Upregulation of Matrix Metalloproteinase-2 in the Arterial Vasculature Contributes to Stiffening and Vasomotor Dysfunction in Patients With Chronic Kidney Disease

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Background—Cardiovascular disease is the leading cause of mortality in chronic kidney disease patients on maintenance dialysis. Given the importance of matrix metalloproteinase-2 (MMP-2) in matrix integrity, vascular cell function, and structural stability, we hypothesized that MMP-2 was elevated in the macrovasculature in dialyzed chronic kidney disease patients compared with chronic kidney disease patients not on dialysis and kidney donors.

Methods and Results—Arteries from live kidney donors (Adonor; n=30) and recipients (nondialysis [Anondialyzed], n=17; dialysis [Adialyzed], n=23 [peritoneal dialysis, n=10; hemodialysis, n=13]) were harvested during the transplantation procedure. Compared with Adonor, MMP-2 upregulation was evident in both recipient groups. Protein expression of latent plus active MMP-2 in Adialyzed was 2-fold that in Anondialyzed. MMP-2 activity increased with length of dialysis (r=0.573, P=0.004). In Adialyzed, medial elastic fiber fragmentation was pronounced, and the ratio of external elastic lamina to media was negatively correlated with MMP-2 activity (r=−0.638, P=0.001). Adialyzed was 25% stiffer than Anondialyzed; this increased stiffness correlated with MMP-2 activity (r=0.728, P<0.0001) and the severity of medial calcium deposition (r=0.748, P=0.001). The contractile function and endothelium-dependent relaxation were reduced by 35% to 55% in Adialyzed and were negatively associated with MMP-2 activity (r=−0.608, P=0.002; r=−0.520, P=0.019, respectively). Preincubation with MMP-2 inhibitor significantly improved contractility and relaxation in Adialyzed.

Conclusions—We describe a strong correlation between MMP-2 activation and elastic fiber disorganization, stiffness, calcification, and vasomotor dysfunction in the arterial vasculature in dialyzed chronic kidney disease patients. These findings may contribute to an improved understanding of mechanisms important in vascular health in chronic kidney disease patients. (Circulation. 2009;120:792-801.)

Key Words: arteries • kidney failure, chronic • dialysis • matrix metalloproteinase 2

M ortality from cardiovascular disease is 15 to 30 times higher in patients with chronic kidney disease (CKD) compared with the general population1–3 and is even higher in patients on peritoneal dialysis (PD) and hemodialysis (HD) therapy. These observations suggest that the disease manifestations of CKD are not only limited to the glomerulus but also generally affect the vascular system. Critical changes in vasomotor function and vessel structure could explain the propensity of cardiovascular disease in association with CKD progression.3

Clinical Perspective on p 801

Vascular function is determined by a set of factors that include endothelial secretion, smooth muscle contractility, and structural integrity. Vascular abnormalities develop early in CKD in parallel with cardiac abnormalities and become more severe as end-stage disease is reached. Endothelial dysfunction and reduced nitric oxide (NO) bioavailability are implicated as the initial steps in the vascular damage in CKD. Exaggerated production of vasoconstrictors (eg, norepinephrine, endothelin-1, and angiotensin) has been suggested to be associated with uremia.4–6 Several studies report decreased vascular responsiveness to adrenergic agonists in CKD and that this factor contributes to the hemodynamic instability and autonomic disturbances.7–9 It is generally accepted that structural alterations, including nonocclusive vascular remodeling, are prevalent in CKD.10,11 Increased aortic stiffness, as measured by pulse-wave velocity (PWV), and vascular calcification have been shown to be associated with unfavorable cardiovascular outcomes such as left ventricular hypertrophy, decreased coronary perfusion, and isolated systolic hypertension in dialyzed patients.10 A recent study has shown that

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medial calcification was evident in both predialyzed and
dialyzed children, but factors specific to dialysis may be
responsible for the extremely rapid calcification accompanied
by extensive vascular remodeling.\textsuperscript{11}

Vascular remodeling represents any enduring change in the
size and/or composition of an adult blood vessel. The pro-
cesses permit blood vessels to adapt to stressors but paradox-
ically may underlie the pathogenesis of cardiovascular
disease.\textsuperscript{12}Physiological and pathological vascular remodeling
entails degradation and reorganization of extracellular
matrix, explaining the recent great interest in matrix metal-
lopeptidases (MMPs). The gelatinase MMP-2 is of particular
importance in vascular biology because it is expressed abun-
dantly in arterial vasculature and its activity is markedly
modified during disease progression.\textsuperscript{12} In addition to the
matrix-degrading property, MMP-2 has been shown to inhibit
smooth muscle contraction, which could be associated with the
development of aortic aneurysm and venous dilata-
tion.\textsuperscript{12–15} MMPs are known to be activated in a cellular
environment under oxidative stress and with reduced NO
bioavailability.\textsuperscript{16} Inflammatory cells in the adventitia or
smooth muscle cells in the media secrete MMP-2, and the
release of degraded elastic fiber highly induces calcium
deposition.\textsuperscript{17} Elastinolysis is the primary event impairing the
arterial mechanical properties,\textsuperscript{15} and serum MMP-2 is asso-
ciated with arterial stiffness.\textsuperscript{18} Serum MMP-2 has been
suggested to be one of the strong independent variables
linked to intima-media thickness in dialyzed patients.\textsuperscript{19} Re-
cently, we have demonstrated that the upregulation of
MMP-2 in human internal mammary artery is associated with
reduced kidney function, ie, declining estimated glomerular
filtration rate.\textsuperscript{20}

We hypothesized that MMP-2 would be upregulated in the
artery from dialyzed patients compared with nondialyzed
CKD patients and kidney donors. Appreciating the potential
different impacts that dialysis procedures themselves would
have, we also compared arteries from PD and HD patients to
determine whether they differ in MMP-2 activation and
vascular function. We demonstrate that elevated MMP-2 in
the artery from dialyzed patients is strongly associated with the
degeneration of elastic fibers, arterial stiffening, calcifi-
cation, and vasomotor dysfunction compared with arteries
from nondialyzed patients. Given the known biological func-
tions of MMP-2 in vascular health and disease progression,
we suggest that the upregulation of MMP-2 may be one of the
important pathological mechanisms by which the presence of
CKD with and without dialysis exposure exacerbates vascular
dysfunction.

Harvested vessels were placed in ice-cold RPMI in the presence of
heparin and delivered to the laboratory for functional experiments
within 1 hour. Medical records were used to obtain demographic data
and information on cardiovascular risk factors and renal function at the
time of transplantation. The estimated glomerular filtration rate
was calculated from the Modification of Diet in Renal Diseases
formula. We use $A_{\text{moddosed}}$ to denote the artery harvested from the
patient who had not been on dialysis at the time of transplantation and $A_{\text{dialyzed}}$ to denote the artery from the patient who had been on
either PD (n=10) or HD (n=13).

Note that a “ring” artery is needed for the functional and
mechanical experiments. Because of this technical limitation, we
were able to collect only 5 renal arteries from the donor group.
However, for the molecular biology experiments, renal arterial
segments could be used; thus, more donor samples were available.

Additional experiments performed included Western immunoblot-
ting, gelatinolytic zymography, reverse zymography, Movat stain-
ing, von Kossa staining, in vivo PWV, isometric force measurement,
and mechanical properties of artery.\textsuperscript{15,20,22-23} The specifics of these procedures are available in the online-only Data Supplement.

### Statistics

Data are reported as mean±SD. Comparisons between 2 groups
were performed by unpaired $t$ test. Comparisons of ≥3 groups were
performed with 1-way ANOVA (with Bonferroni posthoc analysis
for multiple comparisons). Differences between concentration-
response curves (involve multiple parameters per patient) were
analyzed by a linear mixed-effects model that accounted for corre-
lated data using individual patient random effects. Correlations
between MMP-2 activity and the vascular functional/ structural
parameters were calculated with Spearman rank correlation. Corre-
lations were studied when all data points were combined and in
subgroups (ie, PD and HD). To investigate whether 2 regression lines
were significantly different, we tested the equality of slopes and
intercepts by using general linear models. If 2 linear regression lines
had neither a different slope nor a different intercept, a common
regression coefficient and the $y$ intercept were used to describe the
whole population. When ≥3 regression lines were compared, we
tested for equality of all or just some of the lines by using general
linear models. Construction of concentration-response curves and
statistical analysis were performed with GraphPad Prism (version
4.03, GraphPad, San Diego, Calif) software and SPSS version 14.0
(SPSS Inc, Chicago, Ill).

### Results

#### Study Cohorts

Thirty donors and 40 transplant recipients consented to
participate in the study. Of the recipients, 23 were on dialysis,
and 17 were not. Table I of the online-only Data Supplement
describes the demographics of the study groups. Among the
recipients, the only difference was in medication use. More of
the nondialysis patients than dialysis patients used calcium
channel blockers, but there were no significant differences
between these 2 cohorts in any other parameters. Note that the
duration of exposure to CKD, defined as number of months
since first nephrologist consult to dialysis start date or
transplantation, is comparable between the 2 groups, as is the
prevalence of diabetes mellitus and hypertension. On aver-
age, the dialysis group had been exposed to 37.6 months of
dialysis treatment. Importantly, the donor and recipient ages
were not different. We also describe the differences between
the 10 PD and 13 HD patients. The only significant difference
between those 2 groups was female gender.

### Methods

#### Study Population Selection

Patients who were to undergo live donor kidney transplantation at St Paul’s Hospital (Vancouver, British Columbia, Canada) were
approached for participation in the study, which was approved by the
ethics boards of Providence Health Care and the University of British
Columbia. Written informed consent was obtained from donors
(n=30) and recipients (n=40) before surgery.

All patients consented to noninvasive measurements of vascular
stiffness (PWV measurements)\textsuperscript{21} and the use of the discarded inferior
epigastic artery (from recipients) and renal artery (from donors).

### Additional experiments performed included Western immunoblot-
ting, gelatinolytic zymography, reverse zymography, Movat stain-
ing, von Kossa staining, in vivo PWV, isometric force measurement,
and mechanical properties of artery.\textsuperscript{15,20,22-23} The specifics of these procedures are available in the online-only Data Supplement.
Elevated Activation of MMP-2 in \( A_{\text{dialyzed}} \)

The protein expression of latent MMP-2 in \( A_{\text{dialyzed}} \) was greatly increased compared with that in \( A_{\text{donor}} \) and \( A_{\text{ nondialyzed}} \) \((P<0.0001)\). Active MMP-2 expression in \( A_{\text{nondialyzed}} \) and \( A_{\text{dialyzed}} \) was 3.5-fold higher than that in \( A_{\text{donor}} \). The PD group was not significantly different from the HD group with respect to latent \((P=0.09)\) and active \((P=0.23)\) MMP-2 expression (Figure 1A and 1B). Expression of tissue inhibitor of metalloproteinase (TIMP) -1 and TIMP-2, the major endogenous inhibitors of MMP-2 activation,\(^{12}\) was not different among all groups. However, the activities of TIMP-1 and TIMP-2 in \( A_{\text{donor}} \) were significantly higher than those in \( A_{\text{dialyzed}} \) \((P=0.038 \text{ and } P=0.007, \text{ respectively})\). TIMP-1 and TIMP-2 activities in \( A_{\text{dialyzed}} \) were only 75% and 64%, respectively, of those of \( A_{\text{nondialyzed}} \). There was no difference in TIMP activity between the PD and HD groups (Figure 1C through 1E).

Activities of latent and active MMP-2 were significantly \((P<0.001)\) less in \( A_{\text{donor}} \) compared with the recipient groups. Latent and active MMP-2 activities were upregulated in \( A_{\text{dialyzed}} \) by 43% and 146%, respectively, compared with \( A_{\text{nondialyzed}} \) (Figure 2A and 2B). We pooled the activities of the latent and active forms of MMP-2 and observed a positive correlation with the length of CKD before renal replacement therapy (transplantation or dialysis) in the nondialyzed group \((r=0.713, P=0.001)\). This correlation was absent in the PD \((P=0.227)\) and HD \((P=0.521)\) groups, which were not significantly different from each other in slope \((P=0.720)\) and intercept \((P=0.969)\) (Figure 2C). The total MMP-2 activity formed a positive correlation with length of dialysis \((r=0.573, P=0.004)\) when all data were combined. In the subgroup analysis, the linear regression lines between the PD and HD groups were not significantly different in slope \((P=0.555)\) and intercept \((P=0.187)\) (Figure 2D).

Although the total MMP-2 activity was comparable between the PD and HD groups (Figure 2A), of interest, there appeared to be a difference in the increase in MMP-2 level as a function of exposure time to different dialysis modalities. A pronounced increase in MMP-2 activity \((\text{ie}, >325 \text{ arbitrary units})\) was seen in HD patients earlier in the course of their treatment \((P=0.05)\) than in the PD patients \((\text{ie}, \text{after } 145\pm65 \text{ months of exposure}; n=5, P=0.05)\).

Degradation of Elastic Fiber in the Media of \( A_{\text{dialyzed}} \) Correlates With MMP-2 Activity

On Movat histology, elastic fiber was present in the internal elastic lamina, tunica media, and external elastic lamina (EEL) of the artery (Figure 3). Within the tunica media, a uniformly long and continuous structure of elastic fiber was...
found in both A donor and Anondialyzed (Figure 3D and 3F). However, elastic fiber in the tunica media of Adialyzed was thinner and appeared fragmented (Figure 3H and 3J). Along the EEL, a disintegrated organization of elastic fiber was evident in recipient groups, whereas in A donor, it presented as a continuous and intact structure (Figure 3E). Although the intima-media thickness among A donor (0.508 ± 0.024 mm), Anondialyzed (0.502 ± 0.028 mm), and Adialyzed (0.500 ± 0.025 mm) was not significantly different, the ratio of EEL to medial thickness in Anondialyzed was only 77% of that in A donor (P < 0.01). This ratio was further decreased in Adialyzed by 35% compared with that in Anondialyzed (Figure 3L). Intimal thickening was detected in 46% of Adialyzed but absent in Anondialyzed (Figure 3A through 3C).

There was a negative correlation between the activity of MMP-2 and the ratio of EEL to media in Adialyzed (r = -0.638, P = 0.001) (Figure 3M). This correlation was weaker in Anondialyzed (r = -0.42, P = 0.05).

Increased Stiffness in Adialyzed Is Associated With MMP-2 Activity
Arterial stiffness was evaluated in vitro from the J-shaped stress-strain curve (Figure 4A). Measurement of arterial stiffness (the K value) indicated that Adialyzed was 25% and 30% stiffer than Anondialyzed and A donor (P = 0.02) (Figure 4B). When all data points were combined, the K value demonstrated a strong correlation with the in vivo PWV (r = 0.728, P < 0.0001). In the subgroup analysis, this strong correlation was seen in the nondialyzed (r = 0.722, P = 0.002), PD (r = 0.913, P = 0.002), and HD (r = 0.783, P = 0.013) groups (Figure 4C). These 4 linear regression lines were not different in slope (P = 0.157) but intercept (P = 0.039).

To investigate the structural stability, we measured the breaking stress, which represents the breakage of elastic fiber and smooth muscle association, the point at which the vessel could no longer maintain the stable resting tension. The breaking stress was >50% lower in Adialyzed than in A donor and Anondialyzed, the ease with which preservation of stable tension was lost suggested the structural weakening in Adialyzed (Figure 4D).

The K value positively correlated with MMP-2 activity in Adialyzed (r = 0.678, P = 0.001) but not in Anondialyzed (r = 0.326, P = 0.218). In the subgroup analysis, the PD and HD linear regression lines were significantly different in slope (P = 0.05) (Figure 4E).

Increased Calcium Deposition in Adialyzed Is Associated With Increased MMP-2 Activity
From the von Kossa staining, calcium phosphate deposition was evident, to various degrees, in 74% of CKD patients. In Adialyzed, calcium deposition was more severe than in Anondialyzed (Figure 5A through C). The semiquantified calcium deposition positively correlated with the K value in Adialyzed (r = 0.748, P = 0.001) but not in Anondialyzed (r = 0.16, P = 0.54; Figure 5D).

Because vascular smooth muscle cells release not only calcium but also MMPs during the process of calcification, we sought to determine whether the increased MMP-2 was a result of the greater calcification in Adialyzed or whether it was...
associated with exposure to dialysis. We compared \( A_{\text{nondialyzed}} \) and \( A_{\text{dialyzed}} \) with similar graded calcium deposition. Significantly more MMP-2 was present in \( A_{\text{dialyzed}} \) (Figure 5E). We also observed an increase in calcification severity with length of dialysis (Figure 5F).

**Vascular Dysfunction in \( A_{\text{dialyzed}} \) Correlates With MMP-2 Activation**

Vasoconstriction in response to KCl in \( A_{\text{dialyzed}} \) was only 30% and 50% of that in the nondialyzed and donor groups, respectively (Figure 6A). The maximal force (\( E_{\text{max}} \)) induced by 50 \( \mu \text{mol/L} \) phenylephrine in \( A_{\text{dialyzed}} \) was 65% of that in \( A_{\text{nondialyzed}} \) (Figure 6B), although the pEC\(_{50}\) values were not significantly different between 2 groups (nondialyzed, 5.42±0.26; dialyzed, 5.39±0.46). We also stimulated vasoconstriction with angiotensin, serotonin, and endothelin-1, and \( A_{\text{dialyzed}} \) developed 45% to 55% less force regardless of the means of stimulation (Figure 6B).

The KCl-stimulated contraction was negatively correlated with MMP-2 activity in \( A_{\text{dialyzed}} \) (\( r=−0.608, P=0.002; \) Figure 6C) but not in \( A_{\text{nondialyzed}} \) (\( r=−0.22, P=0.65 \)). When \( A_{\text{dialyzed}} \) was preincubated with specific MMP-2 inhibitor, the phenylephrine-induced contraction was significantly (\( P=0.02 \)) improved (Figure 6D).

The maximal response of acetylcholine-stimulated endothelium-dependent relaxation in \( A_{\text{dialyzed}} \) was 48% greater than that in \( A_{\text{nondialyzed}} \) (\( P=0.04 \)). The acetylcholine response in \( A_{\text{dialyzed}} \) was only 66% of that in \( A_{\text{nondialyzed}} \) despite the similar pEC\(_{50}\) (nondialyzed, 6.88±0.25; dialyzed, 6.81±0.47; Figure 7A). In \( A_{\text{dialyzed}} \) and \( A_{\text{nondialyzed}} \), the expression of phosphorylated endothelial NO synthase (eNOS\(_{\text{Ser1177}}\)), which indicates the activation of endothelial NO synthase, was only 50% of that in \( A_{\text{donor}} \) (Figure 7B). The endothelium-dependent relaxation in the \( A_{\text{dialyzed}} \) was negatively correlated with MMP-2 activity (\( r=−0.520, P=0.019; \) Figure 7C), but this relation was absent in \( A_{\text{nondialyzed}} \) (\( P=0.55 \)). Preincubation with specific MMP-2 inhibitor improved the acetylcholine relaxation by 247% in \( A_{\text{dialyzed}} \) with severely deteriorated endothelial function (\(<30\% \) relaxation). However, MMP-2 inhibitor did not improve relaxation in the vessel with better endothelial function (Figure 7D).

**Discussion**

Patients with CKD at any stage are at increased risk of cardiovascular complications, with the highest risk being in
the dialysis group. Various hypotheses and observational studies suggest that dialysis procedures per se may be responsible for detrimental changes in vascular function and structure, although how this occurs remains an area of active investigation. This study explores the hypothesis that MMP-2 upregulation may be one of the key processes explaining the aberrations in structure and function seen in CKD. We have used a cross-sectional study design to examine human tissue from donors and recipients at the time of kidney transplantation. Living kidney transplantation represents a unique opportunity to examine vessels and clinical status in a controlled situation. We describe a number of novel findings that support the hypothesis that CKD patients, more notably those on dialysis, have alterations in structural and functional properties of the arterial vasculature. In Adialyzed, the upregulation of MMP-2 is strongly associated with length of dialysis, elastic fiber degradation and arterial stiffening, medial calcification, and impaired vasocontraction and endothelium-dependent relaxation. Upregulation of MMP-2 appears to begin in the predialysis stage, and those exposed to PD and HD appear to exhibit further activation of MMP-2. Given the variety of events that occur during the dialysis procedure that may influence MMP-2 levels (detailed more fully below), the results also suggest that MMP-2 could be one of the plausible causal mechanisms that contribute to structural remodeling, vascular dysfunction, and ultimately the enhanced cardiovascular burden in adult CKD patients receiving maintenance dialysis.

We have described upregulation of MMP-2 in CKD in general relative to donors and, within the recipient population, differential upregulation between those on and not on dialysis. MMPs play diverse roles in arterial pathological processes during restenosis, calcification, atherosclerosis, and aneurysmal degeneration. The altered regulation of MMPs in the vasculature may be a critical component in linking CKD to vascular changes that cause both microvascular and macrovascular disease and ultimately cardiovascular disease. We have previously demonstrated that MMP-2 activity is associated with reduced renal function in the human internal mammary artery. The present data extend our previous findings (which were in early CKD patients) and show that along a continuum, dialyzed patients have greater activation of MMP-2 activity than the nondialyzed group. Nevertheless, in the nondialyzed group, arterial MMP-2 activity appears to be correlated with the duration of CKD, whereas in the dialyzed group, arterial MMP-2 activity does not relate to the duration of CKD before dialysis initiation. Importantly, MMP-2 activation was associated with the duration of dialysis.

Figure 4. A, Stress-stain curves. B, K values. C, Correlations between K value and PWV (m/s) in subgroups. D, Breaking stress. E, Correlation between MMP-2 activity and K values. *P<0.05; **P<0.01.
ysis, thus leading to the hypothesis that the dialysis procedure may promote MMP-2 activity. Exposure to dialysis treatments would lead to abnormalities in metabolic, humoral, inflammatory, and hemodynamic factors (eg, chronic volume overload, oxidative stress, vitamin D deficiency, hyperphosphatemia, hyperhomocysteinemia, and upregulation of cytokines, growth factors, and C-reactive protein), which are known to be promoters of MMP-2 activation.\textsuperscript{1,12,25–28} Moreover, our observation suggests that MMP-2 may be more profoundly and quickly upregulated by the HD procedure. However, future studies need to measure MMP-2 activity from tissue and blood at the beginning of dialysis in different modalities (eg, HD and PD) and follow those levels over time before definite statements can be made. Taken together, the upregulation of MMP-2 and the downregulation of TIMPs in A\textsubscript{dialyzed} indicate a favorable proteolytic activity in the vasculature.

The strong correlation between MMP-2 activity and elastic fiber degeneration and arterial stiffness in A\textsubscript{dialyzed} supports the hypothesis that MMP-2 would be a crucial dialysis-related factor modifying the matrix component and leading to arterial stiffness. MMPs are secreted by smooth muscle cells and inflammatory cells in the adventitia or media, which consequently leads to degradation of medial elastic fiber.\textsuperscript{12} Elastic fiber is essential for arterial elasticity; therefore, the fragmentation seen in A\textsubscript{dialyzed} may explain the structural weakening and reduction of elasticity. Our observation of an increased incidence of neointimal formation is concordant with the current literature that suggests that MMP-2 activation is a strong independent variable linked to intima-media thickness in dialyzed patients.\textsuperscript{11,12,19,25}

The significant correlation between arterial stiffness and calcium deposition in A\textsubscript{dialyzed} is also concordant with other investigations. It has been shown that vascular calcification in dialyzed patients was associated with increased stiffness of large-capacity, elastic-type arteries like the aorta, brachial artery, and common carotid artery.\textsuperscript{1,11,21,24,29} We suggest, on the basis of published literature, that there may be a potential relationship between MMP-2 and calcification, given that MMP-2 causes elastinolysis and disrupts arterial elasticity. During vascular calcification, smooth muscle cells undergo apoptosis and phenotypic transformation and release a variety of calcification-related factors.
of proteinases, including MMPs. The present data demonstrate that the increased MMP-2 activity in Adialyzed is associated with exposure to dialysis, not calcification per se. Our observations complement the current literature and suggest the detrimental roles of MMP-2 in vascular remodeling and functional aberration during the osteogenic and matrix degenerative processes.

We performed a comprehensive set of functional experiments to elucidate how the structural deterioration in the vasculature influences the vasomotor function in patients with various degrees of CKD. The decreased response to KCl may be explained by the clinical observation that dialyzed patients have an elevated total body potassium and serum potassium, likely causing some degree of desensitization. The reduction of agonist-induced contraction could be due to the paucity of α-smooth muscle actin expression in the arteries and the increased levels of vasoconstrictors causing receptor downregulation and desensitization. Calcium entry for contraction could also be inhibited by the decreased Na⁺/K⁺-ATPase activity in muscle cells and the upregulation of MMP-2 in Adialyzed, which indicates its nonproteolytic property in the vasculature. Patients on dialysis have been reported to exhibit indicators of endothelial injury and reduction of NO synthesis. The reciprocal relation between NO bioavailability and MMP activation corroborates the association between endothelium-dependent relaxation and}

![Figure 6](image6.png)

Figure 6. Maximum force generated in the artery in response to 80 mmol/L KCl (A; *P<0.05) and phenylephrine (PE), angiotensin (ANG), endothelin-1 (ET-1), and serotonin (5-HT) (B). *P<0.05, Student t test for comparison of nondialyzed and dialyzed groups. C, Scatterplot of KCl-induced contraction plotted vs MMP-2 activity in Adialyzed. D, Concentration-response curves of PE contraction in Anondialyzed (triangle), Adialyzed (square with solid line), and Adialyzed with preincubation of MMP-2 inhibitor (square with dotted line). Difference between concentration-response curves was analyzed by linear mixed-effects model.

![Figure 7](image7.png)

Figure 7. A, Maximum acetylcholine (ACh)-induced relaxation (Emax). B, Representative Western blots showing protein expression of phosphorylated endothelial NO synthase (eNOSSer1177). Bar graph is the densitometric analysis. *P<0.05; **P<0.001. C, Scatterplot of acetylcholine relaxation plotted vs MMP-2 activity in Adialyzed. D, MMP-2 inhibitor significantly improved (P<0.05, Student t test for comparison between absence and presence of MMP-2 inhibitor) acetylcholine relaxation in the Adialyzed with severely deteriorated relaxation response (<30%).
MMP-2 activity in the dialyzed group. Nevertheless, from this systematic and comprehensive set of functional experiments, we have demonstrated the improvement in contraction and relaxation by antagonizing MMP-2 activity, which strongly supports our hypothesis that MMP-2 plays a critical role in CKD- and dialyzed-associated vasomotor dysfunction.

This study has limitations. First, the exploration of a single set of hypotheses related to MMP-2 means that we may miss the opportunity to examine other MMPs (e.g., MMP-9, MMP-1, and MMP-3) that are important in vascular remodeling. From our preliminary observation, there was a moderate increase in arterial MMP-9 activity in the dialyzed group, but it did not reach statistical significance (P = 0.19). Second, the exposures of patients to CKD care were not identical (e.g., dialysis and nondialysis; PD and HD). However, we have attempted to analyze the data with appropriate separation of those factors that were overtly different between the groups. Given that this cohort includes only patients receiving live kidney transplantation who were demographically and clinically similar, the issue of “selection of the fittest” arises. The lesions described would likely represent the least severe of all CKD populations, given that they are well enough to undergo transplantation. Thus, the signal we have demonstrated herein may be much more profound if we were to study long-term dialysis patients not eligible for transplantation because of high cardiovascular disease burden or those who were receiving cadaveric transplantation with longer dialysis exposure. Third, the cross-sectional nature of the study does not allow us to confirm or refute that the dialysis procedure leads to worsening of vascular function because it is confounded by time, eg, longer total duration of CKD exposure in those who undergo dialysis before preemptive transplantation. Nonetheless, our results raise interesting questions for further testing using more sophisticated and appropriate methodology. Given that access to tissue at different stages of disease in 1 individual may be difficult, the present study design is appropriate for exploratory and pragmatic purposes. Thus, this is a relative limitation and gives us a conservative estimate of the possible effect of time, dialysis, or both on vascular health. Fourth, in an adult study, all patients have some degree of preexisting confounders for vascular disease (eg, diabetes mellitus, dyslipidemia, and hypertension). However, the incidence of these concomitant diseases was not significantly different between the 2 recipient groups. Note again that all of these individuals had been screened and found to be of sufficiently good cardiovascular health to proceed with transplantation. Ideally, tissue in pediatric patients may lead to further insights because young patients tend to have less vascular disease burden. Finally, the relatively small number of patients in the subgroups limits our ability to infer any statistical difference between groups because of power to detect true differences of moderate magnitude.

Conclusions

In this unique study using human vessels from healthy live kidney donors and transplant recipients, we have described an interesting relationship between MMP-2 activation and elastic fiber degeneration, stiffening, medial calcification, and vasomotor dysfunction in macroarterial vasculature. From described pathophysiological mechanisms, it is possible to postulate that the activation of MMP-2 and the resulting structural and functional changes in the vasculature may be accelerated by the PD and HD procedures. Loss of structural and functional integrity as a result of differences in arterial MMP-2 activity may be an important component in understanding the susceptibility of vascular disease in CKD patients. Furthermore, it may be that the manipulation of MMP-2 activity could be a potential therapeutic strategy for controlling vascular function, calcification, and arterial stiffening in patients with kidney disease. We offer this set of experiments in human tissue as evidence to support further exploration of disease mechanisms, and the possible role of MMP-2 in the pathobiology of vascular disease in CKD warrants serious consideration in future studies.

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Disclosures

None.

References

Cardiovascular disease is the leading cause of mortality in chronic kidney disease (CKD) patients on maintenance dialysis. Given the known biological functions of matrix metalloproteinase-2 (MMP-2) in vascular cell function and structural stability, we hypothesize that MMP-2 upregulation may be responsible for the vascular dysfunction and remodeling seen in CKD and describe differential regulation according to dialysis status. Using a cross-sectional study, we examine human vessels obtained from donors and recipients at the time of kidney transplantation. We demonstrate the pronounced MMP-2 upregulation in the arteries from dialyzed patients, and the results suggest that MMP-2 activation may be an important causal mechanism by which structural remodeling and vascular dysfunction occur in adult CKD patients receiving dialysis. Although numerous animal studies have examined the pathological modifications in the vasculature during CKD development, many have demonstrated inconsistent results in different models and strains and thus may be of limited applicability to the human condition. The complex vascular pathology in CKD seen in middle-aged and elderly patients is impossible to reproduce in laboratory animals. Therefore, this translational study has the advantage of examining human vessels; in doing so, we may begin to characterize some of the fundamental mechanisms of vascular disease in CKD. Given the importance of MMP-2 in maintaining vascular integrity, our findings are highly clinically relevant, and with the increasing interest in linking basic science and clinical science, we believe that the observed MMP-2 upregulation and its interaction with vascular dysfunction will stimulate others to explore this avenue of research further.
Upregulation of Matrix Metalloproteinase-2 in the Arterial Vasculature Contributes to Stiffening and Vasomotor Dysfunction in Patients With Chronic Kidney Disease
Ada W.Y. Chung, H.H. Clarice Yang, Jong Moo Kim, Mhairi K. Sigrist, Elliott Chum, William A. Gourlay and Adeera Levin

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Supplemental Material

Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease

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Gelatinolytic Zymography

The procedures have been extensively described in our previous publications\(^1,2\). Briefly, arterial segments were ground with liquid nitrogen in a stainless-steel motor and pestle. Tissue powder was mixed in 9 volumes of ice-cold lysis buffer (50mmol/L Tris-HCl pH 7.4 with 3.1mmol/L sucrose, 1mmol/L dithiothreitol, 10µg/mL leupeptin, 10µg/mL soybean trypsin inhibitor, 2µg/mL aprotinin and 0.1% Triton X-100). After 20 minutes incubation on ice, samples were homogenized by a glass homogenizer. The gelatinolytic activity was analyzed by separating protein (15µg) on 8% SDS-PAGE gels copolymerized with gelatin (2mg/mL) as substrate. After electrophoresis, the gels were washed with 2.5% Triton X-100, then incubated in incubation buffer (50mmol/L Tris-HCl buffer with 0.15mol/L NaCl, 5mmol/L CaCl\(_2\), and 0.05% NaN\(_3\), pH 7.4) at 37\(^\circ\)C until activities of the enzymes could be determined. After incubation, the gels were stained with 0.05% Coomassie brilliant blue G-250 in a mixture of methanol: acetic acid: water (2.5: 1: 6.5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activity was identified as transparent bands against the background of Coomassie blue-stained gelatin. Images were taken by Canon digital camera, and densitometry was analyzed by Quality One Image software. In order to compare band intensities from different gels, an internal control chosen from one of the samples was run along in all gels and the enzymatic activity from each patient samples was normalized with the internal control.

Reverse Zymography

Activities of TIMPs in tissue protein extract (40µg) were determined by electrophoresis in 13% SDS-PAGE copolymerized with 1mg/mL of gelatin and 50ng/mL human recombinant MMP-2 or -9 (Calbiochem). After staining and destaining procedures as described above, activity of TIMPs was visualized as dark bands against the clear background\(^1,2\).
Western immunoblotting

After homogenization, protein concentration was determined by Bradford reagent using bovine serum albumin as standards (Biorad). Protein extracts (40μg) were diluted to the equivalent amounts of total protein per sample in sample buffer containing 40% glycerol, 8% SDS, 0.5mol/L Tris-HCl (pH 6.8), 0.02% bromophenol blue and 4% β-mercaptoethanol. Samples from donor and recipient arteries were then subjected to 8% (for phospho-eNOS^{Ser1177}) or 12% (for phospho-Akt Ser^{473}, phospho-Akt Thr^{308}, angiostatin, and VEGF), then transferred to polyvinylidifluoride membranes (Biorad). Membranes were blocked overnight with blocking buffer (5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20), then probed with polyclonal antibodies against angiostatin (dilution=1:200; Oncogene), VEGF (dilution=1:300; Calbiochem), eNOS^{Ser1177} (dilution=1:1000; Cell Signaling), Akt^{Thr308} (dilution=1:1000; Cell Signaling) or Akt^{Ser473} (dilution=1:1000; Cell Signaling) overnight at 4°C. After washing three times in Tris-buffered saline containing 0.1% Tween 20, membranes were incubated with anti-rabbit or anti-mouse IgG peroxidase-conjugated secondary antibodies (dilution=1: 2500) for 1 hour. The immunoreactive proteins were then visualized by chemiluminescence (Amersham Biosciences, Buckinghamshire, UK), and densitometric measurement was performed. To ensure equal protein loading, membranes were stripped by incubating in 62.5mmol/L Tris HCl (pH 6.8), 1% SDS, and 100mmol/L β-mercaptoethanol at 55°C, and reprobed with anti-β-actin mouse antibody (dilution=1:5000)^{1,2}.

Movat’s staining

4-mm arterial segments were formalin fixed and embedded in paraffin. 3μm cross-sections were prepared and stained with modified Movat’s pentachrome^{1,2}. Computerized morphometry was performed to determine the intimal and medial thickness. Medial thickness was measured.
between the internal elastic lamina and the edge of the external elastic lamina of the vessel. 

Intimal thickness was measured between the lumen and the internal elastic lamina.

**Mechanical properties of artery**

Vessel elasticity was deduced from the stress-strain curves\(^1,2\). The procedure was repeated until the vessel was unable to maintain its resting tension. The stress at which instability occurred was reported as ‘breaking stress’. The stress-strain relationship was eliciting as ‘J-shaped curves’.

Equation of the exponential growth is \(Y = \text{Start} \times \exp(K \times X)\), where \(Y\) is stress, \(X\) is strain, and \(K\) is a rate constant which \(Y\) increases exponentially. Increased \(K\) value indicates increased stiffness.

**Von Kossa’s staining**

Paraffin-embedded arterial sections (3\(\mu\)m) were deparaffinized and immersed in 5% silver nitrate under bright light until brown-black calcium salts developed. The sections were then treated in 5% sodium thiosulfate, and counterstained with 0.5% neutral red (CI 50040). After being rinsed briefly in distilled water, sections were dehydrated and mounted. Images were captured by a SPOT digital camera (Diagnostic Instrument Inc). Calcium deposit was graded by three observers from the department of pathology who were blinded to patient status. Calcium deposition was assessed on a scale from 0 to 3: 0, no calcium deposit; 1, small calcium deposit on <50% area; 2, small calcium deposit on most of the media; 3, large calcium deposit on the entire vessel accompanying with medial dissection.

**In vivo PWV**

Using noninvasive methods, radial artery waveforms were obtained with a high-fidelity micromanometer (SPC-301; Millar Instruments, Houston) applied at the radial pulse\(^3\). This generated an augmentation index, a composite measure of systemic arterial stiffness and wave-
reflection amplitude or intensity (Sphygmocor; AtCor Medical, Sydney, Australia). Aortic PWV was determined from carotid and femoral pulses. Despite consent to the procedure, not all patients underwent PWV measurement and some data were missing due to logistic issues in the timing of the procedure, availability of the patient prior to transplantation surgery (i.e. some patients are living in other cities), etc.

**Isometric force measurement**

In a small vessel myograph\(^1,2\), segments were stretched for 60 minutes at which the resting force was stabilized, then challenged twice with 80mmol/L potassium chloride (KCl) before experiments. Phenylephrine (PE, 1nmol/L-300μmol/L), endothelin-1 (ET-1, 1-700nmol/L), angiotensin-II (ANG-II, 1-30nmol/L), serotonin (5-HT, 1nmol/L-1μmol/L) were added cumulatively to generate concentration-response curves. To evaluate the endothelium-dependent relaxation, vessels were precontracted with 50µmol/L PE before the cumulative addition of acetylcholine (ACh, 0.01nmol/L-100µmol/L)\(^1,2\). To evaluate the effect of MMP-2 on vasocontraction and relaxation, artery was preincubated with specific MMP-2 inhibitor (1μmol/L, Calbiochem) for 30 minutes before the addition of PE, which was followed by the addition of ACh.

**Materials**

All reagents were purchased from Sigma (Oakville, Canada) unless specifically stated.
Table 1. Demographics and clinical features of the living donors and recipients. Contrast analysis was used to test the statistical significance between non-dialysis and dialysis, and between PD and HD. NA: not available.
<table>
<thead>
<tr>
<th></th>
<th>Donor (n=30)</th>
<th>Non-Dialysis (n=17)</th>
<th>Dialysis (n=23)</th>
<th>P-value</th>
<th>PD (n=10)</th>
<th>HD (n=13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (%)</td>
<td>70</td>
<td>24</td>
<td>43</td>
<td>0.28</td>
<td>70</td>
<td>23</td>
<td>0.02</td>
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<td>Age (y)</td>
<td>51±2.2</td>
<td>54±2.6</td>
<td>49±2.8</td>
<td>0.23</td>
<td>49±4.6</td>
<td>49±3.7</td>
<td>0.95</td>
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<td>Creatinine (μmol/l)</td>
<td>81.4±3.7</td>
<td>423±34</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>74.6±2.6</td>
<td>1.2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Diabetes (%)</td>
<td>0.0</td>
<td>6.0</td>
<td>17.4</td>
<td>0.29</td>
<td>10</td>
<td>23</td>
<td>0.44</td>
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<td>Hypertension (%)</td>
<td>0.0</td>
<td>100</td>
<td>100</td>
<td>NA</td>
<td>100</td>
<td>100</td>
<td>NA</td>
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<tr>
<td>Calcium (mmol/l)</td>
<td>NA</td>
<td>0.04</td>
<td>0.04</td>
<td>0.34</td>
<td>0.04</td>
<td>0.06</td>
<td>0.13</td>
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<td>Phosphate (mmol/l)</td>
<td>NA</td>
<td>0.11</td>
<td>0.12</td>
<td>0.95</td>
<td>0.15</td>
<td>0.17</td>
<td>0.32</td>
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<tr>
<td>PTH (pmol/l)</td>
<td>NA</td>
<td>6.3</td>
<td>4.6</td>
<td>0.51</td>
<td>4.6</td>
<td>6.4</td>
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<td>Hemoglobin</td>
<td>130±3</td>
<td>112±2.9</td>
<td>120±3.0</td>
<td>0.07</td>
<td>115±5.2</td>
<td>126±2.6</td>
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<tr>
<td>Length of dialysis (months)</td>
<td>0.0</td>
<td>0.0</td>
<td>37.6±</td>
<td>NA</td>
<td>50.3±</td>
<td>27.2±</td>
<td></td>
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<tr>
<td>Number of months since GFR&lt;30</td>
<td>0.0</td>
<td>78.4±</td>
<td>68.2±</td>
<td>0.69</td>
<td>19.8±</td>
<td>6.5±</td>
<td>0.24</td>
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<tr>
<td>Number of months since 1st nephrologist consult</td>
<td>0.0</td>
<td>25.6</td>
<td>10.8</td>
<td>0.69</td>
<td>67.4±</td>
<td>62.5±</td>
<td>0.83</td>
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<td>ACE inhibitor (%)</td>
<td>0</td>
<td>35.3</td>
<td>21.7</td>
<td>0.36</td>
<td>11.1</td>
<td>18.2</td>
<td>0.68</td>
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<td>ARB (%)</td>
<td>0</td>
<td>17.6</td>
<td>21.7</td>
<td>0.76</td>
<td>33.3</td>
<td>9.1</td>
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<tr>
<td>Beta Blockers (%)</td>
<td>3.7</td>
<td>41.2</td>
<td>34.8</td>
<td>0.69</td>
<td>55.6</td>
<td>18.2</td>
<td>0.09</td>
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<tr>
<td>Calcium channel blockers (%)</td>
<td>0</td>
<td>64.7</td>
<td>21.7</td>
<td>0.005</td>
<td>11.1</td>
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<td>0.40</td>
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<tr>
<td>Aspirin (%)</td>
<td>3.7</td>
<td>29.4</td>
<td>52.2</td>
<td>0.16</td>
<td>44.4</td>
<td>54.5</td>
<td>0.67</td>
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<tr>
<td>Statins (%)</td>
<td>0</td>
<td>47.1</td>
<td>26.1</td>
<td>0.18</td>
<td>33.3</td>
<td>18.2</td>
<td>0.46</td>
</tr>
</tbody>
</table>
Reference

