



Carbamylation Is Instrumental in End-Stage Kidney Disease Coagulopathies: The Impact on von Willebrand Factor and Platelet Functionality

Janka Babickova^{1,*} Urszula Kałucka^{1,*} Alicja Sochaj-Grzegorzczuk² Jan Potempa²
Carsten Scavenius³ Thomas Knoop^{4,5} Hans-Peter Marti^{4,5} Marta Kaminska^{1,*} Piotr Mydel^{1,2,*}

¹ Broegelmann Research Laboratory, Department of Clinical Science, Faculty of Medicine, University of Bergen, Bergen, Norway

² Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

³ Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark

⁴ Department of Medicine, Haukeland University Hospital, Bergen, Norway

⁵ Department of Clinical Medicine, Faculty of Medicine, University of Bergen, Bergen, Norway

Address for correspondence Marta Kaminska, PhD, Broegelmann Research Laboratory, Department of Clinical Science, Faculty of Medicine, University of Bergen, Jonas Lies vei 91B, 5021 Bergen, Norway (e-mail: marta.kaminska@uib.no).

Thromb Haemost

Abstract

Background Chronic kidney disease (CKD) is a progressive, irreversible, and incurable condition characterized by high morbidity and mortality, affecting approximately one-tenth of the global population. Rise of urea-derived cyanate levels in CKD patients, severalfold higher in comparison to those found in healthy individuals, leads to an increased rate of carbamylation of lysine residues of proteins and peptides. This posttranslational modification plays an important role in the progression of kidney failure but also in the onset of CKD-related complications, including previously reported coagulopathies. In this study, we have explored the impact of carbamylation on the functionality of von Willebrand factor (vWF), a pivotal player in hemostasis, and its implications for platelet adhesion.

Materials and Methods We have explored carbamylated vWF's interactions with its partner proteins via ELISA. Mass spectrometry was employed to identify modified lysine residues. Blood platelets isolated from healthy donors were carbamylated, and their activation, binding to endothelium and thromboxane release were evaluated using flow cytometry, adhesion assays and ELISA, respectively.

Results Using mass spectrometry we detected the vWF's lysine residue most susceptible to carbamylation. This modification has in turn affected vWF's interactions with its key binding partners: decreased binding to collagen types I/III but increased the affinity to factor FVIII, while its binding to fibrinogen remained unchanged. Carbamylation of vWF impeded vWF-blood platelet binding, but carbamylation of platelets led to their increased thrombin-dependent activation as observed by enhanced phosphatidylserine exposure,

Keywords

- ▶ carbamylation
- ▶ von Willebrand factor
- ▶ coagulation
- ▶ platelet
- ▶ chronic kidney disease

* These authors contributed equally.

received

May 14, 2024

accepted after revision

June 19, 2024

accepted manuscript online

July 25, 2024

DOI <https://doi.org/10.1055/a-2373-3792>

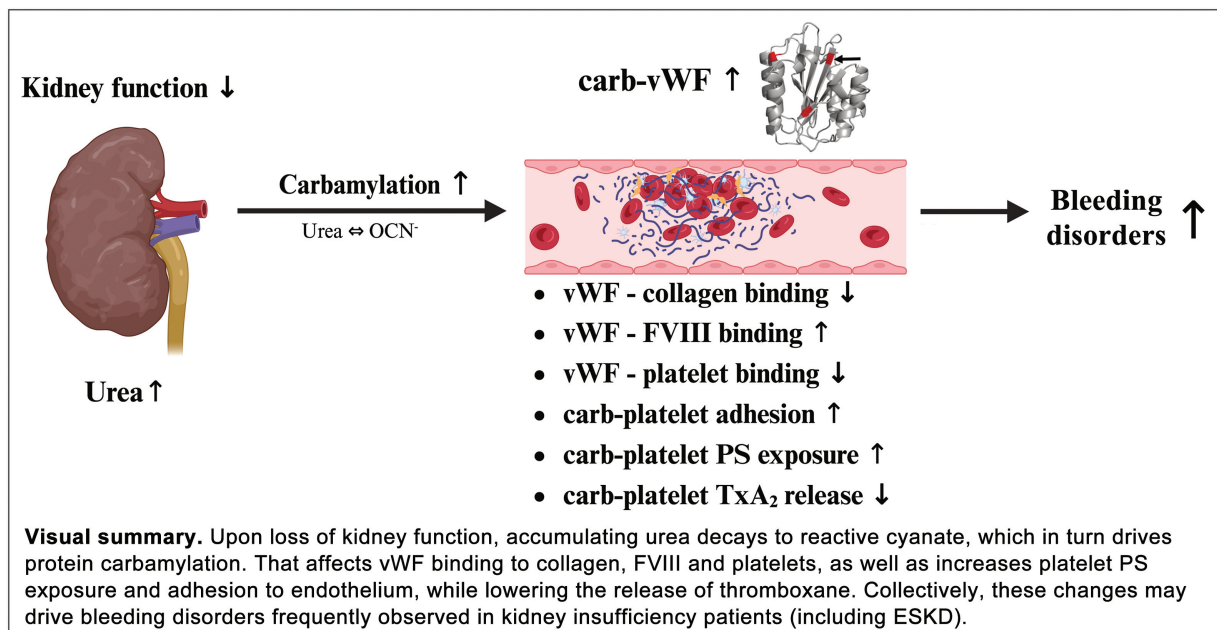
10.1055/a-2373-3792.

ISSN 0340-6245.

© 2024. The Author(s).

This is an open access article published by Thieme under the terms of the Creative Commons Attribution-NonDerivative-NonCommercial-License, permitting copying and reproduction so long as the original work is given appropriate credit. Contents may not be used for commercial purposes, or adapted, remixed, transformed or built upon. (<https://creativecommons.org/licenses/by-nc-nd/4.0/>)

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany



improved their binding to vascular endothelium, at the same time decreasing the production of the prothrombotic mediator, thromboxane A₂.

Conclusion Our findings highlight the multifaceted impact of carbamylation on vWF and platelets, disturbing the delicate balance of coagulation cascade. These alterations could contribute to the complex hemostatic imbalance in ESKD, underscoring the need for further research to fully understand these mechanisms and their clinical implications.

Introduction

Chronic kidney disease (CKD), a complex condition characterized by high morbidity and mortality with no definitive cure, affects approximately one-tenth of the global population.¹ The interplay between the kidney and circulating metabolites is intricate. These metabolites play pivotal roles in the kidney, acting as ligands for specific receptors, substrates for posttranslational modifications (PTMs), or as inflammatory chemoattractants.² Thus, circulating metabolite alterations in CKD are a complex tally of impaired filtration, absorption, and secretion, acting as functional mediators of disease. The reduction of glomerular filtration rate during CKD results in the accumulation of many protein-bound and water-soluble metabolites in plasma, referred to as uremic solutes that can exert devastating effects on almost all organ systems. The improvement of uremic thrombocytopathies by modern hemodialysis techniques indicates that uremic toxins are, at least partially, responsible for the observed platelet dysfunctions,³ e.g., phenolic acids and guanidinosuccinic acid inhibited platelet aggregation, while urea or creatinine did not.^{4,5} Urea, quantitatively the most abundant retention solute in the body, is in equilibrium with the reactive product of its decay, cyanate. Cyanate ions react with amino groups of protein N-terminus and lysine residues, thus converting them to noncharged carbamyl residues

in a process known as carbamylation.⁶ This irreversible nonenzymatic PTM of proteins and peptides can lead to their structural and functional changes. In fact, several carbamylated proteins have been reported to exhibit reduced functional capacity and higher immunogenicity, such as fibrinopeptide B, serum albumin, erythropoietin, matrix metalloprotease-2, and others.⁷⁻⁹

In CKD, cyanate levels can rise severalfold to those found in healthy individuals. This elevation leads to an increased production of carbamylated compounds, which not only play a role in the progression of kidney failure but also in the onset of CKD-related complications. Due to their strong association with cardiovascular diseases and overall mortality, these carbamylated compounds have garnered clinical interest as potential biomarkers. Carbamylated proteins can accumulate across various tissues, including the skin, heart, liver, and kidneys.¹⁰⁻¹² A significant consequence of raised serum cyanate is its detrimental effect on cell-surface proteins. For instance, carbamylation of erythrocyte membrane proteins in patients with uremia has been linked to decreased membrane stability and the premature breakdown of erythrocytes.^{13,14}

Coagulopathies represent a significant complication associated with renal insufficiency, affecting nearly half of all patients with end-stage kidney disease (ESKD).^{15,16} Our previous research demonstrated that the exposure of

platelets to cyanate led to structural changes in integrin $\alpha\text{IIb}\beta\text{3}$, a critical fibrinogen-binding protein, resulting in a fibrinogen-binding defect that impairs the aggregation of uremic platelets.¹⁷ Furthermore, the carbamylation of fibrinogen itself influences the kinetics of fibrin clot formation, affecting the clot's structure and degradation. These changes are thought to contribute significantly to the disrupted hemostasis observed in ESKD patients.¹⁸

von Willebrand factor (vWF), an important ligand of $\alpha\text{IIb}\beta\text{3}$, is one of the largest glycoproteins (GPs) circulating in plasma and plays a pivotal role in hemostasis. In ESKD patients, the reported plasma levels of vWF were either higher than in healthy volunteers or within a normal range, suggesting that the vWF levels do not contribute to uremic diathesis.^{19,20} Interestingly, vWF binding to $\alpha\text{IIb}\beta\text{3}$ was decreased, while binding to GPIb receptor was not altered in ESKD patients.²¹ A reduced protein tethering and platelet adhesion to subendothelial surfaces has been reported, while binding to collagen was normal^{22,23} and the concentration of the vWF:FVIII complex was higher in the ESKD group than in healthy volunteers.²⁴

The impact of carbamylation on vWF functionality and its implications for platelet adhesion remain largely unexplored. This study aims to elucidate whether carbamylation of vWF establishes a mechanistic connection between uremia and coagulation dysfunction in patients with ESKD. Our investigation centers on the susceptibility of vWF to carbamylation, its influence on protein-protein interactions, and subsequent effects on platelet adhesion. Additionally, using cell models we have assessed the direct impact of uremia and carbamylation on platelet activation and their adherence to the endothelium.

Materials and Methods

Study Subjects

Blood from anonymous healthy volunteers was collected at the local blood bank (Department of Immunology and Transfusion Medicine, Haukeland University Hospital, Bergen, Norway) using acid citrate dextrose collection tubes (Greiner Bio-One, Monroe, North Carolina, United States).

Platelet Isolation and Quantification

The collected blood was centrifuged for 20 minutes at 200 g, and platelet-rich plasma was collected, diluted with an equal volume of HEP buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 5 mM EDTA, pH 7.4), and centrifuged for 20 minutes at 100 g to remove red and white blood cells. The supernatant was then centrifuged for additional 20 minutes at 300 g. The platelet-containing pellet was resuspended in 2 mL of platelet buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM Na_2HPO_4 , 6 mM glucose, pH 7.4). All centrifugations were performed at room temperature (RT). For platelet quantification, the density was measured at OD_{800} and platelet number was calculated as described by Tamang et al.²⁵ Platelets were then diluted to a concentration of 2×10^8 /mL, allowed to rest for an hour at RT, and then used in subsequent experiments.

Platelet Carbamylation

Platelets were carbamylated in 1, 5, or 10 mM KCNO (Merck) for 30 minutes at 37°C. Samples were then centrifuged for 5 minutes at 300 g, resuspended in binding buffer (140 mM NaCl, 2.5 mM CaCl_2 , 10 mM HEPES), and allowed to rest for 30 minutes at RT.

Platelet Activation

Samples were incubated with either 0.4 U/mL human thrombin (Merck), 50 ng/mL convulxin (Cayman Chemical, Ann Arbor, Michigan, United States), 25 mM TRAP-6 (Abcam, Cambridge, United Kingdom), or 25 mM of AY-NH₂ (Tocris Bioscience, Bristol, United Kingdom) in binding buffer for 20 minutes at 37°C.

Tandem Mass Spectrometry

vWF (Haematologic Technologies Inc., Essex Junction, Vermont, United States) was carbamylated in 1 or 10 mM KCNO in phosphate-buffered saline (PBS) for 2, 6, and 16 hours at 37°C. Samples were then deglycosylated with PGNase F (Promega, Madison, Wisconsin, United States) and separated via SDS-PAGE. Protein bands were visualized using Coomassie Blue, bands corresponding to vWF were excised, submerged in HPLC-grade water, and further processed at the Proteomics and Mass Spectrometry Core Facility of Małopolska Centre of Biotechnology, Krakow, Poland.

Samples were destained, reduced with 50 mM dithiothreitol for 45 minutes at 37°C, and alkylated with iodoacetamide before digesting with 6.15 ng/ μL chymotrypsin solution overnight (16 hours) at 25°C. Peptides were extracted with 100% acetonitrile (can) by sonication and subsequently lyophilized. Samples were resuspended in 2% ACN and analyzed with a Q-Exactive mass spectrometer coupled with a nanoHPLC UltiMate 3000 RSLCnano System (ThermoFisher Scientific, Waltham, Massachusetts, United States) using an Acclaim PepMap 100 C18 (75 $\mu\text{m} \times 20$ mm, 3 μm particle, 100 Å pore size) trap column (ThermoFisher Scientific) in 2% ACN with 0.05% trifluoroacetic acid at a flow rate of 5 $\mu\text{L}/\text{min}$. Samples were separated on Acclaim PepMap RSLC C18 (75 $\mu\text{m} \times 500$ mm, 2 μm particle, 100 Å pore size) analytical column (ThermoFisher Scientific) at 50°C in a 60 minute 2–40% ACN gradient in 0.05% formic acid at a flow rate of 250 nL/min. The eluted peptides were ionized using a Digital PicoView 550 nanospray source (New Objective, Littleton, Massachusetts, United States). The Q-Exactive was operated in a data-dependent mode using top eight method with 35 seconds of dynamic exclusion. Full-scan mass spectrometry (MS) spectra were acquired with a resolution of 70,000 at m/z 200 with an automatic gain control (AGC) target of 1e6. The MS/MS spectra were acquired with a resolution of 35,000 at m/z 200 with an AGC target of 3e6. The maximum ion accumulation times for the full MS and the MS/MS scans were 120 and 110 milliseconds, respectively. Raw files acquired by the MS system were processed using the Proteome Discoverer platform (v.1.4, ThermoFisher Scientific). The obtained peak lists were searched using an inhouse MASCOT server (v.2.5.1, Matrix Science, London, United Kingdom) against the cRAP database (<https://www>.

thegpm.org/crap/, released August 2019) with manually added sequences of the protein of interest. During search, up to five missed chymotrypsin cleavages were allowed. Peptide mass tolerance and fragment mass tolerance were 10 ppm and 20 mmu, respectively. Carbamidomethyl (C) was set as a fixed modification and oxidation (M), deamidated (NQ), carbamyl (K) as variable modifications.

von Willebrand Factor Adhesion to Collagen, Factor VIII, and Fibrinogen

A 96-well microplate was coated with collagen (30 µg/mL in PBS; MP Biomedicals, Santa Ana, California, United States), factor VIII (FVIII; 10 µg/mL in PBS) for 24 hours at RT, or fibrinogen (50 µg/mL in PBS, Merck) for 2 hours at 4°C, washed twice with PBS and carbamylated with 1 mM KCNO (Merck) for 24 hours at 37°C. Wells were washed with PBS (2×), blocked with 200 µL 5% BSA in 1× PBS for 2 hours at 37°C, and then washed with 200 µL PBS 0.05% Tween-20 (3×). Next, 50 µL of 5 µg/mL of human vWF (Merck) in PBS 0.01% Tween-20 was added and plates were incubated for 2 hours at RT. After washing with 200 µL of PBS 0.05% Tween-20 (3×), wells were incubated with 100 µL of HRP-conjugated rabbit polyclonal anti-human vWF (1:20,000 in 2.5% BSA, 0.01% Tween-20; P0226, Dako) for 1 hour at RT and then washed with 200 µL of PBS 0.05% Tween-20 (3×). The reaction was then started by adding 100 µL of TMB substrate (BD Biosciences, Franklin Lakes, New Jersey, United States), and stopped within 30 minutes by adding 50 µL 2N H₂SO₄. Absorbance was measured at 450 nm (reference wavelength 570 nm) via SpectraMax iD5 microplate reader (Molecular Devices, San Jose, California, United States).

Thromboxane A₂ Release

After activation, the 5×10^7 platelets were centrifuged for 10 minutes, 10,000 g at 4°C to obtain the supernatant. The concentration of thromboxane B₂ (TxB₂), stable product of nonenzymatic hydration of Thromboxane A₂ (TxA₂), was measured using ELISA (R&D Systems, Minneapolis, Minnesota, United States) following the manufacturer's instructions. Results were normalized to TxB₂ released by noncarbamylated controls.

Platelet Exposure of Phosphatidylserine

After carbamylation and activation, 5×10^7 platelets were then stained with FITC-Annexin V (ThermoFisher Scientific) for 10 minutes at RT, diluted with 200 µL binding buffer, and analyzed by flow cytometry using BD LSRFortessa (BD Biosciences). The percentage of positively stained platelets was calculated using FlowJo software (v10.9.0, BD Biosciences).

Platelet Adhesion to von Willebrand Factor

Wells of 96-well microplate were coated with 100 µL of human vWF (10 µg/mL) in coating buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6) overnight at 4°C. Bound vWF was subsequently carbamylated in 1, 5, or 10 mM KCNO overnight at 37°C. Then, wells were washed and blocked with 2.5% BSA in PBS for 2 hours at RT. Wells were washed (3×) and incubated with native or carbamylated thrombin-activated

platelets (1×10^6). After 30 minutes at 37°C, adherent platelets were washed twice, fixed with 1% glutaraldehyde in PBS, and then stained with 0.5% crystal violet in H₂O for 10 minutes at RT. Formed violet crystals were dissolved in 1% SDS. Absorbance was measured at 570 nm using Synergy H1 Hybrid Multi-Mode Reader (BioTek, Bad Friedrichshall, Germany). The results were calculated as percentage of noncarbamylated control.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were maintained on collagen-coated (Advanced BioMatrix, Carlsbad, California, United States) 75 cm² bottles in DMEM/F12, 10% FBS, Large Vessel Endothelial Supplement (ThermoFisher Scientific), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C/5% CO₂. Cells were passaged at 90 to 95% confluence. For all experiments, cell passages between five to eight were used.

Platelet Adhesion to Endothelium

HUVECs were seeded onto clear bottom black 96-well plates (Corning, New York, United States) and, upon reaching full confluence, washed once with PBS, and activated with 50 ng/mL tumor necrosis factor (TNFα; ThermoFisher Scientific) and carbamylated with 1 mM KCNO (Merck) at 37°C for 24 hours.

Native and carbamylated platelets were stained with CellTrace Far Red (1 µM; ThermoFisher Scientific) for 45 minutes at 37°C. The platelets were then activated with thrombin. Platelets were diluted with DMEM/F12 (1.5×10^8 /mL) and allowed to adhere to endothelium for 30 minutes at 37°C. Unbound platelets were washed out with PBS (2×). Cells were fixed with 4% paraformaldehyde for 10 minutes at RT. Cells were visualized using Cytation 5 Cell Imaging Multimode Reader (BioTek) and one image per well was acquired (Texas Red filter cube, 10× magnification). Analysis of fluorescence intensity was performed using Fiji software.²⁶ To identify platelets in the images, "Adaptive Thresholding" plugin [Tseng Q] was used, with threshold calculated as a weighted mean, and C value was empirically determined for each experiment. The thresholded images were used as an input for "analyze particles" command and the result allowed for conversion of identified objects into regions of interests. These were overlaid onto corresponding raw images and the "integrated density" was measured.

Statistical Analysis

ANOVA with Tukey's post-hoc test was used to analyze the results. The results are presented as mean ± standard error of the mean. Statistical significance was evaluated with GraphPad Prism 9.5.1 (La Jolla, California, United States). The differences were considered significant: * if $p < 0.05$, ** if $p < 0.01$, or *** if $p < 0.001$.

Results

von Willebrand Factor Is Efficiently Carbamylated at Multiple Sites

To assess vWF's vulnerability to carbamylation, recombinant vWF was incubated with 1 mM and 10 mM KCNO, and the

carbamyl-lysines were detected through MS/MS. Modified lysines were observed in most vWF domains, with lysines within A1 exhibiting increased susceptibility to carbamylation (►Fig. 1A, ►Supplementary Table S1 [available in the online version]). In total, at 1 mM KCNO, 22 carbamylated lysine residues were identified, increasing to 47 at 10 mM KCNO. This signifies clear concentration- and time-depen-

dence of carbamylation. In the A3 domain, pivotal for vWF interactions with collagen I, type III, and ADAMTS13, we detected three carbamylated lysines, including K1720 and K1850, which were readily modified at 1 mM KCNO (►Fig. 1B, ►Supplementary Table S1 [available in the online version]). The D' and D3 domains, known for their role in FVIII binding, harbored one carbamylated lysine each (K773

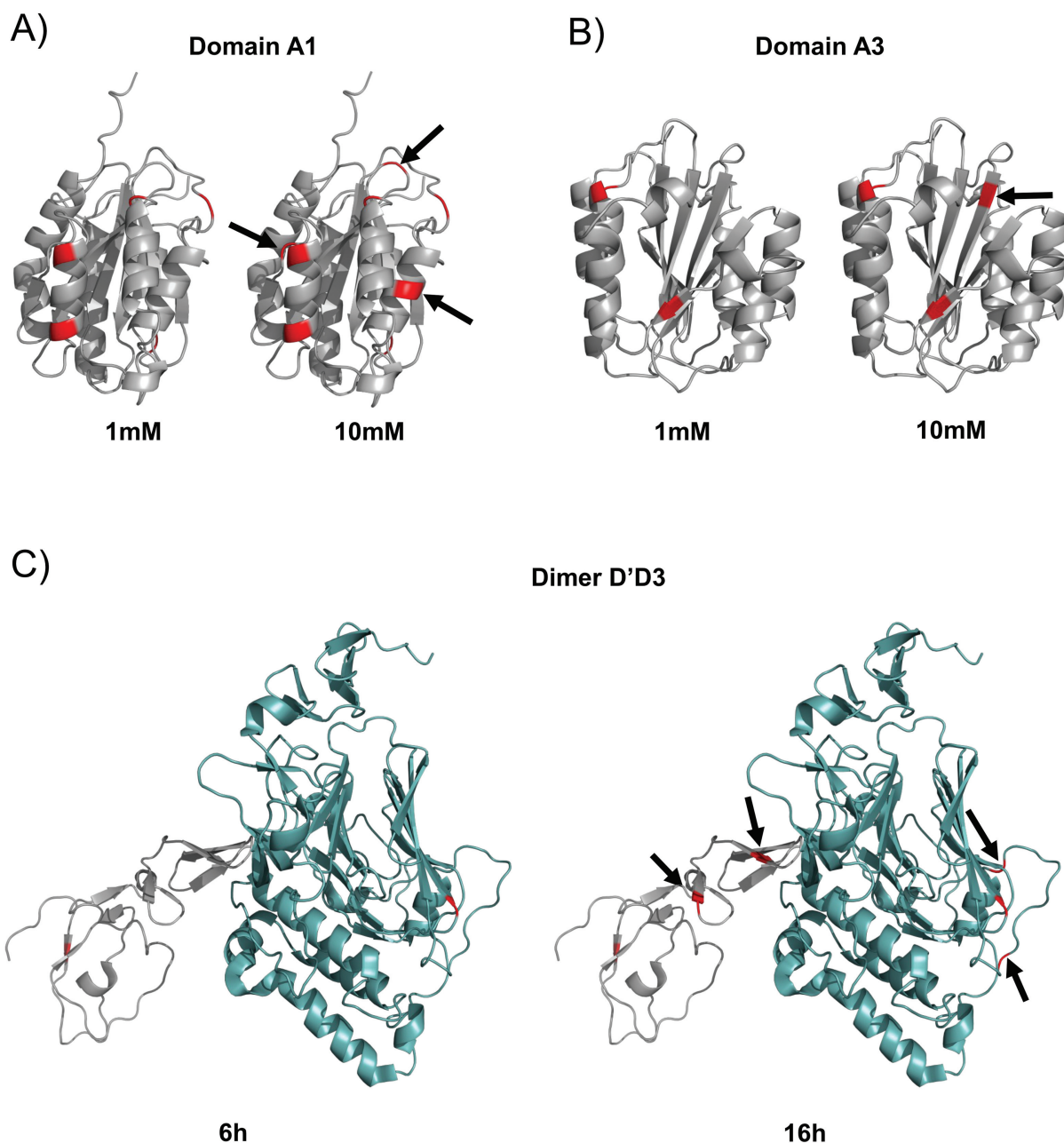


Fig. 1 Carbamylation sites on vWF identified in vitro and modeled in silico. (A) Three-dimensional in silico model of the vWF A1 domain showcasing five carbamylated lysine residues (K1348, K1371, K1408, K1432, K1430; depicted in red) as determined by orbitrap mass spectrometry following 6 hours of carbamylation with 1 mM KCNO (left panel). An increase in KCNO concentration to 10 mM for 6 hours revealed three additional carbamylated lysines (K1312, K1362, K1436; indicated in red with black arrows) (right panel). (B) In silico three-dimensional model of the vWF A3 domain illustrating two carbamylated lysines (K1720, K1850; in red) identified post 6-hour carbamylation with 1 mM KCNO (left panel). Carbamylation with 10 mM KCNO identified an additional lysine (K1794; in red with a black arrow) (right panel). (C) Three-dimensional in silico representation of the D'D3 dimer, highlighting two carbamylated lysines following 6 hours of carbamylation with 10 mM KCNO (vWF domain D': depicted in gray with K773; D3: in light green with K912; both residues marked in red) (left panel). Extended carbamylation for 16 hours at 10 mM KCNO revealed two more carbamylated lysines in each domain (D': K834, K843; D3: K1026, K1036; indicated in red with black arrows) (right panel). vWF, von Willebrand factor.

and K912, respectively) at lower KCNO concentrations (1 mM) and shorter incubation times (2 and 6 hours). Extended incubation at 10 mM KCNO for 16 hours produced additional modifications (K834, K843 in D' and K1026, K1063 in D3; ►Fig. 1C). Normally, vWF has an average circulation half-life of 16 hours^{27,28}; however, the plasma half-life of vWF in ESKD might be increased due to its impaired clearance, as suggested by increased plasma vWF in some studies.²⁹ Herein, even incubation times significantly shorter than the circulation half-life (2–6 hours) produced multiple carbamyl-lysines. Moreover, we have used KCNO concentration of 1 mM, which resembles the uremic conditions *in vivo*,³⁰ thus confirming the high likelihood of carbamylation of circulating vWF.

Carbamylation Affects Binding of von Willebrand Factor to Its Partners

To further investigate impact of the modifications observed via MS/MS analyses, we conducted binding assays with collagen (types I and III), FVIII, and fibrinogen (Fib). Our findings reveal that carbamylation significantly impairs the binding affinity between vWF and collagen, with a notable decrease observed after carbamylation of both proteins

(►Fig. 2A). Interestingly, the reduction in binding affinity was more pronounced when collagen itself was carbamylated, indicating that the collagen carbamylation status plays a critical role in this interaction. The binding assay results for FVIII presented a contrasting outcome. When FVIII was carbamylated in 10 mM KCNO, its binding to vWF was enhanced compared with the native form of FVIII (►Fig. 2B). This suggests that carbamylation may facilitate a stronger association between FVIII and vWF, potentially influencing coagulation dynamics. Contrary to the effects seen with collagen and FVIII, carbamylation of vWF did not alter its binding capacity to fibrinogen, even in the presence of thrombin (►Fig. 2C). This indicates that the carbamylation process does not significantly impact the vWF–fibrinogen interaction, underscoring the specificity of carbamylation effects on protein interactions within the coagulation cascade.

Carbamylation Hampers Adhesion of Platelets to vWF but Increases Rate of Binding to Endothelium

To analyze whether carbamylation influences the platelet–vWF adhesion, we carbamylated platelets, activated them using thrombin, and examined their binding to immobilized vWF. Our results indicate a KCNO concentration-dependent

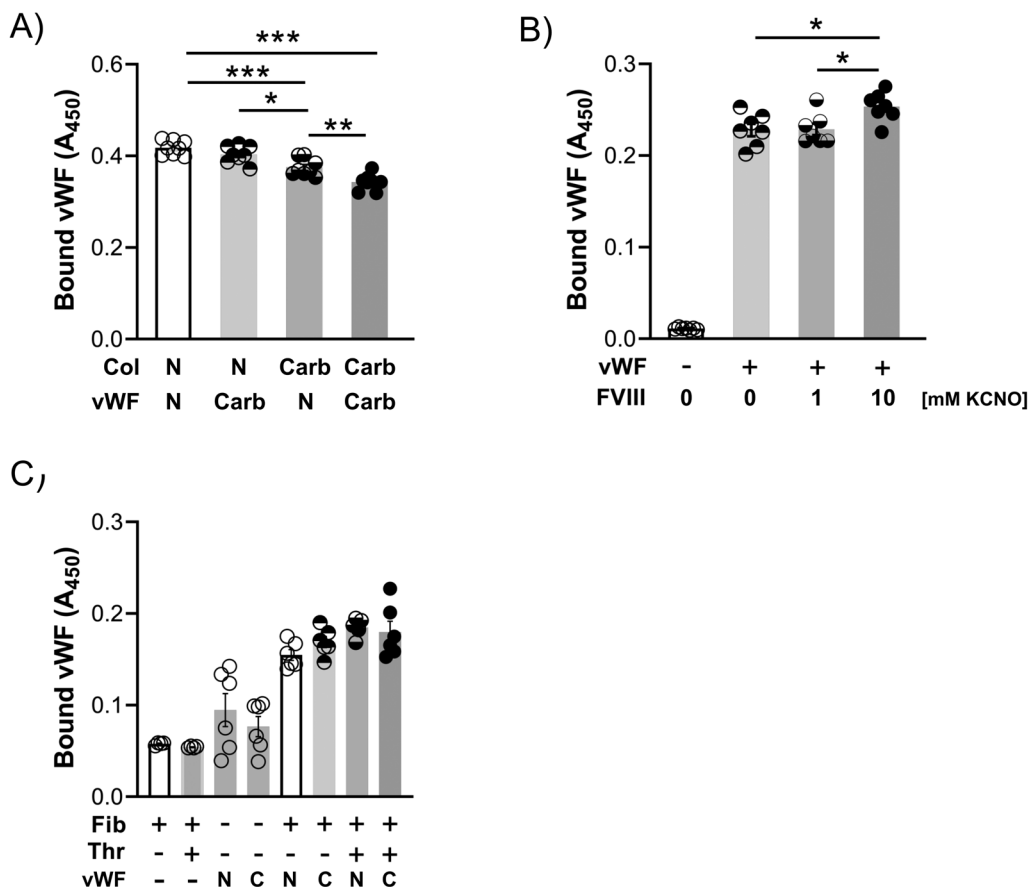


Fig. 2 The effect of carbamylation on protein–protein interactions *in vitro*. (A) Plates were coated with collagen and carbamylated with 1 mM KCNO. Native (N) or carbamylated (Carb; 1 mM KCNO) vWF was added and incubated for 2 hours at 37°C. Bound vWF was determined by HRP-conjugated anti-vWF antibody. (B) Plates were coated with factor VIII and carbamylated by 1 or 10 mM KCNO. Native vWF was added, incubated, and detected as above. (C) Plates were coated with fibrinogen, and native (N) or carbamylated (C; 10 mM) vWF was added. The reaction was performed with or without the presence of thrombin. Data are presented as mean ± SEM, **p* < 0.05, ***p* < 0.01, *n* = 6 (A, C) or 7 (B) per group. HRP, horseradish peroxidase; SEM, standard error of the mean; vWF, von Willebrand factor.

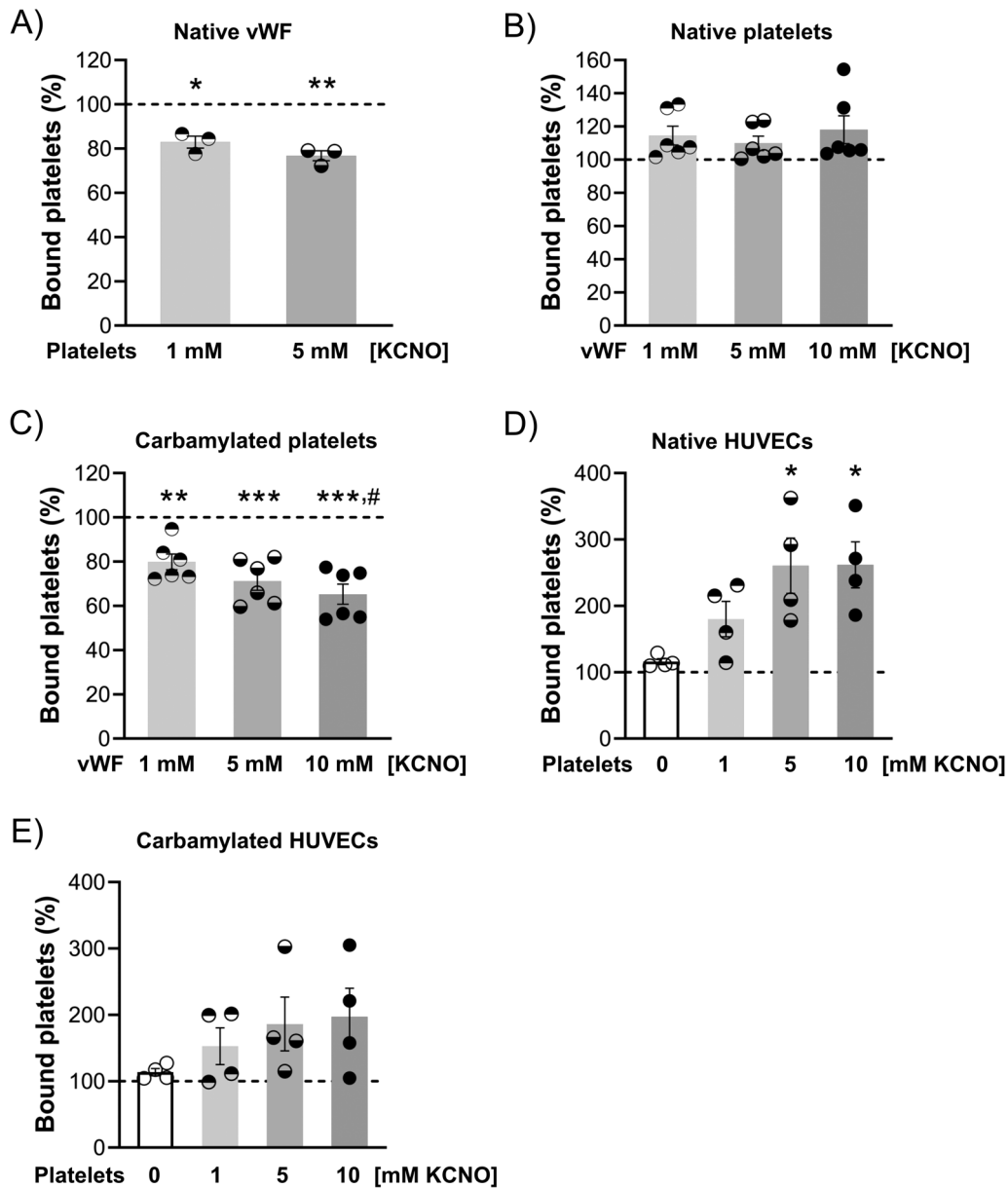


Fig. 3 The effect of platelet carbamylation on their binding capacity. (A) Platelets were carbamylated by either 1 or 5 mM KCNO and incubated with vWF-coated plates for 30 minutes at 37°C. The number of adhered platelets was determined spectrophotometrically after staining the platelets with crystal violet. (B) vWF was carbamylated by 1, 5, or 10 mM KCNO at 37°C for 16 hours. Noncarbamylated, thrombin-activated platelets were incubated for 30 minutes at 37°C and analyzed as before. (C) vWF was carbamylated by 1, 5, or 10 mM KCNO at 37°C for 16 hours. Carbamylated, thrombin-activated platelets were incubated for 30 minutes at 37°C and analyzed as before. (D) Platelets were carbamylated, stained using CellTrace Far Red dye, activated by thrombin, and incubated with cultured HUVECs for 30 minutes. Bound platelets were washed, fixed, and visualized under a fluorescence microscope. (E) Platelets were carbamylated by 1, 5, or 10 mM KCNO and incubated with carbamylated HUVECs (1 mM). Data are presented as mean \pm SEM, dotted line represents noncarbamylated platelets set as 100% (A–C), or nonactivated HUVECs (D, E); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 100%, # $p < 0.05$ vs. 1 mM, $n = 3$ (A), 6 (B), 5 (C, D), or 4 (E) per group. vWF, von Willebrand factor.

reduction in the adhesion of platelets to vWF (\rightarrow Fig. 3A). Subsequently, we examined the binding behavior of noncarbamylated and carbamylated platelets to carbamylated vWF. While noncarbamylated platelets exhibited a trend toward increased adhesion to carbamylated vWF, this difference did not reach statistical significance (\rightarrow Fig. 3B). In contrast, simultaneous carbamylation of both vWF (10 mM KCNO) and platelets led to a significant decline in platelet adhesion, also in a KCNO-concentration-dependent manner (\rightarrow Fig. 3C).

We extended our investigation to examine how carbamylation influences the binding interaction between platelets and endothelial cells. In this series of experiments, platelets were carbamylated, stained, and activated with thrombin before being incubated with TNF α -prestimulated HUVECs. Our findings demonstrate a significant enhancement in the adhesion of carbamylated platelets to the endothelium, with the extent of adhesion increasing in a KCNO-concentration-dependent manner (\rightarrow Fig. 3D). In a subsequent analysis, both

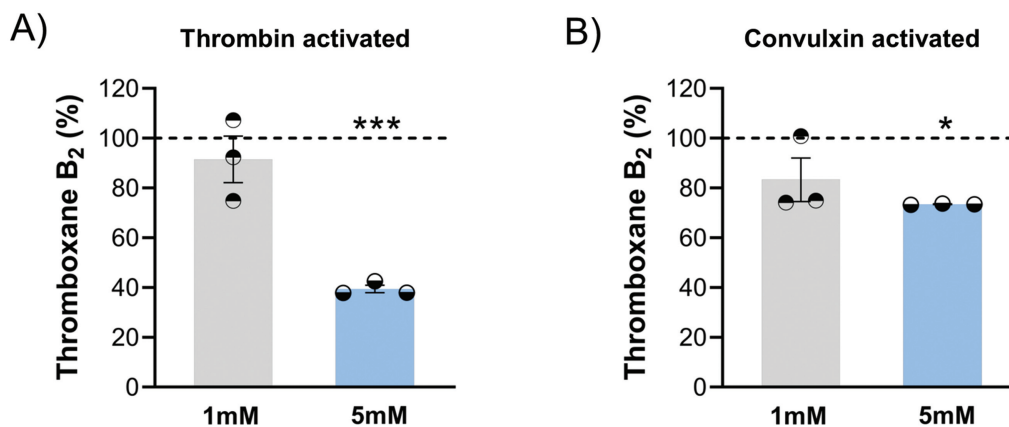


Fig. 4 Carbamylation of platelets reduces the release of TXA₂. (A) Platelets were carbamylated by 1 or 5 mM KCNO for 30 minutes and activated by thrombin for 10 minutes. The platelets were then centrifuged, the supernatant was collected, and Thromboxane B₂ was measured using ELISA. (B) Platelets were carbamylated as before and activated with convulxin for 10 minutes. Data are presented as mean ± SEM, dotted line represents noncarbamylated platelets set as 100%. **p* < 0.05, ****p* < 0.001, *n* = 3 per group.

the endothelium and platelets were subjected to carbamylation. This condition suggested a trend toward increased platelet adhesion compared with native platelets. However, the observed increase in adhesion was markedly lower than that observed when employing native endothelium, suggesting that while carbamylation of platelets promotes adhesion, simultaneous carbamylation of endothelium hampers that interaction (►Fig. 3E)

Carbamylation Limits Release of Thromboxane A₂ upon Platelet Activation

TxA₂, a metabolite of arachidonic acid produced by activated platelets, serves as a critical mediator in platelet activation and aggregation. Therefore, we next measured the concentration of its stable derivative, TxB₂, to assess the impact of carbamylation on TxA₂ release. Platelets underwent carbamylation prior to their activation with either thrombin or convulxin, and TxB₂ concentrations in the supernatants were measured. Our results clearly indicate that carbamylation significantly diminishes the release of TxA₂ following activa-

tion by both thrombin and convulxin, as evidenced by the reduced levels of TxB₂ (►Fig. 4A and B, respectively).

Thrombin-Induced Phosphatidylserine Exposure Is Increased after Platelet Carbamylation

Platelet activation is a critical step in the coagulation cascade, marked by the exposure of phosphatidylserine (PS) on the platelet surface, which plays a pivotal role in creating a procoagulant environment. This study sought to examine the influence of carbamylation on PS exposure following platelet activation. Carbamylated platelets were activated with either thrombin or convulxin and subsequently stained with annexin V. Flow cytometry analysis revealed a significant increase in PS exposure on carbamylated platelets following thrombin activation, with a 190% increase compared with 100% in native platelets (►Fig. 5A). Conversely, the response of carbamylated platelets to convulxin was notably diminished in comparison to the nonmodified counterparts (►Fig. 5B).

Given the specific pathways through which thrombin mediates platelet activation—namely, the protease-activated

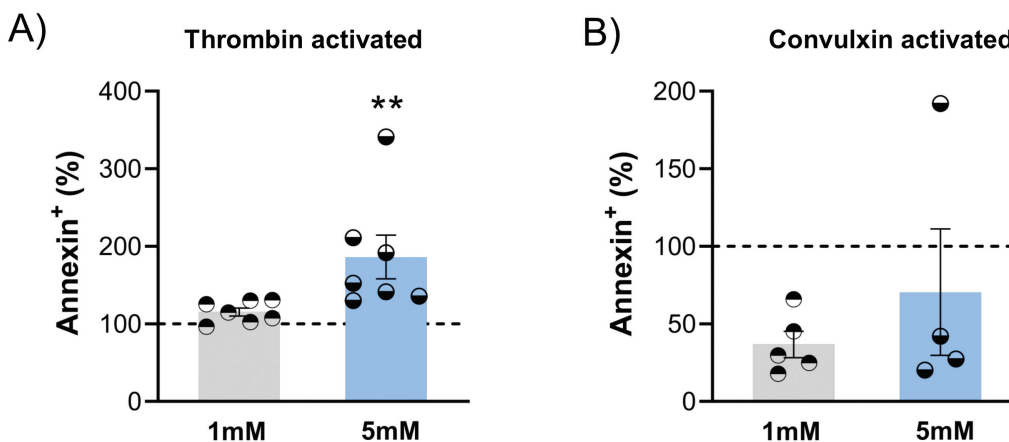


Fig. 5 Carbamylation of platelets alters the surface exposure of phosphatidylserine depending on the activating agent. (A) Platelets were carbamylated by 1 or 5 mM KCNO for 30 minutes, activated by thrombin for 10 minutes, stained with Annexin V and immediately analyzed using flow cytometry. (B) Platelets were carbamylated as before and activated with convulxin. Data are presented as mean ± SEM, ***p* < 0.01, *n* = 4–5 per group.

receptors 1 and 4 (PAR-1 and PAR-4)—we further investigated the effect of carbamylation on platelet activation through these receptors. Utilizing the specific agonists TRAP-6 (PAR-1 agonist) and AY-NH2 (PAR-4 agonist), our investigations revealed no discernible impact of carbamylation on platelet activation via either PAR receptor (data not shown).

Discussion

Carbamylation is an unavoidable outcome of elevated levels of plasma cyanate in patients with ESKD. This PTM impacts a broad spectrum of proteins and peptides in circulation as well as the cell-bound proteins. Among others, the carbamylation targets components of the coagulation cascade, serving as a direct catalyst for the development of coagulopathies frequently seen in individuals with ESKD. Our previous research has demonstrated that carbamylation significantly alters the structure and function of fibrinogen and $\alpha\text{IIb}\beta\text{3}$, two critical components involved in blood clotting and platelet function. These alterations contribute to the impaired function of platelets, a key factor in the abnormal clotting observed in ESKD patients.^{17,18} In this study, we further explore the mechanisms by which carbamylation influences the interaction between vWF and its partner proteins, examining its impact on platelet aggregation and function. Our findings illuminate the complex ways carbamylation disrupts these essential processes, highlighting the multifaceted complications associated with ESKD.

The association between vWF and coagulopathies in patients with ESKD has been predominantly linked to the compromised functionality of vWF, rather than a decrease in its levels. Interestingly, vWF concentrations in the plasma of these patients are often normal or slightly elevated.²⁰ vWF is a large multimeric GP essential for hemostasis and thrombosis regulation. It circulates in plasma, where it binds and stabilizes FVIII and is also found intracellularly within endothelial cells and platelets. Upon vascular injury, vWF plays a pivotal role by recruiting platelets to the site of damage, facilitating their adhesion and aggregation. Consequently, deficiencies or abnormalities in vWF can lead to bleeding disorders. In our research, we identified multiple lysine residues within vWF domains that are susceptible to carbamylation. This modification could impair vWF's function, shedding light on the molecular mechanisms underlying coagulation disorders in ESKD. Our *in vitro* analyses revealed that exposure to cyanate leads to differential carbamylation

across vWF domains, with domain A1 exhibiting the most substantial level of modification, marked by the carbamylation of nine lysine residues. These modifications occurred at pivotal sites within the A1, A3, D3, C4, and CK domains, where lysins have been implicated to take part in the binding or are in the immediate vicinity of crucial vWF-interactions (► **Table 1**). The A1 domain is involved in vWF binding to sulfatides on the surface of platelets, and its readily undergoing carbamylation lysine residues - K1408, K1423, and K1430 - are involved in vWF-sulfatide binding.³¹ K1408 plays a crucial role in vWF's interaction with the scavenger receptor LRP1, instrumental in vWF clearance from circulation.³² Whether carbamylation of K1408 in ESKD patients might contribute to decreased vWF elimination requires further investigation. Whereas within the A3 domain, lysine residues K1720 and K1850, crucial for binding to the GPIb platelet surface receptor, were carbamylated. This modification may disrupt the vWF-GPIb interaction, pivotal for platelet adhesion under high shear conditions. Moreover, lysine K1312, essential for vWF's binding to GPIb, was susceptible to carbamylation under various conditions, potentially impairing native platelet binding to carbamylated vWF, as evidenced in our findings (► **Fig. 3B**).³³ Domain A3 plays a pivotal role in the immobilization of vWF onto the subendothelial matrix, primarily through interactions with collagen type I and type III.²³ This interaction is facilitated by several key amino acids located at the domain's upper epitope, critical for mediating collagen binding.^{33,34} Under shear stress, vWF's domain A2 unfolds, revealing a cleavage site between residues Y1605 and M1606 for the specific metalloproteinase ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motif 13).³⁵ We identified two carbamylated lysine residues, K1518 and K1617, within this domain, with K1617 playing a role in the ADAMTS13 recognition sequence essential for vWF binding. It remains to be determined whether the carbamylation patterns observed *in vitro* mirror those in ESRD patients. Nonetheless, previous studies on albumin¹⁰ and integrin $\alpha\text{IIb}\beta\text{3}$ ¹⁷ strongly suggest that this is highly probable. Overall, carbamylation of vWF at specific lysine residues, observed across various domains, could significantly influence its multifaceted roles, particularly in protein-protein interactions critical for hemostatic processes.

Although the direct role of the K1720 and K1794 residues in collagen interaction has not been previously described,

Table 1 List of vWF domains with identified carbamylated lysines and their respective interacting domains (lysines were identified at two different carbamylation conditions [1 mM and 10 mM KCNO])

Domain	Carbamylated lysine	Interactions
A1	K1312, K1348, K1408	GP1b α , β2 -integrins, β2 -glycoprotein I, PSGL-1, Col VI, ADAMST13
A3	K1720, K1850	Col I and III, ADAMST13, thrombospondin
D3	K1935	ADAMST13, IGFBP7 (insulin growth factor-binding protein-7)
C4	K2537	$\alpha\text{IIb}\beta\text{3}$, $\alpha\text{V}\beta\text{3}$, ADAMST13, fibrin, IGFBP7
CK	K2757	ADAMST13, IGFBP7, CTGF/CCN2 (connective tissue growth factor)

Abbreviation: vWF, von Willebrand factor.

their proximity to crucial amino acids suggests a potential impact on the binding efficiency. In vitro analyses demonstrated a diminished interaction between carbamylated vWF and a collagen mixture (types I and III), hinting at the significant role these modified residues may play in the collagen–vWF binding process, potentially obstructing access to R1726 and H1786 directly interacting with collagens. Intriguingly, this reduction in binding affinity was more pronounced when both vWF and collagen were carbamylated, presenting a contrast to previous research that generally reports unaffected vWF–collagen binding in ESKD patients.³⁶ Furthermore, vWF domains D' and D3 are known to engage with FVIII, serving as a crucial cofactor in the coagulation cascade, with the vWF:FVIII complex circulating in an inactive form in plasma.²⁴ In the context of ESKD, there is an upsurge in circulating levels of the vWF:FVIII complex. Notably, our study revealed that carbamylation of FVIII in vitro enhances its binding to vWF, potentially contributing to the elevated plasma levels of the complex as observed in ESKD. Additionally, the identification of carbamylated lysine K773 within the D' domain of vWF, a residue known to be integral to FVIII binding,³¹ provides further potential mechanisms through which carbamylation could augment the vWF:FVIII interaction.

Contrary to expectations, our study revealed no significant effect of vWF carbamylation on its binding to fibrinogen. This suggests that the carbamylation-induced alterations in vWF do not hinder its competition with fibrinogen for the α IIb β 3 integrin, a key mediator of platelet aggregation. Contrary to the effects observed with immobilized vWF, we demonstrated that carbamylation significantly enhances platelet adhesion to endothelial cells in vitro. The adhesion to HUVECs is primarily mediated through ICAM-1 and Integrin α IIb β 3, offering a potential explanation for the observed discrepancies with vWF.³⁷ While platelets typically adhere to the subendothelial extracellular matrix, our findings indicate that carbamylation facilitates a notable increase in the direct platelet–endothelium binding. This effect is evident in both isolated and carbamylated platelets, as well as under conditions simulating ESKD and uremia.³⁸

Interestingly, when both HUVECs and platelets undergo carbamylation, the rate of platelet adhesion, although still elevated in carbamylated platelets, is marginally reduced compared with their adhesion to noncarbamylated HUVECs. This nuanced result might shed light on the complex and ambivalent nature of hemostasis in ESKD patients—fluctuating between diathesis and prothrombotic states. However, our observations of predominantly reduced platelet adhesion in this context suggest a more intricate interplay at the endothelial interface than previously understood. Our investigations into the effects of carbamylation on platelet–vWF interactions showed that carbamylated platelets exhibit reduced binding to native vWF. Interestingly, native platelets displayed a trend toward increased binding to carbamylated vWF. This suggests that platelet surface carbamylation influences their interaction with vWF potentially more than the carbamylation of vWF itself. However, in vivo, both carba-

mylated platelets and vWF likely contribute to impaired hemostasis in ESKD.

We also explored the effects of carbamylation on the release of TxA₂ and the exposure of PS upon platelet activation. A reduction in TxA₂ secretion following carbamylation and activation of platelets aligned with previous observations of diminished TxA₂ generation in ESKD.³⁹ Additionally, carbamylation led to an increased exposure of PS on activated platelets, supporting in vivo findings of enhanced procoagulant activity in ESKD. The exposure is crucial for creating a procoagulant surface by providing binding sites for factor X leading to assembly of prothrombinase complex and releasing thrombin. In ESKD, the exposure of PS by platelets was increased.⁴⁰

In conclusion, our findings elucidate the multifaceted impact of carbamylation on vWF and platelets, highlighting its significant role in modifying their interactions and functions. These alterations could contribute to the complex hemostatic imbalance in ESKD, underscoring the need for further research to fully understand these mechanisms and their clinical implications.

What is known about this topic?

- Coagulopathies affect almost half of the patients suffering from the ESKD.
- Carbamylation of integrin α IIb β 3 impairs aggregation of uremic platelets.
- Structure and formation of carbamylated fibrinogen is significantly affected.

What does this paper add?

- Carbamylation affects vWF's affinity to its key binding partners.
- Carbamylation has significant impact on the platelet activation and their adhesion to endothelium.

Funding

This work was supported by grants awarded to P.M. (2019/33/B/NZ4/01889 and 2022/47/B/NZ4/01696, National Science Centre, Poland). J.B., U.K., P.M., and M.K. were supported by the Broegelmann Research Foundation. M.K. was supported by the L. Meltzer Research Group.

Conflict of Interest

None declared.

Acknowledgment

We acknowledge Proteomics and Mass Spectrometry Core Facility of the Malopolska Centre of Biotechnology, Jagiellonian University for mass spectrometry analysis.

References

- 1 Chen TK, Knicely DH, Grams ME. Chronic kidney disease diagnosis and management: a review. *JAMA* 2019;322(13):1294–1304

- 2 Husted AS, Trauelsen M, Rudenko O, Hjorth SA, Schwartz TW. GPCR-mediated signaling of metabolites. *Cell Metab* 2017;25(04):777–796
- 3 Linthorst GE, Avis HJ, Levi M. Uremic thrombocytopenia is not about urea. *J Am Soc Nephrol* 2010;21(05):753–755
- 4 Horowitz HI, Stein IM, Cohen BD, White JG. Further studies on the platelet-inhibitory effect of guanidinosuccinic acid and its role in uremic bleeding. *Am J Med* 1970;49(03):336–345
- 5 Rabiner SF, Molinas F. The role of phenol and phenolic acids on the thrombocytopenia and defective platelet aggregation of patients with renal failure. *Am J Med* 1970;49(03):346–351
- 6 Battle S, Gogonea V, Willard B, et al. The pattern of apolipoprotein A-I lysine carbamylation reflects its lipidation state and the chemical environment within human atherosclerotic aorta. *J Biol Chem* 2022;298(04):101832
- 7 Mydel P, Wang Z, Brissler M, et al. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. *J Immunol* 2010;184(12):6882–6890
- 8 Shi J, van Veelen PA, Mahler M, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. *Autoimmun Rev* 2014;13(03):225–230
- 9 Kalim S, Karumanchi SA, Thadhani RI, Berg AH. Protein carbamylation in kidney disease: pathogenesis and clinical implications. *Am J Kidney Dis* 2014;64(05):793–803
- 10 Berg AH, Drechsler C, Wenger J, et al. Carbamylation of serum albumin as a risk factor for mortality in patients with kidney failure. *Sci Transl Med* 2013;5(175):175ra29
- 11 Pietrement C, Gorisse L, Jaisson S, Gillery P. Chronic increase of urea leads to carbamylated proteins accumulation in tissues in a mouse model of CKD. *PLoS One* 2013;8(12):e82506
- 12 Kalim S, Trottier CA, Wenger JB, et al. Longitudinal changes in protein carbamylation and mortality risk after initiation of hemodialysis. *Clin J Am Soc Nephrol* 2016;11(10):1809–1816
- 13 Kraus LM, Elberger AJ, Handorf CR, Pabst MJ, Kraus AP Jr. Urea-derived cyanate forms epsilon-amino-carbamoyl-lysine (homocitrulline) in leukocyte proteins in patients with end-stage renal disease on peritoneal dialysis. *J Lab Clin Med* 1994;123(06):882–891
- 14 Pieniżek A, Gwoździński K. Carbamylation of proteins leads to alterations in the membrane structure of erythrocytes. *Cell Mol Biol Lett* 2003;8(01):127–131
- 15 Hedges SJ, Dehoney SB, Hooper JS, Amanzadeh J, Busti AJ. Evidence-based treatment recommendations for uremic bleeding. *Nat Clin Pract Nephrol* 2007;3(03):138–153
- 16 Lutz J, Menke J, Sollinger D, Schinzel H, Thürmel K. Haemostasis in chronic kidney disease. *Nephrol Dial Transplant* 2014;29(01):29–40
- 17 Binder V, Chruścicka-Smaga B, Bergum B, et al. Carbamylation of integrin $\alpha_{IIb}\beta_3$: the mechanistic link to platelet dysfunction in ESKD. *J Am Soc Nephrol* 2022;33(10):1841–1856
- 18 Binder V, Bergum B, Jaisson S, et al. Impact of fibrinogen carbamylation on fibrin clot formation and stability. *Thromb Haemost* 2017;117(05):899–910
- 19 Boccardo P, Remuzzi G, Galbusera M. Platelet dysfunction in renal failure. *Semin Thromb Hemost* 2004;30(05):579–589
- 20 van der Vorm LN, Visser R, Huskens D, et al. Circulating active von Willebrand factor levels are increased in chronic kidney disease and end-stage renal disease. *Clin Kidney J* 2019;13(01):72–74
- 21 Benigni A, Boccardo P, Galbusera M, et al. Reversible activation defect of the platelet glycoprotein IIb-IIIa complex in patients with uremia. *Am J Kidney Dis* 1993;22(05):668–676
- 22 Casonato A, Pontara E, Vertolli UP, et al. Plasma and platelet von Willebrand factor abnormalities in patients with uremia: lack of correlation with uremic bleeding. *Clin Appl Thromb Hemost* 2001;7(02):81–86
- 23 Watson SP. Platelet activation by extracellular matrix proteins in haemostasis and thrombosis. *Curr Pharm Des* 2009;15(12):1358–1372
- 24 Park HS, Kim MW, Kim TJ, Hwang SD, Lee HB. Platelet function and factor VIII in uremia. *Korean J Intern Med (Korean Assoc Intern Med)* 1987;2(01):74–78
- 25 Tamang HK, Stringham EN, Tourdot BE. Platelet functional testing via high-throughput microtiter plate-based assays. *Curr Protoc* 2023;3(02):e668
- 26 Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012;9(07):676–682
- 27 Lenting PJ, Christophe OD, Denis CV. von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. *Blood* 2015;125(13):2019–2028
- 28 Josefsson EC, Vainchenko W, James C. Regulation of platelet production and life span: role of Bcl-xL and potential implications for human platelet diseases. *Int J Mol Sci* 2020;21(20):7591
- 29 Ocak G, Roest M, Verhaar MC, et al. Von Willebrand factor, ADAMTS13 and mortality in dialysis patients. *BMC Nephrol* 2021;22(01):222
- 30 Delanghe S, Delanghe JR, Speeckaert R, Van Biesen W, Speeckaert MM. Mechanisms and consequences of carbamylation. *Nat Rev Nephrol* 2017;13(09):580–593
- 31 Nakayama T, Matsushita T, Yamamoto K, et al. Identification of amino acid residues responsible for von Willebrand factor binding to sulfatide by charged-to-alanine-scanning mutagenesis. *Int J Hematol* 2008;87(04):363–370
- 32 Rastegarlarri G, Pegon JN, Casari C, et al. Macrophage LRP1 contributes to the clearance of von Willebrand factor. *Blood* 2012;119(09):2126–2134
- 33 Interlandi G, Yakovenko O, Tu AY, et al. Specific electrostatic interactions between charged amino acid residues regulate binding of von Willebrand factor to blood platelets. *J Biol Chem* 2017;292(45):18608–18617
- 34 Romijn RAP, Bouma B, Wuyster W, et al. Identification of the collagen-binding site of the von Willebrand factor A3-domain. *J Biol Chem* 2001;276(13):9985–9991
- 35 Petri A, Kim HJ, Xu Y, et al. Crystal structure and substrate-induced activation of ADAMTS13. *Nat Commun* 2019;10(01):3781
- 36 Niesvizky R, Calandri C, Patel ND, Zhou SL, Potter BJ, Rand JH. von Willebrand factor binding to collagen in patients with end-stage renal disease. *J Lab Clin Med* 1994;123(01):137–142
- 37 Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a GPIIb/IIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alpha5beta1 integrin, and GPIIb/IIIa. *J Exp Med* 1998;187(03):329–339
- 38 Sosińska-Zawierucha P, Bręborowicz A. Uremic serum induces prothrombotic changes in venous endothelial cells and inflammatory changes in aortic endothelial cells. *Ren Fail* 2021;43(01):401–405
- 39 Baaten CCFMJ, Sternkopf M, Henning T, Marx N, Jankowski J, Noels H. Platelet function in CKD: a systematic review and meta-analysis. *J Am Soc Nephrol* 2021;32(07):1583–1598
- 40 Pavlou EG, Georgatzakou HT, Fortis SP, et al. Coagulation abnormalities in renal pathology of chronic kidney disease: the interplay between blood cells and soluble factors. *Biomolecules* 2021;11(09):1309