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Homocitrulline Is Associated with Cardiovascular Outcomes in Nondialysis Patients with CKD

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Key Points

- In nondialysis CKD, baseline serum homocitrulline was positively and independently linked to age, low eGFR, urea, anemia, and diuretics.
- A higher serum homocitrulline concentration was associated with an elevated risk of major adverse cardiovascular event and all-cause mortality rate.
- Targeting elevated levels of protein carbamylation may be a way of modifying the cardiovascular risk in patients with CKD.

Abstract

Background Protein carbamylation contributes to an increase in the cardiovascular risk in certain patient populations (e.g., in patients with CKD because of elevated urea concentrations). Homocitrulline (HCit) is a biomarker of overall protein carbamylation. In a study of a large cohort of nondialysis patients with a confirmed diagnosis of CKD and an eGFR <60 ml/min per 1.73 m², we sought to determine whether the serum HCit concentration was associated with adverse cardiovascular outcomes and all-cause mortality.

Methods CKD-renal epidemiology and information network is a prospective cohort of patients with CKD and an eGFR <60 ml/min per 1.73 m². The baseline serum HCit concentration was centrally measured. The 2195 patients included in the analysis were divided into tertile (T) groups according to the baseline HCit concentration (T1 <292, T2 = [292–429], and T3 ≥430 μmol/mol lysine). Adjusted Cox proportional hazards models were used to estimate hazard ratios for the first major adverse cardiovascular event (MACE) and death before KRT.

Results Among the 2195 included patients, the median age was 68 years and the mean eGFR was 34.6 ml/min per 1.73 m². The median (interquartile range) serum HCit was 352 (266–481) μmol/mol lysine. The HCit concentration was correlated with the eGFR ($r = -0.57$) and the urea concentrations ($r = 0.73$). In an adjusted linear regression model, the HCit concentration was independently and positively associated with age, eGFR decrease, urea, anemia, baseline prescription of diuretics, and negatively associated with male sex and an elevated urinary albumin-to-creatinine ratio. The adjusted hazard ratio (95% confidence interval) for MACEs as a function of the baseline HCit concentration was 1.32 (0.96 to 1.84) for T2 and 1.63 (1.16 to 2.30) for T3, compared with T1. The risk of death before KRT as a function of the baseline serum HCit concentration was 2.09 (1.45 to 3.03) for T3 and 1.48 (1.04 to 2.11) for T2, compared with T1.

Conclusions Our analysis of a large cohort of patients with CKD demonstrated that the serum HCit concentration was associated with a greater likelihood of a MACE and death. To confirm causality, further studies of therapeutic interventions for preventing or reducing carbamylation are now warranted.

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Introduction

In the human body, proteins undergo various chemical reactions that can change their functional properties. Post-translational modifications can influence a protein's charge, structure, and function under various physiologic and pathologic conditions.¹ Protein carbamylation is a nonenzymatic, post-translational modification that occurs when cyanate (mostly generated from the breakdown of urea) reacts spontaneously with the proteins' primary amino groups. Depending on the role of the protein involved, the resulting conformational change may affect cell functions. The serum concentration of homocitrulline (HCit), an amino acid residue produced by the carbamylation of the lysine residue on an ϵ -NH₂ side chain, is a validated marker of protein carbamylation.^{2,3}

Greater formation of carbamylation-derived products is observed in certain disease settings and particularly in CKD, due to the elevated blood urea concentration.⁴ We have previously reported that the serum urea concentration is a predictor of cardiovascular outcomes independently of other cardiovascular risk factors (including the eGFR).⁵ Urea may have direct and/or indirect toxic effects through the carbamylation process. It is therefore important to characterize the role of protein carbamylation in patients with CKD because this process might be a valuable therapeutic target.⁶

The literature data show that circulating markers of carbamylation are associated with the mortality rate and cardiovascular outcomes in patients on hemodialysis.⁷⁻⁹ Furthermore, carbamylated albumin was shown to be an independent risk factor for CKD progression and kidney failure in a predialysis population.^{10,11} The results of pre-clinical studies suggest that carbamylation may have a role in the development of cardiovascular complications by altering lipoproteins, circulating proteins (*e.g.*, fibrinogen), and extracellular matrix components (*e.g.*, collagens, proteoglycans, and fibronectin).¹² For instance, protein carbamylation accentuated the calcification of human vascular smooth muscle cells by downregulating the expression of a key enzyme (ectonucleotide pyrophosphate/phosphodiesterase 1) involved in the production of pyrophosphate—a powerful inhibitor of ectopic calcification¹³ and increased stiffness in elastic fibers.¹⁴ Given that patients with CKD have an elevated cardiovascular risk even before the onset of kidney failure, we decided to evaluate the association between HCit and the cardiovascular risk in a nondialysis CKD population.

The objectives of this study were therefore to (1) describe serum HCit concentrations in nondialysis CKD patients with a confirmed diagnosis of CKD and an eGFR <60 ml/min per 1.73 m², (2) identify factors associated with the serum HCit concentration, and (3) determine whether the serum HCit concentration is associated with serious adverse outcomes, *i.e.*, fatal or nonfatal major cardiovascular events, and death before KRT (defined as kidney transplantation or the initiation of long-term dialysis therapy) after adjustment for the eGFR and other risk factors.

Methods

The results of this cohort study were reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines.¹⁵

Study Design and Participants

CKD-renal epidemiology and information network (REIN) is a prospective cohort study conducted in 40 nephrology outpatient facilities, constituting a representative, nationwide sample of nephrology practices in France. The study protocol has been described in detail elsewhere.¹⁶ The main inclusion criteria were 18 years or older, a confirmed diagnosis of moderate or advanced CKD (stages 2–5), and no KRT. Between July 2013 and April 2016, 3033 patients were recruited during a routine nephrology outpatient visit and were followed for up to 5 years. Outpatient recruitment aimed to avoid the inclusion of participants with acute conditions (*e.g.*, AKI and gastrointestinal hemorrhage) at the time of inclusion. Furthermore, all patients were on stable background therapy. The study protocol was approved by the Institutional Review Board at the French National Institute of Health and Medical Research (reference: IRB00003888) and was registered at ClinicalTrials.gov (NCT03381950). The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki.

For the purposes of the present analysis, we excluded patients with missing data for the serum HCit or urea concentration at baseline or who had an aberrant serum urea value (*i.e.*, <2.5 or >100 mmol/L). To limit the effects of nonrenal factors on HCit concentrations, patients who were on corticosteroids or had a history of metastatic cancer were excluded (Supplemental Figure 1). Hence, a total of 2195 patients were included in the present analysis.

Study Data

Trained clinical research associates (CRAs) collected data from patient interviews and medical records at baseline and then annually. Sociodemographic data (such as age, sex, and smoking status) and medical history (for hypertension, diabetes, cardiovascular disease [CVD], or AKI) were recorded at baseline (for the definitions of the variables, see Supplemental Table 1). BP was measured at baseline, as were height and weight (for calculation of the body mass index). Patients were asked to bring all their drug prescriptions for the preceding 3 months to the enrollment visit. The CRAs used an electronic case report form (linked to the international Anatomical Therapeutic and Chemical classification) to enter standardized drug codes.

All patients were prescribed a set of standard blood and urine tests (as recommended by the French health authorities for routine CKD care), with a sample to be collected at their usual medical laboratory. The GFR was estimated using the CKD Epidemiology Collaboration creatinine equation.¹⁷ The urine albumin-to-creatinine ratio (uACR) was either measured directly or estimated using an equation based on proteinuria values.¹⁸ In addition, serum samples were collected at the time of patient's enrollment, stored at 4°C, and aliquoted within 6 hours, without further processing. These samples were then stored at –80°C at the Biobanque de Picardie biological resource center (French national reference: BB-0033-00017) in Amiens-Picardie University Medical Center (Amiens, France). Serum samples were assayed for urea and high-sensitivity C-reactive protein (hs-CRP) in a central laboratory.

Determination of the HCit Concentration

The level of protein carbamylation in the biologic resource center's samples was assessed by measuring the total serum HCit concentration with a previously described liquid chromatography-tandem mass spectrometry method.¹⁹ The results were expressed in μmol of HCit per mol lysine ($\mu\text{mol}/\text{mol}$ lysine). The reference range for the serum HCit concentration in the general population was 100–170 $\mu\text{mol}/\text{mol}$ lysine.²⁰

Study Outcomes

Cardiovascular events, identified from medical records, hospital reports, and/or the annual patient interview, were assessed according to the Cardiovascular and Stroke Endpoint Definitions for Clinical Trials.²¹ The primary endpoint was the occurrence of a first major adverse cardiovascular event (MACE), defined as cardiovascular death, myocardial infarction, stroke, or hospital admission for heart failure. The secondary outcomes included the first instance of fatal or nonfatal CVD (whether atheromatous or nonatheromatous), the first instance of fatal or nonfatal atheromatous CVD, the first instance of fatal or nonfatal nonatheromatous CVD, and all-cause deaths (Supplemental Table 2). Only events occurring before KRT were considered.

Deaths before KRT were identified from death certificates, hospital records, reported by family members at the annual follow-up visit, and/or by linkage with the national death registry. Preemptive kidney transplantation and the initiation of long-term dialysis therapy were identified from medical records and/or by linking to the French National Kidney Failure Registry.²²

Patients were censored at the date of the competing event (KRT and/or noncardiovascular death, depending on the outcome), the end of their 5-year follow-up period, or if they were lost to follow-up.

Statistical Analysis

The participants' serum HCit concentrations were divided into tertiles (T1 <292, T2 [292–429], T3 ≥ 430 $\mu\text{mol}/\text{mol}$ lysine). The baseline characteristics were described for the overall population ($N=2195$) and for HCit tertile subgroups. Spearman coefficient or Pearson coefficient was calculated to estimate the strength of the correlation between HCit and certain other biochemical variables (eGFR and serum levels of urea, hs-CRP, and albumin). Multivariable linear regressions (based on sociodemographic variables, clinical variables, and prescription drugs) were used to estimate independent associations with the serum HCit concentration (expressed as a non-standardized coefficient [95% confidence interval (CI)]). The selection of variables for inclusion in multivariable models was based on a literature review and a P value < 0.05 in a univariable analysis.

Incidence rates (95% CI) for each event were computed as a function of the HCit concentration. For each outcome studied, we used cause-specific Cox models to estimate the crude and adjusted hazard ratios (HRs, 95% CI) for the association with the HCit concentration (in tertiles). For each outcome, HRs were adjusted for relevant demographic variables and baseline comorbidities that had been preselected in a literature review.

Risks associated with HCit were adjusted for age at baseline, sex, smoking status, baseline eGFR, natural log-transformed uACR, a binary variable indicating whether or not the uACR had been estimated from the proteinuria value, body mass index, diabetes, systolic BP, a history of CVD, anemia, serum albumin, serum hs-CRP (natural log-transformed), and prescriptions of diuretics, statins, and antithrombotic agents at baseline. As a significant interaction between sex and eGFR was previously identified in the modeling of atheromatous CVD risk,²³ this interaction was accounted for in our atheromatous CVD risk model. Each model's validity (according to the proportional hazards assumption) was checked by testing the Schoenfeld residuals. We used penalized splines in fully adjusted Cox models to represent the functional relationship between serum HCit and the CVD risk or mortality.

Missing covariate data were managed by multivariate imputation by chained equations.²⁴ By using the multivariate imputation by chained equations package in R statistical software, we imputed 30 datasets (20 iterations). All covariates present in the Cox models and the baseline serum bicarbonate were included in the imputation model. Fitted Cox models were generated for each dataset, and pooled regression coefficients were obtained using Rubin's rules. All statistical analyses were performed with R software (version 4.4.0).²⁵

Results

Characteristics of the Study Population at Baseline

Of the 3033 patients in the CKD-REIN cohort, 2195 were included in the present analysis (Supplemental Figure 1). The median age was 68 (60–76), and the mean (SD) eGFR was 34.6 (13.3) ml/min per 1.73 m²; 53% of the patients had a history of CVD, 42% had a history of diabetes, and 39% had a history of anemia (Table 1). The median (interquartile range) serum HCit concentration was 352 (266–481) $\mu\text{mol}/\text{mol}$ lysine, which was twice the upper boundary of the reference range. Overall, the serum HCit concentrations increased with the CKD stage (Figure 1). At baseline and compared with patients in HCit T1 and T2, patients in T3 were older and more likely to have a history of CVD, a lower eGFR, and higher serum urea and phosphate concentrations. However, patients in T1 were more likely to be male and had a higher prevalence of diabetes, compared with patients in T2 or T3. The serum HCit concentration was significantly and negatively correlated with the eGFR ($r=-0.57$; $P < 0.001$) and significantly and positively correlated with the urea concentration ($r=0.73$; $P < 0.001$). We also observed a moderate but significant negative correlation between the serum HCit and the albumin level ($r=-0.05$; $P = 0.01$). The HCit and hs-CRP concentrations were not correlated.

Factors Associated with the HCit Concentration

In an adjusted linear regression model, the serum HCit concentration was independently and positively associated with age, the eGFR decrease, urea, anemia, and baseline prescription of diuretics. By contrast, the HCit concentration was negatively associated with male sex and a greatly

Table 1. Baseline characteristics of the study population

Characteristic	Total (N=2195)	Baseline Serum HCit ($\mu\text{mol/mol Lys}$)			Imputed Data (N=2195)
		T1 <292 (n=730)	T2 (292–430) (n=732)	T3 \geq 430 (n=733)	
Serum HCit ($\mu\text{mol/mol Lys}$)	352 (266–481)	237 (201–266)	352 (323–385)	547 (480–684)	—
Serum urea (mmol/L)	13.4 (10.3–18.2)	9.90 (8.10–11.8)	13.6 (11.1–16.4)	19.3 (15.3–24.2)	—
Age (yr)	68 (60–76)	67 (59–73)	68 (60–77)	71 (61–78)	—
Men	66%	71%	66%	62%	—
Educational level, yr					1%
<9	15%	12%	16%	17%	
9–11	49%	48%	48%	51%	
\geq 12	36%	40%	36%	32%	
Smoking status					1%
Current smoker	40%	40%	38%	43%	
Never smoker	13%	13%	12%	13%	
Former smoker	47%	47%	50%	44%	
BMI (kg/m^2)	28.9 (5.8)	29.6 (5.7)	28.7 (6.0)	28.4 (5.8)	2%
Alcohol abuse	6%	7%	6%	6%	1%
Diabetes	42%	44%	43%	40%	0.2%
Dyslipidemia	74%	75%	75%	73%	0.1%
Hypertension	97%	95%	97%	98%	0.1%
Systolic BP (mm Hg)	142 (20)	142 (19)	142 (20)	141 (20)	0.4%
Diastolic BP (mm Hg)	78 (12)	79 (12)	78 (12)	77 (12)	0.5%
History of CVD	53%	48%	54%	57%	0.5%
History of nonatheromatous CVD	14%	12%	15%	16%	0.5%
History of atheromatous CVD	36%	32%	37%	39%	0.7%
Coronary disease	24%	22%	24%	27%	1%
Heart failure	13%	8%	13%	18%	0.1%
Peripheral artery disease	17%	15%	17%	17%	0.7%
Stroke and/or transient ischemic attack	10%	9%	10%	11%	0.7%
History of cerebrovascular disease	12%	10%	12%	13%	0.7%
History of gastrointestinal bleeding	4%	3%	4%	4%	—
eGFR at baseline ($\text{ml/min per } 1.73 \text{ m}^2$)	34.6 (13.3)	43.8 (12.2)	34.0 (11.5)	26.0 (9.6)	—
Albumin-to-creatinine ratio (mg/g)	126 (24.5–667)	75.3 (15.6–492)	110 (21.3–677)	206 (43.8–793)	9.4%
A1 (normal to mildly elevated)	28%	36%	29%	19%	
A2 (moderately elevated)	34%	34%	33%	36%	
A3 (greatly elevated)	38%	31%	38%	44%	
Anemia	39%	20%	39%	57%	1.6%
hs-CRP (mg/L)	2.5 (1.1–5.7)	2.5 (1.1–5.5)	2.4 (1.0–4.9)	2.6 (1.2–7.0)	1.2%
Serum albumin (g/L)	40.7 (3.9)	40.8 (4.0)	40.8 (3.7)	40.3 (4.0)	0.2%
Sodium (mmol/L)	140 (2.7)	140 (2.6)	141 (2.7)	140 (2.9)	1.2%
Potassium (mmol/L)	4.54 (0.5)	4.44 (0.5)	4.58 (0.5)	4.59 (0.6)	0.8%
Calcium (mmol/L)	2.35 (0.1)	2.37 (0.1)	2.35 (0.1)	2.34 (0.1)	3.8%
Phosphate (mmol/L)	1.15 (0.2)	1.08 (0.2)	1.14 (0.2)	1.25 (0.2)	5.9%
Uric acid (mmol/L)	427 (118)	413 (107)	431 (116)	437 (128)	9.7%
No. of prescription drugs taken daily	8 (5–10)	7 (4–10)	8 (5–10)	8 (6–11)	—
Diuretics	55%	45%	53%	67%	—
β -blockers	43%	41%	42%	47%	—
Calcium channel blockers	48%	47%	51%	48%	—
RASi	77%	78%	79%	75%	—
Statins	59%	59%	59%	59%	—
Antithrombotic agents	53%	50%	53%	55%	—
Immunosuppressants	3%	3%	3%	3%	—
Antigout medications	36%	30%	31%	46%	—
Proton pump inhibitors	31%	29%	31%	31%	—

Data are presented as the mean (SD), or the median (first–third quartiles), or percentages. BMI, body mass index; CVD, cardiovascular disease; HCit, homocitrulline; hs-CRP, high-sensitivity C-reactive protein; Lys, lysine; RASi, renin-angiotensin system inhibitors; T1, tertile 1; T2, tertile 2; T3, tertile 3.

elevated uACR (Table 2). Notably, after adjustment for multiple variables, the HCit concentration was not significantly associated with the hs-CRP level or a history of

CVD. Finally, smoking status, diabetes, and the serum albumin level were not associated with the HCit concentration in univariate models.

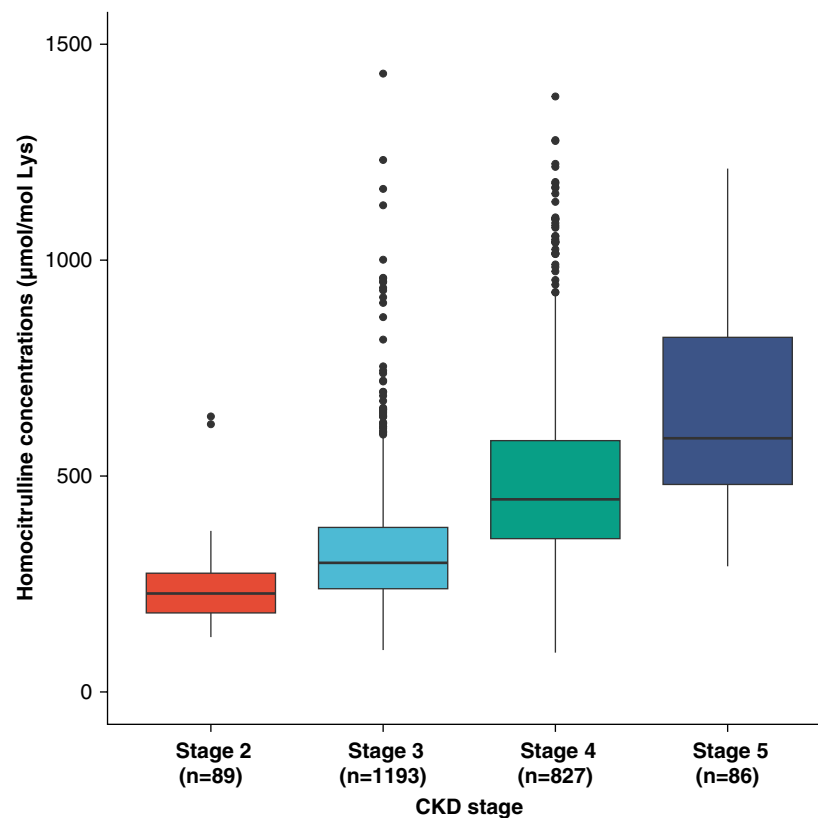


Figure 1. Serum HCit concentrations, by CKD stage. HCit, homocitrulline; Lys, lysine.

MACEs

Over a median (interquartile range) follow-up period of 4.9 (4.8–4.9) years, 324 patients experienced a first MACE. The crude incidence rate (95% CI) was 4.0 (3.5 to 4.4) per 100 person-years. The incidence rate was higher in patients in T3 and in T2 than in patients in T1 (Supplemental Table 3). Relative to patients in T1, the adjusted HR (95% CI) for MACEs associated with the baseline HCit concentration was 1.32 (0.96 to 1.84) for patients in T2 and 1.63 (1.16 to 2.30) for patients in T3 (Figure 2). The results of penalized spline analyses were consistent with the incidence rates and indicated that the risk increased as the HCit concentration rose (Figure 3A).

Fatal and Nonfatal Atheromatous or Nonatheromatous Cardiovascular Events

Of the 2195 patients, 554 experienced a first atheromatous or nonatheromatous cardiovascular event (fatal or nonfatal) at some point during the follow-up period; this yielded a crude incidence rate (95% CI) of 7.3 (6.7 to 7.9) per 100 person-years. The incidence rate was the highest in patients in the HCit T3 (Supplemental Table 3). After multiple adjustments, the risk of fatal and nonfatal atheromatous or nonatheromatous cardiovascular events was found to be significantly greater for patients in T3 than in patients in T1 (HR [95% CI], 1.42 [1.10 to 1.83], Figure 2). A nonsignificant trend toward a greater risk was also noted for patients in T2 (HR [95% CI], 1.09 [0.86 to 1.39]). A penalized spline analysis showed the same trends (Supplemental Figure 2).

A sensitivity analysis failed to show an association between the baseline HCit concentration and atheromatous cardiovascular events (fatal or nonfatal; Supplemental Figure 3). By contrast, a significantly greater risk of a non-atheromatous cardiovascular event (fatal or nonfatal) was observed in patients in T3 (HR [95% CI], 1.57 [1.15 to 2.14]), and a nonsignificantly greater risk was observed in patients in T2 (HR [95% CI], 1.13 [0.84 to 1.53]; Supplemental Figure 3). The results of penalized spline analyses were consistent with these findings (Supplemental Figure 4).

Death before KRT

Over a median follow-up period of almost 5 years in the CKD-REIN cohort, 284 patients died before KRT; this gave an incidence rate of 3.3 (2.9 to 3.7) per 100 person-years. The incidence rate was higher in patients in HCit T3 and T2 than in patients in T1 (Supplemental Table 3). After multiple adjustments and relative to patients in T1, the risk of death before KRT was 1.48 (1.04 to 2.11) for patients in T2 and 2.09 (1.45 to 3.03) for patients in T3 (Figure 2). Again, the results of penalized spline analysis confirmed these trends (Figure 3B).

Discussion

In a study of 2195 nondialysis patients with CKD selected from a large, prospectively monitored cohort, we found that age, a low eGFR, urea, anemia, and the baseline prescription of diuretics were independently and positively

Table 2. Factors associated with the baseline serum homocitrulline concentration (natural log-transformed)

Variable	Crude Analysis		Adjusted Analysis	
	β (95% CI)	P Value	β (95% CI)	P Value
Age (per 5 yr)	0.02 (0.01 to 0.03)	<0.001	0.02 (0.01 to 0.02)	<0.001
Men (versus women)	-0.08 (-0.12 to -0.04)	<0.001	-0.08 (-0.11 to -0.06)	<0.001
Smoking status				
Current smoker versus never smoker	-0.04 (-0.10 to 0.02)	0.23		
Former smoker versus never smoker	-0.02 (-0.06 to 0.02)	0.23		
eGFR (per 5 ml/min per 1.73 m ² decrement)	0.10 (0.09 to 0.10)	<0.001	0.023 (0.017 to 0.029)	<0.001
Log (ACR [mg/g])	0.003 (-0.0002 to 0.007)	0.06	-0.003 (-0.006 to -0.001)	0.02
BMI (per kg/m ²)	-0.01 (-0.01 to -0.003)	<0.001	-0.02 (-0.02 to -0.01)	<0.001
Diabetes	-0.02 (-0.06 to 0.02)	0.31		
History of CVD	0.07 (0.04 to 0.11)	<0.001	0.01 (-0.02 to 0.05)	0.36
Anemia	0.30 (0.26 to 0.33)	<0.001	0.07 (0.05 to 0.10)	<0.001
Urea (per mmol/L)	0.048 (0.046 to 0.05)	<0.001	0.040 (0.038 to 0.043)	<0.001
Serum albumin (per g/L decrease)	0.004 (-0.001 to 0.008)	0.13		
Log(hs-CRP [mg/L])	0.02 (0.0004 to 0.03)	0.04	-0.004 (-0.02 to 0.007)	0.49
No. of prescription drugs taken daily	0.02 (0.01 to 0.02)	<0.001		
Diuretics at baseline	0.19 (0.15 to 0.23)	<0.001	0.06 (0.03 to 0.09)	<0.001
β blockers at baseline	0.06 (0.02 to 0.10)	0.002	-0.001 (-0.03 to 0.02)	0.92
RASi at baseline	-0.03 (-0.08 to 0.01)	0.16		
Calcium channel blockers at baseline	-0.002 (-0.04 to 0.04)	0.92		
Statins at baseline	0.01 (-0.03 to 0.05)	0.59		
Antithrombotic agents at baseline	0.05 (0.009 to 0.08)	0.01	-0.02 (-0.05 to 0.01)	0.18
Proton pump inhibitors at baseline	0.03 (-0.01 to 0.07)	0.20		

The dependent variable (homocitrulline) was natural log-transformed because of its skewed distribution. Therefore, the interpretation is as follows: for one-unit change in the independent variable, homocitrulline level increases by a factor of $\exp(\beta)$. ACR, albumin-to-creatinine ratio; BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; hs-CRP, high-sensitivity C-reactive protein; RASi, renin-angiotensin system inhibitors.

associated with the serum HCit concentration. Male sex and a greatly elevated albumin-to-creatinine ratio were negatively associated with the serum HCit concentration. Furthermore, a high serum HCit concentration was associated with an elevated risk of MACEs and all-cause mortality.

Protein carbamylation has a variety of detrimental effects; it affects renal function, cardiovascular health, and immune responses. We sought to better characterize the factors associated with this process. Consistently with the accumulation of urea, the serum HCit concentration was independently associated with the eGFR and with anemia (a known marker of declining renal function). Interestingly, we found that the median serum HCit concentration was higher in women than in men. However, the urea level did not depend significantly on the sex ($P = 0.99$). This link between sex and HCit persisted after multivariate adjustment. This finding might be relevant, and we are not aware of published data indicating that carbamylation is more intense in women than in men. This phenomenon and its potential impact warrant further research. The positive association between diuretic prescriptions and the HCit concentration might be due to the lower eGFR and higher serum urea concentration observed in patients on diuretics. However, we cannot rule out a potential, direct influence of diuretics on the HCit concentration. Furthermore, we were unable to demonstrate an association with systemic markers of inflammation because myeloperoxidase-driven carbamylation is a localized process that occurs within

atherosclerotic plaques. Our data highlight the prominent role of urea in the carbamylation process in patients with CKD. Furthermore, our findings are in line with literature data from studies of patients with CKD, in which an association between hs-CRP (or myeloperoxidase) and carbamylated-derived products was lacking.^{7,9,26,27} Although our results do not rule out an influence of inflammation on the development of cardiovascular complications (as reported previously), the data on nonuremic patients²⁸ suggest that CVD can be driven by mechanisms other than carbamylation.

In our cohort of nondialysis patients, we found that a high serum HCit concentration is associated with adverse cardiovascular outcomes. We have previously reported on the relationship between an elevated urea concentration and cardiovascular events.⁵ In this study, we shifted our focus to carbamylation—a process known to result from urea accumulation. An investigation of carbamylation might provide a more integrated understanding of the pathophysiologic mechanism of uremic toxin accumulation and offers a broader perspective on the effects of uremia.²⁹ Significantly elevated levels of carbamylated hemoglobin and other circulating proteins (such as albumin, low-density lipoproteins, and high-density lipoproteins) have been observed in patients with CKD,⁴ along with evidence of tissue carbamylation—notably in kidney, heart, and aorta.³⁰ This study focused on HCit as a key marker of overall protein carbamylation. In a previous study of a population of patients on hemodialysis, we found that

	N events	Event rate per 100 person-years	Adjusted HR*
MACEs			
Tertile 1	68	2.2 [1.7; 2.7]	Ref
Tertile 2	110	3.9 [3.2; 4.6]	1.32 [0.96; 1.84]
Tertile 3	146	6.5 [5.5; 7.6]	1.63 [1.16; 2.30]
Fatal and non-fatal atheromatous or nonatheromatous CVD			
Tertile 1	150	5.2 [4.3; 6.0]	Ref
Tertile 2	181	6.9 [5.9; 7.9]	1.09 [0.86; 1.39]
Tertile 3	223	10.8 [9.4; 12.2]	1.42 [1.10; 1.83]
Death before KRT			
Tertile 1	54	1.8 [1.3; 2.3]	Ref
Tertile 2	94	3.2 [2.5; 3.8]	1.48 [1.04; 2.11]
Tertile 3	132	5.5 [4.6; 6.4]	2.09 [1.45; 3.03]

Figure 2. Numbers of events, incidence rates, and adjusted HRs for MACEs (whether atheromatous or nonatheromatous and fatal or nonfatal) and death before KRT as a function of the baseline serum HCit concentration. *HRs were adjusted for age, sex, smoking status, body mass index, diabetes, systolic blood pressure, estimated glomerular filtration rate, urine albumin- or protein-to-creatinine ratio (natural log-transformed), ACR estimated from proteinuria (binary variable), history of CVD, anemia, serum albumin, high-sensitivity C-reactive protein (natural log-transformed), and the use of diuretics, statins, and antithrombotic agents at baseline. ACR, albumin-to-creatinine ratio; CVD, cardiovascular disease; HR, hazard ratio; MACE, major adverse cardiovascular event.

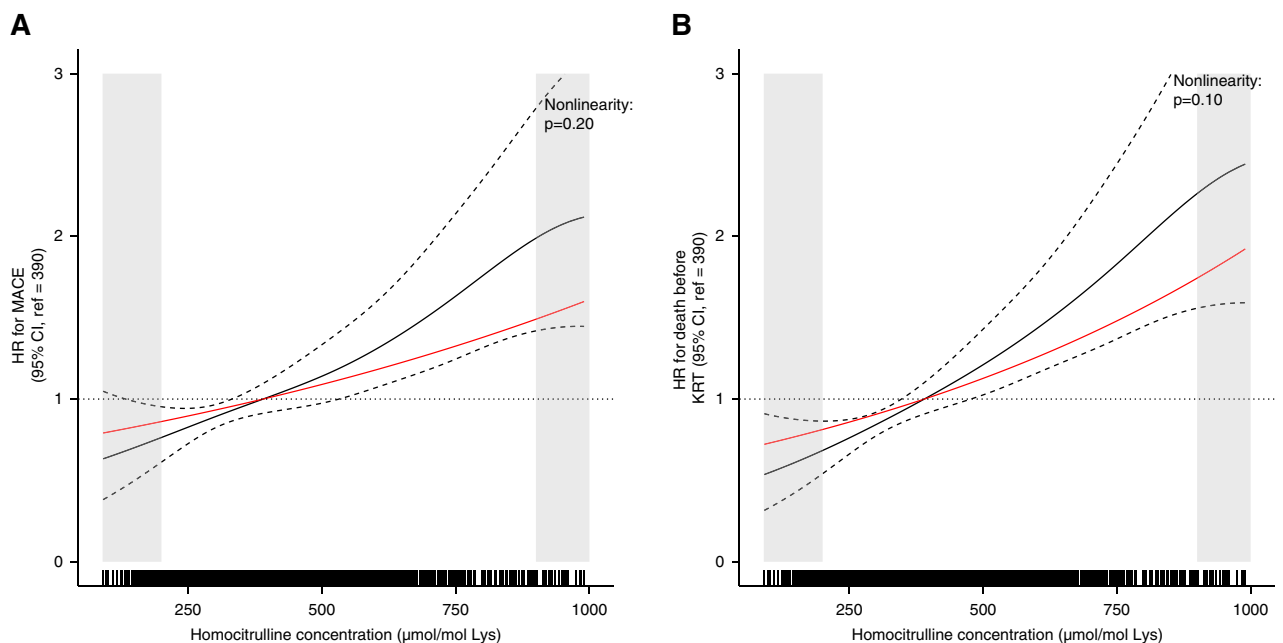


Figure 3. Adjusted HR for the occurrence of the first MACE and death before KRT, as a function of the baseline serum HCit concentration, using penalized spline terms. (A) Adjusted HR for first MACE, as a function of the baseline serum HCit concentration. (B) Adjusted HR for death before KRT, as a function of the baseline serum HCit concentration. CI, confidence interval.

albumin carbamylation was associated with plasma levels of both total HCit and protein-bound HCit.³¹ Various pre-clinical studies have corroborated the association between HCit and cardiovascular outcomes in general and non-atherosclerotic events in particular. Indeed, protein carbamylation exacerbated the calcification of human vascular smooth muscle cells by suppressing the expression of ectonucleotide pyrophosphate/phosphodiesterase 1.¹³ The calcification process (especially medial calcification) is an important factor in the induction of vascular stiffness, which is associated with heart failure. Furthermore, our study of cyanate supplementation in the mouse evidenced greater protein carbamylation in the aortic wall and stiffer elastic fibers.¹⁴ This involvement in medial calcification and aortic stiffness might account for the association between HCit and a nonatheromatous CVD event such as heart failure. Furthermore, it has recently been shown that carbamylation of $\alpha_{IIb}\beta_3$ (a receptor required for platelet aggregation) is associated with low integrin activation, low fibrinogen binding capacity, impaired platelet adhesion to fibrinogen, and impaired platelet aggregation. The level of protein carbamylation was significantly higher in platelets from patients on hemodialysis than in platelets from controls, confirming the presence of platelet carbamylation *in vivo*.³² The consequences of platelet carbamylation remains to be characterized. Protein carbamylation affects various proteins involved in complications of CVD. Indeed, low-density lipoproteins are particularly susceptible to carbamylation, which can lead to endothelial cell apoptosis and smooth muscle cell proliferation *in vitro*³³ and other molecular changes that contribute to vascular damage *in vivo*.³⁴ Most epidemiologic studies showing a link between protein carbamylation, the cardiovascular risk, and the mortality rate were performed in hemodialysis patients⁷⁻⁹; this study extends this finding to a nondialysis population. A *post hoc* analysis of the 4D trial in diabetic hemodialysis patients found an association between protein carbamylation, heart failure, and the mortality rate.⁷ Our present results therefore suggest that nonatherosclerotic events are also associated with protein carbamylation.

Focusing on protein carbamylation (and not just urea accumulation) is important because it might constitute a valuable therapeutic target. Indeed, amino acid concentrations can influence protein carbamylation rates in uremic models *in vitro* and *in vivo*.⁸ Moreover, nutritional therapy with a Mediterranean diet and a very-low-protein diet can reduce protein carbamylation and cyanate production.³⁵ Furthermore, a pilot study in patients on dialysis reported that intravenous amino acid supplementation effectively reduced the carbamylation level of plasma proteins, as evaluated by the percentage of carbamylated albumin.⁶

This study had several strengths. First, our analysis of a large number of patients from a nationally representative, multicenter, prospective cohort enabled comprehensive adjustments for confounding factors. Second, HCit concentrations were measured centrally, using a robust liquid chromatography-tandem mass spectrometry method.

Our study also had some limitations. First, the HCit concentrations were measured once (at baseline), and so we were not able to explore putative associations between

time-varying concentrations and outcomes. Moreover, we did not assess the dietary protein intake at the time of the blood test; this variable might have affected the HCit level. However, our work could be considered to be a hypothesis-generating study. Second, the study's observational design precluded any causal interpretations.

In conclusion, our analysis of a large cohort of patients with moderate-to-advanced CKD demonstrated that an elevated serum HCit concentration was associated with a greater likelihood of MACE and a higher mortality rate. Targeting elevated levels of protein carbamylation might be a promising way of modifying the cardiovascular risk in patients with CKD.

Disclosures

Disclosure forms, as provided by each author, are available with the online version of the article at <http://links.lww.com/KN9/B9>.

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Data Sharing Statement

Partial restrictions to the data and/or materials apply. The data that support the findings of this study are available upon reasonable request by contacting the CKD-REIN study coordination staff at ckdrein@inserm.fr. The funding sources had no roles in study design, conduct, reporting or the decision to submit for publication.

Supplemental Material

This article contains the following supplemental material online at <http://links.lww.com/KN9/B10>.

Supplemental Table 1. Definitions of variables.

Supplemental Table 2. Definitions of cardiovascular outcomes.

Supplemental Table 3. Incidence rates of outcomes, as a function of the baseline serum HCit concentration.

Supplemental Figure 1. Study flow chart.

Supplemental Figure 2. Adjusted HR for the occurrence of the first atheromatous or nonatheromatous event as a function of the baseline serum HCit concentration, using penalized spline terms.

Supplemental Figure 3. Numbers of events, incidence rates, and adjusted HRs for the first atheromatous and nonatheromatous events as a function of the baseline serum HCit concentration.

Supplemental Figure 4. Adjusted HR for the occurrence of the first atheromatous cardiovascular event and the first non-atheromatous cardiovascular event, as a function of the baseline serum HCit concentration, using penalized spline terms. (A) Adjusted HR for the first atheromatous CVD event, as a function of the baseline serum HCit concentration. (B) Adjusted HR for the first nonatheromatous CVD event, as a function of the baseline serum HCit concentration.

Supplemental Appendix 1. List of biologic resources centers.

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