics has already delivered some important contributions to the field of blood-related diseases. For example, proteomics has allowed some progress in the knowledge of Hodgkin's lymphoma (HL); HL is a cancer that originates in lymphocytes and is characterized by the presence of Reed-Sternberg cells (giant multinucleated cells). Fujii *et al.* have provided the first large-scale proteomic study of various lymphoid neoplasms (HL, Burkitt's lymphoma, B-cell acute lymphoblastic leukemia, anaplastic large-cell lymphoma, adult T-cell leukemia and cutaneous T-cell lymphoma). Through 2D-DIGE, they demonstrated that HL cells have a protein profile that is essentially different from any other lymphoid neoplasm cells and control cells (reference B and T cells). Even though HL cells derive from B cells, they appear to develop their own protein expression pattern in the course of disease [29,30]. Recently, Ma *et al.* analyzed the secretome of Hodgkin and Reed-Sternberg (HRS) cells in culture, in order to understand the way HRS cells interact with inflammatory background cells. They identified 1290 proteins in the culture medium, among which 368 were predicted to be secreted. Among secreted proteins, 37 were classified by gene ontology as inflammation or immune response proteins and 26 as cell communication proteins. Based on possible relevance to the disease and antibody availability, 16 proteins of the 'inflammation or immune response' group (including all identified cytokines and chemokines, CD molecules and cathepsin family members) were assessed in cell lines by immunohistochemistry and/or immunoassays. Ten of

these proteins were assessed in patients' plasma. Not only were classical HL associated chemokines identified (such as TARC, which recruits Th2 and CD4⁺CD25⁺ regulatory T cells, IP-10 and RANTES, which attracts T cells, natural killer cells and eosinophils), but also new ones (e.g., fractalkine, whose role is not known). A small group of cytokine-related proteins were also identified (e.g., as IL-1R2, MIF, CD44 and IL-25); their presence in the HRS cells' secretome enables us to hypothesize mechanisms through which HRS cells elude cytotoxic T-lymphocyte response and trigger inflammation and proliferation of reactive cells [31]. These three studies show how proteomic approaches can be used in blood-related diseases to classify disease (compared with reference cell lines or healthy control individuals) and study the pathological mechanisms by which disease develops and escapes physiological defense mechanisms. Beyond this particular example of HL, proteomic approaches have also proved useful in characterizing protein microheterogeneity in amyloidosis [32–35], genetic diseases of coagulation factors [36] and more generally of hemostasis [37] in immunoglobulinopathies [38].

Besides fundamental and biomedical applications, proteomics holds promise in the design and evaluation of clinical practices in hematology and transfusion science."

Besides fundamental and biomedical applications, proteomics holds promise in the design and evaluation of clinical practices in hematology and transfusion science. For example, the preparation of red blood cells or platelet concentrates involves many steps from the donor to a deliverable product (puncture,

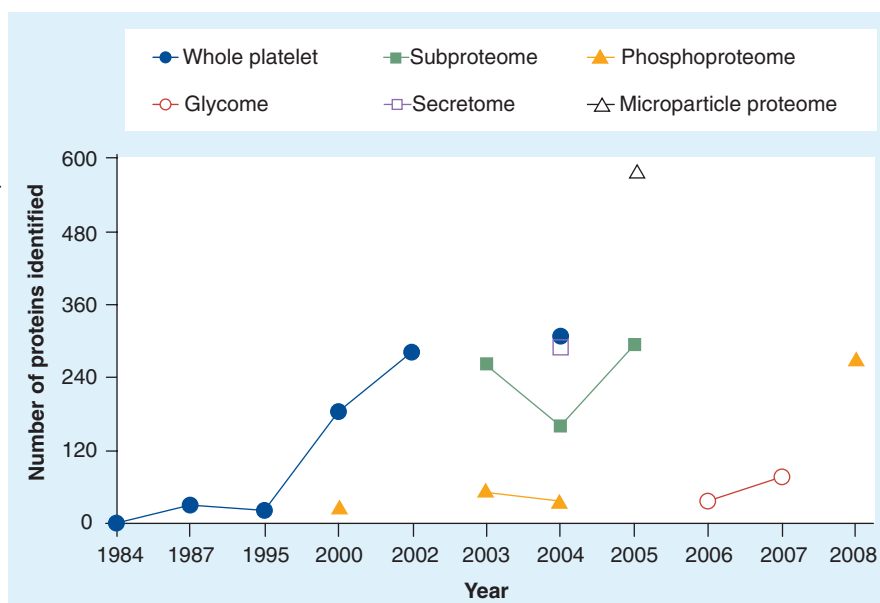


Figure 2. Platelet proteome. The number of proteins identified in a single experiment targeting whole platelets, instrumental subproteomes (e.g., cysteine-containing proteins) and other functional subproteomes.

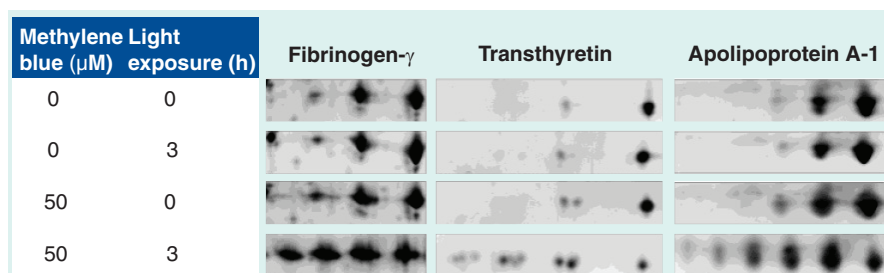


Figure 3. Effect of methylene blue and light treatment for pathogen inactivation on fibrinogen, transthyretin and apolipoprotein A-1, as seen by 2D-gel electrophoresis.

Reproduced with permission from [39].

apheresis, filtration, centrifugation, addition of anticoagulant cocktails, addition of pathogen inactivation reagents and so on), and little is known about the impact of each of these steps on the product proteome. Different platelet apheresis instruments may induce subtle changes in the state of the isolated platelets. However, only macroscopic measurements (e.g., percentage of activated platelets and pH) are routinely measured to qualify apheresis machines and protocols. Similarly, leukoreduction usually involves filtration processes that might not be completely innocuous for surface sensitive cells, such as red blood cells and platelets. Recently, our laboratory studied the effect of a particular pathogen inactivation procedure (methylene blue and light treatment) on the plasma proteome [39].

As illustrated in FIGURE 3, this treatment results in the appearance of altered proteins on the acidic side of native spots, which allows us to hypothesize that methylene blue treatment results in major oxidation of plasma proteins. The effect of such modifications on the transfusion efficiency is unknown to date.

Complementarily, Fortis *et al.* recently analyzed injectable solutions of serum albumin prepared from plasma by a new

enrichment strategy targeted toward trace contaminants. They found that dozens of plasma proteins are still present in the injectable solution, including immunoglobulin fragments [40]. Such results may question the innocuousness of plasma-derived injectable products [41].

As outlined in the aforementioned examples, proteomics is pervading all fields of blood-related disciplines, from basic research about blood components and their physiology, to disease pathology research, biomarker discovery and toxicology, and is beginning to be used as a qualification tool for clinical practices in transfusion sciences. Despite the cost of

proteomic technologies (particularly mass spectrometry instrumentation and the expertise they require), they tend to become more and more accessible to clinical practitioners through the establishment of proteomic analysis platforms in most academic hospitals and universities. In view of its recent achievements, it is almost certain that proteomics (together with all other omic technologies) will continue to gain popularity in hematology. In our opinion, it can be best used to tackle focused, well-formulated questions at an unprecedented speed and comprehensiveness, as well as providing hypotheses that can be further documented by more conventional biochemical studies.

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