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Plasma protein-bound di-tyrosines as biomarkers of oxidative stress in end stage renal disease patients on maintenance haemodialysis

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ABSTRACT

Background: Patients with end-stage renal disease (ESRD) undergoing haemodialysis (HD) experience enhanced oxidative stress and systemic inflammation, which are risk factors for cardiovascular disease, the most common cause of excess morbidity and mortality for these patients. Different pathways producing different types of oxidative stress occur in ESRD. The purpose of our study was to determine the effect of HD on plasma levels of protein-bound dityrosine (di-Tyr), a biomarker of protein oxidation.
Methods: Protein-bound di-Tyr formation was measured by size exclusion HPLC coupled to fluorescence detector. Clinical laboratory parameters were measured by standardized methods.
Results: In most ESRD patients, a single HD session decreased significantly the plasma protein-bound di-Tyr level, although the mean post-HD level remained significantly greater than the one in healthy people. Furthermore, pre-HD plasma protein-bound di-Tyr level was positively correlated with pre-HD serum creatinine and albumin concentrations. No significant correlation was found between plasma protein-bound di-Tyr level and serum concentration of C-reactive protein, a biomarker of systemic inflammation.
Conclusions: This study demonstrates that a single HD session does not increase, rather partially decreases, oxidative pathways producing di-Tyr in the haemodialyzed patient.
General significance: The choice of the most pertinent biomarkers of oxidative stress is critical for the development of novel treatments for ESRD. However, the relative importance of oxidative stress and inflammation in ESRD remains largely undetermined, and several questions concerning oxidative stress and inflammation remain poorly defined. These results could stimulate further studies on the use of plasma protein-bound di-Tyr as a long-lasting oxidative stress biomarker in ESRD.

1. Introduction

Chronic kidney disease (CKD), or chronic renal failure, is an important public health problem since its prevalence has reached epidemic proportions, with 10–13% of the population affected in different countries around the world [1]. Patients affected by CKD are categorized into five stages according to the glomerular filtration rate and presence of signs of kidney damage [2]. Compared with the general population, CKD patients have a higher risk for premature death, primarily as a result of cardiovascular disease (CVD), and their cardiovascular risk increases continuously with the decrease in kidney function [3]. Thus, most patients with mild to moderate (stages 3–4) CKD die of CVD rather than progress to end stage renal disease (ESRD, or CKD stage 5) [4]. ESRD represents the total inability of kidneys to maintain homeostasis and hence is incompatible with life. Therefore, to ensure survival of patients with ESRD, it is necessary to use methods that substitute for kidney function, including haemodialysis (HD), peritoneal dialysis and kidney transplantation. ESRD patients on maintenance HD too experience a higher risk for CVD and its associated mortality compared to the general population [5].

Patients with CKD are at higher risk for CVD because of higher prevalence of traditional (such as age, diabetes mellitus, left ventricular hypertrophy, dyslipidemia, hypertension) and non-traditional cardiovascular risk factors [6,7]. The latter include anaemia, uraemia, altered calcium-phosphate metabolism, malnutrition, inflammation and

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oxidative stress [8–11]. In patients with ESRD, HD may also impose an additional oxidative stress, mainly attributed to loss of circulating low-molecular-mass dialyzable antioxidants and to the activation of neutrophil NADPH oxidase, provoking inflammation with release of reactive oxygen species [11–14]. In fact, the extracorporeal treatment itself represents a bioincompatible event in the patient's life: during the HD session, blood is exposed 3 to 4 h to synthetic material, i.e., blood lines and filter. Historically, the first filters used in HD were composed of cellulose: this treatment was so bioincompatible that patients used to experience fever and chills during HD due to complement activation [15]. Currently, with the use of synthetic filters, patients do not experience fever yet, but sub-clinical activation and degranulation of polymorphonuclear neutrophils still occurs [16]. Moreover, intravenous iron therapy in HD patients, even in recommended doses, could further aggravate oxidative stress and atherosclerotic disease. Furthermore, increased total body iron level exacerbates deficiency of lycopene and other lipophilic antioxidants [17].

Four pathways producing different types of "oxidative" stress can be hypothesized in CKD patients, i.e., classical oxidative stress, carbonyl stress, nitrosative stress, and chlorine stress [18]. Increased oxidative stress in patients with ESRD and CKD stage 3 or higher is demonstrated by increase in plasma thiol-specific oxidative stress [19–23] and protein carbonyls (PCO) [19,20,24–26] and by the presence of plasma advanced oxidation protein products (AOPPs) [26–29].

AOPPs are considered as potential uremic toxins and inflammatory mediators [30], involved in the activation of polymorphonuclear granulocytes, monocytes and vascular endothelial cells [31,32]. Chronic accumulation of AOPPs accelerates atherosclerosis by promoting oxidative stress and inflammation [33]. Furthermore, AOPPs directly impair metabolism of high-density lipoproteins, being potent antagonists of their receptor and, therefore, might be directly involved in the development of CVD [34].

AOPPs are a heterogeneous group of dityrosine (di-Tyr)[1], pentosidine and carbonyl-containing protein products generated in plasma proteins by both myeloperoxidase (MPO)-dependent (e.g., in ESRD patients) and MPO-independent (e.g., in the predialysis phase of CKD) mechanisms during oxidative/chlorine stress [27,35]. AOPPs are considered a generic biomarker of protein oxidation because their molecular composition has not yet been precisely defined and their easy spectrophotometric determination is often invalidated by poor reproducibility and accuracy of most colorimetric methods for their detection. Furthermore, measuring AOPPs in diluted plasma as absorbance at 340 nm is a rather nonselective way to determine the level of oxidized proteins; therefore, it is necessary to take precautions to minimize the contribution of species other than AOPPs. Consequently, reliable, validated AOPP reference values in healthy humans are still lacking [27,34–37]. In addition, measurement of AOPPs during HD session gave contrasting results [20,38,39].

Our preliminary results showed a significant ($p < 0.001$) increase in di-Tyr fluorescence (normalized to protein concentration) in plasma samples of patients with ESRD undergoing regular maintenance HD as compared to healthy controls [37]. As mentioned above, there is concern that the HD session itself can be, at least in part, responsible of this tremendous oxidative burden [38]. The purpose of the present study was to determine the effect of a single HD session on plasma levels of protein-bound di-Tyr, a biomarker of irreversible protein oxidation, in ESRD patients on maintenance HD. We also examined the potential correlation between plasma protein-bound di-Tyr concentration, taken as a biomarker of oxidative stress and creatinine, albumin, and C-reactive protein (CRP) concentration, taken as biomarkers of systemic inflammation.

[1] In this manuscript the term dityrosine will refer to 3,3′-dityrosine (3,3′-bityrosine or o,o′-dityrosine)

2. Materials and methods

2.1. Study participants

All the patients enrolled in the study belong to stage 5 of CKD and are referred to as ESRD patients. These patients do not show any residual renal function and thus require renal replacement therapy. In addition to hemodialysis, patients are treated with a pharmacological treatment that varies upon the clinical necessities and consists mainly on the treatment of ESRD complications. Most of the patients assume drugs for anaemia and bone mineral disorder. In particular, for anaemia they may assume iron endovenous supplementation and/or erythropoietin, and for bone mineral disorder calcium supplementation, phosphate binders, vitamin D, paracalcitol and/or calcimimetics. In addition to these therapies, patients may also take specific drugs for other comorbidities, e.g. hypertension, diabetes mellitus, ischemic cardiopathy and other vasculopathies. Blood samples were collected after informed written consent from ESRD patients undergoing maintenance HD at the Nephrology Unit of the Humanitas Clinical and Research Center (Rozzano, Milan, Italy). The samples have been collected at the arterial line at the beginning and at the end of HD session. All the filters used were made of polyethersulphone (Polyflux™ Gambro-Baxter, Rome, Italy). The presence of a clinically evident infectious process was the only exclusion criteria. For every patient an anamnestic record was collected. A de-identification of the samples was made for the further data treatment. Seventy-three haemodialyzed patients joined the study (Table 1). Control blood samples were collected from 25 (13 male and 12 female) age-matched voluntary healthy donors at the Analysis Laboratory of the University of Milan (Laboratorio Analisi Università di Milano), after obtaining informed verbal consent. Criteria included no known history of CKD or other diseases that could influence the analysis. In particular, healthy subjects were tested for serum creatinine in order to exclude CKD.

Sample collection

From ESRD patients, venous blood samples of 10 ml were collected before HD and 5 ml were obtained after the same HD session. All samples were collected on the long inter-dialytic interval, i.e., two days apart from the previous HD session. Blood was taken from the arteriovenous fistula or central venous catheter. From healthy donors, 10 ml of venous blood was collected from the antecubital vein. K_3EDTA was used as anticoagulant in all the blood samples. All the samples were processed within the first hour from blood sampling through centrifugation for 10 min at 1000*g*, obtaining pre-HD and post-HD plasma aliquots from haemodialyzed patients and plasma aliquots from healthy

Table 1
Characteristics of study group. Data are expressed as mean ± SE.

	Haemodialyzed Patients ($n = 73$)
Age (years)	69.62 ± 1.48
Sex	48 male, 25 female
Diabetes	50 nondiabetic, 23 diabetic
Length of time on dialysis (years)	5.71 ± 0.44
CRP (mg/dL)	0.51 ± 0.06
Albumin (g/dL)	3.50 ± 0.04
Fibrinogen(mg/dL)	355.47 ± 9.01
White blood cells (cells/mm^3)	7293.15 ± 257.90
Haemoglobin (g/dL)	11.02 ± 0.11
Urea (mg/dL)	150.14 ± 4.76
Creatinine (mg/dL)	9.16 ± 0.35
Sodium (mmol/L)	137.80 ± 0.36
Potassium (mmol/L)	5.22 ± 0.09
Calcium (mmol/L)	2.23 ± 0.02
Phosphorus (mmol/L)	1.65 ± 0.05
Ferritin (ng/mL)	199.90 ± 16.03
Total iron-binding capacity (g/L)	184.62 ± 7.18