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Matrix Metalloproteinase (MMP)-2 Genetic Variants Modify the Circulating MMP-2 Levels in End-Stage Kidney Disease

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Key Words

End-stage kidney disease · Haplotypes · Hemodialysis · Matrix metalloproteinase-2 · Polymorphisms · Tissue inhibitor of metalloproteinases-2

Abstract

Background: Matrix metalloproteinases (MMPs) play important roles in the pathophysiology of renal diseases, and imbalanced MMP-2 and its endogenous inhibitor (the tissue inhibitor of metalloproteinases-2; TIMP-2) are implicated in the vascular alterations of end-stage kidney disease (ESKD) patients. We have examined whether MMP-2 gene polymorphisms and haplotypes modify MMP-2 and TIMP-2 levels in ESKD patients as well as the effects of hemodialysis on the concentrations of these biomarkers. ***Methods:*** We determined MMP-2 and TIMP-2 plasma levels by gelatin zymography and ELISA, respectively, in 98 ESKD patients and in 38 healthy controls. Genotypes for two relevant MMP-2 polymorphisms ($C^{-1306}T$ and $C^{-735}T$ in the promoter region) were determined by TaqMan® allele discrimination assay and real-time polymerase chain reaction. The software program PHASE 2.1 was used to estimate the haplotype frequencies. ***Results:*** We found increased plasma MMP-2 and TIMP-2 levels in ESKD patients compared to controls ($p < 0.05$), and hemodialysis decreased MMP-2 (but not TIMP-2) levels ($p < 0.05$). The T allele for the $C^{-735}T$ polymorphism and the C-T haplotype were associated with higher MMP-2 (but not TIMP-2) levels ($p < 0.05$), whereas the $C^{-1306}T$ had no effects. Hemodialysis decreased MMP-2 (but not TIMP-2) levels independently of MMP-2 genotypes or haplotypes ($p < 0.05$). ***Conclusions:*** MMP-2 genotypes or haplotypes modify MMP-2 levels in ESKD patients, and may help to identify patients with increased MMP-2 activity in plasma. Hemodialysis reduces MMP-2 levels independently of MMP-2 genetic variants.

Introduction

Matrix metalloproteinases (MMPs) are a wide family of zinc-dependent proteases that regulate tissue remodeling, cell proliferation and angiogenesis by cleaving many components of the extracellular matrix [1]. Whereas their activities are balanced by their interactions with endogenous inhibitors (the tissue inhibitors of metalloproteinases; TIMPs), there is now clear evidence that they play important roles in the pathophysiology of renal diseases [2].

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End-stage kidney disease (ESKD) patients have unstable atherosclerotic plaques that are prone to rupture [3]. This process includes enhanced arterial calcification and activation of fibroblasts and cytokines, eventually leading to vascular extracellular matrix remodeling [4, 5]. While growing evidence suggests that MMP abnormalities are involved in the vascular changes associated with kidney failure [6], a particular imbalance between MMP-2 and its endogenous inhibitor, TIMP-2, has been implicated in the vascular alterations of ESKD [7]. Previous studies showed altered MMP-2 and TIMP-2 levels in dialysis patients, thus suggesting a mechanism for cardiovascular disease (CVD) complications in these patients [8–12]. However, inconsistent results have been reported with respect to the effects of hemodialysis on the circulating MMP levels. Some studies suggested that plasma MMP-2 and TIMP-2 are unaltered or even reduced in uremic subjects compared to healthy controls [13, 14]. In addition, conflicting results have been reported with respect to the effects of a single hemodialysis session on MMP-2 and TIMP-2 levels [10, 11, 14, 15].

There is now evidence that a single nucleotide polymorphism (SNP) in the MMP-2 gene may affect MMP-2 expression or activity [16, 17]. Two SNPs in the promoter region of the MMP-2 gene apparently affect MMP-2 expression [18, 19] (the $C^{-1306}T$; rs 243865, and the $C^{-735}T$; rs 2285053) and have been associated with malignancies and CVD [20–22]. Nevertheless, no previous study has examined how these functional MMP-2 polymorphisms, or their combinations within haplotypes, affect MMP-2 levels in ESKD patients. Moreover, no previous study has examined how these MMP-2 polymorphisms may modify the effects of a hemodialysis session on MMP-2/TIMP-2 levels.

We compared MMP-2 and TIMP-2 plasma levels in healthy volunteers with those found in ESKD patients on hemodialysis, and we examined the effects of a single hemodialysis session on these biochemical markers. We hypothesized that MMP-2 alleles, genotypes, and haplotypes could alter the circulating levels of MMP-2 and TIMP-2 and modify the effects of a single hemodialysis session on these biochemical markers in ESKD patients.

Subjects and Methods

Patients and Healthy Controls

This cross-sectional, observational study was approved by the Research Ethics Committee of the Pontíficia Universidade Católica do Rio Grande do Sul (PUCRS), and informed consent was obtained from each participant.

We studied 98 patients with end-stage renal disease on chronic hemodialysis followed in three dialysis clinics (ESKD group). We included patients aged between 18 and 65 years who were on regular treatment for at least 3 months and were clinically stable. Patients with previous CVD or using medications were excluded from our sample because we aimed at sampling representative patients on hemodialysis. The etiology for ESKD and concomitant CVD are described in supplementary table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000336108). The patients were routinely dialyzed three times a week for 4 h with a polysulfone hollow-fiber membrane, bicarbonate dialysate, and standard heparin anticoagulation. Reverse osmosis was used for water treatment and the dialysate was regularly checked for the presence of endotoxin. Dialysis adequacy was evaluated by measuring Kt/V. Blood pressure was measured using a calibrated sphygmomanometer with appropriated cuff size.

A group of 38 healthy volunteers with normal renal function was recruited among blood donors at the blood bank of the University Hospital (PUCRS) as a control group. These subjects were matched for age and gender with the patients in the ESKD group. All subjects provided a complete health history and underwent physical examination and laboratory analysis to exclude subjects with hypertension, diabetes mellitus, other concomitant CVDs, respiratory, hepatic, renal, or hematological dysfunction.

Venous blood samples from each subject were collected into EDTA Vacutainer tubes (Becton-Dickinson, São Paulo, Brazil) by venipuncture. Patients in the ESKD group were sampled immediately before and at the end of the hemodialysis session. The blood samples were centrifuged at 1,000 *g* for 10 min and plasma fractions were immediately stored at –70°C until used for biochemical measurements. Venous blood samples were also collected to extract genomic DNA.

Hematological and biochemical parameters were determined by routine techniques using an automated analyzer (Johnson Vitros Chemistry 5.1 SS). LDL cholesterol was calculated using Friedewald's formula.

Assessment of MMP-2 Levels in Plasma by SDS-Polyacrylamide Gel Electrophoresis Gelatin Zymography

Gelatin zymography of MMP-2 of plasma samples was performed as previously described [23]. Briefly, plasma samples were subjected to electrophoresis on 7% SDS-polyacrylamide gel electrophoresis (PAGE) co-polymerized with gelatin (1%) as the substrate. After electrophoresis was complete, the gels were incubated for 1 h at room temperature in a 2% Triton X-100 solution, and incubated at 37°C for 16 h in Tris-HCl buffer, pH 7.4, containing 10 mmol/l $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 for 3 h, and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. The gels were scanned and the digital images were obtained from the scanner. The intensity of the band corresponding to MMP-2 (72 kDa) was analyzed with an image analysis software (Image J 1.43u). The intensity value for the MMP-2 band was calculated as relative activity according to the intensity of related MMP-2 standard [23].

Enzyme Immunoassay of TIMP-2

Plasma TIMP-2 concentrations were measured with commercially available ELISA assay kits (R&D Systems, Minneapolis, Minn., USA), according to the manufacturer's instructions.

Genotyping for MMP-2 Polymorphisms

Genomic DNA was extracted from the cellular component of 1 ml of whole blood and stored at –20°C until analyzed. Genotypes for the $C^{-1306}T$ (rs 243865) and the $C^{-735}T$ (rs 2285053) in the 5′-flanking region of the MMP-2 gene were determined by TaqMan® allele discrimination assay (Applied Biosystems, Carlsbad, Calif., USA). Probes and primers used for the $C^{-1306}T$ genotyping assay were customized as follows: forward 5′-GCCATTGTCAATGTTCCCTAAAACA-3′, reverse 5′-TGACTTCTGAGCTGAGACCTGAA-3′, and probes 5′-CAGCACTC[T/C]ACCTCT-3′. TaqMan polymerase chain reaction (PCR) was performed in a total volume of 12 μl (3 ng of dried DNA, 1× TaqMan master mix, 900 nM of each primer, and 200 nM of each probe) placed in 96-well PCR plates. Fluorescence from PCR amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories, Hercules, Calif., USA) and analyzed with the manufacturer's software. Probes and primers used in the MMP-2 $C^{-735}T$ assay were designed by Applied Biosystems (ID: C_26734093-20). TaqMan PCR and fluorescence reading were performed as described above for the $C^{-1306}T$ polymorphism [24].

Statistical Analysis

Clinical features were compared between the groups using unpaired Student's t or Mann-Whitney's tests. The distribution of the genotypes for each polymorphism was assessed for deviation from the Hardy-Weinberg equilibrium using χ^2 tests. To examine the effects of the MMP-2 genotypes and haplotypes on circulating levels of MMP-2, TIMP-2, or MMP-2/TIMP-2 ratios, we used unpaired Student's t test and one-way ANOVA (followed by Tukey's posttest), respectively. Comparisons of MMP-2, TIMP-2, or MMP-2/TIMP-2 ratios in the ESKD group before and after hemodialysis were made with paired Student's t test.

Haplotype frequencies were estimated using PHASE software (http://depts.washington.edu/uwc4c/express-licenses/assets/phase/). Only haplotypes with frequencies higher than 5% were taken into consideration. The possible haplotypes including genetic variants of two polymorphisms in the MMP-2 gene studied ($C^{-1306}T$ and $C^{-735}T$) were: H1 (C,C); H2 (C,T); H3 (T,C), and H4 (T,T). Because the TT genotype for both polymorphisms was very rare, we grouped the CT and TT genotypes. A value of $p < 0.05$ was considered statistically significant.

Table 1. Demographic and clinical features of healthy controls and ESKD patients

Clinical features	Controls (n = 38)	ESKD patients (n = 98)	p
Age, years	50 ± 9	51 ± 11	NS
Race, white/non-white	30/8	81/17	NS
Male/female	19/19	55/43	NS
Current smokers	6	45	0.0011
SBP, mm Hg	120 ± 11	141 ± 30	<0.0001
DBP, mm Hg	79 ± 9	81 ± 14	NS
Diabetes mellitus	0	33	<0.0001
Hypertension	0	77	<0.0001
BMI	27.7 ± 5.1	25.4 ± 5.8	0.0077
Total cholesterol, mg/dl	198.5 ± 45.2	158.9 ± 55.9	0.0002
HDL cholesterol, mg/dl	50.6 ± 17.6	38.0 ± 12.8	<0.0001
LDL cholesterol, mg/dl	119.5 ± 42.6	86.3 ± 42.6	<0.0001
Triglycerides, mg/dl	115.1 ± 61.6	194.5 ± 151.2	0.0169
Hemoglobin, g/dl	13.4 ± 1.02	10.6 ± 2.5	<0.0001
Hematocrit, %	40.3 ± 3.4	31.7 ± 6.0	<0.0001
Leukocytes, $\times 10^3/\mu l$	6.0 ± 7.5	6.7 ± 2.1	NS
Creatinine, mg/dl	0.86 ± 0.29	9.03 ± 3.22	<0.0001
Calcium, mg/dl	9.20 ± 0.76	8.90 ± 1.10	NS
Phosphorus, mg/dl	3.18 ± 0.70	5.91 ± 1.60	<0.0001
Potassium, mg/dl	4.19 ± 0.33	5.32 ± 0.96	<0.0001
PTH, pg/ml	NA	530 ± 655	–
Albumin, mg/dl	NA	3.91 ± 0.34	–

Results

Clinical features of studied subjects are shown in table 1. Although the groups were matched by age, gender, and race distributions (all $p > 0.05$), significant differences were found in arterial blood pressure, BMI, lipid fractions, hemoglobin, hematocrit, creatinine, phosphorus, and potassium concentrations (all $p < 0.05$). Further details with respect to the etiology of ESKD etiology or previous CVD diagnoses are shown in online supplementary table S1.

The distributions of allele, genotype, and haplotype frequencies in ESKD patients are shown in online supplementary table S2. The distribution of genotypes for each polymorphism showed no deviation from Hardy-Weinberg equilibrium ($p > 0.05$).

The plasma concentrations of MMP-2 and TIMP-2 were evaluated both in controls and in ESKD patients (before and after hemodialysis). While we found higher MMP-2 and TIMP-2 levels in ESKD patients compared to healthy controls (fig. 1a, b, respectively; both $p < 0.0001$), no significant differences between the groups were found in MMP-2/TIMP-2 ratios ($p = 0.0575$; fig. 1c). Interestingly, hemodialysis decreased MMP-2 levels ($p < 0.0001$; fig. 1a) without changing TIMP-2 concentrations ($p = 0.8916$; fig. 1b), thus lowering MMP-2/TIMP-2 ratios ($p < 0.0001$; fig. 1c).

When ESKD patients were divided according to the genotypes for the $C^{-1306}T$ polymorphism, we found no differences in MMP-2 or TIMP-2 levels before hemodialysis (both $p > 0.05$; fig. 2a, b). Hemodialysis decreased MMP-2 (but not TIMP-2) levels independently of the genotypes for the $C^{-1306}T$ polymorphism ($p < 0.0001$; fig. 2a, b).

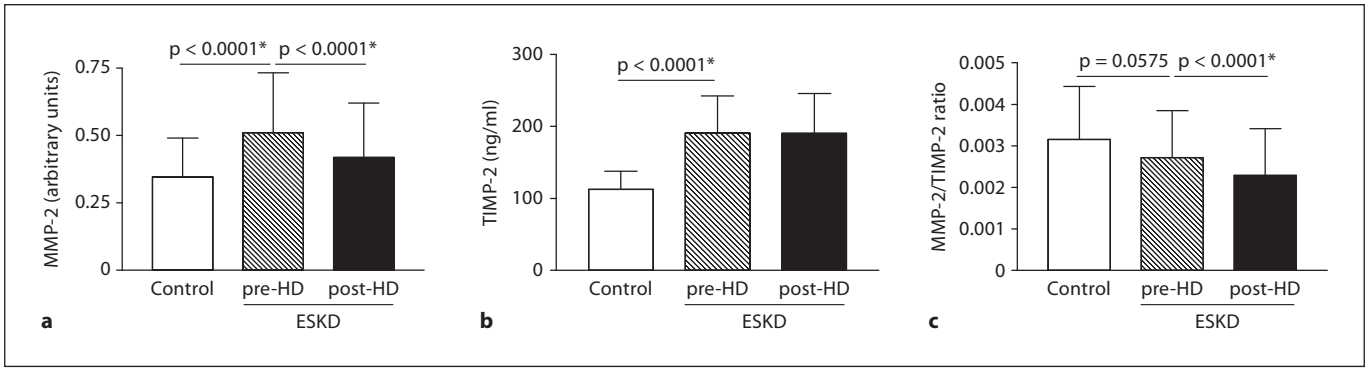

Fig. 1. Effects of hemodialysis on plasma MMP-2 and TIMP-2 levels, and on MMP-2/TIMP-2 ratios. Concentrations of MMP-2, TIMP-2, and MMP-2/TIMP-2 ratios in healthy controls and in ESKD patients before (pre) and after (post) hemodialysis (HD). * Statistically significant.

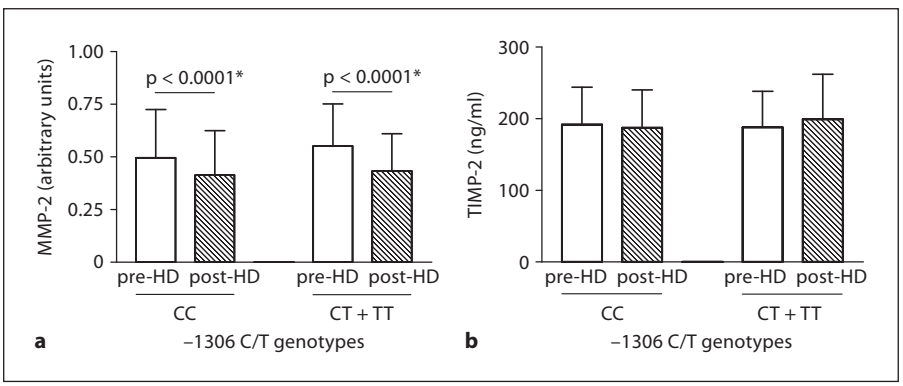

Fig. 2. Plasma concentrations of MMP-2 and TIMP-2 according to *MMP-2* $C^{-1306}T$ genotypes before (pre) and after (post) hemodialysis (HD). * Statistically significant.

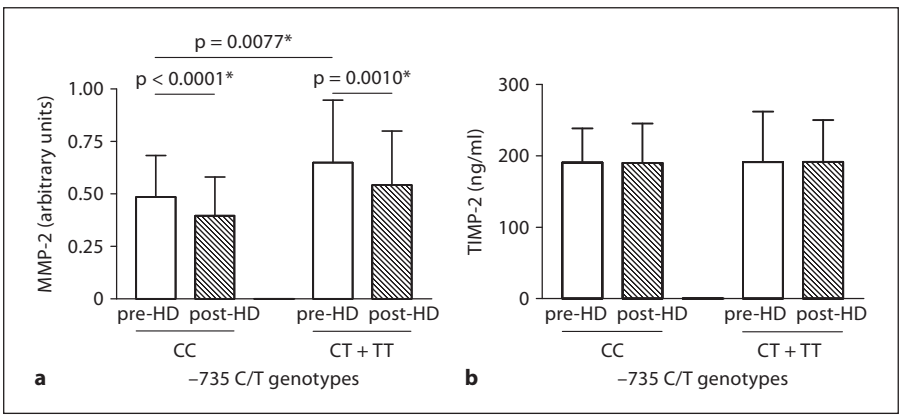

Fig. 3. Plasma concentrations of MMP-2 and TIMP-2 according to *MMP-2* $C^{-735}T$ genotypes before (pre) and after (post) hemodialysis (HD). * Statistically significant.

In contrast to the $C^{-1306}T$ polymorphism, when ESKD patients were divided according to the genotypes for the $C^{-735}T$ polymorphism, we found higher MMP-2 (but not TIMP-2) levels before hemodialysis in subjects with the CT/TT genotypes compared with those found in subjects with the CC genotype (p = 0.0077; fig. 3a, b). However, in parallel with the $C^{-1306}T$ polymorphism, hemodialysis decreased MMP-2 (but not TIMP-2) levels independently of the genotypes for the $C^{-735}T$ polymorphism ($p < 0.0010$; fig. 3a, b).

The analysis of haplotypes showed higher MMP-2 (but not TIMP-2) levels in ESKD patients with the C-T haplo-

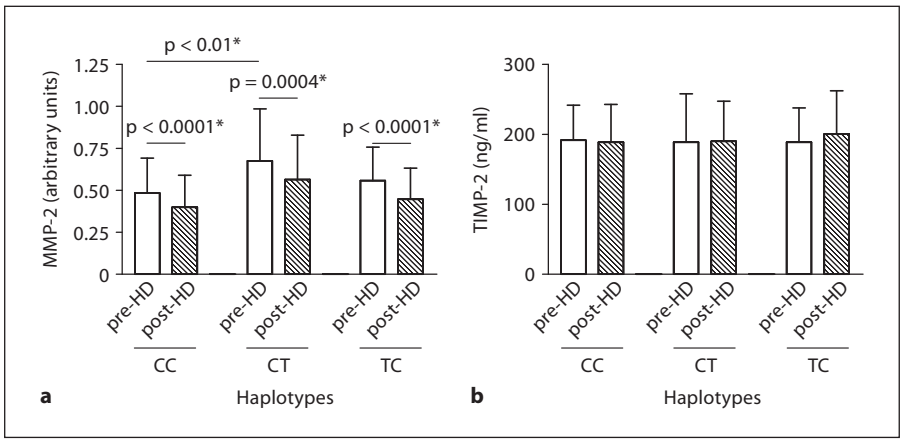

Fig. 4. Plasma concentrations of MMP-2 and TIMP-2 according to MMP-2 haplotypes before (pre) and after (post) hemodialysis (HD). * Statistically significant. The haplotype H4 (T,T) was not observed.

type compared with those with the C-C haplotype (the most common haplotype; $p < 0.01$; fig. 4a, b). Hemodialysis decreased MMP-2 (but not TIMP-2) levels in all MMP-2 haplotype groups ($p < 0.0004$; fig. 4a, b).

Discussion

The main findings of the present study were: (i) patients with ESKD have higher circulating MMP-2 and TIMP-2 levels than healthy controls; (ii) ESKD patients carrying the T allele for the $C^{-735}T$ polymorphism or the C-T haplotype have higher plasma MMP-2 levels than those without these genetic markers, and (iii) hemodialysis decreases plasma MMP-2 (but not TIMP-2) concentrations in ESKD patients. However, these effects are not modified by MMP-2 polymorphisms. This is the first study to examine how genetic MMP-2 variants may affect MMP-2/TIMP-2 levels in ESKD patients or modify the effects of hemodialysis.

Abnormal MMP-2 activity clearly contributes to the vasculopathy found in ESKD patients [7, 25]. Interestingly, experimental evidence showed an early upregulation of MMP-2 expression in areas of elastin degradation and smooth muscle cells phenotype change in chronic kidney disease course, which is associated with increased circulating MMP-2 levels [4–6]. These alterations clearly promote vascular medial layer calcification [6], and the increases in MMP-2 levels correlated positively with vascular stiffness and phosphate concentrations in chronic kidney disease patients [25]. In line with our results showing increased MMP-2 and TIMP-2 levels in ESKD patients, elevated circulating MMP-2 and TIMP-2 levels have been described as an indicator of CVD in dialysis patients [8, 9]. It is possible that TIMP-2 levels increase in order to protect against abnormal proteolytic activity in patients on dialysis, which could promote excessive extracellular matrix remodeling, as previously suggested [9, 15]. While most of the previous studies agree with our findings [8, 10–12], two studies detected no significant changes in these markers [13, 14], and one study showed lower TIMP-2 levels and augmented MMP-2/TIMP-2 ratios in ESKD [14]. Probably, the exclusion of CVD patients from that study may have affected the conclusions drawn by the authors [14]. The explanation for such discrepancies between studies may be explained by differences in the studied populations, ethnicity, age, sample size, and etiology for ESKD [15].

Several studies analyzed the effects of a hemodialysis session on the circulating levels of MMP-2. However, conflicting results have been reported. In line with our findings, most studies showed that hemodialysis reduced MMP-2 levels, with one exception [10, 11, 14, 15]. Variable results have also been reported for the effects of hemodialysis on TIMP-2 levels [10, 14, 15]. However, this study is the first to report significant reductions in MMP-2/TIMP-2 ratios after a dialysis session, whereas MMP-2/TIMP-2 ratios were not affected in another study [14]. It is not clear why TIMP-2 (a 21-kDa molecule) levels are not altered by hemodialysis, whereas the concentrations of MMP-2 (72 kDa) decrease. The larger size of MMP-2 should have precluded its filtration, thus suggesting that a mechanism other than ultrafiltration is certainly involved. Interestingly, although hemodialysis activates inflammatory responses, which promote MMP release [11], no study showed increased MMP-2 levels after a session.

Genetic markers may contribute to the variability in MMP-2 in ESKD patients. Given the importance of

MMP-2 to CVD [6, 26–28], MMP-2 polymorphisms could affect MMP-2 levels in uremic patients and therefore influence the prevalence of CVD in uremic patients. Whereas no previous study has examined this possibility in ESKD patients, we studied the effects of two functionally relevant MMP-2 SNPs in the promoter of MMP-2. The $C^{-735}T$ and $C^{-1306}T$ SNPs disrupt the Sp1 regulatory element in the promoter site (CCACC box), thus affecting MMP-2 expression in an allele-specific manner [18]. In line with the idea that the C allele for the $C^{-1306}T$ polymorphism increases MMP-2 activity, higher MMP-2 levels and MMP-2/TIMP-2 ratios were reported in subjects exposed to mercury and carrying this allele [16]. However, this polymorphism had no significant effects on MMP-2 levels in our uremic patients. This apparent discrepancy between studies is probably explained by differences between clinical conditions, with different factors, possibly with MMP-2 gene variants.

We found that the T allele for the $C^{-735}T$ polymorphism and the C-T haplotype were associated with increased MMP-2 (but not TIMP-2) levels in ESKD patients. While our results may not be consistent with previous molecular findings discussed above [18], little is known about the complex regulation of MMPs, especially MMP-2 in ESKD patients. In vivo clinical findings may differ significantly from in vitro molecular studies. In line with our findings, previous studies showed that the C-C haplotype was more commonly found in hypertensive patients with a lower left ventricular mass index [22], and this clinical finding also contrasts with molecular studies [18]. Although further studies are required to clarify the mechanisms underlying these clinical associations, it is interesting that we found no effects of MMP-2 polymorphisms on the changes in MMP-2 levels associated with a hemodialysis. These findings suggest that hemodialysis decreases MMP-2 levels independently of genetic factors.

The present study has some limitations. First, although this cross-sectional study allows the detection of associations, causality is not properly addressed. Second, we studied a relatively small number of patients, and this may have limited our chances to detect differences between the groups. Third, the patients included in the present study were under pharmacological treatment, and this may have altered MMP-2 and/or TIMP-2 levels [29, 30]. However, it is clearly unacceptable not to treat uremic patients.

In conclusion, we found that the MMP-2 genotypes or haplotypes modify MMP-2 levels in ESKD patients, and may help to identify patients with increased levels of MMP-2 while TIMP-2 remains unaltered. Our findings show that hemodialysis reduces MMP-2 levels independently of MMP-2 genetic variants.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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Erratum

In the above article by Marson et al. entitled 'Matrix Metalloproteinase (MMP)-2 Genetic Variants Modify the Circulating MMP-2 Levels in End-Stage Kidney Disease' [Am J Nephrol 2012;35:209–215], the following error occured on page 210 under Subjects and Methods, right column, third sentence: 'Patients with previous CVD or using medications were excluded.', it should read 'Patients with previous CVD or using medications were not excluded.'