



## 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.

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### 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)<sup>1</sup>, 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.

<sup>1</sup> 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<sub>3</sub>EDTA 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 1000g, 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  $\pm$  SE.

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

controls. Such aliquots were stored at  $-80^{\circ}\text{C}$  until the execution of the assays.

### 2.3. Plasma protein-bound di-Tyr determination

Protein-bound di-Tyr formation was evaluated by Size Exclusion/Gel Filtration High Performance Liquid Chromatography (GF-HPLC, same as SE-HPLC) on a BioSep-SEC-S4000 column (300 mm  $\times$  7.8 mm) with a guard column (SecurityGuard™ GFC-4000, 4 mm length  $\times$  3 mm ID) and UV-VIS detector. Plasma samples were diluted 1:15 in 50 mM Tris-HCl, pH 7.4 and 20  $\mu\text{l}$  was loaded into the column for each sample. The mobile phase consisted of Milli-Q water, containing 0.5% (w/v) SDS and was eluted at 1 ml/min. Eluates were monitored both at 215 nm for measuring absorbance of peptide bonds and at 415-nm emission with 325-nm excitation for measuring di-Tyr fluorescence. In the time range between 6 and 9 min, both the area under the 215-nm absorbance chromatogram ( $A_{215}$ ) and the area under the 415-nm emission fluorescence chromatogram ( $IF_{415\text{nm em}}$ ) were considered (Supplementary Fig. S1). The ratio between total fluorescence and total absorbance ( $IF_{415\text{nm em}}/A_{215\text{nm}}$ ) was calculated for each sample.

### 2.4. Determination of clinical laboratory parameters

CRP, white blood cells count, albumin, creatinine, fibrinogen, haemoglobin, ferritin, total iron-binding capacity, urea, sodium, potassium, calcium, and phosphorus were measured by standardized methods at the clinical laboratory of the Humanitas Clinical and Research Center.

### 2.5. Statistical analysis

Student's *t*-test for independent samples was used to test for differences between the ESRD patients on maintenance HD and the age-matched healthy subjects. The  $\alpha$ -level of the tests was set at  $p < 0.05$ . In Table 2, we reported the mean (standard error, SE) values estimated on the largest sample available for each sex separately. Sex-specific mean values were compared by independent-sample *t*-tests. The data met the assumptions of homogeneity of within-sex variances (Levene's test for homoscedasticity;  $F \leq 1.52$ ,  $p \geq 0.22$  for both markers) and of within-sex normality (Kolmogorov-Smirnov test,  $Z \leq 1.00$ ,  $p \geq 0.27$  for all sex-by-marker combinations). Analyses of covariance were used to test for consistency of any sex-dependent variation in di-Tyr while controlling for the potentially confounding effects of age. Predialysis (pre-HD) and postdialysis (post-HD) di-Tyr values were compared by Student's *t*-tests for paired samples within each sex separately. The relationships between variables were investigated by linear regression and Pearson's correlation coefficient. Estimated parameters are presented with their associate standard error (SE).

**Table 2**

Plasma protein-bound di-Tyr content in male and female ESRD patients. Mean (SE) of plasma protein-bound di-Tyr level in male and female ESRD patients before (pre-HD) and after (post-HD) a single HD session. Sex-specific mean values were compared by independent-sample *t*-tests.

	N	Mean (SE)	t	df	P
di-Tyr pre-HD					
Males	48	0.193 (0.005)	2.23	71	0.029
Females	25	0.170 (0.010)			
di-Tyr post-HD					
Males	47	0.174 (0.0174)	2.11	70	0.039
Females	25	0.154 (0.010)			

## 3. Results

### 3.1. Plasma protein-bound di-Tyr levels in healthy people and ESRD patients on maintenance HD

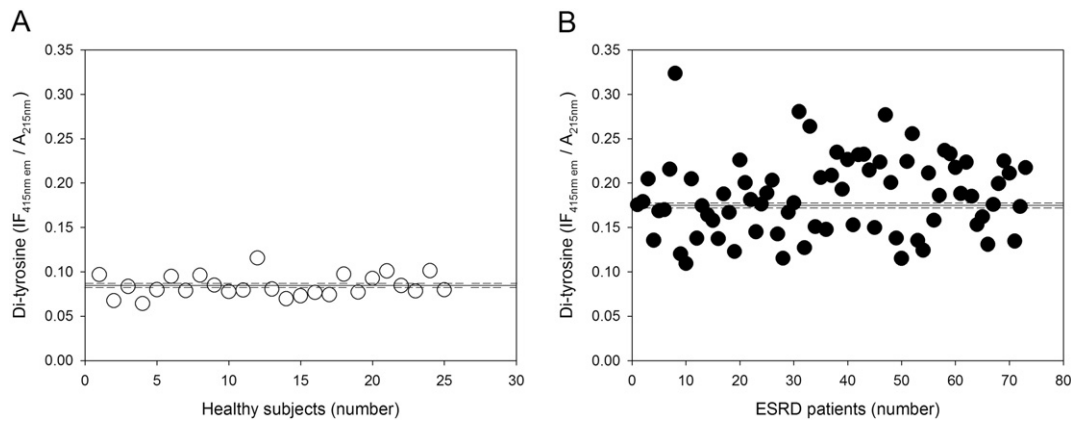
There are significant differences in total plasma protein and albumin concentrations between healthy subjects and patients with ESRD [23, 40], as well as for each ESRD patient before and after a single HD session [23]. The latter is due to ultrafiltration performed during the dialysis session in order to restore the dry body weight of the patient. This deserves close consideration in the analysis of plasma protein-bound di-Tyr concentrations, since any increase or decrease in total plasma protein and albumin concentration can dramatically affect the di-Tyr measured values. Therefore, in this study we express plasma protein-bound di-Tyr content as  $IF_{415\text{nm em}}/A_{215\text{nm}}$  ratio.

Firstly, we determined the plasma protein-bound di-Tyr content in healthy subjects and in ESRD patients before HD (Fig. 1). Plasma protein-bound di-Tyr content ranged from 0.064 to 0.116  $IF_{415\text{nm em}}/A_{215\text{nm}}$  (mean  $0.084 \pm 0.0024 IF_{415\text{nm em}}/A_{215\text{nm}}$ ) in healthy subjects (Fig. 1A) and from 0.109 to 0.324  $IF_{415\text{nm em}}/A_{215\text{nm}}$  (mean  $0.185 \pm 0.0051 IF_{415\text{nm em}}/A_{215\text{nm}}$ ) in ESRD patients before HD session (Fig. 1B), being significantly higher in ESRD patients ( $t = 11.32$ , d.f. = 96,  $p < 0.00001$ ).

We evaluated the discriminative power of plasma protein-bound di-Tyr content in distinguishing ESRD patients from age-matched healthy subjects by means of receiver operating characteristic (ROC) curve analysis (Supplementary Fig. S2). Plasma protein-bound di-Tyr levels from healthy subjects and ESRD patients yielded an area under the curve (AUC) of 0.99 (95% confidence interval 0.9943 to 1.002;  $p < 0.0001$ ) (Supplementary Fig. S2A). The cut-off level for plasma protein-bound di-Tyr content as predictor of ESRD was determined by maximizing sensitivity and specificity, at 0.112  $IF_{415\text{nm em}}/A_{215\text{nm}}$  (Supplementary Fig. S2B). Conversely, plasma protein-bound di-Tyr levels from diabetic ( $n = 23$ ) and non-diabetic ( $n = 50$ ) ESRD patients yielded an AUC of 0.54 (not shown), implying that di-Tyr cannot discriminate between diabetic and non-diabetic ESRD patients.

### 3.2. Effect of a single HD session on the level of plasma protein-bound di-Tyr

To determine the effect of HD on the plasma protein-bound di-Tyr level, we measured protein-bound di-Tyr in the plasma of the same ESRD patients immediately before and immediately after a single HD session. The scatter diagram of plasma protein-bound di-Tyr levels in haemodialyzed patients is presented in Fig. 2A. In most ESRD patients, we observed a small decrease in the plasma protein-bound di-Tyr level after the HD procedure compared to the pre-HD value. Differently, some ESRD patients showed the same or a slightly higher plasma protein-bound di-Tyr level immediately after the HD procedure compared to the pre-HD value. The result of the Student's *t*-test for paired data of plasma protein-bound di-Tyr level measured in ESRD patients pre-HD (mean  $0.185 \pm 0.0051 IF_{415\text{nm em}}/A_{215\text{nm}}$ ) and post-HD (mean  $0.167 \pm 0.0045 IF_{415\text{nm em}}/A_{215\text{nm}}$ ) proved that the means are significantly different ( $t = 7.11$ , d.f. = 71,  $p < 0.0001$ ) (Fig. 2B). However, this decrease did not result in normalization of plasma protein-bound di-Tyr content that remained significantly greater in ESRD patients post-HD than in age-matched healthy subjects (mean  $0.084 \pm 0.0024 IF_{415\text{nm em}}/A_{215\text{nm}}$ ) (Fig. 2B; see also Fig. 1A). It is worth noting that plasma protein-bound di-Tyr level immediately before (pre-HD) and after (post-HD) a single HD session differed between male and female ESRD patients (Table 2). These results were confirmed when we checked for the potentially confounding effect of age on sex-dependent variation in both pre-HD and post-HD plasma protein-bound di-Tyr level in analyses of covariance, where age was entered as a covariate (effect of sex on pre-HD and post-HD plasma protein-bound di-Tyr level:  $F = 5.71$ ,  $df = 1.70$ ,  $p = 0.020$  and  $F = 4.73$ ,  $df = 1.69$ ,  $p = 0.033$ , respectively). Analyses of covariance revealed no significant effect of age



**Fig. 1.** Plasma protein-bound di-Tyr level in healthy subjects and ESRD patients. (A) Plasma protein-bound di-Tyr in individual healthy subjects ( $n = 25$ ). (B) Plasma protein-bound di-Tyr content in individual ESRD patients ( $n = 73$ ) before HD session (pre-HD). In both (A) and (B), the horizontal solid and dashed lines represent, respectively, the mean and the SE of the plasma protein-bound di-Tyr level.

on plasma protein-bound di-Tyr level before and after HD ( $F \leq 1.70$ ,  $p \geq 0.19$ ). Simple linear regression analysis revealed that plasma protein-bound di-Tyr level measured pre-HD was significantly positively correlated with post-HD di-Tyr level in both male (Fig. 3A) and female (Fig. 3B) ESRD patients (panel A:  $r = 0.884$ ,  $p < 0.0001$ ; panel B:  $r = 0.866$ ,  $p < 0.0001$ ).

### 3.3. Correlation between pre-HD serum creatinine and albumin concentrations and plasma protein-bound di-Tyr level measured pre-HD

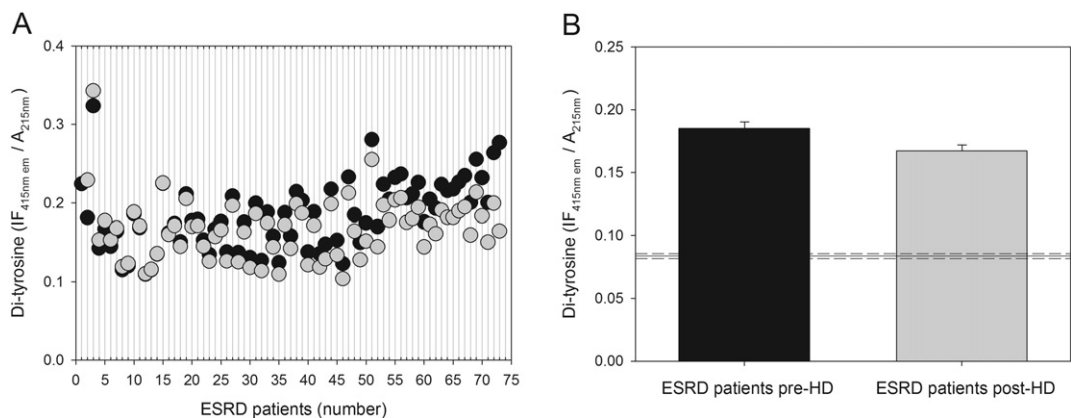
Haemodialyzed patients have <15–10% kidney functions and the severely damaged kidneys do not efficiently remove creatinine from the blood. Therefore, all ESRD patients develop high serum creatinine levels. However, as a small molecule, creatinine is efficiently cleared by dialysis. Thus, we examined the relationship between plasma protein-bound di-Tyr level and predialysis creatinine concentration in ESRD patients before a single HD session (Fig. 4). The scatter diagram of predialysis serum creatinine concentration in individual ESRD patients is presented in Fig. 4A. Predialysis creatinine concentrations in ESRD patients ranged from 2.67 to 19.52 mg/dl (mean  $9.1614 \pm 0.3494$  mg/dl) (Fig. 4A) and were significantly positively correlated with pre-HD plasma protein-bound di-Tyr levels (Fig. 4B) ( $r = 0.318$ ,  $p = 0.006$ ).

The  $IF_{415nm\ em} / A_{215nm}$  ratio relative to total plasma proteins actually mainly measures the di-Tyr content of albumin, because albumin is the most abundant plasma protein, accounting for 50%–60% of total proteins

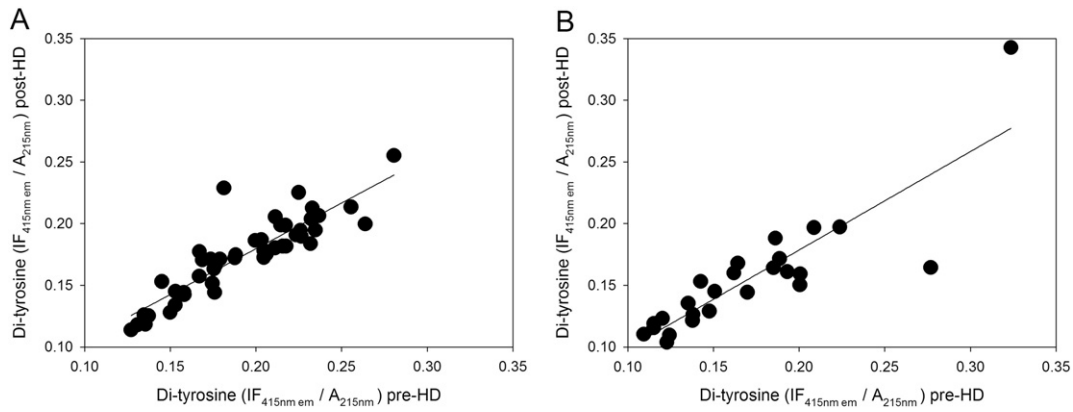
in human plasma [41]. Therefore, we examined the relationship between plasma protein-bound di-Tyr content and albumin concentration in ESRD patients before a single HD session (Fig. 5). The scatter diagram of pre-HD plasma albumin concentration in individual ESRD patients is presented in Fig. 5A. Predialysis albumin concentrations in ESRD patients ranged from 2.6 to 4.2 g/dl (mean  $3.537 \pm 0.0474$  g/dl) (Fig. 5A) and were significantly positively correlated with pre-HD plasma protein-bound di-Tyr levels (Fig. 5B) ( $r = 0.315$ ,  $p = 0.0055$ ).

### 3.4. Correlation between pre-HD serum CRP concentration and plasma protein-bound di-Tyr content measured pre-HD

Persistent inflammation is a prominent characteristic of patients with ESRD, where it is a local process that is also reflected systemically [42]. CRP, which is the most widely used inflammatory biomarker in the clinical setting since high-sensitivity CRP assays are widely available [43], is elevated up to ten-fold in haemodialyzed patients compared to healthy individuals [44]. Hence, we investigated whether plasma protein-bound di-Tyr level was related to the serum CRP concentration in ESRD patients before HD (Fig. 6). The scatter diagram shows that pre-HD serum CRP concentration in ESRD patients ranged from 0.02 to 1.99 mg/dl (mean  $0.515 \pm 0.0609$  mg/dl) (Fig. 6A). As shown in Fig. 6B, no correlation was found between plasma protein-bound di-Tyr content and the serum CRP concentration in ESRD patients ( $r = 0.03$ ;  $p = 0.796$ ).



**Fig. 2.** Effect of a single HD session on the level of plasma protein-bound di-Tyr. (A) Scatter diagram showing plasma protein-bound di-Tyr level in patients with ESRD ( $n = 73$ ) immediately before (black circles) and after (gray circles) a single HD session. (B) Plasma protein-bound di-Tyr content in ESRD patients immediately before (pre-HD) and after (post-HD) a single HD session. Data are expressed as mean  $\pm$  SE. The horizontal solid and dashed lines represent, respectively, the mean and the SE of the plasma protein-bound di-Tyr level in age-matched healthy subjects ( $n = 25$ ).



**Fig. 3.** Correlations between plasma protein-bound di-Tyr levels in ESRD patients ( $n = 73$ ) measured immediately before (pre-HD) and after (post-HD) a single HD session. (A) Males ( $n = 48$ ), (B) females ( $n = 25$ ). Correlations were investigated using simple linear regression analysis.

#### 4. Discussion

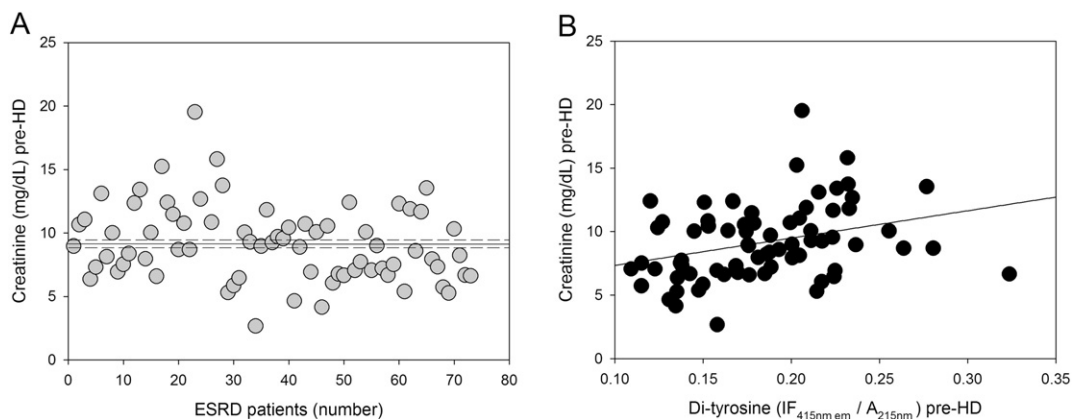
The accessibility of plasma proteins for sampling, the relatively long half-lives of many plasma proteins, and the well-characterized biochemical pathways of protein oxidation make plasma proteins an attractive biomarker of oxidative stress in ESRD patients on HD. Biomarkers of protein oxidation can be classified in two types: (i) generic biomarkers, which include oxidation of multiple residues within protein to form several products, e.g., PCO and AOPPs [37,45]; and (ii) specific biomarkers, which are very specific in both the residue oxidized and the product generated: e.g., oxidation of protein free sulphhydryl groups (P-SH) and oxidation of protein Tyr residues to give di-tyrosines [46].

Single HD sessions have different effects on the different types of biomarkers of protein oxidation. For example, in haemodialyzed patients plasma protein oxidation is revealed by decreased P-SH [23], which might result from S-thiolation, the formation of mixed disulphides between P-SH and low-molecular-mass aminothiols, which is considered to be the mechanism protecting P-SH from losing their biological activity by irreversible oxidation [47,48]. S-thiolated plasma proteins are indeed increased in haemodialyzed patients [21–23,40]. However, a single HD session, by removing solutes responsible for increasing ROS production and the low-molecular-mass aminothiols involved in S-thiolation [21], caused transient return of plasma P-SH to the level equal or close to that occurring in healthy subjects [20,21,23]. De-thiolation of S-thiolated proteins during a single HD session can be explained by considering the reversible reactions involved in S-thiolation [47]. This is very important in the case of albumin, because its Cys34 thiol represents the largest fraction of all free thiols in plasma,

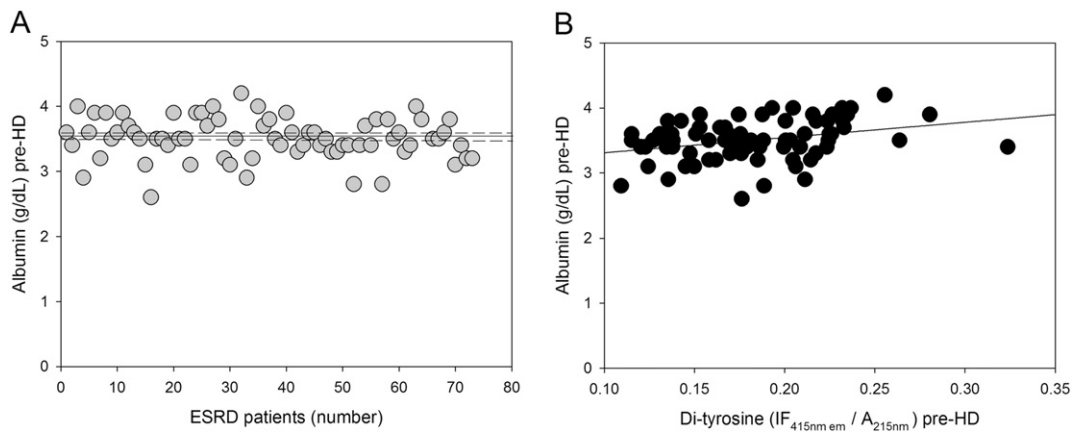
thus attributing to albumin a major role in total plasma antioxidant capacity [41]. Serum albumin de-thiolation during a single HD session may restore transiently its antioxidant activity in haemodialyzed patients [49]. Therefore, S-thiolated proteins represent a useful indicator of thiol-specific reversible oxidative stress in ESRD patients on HD.

Otherwise, by measuring plasma PCO after reaction with 2,4-dinitrophenylhydrazine (DNPH), a number of studies have demonstrated that plasma PCO concentrations increase in ESRD patients [19,20,24,25]. Detection and quantification of PCO by means of DNPH-based methods does not allow for any distinction between primary, or direct, and secondary, or indirect, protein carbonylation [46,50] and also measure sulphenic acids [51]. Therefore, PCO provide a general and widely used biomarker of severe protein oxidation in ESRD patients.

Increased oxidative stress in ESRD patients is also revealed by the formation of plasma AOPPs [27–29], which are considered a generic biomarker of protein oxidation and oxidative stress. In this respect, determination of protein-bound di-Tyr by GF-HPLC with fluorometric detection could be taken as a highly specific biomarker of protein oxidation [23,52–54]. Protein-bound di-Tyr are final, chemically stable and easily detectable products of tyrosine oxidation in response to oxidative stress induced by both non-enzymatic and peroxidase-catalyzed mechanisms [52,55]. Myeloperoxidase (MPO), a haemoprotein present in phagocytes, uses hydrogen peroxide to generate di-Tyr from Tyr residues via its peroxidase cycle, in a manner that functions most efficiently at neutral to slightly alkaline pH (7.5–8), near the physiological concentrations of chloride ions and amino acids [56]. In haemodialyzed patients, plasma levels of MPO are significantly higher than the reference value for healthy subjects and further increase during HD [38,57].



**Fig. 4.** Correlation between pre-HD serum creatinine concentration and pre-HD plasma protein-bound di-Tyr level in ESRD patients. (A) Serum creatinine concentrations in individual ESRD patients ( $n = 73$ ) before HD session (pre-HD). The horizontal solid and dashed lines represent, respectively, the mean and the SE of the serum creatinine concentration. (B) Positive linear correlation between serum creatinine concentration and plasma protein-bound di-Tyr level pre-HD in ESRD patients ( $n = 73$ ).



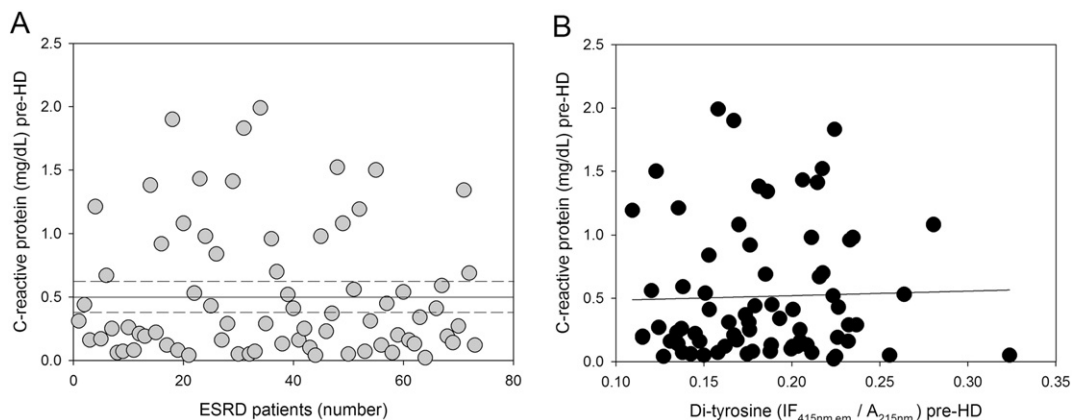
**Fig. 5.** Correlation between pre-HD plasma albumin concentration and pre-HD plasma protein-bound di-Tyr level in ESRD patients. (A) Plasma albumin concentrations in individual ESRD patients ( $n = 73$ ) before HD (pre-HD). The horizontal solid and dashed lines represent, respectively, the mean and the SD of the plasma albumin concentration. (B) Positive linear correlation between plasma albumin concentration and plasma protein-bound di-Tyr content pre-HD in ESRD patients ( $n = 73$ ).

Indeed, the measurement of MPO may serve as a reliable marker of the degree of oxidative stress induced using dialysis membranes of different biocompatibilities [38]. Increased MPO activity could also serve as one mechanistic link between inflammation, oxidative stress and endothelial dysfunction in ESRD [58] and was found to be associated with mortality in ESRD patients undergoing HD [18,59].

We found significantly higher pre-HD levels of plasma protein-bound di-Tyr in haemodialyzed patients compared with di-Tyr level in age-matched healthy subjects (Fig. 1). The AUC (0.99) indicates that the ROC curve has excellent accuracy and that plasma protein-bound di-Tyr level is good indicator of ESRD due to its high sensitivity and specificity in discriminating between ESRD patients and age-matched healthy subjects (Supplementary Fig. S2). However, the usefulness of plasma protein-bound di-Tyr level in clinical practice is questionable, as there is no need for a new biomarker to diagnose ESRD. Rather, the interesting finding is that, in most ESRD patients, a single HD session decreased significantly the plasma protein-bound di-Tyr level, even if the mean level of plasma protein-bound di-Tyr post-HD remained significantly greater in ESRD patients compared to the mean di-Tyr level in age-matched healthy subjects (Fig. 2). Conversely, in a previous study, AOPPs increased during dialysis session, both in patients using a cellulose filter and in patients using polysulphone filters, the latter being the filters also used in our group of patients [38]. In two other studies, a single HD session had no effect on AOPP concentration, which remained significantly greater than normal after the HD session [20,39]. Given that di-Tyr is a more specific biomarker of protein oxidation and oxidative stress than AOPPs [52,55], our data challenge the

previous findings, suggesting that, in general, the HD session itself does not make the patient's oxidative status worse and can even improve it. It is worthy to note that, in our patients, creatinine levels were correlated with pre-HD plasma protein-bound di-Tyr levels (Fig. 4). Although creatinine is influenced by lean body mass in ESRD patients on HD [60], it is one of the historically used biomarkers of uraemia in HD and its levels efficiently predict mortality in this population [61,62]. Thus, creatinine is a faithful biomarker of uraemia that, on turn, is known to affect deeply the oxidative status of the patient: this explains the good correlation that we found between di-Tyr levels and creatinine concentration before dialysis. Therefore, we can speculate that the oxidative damage due to uraemic toxins is efficiently improved by the HD session, as demonstrated by reduction of protein-bound di-Tyr. On the other hand, AOPPs may represent a grosser marker of oxidative stress that, in HD patients, is also due the presence of comorbidities, such as diabetes mellitus, and the occurrence of acute clinical events, such as infections. However, future studies with a greater number of patients, inclusive of patients with CKD stages 1–5, are needed to extend these findings, because the usefulness of the ideal biomarker of oxidative damage lies in its ability to provide early indication of disease and/or its progression. As we expected, plasma protein-bound di-Tyr levels measured pre-HD were significantly positively correlated with post-HD plasma protein-bound di-Tyr levels (Fig. 3).

We also found a moderate positive correlation between plasma protein-bound di-Tyr level and pre-dialysis serum albumin concentration (Fig. 5). This result is particularly interesting considering that serum albumin, which is frequently considered a predictor of nutritional status



**Fig. 6.** Correlation between pre-HD serum CRP concentration and pre-HD plasma protein-bound di-Tyr content in ESRD patients. (A) Serum CRP concentrations in individual ESRD patients ( $n = 73$ ) before HD (pre-HD). (B) Relationship between serum CRP concentration and plasma protein-bound di-Tyr level pre-HD in ESRD patients ( $n = 73$ ).

in patients with ESRD [63], is typically low in ESRD patients and hypoalbuminaemia is associated with mortality in haemodialyzed patients [64–67]. However, other studies suggest that hypoalbuminaemia may be more reflective of inflammation than nutritional status in ESRD patients [58,68–70]. In addition, a linkage of hypoalbuminaemia, inflammation, and oxidative stress has been shown in ESRD patients receiving maintenance HD therapy. Indeed, there is a high prevalence of inflammation and oxidative stress in these patients and levels of inflammatory and oxidative stress biomarkers are increased further in hypoalbuminaemic compared with normoalbuminaemic haemodialyzed patients [71]. Therefore, we hypothesized that plasma protein-bound di-Tyr level could correlate with circulating inflammatory biomarkers.

Among the variety of circulating inflammatory biomarkers, CRP, the major acute phase response protein, is elevated in ESRD patients and is the most widely used inflammatory marker predicting future cardiovascular risk and mortality in ESRD patients [42,72–74]. In contrast to our hypothesis, the results do not show any statistically significant correlation between plasma protein-bound di-Tyr level and serum concentration of CRP (Fig. 6). These results are in line with others showing no correlations between plasma AOPPs and CRP [28,75]. Anyway, these results are somewhat surprising considering that inflammation, which is not confined to the kidney, rather it is systemic [76–78], is a common feature of ESRD patients and both oxidative stress and inflammation are usually inseparably linked and participate in a self-perpetuating vicious circuit. Consequently, the presence and severity of systemic inflammation contribute to ESRD-associated oxidative stress. Indeed, there is evidence to suggest that renal transplantation is associated with almost complete correction of the biomarkers of oxidative stress (measured as PCO) and inflammation (measured as CRP) in patients with ESRD [79,80]. Thus, our results could suggest that, in haemodialyzed patients, oxidative stress and inflammation may also be in part independent of each other. Otherwise, the absence of correlation between plasma protein-bound di-Tyr level and CRP concentration (measured as “pinpoint marker”) could be explained by the fact that CRP concentration fluctuates substantially over time in patients undergoing HD; therefore, reliable CRP levels can be obtained following regular, repeated measurements [74].

This study has strengths and limitations. Strengths include, firstly, the use of a highly specific biomarker of protein oxidation easy to detect: the intrinsic fluorescence properties of di-Tyr and its chemical stability (fairly unreactive to changes in oxygen and/or pH) allow for its sensitive detection in proteins [23,52–55]. Furthermore, plasma protein-bound di-Tyr are obviously not washed out during HD session, unlike some small molecules, such as 4-hydroxynonenal, malonyldialdehyde, and free F<sub>2</sub>-isoprostanes, which were considered promising biomarkers of oxidative stress in ESRD patients [81–83]), which are washed out during HD session. In addition, if we consider that protein-bound di-Tyr are carried mainly by albumin in the blood and that albumin half-life in plasma is ~19 days [84], protein-bound di-Tyr might serve as long-lasting biomarkers of oxidative stress in ESRD patients. The finding that di-Tyr levels decreased at the end of the dialysis session has to be further discussed, since di-Tyr represents an irreversible oxidative product and, of course, dialysis cannot modify it. However, modern dialyzers have an increased molecular cut-off compared to the older ones, and are capable to dialyze low molecular weight proteins, such as beta-2 microglobulin, which has a molecular weight of 11.8 kD and six Tyr residues. Given that we assessed di-Tyr levels as the ratio to total serum proteins, we can speculate that the dialysis of low molecular weight proteins richer in di-Tyr could have improved the overall content of di-Tyr at the end of the dialysis session. This, of course, does not imply that the di-Tyr content of larger proteins that are not dialyzed, such as albumin, has been modified by the dialysis session. Limitations include the relatively small number of ESRD patients, the fact that the study has been performed in only one single HD centre, and measurements have been made at single time points (“pinpoint marker”). Another limitation is that we assessed the effect of a single HD

session using only a single filter type: it would be interesting, in the future, to analyze the influence of different type of dialyzers on plasma protein-bound di-Tyr levels. We thus consider our findings as hypothesis-generating and hope that these results stimulate further studies on the use of plasma protein-bound di-Tyr as a biomarker for oxidative stress in ESRD, with a larger number of haemodialyzed patients enrolled from different dialysis centres.

In conclusion, the choice/indication of the most pertinent biomarkers of oxidative stress is a critical step in the development of novel treatment options for ESRD patients. Furthermore, ESRD is associated with other pro-oxidant conditions such as CVD and diabetes mellitus. In this regard, the relative importance of the different types of oxidative stress and inflammation in ESRD remains largely undetermined, and several questions concerning oxidative stress and inflammation remain poorly defined. Additional large-scale studies with the inclusion of clinically relevant endpoints are required to examine the potential correlations between a panel of biomarkers of inflammation and oxidative stress in ESRD patients on HD. This may pave the way for potential therapeutic intervention aimed at reducing the oxidative stress in hemodialysed patients. The widespread use of anti-oxidants cannot be recommended yet, as large studies with hard end-points are currently lacking. However, when taking into account some surrogate end-points, such as albumin for malnutrition, the already available data are encouraging [85]. Moreover, the use of more biocompatible and anti-oxidant filters, such as vitamin E-coated polysulfone membranes, could potentially change the clinical practice in the future [86,87].

#### Conflict of interest disclosure statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We further confirm that the manuscript has been read and approved by all named authors and that the order of authors listed in the manuscript has been approved by all of us.

#### Abbreviations

AOPPs	advanced oxidation protein products
AUC	area under the curve
CDK	chronic kidney disease
CRP	C-reactive protein
CVD	cardiovascular disease
di-Tyr	dityrosine(s)
DNPH	dinitrophenylhydrazine
ESRD	end stage renal disease
HD	haemodialysis
GF-HPLC	gel filtration high performance liquid chromatography
MPO	myeloperoxidase
PCO	protein carbonyls
P-SH	protein sulphhydryl group(s)
ROC	receiver operating characteristic

#### Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbacli.2016.12.004>.

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