

## Matrix metalloproteinase-1 and matrix metalloproteinase-3 gene promoter polymorphisms are associated with mortality in haemodialysis patients

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### Abstract

**Background.** Vascular calcification and accelerated atherosclerosis are major causes of death in haemodialysis (HD) patients. Matrix metalloproteinases (MMPs) are a family of enzymes, involved in the biology of extracellular matrix and in atherogenesis. MMP1 and MMP3 contribute to the enlargement and instability of atherosclerotic plaque, respectively. The common polymorphisms on MMP1 (2G/2G) and MMP3 (6A/6A) gene promoters have been related to increased coronary artery calcification and to carotid artery stenosis. The aim of this study was to evaluate the association of MMP1 and MMP3 polymorphisms with end-stage renal failure (ESRD) and all-cause mortality risk in HD.

**Methods.** Ninety-nine HD patients, followed-up for 36 months, and 133 matched controls were genotyped for the two polymorphisms. HD patients' characteristics were age  $64 \pm 13$  years, males 64%, diabetic 24%, hypertensive 62%, smokers 38%, dyslipidaemic 28%, all undergoing standard HD thrice weekly.

**Results.** ESRD was strongly associated with the combination of 2G/2G and 6A/6A homozygosity: OR 2.57 (0.95–7.4),  $P = 0.037$ , but not with isolated 2G/2G and 6A/6A homozygosity ( $P = 0.09$  and  $P = 0.11$ , respectively). Isolated 2G/2G was associated with all-cause mortality risk independently from age, gender, diabetes, hypertension, smoking, dyslipidaemia, C-reactive protein, albumin, dialysis vintage and history of cardio-vascular disease: HR 2.96 (1.29–6.80),  $P = 0.01$ . A trend for the association of mortality and isolated 6A/6A homozygosity was also observed: HR 3.01 (0.88–10.26),  $P = 0.078$ . Combination of 2G/2G and 6A/6A homozygosity significantly increased the mortality risk in the same Cox regression model: HR 4.69 (1.72–12.81),  $P = 0.003$ .

**Conclusions.** In this study, we demonstrated for the first time that MMP-1 and MMP-3 gene polymorphisms are negative prognostic risk factors for all-cause mortality in HD patients, independently from traditional risk factors. These data may have important implications for better understanding the pathogenesis of the increased mortality in HD patients.

**Keywords:** atherosclerosis; genetic polymorphism; haemodialysis; metalloproteinases

### Introduction

Cardiovascular (CV) disease is the leading cause of mortality in haemodialysis (HD) patients [1]. The very high CV mortality and morbidity rates in this population are only partially explained by the high prevalence of traditional CV risk factors [2], which are classically related to atherosclerosis. The vascular changes observed in chronic kidney disease (CKD) patients consist in not only atherosclerosis but also arteriosclerosis associated with both medial and intimal vascular calcifications [3]. The degree of arterial stiffening and the extent of calcification are closely related [4], and both of these variables are strong and independent prognostic markers of all-cause and CV mortality in patients on HD [5,6].

Over the last few years, matrix metalloproteinases (MMPs) have been increasingly implicated in connective tissue remodelling during atherogenesis [7]. MMPs are involved in plaque rupture, which is the main pathological cause of myocardial infarction. Interstitial collagenase (MMP-1) is the only MMP that can cleave native collagen types I and III, which are major structural components of the fibrous plaque cap. MMP-1 might play a significant role in fibrous plaque disruption by contributing to the degradation of interstitial collagens and thinning of the fibrous cap [8]. A common insertion polymorphism (an additional guanidine) in the nucleotide sequence of the MMP-1 gene promoter has been reported. The 2G homozygotes show increased transcription activity compared with 1G homozygotes and controls [8–10]. On the other hand, extensive expression of the MMP-3 gene was localized particularly to plaque regions prone to rupture, such as the fibrous cap and its adjacent tissues [7]. A common variant in the promoter of the MMP-3 gene has been described [9]. *In vitro* assays of promoter activity revealed that the 5A allele had 2-fold higher promoter activity than the 6A allele [10].

Genetic studies have demonstrated that MMP-1 1G/2G and MMP-3 5A/6A polymorphisms modify transcriptional activity in allele-specific manners [11]. In this study, we investigated the association of MMP1 and MMP3 polymorphisms with end-stage renal failure (ESRD) and all-cause mortality risk in HD.

## Materials and methods

### Patients

Patients were recruited among prevalent HD population at S. Paolo Hospital in Milan (Italy). All the 99 recruited patients were Caucasian adults aged  $64 \pm 13$  years, of which 64% were male, with a median dialysis vintage of 85 months (range: 9–454 months). All patients were treated by standard bicarbonate dialysis for 4 h three times weekly. The control group consisted of 133 adult subjects with normal renal function. It was specifically assembled by Ghilardi *et al.* [11] from a previous study investigating the association of MMP1 and MMP3 polymorphisms with carotid artery stenosis. Control subjects were all Caucasian adults, aged  $65 \pm 9$  years, 58% male.

Height and body weight were recorded, and the body mass index (BMI) was calculated according to the formula: weight in kg/square of height in metres.

Information on concomitant hypertension, diabetes mellitus and dyslipidaemia was obtained from hospital charts; patients were inquired about their smoking status and previous CV events (myocardial infarction, angina, stroke, transient ischaemic attack, pulmonary thromboembolism). Once enrolled, patients were followed up for 3 years.

Blood pressure was measured at the beginning of the dialysis session. Blood pressure was measured at the beginning of the dialysis session. Hypertension was defined as systolic blood pressure (SBP)  $\geq 140$  mmHg and diastolic blood pressure (DBP)  $\geq 80$  mmHg or normal blood pressure in patients treated with one or more drugs. Patients were treated with different drugs, such as beta-blockers, ACE-inhibitors, angiotensin receptor antagonists, diuretics and calcium-channel blockers. Data about concomitant pharmacological therapy were collected.

Diabetes was defined as fasting blood glucose levels  $\geq 126$  mg/dl. Dyslipidaemia was defined as serum LDL-cholesterol levels  $\geq 110$  mg/dl in at least three measurements in 1 year. Smoking was defined as current use of cigarettes  $> 10$  cigarettes daily for more than 10 years.

A blood sample was drawn from each patient before the beginning of the first HD treatment of the week to measure concentrations of routine biochemical variables.

### Identification of MMP-1 and -3 gene polymorphisms

Patients were genotyped for the common polymorphism on the MMP1 and MMP3 genes.

Whole blood (3 ml) from patients and controls was collected into potassium EDTA. DNA was prepared with the Istage Matrix extraction kit (Bio-Rad Laboratories). The polymerase chain reaction for MMP-1 and MMP-3 was performed in a total volume of 25  $\mu$ l with 5  $\mu$ l of extracted genomic DNA, 100  $\mu$ M of dATP, dGTP, dTTP and dCTP, 1.5 mmol/L of MgCl<sub>2</sub> and 1 U of *Taq* polymerase, with the two primers, forward and reverse, each at a concentration of 80 nM. The primers were designed with the Primer Express software. The MMP-1 primer sequence is as follows: forward: 5'-CCCTCTTGAACACATGTTATG-3'; reverse: 5'-ACTTTCCTCCCCTTATGGATTCC-3'. The MMP-3 primer sequence is as follows: forward: 5'-TCCTCATATCAATGTGGCCAAA-3'; reverse: 5'-CGGCACCTGGCCTAAAGAC-3'. The polymerase chain reaction starts with 5 min of incubation at 94°C to activate the enzyme, followed by 35 cycles of 20 s at 94°C, 20 s at 55°C and 30 s at 72°C. The amplification was verified on an agarose gel (2%) followed directly by sequencing with an automatic sequencer in fluorescent DNA capillary electrophoresis (ABI Prism 310; Applied Biosystems; Foster City, CA, US).

### Statistical analyses & ethics

Categorical and continuous variables were expressed as rates and means  $\pm$  SD, respectively. Odds ratios (ORs) (approximate relative risk) were calculated as an index of the association of the MMP-1 and MMP-3 genotype

**Table 1.** Main characteristics of the study population

Characteristic	Controls (n = 133)	Haemodialysis patients (n = 99)
Gender (male %)	58	64
Age (mean $\pm$ SD, years)	65 $\pm$ 9	64 $\pm$ 13
Diabetes mellitus type II (%)	18	24
<sup>a</sup> Arterial hypertension (%)	34	62
<sup>b</sup> Smokers (%)	48	38
Dyslipidaemia (%)	54	28
<sup>c</sup> C-reactive protein (mean $\pm$ SD, mg/dl)	n.a.	7.54 $\pm$ 9.36
<sup>c</sup> Albumin (mean $\pm$ SD, g/dl)	n.a.	4.09 $\pm$ 0.31
History of cardiovascular events (%)	0	62
<sup>c</sup> Ca $\times$ P product (mean $\pm$ SD, mg <sup>2</sup> /dl <sup>2</sup> )	n.a.	58 $\pm$ 14
<sup>c</sup> iPTH (mean $\pm$ SD, pg/ml)	n.a.	274 $\pm$ 254
<sup>c</sup> URR (%)	n.a.	78 $\pm$ 1
HD vintage (mean $\pm$ SD, months)	–	85 $\pm$ 65

<sup>a</sup>Hypertension was defined as systolic blood pressure (SBP)  $\geq 140$  mmHg and diastolic blood pressure (DBP)  $\geq 80$  mmHg or normal blood pressure in patients treated with one or more drugs. Patients were treated with different drugs, such as beta-blockers, ACE-inhibitors, angiotensin receptor antagonists, diuretics and calcium-channel blockers.

<sup>b</sup>Dyslipidaemia was defined by serum LDL-cholesterol levels  $\geq 110$  mg/dl in at least three measurements in 1 year.

<sup>c</sup>Regular lab tests, at least three measurements in 1 year.

with each phenotype. A logistic regression model was set to investigate the association between genotype and ESRD. Cumulative survival during the 3-year follow-up among ESRD patients was estimated by the Kaplan–Meier method according to different genotypes and compared by the use of the Mantel (log-rank) test. A Cox hazard regression model was then set to test the independent relationship between survival and the following predictors: genotypes, age, gender, hypertension, diabetes, smoking, dyslipidaemia, C-reactive protein, albumin, dialysis vintage and history of CV disease. For each OR and HR, two-tailed probability values and 95% CIs were calculated. We deemed  $P < 0.05$  significant. Statistical analysis was performed by SPSS 13.0 for Windows.

The study was approved by the Ethics Committee of San Paolo Hospital in Milan (Italy). All patients gave their informed consent in writing before they were submitted to any procedures related to the study.

## Results

A total of 99 HD patients and 133 controls were included in the study. Their main characteristics are summarized in Table 1. The prevalence of genotypes is reported in Table 2. The distribution of genotypes in the control group and in HD patients differed significantly from that expected under the Hardy–Weinberg equilibrium, and there was a significant deviation from Hardy–Weinberg proportions in the HD group.

ESRD was not associated with isolated 2G/2G and 6A/6A homozygosity ( $P = 0.09$  and  $P = 0.11$ , respectively) (Table 2). However, ESRD was strongly associated with the combination of 2G/2G and 6A/6A homozygosity: OR 2.57 (0.95–7.4),  $P = 0.037$ .

All-cause mortality rate was 41% at 3 years of follow-up. No patients were lost or transplanted during follow-up. All-cause mortality was associated with the following distribution of genotypes: isolated 2G/2G homozygosity, isolated 6A/6A homozygosity, combination of 2G/2G and 6A/6A

**Table 2.** Distribution of MMP1 and MMP3 gene polymorphisms

	Patients	Controls	OR (95% CI)	P
MMP-1 genotype				
1G/1G	19 (19%)	33 (25%)		
1G/2G	48 (49%)	70 (53%)	0.60 (0.32–1.14)	0.09
2G/2G	32 (32%)	30 (22%)		
Allelic frequency				
2G	0.56	0.49	0.73 (0.49–1.08)	0.10
MMP-3 genotype				
5A/5A	18 (18%)	36 (27%)		
5A/6A	60 (60%)	59 (44%)	0.59 (0.29–1.18)	0.11
6A/6A	21 (21%)	38 (28%)		
Allelic frequency				
6A	0.51	0.51	1.00 (0.67–1.48)	0.98
6A/6A and 2G/2G	14 (14%)	8 (6%)	2.57 (0.95–7.4)	0.037

**Table 3.** Cox regression analysis of all-cause mortality at 3 years follow-up

Variable	Wald	HR (IC 95%)	P
Model 1 ( $\chi^2 = 28.354$ , $P = 0.008$ )			
Isolated 2G/2G	6.515	2.96 (1.29–6.80)	0.011
Isolated 6A/6A	3.100	3.01 (0.88–10.26)	0.078
Combination of 2G/2G and 6A/6A	0.095	4.69 (1.72–12.81)	0.003
History of cardiovascular events	5.421	2.39 (1.15–4.99)	0.020
Model 2 ( $\chi^2 = 28.333$ , $P = 0.005$ )			
Isolated 6A/6A or isolated 6A/6A	0.789	2.97 (1.39–6.35)	0.005
Combination of 2G/2G and 6A/6A	0.095	4.69 (1.72–12.81)	0.003
History of cardiovascular events	5.421	2.39 (1.15–4.99)	0.020

Other variables included in Model 1 and in Model 2: age, gender, hypertension, diabetes, smoking, dyslipidaemia, C-reactive protein, albumin and dialysis vintage.

homozygosity, other genotypes (Figure 1A). A Cox regression analysis was then performed including genotypes, age, gender, hypertension, diabetes, smoking, dyslipidaemia, C-reactive protein, albumin, dialysis vintage and history of CV disease. Mortality was significantly associated with the isolated 2G/2G homozygosity [HR 2.96 (1.29–6.80),  $P = 0.01$ ], the combination of 2G/2G and 6A/6A homozygosity [HR 4.69 (1.72–12.81),  $P = 0.003$ ] and history of CV disease ( $P = 0.02$ ) (Table 3). In the same model, a trend for the association of mortality and isolated 6A/6A homozygosity was also observed [HR 3.01 (0.88–10.26),  $P = 0.078$ ] (Table 3). Kaplan–Meier curves and Cox regression model, including the same covariates, were then performed encompassing isolated 2G/2G homozygosity and isolated 6A/6A homozygosity in a single survival curve (Figure 1B, Table 3). Mortality was independently associated with the isolated 2G/2G or isolated 6A/6A homozygosity [HR 2.97 (1.39–6.35),  $P = 0.005$ ], and the combination of 2G/2G and 6A/6A homozygosity [HR 4.69 (1.72–12.81),  $P = 0.003$ ] (Table 3).

## Discussion

In this study, we demonstrated for the first time that HD patients have a different distribution of MMP-1 and -3 gene polymorphisms as compared to the normal population. Consequently, altered polymorphisms of the MMP1

and MMP3 genes might be considered a negative prognostic factor for the progression to ESRD. Moreover, HD patients who are 2G/2G homozygotes and 6A/6A homozygotes appear to have a significantly worse prognosis in terms of mortality.

The MMPs are a family of enzymes, involved in the biology of extra-cellular matrix and in atherogenesis. MMP1 and MMP3 participate to the enlargement and instability of atherosclerotic plaque, respectively [12,13]. The common polymorphisms on MMP1 (2G/2G) and MMP3 (6A/6A) gene promoters have been related to increased coronary artery calcification and to carotid artery stenosis [14].

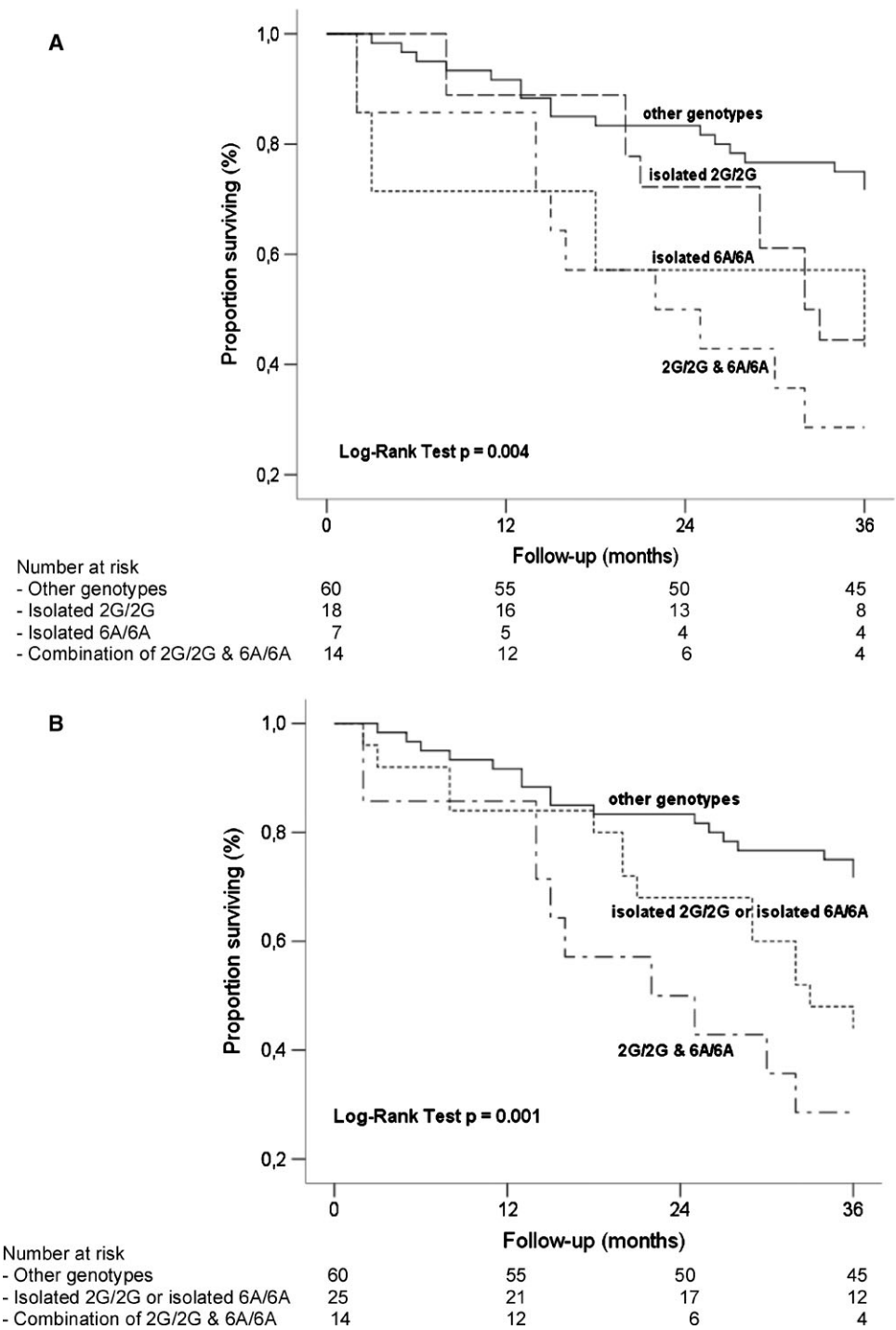
It should be borne in mind that numerous factors contribute towards the marked arterial calcification observed in CKD patients: all the 'classic' risk factors for atherosclerosis plus 'uraemia-specific' risk factors, such as duration of dialysis, uraemic toxins, inflammation, increased serum levels of phosphate, calcium and PTH [5].

Accelerated atherosclerosis and CV disease have been shown to be associated with the MMP3 gene 5A/6A polymorphism. MMP3 has proteolytic activity on different extracellular matrix proteins [14,15] and can activate other MMPs [15]. Therefore, MMP3 has an important role in vascular and cardiac matrix remodelling [15]. Interestingly, Humphries *et al.* [16] have shown that subjects with the 6A/6A genotype have a higher rate of coronary atherosclerotic lesion growth compared with individuals with the 5A/5A or 5A/6A genotype. In agreement, several cross-sectional studies of patients with coronary atherosclerosis documented by coronary angiography show that individuals with the 6A/6A genotype have more significant stenosis of coronary arteries compared to those with the 5A/5A or 5A/6A genotype [17,18].

Furthermore, the most common cause of myocardial infarction is coronary atherosclerotic plaque rupture or erosion [19,20], resulting in exposure to thrombus formation [19,20]. The association between increased MMP3 expression and plaque rupture has been investigated by different authors [21,22]. Others investigated the MMP3 gene 5A/6A polymorphism in relation to the risk of myocardial infarction [23–26], although two studies have not detected any effect of the MMP3 5A allele on the risk of myocardial infarction [18,27].

Vascular calcification increases the risk of CV events [28]. MMP3 expression is co-localized with calcium deposition in atherosclerotic lesions [29]. In an autopsy study of men who died of cardiac disease or other causes, Pollanen *et al.* [30] have found that patients of the 5A/5A or 5A/6A genotype had more calcification in atherosclerotic lesions than subjects of the 6A/6A genotype.

Three independent studies have shown that the 5A/6A polymorphism is associated with carotid intima-media thickness [31–33]. Furthermore, individuals of the 6A/6A genotype more likely have advanced carotid atherosclerosis resulting in significant carotid stenosis. In a study of patients with carotid atherosclerosis and controls with no evidence of the disease, Ghilardi *et al.* [11] showed that the frequency of the 6A/6A genotype was higher in the case group than in the control group, and that among the cases, carriers of the 6A/6A genotype had a higher degree of carotid stenosis.



**Fig. 1.** Kaplan–Meier survival curves for all-cause mortality according to MMP1 and MMP3 genotypes: isolated 2G/2G homozygosity, isolated 6A/6A homozygosity, combination of 2G/2G and 6A/6A homozygosity, other genotypes (A); encompassing isolated 2G/2G homozygosity and isolated 6A/6A homozygosity in a single survival curve (B).

The major constituents of atherosclerotic lesions are matrix proteins (collagen, proteoglycans, elastin, etc.), smooth muscle cells, macrophages and lipids [34]. Since MMP3 is considered to play an important role in the degradation of matrix proteins in atherosclerotic lesions, and since MMP3 expression in vascular tissues is higher in individuals carrying the 5A allele than in individuals of the 6A/6A geno-

type, a possible explanation for our findings, that the 6A/6A genotype is associated with greater mortality risk, is that HD patients with the low MMP3 expression 6A/6A genotype are prone to developing atherosclerotic plaques that are rich in matrix proteins and hence relatively large and stable, whereas individuals with the high MMP3 expression 5A/5A or 5A/6A genotype are predisposed to developing

atherosclerotic plaques that have less matrix proteins and hence are smaller but prone to rupture.

In our previous studies, we reported that other genotypes of inhibitory proteins involved in the pathogenesis of vascular calcification do (matrix Gla protein) or do not (fetuin-A) associate with mortality risk in HD patients [35,36].

In the present analysis, all-cause mortality risk was higher in patients with the association of 2G/2G and 6A/6A homozygosity for MMP1 and MMP3, respectively (Figure 1). This observation appears reasonable considering the specific pathophysiological implications of MMP1 and MMP3 in atherogenesis, as previously described. Simultaneous homozygosity for both 2G/2G (MMP1) and 6A/6A (MMP3) may have an additive effect on the instability of atherosclerotic plaque. Indeed, the 6A/6A genotype may promote the generation of smaller and more friable plaques and 2G/2G may enhance their disruption by degrading the interstitial collagen and thinning the fibrous cap.

We observed that ESRD, compared to controls, was strongly associated with the combination of 2G/2G and 6A/6A but not with isolated 2G/2G and 6A/6A homozygosity (Table 2). Further prospective studies are needed to better investigate if the combination of 2G/2G and 6A/6A may contribute to the onset and progression of CKD.

Our data justify the design of a prospective study in HD patients on the role of MMP1 and MMP3 gene polymorphism in determining development of CV calcification, in the attempt to improve our understanding of the pathogenesis of increased risk of ectopic calcification and CV events in patients with renal failure.

*Conflict of interest statement.* None declared.

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## The plasma retinol levels as pro-oxidant/oxidant agents in haemodialysis patients

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### Abstract

**Background.** Oxidative stress is a process involved in haemodialysis-related pathologies such as cerebrovascular diseases. Retinol is the major circulating form of vitamin A and it is elevated in haemodialysis (HD) patients. It is known that these patients present anaemia that is not totally responsive to erythropoietin. The aim of this study was to evaluate the influence of plasma retinol levels on oxidative stress biomarkers, especially on  $\delta$ -aminolevulinate dehydratase.

**Methods.** Plasma retinol and malondialdehyde (MDA) levels were quantified by HPLC-UV/VIS; blood activities of catalase (CAT), superoxide dismutase (SOD) and  $\delta$ -aminolevulinate dehydratase (ALA-D) were analysed by spectrophotometric methods, in HD patients ( $n = 29$ ) and healthy subjects ( $n = 20$ ).

**Results.** The MDA and retinol levels, SOD and CAT activities were significantly increased in HD patients. ALA-D activity was significantly decreased. Retinol levels were correlated with MDA levels ( $r = 0.68$ ), CAT ( $r = 0.39$ ), SOD ( $r = 0.40$ ) and ALA-D ( $r = -0.55$ ). A partial correlation between retinol levels with ALA-D ( $r = 0.43$ ), SOD ( $r = 0.30$ ) and CAT ( $r = 0.36$ ) activity was found, utilizing MDA levels as co-variable.

**Conclusion.** Higher retinol levels may be associated with the increase of SOD and CAT activities, but this increase was not sufficient to prevent the lipid peroxidation and ALA-D thiolic group oxidation. In this manner, our results

could suggest that high retinol levels contribute as an additional factor to the oxidative tissue damage.

**Keywords:** ALA-D activity; haemodialysis patients; MDA; oxidative stress; plasma retinol levels

### Introduction

Haemodialysis treatment is the main resource for patients in the end-stage of renal disease, who are either waiting for or are not suitable to undergo renal transplantation [1]. In chronic renal failure (CRF) patients under haemodialysis (HD) treatment, the formation of reactive oxygen species (ROS) is amplified and the oxidative stress may be one of the most relevant complications occurring. This problem may not have immediate clinical effects although it may represent a long-term complication derived from the repetitive effects of the blood–membrane interaction [2–4]. Nevertheless, the multifactorial nature of this process [3] might include other factors peculiar to chronic HD treatment, such as the absence of a complete correction of the uraemic toxicity, malnutrition and the progressive worsening of the clinical condition due to ageing and comorbidity [2–4].

Retinol, the major circulating form of vitamin A, was shown to have some antioxidant properties [5] although

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