Cardiovascular effects of uremia in apolipoprotein E-deficient mice

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1. INTRODUCTION

Cardiovascular (CV) mortality is 10 to 20 times higher in patients on dialysis than in the general population, and even mild kidney dysfunction is a risk factor of cardiovascular disease (CVD) [7-11]. Although highly prevalent, the increased risk cannot be explained by the classical CV risk factors[1] alone [15, 16]. In addition, patients with advanced chronic kidney disease (CKD)[2] respond differently than the general population to treatment for CVD. For example, statin treatment, which is effective in the prevention of CVD in the general population, did not show any effect on CVD in hemodialysis patients with diabetes [18]. On the other hand, vitamin E treatment, which is without any effect in the prevention of CVD in the general population, reduced CVD in hemodialysis patients with preexisting CVD [19]. These observations suggest that CKD may mediate increased risk of CVD through nonclassical CV risk factors and/or atypical pathophysiology.

The overall purpose of this thesis work was to describe a new animal model of uremic vascular disease that could provide a tool to identify molecular responses of the arterial wall to uremia, and also help identify new approaches for treatment and prevention of CVD.

1.1 CLINICAL BACKGROUND

CVD in patients with CKD

In the United States Renal Data System Wave 2 study of 3941 patients initiating dialysis, the prevalence of coronary heart disease, congestive heart failure, cerebrovascular disease, and peripheral vascular disease was 32.4%, 24.9%, 10.2% and 16.9%, respectively [20]. Over a 2.2-year follow-up, the incidence of new acute coronary syndrome, congestive heart failure, stroke and peripheral vascular disease was 10.2%, 13.6%, 2.2% and 14%, respectively [20]. A total of 36.1% of patients died during this period [20]. Also in patients with minor reductions of kidney function, the frequency of atherosclerotic events and congestive heart failure was markedly increased as compared to the general population [8, 9, 11].

1) Hypertension, diabetes, hypercholesterolemia, obesity, smoking and physical inactivity in addition to male gender, high age and a family history of CVD, e.g. [12-14].

2) Defined as either kidney damage or glomerular filtration rate (GFR) < 60 mL/min/1.73 m^2 for ≥ 3 months. Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies. Stage 1: Kidney damage with GFR ≥ 90 (mL/min/1.73m^2), stage 2: kidney damage with GFR 60-89, stage 3: GFR 30-59, stage 4: GFR 15-29, stage 5: GFR< 15 (or dialysis) [17].

CKD in patients with CVD

In a cohort study of randomly selected US Medicare patients admitted to hospital with myocardial infarction or heart failure, the prevalence of CKD (creatinine clearance < 60 mL/min/1.73m^2) was very high, 60 and 52%, respectively [21]. Similar observations were made in a heart-function clinic cohort study in Canada [22]. In patients with myocardial infarction or heart failure, CKD is associated with increased risk of adverse outcomes [21-23].

Prevalence of moderate and severe CKD

In a sample representative of the American population [24], the prevalence of CKD stages 3 to 4 was 3.8%. If CKD stages 1 to 2 were included, the prevalence was 9.4%. This is close to the overall CKD prevalence of 10.2% found in a large population-based study in Norway [25]. The incidence of end-stage renal disease (CKD stage 5) among individuals with CKD, however, was 3 times lower in Norwegian than in white patients in the US. This large variation in end-stage renal disease incidence rates may be related to a higher prevalence of obesity and diabetes in the US population, and to different management of patients with existing CKD in European versus US populations [25, 26].

Arterial disease

Atherosclerosis is the most frequent underlying cause of coronary heart disease, cerebrovascular disease, and peripheral vascular disease. It is a focal inflammatory-fibroproliferative disease in the intima of medium-sized and large arteries, predominantly due to deposition of atherogenic lipoproteins [27-30]. In CKD, a high prevalence of atherosclerosis has been shown by histological examination of arterial biopsies from young uremic patients [31, 32] and angiographic studies of asymptomatic patients with CKD considered for renal transplantation [33, 34]. Atherosclerotic disease contributes to the premature stiffening of the conduit arteries seen in CKD [35, 36]; however, this condition is also due to acceleration of the ageing process with fibrous intimal thickening and medial changes such as reduction in elastin content and the number of smooth muscle cells, increase of collagen content, and extensive calcification [35, 37, 38]. Aortic stiffening (as assessed by pulse wave velocity measurements) progresses with decreasing glomerular filtration rate (GFR) [39-40], and is even observed in pediatric dialysis patients [41]. Aortic pulse wave velocity is associated with the presence of arterial calcifications [42-45], which are common and progressive in young and adult patients with stage 5 CKD [46, 47]. Stiffness of the aorta is associated with increased systolic blood pressure (BP) and pulse pressure, thereby increasing left ventricular afterload and hypertrophy [36]. The lower diastolic blood pressure, which is another consequence of arterial stiffening, decreases coronary perfusion pressure. In patients with stage 5 CKD, aortic pulse wave velocity is a strong and independent predictor of CV and all-cause mortality [48, 49].

Heart disease

CKD is also characterized by structural and functional changes of the heart. Left ventricular hypertrophy (LVH) and diastolic dysfunction (as assessed by echocardiography) develop in early kidney failure in both children and adults [50-52]. Accordingly, LVH was present in 27% of patients with a creatinine clearance greater than 50 mL/min [53], and in a Canadian cohort study of 433 patients starting renal replacement therapy [54], 74% had LVH, 36% had left ventricular dilatation, and 15% had systolic dysfunction. LVH is a strong independent prognostic indicator of CV death in CKD [54, 55]. In addition, several studies have demonstrated a high prevalence of cardiac valve calcification and valvular heart disease in patients with stage 5 CKD [56-58].

CV risk factors in CKD

Classical risk factors for CVD are highly prevalent in CKD populations, but cannot alone explain the increased risk of CVD [59-62]. Thus, CKD is also associated with a growing number of "novel" pu-

tative CV risk factors, including markers of oxidative stress and inflammation and abnormal calcium-phosphorus metabolism [59, 60, 62]. The role of these nonclassical risk factors remains, however, to be fully elucidated.

Classical risk factors

Hypertension

Hypertension is present in 60 to 90% of CKD patients, depending on the degree of kidney dysfunction and the cause of kidney disease [63, 64]. Hypertension is associated with increased risk of CVD in patients with stage 3-4 CKD [55, 63], whereas several studies in patients with stage 5 CKD have shown a U-shaped relationship between systolic BP and death [65-67]. These observations may be due to other comorbid conditions, e.g. lower BP seen in patients with cardiac failure. Hence, a prospective Canadian cohort study showed that high BP predicted LVH and development of de novo cardiac failure and de novo ischemic heart disease in dialysis patients [65]. The hypothesis that BP lowering reduces CV events in patients with CKD has not yet been tested in a randomized, placebo-controlled trial (RCT).

Diabetes

Diabetes is present in 20-50% of patients with CKD [8, 63]. Diabetes is related to a higher risk for CVD in all stages of CKD [8, 55, 63]. Moreover, insulin resistance is an independent predictor of CV mortality in stage 5 CKD [68]. A recent study showed that insulin resistance is present even in the earliest stages of CKD [69].

Dyslipidemia

CKD is associated with dysregulation of several key enzymes and receptors involved in the metabolism of lipoproteins, particularly those of high density lipoprotein (HDL) and triglyceride (TG)-rich lipoproteins [70, 71]. Accordingly, uremic dyslipidemia is characterized by an increase in plasma TG and elevated plasma levels of very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and chylomicron remnants, reduced HDL cholesterol, and increased lipoprotein(a) [70, 71]. Total and low density lipoprotein (LDL)-cholesterol levels are usually normal or modestly increased [70, 71]. Qualitative abnormalities in lipoproteins are common, including increased proportions of small dense LDL, oxidized LDL (OxLDL), and cholesterol-enriched TG-rich lipoproteins [70, 71]. Sources of variability in the severity of dyslipidemia include the stage of CKD and the degree of proteinuria. Plasma total cholesterol (and TG and apolipoprotein B), were predictors of increased CVD in stages 3-4 CKD [63], but the relation between total cholesterol and death is U-shaped in patients with stage 5 CKD [72]. A recent study demonstrated, however, that among stage 5 CKD patients without evidence of inflammation (defined by low serum albumin, high interleucin(IL)-6, and high C-reactive protein (CRP) levels), the expected relation between higher cholesterol and death was seen [73]. In RCTs of statins, post hoc analyses of subgroups with impaired kidney function have suggested that statins are efficacious in lowering CVD risk, at least in patients with mildly decreased GFR (mean GFR 65 mL/min/1.73m^2 [74] and 61 mL/min/1.73m^2 [75]). The randomized controlled atorvastatin 4D (Die Deutsche Diabetes Dialyse) study including 1255 hemodialysis patients with type 2 diabetes, on the other hand, showed only a nonsignificant trend toward an improved outcome with respect to the primary composite end point (cardiac death, fatal stroke, nonfatal myocardial infarction, nonfatal stroke) [18]. Two larger randomized trials using statins in patients with CKD are ongoing (The Study of Heart and Renal Protection (SHARP) and The Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Hemodialysis: An Assessment of Survival and Cardiovascular Events (AURORA)). There are no RCTs of fibrates and nicotinic acid in patients with CKD. A post hoc subgroup analysis of a RCT of gemfibrozil in men with established coronary artery disease, HDL cholesterol < 1 mmol/L and LDL cholesterol ≤ 3.6 mmol/L indicated that gemfibrozil may be effective for secondary prevention of CV events in patients with estimated creatinine clearance 30-75 mL/min [76]. Supplementation with n-3 polyunsaturated fatty acids (PUFAs) had a favorable effect on lipoprotein profile in patients with CKD [77]. A small RCT of n-3 PUFA did not show any effect on the total number of CV events and all-cause mortality in hemodialysis patients with established CVD [78]. However, treatment with n-3 PUFA for 2 years significantly reduced the number of myocardial infarctions.

Obesity

Whereas obesity is an independent risk factor for CKD [79] and related to a higher risk for CVD in stage 3-4 CKD [63], a high body mass index and a high body fat index are positively correlated with survival in dialysis patients [79-81].

Smoking

Smoking is associated with increased CVD in all stages of CKD [20, 55, 63]. In dialysis patients smoking is associated with new-onset congestive heart disease, peripheral vascular disease and death [20].

Physical inactivity

In the United States Cardiovascular Health Study, low physical activity (lowest quartile of reported energy expenditure) was a predictor of CV mortality in patients with a mean GFR of 50 mL/min [55], whereas in the United States Atherosclerosis Risk in Communities study physical inactivity (less than 1 hour of sport activity per week) was not associated with CVD in stage 3-4 CKD [63]. Exercise capacity (peak oxygen uptake) was strongly predictive of survival in a cohort of hemodialysis patients [82].

Nonclassical risk factors

Anemia

Anemia is associated with increased risk for CVD in all stages of CKD [63, 83, 84]. Anemia increases the risk of LVH in mild to moderate CKD [85], and the combination of both anemia and LVH markedly increases the risk of adverse CV outcomes [84]. In dialysis patients, anemia was associated with left ventricular dilatation, development of de novo cardiac failure, and mortality [83]. RCTs have not been able to demonstrate a positive effect of correction of anemia on LVH or CV outcome in CKD patients [86-91] .

Homocysteine

Plasma homocysteine levels and kidney function are inversely related [92]. Whether high plasma homocysteine levels contribute to increased CV mortality in CKD patients remains controversial [93]. A recent study demonstrated that hyperhomocysteinemia may be a strong risk factor for mortality in hemodialysis patients without evidence of chronic inflammation-malnutrition (normal serum albumin and CRP) [94]. However, two RCTs of folic acid in predialysis/dialysis patients showed a reduction of plasma homocysteine, but no effect on CV outcomes [95, 96].

Calcium-phosphorus imbalance

Hyperphosphatemia and hyperparathyroidism are associated with increased CV mortality [97]. The link between hyperparathyroidism and hyperphosphatemia and CV complications likely are related to arterial calcifications, since optimized treatment of calcium and phosphate disturbances decreases arterial calcifications [98] and mortality risk in hemodialysis patients [99]. Nevertheless, a recent large multi-center, randomized, open-label trial (DCOR study) [100] failed to show any beneficial effect of sevelamer over calcium-based phosphate binders on overall mortality, cause-specific mortality or morbidity in hemodialysis patients. Current studies explore the potential role of the reduced arterial expression of extracellular calcium sensing receptor in patients with CKD stage 5 [101]. The EVOLVE study is a global, double-blind, RCT evaluating the effect

of cinacalcet (a calcimimetic drug) on mortality and CV events in hemodialysis patients with secondary hyperparathyroidism [102].

Oxidative stress

Increased plasma and tissue levels of lipid, carbohydrate, and protein oxidation products are common in CKD patients [103, 104]. In contrast to the negative outcome of RCTs of antioxidants in the non-renal population at high CV risk [105] and of a post hoc subgroup analysis in patients with mild-moderate CKD [106], two small RCTs in hemodialysis patients showed a reduction in composite CVD end points, but not in total or CVD mortality, with vitamin E 800 IU per day for 17 months and N-acetylcysteine 600 mg twice daily for 14 months, respectively [19, 107].

Inflammation

Abnormal circulating levels of a variety of inflammatory factors [63, 108-110] are common in patients with CKD. Some of these (e.g. CRP, IL-6, albumin, and fibrinogen) have been shown to be predictors of CVD events or CVD mortality [55, 63, 109, 111, 112]. A number of treatments have been shown to lower plasma CRP levels, including statins, aspirin, and angiotensin converting enzyme (ACE) inhibitors [113-115]. However, so far there is no evidence that such therapeutic maneuvers improve survival in CKD patients.

Renin angiotensin system (RAS)

Inappropriate activation of RAS is one of the striking characteristics of kidney failure [116]. A post hoc subgroup analysis of a RCT of ramipril (an ACE-inhibitor) in patients who had preexisting vascular disease or diabetes, suggested that ramipril reduced CVD risk in patients with mild kidney failure, regardless of whether or not the patients had a history of hypertension [117]. Among two randomized trials in hemodialysis patients, one study showed no benefit of fosinopril (another ACE-inhibitor) over placebo in reducing the incidence of CVD events [118], whereas the other small open-label study of candesartan (an angiotensin II type 1 receptor blocker) found a reduction of CVD events and mortality [119]. Noteworthy, there was no difference in mean BP at follow-up between intervention and control groups. However, both studies were underpowered to permit reliable conclusions.

Sympathetic nerve activity

Plasma norepinephrine is associated with mortality and CV outcomes in patients with stage 5 CKD [120]. The stimulus for increased activity of the sympathetic nerve system in CKD appears to be mediated by an afferent signal arising in the failing kidneys [121]. Interestingly, RAS inhibition decreases the sympathetic hyperactivity in CKD [122, 123].

Nitric oxide synthase (NOS)-nitric oxide (NO) system and endothelial function

Endothelial damage may lead to a decreased production of NO[3] or an increased breakdown [124, 125]. Dysfunction of the endothelium[4] has been demonstrated in several conditions associated with increased risk of CVD, e.g. dyslipidemia, diabetes, arterial hypertension, smoking [124, 125, 127], and CKD [128]. In CKD, several factors may interfere with the NOS-NO system [128], e.g. in patients with CKD stage 5 those with the highest plasma concentrations of asymmetrical dimethylarginine (ADMA), an inhibitor of NOS, show the highest risk of CV events [129].

3) NO functions as an endogenous anti-atherogenic molecule by promoting arterial vasodilatation, inhibiting proliferation of vascular smooth muscle cells, attenuating platelet adhesion and aggregation, and inhibiting leucocyte-endothelial interaction, e.g. [124, 125].

4) Arterial endothelial function can be assessed by measuring vasodilatation after stimulation of NO release by acetylcholine infusion, or by increasing flow and shear stress (e.g. by inflation followed by deflation of a proximal upper arm cuff) [126].

1.2 RATIONALE FOR THE ANIMAL EXPERIMENTS

The mechanisms for the increased CV risk associated with CKD is incompletely understood. Investigation of the underlying molecular mechanisms of CVD in humans with CKD is difficult, because CV tissues cannot easily be sampled for testing in living humans. Moreover, the variability in genetic and life style factors, underlying kidney diseases, co-morbidity, and therapeutic regimens among patients with CKD may complicate interpretation of human data. In animal models, however, it is possible to control these sources of variability, and to obtain CV tissue for gene and protein expression analysis at defined time intervals after the induction of uremia.

When the experimental work behind this thesis work was undertaken, there was no established animal model of uremia which was susceptible to the development of atherosclerosis. Also, at that time it was controversial whether the increased atherosclerosis seen in uremia was due alone to the high prevalence of classical CV risk factors, or whether factors associated with kidney dysfunction played a major role. Moreover, limited data was available on the effect of conventional and non-conventional therapy on CVD in uremia. Thus, the overall purpose of this thesis work was to establish an animal model that could be used to study the pathogenesis and potential therapies in uremic atherosclerosis.

Aims

The specific major aims were:

1. To establish an experimental mouse model for studying the pathogenesis of atherosclerosis in uremia.
2. To test whether uremia resulted in accelerated atherosclerosis in this model.
3. To examine whether the composition of uremic plaques differed from classical atherosclerotic lesions.
4. To explore uremia-induced gene expression changes in the arterial wall.
5. To test whether accelerated atherogenesis in uremia was associated with the development of an immune response against oxidized LDL (OxLDL).
6. To examine whether a proatherogenic effect of uremia was preventable by blockade of the renin angiotensin system (RAS).
7. To examine whether a proatherogenic effect of uremia was preventable by blockade of the receptor for advanced glycation end products (RAGE).
8. To examine the effects of uremia on heart structure and function in the apolipoprotein E-deficient (apoE-/-) mouse model.

2. METHODS

2.1 ANIMALS

Male apolipoprotein E-deficient (apoE-/-) mice were used in the present studies (Taconic M&B Laboratory Animals and Services for Research, Ry, Denmark). The mice were fed a standard mouse diet containing: 22.5% protein, 5% fat, 48% carbohydrates, 0.9% calcium, 0.7% phosphorus, and 600 IU/kg of vitamin D_3 (Altromin 1314, Lage, Germany).

2.2 EXPERIMENTAL KIDNEY FAILURE – SURGICAL PROCEDURES

Kidney failure was induced in anesthetized apoE-/- mice by surgical 5/6 nephrectomy. After a modification of the initial procedures [1], kidney failure was induced by a 2-step procedure as described [2]. Briefly, the upper and lower poles of the right kidney were resected leaving an intact kidney segment. Two weeks later the left kidney was removed after ligation of the renal blood vessels and the ureter. The peri-operative mortality was 10-40% [1-5].

2.3 BLOOD PRESSURE

Systolic BP was measured with a tail-cuff system (BP 2000; Visitech Systems, USA) that uses a photoelectric sensor to detect the blood

flow in the tail [130]. This method gives results similar to those obtained with an intraarterial method [130]. The variability in the BP measurement was ~6% [1, 4].

2.4 ANALYSIS OF AORTIC ATHEROSCLEROSIS

Atherosclerosis was assessed in three different ways. For en face examination of the total aortic intimal surface [131], total aortic area and plaque area were determined by computer-assisted image analysis (Multi-Analyst/PC version 1.1., Bio-Rad Laboratories, USA). The inter-observer and intra-observer variability were 8.9% and 5.3%, respectively [5]. For quantification of aortic lipid accumulation, aortic lipids were extracted. The total aortic cholesterol content was quantified with an enzymatic method [132]. Further, lipid composition of aortas was quantified with thin layer chromatography (TLC) [133-135]. Coefficients of variation of the TLC assay were 11-14% [133]. For histological examination, five cross sections taken at defined intervals from the levels of the aortic valves and upward were examined [136]. The sections were stained and plaque areas were measured (in μm^2) with computer-assisted image analysis equipment. Aortic root plaque area was expressed as the mean plaque area of the five sections.

2.5 ECHOCARDIOGRAPHY

The mice were anesthetized before transthoracic echocardiography with a Vivid Five Instrument (GE Ultrasound, Denmark) and a 10-MHz transducer head. To examine cardiac function during cardiac stress, measurements were repeated after an intraperitoneal injection of dobutamine (1.0 μg/g body weight). Echocardiography and data analyses were performed by an experienced examiner (E.B.) in a blinded fashion. The intra-observer variability of measurements was 5% (Bollano E, Sahlgrenska Academy, Goteborg University, Sweden (Doctoral Thesis), 2001).

2.6 RNA ANALYSIS

Total RNA was isolated from aortas (or heart tissue) with TRIzol reagent after homogenization, as described [2-6]. RNA purity and concentration were determined by absorbance measurements at 260 nm and 280 nm. RNA integrity was ensured by analysis of the 28S/18S ribosomal RNA ratio.

Real-time polymerase chain reaction (PCR) in a Light Cycler was used to quantify gene transcripts. The specificity of the PCRs was confirmed by DNA sequencing. MessengerRNA quantifications were done twice (or more) in separate runs. The inter-assay coefficients of variations for gene transcript quantifications were 10-14% [4]. To account for differences in cDNA preparation and cDNA amplification efficiency, all mRNA expression data were normalized with the mRNA expression of the house keeping gene in the same sample.

For mRNA micro-array analysis, aortic cRNA from uremic and control aortas were hybridized to high-density oligonucleotide microarrays containing approximately 12,000 probe sets (6,000 expressed sequence tags and 6,000 characterized genes) (Affymetrix mouse genome U74Av2 array). Three chips (each hybridized with a pool of cRNA made with RNA from 3 mouse aortas) were used to study the global gene expression in each of the study groups. The image files (cel files) were analyzed with the dChip software (www.dchip.org) [137]. Probe sets that were significantly differentially expressed by showing at least 1.5 fold change, an absolute change in expression of ≥ 50 units with $P < 0.05$ on a two-sample Student's t-test were selected. The lists of differentially expressed transcripts were annotated based on Gene Ontology terms using the NetAffx Analysis Center (www.affymetrix.com/index/analysis/index. affx) [138].

2.7 BIOCHEMISTRY

Plasma creatinine, urea, total calcium, and phosphate were measured with automatic analyzers [1-6]. Plasma total cholesterol and triglyceride levels were assayed manually with enzymatic kits [133], or plasma cholesterol was measured with an automatic analyzer [4]. For assessing plasma lipoproteins, pooled plasma samples (200 μl) were subjected to fast-phase liquid chromatography [139]. Plasma homocysteine was analyzed with a fluorescence polarization immunoassay (Abbott Axsym System, Axis-Shield, Oslo, Norway).

Plasma concentrations of soluble (s) portions of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were measured with monoclonal antibody-based sandwich ELISA kits (R&D Systems Europe, Abingdon, Oxon, UK). The intra-assay coefficients of variation were < 5% for both ELISA assays [2, 4, 5]. Plasma ACE activity was measured after centrifugation of the plasma samples at $20.000 \times g$ with a kit (Trinity Biotech Sigma Clinical Chemistry, Bray, Ireland). The intra-assay coefficient of variation was < 5% [4]. Titers of antibodies against OxLDL were determined by chemiluminescent enzyme immunoassays, as described [140]. Plasma levels of oxidized phospholipid (EO6) epitopes present on apolipoprotein B-100 (apoB-100) [4] or in total plasma [5] were measured by a chemiluminescent ELISA [141, 142] using a biotinylated mouse antibody EO6, which specifically recognizes the phosphorylcholine moiety of oxidized phospholipids.

3. RESULTS AND DISCUSSION

3.1 A MOUSE MODEL OF UREMIC ATHEROSCLEROSIS

None of the common laboratory animals spontaneously develop mature atherosclerotic lesions. Accordingly, previous studies of arterial disease in rats and rabbits rendered uremic by partial nephrectomy failed to show intimal accumulation of foam cells and lipids [143, 144].

Since these early studies were undertaken, however, gene manipulation techniques have made it possible to alter genes involved in lipid metabolism. The hyperlipidemic apolipoprotein E-deficient (apoE-/-) and LDL receptor-deficient (LDLR-/-) mouse models were developed in 1992-93 [145-148]. Apolipoprotein E (apoE) serves as a ligand for the LDL and chylomicron-remnant receptors and plays an important role in the hepatic clearance of lipoprotein particles from the plasma [149]. ApoE-/- mice show elevated plasma cholesterol levels (approximately five times normal[5]) as a result of accumulation of chylomicron and VLDL remnant lipoproteins [147, 148]. Atherosclerotic lesions similar to those found in humans develop in the aortas of these mice, even when fed a chow diet [147, 148, 150, 151]. Foam cell lesions begin to appear at 10-15 weeks of age, and advanced lesions consisting of a central necrotic core with cholesterol clefts and foam cells, covered by a fibrous cap containing smooth muscle cells and connective tissue, show after 5-8 months [150, 151]. Interestingly, plaque rupture and superimposed thrombosis is exceedingly rare in apoE-/- mice [152, 153]. Thus, the apoE -/- mouse model provides only intermediate CV end points. LDLR-/- mice accumulate LDL in plasma due to a defect clearance and develop severe atherosclerotic lesions throughout their aortas, when fed a fat and cholesterol enriched diet [145, 154]. The apoE-/- and LDLR-/- mouse models are now widely used for studying the pathogenesis of atherosclerosis. Since 2003 three independent groups (including Bro et al.) have used the apoE -/- mouse model for studies of arterial disease in uremia [1, 155, 156]. A fourth group has presented a study in uremic LDLR -/- mice [157].

Experimental kidney disease may be induced by reduction of the renal mass by surgery [158, 159] or electrocoagulation [144, 160], ligation of renal arterial branches [161], ureteral obstruction [162], renal irradiation [163] or treatment with tubulotoxic substances [164]. In the studies by Bro et al., apoE-/- mice were rendered uremic by surgical 5/6 nephrectomy (NX). This method was preferred, since it allowed a controlled graduation of kidney damage, and a minimal risk of unwanted side-effects on other organ systems. Furthermore, the uremic state seemed to be relatively stable over time.

5) The average plasma cholesterol of wild-type mice is 1-3 mmol/L and most of this cholesterol is carried by HDL. Levels of LDL and VLDL are very low.

The uremic mice were characterized by a ~2.5-fold increase of plasma urea [1], which is similar to findings in other studies [155-157], and a reduction of creatinine clearance to 1/3 of that in mice with normal kidney function [6]. Like in rats [165], diuresis was increased by uremia [6]. The plasma urea levels remained stable from 4 to 22 weeks after NX [1]. Another group reported that proteinuria did not increase after subtotal nephrectomy in apoE-/- mice [166], and a histological study [167] found no progression of CKD 12 weeks after surgery. The uremic apoE-/- mice thrived normally with a moderate (10 to 17%) reduction of body weight [1, 4]. The blood hemoglobin concentration was lower (18 to 23%), and the plasma phosphate level was higher (18 to 27%) in uremic compared with control mice [1, 4]. Uremic mice showed increased plasma total cholesterol, VLDL and IDL/LDL cholesterol concentrations [1, 3, 4].

In contrast to humans [168, 169], the mice showed 11 to 18% higher plasma total calcium levels, and did not develop hyperhomocysteinemia or hypertension upon the induction of uremia [1, 4]. The explanation for the elevated plasma calcium concentration consistently reported after renal mass reduction in mice and dogs [1, 2, 4, 156, 160, 161, 170] remains enigmatic. Despite the hypercalcemia, uremia in both mice and dogs still is accompanied by hyperparathyroidism [156, 161, 170]. Like dogs [171] and rabbits [144], some mouse strains, e.g. the C57BL/6J mouse [1, 4, 155, 156, 172, 173], do not develop hypertension upon renal mass reduction. The reason is unknown.

3.2 UREMIA ACCELERATES ATHEROGENESIS

Twenty-two weeks after NX, the total aortic plaque area fraction, total aortic cholesterol content, and aortic root plaque area were increased in uremic mice [1] (Figure 1). The unilaterally nephrectomized mice showed intermediate increases (Figure 1). The total aortic plaque area fraction was closely associated with the total aortic cholesterol content, whereas the association with the aortic root plaque area was less pronounced [1]. The effect of uremia on atherogenesis in apoE-/- mice was independent of BP and plasma homocysteine levels (which were similar in uremic and non-uremic mice), and it could not be fully explained by changes in total plasma cholesterol [1]. It is likely, however, that the higher total cholesterol concentration contributed to the accelerated formation of atherosclerosis in uremic mice. In support of this idea, normocholesterolemic C57BL/6J mice developed no plaques after subtotal nephrectomy [174], whereas the uremic and hypercholesterolemic mice developed severe and advanced lesions [1, 2, 4, 5]. Similar observations were made by other groups [155-157, 175]. The change in the cholesterol distribution between lipoproteins [1] may also have contributed to the effect of CKD on atherosclerosis. A differential atherogenic response to uremia in different parts of the aorta as shown in Figure 1 (i.e. a less pronounced effect of uremia on plaque area in the aortic root than in the total aorta) was also observed by Massy et al. [156]. The impact of unilateral nephrectomy on atherosclerosis, which has also been demonstrated by others [155, 176], was noteworthy. It may be specific to the apoE-/- mouse model, however, since the apoE-/- mouse spontaneously develops renal lesions with lipid deposits in the glomeruli [177].

In conclusion, the studies by Bro et al. and three other groups support the hypothesis that atherogenesis is markedly increased after induction of kidney failure in mice with genetical hyperlipidemia.

3.3 PLAQUE COMPOSITION IS SIMILAR IN CLASSICAL AND UREMIC ATHEROSCLEROSIS

The studies by Bro et al. [1, 2] suggested that the composition of uremic plaques did not differ from classical atherosclerotic lesions. Thus, early uremic lesions contained lipid-filled macrophages in the intima [2], and advanced uremic lesions showed accumulation of extracellular lipids, lipid-filled macrophages, and collagen-rich connective tissue [1] (Figure 2). Biochemical analyses demonstrated increased free and esterified cholesterol in uremic aortas [2], which is pathognomonic of classical atherosclerosis. These findings are in agreement with those of Buzello et al. [155]. Massy et al. [156] found that the macrophage infiltration (percentage of plaque area) was similar in aortic plaques from uremic and non-uremic apoE-/- mice, whereas the collagen content was higher in the uremic plaques.

In conclusion, both morphologic and biochemical analyses of aortas suggested that accelerated initiation and expansion rather than a specific uremic lesion composition characterize atherosclerosis in the uremic mice.

3.4 UREMIA-INDUCED GENE EXPRESSION CHANGES IN THE ARTERIAL WALL

Effects on vascular inflammation

In uremia, signs of increased inflammation including increased plasma concentrations of soluble portions of major adhesion molecules, such as ICAM-1 and VCAM-1 [110, 178, 179] are seen. Moreover, uremic plasma causes increased expression of mRNA for ICAM-1, VCAM-1 and E-selectin and enhanced shedding of the soluble parts of these adhesion molecules when added to cultures of vascular endothelial cells [180]. Increased expression of adhesion molecules represents a key event in atherogenesis initiation by mediating the recruitment of mononuclear white blood cells to the in-

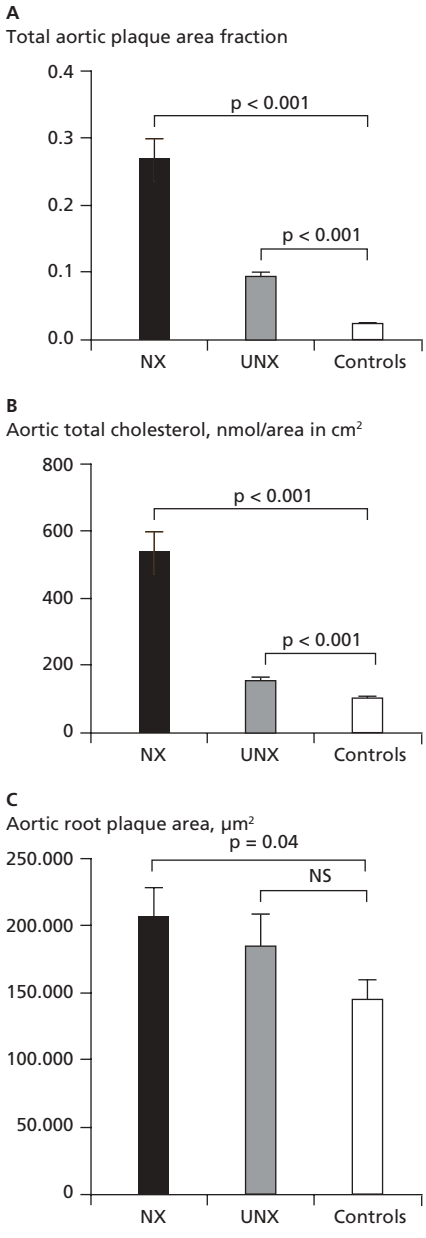

Figure 1. Severity of aortic atherosclerosis in apoE -/- mice with chronic uremia. Aortic atherosclerosis was measured at 22 weeks after 5/6 nephrectomy (NX, n=28), unilateral nephrectomy (UNX, n=24) or no-surgery (Controls, n=23). Aortic plaque area fraction (**A**), aortic total cholesterol content (**B**), and aortic root plaque area (**C**). Values are mean ± SEM. NS = not significant (modified from [1]).

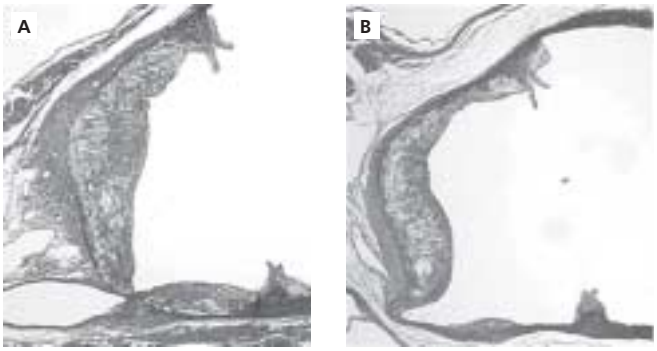

Figure 2. Representative micrographs showing histopathology of atherosclerotic plaques in an apoE -/- mouse with chronic uremia and a control mouse. Aortic atherosclerosis was measured at 22 weeks after 5/6 nephrectomy. (**A**) Elastic trichrome-stained cross-section of the aortic root from an apoE -/- mouse with chronic uremia and advanced plaque formation. The plaque extends into the media and consists of lipid-filled macrophages, extracellular lipids, and collagen-rich connective tissue. (**B**) Elastic trichrome-stained cross-section of the aortic root from a control apoE-/- mouse (no surgery) [1].

tima [181, 182]. Thus, Bro et al. [2] tested the hypothesis that the expression of major adhesion molecules might be upregulated in aortas of uremic mice. Interestingly, uremic aortas had increased expression of ICAM-1 and VCAM-1. The expression of ICAM-1 and VCAM-1 is regulated transcriptionally in a nuclear factor-κB (NF-κB) dependent fashion [183]. NF-κB is a key transcription factor in vascular inflammation and is activated by proinflammatory cytokines, angiotensin II, atherogenic lipoproteins, advanced glycation end products (AGEs), and reactive oxygen intermediates [184]. The above-mentioned stimuli are all characteristically in excess in uremia, suggesting that NF-κB activation may trigger the observed increases of ICAM-1 and VCAM-1 expression.

To identify novel genes of importance to the development of uremic arterial disease, Bro et al. [3] performed micro-array analyses of aortic RNA from uremic and control (sham-operated) apoE -/- mice before and after lesion formation was initiated. With the micro-array analysis, it was possible to examine the expression of 6,000 characterized genes on a single assay. The gene expression changes were verified by real-time PCR quantification of 8 selected genes using RNA from individual mouse aortas.

Two weeks after surgery, i.e. before lesion formation, 23 of the 41 differentially expressed genes were related to inflammation (all were increased). This observation is in close accordance with the above findings. It was striking that 16 of the 41 differentially expressed genes after 2 weeks were related to production of immunoglobulins. Although no B-lymphocytes were seen in sections of non-lesioned uremic aortas, the result could reflect that uremia is accompanied by a humoral immune response. Indeed, a later study by Bro et al. [4] could demonstrate a marked increase of plasma titers of antibodies against OxLDL 2 weeks after the induction of uremia.

In mice with early atherosclerotic lesions, 24 genes were differentially expressed in uremic compared with control mice [3]. Nine transcripts involved in inflammation were all upregulated (1.8- to 8.7-fold) and included osteopontin, matrix metalloproteinases-3 and -12, VCAM-1, and serum amyloid A. All of these genes have been implicated in atherogenesis.

In conclusion, the above findings support the notion that an augmented inflammatory response in the arterial wall may be an important impetus for initiation and expansion of atherosclerosis in uremia.

Effects in the arterial media

In the uremic mice with early atherosclerotic lesions, a prominent change observed with the micro-array analysis of aortic RNA was 3.3- to 142-fold downregulations of transcripts assigned to muscle structure and development, e.g. myosin and α-actin encoding genes. The ≥ 3.3 fold downregulation of expression of muscle cell assigned genes (corresponding to ≥ 70% reductions) suggested that uremia in apoE-/- mice affects smooth muscle cells at all sites within the arterial wall and not only beneath the lesions, since only 3.9% of the aortic surface area contained lesions.

To expand this idea, Bro et al. [3] compared the expression of both macrophage assigned and muscle cell assigned genes in lesioned versus non-lesioned areas of the aortas from non-uremic apoE-/- mice with classical atherosclerosis. Noteworthy, in the lesioned areas ~ 30% was lesion-covered, and the lesions were more advanced than those in the uremic apoE-/- mice. Accordingly, macrophage assigned genes displayed more pronounced increases in expression in lesioned versus non-lesioned areas of the aortas from non-uremic apoE-/- mice with classical atherosclerosis than in the uremic versus sham apoE-/- aortas. In contrast, the downregulation of muscle cell assigned genes was similar or more pronounced in the uremic versus sham apoE-/- aortas as compared with lesioned versus non-lesioned areas of non-uremic apoE-/- mouse aortas.

To further evaluate the structural characteristics of the arterial media in uremic mice, Bro et al. [3] examined aortas with advanced atherosclerosis from uremic and control mice by electron microscopy. The ultrastructure of the media was different in the uremic compared with the sham-operated apoE -/- mice, both underneath

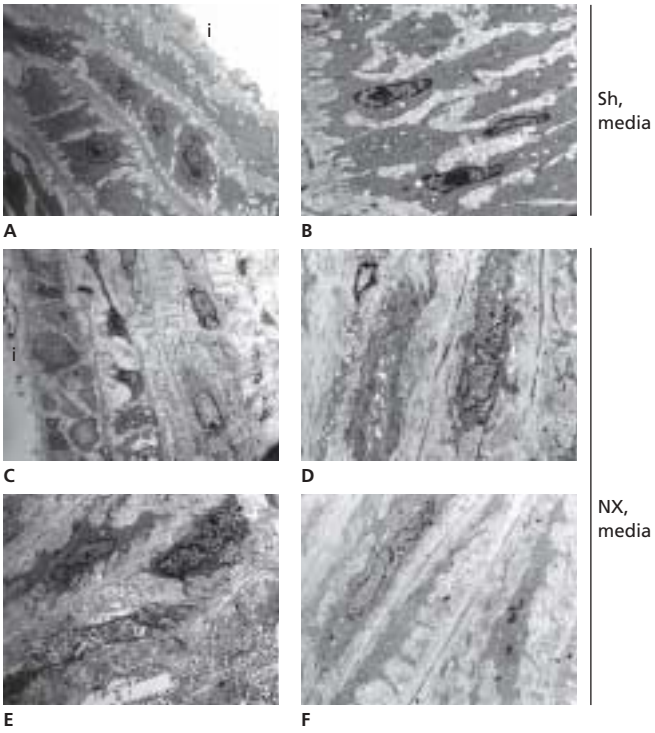

Figure 3. Electron microscopy showing altered ultrastructure of aortic media in apoE-/- mice with chronic uremia. Ultrathin sections of the aortic root from uremic and control mice were examined 37 weeks after 5/6 nephrectomy (NX) or sham-operation (Sh) in apoE-/- mice. **A**, media under nonlesioned intima (i) in a Sh mouse aorta. **B**, media under lesioned intima in a Sh mouse aorta. Note the more vacuolized and irregularly shaped smooth muscle cells and increased deposition of intercellular matrix as compared with A (media under nonlesioned intima in Sh mouse). **C**, media under nonlesioned intima (i) in a NX mouse aorta. The density of the smooth muscle cells was decreased with more intercellular matrix and some of the smooth muscle cells were irregularly shaped and vacuolized, as compared with A (media under nonlesioned intima in Sh mouse). **D** to **F**, media under lesioned intima in NX mouse aorta. In comparison with media under nonlesioned intima in NX mice (C), as well as media under intimal lesions in Sh mice (B), the smooth muscle cells were more vacuolized and surrounded by increased intercellular matrix (D). Under lesioned intima in NX mouse aorta some smooth muscle cells were necrotic (E), and others were irregularly shaped with large projections (F); those alterations were not seen under nonlesioned intima in NX mouse aortas or in sham mouse aortas. Original magnification 3500 × (B, D, E), 2800 × (A and F), 2200 × (C) (modified from [3]).

non-lesioned and lesioned intima (Figure 3). The smooth muscle cells in uremic aortas were characterized by more irregular shaping, vacuolization, and necrosis, and were fewer in number than in control aortas. These observations agree with the results of the micro-array analysis and support the notion that uremia has a marked effect on smooth muscle cells in the arterial media. The morphological alterations in the media of apoE-/- mice were similar to those described by Ejerblad et al. in aortas from uremic rats [143, 185], and in radial arteries from uremic patients [186]. Disorganized arrangement of smooth muscle cells and increased extracellular matrix were also described by Moe et al. [187] in inferior epigastric arteries with medial calcifications from patients with stage 5 CKD undergoing renal transplantation.

Noteworthy, in contrast to the finding in apoE-/- mice, Bro et al. did not observe changes in muscle cell biology related genes in uremic normocholesterolemic wild-type mice [3]. This may reflect that hypercholesterolemia is required to change smooth muscle cell gene expression in uremic mouse aortas and raises the possibility that the changes in smooth muscle cell biology may be associated with the accelerated intimal lesion formation. It is also possible that other metabolic disturbances, e.g. abnormalities in calcium and phosphate metabolism, or apoE in itself, may play a role. Uremic apoE-/- mice [2] and uremic rats [143] display increased plasma calcium × phosphate products, whereas the calcium × phosphate product was not increased by induction of uremia in the wild-type mice in the study by Bro et al. [3]. Interestingly, it was possible to reduce medial smooth muscle cell necrosis (and almost completely prevent medial calcifications) by parathyroidectomy prior to 5/6 nephrectomy in rats [143]. Recently, Shanahan et al. [188, 189] and others [190-192] have emphasized the importance of vascular smooth muscle cell damage (apoptosis) and osteoblastic transformation in uremic vascular disease. Experimental in vitro data suggest that multiple factors, such as elevated levels of circulating calcium and phosphate, reactive oxygen species(ROS), oxidized lipids, AGEs, and inflammatory cytokines, may contribute to the vascular smooth muscle cell changes seen in CKD [193-197].

In conclusion, the gene expression analysis, in addition to showing increased inflammation, suggests that uremic vasculopathy in apoE-/- mice is characterized by a uremia-specific medial smooth muscle cell degeneration. This observation was supported by electron microscopy studies. Noteworthy, other changes in gene expression patterns than those seen by Bro et al. in uremic aortas may have been overlooked due to limitations of the micro-array analysis. Hence, the micro-arrays used in the study by Bro et al. did not cover the complete mouse genome (such micro-arrays did not become available until 2004).

3.5 UREMIA IS ASSOCIATED WITH DEVELOPMENT OF AN IMMUNE RESPONSE AGAINST OXIDIZED LDL

Uremia is associated with increased plasma levels of markers of oxidative stress, and reduced levels of antioxidants [103, 104]. Aortic lesions in uremic apoE -/- mice display a marked accumulation of nitrotyrosine (a marker of ROS-modification of proteins) [1, 155], and express receptors for AGEs (RAGE) [155], which upon activation may lead to prooxidative changes including increased expression of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and formation of ROS [198]. Increased production of ROS may promote generation of OxLDL [199], which has important proatherogenic effects in the vasculature including endothelial damage and accelerated foam cell formation [28, 200-202]. In addition, the formation of oxidized neoepitopes in LDL can elicit an immune response with formation of antibodies against OxLDL [200, 201, 203]. Indeed, patients with CKD display both elevated levels of OxLDL and titers of antibodies against OxLDL [204, 205].

Bro et al. [4] tested the idea that accelerated atherogenesis in uremic mice is associated with development of an immune response against OxLDL. In fact, acute uremia led to a rapid immune response in the uremic apoE-/- mouse model, as indicated by marked increases of titers of IgM antibodies against OxLDL 2 weeks after NX. The formation of antibodies against OxLDL 2 weeks after induction of uremia likely reflects increased generation of OxLDL. Hence, the circulating levels of the oxidized phospholipid epitope EO6 present on apoB-100 were increased in uremic mice [4]. Interestingly, in normocholesterolemic wild-type mice, the antibody response to OxLDL on the induction of uremia by NX was similar to that in apoE-/- mice indicating that this effect was not dependent on hypercholesterolemia [4]. All together, the studies by Bro et al. suggest that uremia is associated with development of an immune response against OxLDL in mice. Even though increased oxidative stress is a known proatherogenic stimulus [201, 202], it is not clear whether the induced antibodies themselves have pro- or antiatherogenic properties. In LDLR-/- mice, titers of antibodies against OxLDL are positively associated with atherosclerosis [206]. Nevertheless, vaccination with OxLDL protects animal models against atherosclerosis [207-209]. Studies are currently in progression to determine the effect of vaccination with OxLDL on atherogenesis in uremic apoE-/- mice (our group).

3.6 THE PROATHEROGENIC EFFECT OF UREMIA IN MICE IS PREVENTABLE BY INHIBITION OF THE RENIN ANGIOTENSIN SYSTEM

Besides the dysregulation of extracellular fluid volume and vasoconstriction, one of the most deleterious actions of RAS is activation of NADPH oxidase by angiotensin II, resulting in formation of ROS, which leads to upregulation of inflammatory mediators including cytokines, chemokines and adhesion molecules, and ROS scavenging of NO, e.g. [210, 211]. These events may promote endothelial dysfunction, and progression of atherosclerosis [210, 211]. Moreover, treatment with ACE inhibitors or angiotensin II receptor antagonists has previously been reported to be anti-inflammatory [212] and to inhibit LDL oxidation in vitro [213, 214]. Bro et al. [4] therefore examined whether the proatherogenic effect of uremia would be preventable by RAS inhibition in apoE-/- mice.

Three different findings supported that this is the case. Firstly, the effect of uremia on atherosclerosis was essentially eliminated by an ACE inhibitor (enalapril 12 mg/kg/d), when the treatment was started 4 weeks after NX, i.e. before or at a very early stage of lesion development (Figure 4A). Secondly, when enalapril treatment was initiated 20 weeks after surgery, i.e. at a time where lesion formation is expected to be extensive, the mean aortic plaque area fraction was reduced by ~30% [4].

Thirdly, enalapril and losartan (an angiotensin II receptor blocker) both reduced aortic atherosclerosis in uremic apoE-/- mice (Figure 4B). Although hydralazine was as effective as enalapril and losartan in lowering the BP, it did not reduce atherosclerosis in uremic apoE-/- mice (Figure 4B). This finding is in agreement with a BP-independent reduction of atherosclerosis by losartan in unilaterally nephrectomized apoE-/- mice [176].

The effect of enalapril on atherosclerosis was parallelled by reductions of the aortic expression of VCAM-1 mRNA (Figure 5A) and the plasma concentrations of sVCAM-1 and sICAM-1 [4]. Also, enalapril attenuated the increase of IgM antibodies against OxLDL (Figure 5B), when treatment was started immediately after induction of uremia.

In conclusion, these results suggest that RAS blockade prevents the proatherogenic effect of uremia in mice. The impact of RAS inhibition on atherosclerosis was at least partly independent of BP-lowering and possibly reflects anti-inflammatory and antioxidative effects.

3.7 THE PROATHEROGENIC EFFECT OF UREMIA IN MICE IS REDUCED BY BLOCKADE OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS

Both diabetes and kidney dysfunction cause increased plasma concentrations of AGEs [215, 216]. AGEs are formed by non-enzymatic

glycation in a series of biochemical reactions between glucose and reactive carbonyl compounds, proteins, lipids or nucleic acids [217, 218]. They bind to and activate the receptor for AGEs (RAGE) [198]. Other RAGE ligands include S100/calgranulins, amphoterin, and the β2-integrin Mac-1 [219, 220]. RAGE is expressed in cultured endothelial cells, monocytes/macrophages, and smooth muscle cells [198]; all three cell types participate in atherosclerotic lesion formation. In vitro, ligand interaction with RAGE leads to prooxidative changes including increased expression of NADPH oxidase and formation of ROS [198]. RAGE stimulation may also activate NFκB and as such increase the expression of adhesion molecules and other proinflammatory molecules [198]. Upregulation of vascular RAGE expression has been demonstrated in both diabetic individuals and uremic individuals without diabetes [221, 222]. A role of RAGE in atherogenesis is suggested by reduced development of atherosclerosis in diabetic apoE-/- mice upon blockade of RAGE with a soluble extracellular ligand-binding domain of RAGE [223, 224].

Previous studies developed a monoclonal RAGE-antibody that blocks RAGE and inhibits signaling events elicited by RAGE ligands in vitro [225] and adverse renal effects in diabetic mice in vivo [226, 227]. To test the involvement of RAGE in development of uremic atherosclerosis, Bro et al. [5] treated uremic apoE-/- mice with the RAGE-antibody or an isotype-matched, control antibody for 12 weeks. The RAGE-antibody reduced the aortic plaque area fraction by 59% (Figure 6A).

Treatment with RAGE-antibody reduced total plasma levels of the oxidized phospholipid epitope (EO6) (Figure 6B). Moreover, the titers of IgG antibodies against both malondialdehyde modified (MDA)-LDL and Cu^{2+}-oxidized (CuOx)-LDL (Figure 6C) were also reduced in the RAGE-antibody treated group. OxLDL have several proatherogenic effects including endothelial damage and accelerated foam cell formation [28, 200-202]. Thus, an antioxidative effect of the RAGE-antibody could explain the reduced development of atherosclerosis in RAGE-antibody treated uremic apoE-/- mice.

It is unknown whether the effect of RAGE blockade reflects a direct effect of RAGE in the arterial wall in addition to a systemic decrease in oxidative stress. Although RAGE mRNA was expressed in the arterial wall and the expression was increased in uremic versus non-uremic mice, the tissue expression was >1000-fold lower in the aorta than in the lung [5]. Treatment of uremic mice with the RAGE-antibody did not affect aortic mRNA expression of VCAM-1 and ICAM-1 or other selected inflammatory genes [5]. Thus, these results contrast the findings in both type 1 and type 2 diabetic apoE-/- mice where treatment with a soluble extracellular ligand-binding domain of RAGE resulted in decreased VCAM-1 protein expression in aorta [224, 228]. The apparent discrepancy might reflect subtle differences in effects of the two modalities used to block RAGE, e.g. that different downstream pathways may be affected depending on the agent used for RAGE blockade. Also, it is possible that the effects of RAGE blockade on atherosclerosis in uremic apoE-/- mice depend on other effects than those mediated through NF-κB. Hence, a previous in vivo study also saw no effect on NF-κB activation, despite effects on renal morphology and function when treating diabetic mice with the RAGE-antibody [226].

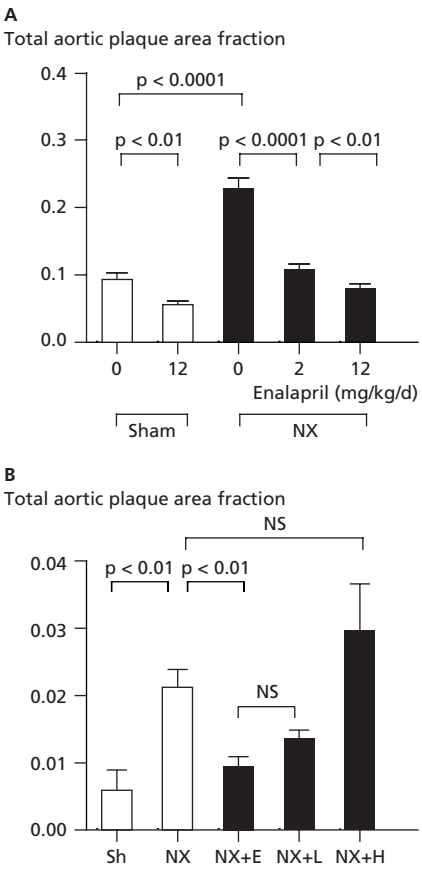

Figure 4. Effect of treatment with enalapril, losartan, and hydralazine on atherosclerosis in uremic apoE-/- mice. (**A**) Aortic atherosclerosis was measured at 36 weeks after 5/6 nephrectomy (NX) or sham-operation in apoE-/- mice. Enalapril administration was started 4 weeks after NX or sham-operation. □, Sham-operated mice treated with 0 or 12 mg/kg/d of enalapril (n = 22 and 24, respectively); ■, uremic mice treated with 0, 2 or 12 mg/kg/d of enalapril (n = 20, 21 and 23, respectively). Values are mean ± SEM. (**B**) Aortic atherosclerosis at 16 weeks after 5/6 nephrectomy (NX) or sham-operation in apoE-/- mice. Drugs were administered from 1 week after NX and continued for 15 weeks. Sh, sham-operated mice receiving no treatment (n = 6); NX, uremic mice receiving no treatment (n = 10); NX+E, uremic mice treated with enalapril 12 mg/kg/d (n = 13); NX+L, uremic mice treated with losartan 30 mg/kg/d (n = 15); NX+H, uremic mice treated with hydralazine 55 mg/kg/d (n = 7). Values are mean ± SEM. NS = not significant [4].

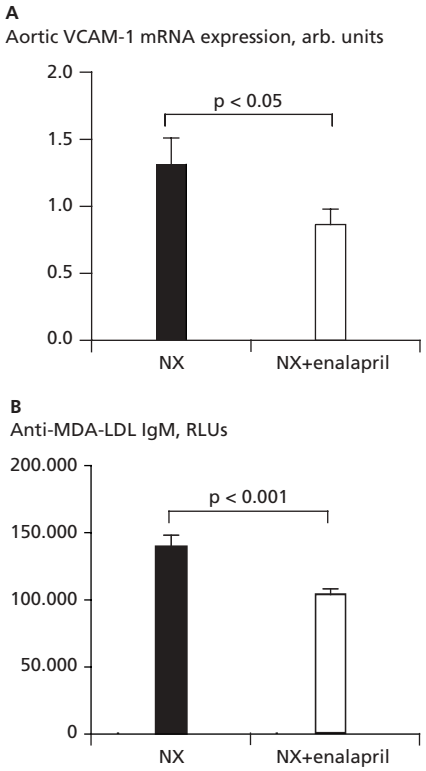

Figure 5. Effect of enalapril on aortic mRNA expression of VCAM-1 and plasma titers of antibodies against oxidized LDL in apoE-/- mice with acute uremia. **A**) Aortic mRNA expression of VCAM-1 two weeks after 5/6 nephrectomy (NX) in apoE-/- mice. NX, uremic mice receiving no medication (n = 9), NX+enalapril, uremic mice treated with enalapril 12 mg/kg/d from day 0 after NX (n = 14). The mRNA expression of VCAM-1 was normalized by GAPDH. **B**) Plasma titers of IgM antibodies (Relative light units, RLUs) reacting with malondialdehyde modified (MDA)-LDL two weeks after NX in apoE-/- mice. NX mice (n = 14); NX+enalapril mice (n = 16) [4].

3.8 NO EFFECT OF UREMIA ON CARDIAC STRUCTURE OR FUNCTION IN APOE-/- MICE

Essential hypertension (with pressure overload of the left ventricle) causes the same type of structural and functional changes in the heart as seen in uremia [229]. Uremia is often accompanied by hypertension and metabolic disturbances such as anemia, hyperphosphatemia, hyperparathyroidism, microinflammation, and activation of the RAS that could also confer negative effects on the heart [230, 231]. The relative importance of hypertension and direct metabolic effects in the development of uremic cardiomyopathy is unknown.

Uremic rats and CD-1 mice that display hypertension after NX develop LVH, diastolic dysfunction, and cardiac fibrosis [232-234]. Since C57BL/6J apoE-/- mice are resistant to development of hypertension after renal mass reduction [1, 4, 155, 156, 172, 173], Bro et al. reasoned that the uremic apoE-/- mouse model could be used to assess the putative role of BP-independent effects of uremia on the heart.

Thirty-six weeks after NX, heart wet weight, echocardiographic estimates of left ventricular mass and left ventricular diastolic and systolic functions were similar in uremic and control (sham-operated) mice [6]. Furthermore, uremia did not increase cardiac fibrosis or cardiac mRNA expression of biglycan or procollagen [6]. Since uremia has no effect on BP in apoE-/- mice, the results may reflect that hypertension is important for development of left ventricular disease in uremia. Indeed, dogs with remnant kidneys are also resistant to induction of hypertension, and according to Tatematsu et al. [235] cardiac dysfunction is not apparent in the uremic dog model.

It should be noted though that echocardiography is observer-dependent and has inherent limitations and thus may have overlooked changes in cardiac function. Indeed, flow Doppler assessments are not as sensitive as tissue Doppler imaging and invasive physiological measurements to detect changes in left ventricular diastolic function. Still, with the same equipment as in the present study it has previously been possible to demonstrate subtle changes in both systolic and diastolic cardiac function in diabetic and obese mice [134, 236]. Bro et al. [6] did not see calcifications in the uremic mouse hearts either by von Kossa staining or by electron microscopy of the myocardium from 3 uremic mice. It has been proposed that myocardial calcifications often encountered in uremic individuals contribute to systolic and diastolic cardiac dysfunction and arrhythmias [237]. Thus, the lack of effect of uremia on cardiac structure and function could also reflect the absence of calcifications in the apoE-/- uremic mouse model. Future studies, e.g. comparing cardiac structure and function in uremic mice with and without hypertension (e.g. in CD-1 versus C57BL/6J wild-type mice), could be useful to shed further light on the pathogenesis of heart dysfunction in uremia.

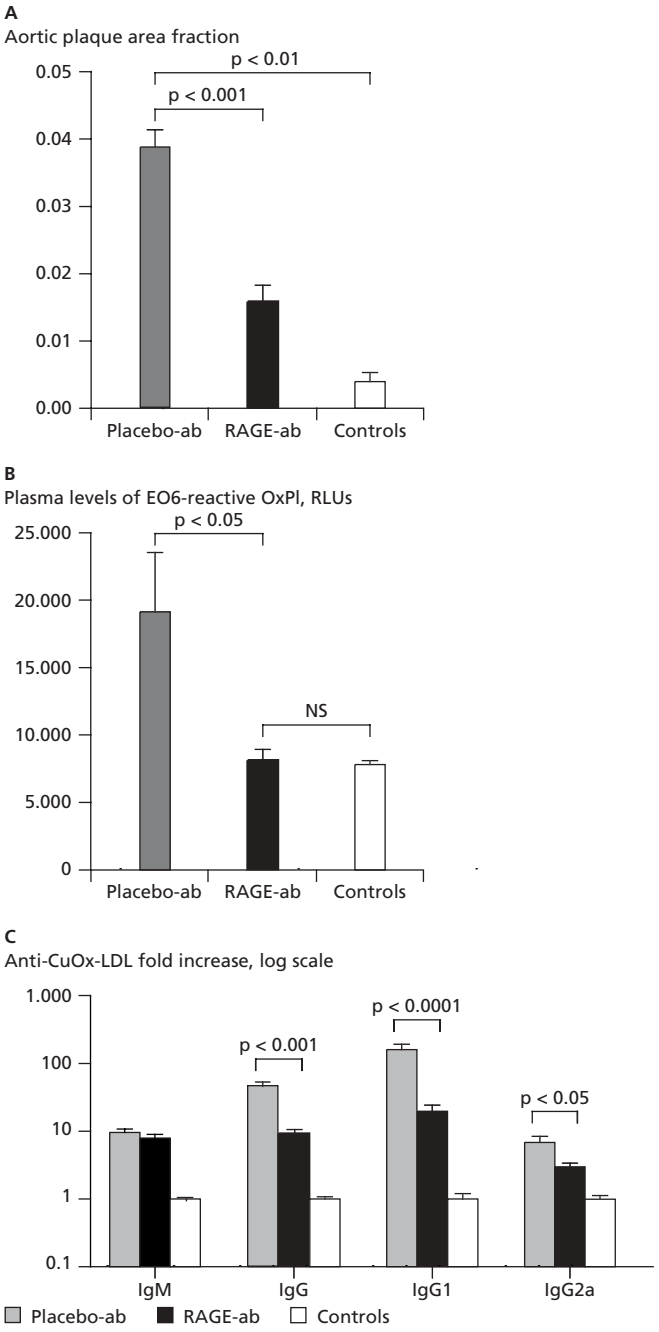

Figure 6. Effects of uremia and RAGE-antibody treatment on development of aortic atherosclerosis, plasma concentrations of EO6-reactive phospholipid epitopes, and formation of antibodies against oxidized LDL in apoE-/- mice. Measurements were performed 16 weeks after 5/6 nephrectomy. RAGE-antibody (RAGE-ab) or placebo-antibody (placebo-ab) were administered during weeks 4 to 16 after 5/6 nephrectomy. Control apoE-/- mice did not undergo surgery, and received no treatment. (**A**) Aortic atherosclerosis (**B**) Plasma concentrations of E06-reactive oxidized phospholipid (OxPL) epitopes (**C**) Plasma titers of antibodies reacting with Cu^{2+}-oxidized (CuOx) LDL. Antibody titers in uremic mice were normalized to control values. Antibody titers and EO6/OxPl reactivity were measured in relative light units (RLUs). All values are mean +SEM [5].

4. LIMITATIONS, CONCLUSIONS, AND PERSPECTIVES

LIMITATIONS

Indeed, the mouse model of uremic atherosclerosis described in the present thesis work has several limitations, including absence of vascular calcifications, unphysiological lipoprotein metabolism, and absence of hard end points such as myocardial infarction or stroke.

In humans [37, 187], rats [143] and rabbits [144], uremia is accompanied by vascular calcifications. Also, in non-uremic apoE-/- mice between 45 and 75 weeks of age calcifications can be found in atherosclerotic lesions [238]. Of note, although the uremic apoE-/- model has increased plasma calcium × phoshate product, the studies of Bro et al. [1] and Buzello et al. [155] of 22 to 29 weeks old male apoE-/- mice did not reveal calcifications of the arterial wall on histological analysis of the aortic roots (including von Kossa stained sections) or by microradiographs of the entire aortas. Neither was any calcium deposition seen in the media on electron microscopy of aortas from 48 weeks old male uremic apoE-/- mice, although matrix vesicles (potential nidus of microcalcification) were observed [3]. Similarly, a histologic study of 38 weeks old female uremic apoE-/- mice could only demonstrate sporadic calcium deposits in the aortic intima (Tanja X. Pedersen, 2007, personal communication). The cause of the lack of vascular calcifications is not clear. It may be related to the marked upregulation of osteopontin, which was seen both within the intimal lesions and in the media underneath the lesions [3]. Osteopontin is believed to inhibit mineralization in bone [239] and vascular tissue [240]. Accordingly, excessive vascular calcifications develop in osteopontin-deficient male apoE-

-/- mice [241]. In contrast to the findings by Bro et al. [1, 3] and Buzello et al. [155], however, Massy et al. [156] showed accentuated calcium deposition both in the intima and media of aortas from 16-weeks-old male and female apoE-/- mice as early as 6 weeks after induction of uremia. A putative explanation of this difference may be a higher vitamin D_3 content and calcium to phosphate ratio in the mouse diet used by the latter group (high carbohydrate Harlan Teklad Global Diet), and perhaps the inclusion of female mice in their study, since Massy et al. [156] found that female mice had faster progression of vascular calcification than their male counterparts. However, the plasma levels of total calcium and phosphate were not higher in uremic mice fed the Harlan Diet as compared to those fed the standard Altromin Diet used by Bro et al. and Buzello et al., nor were the plasma cholesterol concentrations. Interestingly, Professor Massy's group did not see significantly increased osteopontin expression in aortas of uremic mice [242]. Increased intimal and medial calcification of the aortic root with no reported difference between genders was observed by Davies et al. [157] in uremic LDLR -/- mice, when these mice were fed a high-fat diet from Harland Teklad. Studies by our group in a double knock out mouse model (apoE-/-,OPN-/-) are currently in progression to examine the effect of osteopontin (OPN) deficiency on arterial disease in uremic apoE -/- mice.

The unphysiological lipoprotein metabolism due to apoE deficiency implies that the uremic apoE-/- mouse model is not suitable for studying uremia-induced changes of lipoprotein metabolism. Also, macrophage-derived apoE has direct effects on the arterial wall [243].

The apoE-/- mouse model develops human-like advanced atherosclerosis, but plaque rupture and trombosis is exceedingly rare in these mice [152, 153]. Even if atherosclerosis was markedly enhanced by uremia, no myocardial infarctions were observed in the uremic apoE-/-mice studied by Bro et al. Thus, only intermediate CV end points can be studied in this model.

CONCLUSIONS AND PERSPECTIVES

The mouse studies by Bro et al. showed that uremic vasculopathy in apoE-/- mice, besides accelerated intimal atherosclerosis, is characterized by a uremia-specific medial smooth muscle cell degeneration. Further, the studies suggested that vascular inflammation and systemic oxidative stress may explain some of the proatherogenic effects of uremia in mice.

The potential role of the uremia-associated adaptive immune response against neoepitopes in oxidized LDL in the atherogenic process is complex and remains unresolved. Studies are in progress to determine the effects of vaccination with OxLDL on atherogenesis in uremic apoE-/- mice.

Since 2003 three independent groups have been working with the uremic apoE-/- mouse model. A series of interesting intervention studies have been performed (Table 1). Bro et al. showed that accelerated atherosclerosis in uremia could be prevented by RAS inhibition, or markedly reduced by RAGE blockade, probably through anti-inflammatory and antioxidative effects. Accordingly, it was demonstrated that the antioxidant N-acetyl-cysteine [244] and the phosphate binder sevelamer [170] reduced both uremia-enhanced atherosclerosis and the aortic accumulation of nitrotyrosine (a marker of oxidative stress) in aortic lesions. This suggests antioxidative effects of these agents. Certainly, in human studies sevelamer proved to have several effects in addition to phosphate binding, such as lowering of plasma CRP and LDL cholesterol [245]. Remarkably, none of the agents reducing atherosclerosis in uremic mice, had any influence on plasma cholesterol concentrations. The mechanism by which the calcimimetic R-568 reduced the progression of atherosclerosis in apoE-/- mice remains to be elucidated [246]. Neither treatment with a statin (simvastatin) [247], nor repeated injection of human apolipoprotein A-1 (apoA-1), the major protein component in HDL which has been shown to promote reverse cholesterol transport [248, 249], showed any effect on aortic atherosclerosis in uremic mice [250], but the importance of these findings in relationship to human disease is unclear.

Table 1. Invervention studies in the uremic apoE-/- mouse model.

Treatment	Effect on aortic atherosclerosis	Vascular calcification	Reference
Enalapril/losartan	↓	NA	4
Anti-RAGE antibody	↓	NA	5
N-acetylcysteine	↓	NA	244
Sevelamer	↓	↓	170
R-568	↓	↓	246
Simvastatin	↔	↓	247
Human ApoA-1	↔	NA	250
Calcium carbonate	↔	↓	242

NA: not available

In patients with CKD, more RCT trials are needed to establish the role of antioxidants, RAS inhibitors and other agents in the prevention of CVD.

It should be kept in mind that the advances in knowledge about pathogenesis of CVD in CKD obtained in mice may not be valid in relation to patients with CKD. Nevertheless, the new uremic mouse model has provided a tool to identify molecular responses of the arterial wall to uremia, and may help identify new approaches for treatment and prevention of atherosclerotic disease in uremia. Also, the data obtained with the mouse model provide a platform for further studies in humans. Certainly, knowledge is needed to enable identification of modifiable risk factors in patients not yet on dialysis. One way of obtaining such information could be to identify specific serum protein expression patterns, as determined by mass spectrometry, and specific aspects of the humoral immune response against OxLDL, together with specific plasma markers of inflammation and oxidative stress that can be used to stratify uremic patients into those at high and low risk of CVD. The identification of such patterns may provide diagnostic tools as well as outline mechanisms that can be targeted specifically to prevent CVD in uremic patients. Also studies of gene expression changes in radial artery biopsies acquired during arteriovenous fistula formation could help to identify specific arterial gene expression patterns related to the development of CVD in uremia. If the products of some of the differentially expressed genes are secreted into plasma, it will be possible to assess whether plasma concentration measurements can be used to assess the risk of CVD.

The increasing prevalence of CKD calls for continued research efforts, involving both basal, animal based, and human studies to improve and renew patient care. The uremic apoE-/- mouse model provides a valuable animal model to study vascular effects of CKD.

5. SUMMARY

The purpose of this thesis work was to establish an experimental mouse model for studying the pathogenesis and therapy of accelerated atherosclerosis in uremia. Uremia was induced by surgical 5/6 nephrectomy in apolipoprotein E-deficient (apoE-/-) mice and led to development of severe aortic atherosclerosis independently of BP and plasma homocysteine levels. Also, the accelerated atherosclerosis could not be fully explained by changes in total plasma cholesterol. Morphologic and biochemical analyses of aortas suggested that accelerated initiation and expansion rather than a specific uremic lesion composition characterize atherosclerosis in the uremic mice. Increased expression of inflammatory genes in aortas of uremic mice suggests that an augmented inflammatory response in the arterial wall might be an important impetus for accelerated atherosclerosis in uremia. A marked downregulation of expression of smooth muscle cell assigned genes indicates that besides intimal atherosclerosis, uremic vasculopathy in apoE-/- mice is characterized by a uremia-specific medial smooth muscle cell degeneration. Oxidative stress could also be important for the development of

atherosclerotic lesions in uremia. In the mouse model, uremia led to a marked increase of titers of antibodies against oxidized LDL (Ox-LDL), and increased circulating levels of the oxidized phospholipid epitope EO6. Treatment with enalapril (an ACE inhibitor) almost completely prevented the development of accelerated aortic atherosclerosis in uremic mice. This effect was parallelled by reductions of aortic expression of the proinflammatory adhesion molecule VCAM-1, and plasma titers of IgM antibodies against OxLDL, and was at least partly independent of BP-lowering. To test the involvement of the receptor for advanced glycation end products (RAGE) in development of uremic atherosclerosis, uremic mice were treated with a neutralizing RAGE-antibody. This treatment reduced the aortic plaque area fraction by 59% in parallel with reductions of the plasma levels of the oxidized phospholipid epitope EO6, and titers of IgG antibodies against OxLDL. As opposed to rats and CD-1 mice, apoE-/- mice did not have impaired cardiac structure and function (as assessed by echocardiography, histology, gene expression analysis) upon the induction of uremia. Since the uremic apoE -/- mouse is normotensive and did not develop myocardial calcifications, it is possible that these factors may be important for the development of cardiac dysfunction in uremia.

In conclusion, the mice studies by Bro et al. showed that uremic vasculopathy in apoE-/- mice, besides accelerated intimal atherosclerosis, was characterized by a uremia-specific medial smooth muscle cell degeneration. Furthermore, the studies suggested that vascular inflammation and systemic oxidative stress may explain some of the proatherogenic effects of uremia in mice. Interestingly, the accelerated atherosclerosis could be prevented by RAS inhibition, or markedly reduced by RAGE blockade, probably through anti-inflammatory and antioxidative effects. The new uremic mouse model has provided a tool to identify molecular responses of the arterial wall to uremia, and may help identify new approaches for treatment and prevention of atherosclerotic disease in uremia. Also, the data obtained with the mouse model provide a platform for further studies in humans.

LIST OF ABBREVIATIONS:

ACE:	Angiotensin converting enzyme
ADMA:	Asymmetric dimethylarginine
AGEs:	Advanced glycation end products
ApoA-1:	Apolipoprotein A-1
ApoB-100:	Apolipoprotein B-100
ApoE:	Apolipoprotein E
ApoE-/-:	Apolipoprotein E-deficient
BP:	Blood pressure
CKD:	Chronic kidney disease
CRP:	C-reactive protein
CuOx-LDL:	Cu2+-oxidized LDL
CV:	Cardiovascular
CVD:	Cardiovascular disease
ELISA:	Enzyme-Linked ImmunoSorbent Assay
EO6:	Oxidized phospholipid epitope recognized by the mouse EO6 antibody
GFR:	Glomerular filtration rate
HDL:	High density lipoprotein
ICAM-1:	Intercellular adhesion molecule-1
IDL:	Intermediate-density lipoprotein
IL:	Interleukin
LDL:	Low density lipoprotein
LDLR-/-:	LDL receptor deficient
LVH:	Left ventricular hypertrophy
MDA-LDL:	Malondialdehyde modified LDL
NADPH:	Nicotinamide adenine dinucleotide phosphate
NFkB:	Nuclear factor-kB
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NX:	5/6 nephrectomy
OPN:	Osteopontin
OxLDL:	Oxidized LDL
PCR:	Polymerase chain reaction
PUFAs:	Polyunsaturated fatty acids
RAGE:	Receptor for AGEs
RAS:	Renin angiotensin system
RCT:	Randomized placebo-controlled trial
ROS:	Reactive oxygen species
TG:	Triglyceride
TLC:	Thin layer chromatography
VCAM-1:	Vascular cell adhesion molecule-1
VLDL:	Very low density lipoprotein

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