Chronic Renal Failure Accelerates Atherogenesis in Apolipoprotein E–Deficient Mice

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Abstract. Cardiovascular mortality is 10 to 20 times increased in patients with chronic renal failure (CRF). Risk factors for atherosclerosis are abundant in patients with CRF. However, the pathogenesis of cardiovascular disease in CRF remains to be elucidated. The effect of CRF on the development of atherosclerosis in apolipoprotein E–deficient male mice was examined. Seven-week-old mice underwent 5/6 nephrectomy (CRF, n = 28), unilateral nephrectomy (UNX, n = 24), or no surgery (n = 23). Twenty-two weeks later, CRF mice showed increased aortic plaque area fraction (0.266 ± 0.033 versus 0.045 ± 0.006; P < 0.001), aortic cholesterol content (535 ± 62 versus 100 ± 9 nmol/cm² intimal surface area; P < 0.001), and aortic root plaque area (205,296 ± 22,098 versus 143,662 ± 13,302 μm²; P < 0.05) as compared with no-surgery mice; UNX mice showed intermediate values. The plaques from uremic mice contained CD11b-positive macrophages and showed strong staining for nitrotyrosine. Systolic BP and plasma homocysteine concentrations were similar in uremic and nonuremic mice. Plasma urea and cholesterol concentrations were elevated 2.6-fold (P < 0.001) and 1.5-fold (P < 0.001) in CRF compared with no-surgery mice. Both variables correlated with aortic plaque area fraction (r² = 0.5, P < 0.001 and r² = 0.3, P < 0.001, respectively) and with each other (r² = 0.5, P < 0.001). On multiple linear regression analysis, only plasma urea was a significant predictor of aortic plaque area fraction. In conclusion, the present findings suggest that uremia markedly accelerates atherogenesis in apolipoprotein E–deficient mice. This effect could not be fully explained by changes in BP, plasma homocysteine levels, or total plasma cholesterol concentrations. Thus, the CRF apolipoprotein E–deficient mouse is a new model for studying the pathogenesis of accelerated atherosclerosis in uremia.

The mortality from cardiovascular disease is 10 to 20 times higher in patients with end-stage renal failure than in the general population (1). Although structural and functional abnormalities of the heart (2–5) and stiffness of the large arteries as a result of changes in the arterial media (6,7) may play a role, there are several observations that are compatible with the idea that increased atherosclerosis also contributes to the increased cardiovascular mortality. Angiographic studies of small series of patients considered for renal transplantation (8,9) and histologic evaluations of arterial biopsies from renal transplant recipients (10,11) have suggested that the prevalence of atherosclerosis might be higher in uremic compared with nonuremic individuals. Also, chronic renal failure (CRF) enhances a number of well-established risk factors for atherosclerosis (e.g., BP, plasma levels of atherogenic lipoproteins (12)) and affects several additional factors that potentially promote atherogenesis (e.g., homocysteine metabolism (13), inflammatory mediators (14,15), the balance between oxidants and antioxidants (16), plasma concentrations of advanced glycation end products [AGE] (17)). Nevertheless, the mechanism of increased cardiovascular disease in uremia remains to be elucidated.

In the present investigation, we describe a new mouse model that may be useful for studying factors that cause atherosclerosis in CRF. Apolipoprotein-E mediates the clearance of lipoproteins via the liver. Consequently, apolipoprotein E–deficient (apo-E −/−) mice develop hypercholesterolemia as a result of an accumulation of chylomicron remnants, VLDL, and intermediate-density lipoproteins (IDL) (18). Atherosclerotic lesions similar to those found in humans develop in the aortas of these mice when fed a normal mouse diet (18). In the present study, renal failure was induced by surgical removal of kidney tissue (5/6 nephrectomy), and aortic atherosclerosis was studied 22 wk after surgery.

Materials and Methods

Animals

Male apo-E −/− mice (C57BL/6JBom-ApoE<sup>−/−</sup>) backcrossed 10 generations onto the C57BL/6 background; M&B Laboratory Animals and Services for Biomedical Research, Ry, Denmark) were kept (five mice per cage) in a temperature-controlled room at 21°C to 23°C with a 12-h light/dark cycle and allowed free access to water and
a standard mouse diet (Altromin 1314, Altromin, Lage, Germany). Body weights were monitored at regular intervals. The experiments were performed according to the principles stated in the Danish law on animal experiments and approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

**Experimental Renal Failure**

At 7 wk of age, the mice were randomly allocated to 5/6 nephrectomy (CRF), unilateral nephrectomy (UNX), or no surgery. A mixture of fentanyl 0.079 mg/ml, fluanisone 2.5 mg/ml, and midazolam 1.25 mg/ml (Hypnorn/Dormicium) at a dose of 0.08 to 0.10 ml/10 g body wt was given subcutaneously for anesthesia, and buprenorphine (0.001 mg/10 g body wt, subcutaneously, twice daily) was given for analgesia after surgery.

For 5/6 nephrectomy, we made a dorsal midline incision of the skin and dissected each kidney free through a dorsosventral incision of the muscles and fascia near to the costal margin. The upper and lower poles of the right kidney were resected, leaving an intact kidney segment. The left kidney was removed after ligation of the renal blood vessels and the ureter (19,20). The musculofascial incisions were sutured, and the skin incision was closed by metal clips. For unilateral nephrectomy, mice underwent left-sided nephrectomy as for the 5/6 nephrectomy mice.

**BP**

Systolic BP was measured during the last week of the study with a tail-cuff system (BP 2000; Visitech Systems, Apex, NC) that uses a photoelectric sensor to detect the blood flow in the tail (21). The mice were familiarized to the procedure during four consecutive days before BP recordings on the fifth day. In each mouse, at least one set of 10 measurements with nine or more successful readings was obtained. The accuracy of measurements was secured by regular calibration of the pressure transducer. The variability in the BP measurement was 5.7%.

**Plasma Biochemistry**

Blood from the retro-orbital venous plexus was collected in heparinized microtubes (Capiject; Terumo Medical, Elkton, MD). Plasma urea was measured with a Vitros 250 (Ortho-Clinical Diagnostics, Johnson & Johnson, Rochester, NY). At the end of the study, each mouse was fasted overnight, anesthetized, and exsanguinated. Whole blood hemoglobin was determined using an OSM3 hemoximeter (Radiometer, Denmark). Plasma was separated by centrifugation at 2000 × g for 10 min at 4°C and stored at −20°C. Plasma creatinine, total calcium, and phosphate were measured with a Hitachi Automatic analyser 917 with reagents from Roche A/S (Hvidovre, Denmark). Plasma total cholesterol and triglyceride levels were assayed with enzymatic kits (22). For assessing plasma lipoproteins, pooled plasma samples (200 μl) from CRF mice (n = 10), UNX mice (n = 12), and no-surgery mice (n = 11) were subjected to fast-phase liquid chromatography on a Superose 6HR 10/30 column (Amersham Pharmacia Biotech) (23).

Plasma homocysteine was analyzed with a fluorescence polarization immunoassay (Abbott Axsym system; Axis-Shield, Oslo, Norway). For assessing plasma protein fractions involved in the acute-phase response reaction, protein electrophoresis was performed on hydragel β1-β2 15/30 agarose gels and stained with amido black using reagents and instruments from Sebia (Hatier, France). The relative plasma concentrations of α1-1, α2-1, β1-1, β2-1, and γ-migrating protein fractions were determined with densitometric scanning. Acute-phase proteins including orosomucoid, α1 antitrypsin, and haptoglobin migrate in the α zones.

**Analysis of Aortic Atherosclerosis**

For evaluating the degree of atherosclerosis, aorta was removed 22 wk after surgery. Each mouse was anesthetized, and the thorax was quickly opened. A small incision was made in the right cardiac auricle, and a cannula was inserted into the left ventricle. Through the left ventricle, the circulation was perfused with a cardioplegic solution (Kardioplex infusion fluid, item 747501; the Pharmacy of Rigshospitalet, Copenhagen, Denmark) until the eluent became clear, followed by perfusion-fixation at approximately 100 mmHg with phosphate-buffered paraformaldehyde (4% wt/vol [pH 7.0]; Bie & Berntsen, Roedovre, Denmark). Finally, the mice were immersed in the fixative for 6 h before storage (4°C) in phosphate buffer (0.1 mol/L [pH 7.4]; Bie & Berntsen).

A portion of the heart including the proximal ascending aorta was embedded in paraffin. The aortic root was sectioned serially at 4-μm intervals from the cardiac end (24). Once the aortic sinuses appeared, every other section was collected on glass slides. Five sections taken at 80-μm intervals, spanning 320 μm of the aortic root from the commissures of the aortic leaflets and upward, were stained with orcein. An observer without any knowledge of mouse treatment (J.F.B.) measured the plaque area (in μm²) with computer-assisted image analysis equipment from Olympus (BX50 light microscope, digital camera C-3030ZOOM, and DP-Soft Imaging System). Aortic root plaque area was expressed as the mean plaque area of the five sections.

The remaining portion of aorta was removed, fixed in 10% acetone at −20°C, and stored until sectioning. The sections were cut from the cardiac end of the aortic root and transferred to slides (SuperFrost Plus; Menzel-Glaser, Germany) for 8 min before incubation for 60 min at room temperature. The sections were rinsed in TRIS-buffered saline (pH 7.6; Bie & Berntsen) and preincubated in peroxidase blocking solution (DakoCytomation, Glostrup, Denmark) for 8 min before incubation for 60 min at room temperature with either the monoclonal or polyclonal antibodies as mentioned below. After repeated rinsing with TRIS buffer, the sections that had been incubated with biotinylated monoclonal antibodies were treated with peroxidase-labeled streptavidin (DakoCytomation) for 30 min (26), whereas the sections that had been incubated with rabbit polyclonal antibodies were treated with goat anti-rabbit EnVision-peroxidase-enzyme conjugate (DakoCytomation) (27,28) for 30 min. The peroxidase reaction was visualized by incubation with 2% 3,3’-diaminobenzidine (DakoCytomation) in substrate buffer (Dako-
Figure 1. Plasma urea concentrations in apolipoprotein E–deficient (apo-E−/−) mice with chronic renal failure (CRF). Plasma urea concentrations after 5/6 nephrectomy (CRF mice), unilateral nephrectomy (UNX mice), or no surgery at day 0. ■ CRF mice (n = 28); ● UNX mice (n = 24); ○, no-surgery mice (n = 23). Values are mean ± SEM. *P < 0.01 and ** P < 0.001 compared with the no-surgery group.

**Results**

**Effect of CRF on Indices of Uremia, Plasma Lipids, Homocysteine, α-Migrating Protein Fractions, and BP**

In CRF mice, the plasma urea concentration increased to approximately 250% of the baseline concentration within 2 wk after 5/6 nephrectomy and remained elevated throughout the study (Figure 1). The plasma creatinine concentration was 42% higher (0.051 ± 0.001 versus 0.036 ± 0.001 mmol/L; P < 0.001), whereas the body weight was 11% lower (23.5 ± 0.6 versus 26.4 ± 0.3 g; P < 0.001) and the blood hemoglobin concentration was 18% lower (6.9 ± 0.1 versus 8.4 ± 0.1 mmol/L; P < 0.001) in CRF compared with no-surgery mice (Table 1). In addition, the plasma phosphate concentration was 18% higher (2.49 ± 0.13 versus 2.11 ± 0.05 mmol/L; P < 0.05) and the plasma calcium concentration was 11% higher (2.52 ± 0.03 versus 2.27 ± 0.07 mmol/L; P < 0.01) in the CRF mice (Table 1). In the UNX mice, the plasma urea concentration was approximately 20% higher than in the no-surgery mice (12.6 ± 0.4 versus 10.5 ± 0.5 mmol/L; P < 0.01; Figure 1 and Table 1). Plasma creatinine, blood hemoglobin, and plasma calcium concentrations were similar in UNX and no-surgery mice (Table 1).

The total plasma cholesterol concentration was 50% higher in CRF compared with no-surgery mice (19.4 ± 0.6 versus 12.9 ± 0.5 mmol/L; P < 0.001; Table 1), whereas the plasma triglyceride concentrations did not differ among the three groups. In the UNX mice, the plasma cholesterol concentration was 19% higher than in the no-surgery mice (15.4 ± 0.7 versus 12.9 ± 0.5 mmol/L; P < 0.01; Table 1). In CRF mice, VLDL, IDL, and LDL cholesterol concentrations were increased compared with the UNX and the no-surgery mice (Figure 2).

Analysis of plasma obtained 12 wk after nephrectomy or sham operation showed similar plasma homocysteine concentrations in uremic and nonuremic mice (4.9 ± 0.3 μmol/L [n = 14] versus 4.8 ± 0.3 μmol/L [n = 8]; P = 0.84). Plasma protein electrophoresis on agarose gels followed by densitometric scanning of the stained gels did not reveal any significant difference in the concentrations of the α-migrating plasma proteins involved in an acute-phase reaction between uremic and nonuremic mice (α1-globulins, 8.7 ± 0.3 g/L [n = 7] versus 6.9 ± 1.1 g/L [n = 5]; P = 0.08; α2-globulins, 5.0 ± 0.6 g/L [n = 7] versus 4.2 ± 0.7 g/L [n = 5]; P = 0.43). The mean BP was the same in CRF, UNX, and no-surgery mice (Table 1).

**Effect of CRF on Aortic Atherosclerosis**

Twenty-two weeks after induction of renal failure, the total aortic plaque area fraction was increased 5.9-fold (P < 0.001) and the aortic cholesterol content was increase 5.4-fold (P < 0.001) in CRF mice compared with no-surgery mice (Figure 3, A and B). The UNX mice showed intermediate increases of the total aortic plaque area fraction (i.e., a 2.1-fold increase [P < 0.001]) and the aortic cholesterol content (i.e., a 1.5-fold increase [P < 0.001]) compared with no-surgery control mice (Figure 3, A and B). The impact of renal failure on plaque area in the aortic root was less pronounced than the impact on the total aortic plaque area fraction and cholesterol content. The aortic root plaque area was increased 1.4-fold (P < 0.05) in CRF mice compared with no-surgery mice but only 1.2-fold (NS) compared with UNX mice (Figure 3C).

On linear regression analysis, the total aortic plaque area fraction was closely associated with the total aortic cholesterol content (r² = 0.7, n = 74, P < 0.001), whereas the association with the aortic root plaque area was less pronounced (r² = 0.07, n = 74, P < 0.05). On univariate linear regression analysis, the total aortic plaque area fraction was positively associated with both the plasma cholesterol (Figure 4A) and...
Table 1. Effect of chronic renal failure on body weight, BP, plasma indices of uremia, and plasma lipids

<table>
<thead>
<tr>
<th></th>
<th>CRF</th>
<th>UNX</th>
<th>No-surgery</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>23.5 ± 0.6*</td>
<td>27.5 ± 0.3‡</td>
<td>26.4 ± 0.3</td>
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<tr>
<td>BP (mmHg)</td>
<td>115.6 ± 2.5</td>
<td>113.8 ± 1.5</td>
<td>114.0 ± 1.7</td>
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<tr>
<td>Blood hemoglobin (mmol/L)</td>
<td>6.9 ± 0.1*</td>
<td>8.3 ± 0.1</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>Urea</td>
<td>27.1 ± 1.7*</td>
<td>12.6 ± 0.4†</td>
<td>10.5 ± 0.5</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.051 ± 0.001*</td>
<td>0.037 ± 0.001</td>
<td>0.036 ± 0.001</td>
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<tr>
<td>Phosphate</td>
<td>2.49 ± 0.13‡</td>
<td>1.87 ± 0.06†</td>
<td>2.11 ± 0.05</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.52 ± 0.03†</td>
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<td>2.27 ± 0.07</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>19.38 ± 0.61*</td>
<td>15.41 ± 0.70†</td>
<td>12.94 ± 0.49</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.57 ± 0.12</td>
<td>0.56 ± 0.04</td>
<td>0.70 ± 0.06</td>
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Values are 22 wk after 5/6 nephrectomy (CRF, n = 28), or unilateral nephrectomy (UNX, n = 24) as compared with no-surgery mice (n = 23). Unless otherwise indicated, values are plasma concentrations in mmol/L. All plasma concentrations are after fasting. Values are mean ± SEM. ‡P < 0.05, †P < 0.01, *P < 0.001 compared with no-surgery controls.

Discussion

The present study revealed a pronounced effect of CRF on the progression of aortic atherosclerosis: twenty-two weeks after induction of renal failure, the total aortic plaque area fraction and aortic cholesterol accumulation were increased 5.9-fold and 5.4-fold, respectively, in apo-E /−/− mice. The data support the hypothesis that renal failure leads to markedly accelerated atherogenesis. This finding is in agreement with a recent study (29) in which the maximal diameter of plaques in the aortic root was increased 2.5-fold 12 wk after surgery in subtotally nephrectomized as compared with sham-operated apo-E /−/− mice.

It is generally believed that uremia does not develop after unilateral nephrectomy, e.g., in living kidney donors (30). However, both the present and a previous study (29), removal of one kidney in apo-E /−/− mice (UNX group) led to increases in the plasma indices of uremia and an approximately twofold increase in aortic atherosclerosis. The impact of unilateral nephrectomy on atherosclerosis, however, may be specific to the hypercholesterolemic apo-E /−/− mouse model and should not be extrapolated to other species, including humans. Indeed, the apo-E /−/− mouse spontaneously develop renal lesions with lipid deposits in the glomeruli (31). Nevertheless, the combined susceptibility of the apo-E /−/− mouse to development of uremia upon partial nephrectomy and its predisposition to development of human-like atherosclerotic lesions (18) provides an excellent model for studying uremic atherosclerosis.

The effect of CRF on plaque lesion area in cross-sections of macrophages (Figure 6A). Neither CD3e-positive T lymphocytes nor CD22-positive B-lymphocytes could be demonstrated at this time point in the plaques, although a few CD3-positive lymphocytes were found in the adventitia (data not shown). Strong staining for nitrotyrosine was present in all of the uremic plaques that we examined. Nitrotyrosine was predominantly seen within the core of the plaques (Figure 6B) and to a lesser extent in the underlying medial smooth muscle cell layer and adjacent nonlesioned vessel wall.

Figure 2. Plasma lipoprotein cholesterol in apo-E /−/− mice with CRF. Pooled plasma (200 µl) from apo-E /−/− mice was fractionated by fast-phase liquid chromatography. ■, CRF mice (n = 10); ●, UNX mice (n = 12); ○, no-surgery mice (n = 11). IDL, intermediate-density lipoprotein.
the aortic root was much less pronounced than the effect on total aortic plaque area fraction and cholesterol accumulation. This suggests that uremia in apo-E−/− mice affects lesion formation differently in the aortic root compared with the thoracic and abdominal parts of the aorta. Differential atherogenic responses to treatment in different parts of the aorta have been observed previously in mice, e.g., lesion size increased in the aortic root whereas lesion involvement decreased in the thoracic aorta in probucol-treated apo-E−/− mice and in bone marrow–transplanted LDL receptor–deficient mice (32,33). The present results underscore the importance of using more than one measure of atherosclerosis in mouse studies in general and in studies of CRF effects in the apo-E−/− mouse in particular.

Limited data are available on the impact of uremia on atherosclerotic plaque composition. On histologic examina-
tions of hematoxylin-eosin–stained sections, the plaque morphology of the aortic root appeared similar in partially nephrectomized mice and control mice. Immunohistochemistry showed accumulation of lipid-filled macrophages, whereas no T or B cells were seen in early lesions. These observations suggest that uremic lesions share key characteristics with atherosclerosis in general.

Coronary artery plaques in patients with ESRD are more calcified than those in age- and gender-matched patients with coronary artery disease and normal kidney function (34). In addition, calcifications of the arterial wall medial layer are frequently observed in arterial specimens obtained from uremic patients (7,11), rats (35), and rabbits (36). It has been suggested that the arterial wall calcifications are caused by the changes in the calcium/phosphate metabolism that accompanies uremia (7,37). The plasma calcium and phosphate concentrations both were elevated in the CRF mice. The explanation for this remains enigmatic, but a similarly increased plasma calcium concentration has been previously described in mice (38) and dogs (39) with surgically reduced renal mass. Nevertheless, in agreement with a recent study (29), we did not observe calcification of the arterial wall in CRF mice on histologic analysis of the aortic root or on microradiographs of the entire aorta (data not shown).

The mechanism of accelerated atherogenesis in CRF mice remains to be elucidated. Although hypertension and hyperhomocysteinemia occur in approximately 90% of uremic patients at the start of renal replacement therapy (40,41), the BP and plasma homocysteine concentrations in apo-E −/− mice were unaffected by CRF (29,38,42). Thus, BP and hyperhomocysteinemia are not important causative factors of the accelerated atherosclerosis in uremic apo-E −/− mice.

The total plasma cholesterol concentration was higher in CRF mice than in control mice. It is likely that this difference contributed to the accelerated formation of atherosclerosis in uremic mice. Moreover, it is almost certain that hypercholesterolemia is a prerequisite for advanced uremic lesion formation in mice. In support of this idea, only three of eight uremic and normocholesterolemic C57/BL6 mice developed very small plaques after subtotal nephrectomy (29), whereas the uremic and hypercholesterolemic apo-E −/− mice developed severe and advanced lesions. Fast-phase liquid chromatography analysis of plasma revealed that the increase in the total plasma cholesterol concentration of the uremic apo-E −/−
mice primarily reflected an increase in VLDL and IDL/LDL cholesterol. This observation is in accordance with findings in patients with CRF, who frequently display increased levels of VLDL and IDL (43,44). The change in the cholesterol distribution between lipoproteins may also have contributed to the effect of CRF on atherosclerosis. The abnormal function and catabolism of lipoproteins as a result of oxidation or glycation are characteristics of uremia that may further enhance the atherogenicity of plasma lipoproteins (45,46).

Oxidative stress and inflammation are often suggested to play central roles in the pathogenesis of cardiovascular disease in uremia (16). In the present study, we observed marked expression of nitrotyrosine (a marker of reactive oxygen species–protein interaction (47)) in the uremic plaques as well as the underlying smooth muscle cell layer. This suggests that oxidative stress within the arterial wall may be important for progression of atherosclerotic lesions in uremia.

A positive correlation between plasma urea concentration and the severity of atherosclerosis was observed using both uni- and multivariate linear regression analysis. This supports, albeit does not prove, the hypothesis that one or more uremia-related factors (in addition to hypercholesterolemia) affect the development of atherosclerosis. Increasing evidence suggests that uremia has direct adverse effects on the vascular wall, e.g., the plasma concentrations of soluble portions of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1 are increased, and endothelium-dependent vasodilation is impaired in patients with CRF (48,49). Moreover, CRF promotes the accumulation of AGE (e.g., pentosidine, carboxymethyl lysine) in plasma and tissue proteins (50), including apolipoprotein B (17,46). AGE have been detected in atherosclerotic lesions (17,51,52), where they may interact with AGE receptors on endothelial cells, causing increased expression of adhesion molecules (17,53). AGE are also thought to play a central role in development of diabetic complications (17,53). It is interesting that diabetes-induced atherosclerosis in apo-E−/− mice was completely suppressed by the administration of the soluble extracellular domain of the AGE receptor (54), and massive plaque expression of the AGE receptor was recently seen in uremic apo-E−/− mice (29). In the future, it will be of interest to determine whether inhibition of formation or of downstream effects of AGE might also affect the development of uremic atherosclerosis.

In conclusion, the present study showed that uremia markedly accelerates atherogenesis in apo-E−/− mice. This is compatible with the concept of a pronounced increase in cardiovascular mortality in patients with uremia that relates to accelerated development of atherosclerosis. The CRF apo-E−/− mouse model provides a useful tool to explore the mechanisms of uremic atherosclerosis.

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