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► To cite this version:

Touria Mernissi, Stéphane Jaisson, Pierre Spicher, Said Kamel, Anaïs Okwieka, et al.. Exploring the Interactions between Drugs and Metabolic Disturbances in CKD. *Kidney360*, 2025, 6 (12), pp.2145-2156. <10.34067/KID.0000000862>. <hal-05129261>

HAL Id: hal-05129261

<https://u-picardie.hal.science/hal-05129261v1>

Submitted on 20 Apr 2026

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Exploring the Interactions between Drugs and Metabolic Disturbances in CKD

An Example with Oral Anticoagulants

Touria Mernissi,^{1,2} Stéphane Jaisson ,³ Pierre Spicher ,¹ Said Kamel,¹ Anaïs Okwieka ,³ Julien Demagny ,⁴ Gabriel Choukroun,^{1,5} Solène M. Laville ,^{1,6} Sandra Bodeau ,^{1,2} and Sophie Liabeuf ,^{1,6}

Key Points

- Kidney function was independently associated with free drug concentration; the latter concentration was higher in patients with a lower eGFR.
- The conformational changes in albumin induced by carbamylation may alter its binding capacity to vitamin K antagonists.
- The results on the accumulation of free fraction of drugs in patients with low eGFR may be applicable to other drugs that are bound to plasma proteins.

Abstract

Background The metabolic disturbances associated with CKD might alter drug distribution, decrease albumin drug binding, and thus increase the free (unbound) drug concentrations. Uremic toxins (UTs) can affect the pharmacokinetic or pharmacodynamic activity of certain drugs. Vitamin K antagonists (VKAs) are interesting candidates for the evaluation of potential interactions between UTs and drugs. The primary objective of this study was to investigate the association between free VKA concentrations and the eGFR. Furthermore, we sought to determine whether this relationship was mediated by protein carbamylation (measured by homocitrulline levels) and/or the accumulation of protein-bound UTs (PBUTs).

Methods In this prospective cross-sectional study, 389 adult patients treated with VKA were included between May 2021 and June 2023. Levels of free VKAs, total VKAs, homocitrulline, and PBUTs were assayed using liquid chromatography-tandem mass spectrometry. We used a linear regression model to explore the association between kidney function and free VKA levels and mediation analyses to determine whether the association between kidney function and free VKA levels was mediated (at least partly) by PBUTs and/or protein carbamylation.

Results Patients with an eGFR <40 ml/min per 1.73 m² or those on chronic hemodialysis had lower total VKA levels, higher free VKA levels, and thus a higher free/total VKA ratio than those with an eGFR ≥40 ml/min per 1.73 m². Kidney function was independently associated with free VKA levels ($\beta_1=0.31$ [0.19 to 0.42], $P < 0.001$). Twenty-one percent (95% confidence interval, 1% to 35%) of the association between kidney function and free VKA levels was mediated by homocitrulline, but not by PBUTs.

Conclusions Our results showed that a low kidney function was associated with an elevation in the VKA free drug fraction. This association was independent of blood albumin levels and appeared to be partly mediated by protein carbamylation.

Kidney360 6: 2145–2156, 2025. doi: <https://doi.org/10.34067/KID.0000000862>

Introduction

In CKD, the decline in GFR leads to a reduced clearance of renally excreted drugs and their metabolites. This

necessitates careful dose adjustments to avoid toxicity. However, focusing exclusively on elimination may overlook other key contributors to drug accumulation. Indeed,

Due to the number of contributing authors, the affiliations are listed at the end of this article.

Correspondence: Prof. Sophie Liabeuf, email: liabeuf.sophie@chu-amiens.fr

Received: December 18, 2024 **Accepted:** June 13, 2025

Published Online Ahead of Print: June 19, 2025

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drug accumulation in CKD is a multifactorial phenomenon, influenced not only by reduced renal elimination but also by the intricate interactions between medications and uremic toxins (UTs).¹ In CKD, the decline in kidney function leads to the accumulation of compounds that are normally cleared by healthy kidneys. These compounds are known as UTs^{2,3} when they interfere with biologic or biochemical functions. Indeed, urea accumulation in CKD enhances the carbamylation of blood proteins, including albumin. Along with direct toxic effects on tissues, UTs can affect the pharmacokinetic or pharmacodynamic activity of certain drugs.^{1,4–8} It is important to bear in mind that many systemically distributed drugs bind to blood proteins (mainly albumin). The metabolic disturbances associated with CKD may alter drug distribution, decrease albumin drug binding, and thus increase the free (unbound) drug fraction.⁹ Note that the free fraction of a drug constitutes its active form, responsible for its biologic activity. We could hypothesize that the number of drug binding sites on albumin can be reduced by (1) hypoalbuminemia, (2) conformational changes in albumin induced by the carbamylation associated with urea accumulation, and (3) the accumulation of competing endogenous substances.

Oral anticoagulants, especially vitamin K antagonists (VKAs), are excellent candidates for testing this hypothesis. These medications aimed at preventing or treating cardiovascular complications are frequently prescribed to patients with CKD; however, VKA management is complicated by the narrow therapeutic window.^{10–13} VKAs are strongly bound to plasma proteins (primarily albumin), metabolized by the liver, and excreted in an inactive form in the stools and urine. As a class, oral anticoagulants are associated with a high incidence of adverse drug reactions.^{14,15} Achieving effective anticoagulation while minimizing the risk of bleeding is a delicate task, especially in patients with CKD taking VKAs. Furthermore, patients on VKA therapy with CKD are less frequently within the therapeutic range compared with patients without CKD.^{16,17} It is therefore essential to determine why these patients have difficulty in achieving optimal anticoagulation.

Hence, the effect of CKD-associated metabolic disturbances on drug distribution, effectiveness, and safety requires further evaluation. This study was designed to test the hypothesis (Figure 1) whereby a low kidney function in CKD is correlated—through protein carbamylation and the accumulation of UTs—with a high free (plasma) VKA concentration. The study's primary objective was to describe the association between free VKA concentrations and GFR. The secondary objective was to determine whether this association is mediated by CKD-related metabolic disturbances, such as protein carbamylation and the accumulation of protein-bound UTs (PBUTs).

Methods

The results of this study are reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines.¹⁸

Study Design and Participants

The DRUG-AVKTOX single-center, prospective, cross-sectional study was conducted at Amiens University Medical Center (Amiens, France). Between May 2021 and June 2023, we included adult patients (18 years or older) having been treated with VKA (fluidione or warfarin) for at least 5 days and for whom the international normalized ratio (INR) had been monitored at the medical center's hematology laboratory using Thromborel S thromboplastine (Siemens Healthcare Diagnostics, Marburg, Germany) according to manufacture protocol on CS-5100 analyzer (Siemens Healthcare Diagnostics, Erlangen, Germany). We excluded patients treated with a VKA for <5 days, those undergoing kidney transplantation, and those who did not consent to the research use of their personal medical data.

We sought to include 200 patients with an eGFR <40 ml/min per 1.73 m² or hemodialysis patients (because these patients typically have high UT concentrations^{19,20}) and 200 patients with an eGFR ≥40 ml/min per 1.73 m² (as these patients typically have lower UT concentrations). All consecutive patients meeting the inclusion criteria were included in one eGFR category or the other. The eGFR >40 ml/min per 1.73 m² group was the first to attain the recruitment target of 200 patients; after recruiting 224 patients in this group, only consecutive patients with eGFR ≤40 ml/min per 1.73 m² were included in the study. Each patient was included once only. Our selection of the 40 ml/min per 1.73 m² cutoff was based on our previous publications and other literature data on the accumulation of UTs.^{19,20} Indeed, we and others have demonstrated that an eGFR of 40 ml/min per 1.73 m² is the threshold above which UT concentrations begin to increase significantly.

In accordance with the French legislation on noninterventional studies of routine clinical practice, institutional review board approval was neither required nor sought. However, the study was registered with the French National Commission on Data Protection (Commission Nationale de l'Informatique et des Libertés [Paris, France]; registration number: PI2021_843_0223). The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Patients were given information about the study's objectives and procedures and were free to refuse to participate in the study. The French legislation prevents us from sharing the study dataset.

Study Objectives and End Points

The study's primary objective was to describe the association between free VKA concentrations and a low eGFR. The secondary objective was to determine whether this relationship was mediated by protein carbamylation and/or accumulation of PBUTs.

Collected Data

The detailed dataset collected for each patient included sociodemographic variables (age and sex), anthropometric variables (body mass index), clinical variables (a history of dyslipidemia, diabetes, or hypertension), the indication for the VKA, and laboratory test results (including the INR and the creatinine, calcium, phosphate, albumin, hemoglobin, and urea levels). The Modification of Diet in Renal

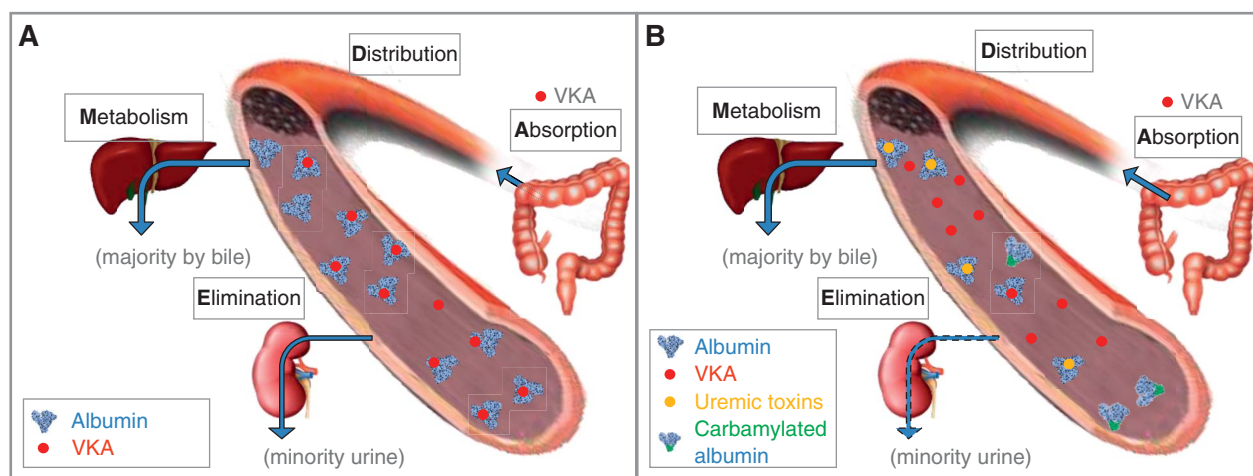


Figure 1. A schematic diagram of the working hypothesis. Low albuminemia, conformational changes in albumin (such as carbamylation), and competition between protein-associated UTs and VKA for albumin binding induce an elevation in the free VKA fraction. Working hypothesis in a person with (A) normal kidney function and (B) late-stage CKD. UT, uremic toxin; VKA, vitamin K antagonist.

Disease equation was used to estimate GFR. CKD was staged according to the Kidney Disease Improving Global Outcomes classification.

Patient Samples

The VKA treatment was monitored routinely in the hematology department by determination of the INR in plasma samples. The remaining volume of plasma was used for the measurement of UT and VKA levels and some other biochemical variables. Patient samples were stored at -20°C in the medical center's toxicology laboratory until the time of analysis. It should be noted that the VKA was administered the evening before the morning on which the blood sample was collected. For patients on hemodialysis, the sample was collected before the start of the dialysis session.

Measurement of the Free and Total Plasma Concentrations of VKAs

The free and total plasma concentrations of fluindione and warfarin were measured using liquid chromatography-tandem mass spectrometry (LCMS-8060, Shimadzu, Marne-la-Vallée, France) method.²¹ The ratio between free/total concentrations was then calculated. Total plasma ($50\ \mu\text{l}$) and plasma ultrafiltrate ($50\ \mu\text{l}$) were used to measure total and free concentrations, respectively. To deproteinize the samples, $100\ \mu\text{l}$ of iced acetonitrile solution containing a mixture of deuterated internal standards of fluindione and warfarin were added. After centrifugation, $30\ \mu\text{l}$ of the supernatant were transferred to vial inserts and diluted before injection into the chromatographic system. Chromatographic separation was achieved using a Raptor Biphenyl column ($2.7\ \mu\text{m}$, $100\times 2.1\ \text{mm}$) with a gradient of mobile phase B (methanol with 0.002% formic acid and 2 mM ammonium formate) and mobile phase A (ultrapure water with 0.002% formic acid and 2 mM ammonium formate) at a flow rate of 0.3 ml/min. The spectrometry acquisition was set to multiple reaction monitoring mode using negative electrospray ionization.

To note, fluindione is exclusively used in France as oral anticoagulant. This molecule shares similar pharmacodynamic and pharmacokinetic properties with warfarin including its elimination.

Measurement of the Plasma Concentrations of PBUTs

We used liquid chromatography-tandem mass spectrometry (3200 QTRAP, Sciex, Les Ulis, France) to determine the plasma levels of PBUTs, including paracresyl sulfate (PCS), indoxyl sulfate (IS), paracresyl glucuronide (PCG), hippuric acid (HA), indole acetic acid (IAA), and 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF).²² These particular PBUTs were selected because of (1) our hypothesis regarding potential interactions with albumin, (2) the availability of assays in our laboratory, and (3) strong evidence of toxicity in people with CKD. To extract these UTs, $200\ \mu\text{l}$ of an ice-cold acetonitrile solution containing the internal standards were added to $50\ \mu\text{l}$ of plasma. The compounds were separated using a pentafluorophenyl propyl column ($5\ \mu\text{m}$, $50\times 2.1\ \text{mm}$, Restek) with a gradient of acetonitrile and ultrapure water (both containing 0.1% formic acid) at a flow rate of 0.8 ml/min. Data were acquired through multiple reaction monitoring mode after negative-mode electrospray ionization (for CMPF, HA, IS, PCG, and PCS) or positive-mode electrospray ionization (for IAA). The plasma concentrations of urea were determined using the Atellica clinical chemistry Urea Nitrogen assay (Siemens).

Albumin Assays

Plasma albumin concentrations were measured using the ALTUR immunoturbidimetry assay (Diagam, Lille, France) on an Atellica clinical chemistry analyzer (Siemens). After albumin has reacted with a specific antibody, the resulting turbidity (measured at a wavelength of 340 nm) is directly proportional to the concentration of albumin in the sample. The results were evaluated using commercial quality controls at three concentration levels (Liquichek Immunology Control, BioRad, Marnes-la-Coquette, France), in

compliance with the International Organization for Standardization 15189 standard.

Determination of the Homocitrulline Concentration

Carbamylation is a nonenzymatic, irreversible, post-translational modification that occurs when a carbamoyl group is added to the functional groups of certain amino acids, peptides, or proteins. The carbamoyl group comes from a reaction with isocyanic acid, which is produced mainly by the decomposition of urea. The concentration of homocitrulline (an amino acid resulting from the carbamylation of lysine residue) is a marker of *in vivo* protein carbamylation. In our study, protein carbamylation was assessed by measuring total homocitrulline with the liquid chromatography-tandem mass spectrometry method described by Jaisson *et al.*²³ The results are expressed in μmol of homocitrulline per mole of lysine.

Statistical Analyses

The participants' baseline characteristics were described for the study group as a whole and by kidney function group (eGFR ≥ 40 versus < 40 ml/min per 1.73 m^2 or chronic hemodialysis). Data were expressed as the mean (SD), the median (interquartile range [IQR]), or the frequency (%). Wilcoxon–Mann–Whitney and chi-squared tests were used to compare groups.

We used a multivariable linear regression model to explore the association between kidney function (defined as a binary variable: eGFR ≥ 40 ml/min per 1.73 m^2 and eGFR < 40 ml/min per 1.73 m^2 or on chronic hemodialysis) and free VKA levels. Variables with $P \leq 0.05$ in a univariable analysis (age, serum albumin, and serum phosphate) were included in the multivariable linear model. Other variables (sex, hypertension, diabetes, body mass index, the hemoglobin level, the calcium level, the VKA indication, and concomitant drugs [Supplemental Table 1]) were not included in the multivariable model as they were not significantly associated with free VKA levels in univariable analyses. Several diagnostic plots and influence statistics were used to assess the fit of the model, including residuals versus fitted values, Q-Q plots, scale-Location plots, and Cook's distance plots. The result of the linear regression results was quoted as the coefficient (95% confidence interval [CI]).

We used two separate conventional mediation analyses^{24,25} (Baron and Kenny method) to determine whether or not the association between kidney function and free VKA levels was mediated by PBUTs or by protein carbamylation. A mediation analysis consists of three series of regressions: $X \rightarrow Y$, $X \rightarrow M$, and $X + M \rightarrow Y$, where X is the exposure, Y is the outcome, and M is the mediator. The mediation models used in this study were (1) $Y = \beta_0 + \beta_1 X + e$, (2) $M = \beta_0 + \beta_2 X + e$, and (3) $Y = \beta_0 + \beta_3 X + \beta_4 M + e$.

First, the multivariable linear regression exploring the association between kidney function and free VKA levels provided an estimate of the total effect of kidney function on free VKA levels (equation [1], yielding an adjusted coefficient β_1).

In the first mediation analysis, we sought to determine whether or not PBUTs mediated the association between kidney function and free VKA levels. The overall PBUT

level was the sum of the levels of the individual PBUTs, namely, PCS, IS, PCC, HA, IAA, and CMPF (in mmol/L). Multivariable linear regression modeling of the association between PBUT and kidney function (equation [2]) yielded an adjusted coefficient β_2 . If the association between the mediator (*i.e.*, PBUTs) and the exposure (*i.e.*, kidney function) was statistically significant, we used equation (3) to evaluate the indirect effect (obtaining a coefficient β_3 for kidney function and a coefficient β_4 for PBUTs). The total indirect effect was calculated as the product of β_2 and β_4 . Finally, we calculated the proportion mediated by PBUTs by dividing the total indirect effect ($\beta_2 \times \beta_4$) by the total effect (β_1).

In the second mediation analysis, we sought to determine whether or not protein carbamylation (as estimated from the homocitrulline level) mediated the association between kidney function and free VKA levels. The methodology used in the first mediation analysis was applied to this second analysis.

Continuous variables that were not normally distributed (*i.e.*, free VKA concentration, PBUTs, homocitrulline, phosphate, and urea) were log-transformed for use in the regression models. Bootstrap resampling ($n = 5000$) was used to compute 95% CIs for the linear regression coefficients. Spearman correlation coefficient was calculated as a guide to the strength of the association between the urea level and the homocitrulline level.

Missing data were handled through multiple imputation by chained equations (20 datasets), using all the patient variables listed in Table 1.²⁶ The data patterns suggested that the “data missing at random” assumption was plausible. Seventeen percent of the patients had at least one missing data item. The proportions of missing data for each variable are listed in Table 1. Fitted linear regression models were generated for each dataset, and pooled regression coefficients were obtained using Rubin rules. All statistical analyses were performed with R software.²⁷

Results

Patient Characteristics

Of the 389 patients included in the analysis (Supplemental Figure 1), 229 (59%) were men. The median age (IQR) was 75 (66–83), 157 (40%) participants had diabetes, and 276 (71%) had hypertension. Seventy patients were on chronic hemodialysis. The median (IQR) eGFR of nondialyzed patients was 63 (33–91) ml/min per 1.73 m^2 . Patients with an eGFR < 40 ml/min per 1.73 m^2 or on chronic hemodialysis ($n=165$) had higher levels of UTs (including urea and PBUTs) and were more likely to have comorbidities, when compared with patient with an eGFR ≥ 40 ml/min per 1.73 m^2 (Table 1).

Characteristics of the VKA Prescriptions

A total of 176 patients (45%) were being treated with warfarin, and 213 (55%) were being treated with flutidione. Patients with an eGFR < 40 ml/min per 1.73 m^2 or those on chronic hemodialysis were more likely to be treated with warfarin than treated with flutidione and were receiving lower dose of VKAs, relative to patients with an eGFR ≥ 40 ml/min per 1.73 m^2 (Table 2). The most common indications for VKA treatment were atrial

Table 1. Baseline characteristics of the study participants

Variables	Overall (N=389)	≥40 ml/min per 1.73 m ² (n=224)	<40 ml/min per 1.73 m ² or on Chronic Hemodialysis (n=165)	P Value	Imputed Data (N=389)
Age (yr)	75 (66–83)	74 (62–81)	76 (70–84)	0.01	—
Men	229 (59%)	134 (60%)	95 (58%)	0.73	—
Diabetes	157 (40%)	60 (27%)	97 (59%)	<0.001	—
Hypertension	276 (71%)	133 (59%)	143 (87%)	<0.001	—
Dyslipidemia	228 (59%)	115 (51%)	113 (68%)	0.001	—
BMI (kg/m ²)	27.7 (24.4–31.9)	27.3 (24.4–31.4)	28.4 (24.5–32.6)	0.15	6%
Kt/V	—	—	1.20 (0.28) ^a	—	—
Hemoglobin (g/dl)	12.0 (2.1)	12.6 (2.0)	11.1 (2.0)	<0.001	5%
Serum albumin (g/L)	30.3 (5.7)	31.6 (5.5)	28.5 (5.5)	<0.001	1%
Calcium (mmol/L)	2.31 (0.16)	2.34 (0.14)	2.27 (0.17)	<0.001	11%
Phosphate (mmol/L)	1.06 (0.89–1.28)	0.98 (0.82–1.13)	1.23 (1.02–1.59)	<0.001	12%
UTs					
Urea (mmol/L)	10.0 (5.9–16.0)	6.5 (4.9–9.2)	17.8 (12.4–25.3)	<0.001	1%
Homocitrulline (μmol/mole of lysine)	405 (270–633)	296 (228–429)	602 (462–897)	<0.001	—
PBUTs (mmol/L)	34.7 (17.1–86.4)	19.4 (13.0–34.6)	93.80 (48.30–214)	<0.001	—
HA	5.86 (2.73–15.50)	3.63 (1.80–5.86)	19.20 (7.81–55.80)	<0.001	—
PCS	7.33 (1.68–22.80)	3.59 (1.14–13.40)	14.70 (6.11–37.90)	<0.001	—
CMPE	3.66 (0.88–8.41)	2.05 (0.42–5.04)	6.24 (2.49–12.50)	<0.001	—
PCG	0.35 (0.35–1.08)	0.35 (0.35–0.37)	1.08 (0.38–4.29)	<0.001	—
IS	5.53 (3.18–15.7)	3.71 (2.03–5.04)	18.9 (8.3–52.1)	<0.001	—
IAA	2.73 (1.64–4.41)	1.94 (1.24–2.86)	4.42 (3.04–6.28)	<0.001	—
Drug prescription					
Allopurinol	26 (7%)	11 (5%)	15 (9%)	0.15	—
Amiodarone	62 (16%)	23 (10%)	39 (24%)	<0.001	—
Statins	186 (48%)	96 (43%)	90 (55%)	0.03	—
Acetylsalicylic acid	61 (16%)	26 (12%)	35 (21%)	0.01	—
Proton pump inhibitor	212 (54%)	111 (50%)	101 (61%)	0.03	—
Antibiotics	82 (21%)	36 (16%)	46 (28%)	0.007	—
Use of at least one strong CYP3A4 inhibitor ^b	68 (17%)	33 (15%)	35 (21%)	0.13	0.3%
Use of at least one strong CYP3A4 inducer ^b	4 (1%)	2 (1%)	2 (1%)	1	0.3%
Use of at least one strong CYP2C9 inhibitor ^b	63 (16%)	26 (12%)	37 (22%)	0.007	0.3%
Use of at least one strong CYP2C9 inducer ^b	4 (1%)	2 (1%)	2 (1%)	1	0.3%
Use of at least one drug with strong protein binding ^b	354 (91%)	195 (87%)	159 (96%)	0.004	0.3%

The results are expressed as the *n* (%), mean (SD), or median (interquartile range). BMI, body mass index; CMPE, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; HA, hippuric acid; IAA, indole acetic acid; IS, indoxyl sulfate; PBUT, protein-bound uremic toxin; PCG, paracresyl glucuronide; PCS, paracresyl sulfate; UT, uremic toxin.

^aOnly for dialysis patients with available data (*n*=49).

^bFor drug list, refer to Supplemental Table 1.

fibrillation (51%) and thrombosis (18%). The two groups differed regarding the indications; the proportion of patients with a prosthetic valve was higher in the group with eGFR ≥40 ml/min per 1.73 m² than in the other group. The median (IQR) INR was 2.7 (2.2–3.2); however, around one third of the patients had an INR >3, and 17% had an INR <2. The INR was not statistically different between the two groups.

The Association between VKA Concentrations and Kidney Function

Patients with an eGFR <40 ml/min per 1.73 m² or those on chronic hemodialysis had lower total levels of VKA, a higher free VKA concentration, and thus a higher free/total VKA ratio than those with an eGFR ≥40 ml/min per 1.73 m²; this was true for both warfarin and fluindione (Figures 2 and 3).

Table 2. Description of blood levels and indications and the international normalized ratio, as a function of the eGFR and hemodialysis status

Variables	Overall (n=389)	≥40 ml/min per 1.73 m ² (n=224)	<40 ml/min per 1.73 m ² or on Chronic Hemodialysis (n=165)	P Value	Imputed Data (N=389)
Types and blood levels of VKA					
Fluindione	213 (55%)	148 (66%)	65 (39%)	<0.001	
Total fluindione (ng/ml)	3290 (2400–5000)	3530 (2550–5040)	2770 (1950–4550)	0.02	
Free fluindione (ng/ml)	2.86 (2.05–4.03)	2.67 (1.95–3.78)	3.17 (2.34–5.43)	0.01	
Free/total fluindione ratio (×10 ⁻⁴)	8 (6–11)	8.00 (6.00–10.0)	11.0 (9.00–16.0)	<0.001	
Warfarin	176 (45%)	76 (34%)	100 (61%)	<0.001	
Total warfarin (ng/ml)	1080 (751–1500)	1280 (921–1580)	1010 (625–1320)	<0.001	
Free warfarin (ng/ml)	4.21 (2.91–5.97)	3.54 (2.60–5.23)	4.71 (3.38–6.93)	0.01	
Free/total warfarin ratio (×10 ⁻⁴)	39.0 (28.0–55.8)	29.5 (22.8–38.3)	47.0 (35.5–69.0)	<0.001	
Duration of VKA therapy (yr)	4.2 (0.6–10.8)	7.3 (1.9–12.8)	2.0 (0.2–5.9)	<0.001	
Missing	36 (9%)	26 (12%)	10 (6%)		
VKA indications					
Atrial fibrillation	53%	44%	65%	<0.001	3%
Valve prosthesis	17%	24%	7%		
Thrombosis	19%	17%	20%		
Antiphospholipid syndrome	5%	7%	4%		
Factor V Leiden mutation	2%	3%	2%		
Other	4%	5%	2%		
VKA daily dose (mg)					
Fluindione	15 (10–20)	15 (10–20)	10 (15–19)	0.08	8%
Warfarin	4 (3–6)	5 (3–7)	4 (3–5)	0.01	
INR					
<2	2.65 (2.17–3.18)	2.66 (2.18–3.18)	2.64 (2.14–3.17)	0.84	
2–3	67 (17%)	37 (17%)	30 (18%)		
3–4	192 (49%)	110 (49%)	82 (50%)		
>4	130 (33%)	77 (34%)	53 (32%)		

Results are expressed as the n (%), mean (SD), and median (interquartile range). INR, international normalized ratio; VKA, vitamin K antagonist.

In a univariable linear regression model, kidney function (*i.e.*, <40 ml/min per 1.73 m² or hemodialysis versus ≥40 ml/min per 1.73 m²) was associated with the free VKA concentration ($P < 0.001$). After adjustment in a multiple regression, the association persisted ($\beta_1=0.31$ [0.19 to 0.42], $P < 0.001$); this corresponded to the total effect of kidney function on levels of free VKA (model [a], Tables 3 and 4).

The Mediation Analysis with PBUTs

We first evaluated the association between PBUT levels and kidney function (Supplemental Figure 2). In a linear regression model, kidney function was significantly associated with PBUT levels after adjusting for age, sex, hypertension, diabetes, the phosphate level, and the urea level ($\beta_2=1.12$ [0.88 to 1.36], $P < 0.001$; Supplemental Table 2).

After the addition of the PBUT variable to the model of the total effect of kidney function on free VKA levels, the coefficient for kidney function remained significant ($\beta_3=0.31$ [0.16 to 0.46], $P < 0.001$) but the coefficient for PBUTs was weak and nonsignificant ($\beta_4=0.001$ [-0.06 to 0.06], $P = 0.98$; Table 3).

The Mediation Analysis with Homocitrulline

Our results indicated that the urea and homocitrulline levels were correlated ($r=0.73$ [$P < 0.001$], Supplemental Figure 3). In a linear regression model, kidney function was significantly associated with homocitrulline levels after adjusting for age, hypertension, diabetes, serum albumin, hemoglobin, and phosphate levels ($\beta_2=0.54$ [0.43 to 0.64], $P < 0.001$; Supplemental Table 3).

After the addition of the variable homocitrulline to the model of the total effect of kidney function on free VKA levels, the coefficient for kidney function fell but remained statistically significant ($\beta_3=0.24$ [0.11 to 0.37], $P < 0.001$). The coefficient for homocitrulline was ($\beta_4=0.12$ [0.004 to 0.23], $P = 0.04$; Table 4).

According to our analysis, 21% (95% CI, 1% to 35%) of the association between kidney function and free VKA levels was mediated by homocitrulline.

Discussion

In this cross-sectional study of patients treated with a VKA and at various renal function levels, we observed an independent association between kidney function and the free VKA concentration. The latter concentration was

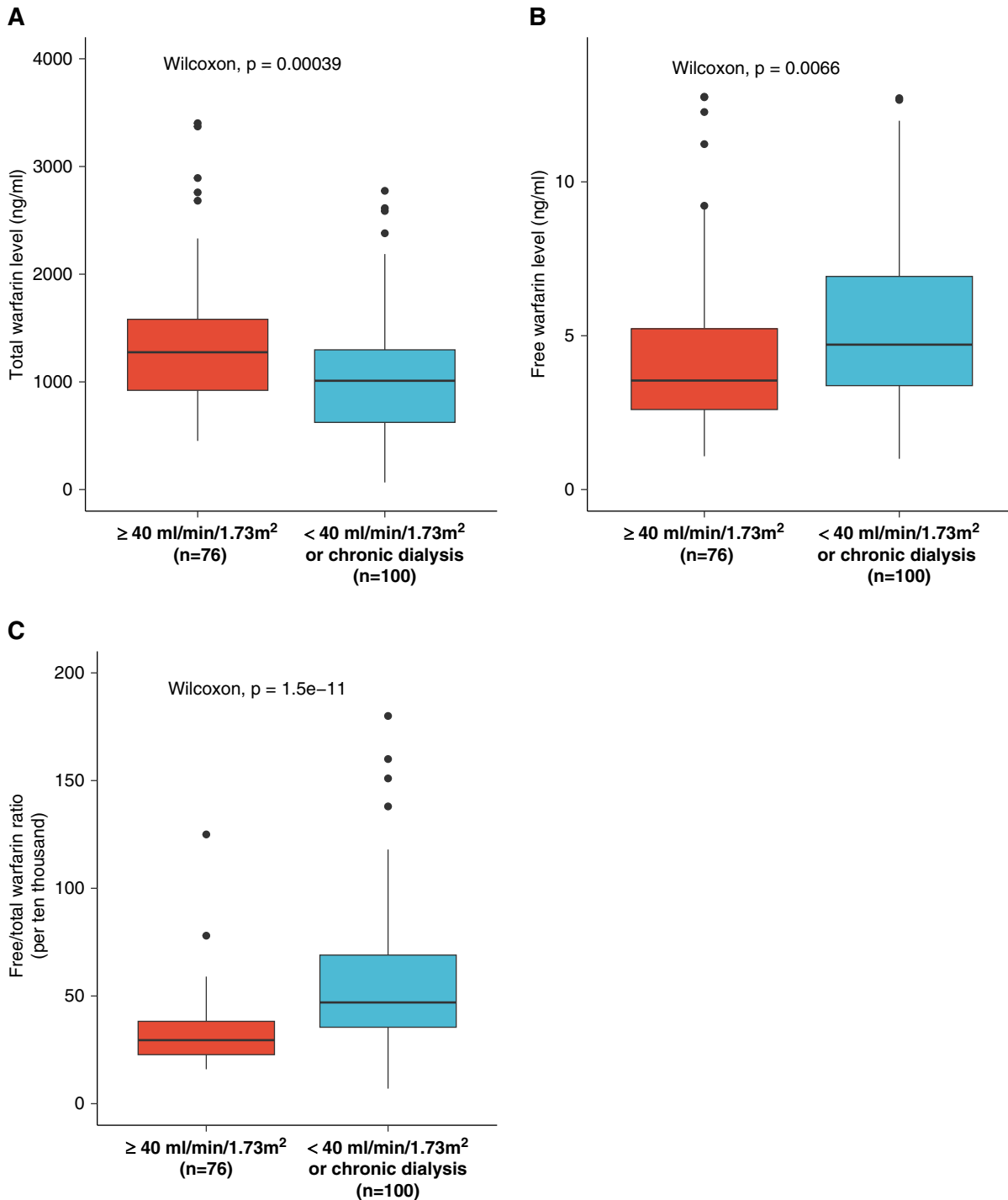


Figure 2. Boxplot of warfarin as a function of the eGFR and dialysis status. (A) Total warfarin level, (B) free warfarin level, and (C) free/total warfarin ratio.

higher in patients with a lower eGFR. Our mediation analysis revealed that the association between the eGFR and the free VKA concentration was mediated in part by protein carbamylation.

It is generally accepted that only the free (unbound) fraction of a drug exerts a pharmacologic action.²⁸ For drugs that bind strongly to albumin, the free fraction is small but can be influenced significantly by changes in

albumin concentration, conformational changes in the albumin molecule, or competitive ligand-ligand interactions²⁹; *e.g.*, a decrease in the bound fraction from 98% to 96% would double the pharmacologically active, unbound fraction.³⁰ Evaluating free drug concentrations can be particularly useful for drugs with a narrow therapeutic index and in certain patient populations, such as those with CKD. In this study, we evaluated free VKA

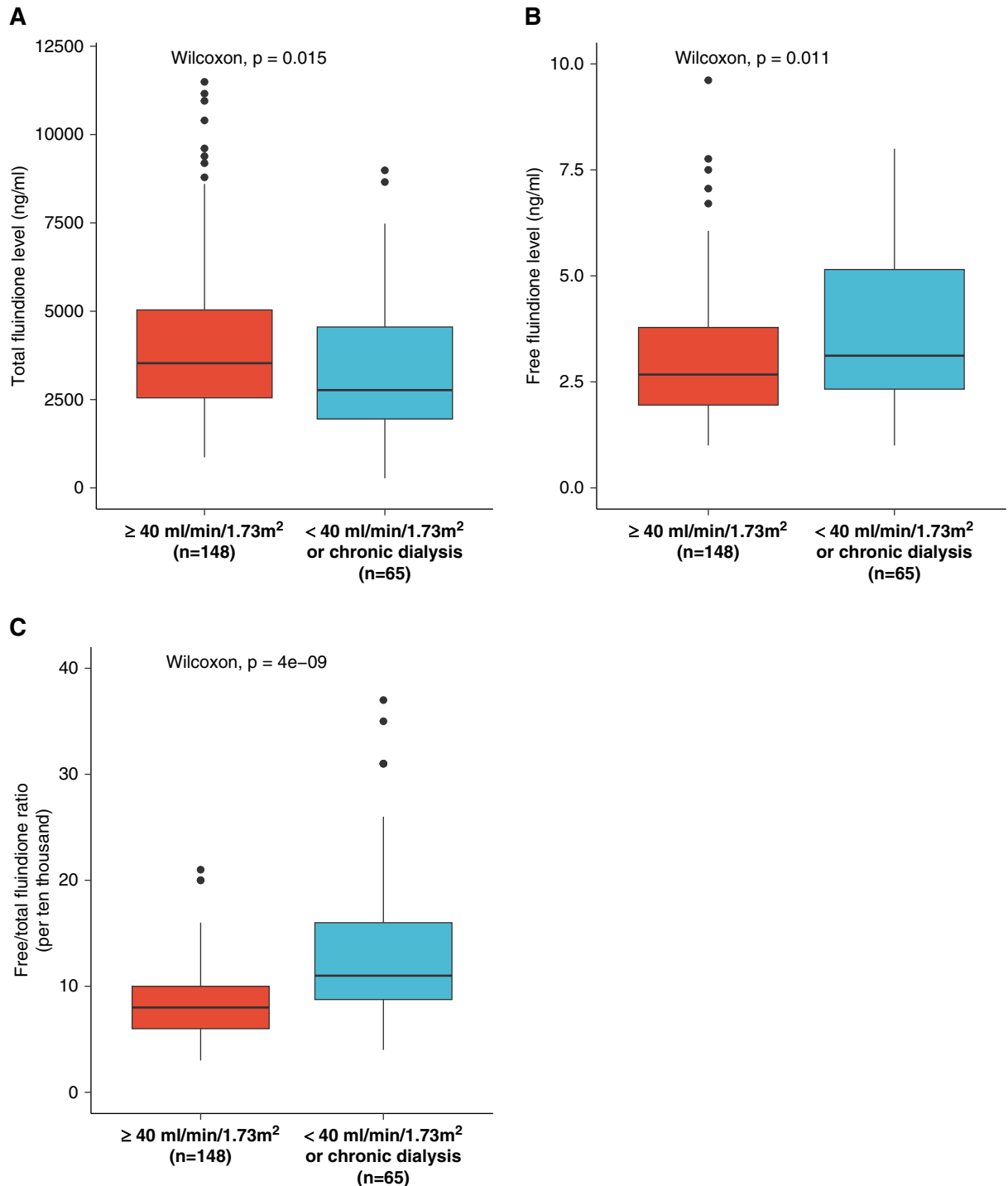


Figure 3. Boxplot of fluidione as a function of the eGFR and dialysis status. (A) Total fluidione level, (B) free fluidione level, and (C) free/total fluidione ratio.

concentrations. VKAs are excellent candidates for this purpose because 97% of the total drug is bound to albumin. Although the use of this type of anticoagulant is common in patients with CKD (due to the high prevalence of cardiovascular disease in general and atrial fibrillation in particular), VKA management is complicated by the narrow therapeutic index. VKAs are metabolized by the liver,

and the inactive metabolites are excreted in the stools and urine. As mentioned above, VKAs bind strongly to plasma proteins (albumin, primarily). Most importantly, the VKAs belong to the drug class that has the greatest incidence of adverse drug reactions, the most prominent of which are hemorrhagic events.³¹ The bleeding risk associated with VKAs increases as kidney function declines.^{15,32–34} Here,

Table 3. Association between the free vitamin K antagonist concentration and kidney function, as potentially mediated by protein-bound uremic toxins

Variables	Crude Analysis		Adjusted Analysis (a)		Adjusted Analysis (c)	
	β (95% CI)	P Value	β (95% CI)	P Value	β (95% CI)	P Value
eGFR (ml/min per 1.73 m²) and hemodialysis status						
≥ 40	Ref		Ref		Ref	
<40 or chronic hemodialysis	0.30 (0.19 to 0.41)	<0.001	0.31 (0.19 to 0.42)	<0.001	0.30 (0.16 to 0.43)	<0.001
Age (per 5-yr increment)	-0.04 (-0.06 to -0.02)	<0.001	-0.03 (-0.05 to -0.02)	<0.001	-0.03 (-0.05 to -0.02)	<0.001
Serum albumin (per g/L)	0.01 (0.002 to 0.02)	0.02	0.02 (0.008 to 0.03)	<0.001	0.02 (0.008 to 0.03)	0.003
Phosphate (per mmol/L) ^a	0.40 (0.24 to 0.56)	<0.001	0.25 (0.08 to 0.42)	0.004	0.24 (0.08 to 0.42)	0.005
PBUTs (per mmol/L) ^a	0.10 (0.05 to 0.15)	<0.001			0.001 (-0.06 to 0.06)	0.98

The variable free vitamin K antagonist concentration was log-transformed due to its non-normal distribution. The association between kidney function and free vitamin K antagonist levels was not mediated by protein-bound uremic toxins, as no change was observed in the coefficient for kidney function when the protein-bound uremic toxin variable was added. Protein-bound uremic toxin was not significantly associated with free vitamin K antagonist levels. CI, confidence interval; PBUT, protein-bound uremic toxin.

^alog(var) was used in the models.

we found that despite having a lower total VKA concentration (due to a reduction in drug dosage), patients with poor kidney function exhibited a higher free VKA concentration. Hence, patients with a low eGFR had low VKA daily dose and high VKA concentrations; this suggests that exposure to lower daily doses is sufficient for anticoagulation. However, specific clinical studies are needed to confirm this hypothesis. The association between free VKA concentration and kidney function was independent of confounders such as age and serum albumin. To the best of our knowledge, the present clinical study is the first to have evaluated this relationship. *In vitro* studies of the sera of patients at different CKD stages found a progressive loss of the albumin binding capacity as the CKD worsened.⁹ In

contrast to the free VKA concentrations, the INR did not seem to be correlated with kidney function. However, the value of the free VKA concentration as a predictor of bleeding events will need to be evaluated.

We also sought to determine how the metabolic disturbances associated with CKD (*i.e.*, the accumulation of urea through protein carbamylation and PBUTs) might alter drug distribution and increase the free VKA fraction. Protein carbamylation is a nonenzymatic, irreversible, post-translational modification that is exacerbated by the accumulation of urea in CKD.³⁵ In an *in vitro* study, Erill *et al.* demonstrated that salicylate protein binding decreased when protein carbamylation was induced.³⁶ In this study, we measured levels of homocitrulline as a

Table 4. Association between free vitamin K antagonist concentration and kidney function, as mediated by homocitrulline

Variables	Crude Analysis		Adjusted Analysis (a)		Adjusted Analysis (c)	
	β (95% CI)	P Value	β (95% CI)	P Value	β (95% CI)	P Value
eGFR (ml/min per 1.73 m²) and dialysis status						
≥ 40	Ref		Ref		Ref	
<40 or chronic hemodialysis	0.30 (0.19 to 0.41)	<0.001	0.31 (0.19 to 0.42)	<0.001	0.24 (0.11 to 0.37)	<0.001
Age (per 5-yr increment)	-0.04 (-0.06 to -0.02)	<0.001	-0.03 (-0.05 to -0.02)	<0.001	-0.04 (-0.06 to -0.02)	<0.001
Serum albumin (per g/L)	0.01 (0.002 to 0.02)	0.02	0.02 (0.008 to 0.03)	<0.001	0.02 (0.009 to 0.03)	<0.001
Phosphate (per mmol/L) ^a	0.40 (0.24 to 0.56)	<0.001	0.25 (0.08 to 0.42)	0.004	0.22 (0.05 to 0.39)	0.01
Homocitrulline (per μ mol/mole of lysine) ^a	0.14 (0.05 to 0.23)	0.003			0.12 (0.004 to 0.23)	0.04

The variable free vitamin K antagonist concentration was log-transformed due to its non-normal distribution. The association between kidney function and free vitamin K antagonist levels appeared to be mediated partly by homocitrulline, as the coefficient for kidney function decreased after the addition of the homocitrulline level, which was significantly associated with free vitamin K antagonist levels. The estimated mediated proportion was 20% (95% confidence interval, 0.7% to 34%). CI, confidence interval.

^alog(var) was used in the models.

carbamylation-derived product that reflects the average carbamylation rate for blood proteins. The homocitrulline concentration is known to be correlated with the level of carbamylated albumin.³⁷ In a recent epidemiologic study, carbamylated albumin and homocitrulline had similar prognostic values.³⁸ Here, we observed a strong correlation between urea and homocitrulline levels and found that homocitrulline mediated 21% of the association between kidney function and free VKA concentrations. This suggests that the conformational changes in albumin induced by carbamylation may alter its binding capacity to VKAs, which could partially explain the observed link between a low eGFR and a high free VKA concentration. By contrast, the accumulated PBUTs did not appear to mediate this association—particularly once the analysis was adjusted for several confounders. Indeed, the association between the progressive loss of albumin binding capacity and the accumulation of PBUTs has only been demonstrated *in vitro* with various drugs, including phenytoin, phenobarbital,³⁵ and aripiprazole.³⁶ Therefore, factors other than those studied here might explain the observed association between free VKA concentrations and the eGFR. Indeed, a low albumin concentration influences albumin-ligand binding. Hypoalbuminemia is frequent in patients with CKD and results from the complex interplay between several factors, including malnutrition and inflammation. However, all of our statistical models were adjusted for the serum albumin level.

Direct oral anticoagulants (another drug class that directly inhibits coagulation factors) are increasingly prescribed for cardioembolic prophylaxis in patients with nonvalvular atrial fibrillation. However, VKAs are still indicated in patients with valvular disease or impaired renal function. In Europe, direct oral anticoagulants are not approved for use in patients on dialysis. Although the free VKA assay is not readily available in most centers and might be difficult to implement in routine practice, it might be valuable for studying patients with difficult-to-stabilize anticoagulation and thus for better understanding interactions with VKAs. In addition to the implications for anticoagulation in patients with impaired renal function, our results demonstrate that the metabolic disturbances associated with CKD (protein carbamylation, specifically) may be associated with an elevation in the free drug fraction.

This study had several strengths. First, we use validated chromatography methods to measure levels of PBUTs, homocitrulline, and free VKAs. Second, we explored an area that remains largely unexamined *in vivo*; most studies of protein binding in CKD have been conducted *in vitro*. Our study also had several limitations. First, our observational study was not designed to evaluate the potential effect of elevated VKA free concentrations on the bleeding risk. Second, we evaluated only one of the several pharmacokinetic steps that might conceivably be affected by CKD-associated metabolic disturbances. UTs might influence the metabolism by acting on cytochromes. Third, we did not have data on the diet and the microbiota in our cohort; these variables might modulate UT concentrations. Finally, the present results do not include patients undergoing peritoneal dialysis, as none were treated with

this modality at the inclusion center. Consequently, the findings may not be generalizable to this population, highlighting the need for further investigation.

In conclusion, the results of our cross-sectional clinical study showed that a low eGFR was associated with an elevation of the free VKA concentration. This association was independent of albumin levels and appeared to be mediated in part by protein carbamylation. Future research should assess the effect of the elevated free VKA fraction on patients with CKD determining whether the direct measurement of free VKA levels can improve patient monitoring and avoid the occurrence of bleeding events. In addition, these results regarding the potential accumulation of the free fraction of drugs in patients with low eGFR are applicable to other drugs that are strongly bound to plasma proteins. This emphasizes the need for caution regarding the safety of these medications.

Disclosures

Disclosure forms, as provided by each author, are available with the online version of the article at <http://links.lww.com/KN9/B138>.

Funding

Amiens University Medical Center and Santély.

Author Contributions

Conceptualization: Sandra Bodeau, Gabriel Choukroun, Solène M. Laville, Sophie Liabeuf.

Data curation: Sandra Bodeau, Julien Demagny, Touria Mernissi, Anaïs Okwieka, Pierre Spicher.

Formal analysis: Sandra Bodeau, Julien Demagny, Stéphane Jaisson, Solène M. Laville, Sophie Liabeuf, Anaïs Okwieka.

Funding acquisition: Sophie Liabeuf.

Investigation: Gabriel Choukroun, Sophie Liabeuf.

Methodology: Stéphane Jaisson, Sophie Liabeuf.

Project administration: Sophie Liabeuf.

Supervision: Solène M. Laville, Sophie Liabeuf.

Validation: Sandra Bodeau, Solène M. Laville, Sophie Liabeuf.

Writing – original draft: Sophie Liabeuf.

Writing – review & editing: Sandra Bodeau, Gabriel Choukroun, Julien Demagny, Stéphane Jaisson, Said Kamel, Solène M. Laville, Sophie Liabeuf, Touria Mernissi, Pierre Spicher.

Data Availability Statements

Partial restrictions to the data and/or materials apply. The deidentified or aggregated data that support the findings of this study are available from the corresponding author upon reasonable request, and are subject to institutional and ethical restrictions.

Supplemental Material

This article contains the following supplemental material online at <http://links.lww.com/KN9/B139>.

Supplemental Figure 1. Study flow chart.

Supplemental Figure 2. PBUT levels, by CKD stage.

Supplemental Figure 3. Relationship between the urea level and the homocitrulline level (both log-transformed).

Supplemental Table 1. List of drugs described as CYP3A4 inhibitors, CYP3A4 inducers, CYP2C9 inhibitors, CYP2C9 inducers, drugs with strong protein binding.

Supplemental Table 2. Factors associated with PBUTs.

Supplemental Table 3. Factors associated with homocitrulline.

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AFFILIATIONS

¹MP3CV Laboratory, Jules Verne University of Picardie, Amiens, France

²Toxicology Unit, Department of Clinical Pharmacology, Amiens-Picardie University Medical Center, Amiens, France

³MEDyC Unit, CNRS, Reims University Medical Center (Department of Biochemistry-Pharmacology-Toxicology), University of Reims Champagne-Ardenne, Reims, France

⁴Department of Hematology, Amiens-Picardie University Medical Center, Amiens, France

⁵Department of Nephrology, Dialysis and Transplantation, Amiens-Picardie University Medical Center, Amiens, France

⁶Pharmacoepidemiology Unit, Department of Clinical Pharmacology, Amiens-Picardie University Medical Center, Amiens, France