Carbamylated sortilin associates with cardiovascular calcification in patients with chronic kidney disease

Vera Jankowski1,12, Turgay Saritas2,3,12, Mads Kjolby4,5,6, Juliane Hermann1, Thimoteus Speer7, Anika Himmelsbach8, Kerstin Mah8, Marina Augusto Heuschkel8, Stefan J. Schunk9, Soren Thirup9, Simon Winther10, Morten Böttcher10, Mette Nyegard11, Anders Nykjaer4, Rafael Kramann2,3, Nadine Kaesler2, Joachim Jankowski1, Juergen Floege2, Nikolaus Marx8 and Claudia Goettsch8

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1 IMCAR, Medical Faculty, RWTH Aachen University, Aachen, Germany; 2 Division 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 PROMEO and DANDRITE, Department of Biomedicine, Aarhus University, Aarhus, Denmark; 5 Danish Diabetes Academy, Novo Nordisk Foundation, Denmark; 6 Department of Clinical Pharmacology, Aarhus University Hospital, Aarhus, Denmark; 7 Department of Internal Medicine 4, Translational Cardio-Renal Medicine, Saarland University, Saarland University Hospital, Homburg/Saar, Germany; 8 Department of Internal Medicine I, Cardiology, University Hospital, Medical Faculty, RWTH Aachen, Aachen, Germany; 9 Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark; 10 Department of Cardiology, Gedstrup Hospital, NIDO, Herning, Denmark; and 11 Department of Health Science and Technology, Aalborg University, Aalborg, Denmark

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

Received 23 March 2021; revised 20 September 2021; accepted 1 October 2021


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

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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 posttranslationally 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). Impaired kidney function is a major independent risk factor for cardiovascular (CV) morbidity and mortality and all-cause mortality. In fact, patients with CKD are much more likely to die from CV events than to develop dialysis-requiring end-stage renal disease. The excess calcific mineral deposition within vascular tissue observed in CKD patients contributes mainly to increased CV risk. CKD facilitates post-translational modification (PTM) of proteins. PTMs, in turn, have been linked to CV calcification, 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. 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. 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. 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. The ectodomain of plasma membrane-bound sortilin can be shed and secreted into the circulation. 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. 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. 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. A subset of 97 patients was used for the analysis.

Dan-NICAD 1 cohort
The study design of the Danish study of noninvasive testing in coronary artery disease (Dan-NICAD 1) trial has been described previously. 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 study of the German Chronic Kidney Disease study. 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. 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 2019/2020.

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 carboxylation
 Recombinant proteins were carboxylated in vitro by O-methylisourea bisulfate solution (pH 11.0) at 25 °C for 3 hours, as previously described.

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-Daltonics), as previously described.

MS imaging of human vessel sections
Tissue sections were analyzed with Rapiflex (Bruker-Daltonics) 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 test 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 χ² 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.

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 carboxylation (yes vs. no) was used for the analysis of CAC progression. In addition, this model was
Therefore, we performed a detailed

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

<table>
<thead>
<tr>
<th>VCh</th>
<th>Control*</th>
<th>CKD*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>97</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>63.3 ± 6.9</td>
<td>64.1 ± 11.3</td>
<td>0.569</td>
</tr>
<tr>
<td>Male</td>
<td>62 (64)</td>
<td>66 (68)</td>
<td>0.544</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.5 ± 4.0</td>
<td>30.2 ± 4.6</td>
<td>0.258</td>
</tr>
<tr>
<td>Smoking</td>
<td>14 (14)</td>
<td>14 (14)</td>
<td>1.000</td>
</tr>
<tr>
<td>CVD</td>
<td>28 (29)</td>
<td>35 (36)</td>
<td>0.303</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>28 (29)</td>
<td>38 (39)</td>
<td>0.130</td>
</tr>
<tr>
<td>eGFR&lt;sub&gt;cys-crea&lt;/sub&gt;, ml/min per 1.73 m²</td>
<td>92.3 ± 9.9</td>
<td>44.8 ± 23.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sortilin, ng/ml</td>
<td>32.9 ± 8.7</td>
<td>51.2 ± 23.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

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 S7.

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 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. 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 Table S1) using MALDI-TOF/TOF-MS. Compared with control subjects, CKD patients had 8 of the 30 lysine residues that were predominantly carbamylated in the extracellular domain of sortilin (Supplementary Table S3 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 S2) 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 creatinin and creatinine (eGFR<sub>cys-crea</sub>), 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 gender (Table 2). CKD patients under aldosterone antagonist medication displayed reduced sortilin carbamylation (Table 2), whereas there was no difference in eGFR<sub>cys-crea</sub> (no, 50.6 ± 23.2 ml/min per 1.73 m²; yes, 43.5 ± 22.9 ml/min per 1.73 m²; 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. 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 SEDYGNFK (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 calcification in vitro carbamylation of recombinant sortilin (SortCarb) by urea and detected a similar carbamyl-lysine residue pattern as detected in humans in vivo (Supplementary Figure S4A and Supplementary Table S4). In vitro, carbamylation did not alter the protein integrity of SortCarb compared with control mock-modification (SortCo), as assessed by gel electrophoresis and Western blot (Supplementary Figure S4B and C).

Neither SortCo nor SortCarb exhibited cytotoxic effects on hSMCs (Supplementary Figure S5A). SortCo and SortCarb were equally taken up by hSMCs (Supplementary Figure S5B). In calcifying hSMCs, SortCarb induced the pro-osteogenic transcripts ALPL (+56%; P = 0.039) and 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 SortCo. On a functional level, SortCarb significantly augmented matrix calcification (Figure 2d and e). Finally, in an ex vivo organ culture model, SortCarb 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)

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. carbamyl residues</td>
<td>Unadjusted</td>
<td>Adjusted*</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>38.13</td>
<td>0.90–1.19</td>
<td>0.030</td>
</tr>
<tr>
<td>60–72</td>
<td>32.216</td>
<td>1.51–2.80</td>
<td>0.008</td>
</tr>
<tr>
<td>&gt;72</td>
<td>37.237</td>
<td>2.00–2.74</td>
<td>0.008</td>
</tr>
<tr>
<td>eGFR, ml/min per 1.73 m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30</td>
<td>38.287</td>
<td>2.49–3.24</td>
<td>0.003</td>
</tr>
<tr>
<td>30–60</td>
<td>31.203</td>
<td>1.62–2.45</td>
<td>0.000</td>
</tr>
<tr>
<td>&gt;60</td>
<td>28.500</td>
<td>0.06–0.94</td>
<td>0.000</td>
</tr>
<tr>
<td>Urea, mg/dl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;54</td>
<td>46.115</td>
<td>0.77–1.54</td>
<td>0.000</td>
</tr>
<tr>
<td>&gt;55</td>
<td>51.261</td>
<td>2.24–2.98</td>
<td>0.000</td>
</tr>
<tr>
<td>NT-proBNP, pg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;209.9</td>
<td>49.143</td>
<td>1.02–1.83</td>
<td>0.000</td>
</tr>
<tr>
<td>&gt;209.9</td>
<td>48.242</td>
<td>2.01–2.82</td>
<td>0.000</td>
</tr>
<tr>
<td>Aldosterone Antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>80.206</td>
<td>1.74–2.39</td>
<td>0.000</td>
</tr>
<tr>
<td>Yes</td>
<td>17.124</td>
<td>0.53–1.94</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**CARE FOR HOMe, Cardiovascular and Renal Outcome in CKD 2–4 Patients.** The Fourth Homburg evaluation; CI, confidence interval; eGFR, estimated glomerular filtration rate based on serum creatinine and creatinine; KDIGO, Kidney Disease: Improving Global Outcomes; NT-proBNP, N-terminal pro-brain natriuretic peptide.

*aAge and gender adjusted.

*bValue versus aged ≤60 years.

*cValue versus eGFR <30 ml/min per 1.73 m².

**Age was categorized into tertiles. eGFR was stratified with 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 S2 were tested. Only significant variables were shown.

**Carbamylated sortilin alters ligand binding**

Sortilin is a coreceptor for several ligands. 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), Lys320(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 (PDB file: 4PO7; Figure 3a) and the ligand-free form at acidic pH (PDB file: 6EH0; Supplementary Table S5).

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 carbonyl 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(111) at neutral pH, whereas no interactions are found at acidic pH. Thus, carbamylation at Lys205 would possibly favor the acidic pH conformation of sortilin. Lys924(261) has no interactions at both neutral and acidic pH.

Finally, at neutral pH, Lys206(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, 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.

Subsequent binding studies using surface plasmon resonance spectroscopy and SortCo or SortCarb revealed more efficient binding of IL-6 to SortCarb (KD = 23 nM) compared to SortCo, with nonmodified SortCo (KD = 141 nM), suggesting carbamylation of sortilin as a potential IL-6 binding partner (Figure 3b and c). Progranulin, another known sortilin ligand, bound to SortCo but a binding to SortCarb could not be detected (Supplementary Figure S7A and B). Both SortCo and SortCarb are bound to human receptor-associated protein fused to a GST tag, 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 hMSCs, the addition of IL-6 to SortCarb promoted ALPL and RUNX2 mRNA expression (Figure 3d and e) as well as TNSP activity (Figure 3f), whereas it had no effect in combination with SortCo. 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 S6). Ln-(CAC + 1) at baseline correlated with the presence and increase of post-translationally carbamylated sortilin (Supplementary Table S7). Furthermore, Ln-(CAC + 1) was also associated with male gender, 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 S7). Nevertheless, the association between carbamylated sortilin residues and baseline Ln-(CAC + 1) remained significant after adjustment for all variables mentioned above \((P = 0.029; \text{Table 3})\).

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 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 (interquartile range = 84.0) (Figure 4C).
contrast, CKD patients without sortilin carbamylation (n = 8) revealed an annual change in median CAC volume of 1.63 (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, gender, 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. We showed that CKD patients with higher urea levels due to reduced kidney function exhibited more sortilin carbamylated 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. It has been shown that the antihypertensive drugs hydrochlorothiazide and amiodipine affect homocitrulline levels, a urea cycle-related amino acid, and carbamylation-derived product. 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.\textsuperscript{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.\textsuperscript{2} Circulating sortilin may originate from cellular shedding of their luminal domain, as demonstrated \textit{in vitro},\textsuperscript{9,31} and from secreted sortilin-packed extracellular vesicles.\textsuperscript{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.\textsuperscript{1} 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 \textit{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.\textsuperscript{2,33}

Lysine 260 forms a hydrogen bond to a tyrosine of neutrotransin inside the β-propeller tunnel, intimating that other ligands that bind similarly to neutrotransin 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.\textsuperscript{12,24,54,55}

Previously, a study on cellular sortilin found that phosphorylation of the intracellular domain of sortilin promoted vascular calcification.\textsuperscript{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 \textit{in vitro} and \textit{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,\textsuperscript{36} oxidative stress,\textsuperscript{25} endothelial dysfunction,\textsuperscript{37} and atherosclerosis\textsuperscript{38} all involved in calcification initiation and progression. Although Mori et al. reported that protein carbamylation promotes vascular calcification through carbamylation of mitochondrial proteins,\textsuperscript{2} no specific target proteins were identified. A recent study demonstrated that carbamylation of uromodulin resulted in a loss of its anti-calcific properties \textit{in vitro}.\textsuperscript{59} Both studies used Western blot to detect carbamylation in general. We demonstrated that \textit{in vitro} carbamylated sortilin mimics the specific \textit{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.\textsuperscript{10} In low- to intermediate-risk chest pain patients, sortilin levels did not associate with the severity of coronary artery disease.\textsuperscript{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\textsuperscript{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.\textsuperscript{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 \textit{in vitro}, \textit{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 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 carbamylation

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-3.058</td>
<td>2.373</td>
<td>0.203</td>
</tr>
<tr>
<td>Carbohydrated sortilin residues, yes vs. no</td>
<td>1.063</td>
<td>0.476</td>
<td>0.029</td>
</tr>
<tr>
<td>Age, yr</td>
<td>1.452</td>
<td>0.236</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female vs. male</td>
<td>-1.086</td>
<td>0.451</td>
<td>0.019</td>
</tr>
<tr>
<td>BMI, kg/m(^2)</td>
<td>-0.188</td>
<td>0.230</td>
<td>0.417</td>
</tr>
<tr>
<td>eGFR(_{crea}), ml/min per 1.73 m(^2)</td>
<td>0.113</td>
<td>0.220</td>
<td>0.609</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>0.003</td>
<td>0.192</td>
<td>0.987</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>0.142</td>
<td>0.255</td>
<td>0.580</td>
</tr>
<tr>
<td>Diabetes mellitus, yes vs. no</td>
<td>0.185</td>
<td>0.433</td>
<td>0.671</td>
</tr>
<tr>
<td>Lipid-lowering medication, yes vs. no</td>
<td>0.691</td>
<td>0.385</td>
<td>0.078</td>
</tr>
</tbody>
</table>

BMI, body mass index; CAC, coronary artery calcification; eGFR\(_{crea}\), 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 \(\text{ln}(\text{CAC} + 1)\), \(F(9, 61) = 12.840\), \(P < 0.001\). Bold values denote \(\text{xxx}\).
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. 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 06/20 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)


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. Characteristics of healthy control subjects with normal kidney function for mass spectrometry.
Table S2. 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 S3. Identified and quantified peptides analyzed for in vivo carbamylation status of serum sortilin.
Table S4. Identified carbamylated peptides after in vitro carbamylation of recombinant sortilin.
Table S5. In silico analysis of the interaction within the sortilin structure and known ligands.

Table S6. 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.

Table S7. Univariable correlations between Ln-transformed coronary artery calcification (Ln-CAC) volume and other variables.

REFERENCES


