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Research Article

Quantification of carbamylated albumin in serum based on capillary electrophoresis

Protein carbamylation, a nonenzymatic posttranslational modification promoted during uremia, is linked to a poor prognosis. In the present study, carbamylation of serum albumin was assayed using the symmetry factor on a capillary electrophoresis instrument (Helena V8). The symmetry factor has been defined as the distance from the center line of the peak to the back slope, divided by the distance from the center line of the peak to the front slope, with all measurements made at 10% of the maximum peak height. Serum albumin, creatinine, and urea concentrations were assayed using routine methods, whereas uremic toxins were determined using HPLC. In vitro carbamylation induced a marked albumin peak asymmetry. Reference values for the albumin symmetry factor were 0.69–0.92. In kidney patients, albumin peak asymmetry corresponded to the chronic kidney disease stage ($p < 0.0001$). The symmetry factor correlated well with serum urea ($r = -0.5595$, $p < 0.0001$) and creatinine ($r = -0.5986$, $p < 0.0001$) concentrations. Several protein-bound uremic toxins showed a significant negative correlation with the symmetry factor. Morphology of the albumin fraction was not affected by presence of glycated albumin and protein-bound antibiotics. In conclusion, the presented method provides a simple, practical way for monitoring protein carbamylation.

Keywords:
Capillary electrophoresis / Carbamylation / Chronic kidney disease

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1 Introduction

Chronic kidney disease (CKD) is a global public health problem with a prevalence varying between 3 and 18%, depending on the population [1]. Patients who reach end-stage renal disease (ESRD) have an annual mortality of 15–20%, which is largely attributable to cardiovascular disease [2–4]. An enhanced degree of carbamylation of serum albumin has been identified as an important risk factor for accelerated atherogenesis and mortality in patients on dialysis [2].

Carbamylation is a nonenzymatic posttranslational reaction of either a primary amino or a free sulfhydryl group of a protein with isocyanate. Urea dissociates spontaneously into ammonia and cyanate, the latter being in equilibrium with its tautomer isocyanate. Albumin is the most abundant serum protein with an in vivo half-life of approximately 20 days [5], which makes it an ideal target to study carbamylation. Albumin carries a four to fivefold higher molar amount of carbamylated groups than hemoglobin in uremic patients [6]. In uremia, the secondary structure of albumin remains intact, but there is a modified environment of Asp/Glu residues in the protein globule after carbamylation. There is a good correlation between the percentage of carbamylated albumin and the global carbamylation of blood proteins in uremic subjects [2]. The preferred site for carbamylation on albumin is lysine-549 (Lys-549). Carbamylation of albumin reduces its ability to bind ligands, especially drugs [7]. Carbamylated albumin is a potent inhibitor of the polymorphonuclear neutrophil respiratory burst [8]. Carbamylated albumin has an enhanced nephrotoxicity compared to unmodified albumin, resulting in tubular epithelial and interstitial lesions [9].

At present, assessment of carbamylation requires analysis of characteristic carbamylation-derived compounds such as ε-carbamyllysine (homocitrulline) [10]. In the present study, we explored the possibility of using capillary electrophoresis of albumin to assess carbamylation in CKD. Capillary electrophoresis is available in most routine clinical laboratories and is characterized by a high resolution, which makes is well suited to detect posttranscriptional changes.

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Abbreviations: **β2-M**, beta 2-microglobulin; **CKD**, chronic kidney disease; **CMPF**, 3-carboxy-4-methyl-5-propyl-2-furane propionate; **ESRD**, end-stage renal disease; **HA**, hippuric acid; **IAA**, indol-3-acetic acid; **IxS**, indoxyl sulfate; **Lys**, Lysine; **pCG**, p-cresyl-glucuronide; **pCS**, p-cresyl sulfate

Colour Online: See the article online to view Figs. 1 and 2 in colour.

2 Materials and methods

2.1 Study population

The study population group consisted of 200 patients (median age: 68 years, IQR: 56–79 years, 36.5% diabetics) with different stages of CKD: 20 patients with CKD 2 (11 men, nine women), 20 patients with stages CKD 3A (16 men, four women), 21 patients with stages CKD 3B (15 men, six women), 19 patients with stages CKD 4 (13 men, six women), and 120 ESRD patients treated with chronic hemodialysis (CKD 5d) (75 men, 45 women). The control group consisted of 39 healthy volunteers (20 men, 19 women, median age: 34 years, IQR: 28–44 years). The study was approved by the local ethics committee (2015/0932, Belgian registration number B670201525559). The authors complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

2.2 Determination of routine laboratory parameters

Blood was drawn, tubes were centrifuged (10 min, 3000 rpm, room temperature) and serum was obtained, aliquoted, and stored at −80°C until analysis. In ESRD patients, blood was sampled before the start of a hemodialysis session. Routine parameters (urea, creatinine, and albumin) were determined in serum. Albumin was assayed using immunonephelometry on a BN II Nephelometer (Siemens, Medical Solutions, Erlangen, Germany). Creatinine was assayed using a compensated rate-blanked picrate assay [11]. Urea was assayed on a Modular analyzer (Roche, Mannheim, Germany). The estimated glomerular filtration rate was calculated using the Chronic Kidney Disease Epidemiology Collaboration 2009 formula [12]. In the group of hemodialysis patients, standardized Kt/V (stdKt/V) was calculated according to the formula [13]: $std\frac{Kt}{V} = 7.24.60.60.\frac{m}{C_0.V}$, in which K stands for urea clearance (m^3/s), t for dialysis time (s), V for the distribution volume (m^3), m for the mass generation rate of urea (mol/s) and C_0 for the urea concentration at the start of dialysis (mol/m^3).

2.3 Detection of uremic toxins

Besides urea (molecular weight: 60 Da, water-soluble compound), eight uremic toxins were determined in serum of 78 hemodialysis patients: indoxyl sulfate (IxS, molecular weight: 213 Da, protein binding ~90%), p-cresyl sulfate (pCS, molecular weight: 187 Da, protein binding ~95%), p-cresyl-glucuronide (pCG, molecular weight: 284 Da, protein binding ~10%), indole-3-acetic acid (IAA, molecular weight: 175 Da, protein binding ~65%), 3-carboxy-4-methyl-5-propyl-2-furane propionate (CMPF, molecular weight: 240 Da, protein binding ~100%), hippuric acid (HA, molecular weight: 179 Da, protein binding ~50%), uric acid (molecular weight: 168 Da, nonprotein-bound), and beta 2-microglobulin (β2M, molecular weight: 11818 Da, nonprotein-bound). For the determination of uremic toxins, a HPLC assay was used. Prior to analysis, serum samples were denaturated for 30 min at 95°C, followed by filtration using a 30 kDa cut-off molecular filter (Centri-free Micropartition Devices, Amicon Inc, Beverly, MA). For the determination of the free fractions, denaturation was preceded by filtration (Millipore Billerica, Massachusetts). The HPLC analysers consisted of a Waters Alliance 2695 device (Waters, Zellik, Belgium) and two detectors in series: a Waters 996 photodiode array detector and a Waters 2475 fluorescence detector. The separation was performed at room temperature on a reversed-phase XBridge C8 column (3.5 μm, 150 mm × 4.6 mm, Waters) with an Ultrasphere ODS guard column (5 μm, 45 mm × 4.6 mm, Beckman Instruments). The mobile phase consisted of a 50 mM ammonium formate buffer (mobile phase A, pH 3.0) and methanol (mobile phase B). HA and CMPF were analyzed by UV detection at 245 nm and 254 nm, respectively, whereas PCS and PCG (λex = 264 nm, λem = 290 nm), and IAA and IxS (λex = 272 nm, λem = 340 nm, and 374 nm, respectively) and the internal standard fluorescein (λex: 443 nm, λem: 512 nm) were determined by fluorescence detection [14]. For β2M, ELISA kits of Orgentec Diagnostika GmbH (Mainz, Germany) were used.

2.4 Capillary electrophoresis

Capillary electrophoresis of serum was carried out using a Helena V8 (Helena, Newcastle, UK). Two microliters of each serum sample was 50-fold diluted (0.9% saline) and was automatically injected. Capillaries were automatically washed between each sample migration. Separation of proteins was obtained by applying a voltage of 9 kV in eight 30 cm fused-silica capillaries controlled by Peltier effect. Capillaries had an effective length of 23 cm and an internal diameter of 50 μm. During separation, temperature was maintained at 20°C. A borate based buffer was used, pH 10.1. A ninth capillary was used as reference (buffer only). In the standard operating mode, direct detection of proteins was performed by measuring UV absorbance of the separated fractions at 214 nm. Throughput was 90 samples/h. The Helena V8 capillary electrophoresis (Zoom mode) was used in a standard setting for serum electrophoresis [15]. Following electrophoresis, the morphology of the albumin peaks was analyzed by calculating the asymmetry factor, making use of the Platinum 4.1® software. The asymmetry factor is a measure of peak tailing. It is defined as the distance from the center line of the peak to the back slope, divided by the distance from the center line of the peak to the front slope, with all measurements made at 10% of the maximum peak height. Figure 1 represents the spectrum following capillary electrophoresis of serum, showing the calculation of the symmetry factor. The *x*-axis depicts the migration time (s), the *y*-axis was normalized on the albumin peak. The reproducibility of the assay was evaluated by repeating a series ($n = 10$) of three different serum pools, showing respectively pronounced, average, and limited fronting.

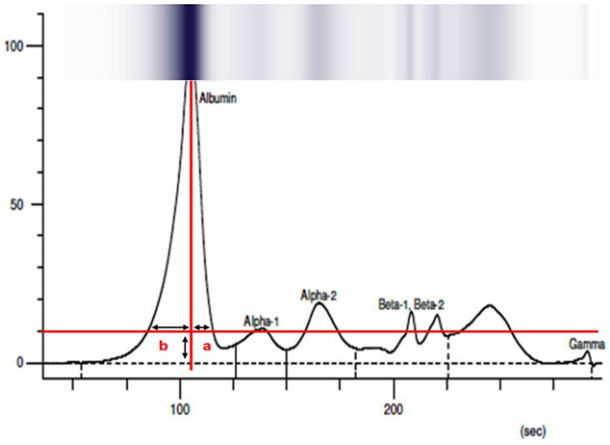

Figure 1. Spectrum following capillary electrophoresis of serum showing the calculation of the symmetry factor. The *x*-axis depicts the migration time (s), the *y*-axis was normalized on the albumin peak.

2.5 In vitro carbamylation and investigation of confounding factors

In vitro carbamylation of serum from healthy subjects was achieved by adding 50 μL potassium cyanate (Sigma, St. Louis, Missouri, USA) in PBS (0.1 mol/L, pH 7.4) to 500 μL of serum. Solutions with final concentrations of 10 to 100 mmol/L potassium cyanate were used. The potential confounding effect of antibiotics was investigated by incubating serum from healthy subjects with 10 mg/mL amoxicillin at room temperature for 60 min. In addition, as Lys-549 on albumin is susceptible to both carbamylation and glycation by glucose [16], we evaluated the interference of glycated albumin on the shift in electrophoretic mobility by comparing the electrophoresis in diabetics ($n = 73$) with nondiabetics ($n = 166$) matched for CKD stage.

2.6 Statistics

Statistical analysis was carried out using the program MedCalc (MedCalc, Mariakerke, Belgium). Normality of distributions was tested using the D'Agostino Pearson test. Differences between patient groups were assessed using the Mann–Whitney U test and the Kruskall–Wallis test. To investigate the correlation between two nonnormal continuous variables, a rank correlation test was carried, whereby Spearman's rho was calculated. A p value < 0.05 was considered a priori to be statistically significant.

3 Results

In vitro carbamylation (final isocyanate concentration: 10–100 mmol/L) resulted in a dose-dependent alteration of the albumin peak symmetry. Figure 2 depicts the pherograms of noncarbamylated and carbamylated serum and shows the gradual change in albumin peak morphology. The assay showed a good reproducibility with a within-run coefficient of variation was 1.1%. The reference range for the albumin symmetry factor was found to be 0.72–0.92.

In vivo carbamylated serum samples showed similar changes as observed in the in vitro experiments. Figure 3 compares the symmetry of the albumin peaks between controls and the various CKD stages. A significant lower albumin peak symmetry was found with increasing stage of CKD ($p < 0.0001$). In the overall group, the albumin peak asymmetry correlated well with the average serum urea concentration (y (symmetry factor; %) $= -0.001821 \times$ (average serum urea concentration) $+ 0.8307$; $r = -0.5595$, $p < 0.0001$) (Fig. 4). A similar correlation was found with serum creatinine (y (symmetry factor; %) $= -0.02287 \times$ (average serum creatinine concentration) $+ 0.7898$; $r = -0.5986$, $p < 0.0001$).

Besides urea, which is a water-soluble compound, the serum concentrations of several protein-bound uremic toxins showed a significant negative correlation with the albumin symmetry factor: the free and total HA (Spearman's rho: -0.4124; $p = 0.0002$; Spearman's rho: $-0,4134$; $p = 0.0002$), the free and total I × S (Spearman's rho: -0.8179; $p < 0.0001$; Spearman's rho: -0.9943; $p < 0.0001$), the free and total concentration of pCG (Spearman's rho: -0.4144; $p = 0.0002$; Spearman's rho: -0.3984; $p = 0.0003$), the free pCS (Spearman's rho: -0.2308; $p = 0.0435$) and CMPF (Spearman's rho: -0.2712; $p = 0.0163$). Also the patients' age showed a negative correlation (Spearman's rho: -0.4363; $p < 0.0001$). A positive correlation was found between the albumin symmetry factor and stdKt/V (Spearman's rho: 0.2997; $p = 0.0364$).

Evaluation of potential confounding factors showed that in vitro addition of amoxicillin to serum samples from healthy subjects had no influence on the albumin symmetry factor. Comparison of the pherograms of carbamylated serum in diabetics and nondiabetics showed no statistically significant influence of glycated albumin on the observed shift in electrophoretic mobility.

4 Discussion

In the present paper, we have demonstrated for the first time the use of capillary electrophoresis of albumin to assess carbamylation in CKD patients. The carbamylation-induced charge difference is the physical basis of the observed shift in electrophoretic mobility. The broadening of the albumin peak in patients with different stages of CKD points toward an increased heterogeneity of albumin due to posttranslational protein modifications in uremia.

Lys-549 is the preferential locus of carbamylation. Lysine residues carry a positive charge at physiological pH. Attaching a -$CONH_2$ group induces a loss of this positive charge, leading to a change in isoelectric point. Albumin, which is negatively charged at physiological pH migrates during electrophoresis towards the positive electrode because the electro-osmotic flow outweighs the electrophoretic mobility. As albumin

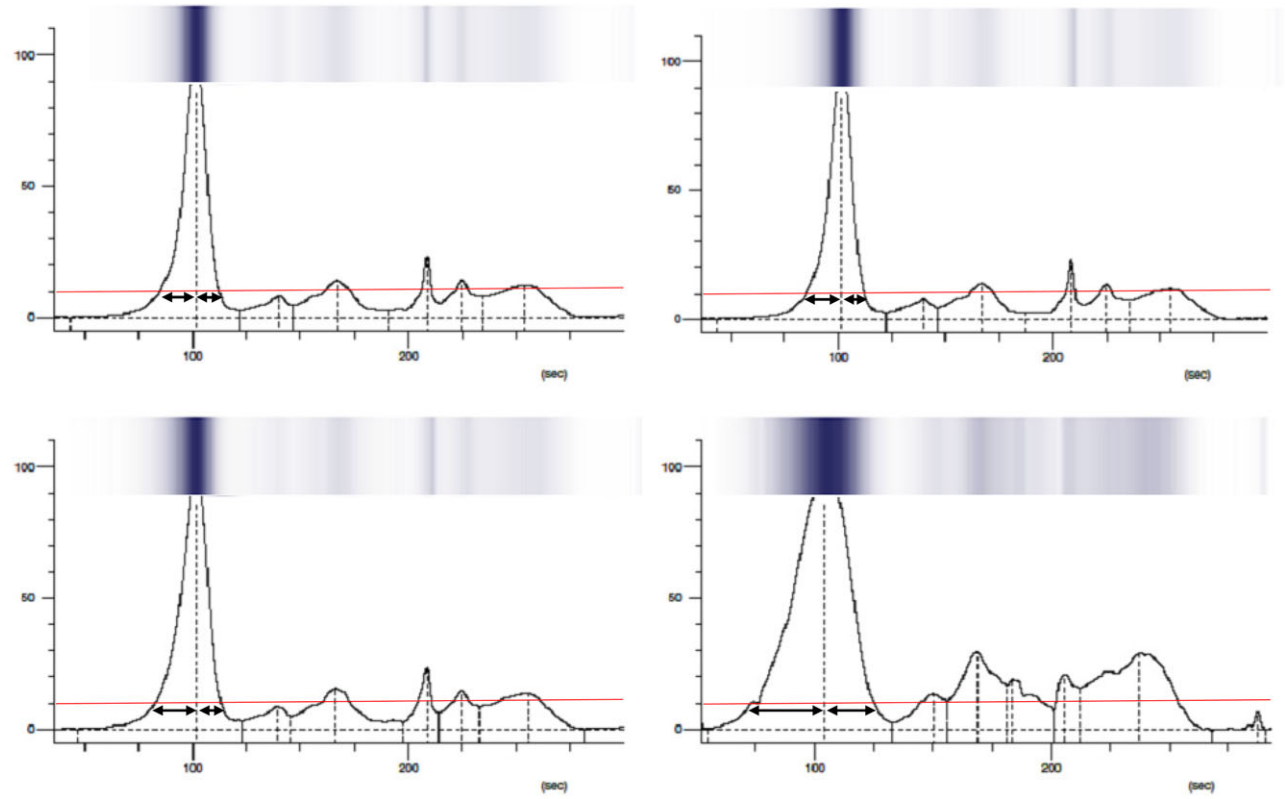

Figure 2. In vitro carbamylation of serum albumin (top left: control sample: symmetry factor 0.630; top right: following incubation with 1 mmol/L KCNO; bottom left: following incubation with 10 mmol/L KCNO; bottom right: following incubation with 150 mmol/L KCNO).

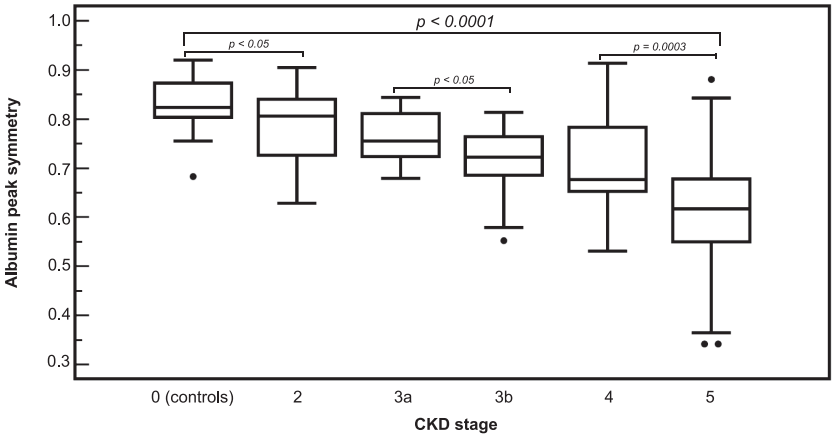

Figure 3. Symmetry factor of serum albumin in controls and CKD stages (2–5) ($n = 239$).

contains Lys-549 residues, carbamylation induces a mixture, in which not every albumin molecule is carbamylated to the same extent or even on the same residues. The various components of such a complex mixture will show a slightly different mobility, which explains the shouldered appearance of the albumin fraction. The elimination of a positive charge, associated with the formation of ε-carbamyl-lysine of homocitrulline, increases the electrophoretic mobility, resulting in a slower migration. Fronting is partially due to the more slowly migration of albumin [17–22]. As Lys-549 is also a common target for glycation by glucose, we evaluated the potential interference of glycated albumin on the albumin symmetry factor. Comparable results were reported in diabetics as in nondiabetics, suggesting that glycation at Lys-549 does not interfere in the observed shift in electrophoretic mobility [16].

Simultaneously, S-carbamylation of free sulfhydryl groups on cysteine and glutathion takes place. The combination of events induces a loss of structural homogeneity, causing peak broadening and explains a lowered symmetry factor [17, 20, 21, 23]. When BSA is incubated with potassium

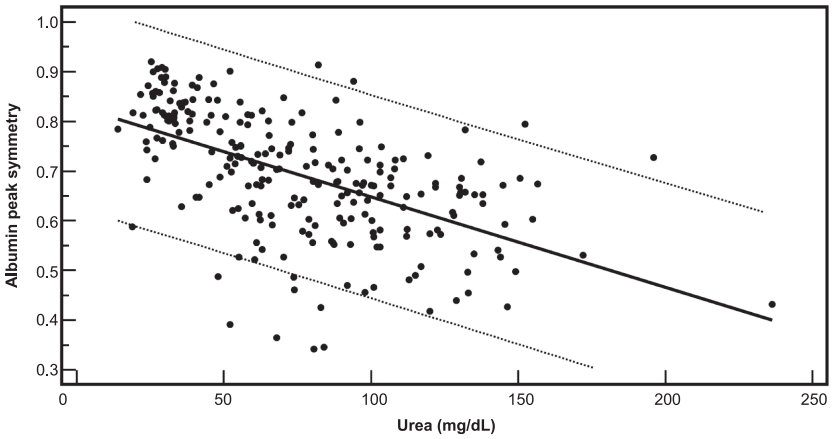

Figure 4. Scatter plot showing the correlation between the symmetry factor of serum albumin (*y*-axis) and the serum urea concentration (*x*-axis) ($n = 239$). The dotted lines represent the 95% prediction-interval. $r = -0.5595$, $p < 0.0001$.

cyanate, an increased Stokes' radius is observed. The elimination of positive charge implies conformational changes, which are due to the disruption of ionic interactions. Mobility of proteins, reflected by electrophoretic mobility, is inversely proportional to the Stokes' radius [24]. As demonstrated in the in vitro experiment, the mobility was not affected by drug-binding, as evidenced by the addition of amoxicillin.

As albumin has a plasma half-life of about 20 days [5], the presented method allows to study plasma protein carbamylation, indicating the average urea concentration with a window of two to four weeks. As albumin concentration is characterized by a very low intraindividual variation (as compared to other plasma protein fractions) [25], interpretation of the test is hardly affected by intraindividual variability. This method is much simpler than the methods classically used in the assessment of carbamylation of serum proteins. The use of capillary electrophoresis to monitor protein carbamylation is innovative. The current used analytical methods are MS, agarose gel electrophoresis and HPLC. As compared to MS, capillary electrophoresis is a very simple, fast, and cheap method. Agarose gel electrophoresis is more labor intensive and time consuming. In addition, capillary electrophoresis is more sensitive and precise, with a comparable specificity. Capillary electrophoresis is also a very efficient and reproducible method for plasma protein separations. Separation is extremely efficient, as evidenced by theoretical plate numbers exceeding 10 000. When comparing capillary electrophoresis to HPLC, a better resolution, a reduced sample preparation, a faster analysis time, reduced reagents consumption and a reduced cost are reported. Another advantage of capillary electrophoresis is the absence of fronting. In HPLC, fronting may occur when the column capacity is exceeded. Moreover, capillary electrophoresis combines high throughput and sensitive analyses, and the technique can be automated. Disadvantages are a lower sensitivity and reproducibility compared to HPLC [26].

Besides urea, which is a water-soluble uremic toxin that mediates its toxicity through carbamylation, several protein-bound uremic toxins (HA, IxS, pCS, pCG, CMPF) showed a negative correlation with the albumin peak symmetry. Worsening urea clearance is associated with an increased protein carbamylation and with the accumulation of uremic toxins [27]. As expected, a rather poor correlation with stdKt/V was observed. Although stdKt/V is considered as a baseline parameter of dialysis adequacy, stdKt/V is a too simple concept for the complexities of uremia and of today's dialysis [28]. Based on the findings in the present study, quantification of carbamylated albumin using capillary electrophoresis may be an alternative method to monitor long-term complications of carbamylation and mortality over an extended period. The observed negative correlation between age and symmetry factor can be explained by the fact that protein carbamylation may be considered as a hallmark of aging in mammalian species with a significant influence on the structural and functional tissue damages encountered during aging [29].

A first limitation of the presented method is that although the in vitro experiments have clearly demonstrated that carbamylation is responsible for the shift in the albumin peak symmetry, quantification of carbamylation-derived products (e.g. homocitrulline) was not performed [17, 30]. A second limitation is that recent administration of albumin infusions could theoretically affect the obtained results.

In conclusion, the symmetry factor of serum albumin determined by capillary electrophoresis is a promising alternative biomarker for monitoring protein carbamylation in patients with renal insufficiency. Determining conventional plasma biomarkers like urea and creatinine do not allow clinicians to precisely evaluate the risks due to carbamylation in individual patients. Implementation of simple, affordable methods like the described one may therefore be very helpful in the management of advanced chronic kidney disease.

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The authors have declared no conflict of interest.

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