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Phenotypic features of vascular calcification in chronic kidney disease

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Abstract. Dai L, Debowska M, Lukaszuk T, Bobrowski L, Barany P, Söderberg M, Thiagarajan D, Frostegård J, Wennberg L, Lindholm B, Qureshi AR, Waniewski J, Stenvinkel P (Karolinska Institutet, Stockholm, Sweden; Nalecz Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences, Warsaw; Bialystok University of Technology, Bialystok, Poland; AstraZeneca R&D, Gothenburg, Sweden). Phenotypic features of vascular calcification in chronic kidney disease. *J Intern Med* 2020; **287**: 422–434.

Background. Patients with chronic kidney disease stage 5 (CKD5) are predisposed to vascular calcification (VC), but the combined effect of factors associated with VC was sparsely investigated. We applied the relaxed linear separability (RLS) feature selection model to identify features that concomitantly associate with VC in CKD5 patients.

Methods. Epigastric arteries collected during surgery from living donor kidney transplant recipients were examined to score the histological extent of medial VC. Sixty-two phenotypic features in 152 patients were entered into RLS model to differentiate between *no–minimal* VC ($n = 93$; score 0-1) and *moderate–extensive* VC ($n = 59$; score 2-3). The subset of features associated with VC was selected on the basis of cross-validation procedure. The strength of association of the selected features with VC was expressed by the absolute value of 'RLS factor'.

Results. Among 62 features, a subset of 17 features provided optimal prediction of VC with 89% of patients correctly classified into their groups. The 17 features included traditional risk factors (diabetes, age, cholesterol, BMI and male sex) and markers of bone metabolism, endothelial function, metabolites, serum antibodies and mitochondrial-derived peptide. Positive RLS factors range from 1.26 to 4.05 indicating features associated with increased risk of VC, and negative RLS factors range from −0.95 to −1.83 indicating features associated with reduced risk of VC.

Conclusion. The RLS model identified 17 features including novel biomarkers and traditional risk factors that together concomitantly associated with medial VC. These results may inform further investigations of factors promoting VC in CKD5 patients.

Keywords: chronic kidney disease, vascular calcification, biomarkers, relaxed linear separability (RLS) method.

Introduction

Arterial hardening with medial vascular calcification (VC), a typical early vascular ageing (EVA) phenotype, leads to high risk of premature cardiovascular morbidity and mortality in patients with chronic kidney disease (CKD) [1–4]. Calcification occurring within the medial wall (Mönckeberg's sclerosis) is a characteristic feature of arteriosclerosis, whereas atherosclerosis is mainly due to build-up of plaques in the intimal layer of the artery with subsequent narrowing of the vessels [5]. The poor clinical outcome in patients with CKD stage 5 (CKD5) is to a large extent linked with medial VC [4, 6, 7]. The severity and extent of VC can be used as a measure of EVA and as a predictor of mortality risk in CKD [8, 9]. Apart from the key roles of calcium and phosphate [10], VC has been

demonstrated as a highly regulated cell-mediated active process involving numerous mediators and effector proteins [11, 12] that share similarity with osteogenesis [13–15]. While key regulators of bone metabolism may potentially explain underlying pathophysiological mechanisms of VC [16, 17], a complete set of clinical features and circulating biomarkers is still not fully identified-partly due to the lack of comprehensive clinical data sets as well as lack of appropriate statistical methods.

There is a huge clinical need to identify features that could optimally predict the presence of VC with high specificity and sensitivity. In the uraemic milieu, apart from traditional risk factors, the simultaneous increase in arterial osteochondrocytic programming and defective anti-ectopic osteogenesis defence systems create a 'perfect storm' for VC [13, 18–21]. The cellular interactions beyond these processes provide additional layers of complexity that make it difficult to identify relevant circulating biomarkers of VC in clinical research. One approach to tackle this type of complex data sets is to use the relaxed linear separability (RLS) method, a predictive model based on available phenotype or genotype feature input and a dichotomous outcome. We have reported that the RLS model successfully identified a panel of predictors of inflammation in patients with CKD [22, 23].

The overall objective of the current analysis is to identify factors associated with histologically verified VC in arterial biopsies obtained from patients with CKD5 undergoing living donor kidney transplantation (LD-RTx). A large set of phenotypic 'features', that is, demographic, clinical and laboratory parameters potentially linked to VC, was entered into the RLS model to select those features that could best discriminate patients with extensive or moderate VC (defined by scoring of VC in the vascular specimens) from those without or with less severe signs of VC.

Materials and methods

Patients

We reviewed demographic, clinical and biochemical data of CKD5 patients undergoing LD-RTx from March 2009 through April 2018 at the Department of Transplantation Surgery at Karolinska University Hospital. The protocol, selection criteria and clinical procedures used in the study of the LD-RTx cohort were described previously [24]. Informed consent was obtained from each individual, and the study was approved by the regional committee of ethics in Stockholm and adhered to the statutes of the Declaration of Helsinki.

In the present study, 152 patients (with 63 features) were included among 174 patients (with 82 features) of the LD-RTx cohort who had undergone vascular biopsy (see below) and histological media VC scoring (Fig. 1). The selection of patients and features entering the RLS model was based on the following criteria: only those patients (152 out of 174 patients) for whom at least 65% of the investigated features were measured were included; only measurements (63 out of 82 features) that were available in at least 70% of the patients were selected. The final data set thus consisted of 152 patients and 63 features (counting VC score), with 8% of missing data (Fig. 1 and Table S1).

A comparison of clinical and demographic characteristics among 152 patients included in the data set and 22 patients excluded showed-except for the proportion of smokers, which was higher among those with severe/moderate VC-no significant differences between the two groups (Table S2).

Among the 152 patients (median age 46 year, 66% males), 55 patients (36%) received conservative treatment before undergoing LD-RTx (pre-emptive Rtx) while 97 patients (64%) underwent dialysis treatment (median length of time on dialysis of 12 (3-43) months) before LD-RTx, by haemodialysis (n = 47, 31%) or peritoneal dialysis (n = 45, 30%), or both, as two patients who initially received peritoneal dialysis later on were switched to haemodialysis and three patients who started with haemodialysis later on were switched to peritoneal dialysis.

Clinical and biochemical data collection

All patients selected for LD-RTx underwent extensive clinical and laboratory investigations at the baseline visits prior to renal transplantation [24]. Clinical investigations included anthropometric body composition measurements, coronary artery calcification (CAC) score by computed tomography (CT) (Light Speed VCT or Revolution CT; GE Healthcare, Milwaukee, WI, USA), pulse wave analysis by SphygmoCor System (AtCor Medical, Sydney, Australia) and estimated skin content of advanced glycation end products (AGEs) by autofluorescence (Autofluorescence AGE-ReaderTM, DiagnOptics Technologies BV, Groningen, the

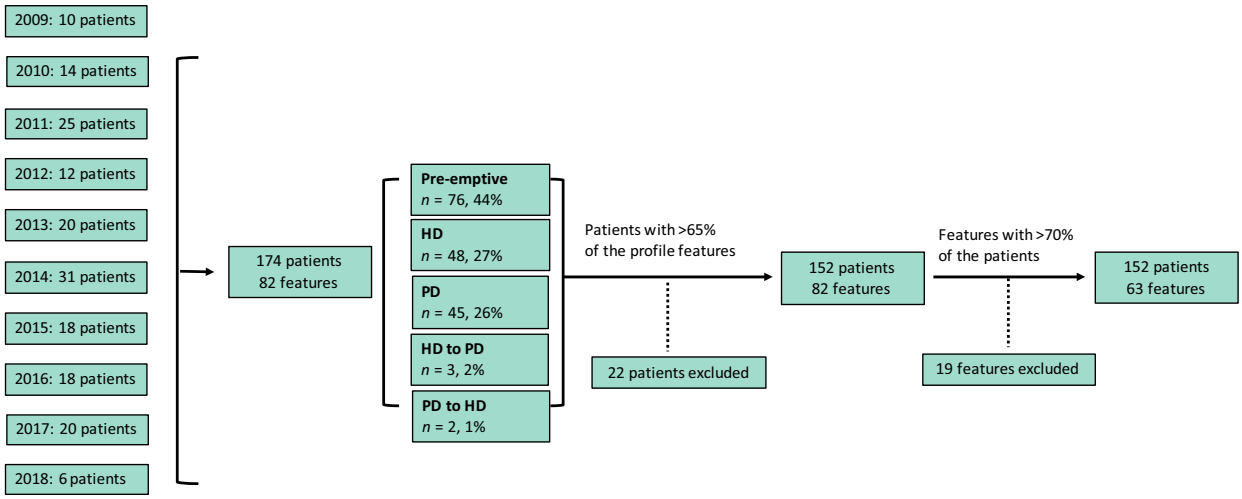

Fig. 1 *Flow chart showing recruitment of patients and criteria for selection of patients and features that were included in the analysis by the relaxed linear separability method. Recruitment period, March 2009 through April 2018. Abbreviations: Pre-emptive, denotes patients that pre-emptively underwent living donor kidney transplantation (LD-Rtx) without undergoing dialysis; HD, haemodialysis; PD, peritoneal dialysis; HD to PD, patients initiating dialysis on HD and subsequently switching to PD; PD to HD, patients initiating dialysis on PD and subsequently switching to HD.*

Netherlands). In addition, we analysed a large panel of circulating biomarkers (n = 49) that are considered to reflect inflammation, nutritional status, bone metabolism and VC in CKD5. The investigated parameters are listed in Table S1.

Assessment of presence and extent of media calcification in arterial biopsies

The outcome of the present study was the presence of histological proven (by scoring of an experienced pathologist) VC at the time of LD-RTx. The preparation of epigastric artery biopsies and histological evaluation procedures were described previously [24]. The degree of media calcification was semi-quantified on von Kossa-stained sections and graded 0-3, where 0 indicated no calcification and 3 the highest degree of calcification.

Method for feature selection and classification

The classification of patients was based on presence of histological signs of calcification: patients graded as 0 (n = 25) and 1 (n = 68) were combined into Group 1 representing no–minimal vascular calcification (n = 93), and those having moderate (score 2; n = 38) or extensive (score 3; n = 21) signs of VC were combined into Group 2, representing moderate–extensive vascular calcification (n = 59).

The demographic and clinical characteristics of the two studied groups are outlined in Table 1. Vascular calcification and 62 other phenotypic features were analysed, including 6 clinical and demographic characteristics, 4 anthropometric parameters, 49 circulating markers and 3 other measurements, that is, CAC, pulse wave analysis and AGEs (Table S2).

The RLS method was applied to select the subset of features associated with VC. The term 'relaxed' in the name of the method means the deterioration of the linear separability (between two groups of patients) due to the gradual neglecting of selected features. Initially, in the RLS algorithm the optimal hyperplane that separates patients from two groups is determined. This hyperplane is usually described by a large number of features. The repeated minimization of criterion function with a gradual increase in regularization parameter of RLS method allows to generate in a deterministic manner the descending sequence of feature subsets. In the process of evaluation of each feature subset, the cross-validation (leave one out) procedure was used. The apparent error (AE) and the cross-validation error (CVE) determined the errors on the training and testing parts of the data, respectively, and both denote the proportion of misclassified patients. The feature subset with minimal

Table 1. *Clinical and demographic characteristics of 152 ESRD patients according to the classification of medial VC score*

	No–minimal VC(n = 93)	Moderate–extensive VC(n = 59)	P value
Age, years	39 [21-61]	51 [37-67]	<0.001
Gender (male), n (%)	52 (56)	48 (81)	0.002
BMI, kg/m^2	23.5 [19.6–28.0]	25.7 [22.3-29.8]	<0.001
DM, n (%)	0 (0)	14 (24)	<0.001
CVD, n (%)	5 (5)	17 (29)	<0.001
MAP, mmHg	103 [84-116]	104 [88-123]	0.38
Current smoker, n (%)	32 (36)	25 (47)	0.25
HGS%	100 [63-123]	90 [56-116]	0.31
Haemoglobin, g L^{-1}	114 [96-132]	114 [98-132]	0.93
Albumin, g L^{-1}	36 [31-40]	35 [29-40]	0.14
Calcium, mmol L^{-1}	2.3 [2.1-2.5]	2.3 [2.0-2.5]	0.37
Phosphate, mmol L^{-1}	1.7 [1.1-2.3]	1.7 [1.0-2.5]	0.73
iPTH, ng L^{-1}	226 [94-562]	302 [80-594]	0.29
HDL, mmol L^{-1}	1.4 [1.0-1.9]	1.3 [0.8-2.1]	0.15
Cholesterol, mmol L^{-1}	4.6 [3.4-5.9]	4.3 [3.0-6.4]	0.19
TG, mmol L^{-1}	1.3 [0.7-2.3]	1.4 [0.8-2.5]	0.22
hsCRP, mg L^{-1}	0.6 [0.2-4.2]	0.9 [0.3-6.1]	0.06
IL-6, pg mL^{-1}	1.2 [0.1-3.8]	1.1 [0-4.2]	0.85
CAC, AU	0 [0-336]	111 [0-1981]	<0.001

ESRD, end-stage renal disease; VC, vascular calcification; BMI, body mass index; DM, diabetes; CVD, cardiovascular disease; MAP, mean arterial blood pressure; HGS%, handgrip strength, % of sex-matched controls; iPTH, intact parathyroid hormone; HDL, high-density lipoprotein; TG, triglyceride; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; CAC, coronary artery calcification; AU, Agatston units.
Data presented as median (10^{th}-90^{th} percentile), number or percentage.

CVE was selected as optimal and applied on data of all patients to determine receiver operating characteristic (ROC) curve and check classification accuracy. The details of RLS feature selection method were presented elsewhere [25, 26].

Statistical methods and other calculations

Statistical significance was set at the level of P-value <0.05 unless otherwise indicated. Nonparametric Wilcoxon rank-sum test was used to compare continuous features and Fisher's exact test to compare categorical features between two groups. Feature dependencies were investigated using nonparametric Spearman's rank correlation test. In multivariate logistic regression, the backward stepwise procedure was used to select features. Sensitivity, specificity and the correctness of classification are presented for the maximal value of Youden index.

Missing values were assigned using k-nearest neighbour algorithm at k = 1 using 'knnimpute' function from Bioinformatics Toolbox (MATLAB 2018b, MathWorks, Natick, MA, USA). In total, 8% of missing values were imputed. The mean values of features in the resultant data set differed on average of 1.21% ± 1.73% from the original data, and none of the features were statistically different. The imputation of missing values did not involve the outcome variable. The RLS model was applied for the final data set with complete set of values, whereas all the other methods operated on the original data set.

Results

When entering all of the 62 features into the RLS model, the smallest CVE was achieved for a subset of 17 features (CVE = 0.16, AE = 0.14, Figure S1). For the selected features, the area under ROC curve was 0.91 (Fig. 2). At the highest value of Youden index, 89% of the patients were correctly classified into their groups at sensitivity and specificity of 81% and 95%, respectively. The

selected features are listed in Table 2 in descending order of the absolute value of the 'RLS factor' that describes the relative importance of each feature in the selected subset; positive RLS factors indicate features associated with increased risk of VC, and negative RLS factors indicate features associated with reduced risk of VC.

The selected features were as follows: (i) soluble receptor activator of nuclear factor-κB ligand (sRANKL) (factor 4.05), (ii) diabetes mellitus (factor 3.66), (iii) age (factor 3.55), (iv) angiopoietin 2 (factor 3.22), (v) cholesterol (factor 3.15), (vi) body mass index (BMI) (factor 2.95), (vii) sex (factor 2.54), (viii) uric acid (factor 2.48), (ix) IgM antibodies against phosphorylcholine-A (IgM anti-PC) (factor −1.83), (x) free tri-iodothyronine (fT3) (factor −1.59), (xi) tartrate-resistant acid phosphatase 5a (TRAP 5a) (factor −1.51), (xii) mitochondrial open reading frame of the 12S rRNA-c (MOTSc) (factor 1.48), (xiii) carboxy-terminal collagen cross-links (CTX) (factor 1.40), (xiv) IgM antibodies against malondialdehyde (IgM anti-MDA) (factor 1.40), (xv) osteoprotegerin (OPG) (factor 1.30), (xvi) intact parathyroid hormone (iPTH) (factor 1.26) and (xvii) betaine (factor −0.95).

Univariate analysis and multivariate logistic regression

Univariate Spearman's correlation analysis was performed on all 63 features, and the results from this analysis are included in Table 3. A total of 17 features showed a significant correlation with the presence of VC; 7 were among the 17 features identified using RLS method: age (rho = 0.41), OPG (rho = 0.38), diabetes mellitus (rho = 0.40), BMI (rho = 0.32), sex (rho = 0.26), IgM anti-PC (rho = −0.22) and angiopoietin 2 (rho = 0.18). However, 10 features with significant correlation (rho) were not recognized by the RLS model as predictors of VC: CAC score (rho = 0.50), CVD (rho = 0.32), copeptin (rho = 0.28), sclerostin (rho = 0.26), IGF-1 (rho = −0.25), choline (rho = 0.25), troponin T (rho = 0.25), fat body mass index (rho = 0.24), AGEs (rho = 0.21) and dephosphorylated and uncarboxylated matrix Gla-protein (duMGP) (rho = 0.19).

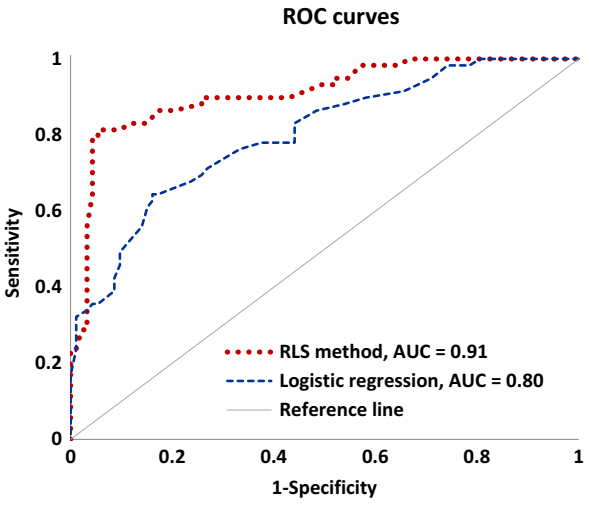

Fig. 2 *Receiver operating characteristic (ROC) curves with area under curves (AUC) for relaxed linear separability (RLS) and multivariate logistic regression methods.*

In the following analysis, we entered features with significant rho correlations into traditional multivariate logistic regression model (Table 4). Four statistically significant features came out from these 17 features in the final model as determinants of VC (pseudo $r^2 = 0.32$): 1-SD higher age [OR: 1.06 (95% CI: 1.03,1.10)], male versus female sex [OR: 6.67 (95% CI: 2.53,17.58)], 1-SD higher BMI [OR: 1.17, (95% CI: 1.04,1.31)] and 1-SD higher OPG [OR: 1.23, (95% CI: 1.05-1.44)]. The area under ROC curve of logistic regression was 0.80 (Fig. 2). At the best cut-off, as measured by Youden statistics, the accuracy of patient classification (based on the coefficients derived from the logistic regression model) was 76% with sensitivity and specificity equal to 64% and 84%, respectively.

Discussion

The identification of risk factors promoting EVA processes in patients suffering from a complex disease condition, such as CKD, is challenging as it requires handling of a complex multifactorial panel of interactive factors and mechanisms involved in VC. In the present study, we applied the multifactorial RLS model to identify factors associated with VC in a clinical data set of CKD5 patients undergoing LD-RTx. From 62 features, a set comprising 17 factors was obtained as the best feature panel allowing 89% of the patients to be classified correctly to their respective groups. In contrast to traditional methods, where factors are tested one by one or in preselected groups versus an outcome, the current approach, using the RLS method, provides a broader as well as less biased view of potential risk factors that-in combination and

Table 2. *List of 17 features derived from RLS method as predictors of VC in 152 CKD5 patients*

No.	Feature Name	RLS factor	Potential clinical and pathophysiological interpretations associated with VC
1	sRANKL	4.05	Member of TNF superfamily; RANKL/RANK signalling regulates osteoclast formation and activation in the physiological bone remodelling process as well as increased bone turnover in pathologic conditions [32]. Together with bone regulatory protein OPG, the RANKL/OPG system is a key mediator of bone metabolism participating in the bone–vascular interplay affecting coronary arteries, atherosclerotic lesions and media vascular layers [17, 33–36]
2	Diabetes	3.66	Patients with type 2 diabetes commonly display media VC (most commonly observed as Mönckeberg's medial sclerosis) accompanied by increased expression of bone matrix proteins in the medial layers, partially due to the direct effects of hyperglycaemia on VSMC osteogenesis via multiple mechanisms [37, 38].
3	Age	3.55	VC is a well-known age-related process. The impact of physiological ageing on VC is complex and multifactorial; underlying mechanisms include cellular senescence and decreased autophagy, prelamin A accumulation, Klotho deficiency and oxidative stress [39]
4	Angiopoietin-2	3.22	A ligand of the Tie-2 receptor modulating activities of inflammatory and angiogenetic cytokines that correlate with disease progression and cardiovascular outcome in CKD [40, 41] and associate with severity of arterial stiffness in CKD stage 3-5 nondialysis patients [42]
5	Cholesterol	3.15	A well-known traditional risk factor for cardiovascular calcification; yet, clinical evidence is needed to demonstrate whether lipid-lowering strategies reduce the progression of calcification [43, 44]. *In vitro*, cholesterol-lowering drugs could enhance osteoblastic differentiation in osteoblast 2T3 cells [45] and VSMCs isolated from LDL receptor (LDLR) knockout mice showed a reduced ALP activity and matrix mineralization with concomitantly decreased intracellular cholesterol levels [46], indicating the role of cellular cholesterol in bone remodelling and VC. It has been hypothesized that statins may promote VC by inhibiting synthesis of vitamin K [47]
6	BMI	2.95	While obesity is assumed to be a precursor of dyslipidaemia and inflammation accounting for increased risk of subclinical atherosclerosis in general population [48], the 'obesity paradox' suggests higher BMI may be associated with better cardiovascular outcome in patients with CKD and other chronic diseases [49]. Data from observational studies were, however, inconsistent [50–52], and studies are warranted to better understand the paradox. Recent data suggest that obesity with systemic inflammation is associated with a different prognosis than obesity without inflammation [53]
7	Male sex	2.54	Despite the long-acknowledged disparity between males and females as regards cardiac disease, the aetiology of the female gender advantage in cardiovascular calcification remains to be delineated. Possible factors include differences in vascular beds, hormonal variations, as well as lifestyle issues

Table 2 (*Continued*)

No.	Feature Name	RLS factor	Potential clinical and pathophysiological interpretations associated with VC
8	Uric acid	2.48	Uric acid suppresses 1α-hydroxylase activity and subsequently results in decreased 1,25(OH)2D and increased PTH levels in rats [54]; in humans, allopurinol lowers serum uric acid with a concurrent increase in 1,25(OH)2D and a reduction in PTH [55]. Epidemiological data demonstrated hyperuricaemia as an independent predictor of moderate CAC score in various study populations [56–58]; transporters of uric acid were expressed both in rat and in human VSMCs [59, 60], which could potentially elucidate its direct or indirect vascular effect
9	IgM anti-PC	−1.83	High circulating levels of IgM anti-PC are negatively associated with atherosclerosis development, cardiovascular risk and mortality in CKD [61–63]. Underlying mechanisms, such as anti-inflammatory effects promoting T regulatory cell polarization and clearance of dead cells, support the protective role of anti-PC in vascular ageing [61, 64, 65]
10	fT3	−1.59	fT3 protects vasculature against calcification [66]. The inverse association between serum fT3 and cardiovascular calcification/outcome has been widely demonstrated [67, 68]. *In vitro*, T3 suppresses Ca/P-induced calcification of rat VSMCs via PI3K/Akt signalling pathway [66] as well as upregulates MGP gene expression and prevents VC [69]
11	TRAP 5a	−1.51	Serum TRAP5a, a novel inflammatory marker, is highly prevalent in patients with rheumatoid arthritis and ESRD [70, 71]. TRAP5a could be an indicator of inflammatory vulnerable plaques that are prone to rupture [72, 73]. The current paradoxical correlation between TRAP5a and epigastric media VC implies that presence of atherosclerosis with vulnerable plaques was present in the subset of non-VC patients, whereas patients in the media VC subset may have co-existent relatively more stable calcified plaque lesions.
12	MOTSc	1.48	MOTSc is a mitochondrial-derived peptide (MDP) which acts as a metabolic hub regulating cellular functions in response to cellular stress [74]. Cellular MDPs induce the senescence-associated secretory phenotype cytokines, such as IL-6, IL-8, IL-10 and TNF [75, 76]. The present finding of high circulating levels of MOTSc in patients with severe VC could represent a compensatory mechanism against VSMC senescence and calcification process in uraemia
13	CTX	1.40	CTX is produced by osteoclasts as a marker of bone resorption. The 'calcification paradox' illustrates the contradictory association among ectopic artery mineralization and disturbed bone turnover [69]. High bone resorption with elevated serum CTX levels could be linked with increased VC
14	IgM anti-MDA	1.40	Less is known about antibodies against MDA conjugated with albumin, which functions as a DAMP [61]. Studies from a few groups have reported its protective property in CVD and SLE [64, 77]. Further studies are needed to elucidate the difference between IgM anti-PC and IgM anti-MDA

Table 2 (*Continued*)

No.	Feature Name	RLS factor	Potential clinical and pathophysiological interpretations associated with VC
15	OPG	1.30	OPG acts as a soluble receptor for RANKL and inhibits binding of RANKL to RANK, thereby preventing osteoclast activation and subsequent bone resorption [32]. OPG-/- mice develop early-onset osteoporosis and VC [78]. Clinically, high circulating levels of OPG associate with increased VC as a compensatory mechanism against VC [34]
16	iPTH	1.26	Despite the impact of high PTH on bone loss, cardiovascular events and CAC progression in various populations [79–81], the direct role of PTH in VC remains to be determined. Disputed preclinical data [82, 30] exist as to whether high PTH exacerbates VSMC osteogenesis and calcium deposition through PTH 1 receptor signalling, possibly due to the heterogeneity of calcification inducement, PTH ($PTH_{1\text{-}34}$ fragments or PTH-related peptides), medium composition and VSMC types
17	Betaine	-0.95	Betaine is a nutrient that acts as osmolyte and methyl group donor to maintain organ function [83]. Low plasma levels of betaine associate with cardiovascular events [84]. While the direct vascular effect of betaine remains to be explored [85, 86], an indirect protective role on vascular health could be speculated given its role of in the methionine cycle

RLS, relaxed linear separability; VC, vascular calcification; CKD5, chronic kidney disease stage 5; sRANKL, soluble receptor activator of nuclear factor-κB ligand; TNF, tumour necrosis factor; RANK, receptor activator of nuclear factor-κB; OPG, osteoprotegerin; VSMC, vascular smooth muscle cell; LDL, low-density lipoprotein; BMI, body mass index; PTH, parathyroid hormone; CAC, coronary artery calcium; IgM anti-PC, IgM antibodies against phosphorylcholine-A; fT3, free tri-iodothyronine; MGP, matrix Gla-protein; TRAP 5a, tartrate-resistant acid phosphatase 5a; ESRD, end-stage renal disease; MOTSc, mitochondrial open reading frame of The 12S rRNA-c; CTX, carboxy-terminal collagen cross-links; IgM anti-MDA, IgM antibodies against malondialdehyde; DAMP, damage-associated molecular pattern; CVD, cardiovascular disease; SLE, systemic lupus erythematosus; OPG, osteoprotegerin; iPTH, intact parathyroid hormone

analysed concomitantly-associate with presence of VC in the uraemic milieu.

Five traditional risk factors were placed among the top seven in the resulting ranking feature set derived from RLS method. Despite the common view that traditional risk factors may be inferior as compared to novel biomarkers predicting cardiovascular outcomes in CKD [27, 28], our results indicate that conventional risk factors, that is age, sex, diabetes, BMI and cholesterol, outperformed many novel biomarkers, suggesting that traditional risk factors are of paramount importance for VC in uraemia. Five biomarkers selected by the RLS algorithm were bone metabolism-related markers, that is, sRANKL, fT3, CTX, OPG and iPTH, suggesting the close interplay between disturbed bone metabolism and ectopic VC [29, 30]. In addition, RLS yielded several novel VC biomarkers for which clinical and potential pathophysiological implications are noted in Table 2.

Interestingly, some factors included in the RLS model did not turn out to have any predictive value in the final feature set. For instance, the presence of CVD, CAC score and the systemic inflammatory markers, hsCRP and IL-6, were not associated with VC. Thus, we would assume that the commonness of these features is not conducive for separating between patients with and without VC. Also, while the presence of CVD and the high inflammatory burden are involved with the pathogenesis of VC, these factors may not be direct promotors of VC in the context of uraemic milieu. In the univariate analysis, though CAC score was significantly correlated with VC scores, it did not show up as a predictive feature of media VC verified in epigastric arteries. One possible explanation could be that the aetiology of calcification process in diverse vasculatures, that is coronary arteries and epigastric artery, might be different, resulting in a discrete extent of calcification. Also, by CT heart scanning, we cannot disclose the calcified plaques

Table 3. *Spearman's correlation between vascular calcification and other features*

No.	Variables	Rho	*P* value
1	Age, years	0.41	<0.001
2	Sex, male	0.26	0.001
3	DM, yes	0.40	<0.001
4	CVD, yes	0.32	<0.001
5	BMI, kg m^{-2}	0.32	<0.001
6	AGEs, skin autofluorescence	0.21	0.02
7	Angiopoietin-2, pg mL^{-1}	0.18	0.04
8	CAC, AU	0.50	<0.001
9	Choline, μmol L^{-1}	0.25	0.003
10	Copeptin, pg mL^{-1}	0.28	0.001
11	duMGP, pmol L^{-1}	0.19	0.03
12	FBMI, kg m^{-2}	0.24	0.01
13	IGF-1, ng mL^{-1}	−0.25	0.004
14	IgM anti-PC, U mL^{-1}	−0.22	0.01
15	Osteoprotegerin, pg mL^{-1}	0.38	<0.001
16	Sclerostin, pg mL^{-1}	0.26	0.001
17	Troponin T, μg L^{-1}	0.25	0.006

DM, diabetes; CVD, cardiovascular disease; BMI, body mass index; AGEs, advanced glycation end products; CAC, coronary artery calcification; duMGP, dephosphorylated and uncarboxylated matrix Gla-protein; FBMI, fat body mass index; IGF-1, insulin growth factor 1; IgM anti-PC, IgM antibodies against phosphorylcholine-A

Table 4. *Logistic regression analysis of determinants of vascular calcification in 152 CKD5 patients*[a]

Variables	OR (95% CI)
1-SD higher age, years	1.06 (1.03, 1.10)
Sex, male versus female	6.67 (2.53, 17.58)
1-SD higher BMI, kg/m^2	1.17 (1.04, 1.31)
1-SD higher osteoprotegerin, pg/mL	1.23 (1.05, 1.44)

BMI, body mass index; OR, odds ratio; SD, standard deviation.
[a]Logistic regression was applied by backward stepwise selection procedure, and the significant determinants were presented in this final model.

in coronary arteries as a result of medial or intima calcification.

It is crucial to understand that the features identified by the RLS model-based on their ability to separate the two subgroups characterized by calcification and noncalcification, respectively-are derived taking all investigated features into account simultaneously. The comprehensive results-including some apparently counterintuitive findings among the resulting feature set-require nonbiased interpretations of the mechanisms involved in VC. Of note, in this feature selection analysis, while current knowledge of the biological or pathological implications of certain identified factors may help to elucidate their roles in the studied phenomena, each individual feature should not be considered separately but in combination with all other features in the resulting model. Whereas several of the identified factors are already well-established VC risk factors, the added value of the applied RLS feature selection model is that it provides nephrologists with novel aspects of factors associated with VC, which appear to fit well with the intricate scenario of VC as observed in the clinical situation.

To compare RLS with traditional statistical methods, we performed univariate Spearman's rank correlation and multivariate logistic regression. These analyses revealed certain deficiencies and strengths. Univariate analysis is clearly not ideal for this type of data set. Given that the number of factors resulting in a complex clinical scenario is presumably very large, the number of statistical tests performed would inevitably generate increased risks of false-positive findings; meanwhile, correction for multiple testing would consequently induce false-negative results. Establishing a model such as RLS, taking all available factors into account concomitantly, rather than testing them one by one, minimizes the risk of statistical errors and provides a biologically more relevant way of analysing the data. Nevertheless, most of the significant correlation coefficients resulting from the univariate analysis appear to be biologically plausible based on our current knowledge on factors associated with VC. For example, VC correlated with well-established markers of VC such as sclerostin, duMGP and CAC, while there was a negative correlation between VC and IGF-1 (Table 3). A potential drawback of the RLS method can occur when two features strongly related to the outcome are dependent on each other. In this case, only one feature will enter the model and potentially the second highly predictive feature will be missed. In such situation, univariate analysis has the advantage over the RLS method. It is reassuring that four of the features that associated with VC in the multivariate logistic regression analysis, that

is, age, sex, BMI and OPG, were also present among the 17 features identified by the RLS analysis. However, the predictive performance of VC using logistic regression was lower than that obtained by RLS approach with 76% vs. 89% patients, respectively, correctly classified into their groups. Simultaneously, the proportion of true-positive to false-positive rate was less advantageous in logistic regression than in RLS model with area under ROC curve being 0.80 in logistic regression and 0.91 using RLS method.

Several limitations of the study should be considered. This model is based on a specific data set of CKD patients undergoing LD-RTx who underwent specific measurements of phenotypes and biomarkers with clinical characteristics unique to this cohort. Thereby, the preselection of features was to a large extent based on already available knowledge about causes and implications of VC; *vice versa*, unmeasured biomarkers recently proven to be relevant to VC, such as uromodulin [31], were not included in the analysis. In addition, the definition of VC was based on the established presence-according to scoring by one experienced pathologist-of pathological media VC of the epigastric artery and one single vasculature bed would not be sufficiently representative for the entire vascular system. On the other hand, this study represents a novel approach that may have the capacity to reveal unknown combined effects of individual phenotypic features. Additional tests in replication cohorts as to reinforce and confirm the current findings are warranted.

In conclusion, premature VC as defined by pathological medial calcification score of epigastric arteries in CKD5 patients is-to a large extent-associated with traditional risk factors and bone turnover markers as well as with some novel markers. The current analysis provides a mapping of selected features that-when all investigated features are analysed concomitantly-differentiate patients with VC from those without. Also, using the multifactorial approach characterizing the RLS method and evaluating several features simultaneously makes it possible to identify factors associated with VC in the complex uraemic milieu. Although the identification of phenotypic features that associated with biopsy-verified vascular media calcification needs replication in other cohorts, our findings-if confirmed-may inform future investigations on premature vascular ageing in this patient population without the need of arterial biopsies.

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Conflict of interest statement

BL is affiliated with Baxter Healthcare. MS is an employee of AstraZeneca. PS is on the scientific advisory board of REATA, Baxter and Corvidia.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of features in the datasets.

Table S2. Clinical and demographic characteristics of 152 CKD5 patients included in the analysis and 22 patients not in the final analysis.

Figure S1. Cross-validation error (CVE) and apparent error (AE) during RLS procedure.■

