Mechanisms and consequences of carbamoylation

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Abstract | Protein carbamoylation is a non-enzymatic post-translational modification that binds isocyanic acid, which can be derived from the dissociation of urea or from the myeloperoxidase-mediated catabolism of thiocyanate, to the free amino groups of a multitude of proteins. Although the term 'carbamoylation' is usually replaced by the term "carbamylation" in the literature, carbamylation refers to a different chemical reaction (the reversible interaction of CO_2 with α and ε-amino groups of proteins). Depending on the altered molecule (for example, collagen, erythropoietin, haemoglobin, low-density lipoprotein or high-density lipoprotein), carbamoylation can have different pathophysiological effects. Carbamoylated proteins have been linked to atherosclerosis, lipid metabolism, immune system dysfunction (such as inhibition of the classical complement pathway, inhibition of complement-dependent rituximab cytotoxicity, reduced oxidative neutrophil burst, and the formation of anti-carbamoylated protein antibodies) and renal fibrosis. In this Review, we discuss the carbamoylation process and evaluate the available biomarkers of carbamoylation (for example, homocitrulline, the percentage of carbamoylated albumin, carbamoylated haemoglobin, and carbamoylated low-density lipoprotein). We also discuss the relationship between carbamoylation and the occurrence of cardiovascular events and mortality in patients with chronic kidney disease and assess the effects of strategies to lower the carbamoylation load.

Chronic Kidney Disease (CKD) is a global public health issue with an estimated prevalence that varies between 3% and 18% depending on the region and population studied[1]. This percentage is likely to further increase in the near future owing to the growing numbers of patients with diabetes mellitus, arterial hypertension and obesity[1–3]. Individuals with CKD have a 10–20-fold higher cardiovascular mortality compared to that of age-matched and sex-matched controls with normal renal function[4]. Patients who reach end-stage renal disease (ESRD) suffer from an annual mortality of 15–20%, which is largely attributable to cardiovascular disease and infection. This excess of cardiovascular mortality cannot be solely explained by traditional risk factors such as smoking, arterial hypertension and hypercholesterolaemia[5]. In studies published in the past 10 years[6–8], the degree of carbamoylation was identified as an important risk factor for mortality in patients undergoing dialysis or with accelerated atherogenesis.

The addition of functional groups to proteins leads to the formation of post-translational modification-derived products (PTMDPs)[9]. Post-translational modifications, such as oxidation, glycation, glycoxidation, methylation and carbamoylation have different effects on the structure and function of proteins. In particular, carbamoylation is involved in the pathogenesis of various diseases such as atherosclerosis[10–12], thrombus formation[13], infections[14], autoimmune diseases[15] and kidney diseases[7,16]. Carbamoylation occurs only on a minor proportion (generally <2%) of medium-size proteins such as haemoglobin (Hb) or albumin in patients with CKD. However, proteins with extremely long half-lives, such as crystallines in the eye lens during the development of cataracts[17] or collagen during the ageing process[18] can accumulate high levels of carbamoylated lysines, which can markedly impair their function.

This Review provides an overview of the potential links between protein carbamoylation and the uraemic syndrome. We discuss the basic biochemical processes that underlie carbamoylation and their potential biological impact, and highlight the potential role of protein carbamoylation as a biomarker in risk stratification.

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doi:10.1038/nrneph.2017.103
Published online 31 Jul 2017

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Key points

- Carbamoylation is a non-enzymatic reaction during which a carbamoyl moiety is added to proteins, peptides or amino acids; this post-translational molecular modification contributes to the molecular ageing of proteins
- Carbamoylation-derived products are formed by the reaction of proteins, peptides or amino acids with isocyanic acid, which originates from urea or by oxidation of thiocyanate by myeloperoxidase in inflammatory conditions and in atherosclerotic plaques
- Carbamoylation has important effects on the immune system, atherosclerosis, lipid metabolism, and the progression of chronic kidney disease
- Carbamoylation-derived products are emerging as important markers of survival in the general population as well as in patients on dialysis

Different pathways of carbamoylation

In vivo, urea dissociates spontaneously into ammonia and cyanate, the latter being in equilibrium with its tautomer isocyanic acid[19] (FIG. 1). This process is pH and temperature-dependent. Isocyanic acid is a highly reactive electrophile that can quickly react with nucleophilic groups and modify the α-amino group of primary amines, the ε-amino group of lysines or the free sulfhydryl groups of proteins, peptides or amino acids irreversibly and thiols, hydroxyls, phenols, and imidazole groups reversibly[20,21]. The rate of these reactions increases with decreasing p*Ka* values, which are in general lower for α-amino groups than for lysine side-chain amino groups. Thus α-amino groups of free amino acids react about 100 times faster than the lysine side chain ε-amino groups on proteins at physiologic pH levels[20]. *In vivo*, however, the p*K*a values of lysine side chains present in proteins can vary depending on their microenvironment. This variability can result in lower p*K*a values in comparison with that of N-terminal amino groups, with faster binding capacity[22].

The reaction between isocyanic acid and proteins in a process called 'carbamoylation' results in the addition of a carbamoyl moiety (-$CONH_2$) to a functional group and causes changes in the electric charge of proteins or amino groups as positively charged lysines are transformed into homocitrulline, and in their molecular weight and function (FIG. 1). The term 'carbamoylation' is usually replaced by 'carbamylation' in the literature. However, carbamylation actually describes another reaction that involves the reversible interaction of CO_2 with α and ε-amino groups of proteins[23]. In this Review, we use the term 'carbamoylation'.

Isocyanic acid is produced by two reactions: urea deamination and thiocyanate (SCN^-) oxidation by myeloperoxidase (MPO) or by environmental factors[8,24–26] (FIG. 1). In physiological conditions, the levels of urea and isocyanic acid are at equilibrium[27]. The blood and serum concentrations of urea as well as the levels of serum isocyanic acid increase with the progression of renal insufficiency. For example, isocyanic acid levels can vary from ~45 nmol/l in healthy controls to 140 nmol/l in uraemic patients[28]. However, this increase is smaller than the much larger increase in serum urea concentrations between healthy controls and patients with ESRD[20]. This discrepancy is probably due to the high reactivity between isocyanic acid and amino groups[29], which causes a lower isocyanic acid concentration than expected in relation to the amount of urea present.

The serum concentration of thiocyanate, the other source of isocyanic acid generated by MPO activity, is influenced by smoking and diet as it is contained in cruciferous vegetables, certain fruits, milk products and almonds[26]. MPO is a haeme-containing enzyme that is present in the granules of neutrophils, monocytes and certain tissue macrophages (for example, peritoneal macrophages, microglia and Kupffer cells)[30]. MPO levels are a predictor of cardiovascular disease[31–34] and the enzyme is present in catalytically active atherosclerotic lesions[8]. In addition, antineutrophil cytoplasmic antibodies (ANCAs) directed against MPO have an important role in the pathogenesis of ANCA-associated glomerulonephritis and vasculitis[35]. MPO catalyses the reaction of hydrogen peroxide (H_2O_2) and thiocyanate (among other halides and pseudo-halides) to form the corresponding hypothiocyanous acid (HOSCN) and cyanate (FIG. 1)[36].

The MPO pathway is a major contributor to oxidative stress in ESRD as serum or plasma thiocyanate concentrations are 6–7-fold higher in patients on haemodialysis than in non-smoking controls (to avoid the confounding effect of smoking on MPO activity) and they decrease only modestly following dialysis[37–39]. This pathway is also involved in the pathogenesis of diabetic nephropathy, as patients with this disease have higher MPO concentrations than healthy controls[40]. However, patients with type 2 diabetes mellitus and normal renal function also have elevated plasma MPO concentrations[41,42], which correlate strongly with the plasma concentrations of carbamoylated low-density lipoprotein (cLDL)[41]. Plasma MPO concentration is an independent determinant of the plasma cLDL concentration; however, the correlation between MPO and carbamoylation-derived products has not been observed in all studies, probably due to the use of different patient populations or different MPO assays[7,43] or due to differences in the local production of MPO within the atherosclerotic plaques[8].

In addition to the urea deamination and MPO pathway, other small environmental sources of isocyanic acid exist and could cause carbamoylation. Cyanate can be inhaled from urban air; its estimated concentration is 200 parts-per-trillion by volume, which is mainly derived from biofuel usage, cooking or tobacco smoke[24]. Cyanate can also be ingested through food such as cassava root, which contains cyanogens that are metabolized to cyanate. The consumption of cassava is associated with an increased level of protein carbamoylation[25].

Quantifying carbamoylation levels

Total levels of carbamoylated proteins

The carbamoylation burden can be assessed by two methods: through the assessment of overall levels of carbamoylated plasma proteins or the quantification of specific carbamoylated proteins. The total concentration of carbamoylated proteins can be measured by a colorimetric assay based on the reactivity of carbamoyl groups with diacetyl monoxime[44]. This assay has

Tautomer
Constitutional isomer of organic compounds that readily interconvert into another tautomer, usually by the realocation of a proton.

p*Ka* values
Negative logarithm of the ionization constant of an acid, which is a measure of the strength of an acid.

Halides
Binary compounds consisting of a halogen atom and an element or radical that is less electronegative (or more electropositive) than the halogen.

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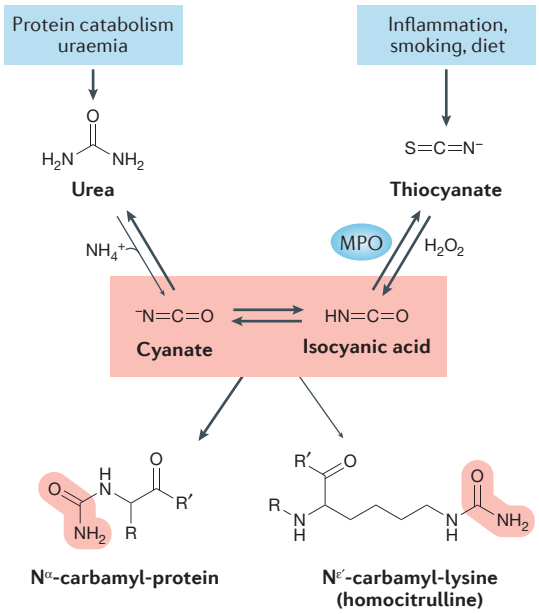

Figure 1 | **Carbamoylation.** Carbamoylation is the non-enzymatic reaction of either a primary amino or a free sulfhydryl group of a protein, peptide or amino acid with isocyanic acid, resulting in the formation of an irreversible covalent bond. Isocyanic acid can have multiple origins: urea deamination or thiocyanate oxidation by myeloperoxidase (MPO), an enzyme that is released from sites of inflammation, including atherosclerotic plaques, and by environmental factors. The deamination of urea to isocyanic acid happens under physiological conditions in an equilibrium with a ratio of 100:1. In chronic kidney disease, the concentration of urea is greatly increased, augmenting the concentration of isocyanic acid. Isocyanic acid quickly reacts with either the α-amino group of peptides, proteins or amino acids, or the ε-amino group of lysine. The latter reaction results in the formation of homocitrulline, which is one of the carbamoylation derived products. Figure adapted with permission from Elsevier © Verbrugge F. H. *et al.*, *Kidney Int.* **88**, 474–478 (2015).

Stable-isotope-dilution
Method to determine the quantity of chemical substances in a sample, involving the addition of known amounts of an isotopically-enriched substance to the sample to be analysed.

High-performance liquid chromatography
(HPLC). Analytical method to separate, identify and quantify each component present in a mixture.

Tandem mass spectrometry
(MS/MS). Multiple step mass spectrometry, with some form of fragmentation occurring between the stages, to determine the mass of a sample.

Linearity
The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Coefficient of variation
(CV). Widely used term in analytical chemistry to express the precision and repeatability of an assay.

Kt/V
Baseline parameter of dialysis adequacy that represents the blood volume cleared of urea relative to the distribution volume of urea..

disadvantages, however: it is not specific and has analytical interferences from substances other than the analyte when performed in complex matrices[45]. Homocitrulline (or ε-amino-carbamoyl-lysine) is considered to be a reliable marker of global protein carbamoylation[46]. The levels of protein-bound homocitrulline (~90% of the total amount of homocitrulline[46]) can be specifically quantified in plasma and tissues with a high analytical specificity and a low detection threshold using stable-isotope-dilution high-performance liquid chromatography (HPLC) with online tandem mass spectrometry (MS/MS) after protein acid hydrolysis[8]. In addition, hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectrometry was developed in 2012 to measure total, protein-bound and free homocitrulline in plasma samples[47]. This simple, precise, accurate and fast method has a good linearity from 10 nmol/l to 1.6 µmol/l. As the amino acid composition of proteins is not altered in patients with CKD, circulating homocitrulline can be measured in parallel with protein-bound lysine and expressed as the ratio of homcitrulline (µmol) to lysine (mol), which represents the proportion of carbamoylated proteins[10,46,48].

Specific carbamoylated proteins

Albumin. The levels of specific carbamoylated proteins such as carbamoylated albumin, carbamoylated haemoglobin (cHb) and carbamoylated LDL (cLDL) can be quantified. Protein carbamoylation on multiple side-chain lysines is promoted by urea, with a predominant carbamoylation site on lysine-549 of albumin[6]. Lysine-549 is also a common target for glycation, although no interference of glycation with carbamoylation at that same site has been reported. Other albumin lysines are less susceptible to carbamoylation. Carbamoylated albumin levels can be quantified using HPLC–MS/MS based on the identification of the carbamoylated and non-carbamoylated forms of the peptide encompassing lysine-549. This assay has an excellent linearity with a coefficient of variation of 4.2%, which is particularly important for assessing the precision and reproductibility of the assay. As blood urea levels can fluctuate by 40–70% before and after any given dialysis session, the percentage of carbamoylated albumin could provide a clearer index of the average urea load than blood urea levels. This percentage could also be an index of the time-averaged urea–amino acid balance, as the half-life of albumin is of approximately 2 weeks in patients with ESRD[6].

Haemoglobin. cHb was detected in uraemic patients as early as 1981 (REF. 49). Each valine of the α or β-subunits of Hb can be altered by this reaction[50]. Up to 2% of total Hb can be carbamoylated[45] and this percentage depends on the duration of urea exposure and the absolute blood concentration of urea[51]. The effect of urea on cHb levels could explain why proteins are less carbamoylated in patients with acute renal failure than in those with CKD, which allows discrimination between the two pathologies[52]. Blood cHb levels can be measured with three different methods: ELISA[53], HPLC[54] or capillary electrophoresis[54,55]. Although these methodologies are straightforward, the quanitification of cHb is not yet routinely performed in the clinic.

Similarly to glycated haemoglobin (HbA1c), which is a long-term indicator of proper glycaemic control, cHb could also be used as a long-term indicator of the carbamoylation load as it has a half-life of ~2 months[56]. As is the case with homocitrulline, cHb levels are higher in patients with advanced stages of nondialysis CKD than in patients undergoing haemodialysis[57]. Overall, cHb levels correlate strongly with the time-averaged concentration of serum urea[49,58,59] but their relationship with other dialysis efficiency parameters such as Kt/V is far less clear[56–58,60]. cHb levels correlate with averaged blood urea nitrogen (BUN) values, as determined in pre-dialysis urine samples from patients on haemodialysis and from nondialyzed individuals (healthy individuals and patients with CKD not on dialysis)[57,60], although these findings have not been confirmed in all studies[58]. Based on kinetic analyses, the lifespan of cHb seems to be the same as that

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of its uncarbamoylated counterpart[61] and no significant difference exists between pre-haemodialysis and post-haemodialysis cHb concentrations[57].

Under physiological conditions, cHb has a higher affinity for oxygen than does non-carbamoylated Hb; this knowledge has been applied to the clinic by administrating urea to patients with sickle cell disease to increase the proportion of cHb and reduce the formation of sickle-shaped cells[62]. Urea can disrupt hydrophobic bonds, which might affect the polymerization and gelation of the Hb molecule. In addition, urea-derived cyanate can have two effects: small quantities of cyanate inhibit sickling through an oxygen affinity-dependent mechanism, whereas high concentrations of cyanate block the intracellular polymerization of Hb more directly[63]. In addition, carbamoylation also decreases the aggregation of sickle Hb[64].

The levels of cHb in patients with CKD can be altered by anaemia, the shortened lifespan of erythrocytes and by dilution following transfusions. Erythropoietin (EPO) administration, which results in a rapid generation of new red blood cells, can also influence the percentage of cHb. One study reported a marked difference in cHb concentrations among patients undergoing haemodialysis between those who received EPO treatment and those who did not[58]. In a small subpopulation of patients on haemodialysis, blood transfusions did not alter cHb levels[60]. However, most studies in this field have excluded patients who have received recent blood transfusions[56,58].

cLDL. cLDL is generated by carbamoylation of apolipoprotein B, the protein component of the LDL particle. Numerous modified forms of LDL exist (for example, cLDL, glycated LDL and oxidated LDL), of which cLDL is the most prevalent in the general population and in patients on haemodialysis[65–71]. Blood cLDL levels can be determined by ELISA or by colorimetric measurement of protein carbamoylation after isolation of the LDL fraction by ultracentrifugation[44]. Although the ELISA assay is simple, its incorporation into clinical routine practice has been hindered by a lack of standardized antibodies against cLDL. Patients with uraemia have a threefold increase in cLDL levels compared to those of control individuals (280 mg/l versus 90 mg/l)[65]. They also have an increased number of cLDL units per units of native LDL (percentage cLDL/LDL). In patients with CKD, cLDL induces endothelial cell dysfunction and has been linked to atherosclerotic vascular disease[12,72–74]. The levels of circulating cLDL are also generally elevated in the elderly population[65].

Effects of carbamoylation

Uraemic toxicity and cardiovascular diseases

Direct effects. Several studies have suggested a direct link between high levels of urea and the development of cardiovascular disease[75–79]. In mice and primary human smooth muscle cells, exposure to uraemic toxins causes sensitization to the pro-apoptotic effects of oxidative stress caused by oxidized cholesterol (oxLDL)[75], which might contribute to increased levels of apoptosis in the arterial wall, promoting vascular calcification[77]. Incubation of human aortic endothelial cells with 20 mM urea was associated with an increased production of mitochondrial reactive oxygen species (ROS) and activation of proinflammatory pathways through induction of NF-κB expression, accumulation of intracellular advanced glycation end-products (AGEs), and increased levels of protein kinase C and hexosamine activity[79].

Apolipoprotein E-deficient ($ApoE^{-/-}$) mice with surgically induced CKD (using a two-step surgery) and fed a high-fat diet (21% total fat, 0.15% cholesterol; Harlan Teklad diet) had 2–3-fold higher plasma concentrations of cLDL and oxLDL and higher levels of atherosclerosis compared to that of $ApoE^{-/-}$ mice without CKD (sham-operated) and to that of $ApoE^{-/-}$ mice with CKD but fed a low-fat diet[74]. In addition, oral administration of urea to $ApoE^{-/-}$ mice subjected to unilateral nephrectomy and fed a high-fat diet led to an eightfold increase in cLDL levels, but not in oxLDL levels[74]. The production of cLDL in the presence of elevated levels of urea was associated with accelerated atherogenesis. This study showed the direct atherogenic capacity of urea and its metabolites, including cLDL.

Carbamoylation might also contribute to the high levels of oxidative stress observed in patients with CKD by reducing the effects of antioxidant molecules[80]. For example, *S*-carbamoylation of free cysteine and glutathione sulfhydryl groups interferes with their antioxidant functions[81]. In addition, *S*-carbamoylation abrogates the antioxidant, anti-atherosclerotic, cytoprotective, vasodilator and proangiogenic properties of hydrogen sulfide (H_2S) by strongly inhibiting its free radical scavenging capacity[82]. Chronic inflammation and oxidative stress can, in turn, enhance protein carbamoylation by promoting the formation of plaques within the cardiovascular wall[8].

Indirect effects. Urea also has indirect effects, through protein carbamoylation, on the pathophysiology of atherosclerosis[76] (FIG. 2). One of the first events in atherosclerosis is endothelial dysfunction[78]. The endothelium has vasoprotective effects, which are largely mediated by nitric oxide (NO)[78]. For example, the endothelium induces vasodilation and suppresses smooth muscle cell growth, which increases blood flow in the vessels and protects against plaque formation[83]. cLDL causes endothelial dysfunction by activating the endothelial lectin-like oxidized LDL receptor-1 (LOX-1), which induces the expression of tissue factor and plasminogen activator inhibitor type 1 and the activation of NF-κB. These pathways lead to reduced NO bioavailability through the p38-dependent activation of nicotinamide adenine dinucleotide phosphate-oxidase, the production of ROS and *S*-glutathionylation-dependent endothelial nitric oxide synthase (eNOS) uncoupling[12]. In addition, *in vitro* experiments have shown that cLDL causes endothelial cell death in a dose and time-dependent manner as well as vascular smooth muscle proliferation[84]. cLDL also induces oxidative stress and accelerates senescence in human endothelial progenitor cells[65], and enhanced monocyte binding to endothelial cells through dose-dependent upregulation

Uraemic toxins
Chemical solutes that accumulate in patients with chronic kidney disease and have negative effects on the body.

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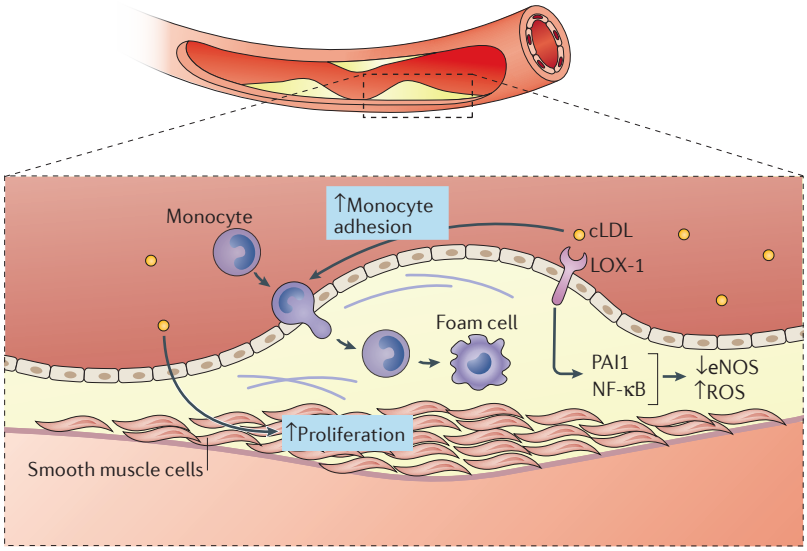

Figure 2 | **Effects of carbamoylation on endothelial function.** Carbamoylated LDL (cLDL) causes endothelial dysfunction by interacting with the endothelial lectin-like-oxidized LDL receptor-1 (LOX-1), leading to reduced nitric oxide (NO) bioavailability through p38-dependent NADPH (nicotinamide adenine dinucleotide phosphate)-oxidase activation, the production of reactive oxygen species (ROS), and S-glutathionylation-dependent endothelial nitric oxide synthase (eNOS) uncoupling. cLDL enhances endothelial cell death as well as vascular smooth muscle proliferation and inflammatory macrophage signalling. cLDL also enhances monocyte binding to endothelial cells through a dose-dependent upregulation of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). cHDL colocalizes with myeloperoxidase and macrophages in human atherosclerotic lesions and impairs endothelial repair. Monocytes are key players in the initiation and progression of atherosclerosis thorugh the synthesis of, for example, ROS and matrix metalloproteinases that influence extracellular matrix plaque remodelling. Carbamoylation leads to an increased fragmentation of collagen type I by disturbing the triple-helix structure, increasing susceptibility to collagenases.

of the two adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)[85]. Monocyte adhesion to the endothelial lining has a key role in endothelial dysfunction by initiating neo-intimal invasion with foam cell formation during plaque formation and by modulating plaque stability[86].

Carbamoylation alters the intracellular trafficking of LDL[87–90]. *In vitro*, the binding of mildly carbamoylated LDL to fibroblast receptors (such as apolipoprotein B receptor and apolipoprotein E receptor) was decreased compared to that of unmodified LDL[88], resulting in reduced cLDL clearance by as much as 41%[87–89]. However, extensive LDL carbamoylation accelerated cLDL clearance[89]. This effect is caused by the binding of cLDL to a unique pattern of scavenger receptors such as LOX-1, CD36 (also known as platelet glycoprotein 4), scavenger receptor expressed by endothelial cells I (SREC-1) and scavenger receptor class A member 1 (also known as CD204), which have proatherogenic effects on human endothelial cells[8,84,89,90].

MPO can generate multiple atherogenic LDL species such as cLDL, nitrogen dioxide (NO_2)-LDL[91], oxLDL[92], hypochlorous acid (HOCl)-modified LDL[92] and acetylated LDL[93] *in vivo*[94]. The uptake and degradation of NO_2-LDL by macrophages leads to massive cholesterol deposition and foam-cell formation[91]. Mildly oxidized LDL and HOCl-modified LDL disturb anti-atherogenic reverse cholesterol transport through effects on the activity of lecithin–cholesterol acyltransferase[92]. In transgenic mMPO-knockout mice that express human MPO, atherosclerosis was accelerated[95,96] and the concentration of protein-bound homocitrulline was increased in aortic tissue, which correlated strongly with total aortic cholesterol content. In addition, MPO can mediate protein carbamoylation in atherosclerotic plaques in the human artery wall, where cLDL and MPO are colocalized[8].

Carbamoylation of HDL (cHDL) impairs HDL-mediated activation of lecithin-cholesterol acyltransferase, which results in loss of its anti-inflammatory properties by inhibiting the activity of paraoxonase, a major HDL-associated anti-inflammatory enzyme. Carbamoylation also decreases the antioxidative activity of HDL[97]. cHDL colocalizes with MPO and macrophages in human atherosclerotic lesions[98]. Using physiological and pathological levels of carbamoylation, two studies showed that modification of one carbamoyl-lysine residue per HDL-associated apolipoprotein A-I was sufficient to induce cholesterol accumulation and lipid-droplet formation in human macrophages[98]. This phenotype resulted from a reduction in cholesterol efflux, which is mediated by the HDL-receptor scavenger receptor class B member 1 (SRB1).

In vitro studies with human arterial endothelial cells showed that cHDL impaired endothelial repair by down-regulating the expression of vascular endothelial growth factor receptor 2 and inhibiting the SRB1-mediated signalling pathway. Based on these findings, the presence of cHDL could be expected to accelerate the development and progression of cardiovascular disease in patients with ESRD[10].

Monocytes are key players in the initiation and development of atherosclerosis through the synthesis of ROS and matrix metalloproteinases that influence extracellular matrix plaque remodelling[99]. Monocytes are attracted by several chemokines and cytokines to atherogenic sites in vessel walls. They then undergo transendothelial migration to the arterial wall, mediated by their interaction with type I collagen through integrin-β2 (also known as CD18) and integrin-αX (also known as CD11c)[100]. Finally, the differentiation of monocytes into macrophages contributes to the constitution of the plaque[101]. *In vitro* carbamoylation experiments have shown that monocytes adhere better to carbamoylated type I collagen than to normal collagen[102]. Carbamoylation could therefore lead to a high number of monocytes in the arterial wall, which are able to degrade the extracellular matrix by producing high amounts of matrix metalloproteinase-9 and could increase the risk of fibrous-cap rupture, triggering a thrombotic event or acute coronary syndrome[102].

Based on growing evidence from experimental *in vitro* and *in vivo* studies, carbamoylation has a clear mechanistic link with atherosclerosis by inducing endothelial dysfunction, and high levels of carbamoylated proteins increase the risk of cardiovascular

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disease, independent of traditional risk factors. These effects might contribute to the increased cardiovascular morbidity and mortality in patients with uraemia.

Renal cell carcinoma

Carbamoylation can also alter the turnover of extracellular matrix proteins by increasing their resistance to various collagenases or by increasing their sensitivity to matrix metalloproteinase-2 (REF. 103). This post-translational modification reduces the ability of collagen to form fibrils *in vitro*[104] and could therefore prevent tumour spreading. In an *in vitro* model of a pre-intravasation tumoural microenvironment, collagen type I glycation and carbamoylation inhibited invasive cell migration of human HT1080 fibrosarcoma cells without affecting their proliferation[105]. Accordingly, ESRD-induced renal cell carcinoma in patients undergoing dialysis is less metastatic than sporadic renal cell carcinoma[106,107]. The inhibitory effect of carbamoylation on tumour spreading could help the development of novel anticancer strategies in the future.

Immune system

The carbamoylation process has several consequences for the immune system. Lysine carbamoylation of human IgG1 via the MPO–H_2O_2–SCN^- pathway in activated neutrophils inhibits the classical complement pathway[14]. Carbamoylation of five of the 26 lysine residues in the human IgG1 heavy chain (located in the hinge region and the N-terminal fragment of the CH_2 domain) hinders the binding of IgG1 to the complement component C1q. Carbamoylation also selectively inhibits the complement-mediated cytotoxicity of rituximab without affecting its antibody-dependent cytotoxicity[14]. This finding illustrates that carbamoylation could reduce the effectiveness or toxicity of certain treatments for inflammatory diseases.

In vitro studies have shown that carbamoylated albumin[108] and carbamoylated collagen[104] inhibit the respiratory burst of polymorphonuclear neutrophils, which is characterized by a release of oxygen free radicals and contributes to their antibacterial activity. This process depends on the inhibition of p125 focal adhesion kinase 1 phosphorylation[108], which suggests that carbamoylation could be linked to the increased occurrence of infections in patients with CKD.

Carbamoylation therefore has negative effects on nonspecific innate immunity as well as on specific memory-based adaptive immunity, indicating that carbamoylation contributes to neutrophil dysfunction and to the increased vulnerability of patients with CKD or ESRD to inflammation and infection. In patients with uraemia, polymorphonuclear white blood cells have reduced phagocytic activity, which is directly attributable to uraemic toxicity[109]. In addition, several other factors such as CKD-indued anaemia, iron overload, dialysis and dialyser bioincompatibility can exacerbate these cellular dysfunctions[109].

The formation of PTMDPs contributes to autoimmune diseases such as rheumatoid arthritis, which are characterized by the generation of antibodies against citrullinated and carbamoylated peptides (anti-carP antibodies)[15,110]. The heterogeneity of citrullinated and carbamoylated proteins and their autoantibodies hinders elucidation of the molecular mechanisms underlying their pathogenicity[15]. Anti-carP antibodies are present years before rheumatoid arthritis can be diagnosed clinically, and their presence is associated with a more aggressive disease course[15,110]. These antibodies are primarily directed against specific peptides of the carbamoylated human fibrinogen β-chain (nine of 34 lysines)[111]. Peptides containing a homocitrulline at position 83 were immunodominant epitopes in the sera of 35% of patients with rheumatoid arthritis[15,110]. In addition, anti-carP antibodies targeted carbamoylated lysines at positions 52, 264, 351, 367 and 374 of the fibrinogen β-chain. As anti-carP antibodies recognize a single post-translationally modified protein (fibrinogen), they might be a good indicator of disease risk and disease progression compared to anti-citrullinated protein antibodies, which are polyreactive[112]. However, carbamoylated albumin[113], carbamoylated vimentin[114], α-enolase[115] and a complex mixture of carbamoylated proteins in fetal calf serum[116,117] have also been identified as anti-CarP antibody targets in rheumatoid arthritis.

Endothelial function was impaired in patients with rheumatoid arthritis, even in those without traditional risk factors. These patients had anti-carP antibodies, which could potentially hamper endothelial function[118]. Anti-carP antibodies are also present in 50% of patients with systemic lupus erythematosus[119].

Carbamoylation is therefore a novel molecular pathway that leads to the generation of new potentially pathogenic epitopes and to the production of autoantibodies. Future studies should investigate the contribution of anti-carP antibodies to renal injury in patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis.

Renal fibrosis

Carbamoylated albumin can alter renal function as it is more nephrotoxic than glycated or unmodified albumin. In an amphibian axolotl model, carbamoylation enhanced the severity of tubular cell damage and interstitial fibrosis caused by albumin[16]. These pathological changes in the interstitium were mediated by an upregulation of factors involved in the AGE–NF-κB pathway. In addition, injection with carbamoylated albumin stimulated the expression of pro-fibrogenic factors such as TGFβ (REF. 16) in axolotl tubular epithelial cells, leading to an excessive accumulation of extra-cellular matrix and widespread tissue scarring[120,121].

Carbamoylation locally destabilized the triple helix structure of acid-soluble type collagen molecules prepared from Sprague–Dawley rat tail tendons, making them less prone to collagenases[103]. This post-translational modification leads to a loss of the structural and functional properties of collagen and is a hallmark of fibrosis. In addition, *in vitro* carbamoylated serum proteins could activate mesangial cells and lead them to a profibrogenic phenotype by increasing the deposition of collagens I and IV[122].

Intravasation
Invasion of cancer cells through the basal membrane into a blood or lymphatic vessel.

Immunodominant
Epitope that elicits the stronger immune response.

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These findings suggest that carbamoylation decreases the susceptibility of extracellular matrix proteins to proteolytic degradation, which will modify their turnover. Novel insights in the role of carbamoylation in the pathophysiology of renal fibrosis could open pathways to prevent or decelerate progression of CKD[123].

Haemostasis and coagulation

CKD is associated with a variety of haemostatic dysfunctions and with a prothrombotic tendency in early stages of the disease, leading to frequent cardiovascular events[124]. Patients with advanced stages of CKD also have an increased incidence of bleeding diathesis[124]. Fibrinogen, which is crucial for coagulation, is a target for cyanate-induced modifications in patients with CKD. Although data on fibrinogen carbamoylation in patients with non-dialysis CKD are lacking, ESRD is associated with elevated plasma concentrations of carbamoylated fibrinogen[125].

In vitro experiments have shown that fibrinogen carbamoylation alters the morphology of the fibrin clot, reduces its mechanical stability and increases its resistance to fibrinolysis[125]. Carbamoylation impairs the aggregation and crosslinking of fibrin monomers, presumably as a result of structural alterations at the lysine residues, which are located upstream of the polymerization signals on the α and β-chains of fibrinogen[125]. In addition, the modification of more distant residues could also have an effect on the kinetics of fibrinogen polymerization. Carbamoylation might also be associated with conformational changes in the C-terminal γ-chain of fibrinogen, although this hypothesis has not yet been tested.

Thrombin-mediated cleavage of unmodified and carbamoylated fibrinogen *in vitro* yielded comparable amounts of fibrinopeptides A and B, suggesting that carbamoylation has no effect on this process[125]. However, in contrast to fibrinopeptide B, which is protected by pyroglutamination of the N-terminal amino acid, the N-terminal amino group of fibrinopeptide A was highly carbamoylated *in vitro*, as shown by mass spectroscopy[125]. This modification is responsible for the conversion of fibrinopeptide A into a chemotaxin that recruits neutrophils, potentially contributing to the migration of inflammatory cells to sites of fibrinogen turnover. As fibrinogen and its derived peptides have many pro-inflammatory functions[126], the effects of carbamoylation on the interaction between fibrinogen and immune cells should be further investigated.

Fibrinogen also links platelets to adjacent platelet aggregates[127]. Carbamoylation might thus affect fibrinogen binding to platelet receptors, potentially leading to destabilization of the platelet plug with prolongation of bleeding periods as a consequence. In the presence of carbamoylated fibrinogen, fibrin clots develop large pores under shear stress and are prone to fragmentation[125]. Detachment of the clot from the vascular wall can lead to embolism and ischaemia.

Bleeding diathesis
Increased tendency to bleed mostly due to hypocoagulability, which in turn is caused by a coagulopathy.

Erythropoietin activity

In vivo studies in healthy Sprague–Dawley rats showed that exposure of EPO to high concentrations of cyanate reduced its biological activity. Carbamoylation of EPO diminished its ability to stimulate erythropoiesis and its non-erythropoietic effects on renal proximal and distal tubular cells[128–130].

In patients with ESRD, the level of resistance to EPO correlated positively with the presence of carbamoylation products[131]. Carbamoylated EPO (cEPO) has gained a lot of attention in the past 10 years owing to its cytoprotective effects against renal injury[132–134]. Administration of cEPO in rat models of acute kidney injury (AKI) improved recovery of renal function, increased body and kidney weight and extended survival without substantially increasing haematocrit levels or blood pressure, as seen with native EPO[132]. Treatment with cEPO also stimulated angiogenesis, reduced tubular apoptosis and increased the growth of peritubular capillary endothelial cells in a rat model of ischaemia–reperfusion injury[135].

In contrast to native EPO, cEPO does not have erythropoietic and vasoconstrictive effects as it activates the heterodimeric form and not the homodimeric form $(EPO\text{-}R)_2$ of the erythropoietin receptor (EPO-R)[136]. In addition, EPO and its carbamoylated derivative have different effects on endothelial cell migration. For example, exposure of human endothelial cells (EA.hy926 cells) to native EPO, but not to cEPO, markedly increased cell motility. After binding to $(EPO\text{-}R)_2$, EPO activates janus kinase 2 (JAK2)[137]. JAK2-mediated phosphorylation of EPO-R leads to activation of the phosphatidylinositol 3-kinase (PI3K) and RACα serine/threonine-protein kinase (AKT) pathways, as well as stimulation of the RAS–RAF–MEK–MAPK pathway, phosphorylation and dimerization of STAT transcription factors, and activation of NF-κB[138]. Human leukaemic cells (UT-7 cells) exposed to cEPO had a higher expression and activity of protein tyrosine phosphatase 1B (PTP1B), which terminates the signal induced by EPO. These findings could explain the lack of proliferative effects of cEPO in erythroid cells. *In vitro*, however, cEPO retained its protective action against ROS generation[137]. In rats fed a high-fat, high-carbohydrate diet, administration of cEPO protected against diabetic cardiomyopathy without markedly affecting erythropoiesis through activation of the PI3K–AKT pathway[133].

In mice with ciclosporin-induced nephropathy, administration of cEPO inhibited tubular apoptosis and was associated with elevated PI3K activation and AKT phosphorylation[139]. cEPO administration also reduced ciclosporin-induced interstitial fibrosis, decreased cortical mRNA levels of TGFβ1 and type I collagen and suppressed interstitial macrophage infiltration.

In a model of ischaemia–reperfusion injury after kidney transplantation, treatment of the donor and recipient with cEPO prevented renal graft dysfunction, tubular oxidative stress and apoptosis[140]. cEPO administration prevented the drop in AKT expression in the tubuli that is usually caused by ischaemia–reperfusion injury and preserved capillary density in the tubulointerstitium of the graft. Although cEPO has no effect on haematocrit levels, the renoprotective potential of this EPO derivative could be harnessed to treat different clinical conditions such as AKI, ischaemia–reperfusion injury and ciclosporin-induced nephropathy.

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Carbamoylation predicts cardiovascular disease

Two separate, relatively small, case-control studies ($n = 1{,}000$ in both studies combined) examined individuals involved in the Genebank initiative, a large clinical repository generated from sequential consenting individuals undergoing diagnostic left heart catheterization[8]. In one of the studies — an age and gender-matched case-control study of 150 patients with cardiovascular disease and 300 controls — plasma concentrations of carbamoylation products, as measured by protein-bound homocitrulline levels, correlated with the prevalence of cardiovascular disease, defined as coronary artery disease (CAD) or peripheral artery disease (PAD)[8]. The quartile with the highest plasma concentrations of protein-bound homocitrulline (≥300 μmol homocitrulline per mol of lysine) had an approximately 7–8-fold higher risk of having clinical or angiographic evidence of cardiovascular disease and major adverse cardiac events compared to that of individuals in the first-quartile (≤30 μmol homocitrulline per mol of lysine). In the second age and gender-matched case-control study, the plasma concentration of protein-bound homocitrulline was an independent predictor of increased risk of CAD, future myocardial infarction, stroke and death[10]. These findings have been confirmed by other studies[11,141] (TABLE 1). Patients with CAD had a markedly higher serum level of protein-bound homocitrulline than those without CAD, which was independently associated with the presence and severity of CAD, poor collateral coronary growth and the number of stenotic arteries[11,141]. As all patients included in this study had normal renal function, the elevated levels of protein-bound homocitrulline could result from the local production of isocyanic acid (in the atherosclerotic plaques for example). However, no measurements of MPO levels were performed in this study. In a cohort study of patients with chronic systolic heart failure, increasing concentrations of plasma protein-bound homocitrulline correlated with long-term adverse clinical outcomes such as death and cardiac transplantation, even after adjustment for traditional cardiorenal indices of adverse prognosis[43].

The reserachers of the Genebank initiative cohort study not only demonstrated the diagnostic potential of protein-bound homocitrulline for cardiovascular disease, but also supported their clinical findings with *in vitro* chemistry and well-defined animal experiments[8]. In particular, the increased risk of atherogenicity due to the local production of protein-bound homocitrulline by MPO was clearly supported by experimental findings. In a peritonitis mouse model, the levels of protein-bound homocitrulline were markedly increased in wild-type mice but not in MPO-deficient mice. In a second experiment, human MPO overexpression in hyperlipidaemic LDL-receptor-null mice fed a high-fat, atherogenic diet dramatically increased the levels of protein-bound homocitrulline in atherosclerotic plaque-laden aortas. This study highlighted MPO as a key player in the development of atherosclerosis but also suggested that it is crucial for other diseases associated with oxidative stress and inflammation such as multiple sclerosis or rheumatoid arthritis, and in smokers, who have an increased risk of cardiovascular diseases owing to higher blood concentrations of thiocyanate.

In patients with ESRD on haemodialysis, levels of serum protein-bound homocitrulline have potential for predicting overall survival[7]. A 5 year follow-up study of 347 patients undergoing haemodialysis showed a strong correlation between the levels of serum protein-bound homocitrulline and all-cause and cardiovascular mortality. Serum protein-bound homocitrulline remained an independent predictor of survival, even after adjusting for several other covariates. Mortality of patients in the highest tertile of serum protein-bound homocitrulline levels was twice as high as that of patients in the middle and the lowest tertile. However, no statistical significant relationship between levels of protein-bound homocitrulline and MPO was found in that study.

In the prospective, observational ARMORR study, which investigated blood markers of early (<90 days) and late (≥1 year) mortality among 187 patients on haemodialysis, and in the randomized, controlled 4D study, which investigated the benefits of cholesterol-lowering agents in 1,161 patients with diabetes mellitus on haemodialysis, the baseline percentage of serum carbamoylated albumin was markedly lower in survivors than in non-survivors in the first year of dialysis[6]. The percentage of carbamoylated albumin in serum associated strongly with the increased 1-year adjusted risk of cardiovascular mortality, sudden cardiac death and the 4-year risk of death from congestive heart failure, but not with myocardial infarction or stroke[6,142]. The latter finding contradicts that of the Genebank case-control study. However, the fact that patients with severe heart disease could have died from an acute event (sudden cardiac death) before they reached ESRD and were thus not included in these studies, should be taken into account. In addition, congestive heart failure is a larger cause of morbidity and mortality in patients on haemodialysis than in patients with nondialysis CKD due to the ever changing volume status in these often anuric patients. In the 4D study, the baseline serum percentage of carbamoylated albumin correlated with serum levels of cardiac stress markers (for example, troponin T and N-terminal pro-B-type-natriuretic peptide) in patients with type 2 diabetes mellitus on dialysis[142].

The link between carbamoylation, uraemia and mortality is not completely understood. The finding that uraemic cardiomyopathy is one of the risk factors for sudden death in patients on haemodialysis could be explained by the hypothesis that 'hypercarbamoylation' of cardiac proteins is an acute insult to the cardiac conduction system[6,143]. Carbamoylation could alter the interaction between proteins and inflammatory cells and increase the susceptibility of proteins to proteolysis and cause aberrant tissue remodelling in target organs. As shown in a mouse model of CKD, the progressive accumulation of carbamoylated residues in extracellular matrix proteins, such as collagen in the heart and kidney, leads to aberrant tissue remodelling of the extracellular matrix by activated monocytes[48], which could be associated with a high resistance of electrical conduction pathways, contributing

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Table 1 | **The association of carbamoylation with cardiovascular disease, myocardial dysfunction, heart failure and mortality**

Marker of carbamoylation	Detection method	Study design	Number of patients	End point	Key findings	Refs
Serum levels of protein-bound homocitrulline	HPLC–MS/MS	Cohort study	347 patients on HD	All-cause mortality, cardiovascular mortality; follow-up at 5 years	The level of protein-bound homocitrulline was an independent predictor of cardiovascular and all-cause mortality	9
Plasma levels of protein-bound homocitrulline	HPLC–MS/MS	Case-control study	300 control individuals (no clinical evidence of CVD* and no angiographic evidence of atherosclerosis) and 150 patients with CVD	Risk of MI, stroke and death	The level of protein-bound homocitrulline was an independent predictor of increased risk of CAD, MI, stroke and death	10
Plasma levels of protein-bound homocitrulline	HPLC–MS/MS	Cohort study	115 patients with chronic systolic HF	Development and progression of cardiorenal syndrome and adverse long-term clinical outcomes (death, cardiac transplantation); follow-up at 5 years	• High levels of protein-bound homocitrulline were associated with poor renal function, but not with indices of cardiac structure or function except modestly with increased right atrial volume index • Levels of protein-bound homocitrulline predicted adverse long-term events	31
Serum levels of homocitrulline	ELISA	Cohort study	117 patients with stable angina and chronic total occlusion of at least one major epicardial coronary artery	Formation of coronary collaterals	• Increased homocitrulline concentrations in patients with poor coronary collateralization compared with those with high collateralization • Elevated homocitrulline concentration was a strong predictor of poor coronary collateral growth	117
Total (free and protein bound) levels of homocitrulline	HPLC–MS/MS	Cross-sectional study	45 control individuals (that is, with no cardiovascular risk factors) and 109 patients who were referred for coronary angiography	Association with CAD	Homocitrulline levels were increased during CAD and were positively associated with disease severity	118
Serum percentage of carbamoylated albumin	HPLC–MS/MS	Case-control study and cohort study	20 non-uraemic individuals, 122 patients with CKD (stage 3 or 4), 187 patients on HD from the ARMORR study and 1,161 patients on HD with diabetes mellitus from the 4D trial	All-cause mortality; follow-up at 1 year	• The serum percentage of carbamoylated albumin correlated with time-averaged blood urea concentrations and was twice as high in patients with ESRD than in non-uraemic individuals • The serum percentage of carbamoylated albumin was a risk factor for all-cause mortality • Decreased serum concentrations of amino acids correlated with higher serum percentages of carbamoylated albumin in patients with ESRD	8
Serum percentage of carbamoylated albumin	HPLC–MS/MS	Cohort study	1,161 patients on HD with diabetes mellitus from the 4D trial	Incidence of cardiovascular mortality, sudden death, MI (fatal and non-fatal), stroke (fatal and non-fatal), all-cause mortality, and deaths due to infection; follow-up at 4 years	• The serum percentage of carbamoylated albumin was strongly associated with 1-year adjusted risk of cardiovascular mortality, sudden cardiac death, and the 4-year risk of death from congestive HF, but not with MI or stroke • Patients with low serum percentage of carbamoylated albumin, treated with atorvastatin, experienced a marked improvement in their 4-year survival • The percentage of carbamoylated albumin in serum was associated with ongoing cardiac damage, risk of congestive HF, and sudden cardiac death	119
Serum percentage of carbamoylated albumin	HPLC–MS/MS	Case-control study	244 patients on HD who survived at least 1 year of treatment (controls), 122 patients on HD who survived at least 90 days but died within 1 year of initiating HD (ARMORR study)	All-cause mortality; follow-up at 1 year	• Protein carbamoylation decreased with dialysis initiation, and an increased reduction over time was associated with a lower risk of mortality. • Changes in carbamoylation levels predicted mortality risk beyond traditional variables, including markers of dialysis adequacy and nutrition	121

*CVD defined as documented history of CAD, PAD, MI, stroke, revascularization procedure, or angiographic evidence of atherosclerosis (>50% stenosis) in one or more major coronary vessels. CAD, coronary artery disease; CKD, chronic kidney disease; CVD, cardiovascular disease; ESRD, end-stage renal disease; HD, haemodialysis; HF, heart failure; HPLC–MS/MS, high-performance liquid chromatography with tandem mass spectrometry; MI, myocardial infarction; PAD, peripheral artery disease.

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to intraventricular conduction disturbance, arrhythmia and sudden death. The lack of association between the percentage of carbamoylated albumin and myocardial infarction or stroke could be explained by the fact that the study population consisted of patients with diabetes mellitus and ESRD. In this population, levels of carbamoylated albumin might not be the most important risk factor for atherosclerosis in comparison with the large variety of novel and traditional cardiovascular risk factors that exist in the general population. Although uraemia is a systemic source of cLDL, local MPO activation in the atherosclerotic plaques probably contributes more cLDL than does uraemia[8]. The strong correlation between MPO activity in atherosclerotic plaques and plasma cLDL levels, and the lack of such correlation with the percentage of carbamoylated albumin in serum increases the plausibility of this hypothesis.

In a nested patient-control study that included 122 participants from a large incident dialysis cohort who survived at least 90 days, but died within 1 year of initiating haemodialysis (patients) and 244 patients on haemodialysis who survived for at least 1 year (controls), the percentage of carbamoylated albumin in plasma was measured at dialysis initiation and every subsequent 90-day period until 1 year or death. Comparable baseline percentages of carbamoylated albumin were observed between controls and patients[144]. Adjusted repeat measurements of percent carbamoylated albumin showed a greater mean decrease after initiation of haemodialysis from baseline to final measure in the surviving control group than in the group of patients who died before the 1-year end point[144]. These findings suggest that a greater reduction in carbamoylated albumin over time is associated with a lower risk of mortality. As these findings are based on relatively small study populations comprised only of patients undergoing haemodialysis, the pathogenic role of carbamoylated albumin in early stages of CKD has not yet been demonstrated.

Several studies have shown that statins are not effective in reducing the risk of cardiovascular death, nonfatal myocardial infarction or stroke in patients receiving haemodialysis[145,146]. In an analysis of the carbamoylation patterns of patients in the 4D study, the use of atorvastatin was associated with reduced mortality in the tertile of patients with the lowest percentage of carbamoylated albumin in serum, but not in the middle or upper tertiles. These findings could be explained by the inverse correlation between the percentage of carbamoylated albumin and LDL concentration[142]. The pathogenesis of atherosclerosis and CAD is different in patients with CKD than in the general population. Although the concentration of LDL-cholesterol is an independent risk factor for atherosclerotic events in the general population[147], the utility of reducing LDL-cholesterol levels in patients with CKD, particularly in those undergoing dialysis, is controversial[148–158]. In addition to atherosclerotic changes and calcification, carbamoylation might potentially have a stronger causative effect than hypercholesterolaemia in this specific population than in the general population[142]. The hypothesis according to which hypercarbamoylation of cardiac proteins can lead to an acute insult to the heart conduction system (sudden cardiac death) fits with the observation that cholesterol-lowering treatments only have beneficial effects in patients with well-controlled carbamoylated protein concentrations.

Several studies have demonstrated that lipoprotein carbamoylation explains the cardiovascular risks that are not captured by traditional cardiovascular risk factors in patients with CKD. Lipoprotein carbamoylation could therefore be a target for the development of novel drugs to treat and prevent cardiovascular diseases.

Reducing the carbamoylation load

Several studies mentioned above have linked carbamoylated proteins to the adverse outcomes of kidney disease. Reducing the rate of carbamoylation could therefore be an interesting therapeutic approach. Several options to lower the rate of carbamoylation exist: nutritional approaches (amino acid supplementation), modified dialysis prescriptions and anti-inflammatory therapies. To date, however, only a small number of studies of limited size have investigated the effectiveness of strategies to reduce the carbamoylation load and their impact on morbidity and mortality.

Amino acid supplementation

Free amino acids can act as scavengers for carbamoylation and reduce the carbamoylation of proteins such as albumin[159]. *In vitro*, preclinical and human studies have shown that the formation of carbamoylated albumin is stimulated by low levels of free amino acids and high concentrations of urea in the blood[6,160]. Supplementation with certain amino acids (for example, cysteine and histidine) or some nucleophilic biomolecules (for example, cysteamine, glycylglycine and taurine) can inhibit protein carbamoylation[6].

Haemodialysis

The identification of individuals who are at risk of developing uraemic complications by measuring the carbamoylation load could lead to adaptation and intensification of haemodialysis regimens (daily or extended). In a small study of 33 patients with ESRD, extended haemodialysis (7–8 h of dialysis three times a week) versus standard-duration haemodialysis reduced the average change in the percentage of carbamoylated albumin (–3.20 mmol/mol versus +0.21 mmol/mol) after 52 weeks of treatment[161]. Patients with a >25% reduction in carbamoylated albumin levels had decreased left ventricular mass and increased total serum albumin concentrations compared to those of patients who did not achieve such reductions in carbamoylated albumin levels, leading to the suggestion that the reduction in carbamoylated albumin levels resulted from a decrease in average blood urea concentrations as a consequence of dialysis. Although this study has several limitations including small sample size, lack of matching between the two groups (cause of ESRD, baseline access type line and length of dialysis), it shows that reducing protein carbamoylation could be an novel therapeutic strategy to prevent CKD-associated cardiovascular disease[144].

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Amino acid supplementation and haemodialysis

Patients with CKD have high levels of carbamoylated amino acids; in fact, the percentage of carbamoylated amino acids can even exceed that of unmodified amino acids[162]. As carbamoylated amino acids cannot be used for protein synthesis, carbamoylation might contribute heavily to amino acid deficiencies and, thus, to protein malnutrition. Patients undergoing haemodialysis have reduced plasma concentrations of amino acids[163], which can easily be removed by diffusive strategies such as haemodialysis owing to their size. The removal of carbamoylated amino acids with haemodialysis varies between 25% and 65% depending on dialysis duration and intensity[160]. As more intense dialysis also removes non-carbamoylated amino acids, the anabolic effects of intensive dialysis regimes could be attributed to more efficient protein synthesis resulting from the removal of carbamoylated amino acids than to higher levels of amino acids *per se*. To maximize the positive effects of protein synthesis, intradialytic oral nutritional supplements can be provided to patients undergoing maintenance haemodialysis[164,165].

In a small trial, levels of carbamoylated albumin were 15% lower among patients who received intravenous administration of amino acids during thrice weekly dialysis sessions over 8 weeks than in patients who did not receive amino acid supplementation[159], despite a lack of difference in BUN levels between the interventional and the control group. Combining intense dialysis to remove carbamoylated amino acids and administration of additional non-carbamoylated amino acids can therefore substantially reduce the carbamoylation load; however, no studies to date have evaluated whether this strategy actually results in enhanced protein synthesis.

Anti-inflammatory therapies

Uraemia is a state of oxidative stress with decreased serum antioxidant activity. Although the effects of vitamin administration in patients with CKD are unclear, *in vitro* antioxidants can inhibit LDL carbamoylation[166].

The potential of the above-described carbamoylation lowering strategies should be tested in large double-blind, randomized clinical trials using patient-relevant end points such as improved nutritional status and/or decreased cardiovascular morbidity and mortality. The optimal combination of amino acids for the prevention of protein carbamoylation should be identified, taking into account differences in the effectiveness, pharmacokinetics, safety and tolerability of the different amino acids.

Conclusions

The process and consequences of carbamoylation might be more complex than initially thought. Urea has been regarded for decades as a relatively non-toxic compound based on data from the Haemodialysis Study Group[167] and the ADEMEX trial[168], which showed that urea removal was not associated with improvements in hard outcomes. Accumulating evidence supports the notion that protein carbamoylation, which is inherently associated with urea accumulation and renal failure, results in a broad spectrum of biological and clinical consequences. As demonstrated by *in vitro*, *in vivo* and clinical studies, carbamoylation has detrimental effects at every level of physiology and its special relationship with inflammation and uraemia sheds light on risk factors in patients with ESRD. One should keep in mind, however, that *in vitro* experiments sometimes use non-physiological concentrations of cyanate or thiocyanate to induce carbamoylation, and that the relevance of findings from such studies require confirmation in *in vivo* studies.

The effects of protein carbamoylation in kidney disease and inflammation might be mediated by the pathologic gain-of-function of carbamoylated proteins. Carbamoylation could generate new ligands in the same way that protein glycation creates pathophysiologic protein AGEs. As only a low proportion of lysines are modified, carbamoylation is, however, unlikely to result in large amounts of proteins with altered function. Further research is therefore needed to unravel the pathophysiological mechanisms underlying the effects of protein carbamoylation in kidney disease and inflammation.

Various biomarkers to assess carbamoylation levels have been assessed, among which homocitrulline levels and percentage of carbamoylated albumin are the most used. The majority of potential markers can only be assayed using rather complex analytical methods, hampering their use in clinical practice. Attempts should be made to develop simpler tests, which can be implemented into routine clinical practice. We have developed a simple and fast electrophoretic assay to quantify carbamoylated albumin[169]. The development of such high-throughput assays could reduce analysis time, although standardizing the quantification of carbamoylation biomarkers remains another demanding task.

Carbamoylation was described in 1960 (REF. 7) but the importance of this non-enzymatic process as a prognostic marker for cardiovascular events in the general population and in patients undergoing dialysis in particular has only been addressed in the past 10 years. Assessing the degree of carbamoylation could provide a robust link between uraemic toxicity and cardiovascular outcomes, and could potentially be used to monitor the adequacy of dialysis and reduce uraemic toxicity. The addition of carbamoylation products to a multivariate models (adjusted for age, sex, race, history of cardiovascular disease, diabetes mellitus, Charlson comorbidity index, dialysis vintage, levels of MPO, IL-6, BUN, creatinine, albumin, C-reactive protein and TNF, body mass index, levels of EPO and normalized protein catabolic rate) improved mortality risk prediction in patients with ESRD, independent of traditional risk factors[7].

Finally, the quest for protein carbamoylation modifying mechanisms is far from complete. The enigmatic biochemistry of elasmobranchs, the physiology of which is characterized by urea retention, resulting in body fluids that contain extremely high concentrations of urea (350–500 mM), is still incompletely resolved[170,171]. Intriguing questions regarding the handling of carbamoylated proteins by sharks[172], which have very high urea concentrations but do not seem to experience harmful carbamoylation effects, and the existence of decarbamoylases, which could be harnessed for therapeutic purposes, also remain open.

Elasmobranchs
Subclass of Chondrichthyes or cartilaginous fish.

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Author contributions
All authors contributed substantially to researching data for the article, discussing of the article's content, writing, and reviewing or editing the manuscript before submission.

Competing interests statement
The authors declare no competing interests.

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