REVIEW ARTICLE

Blood Cell Proteomics in Chronic Kidney Disease

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Abstract:

Background:

The uremic syndrome mimes a systemic poisoning with the retention of numerous compounds which are normally removed by the kidney. The study of proteins and peptides, or proteomics, represents an important field of research for the investigation of blood and blood diseases.

Methods and Materials:

We focused our review on the results of proteomic investigations on blood cells of uremic patients with particular regard to the study of red blood cells, platelets, and monocytes.

Results:

In literature there are few, preliminary studies on platelets and monocytes while the knowledge on uremic erythrocytes is much wider. Proteomic investigations showed that erythrocyte membrane proteome of uremic patients, differs significantly from the proteome of healthy subjects, being characterized by an extensive remodeling which may influence visco-elastic properties of RBC such as deformability and involve diverse molecular pathways driving red blood cell signaling and removal.

Conclusion:

Proteomic technologies emerged as a useful tool in defining and characterizing both physiological and disease processes being able, among others, to give important insights into uremic anemia.

Keywords: Chronic kidney disease, Proteomic, Red blood cell, Platelet, Monocyte, Anemia, Hemodialysis.

1. INTRODUCTION

Chronic Kidney Disease (CKD) is a worldwide major public health problem, due to the increasing number of affected patients. The disease is characterized by a progressive loss of renal function, paralleled by deterioration of biochemical and physiological functions, and by a progressive rise in both morbidity and mortality mainly due to cardiovascular complications [1]. A number of CKD patients reach End-Stage Renal Disease (ESRD), a stage that usually requires chronic renal replacement therapy, *i.e.* Hemodialysis (HD), peritoneal dialysis or transplantation.

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Most of the biochemical and clinical characteristics of uremia have been defined in ESRD. The uremic syndrome resembles a systemic poisoning [2], and it is characterized by the retention of various solutes which are normally cleared by the kidney. Uremic retained solutes include several proteins, and may cause biochemical/biological toxic effects [3]. More than 150 uremic toxins have been identified and quantified to date [4, 5], and more are expected. The uremic syndrome is however not only the result of compound retention but also of deranged hormonal, metabolic, and enzymatic homeostasis [6].

Uremia is a quite complex syndrome, and the molecular mechanisms involved in its pathophysiological disturbances are still not fully identified.

In the last decade, several *omics* technologies have been developed to study specific subgroup of molecules (genomic, transcriptomic, metabolomic, and proteomic). Interestingly, every *omics* approach allows to perform unsupervised survey analysis to have a broad picture of the modulation of a molecule subset within a particular pathological condition, in contrast with traditional experimental design where single analytes are studied to unravel their specific function.

Proteomic is the –omic technology suited for the study of proteins and peptides and holds promise to significantly improve our understanding and treatment of the molecular basis of changes caused by renal failure and/or related replacement therapy [7 - 10].

Characterization of complex protein repertoire takes advantage of the powerful performance of mass spectrometry based technology which, coupled to efficient pre-analysis sample separation procedure (e.g, 2D-Electrophoresis-2DE- or Reverse Phase Liquid Chromatography-RPLC-) and post-acquisition computational analysis of mass spectrometry datasets, represents the most reliable and accepted approach in the field [11].

Such developments in proteomic strategies and bioinformatics tools now allow identification, relative and absolute quantification, and functional classification of a multitude of proteins in complex samples. Moreover, mass spectrometry allows to identify single aminoacid modification hence giving the possibility to investigate the perturbation of the proteome consequent to protein post–translational modifications.

Another novel advance of recent years is the tendency of making -omics data freely available to the scientific community. The ProteomXchange Consortium is an example of centralized database, which complies with standards and guidelines, to which proteomics data can be deposited. The datasets, handled by expert biocurators, include protein and peptide identifications, post-translational modifications and supporting spectral evidence [12] and are associated to a published manuscript. The power of this approach is the possibility for every researcher worldwide to access the data and reprocess it, maybe tackling a different biological question, or perhaps using novel algorithms or updated tools at a later time.

In ESRD, proteomic investigations have been successfully applied to describe the protein uremic milieu [13 - 15], to assess the blood purification efficiency of different hemodialysis dialyzers and systems [15 - 21], and to examine the biocompatibility and adsorptive properties of biomaterials used for HD membranes [22 - 24].

Over the last decade, proteomics proved to represent an important field of research for the study of blood and blood diseases [25, 26]. Uremia adversely affects a number of hematological parameters, including red cell production and destruction, platelet, granulocyte and lymphocyte function, and coagulation. When traveling through the patient's body, uremic blood cells encounter toxic molecules that can accumulate not only in plasma but also within the cell itself. Many cellular changes in protein repertoire in response to an external stimulus can only be characterized at the proteome level [27]. Thus, proteomic analysis of changes in protein amount or protein species might help to uncover the underlying pathophysiological abnormalities.

In this article, we review the results of proteomic investigations on blood cells of uremic patients. We highlight the recent promising findings, and which future developments can be expected.

2. EVIDENCE ACQUISITION

We systematically searched for studies published in English in the MEDLINE (from 1956 to January 2018). For this search, we used the following terms in various combinations: chronic kidney disease, end stage renal disease, red blood cell, erythrocyte, inflammation, oxidative stress, uremia, hemodialysis, platelet, leukocyte, proteomic, mass spectrometry, anemia.

3. PROTEOMICS OF UREMIC BLOOD CELLS

3.1. Red Blood Cell

Red Blood Cells (RBCs) represent the most abundant cell type in the human body. The RBC is a fairly simple cell devoid of a nucleus, main components being the cell membrane and the hemoglobin-rich cytoplasm, and has always been regarded as a circulating bag of Hemoglobin (Hb). However, the development of advanced proteomic technologies and of bioinformatics has challenged this assumption, revealing unexpected complexity and novel roles for erythrocytes not just in gas transport [28, 29]. A recent, in depth quantitative analysis of the RBC proteome, identified 2650 proteins, 1890 of them occurring at more than 100 copies per cell, findings which were validated by a targeted analysis using labelled standards and which can serve as a useful tool for further studies on RBC pathologies and aging [30]. Comparative proteomic inventories of RBC membrane have yielded new clues to the mechanisms regulating membrane-cytoskeleton interactions in health and disease, and to the ways by which erythrocytes communicate with their environment [31]. In addition, proteomic data have indicated that many, hitherto unsuspected, metabolic processes may be active in the RBC cytoplasm [31], as suggested by the presence of new receptor/signaling pathways, stress proteins, transport systems, and proteases [28, 32]. Interestingly, erythrocyte metabolism does not seem to be restricted to energy production and redox status, since amino acid and lipid metabolism may be particularly active as well [33, 34]. However, some limitations of proteomics in RBC studies must be considered as well, due for instance to the broad dynamic range of protein molecular weight, the abundance of membrane proteins or the effect of post-translational modifications, though other recently developed proteomic-related techniques, such as lipidomics, interactomics or glycomics can contribute to unravel the global snapshot of the whole RBC protein repertoire.

In ESRD, normochromic normocytic anemia is a typical, common, and serious complication, which reduces the quality of life and has been associated with a number of adverse clinical outcomes [35]. The main cause of renal anemia is a reduced erythropoietin (EPO) production, though shortened RBC lifespan, uremic toxins, and inflammation also play a role [36]. Besides a reduction in their number, erythrocytes in the uremic milieu are also prone to many structural and functional impairments, including alterations in the production of nitric oxide, reduced antioxidant activity, increased adhesion to the endothelium and pro-coagulant activity, and modification in the composition of plasma membrane, all aspects that may jeopardize their properties [37].

One of the far from negligible pathogenic features of CKD anemia is the reduced lifespan of RBCs [38]. This is essentially due to the toxic action of the uremic plasma environment upon the RBC [36], but the implicated molecular pathways are poorly understood [39]. RBC membrane composition modulates the cell's visco-elastic properties, which are of fundamental importance for survival of RBC in the circulation. Deformability is an intrinsic characteristic of normal erythrocytes, which enables red cell passage through the microcirculation for oxygen delivery. A reduced deformability triggers hemolysis in the capillaries and premature sequestration of RBCs by the reticulo-endothelial system [40].

Uremic RBCs exhibit reduced surface charge and deformability [40]. Alvarez-Llamas *et al*. investigated and compared by a non-biased approach based on 2D fluorescence difference gel electrophoresis and mass spectrometry, the RBC membrane proteome of normal subjects, non-dialyzed CKD patients and EPO/dialysis-treated ESRD patients [41]. They found nine differentially expressed spots among study populations, corresponding to five proteins (ezrin, radixin, beta-adducin, tropomodulin-1, and HSP 71/72). Both uremia itself and its treatment (HD-EPO) may modulate the amount of certain RBC membrane proteins. Thus, ezrin and radixin, two proteins which link the plasma membrane and the actin cytoskeleton and are involved in cell adhesion, microvilli formation, and membrane ruffling [42], were increased in dialysis patients compared to the other two groups. Differently, levels of beta-adducin, a membrane skeletal protein involved in the mechanical properties of erythrocyte membrane [43, 44], proved to be higher in RBC from both CKD patients (dialyzed or not) compared with the control group, suggesting an alteration induced by uremia which may be interpreted as a compensatory response to the increased erythrocyte osmotic fragility, and disturbed calcium homeostasis [39]. Also, dialysis/EPO combination could modulate the effect of uremia on a sub-group of RBC membrane proteins such as tropomodulin, a cytoskeleton-associated protein which contributes to define the visco-elastic properties of RBCs [45]. Indeed, increased levels of tropomodulin in RBC from CKD patients were normalized in dialysis/EPO treated patients [41]. In a previous study analyzing the expression of certain key proteins of the erythrocyte membrane of HD patients, low levels of spectrin were found [46]. Since the cytoskeletal network is a chief factor behind erythrocytes' visco-elastic properties such as deformability [47], observed changes to the membrane protein component [41, 47] might be part of the pathogenesis affecting deformability and reduced survival of

erythrocytes in CKD.

The difference between CKD erythrocyte membrane proteome and healthy subjects' proteome has been demonstrated also by other investigations. Antonelou *et al.* examined the RBC membrane proteome of twelve non-diabetic EPO-responsive ESRD patients on HD with biocompatible hemodialyzers by electrophoresis and immunoblotting [48]. Uremic RBC membrane proteome demonstrated a remarkable remodeling, characterized by loss, fragmentation, aggregation and carbonylation of critical components, and by over-expression of cellular stress markers [48]. Erythrocyte membrane was found to be deficient in spectrin, band 3, pallidin, actin, stomatin, and CD47, the latter being a marker preventing erythrophagocytosis [49]. There was instead a marked increase in membrane-bound HSP70, calpain-1, peroxiredoxin-2 (a cytosolic component) and immunoglobulins G, associated to the presence of the spectrin-Hb complex and the band 3- and spectrin-related degradation products. Levels of ubiquitinated proteins also were for the first time found to be highly increased. Observed changes are indicative of defense responses to increased oxidative and/or carbonyl stress, as detected in uremia [50, 51], as well as of cell senescence. The results of this study might add knowledge to the progression of anemia and the premature death of RBCs in uremia [48].

The same group of patients [48] was followed-up for 36 months, in order to examine for blood modifications associated with the progression of disease and duration of HD treatment, and to gain more insights into the pathophysiology of uremic erythrocytes [52]. During the follow-up period, half of the patients died from cardiovascular complications. As compared to patients who survived, patients who died had higher levels of uremic toxins and of RBC indexes, longer HD vintage, more frequent RBC spherocytic modifications and degenerative shapes, a high membrane proteome carbonylation index (measuring the protein oxidative assaults) further increasing after HD session, and a more sustained oxidative stress. RBC membrane proteome of deceased patients was characterized by deficiency of band 3 and pallidin proteins, and by excess of band 8, aquaporin-1, calpain 1, and peroxiredoxin 2 proteins. Interestingly, intra-erythrocyte Hb concentration proved to be increased as compared to the survival patients, which may lead to problematic oxygen access, Hb-mediated oxidative reactions to cells, and increased rate of Hb auto-oxidation. Survivor patients, as compared to both died patients and healthy controls, also displayed low RBC membrane expression of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), glucose transporter 1 (GLUT1), and clusterin. Such remodeling is indicative of a pathologic regulation of cell hydration and susceptibility to oxidation changes induced by HD [52].

During the 3-year follow-up study, substantial stability of pre-dialysis levels of uremic solutes and of plasma antioxidant capacity, a trend toward increased RBC indexes, and higher normally shaped discocytes, were found in HD patients, whereas the respective profiles in co-studied healthy subjects showed negligible changes [52]. In addition, as compared to the protein modification rate of healthy RBC membrane, the majority of HD patients showed statistically significant increases of membrane-bound Hb, GLUT1, and stomatin. Overexpression of GLUT1 as dialysis duration goes on is a novel observation, which could indicate increased glucose uptake for metabolic purposes [53]. It might also be compensatory for the loss of band 3 protein (the main structural RBC membrane component), since GLUT1 accomplishes the link of skeletal components to the membrane [54]. Moreover, since GLUT1 transports dehydroascorbic acid derived from plasma ascorbic acid oxidation into RBC [55], where it is reduced to ascorbic and then diffuses back to plasma, increase of GLUT1 in the ESRD RBC membrane might assure plasma ascorbic regeneration [52]. The novel blood modifications reported in the above study would indicate compensatory responses to the chronic challenges imposed by the uremic milieu in patients on prolonged HD [52].

The results of a recent study indicate that also the type of extracorporeal renal replacement therapy might play a role on the physiological features of ESRD RBCs [56]. The authors compared a number of RBC-related parameters in ESRD patients undergoing HD (a technique based mainly on the principle of diffusion) or hemodiafiltration (a technique based on both diffusion and convection). While hemodiafiltration was associated with a better redox potential and a suppression of RBC exovesiculation before dialysis, it was also characterized by an acute, temporary, oxidative stress-driven increase in RBC removal signaling and hemolysis. This abnormality may be attributed to the removal of dialyzable antioxidant compounds from the uremic plasma and suggests the use of antioxidant therapy during the hemodiafiltration session [56], a concept that requires further investigation.

Georgatzakou *et al.* [57] investigated the physiological profile of RBCs in HD patients, responsive (n=16) or non-responsive (n=12) to standard doses of recombinant human EPO (rhEPO). Resistance to rhEPO was defined as the use of maintenance rhEPO doses > 300 IU/kg/week [46]. There were no significant differences between responders and non-responders before dialysis in the structural and physiological characteristics of RBCs including severe shape distortions, reduced fragility, and increased exposure of PhosphatidylSerine (PS), as compared to controls [57]. The HD

session triggered the formation of reactive oxygen species in RBCs from both groups, and enhanced the exposure of PS in non-responders. RBC membrane protein composition, however, proved to be quite different between the two groups of HD patients. Erythrocytes of non-responders exhibited lower expression of CD59 and clusterin proteins (complement inhibitors), and increased peroxiredoxin-2 levels tipically induced by oxidative stress [58]. Differently, RBCs of responders showed excess of membrane-bound IgGs and severe deficiency of the CD47 "marker-of-self". Further differences were observed with regard to the effect of the HD session. In fact, a marked decrease in the accumulation of RBC-derived microvesicles was found in responsive patients, while in non-responders HD did not affect RBC-derived microvesicles or RBC protein membrane composition. These findings suggest that deregulation of erythrocyte homeostasis might involve different molecular pathways during RBC signaling and removal in patients responsive to ESA treatment compared to non-responsive patients. In the former group, anemia might at last partly results from increased opsonization, increased susceptibility to Hb loss, and erythrophagocytosis through IgGs – and CD47-mediated changes. On the other side, in patients not adequately responding to ESA, RBCs proved to be characterized by an increased susceptibility to complement-mediated injury and by HD-triggered PS exposure. To be noted that exposure of PS on uremic RBC membrane is expected to promote not only cell removal [59], but also procoagulant properties [60], and increased adhesion to endothelium decreasing NO release [61], an effect associated with CV mortality in ESRD. These findings may shed some light on the erythrocyte pathophysiology in ESRD, and further support the potential role of uremic RBCs *per se* as CV risk factor [37].

3.2. Platelet Cell

Platelets are the smallest of the formed elements in blood. They average 2-4 mm in diameter, have a disk-shape, and are produced and released into the bloodstream by fragmentation of megakaryocytes [62], with a life span of 7-10 days. The main function of platelets is to contribute to hemostasis stopping bleeding at the site of interrupted endothelium through adhesion to various extracellular matrix components. Besides their physiological role, it is well established that platelets also contribute to thrombotic disorders, tumor metastasis, and inflammation [63].

The association of both physiological and pathological roles, makes platelets a very attractive model for biomedical investigation. Furthermore, as platelets do not have a nucleus, proteomics represent an ideal technology to study their function, and complementary proteomic approach such as 2DE-mass spectrometry and nRPLC-mass spectrometry have been shown to offer the possibility to obtain a comprehensive picture of the whole platelet proteome and to compare healthy and diseased platelets [64, 65].

Uremic patients develop an acquired platelet dysfunction that results in bleeding complications [66]. Although the frequency of severe hemorrhage has decreased with the advent of modern dialysis techniques, this complication limits surgery and invasive procedures in patients on dialysis. Several studies have also demonstrated that patients with renal failure on hemodialysis actually live in a state of chronic platelet activation related to both uremia and the dialysis procedure [67 - 69]. At present the nature of platelet dysfunction in these subjects is not yet fully understood, so the analysis of protein changes occurring in uremic diseased platelets might shed some light about pathophysiological mechanisms.

The first proteomic evaluation performed in uremic platelets was carried out by Walkowiak *et al*. [70]. In this preliminary work, the authors performed a comparative analysis using 2DE (IPG strips pH 3-10, 11 cm and Excel gels 12.5) to separate the platelet proteins of uremic patients, both dialyzed (n=9) and non dialyzed (n=9), and healthy controls (n=9). The authors determined changes in the global number of proteins between study populations but did not perform quantitative measurements. A grand array of low molecular weight proteins appeared in dialyzed samples, related to the contact suffered by platelets with the artificial membrane packed into the hemodialyzer during extracorporeal HD treatment.

Fifteen non dialyzed uremic patients (NKF-K/DOQI stage IV chronic kidney disease) were enrolled in another study whose purpose was to evaluate changes in the expression of proteins in functional (n=7) and dysfunctional platelets (n=8) [71]. Using the platelet function test PFA-100 assay through the use of 2DE and mass spectrometry, Marques and coll. found alterations of proteins related to cytoskeleton (actin-interacting protein-1 isotype 1 was significantly down-regulated in the dysfunctional uremic platelets) which correlate with the abnormal cytoskeletal assembly in uremic platelets [66], oxidative stress (glutathione S-transferase isotypes 1 and 2 and peroxiredoxin VI, all antioxidant-related enzymes, were up-regulated in the abnormal cells), cell interactions and energy metabolism.

These studies confirm that proteomics may be a valuable aid in better understanding platelet alterations that may be

associated with clinical alterations in patients suffering from renal failure.

3.3. Monocyte Cell

Monocytes are a type of white blood cell derived from hemopoietic stem cells in bone marrow. They can usually be distinguished in stained smears by their large kidney shaped or notched nuclei. Monocytes play fundamental roles in human physiology: for example, they have a central role in regulating host inflammatory processes through chemotaxis, phagocytosis and cytokine production [72], and are also involved in muscle system processes [73] and nervous system development [74]. Despite the significance of monocytes in human physiology and pathology, the molecular bases underlying their diverse functions are still poorly understood.

In the literature there is only a single work that have applied proteomic profiles in human monocytes of patients suffering from CKD. Scholze *et al*. [75] have analyzed superoxide dismutase type 1 (SOD1) in 98 chronic hemodialysis patients, 211 CKD non dialyzed patients, and 34 control subjects. SOD1 protein amount was significantly lower in HD compared to CKD patients or control subjects.

Serum SOD could be a marker of cardiovascular alterations in hypertensive and diabetic patients [76] since changes in its serum levels are correlated with alterations in vascular structure and function. Authors enrolled 255 consecutive hypertensive and diabetic patients and 52 non diabetic and non hypertensive controls, and they detected for the first time a negative correlation between SOD and pressure wave velocity, pulse pressure, peripheral and central augmentation index, and ambulatory arterial stiffness index, suggesting the enzyme to represent a major antioxidant defense mechanism.

CONCLUSION

Proteome approaches have been recently used to investigate blood cells of patients suffering from CKD. While proteomic examination of platelet and leukocyte uremic populations is still preliminary, the knowledge on uremic erythrocytes is much wider (Table **1**).

Table 1. Main modifications in the expression of membrane proteins in end-stage renal disease blood cells: the "top ten proteins".

Protein	Blood Cell	Expression (vs. control)	Cell Pathway
Actin	RBC	Lower	Structural integrity
Band-3	RBC	Lower	Aging, cell removal, oxidative stress
Calpain-1	RBC	Higher	Eryptosis, calcium stress
Clusterin	RBC	Lower	Cell aging, oxidative stress
Pallidin	RBC	Lower	Structural integrity
Peroxiredoxin-2	RBC	Higher	Calcium stress, oxidative stress
Spectrin	RBC	Lower	Structural integrity
Ubiquitinated proteins	RBC	Higher	Structural integrity, proteome stress
Actin-interacting protein-1 isotype 1	Platelet	Lower	Structural integrity
Superoxide dismutase type 1	Monocyte	Lower	Vascular function, structural integrity

Analyses of uremic RBC proteome have shown a number of changes in proteins involved in cellular shape modifications, inflammatory signaling, oxidative stress, energy metabolism, premature aging, cellular clearance, and ion transport [39]. These alterations, which may be relevant to the pathogenesis of RBC abnormalities in uremia, appear to stem from a complex network of interacting pro-oxidant and pro-inflammatory factors within the context of the uremic toxic milieu, and may be significantly influenced by the extracorporeal dialysis procedure.

As for the future perspectives, omics approach of uremic blood components holds promise for characterizing molecular changes associated with the uremic milieu, and for the development of new therapies directly targeting the underlying pathophysiologic mechanisms. Moreover, novel proteomic factors might be considered in future large scale studies on cardiovascular morbidity and mortality as candidate biomarkers in ESRD patients.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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