Carbamylation-Derived Products: Bioactive Compounds and Potential Biomarkers in Chronic Renal Failure and Atherosclerosis

Stéphane Jaisson, Christine Pietrement, and Philippe Gillery

BACKGROUND: Carbamylation is a posttranslational modification of proteins resulting from the nonenzymatic reaction between isocyanic acid and specific free functional groups. This reaction alters protein structural and functional properties and thus contributes to molecular ageing. Many studies have shown the involvement of carbamylated proteins in diseases, especially in chronic renal failure and atherosclerosis.

CONTENT: In this review we describe the biochemical basis of the carbamylation process and its role in protein molecular ageing. We summarize the current evidence of protein carbamylation involvement in disease, identify available biomarkers of the carbamylation process and their related analytical methods, and discuss the practical relevance of these biomarkers.

SUMMARY: Carbamylation-induced protein alterations are involved in the progression of various diseases, because carbamylation-derived products (CDPs) are bioactive compounds that trigger specific and inappropriate cellular responses. For instance, carbamylation may promote hormone and enzyme inactivation, and carbamylated proteins, as diverse as collagen or LDLs, induce characteristic biochemical events of atherosclerosis progression. CDPs are potential biomarkers to monitor diseases characterized by an increased rate of carbamylation (e.g., chronic renal failure and atherosclerosis). Different methods (e.g., liquid chromatography–tandem mass spectrometry and immunoassays) to measure specific carbamylated proteins or general markers of carbamylation, such as protein-bound homocitrulline, have been described.

Their use in clinical practice must still be validated by appropriate clinical studies.

Carbamylation results from the covalent binding of isocyanic acid to proteins and is one of the nonenzymatic reactions involved in protein molecular ageing (i.e., glycation, oxidation, or carbonylation). These posttranslational modifications are characterized by the formation of covalently bound adducts on proteins, called posttranslational modification-derived products (PTMDPs), that are responsible for alterations of protein structural and functional properties (1).

Because most PTMDPs progressively accumulate in the organism during ageing and in various diseases [e.g., diabetes mellitus, atherosclerosis, chronic renal failure (CRF)], they are considered potential biomarkers in clinical practice (2). Some PTMDPs, such as hemoglobin A1c (Hb A1c) in diabetes monitoring (3), are already routinely used as therapeutic efficiency indicators in patient care. In prospective studies, other PTMDPs have been considered predictors or markers of complications, as advanced glycation end products, involved in the pathogenesis of many metabolic diseases (4, 5).

Results of in vitro studies have clearly shown that carbamylation alters protein properties, thus suggesting its involvement in molecular and cellular dysfunction (1, 6). However, the question of carbamylation responsibility in pathophysiological processes, such as CRF and atherosclerosis (6, 7), is still a matter of debate, and the quantification of carbamylation-derived products (CDPs) remains sparsely used in clinical practice (1). The purpose of this review is to summarize the current evidence relative to the involvement of protein carbamylation in human pathophysiology, with a special emphasis on renal failure, and to

1 Laboratory of Pediatric Biology and Research and 2 Department of Pediatrics (Nephrology Unit), American Memorial Hospital, University Hospital of Reims, France; 3 Laboratory of Biochemistry and Molecular Biology, Faculty of Medicine, Reims, France.

* Address correspondence to this author at: Laboratoire de Biochimie Médicale et Biologie Moleculaire, UMR MEDyC CNRS/URCA n 6237, Faculté de Médecine, 51 Rue Cognacq-Jay, F-51095 Reims, France. Fax +33-3-26-78-38-82; e-mail pgillery@chu-reims.fr.

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Nonstandard abbreviations: PTMDPs, nonenzymatic posttranslational modifications derived products; Hb A1c, hemoglobin A1c; CRF, chronic renal failure; CDPs, carbamylation-derived products; EPO, erythropoietin; cLDL, carbamylated LDL; cHb, carbamylated hemoglobin; VH, valine hydantoin.

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revalue the clinical relevance of carbamylation-related biomarkers.

Biochemistry of the Carbamylation Reaction

Carbamylation refers to the “carbamoyl” moiety (-CONH$_2$) addition on protein or amino acid functional groups. This reaction mainly results from interaction between isocyanic acid and proteins (Fig. 1), although carbamoyl-phosphate may also act as a carbamylating agent (8, 9). In vitro, isocyanic acid can interact with amino, thiol, carboxyl, or phenol, but not hydroxyl groups (10). Thiol and phenol groups exhibit the highest reactivity with isocyanic acid, but in a reversible manner, thus limiting its impact on protein properties. By contrast, amino groups of proteins or amino acids irreversibly bind isocyanic acid, and the α-amino groups react about 100 times faster than lysine side chain ε-amino groups because of their lower pK$_{a}$. Two terms are used in literature to describe this reaction: carbamoylation and carbamylation. The former has been recommended by the International Union of Pure and Applied Chemistry (11) but the latter one remains the most commonly used terminology in medical and biochemical reports.

In humans, isocyanic acid is mainly formed by the spontaneous decomposition of urea into cyanate and ammoniac, which occurs in aqueous solutions according to an equilibrium that favors urea more than 99% (12). Cyanate is rapidly converted into its reactive form, isocyanic acid (6). Thus, CRF, which is characterized by chronic hyperuremia, is a pathological state that promotes the formation of isocyanic acid. The plasma concentration of isocyanic acid in healthy individuals is about 50 nmol/L, and in uremic patients it reaches 150 nmol/L (13). According to the kinetic parameters of urea decomposition, these marginal concentrations are about 1000 times lower than expected. This phenomenon may be explained by the high reactivity of isocyanic acid toward amino groups. Indeed, several authors have claimed that protein carbamylation actively contributes to decreased plasma concentrations of isocyanic acid (14).

Urea decomposition, however, is not the sole pathway leading to isocyanic acid formation, especially in nonuremic patients. Cyanate may also derive from thiocyanate metabolism (15), because myeloperoxidase catalyzes thiocyanate oxidation in the presence of hydrogen peroxide and then promotes protein carbamylation at inflammation sites (16, 17). At this time, the relative contribution of each of those mechanisms in uremic and nonuremic individuals is still unknown.

Theoretically, all proteins may be carbamylated in vivo. However, the carbamylation potential of each protein depends on various parameters, such as the number and accessibility of amino groups, and the protein lifespan. Various CDPs are formed, among them α-carbamyl-amino acids (or α-carbamyl-proteins), when α-amino groups are involved, and ε-carbamyl-lysine, also called homocitrulline, when ε-amino groups are involved.

Rationale for the Involvement of Protein Carbamylation in Pathophysiology

The first demonstration of the deleterious effects of protein carbamylation in humans was made in the 1970s. At that time, urea was used as a therapeutic agent in sickle cell disease because of its ability to block and even reverse sickling. Two mechanisms had been suggested to explain its beneficial action: direct interference of the urea molecule per se with sickling (18), and hemoglobin S carbamylation by urea-derived isocyanic acid, which increases oxygen affinity and decreases aggregation properties (19). In this regard, sodium cyanate has also been suggested as an oral treatment. However, unexpected side effects have been observed, with some patients developing cataracts during the course of oral cyanate administration (20). This phenomenon has been explained by the carbamy-

![Fig. 1. Biochemical pathways leading to protein carbamylation by isocyanic acid.](image-url)
Carbamylation alters the structural properties of proteins. A prominent effect is positive-charge elimination, which changes protein–water interactions and disrupts ionic interactions on the protein surface. Because these interactions are thought to stabilize secondary and tertiary structures of proteins, their loss leads to dramatic conformational changes. For instance, incubation of BSA with potassium cyanate results in a significant increase of its Stoke’s radius and in changes in protein stability (27). During cataract progression, carbamylation is responsible for alterations of α-crystallin secondary structure, increasing protein thiol reactivity and thus interchain disulfide bonding (28). Carbamylation also disturbs protein–protein interactions. For example, the carbamylation of type I collagen induces conformational changes within its triple helix structure, which leads to a decreased ability to polymerize into normal fibrils (29). Another example is actin, which fails to self-associate into ordered filaments after carbamylation (30).

Carbamylation-induced structural changes are associated with partial or complete loss of protein functions. Many studies have provided evidence that carbamylation inhibits enzymatic activities (31–33) and hormonal actions. For instance, carbamylation decreases insulin activity by 80% with respect to glucose oxidation or to receptor binding (34). Another example is the decreased biological activity of carbamylated erythropoietin (EPO), which may contribute to the suboptimal erythropoietic responses to EPO therapy during CRF (35). Surprisingly, EPO carbamylation leads to a loss of its erythropoietic effects but does not alter its cytoprotective properties (36).

Moreover, carbamylated proteins also interact with various cell types, inducing inappropriate cellular responses, as illustrated by the enhanced secretion of proteolytic enzymes by monocyte interaction with carbamylated collagen (37) or by the carbamylated albumin-induced overexpression of specific microRNAs in mesangial cells (38).

However, in many studies, carbamylation has been achieved by in vitro incubation of purified proteins with high concentrations of KCNO, which could lead to a nonphysiological carbamylation rate, and to an irrelevant interpretation of carbamylation-induced effects. For this reason, the carbamylation rate of proteins prepared in vitro should be checked to be consistent with in vivo values before use in experimental models.

### Protein Carbamylation in Disease

Because of the pathways of isocyanic acid formation in vivo, carbamylation is preferentially involved in 2 pathophysiological processes: CRF and atherosclerosis (26, 39, 40).

In CRF, carbamylation, which is a consequence of increased uremia, may also be an aggravating factor of the disease. For example, carbamylated albumin may cause enhanced tubular cell damage and promote peritubular fibrosis (41). Furthermore, carbamylated plasma proteins stimulate glomerular cell proliferation as well as collagen synthesis, thus favoring renal fibrosis (42). Fibrosis could also result from the inactivation of enzymes involved in extracellular-matrix remodeling (26) and from the unbalanced remodeling of collagen (43). Indeed, the carbamylation-induced destabilization of collagen triple-helix conformation is responsible for an altered sensitivity to proteolysis, because carbamylated collagen exhibits greater resistance to collagenases, but increased sensitivity to gelatinases (43). Moreover, carbamylated proteins modulate inflammatory cell functions, as illustrated by the inhibitory effect of carbamylated albumin on polymorphonuclear neutrophil respiratory burst (29, 44). Such a dysregulation of polymorphonuclear neutrophil oxidative functions might contribute to the inflammatory and infectious disorders observed in uremic patients. Uremia favors the conversion of free amino acids into carbamylated amino acids, which have been identified as uremic toxins because they interfere with protein synthesis and transamination reactions and contribute to insulin resistance (45, 46).

In atherosclerosis, carbamylation of LDLs seems to play a pivotal role (7, 39). Carbamylated LDLs (cLDLs) exhibit decreased clearance in vivo (47) and thus might be more prone to oxidation (48). In addition, macrophage scavenger receptors recognize cLDLs (17, 49). cLDLs have been reported to promote the adhesion of monocytes on endothelial cells (50), induce endothelial cell death (51, 52), stimulate the proliferation of vascular smooth muscle cells (53), and induce oxidative stress and accelerated senescence in human endothelial progenitor cells (54). Carbamylated HDLs have been reported to contribute to the atherosclerotic process by promoting foam cell formation (55). Moreover, other carbamylated proteins, located within the extracellular matrix, may act as causative agents of thromboembolic events. For instance, carbamylated collagen stimulates the production of active matrix...
metalloproteinase-9 by monocytes, thus potentially enhancing extracellular matrix turnover within the arterial wall and favoring the disruption of atherosclerotic plates (37).

Carbamylation is involved in the pathogenesis of other diseases. For example, carbamylation generates new epitopes on tau protein, promoting its self-association into abnormal polymers found in Alzheimer disease (56). Similarly, carbamylation plays a role in the induction of arthritis by promoting the production of anti-homocitrulline autoantibodies and triggering subsequent immune responses (57).

This wide range of data leads to the proposal of a common mechanism of protein carbamylation involvement in pathophysiological processes: although carbamylation never constitutes the sole initial causative factor of a disease, it first is a consequence of the process, and then becomes an aggravating factor by promoting the development of long-term complications (Fig. 2).

**Available Biomarkers and Their Clinical Relevance**

Several CDPs have been suggested as biomarkers to evaluate the rate of protein carbamylation in biological samples, especially in plasma and whole blood (1).

Two approaches can be used to assess the extent of protein carbamylation: the quantification of a specific carbamylated protein, and the overall assessment of carbamylated plasma proteins (Table 1). For the first approach, assays have been developed for 2 carbamylated proteins: carbamylated hemoglobin (cHb) and cLDL. cHb mainly results from the covalent binding of isocyanic acid to the N-terminal valine residue of the globin β chains (58), and can reach 2% of total hemoglobin. cHb was initially identified as an analytical interference in the measurement of Hb A₁c by ion-exchange chromatography. cHb also constitutes a “pathophysiological interference” during Hb A₁c formation, because isocyanic acid competes with glucose for the binding to the N-terminal valine residue. The implication is that Hb A₁c values obtained in uremic patients should be carefully interpreted (59, 60). cHb is generally assayed by liquid chromatography coupled to ultraviolet detection of the hydantoin derivative of N-carbamoylvaline obtained during acid hydrolysis (61). The concentrations of cHb, expressed as microgram of valine hydantoin (VH) per gram of total hemoglobin (μgVH/gHb), range between 27 and 38 μgVH/gHb in healthy individuals, and are about 5 times higher in patients with CRF (61, 62). The increase in cHb correlates with both the duration and the degree of exposure to high blood urea concentrations (25, 62), suggesting that cHb could be a useful biomarker in differentiating patients with acute or chronic renal failure (63, 64).
More generally, cHb has been described as a biomarker of protein carbamylation, and could constitute an index of uremic balance, used in a similar way as Hb A1c, which reflects glycemic control in diabetic patients. For example, a threshold value of 100 μgVH/gHb has been suggested for determining the efficiency of hemodialysis sessions (65).

cLDLs may also be evaluated, but only 1 ELISA has been developed to determine serum cLDL concentrations (66). Concentrations are about 90 mg/L in healthy individuals and are significantly increased in patients with end-stage renal disease, in whom mean concentrations reach 280 mg/L. This method is a simple and easy-to-use assay, but it has not been implemented in routine practice because it has not been standardized.

The second analytical approach consists of assessing overall plasma protein carbamylation. A colorimetric assay based on the reactivity of carbamoyl groups with diacetylmonoxime may be used (22). However, this assay appears nonspecific and suffers from analytical interferences when performed in complex matrices such as plasma. More recently, a method allowing the specific quantification of protein-bound homocitrulline has been developed (17). Homocitrulline is a well-described CDP and thus constitutes the best candidate marker for the global evaluation of protein carbamylation. Homocitrulline is quantified by use of HPLC coupled to tandem mass spectrometry after protein acid hydrolysis. This method is recognized for its high analytical specificity and low limits of quantification (2) and made it possible to show that protein-bound homocitrulline is an independent biomarker of coronary disease risk, future myocardial infarction, and death (17). However, unlike the assessment of specific modified proteins, this second approach, which reflects the carbamylation of a large spectrum of proteins, does not provide information on the molecular mechanisms involved in carbamylation-related pathological events.

The literature supports the pathophysiological involvement of protein carbamylation. The accumulation of CDPs may contribute to the pathogenesis and progression of CRF and atherosclerosis, which are major public health problems in many countries. There is evidence for the usefulness of CDPs as biomarkers, although their use is still limited in current clinical practice. Nevertheless, the recent availability of analytical methods, along with additional clinical studies (which could provide reference values and indications that would facilitate clinical interpretation) will likely show CDPs to be relevant biomarkers for the management of various diseases, including CRF and atherosclerosis.

### Table 1. Available biomarkers for the assessment of protein carbamylation.

<table>
<thead>
<tr>
<th>CDPs</th>
<th>Assay</th>
<th>Information provided</th>
<th>References</th>
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</thead>
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<tr>
<td>cHb</td>
<td>HPLC with ultraviolet detector</td>
<td>Uremic state differentiation between acute and chronic renal failure</td>
<td>Kwan et al. (61), Stim et al. (62), Tarif et al. (65), Kwan et al. (67)</td>
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<tr>
<td>cLDLs</td>
<td>ELISA</td>
<td>Atherosclerosis risk factor; uremic state</td>
<td>Apostolov et al. (66)</td>
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<tr>
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<tr>
<td>Protein-bound homocitrulline</td>
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<td>Coronary disease risk factor</td>
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