Guanidinylation compromises the anti-inflammatory and anti-oxidative properties of apolipoprotein A-I in chronic kidney disease progression

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Chronic kidney disease (CKD) substantially heightens the likelihood of cardiovascular events, in part due to the impaired functionality of high-density lipoprotein (HDL) and its connection with atherosclerosis. Here, 82 patients with CKD stages 2-5 had their plasma isolated and analyzed using mass spectrometry to detect post-translational modifications of apolipoprotein A-I (apoA-I), the main protein component of HDL. Guanidinylation, a non-enzymatic post-translational modification, led to increased levels of apoA-I with CKD progression. The increase in guanidinylated apoA-I became significant from CKD stage 3 onwards. The modification patterns of apoA-I in patients with CKD were mimicked *in vitro* by exposure to O-methylisourea bisulfate. The thus modified apoA-I was used for functional assays which revealed that guanidinylation compromised the anti-inflammatory and anti-oxidative properties of apoA-I, of potential relevance for clinical findings. Specifically, guanidinylated apoA-I activated inflammatory kinases in macrophages, suggesting a mechanistic link between apoA-I modifications and inflammatory responses. These findings are in favor of alterations in the functional properties of apoA-I in patients with CKD due to guanidinylation. The identification of high guanidinylated apoA-I peptide levels in plasma highlights a novel aspect of protein modification in CKD pathophysiology. The results of our study may provide a better understanding of the molecular mechanisms underlying CKD-related cardiovascular complications and highlight the importance and the need to minimize post-translational modifications in patients with CKD.

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Chronic kidney disease (CKD) is increasingly recognized as a global health burden.[1] However, most patients with CKD do not succumb to end-stage kidney disease, but rather to cardiovascular events as the risk for a cardiovascular event increases 500- to 1000-fold on CKD development.[2] This increased risk shows that CKD and cardiovascular diseases (CVDs) are closely interconnected, and the development of one disease can often lead to the progression of the other.[2]

More specifically, CKD has been directly linked with the progression of atherosclerosis, which is the leading underlying cause of CVD.[3] Atherosclerosis is a chronic inflammatory disease initiated by damage of the endothelium due to turbulent flow, causing endothelial activation and, thereby,

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Lay Summary

Chronic kidney disease (CKD) is a global health burden that coincides with a strongly increased risk for cardiovascular events. Although the underlying mechanisms behind this interaction remain rather elusive, the impairment of the protective role of high-density lipoproteins (HDLs) seems to play an important role. The main protein component of HDL, apolipoprotein A-I (apoA-I), is known to be a main driver of these protective effects. However, in this study, we identified that apoA-I gets modified (guanidinylated) on CKD progression, which was determined using mass spectrometry in plasma samples from 82 patients with CKD stages 2 to 5. We were able to mimic this modification of apoA-I *in vitro* and determined that guanidinylated apoA-I has an abolished anti-inflammatory and antioxidative capacity. Therefore, this study shows that modified apoA-I may be responsible for the increased cardiovascular risk in patients with CKD, highlighting the importance and the need to prevent such post-translational modifications in patients with CKD.

monocyte recruitment to the injured site.[3] Infiltrated monocytes subsequently differentiate into macrophages and phagocytize oxidized low-density lipoproteins, forming foam cells, the primary driver of disease progression.[4] Although low-density lipoprotein exerts a proatherogenic role by delivering lipids to the vessel wall, its counterpart, high-density lipoprotein (HDL), extracts lipids from the vessel wall in an atheroprotective process called reverse cholesterol transport.[5] Additionally, HDL has anti-inflammatory and antioxidative properties. As a result, HDL is typically inversely correlated with cardiovascular risk. However, this association is completely lost in patients with CKD, who exhibit no association between HDL cholesterol and risk of mortality or severity of coronary artery disease.[6]

Over the past few years, HDL-raising therapies in patients with CKD have failed to achieve a marked enhancement of cardiovascular outcomes, suggesting that HDL's functionality seems more important than HDL's quantity.[7] Indeed, dysfunctional HDL has been recently characterized by a reduced adenosine triphosphate–binding cassette-1–dependent cholesterol efflux capacity, which thus mitigates reverse cholesterol transport, leading to a loss of its atheroprotective function.[8]

Numerous proposed underlying mechanisms for impaired HDL function in patients with CKD are currently under consideration. These include nonenzymatic post-translational modifications (PTMs) of HDL, caused by the accumulation of reactive uremic toxins in the circulation of patients with CKD.[9] For example, a recent study has shown increased carbamylated HDL levels in patients diagnosed with CKD stage 5.[10] Furthermore, carbamylated apolipoprotein A-I (apoA-I), the predominant protein in HDL, has been isolated from human atheromas and inhibits cholesterol efflux from macrophages, indicating a role in atherosclerotic vascular disease.[11] This altered functionality was also evident in carbonylated HDL from hemodialysis patients and a CKD rabbit model. These exhibited a diminished capacity to inhibit platelet aggregation, which may contribute to the heightened cardiovascular risk observed in patients with CKD.[12]

Previously, we showed that guanidinylations of albumin occur in patients with CKD stage 5, impairing the binding of hydrophobic substances to albumin.[13] However, guanidinylations of apoA-I have not yet been described *in vivo* in humans. Investigating the quality rather than the quantity of HDL and exploring potential interventions to restore HDL function hold promise for addressing cardiovascular risk in patients with CKD. Therefore, we used a proteomic approach to identify and quantify guanidinylations of human apoA-I in patients with CKD, as this is the main protein component of HDL. Furthermore, we evaluated the functional effects and underlying mechanisms of guanidinylation on the anti-inflammatory and antioxidant properties of apoA-I and how this is affected on guanidinylation of apoA-I. This study addresses aspects of basic research and clinical practice relevant to improving our understanding of HDL dysfunction in CKD.

METHODS

Detailed methods are provided as Supplementary Methods.

Study population

A total of 82 patients were recruited from the inpatient area of Medical Clinic I of the University Hospital Aachen (Germany) and the CARE FOR HOMe (Cardiovascular and Renal Outcome in CKD 2–4 Patients—The Fourth Homburg evaluation) study at Saarland University. All study participants were classified according to Kidney Disease: Improving Global Outcomes Clinical Practice Guidelines into 5 CKD stages[14] (Table 1).

Proteomic analyses of apoA-I

Isolated plasma proteins were digested by trypsin and analyzed by matrix-assisted laser-desorption/ionization–time-of-flight mass spectrometry, as previously described.[15]

In vitro guanidinylation of apoA-I

Purified native apoA-I was guanidinylated in the procedure adapted from Kimmel.[16]

Impact of kidney failure on apoA-I

To analyze the direct impact of kidney failure on apoA-I, blood samples were collected from Alport mice (*Col4a3*$^{-/-}$), and mice with 2,8-dihydroxyadenine A nephropathy or 5/6 nephrectomy and respective controls.

Cell culture and treatments

Differentiated bone marrow–derived macrophages (BMDMs) and resident peritoneal macrophages of C57Bl6/J mice were prestimulated for 24 hours with apoA-I or guanidinylated

Table 1 | Descriptive statistics of the patients with CKD at stages 2, 3, 4, and 5 involved in the study

Variable	CKD stage 2 (n = 16)	CKD stage 3a (n = 15)	CKD stage 3b (n = 16)	CKD stage 4 (n = 20)	CKD stage 5 (n = 15)
Women/men, n	4/12	8/7	6/10	5/15	5/10
Age, yr	61 ± 3	66 ± 3	70 ± 3	70 ± 2	81 ± 3
BMI, kg/m^2	31 ± 1	29 ± 1	32 ± 2	29 ± 1	30 ± 1
CRP, mg/l	50 ± 10	23 ± 4	25 ± 5	75 ± 17	24.3 ± 6.0
Diabetes, n	7	6	7	8	4
eGFR, ml/min per 1.73 m^2	62 ± 2	51. 6 ± 1.1	39.4 ± 1.7	26.6 ± 1.5	NA
Urea, mmol/l	16.1 ± 0.7	18.1 ± 1.4	26.1 ± 1.8	37.9 ± 2.1	25.7 ± 3.9
Creatinine, µmol/l	97.2 ± 0.0	106.1 ± 0.0	141.4 ± 8.8	2.43 ± 0.14	NA
Albuminuria, mg/mmol	59.6 ± 25.7	351.3 ± 214.9	511.6 ± 421.3	406.2 ± 139.6	NA
Cholesterol, mmol/l	5.0 ± 0.29	5.1 ± 0.3	4.8 ± 0.4	4.7 ± 0.3	4.4 ± 0.2
HDL, mmol/l	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.2 ± 0.2	1.4 ± 0.1
LDL, mmol/l	3.2 ± 0.2	3.0 ± 0.3	2.7 ± 0.3	2.8 ± 0.2	2.2 ± 0.3
Triglycerides, mmol/l	1.6 ± 0.2	1.7 ± 0.3	1.9 ± 0.2	2.3 ± 0.3	1.8 ± 0.3

BMI, body mass index; CKD, chronic kidney disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not available; SI, International System of Units.
Results represent mean ± SEM unless otherwise indicated.
Conversion factors from conventional to SI units: CRP, 1 mg/dl = 10 mg/l; urea, 1 mg/dl = 0.36 mmol/l; creatinine, 1 mg/dl = 88.4 µmol/l; albuminuria, 1 mg/g = 0.11 mg/mmol; cholesterol, 1 mg/dl = 0.03 mmol/l; HDL, 1 mg/dl = 0.03 mmol/l; LDL, 1 mg/dl = 0.03 mmol/l; triglycerides, 1 mg/dl = 0.01 mmol/l.

apoA-I, followed by a phosphate-buffered saline wash and stimulation with 1 ng/ml of lipopolysaccharides for 6 hours (enzyme-linked immunosorbent assays) or 30 minutes (kinases).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays were performed according to the manufacturer's instructions. Cytokine levels were subsequently normalized to protein concentrations.

Kinase activity profiling

The PamChip peptide Ser/Thr Kinase assay microarray system was used on the PamStation12 (PamGene International) to define the serine-threonine kinase activity profiles.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 10.1.1 (GraphPad Software). A 2-tailed $P < 0.05$ was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$).

RESULTS

Stage-dependent increased guanidinylation of apoA-I in patients with CKD

We analyzed the plasma of 82 patients with CKD at different disease stages to evaluate the extent of post-translational guanidinylation in patients with CKD (Figure 1a and Table 1). After tryptic digestion of the plasma samples, the resulting peptides were analyzed using micro–reversed-phase chromatography and matrix-assisted laser-desorption/ionization—time-of-flight–based proteomics. Indeed, we successfully identified evidence of post-translational guanidinylation of apo-AI within the samples derived from individuals with CKD. Quantitative analysis of the post-translational guanidinylations in apoA-I revealed a significant correlation with CKD disease progression (Figure 1b). This remarkable increase emphasizes the potential link between disease progression and increased guanidinylation levels in apoA-I. In patients with CKD stage 2, the average number of guanidinylations in apoA-I was only 0.19 ± 0.14 (normalized mass-signal intensity [arbitrary unit]), whereas 1.6% of available modification positions were modified across patients. However, this number increased significantly with the progression of the disease. For patients with CKD stage 3a and 3b, the guanidinylations were 2.33 ± 0.50 and 1.81 ± 0.46, respectively (19.4% and 15.1%, respectively, of available modification positions). The increase was even more pronounced in stages 4 and 5, with 3.05 ± 0.30 and 3.20 ± 0.45 (each normalized mass-signal intensity [arbitrary unit]) guanidinylations, respectively (25.4% and 26.7%, respectively, of available modification positions; Figure 1b). Interestingly, bivariate correlation analysis revealed that the amount of modifications were not only correlating with estimated glomerular filtration rate, but also with urea and creatinine levels (Table 2). Additionally, multivariate linear regression analysis revealed that estimated glomerular filtration rate is the main predictor of guanidinylation (Table 3). Furthermore, it could be shown that the levels of native apoA-I decrease on CKD progression, whereas the levels of guanidinylated apoA-I increase as shown for example for the modification at position 176 (Supplementary Figure S1).

Interestingly, post-translational guanidinylated sites were observed at different positions in the amino acid sequence of apoA-I (Figure 2). The post-translational guanidinylations were confirmed at 16 positions in the apoA-I sequence, corresponding with the mass signals of modified peptides detected in the fragment spectra. Using tandem mass spectrometry peptide fragmentation, we identified

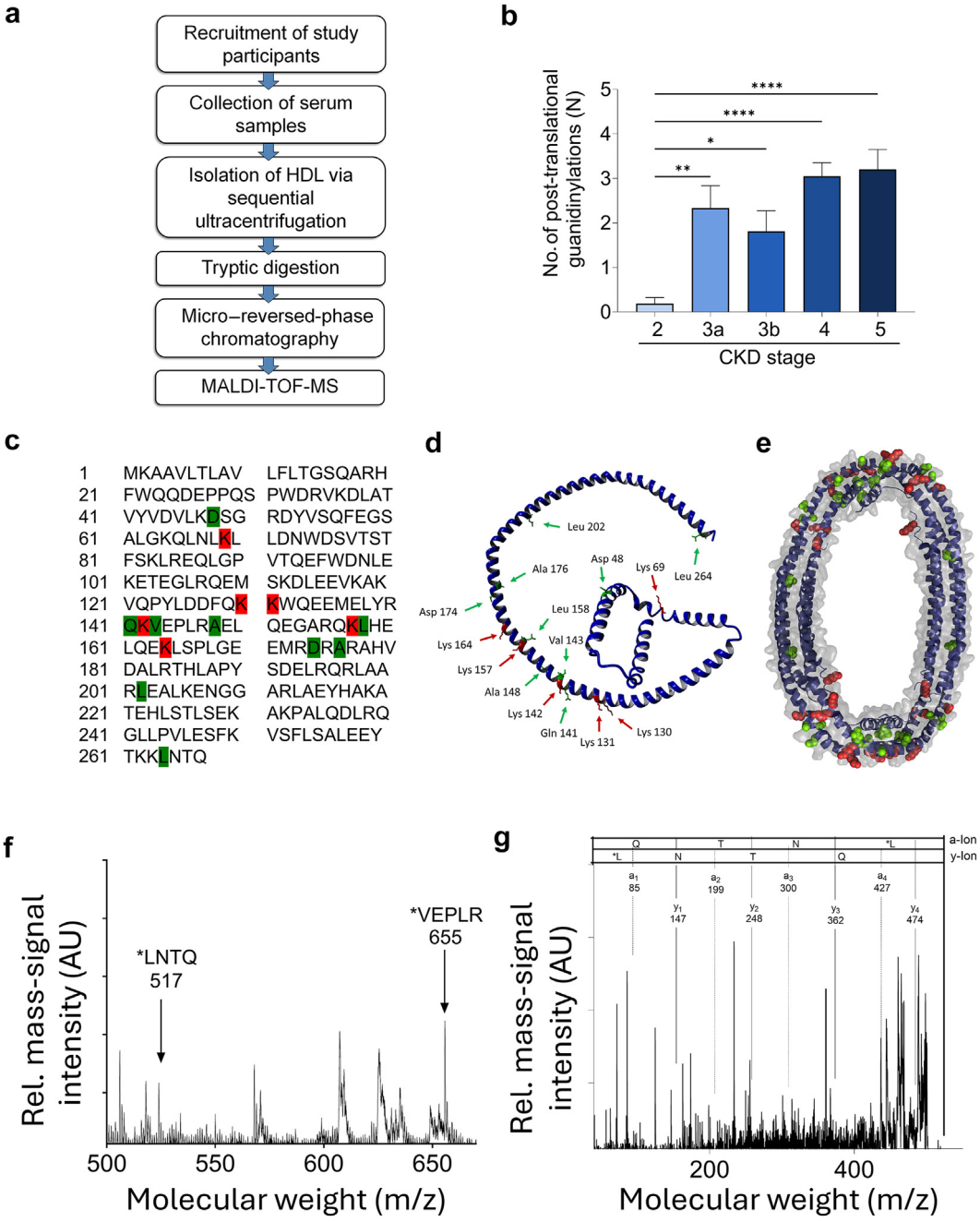

Figure 1 | Isolation and identification of post-translationally modified apolipoprotein A-1 (apoA-I). (**a**) Scheme for isolating and identifying post-translational modifications (PTMs) in apoA-I from patients with chronic kidney disease (CKD) and control subjects. (**b**) Comparison of the number of modifications (guanidinylation) between CKD stages 2 (n = 16), 3a (n = 15), 3b (n = 16), 4 (n = 20), and 5 (n = 15). The number indicated on the *y* axis reflects the total occurrences of guanidylation across the entire apo-A1 protein. (**c**) Amino acid sequence of apoA-I shows 2 different types of modifications. Red-marked amino acids show PTMs of the lysins, whereas green-marked amino acids show PTMs of other amino acids. (**d**) Three-dimensional structure of human apoA-I. The guanidinylated amino acids are marked in green, and the guanidinylated lysins are marked in red. (**e**) Crystal structure of truncated human apoA-I (protein data bank identifier: 1av1) consisting of 4 molecules seen in blue and its associated surface area in gray. The atoms of residues of interest are shown as spheres with lysines colored in red and other amino acids in green. (**f**) Matrix-assisted laser-desorption/ionization (MALDI) mass spectrum of apoA-I isolated from plasma of a patient with CKD (Kidney Disease: Improving Global Outcomes [KDIGO], CKD stage 4). The arrows show peaks of guanidinylated apoA-I fragments indicating modified fragment *L(264)NTQ at m/z 517 Da and modified fragment *V(143)EPLR at m/z 655 Da. (**g**) MALDI tandem mass spectrometry (MS/MS) tandem time-of-flight (TOF/TOF) mass spectrum of the molecular mass of 517 (m/z) from plasma of a patient with CKD (KDIGO stage 4). Bar graph represents mean ± SEM. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. AU, arbitrary unit; HDL, high-density lipoprotein; Rel, relative.

Table 2 | Bivariate correlation analysis between the number of guanidinylations and various parameters

Parameter	No. of patients	Spearman coefficient (ρ)	*P* value
Gender	82	0.089	0.425
Age, yr	82	0.240	0.030
BMI, kg/m^2	82	−0.079	0.478
CRP, mg/l	75	−0.014	0.907
Diabetes	82	0.054	0.631
eGFR, ml/min per 1.73 m^2	67	−0.469	<0.001
Urea, mg/dl	75	0.416	<0.001
Creatinine, mg/dl	67	0.393	<0.001
Albuminuria, mg/g	67	0.108	0.385
Total cholesterol, mg/dl	75	−0.102	0.383
HDL, mg/dl	80	−0.255	0.023
LDL, mg/dl	75	−0.121	0.300
Triglycerides, mg/dl	75	0.204	0.080

BMI, body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

guanidinylations in 7 lysine residues (Figure 1c-d, highlighted in red, and Figure 2) and 9 other amino acids (Figure 1c-d, highlighted in green, and Figure 2). A molecular model of apoA-I revealed the surface accessibility of the modified residues (Figure 1e). The lipid-free structure of truncated apoA-I (protein data bank identifier: 1av1)[17] was examined, and the residues appeared to be accessible to the surface and able to interact with guanidino compounds and, therefore, have the potential to be guanidinylated. The amino acid Asp 48 was an exception as it is absent in the truncated crystal structure used for modeling.

Figure 1f shows an example of a matrix-assisted laser-desorption/ionization mass spectrum of apoA-I of a patient with CKD stage 4. The arrows indicate the mass-to-charge ratios of 2 modified apoA-I peptide fragments, being *L(264)NTQ at m/z 517 and *V(143)EPLR at m/z 655. These modifications were not observed in patients with CKD stage 2 (data not shown). Focusing further on the *L(264)NTQ fragment at m/z 517, the corresponding tandem mass spectrometry spectrum obtained by fragmentation of this peptide elucidated a characteristic guanidinylation of the leucine residue at position 264 in the amino acid sequence, indicated by a mass shift of 42 Da, reflecting the molecular mass of the guanidine group, from the unmodified peptide (m/z 475) to the modified peptide (m/z 517; Figure 1g).

Combined, these data demonstrate that apoA-I becomes increasingly guanidinylated on the progression of CKD, characterized by multiple amino groups of amino acids and lysine residue modifications.

Chronic renal insufficiency leads to elevated plasma concentrations and tissue levels of guanidinylated apoA-I in mice

To confirm that the observed guanidinylation is directly attributable to chronic renal insufficiency, apoA-I was extracted from the plasma of mice with healthy kidney function and those with chronic kidney insufficiency. Chronic renal insufficiency in (Alport) mice results in significantly higher serum creatinine levels (85.7 ± 15.1 µmol/l) compared with wild-type mice (12.8 ± 7.6 µmol/l). Post-translational guanidinylated apoA-I in the plasma of (Alport) mice with chronic kidney insufficiency was identified via mass spectrometry (Figure 3a) and tandem mass spectrometry (Figure 3b). Three different animal models (Alport, 2,8-DHA nephropathy, and 5/6-nephrectomy) of chronic kidney insufficiency were used to distinctively demonstrate that guanidinylated apoA-I is clearly accumulated in the plasma on disease development, compared with sham, healthy, or wild-type mice (Figure 3c–e). Additionally, renal biopsies taken from the mice revealed the presence of guanidinylated apoA-I and, more specifically, a significantly increased accumulation of guanidinylated apoA-I in the renal tissue of mice with chronic renal insufficiency (Figure 3f and g). Interestingly, this accumulation of guanidinylated apoA-I was not only restricted to the kidneys as we could also identify an accumulation of guanidinylated apoA-I in the aorta of mice with chronic renal insufficiency, suggesting a potential mechanistic link between PTMs of apoA-I and vascular alterations associated with impaired renal function (Figure 3h).

The molecular structure of *in vitro* modified apoA-I corresponds to the structure of apoA-I isolated from the plasma of patients with CKD

To study the functional consequences of the guanidinylation of apoA-I in detail, we mimicked the PTMs observed in patients with CKD by modifying apoA-I *in vitro*.

Although native apoA-I did not show any mass signals related to guanidinylations (Figure 4a), the *in vitro* modification of apoA-I by O-methylisourea bisulfate resulted in an identical modification pattern as observed in patients with CKD (e.g., the identical modifications were observed at molecular masses [m/z] of 517 [L{264}NTQ] and 655 [V{143}EPLR]; Figure 4b). The number and position of *in vitro* guanidylated apoA-I and apoA-I isolated from patients in the CKD stadium showed high homology. For example, the apoA-I fragment *LNTQ exhibited a guanidinylation rate of 66.6% in both patients with stage 5 CKD and in *in vitro* modified apoA-I, whereas no guanidinylation (0%) was detected in patients with stage 2 CKD. Overall, 25.0% of available modification positions were modified in *in vitro* modified apoA-1, which closely resembles the amount of modification in patients with stage 4 and stage 5 (25.4% and 26.7%, respectively, of available modification positions). The corresponding fragmentation pattern involving a-ions and y-ions originating from the fragment at m/z 517 and the amino acid sequence *LNTQ are given in Figure 4c. In detail, a-ions are generated by cleavage between an α-carbon atom and an adjacent carbonyl-carbon atom, whereas y-ions result from peptide bond cleavage. This fragmentation pattern demonstrates the post-translational guanidinylation of apoA-I, as

Table 3 | Multiple regression analysis of guanidinylation amount with various parameters, modeling with backward elimination

		Coefficients						ANOVA		Model summary	
Model	Indicators	B	SE	β coefficient	t Value	*P* value	VIF	F	*P* value	R^2	Adjusted R^2
Model 1	*y* Intercept	4.396	2.435	NA	1.805	0.076	NA	3.279	0.011	0.212	0.147
	eGFR, ml/min per 1.73 m^2	−0.052	0.015	−0.419	−3.502	0.001	1.109				
	Age, yr	0.015	0.020	0.101	0.771	0.444	1.334				
	Diabetes	0.252	0.452	0.066	0.557	0.579	1.093				
	Albuminuria, mg/g	0.000	0.000	−0.063	−0.468	0.641	1.396				
	BMI, kg/m^2	−0.037	0.047	−0.098	−0.780	0.438	1.231				
Model 2	*y* Intercept	3.772	2.024	NA	1.863	0.067	NA	4.096	0.005	0.209	0.158
	eGFR, mL/min per 1.73 m^2	−0.050	0.014	−0.405	−3.522	0.001	1.035				
	Diabetes	0.019	0.017	0.130	1.124	0.265	1.045				
	Albuminuria, mg/g	0.245	0.449	0.064	0.546	0.587	1.092				
	BMI, kg/m^2	−0.029	0.044	−0.079	−0.669	0.506	1.103				
Model 3	*y* Intercept	3.638	1.998	NA	1.821	0.073	NA	5.423	0.002	0.205	0.167
	eGFR, ml/min per 1.73 m^2	−0.050	0.014	−0.403	−3.531	0.001	1.034				
	Albuminuria, mg/g	0.020	0.017	0.131	1.141	0.258	1.045				
	BMI, kg/m^2	−0.023	0.042	−0.061	−0.537	0.593	1.010				
Model 4	*y* Intercept	2.894	1.433	NA	2.020	0.048	NA	8.080	<0.001	0.202	0.177
	eGFR, ml/min per 1.73 m^2	−0.050	0.014	−0.403	−3.549	0.001	1.034				
	BMI, kg/m^2	0.020	0.017	0.137	1.208	0.232	1.034				
Model 5	*y* Intercept	4.446	0.637	NA	6.982	0.000	NA	14.597	<0.001	0.183	0.171
	eGFR, ml/min per 1.73 m^2	−0.053	0.014	−0.428	−3.821	0.000	1.000				

ANOVA, analysis of variance; BMI, body mass index; eGFR, estimated glomerular filtration rate; NA, not available; VIF, variance inflation factor.
The dependent variable = number of guanidinylations.

Molecular weight (m/z)	Post-translational modified amino acid of the peptide fragment
476	D(48)SGR
657	QLNLK(69)
1465	VQPYLDDFQK(130)/K(131)
954	Q(141)K(142)VEPLR
655	V(143)EPLR
1214	A(148)ELQEGARQK
1236	QK(157)LHELQEK(164)*)
938	L(158)HELQEK
938	LHELQEK(164)*)
560	D(174)RAR
1049	A(176)RAHVDALR
615	L(202)EALK
517	L(264)NTQ

Figure 2 | Guanidinylated peptide fragments of apolipoprotein A-1 detected using matrix-assisted laser-desorption/ionization–tandem time-of-flight mass spectrometry, sorted by the mass-to-charge ratio. Red label: modification of lysine (K). Green label: modification of other amino acids. *Same modification at lysine (K) at position 164.

indicated by the mass shift (m/z) of 42 corresponding to the guanidino group added to the native peptide fragment LNTQ, observed at m/z 517.

In short, we generated guanidinylated apoA-I *in vitro* that mimics the apoA-I modifications we found in patients with CKD, allowing follow-up investigations on its functionality.

Guanidinylation of apoA-I causes the loss of its anti-inflammatory and antioxidative properties

To assess changes in the functionality of apoA-I on guanidinylation, we evaluated the effects of this modification on the anti-inflammatory and antioxidative properties of apoA-I. Because macrophages play an instrumental role in atherosclerosis and thus CVD,[18] we focused on these cells for the functional evaluation of guanidinylated apoA-I. In line with the notion that apoA-I has anti-inflammatory effects on macrophages,[19,20] it could be confirmed that unmodified apoA-I significantly reduced the secretion of inflammatory cytokines interleukin-6, tumor necrosis factor, and CC chemokine ligand 2 in BMDMs (Figure 5a–c). Strikingly, however, guanidinylated apoA-I completely lost this anti-inflammatory potential as the secretion of inflammatory cytokines was unchanged and even slightly increased compared with untreated control cells (Figure 5a–c).

Additionally, the antioxidative potential of apoA-I in BMDMs was evaluated, showing that unmodified apoA-I, as expected, reduces oxidative stress in the cells. At the same time, this effect was again completely abrogated in cells treated with guanidinylated apoA-I (Figure 5d and e). Finally, the loss of the anti-inflammatory properties of apoA-I on guanidinylation was also confirmed in resident peritoneal macrophages. Peritoneal macrophages treated with unmodified apoA-I showed again a strongly reduced secretion of inflammatory cytokines, whereas this effect was abolished if cells were treated with guanidinylated apoA-I (Figure 5f and g). These findings unequivocally demonstrate that the guanidinylation of apoA-I attenuates its favorable attributes, notably the diminution of its anti-inflammatory and antioxidative properties.

Guanidinylated apoA-I induces inflammatory signaling cascades in macrophages

Because kinases play a crucial role in cellular responses, like inflammation and oxidative stress, by phosphorylating proteins involved in signaling cascades, a kinomics approach focusing on serine-threonine kinases was deployed to evaluate the effect of apoA-I and its guanidinylation on intracellular signaling. The degree of phosphorylation of peptides coated on serine-threonine kinase arrays was determined to evaluate differentially activated kinases. Treatment of BMDMs with unmodified apoA-I resulted in a predominant down-regulation of peptide phosphorylation compared with untreated control cells (Figure 6a and Supplementary Figure S2A and B). Furthermore, several kinases were significantly less activated in BMDMs treated with apoA-I, many of which are involved in inflammatory pathways, such as IκB kinases and p38, and cell cycle, such as cyclin-dependent kinases (Figure 6b). This was also further corroborated by pathway analysis, showing enrichment in pathways related to inflammatory signaling (e.g., toll-like receptor signaling, tumor necrosis factor signaling) and cell cycle (e.g., G_1 phase, cyclin D–associated events in G_1; Figure 6c).

BMDMs treated with guanidinylated apoA-I showed again increased peptide phosphorylation compared with cells treated with unmodified apoA-I (Figure 6d and Supplementary Figure S2A and C). Principal component analysis demonstrated that BMDMs treated with guanidinylated apoA-I resembled untreated control cells more closely

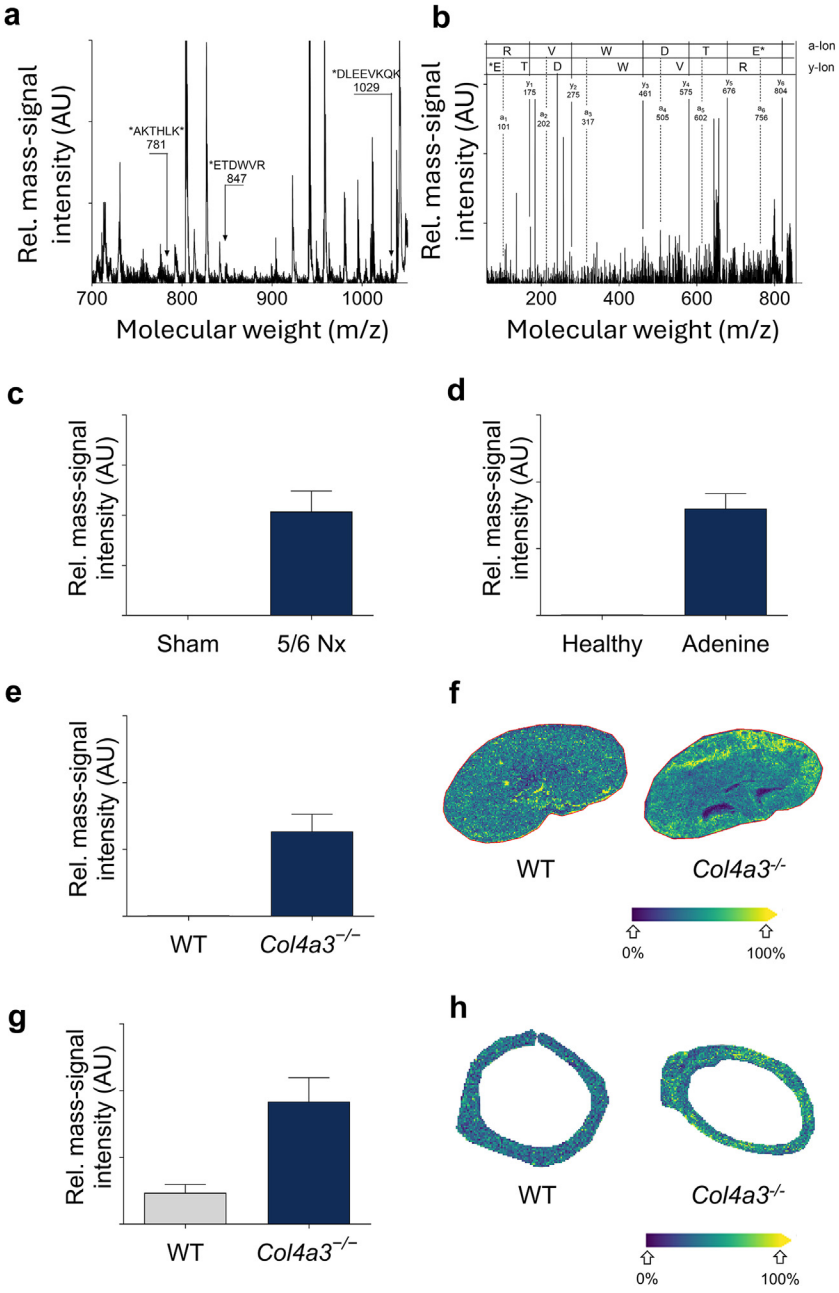

Figure 3 | Post-translational guanidinylated apolipoprotein A-1 (apoA-I) is detectable in plasma and renal biopsies obtained from mice with chronic kidney insufficiency. (**a**) Matrix-assisted laser-desorption/ionization (MALDI) mass spectrum of apoA-I isolated from plasma of a mouse with chronic kidney insufficiency. (**b**) MALDI tandem mass spectrometry tandem time-of-flight mass spectrum of the molecular mass of 847.30 (m/z) from plasma of a mouse with chronic kidney insufficiency. (**c**) Quantification of the mass signal of guanidinylated apoA-I in the plasma samples obtained from C57BL/6N mice that underwent a 5/6 nephrectomy (Nx) or sham operation, followed by a 10-week observation period. (**d**) Quantification of the mass signal of guanidinylated apoA-I in the plasma samples obtained from FVB/N wild-type mice fed 0.2% adenine-containing chow food (Adenine) or regular chow food (Healthy) for 6 weeks. (**e**) Quantification of the mass signal of guanidinylated apoA-I in the plasma samples obtained from wild-type mice (WT) and mice with chronic kidney insufficiency (*Col4a3*$^{-/-}$). (**f**) Spatial proteomics heat map image shows the distribution/presence of post-translational guanidinylated apoA-I peptide (m/z = 1108.39) in renal biopsies of the WT mice and mice with chronic kidney insufficiency. Intensity relative within 1 image in arbitrary units (AUs). Representative images from 3 cases per group. (**g**) Quantification of the spatial proteomics mass signal of guanidinylated apoA-I in renal biopsies from WT and mice with chronic kidney insufficiency (*Col4a3*$^{-/-}$). (**h**) Characteristic MALDI images of an aorta of (right image) an Alport mouse and (left image) control mouse demonstrating the spatial distribution of guanidinylated apoA-I (m/z: 1108.2). Bar graphs represent mean ± SEM. Rel, relative.

than cells treated with unmodified apoA-I (Supplementary Figure S2D). The kinase activity of 43 kinases was significantly upregulated in BMDMs treated with guanidinylated apoA-I, compared with cells treated with unmodified apoA-I (Figure 6e). As demonstrated by the pathway analysis, there is again a strong enrichment in pathways related to

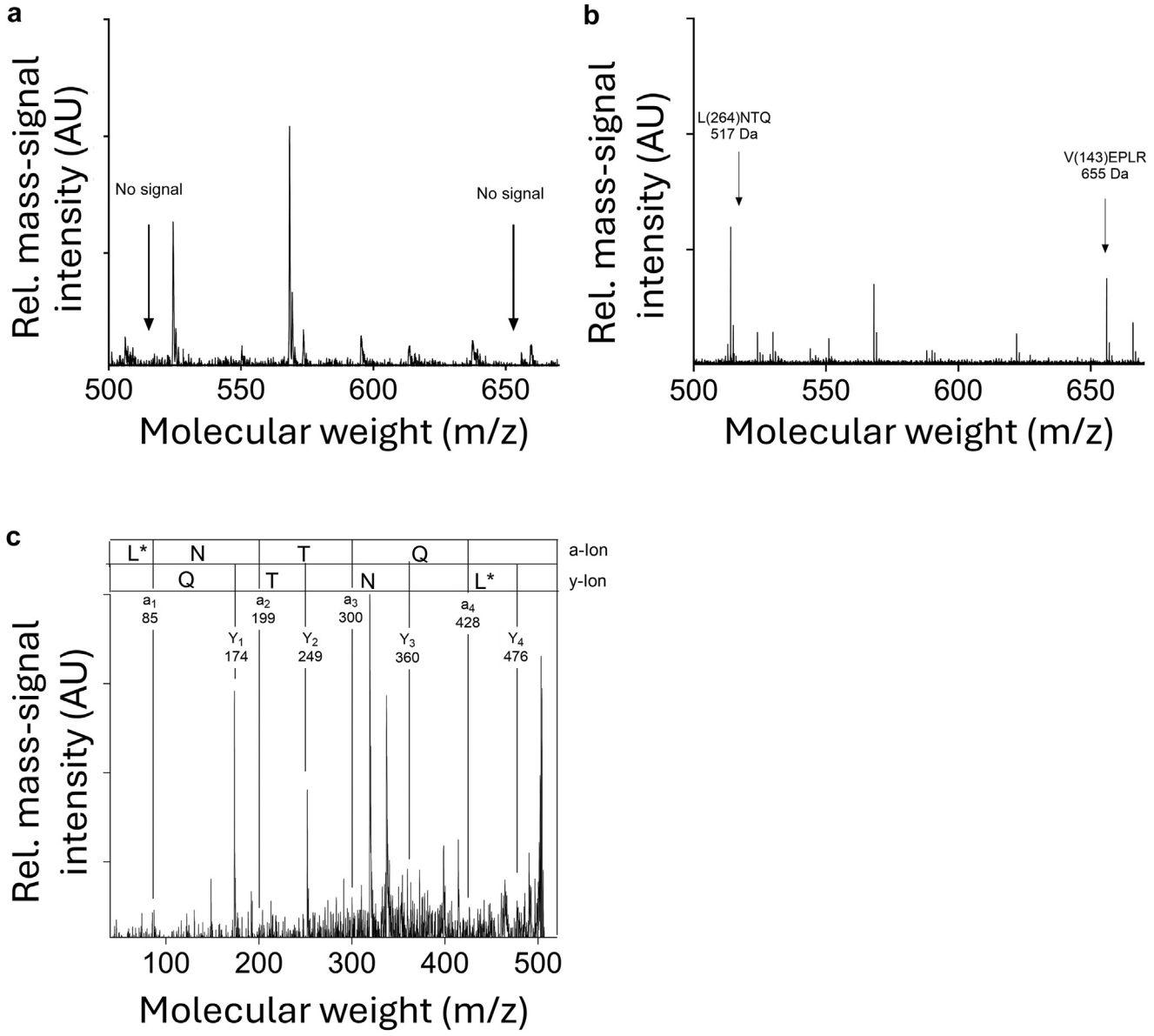

Figure 4 | *In vitro* guanidinylation and quantification of apolipoprotein A-I (apoA-I) in chronic kidney disease. (**a**) Matrix-assisted laser-desorption/ionization (MALDI) mass spectrum of commercially available unmodified apoA-I. (**b**) MALDI mass spectrum of commercially acquired apoA-I modified with o-methylisourea bisulfate. (**c**) MALDI tandem mass spectrometry tandem time-of-flight mass spectrum of the molecular mass of 517 (m/z) labeled by an arrow in b. AU, arbitrary unit; Rel, relative.

inflammation (e.g., toll-like receptor signaling and mitogen-activated protein kinase pathways; Figure 6f), which is also in line with our functional observations that guanidinylated apoA-I results in the loss of anti-inflammatory properties of apoA-I.

In summary, unmodified apoA-I demonstrated a capacity to mitigate the activity of inflammatory kinases, whereas post-translational guanidinylation of apoA-I elicited a robust induction of inflammatory signaling cascades. The observed augmentation in inflammatory signaling pathways consequent to guanidinylation suggests a plausible mechanistic underpinning for the discernible functional effects associated with guanidinylated apoA-I.

DISCUSSION

The mechanisms underlying the significantly increased cardiovascular risk in patients with CKD, especially in the early stages, are still poorly understood. Increasing evidence suggests that PTMs are crucial in developing CVD in CKD. Because apoA-I, as the main protein of HDL, plays an important role in both pathologies, our study focused on examining the presence and characteristics of apoA-I obtained from subjects afflicted with CKD. Using a novel proteomics approach, we detected a progressive increase in the occurrence of guanidinylation modifications on apoA-I with the progression of CKD. This increase reinforces the assumption of a possible link between the progression of CKD and the accumulation of PTMs, which

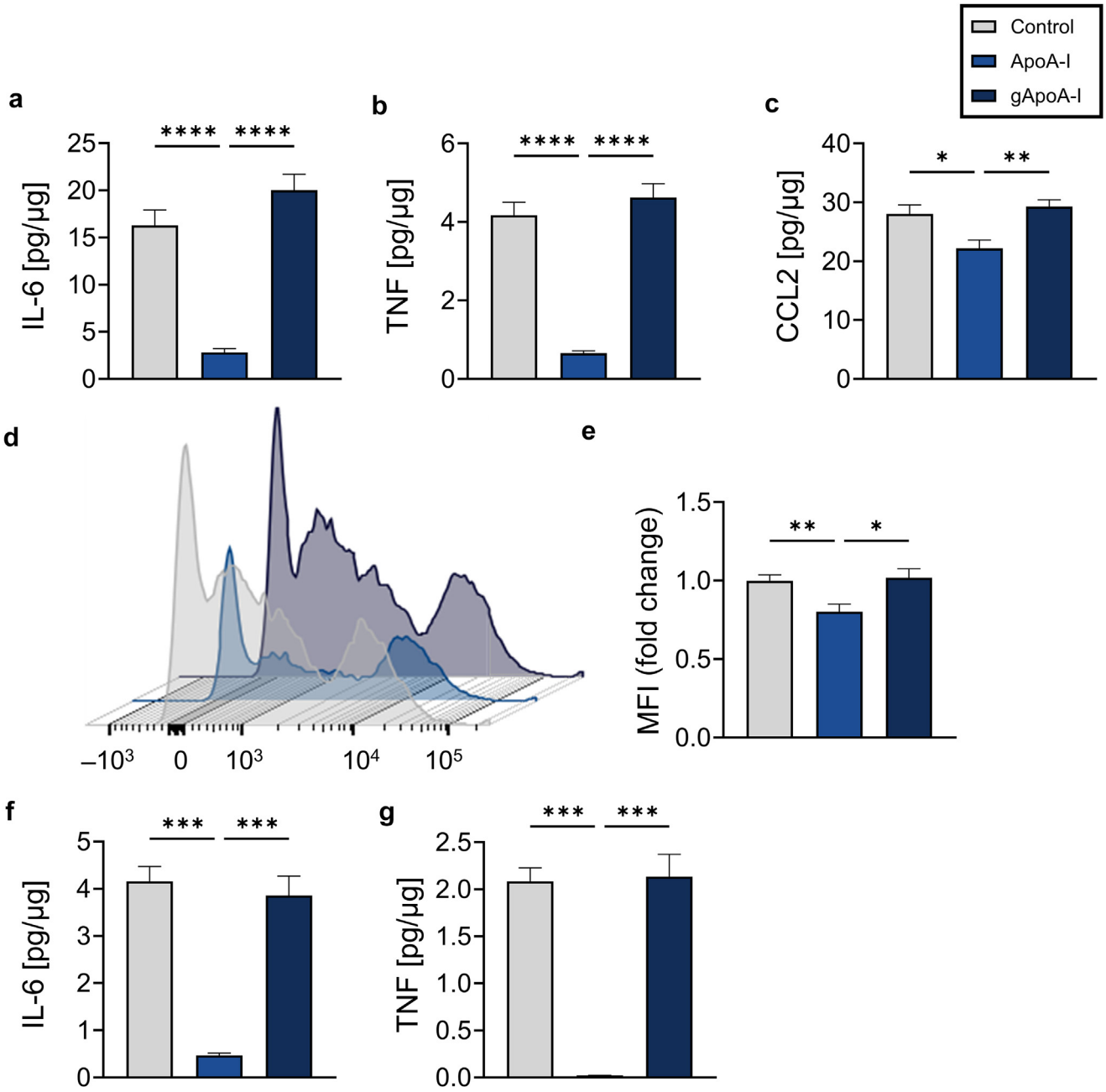

Figure 5 | Guanidinylation of apolipoprotein A-I (apoA-I) results in the loss of its anti-inflammatory and antioxidant properties. (**a–c**) Cytokine levels of (**a**) interleukin 6 (IL-6), (**b**) tumor necrosis factor (TNF), and (**c**) CC chemokine ligand 2 (CCL2) were measured using enzyme-linked immunosorbent assay (ELISA) in bone marrow–derived macrophages (BMDMs; n = 5) stimulated with vehicle and lipopolysaccharide (LPS; control), unmodified apoA-I and LPS (ApoA-I), and guanidinylated apoA-I and LPS (gApoA-I). (**d,e**) Representative flow cytometry plot and quantification of 2′,7′- dichlorodihydrofluorescein diacetate signal in BMDMs (n = 3) stimulated with a vehicle and phorbol 12-myristate 13-acetate (PMA [control]), unmodified apoA-I and PMA (ApoA-I), and guanidinylated apoA-I and PMA (gApoA-I). (**f,g**) Cytokine levels of (**f**) IL-6 and (**g**) TNF were measured using ELISA in murine peritoneal macrophages (n = 3) stimulated with vehicle and LPS (control), unmodified apoA-I and LPS (ApoA-I), and guanidinylated apoA-I and LPS (gApoA-I). Bar graphs represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. MFI, mean fluorescence intensity.

is reflected by the impact of renal dysfunction on apoA-I function. Using guanidinylated apoA-I *in vitro*, we could demonstrate that these guanidinylations abolished the anti-inflammatory and antioxidant properties typically associated with apoA-I. Because inflammation and oxidation play a causal role in CVD, guanidinylated apoA-I might at least be partly responsible for the increased cardiovascular risk in patients with CKD. Therefore, a comprehensive understanding of the involvement of guanidinylated apoA-I in this context is essential for developing targeted therapeutic interventions and prevention strategies tailored to patients with CKD at increased cardiovascular risk.

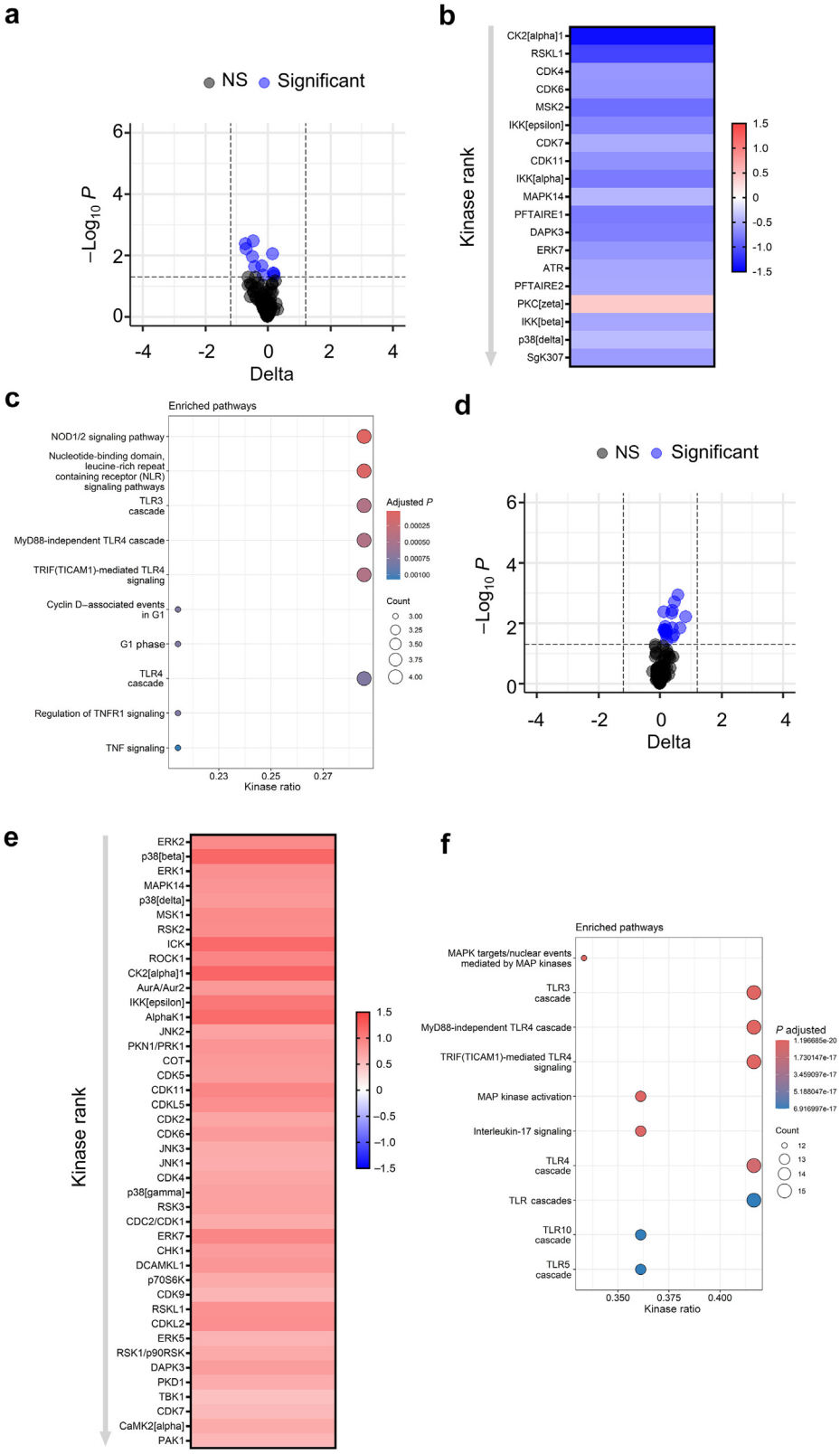

Figure 6 | Inflammatory kinase activity is downregulated in bone marrow–derived macrophages (BMDMs) treated with apolipoprotein A-I (apoA-I), an effect that is lost on apoA-I guanidinylation. (**a**) Volcano plot visualizing fold change and *P* value for phosphorylated peptides from serine-threonine kinase (STK) array. Blue dots represent significantly altered phosphopeptides in BMDMs treated with apoA-I and lipopolysaccharide (LPS; apoA-I) compared with only LPS treatment (control). (**b**) Heat maps of significantly changed kinases are ranked on the basis of the median final score (cutoff value of 1.2). Only significantly regulated kinases are shown. Color corresponds to the median kinase statistic, which represents effect size. Red indicates increased, and blue indicates decreased, kinase activity in BMDMs treated with apoA-I compared with those treated with LPS only. (**c**) Top 10 enriched pathways in BMDMs of cells (continued)

Post-translational guanidinylation observed in proteins and peptides arises from their interaction with guanidine.[21] The association between protein guanidinylation and both cardiovascular and all-cause mortality in patients with CKD has become increasingly evident. For example, recent studies have demonstrated that guanidinylation of proteins, such as fibrinogen,[22] apolipoprotein C3,[15] and albumin,[13] has negative effects by increasing clot density, promoting renal fibrosis, and reducing the detoxification capacity of albumin, respectively. These results emphasize the overall potential role of guanidinylation as a mediator of increased morbidity and mortality in CKD. In the context of CKD, the effects of PTMs, like guanidinylation, have recently been reviewed.[21]

In patients with CKD and comorbid diabetes, the function of apoA-I is altered not only by post-translational guanidinylation, but also by other modifications. Hyperglycemia and resulting oxidative stress cause post-translational glycation[23] and oxidation[24] of apoA-I, reducing the functional capacity to promote cholesterol efflux and anti-inflammatory effects in these patients. Additionally, diabetes increases the formation of advanced glycation end products, further impairing the protective functions of apoA-I.[25] Therefore, diabetes also exacerbates vascular dysfunction and inflammation in patients with CKD, contributing to accelerated atherosclerosis and cardiovascular complications.

The method for the *in vitro* generation of guanidinylations is based on established biochemical methods (e.g., O-methylisourea bisulfate is used for guanidinylation reactions in aqueous solution).[16,26] Therefore, in addition to urea's mechanistic involvement in carbamylation, the participation of urea in guanidinylation reactions is also conceivable. The *in silico* analysis of the protein surface revealed that the modified residues are accessible, suggesting that they can readily interact with guanidino-containing compounds and are, therefore, susceptible to guanidinylation. The chemical similarity between guanidine and urea molecules allows conclusions to be drawn about the potential mechanism of guanidinylation. Urea causes post-translational carbamylation by reacting cyanates with the specific protein or peptide amino groups. The reactive cyanates are dissociation products of urea,[27] and reactive intermediates from urea may be formed upstream of guanidinylation reactions. The molecular similarities between guanidine and urea could indicate a common biological origin. Analyzing the urea cycle within the human body elucidates the pivotal role of urea as a key molecule in this metabolic pathway. Interestingly, guanidine compounds are also involved in different parts of the urea cycle. In addition to urea, there are other toxins, particularly guanidino-containing compounds, that are accumulated in patients with CKD and that are likely responsible for guanidinylation in patients. Guanidino-containing compounds are a large group of small water-soluble compounds, including guanidine, creatinine, guanidinosuccinic acid, symmetric dimethylarginine, asymmetric dimethylarginine, and methylguanidine. On their own, various CGs have been linked to increased cardiovascular risk.[28–30] For example, symmetric dimethylarginine in HDL from patients with CKD is associated with increased mortality.[31] Interestingly, creatinine is one of the most abundant uremic toxins and, similarly to urea, it is not well characterized for its biological toxicity.[32] However, based on its potential to guanidinylate, it may be interesting to further characterize its potential for biological damage. As expected, our study revealed a clear association between the amount of guanidinylations and both creatinine and urea levels, supporting the notion that these 2 are likely involved in the guanidinylation process.

Despite proteomic studies doing more extensive scale analysis and characterizing various proteoforms of apoA-I resulting from modifications,[33,34] this is the first time, to our knowledge, that apoA-I guanidinylations have been identified and its (patho-) physiological effects characterized. Previously, we demonstrated the occurrence of guanidinylations of albumin and fibrinogen in patients with CKD.[13,22] Furthermore, we recently demonstrated that apolipoprotein C-III also gets guanidinylated by guanidine and urea in patients with CKD.[15] This guanidinylated apolipoprotein C-III promoted inflammatory responses and kidney tissue fibrosis, demonstrating a detrimental phenotype on modification.[15] This aligns with our current results, demonstrating that

Figure 6 | (continued) treated with apoA-I compared with LPS-treated cells. (**d**) Volcano plot of phosphorylated peptides (fold change and *P* values) from STK array. Significantly altered phosphopeptides are depicted in blue in BMDMs treated with guanidinylated apoA-I compared with apoA-I (both LPS stimulated). (**e**) Significantly changed kinases in a heat map are ranked on the basis of the median final score (cutoff value 1.2). Only significantly regulated kinases are shown. Red indicates increased and blue decreased kinase activity in BMDMs treated with guanidinylated apoA-I compared with cells treated with apoA-I (both LPS stimulated). (**f**) Top 10 enriched pathways in BMDMs of cells treated with guanidinylated apoA-I compared with apoA-I–treated cells (both LPS stimulated). The presented data are an average of 4 individual samples per treatment. ATR, ataxia telangiectasia and Rad3-related; AurA, aurora kinase A; CaMK2, calcium/calmodulin-dependent protein kinase II; CDC2, cell division cycle 2; CDK, cyclin-dependent kinase; CDKL2, cyclin-dependent kinase-like 2; CHK1, checkpoint kinase 1; CK, creatine kinase; COT, cancer osaka thyroid kinase; DAPK3, death-associated protein kinase 3; DCAMKL1, doublecortin-like and calcium/modulin kinase-like 1; ERK, extracellular signal-regulated kinase; ICK, intestinal cell kinase; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MSK, mitogen- and stress-activated kinase; NOD, nucleotide-binding oligomerization domain-containing protein; NS, not significant; PAK1, p21-activated kinase 1; PFTAIRE, designation for a subgroup of cyclin-dependent kinases characterized by the PFTAIRE amino acid sequence; PKC, protein kinase C; PKD1, protein kinase D1; PKN1, protein kinase N1; PRK1, protein kinase N1; ROCK1, Rho-associated coiled-coil–containing protein kinase 1; RSK, ribosomal S6 kinase; RSKL1, ribosomal protein S6 kinase C1; SgK, serum and glucocorticoid-regulated kinase 1; TBK1, TRIF/TANK-binding kinase 1; TICAM, Toll–interleukin 1 receptor domain-containing adaptor molecule; TLR, toll-like receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

apoA-I also obtains an unfavorable and pathogenic phenotype on guanidinylation.

So far, guanidinylations have been detected at lysine residues of proteins *in vivo*.[13,22] Additionally, guanidinylations were observed in the glycine residues of proteins after *in vitro* modification.[35] However, the guanidinylations of apoA-I determined in the present study are not only located in lysine residues, but were also found on aspartic acid, leucine, alanine, and glutamine in apoA-I.

An increase in post-translational guanidinylations of the HDL-associated apoA-I was observed on CKD progression. These findings of modified HDL concur with various studies describing an altered HDL proteome in patients with CKD,[36–38] resulting in dysfunctional HDL. However, because of the study design of a unicenter cross-sectional study, only a correlation between the presence of a dialysis requirement and the changes mentioned above was described, in contrast to our current study. For patients with CKD stage 5, a decreased apoA-I plasma concentration has been demonstrated in the past. Increased degradation of apoA-I can explain this; consequently, HDL concentration is decreased in this patient population.[39,40] These findings raised the question of the impact of the post-translational guanidinylation of apoA-I on HDL stability, which must be addressed in future longitudinal studies. In line with this, antibody levels against apoA-I are increased in patients with CKD stage 5 in correlation with dialysis duration,[41] probably resulting in this increased degradation, which may explain the slight decrease of apoA-I modification in patients with CKD stage 5 in our study.

These molecular modifications of apoA-I can particularly impact the cardiovascular state of patients with CKD. PTMs of HDL in CKD have a significant role in reduced inhibition of reactive oxygen species production and, thus, in the loss of antioxidant properties of HDL.[42,43] Post-translationally modified lipoproteins from patients with CKD increase oxidative stress, which has, for example, been extensively described for carbamylated low-density lipoprotein.[42] Carbamylated low-density lipoprotein is highly abundant in patients with CKD and correlates with poor cardiovascular outcomes.[44,45] In addition, there is evidence for the pathophysiological effects of carbamylated HDL on the endothelium.[10] The effect of modified apoA-I on oxidative processes involved in endothelial dysfunction has also already been described. For example, glycated apoA-I showed a reduced inhibitory effect on reactive oxygen species formation in *in vitro* experiments compared with apoA-I.[46] These findings align with our results, demonstrating reduced apoA-I functionality following guanidinylation.

A main strength of this study was the use of mass spectrometry to detect post-translational guanidinylation with high sensitivity and specificity. However, because PTMs are diverse and complex, other important modifications may have been missed while focusing on guanidinylation. Furthermore, the study was performed in a small group of $\approx$82 patients, which limits the generalizability of the results to a larger patient group. A larger study is now essential to validate the reliability and generalizability of the results. Additionally, the study's cross-sectional design limits the possibility of concluding direct causal links between the observed post-translational guanidinylations and their effects. Because the data were collected at a single time point, it is difficult to determine whether the detected modifications directly caused changes in the anti-inflammatory properties in patients with CKD. Long-term studies are needed to understand these causal relationships in more detail. Such studies would also allow for a more comprehensive evaluation of external factors, like diet, medication, and the patient's clinical conditions, and how they affect these modifications.

In conclusion, this study provides significant insights into the role of apoA-I guanidinylation in patients with CKD, highlighting a potential mechanistic link to cardiovascular complications. The PTMs in apoA-I are evident across multiple stages of CKD and are associated with a loss of anti-inflammatory and antioxidant properties. Thereby, this dysfunctional apoA-I is thus a potential culprit in the increased risk of patients with CKD for major cardiovascular events. A limitation of our study is the cross-sectional design and a limited number of patients. This small sample size restricts the ability to analyze potential confounding factors, such as patient medication, comorbidities, and lifestyle factors. However, the exciting relationship between guanidinylation and apoA-I offers a broad field for future research and findings. Furthermore, this study demonstrates the need to prevent or reduce PTMs to improve the cardiovascular situation in patients with CKD.

DISCLOSURE

All the authors declared no competing interests.

DATA STATEMENT

The study includes sensitive patient data, which were collected under strict confidentiality agreements. Releasing these data without appropriate safeguards compromises patient privacy and violates ethical guidelines surrounding patient data protection. Although anonymization of data sets is a common practice, our consent form did not anticipate such broad dissemination, and we are committed to respecting the wishes of the participants. In addition, certain aspects of the data are integral to ongoing proprietary analyses. As such, releasing these data would hinder future scientific progress.

Although we are unable to make the data publicly available at this time, we are open to considering specific requests for data sharing on a case-by-case basis. Interested parties can contact the corresponding author directly for further discussion.

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AUTHOR CONTRIBUTIONS

AB-M, SLM, JH, FK, ML, ES, MB, SK, EPCvdV, JJ, and VJ acquired the data. DF, SS, TS, and DWLW provided key materials. AB-M wrote the manuscript. JJ, EPCvdV, and VJ revised the manuscript.

Supplementary material is available online at www.kidney-international.org.

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