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Functional matrix metalloproteinase (MMP)-9 genetic variants modify the effects of hemodialysis on circulating MMP-9 levels

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ABSTRACT

Background: Altered levels of matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), are involved in cardiovascular alterations associated with end stage kidney disease (ESKD). Genetic polymorphisms in *MMP-9* gene affect MMP-9 levels. We examined how *MMP-9* polymorphisms and haplotypes affect the changes in plasma MMP-9 and TIMP-1 levels found in patients with ESKD undergoing hemodialysis.

Methods: We studied 94 ESKD patients undergoing hemodialysis for at least 3 months. MMP-9 and TIMP-1 were measured by ELISA in plasma from blood samples collected before and after a session of hemodialysis. Genotypes for three MMP-9 polymorphisms ($C^{-1562}T$, rs3918242; −90 $(CA)_{14-24}$, rs2234681; and Q279R, rs17576) were determined by Taqman® Allele Discrimination Assay and real-time polymerase chain reaction. Haplotype frequencies were determined with the software program PHASE 2.1.

Results: Hemodialysis increased MMP-9 and TIMP-1 levels ($P<0.05$). Genotypes had no effects on baseline MMP-9 and TIMP-1 levels ($P>0.05$). Hemodialysis increased MMP-9 and TIMP-1 levels in subjects with the CC (but not CT or TT) genotype for the $C^{-1562}T$ polymorphism ($P<0.05$), and increased MMP-9 levels :0.05), whereas the effects on baseline in subjects carrying the CLQ haplotype ($P=0.0012$ and $P=0.0045$, respectively).

Conclusion: ESKD patients with the QQ genotype for the Q279R polymorphism or with the CLQ haplotype are exposed to more severe increases in MMP-9 levels after hemodialysis. Such patients may benefit from the use of MMP inhibitors.

1. Introduction

Patients with end stage kidney disease (ESKD) bear a heavy burden of cardiovascular diseases (CVD) [1], and hemodialysis may trigger a maladaptive process leading to arterial medial calcification, stiffness, and loss of function mimicking the atherogenic effects of uremic factors and inflammation [1,2]. These vascular alterations found in ESKD are linked to extracellular matrix remodeling and elastocalcinosis [2,3] and involve imbalanced matrix metalloproteinase (MMP) activity [4].

MMPs are structurally related, zinc dependent, enzymes that degrade the extracellular matrix and other non-extracellular matrix-related substrates [5]. They are regulated at transcriptional and post-translational levels, and their activity is also dependent on endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) [6]. Mounting evidence indicates that imbalanced MMP activity contributes to diseased vessels in patients with renal failure [3,7–9], particularly MMP-9, which is associated with atherosclerosis in non-dialytic chronic kidney disease (CKD) [7,8]. Moreover, increased MMP-9 expression has been reported in monocytes from patients with ESKD [10], and recent studies have shown altered MMP-9 and TIMP-1 levels in patients on dialysis [11–15]. Nevertheless, inconsistent findings have been reported regarding the effects of hemodialysis on the circulating MMP levels. Some studies suggest that plasma MMP-9 remains unaltered [11,12,16] or decreases after a hemodialysis session [13,17].

MMP-9 activity is highly dependent on its expression levels [6,18], and functional genetic polymorphisms in the *MMP-9* gene may affect MMP-9 concentrations [19], possibly modifying the susceptibility to cardiovascular diseases [18,20,21]. Therefore, we hypothesized that

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2 functional polymorphisms in the promoter region of *MMP-9* gene (the $C^{-1562}T$ — rs3918242, and the microsatellite $(CA)_{14-24}$ in −90 position — rs2234681), and one polymorphism in exon 6 (the Q279R; rs17576) could affect the changes in MMP-9 levels associated with hemodialysis. In support of this hypothesis, these polymorphisms have been associated with variable MMP-9 levels in other clinical conditions [22–24]. While previous works have studied MMP-2 [25] and MMP-9 [26] polymorphisms in dialysis, no previous study has examined how functional MMP-9 polymorphisms, or their combinations within haplotypes, affect the changes in MMP-9 and TIMP-1 levels during a hemodialysis session.

2. Materials and methods

2.1. Patients

The present study was carried out in accordance with the Helsinki Declaration ethical guidelines. Approval for use of human blood was obtained from the Research Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, and informed consent was obtained from each participant. Ninety-four patients with ages from 18 to 65 y were studied in 3 hemodialysis units. ESKD was defined as glomerular filtration rate <15 ml/min associated with clinical signs of uremic syndrome requiring dialysis. The patients were on continuous therapy for at least 3 months and were stable (without clinical complications). Clinical data were based on medical history, physical examination, and routine analytical tests. While the patients included in the present study had different causes for their clinical condition, diabetes mellitus, hypertension, glomerulonephritis, and polycystic kidney disease were the main causal diseases. The dialysis schedule included three 4-h dialysis sessions per week with a polysulfone hollow-fiber membrane, bicarbonate dialysate, and regular 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.

Blood samples were collected into EDTA vaccutainer tubes (Becton-Dickinson, São Paulo, Brazil) by venipuncture of the arteriovenous fistula before and after a hemodialysis session. The blood samples were centrifuged at 1000×*g* for 10 min and plasma fractions were immediately stored at −70 °C until used for biochemical measurements. Blood samples were also collected to extract genomic DNA. The biochemical and hematological parameters were determined by routine techniques using an automated analyzer (Johnson Vitros Chemistry 5.1 SS). LDL-cholesterol was calculated using the Friedewald's formula.

2.2. Genotyping

Genotypes for the $C^{-1562}T$ (rs3918242) polymorphism in the 5′-flanking region of MMP-9 were determined by polymerase chain reaction (PCR) amplification using the primers: 5′-GCCTGGCAC ATAGTAGGCCC-3′ (sense) and 5-CTTCCTAGCCAGCCGGCATC-3 (antisense), and PCR conditions as described elsewhere [27]. The amplified products were digested with SphI (New England Biolabs, Ipswich, MA) overnight at 37 °C, producing fragments of 247 and 188 bp in the case of a polymorphic variant (allele T), or an undigested 435-bp band in the case of a wild type allele (allele C). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized after silver staining.

The −90 $(CA)_{14-24}$ (rs3222264) microsatellite was detected by polymerase chain reaction as described previously [19], using the primers 5′-GACTTGGCAGT GGAGACTGCGGGCA-3′ (sense) and 5′-GAC CCCACCCCTCCTTGACAGGCAA-3′ (antisense). Amplified products were separated in a 7% polyacrylamide–urea gel and visualized after silver staining. Differences in molecular weight (or number of bases), from 144 bp to 168 bp, were determined by a comparison with migration of a 10-bp DNA ladder (Invitrogen, Carlsbad, CA) and to homozygous samples previously sequenced. In order to make easier the analysis of the bands in the gel, the alleles were classified in accordance with the biallelic distribution of this polymorphism: alleles were grouped as "low" (L) when the number of CA repeats was less than 21, and as "high" (H) when the number of CA repeats was ≥21 [28].

Genotypes for the MMP-9 Q279R (rs17576) polymorphism were determined by Taqman® Allele Discrimination assay. Probes and primers used in the MMP-9 assay were designed by Applied Biosystems (ID: C_11655953_10). TaqMan polymerase chain reaction was performed in a total volume of 12 μl (3 ng of DNA, 1× TaqMan master mix, 900 nmol/l of each primer and 200 nmol/l of each probe) placed in 96-well PCR plates. Fluorescence from polymerase chain reaction amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA) and analyzed with the manufacturer's software [23].

2.3. Measurement of plasma MMP-9 and TIMP-1 concentrations

The plasma MMP-9 and TIMP-1 concentrations were measured with commercially available enzyme-linked immunosorbent assay kits (DY911 and DY970, respectively; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.4. Statistical analysis

Statistical analysis was carried out using the Stat-View (SAS Institute, Cary, NC). The clinical characteristics, plasma MMP-9 and TIMP-1 concentrations, and MMP-9/TIMP-1 ratios were compared with Student's paired *t*-test (parametric data), Mann–Whitney *U*-test (non-parametric data) or chi-square (categorical data). The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium by using chi-squared tests. Difference in the alleles, genotypes, and haplotype frequencies was assessed with chi-squared tests. Because the TT genotype for the $C^{-1562}T$ polymorphism was very rare, we combined this genotype with the heterozygous CT genotype. A $P<0.05$ was considered statistically significant.

The Bayesian statistical-based program Phase 2.1 was used to estimate the haplotype frequencies in each group (http://depts.washington.edu/uwc4c/express-licenses/assets/phase/). The possible haplotypes including genetic variants of three MMP-9 polymorphisms studied ($C^{-1562}T$, $90(CA)_{14-24}$, and Q279R) were H1 (C, L, Q); H2 (C, L, R); H3 (C, H, Q); H4 (C, H, R); H5 (T, L, Q); H6 (T, L, R); H7 (T, H, Q); and H8 (T, H, R). Differences in haplotype frequency distributions were further tested using contingency tables, and to compare specific haplotype frequencies, a value of $P<0.01$ (0.05/number of observed haplotypes) was considered significant to correct for multiple comparisons. We excluded the rare haplotypes H5, H6 and H7 from the analysis.

3. Results

Clinical characteristics of patients are shown in Table 1. Detailed information regarding the etiology of renal failure and coexistent cardiovascular diseases and medications is provided in Supplementary Table S1. The distribution of allele, genotype, and haplotype frequencies in hemodialysis subjects are shown in Supplementary Table S2. The distribution of genotypes for each polymorphism showed no deviation from Hardy–Weinberg equilibrium ($P>0.05$).

The plasma MMP-9 and TIMP-1 concentrations were evaluated in ESKD patients, both before and after hemodialysis. While the session of hemodialysis increased MMP-9 and TIMP-1 plasma concentrations (Fig. 1; $P=0.0087$ and $P=0.0148$, respectively), the MMP-9/TIMP-1

Table 1
Clinical and demographic characteristics of the patients.

Clinical characteristics	Patients, N = 94
Age, y	51 ± 10.8
Race (white/non-white)	79/15
Sex (masculine/feminine)	52/42
Smoking	44.39
SAP, mm Hg	141.4 ± 40.3
DAP, mm Hg	78.8 ± 12.5
Diabetes mellitus	34.6
Hypertension	79.6
BMI, kg/m^2	25.29 ± 5.18
Total cholesterol, mg/dl	157.8 ± 54.6
HDL cholesterol, mg/dl	38.4 ± 11.9
LDL cholesterol, mg/dl	86.6 ± 40.9
Triglycerides, mg/dl	196.1 ± 150.6
Hemoglobin, g/dl	10.4 ± 2.8
Hematocrit, %	31.9 ± 6.1
Leukocytes, ×10^3/μl	6518 ± 2137
Creatinine, mg/dl	9.6 ± 3.2
Calcium, mg/dl	8.8 ± 1
Phosphorus, (mg/dl)	6.6 ± 4.2
Potassium, (mg/dl)	5.2 ± 1.2
PTH (pg/ml)	519.2 ± 452
Albumin (mg/dl)	3.82 ± 0.32

ratios tended to increase after hemodialysis (Fig. 1; P = 0.0561). We found no significant differences when patients with primary kidney diseases and secondary kidney diseases were compared (data not shown).

When ESKD patients were divided according to genotypes, we found no significant effects of genotypes on MMP-9 and TIMP-1 levels measured before hemodialysis (Figs. 2, 3, and 4; all P > 0.05). However, hemodialysis increased MMP-9 levels in some genotype groups. Specifically, we found significant increases in both MMP-9 and TIMP-1 levels in subjects with the CC genotype for the C^{-1562}T polymorphism (Fig. 2; both P < 0.05), but not in subjects with CT or TT genotypes for this polymorphism. When the patients were divided according the genotypes for the CA(n)$_{14–24}$ polymorphism, we found that TIMP-1 levels increased in subjects with the HH genotype (Fig. 3; P = 0.0375). However, this increase in TIMP-1 levels was not associated with significant changes in MMP-9/TIMP-1 ratios (Fig. 3; P > 0.05). Interestingly, we found a trend for increased MMP-9 after hemodialysis in subjects with the LL or the HL genotypes for this polymorphism (Fig. 3; P = 0.0668 and P = 0.0730, respectively). When the patients were divided according to genotypes for the Q279R polymorphism, we found significant increases in MMP-9 levels and in MMP-9/TIMP-1 ratios in subjects with the QQ genotype for this polymorphism (Fig. 4; both P < 0.05). While we found significant increases in TIMP-1 levels in subjects with the QR genotype, this change was not associated with significant changes in MMP-9/TIMP-1 ratios (Fig. 4; P > 0.05).

The analysis of haplotypes showed no effects of MMP-9 haplotypes on both MMP-9 and TIMP-1 levels measured before hemodialysis (Fig. 5; P > 0.05). However, we found significant increases in MMP-9 (but not TIMP-1) levels in subjects carrying the CLQ haplotype (Fig. 5; P = 0.0012), thus resulting in increased MMP-9/TIMP-1 ratio in this particular haplotype group (Fig. 5; P = 0.0045). While hemodialysis was associated with increased TIMP-1 levels in subjects with the CHR haplotype, no significant changes were found in MMP-9/TIMP-1 ratio (Fig. 5; P > 0.05).

4. Discussion

This study examined how MMP-9 genetic polymorphisms affect the changes in MMP-9 and TIMP-1 levels associated with a hemodialysis session in ESKD patients. While we found that MMP-9 polymorphisms do not significantly affect baseline MMP-9 or TIMP-1 levels in ESKD patients, we found a significant genetic contribution of MMP-9 polymorphisms and haplotypes to hemodialysis-induced changes in MMP-9 levels and MMP-9/TIMP-1 ratios. Our findings may help to understand the relevance of MMP-9 genetic polymorphisms to the pathophysiology of cardiovascular complications associated with ESKD and hemodialysis.

MMPs (particularly MMP-9) are important for extracellular matrix remodeling and play key roles in cardiovascular diseases [5,15,20,29]. MMP-9 activation promotes TGF-B signaling and a sequence of events

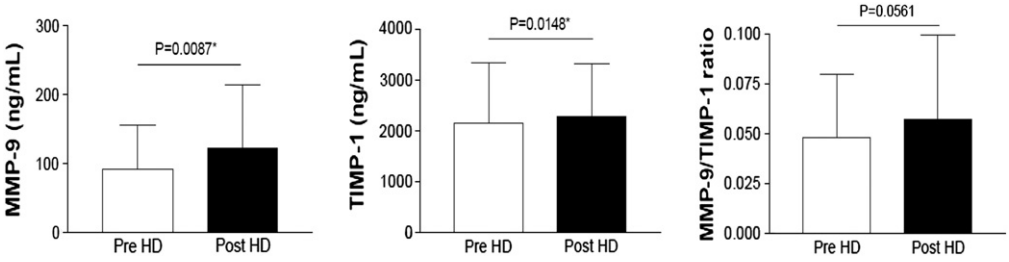

Fig. 1. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios in end stage kidney disease patients before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

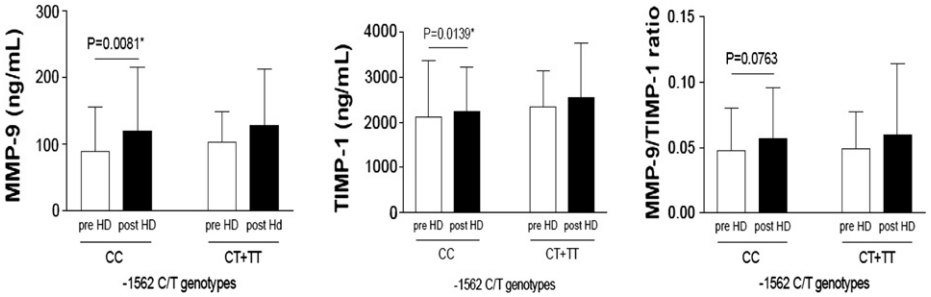

Fig. 2. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their genotypes for the *MMP-9* C^{-1562}T polymorphism. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

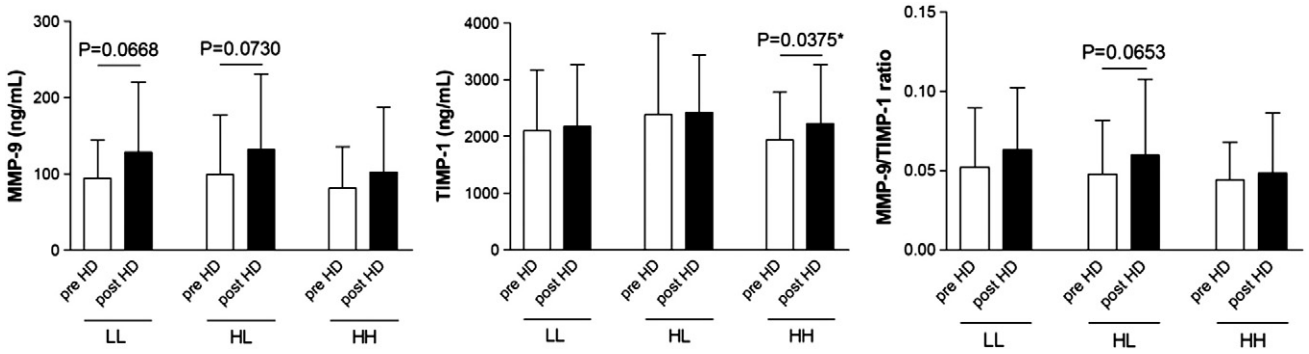

Fig. 3. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their genotypes for the *MMP-9* 90(CA)$_{14–24}$ polymorphism. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

that leads to elastocalcinosis and vascular stiffness [29]. Indeed, recent studies showed that upregulated MMP-9, without proper balance of TIMP-1, disrupts elastin and affects normal vascular smooth muscle cells allowing the extracellular matrix to expand and calcify, eventually leading to overt hydroxyapatite deposition and vascular dysfunction [3,7,9].

Our results showing that hemodialysis elevated the circulating levels of MMP-9 and TIMP-1 differ from previous reports, which described reduced or unaltered MMP-9 and TIMP-1 levels after hemodialysis [11,13,16,17]. We found minor increases in MMP-9/TIMP-1 ratios, and this finding agrees with previous results [12]. It is likely that inconsistencies in reported plasma MMP levels reflect the complex regulation of inflammation and MMPs, as well as differences in sample size, populations, anticoagulation regimen, vascular access, type of membrane and causal diseases for ESKD. In addition, pre-analytical issues may also explain dissimilarities, since modifications in processing the samples may alter MMP-9 levels [30,31]. MMP-9 is expressed constitutively at very low levels in bone marrow-derived cells, however it is highly inducible under oxidative and inflammatory conditions [6,10]. Since hemodialysis activates inflammatory and coagulation pathways, it is plausible to find increased MMP-9 levels after hemodialysis.

MMP-9 gene polymorphisms may modify MMP-9 concentrations and affect the development of cardiovascular complications in ESKD, as previously suggested [1]. Indeed, the C^{-1562}T polymorphism results in loss of a nuclear repressor protein binding site and enhanced MMP-9 mRNA and protein levels when the T allele is present [18,21]. The microsatellite 90(CA)14–24 in the promoter region of the MMP-9 gene was shown to reduce MMP-9 promoter activity when the (CA) 14 allele is present as compared with the (CA)21 allele [32]. The Q279R polymorphism causes an aminoacid residue substitution, thus affecting MMP-9 activity [22,33]. Our results showing similar baseline MMP-9 and TIMP-1 levels suggest that these polymorphisms do not have major effects in ESKD patients, at least in terms of circulating MMP-9 levels.

We found that hemodialysis increased MMP-9 and TIMP-1 levels in subjects with the CC (but not CT or TT) genotypes for the C^{-1562}T polymorphism, and increased MMP-9 (but not TIMP-1) levels in subjects with the QQ genotype for the Q279R polymorphism, thus increasing MMP-9/TIMP-1 ratios. However, the haplotypic analysis may be much more effective and informative than the single locus analysis [20]. While baseline MMP-9 and TIMP-1 levels were not affected by MMP-9 haplotypes, subjects with the CLQ haplotype showed increased MMP-9 (but not TIMP-1) levels after hemodialysis. Interestingly, this particular haplotype combines the C allele for C^{-1562}T polymorphism, and L allele for the 90(CA)$_{14–24}$ polymorphisms, which have been associated with lower MMP-9 upregulation [18,21,32]. While we have not examined the molecular mechanisms possibly explaining these findings, our results suggest that the CLQ haplotype combines a group of MMP-9 genetic markers that may lead to the highest rates of cardiovascular complications associated with abnormal MMP-9 activity in ESKD patients undergoing hemodialysis. It remains to be determined whether MMP-9 inhibition is a suitable pharmacologic approach to prevent or postpone cardiovascular in this highly susceptible population. In fact, pharmacological blockade of MMPs has been suggested in hemodialysis patients [4] and experimental evidence suggests that non-specific MMP inhibition may prevent cardiovascular alterations through complex mechanisms [34,35].

This study has some limitations that should be taken into consideration. The findings reported here may be quantitatively small because the differences that we found were not of major magnitude and some of our findings may not resist correction for multiple comparisons.

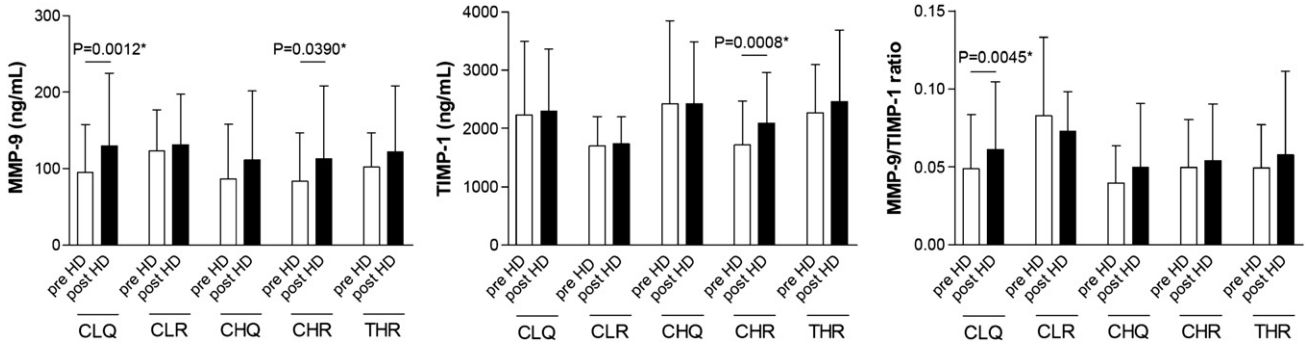

Fig. 4. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their genotypes for the *MMP-9* Q^{-279}R polymorphism. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

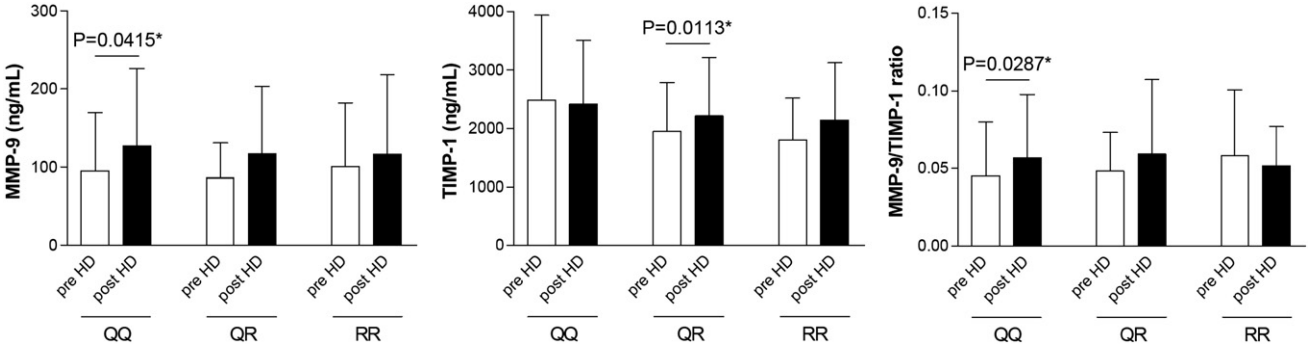

Fig. 5. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their *MMP-9* haplotypes. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

However, it is common sense that the contribution of a single polymorphism to any clinical condition is usually of minor magnitude, and our findings suggest that MMP-9 polymorphisms may have some minor effects on MMP-9 changes induced by dialysis. Moreover, the assays used in the present study are based on antigen detection and may not reflect activity, particularly where a genotypic difference may affect activity.

In conclusion, we found evidence supporting the idea that MMP genetic polymorphisms affect MMP alterations in ESKD patients [25] undergoing hemodialysis. ESKD patients with the QQ genotype for the Q279R polymorphism or with the CLQ haplotype are exposed to more severe increases in MMP-9 levels after hemodialysis. These findings may indicate a group of patients that will have worse cardiovascular prognosis when undergoing hemodialysis. Whether such patients would benefit from the use of MMP inhibitors remains to be elucidated.

Acknowledgments

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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.cca.2012.08.014.

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