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Salam, S. orcid.org/0000-0001-9236-3906, Gallagher, O., Gossiel, F. et al. (3 more authors) (2021) Vascular calcification relationship to vascular biomarkers and bone metabolism in advanced chronic kidney disease. Bone, 143. 115699. ISSN 8756-3282

https://doi.org/10.1016/j.bone.2020.115699

Title: Vascular Calcification Relationship to Vascular Biomarkers and Bone Metabolism in Advanced Chronic Kidney Disease

Authors: Syazrah Salam, [1,2] Orla Gallagher,[2] Fatma Gossiel,[2] Margaret Paggiosi,[2] Richard Eastell,[2] Arif Khwaja[1]

Affiliations: [1]Sheffield Kidney Institute, Sheffield Teaching Hospitals NHS Foundation Trust and [2]Academic Unit of Bone Metabolism and Mellanby Centre for Bone Research, University of Sheffield, United Kingdom.

Correspondence: Dr Syazrah Salam, Sheffield Kidney Institute, Northern General Hospital, Sheffield Teaching Hospitals NHS Foundation Trust, Herries Road, Sheffield S5 7AU, United Kingdom. Tel: (+44) 114 2715788. Fax: (+44) 114 2714604. E-mail: syazrah.salam@nhs.net

Running title: Vascular Calcification in CKD

Word count for abstract: 312

Word count for text: 4171

Abstract

Background

Vascular calcification (VC) and renal osteodystrophy are important complications of advanced chronic kidney disease (CKD). High resolution peripheral quantitative computed tomography (HRpQCT) is able to assess bone microstructure in renal osteodystrophy and lower leg arterial calcification (LLAC) is usually seen as an incidental finding. LLAC can be a useful quantitative assessment of VC in CKD but the relationship between LLAC and vascular biomarkers and bone is unknown. We aimed to assess the relationship between LLAC and biomarkers, bone turnover and microstructure.

Methods

In this cross-sectional study, fasting blood samples were taken from 69 CKD stages 4-5D patients and 68 healthy controls. HRpQCT of distal tibia and radius were performed. 43 CKD patients had trans-iliac bone biopsy after tetracycline labelling.

Results

LLAC was more severe in CKD than controls (median [IQR] 1.043 [0.05 – 16.52] vs 0 [0 - 0.55] mgHA, $p<0.001$). CKD patients with diabetes (28%) had significantly higher LLAC compared to non-diabetic CKD (median [IQR] 24.07 [3.42 – 61.30] vs 0.23 [0- 3.78] mgHA, $p<0.001$). LLAC mass in CKD correlated with serum phosphate (rho = 0.29, $p<0.05$), calcium x phosphate product (rho = 0.31, $p<0.05$), intact parathyroid hormone (rho = 0.38, $p<0.01$), intact fibroblast growth factor-23 (iFGF23) (rho = 0.40, p= 0.001), total alkaline phosphatase (rho = 0.41, $p<0.001$), bone alkaline phosphatase (rho = 0.29, $p<0.05$), osteocalcin (rho = 0.32, $p<0.05$), osteoprotegerin (rho = 0.40, p=0.001) and dephosphorylated-uncarboxylated matrix Gla protein (rho = 0.31, $p<0.05$). LLAC in CKD also correlated with worse distal tibia cortical bone mineral density, thickness and porosity. No association was found between LLAC and

bone turnover, mineralization or volume on biopsy in CKD. In multivariate analysis, only age, diabetes, iPTH and iFGF23 were independently associated with LLAC in CKD.

Conclusions

High levels of PTH and FGF23, along with older age and the presence of diabetes may all play independent roles in the development of LLAC in advanced CKD.

Keywords: arterial calcification, dialysis, renal osteodystrophy, diabetes mellitus, FGF23

Introduction

Vascular calcification (VC) is part of chronic kidney disease-mineral and bone disorder (CKD-MBD), which is an important complication of chronic kidney disease (CKD).[1] VC is highly prevalent in CKD and its severity increases with worsening CKD.[2,3] 50 - 90% of CKD stages 3-5D have evidence of VC and it is associated with increased cardiovascular and all-cause mortality.[3,4] VC is also associated with fractures which compounds increased mortality risk in these patients.[4,5]

Calcification in the media layer of arteries is the predominant pattern of VC in CKD and it is present in the coronary arteries, large aorta and peripheral arteries.[6-8] Lateral abdominal X-ray is the routine test to detect abdominal aortic calcification (AAC) and is considered a reasonable alternative to computed tomography (CT) imaging.[9] Meanwhile, high resolution peripheral quantitative CT (HRpQCT) has been increasingly used as a research tool into the effects of CKD on bone microstructure. Peripheral arterial calcification is often seen as an incidental finding.

A quantitative method to measure lower leg arterial calcification (LLAC) using HRpQCT images of the distal tibia has been validated using coronary artery calcification (CAC) quantified by multi-detector CT in 46 haemodialysis patients.[10] There was a significant positive association between LLAC and CAC. However, it is unclear if HRpQCT would identify more advanced CKD patients with VC compared to X-ray based detection of AAC.

LLAC assessment by HR-pQCT is also well placed to assess the bone-vascular axis. A study by Paccou et al showed that the presence of LLAC was associated with bone microstructure abnormalities of distal tibia and radius in elderly women.[11] Very few studies have assessed this relationship in advanced CKD using HR-pQCT.

VC in CKD is a complex process characterised by vascular smooth muscle cells (VSMCs) transformation into osteoblast-like cells which promotes mineral deposition and hydroxyapatite crystal growth.[12,13] VC process is under the influence of several potential factors such as mineral abnormalities of calcium and phosphate; VC promoters such as alkaline phosphatase (ALP); VC inhibitors such as matrix Gla protein (MGP) and fetuin A; bone abnormalities of renal osteodystrophy; local and systemic inflammation; oxidative stress and various uremic toxins.[14] The role of bone-related markers in VC such as osteocalcin and osteoprotegerin remain uncertain. The relationship between LLAC in advanced CKD and vascular biomarkers and bone is unknown. This study aimed to assess the relationship between LLAC and AAC, vascular biomarkers, bone turnover and bone microstructure. We also aimed to explore if these factors could be used in a classification system to identify advanced CKD patients with LLAC.

Materials and Methods

Study Design and Participants

This was a cross-sectional study in CKD stages 4-5D patients aged 30-80 years old and was part of a diagnostic test study which recruited participants between July 2013 and May 2015.[15] The exclusion criteria included fracture/orthopaedic surgery in the preceding six months; started/changed the dose of phosphate binders, vitamin D or calcimimetics within four weeks of study entry; and received anti-resorptive, anabolic agent or systemic glucocorticoid in the preceding six months. We also recruited age and gender-matched controls with estimated glomerular filtration rate (eGFR) ≥60 ml/min/1.73m². The exclusion criteria were similar to CKD group and individuals with known osteoporosis were also excluded. Participants' demographics were collected including diabetes status and cardiovascular disease history

defined as previous myocardial infarction, angina, coronary artery bypass graft, coronary and non-coronary angioplasty, intermittent claudication, transient ischaemic attack and cerebrovascular accident. The study adhered to the *Declaration of Helsinki* and was approved by the South Yorkshire Research Ethics Committee. All participants gave written informed consent. All samples and imaging studies were obtained purely for research.

Vascular Calcification and Bone Imaging

HRpQCT of distal radius and tibia were performed using XtremeCT (Scanco Medical AG, Switzerland) using standard protocol. Images with unacceptable degree of movement artefact (motion grade 4) were excluded from further analysis. The images were analysed with standard software (Scanco Medical AG, version 6.0) for volumetric bone mineral density (vBMD) and microstructure parameters.

For LLAC assessment, a semi-automated software (Scanco Medical AG, version 6.5) determined the presence of arterial calcification in the areas corresponding to the anatomical position of lower leg arteries (Figure 1). Tibia, fibula, cutaneous calcifications and other non-vascular soft tissue calcifications were excluded from the region of interest. The calcification mass for the length of the scan image (9.02mm) was calculated using the formula: VC mass (mgHA) = [total volume of vascular calcification (mm^3) x mean calcification density ($mgHA/cm^3$)]/1000. Scans with absent vascular calcification were recorded as zero mgHA.

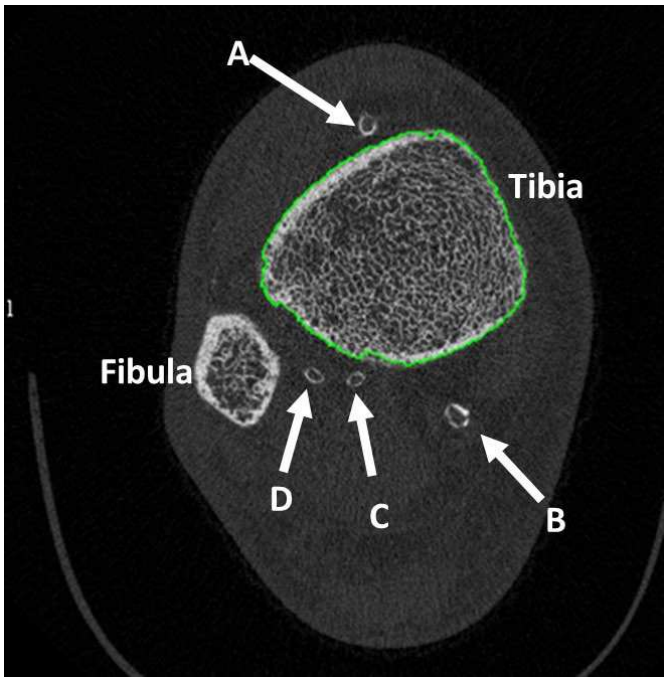

Figure 1. A cross-sectional axial image from distal tibia HR-pQCT which shows vascular calcification in the ankle arteries. This image shows circular hyperdensity shapes corresponding to anatomical territory of arteries in the ankle. Symbols: A, anterior tibial artery; B, posterior tibial artery; C, perforating branch of peroneal artery; D, peroneal artery.

Dual energy X-ray absorptiometry (DXA) of the lumbar spine (L1-4), hip and forearm were performed using Hologic Discovery A densitometer (Hologic Inc, USA). Mean areal BMD and T-score were calculated using Hologic APEX software (version 3.4.2). AAC was assessed from lateral spine images acquired using DXA. The images were scored using an 8-point scale (AAC-8) as described by Schousboe et al.[16]

Serum Biochemistry and Biomarkers

Fasting blood samples were taken, centrifuged and stored at -80^{0}C for analysis at the end of the study. For haemodialysis patients, blood samples were taken on the day after their haemodialysis session. We measured serum adjusted calcium, phosphate, total ALP (tALP), and creatinine on Roche Cobas c701/702 analyser (Roche Diagnostics, England) on the same day as sample collection. The intra-assay and inter-assay coefficient of variation (CVs) for all

the biochemistry tested were ≤5%. eGFR was calculated using Modification of Diet in Renal Disease equation which was used during the study period, preceding the use of CKD-EPI equation. eGFR for dialysis patients were imputed as 5 ml/min/1.73m².

Each biomarker analysis using automated assays were done as single batch. The sample analysis for manual assays were done in duplicates over a period of 2 days. We measured serum iPTH, bone ALP (bALP), N-mid osteocalcin, 25-hydroxyvitamin D, total uncarboxylated (t-uc) MGP and dephosphorylated-uncarboxylated (dp-uc) MGP using the IDS-iSYS auto-analyser (Immuno Diagnostic Systems, U.K.). All assays had an inter-assay CVs of <7% except for t-uc MGP which had an inter-assay CV of <11%. We measured serum OPG using manual ELISA by Biomedica (Vienna, Austria) (intra-assay and inter-assay CVs were <5% and <7% respectively), iFGF23 using manual ELISA by Immutopics (California, US) (intra-assay and inter-assay CVs were <5% and ≤9% respectively) and fetuin A using manual ELISA by BioVendor (Brno, Czech Republic) (intra-assay and inter-assay CVs were <5% and <7% respectively).

Trans-iliac Bone Biopsy and Histomorphometry

Bone biopsy was performed in CKD patients after double tetracycline labelling. A trans-iliac bone biopsy was performed using an 8-gauge Jamshidi 4mm trephine and needle. Trabecular bone analysis was performed using the Bioquant Osteo histomorphometry system (Bioquant Image Analysis Corporation) which uses standardised nomenclature. The samples fulfilled the minimum acceptable total section area in the standard analysis region of 30 mm^2 for quantitative histomorphometry analysis.[17] Normal bone turnover was defined as bone formation rate/ bone surface (BFR/BS) of 18 - 38 $\mu m^3/\mu m^2$/year.[18] Normal mineralization was defined as osteoid thickness (O.Th) <20 µm and mineralization lag time (MLT) <100 days. Low trabecular bone volume was defined as bone volume/tissue volume (BV/TV) <16.8%.

Statistical Analysis

Descriptive statistics are presented as mean ± standard deviation (SD) or median and interquartile range (IQR). Group differences were tested using Student's t-test or Mann-Whitney U test depending on the distribution of the continuous variable and Chi-squared for categorical variables. LLAC had a skewed distribution, therefore its relationship with biomarkers and imaging measurements was tested using Spearman's rank correlation for univariate analysis.

For multivariate analysis, all variables with skewed distribution were firstly $\log_{10}$ transformed. LLAC with zero values were assigned a value by taking the smallest detectable LLAC mass and divided it by two. The LLAC dataset was then $\log_{10}$ transformed. Multivariate analysis was performed using linear regression analysis with stepwise backward rejection to identify independent variables associated with LLAC. All independent variables were tested for co-linearity. All statistical analysis were performed using IBM SPSS Statistics 22. $p<0.05$ indicates statistical significance.

To explore if independent variables identified in the multivariate analysis will be useful for a classification system to identify those with LLAC, we used the Principal Component Analysis (PCA). PCA is an exploratory statistical analysis which can be used to visualize the relationship between groups based on a number of variables. PCA was performed using the ClustVis software (https://biit.cs.ut.ee/clustvis). Standardised Z-scores using the control participants mean and SD were imputed for continuous variables to fulfil the PCA assumption that continuous variables are of the same scale. Singular Value Decomposition with imputation was used to calculate the principal components (PCs). Each PC explains a percentage of the total variation in the dataset. The dot plot with PC1 and PC2 gives an overview of the data spread using the combinations of imputed variables. An automated prediction ellipses were

constructed based on 95% probability that a new observation from the same group will fall inside the ellipse. Ellipses which do not or have little overlap suggest that the variables may be useful in a classification system.

Results

Vascular Calcification in CKD and Controls

The demographics of 69 CKD patients and 68 control participants who took part in the study are shown in Table 1. Primary causes of renal disease in CKD were polycystic kidney disease (19%), glomerulonephritis (17%), diabetic nephropathy (17%), hypertensive nephropathy (16%), ischaemic nephropathy (12%), obstructive nephropathy (4%) and unknown (15%). There were 44 pre-dialysis CKD stages 4-5 patients with median (IQR) eGFR of 13 (11 – 16) ml/min/1.73m^2 and 25 dialysis patients (15 on haemodialysis and 10 on peritoneal dialysis). Two distal tibia HRpQCT scans from CKD could not be assessed due to movement artefact.

A higher proportion of CKD patients had LLAC than healthy controls (Table 1). CKD patients had significantly higher LLAC mass compared to controls. Higher proportion of CKD had detectable LLAC (75%) than AAC (48%) but the difference was not statistically significant. LLAC mass and AAC score were weakly correlated in CKD (rho = 0.28, p<0.05 in CKD) (Figure 2). Meanwhile, there was only a trend in the control group (rho =0.24, p=0.05).

	CKD (N=69)	**Controls (N=68)**	**p value**
Demographics			
Age, years	62 ± 12	62 ± 12	
Male, N	53	53	
BMI (kg/m^2)	27 ± 4.1	28 ± 4.3	0.3
Diabetes	28%	0%	**<0.001**

Cardiovascular disease	32%	6%	**<0.001**
Vascular calcification			
LLAC detected, N (%)	52 (75%)	21 (31%)	**<0.001**
LLAC, mgHA	1.043 (0.05 – 16.52)	0 (0 - 0.55)	**<0.001**
AAC detected, N (%)	33 (48%)	15 (22%)	**0.001**
AAC score	1 (0 – 3)	0 (0 – 0)	**<0.001**
Biochemistry			
Adjusted calcium, mmol/L	2.28 ± 0.15	2.28 ± 0.07	0.9
Phosphate, mmol/L	1.53 ± 0.3	1.06 ± 0.15	**<0.001**
$CaxPO^4$, $mmol^2/L^2$	3.33 (2.97 – 3.91)	2.39 (2.17 – 2.64)	**<0.001**
25-hydrovitamin D, ng/ml	22.9 ± 9.4	23.9 ± 7.0	0.5
iPTH, pg/ml	188 (121 – 280)	32 (27 – 45)	**<0.001**
iFGF23, pg/ml	484 (258 – 2437)	59 (47 – 72)	**<0.001**
tALP, IU/L	88 (73 – 126)	66 (55 – 78)	**<0.001**
bALP, µg/L	22 (17 – 33)	17 (13 – 20)	**<0.001**
Osteocalcin, ng/ml	105 ± 59	16 ± 5	**<0.001**
OPG, pmol/L	8.16 (5.60 – 11.17)	4.06 (3.23 – 5.03)	**<0.001**
t-uc MGP, nmol/L	2064 (1257 – 2903)	4919 (3194 – 6773)	**<0.001**
dp-uc MGP, pmol/L	1479 (1055 – 2148)	443 (346 – 557)	**<0.001**
Fetuin A, µg/ml	239 ± 39	266 ± 49	**0.001**

Table 1. Demographics, vascular calcification and biochemical characteristics in CKD and controls. Abbreviations: BMI, body mass index; LLAC, lower leg arterial calcification; AAC, abdominal aortic calcification; $CaxPO^4$, calcium x phosphate product; iPTH, intact parathyroid hormone; iFGF23, intact fibroblast growth factor-23; tALP, total alkaline phosphatase; bALP, bone alkaline phosphatase; OPG, osteoprotegerin; t-uc MGP, total uncarboxylated matrix Gla protein; dp-uc MGP, dephosphorylated-uncarboxylated matrix Gla protein.

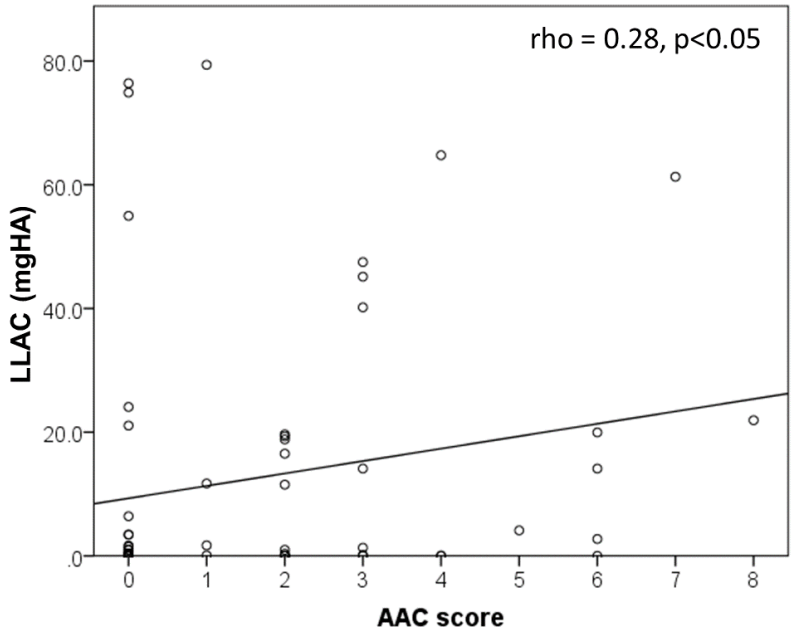

Figure 2. There is a positive association between AAC score and LLAC in CKD patients.

LLAC positively correlated with age (rho = 0.41, p≤0.001) and negatively correlated with eGFR (rho = -0.29, p<0.05) in CKD. 28% of CKD patients had diabetes mellitus (type 1 and type 2) whereas none in the control group had diabetes. CKD patients with diabetes had significantly higher LLAC compared to non-diabetic CKD (median [IQR] 24.07 [3.42 – 61.30] vs 0.23 [0- 3.78] mgHA, p<0.001) (Figure 3).

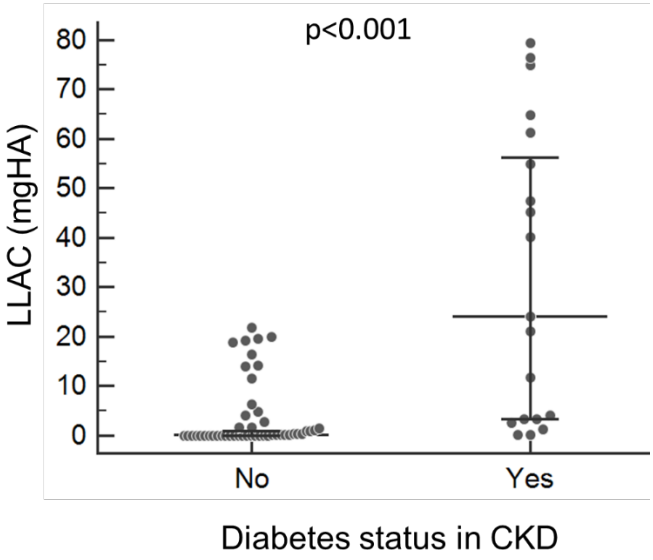

Figure 3. LLAC is more severe in advanced CKD patients with diabetes.

Vascular Calcification Biomarkers

CKD had significantly higher levels of serum phosphate, calcium x phosphate product ($CaxPO^4$), iPTH and iFGF23 than the controls (Table 1). VC biomarkers such as tALP, bALP, osteocalcin and OPG were also significantly higher in CKD than controls. Meanwhile, VC inhibitors such as fetuin A and t-uc MGP were significantly lower in CKD. The level of dp-uc MGP (inactive MGP) was significantly higher in CKD. LLAC in CKD was positively correlated with serum phosphate, $CaxPO^4$, iPTH, iFGF23, tALP, bALP, osteocalcin, OPG and dp-uc MGP (Table 2).

Biomarkers	**rho**	**p values**
Adjusted calcium	0.032	0.8
Phosphate	0.286	**<0.05**
$CaxPO^4$	0.308	**0.01**
25-hydroxyvitamin D	-0.083	0.5
iPTH	0.381	**<0.01**
iFGF23	0.397	**0.001**
tALP	0.411	**<0.001**
bALP	0.286	**<0.05**
Osteocalcin	0.317	**<0.05**
OPG	0.395	**0.001**
t-uc MGP	0.03	0.8
dp-uc MGP	0.308	**<0.05**
Fetuin A	-0.229	0.07

Table 2. Relationship between LLAC and vascular biomarkers in CKD (N=67)

Vascular Calcification and Bone Microstructure

LLAC in CKD was significantly correlated with lower cortical bone vBMD and BV/TV, and thinner and more porous cortical bone at the distal tibia as measured by HRpQCT (Table 3). LLAC was also weakly correlated with distal radius cortical BV/TV. LLAC did not correlate with DXA BMD T-score for total hip, lumbar spine or 1/3 radius ($p>0.05$).

Imaging sites	**LLAC** (N=67)	
Distal radius	**rho**	**p values**
Total vBMD	-0.24	0.08
Cortical vBMD	-0.23	0.09
Trabecular vBMD	-0.12	0.4
Cortical thickness	-0.24	0.08
Cortical porosity	0.23	0.09
Cortical BV/TV	-0.27	**<0.05**
Trabecular thickness	-0.12	0.4
Trabecular number	-0.10	0.5
Trabecular separation	0.12	0.4
Trabecular BV/TV	-0.12	0.4
Distal tibia	**rho**	**p values**
Total vBMD	-0.23	0.07
Cortical vBMD	-0.44	**<0.001**
Trabecular vBMD	0.05	0.7
Cortical thickness	-0.35	**<0.01**
Cortical porosity	0.40	**0.001**
Cortical BV/TV	-0.50	**<0.001**
Trabecular thickness	0.03	0.8
Trabecular number	0.02	0.9
Trabecular separation	-0.06	0.6

Trabecular BV/TV	0.05	0.7

Table 3. Relationship between LLAC and bone characteristics on HRpQCT in CKD. Abbreviations: vBMD, volumetric bone mineral density; BV/TV, bone volume/tissue volume.

Vascular Calcification and Bone Biopsy

43 CKD patients had evaluable bone biopsy samples for histomorphometry. 26% of patients had low, 34% had normal and 40% had high bone turnover as assessed by BFR/BS. There was no correlation between LLAC and BFR/BS. All patients had normal mineralization and LLAC did not correlate with O.Th or MLT. 16% of patients had low trabecular BV/TV and BV/TV did not correlate with LLAC. All correlations remained non-significant when patients without LLAC were excluded.

Independent Factors for Vascular Calcification

Univariate analyses so far have identified a number of factors which significantly correlated with LLAC in CKD such as age, diabetes, biomarkers and bone microstructure. In the multivariate analysis, age, diabetes status, eGFR, log $CaxPO^4$, log iPTH, log iFGF23, log tALP, osteocalcin, OPG, log dp-uc MGP, and distal tibia cortical vBMD on HRpQCT were included. Serum phosphate, log bALP and other distal tibia cortical bone measurements on HRpQCT were excluded due to co-linearity with log $CaxPO^4$, log tALP and distal tibia cortical vBMD respectively. The independent variables included in the model did not show co-linearity. Age, diabetes, log iPTH and log iFGF23 were significantly associated with LLAC in the multivariate analysis (Table 4). However, dialysis patients are known to have more severe LLAC, higher iPTH and iFGF23 levels. Therefore, we performed a sensitivity analysis to assess if the findings are similar in the pre-dialysis CKD subgroup (N=44). The

multivariate analysis for this subgroup also showed that age, diabetes, log iPTH and log iFGF23 were significantly associated with LLAC (adjusted $R^2 = 0.59$).

In the Principal Component Analysis using diabetes status, age Z-score, log iPTH Z-score and log iFGF23 Z-score in CKD, the ellipses for LLAC and non-LLAC overlapped (Figure 4).

Variables	Standardised coefficients, β	p values
Age	0.32	**0.001**
Diabetes	0.42	**<0.001**
Log iPTH	0.23	**<0.05**
Log iFGF23	0.25	**<0.05**

Table 4. Independent factors which are associated with LLAC in CKD in a multivariate analysis. Adjusted R^2= 0.58

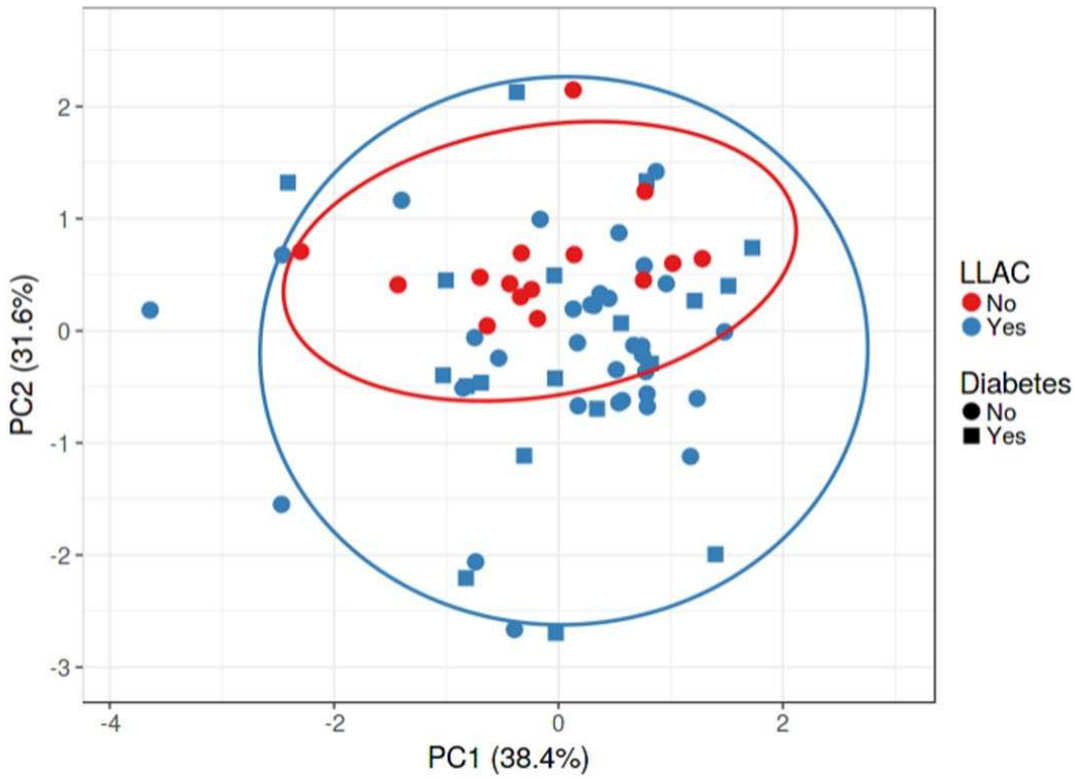

Figure 4. Independent factors associated with LLAC are not able to classify CKD patients with and without LLAC. Independent factors in the model are diabetes status (yes/no), Z-

scores for age, Z-scores for log iPTH and Z-scores log iFGF23. X-axis and Y-axis show that Principal Component 1 (PC1) and Principal Component 2 (PC2) explain 38.4% and 31.6% of the total variance respectively. Prediction ellipses overlap which indicates that these independent factors are not useful for a classification system. N=67.

Discussion

Advanced CKD patients had more severe LLAC than healthy controls, particularly CKD patients with diabetes. After a comprehensive assessment involving a number of biochemical markers, vascular biomarkers, bone turnover and bone microstructure, only age, diabetes, PTH and iFGF23 levels were found to be independently associated with LLAC severity. This is the first study to have comprehensively assessed the relationship between calcification of peripheral arteries using HR-pQCT and CKD-MBD parameters and vascular biomarkers which have been implicated in VC process.

More severe LLAC in CKD compared to healthy controls in our study is consistent with previous studies.[4,19] A study involving 193 dialysis patients showed that the prevalence of VC was 79% compared to 38% in the general population.[4] However, LLAC mass in the CKD group of our study is lower than LLAC mass reported by Patsch et al (median of 1.04 mgHA vs 6.65 mgHA).[10] This is expected because the study involved dialysis patients only and we included pre-dialysis patients in our study. VC has been shown to be worse in dialysis than in pre-dialysis patients.[2]

Studies in dialysis patients showed increasing VC severity with age, often regardless of factors such as dialysis vintage, dyslipidaemia, PTH level and hypertension.[19-23] Diabetes mellitus is another strong risk factor where studies in dialysis and non-dialysis CKD showed that diabetes is associated with 2 - 5 times increased likelihood of having more severe VC.[10,24] Consistent with these studies, we found that age and diabetes were independently associated with LLAC in CKD.

In our study, serum phosphate and $CaxPO^4$ were positively associated with LLAC. Hyperphosphataemia is known to be positively associated with VC.[25,26] The Chronic Renal Insufficiency Cohort (CRIC) study involving 1500 patients with mild to moderate CKD showed that serum phosphate was associated with the prevalence and severity of CAC.[27] Experimental studies have shown that hyperphosphataemia encourages VSMCs to differentiate into osteogenic phenotype, leading to mineral deposition.[28,29]

We also found that tALP and bALP were positively associated with LLAC. High ALP promotes VSMCs transformation into osteoblast-like cells in experimental model.[30] Our finding is consistent with other studies in advanced CKD.[31,32] Furthermore, ALP is associated with VC progression in advanced CKD.[33]

There are also other circulating markers released from bone which may have a role in VC, namely osteocalcin and OPG. High osteocalcin level has been shown to stimulate VSMCs differentiation and mineralization in experimental model and osteocalcin has been found in calcified atherosclerotic plaque and calcified aortic valve.[34-36] Although osteocalcin is produced primarily by osteoblasts in bone, it is also produced locally by VSMCs.[37] Osteocalcin was positively associated with LLAC in our study, thus suggesting that osteocalcin is a VC promoter. However, other studies found no relationship between osteocalcin and VC in dialysis patients.[32,38] In contrast, studies in elderly men and haemodialysis patients showed that low osteocalcin level was associated with VC, suggesting a protective effect of osteocalcin in VC. [39,40]

Another bone-related marker is OPG which is also produced mainly by osteoblasts but it can be produced locally by VSMCs.[41] Experimental studies showed that OPG reduces osteoblastic transformation of VSMCs and blocks ALP activity in the vasculature.[42,43] Consistent with the mechanisms described, an animal study showed that OPG is inversely associated with VC.[44]

However, human studies including our study showed that OPG is positively associated with VC in pre-dialysis and dialysis patients.[31,45,46] There are two possible explanations for these conflicting observations. OPG secreted by VSMCs may be a local VC inhibitor but higher circulating OPG level may not have the same protective effect.[47] OPG production by VSMCs could also be a compensatory mechanism to inflammation from pre-existing VC.[48]

PTH and FGF23 levels rise in response to reduced renal function, reduced renal phosphate excretion, and abnormal vitamin D metabolism in CKD. Thus it has been difficult to infer causal relationship between these markers and VC. In our study, iPTH and iFGF23 were positively and independently associated with LLAC. FGF23 is produced primarily by osteocytes but the presence of its co-receptor (klotho) in VSMCs have been inconsistent.[27,49,50] Two *in vitro* studies showed that it is phosphate, and not FGF23, which induces VC.[27,50] However, there is evidence that FGF23 have a more direct role in VC process. FGF23 has been shown to reduce osteoblastic gene expression in VSMCs which express klotho, suggesting a protective effect of FGF23 in VC.[51] A number of clinical studies have shown that VC is associated with high FGF23 level, not just in CKD and dialysis patients, but also in non-CKD.[3,51-54] Questions remain whether high FGF23 level is part of a compensatory mechanism to prevent overt VC.[55,56]

An experimental study showed that high PTH medium promotes osteoblastic transformation of endothelial cells.[57] Animal study of CKD also showed that high PTH level promotes VC independent of phosphate.[58] It appears that PTH regulates VC via PTH1 receptor supporting a direct role of PTH in VC.[59] PTH effect on VC may also be indirect via increased bone turnover which releases phosphate from bone. However, the relationship between PTH and VC prevalence and progression in clinical studies have been mixed.[53,54,60-62]

There is growing knowledge on circulating VC inhibitors in CKD. MGP is produced by VSMCs and endothelial cells. It inhibits calcium crystal growth locally and prevents VSMCs differentiation to osteoblast-like cells.[63-65] MGP is dependent on vitamin K for its activation and undergoes two post-translational modifications: glutamate carboxylation and serine phosphorylation.[66] The active forms of this inhibitor is the carboxylated and/or phosphorylated MGP. We measured two different forms of MGP; total uncarboxylated (t-uc) MGP and dephosphorylated-uncarboxylated (dp-uc) MGP. The t-uc MGP is mainly in the phosphorylated form which is an active VC inhibitor. We found that CKD patients had lower t-uc MGP level compared to healthy controls but it was not associated with LLAC severity in CKD. Cranenburg et al showed that t-uc MGP level was also lower in dialysis patients compared to healthy controls but in contrast to our study, t-uc MGP was inversely associated with VC.[67] The dp-uc MGP is an inactive MGP molecule as it has not undergone gamma-glutamyl carboxylation and serine phosphorylation which contribute to its function as a calcification inhibitor.[66] The dp-uc MGP level was higher in CKD compared to healthy controls in our study and it was positively associated with LLAC in CKD. This is consistent with findings by Schurgers et al where dp-uc MGP was significantly associated with aortic calcification in 107 patients with CKD stages 2-5D.[68] Our finding suggests that dp-uc MGP may be a better marker for VC compared to t-uc MGP in advanced CKD. Fetuin A is a potent systemic VC inhibitor which is produced by the liver.[69] Studies in predominantly dialysis patients showed that low serum fetuin A was associated with VC.[5,52] We only found a trend towards lower fetuin A with increasing LLAC severity.

The bone-vascular abnormalities are an important sequelae of CKD-MBD. In a study involving 193 dialysis patients, Rodriguez-Garcia et al showed that patients with VC had 6 times higher risk of vertebral fractures than those without.[4] Chen et al reported a study involving 685 dialysis patients which found that patients with VC had double the risk of fractures compared

to those without VC.[5] This may be partly explained by the association between lower BMD and VC in these patients.[62,70,71] A longitudinal study also showed that VC progression was associated with bone loss in CKD.[72] Simultaneous assessment of bone and LLAC at the distal tibia in our study revealed that lower cortical BMD and worse cortical bone microstructure were associated with LLAC severity. Our findings are similar to a study by Cejka et al which found that high CAC was associated with lower BMD and trabecular bone volume at the distal tibia using HRpQCT in 66 dialysis patients.[73] The study and ours found no association between VC and DXA BMD. It is important to note that the association of LLAC and worse bone microstructure may not be unique to advanced CKD.[11]

Studies assessing direct relationship between VC and bone turnover, mineralization and volume using bone biopsy is limited. Two studies in dialysis patients found that low bone volume, but not bone turnover, was associated with increased VC.[20,74] However, VC has also been associated with low bone turnover.[75,76] Malluche et al showed that the presence and progression of VC was associated with iPTH>540pg/mL in a study of 213 dialysis patients.[62] Although bone biopsy was not performed, this level of iPTH is almost certainly associated with high bone turnover. We did not find any relationship between VC and bone turnover, mineralization or volume on bone biopsy in our study. This could be due to small number of patients with bone biopsy data and the inclusion of pre-dialysis and dialysis CKD. Dialysis patients tend to have worse VC and worse bone characteristics than pre-dialysis CKD.[2,77]

Despite positive associations between LLAC and demographics, a number of biochemistry, vascular biomarkers and bone microstructure in this study, only age, diabetes, iPTH and iFGF23 were independently associated with LLAC. Accelerated VC progression, worse secondary hyperparathyroidism and higher level of FGF23 are well recognised in dialysis patients and our findings may be a reflection of dialysis status.[78] However, we have also

shown that these findings are similar in pre-dialysis CKD, suggesting that the same factors play important role in LLAC severity in pre-dialysis and dialysis patients.

The ability of risk factors in classifying patients with VC is an interesting prospect. However, we have shown that the independent factors identified in this study are not able to classify patients with or without LLAC. This may be due to the fact that the independent factors only explains 58% of the change in LLAC severity. The other factors assessed in this study are likely to play a complex inter-connected role in the VC process. Therefore, imaging of the arteries is still required to assess VC in CKD. Quantitative and highly reproducible imaging of the arteries is needed and HR-pQCT can be a useful alternative to cardiac or abdominal aorta CT with the advantage of lower radiation dose (3µSV for HR-pQCT vs 1-10mSV for quantitative cardiac CT).[79]

There are several strengths in this study. We have carried out a comprehensive assessment of biochemical, vascular biomarkers, bone turnover and bone microstructure in a group of unselected CKD stages 4-5D who are representative of real-world clinical practice. Assessment of LLAC using HRpQCT is relatively new but it has been validated using coronary arteries CT and it allows simultaneous bone microstructure and VC assessment.[10] HRpQCT also provides a quantitative assessment of VC whereas AAC assessment using routine X-ray-based imaging is semi-quantitative. We have shown that the weak relationship between the two imaging techniques is not just in CKD but also in healthy controls. We also assessed the relationship between VC and bone in CKD using bone biopsy.

There are also several limitations; we did not assess CAC using CT but VC is a systemic process. We assessed pre-dialysis CKD and dialysis patients together but they may be a heterogeneous group. Dialysis patients are known to have worse VC than pre-dialysis CKD patients. The small sample size may have also limited the statistical power to detect other

independent factors associated with LLAC beyond the ones already identified. Inflammation, a known VC promoter, was not assessed but it is well established that CKD is a pro-inflammatory state.[80] We also did not assess other factors such as hyperlipidaemia and hypercholesterolemia but evidence showed that VC in advanced CKD is independent of these traditional atherosclerosis risk factors.[19,21] Finally, we used thinner 4mm diameter bone biopsy trephine for histomorphometry but a recent study showed that ROD diagnosis was as accurate as samples obtained using the larger 8mm trephine.[81]

Conclusions

LLAC in advanced CKD was independently associated with age, diabetes, PTH and FGF23 level but not with other known circulating VC inhibitors and promoters or bone turnover and microstructure. The finding explains the reason for unsuccessful interventional approach so far as these independent factors are either difficult or non-modifiable. Furthermore, the other biochemical markers, vascular biomarkers and bone microstructure probably still contribute to VC in a complex inter-connected process. Thus future research may have to focus on multi-interventional approach to prevent VC in CKD. HRpQCT can be used in such studies for simultaneous assessment of the bone-vascular axis.

Acknowledgement

This research was funded by Kidney Research UK, Immuno Diagnostic Systems and Sheffield Hospitals Charity. The study was carried out at the National Institute for Health Research (NIHR) Sheffield Clinical Research Facility. The views expressed are those of the authors and not those of the funders, NHS, the NIHR or the Department of Health. The funders had no roles in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

We thank Sister Angela Green (Lead Research Nurse), Selina Bratherton (Bone Imaging Technician and Project Manager), Louese Dunn (Renal Research Co-ordinator), Professor Mark Wilkinson (Consultant Orthopaedic Surgeon for bone biopsy training), Dr David Hughes (Consultant Histopathologist for bone histology support) and all the study participants. Abstracts relating to this manuscript were presented at the European Calcified Tissue Society Congress 2019, UK Kidney Week Conference 2019 and ERA-EDTA Congress 2019.

Disclosures

SS received research grant from Immuno Diagnostic Systems (IDS). RE receives consultancy funding from IDS, Roche Diagnostics, GSK Nutrition, FNIH, Mereo, Lilly, Sandoz, Nittobo, Abbvie, Samsung, Haoma Medica, CL Bio, Biocon, Lyramid and Viking; and grant funding from Nittobo, IDS, Roche, Amgen and Alexion. OG, FG, MP and AK have no financial conflict of interest to declare.

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