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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 ESRD patients and their effect on vascular calcification is unclear. We explored associations between CAC, inflammatory biomarkers, statins and mortality in ESRD.

Material and methods: In 240 ESRD patients (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.

Introduction

Increased coronary artery calcification (CAC), a common feature in end-stage renal disease (ESRD) patients [1], is a predictor of increased risk of cardiac events in dialysis [2] and non-dialysis [3, 4] ESRD patients. In non-renal 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 ESRD patients 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 chronic kidney disease (CKD) patients 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 non-renal 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 ESRD patients. We also assessed the relation between statin use and changes in CAC score in a subgroup of 35 ESRD patients undergoing repeated CT scans.

Methods

Study cohorts

A CT scan for CAC imaging was performed in 240 adult ESRD patients (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] (**Suppl. Table 1**). The patients were recruited between March 2008 and December 2014 at the Dept. 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 ESRD patients (58% male, median age 53 years) allowing estimation of absolute ($CAC_{FOLLOW\text{-}UP} - CAC_{BASELINE}$) and relative ($[CAC_{FOLLOW\text{-}UP} - CAC_{BASELINE}]/CAC_{BASELINE}$) progression rate [5]. No clinically meaningful significant differences in demographic or biochemical characteristics were observed in comparison to 205 ESRD patients who completed only one CAC imaging (**Suppl. Table 2**).

Coronary calcification imaging and quantification

CAC was assessed by CT, an accurate non-invasive approach, performed on a 64-channel detector scanner (LightSpeed VCT; General Electric (GE) Healthcare, Milwaukee, WI, USA) in cine mode. Scans were ECG-gated and a standard non-contrast 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 analyzed 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 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 Dept. 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 (BP) 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 as described in detail previously [28].

Vitamin K metabolism in vascular smooth muscle cells

Human aortic VSMCs (hVSMC) 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% fetal calf serum (FCS) and antibiotics. Cells were seeded 1:3 from T25 cm2 flasks into six-wells plates. When reaching 80-90% confluence the medium was changed to medium supplemented with phylloquinone (2µM) or menadione (2µM) either with or without Simvastatin (2.5□M). After 20 hours of incubation cells were washed with Hepes buffer. Cells were collected and sonicated in 1mL triton X-100 (1%) in Hepes. Protein determination was done 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-phylloquinone. The mixture was homogenized in a blender (Ultra Turrax T25), 3 x 10 seconds cycles of 18000 rpm at 0^{o}C. Extraction of the K vitamins was done using hexane. Vitamin K was analyzed by HPLC using a C-18 reversed phase column and fluorometric detection after post-column zinc reduction. All values are expressed as means and standard deviation for six wells.

Statistical analysis

Data are expressed as median (range of 10th to 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 non-parametric Wilcoxon 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 ESRD patients 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, 1,25-OH vitamin D and 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 higher levels of systolic BP, HbA1c, triglycerides, phosphate, inflammatory biomarkers and CAC score compared to patients in the LD-Rtx cohort (**Suppl. Table 1**). A total of 129 (53.4%) patients had CAC >100 AUs. They were older and had higher BMI, $HbA1_c$, triglycerides, hsCRP, IL-6 and TNF, and higher prevalence of both clinical CVD and diabetes. The median level of 1,25(OH) D-vitamin 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 AUs (**Table 1**).

Basal CAC score independently predicted mortality in 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 AUs as compared to those with low CAC (**Figure 1A**), regardless of use of statins (**Figure 1B**) or presence of inflammation (**Figure 1C**). In multivariate Cox proportional hazards 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.

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

In Spearman rank (rho) analysis, the baseline CAC score correlated significantly with age (**Suppl. Fig. 1**), gender, diabetes, CVD, BMI, $HbA1_c$, triglycerides, HDL-cholesterol, albumin, 1,25-OH vitamin D, inflammation markers, statins, β-blockers and cohort (**Suppl. Table 3**). 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 non-statin users are depicted in **Figure 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 ESRD patients not on statins (**Table 3).** Significantly higher median iPTH (314 vs. 252 ng/L; p<0.05) and $HbA1_c$ levels (44 vs. 38 mmol/mol; p<0.05) in statin users likely reflect the higher prevalence of diabetes. As expected, statins users had lower median cholesterol (4.2 vs. 4.8 mmol/L; 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 non-statin 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 clinical CVD (considered a result rather than the cause of elevated CAC score), cholesterol and apoB (considered related to statin treatment), nor $HbA1_C$ nor triglyceride levels (considered related to diabetes mellitus) were included. Since 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 ESRD patients 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 non-statin users (p=0.01). Statin use was associated with a larger (p=0.001) absolute change (Δ) in CAC score (ΔCAC) during follow-up (**Figure 3**). Also the relative CAC progression was significantly (p=0.008) higher in statin (22.6%/year (IQR 3.3-78.5) vs. non-statin (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 ΔCAC 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 non-statin 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 (**Figure 4**). Extracts of control hVSMCs showed a small but significant peak for K (<0.2 pmol/mg protein) whereas no other K vitamins, i.e. menaquinones could be detected. The cellular K1 probably originates from fetal calf serum as it contains around 0.06-0.10 pmol/ml. Human VSMCs cultured in the presence of phylloquinone did not show any MK-4 accumulation. However, culturing hVSMCs in the presence of menadione resulted in a significant cellular accumulation of MK-4 (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).

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 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 AUs were older, more often on statins, more often diabetics and with clinical signs of 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 aging [36] and in PD

patients only age was independently associated with CAC [15]. Whereas we confirm that a high CAC score associated with diabetes 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. Since statin users were older, had higher BMI and 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, Marcechal et al [43], who reported that statin use was an independent predictor of calcification progression after RTx, attributed their observation to this phenomenon However, since randomised 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 non-renal 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. [44] found that statin treatment 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 [45] reported a 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 [46]. Finally, pooled data from two clinical trials showed that atorvastatin caused a greater progression of CAC compared to placebo [47]. 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 [48] and mesenchymal stem cells [49]. The pathophysiological mechanisms by which statins promote vascular calcifications are not evident. Since statins induce dose-dependent apoptosis [50], it could be speculated that apoptotic bodies serve as a nidus for calcium deposition.

Vitamin K-deficiency is common in ESRD [51] and vitamin K supplementation has been proposed to hold progression of vascular calcification [52]. 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 [53]. 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) [54, 55]. Indeed, our *in vitro* data demonstrate that synthesis of vitamin K2 is impaired in the presence of statins (**Figure 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 accumulation of extracellular matrix made of sulphated proteoglycans and collagen, which can serve as an ideal environment for calcium deposition [56, 57]. 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 pre-

existing lesions than those patients not receiving statins. In addition, further studies should clarify the possible contribution of vitamin K2 depletion [58] and the potential differences in the putative pro-calcifying effects of lipophilic vs. hydrophilic statins [59]. Since 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 [57].

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, 60-62]. Whereas Wang et al. [62] 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. [61] and Kullo et al. [60] 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 needs 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 [63].

We report that high CAC score was an independent predictor of all-cause mortality risk. Since 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 [64]. Berry et al. [65] 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 [66]. As the CAC score is defined by volume × density of calcification it is of interest that a study in 3398 non-renal patients showed that whereas a high CAC volume score predicted CVD risk, high CAC density was inversely associated with CVD risk [67]. Thus, in future studies of ESRD patients, the impact of the volume and density scores should be evaluated separately. Since part of the beneficial effects of statins have been attributed to plaque stabilization by calcification [27, 47] studies should evaluate if statin-induced calcification in uremic coronary arteries is protective or confer higher risk [68].

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 if 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 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 ESRD patients 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. Since our results imply that use of statins associates with progression of CAC in ESRD, further studies are warranted to confirm this finding and to clarify if this translates into greater or less risk of cardiovascular events. Based on the current *in vitro* experiments we hypothesize that statins-induced inhibition of vitamin K synthesis might be one factor contributing to statins´ lack of effect on outcome in ESRD.

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Figure legends

Figure 1. Coronary artery calcification defined as high CAC score (>100AUs) associates with lower survival among ESRD patients independent on use of statins and inflammation (defined as median hsCRP). Kaplan-Meier survival curves for all-cause mortality (5 years of follow-up) among 240 ESRD patients are shown for three scenarios. A. Two groups of ESRD patients with high CAC (>100AUs) and low CAC (≤100AUs) scores, respectively ($p<0.001$). B. Four groups of ESRD patients with: low CAC and non-statin, low CAC and statin treatment, high CAC and non-statin, and high CAC and statin treatment, respectively ($p<0.001$). C. Four groups of ESRD patients 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 ($p<0.001$).

Figure 2. Statin treatment associated with coronary artery calcification defined as high CAC score (>100AUs) among 240 ESRD patients.

Figure 3. Changes (Δ) in CAC score per year in statin users and non-statin users in 35 ESRD patients who underwent repeated cardiac CT imaging after a median period of 1.5 years.

Figure 4: The formation of MK-4 in growing primary hVSMCs. Human VSMCs, $1x10^6$ cells, were seeded onto 6 well culture plates and grown in medium containing menadion (1 mol/L) or medium containing menadion with simvastatin (25mol/L). Cells were harvested after 20 hours incubation and formation of MK-4 was measured using RP-HPLC. Data are presented as triplicate experiment.

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

	Total ESRD patients (n=240)	**Low CAC (≤ 100AUs)** (n=111)	**High CAC (> 100AUs)** (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
Hemoglobin, g/L	113 (96, 130)	111 (94, 130)	114 (98, 130)	0.076
HbA1c, mmol/mol [a]	40 (28, 58)	35 (23, 47)	45 (33, 68)	**< 0.001**
Triglycerides, mmol/L	1.5 (0.9, 2.8)	1.4 (0.8, 2.6)	1.8 (0.9, 3.0)	**0.011**
Cholesterol, mmol/L	4.6 (3.2, 6.3)	4.6 (3.4, 6.0)	4.7 (3.2, 6.6)	0.829
HDL-cholesterol, mmol/L	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, µmol/L	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, % [b]	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 [c]	271 (96, 653)	250 (102, 610)	302 (79, 689)	0.261
Calcium, mmol/L	2.28 (2.02, 2.54)	2.27 (2.05, 2.52)	2.31 (1.97, 2.54)	0.559
Phosphate, mmol/L	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, nmol/L [d]	16 (9, 30)	17 (10, 34)	14 (9, 29)	**0.002**
25-OH vitamin D, ng/L [d]	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 [e]	3.6 (0.5, 13.2)	1.9 (0.1, 8.3)	5.5 (1.7, 15.0)	**<0.001**
TNF, pg/ml [f]	14.8 (9.0, 20.1)	12.2 (8.2, 18.4)	16.2 (10.8, 21.7)	**<0.001**
PTX3 ng/mL [g]	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
Vitamin D analogs, %	85	86	84	0.723
Phosphate binders, %	84	86	82	0.489

Data presented as median (range of 10^{th} - 90^{th} percentile) or percentage.
Abbreviations: BP, blood pressure; HbA1c, hemoglobin 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, tumor necrosis factor; IL-6, interleukin-6; PTX, pentraxin.
[a]; n=236, [b]; n=219, [c]; n=238; [d]; n=165; [e]; n=196, [f]; n=178, [g]; n=157

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/non-use)	1.33 (0.68-2.62)	0.39
Modality, (dialysis /non-dialysis)	1.75 (0.85-3.58)	0.12

Table 3. Demographic, clinical and biochemical characteristics in statin and non-statin users.

	Statin users (n=89)	**Non-statin 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
Hemoglobin, g/L	114 (98, 129)	113 (95, 131)	0.971
$HbA1_c$, mmol/mol [a]	44 (31, 72)	38 (26, 51)	**0.017**
Triglycerides, mmol/L	1.6 (0.9, 2.9)	1.4 (0.8, 2.8)	0.103
Cholesterol, mmol/L	4.2 (3.1, 5.2)	4.8 (3.4, 6.6)	**<0.001**
HDL-cholesterol, mmol/L	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, µmol/L	729 (493, 951)	738 (464, 993)	0.841
S-albumin, g/L	34 (27, 38)	35 (28, 40)	0.163

SGA >1, % [b]	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 [c]	314 (149, 729)	252 (82, 574)	**0.026**
Calcium, mmol/L	2.31 (2.01, 2.54)	2.27 (2.03, 2.54)	0.839
Phosphate, mmol/L	1.8 (1.2, 2.5)	1.8 (1.2, 2.4)	0.459
1,25-OH vitamin D, nmol/L [d]	15 (9, 26)	17 (9, 34)	0.053
25-OH vitamin D, ng/L [d]	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 [e]	4.9 (0.6, 14.5)	2.8 (0.4, 12.5)	**0.048**
TNF, pg/ml [f]	16.0 (9.9, 20.9)	13.7 (8.5, 19.8)	**0.007**
PTX3 ng/mL [g]	1.5 (0.6, 5.2)	2.1 (0.7, 7.0)	0.115
Medications			
Statins, %	100	0	•
ACEI/ARB, %	69	62	0.330
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

Data presented as median (range of 10^{th} - 90^{th} percentile) or percentage.
Abbreviations: 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, tumor necrosis factor; IL-6, interleukin-6; PTX, pentraxin.
[a]; n=236, [b]; n=219, [c]; n=238; [d]; n=165; [e]; n=196, [f]; n=178, [g]; n=157

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 yrs) (<45 yrs reference)	0.95	0.14	**<0.001**
Age (45-65 yrs) (<45 yrs 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/non-use)	0.29	0.11	**0.009**

Multivariate model (GLM)	Estimate	Standard error	P value
Age group			
Age (>65 yrs) (<45 yrs reference)	0.95	0.16	**<0.001**
Age (45-65 yrs) (<45 yrs 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/non-use)	0.44	0.14	**0.001**

Multivariate model (GLM)	**Estimate**	**Standard error**	**P value**
Age group			
Age (>65 yrs) (<45 yrs reference)	0.94	0.18	**<0.001**
Age (45-65 yrs) (<45 yrs 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**

Figure 1.

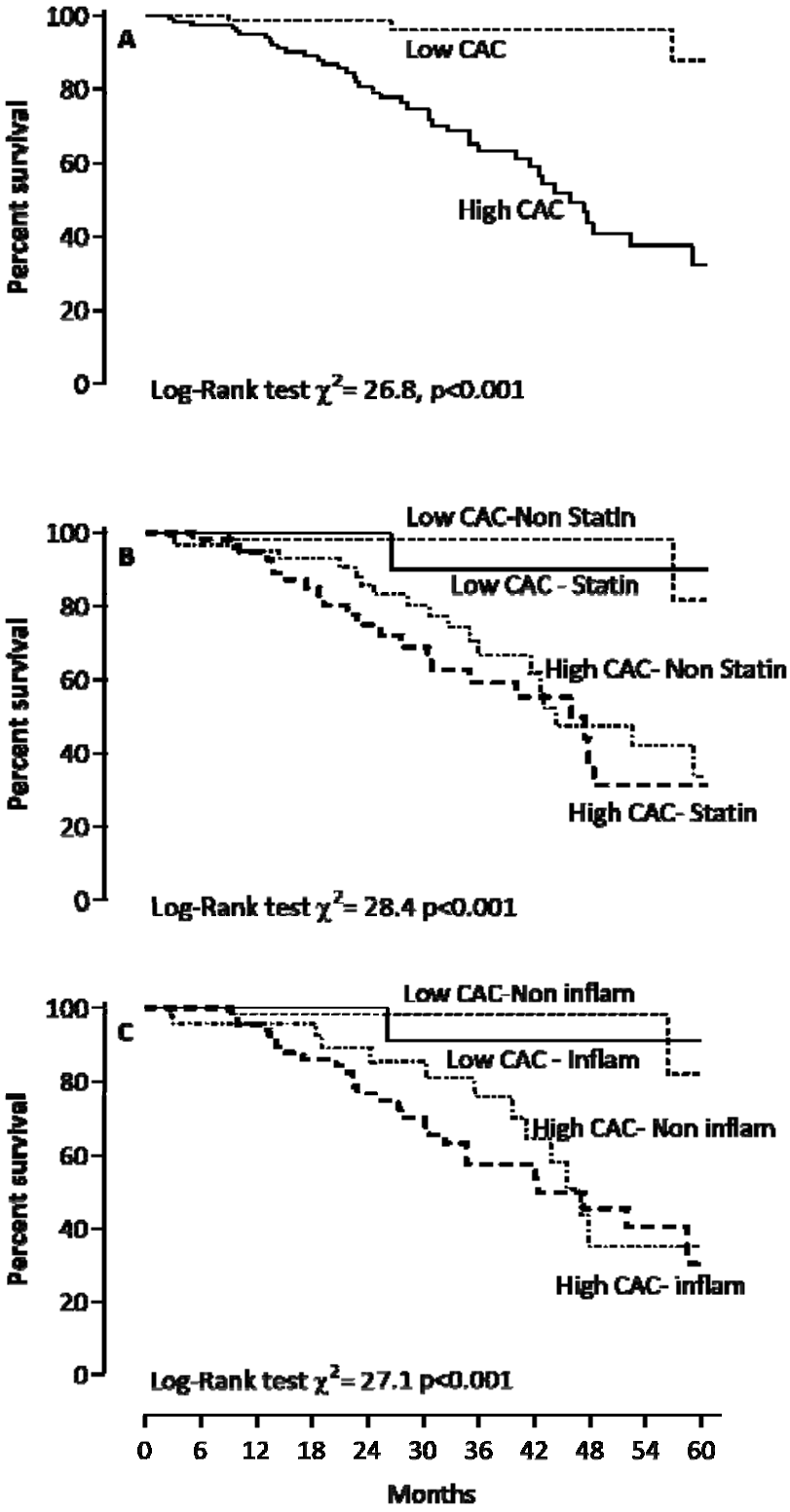

Figure 2.

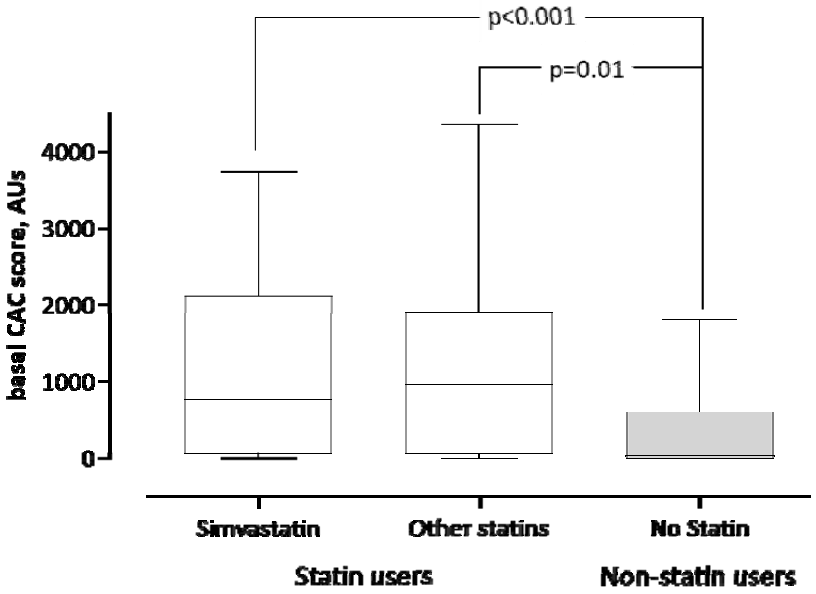

Figure 3.

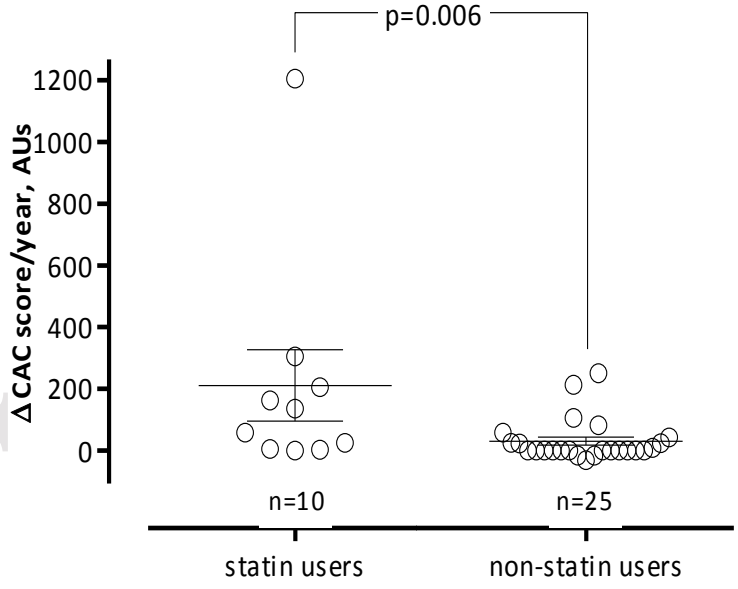

Figure 4