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Published in:
Kidney International

DOI (link to publication from Publisher):
10.1016/j.kint.2021.10.018

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

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Vera Jankowski[1,12], Turgay Saritas[2,3,12], Mads Kjolby[4,5,6], Juliane Hermann[1], Thimoteus Speer[7], Anika Himmelsbach[8], Kerstin Mahr[8], Marina Augusto Heuschkel[8], Stefan J. Schunk[7], Soren Thirup[9], Simon Winther[10], Morten Bottcher[10], Mette Nyegard[11], Anders Nykjaer[4], Rafael Kramann[2,3], Nadine Kaesler[2], Joachim Jankowski[1], Juergen Floege[2], Nikolaus Marx[8] and Claudia Goettsch[8]

[1]Institute for Molecular Cardiovascular Research (IMCAR), University Hospital RWTH Aachen, Aachen, Germany; [2]Department of Nephrology and Clinical Immunology, University Hospital RWTH Aachen, Aachen, Germany; [3]Institute of Experimental Medicine and Systems Biology, University Hospital RWTH Aachen, Aachen, Germany; [4]Center for Proteins in Memory (PROMEMO) and Danish Research Institute of Translational Neuroscience (DANDRITE), Department of Biomedicine, Aarhus University, Aarhus, Denmark; [5]Danish Diabetes Academy, Novo Nordisk Foundation, Hellerup, Denmark; [6]Department of Clinical Pharmacology, Aarhus University Hospital, Aarhus, Denmark; [7]Department of Internal Medicine 4, Translational Cardio-Renal Medicine, Saarland University, Homburg/Saar, Germany; [8]Department of Internal Medicine I, Cardiology, University Hospital RWTH Aachen, Medical Faculty, Aachen, Germany; [9]Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark; [10]Department of Cardiology, Gødstrup Hospital, NIDO, Herning, Denmark; and [11]Department of Health Science and Technology, Aalborg University, Aalborg, Denmark

Sortilin, an intracellular sorting receptor, has been identified as a cardiovascular risk factor in the general population. Patients with chronic kidney disease (CKD) are highly susceptible to develop cardiovascular complications such as calcification. However, specific CKD-induced posttranslational protein modifications of sortilin and their link to cardiovascular calcification remain unknown. To investigate this, we examined two independent CKD cohorts for carbamylation of circulating sortilin and detected increased carbamylated sortilin lysine residues in the extracellular domain of sortilin with kidney function decline using targeted mass spectrometry. Structure analysis predicted altered ligand binding by carbamylated sortilin, which was verified by binding studies using surface plasmon resonance measurement, showing an increased affinity of interleukin 6 to *in vitro* carbamylated sortilin. Further, carbamylated sortilin increased vascular calcification *in vitro* and *ex vivo* that was accelerated by interleukin 6. Imaging by mass spectrometry of human calcified arteries revealed *in situ* carbamylated sortilin. In patients with CKD, sortilin carbamylation was associated with coronary artery calcification, independent of age and kidney function. Moreover, patients with carbamylated sortilin displayed significantly faster progression of coronary artery calcification than patients without sortilin carbamylation. Thus, carbamylated sortilin may be a risk factor for cardiovascular calcification and may contribute to elevated cardiovascular complications in patients with CKD.

Correspondence: *Claudia Goettsch, Department of Internal Medicine I, Cardiology, University Hospital RWTH Aachen, Medical Faculty, Pauwelsstrasse 30, 52074 Aachen, Germany. E-mail: cgoettsch@ukaachen.de*

[12]VJ and TSa contributed equally to this study.

Received 23 March 2021; revised 20 September 2021; accepted 1 October 2021; published online 9 November 2021

Kidney International (2022) **101,** 574–584; https://doi.org/10.1016/j.kint.2021.10.018

KEYWORDS: carbamylation; cardiovascular calcification; cardiovascular disease; chronic kidney disease; post-translational modification; sortilin

Translational Statement

Patients with chronic kidney disease (CKD) are highly susceptible to developing cardiovascular (CV) disease. CKD-specific CV risk factors are hitherto mainly unknown. We demonstrate that circulating sortilin is post-translationally modified by carbamylation in patients with reduced kidney function. Sortilin carbamylation leads to higher binding affinity to interleukin-6 and promotes arterial calcification *ex vivo*. Moreover, carbamylated sortilin is a risk factor for the presence and progression of coronary artery calcification. Our results point to carbamylated sortilin as a potential therapeutic target for hindering CV calcification in patients with CKD.

About 1 in 10 people worldwide experience chronic kidney disease (CKD).[1] Impaired kidney function is a major independent risk factor for cardiovascular (CV) morbidity and mortality and all-cause mortality.[1,2] In fact, patients with CKD are much more likely to die from CV events than to develop dialysis-requiring kidney failure.[3] The excess calcific mineral deposition within vascular tissue observed in CKD patients contributes mainly to the increased CV risk.[4] CKD facilitates post-translational modification (PTM) of proteins.[5] PTMs, in turn, have been linked to CV calcification,[6] suggesting that a better understanding of PTMs in CKD-induced calcification processes could reveal novel therapeutic targets. However, hitherto, specific CKD-associated protein modifications linked to CV calcification are unknown.

Sortilin is a ubiquitously expressed member of the vacuolar protein sorting 10 protein family of intracellular sorting receptors.[7] It is a single-pass type I transmembrane protein with various roles in protein sorting, trafficking, and cell signaling. As an endocytosis receptor, sortilin can trigger the internalization of ligands from the cell surface via endocytosis and sort ligands between intracellular compartments, such as trans-Golgi network, endosome, lysosome, and secretory pathway.[8] Preclinical *in vivo* evidence suggests an important role of sortilin in the pathogenesis of vascular and metabolic disorders through contributions to arterial wall inflammation and calcification, dysregulated lipoprotein metabolism, and type 2 diabetes mellitus, all CV risk factors.[7] In human vascular smooth muscle cells (hSMCs), intracellular sortilin regulates the loading of the procalcific protein tissue nonspecific alkaline phosphatase (TNAP) into extracellular vesicles, thereby conferring the calcification potential that contributes to microcalcification formation.[9] The ectodomain of plasma membrane-bound sortilin can be shed and secreted into the circulation.[7] In a community-dwelling cohort of men aged >50 years, we reported an association of high sortilin serum levels with aortic calcification and CV events, suggesting sortilin as a CV risk factor in the general population.[10] A role of sortilin in CKD, a patient population with a marked increase in CV calcification, continues to defy elucidation. Most studies have focused on the function of cellular sortilin rather than exploring the biological function of the circulating soluble form.[7] Therefore, achieving a better understanding of the mechanistic relationship between circulating sortilin and the regulatory impact of PTM in CKD will broaden the knowledge of sortilin in CV calcification.

This study interrogates the hypothesis that PTM of circulating sortilin is involved in the development of CV calcification in patients with CKD.

METHODS

Cardiovascular and Renal Outcome in CKD 2–4 Patients–The Fourth Homburg evaluation (CARE FOR HOMe) cohort

The CARE FOR HOMe study has been previously described in detail.[11] A subset of 97 patients was used for the analysis.

Danish study of noninvasive testing in coronary artery disease (Dan-NICAD 1) cohort

The study design of the Dan-NICAD 1 trial has been described previously.[12] For the analysis, we identified a subset of 97 enrolled patients with estimated glomerular filtration rate (eGFR) >60 ml/min and performed frequency matching based on age, sex, body mass index, smoking status, diabetes mellitus, and CV disease. Identification was performed blinded for sortilin levels.

Cardiovascular In Depth Assessment (CARVIDA) cohort

The CARVIDA is a substudy of the German Chronic Kidney Disease study.[13] Only samples of CARVIDA patients included in the trial in Aachen, Germany, were used (n = 78). Computed tomographic imaging was performed on a Dual Source CT scanner (SOMATOM Definition Flash or Force; Siemens), as previously described.[14] With a median follow-up time of 4.4 years, 41 of 78 patients agreed or were available for a second computed tomographic scan during the second CARVIDA visit in 2019 and 2020.

Human tissue

Femoral arteries were obtained during autopsies from patients with and without CKD from RWTH Aachen University, Germany. The study was approved by the ethical committee of the RWTH Aachen University (ethical votes EK180/14 and EK239/11) and performed according to the Declaration of Helsinki.

Ex vivo carbamylation

Recombinant proteins were carbamylated *in vitro* by O-methylisourea bisulfate solution (pH 11.0) at 25 °C for 3 hours, as previously described.[15]

Matrix-assisted laser desorption/ionization (MALDI)–time-of-flight mass spectrometry

PTM was identified using MALDI–time-of-flight (TOF) mass spectrometry (MS; Ultraflex III; Bruker-Daltonic), as previously described.[16]

MS imaging of human vessel sections

Tissue sections were analyzed with Rapiflex (Bruker-Daltonic) in positive reflector mode, in a 600- to 3000-dalton mass range and a grid size of 30 μm.

Statistical analysis

Experimental study data are presented as mean ± SD; n indicates the number of independent experiments or number of patients. Normality was tested using the Shapiro-Wilk test, and quantile-quantile plot and variance heterogeneity were tested using the Brown-Forsythe test. A paired or unpaired 2-tailed Student *t* test with equal or unequal variances was performed to compare 2 groups. For comparison among ≥3 treatment groups, 1- or 2-way analysis of variance followed by Tukey *post hoc* was performed for data with normal distribution and equal variance. Data with skewed distribution were assessed by the Kruskal-Wallis test followed by Dunn *post hoc* test. Data with unequal variances were tested by Welch analysis of variance followed by Dunnett T3 *post hoc* test.

In the clinical studies, continuous data are presented as mean ± SD when normally distributed or as median and interquartile range for variables with skewed distribution. Categorical data are presented as percentage. Pearson/Fisher χ^2 test was used to study the association between categorical variables and unpaired Student *t* test or Mann-Whitney *U* test for continuous variables. Differences between 3 groups were compared using 1-way analysis of variance followed by Sidak *post hoc* test. Least-square means multivariate-adjusted numbers of carbamylated sortilin residues were calculated using generalized linear models, as described previously.[11,17,18]

Coronary artery calcification (CAC) volume was log-transformed (i.e., natural logarithm, ln; *ln*[CAC + 1]) to reduce skewness. Bivariate correlation was assessed using Eta (1 nominal variable and 1 metric variable) or Pearson correlation coefficients (if both variables were metric). Change in CAC volume per year was calculated as follows: [(CAC follow-up – CAC baseline) / follow-up time in months] * 12. Analysis of variance with change in CAC volume as the dependent variable and a fixed-effect term for carbamylation (yes vs. no) was used for the analysis of CAC progression. In addition, this model was

Table 1 | Sortilin levels and demographics of subjects with normal and impaired kidney function

Parameter	Control[a]	CKD[b]	*P* value
N	97	97	
Age, yr	63.3 ± 6.9	64.1 ± 11.3	0.569
Male	62 (64)	66 (68)	0.544
BMI, kg/m^2	29.5 ± 4.0	30.2 ± 4.6	0.258
Smoking	14 (14)	14 (14)	1.000
CVD	28 (29)	35 (36)	0.303
Diabetes mellitus	28 (29)	38 (39)	0.130
$eGFR_{cys\text{-}crea}$, ml/min per 1.73 m^2	92.3 ± 9.9	44.8 ± 23.0	<0.001
Sortilin, ng/ml	32.9 ± 8.7	51.2 ± 23.4	<0.001

BMI, body mass index; CARE FOR HOMe, Cardiovascular and Renal Outcome in CKD 2–4 Patients–The Fourth Homburg evaluation; CKD, chronic kidney disease; CVD, cardiovascular disease; Dan-NICAD 1, Danish study of noninvasive testing in coronary artery disease; $eGFR_{cys\text{-}crea}$, estimated glomerular filtration rate based on serum cystatin c and creatinine.
[a]Control is the group with normal kidney function (Dan-NICAD 1 cohort).
[b]CKD is the group with impaired kidney function (CARE FOR HOMe cohort).
Continuous variables are presented as mean ± SD, and absolute number as n (%).

adjusted by the use of analysis of covariance for the covariates CAC at baseline and the variables listed in Supplementary Table S1.

$P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism (Prism Software Inc., version 9) or SPSS (version 26.0).

Supplementary methods

Detailed methods are in Supplementary Methods.

RESULTS

Carbamylated sortilin increases with kidney function decline

Initially, we assessed sortilin serum levels in patients with CKD from the CARE FOR HOMe study[11] and found increased sortilin levels compared with a matched control group with normal kidney function from the Dan-NICAD 1 trial (Table 1). Circulating proteins are prone to PTM in patients with CKD.[5] Therefore, we performed a detailed mapping of PTM residues of circulating sortilin in participants of the CARE FOR HOMe study and healthy control subjects (Supplementary Tables S2 and S3) using MALDI-TOF/TOF-MS. Compared with control subjects, CKD patients had 8 of the 30 lysine residues that were predominately carbamylated in the extracellular domain of sortilin (Supplementary Table S4 and Supplementary Figure S1). Representative MS spectra from a control subject and a patient with CKD are illustrated in Figure 1a and b. The specificity of the signal was supported by MALDI-TOF/TOF-MS/MS spectra (Supplementary Figure S2A). Quantification revealed a CKD stage-dependent increase of carbamylated residue number (Figure 1c) and intensity of carbamylated sortilin peptides (Figure 1d). Lysine residue 205 was equally modified in all CKD stages, whereas residues 95, 260, and 294 were more often modified in advanced CKD stages (Figure 1e).

Next, we assessed associations between sortilin carbamylation and baseline characteristics (Supplementary Table S3) in the CARE FOR HOMe study. We observed an age-dependent increase of sortilin carbamylation residues (Table 2). Lower kidney function, based on eGFR (based on serum cystatin c and creatinine, $eGFR_{cys\text{-}crea}$), and higher urea and N-terminal pro-brain natriuretic peptide (NT-proBNP) levels were associated with higher carbamylated sortilin residues (Table 2). The associations remained significant after adjustment for age and sex (Table 2). CKD patients under aldosterone antagonist medication displayed reduced sortilin carbamylation (Table 2), whereas there was no difference in $eGFR_{cys\text{-}crea}$ (no, 50.6 ± 23.2 ml/min per 1.73 m^2; yes, 43.5 ± 22.9 ml/min per 1.73 m^2; $P = 0.236$) and urea (no, 68.6 ± 40.1 mg/dl; yes, 72.3 ± 40.9 mg/dl; $P = 0.729$) between patients with or without aldosterone antagonists. Besides urea, myeloperoxidase may mediate protein carbamylation in CV disease.[19] However, we found no association between total levels and activity of myeloperoxidase and sortilin carbamylation (Supplementary Figure S3A and B).

Furthermore, we assessed the presence of carbamylated sortilin in human femoral arteries using MS imaging. Carbamylated peptide SEDYGK*NFK* (m/z 1172) was highly present in calcified femoral arteries from CKD patients and absent in noncalcified femoral arteries (Figure 1f). MS/MS spectra supported the identification of SEDYGK*NFK* (Supplementary Figure S2B). SEDYGK*NFK* is located close to calcified areas in the tunica media (Figure 1g). In contrast, the mass-signal intensity of non–post-translationally modified peptide SEDYGKNFK (m/z 1086) was higher in control arteries (Figure 1f).

Taken together, compared with controls with normal kidney function, patients with CKD have higher sortilin serum levels and exhibit post-translational carbamylated sortilin in the circulation, which can also be detected in the vasculature.

Carbamylated sortilin promotes smooth muscle cell calcification

Given our finding that carbamylated sortilin localized to calcified areas, we next assessed the effect of sortilin carbamylation on vascular calcification *in vitro* and *ex vivo*. To determine the functional relevance of sortilin carbamylation, we induced *in vitro* carbamylation of recombinant sortilin ($Sort_{Carb}$) by urea and detected a similar carbamyl-lysine residue pattern as detected in humans *in vivo* (Supplementary Figure S4A and Supplementary Table S5). *In vitro*, carbamylation did not alter the protein integrity of $Sort_{Carb}$ compared with control mock-modification ($Sort_{Co}$), as assessed by gel electrophoresis and Western blot (Supplementary Figure S4B and C).

Neither $Sort_{Co}$ nor $Sort_{Carb}$ exhibited cytotoxic effects on hSMCs (Supplementary Figure S5A). $Sort_{Co}$ and $Sort_{Carb}$ were equally taken up by hSMCs (Supplementary Figure S5B). In calcifying hSMCs, $Sort_{Carb}$ induced the pro-osteogenic transcripts tissue-nonspecific alkaline phosphatase (ALPL; +56%; $P = 0.039$) and runt-related transcription factor 2 (RUNX2; +25%; $P = 0.014$; Figure 2a and b), as well as tissue nonspecific alkaline phosphatase activity (+46%; $P = 0.007$; Figure 2c), compared with $Sort_{Co}$. On a functional level, $Sort_{Carb}$ significantly augmented matrix calcification

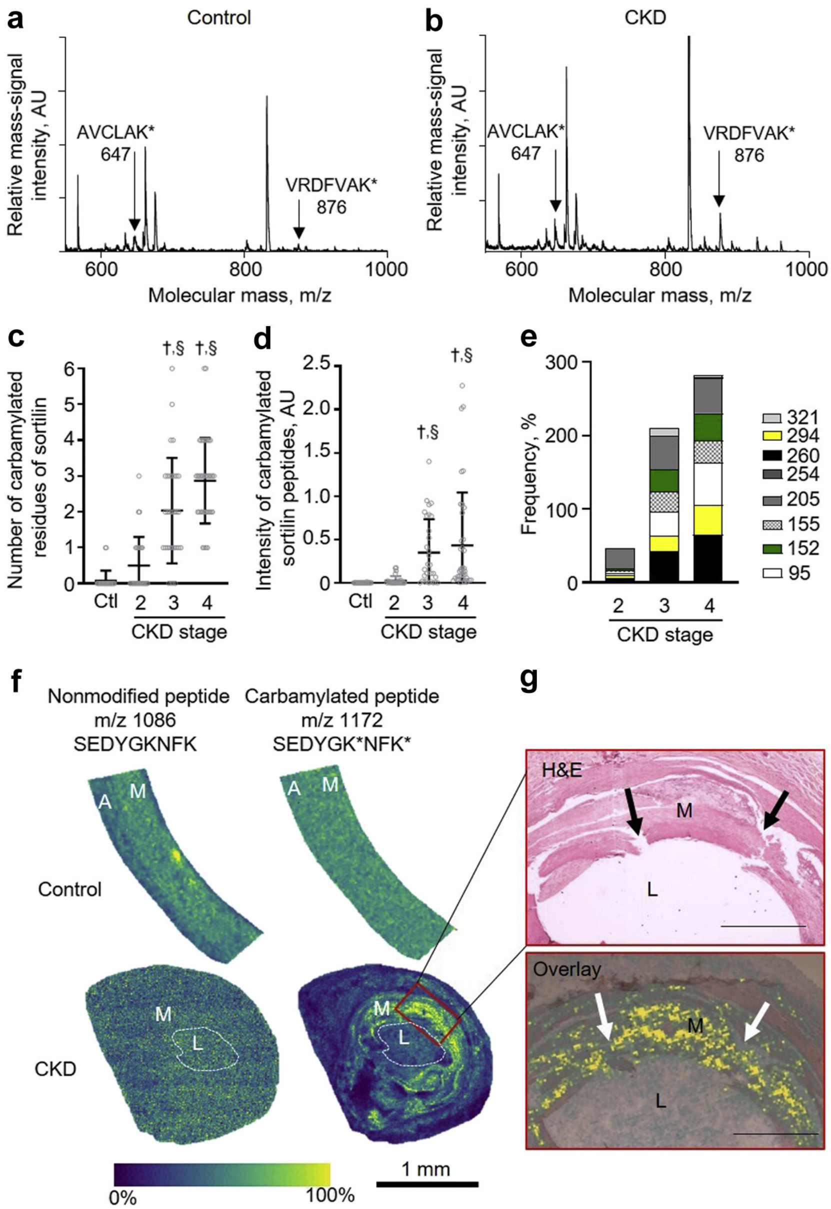

Figure 1 | Sortilin is carbamylated in the circulation and calcified arteries in patients with chronic kidney disease (CKD). (**a,b**) Mass spectrometry spectra from 2 example sortilin peptides from a non-CKD subject (**a**) and CKD patient stage 4 (**b**). The asterisk indicates lysine modification. (**c,d**) Quantification of carbamylated residues within sortilins' extracellular domain isolated from serum from CKD patients stage 2 ($n = 28$), 3 ($n = 31$), and 4 ($n = 38$), and non-CKD subjects (control [Ctl]; $n = 18$) by number of carbamylated sortilin residues (**c**) and signal intensity of carbamylated sortilin peptides (**d**). Data are given as mean ± SD. $^{\dagger}P < 0.01$ versus Ctl, $^{\S}P < 0.01$ versus stage 2 by Kruskal-Wallis test with Dunn *post hoc* test. Each dot represents an individual subject. (**e**) Distribution of sortilin's carbamylated lysine residues in the whole CARE FOR HOMe (Cardiovascular and Renal Outcome in CKD 2–4 Patients–The Fourth Homburg evaluation) cohort, depending on the stage of CKD. Numbers indicate the carbamylated amino acid in the sortilin sequence (see Supplementary Figure S1). (**f,g**) Matrix-assisted laser desorption ionization–imaging mass spectrometry heat map image shows the distribution/presence of the indicated sortilin peptides (m/z 1086, SEDYGKNFK; m/z 1172, SEDYGK*NFK*; asterisk indicates lysine carbamylation) in a calcified femoral artery from CKD patients and non-CKD controls. Intensity relative within one image in arbitrary units (AUs). Representative images from 3 cases per group. Bar = 1 mm. (**g**) Hematoxylin and eosin (H&E) staining and overlay from the red-marked area. Arrows indicate calcified areas. Of note, samples were decalcified before cutting. Bar = 100 μm. A, adventitia; L, lumen; M, media. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

(Figure 2d and e). Finally, in an *ex vivo* organ culture model, $Sort_{Carb}$ increased calcification in rat aortic rings (Figure 2f and g).

Carbamylated albumin and carbamylated collagen type I did not affect ALPL and RUNX2 mRNA expression as well as TNAP activity compared with nonmodified controls (Supplementary Figure S6).

Our data indicate that carbamylated soluble sortilin directly affects vascular cells and promotes vascular calcification *in vitro* and *ex vivo*.

Table 2 | Association of the mean number of sortilin carbamyl residues with baseline characteristics in the CARE FOR HOMe study (n = 97)

Variable	n	No. carbamyl residues Mean	95% CI	*P* value Unadjusted	Adjusted[a]
Age, yr					
<60	38	1.39	0.90–1.89		
60–72	32	2.16	1.51–2.80	0.030[b]	
>72	37	2.37	2.00–2.74	0.008[b]	
$eGFR_{cys\text{-}crea}$, ml/min per 1.73 m^2					
<30	38	2.87	2.49–3.24		
30–60	31	2.03	1.62–2.45	0.003[c]	0.008[c]
>60	28	0.50	0.06–0.94	<0.001[c]	0.000[c]
Urea, mg/dl					
<54	46	1.15	0.77–1.54		
>55	51	2.61	2.24–2.98	<0.001	0.000
NT-proBNP, pg/ml					
<209.9	49	1.43	1.02–1.83		
>209.91	48	2.42	2.01–2.82	0.001	0.007
Aldosterone antagonist					
No	80	2.06	1.74–2.39		
Yes	17	1.24	0.53–1.94	0.038	0.040

CARE FOR HOMe, Cardiovascular and Renal Outcome in CKD 2–4 Patients–The Fourth Homburg evaluation; CI, confidence interval; $eGFR_{cys\text{-}crea}$, estimated glomerular filtration rate based on serum cystatin c and creatinine; KDIGO, Kidney Disease: Improving Global Outcomes; NT-proBNP, N-terminal pro-brain natriuretic peptide.
[a]Age- and sex-adjusted.
[b]Value versus aged <60 years.
[c]Value versus $eGFR_{cys\text{-}crea}$ <30 ml/min per 1.73 m^2.
Age was categorized into tertiles. eGFR was stratified on the basis of the chronic kidney disease classification, according to the KDIGO guidelines. The remaining continuous variables were stratified by median split. All variables from Supplementary Table S3 were tested. Only significant variables were shown.

Carbamylated sortilin alters ligand binding

Sortilin is a coreceptor for several ligands.[8] Carbamylation of lysine significantly alters the properties of the lysine sidechain, whereby the positively charged amino group is replaced by a bulky polar group, potentially affecting ligand binding. We examined the interactions of the most frequently modified sortilin lysine sidechains found in CKD (amino acid sequence [crystal structure sequence]), Lys95(62), Lys205(172), Lys260(227), and Lys294(261), by *in silico* modeling to predict possible structure-function consequences of lysine carbamylation. We used the existing sortilin ectodomain crystal structure from both the neurotensin-bound form at neutral pH (program database [PDB] file: 4PO7; Figure 3a) and the ligand-free form at acidic pH (PDB file: 6EHO; Supplementary Table S6).

At neutral pH, Lys95(62) forms a salt-bridge with Glu609(576) and a hydrogen bond with the main-chain carbonyl group of Arg90(57). At acidic pH, the interaction with Glu609(576) is tighter as the distance between the amino group of Lys95(62) and the carboxyl group of Glu609(576) now allows the formation of a hydrogen bond, whereas the interaction with Arg90(57) is not present. Therefore, carbamylation at Lys95(62) may destabilize both sortilin conformations.

Lys205(172) is forming a salt bridge with Glu148(115) at neutral pH, whereas no interactions are found at acidic pH. Thus, carbamylation at Lys205 would possibly favor the acidic pH conformation of sortilin. Lys294(261) has no interactions at both neutral and acidic pH.

Finally, at neutral pH, Lys260(227) interacts with neurotensin-Tyr11 in the ligand-binding site in the β-propeller tunnel of sortilin. As the acidic pH sortilin structure has no ligand affinity, we find no interactions of the Lys260(227) sidechain in this structure. The interaction with neurotensin-Tyr11 will be affected by carbamylation and might lead to a lower affinity for neurotensin. Interleukin 6 (IL-6), a known sortilin ligand,[20] binds sortilin through its C-terminal tail, which contains an arginine at the equivalent position of neurotensin-Tyr11. Thus, carbamylation of Lys260(227) might probably increase the affinity for IL-6, a potential contributor to vascular calcification.[21]

Subsequent binding studies using surface plasmon resonance spectroscopy and $Sort_{Co}$ or $Sort_{Carb}$ revealed more efficient binding of IL-6 to $Sort_{Carb}$ (dissociation constant [KD] = 23 nM) compared with nonmodified $Sort_{Co}$ (KD = 141 nM), suggesting carbamylated sortilin as a potential IL-6 binding partner (Figure 3b and c). Progranulin, another known sortilin ligand,[22] bound to $Sort_{Co}$, but a binding to $Sort_{Carb}$ could not be detected (Supplementary Figure S7A and B). Both $Sort_{Co}$ and $Sort_{Carb}$ are bound to human receptor-associated protein fused to a glutathion-S-Transferasen (GST) tag,[23] but not to GST alone, supporting the structural integrity of sortilin regardless of its carbamylation status and demonstrating the specificity of our experimental setting (Supplementary Figure S7C–F).

Next, we assessed whether the altered affinity to IL-6 might affect signaling pathways involved in vascular calcification. In calcifying hSMCs, the addition of IL-6 to $Sort_{Carb}$ promoted ALPL and RUNX2 mRNA expression (Figure 3d and e) as well as TNAP activity (Figure 3f), whereas it had no effect in combination with $Sort_{Co}$.

These data indicate that sortilin carbamylation increases the ability of ligand binding to IL-6, which causes increased smooth muscle cell calcification.

Sortilin carbamylation is associated with vascular calcification in CKD patients

On the basis of our data that carbamylated sortilin residues were found to associate with CKD and calcification, we next studied CKD participants of the CARVIDA study, in whom computed tomographic scans quantified CAC. Like the CARE FOR HOMe study participants, most CARVIDA participants had 2 and 3 PTMs, with lysine residues 95 and 260 frequently affected (Supplementary Figure S8A). Compared with patients without carbamylated residues, patients with ≥1 sortilin carbamyl-lysine residues were older ($P = 0.002$), had lower eGFR ($P = 0.008$), were more frequently (former) smokers ($P = 0.023$), and had significantly higher CAC volume ($P < 0.001$; Figure 4a and Supplementary Table S7).

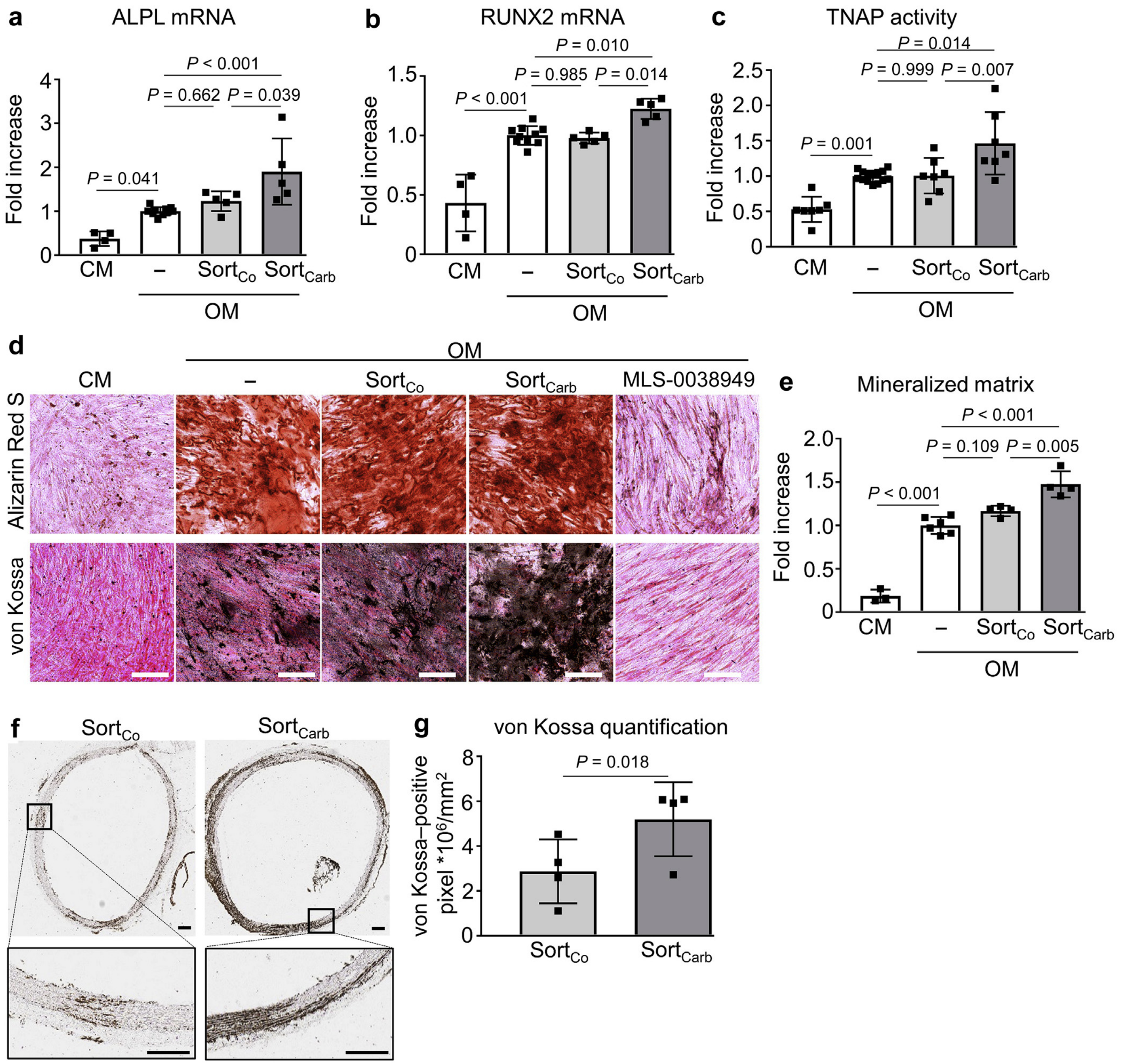

Figure 2 | Carbamylated sortilin induces vascular calcification *in vitro* and *ex vivo*. Human vascular smooth muscle cells were cultured up to 21 days in control medium (CM) or osteogenic medium (OM) supplemented with 200 ng/ml nonmodified ($Sort_{Co}$) or carbamylated ($Sort_{Carb}$) sortilin. (**a,b**) mRNA expression of (**a**) tissue-nonspecific alkaline phosphatase (APLP) and (**b**) runt-related transcription factor 2 (RUNX2) at day 14. n = 4 to 5 independent cell donors. One-way analysis of variance (ANOVA) with Tukey *post hoc* test was used. (**c**) Tissue nonspecific alkaline phosphatase (TNAP) activity at day 14. n = 4 independent cell donors. One-way ANOVA with Tukey *post hoc* test was used. (**d**) Representative images of calcification detected by Alizarin Red S– and von Kossa–stained mineralized matrix at day 21. The TNAP inhibitor (MLS-0038949) served as control. n = 3. Bar = 200 μm. (**e**) Quantification of eluted Alizarin Red S stain. n = 4. One-way ANOVA with Tukey *post hoc* test was used. (**f,g**) Calcification after *ex vivo* aortic rat ring culture with 3 mM Ca/2 mM P and 200 ng/ml $Sort_{Co}$ or $Sort_{Carb}$ for 7 days. (**f**) Representative von Kossa image. Bar = 150 μm. (**g**) Quantification of (**f**). n = 4 rats per group. Each dot represents 1 rat aorta. Paired *t* test was used. n = 3 independent modification preparations were used. Data are given as mean ± SD. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Ln-(CAC + 1) at baseline correlated with the presence and increase of post-translationally carbamylated sortilin (Supplementary Table S1). Furthermore, Ln-(CAC + 1) was also associated with male sex, diabetes mellitus, antidiabetic and lipid-lowering medication, higher age, serum IL-6 and hemoglobin A1c, and lower eGFR and serum high-density lipoprotein (Supplementary Table S1). Nevertheless, the association between carbamylated sortilin residues

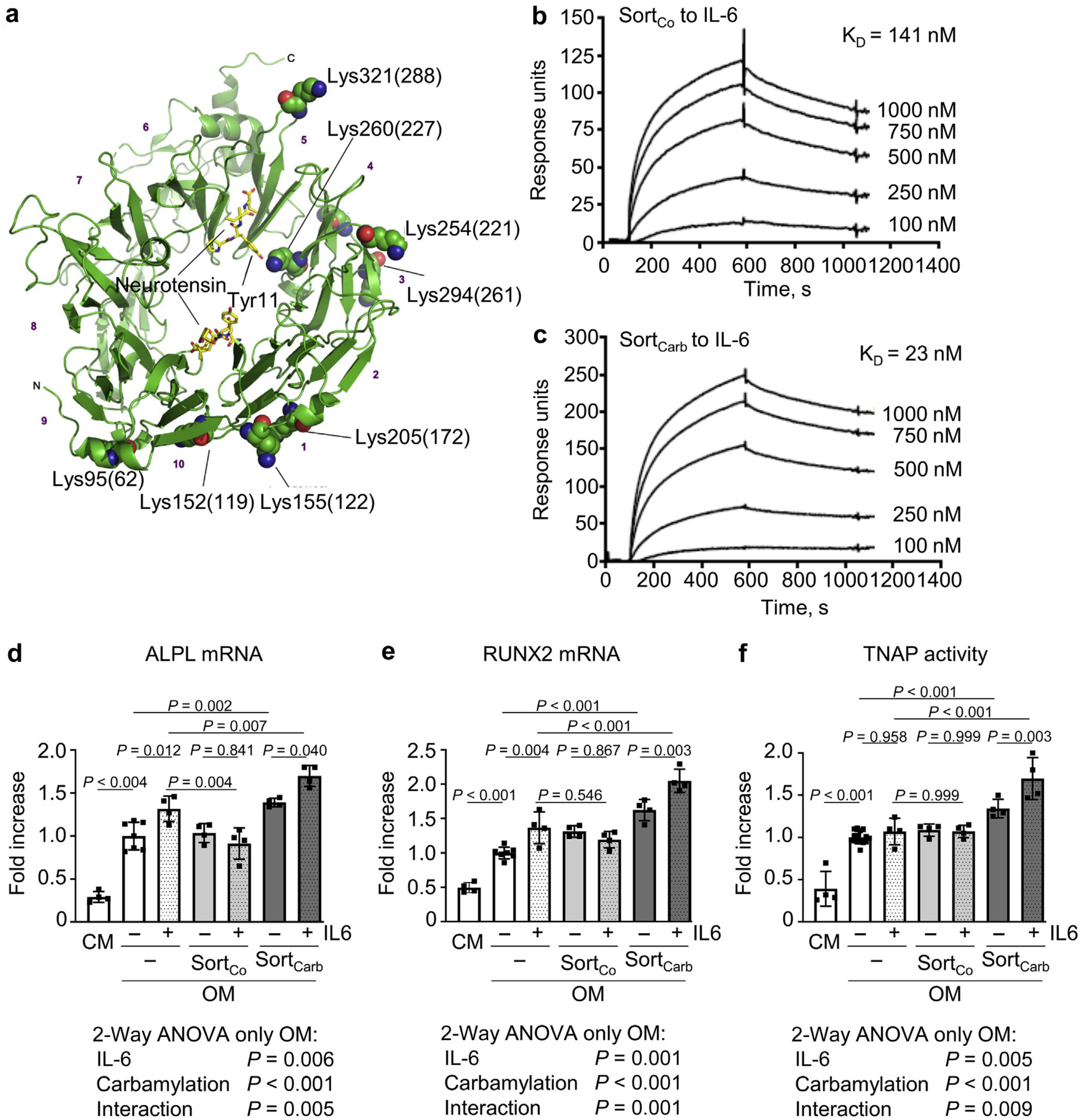

Figure 3 | Carbamylated sortilin alters ligand binding. (**a**) Sortilin bound to neurotensin (yellow) crystal structure (green) at neutral pH (program database [PDB] file: 4PO7), highlighting carbamylated lysines that are named with their corresponding amino acid position in the full protein sequence. The number in parenthesis denotes the amino acid number in the crystal structure protein sequence. Numbers 1 to 10 reference each blade in the β-propeller. (**b,c**) Surface plasmon resonance curves depict binding of human interleukin 6 (IL-6) to immobilized soluble nonmodified (Sort$_{Co}$; **b**) or carbamylated (Sort$_{Carb}$; **c**) sortilin (0–600 s). After 600 seconds, buffer was added and ligand dissociated from the immobilized Sort$_{Co}$ or Sort$_{Carb}$. (**d–f**) Human vascular smooth muscle cells were cultured for 14 days in osteogenic/calcifying medium supplemented with 200 ng/ml Sort$_{Co}$ or Sort$_{Carb}$ with and without 10 ng/ml IL-6. mRNA expression of (**d**) tissue-nonspecific alkaline phosphatase (APLP) and (**e**) runt-related transcription factor 2 (RUNX2). (**f**) Tissue nonspecific alkaline phosphatase (TNAP) activity. n = 4 independent cell donors. Two-way analysis of variance (ANOVA) with Tukey *post hoc* test was used. n = 3 independent modification preparations were used. Data are given as mean ± SD. CM, control medium; K_D, dissociation constant; OM, osteogenic medium.

and baseline Ln-(CAC + 1) remained significant after adjustment for all variables mentioned above ($P = 0.029$; Table 3).

Next, we assessed whether the presence of carbamylated residues has an impact on CAC progression. The presence of carbamylated sortilin (vs. no carbamylation) increased the

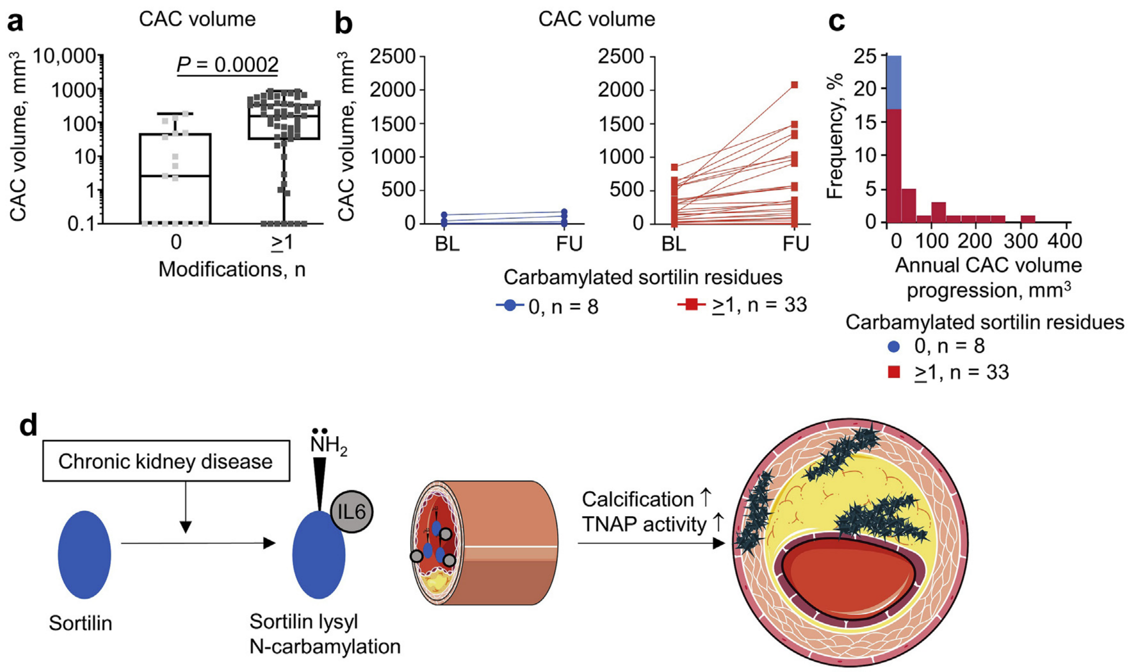

Figure 4 | Carbamylated sortilin associates with cardiovascular calcification in chronic kidney disease (CKD) *in vivo*. (**a**) Coronary artery calcification (CAC) volume of CKD patients without sortilin carbamylation (n = 17) and ≥1 carbamylated sortilin residue (n = 61). Box blot (minimum; maximum) is given. Each dot represents an individual subject. Mann-Whitney test was used. (**b**) CAC volume at baseline (BL) and median 4.4 years follow-up (FU) of CKD patients without sortilin carbamylation (blue; n = 8; $P = 0.964$) and ≥1 carbamylated sortilin residue (red; n = 33; $P < 0.001$). Each dot/line represents an individual subject. (**c**) Annual CAC volume progression as frequencies of 33.3 ranges. No sortilin carbamylation (blue; n = 8) and ≥1 carbamylated sortilin residue (red; n = 33) are shown. (**d**) In chronic kidney disease, circulating sortilin is carbamylated, which leads to vascular calcification. TNAP, tissue nonspecific alkaline phosphatase.

progression of CAC ($P = 0.047$). In detail, CKD patients with carbamylated sortilin (n = 33) exhibited a significantly increased CAC progression over a median follow-up time of 4.4 years (Figure 4b), with an annual increase in median CAC volume of 30.32 mm^3 (interquartile range = 84.0; Figure 4c). In contrast, CKD patients without sortilin carbamylation (n = 8) revealed an annual change in median CAC volume of 1.63 mm^3 (interquartile range = 8.54) and no significant CAC progression over the follow-up period (Figure 4b and c). After adjusting for covariates associated with CAC progression (baseline CAC volume, age, sex, body mass index, eGFR, diabetes mellitus, serum IL-6, serum high-density lipoprotein, and lipid-lowering medication), carbamylation status was no longer associated with CAC progression ($P = 0.286$).

Patients with carbamyl-lysine residue 260 exhibited the highest increase in CAC volume than patients with no carbamylated residue or carbamyl-lysine residues other than 260 (Supplementary Figure S8B).

Overall, the data indicate that post-translational carbamylated sortilin associates with CAC volume and its progression in patients with CKD.

DISCUSSION

In various experimental *in vitro* and *in vivo* studies and 2 independent prospective cohorts, we demonstrate that patients with CKD exhibit a specific pattern of post-translational carbamylated sortilin lysine residues in the circulation, which can also be detected in the vascular wall. Furthermore, sortilin carbamylation was associated with CAC in CKD patients, independent of age, kidney function, and other risk factors for calcification. Mechanistically, we could show that sortilin carbamylation increases its ability to bind IL-6 and acts directly on vascular cells to promote vascular calcification (Figure 4d). Taken together, this study revealed a novel nontraditional risk factor for calcification, which potentially contributes to the high burden of CV diseases in CKD.

Protein carbamylation can be mediated by cyanate, which is developed during the spontaneous decomposition of urea or is generated by myeloperoxidase-catalyzed thiocyanate oxidation at sites of inflammation.[24] We showed that CKD patients with higher urea levels due to reduced kidney function exhibited more sortilin carbamylation residues than healthy controls. However, we found no association between total levels and activity of myeloperoxidase and sortilin carbamylation, suggesting uremia as a primary driver for sortilin carbamylation in CKD. In addition, our data demonstrate that age, NT-proBNP, and a lower intake of aldosterone antagonists are associated with more carbamylated residues. Our data are consistent with previous reports that identified aging as a significant mediator of protein carbamylation.[25] It has been shown that the antihypertensive drugs hydrochlorothiazide and amlodipine affect homocitrulline levels, a urea cycle-related amino acid, and carbamylation-derived

Table 3 | Variables associated with Ln-transformed CAC (Ln-CAC + 1) volume, as obtained by multiple linear regression

Variable	Estimate	SE	*P* value
Intercept	−3.058	2.373	0.203
Carbamylated sortilin residues, yes vs. no	1.063	0.476	**0.029**
Age, yr	1.452	0.236	**<0.001**
Female vs. male	−1.086	0.451	**0.019**
BMI, kg/m^2	−0.188	0.230	0.417
eGFR$_{cys\text{-}crea}$, ml/min per 1.73 m^2	0.113	0.220	0.609
IL-6, pg/ml	0.003	0.192	0.987
HDL, mg/dl	0.142	0.255	0.580
Diabetes mellitus, yes vs. no	0.185	0.433	0.671
Lipid-lowering medication, yes vs. no	0.691	0.385	0.078

BMI, body mass index; CAC, coronary artery calcification; eGFR$_{cys\text{-}crea}$, estimated glomerular filtration rate based on serum cystatin c and creatinine; HDL, high-density lipoprotein; IL-6, interleukin 6.
The model has no autocorrelation as the value of the Durbin-Watson statistic is 2.159. The R^2 for the overall model was 0.655 (adjusted $R^2 = 0.604$), indicative for a high goodness of fit. All variables were able to predict ln(CAC + 1), $F(9, 61) = 12.840$, $P < 0.001$. Bold *P* values denote significant association.

product.[26] Whether intake of aldosterone antagonists lowers homocitrulline, and thus reduces carbamylated residues in CKD patients, is unknown. Drechsler *et al.* demonstrated a correlation between serum carbamylated albumin and NT-proBNP, which was associated with heart failure in dialysis-dependent CKD patients.[27] Whether carbamylation of sortilin may also serve as a risk factor for heart failure is not known, but increased coronary calcification with potentially subsequent harmful effects on heart function could explain the association between carbamylated sortilin residues and NT-proBNP levels in our study.

Recent studies identified the role of sortilin in the pathogenesis of vascular and metabolic disorders but mainly focused on tissue expression of sortilin rather than exploring the function of the soluble form.[7] Circulating sortilin may originate from cellular shedding of their luminal domain, as demonstrated *in vitro*,[28–30] and from secreted sortilin-packed extracellular vesicles.[9,31] Carbamylation leads to alterations in charge, structural, and functional properties of proteins, mediating loss of function and potentially pathophysiological cellular and molecular responses.[5] We established for the first time a specific lysine residue carbamylation pattern of circulating sortilin in CKD, using a targeted MS approach, and predicted functional and structural alterations by *in silico* modeling. We propose that lysine carbamylation will favor the acidic pH conformation of sortilin, affecting the pH-dependent ligand affinity of sortilin. Previous crystal structure studies demonstrated that at pH 5.5, which represents an environment similar to that of late endosomes, sortilin undergoes conformational changes and dimer formation, making known binding sites unavailable for ligand binding.[32,33]

Lysine 260 forms a hydrogen bond to a tyrosine of neurotensin inside the β-propeller tunnel, intimating that other ligands that bind similarly to neurotensin would also be affected. Our investigation found increased affinity of IL-6 to carbamylated sortilin, suggesting participation in IL-6 signaling pathways. Several studies suggested that IL-6 may contribute to vascular calcification in CKD.[22,34,35]

Previously, a study on cellular sortilin found that phosphorylation of the intracellular domain of sortilin promoted vascular calcification.[9] Herein, we found that post-translational modification of soluble sortilin plays a functional role in calcific vascular pathology. Soluble carbamylated sortilin promoted vascular calcification *in vitro* and *ex vivo* by increasing osteogenic target genes, TNAP activity, and matrix mineralization. Moreover, the addition of IL-6 to carbamylated sortilin further increased osteogenic target genes and TNAP activity.

Carbamylation has been associated with extracellular matrix alteration,[36] oxidative stress,[25] endothelial dysfunction,[37] and atherosclerosis,[38] all involved in calcification initiation and progression. Although Mori *et al.* reported that protein carbamylation promotes vascular calcification through carbamylation of mitochondrial proteins,[6] no specific target proteins were identified. A recent study demonstrated that carbamylation of uromodulin resulted in a loss of its anti-calcific properties *in vitro*.[39] Both studies used Western blot to detect carbamylation in general. We demonstrated that *in vitro* carbamylated sortilin mimics the specific *in vivo* lysine modification pattern found in CKD patients using MS. Thus, our study provides clues to specific mechanisms connecting CKD, protein carbamylation, IL-6, and calcification.

Previously, high serum sortilin levels were associated with both abdominal aortic calcification and CV events independent of traditional Framingham risk factors in a community-dwelling cohort of men aged >50 years.[10] In low- to intermediate-risk chest pain patients, sortilin levels did not associate with the severity of coronary artery disease.[40] In this study, we could show that patients with CKD have higher sortilin levels than controls with normal kidney function. Sortilin levels did not increase with decreased kidney function once a patient had a CKD with eGFR <60 ml/min per 1.73 m^2. However, we observed an increase of carbamylated sortilin residues with decreased kidney function. Similarly, Kalim *et al.* reported no association between total and carbamylated albumin levels in CKD patients.[41] Sortilin carbamylation, resulting from impaired kidney function, might further augment vascular calcification related to CKD-associated mineral disturbances. Our study has several strengths and limitations. Strengths include analyzing data obtained *in vitro*, *ex vivo*, and in patients using different experimental approaches. The careful characterization of patients with CKD, including standardized questionnaires to assess participants' characteristics, in-person study visits conducted by trained study nurses, and measurements of many laboratory values and calcification, is a particular strength of the study. The small cohort may appear to limit our study; however, we used independent CKD cohorts with follow-up data for calcification to validate the carbamylation status of sortilin by laborious targeted MS. The clinical findings were made in German CKD patients and healthy Danish subjects, and may thus restrict the generalizability of the findings to other countries or ethnicities. Finally, although the study design allowed adjustment for many important confounders, residual confounding, as in any observational study, cannot entirely be ruled out.

In conclusion, this is the first study demonstrating specific amino acids that are post-translationally modified in circulating sortilin. Our data suggest that the presence of CKD promotes the carbamylation of circulating sortilin and that carbamylated sortilin exhibits procalcific properties, as demonstrated *in vitro*, *ex vivo*, and by the presence in calcified tissue. Sortilin carbamylation was associated with CAC, even after adjustment for age, kidney function, and several other risk factors, suggesting that prevention of sortilin carbamylation might reduce the risk for CV calcification and thus CV complications in patients with CKD. Thus, our results point to carbamylated sortilin as a contributor to CV disease burden in CKD patients.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank Birgit Gittel and Bas van der Heijden for their excellent technical assistance. We thank Gunnar Heine, Vincent Brandenburg, Insa Emrich, Kyrill Rogacev, Sarah Seiler-Mußler, and Fabio Lizzi for the technical and scientific support as investigators of the CARE for HOMe study. This work was funded by the Deutsche Forschungsgemeinschaft (German Research Foundation) GO1804/5-1 to CG and Transregional Collaborative Research Centre (TRR 219; Project-ID 322900939) to CG, VJ, TSp, NM, JF, JJ, and RK, by the START-Program of the Faculty of Medicine, RWTH Aachen (11/17 to CG, 21/20 to TSa), the clinician-scientist program of the German society of internal medicine (to TSa), and the Danish Diabetes Academy, Novo Nordisk Foundation, and the Lundbeck Foundation (to MK).

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods. Detailed methods (Cardiovascular and Renal Outcome in CKD 2–4 Patients–The Fourth Homburg evaluation [CARE FOR HOMe] cohort, Cardiovascular In Depth Assessment [CARVIDA] cohort, Danish study of noninvasive testing in coronary artery disease [Dan-NICAD 1] cohort, human tissue, human primary coronary artery smooth muscle cell [SMC] culture and osteogenic transition, *ex vivo* aortic calcification, cell viability, *ex vivo* carbamylation, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry, mass spectrometry imaging, visualization of calcification, tissue nonspecific alkaline phosphatase activity, sortilin enzyme-linked immunosorbent assay [ELISA], myeloperoxidase ELISA, RNA preparation and real-time polymerase chain reaction [PCR], Western blot, surface plasmon resonance measurement, *in silico* modeling, and statistics).

Figure S1. Amino acid sequence of the sortilin protein.

Figure S2. Mass spectrometry (MS)/MS spectra.

Figure S3. Sortilin carbamylation does not associate with myeloperoxidase activity.

Figure S4. *Ex vivo* carbamylation of soluble recombinant sortilin.

Figure S5. *In vitro* carbamylated sortilin does not affect cell viability and is taken up by vascular smooth muscle cells.

Figure S6. Carbamylated albumin and collagen type I do not alter vascular calcification.

Figure S7. Surface plasmon resonance control curves.

Figure S8. Carbamylated sortilin associates with cardiovascular calcification in patients with chronic kidney disease (CKD).

Table S1. Univaribale correlation between Ln transformed-coronary artery calcification (Ln-CAC) volume and other variables.

Table S2. Characteristics of healthy control subjects with normal kidney function for mass spectrometry.

Table S3. Baseline characteristics of patients with chronic kidney disease (CKD) stratified by glomerular filtration rate (GFR) categories: Cardiovascular and Renal Outcome in CKD 2–4 Patients–The Fourth Homburg evaluation **(**CARE FOR HOMe) cohort.

Table S4. Identified and quantified peptides analyzed for *in vivo* carbamylation status of serum sortilin.

Table S5. Identified carbamylated peptides after *in vitro* carbamylation of recombinant sortilin.

Table S6. *In silico* analysis of the interaction within the sortilin structure and known ligands.

Table S7. Characteristics of patients with chronic kidney disease (CKD) according to the presence of post-translationally carbamylated sortilin residues (n = 78) from the Cardiovascular In Depth Assessment (CARVIDA) cohort.

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