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Carbamylation of Serum Albumin as a Risk Factor for Mortality in Patients with Kidney Failure

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Abstract

Urea, the toxic end-product of protein catabolism, is elevated in end-stage renal disease (ESRD), although it is unclear whether or how it contributes to disease. Urea can promote the carbamylation of proteins on multiple lysine side chains, including human albumin, which has a predominant carbamylation site on lysine 549. The proportion of serum albumin carbamylated on Lys-549 (%C-Alb) correlated with time-averaged blood urea concentrations and was twice as high in ESRD patients than in non-uremic subjects (0.90% vs. 0.42%, P<0.0001). Baseline %C-Alb was higher in ESRD subjects who died within 1-year than in those who survived longer than 1 year (1.01% vs. 0.77%, P<0.001) and was associated with an increased risk of death within 1 year (HR of 3.76, 95% CI: 2.20–6.43, P<0.0001). These findings were validated in an independent cohort of diabetic ESRD subjects (HR 3.73, 95% CI: 2.00–6.96, P<0.001). Decreased concentrations of serum amino acids correlated with higher %C-Alb in ESRD patients, and mice with diet-induced amino acid deficiencies exhibited greater susceptibility to albumin carbamylation than did chow-fed mice. *In vitro* studies showed that amino acids such as cysteine, histidine, arginine, lysine, as well as other nucleophiles such as taurine, inhibited cyanate-induced

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C-Alb formation at physiologic pH and temperature. Together, these results suggest that chronically elevated urea promotes carbamylation of proteins in ESRD, and that serum amino acid concentrations may modulate this protein modification. In summary, we have identified serum %C-Alb as a risk factor for mortality in patients with ESRD and propose that this risk factor may be modifiable with supplemental amino acid therapy.

INTRODUCTION

Chronic kidney disease (CKD) affects 5–10% of adults in industrialized countries (1). For reasons that remain unclear, individuals with CKD are 10–20 times more likely to die from cardiovascular causes than to survive until renal function is completely lost (2). Those who reach end stage kidney disease (ESRD) suffer an annual mortality of 15–20% that is largely attributable to cardiovascular disease (CVD) (3). Yet, efforts to treat the most modifiable cardiovascular risk factor, hypercholesterolemia, with statins have not improved outcomes in ESRD (4, 5). This finding suggests that other mechanisms link ESRD to CVD (6).

One possible mechanism for how ESRD increases the risk of CVD is the accumulation of urea in the blood of ESRD patients. Urea is generated in the liver during catabolism of amino acids and other nitrogenous metabolites and is normally excreted into the urine by the kidneys as rapidly as it is produced. Patients with ESRD cannot make urine, however, and thus increasing concentrations of blood urea will steadily accumulate, a condition that can only be treated with intermittent hemodialysis (HD) or kidney transplantation. Although HD ameliorates ESRD patients' uremia, it replaces only ~10% of normal renal function, however, so these patients still have chronic urea overload (uremia).

Despite the strong association between ESRD and CVD, the role of chronically elevated urea in this disease is controversial. For example, average urea concentration does not predict mortality in CKD, and the HEMO study found no benefit for survival when frequency of HD was increased beyond the current standards (7–10). This lack of effect could have been because the ~40% relative increase in waste removal only replaces ~14% of normal kidney filtration and still leave patients overloaded with waste products. Recent evidence suggests that chronically elevated blood urea contributes directly to cardiovascular risk via a pro-atherogenic protein modification called carbamylation. In one study of patients undergoing diagnostic cardiac catheterization, subjects in the highest quartile of serum protein-bound carbamylated lysine had a 7–8 times higher risk of CVD; these authors also demonstrated that low density lipoprotein (LDL) was a target for protein carbamylation and that carbamylated LDL binds scavenger receptors and produces lipid accumulation in macrophages. (11) In addition, feeding urea to ApoE-deficient mice accelerated their rate of atherosclerosis nearly two-fold and increased accumulation of carbamylated LDL within atherosclerotic plaques (12). Together these findings suggest a potential mechanism for urea's direct contribution to atherogenesis.

Protein carbamylation is an unavoidable consequence of excess urea (13, 14). Urea is in equilibrium with cyanate (HNCO), a product of urea deamination whose central carbon is susceptible to nucleophilic attack from amines and thiols at the N-termini or side chains of proteins in vivo (15). The degree to which proteins with long half-lives are carbamylated should therefore provide a time-averaged indicator of urea concentration, analogous to the relationship between serum glucose and glycated hemoglobin (16). Thus measurements of carbamylated proteins in circulation might provide a quantitative biomarker that is mechanistically linked to urea kinetics and the effectiveness of urea reduction therapy. If carbamylated proteins are indicators of chronic uremia and also direct contributors to atherogenesis and cardiovascular pathology (11, 12), then concentrations of circulating

carbamylated proteins should be closely correlated with ESRD patients' cardiovascular health and overall mortality. We therefore measured carbamylation of the long-lived circulating protein albumin and tested whether albumin carbamylation correlated with outcomes in two independent cohorts of patients with ESRD. Furthermore, we tested whether higher protein carbamylation is associated with increased average blood urea concentrations and whether protein carbamylation can be competitively inhibited by free amino acids.

RESULTS

A preferred carbamylation site on albumin and validation of a high-throughput assay

To map sites on circulating albumin susceptible to carbamylation, cyanate-treated albumin was digested with proteinase, digested peptides were separated by high performance liquid chromatography (HPLC), and peptides were analyzed for carbamylation modifications with tandem mass spectrometry. Several carbamylation modifications were detected, the most abundant on lysine 549 (Fig. 1). The relative amount of carbamylation at each site was quantified by measuring its carbamylated and noncarbamylated peptide forms with multiple reaction monitoring (MRM) and then calculating their ratio (Table S1). The susceptibility to carbamylation at Lys-549 is consistent with reports that Lys-549 is also a common target for glycation (17). We then developed a high-throughput assay that incorporated isotopic peptide standards to reproducibly quantitate the proportion of albumin carbamylated on Lys-549 (%C-Alb) as described in the methods (Fig. S1). Using this assay, we demonstrated that average %C-Alb was approximately 2-fold higher in patients with CKD and ESRD than in non-uremic subjects (Fig. 2). Furthermore, %C-Alb values were highly correlated with the proportional amounts of carbamylated lysine in the unfractionated serum proteins of uremic patients and were correlated with amounts of % carbamylated hemoglobin in these patients (Fig. S2). These correlations corroborate the association between %C-Alb and global carbamylation of blood proteins in uremic subjects.

Thus, Lys-549 is the most frequently carbamylated amino acid on human albumin, although there are additional sites that are less susceptible to carbamylation. Furthermore, we developed a high-throughput assay for measuring carbamylated albumin that can be used in large clinical studies and confirmed that %C-Alb values were elevated in patients with uremia.

Association of %C-Alb with mortality in patients on hemodialysis

%C-Alb was measured in serum from 187 participants from the Accelerated Mortality in Renal Replacement (ArMORR) study (18, 19). Subjects included 81 ArMORR participants who died from any cause during 12 months of follow-up (ArMORR cases) and 106 specimens from ArMORR subjects who survived longer than 365 days (ArMORR survivors). The 12 month follow-up period began 90 days after initiation of HD. Subjects' baseline clinical characteristics and biochemical analyses were measured on day 90 of the study run-in period and are presented in Table S3. As shown in Fig. 2, ArMORR cases who died during follow-up had significantly higher average baseline %C-Alb values than did ArMORR survivors (1.01% vs. 0.77%, P = 0.0009, Fig. 2). Nevertheless, the concentration of urea in their blood was not different from that of ArMORR survivors (16.9 vs. 17.3mmol/L, respectively, P = 0.72), suggesting that factors other than urea concentration may also regulate %C-Alb values.

We analyzed %C-Alb values for their association with 12-month mortality by using Kaplan-Meier survival analysis (20), which showed statistically significant higher mortality for ArMORR subjects in the highest %C-Alb tertile than for subjects with less %C-Alb (Fig. 3).

Using a Cox proportional hazards model (21), we found that increased %C-Alb values were associated with significantly increased risk of death (HR 3.76 (95% CI: 2.20–6.43), P<0.0001) (Table 1). Statistically significant risks of death were also associated with decreased albumin, decreased hemoglobin, and a history of hypertension, which are all known risk factors for death in patients with ESRD. Nevertheless, the risk of death associated with %C-Alb remained strong and statistically significant even after adjusting for these other risk factors (HR 3.23 (95% CI: 1.74–6.00), P = 0.0002). There was no significant risk associated with the subjects' baseline equilibrated Kt/V values, a measure of urea reduction and dialysis adequacy (22). Furthermore, when we excluded ArMORR survivors from the model and stratified just the ArMORR cases by their %C-Alb values, we observed that the highest %C-Alb tertile within this subgroup was associated with an increased hazard rate compared to the cases in the bottom two tertiles (univariate HR 1.56 (95% CI: 1.00–2.43), P = 0.04). This indicated that even amongst the cohort ArMORR subjects that died during the 12-month study there was an association between higher %C-Alb values and shorter survival times.

To validate the major findings from the ArMORR cohort, we tested whether baseline %C-Alb was associated with mortality in a second independent study of HD subjects. We obtained frozen specimens from 1,161 ESRD patients who had participated in the 4D hemodialysis trial, a randomized controlled trial of the benefits of cholesterol-lowering agents in HD patients with diabetes mellitus (4, 23). Baseline characteristics of 4D subjects are shown in Table S4. Similar to the ArMORR study, %C-Alb was measured in specimens sampled at the beginning of the study and analyzed for associations to survival during a 12 month follow-up period. Kaplan Meier survival analysis and multivariable HR analysis of %C-Alb and Kaplan Meier survival analysis again found significant risk of death among 4D subjects with elevated %C-Alb (Table 2 and Fig. 3), with HR estimates similar to those observed in the ArMORR study. When we analyzed the risk of death in ArMORR and 4D subjects stratified into tertiles according to their %C-Alb values, there was again a gradation of risk between subjects stratified by their %C-Alb values (Table S5).

Kidney disease is frequently associated with diabetes mellitus; it is thus possible that risks associated with diabetes mellitus may confound the association between %C-Alb and risk of death. Comparison of the univariate and multivariate HRs associated with %C-Alb showed that adjustment for ArMORR subjects' history of diabetes mellitus or 4D subjects' duration of diabetes mellitus did not significantly change the risk associated with %C-Alb for either of these studies (Tables 1 **and** 2), however. In summary, data from two large independent clinical trials suggested that %C-Alb may be an independent risk factor for mortality in ESRD patients.

%C-Alb as an Index of urea overload and circulating free amino acids

To test whether nucleophilic amines and thiols of free amino acids compete with the side chains of albumin for carbamylation, we measured free amino acids and their carbamylated forms in ArMORR study subjects and analyzed their %C-Alb values as a composite function of serum free amino acid and urea concentrations. We observed a modest positive correlation between time-averaged urea and %C-Alb in ArMORR subjects (Fig. 4A) and a similar correlation between urea and %C-Alb in 4D study subjects (Fig. 4B). The correlation between urea and %C-Alb was significantly stronger in patients with stage 3 or 4 CKD than in either ESRD cohort (Fig. 4C).

In contrast to the positive correlation between blood urea concentrations and %C-Alb, individual serum free amino acid concentrations in our ArMORR subjects were negatively correlated with %C-Alb, suggesting an association between low free amino acid concentrations and high serum protein carbamylation (Table 3). To test whether free amino

acid levels could influence the correlation between uremia and higher %C-Alb, we examined these relationships whle adjusting for the other variables. In most cases we found stronger correlations between %C-Alb and urea after adjusting for amino acid concentrations (Table 3). Together, these studies suggest that increased urea and decreased amino acid concentrations are independently correlated with protein carbamylation in ESRD patients.

Susceptibility of different amino acids to carbamylation in vitro

We postulated that serum amino acids may correlate with %C-Alb because they are alternative substrates for carbamylation by cyanate. To differentiate the cyanate-scavenging ability of individual amino acids, we added cyanate to an equimolar mixture of all twenty amino acids. As expected, increasing concentration of cyanate produced linear increases in all carbamylated amino acids (Fig. S3(A)) and depletion of their unmodified forms (Fig. S3(B and C)). The percent depletion for each amino acid in the presence of 5 mM cyanate is a quantitative index of their relative avidities within this competitive carbamylation reaction (Fig. S3(D). The dipeptide glycylglycine was more strongly carbamylated than glycine or other amino acids. These experiments demonstrated the comparative susceptibilities and competition among individual amino acids for carbamylation and suggested that amino acids may compete with proteins for carbamylation as well.

Prevention of albumin carbamylation by amino acids and other nucleophiles

To test whether competing nucleophiles can prevent albumin carbamylation by cyanate, we then tested whether individual amino acids can reverse carbamylation of purified serum albumin. Although all amino acids that we tested were able to inhibit carbamylation, some amino acids (cysteine, histidine, and arginine) were more effective inhibitors than others(Table 4). The dipeptide glycylglycine reduced the amount of albumin carbamylation by 63%. Taurine, the most abundant intracellular amine, also effectively scavenged cyanate. These results demonstrated that carbamylation of albumin can be counteracted by free amino acids and other endogenous nucleophilic biomolecules.

Protein carbamylation in Mice with amino acid deficiencies

To further test our hypothesis that amino acid deficiencies cause animals to be more susceptible to protein carbamylation, we induced amino acid deficiencies by feeding mice low protein diets for 15 days. Mice were also treated with either cyanate or urea to induce protein carbamylation. In the first experiment, we tested the effect of a low protein diet on acute protein carbamylation: on day 15 animals were injected with cyanate; 30 minutes after injection blood was drawn and tested for serum %C-Alb and free amino acid concentrations. Animals on the low protein diet displayed significantly lower serum essential amino acid concentrations than did control animals fed a normal protein diet (Fig. 5A). When the amino acid-deficient mice were then injected with cyanate, they showed 2-fold greater increases in %C-Alb values than did the animals on the normal protein diet (Fig. 5(B)).

In the second mouse experiment, the effects of chronically elevated blood urea were tested by adding urea to the animals' food. Four groups of mice were fed for 15 days with low or normal protein diets mixed with urea in doses roughly equivalent to the animals' normal daily urea output (24). On day 15, blood was drawn and serum was analyzed for %C-Alb and free amino acids. As shown in the amino acid-deficient mice fed the low protein diet along with urea had significantly increased %C-Alb compared to urea-fed animals on the normal protein diet Fig. 5(C),. In summary, mouse models of both acute and chronic albumin carbamylation confirmed that animals with amino acid deficiencies experience dramatically increased degrees of carbamylation in the presence of equivalent doses of cyanate or urea.

Potential confounders of %C-Alb measurements

Kidney disease is frequently associated with diabetes mellitus and hyperglycemia. Lys-549 on albumin is susceptible to both carbamylation and glycation by glucose (17). To evaluate whether glycated albumin interferes with accurate assessment of %C-Alb values, we modified our %C-Alb assay to also measure glycation of Lys-549. We analyzed %C-Alb and % glycated albumin in 40 serum samples from patients with both diabetes mellitus and chronic or end-stage renal disease. We observed no statistically significant correlation between carbamylated and glycated albumin values, however, suggesting that glycation at Lys-549 does not interfere with the carbamylation reaction at that same site (Fig. S4). Furthermore, multivariable-adjusted HR analysis demonstrated that the association between %C-Alb and mortality was independent of history or duration of diabetes mellitus (Tables 1 **and** 2).

Calculation of %C-Alb includes the measured concentration of non-carbamylated albumin in the denominator. Low serum albumin concentration (hypoalbuminemia) is a known risk factor in patients with renal disease (25), and so we considered whether the risk associated with %C-Alb was confounded by the effects of low serum albumin concentrations. ArMORR subjects' %C-Alb values and serum albumin concentrations showed no significant correlation to each other, however (Fig. S5).

In summary, evaluation of important risk factors associated with ESRD (diabetes mellitus and hypoalbuminemia) showed no correlations between these variables and %C-Alb values and no confounding effects by these variables on the association between %C-Alb and risk of death.

DISCUSSION

We hypothesized that serum protein carbamylation reflects a balance between urea load from kidney filtration failure and amino acid deficiency arising from multiple factors, including the under-nourishment common to CKD (26). To study this, we developed an assay to quantify the carbamylation of serum albumin *in vivo* and applied this method to subjects with ESRD, a condition of chronic urea overload. We analyzed samples from two previously published large independent cohorts and found that %C-Alb was strongly associated with one-year mortality in patients with ESRD undergoing hemodialysis. Furthermore, the association between %C-Alb and 1-year mortality was undiminished even after adjustment for other significant risk factors for mortality (age, body mass index, history of coronary artery disease, congestive heart disease, peripheral vascular disease, atrial fibrillation, hypertension, systolic blood pressure, history and duration of diabetes mellitus, blood hemoglobin concentration, serum albumin, transferrin saturation, and blood leukocyte counts. In contrast, we found that standard measures of dialysis adequacy (eKt/V and urea reduction ratio) were not associated with risk of death. We also examined the effect of free amino acids on %C-Alb in vitro. In studies in human subjects, amino-acid deficient mice, and *in vitro* experiments, we have gathered evidence in support of the conclusion that low free amino acid concentrations and high urea load combine to favor formation of carbamylated albumin. We have identified select amino acids (cysteine, histidine) and similar nucleophilic biomolecules (glycylglycine, taurine, cysteamine) that can act as potent inhibitors of protein carbamylation. Together, these data identify %C-Alb as a possible prognostic indicator for adverse outcomes in ESRD and suggest that the levels of this indicator may be modified by free amino acid supplementation in patients.

Carbamylation of free amino acids and proteins by urea-derived cyanate was first described in 1960 (15), has been studied primarily in kidney failure and uremia (11, 12, 14, 27–40) and was reviewed recently in (41). Whether excess urea is pathogenic has been a

controversial issue. Medical textbooks often assert that urea is relatively non-toxic, but the evidence most often cited for this claim is a 40-year-old pilot trial of three dialysis subjects who were loaded with excess urea over a period of 90 days and monitored for acute symptoms of uremia. Although the acute symptoms of urea loading were relatively minor, the study did not include any follow-up for long term effects (7). Opinions regarding urea's toxicity by the medical community were also influenced by the HEMO clinical trial, in which it was found that increasing hemodialysis dose (as indicated by eKt/V) was ineffective for improving survival in patients (10, 42). The results from HEMO may be uninformative because more frequent dialysis had a relatively small effect on urea removal, however, so it is not surprising that no survival benefit could be detected (43). More recent studies testing the effects of increased frequency of hemodialysis—comparing daily dialysis to the standard prescription of 3 times per week—did show benefits for the subjects (44, 45). Thus, it now appears that the toxicity of urea may have been dismissed prematurely.

The most common cause of death in our study subjects (and in ESRD patients in general) was a cardiac event, and a growing body of evidence has demonstrated that protein carbamylation is associated with risk of cardiovascular disease in patients. Patients undergoing cardiac catheterization who had increased serum carbamylated proteins were at significantly increased risk of cardiovascular disease, coronary heart disease, and peripheral vascular disease (11), and higher carbamylated lysine content in serum proteins—a marker of protein carbamylation—was significantly associated with 5-year mortality in uremic patients on hemodialysis (46). Studies from tissue culture and animal models also suggest that urea and protein carbamylation may contribute to cardiovascular disease. First, carbamylated albumin appears to be pro-inflammatory (47). Second, carbamylated proteins are highly enriched in atherosclerotic plaques, and carbamylated LDL (cLDL) may be a pathogenic ligand for foam cells (11, 28). In addition to transporting cholesterol into atherosclerotic plaques, cLDL also induces endothelial cell death, vascular smooth muscle cell proliferation, monocyte inflammatory signaling and endothelial/monocyte cell adhesion, each of which may exacerbate atherogenesis even further (27, 28, 35). More significantly, when nephrectomized ApoE-null mice were given supplemental urea in their drinking water, their rate of atherogenesis increased 2-fold. (12).

We have shown that amino acid concentrations can modify protein carbamylation rates *in vitro* and in animals. If protein carbamylation is contributing to cardiovascular disease in ESRD patients, amino acid supplementation may represent a valuable therapeutic approach to improving survival in these patients. Free amino acids are important nucleophiles that may be quantitatively consumed by carbamylation in patients with ESRD (33, 34). N-carbamylation neutralizes amino acids' ability to scavenge oxidants such as hypochlorous acid (48). S-carbamylation also neutralizes the antioxidant properties of thiol amino acids and glutathione (48). Consumptive amino acid carbamylation may thus contribute to oxidative stress. Conversely, treatment of patients with free amino acid scavengers may counteract not only protein carbamylation but also combat the buildup of oxidants in CKD and ESRD.

Herein we have also presented evidence that %C-Alb values correlate significantly with concentrations of urea in the blood. %C-Alb may provide a clearer index of average urea concentrations than isolated blood urea measurements because blood urea can fluctuate 40–70% around any given dialysis session. Albumin has a half-life of approximately 2 weeks in ESRD patients, and carbamylated albumin would therefore represent an index of the time-averaged urea/amino acid balance (49).

It is important to note the limitations of the present study as well as the remaining questions that warrant further investigation: First, our experiments do not test whether circulating

carbamylated albumin has any direct pathologic role in cardiovascular disease or mortality. Recent experiments have shown pathogenic effects by carbamylated albumin ligand on certain tissues (47), but further investigation is required to determine if a receptor-mediated signaling cascade is involved or if the effects of carbamylated albumin contribute to uremia-associated sequelae *in vivo*. Second, our outcome studies focused on 1-year mortality in ESRD patients receiving hemodialysis. Further studies are required to determine whether %C-Alb is also a risk factor for patients in earlier stages of CKD. As shown in Fig. 2A, average %C-Alb values are elevated in CKD and ESRD patients compared to non-uremic controls, and were largely equivalent between the hemodialysis and pre-dialysis CKD cohorts . This indicates that "hyper-carbamylation" is present throughout the stages of kidney disease before and after initiation of hemodialysis. Further studies are required to determine whether carbamylation is pathogenic at earlier stages of disease and whether pre-dialysis CKD patients may also benefit from amino acid therapy or treatment with pharmacological carbamylation scavengers. Third, although the human observational studies, animal model and *in vitro* experiments described here suggest a role for free amino acids in regulating protein carbamylation, they do not demonstrate whether amino acid therapy in human patients will reduce protein carbamylation. Human interventional studies in human ESRD subjects are needed to test whether protein carbamylation (high %C-Alb) decreases in response to free amino acid supplementation or other nucleophilic scavengers and whether these reductions in protein carbamylation are associated with improved patient survival. Furthermore, although our *in vitro* studies of albumin carbamylation suggest that certain amino acids are more effective scavengers than others, our studies do not address what kinds of scavengers would be most effective in human patients. Differences in nucleophilicity and reactivity to cyanate, differences in pharmacokinetics, and differences in the safety and tolerability of various amino acid scavengers could affect their therapeutic effectiveness. Additional studies are needed to find the optimal combination of amino acids for the prevention of protein carbamylation. Finally, although our results show that high %C-Alb is a significant risk factor for mortality in HD patients, our results cannot be used to interpret %C-Alb measurements in the clinical setting or predict the risk of death in individual ESRD patients. Additional prospective clinical trials to better define the prognostic value of this test and it's ability to predict response to therapy are needed for this blood test to be translated for clinical use.

In summary, our study describes an assay for protein carbamylation (%C-Alb) that was strongly and more reproducibly associated with risk of death than other standard risk factors in two large independent ESRD cohorts. Amino acid deficiencies in ESRD patients may exacerbate protein carbamylation, suggesting an easily modifiable mechanism that contributes to protein carbamylation. Protein carbamylation may thus represent a new modifiable risk factor for mortality in ESRD, providing an attractive target for future therapeutic trials.

MATERIALS AND METHODS

Materials: All chemicals, except when noted, were purchased from Sigma-Aldrich.

Study Populations

Frozen specimens from 187 participants of the Accelerated Mortality on Renal Replacement (ArMORR) study and 1,161 subjects of Die Deutsche Diabetes Dialyze Studie (4D trial) were used for analysis (4, 18, 19, 50). ArMORR subjects' baseline biochemical data and characteristics (Table S3) were measured after the 90 day run-in period and just prior to initiation of the survival study. 4D subjects baseline characteristics and chemistries (Table

S4) were collected one week prior to subject randomization. Additional details on ArMORR and 4D study populations can be found in Supplementary Materials.

Biochemical Analyses

Instrumentation: All LC-MS/MS analyses were performed using reverse phase chromatography coupled to tandem mass spectrometry. The experiment shown in Fig. 1 was performed on an API 5600 time-of-flight mass spectrometer; experiments shown in Table S1 were performed on an API 5000 triple quadrupole mass spectrometer. All other measurements of carbamylated albumin, glycated albumin, carbamylated hemoglobin, and serum amino acids were measured on an API 3200 mass spectrometer. All instruments were purchased from AB Sciex. **Measurement of human serum %C-Alb:** Serum samples were mixed with isotopic peptide standards then digested with Glu-C protease for peptide analysis by LC-MS/MS. Digested serum proteins were injected onto a C18 reverse phase column and peptides were separated by gradient elution using conditions described in Table S9. The carbamylated and non-carbamylated peptides of interest and their isotopic standards were measured by MRM monitoring using MS/MS mass transitions and settings are shown in Table S9, and the and quantified according to their area under the curve (AUC) of the eluting peaks (Fig. S1). Intra-assay measurement of the 5% carbamylated isotopic peptide standard mix was used to adjust and calibrate assay measurements so that the absolute %C-Alb could be calculated using the following equation: ***%C-Alb = 5% × [Carbamylated albumin AUC ÷ Non-carbamlyated albumin AUC] ÷ [Carbamylated isotopic standard AUC ÷ Non-carbamylated isotopic standard AUC]***. The %C-Alb assay demonstrated excellent linearity ($r^2 = 0.9964$, $y = 0.9826x - 0.0002$) (51) and coefficient of variation of 4.2% (Fig. S1). Replicate measurements (N = 8) of a random serum sample demonstrated a standard deviation of 0.06% (CV = 4.2%). Details describing specimen processing and additional methods for biochemical analysis of mouse %C-Alb, glycated albumin, carbamylated albumin, and amino acid analysis can be found in the Supplementary Materials.

In vitro experiments

Search for carbamylation sites on human albumin: Purified human albumin (40 g/L) dissolved in phosphate-buffer pH 7.4 was carbamylated overnight with 1 mM potassium cyanate. Albumin was reduced with DTT, heat denatured, and digested with Glu-C protease. Digested peptides were analyzed on an API 5600 mass spectrometer. MS/MS fragmentation spectra were matched against the SwissProt human proteome using Protein Pilot software (AB Sciex). **Relative quantification of carbamylation sites on cyanate-treated and untreated albumin:** Serum from an ESRD patient was treated with and without 10 mM potassium cyanate and digested with Glu-C. % carbamylation on different lysines within albumin were performed by measuring each lysine-containing albumin peptide and its carbamylated form using mass settings shown in Table S1, integrating their AUC values and calculating the ratios of the carbamylated and non-carbamylated forms. **Carbamylation of amino acid mixtures *in vitro*:** Cyanate was titrated into equimolar mixtures of purified amino acids (250 micromoles/L) in 100 mM phosphate buffer pH 7.4 and carbamylated amino acids were analyzed by LC-MS/MS. ***In vitro* inhibition of albumin carbamylation by amino acids:** Purified albumin (40 g/L) was pre-mixed with phosphate buffer pH 7.4 (100 mM final conc.) and 10 mM of individual amino acids, followed by addition of 0.5 mM cyanate and overnight incubation at 37°C, then analysis of %C-Alb by LC-MS/MS.

Mouse model of amino acid deficiency and cyanate-induced carbamylation: Two groups of 10-week old male C57BL/6J mice (n = 6 per group) were given a low protein diet for 15 days in order to induce serum amino acid deficiencies (52). Two additional control groups were given feed with normal protein content. Diets were prepared by Research Diets,

Inc. and were based on rodent diet AIN-76A. The normal protein diet contained 20% casein. The low protein diet contained 2% casein and was made isocaloric to the normal diet by supplementing with sucrose. *Chronic carbamylation by urea:* One group of mice on low protein and one group on normal protein diet also had urea added to their feed (67 mg urea per gram of feed) throughout the 15 day study. *Acute carbamylation by cyanate:* On day 15 of the study, one group of mice on low protein diet and one group on normal protein diet were were anesthetized with isoflurane, had blood drawn, and were injected intraperitoneally with sodium cyanate in order to induce carbamylation (100 mg cyanate per kg body weight) (53). 30 minutes after injection animals had blood drawn again followed by euthanasia by carbon dioxide asphyxiation. Animals were anesthetized throughout the procedure. After blood was drawn, samples were allowed to clot and serum was separated, snap frozen in liquid nitrogen, and frozen at −80°C for later analysis. Samples were analyzed for %C-Alb and free amino acid concentrations. All procedures were performed according to the IACUC-approved protocol no. 051–2012 / 101118 (Animal Welfare Assurance #A3153-01).

Statistical Analysis: SAS software, version 9.2 (SAS Institute), was used for all analyses. Comparisons between characteristics of subject groups were analyzed using Student's t-test or Mann-Whitney-U tests or Chi-Squared Tests where appropriate. The Kaplan–Meier product-limit method was used for visualization of time-to-event variables. Analysis of all-cause mortality was performed with the use of Cox proportional-hazards models. Proportional hazard assumptions were checked for all models. %C-Alb values were transformed to their natural log and analyzed as a continuous variable for HR analysis. The variables included in the final multivariate model were limited to just those which demonstrated statistically significant risks ($P<0.05$) during univariate HR analysis. Associations between carbamylated albumin and total amino acids were analyzed using Spearman's rank correlation coefficients and partial correlations were used to adjust for relevant covariates. Multiple correlations were assessed using multiple linear regression for natural log transformed outcomes and covariates. Overall significance of comparisons of carbamylated albumin by amino acid tertile were performed using Kruskal-Wallis Tests, and significant associations were further explored using Sidak adjusted, Wilcoxon Rank Sum Tests. Two-tailed P-values of <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A *Protein Pilot MS/MS Peptide Identification Summary Report*

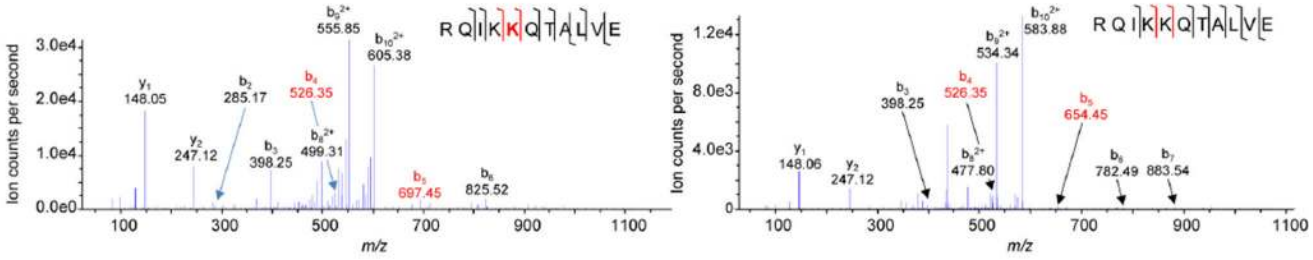
Untreated human albumin

Contrib	Conf	Sequence	Modifications	ΔMass	Prec MW	z	Spectrum
2.00	99	RQIKKQTALVE		0.0086	1312.7914	3	1.1.1.4129.4

Human albumin treated with 10 mM cyanate

Contrib	Conf	Sequence	Modifications	ΔMass	Prec MW	z	Spectrum
2.00	99	RQIK**K**QTALVE	Carbamyl(K)@5	0.0047	1355.7931	3	1.1.1.4161.4

B



C

Carbamylated peptide @ m/z = +1355.79

Residue	b	b+2	y	y+2
R	157.1084	79.0578	1356.7958	678.9015
Q	285.1670*	143.0871	1200.6947	600.8510
I	398.2510	199.6292	1072.6361	536.8217
K	526.3460	263.6766	959.5520	480.2796
K[CRM]	697.4468	349.2270	831.4571	416.2322
Q	825.5053	413.2563	660.3563	330.6818
T	926.5530	463.7802	532.2977	266.6525
A	997.5901	499.2987	431.2500	216.1287
L	1110.6742	555.8407	360.2129	180.6101
V	1209.7426	605.3749	247.1288	124.0681
E	1338.7852	669.8962	148.0604	74.5339

Unmodified peptide @ m/z +1312.79

Residue	b	b+2	y	y+2
R	157.1084	79.0578	1313.7900	657.3986
Q	285.1670	143.0871	1157.6888	579.3481
I	398.2510	199.6292	1029.6303	515.3188
K	526.3460	263.6766	916.5462	458.7767
K	654.4410	327.7241	788.4512	394.7293
Q	782.4995	391.7534	660.3563	330.6818
T	883.5472	442.2772	532.2977	266.6525
A	954.5843	477.7958	431.2500	216.1287
L	1067.6684	534.3378	360.2129	180.6101
V	1166.7368	583.8720	247.1288	124.0681
E	1295.7794	648.3933	148.0604	74.5339

*Predicted ions found in spectra are shown in red

Fig. 1.

Identification of carbamylation on Lys-549 of cyanate-treated albumin. Cyanate-treated and untreated purified human albumin from a commercial source was digested with glutamyl endoproteinase and analyzed by LC-MS/MS. Peptides were identified by matching fragmentation spectra to sequences from the human SwissProt proteome with the AB Sciex Protein Pilot software programmed to search for both carbamylated and non-carbamylated peptide forms. (A) Summary reports for the carbamylated and non-carbamylated forms of the peptide encompassing lysine residue 549 (sequence **RQIKXQTALVE** where **X** = N-ε-carbamoyl-L-lysine). Contrib, contribution of identified peptide (in ProtScore units) to protein identification; Conf, percent confidence of peptide identification; z, peptide charge; Spectrum, within-run identifier for MS/MS spectra used for peptide identification. (B) MS/MS spectra for carbamylated and non-carbamlyated digested albumin peptides encompassing Lys-549; the modification state-revealing fragment ions are shown in red. (C) Predicted ion fragments of carbamylated and non-carbamylated peptide forms; detected fragment ions matching the predicted fragmentation spectra are highlighted in red. Both peptides were identified with 99+% confidence using the Paragon algorithm, which compares the confidence of the match between the observed spectra and the identified peptide sequence to the combined confidence for all other possible peptide matches.(54).

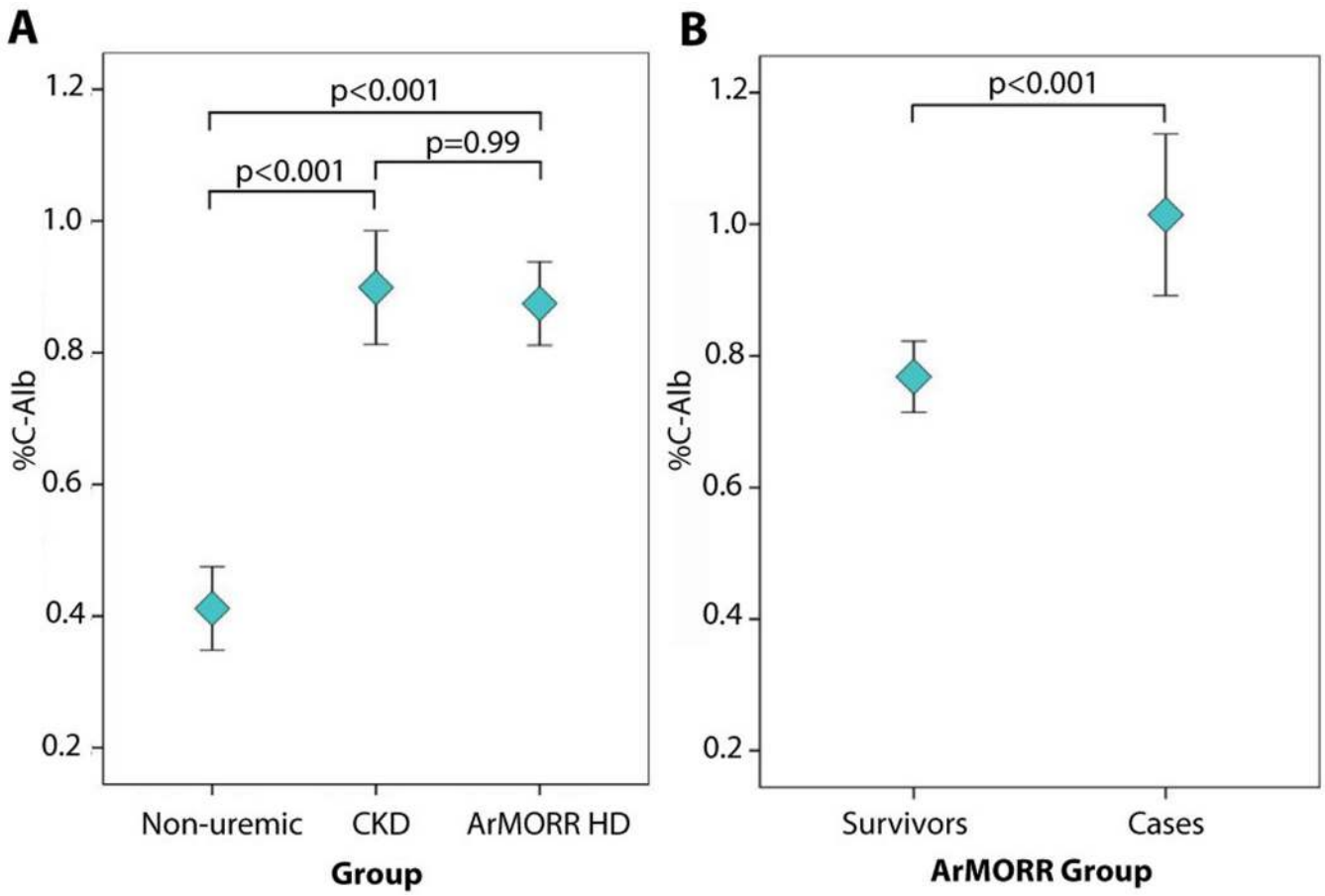
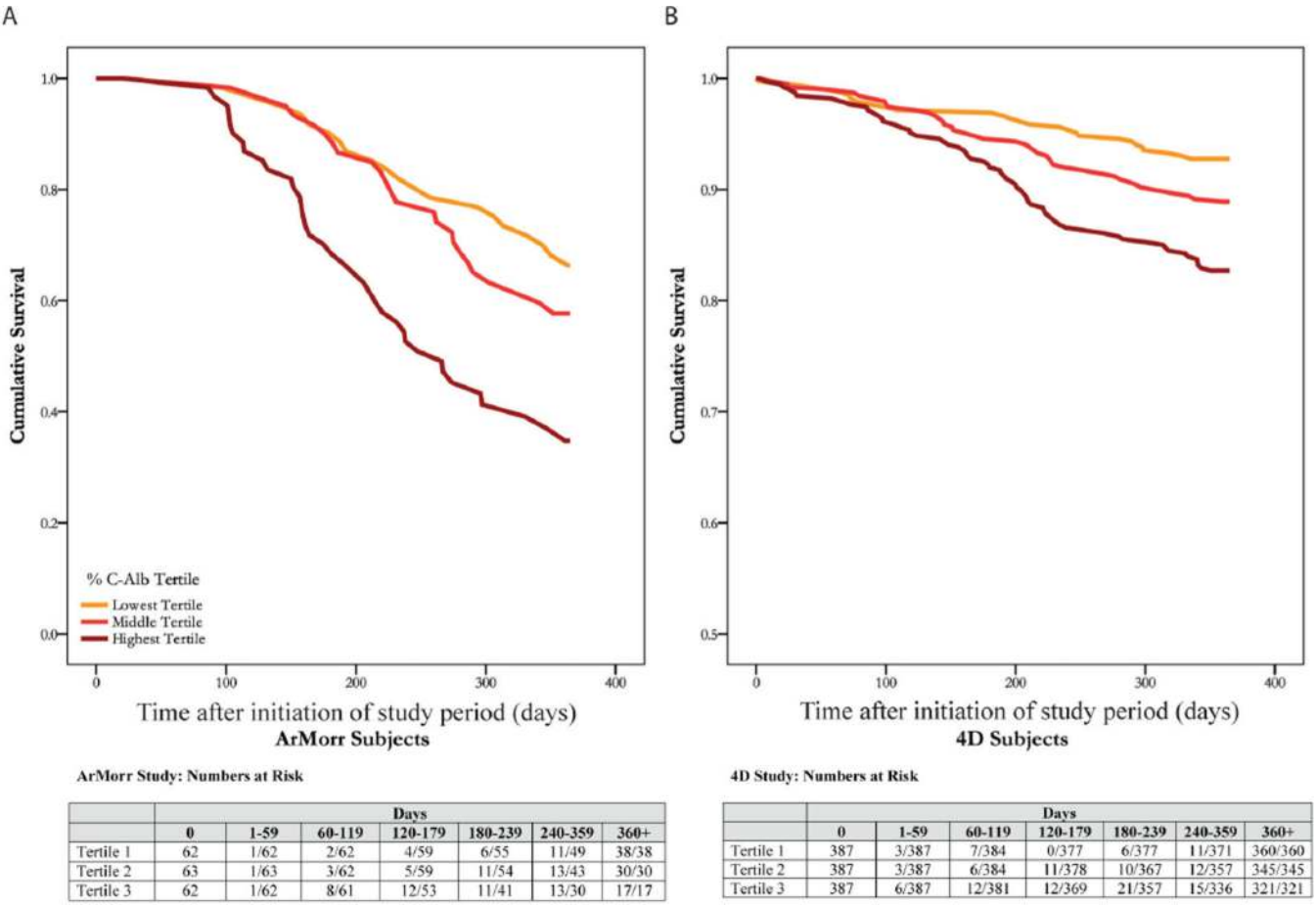


Fig. 2.

Average carbamylated albumin values in uremic and non-uremic patients. (A) Average %C-Alb values in non-uremic subjects (n = 20) and in patients with stage 3 or 4 chronic kidney disease (CKD) (n = 122) and ArMORR HD subjects (n = 187). (B) Average %C-Alb in ArMORR survivors who lived longer than 12 months (n = 106) and in ArMORR cases who died during the 12-month study period (n = 81) . Individual %C-Alb values for each group are shown in Table S2. Data are expressed as average carbamylated albumin as a % of total; error bars, 95% confidence intervals of the mean; Student's t-test P-values shown.



ArMorr Study: Numbers at Risk

	Days						
	0	1-59	60-119	120-179	180-239	240-359	360+
Tertile 1	62	1/62	2/62	4/59	6/55	11/49	38/38
Tertile 2	63	1/63	3/62	5/59	11/54	13/43	30/30
Tertile 3	62	1/62	8/61	12/53	11/41	13/30	17/17

4D Study: Numbers at Risk

	Days						
	0	1-59	60-119	120-179	180-239	240-359	360+
Tertile 1	387	3/387	7/384	0/377	6/377	11/371	360/360
Tertile 2	387	3/387	6/384	11/378	10/367	12/357	345/345
Tertile 3	387	6/387	12/381	12/369	21/357	15/336	321/321

Fig. 3.

Kaplan-Meier curve estimates of the incidence of all-cause mortality in ESRD patients. Subjects were categorized into upper, middle, and lower tertiles according to serum %C-Alb values measured at the outset of the study. (A) 12 month survival rates in ArMORR study subjects. (B) 12 month survival rates in 4D study subjects. Numbers of subjects at risk at different time points during each study shown in the tables at bottom.

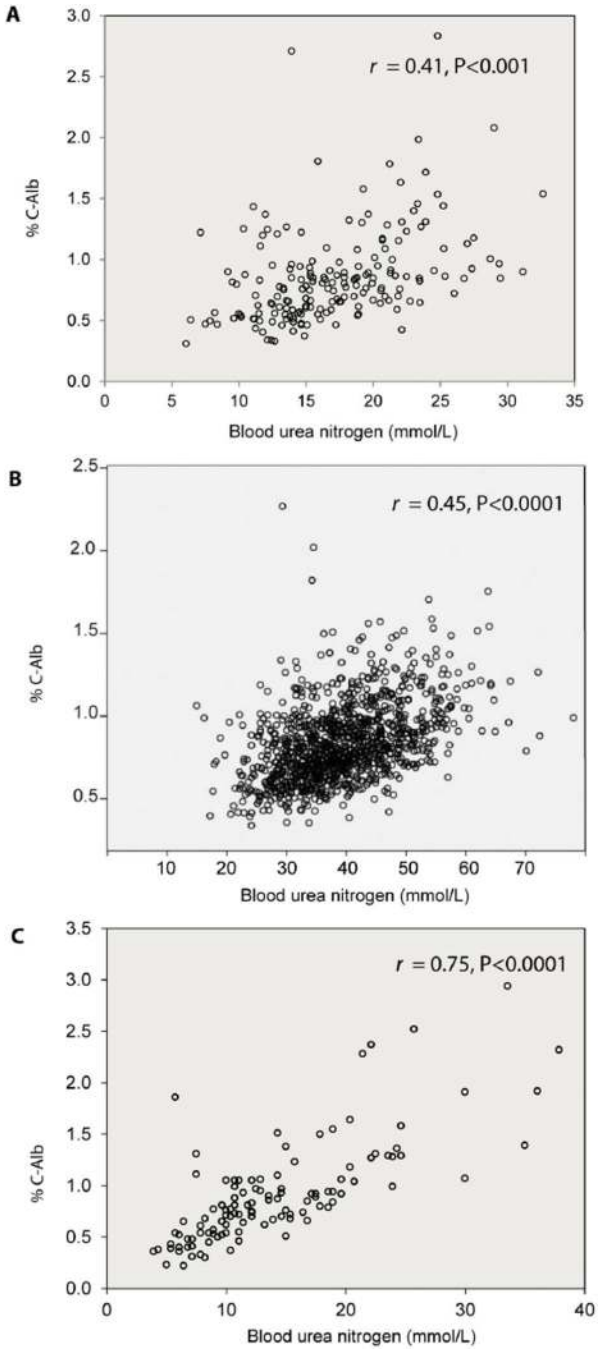


Fig. 4.

Correlation between %C-Alb and blood urea concentrations. (A) Correlation between blood urea and %C-Alb in ArMORR study subjects with ESRD (n = 187). (B) Correlation between blood urea and %C-Alb in 4D study subjects with ESRD (n = 1,161). (C) Correlation between %C-Alb and blood urea in non-hemodialysis CKD subjects (n = 122). Pearson correlation coefficients (*r*) and P-values are shown.

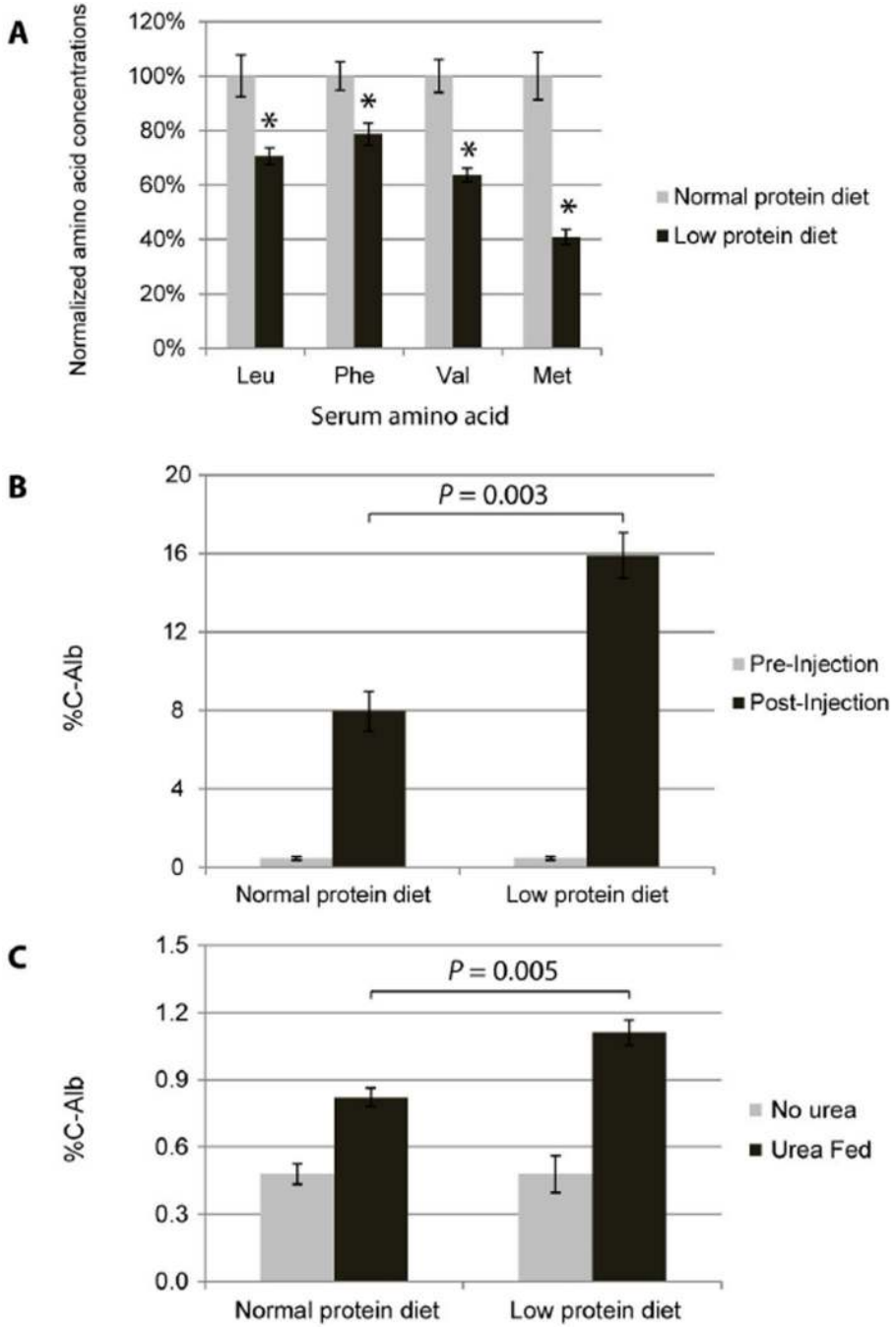


Fig. 5.

Effects in mice of low protein diet on albumin carbamylation by urea or cyanate. (A) Serum amino acid concentrations after 15 days of low- or normal-protein diets. Values are normalized to average concentrations of amino acid in animals on a normal protein diet. **P* < 0.05 using unpaired T test, comparing average amino acids in animals on low and normal protein diets. (B) %C-Alb values in low- or normal-protein fed animals before and 30 minutes after cyanate injection (100 mg/kg body weight). (C) %C-Alb values in animals fed low or normal protein diets supplemented with or without urea (67 mg per gram of feed). Bars indicate mean ± SD, n = 6 animals per group. *P*-values were calculated using unpaired T test.

Table 1

In the ArMORR study, HR estimates for 12-month mortality[1] associated with % C-Alb and other parameters. Values were measured at baseline (start of the study). Additional patient characteristics were included in this analysis but demonstrated no statistically significant risk in univariate analysis. These variables included body mass index, leukocyte count, platelet count, serum concentrations of potassium, phosphorus, calcium, ferritin, alkaline phosphatase, parathyroid hormone, and percentage of subjects with histories of diabetes mellitus, coronary artery disease/myocardial infarction, chronic obstructive pulmonary disease, cancer, dyslipidemia, anemia, peripheral vascular disease, cerebrovascular accident, congestive heart failure, atrial fibrillation, and liver disease.

	Univariate		Multivariable	
Variables	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
% C-Alb[2]	**3.76 (2.20–6.43)**	**<0.0001***	**3.23 (1.74, 6.00)**	**0.0002***
Albumin	0.43 (0.26–0.71)	0.001*	0.53 (0.30, 0.93)	0.03*
Hemoglobin	0.79 (0.69–0.91)	0.001*	0.96 (0.82, 1.14)	0.65
History of hypertension	0.42 (0.25–0.71)	0.001*	0.42 (0.23, 0.74)	0.003*
Equilibrated Kt/V	0.74 (0.30–1.80)	0.50	0.95 (0.37, 2.41)	0.91
Systolic blood pressure	0.98 (0.97–0.99)	<0.0001*	0.99 (0.98, 1.00)	0.05
History of diabetes mellitus	0.69 (0.40, 1.17)	0.17	1.03 (0.55, 1.91)	0.94

*P-values considered significant at P<0.05.

[1]Risk of death from all causes over the 12-months following the 90 day study run-in period

[2]Continuous variable, natural log transformed

Table 2

In the 4D study, HR estimates for 12-month mortality[1] associated with % C-Alb and other parameters. Values were measured at baseline (start of the study). Additional patient characteristics were also included in this analysis but demonstrated no statistically significant differences between cases and controls. These variables included gender, systolic and diastolic blood pressure, platelet count, serum concentrations of parathyroid hormone, potassium, calcium, phosphorus, ferritin, LDL cholesterol, and percentage of subjects with histories of diabetes mellitus, hypertension, or cerebrovascular accident.

	Univariate		Multivariate	
Variables	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
% C-Alb[1]	**3.73 (2.00–6.96)**	**<0.001***	**3.17 (1.55–6.46)**	**0.002***
Age	1.04 (1.02–1.06)	<0.001*	1.03 (1.00–1.05)	0.050
Body mass index	0.94 (0.91–0.97)	0.001*	0.97 (0.93–1.01)	0.122
Alkaline phosphatase	1.004 (1.002–1.005)	<0.001*	1.002 (1.001–1.004)	0.008*
Albumin	0.50 (0.30–0.82)	0.006*	0.90 (0.50–1.63)	0.723
Hemoglobin	0.87 (0.77–0.97)	0.017*	0.94 (0.82–1.08)	0.366
Transferrin saturation	0.98 (0.97–1.00)	0.016*	0.99 (0.97–1.01)	0.180
Leukocyte count	1.12 (1.06–1.19)	<0.001*	1.09 (1.02–1.16)	0.007*
Coronary artery disease	1.54 (1.11–2.13)	0.009*	1.22 (0.83–1.77)	0.312
Congestive heart failure	1.61 (1.18–2.21)	0.003*	1.20 (0.83–1.75)	0.338
Peripheral vascular disease	2.01 (1.46–2.78)	<0.001*	1.82 (1.25–2.66)	0.002*
Atrial fibrillation	2.24 (1.48–3.39)	<0.001*	1.57 (0.92–2.66)	0.097
Duration of diabetes (*years*)	1.03 (1.01–1.05)	0.003*	1.03 (1.01–1.05)	0.010*
Urea reduction ratio	1.00 (0.97–1.02)	0.857		
% Hemoglobin A_{1c}	1.05 (0.93–1.19)	0.408		

*P-values considered significant at P<0.05.

[1]Continuous variable transformed to its natural log

Table 3

Spearman correlations and partial correlations between %C-Alb, blood urea, and free amino acids in ArMORR study subjects

	Unadjusted correlation†		Partial correlation to urea, adjusted for individual amino acids††	
Amino acids	**r_s**	**P-value**	**Partial r_s**	**Partial P -value**
Average urea	**0.431**	**<0.0001***		
Arginine	−0.357	0.0004*	0.507	<0.0001*
Lysine	−0.310	0.0022*	0.454	<0.0001*
Histidine	−0.270	0.0082*	0.477	<0.0001*
Alanine	−0.341	0.0007*	0.434	<0.0001*
Glycine	−0.216	0.0354*	0.406	<0.0001*
Threonine	−0.105	0.3121	0.430	<0.0001*
Serine	−0.270	0.0081*	0.464	<0.0001*
Proline	−0.174	0.0914	0.425	<0.0001*
Glutamine	−0.238	0.0204*	0.474	<0.0001*
Methionine	−0.094	0.3672	0.423	<0.0001*
Tyrosine	−0.080	0.4384	0.433	<0.0001*
Valine	−0.240	0.0193*	0.488	<0.0001*
Leucine/Isoleu	−0.388	0.0001*	0.484	<0.0001*
Aspartic acid	−0.218	0.0338*	0.450	<0.0001*
Glutamic acid	−0.060	0.5629	0.429	<0.0001*
Phenylalanine	−0.134	0.1939	0.458	<0.0001*
Tryptophan	−0.100	0.3352	0.423	<0.0001*

*P-values considered significant at P<0.05. r_S indicates Spearman correlation coefficients with %C-Alb.

†Unadjusted Spearman rank correlations compared to %C-Alb.

††Partial Spearman correlations between average pre-dialysis urea values and %C-Alb after adjustment for amino acid concentrations measured at start of the study.

Table 4

Inhibition of *in vitro* carbamylation by amino acid scavengers. Purified albumin was pre-mixed with or without individual amino acids (10 mM) and carbamylated overnight at 37°C with 0.5 mM cyanate, then analyzed for %C-Alb.

Amino acid	%C-Alb Mean ± SD	P-value†	% Decrease compared to no inhibitor††
Glycylglycine	15 ± 1.2 %	<0.0001	64%
Cysteine + cysteamine	17 ± 0.9 %	<0.0001	59%
Taurine	22 ± 1.7 %	<0.0001	46%
Cysteamine	23 ± 1.0 %	<0.0001	42%
Cysteine	23 ± 1.9 %	<0.0001	42%
Histidine	26 ± 1.0 %	<0.0001	35%
Arginine	27 ± 1.6 %	<0.0001	34%
Glutathione	29 ± 1.4 %	<0.0001	28%
Lysine	31 ± 0.9 %	0.0002	23%
Glycine	31 ± 1.8 %	0.006	23%
Glutamine	32 ± 2.9 %	0.009	21%
Tryptophan	32 ± 2.7 %	0.012	21%
Alanine	32 ± 1.7%	0.018	20%
Valine	33 ± 1.1 %	0.001	18%
Proline	34 ± 1.6 %	0.006	16%
Leucine	37 ± 2.2 %	0.035	10%
Glutamate	38 ± 1.2 %	0.20	6%
No amino acid†	41 ± 2.5 %†		
No cyanate	0.17 ± 0.2 %		

†P-values indicate significance of differences between the average %C-Alb values in samples pre-mixed with amino acid inhibitors compared to no amino acid added.

††% Decrease in carbamylation was calculated by taking the difference between %C-Alb in samples with no amino acid added (41%) and samples with amino acid inhibitors added and divided by 41%. For example, the % decrease in carbamylation for alanine is (41% – 32%) ÷ 41% = 20% inhibition of protein carbamylation.