Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease

Ada W.Y. Chung1*, H.H. Clarice Yang1, Mhairi K. Sigrist2, Genevieve Brin2, Elliott Chum2, William A. Gourlay3, and Adeera Levin2

1Department of Cardiovascular Science, Child and Family Research Institute, University of British Columbia, Room 2099, 950 28th W Ave, Vancouver, BC, Canada V5Z 4H4; 2Division of Nephrology, University of British Columbia, Vancouver, Canada; and 3Division of Urologic Science, University of British Columbia, Vancouver, Canada

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Aims Chronic kidney disease (CKD) and diabetes are the prominent risk factors of cardiovascular disease (CVD). Matrix metalloproteinase (MMP)-2 and -9 regulate vascular structure by degrading elastic fibre and inhibit angiogenesis by generating angiostatin. We hypothesized that MMP-2 and -9 were up-regulated in the arterial vasculature from CKD patients with diabetes, compared with those without diabetes.

Methods and results During living donor transplantation procedures, arteries from donors (n = 8) and recipients (non-diabetic, n = 8; diabetic, n = 8; matched in age, gender, and dialysis treatments) were harvested. Diabetic arteries had increased MMP-2 and -9 activities by 42 and 116% compared with non-diabetic ones. Diabetic arteries were the stiffest, and the stiffness measurement was highly correlated with the summation of MMP-2 + MMP-9 activities (r = 0.738, P = 0.0002). Pulse wave velocity measurements correlated with MMP activity (r = 0.683, P = 0.005). Elastic fibre degradation and calcification were worst in diabetic vessels. The phosphate level, which was 25% higher in diabetic patients, correlated with MMP activity (r = 0.513, P = 0.04) and in vitro stiffness (r = 0.545, P = 0.03), respectively. Angiostatin expression was doubled, whereas vascular endothelial growth factor was 50% reduced in diabetic compared with non-diabetic vessels. Microvascular density in diabetic vessels was 48% of that in non-diabetic ones, and it was strongly associated with MMP activity (r = −0.792, P < 0.0001) and vasorelaxation (r = 0.685, P = 0.0009).

Conclusion Using a matched case-control design, we report up-regulation of MMP-2 and -9 in diabetic CKD arteries and correlate those with stiffening, impaired angiogenesis, and endothelial dysfunction. These findings may help to explain the high susceptibility of CVD in diabetic and non-diabetic CKD patients.

KEYWORDS
Chronic kidney disease; Diabetes; Matrix metalloproteinase; Angiogenesis; Endothelial dysfunction

1. Introduction

Diabetic nephropathy is the leading cause of end-stage chronic kidney disease (CKD) and the most common reason for renal-replacement therapy. Both CKD and diabetes are considered to be the prominent risk factors of cardiovascular disease (CVD). Therefore, improved understanding of vascular function and structure in these individuals might help to explain the underlying mechanisms linking this complex renal–cardiac–metabolic system.

Physiological and pathological vascular remodelling entails degradation and reorganization of extracellular matrix, explaining the great interest in matrix metalloproteinases (MMPs). Activities of MMP-2 and -9 are highly associated with the progression of CKD, diabetes, and coronary arterial disease. They are secreted by inflammatory cells in the adventitia or smooth muscle cells in the media. The degraded elastic fibre induces calcium deposition, which in conjunction with altered vascular structure is associated with vessel stiffening. In CKD and diabetic patients, arterial stiffening increases cardiac afterload that drives left ventricular hypertrophy, reduces coronary artery perfusion that causes myocardial ischaemia, and increases pulse pressure that promotes atheroma formation and vascular remodelling. Although circulating MMP-2 and -9 levels have been found to be associated with large artery stiffness in humans, they cannot be directly related to vessel concentration and do not necessarily reflect arterial matrix degradation. Recently, we had a unique opportunity to study arteries from patients undergoing

* Corresponding author. Tel: +1 604 875 3852; fax: +1 604 875 3120. E-mail address: adawingyee@yahoo.ca

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coronary arterial bypass grafting and demonstrate that MMP-2 up-regulation in vasculature is associated with reduced kidney function.9 Given these associations it is possible that MMP-2 could play an important role in contributing to arterial stiffening, vascular remodelling, and cell dysfunction in end-stage CKD patients.10

Angiogenesis is a compensatory mechanism in response to the obstructive arterial diseases which cause myocardial or lower extremity ischaemia.11 It is initiated by proliferation of endothelial cells which penetrate into the surrounding tissue, and induced by a potent angiogenic cytokine vascular endothelial growth factor (VEGF).12,13 Through the proteolytic cleavage of the non-matrix protein plasminogen, MMP-2 and -9 generate an angiogenic inhibitor angiostatin.12,13 We have reported that up-regulation of MMP-2 and -9 in the arterial vasculature resulted in increased angiostatin production, causing impaired angiogenesis in diabetic patients.14

The purpose of this study was to investigate whether the presence of diabetes, in conjunction with CKD, is associated with more disrupted processes that are regulated by the matrix and non-matrix degrading properties of MMP-2 and -9: (i) arterial stiffening and (ii) angiogenesis. We hypothesized that MMP-2 and -9 would be up-regulated in diabetic CKD vessels, compared with those from donor and non-diabetic ones. Given the potential for confounding due to ageing, gender, and dialysis procedures, diabetic and non-diabetic ones. Given the potential for confounding due to ageing, gender, and dialysis procedures, diabetic and non-diabetic patients were matched. We demonstrate that the presence of diabetes, in conjunction with CKD, is associated with reduced kidney function.9 Given these associations it is possible that MMP-2 up-regulation may be one of the important pathological mechanisms by which diabetes worsens vascular function and clinical outcomes in CKD patients.

2. Methods

2.1 Study population selection

Details of sample collection and preparation are available in the Supplementary material online. Briefly, 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 Board of Providence Health Care and the University of British Columbia. The investigation conforms to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from donors (n = 8) and recipients (n = 16) prior to the surgery. All subjects consented to undergo non-invasive pulse wave velocity (PWV) measurements of vascular stiffness, and consented to the use of the discarded inferior epigastric artery (from recipients) and renal artery (from donors). This study examines a subpopulation of diabetic and non-diabetic recipients who were matched for age, gender, and dialysis treatments [i.e. peritoneal dialysis (PD), haemodialysis (HD), or no dialysis] (Table 1).

We also investigated the vessel morphology, stiffness, and endothelial-dependent relaxation in microrcirculation. Resistance-sized arterioles were carefully dissected from an ellipse of skin from both donors and recipients at the incision site.

2.2 Gelatinolytic zymography

The gelatinolytic activity was analysed by separating protein (15 μg) on 8% SDS-PAGE gels copolymerized with gelatin (2 mg/mL).9,14

2.3 Reverse zymography

Activities of tissue inhibitors of MMP (TIMPs) (50 μg protein) were determined by electrophoresis in 13% SDS-PAGE copolymerized with 1 mg/mL of gelatin and 50 ng/mL human recombinant MMP-2 or -9 (Calbiochem).9,14

Table 1: Demographics and clinical features of the case-control cohort

<table>
<thead>
<tr>
<th>Donors (n = 8)</th>
<th>Non-diabetic recipients (n = 8)</th>
<th>Diabetic recipients (n = 8)</th>
<th>P-value (T-test or Wilcoxon or χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (%)</td>
<td>3 (38%)</td>
<td>3 (38%)</td>
<td>3 (38%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 ± 8</td>
<td>57 ± 11</td>
<td>55 ± 8</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td>6.7 ± 0.6</td>
<td>8.0 ± 1.1</td>
<td>11.9 ± 4.6</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>96 [63–114]</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>74 ± 18</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>—</td>
<td>2.39 ± 0.20</td>
<td>2.39 ± 0.18</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>—</td>
<td>1.42 ± 0.58</td>
<td>1.77 ± 0.31</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>—</td>
<td>22 (9–32)</td>
<td>31 (11–50)</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>131 ± 8</td>
<td>116 ± 13</td>
<td>121 ± 12</td>
</tr>
<tr>
<td>Dialysis modality (PD/HD/pre-emptive)</td>
<td>—</td>
<td>—</td>
<td>1/5/2</td>
</tr>
<tr>
<td>K value</td>
<td>2.1 ± 0.1</td>
<td>2.5 ± 0.6</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>Length of dialysis (months)</td>
<td>—</td>
<td>20 ± 15</td>
<td>18 ± 11</td>
</tr>
<tr>
<td>Number of months since 1st nephrologist consult</td>
<td>—</td>
<td>105 ± 90</td>
<td>73 ± 66</td>
</tr>
<tr>
<td>Duration of CKD</td>
<td>—</td>
<td>86 ± 92</td>
<td>55 ± 72</td>
</tr>
<tr>
<td>ACEi/ARB</td>
<td>0 (0%)</td>
<td>2 (25%)</td>
<td>5 (63%)</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>0 (0%)</td>
<td>2 (25%)</td>
<td>3 (38%)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>0 (0%)</td>
<td>1 (13%)</td>
<td>5 (63%)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0 (0%)</td>
<td>3 (38%)</td>
<td>6 (75%)</td>
</tr>
<tr>
<td>Statins</td>
<td>0 (0%)</td>
<td>1 (13%)</td>
<td>6 (75%)</td>
</tr>
</tbody>
</table>

Comparisons were made between non-diabetic and diabetic recipients. Duration of CKD is the number of months since the first nephrologist consult until the initiation of dialysis or transplantation. PTH, parathyroid hormone; ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker.
2.4 Western blotting
The procedures of western blotting were extensively described previously.14,15

2.5 Movat’s staining
Vessel segments (4 mm) were formalin-fixed and embedded in paraffin. Cross-sections (3 μm) were stained with modified Movat’s pentachrome, and computerized morphometry was performed.

2.6 von Kossa’s staining
Paraffin-embedded arterial sections (3 μm) were immersed in 5% silver nitrate under bright light until brown-black calcium salts developed.

2.7 In vivo PWV
Using non-invasive methods, radial artery waveforms were obtained with a high-fidelity micromanometer (SPC-301; Millar Instruments, Houston, TX, USA) applied at the radial pulse. This generated an augmentation index, a composite measure of systemic arterial stiffness and wave-reflectation amplitude (Sphygmocor; AtCor Medical, Sydney, Australia). Aortic PWV was determined from measurements obtained from carotid and femoral pulses.

2.8 In vitro arterial stiffness
Vessel elasticity was deduced from the ‘J-shaped’ stress-strain curve. 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. Increased \( K \) value indicates increased stiffness.

2.9 Endothelium-dependent relaxation
To evaluate the endothelium-dependent relaxation, vessels were pre-contracted with phenylephrine before the cumulative addition of acetylcholine in a small vessel myograph.15

2.10 Immunohistochemistry and microvascular density evaluation
Vessels embedded in paraffin blocks were cut into 3 μm thick cross-sections, which were stained with antibodies against von Willebrand factor (dilution = 1:400) for labelling endothelial cells. Microvascular density was calculated by dividing the total number of capillaries on the slides by the area of the stained sections (mm²).14,15
Details of these procedures are available in the Supplementary material online.

2.11 Statistics
Data were reported as mean ± SD for normally distributed data (or median ± SE for those with non-normal distribution). T-tests were used for unpaired data, Wilcoxon tests for matched pair data, and \( \chi^2 \) tests for nominal data. One-way analysis of variance (ANOVA) and Bonferroni’s post hoc analysis for multiple comparisons were used to evaluate differences among groups. Differences between concentration–response curves (involve multiple parameters per patient) were analysed by linear-mixed effects model which account for correlated data using individual patient random effects. Correlations between two parameters were generated using the Spearman rank correlations. To investigate whether multiple regression lines were significantly different, we tested the equality of slopes and intercepts by using ANOVA. If linear regression lines had neither different slope nor different intercept, common regression coefficient and y-intercept were used to describe the whole population. Statistical analysis and construction of concentration–response curves were performed using GraphPad Prism (version 4.03, San Diego, CA, USA) software and SPSS version 14.0 (SPSS Inc, Chicago, IL, USA).

3. Results
3.1 Study cohorts
Table 1 describes the demographics of the case–control cohort. This cohort of interest includes eight diabetic CKD patients, one was on PD, five were on HD, and two were not receiving dialysis at the time of transplantation. A matched cohort of eight non-diabetic patients was chosen for comparison. An age-matched control group (n = 8) of kidney donors was also included. Although there was no difference in the length of dialysis between two recipient groups, diabetic CKD patients had shorter CKD exposure and used more medications. Details are presented in Table 1.

3.2 Elevated MMP-2 and -9 activities in the diabetic vasculature
We pooled the activities of the latent and active forms of MMPs and found that there was an increase in activities of MMPs in the arterial samples: donor < non-diabetic < diabetic. Activities of MMP-2 and -9 in diabetic vessels were increased by 42 and 116%, respectively, when compared with those in the non-diabetic group. MMP-2 activity in non-diabetic vessels was significantly higher compared with that from donors by 78% (\( P < 0.0001 \)) (Figure 1A).
Activation of MMP is regulated by TIMPs.6 Arteries from non-diabetic recipients had comparable TIMP-1 and -2 activities to those seen in the donors. TIMP-1 and -2 activities in diabetic vessels were 37 and 55% of those in non-diabetic ones (Figure 1B).

3.3 Vascular remodelling, arterial stiffening, and calcification in diabetic vasculature
3.3.1 Increased medial/lumen ratio
From the histological assessment, we showed that the medial/lumen ratio was doubled in the conduit arteries from recipient groups compared with the donors (Table 2). In the resistance vessels, the medial/lumen ratio of recipient groups was increased by 60–122%, compared with donors. The medial/lumen ratio of diabetic resistance vessels was 38% higher than that of non-diabetic ones (Table 2).

3.3.2 Elastic fibre degeneration in diabetic vessels
As shown on Movat’s histology, although the medial thickness between non-diabetic and diabetic conduit arteries was not significantly different (\( P = 0.41 \); Table 2), the ratio of external elastic lamina/medial thickness in diabetic vessels was 31 ± 4.0% less compared with that in non-diabetic ones (43 ± 3.5%) (\( P = 0.04 \)) (Figure 2A and B).

3.3.3 Profound arterial stiffening in diabetic vessels
The arterial physical property was assessed by the in vitro stiffness measurement denoted as the ‘K value’. Both conduit artery and small arteriole from the diabetic CKD group exhibited the greatest stiffness (Figure 2C). The stiffness measurement was strongly correlated with the summation of MMP-2 + MMP-9 activities (\( r = 0.738, P = 0.0002 \)) (Figure 2D). The in vivo arterial stiffness was assessed by measuring PWV. PWV in the diabetic group was
Figure 1  (A) Gelatin zymograms showing activities of MMP-2 and -9 from donors (n = 8, representatives from four samples) and recipients (n = 16) arterial samples. We pooled the latent and active forms of MMPs and the bar graph is the densitometric analysis. (B) Representative reverse zymograms showing activities of TIMP-1 and -2 in the arterial samples. Bar graph is the densitometric analysis. Comparisons were performed by one-way ANOVA with Bonferroni’s post hoc analysis. *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2  Vessel morphological characteristics

<table>
<thead>
<tr>
<th></th>
<th>Medial thickness (μm)</th>
<th>Internal diameter (μm)</th>
<th>Medial/lumen ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conduit arteries</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donors (n = 3)</td>
<td>380 ± 40</td>
<td>6150 ± 550</td>
<td>0.062 ± 0.007</td>
</tr>
<tr>
<td>Non-diabetic recipients (n = 6)</td>
<td>475 ± 52</td>
<td>3160 ± 332</td>
<td>0.150 ± 0.016*</td>
</tr>
<tr>
<td>Diabetic recipients (n = 6)</td>
<td>410 ± 45</td>
<td>2652 ± 340</td>
<td>0.155 ± 0.017*</td>
</tr>
<tr>
<td><strong>Subcutaneous resistance vessels</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donors (n = 8)</td>
<td>12.3 ± 0.5</td>
<td>149 ± 9</td>
<td>0.083 ± 0.008</td>
</tr>
<tr>
<td>Non-diabetic recipients (n = 8)</td>
<td>21.3 ± 2.4*</td>
<td>160 ± 17</td>
<td>0.133 ± 0.017*</td>
</tr>
<tr>
<td>Diabetic recipients (n = 8)</td>
<td>23.0 ± 2.2*</td>
<td>125 ± 12</td>
<td>0.184 ± 0.018*#</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with donors.
#P < 0.05 compared with non-diabetics.
49 and 78% higher than that in non-diabetic and the donor groups, respectively (Table 1). A linear relation between PWV and MMP-2 + MMP-9 activities was demonstrated \( (r = 0.683, P = 0.005) \) (Figure 2E).

### 3.3.4 Severe calcification in diabetic vessels

Arterial stiffness is also impacted by the degree of vascular calcification, during which MMPs are abundantly secreted from smooth muscle cells and inflammatory cells. Using von Kossa’s staining which detects the calcium/phosphate deposition, we found that in the matched samples (eight matched pairs with similar age, same gender, and renal replacement therapy), diabetic vessels exhibited more severe calcification than non-diabetic ones (Figure 3A and B). The phosphate level was 25% higher in the diabetic group (Table 1) and found to be correlated with large arterial stiffness \( (r = 0.545, P = 0.03) \) and MMP-2 + MMP-9 activities \( (r = 0.513, P = 0.04) \) (Figure 3C and D).

The parathyroid hormone (PTH) level was 41% higher in the diabetic than the non-diabetic group (Table 1). It formed a linear relation with MMP activity in the diabetic group, although it did not reach statistical significance \( (P = 0.17) \) (Figure 3E).

### 3.4 Increased expression of angiostatin in the diabetic vasculature

In diabetic vessels, the angiostatin protein level was doubled compared with that in non-diabetic ones (Figure 4A and B).
VEGF protein expression in diabetic arteries was only 30% of that in donors ($P < 0.0001$) and was 37% less than that in non-diabetic vessels ($P = 0.02$) (Figure 5C).

### 3.5 Reduced microvascular density in the diabetic vessel associated with enhanced MMP activities

From the von Willebrand factor-stained cross-sections, we observed a gradual reduction in microvascular density: donor > non-diabetic > diabetic (Figure 5A–C). The microvascular density in the diabetic vessel media was only 33 and 48% of that in the donor and non-diabetic groups, respectively. The microvascular density in the non-diabetic group was 30% less compared with the donor group (Figure 5D). There was a very strong linear correlation between microvascular density and MMP activity ($r = -0.792, P < 0.0001$) (Figure 5E).

### 3.6 Impaired endothelial-dependent relaxation in the diabetic vasculature

*Figure 6A* describes a gradual reduction in ACh-stimulated endothelium-dependent relaxation in conduit arteries: donor > non-diabetic > diabetic. Diabetes-impaired endothelium function was also evident in microvasculature, as the ACh-stimulated relaxation was significantly suppressed compared with that in donors ($P < 0.05$). The pEC50 value was significantly reduced in diabetic (6.91 ± 0.22) compared with non-diabetic ones (8.36 ± 0.60) ($P < 0.05$) (Figure 6B).

In diabetic arteries, there was a pronounced decrease in protein levels of phosphorylated eNOSSer1177, AktThr308, and AktSer473, compared with those in donors. Non-diabetic vessels exhibited reduced AktThr308 expression, but had comparable levels of eNOSSer1177 and AktSer473 to those in donors (Figure 6C).

The ACh-induced relaxation was negatively correlated with MMP-2 + MMP-9 activities ($r = -0.738, P = 0.002$) (Figure 6D), but positively related to the microvascular density ($r = 0.685, P = 0.0009$) (Figure 6E).

### 4. Discussion

This translational study has the advantage of examining human vessels, from well-characterized individuals at different stages of CKD, and healthy donors so as to examine the impacts of diabetes on vascular function and structural property. The comparable demographic parameters such as age, gender, and dialysis procedure among
angiostatin and (i) increased stiffness and extensive elastic fibre degradation, (ii) severe calcification, (iii) decreased VEGF expression and increased angiostatin level, and (iv) reduced microvascular density and impaired endothelial-dependent relaxation. Taken together, these findings present plausible mechanisms (matrix and non-matrix degrading properties of MMPs) which might explain the high incidence of CVD in diabetic CKD patients.

We have previously demonstrated that MMP-2 activity is associated with reduced renal function in the human internal mammary artery. The current data extend our previous findings (which were in early CKD patients) and suggest that MMP-2 and -9 are substantially up-regulated in end-staged CKD patients, although more so in those with diabetes. CKD without diabetes induced less MMP activation, which might be related to the better preserved endothelial function and nitric oxide signalling than those seen in diabetic CKD vasculature (discussed below). Additionally, the comparable TIMP activity in the non-diabetic group to that in donors may indicate a preserved inhibitory mechanism on MMP activation in CKD vasculature, which is not seen in diabetic patients. Although we have reported the differential regulation of TIMP-1 and -2 by diabetes in human internal mammary arteries, how the diabetic state per se suppresses TIMP activity during CKD is not known. It has been described that in patients with diabetic nephropathy, the serum levels of MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios were two-fold increased. An increased circulating MMP-2 and -9 was also suggested to be the earliest marker of diabetic kidney disease and was critical for graft survival. However, measurements of MMP/TIMP in the circulation and renal biopsy specimens cannot be directly related to vessel concentration and do not necessarily reflect arterial matrix degradation as well as arterial stiffness. Therefore, the uniqueness of our current findings is the direct comparisons between arterial MMP levels and the vascular structural and functional parameters.

MMP up-regulation, without appropriate balance of TIMP production, would result in excessive degradation of elastic fibre, which provides elasticity and stabilizes arterial structure. We demonstrate the profound degeneration of external elastic lamina and an increase in vessel stiffness in diabetic CKD patients, in relation to MMP tissue activity. Given that the in vitro molecular and structural abnormalities and in vivo stiffness measurement are highly correlated and that arterial stiffness has been suggested to be a strong independent predictor of CVD,13,14 these findings are clinically important. Pronounced reduction of nitric oxide bioavailability (discussed below) may explain the accelerated arterial stiffening seen in diabetes even before the development of atherosclerosis.15 As diabetic patients are subject to a myriad of abnormalities, e.g. poor glycaemic control, use of insulin and other hypoglycaemic agents, formation of advanced glycation end product, dyslipidaemia, etc., it is difficult to determine which abnormality is accounting for arterial stiffening.16

Arterial disease in CKD is also characterized by vascular calcification. MMPs promote elastinolysis, and the degraded elastic fibre highly induces calcium deposition. Elevated phosphate has been identified as the key driver for calcification in CKD and linked to the increased CVD mortality in both dialysis-dependent and dialysis-independent CKD patients. Given the cross-sectional nature of this study, we cannot determine whether the increase in MMP was a result of the greater calcification in diabetic vessels or whether it was induced by the exposure to diabetic milieu. Indeed, MMP elevation in diabetic vessels was associated with increased PTH. It is important to investigate factor(s) protecting against PTH elevation in non-diabetic vessels. It is interesting to note that the most severe calcification was seen in diabetic CKD vessels, and associated with pronounced increase in MMP activity and phosphate level, which in turn were strongly correlated with vessel stiffness. We have also reported serum phosphate values in association with in vivo arterial stiffness in a mixed cohort including those with and without diabetes. The higher phosphate level in patients with CKD and diabetes than those with CKD alone adds credence to some of these observations. Hyperphosphataemia could accelerate calcification through phosphate-induced release of matrix vesicles and apoptosis.22 Phosphate could also augment the osteogenic changes in vascular smooth muscle cells treated with proteinase-degraded elastic fibre.23

In addition to the matrix degrading property, the proteolytic functions of MMPs on non-matrix proteins are of great interest in vascular biology. Proteolytic cleavage of plasminogen by MMP-2 and -9 yields a variety of related molecular species of ~40–45 kDa containing Kringle 1–4 or 1–3 of plasminogen, collectively termed angiostatin, a potent inhibitor of angiogenesis. In agreement with our previous finding, the current study demonstrated that the presence
of diabetes is associated with much higher angiostatin expression in the vasculature than those from non-diabetic CKD patients and donors. We found that in patients with CKD alone, the generation of angiostatin was comparable to that in donors, implicating the mild reduction in microvascular density. It also suggests that in non-diabetic CKD patients, there might not be a trigger for angiostatin production, despite the moderate increase in MMP-2 and -9 in the vasculature. However, in CKD patients, the reduced microvascular density in myocardium could be associated with rapid progression of atherosclerosis.

Others have showed that diabetic patients with coronary artery disease exhibit increased myocardial angiostatin expression which negatively correlated with coronary collateralization.

Therefore, if our findings in diabetic CKD patients are correlated with clinical findings, it would be tempting to postulate that suppression of angiogenesis in these populations may account, in part, for the higher mortality related to CVD and the higher prevalence of limb ischaemia. Importantly, we demonstrated the negative correlation of microvascular density with MMP activity. Therefore, although MMP activation is essential for angiogenesis by allowing extracellular matrix degradation and facilitating new blood vessel expansion, increased MMP activity seems to have a strong inhibitory role in angiogenesis by generating angiostatin.

Angiostatin reduces VEGF expression and antagonizes VEGF effects on endothelial cell proliferation, migration, survival, and sprouting. Vessels from those with CKD alone exhibited reduction of VEGF, which could be the result of reduced vasorelaxation, eNOS down-regulation, and decreased nitric oxide bioavailability. A further decrease in VEGF level in the diabetic vessel could be due to the compromised

Figure 5 Representative von Willebrand factor-stained cross-sections from (A) non-diabetic, (B) diabetic, and (C) donor arteries showing the staining of endothelial cells which represents the growth of capillaries in the media. Vessels with a diameter between 5 and 10 μm were considered as capillaries which are indicated by arrows. (D) Bar graph shows the microvascular density (mm⁻²). *P < 0.05; ***P < 0.001. (E) Linear correlation between microvascular density and the sum of MMP-2 + MMP-9 activities.
insulin-mediated PI3K/Akt activation. We demonstrated that the reduction of microvascular density in diabetic vessels was accompanied by compromised endothelial-dependent relaxation in both micro- and macrovasculature. The elevated angiostatin level in diabetic vessels could impair acetylcholine-induced vasodilation and decrease eNOS activation. Vasodilatation is a positive regulator of neovascularization, and nitric oxide mediates VEGF angiogenic responses. However, as reported in our previous finding, vasorelaxation, nitric oxide bioavailability, and its downstream signalling were significantly down-regulated in vessels from diabetic patients. The pronounced endothelial dysfunction in diabetic vessels could also be due to insulin resistance, as the insulin-up-regulated VEGF in smooth muscle cells via phosphorylation of Akt and eNOSSer1177 is dramatically reduced in diabetic vascular tissue. Furthermore, hyperglycaemia leads to formation of advanced glycation end products, which quench nitric oxide and impair endothelial function. This turns into a vicious circle in CKD, as clearance of advanced glycation end product is delayed, that further promotes vascular and renal injury.

We acknowledge the following limitations in this study: (i) this study specially examined a relatively small number of patients. Despite this, we were able to demonstrate statistically significant differences in key biological variables between diabetic and non-diabetic groups. Furthermore, our conclusion is based not on the analysis of single variable but on the use of a panel of molecular (MMP/TIMP activities), functional (in vivo and in vitro arterial stiffness and endothelial-dependent relaxation), histological (elastic fibre integrity, calcification, and microvascular density), and clinical data. This analysis clearly shows the additional detrimental impacts of diabetes on CKD vasculature. (ii) All recipients are hypertensive, which likely causes additive or synergistic adverse effects on vascular function. Therefore, the observed differences could not be attributed to diabetes and renal insufficiency. Hypertension is well characterized to be associated with endothelial dysfunction in coronary and peripheral vasculature. Vascular remodeling (i.e. increased media/lumen ratio) is evident in both hypertensive recipients. (iii) Many patients are on medications such as ACEi/ARB/statin prior to the transplantation for different lengths of time, the impact of these drugs on...
our findings cannot be determined. Numerous clinical studies have reported the beneficial effects of renin-angiotensin system blockers and statins on improving endothelial function and arterial stiffening. Interestingly, although more diabetic patients were on those medications, abnormalities on vascular functions remained prominent. (iv) It should be emphasized that a number of other MMPs can lead to angiotatin generation such as MMP-3, MMP-12, and MMP-7, which were not addressed in this study. (v) As this is a cross-sectional study, it is not possible to determine cause and effect, but rather to describe the associations of these findings in CKD patients with and without diabetes, and to interpret those findings within an appropriate and plausible framework.

In summary, we described that in the arterial vasculature from CKD patients, the presence of diabetes markedly up-regulated MMP-2 and -9, which was strongly associated with elastic fibre degradation, arterial stiffening, and calcification. The increase in MMP in diabetic vessels was also accompanied by pronounced generation of angiotatin, and the reduction of microvascular density was associated with impaired vasorelaxation. This is the first study using human vessels from matched diabetic and non-diabetic patients to describe the up-regulation of MMPs in association with vascular structural and functional changes, which support the possibility that these aberrations may be one of the plausible mechanisms contributing to arterial stiffening, vascular dysfunction, and impaired angiogenesis. Disregulation of these important mediators of vascular health could contribute to the increased cardiovascular risk burden and poor clinical outcomes in CKD patients with diabetes.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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