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Does statins promote vascular calcification in chronic kidney disease?

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ABSTRACT

Background In end-stage renal disease (ESRD), coronary artery calcification (CAC) and inflammation contribute to cardiovascular disease (CVD). Statins do not improve survival in patients with ESRD, and their effect on vascular calcification is unclear. We explored associations between CAC, inflammatory biomarkers, statins and mortality in ESRD.

Materials and methods In 240 patients with ESRD (63% males; median age 56 years) from cohorts including 86 recipients of living donor kidney transplant (LD-Rtx), 96 incident dialysis patients and 58 prevalent peritoneal dialysis patients, associations of CAC score (Agatston Units, AUs), interleukin-6 (IL-6) with high-sensitivity C-reactive protein (hsCRP), tumour necrosis factor (TNF), use of statins and all-cause mortality were analysed. Cardiac CT was repeated in 35 patients after 1.5 years of renal replacement therapy. *In vitro*, human vascular smooth muscle cells (hVSMCs) were used to measure vitamin K metabolism.

Results Among 240 patients, 129 (53%) had a CAC score > 100 AUs. Multivariate analysis revealed that independent predictors of 1-SD higher CAC score were age, male gender, diabetes and use of statins. The association between CAC score and mortality remained significant after adjustment for age, gender, diabetes, CVD, use of statins, protein-energy wasting and inflammation. Repeated CAC imaging in 35 patients showed that statin therapy was associated with greater progression of CAC. *In vitro* synthesis of menaquinone-4 by hVSMCs was significantly impaired by statins.

Conclusion Elevated CAC score is a mortality risk factor in ESRD independent of inflammation. Future studies should resolve if statins promote vascular calcification and inhibition of vitamin K synthesis in the uremic milieu.

Keywords Chronic kidney disease, inflammation, statin, vascular calcification, vitamin K.

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Introduction

Increased coronary artery calcification (CAC), a common feature in patients with end-stage renal disease (ESRD) [1], is a predictor of increased risk of cardiac events in dialysis [2] and nondialysis [3,4] patients with ESRD. In nonrenal patient populations, individual CAC progression is associated with traditional risk factors including smoking, hypertension and diabetes [5]. Inflammation, another common feature in ESRD [6], plays a pivotal role in processes leading to atherosclerosis

[7] and associates with cardiovascular disease (CVD) [8,9] and poor outcome [10] in ESRD.

Arterial calcification develops at two sites of the arterial wall, the intima and media. Intima calcification represents an advanced stage of atherosclerosis and is associated with the development of plaques and occlusive lesions [11]. Media calcification (arteriosclerosis) is common in ESRD [11] and associates with increased arterial stiffness, an independent predictor of mortality [12] that adds to the predictive value of vascular calcification [13]. Although the two arterial wall sites

of calcification coexist, arterial media calcification is more often observed in patients with ESRD without conventional risk factors [14].

As vascular calcification is associated with higher serum levels of inflammatory biomarkers, such as C-reactive protein (CRP) [15], interleukin-6 (IL-6) [16] and tumour necrosis factor (TNF) [17], a link between inflammation and calcification has been proposed. Whereas Jung *et al.* [18] reported that both altered mineral metabolism and chronic inflammation contribute to rapid progression of CAC, others refuted associations between inflammation and vascular calcification in ESRD [19]. Arteriosclerosis affects the walls of the arteries primarily due to processes linked to ageing, whereas atherosclerosis has more complicated risk factors, such as dyslipidaemia, diabetes, obesity and smoking [20]. Although both arterio- and atherosclerosis are chronic inflammatory conditions [21], the association between inflammation and CAC was reported to be weak [22] and mostly explained by concomitant burden of traditional CVD risk factors.

Despite being effective in lowering LDL cholesterol levels, the 4D [23] and AURORA [24] studies failed to demonstrate a survival benefit of statins in dialysis patients. Moreover, although the SHARP trial [25] demonstrated that simvastatin plus ezetimibe reduced the incidence of major atherosclerotic events in patients with chronic kidney disease (CKD) over a wide range of reduced renal function, no protective effect was observed in dialysis patients. However, as SHARP was analysed as an 'intention to treat' analysis, it should be acknowledged that a substantial proportion of patients who started the trial as CKD stage 3–5 progressed and became incident dialysis patients during the course of the study. The lack of beneficial effects of statins in dialysis patients suggests either that LDL lowering does not confer a benefit in this specific patient population or alternatively that statins have pleiotropic effects that counteract the beneficial effects of LDL lowering. Recent studies in nonrenal patients show that statins associate with an increased prevalence and extent of coronary calcification [26,27].

We investigated potential predictors of CAC score by cardiac computed tomography (CT) scan including inflammation, statins and associations between CAC score and all-cause mortality in 240 patients with ESRD. We also assessed the relation between statin use and changes in CAC score in a subgroup of 35 patients with ESRD undergoing repeated CT scans.

Methods

Study cohorts

A CT scan for CAC imaging was performed in 240 adult patients with ESRD (63% males; median age 56 years) from three ESRD cohorts including 86 patients receiving living donor

kidney transplant (LD-Rtx) [28], 96 incident dialysis patients [10] and 58 prevalent peritoneal dialysis (PD) patients [29] (Table S1). The patients were recruited between March 2008 and December 2014 at the Department of Renal Medicine at Karolinska University Hospital, Stockholm. The Ethics Committee of Karolinska University Hospital approved the study protocols, and informed consent was obtained from all patients. As part of the protocol in LD-Rtx and incident dialysis patients, a second CT scan for CAC imaging was performed after 1.5 years (0.7–2.5 years) in 35 patients with ESRD (58% male, median age 53 years) allowing estimation of absolute ($CAC_{\text{FOLLOW-UP}} - CAC_{\text{BASELINE}}$) and relative ($(CAC_{\text{FOLLOW-UP}} - CAC_{\text{BASELINE}})/CAC_{\text{BASELINE}}$) progression rate [5]. No clinically meaningful significant differences in demographic or biochemical characteristics were observed in comparison with 205 patients with ESRD who completed only one CAC imaging (Table S2).

Coronary calcification imaging and quantification

CAC was assessed by CT scan, an accurate noninvasive approach, performed on a 64-channel detector scanner (Light-Speed VCT; General Electric (GE) Healthcare, Milwaukee, WI, USA) in cine mode. Scans were ECG-gated, and a standard noncontrast protocol was used with a tube voltage of 100 kV, tube current of 200 mA, 350 ms rotation time, 2.5 mm slice thickness and display field of view (DFOV) 25 cm. CAC data were processed and analysed using Advantage Workstation (GE Healthcare). SMARTSCORE 4.0 (GE Healthcare) was used to assess CAC score. CAC was quantified as a lesion with an area $> 1 \text{ mm}^2$ and a peak intensity > 130 Hounsfield Units (HUs) based on the Agatston method previously described in detail and expressed in Agatston units (AUs) [30]. Total CAC score was calculated as the sum of the CAC score in the left main artery, the left anterior descending artery, the left circumflex artery and the right coronary artery. As a CAC score > 100 AUs is associated with an increased risk of myocardial ischaemia and coronary heart disease-related events [31], we used this threshold to identify patients with definite to extensive plaque burden.

Laboratory analyses and other measurements

All blood samples were obtained in the morning after an overnight fast. Serum samples were immediately analysed for standard serum analyses, and the samples were kept frozen at -70°C if not analysed immediately. Plasma IL-6 and TNF were analysed by commercial kits available for an Immulite Automatic Analyzer (Siemens Medical Solutions, Los Angeles, CA, USA) according to the instructions of the manufacturer. Pentraxin-3 (PTX3) was analysed with ELISA kits from R&D systems (Abingdon, UK). Other analyses, including high-sensitivity CRP (hsCRP), intact parathyroid hormone (iPTH), serum lipids and lipoproteins, mineral and electrolytes, were

analysed at the Department of Laboratory Medicine, Karolinska University Hospital, Sweden.

Height and body weight were obtained at the baseline, and body mass index (BMI) was calculated. Subjective global assessment (SGA) was used to evaluate protein-energy wasting (PEW) as previously described [32]. PEW was defined as SGA > 1. Systolic and diastolic blood pressures (BPs) were measured in the morning after a 15-min resting period. Clinical signs of CVD defined as earlier or present occurrence of cerebrovascular, cardiovascular or peripheral vascular disease were described in detail previously [28].

Vitamin K metabolism in vascular smooth muscle cells

Human aortic VSMCs (hVSMCs) were derived from tissue explants as described previously [33] and used between passages 4 and 12. hVSMCs were cultured in M199 medium (Gibco; Invitrogen, Breda, the Netherlands) supplemented with 20% foetal calf serum (FCS) and antibiotics. Cells were seeded 1 : 3 from T25 cm² flasks into six-well plates. When reaching 80–90% confluence, the medium was changed to medium supplemented with phylloquinone (2 µM) or menadiolone (2 µM) either with or without simvastatin (2.5 µM). After 20 h of incubation, cells were washed with Hepes buffer. Cells were collected and sonicated in 1 mL Triton X-100 (1%) in Hepes. Protein determination was carried out by micro-BCA (Thermo Fisher Scientific, Frankfurt Am Main, Germany). For vitamin K analysis, cells were extracted as follows: 0.5 mL of the cell suspension was diluted 1 : 1 with water and then mixed with 2 mL of ethanol containing 400 pg of the internal standard 2',3'-dihydro-phyloquinone. The mixture was homogenized in a blender (UltraTurrax, Janke and Kunkel, Staufen, Germany), 3 × 10 s cycles at 0 °C. Extraction of the K vitamins was carried out using hexane. Vitamin K was analysed by HPLC using a C-18 reversed phase column and fluorometric detection after postcolumn zinc reduction. All values are expressed as means and standard deviation for six wells.

Statistical analysis

Data are expressed as median (range of 10th–90th percentile) or percentage or relative risk ratio (95% CI, confidence intervals), as appropriate. Statistical significance was set at the level of $P < 0.05$. Comparisons between two groups were assessed with the nonparametric Wilcoxon signed-rank test for continuous variables and Fischer's exact test for nominal variables. Comparisons between three groups were performed using non-parametric Kruskal–Wallis ANOVA followed by Dunn's test for multiple comparisons. In the combined cohort of all 240 patients, comparisons between high CAC group and low CAC group were assessed with the generalized linear model (GLM) adjusted by different cohorts; continuous variables were

transformed by the ranks before performing GLM. Spearman's rank correlation analysis was used to determine associations between selected parameters. In patients with ESRD undergoing repeated CT scans, CAC scores at baseline and after a median period of 1.5 years of renal replacement therapy were compared using Wilcoxon matched-pairs signed-rank test. To study the predictors of 1-SD higher CAC score with other parameters, a GLM analysis was performed. Survival analyses were made with Kaplan–Meier survival curve, and the Cox proportional hazard regression model was used to estimate hazard ratios (HRs and 95% CI) for all-cause mortality adjusting for covariates. Statistical analyses were performed using statistical software SAS version 9.4 (SAS Campus Drive, Cary, NC, USA).

Results

Clinical and biochemical characteristics

Demographics and clinical characteristics are shown in Table 1. In the combined cohort, CVD was present in 21% and diabetes in 22% of the patients. As expected, compared to the selected patients in the LD-Rtx cohort, prevalent PD patients were older, had lower levels of serum albumin, 25-OH vitamin D and 1,25-OH vitamin D and had higher levels of HbA1c, triglycerides, cholesterol, inflammatory biomarkers and CAC score at the time of inclusion. The incident dialysis patients were older, had lower levels of albumin and had higher levels of systolic BP, HbA1c, triglycerides, phosphate, inflammatory biomarkers and CAC score compared to patients in the LD-Rtx cohort (Table S1). A total of 129 (53.4%) patients had CAC > 100 AU. They were older and had higher BMI, HbA1c, triglycerides, hsCRP, IL-6 and TNF and had higher prevalence of both clinical CVD and diabetes. The median level of 1,25(OH) vitamin D was lower in patients with CAC score > 100 Au. Patients with CAC score > 100 Au were more often treated by β-blockers and statins than patients with CAC < 100 AU (Table 1).

Basal CAC score independently predicted mortality in the presence of inflammation

During 5 years of follow-up, 44 patients (18%) died. The all-cause mortality rate was higher among patients with CAC > 100 AU as compared to those with low CAC (Fig. 1a), regardless of the use of statins (Fig. 1b) or the presence of inflammation (Fig. 1c). In multivariate Cox proportional hazard analysis, the CAC score was independently associated with all-cause mortality after adjustment for risk factors including age, gender, diabetes, CVD, PEW, statins, hsCRP and modality (Table 2). Neither diabetes nor CVD predicted mortality when CAC score, age, hsCRP and PEW were included in the model.

Table 1 Demographic, clinical and biochemical characteristics in 240 patients with ESRD divided according to CAC score

	Total patients with ESRD (n = 240)	Low CAC (≤ 100 AUs) (n = 111)	High CAC (> 100 AUs) (n = 129)	P-values (Adjusted by cohorts*)
Demography, clinical characteristics and metabolic biomarkers				
Age, years	56 (29, 77)	43 (23, 62)	64 (50, 78)	< 0.001
Male, %	63	57	68	0.052
Diabetes mellitus, %	22	8	33	< 0.001
Cardiovascular disease, %	21	4	36	< 0.001
Body mass index, kg/m ²	24.8 (20.2, 30.3)	24.2 (19.7, 30.1)	25.2 (20.9, 30.8)	0.012
Systolic BP, mmHg	140 (116, 169)	138 (115, 164)	143 (113, 179)	0.135
Diastolic BP, mmHg	83 (67, 97)	85 (67, 98)	80 (67, 96)	0.118
Haemoglobin, g/L	113 (96, 130)	111 (94, 130)	114 (98, 130)	0.076
HbA1c, mmol/mol [†]	40 (28, 58)	35 (23, 47)	45 (33, 68)	< 0.001
Triglycerides, mM	1.5 (0.9, 2.8)	1.4 (0.8, 2.6)	1.8 (0.9, 3.0)	0.011
Cholesterol, mM	4.6 (3.2, 6.3)	4.6 (3.4, 6.0)	4.7 (3.2, 6.6)	0.829
HDL cholesterol, mM	1.3 (0.9, 1.9)	1.3 (0.9, 1.9)	1.2 (0.8, 2.0)	0.104
ApoA, g/L	1.42 (1.09, 1.83)	1.40 (1.10, 1.80)	1.44 (1.08, 1.87)	0.633
ApoB, g/L	0.86 (0.60, 1.31)	0.86 (0.60, 1.31)	0.88 (0.57, 1.35)	0.824
Creatinine, μ M	731 (479, 981)	740 (466, 1079)	716 (491, 931)	0.162
S-albumin, g/L	34 (27, 40)	35 (28, 40)	33 (26, 38)	0.190
SGA > 1, % [‡]	37	36	35	0.379
Coronary artery calcification and inflammation biomarkers				
CAC total score, AUs	164 (0, 2884)	0 (0, 54)	1042 (92, 3633)	–
iPTH, ng/L [§]	271 (96, 653)	250 (102, 610)	302 (79, 689)	0.261
Calcium, mM	2.28 (2.02, 2.54)	2.27 (2.05, 2.52)	2.31 (1.97, 2.54)	0.559
Phosphate, mM	1.8 (1.2, 2.4)	1.7 (1.0, 2.4)	1.8 (1.3, 2.5)	0.538
1,25-OH vitamin D, nM	16 (9, 30)	17 (10, 34)	14 (9, 29)	0.002
25-OH vitamin D, ng/L [¶]	35 (13, 72)	39 (14, 72)	30 (13, 72)	0.186
hsCRP, mg/L	1.7 (0.4, 14.0)	1.2 (0.2, 6.3)	3.6 (0.6, 22.3)	< 0.001
IL-6, pg/mL**	3.6 (0.5, 13.2)	1.9 (0.1, 8.3)	5.5 (1.7, 15.0)	< 0.001
TNF, pg/mL ^{††}	14.8 (9.0, 20.1)	12.2 (8.2, 18.4)	16.2 (10.8, 21.7)	< 0.001
PTX3 ng/mL ^{‡‡}	1.9 (0.7, 6.4)	2.2 (0.6, 7.2)	1.7 (0.7, 5.4)	0.140
Medications				
Statins, %	38	23	50	< 0.001
ACEI/ARB, %	64	66	66	1.000
β -blockers, %	60	53	67	0.006
Ca-blockers, %	49	44	53	0.157

Table 1 Continued

	Total patients with ESRD (n = 240)	Low CAC (\leq 100 AUs) (n = 111)	High CAC (> 100 AUs) (n = 129)	P-values (Adjusted by cohorts*)
Vitamin D analogs, %	85	86	84	0.723
Phosphate binders, %	84	86	82	0.489

BP, blood pressure; HbA1c, haemoglobin A1c; HDL, high-density lipoprotein; Apo A and Apo B, apolipoproteins A and B, respectively; CAC, coronary artery calcification; iPTH, intact parathyroid hormone; SGA, subjective global assessment; hsCRP, high-sensitivity C-reactive protein; TNF, tumour necrosis factor; IL-6, interleukin-6; PTX, pentraxin.

Data presented as median (range of 10th–90th percentile) or percentage.

*dialysis vs. nondialysis

[†]n = 236.

[‡]n = 219.

[§]n = 238.

[¶]n = 165.

**n = 196.

^{††}n = 178.

^{†††}n = 157.

Relation between statin use, basal CAC score and change (Δ) of CAC score

In Spearman's rank (ρ) analysis, the baseline CAC score correlated significantly with age (Figure S1), gender, diabetes, CVD, BMI, HbA1c, triglycerides, HDL-cholesterol, albumin, 1,25-OH vitamin D, inflammation markers, statins, β -blockers and cohort (Table S3). The majority ($n = 70$) of the 89 statin-treated patients received simvastatin; the remaining patients were on atorvastatin ($n = 16$), fluvastatin ($n = 2$) and pravastatin ($n = 1$). The CAC scores in statin and nonstatin users are depicted in Fig. 2. As expected, 89 statin users were older (61 vs. 51 years; $P < 0.001$) and had higher prevalence of diabetes mellitus (34% vs. 15%; $P = 0.002$) and CVD (33% vs. 15%; $P = 0.002$) than 151 patients with ESRD not on statins (Table 3). Significantly higher median iPTH (314 vs. 252 ng/L; $P < 0.05$) and HbA1c levels (44 vs. 38 mmol/mol; $P < 0.05$) in statin users likely reflect the higher prevalence of diabetes. As expected, statin users had lower median cholesterol (4.2 vs. 4.8 mM; $P < 0.001$) and ApoB (0.80 vs. 0.91 g/L; $P < 0.001$) levels as a result of ongoing treatment. Whereas CRP levels did not differ significantly between statin and nonstatin users, median levels of TNF (16.0 vs. 13.7 pg/mL; $P = 0.007$) and IL-6 (4.9 vs. 2.8 pg/mL; $P < 0.05$) were higher in statin users. After adjustments for confounders by GLM (age, gender, BMI, diabetes, inflammation), only age, male gender, diabetes and statins remained significantly related to high CAC score (Table 4). Statins were independently associated with higher CAC score irrespective of the inflammatory biomarker (CRP, IL-6, TNF) included in the model (Table 4). In the multivariate model, neither clinical CVD (considered a result rather than the cause of elevated CAC score), cholesterol and apoB (considered related to statin treatment), nor HbA1c nor triglyceride levels (considered related to diabetes mellitus) were included. As categorization of age may reduce the adjustment power and

leave room for residual confounding, GLM was also performed with age as a continuous variable. The relation between statins and high CAC score remained significant.

In 35 patients with ESRD who underwent repeated cardiac CT scan after a median period of 1.5 years (0.7–2.5 years), median CAC score increased significantly ($P < 0.001$) from 0 AUs (0–531 AUs) to 273 AUs (0–1256 AUs) at follow-up in both statin users ($P < 0.001$) and nonstatin users ($P = 0.01$). Statin use was associated with a larger ($P = 0.001$) absolute change (Δ) in CAC score (Δ CAC) during follow-up (Fig. 3). Also, the relative CAC progression was significantly ($P = 0.008$) higher in statin (22.6%/year; IQR 3.3–78.5) vs. nonstatin (0%/year; IQR 0–13.8) users. In a multiple regression model, statin therapy was independently (Estimate 0.74; $P = 0.03$) associated with 1-SD higher Δ CAC score per month following correction for age (Estimate -0.01 ; $P = 0.42$), gender (Estimate -0.02 ; $P = 0.92$), diabetes (Estimate 0.57; $P = 0.08$) and hsCRP (Estimate 0.04; $P = 0.003$). No association was observed between dose of statins and CAC score ($\rho = 0.07$, $P = 0.52$). All-cause mortality did not differ between statin users and nonstatin users ($P = 0.96$) among the 35 patients.

Menaquinone-4 synthesis by hVSMCs *in vitro* is inhibited by statins

To examine the role of hVSMCs in the synthesis of MK-4, either vitamin K1 or menadione was added to VSMC cultures (Fig. 4). Extracts of control hVSMCs showed a small but significant peak for K (< 0.2 pmol/mg protein), whereas no other K vitamins, that is menaquinones, could be detected. The cellular K1 probably originates from foetal calf serum as it contains around 0.06–0.10 pmol/mL. Human VSMCs cultured in the presence of phyloquinone did not show any MK-4 accumulation. However, culturing hVSMCs in the presence of menadione resulted in a significant cellular accumulation of MK-4

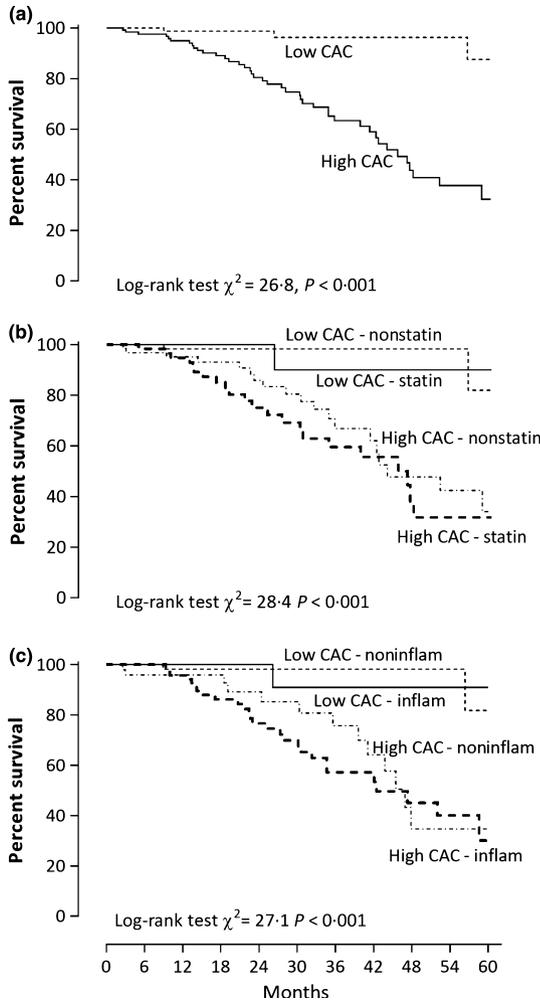


Figure 1 Coronary artery calcification defined as high coronary artery calcification (CAC) score (> 100 AU) associates with lower survival among patients with end-stage renal disease (ESRD) independent on use of statins and inflammation [defined as median high-sensitivity C-reactive protein (hsCRP)]. Kaplan–Meier survival curves for all-cause mortality (5 years of follow-up) among 240 patients with ESRD are shown for three scenarios. (a) Two groups of patients with ESRD with high CAC (> 100 AU) and low CAC (≤ 100 AU) scores, respectively ($P < 0.001$). (b) Four groups of patients with ESRD with: low CAC and nonstatin, low CAC and statin treatment, high CAC and nonstatin and high CAC and statin treatment, respectively ($P < 0.001$). (c) Four groups of patients with ESRD with: low CAC and hsCRP < median (1.7 mg/L), low CAC and hsCRP > median, high CAC and hsCRP < median and high CAC and hsCRP > median, respectively ($P < 0.001$).

(2.02 ± 0.30 pmol/mg). Addition of simvastatin together with menadione resulted in a significant inhibition of MK-4 synthesis (0.34 ± 0.13 pmol/mg).

Table 2 Cox regression analysis of hazard ratios (HRs) for all-cause mortality risk

Variable	HRs (95% CI)	P-value
Higher CAC score, per SD	1.52 (1.12–2.06)	0.006
Higher age, per SD	1.80 (1.16–2.81)	0.008
Higher hsCRP, per SD	1.20 (0.97–1.49)	0.08
SGA > 1 (yes/no)	2.90 (1.43–5.88)	0.003
Gender (male/female)	0.85 (0.41–1.74)	0.65
Diabetes mellitus (yes/no)	1.28 (0.67–2.46)	0.44
CVD (yes/no)	1.05 (0.51–2.16)	0.88
Statins (use/nonuse)	1.33 (0.68–2.62)	0.39
Modality (dialysis/nondialysis)	1.75 (0.85–3.58)	0.12

CAC, coronary artery calcification; hsCRP, high-sensitivity C-reactive protein; SGA, subjective global assessment; CVD, cardiovascular disease.

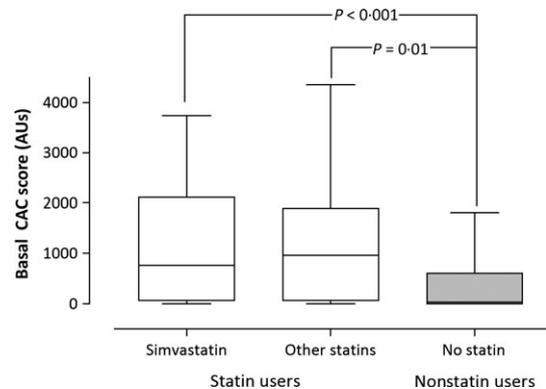


Figure 2 Statin treatment associated with coronary artery calcification defined as high coronary artery calcification (CAC) score (> 100 AU) among 240 patients with end-stage renal disease.

Discussion

We examined the mortality predictive significance of CAC score and its possible links with statins and inflammatory biomarkers. A higher CAC score predicted all-cause mortality independent of systemic inflammation. Use of statins were associated with a higher baseline CAC score, independently of age, gender and diabetes, as well as a more rapid progression of CAC score in a longitudinal evaluation.

The CAC score serves as a composite index of the degree of coronary intima and media calcification [34]. As expected, ESRD patients with CAC score > 100 AU were older, more often on statins, more often diabetics and with clinical signs of

Table 3 Demographic, clinical and biochemical characteristics in statin and nonstatin users

	Statin users (n = 89)	Nonstatin users (n = 151)	P-values
Demography and metabolic biomarkers			
Age, years	61 (44, 75)	51 (26, 75)	< 0.001
Male, %	65	62	0.678
Diabetes mellitus, %	34	15	0.001
Cardiovascular disease, %	33	15	0.002
Body mass index, kg/m ²	25.2 (21.3, 30.8)	24.5 (19.8, 30.2)	0.008
Systolic BP, mmHg	140 (118, 168)	141 (115, 169)	0.338
Diastolic BP, mmHg	83 (68, 95)	83 (67, 100)	0.870
Haemoglobin, g/L	114 (98, 129)	113 (95, 131)	0.971
HbA1c, mmol/mol*	44 (31, 72)	38 (26, 51)	0.017
Triglycerides, mM	1.6 (0.9, 2.9)	1.4 (0.8, 2.8)	0.103
Cholesterol, mM	4.2 (3.1, 5.2)	4.8 (3.4, 6.6)	< 0.001
HDL-cholesterol, mM	1.2 (0.8, 1.8)	1.3 (0.9, 2.0)	0.028
ApoA, g/L	1.42 (1.07, 1.83)	1.41 (1.10, 1.84)	0.803
ApoB, g/L	0.80 (0.56, 1.13)	0.91 (0.60, 1.41)	< 0.001
Creatinine, µM	729 (493, 951)	738 (464, 993)	0.841
S-albumin, g/L	34 (27, 38)	35 (28, 40)	0.163
SGA > 1, % †	29	39	0.134
Coronary artery calcification and inflammation biomarkers			
CAC total score, AUs	857 (0, 3769)	40 (0, 1812)	< 0.001
iPTH, ng/L ‡	314 (149, 729)	252 (82, 574)	0.026
Calcium, mM	2.31 (2.01, 2.54)	2.27 (2.03, 2.54)	0.839
Phosphate, mM	1.8 (1.2, 2.5)	1.8 (1.2, 2.4)	0.459
1,25-OH vitamin D, nM§	15 (9, 26)	17 (9, 34)	0.053
25-OH vitamin D, ng/L§	37 (13, 79)	34 (13, 70)	0.802
hsCRP, mg/L	1.8 (0.4, 22.1)	1.6 (0.3, 10.5)	0.425
IL-6, pg/mL¶	4.9 (0.6, 14.5)	2.8 (0.4, 12.5)	0.048
TNF, pg/mL**	16.0 (9.9, 20.9)	13.7 (8.5, 19.8)	0.007
PTX3 ng/mL††	1.5 (0.6, 5.2)	2.1 (0.7, 7.0)	0.115
Medications			
Statins, %	100	0	–
ACEI/ARB, %	69	62	0.330

CVD than patients with lower CAC score. In addition, as higher CAC score was associated with higher levels of hsCRP, IL-6 and TNF irrespective of treatment modality, this corroborates previous studies [1,35]. Studies in the general population show that CAC progresses with ageing [36], and in PD patients, only age was independently associated with CAC [15]. Whereas we confirm that a high CAC score was associated with diabetes

Table 3 Continued

	Statin users (n = 89)	Nonstatin users (n = 151)	P-values
β-blockers, %	66	57	0.173
Ca-blockers, %	55	46	0.182
Vitamin D analogs, %	84	85	1.000
Phosphate binders, %	79	87	0.107

BP, blood pressure; HDL, high-density lipoprotein; Apo A and Apo B, apolipoproteins A and B, respectively; SGA, subjective global assessment; CAC, coronary artery calcification; iPTH, intact parathyroid hormone; hsCRP, high-sensitivity C-reactive protein; TNF, tumour necrosis factor; IL-6, interleukin-6; PTX, pentraxin.

Data presented as median (range of 10th–90th percentile) or percentage.

*n = 236.

†n = 219.

‡n = 238.

§n = 165.

¶n = 196.

**n = 178.

††n = 157.

and male gender [28,37], the observed link between use of statins and higher CAC score has previously received little attention in nephrology.

The benefits of statins have been disputed [38,39], and a statin-related increased risk of new-onset diabetes have been reported [40]. Moreover, a recent report by Hanai *et al.* [41] demonstrated that lipophilic statins might have harmful effects on kidney function. Statin treatment was an independent predictor of a high CAC score, which suggests that statins may promote vascular calcification in predisposed individuals. As statin users were older, had higher BMI and had higher burden of diabetes and CVD (Table 3), our immediate reflex was that the observed link is a reflection of 'confounding by indication' [42]. Indeed, Maréchal *et al.* [43], who reported that statin use was an independent predictor of calcification progression after RTx, attributed their observation to this phenomenon. However, as randomized controlled trials have failed to show a survival benefit of statins in dialysis patients [23–25], the possibility that statins promote vascular calcification can not be excluded. In the present study, the link between statins and increased CAC score remained significant after adjustment for age (also when age was included in the model as a continuous variable), diabetes, BMI and inflammation. Recent studies in nonrenal patients also suggest that statins promote vascular calcification. Puri *et al.* [27] reported that although statins reduced the atheroma volume, they promoted coronary atheroma calcification. Nakazato *et al.* [26] found that statin treatment is associated with an increased prevalence and extent of coronary plaques possessing calcium. Moreover, in a recent study of gene expression signatures, pathways and networks in patients with carotid atherosclerosis, Perisic *et al.* [44] reported a

Table 4 Predictors of 1-SD higher CAC score by GLM regression analysis

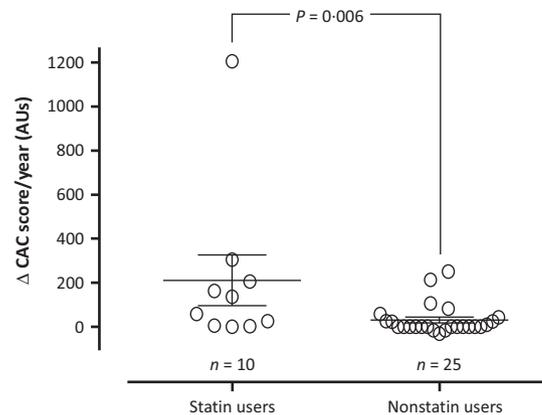
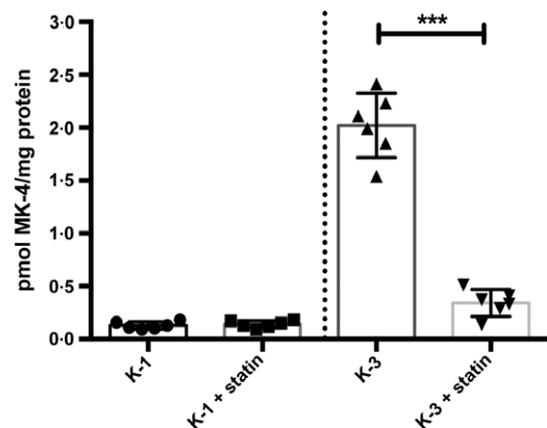
Multivariate model (GLM)	Estimate	Standard error	P value
Age group			
Age (> 65 years) (< 45 years reference)	0.95	0.14	< 0.001
Age (45–65 years) (< 45 years reference)	0.31	0.3	0.01
Gender (male/female)	−0.38	0.11	0.005
BMI, per SD	0.01	0.10	0.93
Diabetes mellitus (yes/no)	−0.46	0.13	0.007
Higher hsCRP, per SD	0.06	0.05	0.29
Statins (use/nonuse)	0.29	0.11	0.009

Age group			
Age (> 65 years) (< 45 years reference)	0.95	0.16	< 0.001
Age (45–65 years) (< 45 years reference)	0.27	0.15	0.08
Gender (male/female)	0.40	0.13	0.002
BMI, per SD	−0.04	0.07	0.57
Diabetes mellitus (yes/no)	0.31	0.16	0.05
Higher IL-6, per SD	0.12	0.07	0.08
Statins (use/nonuse)	0.44	0.14	0.001

Age group			
Age (> 65 years) (< 45 years reference)	0.94	0.18	< 0.001
Age (45–65 years) (< 45 years reference)	0.28	0.16	0.08
Gender (male/female)	0.35	0.13	0.008
BMI, per SD	−0.09	0.07	0.18
Diabetes mellitus (yes/no)	0.21	0.16	0.18
Higher TNF, per SD	0.13	0.06	0.04

CAC, coronary artery calcification; GLM, generalized linear model; hsCRP, high-sensitivity C-reactive protein; TNF, tumour necrosis factor.

possible stabilizing calcifying mechanism by statins. Another recent report based on 3483 participants showed that statin intake was associated with a 31% higher progression of CAC even after adjustment for cardiovascular risk factors [45]. Finally, pooled data from two clinical trials showed that atorvastatin caused a greater progression of CAC compared to

**Figure 3** Changes (Δ) in coronary artery calcification (CAC) score per year in statin users and nonstatin users in 35 patients with end-stage renal disease who underwent repeated cardiac CT imaging after a median period of 1.5 years.**Figure 4** The formation of MK-4 in growing primary human vascular smooth muscle cells (hVSMCs). hVSMCs, 1×10^6 cells, were seeded onto six-well culture plates and grown in medium containing menadion (1 M) or medium containing menadion with simvastatin (25 M). Cells were harvested after 20-h incubation, and formation of MK-4 was measured using RP-HPLC. Data are presented as triplicate experiment. ● K-1 treated hVSMC. ■ K-1 in combination with statin treated hVSMC. ▲ K-3 (menadione) treated hVSMC. ▼ K-3 (menadione) in combination with statin treated hVSMC. $P < 0.001$.

placebo [46]. However, the greater progression of CAC with statin therapy was not associated with greater risk of cardiovascular events, which could imply that calcified plaques are more stable and less prone to rupture. Also, *in vitro* cell culture studies suggest that statins stimulate calcium deposition in smooth muscle vascular cells [47] and mesenchymal stem cells [48]. The pathophysiological mechanisms by which statins promote vascular calcifications are not evident. As statins

induce dose-dependent apoptosis [49], it could be speculated that apoptotic bodies serve as a nidus for calcium deposition.

Vitamin K deficiency is common in ESRD [50], and vitamin K supplementation has been proposed to arrest progression of vascular calcification [51]. Lipophilic statins inhibit the enzymatic activity of UbiA prenyltransferase domain-containing protein 1 (UBIAD1) and enzyme which plays a significant role in vitamin K2 synthesis [52]. Therefore, it is tempting to speculate that statins may accelerate artery calcification [38,39] via depletion of vascular vitamin K2 levels, thereby increasing uncarboxylated matrix gla protein (MGP) [53,54]. Indeed, our *in vitro* data demonstrate that synthesis of vitamin K2 is impaired in the presence of statins (Fig. 4). Whether statins also inhibit vitamin K2 syntheses *in vivo* and subsequent MGP carboxylation has to be demonstrated. One alternative explanation is that the treatment with statins was mainly confined to selected patients who had more extensive atherosclerotic lesions because they had been exposed for longer periods to high levels of LDL. Atherosclerosis causes intima-media expansion due to the accumulation of extracellular matrix made of sulphated proteoglycans and collagen, which can serve as an ideal environment for calcium deposition [55,56]. Our observations require further studies to discriminate between true pro-calcifying effects of statins and epiphenomena linked to – but not causally related to – statin treatment in selected patients with more extensive preexisting lesions than those patients not receiving statins. In addition, further studies should clarify the possible contribution of vitamin K2 depletion [57] and the potential differences in the putative pro-calcifying effects of lipophilic vs. hydrophilic statins [58]. As 97% of the patients who received statins in the present study were on lipophilic statins (simvastatin and atorvastatin), it is of interest that in a rat model of CKD, the hydrophilic pravastatin (and olmesartan) synergistically ameliorated vascular calcification [56].

Another aim of the present study was to evaluate the relationship between systemic inflammation and CAC. Although we observed univariate associations between inflammatory biomarkers and CAC score, these associations lost statistical significance following correction for traditional risk factors. Atherosclerosis, the major cause for coronary artery disease, is a chronic inflammatory disease [21]. The reported associations between systemic inflammatory biomarkers and media calcification (i.e. arteriosclerosis) are conflicting [16,34,59–61]. Whereas Wang *et al.* [61] reported a significant correlation between CRP and CAC score even after adjustment for age and Framingham Risk Score parameters, Anand *et al.* [34] found neither CRP nor IL-6 to be predictive of prevalent CAC or CAC progression. Similar to our study, Khera *et al.* [60] and Kullo *et al.* [59] reported that while CRP was significantly correlated with CAC score, the relationship lost its significance in

multivariate analysis. Variations in population sample sizes, inclusion criteria, inflammatory biomarkers studied and analysis techniques may contribute to the observed differences [22]. The reason(s) for the lack of independent association between inflammatory biomarkers and CAC score are not evident. As the inflammatory process is complex, differences between systemic and local vascular inflammation need consideration. It is also possible that inflammatory biomarkers and CAC reflect distinct different pathophysiology [16]. While inflammation plays a central role in atherosclerosis, the mechanisms of factors that predispose to arteriosclerosis may involve many other pathways, such as diabetes, endothelial dysfunction, dyslipidaemia, autonomic dysfunction and disturbed mineral metabolism [62].

We report that high CAC score was an independent predictor of all-cause mortality risk. As neither diabetes nor clinical CVD predicted death when CAC score and age were accounted for, the results suggest that in the toxic uremic milieu, the degree of coronary calcification represents the major mortality risk factor associated with diabetes and CVD. Cross-sectional and prospective studies have confirmed that CAC is an independent risk factor for cardiovascular risk beyond that provided by conventional risk factors [63]. Berry *et al.* [64] reported that patients with CAC score > 100 AUs had greater hazards for major coronary events even after adjustment for traditional risk factors. Calcification may reflect stabilization and maturation of atherosclerotic plaques and may lead to fewer coronary heart disease deaths [65]. As the CAC score is defined by volume × density of calcification, it is of interest that a study in 3398 nonrenal patients showed that whereas a high CAC volume score predicted CVD risk, high CAC density was inversely associated with CVD risk [66]. Thus, in future studies of patients with ESRD, the impact of the volume and density scores should be evaluated separately. As part of the beneficial effects of statins has been attributed to plaque stabilization by calcification [27,46], studies should evaluate whether statin-induced calcification in uremic coronary arteries is protective or confers higher risk [67].

Some strengths and limitations should be considered when the results of the present study are interpreted. Although the sample size and the number of events during follow-up were limited, this is the first study exploring whether the mortality predictive role of a high CAC score is modified by uremic inflammation. Among the limitations, the observational nature of the study precludes conclusions regarding causality. Moreover, the use of cardiac CT for the measurement of CAC does not allow differentiation of medial from intimal calcification and does not provide information about the composition of plaques. However, we have reported on a strong association between the magnitude of medial vascular calcification in the

epigastric artery and degree of CAC [28]. Our study comprised patients with ESRD from three different cohorts with somewhat different inclusion criteria; however, in the multivariate analysis, treatment modality was corrected for. The lack of data on duration of CKD and statin treatment prior to inclusion also limits the interpretation of the results. Finally, analyses of inactive and active MGP and vitamin K would have benefited the study.

In conclusion, increased CAC score predicts mortality risk independently of inflammation in ESRD. As our results imply that the use of statins associates with progression of CAC in ESRD, further studies are warranted to confirm this finding and to clarify whether this translates into greater or less risk of cardiovascular events. Based on the current *in vitro* experiments, we hypothesize that statin-induced inhibition of vitamin K synthesis might be one factor contributing to statins' lack of effect on outcome in ESRD.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. The relationship between age groups, cohorts and CAC score among ESRD patients ($P < 0.001$, respectively).

Table S1. Baseline characteristics of the different ESRD cohorts.

Table S2. Baseline demographic and biochemical characteristics between the subgroup of ESRD patients evaluated longitudinally with repeated CT scans and the cohort with only one basal CT heart scan.

Table S3. Univariate Spearman's Rho correlations of CAC score with selected variables.