Protein damage and inflammation in uraemia and dialysis patients

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Abstract
The presence of high molecular weight toxins in the uraemic blood had largely been ignored until the beginning of the era of proteomics. In the last decade, increasing interest focused on these solutes has yielded evidence to suggest that some of these proteinaceous uraemic compounds can play pathogenic roles in the inflammatory and vascular comorbidities and mortality in patients with end-stage renal disease (ESRD). The finding that the blood levels of a number of glycation and oxidation markers are simultaneously increased in patients with uraemia suggests that several uraemic and inflammatory pathways may conspire to sustain the protein damage. Although the molecular characteristics and biological roles of the solutes derived from this abnormal chemistry remain poorly understood, it is proposed that they are not only laboratory hallmarks, but that their accumulation might also be the key underlying event in the establishment of a vicious and self-propelled ‘inflammatory loop’. The ‘switch’ is believed to be turned on by the scavenger receptor-dependent recognition of these protein damage products and activation of inflammatory and vascular reactions, which once activated generate further and even more marked protein injuries and inflammatory mediators, establishing a vicious loop together with the contribution of the uraemic intoxication and dialysis-related events. This paper provides the description of this inflammatory model, along with an overview of the literature on the proteinaceous solutes associated with inflammation and oxidative stress reactions, their biological roles, expected therapeutic tools—either pharmacological or dialytic—and investigation strategies based on the most recently introduced proteomic approaches.

Keywords: 3'-nitro-tyrosine; AGEs; dialysis; glycation; inflammation; nitric oxide; oxidation; protein damage; protein-leaking dialysers; proteomics; reactive oxygen species; uraemic toxins

Introduction
Chronic inflammation is considered to strongly influence the morbidity and mortality of patients with end-stage renal disease (ESRD) through its multiple pathogenic roles, in association with oxidative stress, accelerated aging, endothelial dysfunction and atherosclerosis, malnutrition, muscle wasting, anaemia, dialysis-related amyloidosis, leucopenia and immune dysfunction [1–3].

Among the several causes of such inflammatory disease, uraemia with the accumulation of pro-inflammatory toxins and dialysis-related leucocyte activation are considered the most important ones [4]. The pro-inflammatory and pro-oxidant effects of dialysis therapies have been widely explored [5], and it is now well-established that these effects are particularly pronounced in extracorporeal treatments, with the main underlying events being material biocompatibility and contamination of the dialysis fluid by bacterial wall components. On the other hand, there is evidence in the literature to suggest that renal transplantation is associated with almost complete correction of the biochemical indices of inflammation and oxidative stress in patients with ESRD [6].

In general, the severity of the clinical signs and biochemical indices of inflammation and oxidative stress (such as the serum levels of cytokines, acute-phase reactant proteins, markers of oxidative stress) in renal failure patients are influenced by the extent of kidney damage and the type of therapy employed, with the following order of association between the magnitude of the abnormalities and the type of therapy: transplantation < conservative pharmacological therapy < peritoneal dialysis (PD) < haemodialysis (HD). As an example, Figure 1 shows the plasma levels of the advanced glycation end-product (AGE) hallmark, pentosidine, in groups of patients treated by these modalities. This marker is considered to reliably reflect the abnormal chemistry associated with uraemia, since its levels are closely correlated with other indices of damage by oxidation and glycation reactions, and inflammatory markers [7].

The message from these observations is that a major limitation of the currently employed therapeutic...
approaches for ESRD is that they cannot surrogate the homeostatic role played by the kidney in concert with other organ systems to constrain such pro-inflammatory and pro-oxidant factors by the contribution of clearance and/or metabolic processing functions. Rather, HD may be regarded as a pro-inflammatory event. However, recent studies have clearly demonstrated that improvements in the biocompatibility and solute removal capability of dialysis therapies may contribute to slackening of the inflammatory burden in HD patients. For instance, Figure 1 shows the significant influence of the dialysis rhythm and membrane permeability on the pre-HD serum levels of the AGE product pentosidine [8,9].

These findings in literature clearly show that between the products of protein damage there are reliable markers of inflammation, which might help to investigate the clinical consequences of the inflammatory syndrome encountered in ESRD patients. Moreover, a recent and emerging role as inflammatory mediators has been proposed for at least some of these products [4], which, however, remain poorly characterized in terms of both their molecular identities and their biological roles in uraemia and dialysis patients.

In this review the most relevant studies in literature are examined, which describe such uraemic solutes, providing a mechanistic model of the active roles that they could play as pro-inflammatory agents.

In addition, preliminary findings of a proteomic approach addressed to identify some proteinaceous solutes in uraemic plasma and ultrafiltrates and to characterize their removal by conventional and protein-leaking dialysers are presented.

**Leucocyte activation, inflammatory mediators and oxidative stress**

With regard to the central role of chronic leucocyte activation in the inflammatory syndrome in HD patients, lymphocyte subsets and monocytes have been demonstrated to assume a pro-inflammatory phenotype [10]. This results in unbalanced production of pro- and anti-inflammatory cytokines, and sustained generation of other inflammatory mediators (such as arachidonic acid metabolites and PAF), which contribute, along with the uraemic intoxication, to the target organ dysfunction and chronic degenerative events commonly encountered in ESRD. These include the hepatic synthesis of acute-phase proteins, reduced albumin synthesis and malnutrition, muscle wasting, defective lipid metabolism because of lipoprotein lipase inhibition in adipocytes, and above all the overproduction and release of adhesion molecules and chemokines (such as ICAM-1 and VCAM, selectins, MCP-1 and others), which promote leucocyte activation, adhesion and migration into the subendothelial space, and together with the chronic activation of platelets and endothelial cells, contribute as main underlying events in the pathogenesis of atherosclerotic lesions and haemorrhagic and/or thrombotic manifestations [11–14].

Another important consequence of the chronic stimulation of circulating mononuclear and polymorphonuclear leucocytes is the onset of a state of energy and higher susceptibility to apoptosis of these cell populations [15]. These defects, which result in leucopenia and defective cell-mediated immune responses, underlie the immune dysfunction encountered in ESRD and contribute to sustenance of the inflammatory reactions by increasing the susceptibility of the patients to recurrent infections.

Complement-dependent and independent activation of neutrophils in the extracorporeal circulation during HD results in chronic and increased production of reactive oxygen species (ROS) and release of pro-oxidant agents contained in cell granules, such as the haeme protein myeloperoxidase (MPO) and lactoferrin [1,9]. Systemic production of ROS is sustained by other factors, such as interleukins and anaphylatoxins [3], and may also involve tissue macrophages and other cell, such as vascular endothelial cells and platelets. Mononuclear leucocytes and tissue macrophages contribute to sustenance of the oxidant stress through the activity of the enzymes NADPH oxidase and inducible nitric oxide synthase (iNOS) contained in them. This activity is responsible for the overproduction of reactive oxygen and nitrogen species (ROS/RNS), such as superoxide (O$_2^-$) and
H$_2$O$_2$, that together with the MPO-derived product HOCI and the nitric oxide (NO$^*$) and O$_2^*$ reaction-product peroxynitrite (ONOO$^-$) are among the most relevant pro-oxidant molecules produced by phagocytic cells and the main cause of tissue injury in inflammatory reactions. Being a non-conventional risk factor for cardiovascular disease and a possible contributor to the general comorbidity and accelerated aging associated with ESRD, oxidative stress has been intensively investigated in these patients and its presence has been undeniably demonstrated by the accumulation of indices of biomolecular damage, such as of the lipid oxidation products F$_2$-isoprostanes and oxidized LDL, the DNA oxidation and glycation products 8-OH-dG and N2-carboxyethyl-2'-dG, respectively, and several protein oxidation and glycation products (reviewed in [9,16] and further described in the next sections). The impaired NO$^*$ metabolism and biological functions observed in uraemia may further contribute to the increased susceptibility of uraemic tissues to oxidative stress and biomolecular damage [17].

**Protein damage in ESRD**

Protein damage may be a major source of large solute accumulation in ESRD. Indeed, protein modifications by ROS/RNS, reactive carbonyls, reducing sugars and other glyc(oxid)ation agents lead to a series of molecular changes, including hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulphhydryl groups, sulphonoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, causing conversion of amino acid residues to carbonyl derivatives and formation of intra- and inter-molecular cross-links (reviewed in [18]). These changes result in different types of structural defects, modified protein turnover by proteolytic systems and functional alterations with adverse impacts on human biology and health.

The most relevant classes of protein damage products encountered in ESRD include (i) tyrosine damage products and advanced oxidation protein products (AOPP); (ii) protein carbonyls; (iii) AGEs and (iv) miscellaneous products, mostly associated with albumin thiol oxidation.

**Tyrosine damage products and AOPP**

Tyrosine modification by ROS/RNS can lead to the formation of 3-chloro-tyrosine, 3-nitro-tyrosine or di-tyrosine, which represent some of the most reliable and sensitive indices of oxidative stress in biological systems and in clinical studies. Plasma levels of 3-chlorotyrosine are increased in HD patients, suggesting a prominent role of neutrophil activation and MPO-catalysed reactions in protein damage [19]. Similarly, a 2- to 10-fold increase in the blood 3-nitrotyrosine levels has been found in these patients as compared with those in healthy individuals, providing evidence for increased production of RNS by inflammatory cells and/or defective scavenging by antioxidant systems [20]. Plasma proteins containing di-tyrosine have been isolated from patients on regular dialysis and their biochemical and immunological features have been characterized [21]. This type of protein modification creates cross-links that have been designated as AOPP. In chronic renal failure patients, the plasma AOPP levels are correlated with markers of uraemia and oxidative stress, such as the creatinine clearance and blood levels of the macrophage activation product, neopterin. MPO-derived HOCI seems to play a role also in the generation of AOPP. In fact, when serum albumin is treated in vitro with HOCI, it acquires the same leucocyte-activating capability as that of AOPP generated in vivo [22].

**Protein carbonyls**

Protein carbonyls are formed directly by ROS/RNS or through reaction with oxidative stress by-products and free carbonyls. Sulphur-containing residues, such as cysteine and methionine, are particularly prone to oxidation, but other changes can also be induced by direct oxidation of Lys, Arg or Thr or by secondary reactions with the reactive carbonyl compounds of Cys, His or Lys residues [18]. Protein carbonyls can also be generated through oxidative cleavage of proteins, via the α-amidation pathway or through oxidation of glutamine side chains, leading to the formation of peptides with the N-terminal blocked by a γ-ketoacetyl derivative.

The introduction of carbonyl groups into proteins can occur by Michael addition reactions of α-, β-unsaturated aldehydeydes, such as 4-hydroxy-2-nonenal, malondialdehyde and 2-propanal to the amino group of lysine, imidazolone moiety of histidine or the sulphhydral group of cysteine. These adducts have been identified as advanced lipoxidation end-products (ALEs) [23]. Carbonyl groups can also be introduced into proteins by the addition of reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxysons) produced by the reactions of reducing sugars or their oxidation products, and into the amino group of amino acid residues by mechanisms such as glycation and glycoxidation, that would eventually result in the formation of AGEs (see below).

Several studies have demonstrated significantly increased plasma levels of reactive carbonyl compounds (measured as hydrazone formation after reaction with 2,4-dinitrophenylhydrazine) in patients with ESRD [7,24].

**Advanced glyc(oxid)ation end-products (AGEs)**

This class includes a heterogeneous group of compounds that reflect the extent of in vivo protein-aging and can be produced by non-enzymatic glycation (the Maillard or Browning reaction), oxidation and
carbonylation reactions. Widely investigated by food chemists and in diabetes, these products include a number of intermediates and AGE structures formed by enolization, dehydration, cyclization, fragmentation and oxidation reactions [23].

Maillard intermediates and end-products include fluorescent epitopes, such as furosine, pentosidine and vespelysine, which have often been used as in vivo markers of metabolic stress occurring as a result of glycation reactions. However, non-fluorescing products quantitatively predominate, and include glyoxal-lysine-dimer (GOLD), methylglyoxal-lysine dimer (MOLD), N\(^{\alpha}\)-(Carboxymethyl)lysine (CML), N\(^{\alpha}\)-(Carboxyethyl)lysine (CEL), argpyrimidine and imidazolone. In fact, many of these products are found at several-fold higher concentrations in uraemia and diabetes than the most commonly assessed fluorescing epitope, pentosidine. It is noteworthy that uraemic serum shows marked accumulation of AGEs, either in the free or protein-associated forms, independent of the presence of diabetes, which, nonetheless, exacer-
bates their accumulation. Dietary intake and intestinal absorption could contribute to sustained elevations of the serum levels of AGEs in these patients [25].

The role of reactive carbonyls and oxidative stress in the genesis of AGEs and ALEs has been demonstrated beyond doubt and can contribute to inflammatory damage of uraemic tissues [19]. Uraemia and HD patients may show a massive accumulation of free carbonyls, a condition described as ‘carbonyl stress’ [24], which can be derived from polysaturated fatty acids, sugars, glycolytic intermediates, ascorbic acid and free amino acids through the MPO-H\(_2\)O\(_2\)-chloride system and oxidative stress co-factors such as transition metals [26].

Several Maillard intermediates and AGEs have been detected in ESRD [7,8,27]. Glycation reactions and oxidative stress are known to contribute to increased cardiovascular risk in ESRD patients, and may also contribute to the genesis and progression of other comorbidities such as uraemic anaemia and amyloidosis. As a consequence, there is growing clinical interest on the mechanisms that drive the formation and accumulation of AGEs in ESRD.

Importantly, AGEs undergo in vivo proteolytic cleavage, that results in the formation of circulating by-products described as free glycation adducts [28,29] or low molecular mass (LMM)-AGEs [8] that exhibit biological properties similar to the AGE products that they originate from. Together with free carbonyls and pro-oxidant species, they can contribute to glycation reactions and protein damage in tissues. It is important to highlight that as compared with the AGE pre-
cursors, it might be easier to remove LMM-AGEs during dialysis.

The precise identification and quantification of AGEs in biological fluids and tissues has been conducted using HPLC and GC techniques coupled with spectroscopy [8,30] or mass detection systems (reviewed in [29,31,32]). Analysis of the fluorescing epitopes has been largely adopted in clinical studies, furosine and pentosidine being the most investigated. The fact that they are present at much lower levels than other non-fluorescing AGEs (such as MOLD, GOLD or CML) is not a realistic problem, since the sensitivity of the methods employed for measuring the blood levels of these indices yield reliable results and are easily applicable. Pentosidine fluorescence in uraemic plasma is mainly associated with albumin [7]. However, MALDI-MS analysis of fatty acid-free albumin from uraemic individuals has shown minimal modifications of this protein that might be consistent with AGE formation through reaction with \(\alpha\)-oxoaldehydes (molecular shift of +498 Da in ESRD and +438 Da in patients with ESRD on HD) [31].

Clinical investigation of AGE levels in uraemic serum has been performed using direct spectrofluorimetric analysis of fluorescing epitopes (without chromatographic separation of the test molecules). This method may require considerable validation to avoid possible false results. Immunodetection methods have been developed to trace epitopes such as CML and pentosidine, or imidazolone that has been detected in the aorta of uraemic subjects [33]. More recently, a skin autofluorescence assay, which was validated by correlation with the results of skin biopsies, was proposed as a non-invasive test to quantify tissue AGEs. Consistent with the view that tissue AGEs may represent a measure of cumulative metabolic stress and of the pro-inflammatory status in uraemia, it has been observed that skin autofluorescence is a strong, independent predictor of either total or cardiovascular-related mortality in patients with ESRD [34].

Miscellaneous protein damage products

Other (miscellaneous) oxidation products are mostly related to serum albumin damage reactions. Actually, other than being the preferential (sacrificial) target of glycation and oxidation reactions in the circulation [7,19,35,36], this protein is also highly susceptible to metal-catalysed oxidation [37,38], and plays an important antioxidant role in that it contains a highly oxidized product than the mixed disulphide, in combination with cysteine or glutathione and the other is a more advanced oxidant than the mixed disulphide, in which the thiol group is oxidized to the sulphenic (SOH), sulphinic (SO\(_2\)H) or sulphonic (SO\(_3\)H) state, and accounts for a very small proportion in the extracellular fluid compartment.

The overall blood thiol redox status is impaired in uraemia and HD patients [39] and this may contribute to enhanced albumin damage due to carbonyl and
oxidative stress. In fact, increased levels of carbonyl compounds have been reported to be correlated with the oxidation of albumin in uraemic patients. Accordingly, in the uraemic plasma, pentosidine fluorescence [7] and the markers of protein oxidation [19,35] are mainly associated with albumin.

Uraemic solute classification

Besides the large solutes formed as a result of oxidative and glycation processes and reflecting accelerated molecular aging of biological fluid and tissue proteins, large solutes of various other origins also accumulate in the uraemic serum and might contribute to the sustenance of inflammatory reactions. These solutes comprise complement-derived components, hormone-related proteins and peptides, soluble receptors, IgG chains, PMN granule components and other proteins derived from blood cell activation [4,40].

A recent analysis by the European Uraemic Toxin (EUTox) Work Group of the information available in the literature revealed 90 compounds classified under the definition of ‘uraemic toxins’ [41,42]. Toxin accumulation in patients with ESRD clearly shows extreme heterogeneity in terms of the chemical and physical properties of the toxins, as well as from the biochemical and clinical aspects. Uraemic toxins identified so far distribute over a wide range of molecular weight (from small to large solutes), and show different blood concentrations and degree of hydrophobicity. These aspects may be expected to influence the efficacy of dialysis strategies in the removal of these solutes [4,40]. With regard to the molecular weight, of the 90 compounds identified as uraemic toxins, 68 are smaller than 500 Da (small molecules), 10 are between 500 and 12 000 Da (middle) and 12 exceed 12 000 Da (large). Twenty-five of the solutes (28%) are protein-bound small molecules (except for leptin and retinol-binding protein).

However, in the taxonomy of uraemic toxins, the term ‘protein-bound’ can generate confusion. In this circumstance, it may be advisable to identify the type of binding, distinguishing between covalently bound small solutes and irreversible (end-products) or reversible protein modifications. For instance, Hcy may be included in the category of reversibly protein-bound small solutes. In fact, Hcy forms a reversible disulphide bond with the Cys-34 residue of albumin, the most abundant and redox active thiol in the plasma [43]. In contrast, pentosidine similar to other AGEs, becomes an integral part of the protein structure, in which Arg and Lys (as well as other nucleophilic groups in proteins, such as the side-chains of His, Ser, Thr, Trp and Tyr) are stably and irreversibly modified by glycoxidation to generate intra- or inter-molecular cross-links [23]. Thus, in this case, there is no reversible binding, but an irreversible structural change with the formation of an end-product different from the original substrates (reducing sugars and glucose products, short-chain sugars, reactive carboxyls and several other glycation and lipoxidation intermediates). The same is valid for the case of oxidative stress-generated end-products, such as di-Tyr or Lys and Hys oxidation products, in which again there is no binding but rather irreversible molecular changes. Thus, we suggest abolition of the misused general term ‘protein-bound’, use of which, for taxonomic purposes, should be limited to reversibly bound small solutes. In the other cases, the classification of solutes should be based on a correct identification of the individual end-product categories (e.g. mixed disulphides, AGEs, ALEs, AOPP, etc.), together with the reaction species and pathway(s) involved in their formation (e.g. AGE-pentosidine, AGE-CML, ALE-MDA). Where appropriate, the names of the individual amino acid residues or proteins involved should be specified (e.g. MDA-Lys or MDA-albumin, 4-HNE-albumin, etc.).

Protein damage products and the ‘inflammatory loop’ model of ESRD

It is now widely accepted that leucocyte-activating pro-inflammatory solutes accumulate in the uraemic blood, but only a few of these solutes have been characterized and quantified [4,44]. As a consequence, the biological mechanisms and inflammatory activities of these solutes in vivo remain poorly understood. However, it can be reasonably hypothesized that large solutes may play important roles in the genesis and progression of the inflammatory syndrome in patients with ESRD [4]. A model to explain this hypothesis is presented subsequently.

A robust and integrated system of control and defences intimately connected with inflammation, foreign and cancer cell killing, tissue remodelling and repair during normal growth or degenerative processes is present and widely distributed within the human organism [45,46]. The cell components responsible for these functions (often defined as ‘scavenger cells’) are mostly associated with vascular components and include inflammatory and vascular cells (such as PMN, monocyte-macrophages, endothelial cells and vascular smooth muscle cells).

The pleiotropic functions of this system comprise recognition and removal of protein damage products from tissues and biological fluids, which are mediated by a class of highly inducible membrane proteins known by the general name of scavenger receptors (SR).

The interaction between AGE proteins and scavenger cells such as inflammatory cells, liver sinusoidal cells and Kupffer cells, has been intensively investigated and is known to be mediated by a family of receptors that include the AGE receptor (or RAGE) belonging to the immunoglobulin superfamily, the galectin-3/80K-H/oligosaccharyltransferase-48 (OST-48) complex and other receptors [45]. Scavenging activity against AGE proteins was also...
identified for receptors such as CD36 and other members originally recognized to specifically mediate the endocytosis and metabolism of modified and native lipoproteins, such as the macrophage type I and II class A SR (MSR-A), SR-BI and lectin-like ox-LDL receptor 1 (LOX-1). The recognition of AGEs and possibly of other damage proteins by different members of the SR family highlight the pleiotropic nature of the SR responses, which can assume exquisite clinical relevance in cardiovascular diseases and other conditions associated with inflammation and aging (reviewed in [14,46]). In fact, they trigger biological responses, including endocytic uptake and degradation of AGE proteins, metabolic activation with abundant ROS/RNS production, secretion of inflammatory cytokines by phagocytes and endothelial cells, stimulation of monocyte chemotaxis, proliferation of vascular smooth muscle cells, quenching of NO activity with impaired smooth muscle relaxation in the arterial wall, collagenase secretion in synovial cells and osteoclast-induced bone resorption.

These aspects clearly indicate how the homeostatic role exerted by SR-bearing cells through ‘molecular cleaning’ of protein damage products could spill over to cause inflammation and endothelial dysfunction when these products are formed and accumulate at an abnormal rate in fluids and tissues. Therefore, in ESRD, chronic overstimulation of the SRs can initially act as a switch and then as a feeder of the ‘inflammatory loop’ observed in these patients (Figure 2). Other factors that feed the loop may also be suggested. Uraemic toxicity is clearly the earliest player, in that it contributes to the commencement of protein damage accumulation by carbonyl stress and other biochemical events, which then conspires with inflammation and oxidative stress. In this way, with progression of the uraemic condition, the loop assumes a self-propelling nature that can be further enhanced by HD, particularly when bioincompatible materials are used. In fact, leucocyte activation stimulates inflammatory and oxidative pathways, which cause systemic inflammation and abnormal metabolic responses in peripheral (non-inflammatory or target) tissues by inducing activation of resident inflammatory cells and chronic degenerative events (such as endothelial lesions and atherosclerotic plaque formation, muscle wasting, etc.). This may result in further generation of protein damage products, which then

Fig. 2. The ‘inflammatory loop’ sustained by scavenger receptor-dependent recognition of the proteinaceous toxins that accumulate in the uraemic blood. Further details are reported in the text.
‘keep the switch on’ and at the same time promote inflammation by impaired negative/anti-inflammatory feedback mechanisms (ACTH, glucocorticoids and cytokines, such as IL-4, IL-10, and TGF-β), leucopenia and immune cell anergy, defective humoral immunity and increased susceptibility to infections.

In support of this model, HOCl-treated albumin (an *in vitro* source of AOPP) and *in vivo*-generated AOPP (isolated from uraemic blood) have been demonstrated to trigger neutrophil and monocyte oxidative burst, thereby acting as true inflammatory and oxidative stress mediators [22,47]. Further and direct confirmation of this model based on SR-recognition of modified proteins has been obtained from *in vitro* evidence that selective removal with sorbent resins of pentosidine-like fluorescing HMWs markedly decreases the pro-inflammatory power of uraemic serum, as assessed by TNF-α production in U937 monocyte-macrophages [48]; importantly, the pro-inflammatory activity of the solutes removed was demonstrated to be largely RAGE-dependent. Also, HD therapy has been found to enhance RAGE expression in circulating mononuclear leucocytes [49,50], and together with uraemic intoxication, it can also affect RAGE expression in solid tissues. In non-diabetic uraemic subjects, the expression of arterial RAGE was suggested to increase the susceptibility to atherosclerosis in these patients [51].

### Large solutes: a class of toxins in quest of a dialysis therapy

Conventional HD techniques (cut-off limit of dialysis filters approx. ≤30 kDa) carried out with synthetic (highly biocompatible) dialysis membranes and ultra-pure dialysate cannot satisfactorily correct the biochemical or clinical signs of inflammation. Dialysis carried out with standard low- (LF) and high-flux (HF) dialysers efficiently, but almost exclusively, removes small-/middle-molecular-weight molecules (<5000 Da) (i.e. the ‘classical uraemic toxins’) from the uraemic blood, and only small amounts of larger solutes such as β2-microglobulin (MW = 11 000 Da). These latter molecules, once modified by glycation, show further lowering of mass transfer through either neutral or charged membranes [52]. Protein modifications can affect solute filterability by changing both the solute MW and the net charge, which may further contribute to increased physical hindrance to the passage of solutes through the pores of charged dialysis membranes. Accordingly, blood levels of AGEs and their proteolytic by-products, as well as ROS/RNS-derived protein damage products, cannot be efficiently reduced by conventional dialysis methods [4,7,8,27]. Furthermore, some small solutes with pro-inflammatory activity cannot also be efficiently cleared because of their physical and chemical behaviour in uraemic blood, which can contribute to increase large solute formation. This is the case for some glycation intermediates and free carbonyls discussed above in other sections of this paper. In addition, urates may also be present as mononuclear cell-activating crystals [53] and p-cresol has been reported to enhance the susceptibility to oxidation of serum proteins [38].

Future strategies should be oriented towards better characterization, and hopefully, prevention, as early as possible of the causes of inflammation in ESRD. Accumulation of pro-inflammatory toxins must be regarded as one of the causes for which preventive measures, pharmacological therapy and improved dialysis efficacy should be developed (Figure 3).
In this context, carbonyl and oxidative stress indices should be monitored from the earliest stages after the diagnosis of renal failure, in view of the fact that they can provide important information on the clinical evolution and metabolic consequences of inflammation and of the kidney disease. The most relevant preventive measure against inflammatory solute accumulation in CRF is to preserve renal function for as long as possible and to identify, and if feasible, to appropriately treat recurrent infections and immune dysfunction.

In HD patients, pro-inflammatory toxin accumulation can be treated by using different approaches, but it is of primary importance to improve the overall quality of the dialysis therapy. Material biocompatibility and dialysate quality have been improved enormously in recent years and ‘high-performance’ dialysis techniques have been developed. These include strategies based on mixed diffusive-convective methods, adsorption techniques [54-55] and ultra-HF protein-leaking dialysers (PLD) [7,42,56-58]. Among the HF synthetic membranes, polymethylmethacrylate (PMMA) has been used to produce PLD with superior performance, based on a combination of direct removal and adsorption of large solutes. These membranes, besides providing the highest standard of biocompatibility, can be used with dialysis techniques commonly adopted in the majority of HD centres, without adverse effects associated with malnutrition and hypoalbuminaemia or the risk of backfiltration, either in the short- or the long-term [7]. Table 1 shows the significant and steady decrease (30-42% of the baseline values) of the pre-HD levels of several plasma protein damage markers observed after 6 months’ dialysis treatment using PMMA-based PLD. These markers included total pentosidine (or AGE-pentosidine), AOPP, protein carbonyls, polyaminated proteins and a classical protein-bound solute, i.e. Hcy [7,43]. A comparable decrease was also observed in the inflammatory indices, such as IL-6 and CRP. On the other hand, HF non-protein-leaking dialysers (NPLD) did not modify either the protein damage or inflammatory indices (not shown).

According to evidence presented by others [58], an intriguing aspect revealed by these studies on PLD is that the amount of large solutes recovered in the dialysate samples (pentosidine and Hcy were evaluated in detail) was not sufficient to explain the acute intra-HD decreases in the plasma levels of these solutes, and consequently the steady correction of their pre-HD levels. Even when the membrane adsorption (in the case of PMMA membranes) was taken into account, this phenomenon could not be satisfactorily explained. This led to the suggestion of a common mechanism or factor underlying the depurative effect of dialysis using PLDs on such a heterogeneous group of proteinaceous compounds. It has been speculated that the uraemic blood may contain a class of large solutes, which cause a common metabolic defect that ultimately generates different classes of large solutes (e.g. by oxidative and carbonyl stress, and increased SR activity). Albumin damage products, which are present in relative abundance, with the multiple damage pathways (see earlier) serve as the best candidates for such a solute class. In fact, albumin is the most abundant proteinaceous solute removed by dialysis using PLDs (in the range of 1–4 g/dialysis session, an amount similar to or even lower than the amount of proteins lost during PD) [7,43], and might significantly contribute to the mechanism proposed above to sustain a large solute accumulation and the inflammatory loop in patients with ESRD (Figure 2).

Table 1. Plasma homocysteine, some indices of protein damage by glycation and oxidation reactions, and the inflammatory markers IL-6 and CRP, in the plasma of 13 ESRD patients at baseline and after 6 months’ dialysis treatment with a PMMA-based protein-leaking dialysier (BK-F series, Toray Co., Japan)

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<th>Baseline</th>
<th>6 months</th>
<th>Difference (%)</th>
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<tr>
<td>Hcy (µmol/l)</td>
<td>25.3 ± 5.9</td>
<td>17.2 ± 4.2°</td>
<td>-32</td>
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<tr>
<td>Free PT (pmol/ml plasma)</td>
<td>106.1 ± 24.0</td>
<td>60.2 ± 18.9°</td>
<td>-43</td>
</tr>
<tr>
<td>HSA-PT (pmol/mg albumin)</td>
<td>36.3 ± 14.2</td>
<td>23.8 ± 8.9°</td>
<td>-34</td>
</tr>
<tr>
<td>Carboxyls (AU/mg prot)</td>
<td>229.8 ± 156.8</td>
<td>132.2 ± 117.5°</td>
<td>-42</td>
</tr>
<tr>
<td>AOPP (AU/mg prot)</td>
<td>104.7 ± 60.2</td>
<td>64.6 ± 56.6°</td>
<td>-38</td>
</tr>
<tr>
<td>hs IL-6 (pg/ml)</td>
<td>5.0 ± 1.9</td>
<td>3.1 ± 0.6°</td>
<td>-38</td>
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<tr>
<td>CRP (mg/l)</td>
<td>22.7 ± 33.9</td>
<td>12.1 ± 9.1°</td>
<td>-47</td>
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Reference values: Hcy = 5–15, free PT, not detectable; HSA-PT < 2; carbonyls < 35, AOPP < 20. hs IL-6 < 4; CRP < 12.
A control group (n = 13) was maintained on treatment with NPLD, which do not significantly affect any of the parameters over the 6 months’ study period.
Treatment with PLD did not alter the levels of the total protein, albumin or other major protein sub-fractions.
Data obtained from [8; p. 94].
*P < 0.05 and **P < 0.01 vs baseline.
ineffectiveness in efficiently removing larger molecules could represent a major limitation of their performance. To address this limitation, adoption of mixed diffusive-convective methods has been proposed, which involve the use of specific materials, such as sorbent resins. These methods are under preliminary evaluation, aimed at establishing innovative haemoperfusion techniques with specific applications in acute and chronic inflammatory conditions [62]. In fact, these resins can remove several peptides and small proteins such as β2-microglobulin, leptin, complement factor D, angiogenin and a number of cytokines.

Pharmacological approaches to attenuate protein damage, and thereby, interrupt the inflammatory loop in HD patients (Figure 3) could be based on AGE inhibitors or disruptors, such as aminoguanidine [63]. This drug and also others, such as ALT-711, inhibit AGE-induced cardiac hypertrophy and stiffening of large arteries [46]. At the same time, aminoguanidine and metformin have been shown to prevent the impairment of HDL-mediated cholesterol removal from peritoneal macrophages and SR-BI transfected cells induced by AGE-albumin and cell glycoxidation, a biological event which could facilitate the development of premature atherosclerosis in diabetes mellitus and other disease-states associated with carboxyl and oxidative stress [64]. However, these drugs are still far from being approved for clinical use. At present, safety concerns and an apparent lack of efficacy have resulted in the discontinuation of a clinical trial of aminoguanidine in patients with diabetic nephropathy [63].

Proteomics of HMWs

Apart from studies on individual protein damage products, only a few papers have been published on systematic analysis (proteomics) of uraemic plasma and ultrafiltrate or spent dialysate proteins [65]. Future developments can lead proteomic studies to provide important clinical information for both acute and chronic treatments, and a valid support to industries and dialysis technologists.

The study of ultrafiltrate-proteins obtained using different types of NPLD, instead of whole serum proteins, is often adopted in these studies as a useful method for selective depletion of high-MW proteins (mostly albumin) that can interfere with protein and peptide analysis with relative concentrations set in the lowest range.

An early proteomic approach was employed by the group of Vanholder [66], who analysed peptide maps in ultrafiltrates generated after albumin removal using LF and HF PS-based dialysers. This analysis was performed by capillary electrophoresis coupled with MS spectrometry, which allowed solutes with a MW of up to 10 kDa to be investigated. Analysis of ultrafiltrates from uraemic plasma obtained using HF and LF membranes revealed the presence of 1394 and 1046 polypeptides, respectively. These numbers were reduced by more than half in ultrafiltrates prepared from the plasma samples of healthy donors. Only a few proteins were tentatively identified by MALDI-TOF-TOF analysis in this study, which also demonstrated the superiority of HF haem LF membranes in the removal of middle- and large-molecular weight polypeptides.

In another study by Lefler et al. [67], MALDI-TOF-TOF analysis was applied for characterization of the ultrafiltrate-proteins from a patient with acute renal failure. The authors identified 10 different proteins, including albumin, apolipoprotein A-IV, beta-2-microglobulin, lithostathine, mannose-binding lectin associated serine protease 2 associated protein, plasma retinol-binding protein, transferrin, transthyretin, vitamin D-binding protein and Zn-z glycoprotein.

Another attempt to identify ultrafiltrate-proteins was made by Molina et al. [68]. Dialysis fluid enriched with proteins with MWs in the range of 10–50 kDa was obtained from a patient with acute renal failure by dialysis using a polyacrylonitrile-based AN69 membrane (nominal cut-off <50 kDa, i.e. NPLD). Electrophoretic separation of the ultrafiltrate-proteins followed by in-gel digestion and LC-MS/MS analysis led to the identification of 292 proteins, 205 of which had never been reported before in the human serum or plasma. Additional biochemical and immunoenzymatic analysis revealed that many of these proteins had probably never been identified previously because of their presence at very low concentrations. Several proteins showed post-translational modifications useful to map mature forms of the N-termini. In vivo proteolytic fragmentation was tentatively identified in some cases. New proteins, including cytokines, have been identified as predicted transcripts on databases.

Recently, protein removal and adsorption have been evaluated by SELDI-TOF-MS, and ProteinChip arrays have been evaluated in patients dialysed using PS or PMMA moderate-flux membranes (both are included in the category of NPLD) [61]. Mass-to-charge ratios (m/z) between 2000 and 120 000 were analysed. β2-microglobulin (identified as m/z 11 730) was predominantly adsorbed onto, and only a small amount was filtered through, PMMA membranes, which were found to adsorb a total of 149 polypeptides. Sixty-eight peptides were adsorbed more efficiently by PS than by PMMA membranes, and the majority of these showed m/z values <11 730. The dominant peaks adsorbed onto PS showed m/z values of 6629 and 6431 and were identified as apolipoprotein CI and truncated apolipoprotein CI, respectively. Thirty-seven proteins with a MW > m/z 11 730 were more efficiently filtered by PMMA than by PS membranes.

In this laboratory, comparative proteomic analyses of plasma and ultrafiltrate samples obtained from patients dialysed using PMMA-based PLD (BK-F series, Toray Co., Japan; nominal cut-off 70 kDa) and standard PS-based HF NPLD (two types of
membranes were tested: NPLD1 = FX-80 dialysers, Fresenius, Germany and NPLD2 = Toraysulphone dialysers, Toray Co., Japan) have been carried out [65]. Consistent with the results of previous experiments [7], it was confirmed that these two types of membranes produced a net protein-leakage of ~3–5 g and <0.3 g per dialysis session, respectively. For early characterization, mono-dimensional polyacrylamide gel electrophoresis (1D-PAGE) analyses were performed on the plasma and ultrafiltrate-proteins (20 µg total protein/gel line) obtained using NPLD and PLD membranes (n = 4 each). Qualitative and semi-quantitative analyses revealed major differences in the ultrafiltrate protein patterns obtained between the two kinds of dialysers (Figure 4), whereas the plasma protein patterns were not different (data not shown). Qualitative differences confirmed by densitometry and Quantity-One software (Bio-Rad) analysis revealed that, in accordance with the nominal cut-off value declared by the manufacturer, PLD ultrafiltrates mostly contained proteins with a MW ≥60 kDa. As expected, these PAGE analyses also confirmed that albumin predominated over the other proteins removed using these dialysers, but that other proteins were also abundantly removed, including pre-albumin, transferrin and IgG chains. In contrast, the protein patterns of the NPLD ultrafiltrates were characterized by the presence of a much higher proportion a solutes with MW in the range of 10–66 kDa, i.e. the MW of albumin. Taking into account the nominal cut-off values of these membranes, these results clearly show that protein leakage through NPLD, although limited as an entity, is non-specific and affects proteins present in relatively low abundance.

Another objective of our studies was to investigate the presence of damaged proteins in the ultrafiltrates of uraemic blood obtained using these dialysers. Preliminary immunoblotting analyses carried out with antibodies against the ALE marker 4-hydroxy-2-nonenal (4-HNE) and the RNS-dependent and inflammation-related protein damage marker, 3-nitrotyrosine (Figure 4), revealed positive immunostaining of several proteins: albumin was a preferential target of damage by carbonylation and nitration together with transferrin, and other bands in the MW region of 10–30 kDa were immunostained by both the antibodies. Together with several unidentified proteins, this region included IgG light chains as one of the most abundant immunoreactive elements. Faint signals were observed for proteins with higher MW, such as fibrinogen (see line at the origin; MW = 340 kDa), which has been reported to be extensively carbonylated in the uraemic serum [19].

These early results confirm the wide extent of injury of the plasma proteins in uraemic patients caused by inflammation and oxidative stress, and demonstrate how several damaged pathways may be simultaneously active and overlap to amplify protein injury.

In the comparison between NPLD and PLD ultrafiltrates, it was possible to determine that PLD removed a large amount of 4-HNE- and 3-nitrotyrosine-containing proteins, being able to selectively remove albumin. Although protein leakage in PS-based NPLD was limited, these dialysers may find specific application for the selective removal of protein damage products with a low MW. This aspect of possible relevance for dialysis technologists may also have important implications for research strategies aimed at characterizing the immunostained (damage product) proteins with a lower MW.

These results clearly reveal the importance of such proteomic strategy based on the immunodetection of proteins modified by ROS/RNS (also referred to as ‘redoxomics’ [69]) for the characterization of inflammation-related large uraemic solutes and dialyzer performance.

The coupling of 2D-PAGE and immunodetection methods with mass spectrometry analysis provides the highest specificity in protein identification. This type of approach is currently used in this laboratory to further characterize uraemic plasma and ultrafiltrate-proteins. In this respect, albumin removal is widely employed during preparative steps to increase the relative abundance of less expressed plasma proteins. Preliminary experiments to evaluate the impact of this sample preparation procedure have been carried out using an immunoadfinity method (Figure 5A). The results clearly demonstrated that there are some major differences in the relative abundance of some proteins in the whole and albumin-depleted plasma (Figure 5A, panel 2 and 3, respectively; for comparison, a reference plasma protein map available in the SWISS-PROT database is reported in panel 1; see the EXPASY website at: http://www.expasy.org/cgi-bin/map2/def?PLASMA_HUMAN). Moreover, these differences were affected by a high degree of inter-assay variability (data not shown), thus leading to the conclusion that albumin removal can critically influence the assay reproducibility and reliability of semi-quantitative and qualitative evaluations of plasma and ultrafiltrate samples.

Figure 5B shows an example of protein maps obtained without albumin depletion and corresponding to the samples of plasma (left panel) and ultrafiltrate (right panel) obtained from patients dialysed using a PLD membrane (BK-F series, Toray Co., Japan). Image analysis performed using the PDQuest 2D gel software (Bio-Rad) revealed a higher number of proteins in the ultrafiltrate (approximately 25% higher) than in the plasma specimens. Moreover, densitometry data showed several proteins in the ultrafiltrates that were present at least 2-fold higher concentrations than in the plasma (~15 of the spots identified). High-resolution imaging allowed identification of a maximum of 314 and 230 spots in the ultrafiltrates obtained using NPLD and PLD, respectively, with differences in either the presence/absence of the spot or in the signal intensity (e.g. 2-fold difference) for 143 proteins, mostly in the lower range of MWs (<60 kDa), which is in agreement with the findings of 1D-PAGE analysis.
Fig. 4. One-dimension SDS-PAGE gradient analysis (%T range = 9–16; panel A) and Western blotting (%T = 12, panel B) of anti-3-nitrotyrosine (upper) and anti-4-hydroxy-2-trans-nonenal immunoreactive proteins in the ultrafiltrate samples obtained from subjects treated with two types of polysulphone-based non-protein-leaking dialysers (NPLD 1 = FX-80 series, Fresenius, Germany and NPLD 2 = BS-1.8 UL series, Toray Medical Co., Japan) and a polymethylmethacrylate-based protein-leaking dialyser (PLD; BKF series, Toray Medical Co., Japan). For comparison of the ultrafiltrate-proteins, a sample of uraemic plasma was also assessed (lane P). Total proteins were loaded in amounts of 20 μg and 100 μg for the SDS-PAGE and WB analysis, respectively. In the WB analysis, the proteins were immunodetected using a mouse monoclonal anti-3-nitrotyrosine antibody (Upstate, Lake Placid, NY, USA) and subsequently, a goat anti-mouse antibody—HRP conjugate (Upstate, Lake Placid, NY, USA). Qualitative and semi-quantitative image analyses of 1D patterns were performed using the Quantity-one 1-D analysis software (Bio-Rad).
Further analysis of the ultrafiltrates obtained with PLD, revealed that 121 spots can be resolved by 2D-PAGE, to a degree useful to proceed with isolation, trypsinization and mass analysis. This number included at least 14 proteins that exhibited a 2-fold higher signal intensity in the ultrafiltrate than in the plasma, in which a total of 91 spots were identified.

A preliminary list of proteins isolated from the 2D maps of the ultrafiltrates obtained using PLD and NPLD (Figures 6A and B, respectively) and identified by mass spectrometry is shown in Table 2 and includes some of the main classes present in plasma: albumin, prealbumin (transthyretin) and pro-prealbumin, transferrin, complement factors, α-1-antitrypsin...
of patients with ESRD is a large group of proteinaceous solutes that are products of oxidation and/or glycation reactions of proteins that are catalysed by the abnormal chemistry of the uraemic milieu and chronic inflammation.

However, these solutes do not seem to be just hallmarks useful in laboratory evaluations. Increasing evidence is accumulating to suggest that these solutes may have relevant biological roles in the complex pathogenesis of the inflammatory and vascular comorbidities of patients with ESRD. As the mechanism underlying the pathogenic roles of these solutes, it has been proposed that their accumulation can lead to a sustained and chronic activation of inflammatory and vascular cell components. Indeed, these cells express specific classes of SR that are responsible of the binding and processing of oxidatively damaged and glycated proteins. This receptor-dependent mechanism in turn ‘switches on’ and sustains an inflammatory loop, which conspires with other uraemic events to produce a vicious and self-feeding cycle of protein damage and SR-dependent reactions (Figure 2). The generation/accumulation of oxidatively modified and glycated forms of albumin may play a major role in the onset and sustenance of this loop being the most abundant group of proteinaceous toxins in the uraemic blood, and effective SR ligands and pro-inflammatory triggers.

While transplantation seems to almost completely resolve the problem of protein damage, even the most efficient and advanced dialysis techniques and drugs that may be useful for decreasing the blood levels of reducing sugars and free-carbonyl reactivity have failed to prevent/correct the accumulation of the aforementioned protein damage products, which reaches a zenith in patients undergoing maintenance HD.

Future work is needed to develop dialysis techniques to correct the accumulation of large solutes. Even the most recent generations of materials and techniques appear to fall short of the requirements. Mixed diffusive/convective techniques with HF membranes seem to provide maximal efficacy in the removal of small- and middle-molecular weight solutes, and suitable alteration of the rhythm of dialysis may also be considered as another relevant approach to control the accumulation of these toxins. However, large solutes seem to be only marginally affected by any of the available dialysis methods, although some promising, but preliminary, results have been obtained with the use of PLD in standard HD. Adsorption may also be another useful approach for increased and/or selective removal of some large solutes. Thus, early evidence suggests that protein leakage and adsorption are approaches that deserve future investigation in order to determine their potential and applicability in dialysis strategies specifically aimed at reducing the pro-inflammatory mediator burden in uraemia and HD patients.

Proteomic methods represent an effective tool for the identification and molecular characterization of protein damage products in uraemic plasma and

**Conclusion**

Among the biochemical hallmarks of protein damage products that accumulate in the blood and solid tissues precursor and Ig gamma chains. Interestingly, the neutrophil gelatinase-associated lipocalin precursor (p25) was also identified. Between the main spots detected in the 2D analysis of the ultrafiltrate obtained using PLD, but not in the plasma, MS identification was accomplished for α-1-antitrypsin precursor, pre-albumin, some complexes of albumin with small molecules, and the Ig gamma-1 and -2 chain C region. Ongoing MS, immunoblotting and sequencing analyses are expected to yield further protein identification in these samples.
Table 2. Mass spectrometry analysis for the identification of ultrafiltrate proteins obtained from patients dialysed using PMMA-based PLD (BK-F series, Toray Co. Japan) and PS-based NPLD (FX-80, Fresenius, Germany)

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NPLD ultrafiltrate (PS-based membrane, FX-80 series)

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*Proteins present in the ultrafiltrate obtained using PLD, but not in the uraemic plasma.

Electrospray-mass spectrometry (ESI-MS) analysis of oligopeptides obtained from in-gel trypsinization of isolated proteins was performed using a ProteomeX apparatus (Thermo Electron Corporation, San Jose, CA, USA) equipped with a 20 μl loop and two Hypersil-Keystone BioBasic C18 capillary columns (0.18 x 100 mm), using the high-throughput mode configuration. Eluted oligopeptides were electrosprayed directly into the LCQ Deca-XP Plus ion-trap mass spectrometer.

ultrafiltrate samples. This paper shows an original application of proteomics to the characterization of large uraemic solutes removed by PMMA-based PLD membranes and standard PS-based NPLD membranes. The analyses also included a redoxomic approach, with preliminary characterization of ROS/RNS-derived protein damage products investigated by immunological analysis of the epitopes 4-HNE and 3′-N-Tyr.

These results and the available literature on protein damage products that accumulate in the blood and tissues of uraemia and dialysis patients provide an impetus for aggressively pursuing work that is needed to identify the biochemical features, biological roles and toxicity of these protinaceous solutes. Such studies can represent an important prerequisite for the development of more effective therapies against chronic inflammation and vascular dysfunction in patients with ESRD.

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Conflict of interest statement. None declared.

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