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# Protein carbamylation in ESRD: Is there a mortality effect?

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## Abstract

**Purpose of review:** Protein carbamylation is a post-translational protein modification caused, in part, by exposure to urea's dissociation product cyanate. Additional modulators of protein carbamylation include circulating free amino acid levels, inflammation, diet, smoking, and environmental pollution exposures. Carbamylation reactions can modify protein charge, structure, and function, leading to adverse molecular and cellular responses. These changes have been linked to several pathologic biochemical pathways relevant to patients with end stage renal disease (ESRD) such as accelerated atherosclerosis and dysfunctional erythropoiesis, among others. This review examines the consequences of human protein carbamylation and the clinical impact this is thought to have in patients with ESRD.

**Recent findings:** Recent well conducted studies across diverse cohorts of patients have independently associated elevations in protein carbamylation to mortality and morbidity in patients with ESRD. Studies are now examining the best strategies to reduce carbamylation load, including interventions aimed at lowering urea levels and restoring amino acid balance. Whether such carbamylation lowering strategies yield clinical improvements remains to be determined.

**Summary:** Numerous fundamental studies provide plausible mechanisms for the observed association between protein carbamylation burden and adverse clinical outcomes in ESRD. Studies employing nutritional and dialytic interventions to lower carbamylation may mitigate this risk but the net clinical benefit has not been established.

### Keywords

protein carbamylation; uremic toxicity; ESRD; dialysis

## Introduction

In 2018, end stage renal disease (ESRD) remains a significant public health problem reaching a prevalence of over 700,000 in the US population alone, with nearly 500,000 individuals receiving maintenance dialysis therapy to survive [1, 2]. The presence of ESRD increases an individual's risk of death, particularly cardiovascular (CV) death, 10 to 30-fold compared to individuals with preserved kidney function [3, 4]. Traditional risk factors such as hypertension, atherosclerosis, and left ventricular hypertrophy are highly prevalent in

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**Conflicts of Interest:** None

ESRD and contribute to CV risk, yet studies employing conventional treatments such as angiotensin converting enzyme inhibitors [5] and statins [6] have failed to change key outcomes in the ESRD population suggesting novel pathophysiology exists.

Human proteins, during both health and disease, are exposed to a variety of chemical reactions capable of altering their structural and functional properties. Spontaneous post-translational protein modifications can occur through the non-enzymatic binding of reactive molecules to protein functional groups, as seen, for example, in glycation reactions which can increase due to hyperglycemia in diabetes mellitus. Protein carbamylation describes a post translational protein modification that is driven by cyanate, the reactive dissociation product of urea (Figure 1). While generally found in low concentrations *in vivo*, urea, and thus cyanate and carbamylation, all naturally increase when kidney function declines. Protein carbamylation is not solely related to urea, however; it has also been shown that free amino acids compete with proteins for reaction with cyanate, in essence shielding proteins from carbamylation, and amino acid deficiencies from a variety of causes can exacerbate carbamylation burden [7]. Additionally, the heme protein myeloperoxidase (MPO), which is secreted in high concentrations at inflammatory sites from stimulated neutrophils and monocytes, can catalyze conversion of thiocyanate or cyanide (derived from diet, smoking, or environmental pollution) into cyanate, also contributing to carbamylation load (Figure 1) [8, 9]. Protein carbamylation can therefore stem from such divergent exposures as kidney disease, diet, smoking, and air pollution, ultimately leading to common pathways of molecular and cellular dysfunction.

Because carbamylation is a protein modification that can result from exposure to urea and its byproduct, cyanate, it has become an increasing focus of nephrology research, particularly in the ESRD population. This report reviews our current understanding of the consequences of protein carbamylation in humans, followed by clinical studies of carbamylation in ESRD with attention to the possible therapeutic approaches being proposed.

## Consequences of carbamylation

The net result of the carbamylation reaction is the addition of a “carbamoyl” moiety (-$CONH_2$) to a functional group (Figure 1). A major chemical effect of this binding of a carbamoyl group to protein amino groups is the neutralization of a positive charge, which in turn alters ionic protein-water interactions on the protein surface [10]. This disturbance has the potential to subsequently destabilize secondary and tertiary protein structures resulting in conformational and functional changes. Dozens of studies through the years have shown results implicating carbamylation in changes in protein charge, conformation, and stability, with subsequent alterations in enzyme and hormone activity, binding properties, receptor-drug interactions, and cellular expression and responses [11]. Descriptions of the numerous demonstrations of these phenomena are beyond the scope of this report and the reader is referred to other excellent comprehensive reviews of carbamylation recently published [10, 12].

Given the ubiquity of urea in all tissue compartments, the potential disease implications of carbamylation are far reaching. This was clearly demonstrated in a chronic kidney disease (CKD) animal model where the authors were able to quantitatively demonstrate, that

impaired kidney function accelerated carbamylation across a diverse set of tissues including aorta, kidney, bone, skin, liver, and heart, with greater accumulations in the long-lived extracellular matrix proteins [13]. While there appeared to be a basal level of carbamylation occurring in control mice without renal disease, there was a 2-fold increase in carbamylation burden in 75% nephrectomized mice at 20 weeks across these tissues. Thus, at physiological conditions and normal kidney function, carbamylation still occurs, and proteins with long half-lives will accumulate the modification [14]. Indeed, numerous disciplines beyond nephrology have implicated protein carbamylation in specific human disease processes such as cataract formation [15–17], arthritis and autoimmune disease [18–20], and neurological diseases [21, 22]. However, given the relationship between urea, cyanate, and decreased kidney function, the nephrology community may still hold the greatest interest. Carbamylation's link to mechanisms of CV disease and erythropoietin resistance are two particularly relevant examples why.

## Mechanisms of cardiovascular disease and outcomes

Multiple in vitro, animal, and human studies suggest carbamylation can accelerate the biochemical events of atherosclerosis, vascular calcification, and thrombosis via its effects on lipoproteins [23–26], collagen [27], fibrin [28], mitochondrial proteins [29], and proteoglycans and fibronectin [30]. Possibly because of such mechanisms, one of the earliest clinical outcome carbamylation studies linked plasma protein bound homocitrulline levels (carbamylated lysine resides measured using mass spectrometry) to CV outcomes in 1,000 individuals with relatively preserved kidney function (average estimated glomerular filtration rate [GFR] > 80 ml/min/1.73 $m^2$) [8]. Carbamylation levels significantly associated with coronary or peripheral artery disease as diagnosed on angiography, with subjects in the highest quartile of carbamylation showing a 7–8 fold higher risk compared to the lowest quartile. Similarly, over a 3-year period, individuals experiencing revascularization procedures (angioplasty, stent, or bypass grafting), nonfatal myocardial infarction (MI), stroke, or death had significantly higher carbamylation levels than those not experiencing these events. These findings remained significant following adjustment for traditional CV risks factors, GFR, and inflammatory markers. Others have shown similar results in smaller studies [31, 32]. Thus, it seems measures of systemic protein carbamylation burden can independently predict CV outcomes, and advanced kidney disease is not a pre-requisite.

## Erythropoietin resistance mechanisms and outcomes

Carbamylation of erythropoietin abrogates its erythropoietic activity both in vitro and in vivo and, thus, carbamylation of erythropoietin or proteins in the erythropoietic pathway may directly contribute to the pathophysiology of erythropoietin resistance in CKD [33]. Indeed, we showed among 161 hemodialysis patients, that subjects in the highest quartile of carbamylated albumin (a global marker of carbamylation burden analogous to hemoglobin A1C being a marker of glycemic control in diabetes) had the lowest hemoglobin levels, yet used the greatest recombinant erythropoietin (EPO) doses.[34] Interestingly, increasing EPO resistance was associated with a higher risk of death as previously established by others [35], yet the association between EPO responsiveness and mortality was no longer statistically significant when carbamylation was included in the analysis, as carbamylation showed the dominant association with death. This so-called 'hyporesponsiveness' to EPO

that independently associates with carbamylation (independent of traditional risks such as iron stores and inflammatory markers) has become a promising intermediary clinical endpoint in interventional studies that aim to ameliorate carbamylation levels in chronic dialysis patients (e.g. NCT02472834, see next section) [36]. Changes in EPO responsiveness are likely to manifest on the order of months verses the years it takes to chart differences in mortality. The detrimental, mechanistic effects of carbamylation stretch beyond the pathways discussed herein. From accelerating kidney fibrosis to triggering autoimmunity, the reader is directed to broader literature reviews of protein carbamylation [12].

## Clinical association studies of carbamylation in ESRD

The plasma concentration of cyanate in healthy individuals is about 45 nmol/L, and in patients with ESRD on dialysis it reaches 140 nmol/L [37]. While this increase may seem mild, recall that urea dissociation is constantly generating more cyanate, which rapidly binds to nearby proteins and amino acids, and thus the rate of protein and amino acid carbamylation may become quite significant. Moreover, thiocyanate also elevates in ESRD patients, introducing another pathway by which carbamylation can accelerate in this population [38–40]. Kraus et.al. demonstrated that serum concentrations of many carbamylated free amino acids in patients with ESRD exceeded the concentrations of their unmodified precursors, a finding that could have implications for the nutritional depletion, specifically protein energy malnutrition, well described in ESRD patients [41, 42]. While it seems urea accumulation would play a dominant role in the carbamylation burden of patients with ESRD, comparative assessments of the relative impacts of amino acid balance or the MPO catalyzed pathway on carbamylation have not been established.

Early studies looking at carbamylation burden in patients with ESRD requiring dialysis were partially motivated by identifying a reliable marker of dialysis adequacy. It was shown that carbamylated hemoglobin was negatively correlated with both Kt/V and urea reduction ratios [43], and that carbamylated hemoglobin was a more accurate indicator of time averaged urea than the conventional indices of dialysis adequacy [44, 45]. Notably, these studies did not ultimately test if carbamylation measures could predict clinical outcomes such as mortality better than the standard dialysis adequacy measures. However, in contemporary dialysis, we've found adequacy measures often fail to demonstrate a mortality association as most patients meet minimum recommendations and, unfortunately, doses beyond these requirements have failed to show significant survival benefit nor improvement in uremic toxin clearance [46–49]. In this context, we have shown that carbamylation levels are indeed stronger predictors of mortality in ESRD patients on hemodialysis than the conventional urea kinetic based markers [7, 50]. Such findings reiterate that carbamylation should not be considered a simple urea equivalent, rather its levels likely integrate multiple pathologic pathways including nutritional status, inflammation, and time averaged solute clearance and accumulation, making it a potent assessment of health risk.

In 2013, two groups independently and simultaneously reported on the all-cause mortality risk associated with elevated protein carbamylation levels in hemodialysis patients. It was noteworthy that the investigator groups were completely independent, used different measures of carbamylation burden (carbamylated albumin and protein bound

homocitrulline) and studied 3 distinct hemodialysis cohorts in total, each with unique attributes (incident dialysis, prevalent dialysis, and prevalent dialysis patients with diabetes). Even after adjusting for relevant covariates, in each of these cohorts, baseline protein carbamylation was found to be an independent risk factor for death in follow up periods ranging from 1 – 5 years [7, 51].

In the first of these studies, Koeth et al. looked at serum protein bound homocitruline levels in a group of 347 patients undergoing maintenance hemodialysis with 5 years of follow up [51]. After multivariable adjustments, carbamylation levels in the highest tertile conferred a more than double risk of death compared to patients with carbamylation levels in the middle or lowest tertiles. Nearly simultaneously, we reported similar findings using 2 distinct cohorts of hemodialysis patients [7]. The first cohort consisted of 187 participants from the Accelerated Mortality on Renal Replacement cohort which included only incident dialysis patients followed for up to 1 year. Like the Koeth et al study, we found a risk ratio 3.23; 95% confidence interval (CI), 1.74 to 6.00 for the top tertile of carbamylation (measured using carbamylated albumin) compared to the lowest. In the largest hemodialysis cohort in which carbamylation has been studied, we employed data and blood samples form the randomized controlled “German Diabetes and Dialysis study” which investigated the benefits of the cholesterol lowering drug atorvastatin in diabetic dialysis patients (n= 1,161) [6]. In this group of prevalent diabetic hemodialysis subjects, we again found a significant risk of death among subjects with the highest tertile of carbamylated albumin (HR 2.25; 95%CI, 1.42–3.56), even after multivariable adjustments [7].

The actual cause of death in these carbamylation association studies next became of interest to shed light onto any mechanistic underpinnings from the findings. Dreschler et al. addressed this by reanalyzing the aforementioned German Diabetes and Dialysis study data [52]. Carbamylated albumin levels were strongly associated with 1-year adjusted risk of CV mortality, sudden cardiac death, and the 4-year risk of death from congestive heart failure. This has been the only report of cause of death in ESRD in association with carbamylation to date and further work is required. While it may seem at odds with the earlier studies in subjects with preserved renal function that strongly implicated carbamylation in pathways of atherosclerosis, ischemia, and need for revascularization, the differences in the patient populations (ESRD vs. largely preserved renal function) could account for thi s. It is reported that with declining kidney function, there is a progressive shift from ischemic to non-ischemic etiologies of cardiac death, particularly sudden death in the absence of evidence of an acute MI [2]. Notably, carbamylation also associated with cardiac stress markers and adverse long-term events in in a smaller cohort of non- ESRD patients with chronic systolic heart failure [53]. The comparative rates of carbamylation-linked accelerated atherosclerosis vs. cardiomyopathy are unknown.

Another key observation that came of the post-hoc study of the German Diabetes and Dialysis statin trial, was that in subgroup analysis, subjects in the lowest carbamylation tertile appeared to derive a survival benefit from the cholesterol lowering drug whereas subjects in the second and top tertile of carbamylation showed no benefit from statin therapy [52]. As the original trial showed that statins, one of the most potent and efficacious CVD risk modifiers in the general population, conferred no benefit to dialysis patients, detecting a

signal of benefit in an ESRD sub-group is noteworthy. It is plausible that a statin benefit is only evident in patients free from the competing risk of excessive carbamylation, which may be a comparatively stronger risk factor. This hypothesis needs additional work to determine its merit. For example, it remains unclear what effect a patients' carbamylated LDL levels have on the net efficacy of statin therapy. Perhaps greater carbamylated LDL modifies the typical beneficial effects of statin therapy seen in non-ESRD patients. If true, this could potentially introduce a new therapeutic target in the ESRD population (either the reduction of global carbamylation load or carbamylated LDL specifically).

Interest in reducing protein carbamylation burden further increased when we began to better understand the natural history of carbamylation in ESRD overtime. In another case-control study of 122 incident ESRD hemodialysis subjects who survived at least the first 90 days of dialysis but died within 1 year (cases) and 244 incident dialysis patients who survived beyond 1 year (controls), carbamylated albumin levels measured at the start of dialysis and every 90-day period until 1-year or death, showed that at the time of dialysis initiation, carbamylation levels in both groups were markedly higher than levels observed in prevalent maintenance dialysis [50]. This suggests that as CKD progresses and before dialysis restores some degree of solute clearance, patients are likely in a 'peak' carbamylation state. Nevertheless, the high levels of carbamylation at dialysis initiation were similar between the cases and controls. Adjusted repeated measures analysis of carbamylation changes over time then showed that 1-year survivors experienced a significantly greater mean carbamylation decrease from baseline compared to cases. Interestingly, similar analysis of urea levels, serum albumin, or standard dialysis metrics such a Kt/V or normalized protein catabolic rate did not show such any differences between the groups. This implies that a greater reduction in carbamylation over time is associated with a lower risk of mortality and this is a phenomenon independent of traditional risk factors. Indeed, multiple studies aimed at understanding how carbamylation can be modulated seek to prove such a benefit.

## Possible targeted therapies to mitigate carbamylation in kidney disease

Given the associations between carbamylation and poor clinical outcomes, focus has turned to determining precisely *how* to modulate carbamylation. Nutritional interventions and dialysis modifications have been the 2 most promising avenues studied thus far. The transition from late stage CKD not on dialysis to ESRD on maintenance dialysis has been shown to confer a substantial reduction in protein carbamylation presumably from substantial lowering of circulating urea levels [50]. However, maintenance hemodialysis remains a state of excess carbamylation relative to healthy controls and studies looked if more hemodialysis (i.e. extended duration), might reduce carbamylation levels. Subjects receiving routine thrice weekly hemodialysis who converted to extended duration (double treatment time), thrice weekly, in-center hemodialysis showed significant reduction in protein carbamylation compared to a non-randomized control group that did not change from their conventional dialysis prescription [54]. This study went on to show that individuals who experienced the greatest reductions in carbamylation in the extended dialysis group also demonstrated reductions in left ventricular mass as assessed by cardiac MRI. Also noteworthy was subjects undergoing the intensive dialysis strategy demonstrated an increase in serum amino acid levels, possibly related to nutritional improvements in the

more dialyzed patients. Given the background mechanisms of elevated urea driving increases in cyanate and carbamylation, and the demonstration that serum free amino acid levels effectively compete with proteins for carbamylation, the mechanisms may have acted together to produce the observed reduction in carbamylation.

The notion of elevating circulating amino acid levels to effectively shield proteins from carbamylation has been more directly studied as well. Based on in vitro and animal studies demonstrating that amino acid supplementation can reduce protein carbamylation load in the presence of urea or cyanate [7], in human studies of amino acid supplementation during hemodialysis are being pursued. We reported the first proof-of-concept investigation of amino acid therapy aimed at reducing carbamylation in a small pilot study of hemodialysis subjects [36]. Carbamylated albumin levels measured across an 8-week period (2 half-lives of normal serum albumin) fell by 15% in individuals receiving a commercially available amino acid infusion during routine thrice weekly dialysis treatments when compared to individuals receiving no treatment. Notably, there was not an appreciable change in blood urea levels in the treated group which was a concern given the additional nitrogen load amino acids carry as it could counter carbamylation decreases. While such results are promising, it is important to note that no study has yet examined if the modulation of carbamylation will result in actual changes in clinical outcomes. This point is being addressed through an ongoing randomized controlled clinical trial examining amino acid therapy on hemodialysis to reduce carbamylation and assess intermediary clinical end-points such as changes in erythropoietin responsiveness (NCT02472834).

Amino acid strategies were also studied in a post-hoc analysis from the IMPENDIA trial which originally compared the metabolic effects of low-glucose peritoneal dialysis solutions (incorporating icodextrin and intraperitoneal amino acids) to a control group (dextrose-only solutions) [55]. Researchers examined the impact the 2 treatment strategies had on carbamylation levels. First, this report noted that when carbamylated albumin levels of diabetic peritoneal dialysis patients were compared to matched hemodialysis subjects, peritoneal dialysis patients had significantly higher baseline urea levels and higher carbamylated albumin levels (mean ± standard deviation [SD] urea 58.6 ± 16.7 vs 48.4 ± 15.2 mg/dL; carbamylated albumin 11.4 ± 4.0 vs 10.1 ± 4.1 mmol/mol). Among IMPENDIA participants, there was no difference in carbamylation change in either arm, but amino acid treated subjects showed a trend towards increased carbamylation. Notably, the treated subjects also demonstrated an increase in urea, possibly explaining the carbamylation trend. This data suggests intraperitoneal amino acid treatment might not be efficacious to reduce carbamylation in this population [55].

Additional therapeutic insights were gained through a well-designed and rigorous study of dietary modifications to modulate carbamylation. Di Iorio and colleagues achieved significant carbamylation reductions through dietary interventions employing either a very low protein diet supplemented with ketoanalogues (VLPD + KA) or a Mediterranean diet (MD), compared to a free diet (FD) [56]. After 6 months on a given diet intervention, when compared with FD, both MD and VLPD were associated with a decrease in carbamylation levels. While urea reduction is presumably the primary effect these dietary interventions had on carbamylation, it is notable that the VLPD +KA showed the most dramatic carbamylation

reduction and the impact of KA supplementation on circulating AA's is not entirely known. In many ways, this study corroborates the studies in dialysis that suggest urea reduction and AA supplementation (without increasing urea) are effective strategies to reduce carbamylation. To date there have not been association studies linking carbamylation to clinical outcomes in a CKD cohort not on dialysis. Lastly, in vitro and animal studies have shown additional potential through drugs that appear to shield proteins from carbamylation including ascorbic acid [57], flavonoids [58], eicosapentaenoic acid [59], ibuprofen [60], aspirin [61], bendazac [62], and triclosan [63]. Translation of these bench experiments to possible clinical applications is yet to occur.

## Conclusion

Decades of basic science work have created a rich literature demonstrating how protein carbamylation could be mechanistically linked to the novel pathophysiology of ESRD. Observational studies persuasively show that protein carbamylation is an independent risk factor for adverse clinical outcomes in patients with ESRD on dialysis including mortality. The mediators of carbamylation appear diffuse, but include elevations in blood urea levels and decrements in circulating amino acid levels. Interventions to reduce urea load and restore amino acid balance appear the most promising, thus far, at reducing systemic carbamylation burden. However, the uremic milieu is exceedingly complex and the net clinical impact of such maneuvers still needs further investigation prior to changing practice.

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Key study showing that carbamylation levels are at a peak during the stage 5 CKD to ESRD-on-dialysis transition, and carbamylation levels markedly fall with dialysis initiation. Also, when followed serially, individuals who show the greatest lowering of carbamylation, experience the best survival outcomes independent of traditional risk factors.

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Important clinical study showing the association between protein carbamylation burden and mortality risk in ESRD. This was one of the first reports showing this association in the ESRD population.

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This study showed carbamylation associates with cardiovascular death, sudden cardiac death, and heart failure related death in ESRD patients possibly giving mechanistic insights into carbamylation's most relevant role in ESRD mortality risk. Also, this study found while large trials failed to show a benefit to statin therapy in the ESRD population, subjects with low carbamylation levels appear to derive a survival benefit from statins. This has a variety of therapeutic implications motivating studies aimed at lowering carbamylation.

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This study showed the effect of increasing dialysis time and solute clearance on protein carbamylation. While carbamylation was indeed reduced, other studies have failed to find significant benefits to more intensive dialysis tempering excitement for this finding. Interestingly, the mechanism of carbamylation reduction may not only have been the observed reduction in urea, but also the significant increase in plasma amino acid levels that were noted in conjunction with more dialysis time.

[PubMed: 27329430]

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First study to compare carbamylation levels between matched peritoneal and hemodialysis subjects. Peritoneal dialysis patients had higher average urea and carbamylation levels. Also, low glucose plus amino acid peritoneal dialysis solutions did not reduce carbamylation.

[PubMed: 29563277]

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Very well conducted study of nutritional interventions to reduce carbamylation in CKD patients. Very low protein diet plus ketoanalogues and a Mediterranean diet both were effective at reducing carbamylation compared to a free diet. Notably, clinical studies of carbamylation in CKD looking at hard clinical endpoints are still lacking.

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**Key points**

- Protein carbamylation describes a non-enzymatic post-translational protein modification driven by cyanate which can derive from urea, certain dietary sources, smoking, and environmental exposures. Amino acid deficiencies can increase protein carbamylation.
- The carbamylation reaction is capable of changing the charge, structure, and function of proteins and ongoing reports show in exquisite mechanistic detail how these changes can result in pathologic sequelae such as accelerated atherosclerosis, vascular calcification, and dysfunctional erythropoiesis.
- Recent clinical studies of protein carbamylation have sought to reveal the unexplained excess risk of morbidity and mortality in ESRD patients and have yielded compelling results suggesting carbamylation is an independent risk factor for adverse outcomes such as death and erythropoietin resistance.
- Small interventional studies suggest extended duration dialysis, amino acid therapy, or a low protein diet supplemented with ketoanalogues may be effective at reducing carbamylation burden, but the clinical impact of this remains unknown.

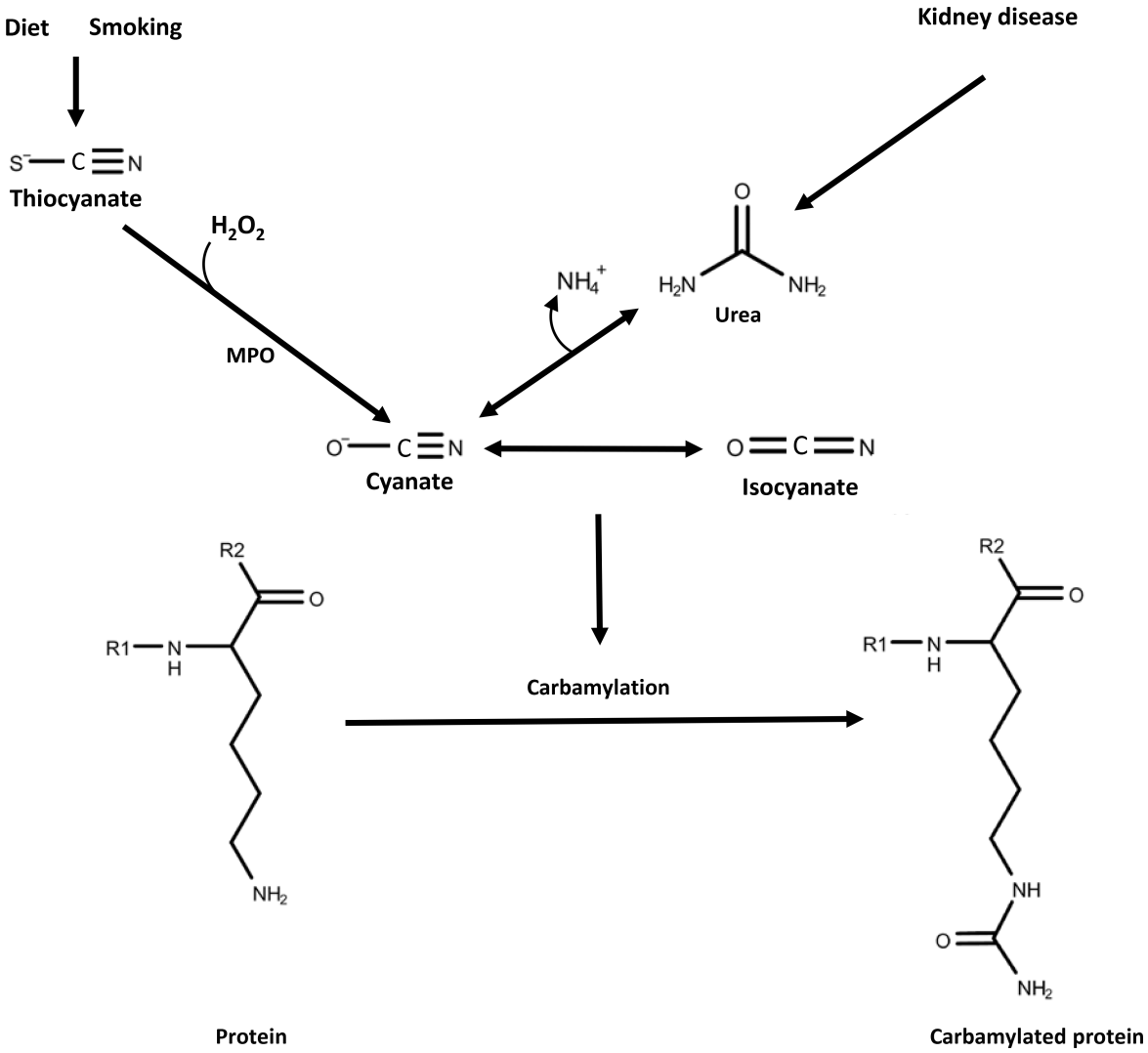


**Figure 1.**

Pathways that promote the protein carbamylation reaction. Cyanate and its tautomer isocyanate can be derived from sources such as diet or smoking, and from urea which accumulates with impaired kidney function. The reactive species can carbamylate the N-terminal of a protein or an amino acid side chain. This reaction can also occur on free individual amino acids in a similar fashion. Carbamylated proteins may undergo change in charge, structure, and function leading to molecular and cellular dysfunction.