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Irreversible Post-translational Modifications – Cardiovascular Risk Factors and Avenue for Therapy

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Irreversible Post-translational Modifications – Cardiovascular Risk Factors and Avenue for Therapy

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Abstract

With continuous identification of non-enzymatic post-translational modified isoforms of proteins and lipoproteins, it is becoming increasingly clear that non-enzymatic post-translational modifications (nPTMs) limit or modify the biological functions of native proteins, peptides and lipoproteins and are majorly involved in development of various chronic disease.

These cumulative and irreversible modifications occur progressively during aging when endogenous levels of reactive metabolite drift toward a physiological imbalance, and more importantly are strongly amplified in the human pathology of various chronic diseases such as chronic kidney disease, metabolic disorders.

This article reviews the recent knowledge on the genesis, pathological consequences and clinical impact of nPTMs and focuses on the role of nPTM in progression of cardiovascular disease.

Abbreviations

AGE	advanced glycation end product
Apo-B	apolipoprotein B
ApoA-I	apolipoprotein A1
CKD	chronic kidney disease
CVD	cardiovascular disease
CVE	cerebrovascular event
ECM	extracellular Matrix
ESRD	end stage renal disease
GFR	glomerular filtration rate
HbA1c	carbamylated hemoglobin
HCit	homocitrulline
HDL	high density lipoprotein
HMG CoA	β-hydroxy β-methylglutaryl-CoA
HPLC	high pressure liquid chromatography
ICAM-1	intracellular adhesion molecule 1
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LOX-1	lectin-like oxidized LDL receptor
MS	mass spectrometry
NHS	n-hydroxysuccinimide
NO	nitric oxide
nPTMs	non-enzymatic post-translational modifications
NSAIDS	nonsteroid anti-inflammatory drugs
oxLDL	oxidized low density lipoprotein
PTM	post-translational modification
RAGE	receptor for Advanced glycation end product
ROS	reactive oxygen species
SGLT2	sodium-glucose co-transporter-2
sRAGE	soluble receptor for Advanced glycation end product
SRB1	scavenger receptor type B1
TBARS	thiobarbituric acid reactive substances
TEAC	trolox equivalent antioxidant capacity
VCAM-1	vascular adhesion molecule 1

Post-translational modifications: essential regulators and disease mediators

Post-translational modifications (PTMs) are chemical modifications of proteins, peptides or lipoproteins after their biosynthesis, thereby altering their original chemical composition and conformation [1]. These PTMs can be regulated by enzyme-catalyzed or spontaneous chemical reactions, hence PTMs are generally divided into two categories: (1) enzymatic and (2) non-enzymatic PTMs. **Enzyme-catalyzed PTMs (ePTM)** are highly regulated, specific for its substrate and mostly reversible [2]. Enzyme-catalyzed PTMs like phosphorylations are essential physiological tools for the regulation of cellular functions, modulation of protein functions and the maintenance of cellular homeostasis. Most prominently, protein kinases are known to regulate protein functions via post-translational phosphorylation.

In contrast, **non-enzymatic PTMs (nPTM)** are spontaneous chemical reactions between electrophilic metabolites and nucleophilic amino side chains of proteins and lipids in close proximity, leading to the addition reaction of chemical groups [2]. With growing numbers of PTMs identified, it has become increasingly evident that especially non-enzymatic post-translational modifications (nPTMs) are strongly involved in the aging process [3] and their frequency is strongly enhanced by metabolic disorders [4] and kidney disease [5] with detrimental consequences for the cardiovascular system [6]. **These novel insights represent potential avenues for prevention, diagnosis and therapy.**

Genesis of nPTMs

The likelihood of nPTMs is influenced by the concentration of reaction partners and increases with prolonged exposure periods [7]. However, the kinetics of non-enzymatic PTMs in living organisms is in constant flux due to the fluctuating ambient concentrations of reactive metabolites as well as the half-life and abundance of each protein, peptide and lipoprotein. Based on the nature of nPTMs, highly abundant proteins and lipoproteins with relative long blood half-life are in particular vulnerable to nPTM [8]. Particular disease conditions such as renal insufficiency, cardiovascular diseases or metabolic disorders or reduced physiological capacity to compensate e.g. metabolic stress due to aging can generate chronic excess of reactive metabolites

seeking nucleophilic side chains, thus promoting the non-enzymatic post-translational modification of proteins, peptides, and lipoproteins [2].

The likelihood of an individual protein, peptide or lipoprotein to undergo nPTMs extends beyond just ambient concentrations of reactive partners or its plasma and/or tissue half-life, but is highly dependent on its amino acid composition, conformation and the sterical availability of nucleophilic side chains [9]. While not each amino acid is vulnerable to non-enzymatic modifications, arginine, lysine, histidine, cysteine, aspartic acid, glutamic acid and tyrosine have strong nucleophilic functional side chains and therefore are able to react with electrophilic metabolites in their free form [10]. However, the **majority of nPTMs** occur at the positively charged amino acid residues of polar amino acids such as histidine, arginine and lysine (**Table 1**). Due to the polarity of these amino acids, they are prone to being located on the outer, hydrophilic surface of proteins, peptides and lipoproteins, with their nucleophilic side chains sterically exposed for electrophilic metabolites [11]. In contrast, cysteine residues within proteins, such as albumin are often buried within the folded protein as disulfide bonds, maintaining the structural integrity of proteins (**Figure 1**) and are therefore less accessible [12]. Notably, lysine is the most affected amino acid by nPTMs (**Table 1**). In addition to the natural high abundance of lysines in proteins (5.9%), lysine contains a long linear hydrophobic C4 hydrocarbon chain, sterically exposing a highly nucleophilic terminal ε-amino group prone for electrophilic attack (**Figure 1**). The primary amine contributes to the protein charge [13] and the stabilization of the protein conformation via salt bridges and/or hydrogen bonds under physiological conditions [14]. With essential roles for protein conformation, nPTMs of these amino acids affect protein fold and final conformational, potentially affecting the biochemical activity of proteins and lipoproteins [3].

Table 1: Amino acids most frequently affected by nPTMs

PTM	Amino acid	Ref
Oxidation	cysteine, histidine, lysine, methionine, tryptophan	[15]
Nitration	tyrosine	[16]
Chlorination	tyrosine	[17]
Carbonylation	lysine, cysteine, histidine, arginine, proline, threonine	[18]

Glycation	cysteine, histidine, lysine, threonine	[19-21]
Carbamylation	lysine, arginine	[22]
Guadinylation	lysine	[23]
Homocysteinylation	lysine	[24]

Pathophysiological cause for nPTMs

Reactive electrophilic molecules, such as free radicals are generated during physiological metabolism, which are commonly removed by endogenous scavengers and/or are cleared by the glomerular filtration [25]. However, endogenous defenses against reactive metabolites deteriorate with the aging process [26]. Moreover, various pathological conditions promote the accumulation of reactive metabolites and hereby contribute to the increased frequency of nPTMs of proteins and lipoproteins [2]. nPTMs promote and contribute to crosstalk between different pathologies, such as a renal impairment, metabolic disorder and oxidative stress, initiating multi-morbid disease development, in particularly CVD [27-32].

Renal impairment and uremia

Decrease of glomerular filtration rate (GFR) and renal blood flow progress gradually with advanced age; with approximately 50% of all adults over the age of 70 having an estimated GFR below 60 mL/min/1.73 m^2, which is in the diagnostic range of chronic kidney disease (CKD). As a result of renal insufficiency, urea is the compound that has the highest serum concentration in CKD patients [33].

In human blood, urea is in equilibrium with ammonium and isocyanate, a highly reactive electrophile that can react with lysine and cysteine to form carbamyl derivatives, a process termed *carbamylation* (**Figure 2**). Even though the equilibrium between urea and isocyanate heavily favors the non-reactive urea by 100:1, significantly elevated urea levels during kidney insufficiency contributes mainly to increased levels of this reactive metabolites [34].

Post-translational **carbamylation** leads to the destabilization of the native secondary and tertiary structures and ultimately conformational changes [35-37], which

are associated with enhanced immunogenicity and autoimmune reactions, including the proliferation and chemotaxis of CD4-positive T-cells, the production of proinflammatory mediators [38] as well as antibody production against carbamylated proteins and lipoproteins [39]. Most noticeably, carbamylation modifications leads to reduction or complete loss-of-function in a broad spectrum of proteins and lipoproteins such as enzymes [40], hormones [41], extracellular matrix [37] and cholesterol[42].

The protein carbamylation level, quantified by plasma level of protein-bound homocitrulline (carbamylated lysine) is associated with increased cardiovascular risk [28]. In an age- and gender-matched case-control study, plasma levels of protein-bound homocitrulline independently predicted increased risk of coronary artery disease, future myocardial infarction, stroke and death [43]. **Therefore, the reduction of urea and hereby carbamylated proteins in patient with renal insufficiency is highly critical for the prevention and treatment of cardiorenal comorbities**.

In parallel to the drastic increase of the uremic burden, which is causative for protein and lipoprotein carbamylation, recent studies demonstrated the pathophysiological relevance of elevated **homocysteine** [44] and **guanidine** [23] levels in renal impaired patients. Proteins and lipoproteins modified by homocysteine and guanidine (**Figure 2**) lose -at least partly- their biological function, acquire cytotoxic, proinflammatory and pro-atherothrombotic properties, which can account CVD development associated with hyperhomocysteinemia [27].

Hyperglycemia: free sugar promotes nPTM

It is becoming increasingly clear that non-enzymatic post-translational **glycations** are highly relevant also in patients with metabolic disorder, which are disproportionately affected by cardiovascular mortality [45]. For example, in diabetic patients, the cardiovascular risk is increased by a factor of 2-4 fold [46]. Monosaccharides, such as glucose, fructose and galactose are reducing sugar, due to their free ketone or aldehyde group, which react with the nucleophilic amino groups, typically the primary amino group of lysine and arginine residues and at the N-terminus of a protein, resulting in the **formation of early-stage glycation adducts** [47]. This first reversible step of protein and lipoprotein glycation is caused by the ambient sugar concentration and accelerated by hyperglycemic burden[48]. Due to the chemical instability of Schiff

bases, these initial modifications undergo isomerization steps to form the more stable Amadori product (**Figure 2**), which can either fragment into reactive intermediaries and/or form irreversible **advanced glycation end products** (**AGEs**)[49].

The nPTMs of protein and lipoproteins by glycation has several pathological effects in the context of metabolic disorders: (1) structural modification by reducing sugars resulting in functional impairment and increased immunogenicity of the post-translational glycated proteins and lipoproteins, (2) generation of reactive *glycation* intermediaries, and (3) AGE associated molecular and cellular effects.

The modification of proteins and lipoproteins by the addition of sugar residues can induce an alteration of the tertiary fold structure, promoting misfolding, protein aggregation and protein dysfunction [50, 51], i.e. impairment of insulin to regulate glucose levels or modulation of hemoglobin affinity to molecular oxygen leading to reduced oxygen delivery to the tissues [52]. Proteins and lipoproteins are glycated via their lysine residues, therefore ePTMs like ubiquitination of the same residues is not feasible and thus impairs physiological degradation and clearance pathways such as the ubiquitin-proteasome pathway, leading to the accumulation of glycated intermediary and end products [53]. The complex transition from early-stage glycan-adducts to AGEs generates the reactive glycation intermediaries glyoxal, methylglyoxal and 3-desoxyglucosione. These *glycation* intermediaries are highly reactive and can themselves react with amino acid residues of proteins and lipoproteins to form additional AGEs, therefore potentiating the initial *glycation* effect induced by hyperglycemia. Although the formation of AGEs is a physiological metabolic process resulting, it increases with progressive aging [54], under hyperglycemic conditions as well as under conditions of increased oxidative stress and hyperlipidemia [55].

Advanced glycation end product (AGE)

AGE accumulation is strongly associated with diabetes mellitus, CKD and CVD[56]. It facilitates protein cross-linking, induces the intracellular production of reactive oxygen species (ROS) and pro-inflammatory mediators via its specific **receptor for advanced-glycation-end-product (RAGE)**. The cross-linking of extracellular matrix of arteries and myocardium reduce tissue elasticity and promote both systolic and diastolic cardiac dysfunction as well as hypertension. The activation of endothelial

RAGE by AGE causes downstream activation of the NADPH oxidase, MAP kinases p38 and NF-κB, initiating the production of ROS, pro-inflammatory cytokines and cell adhesion molecules. The resulting endothelial dysfunction and pro-inflammatory state play pivotal roles in the onset and progression of atherosclerosis. Preclinical studies have shown the inhibition of AGE formation to attenuate atherosclerosis progression [57]. In addition to the proatherogenic features, the AGE/RAGE signaling pathways are also implicated e.g. in the progression of hypertension inducing vascular calcification, downregulating vascular smooth muscle cell markers and upregulating bone matrix proteins, thereby limiting vasoregulation [58].

In line with the preclinical findings, clinical studies confirmed the accumulation of AGE in atherosclerotic plaques and its correlation with arterial stiffness particular in hypertensive patients, independently of diabetes, renal failure or age, implicating its role in the progression of atherosclerosis and CVD [59]. Due to its clinical relevance, **AGE have been suggested as a risk-predictor of both re-hospitalization and mortality in heart failure patient independent from traditional risk markers** [20]. AGE accumulation is also associated with reduced survival in patients with type 2 diabetes mellitus and renal failure, and may indicate disease cross-talk contributing to the increased prevalence of heart failure in these patient populations [29]

Oxidative stress & Aging – nPTM in the dysfunction of proteostasis

Oxidative stress and progressive deterioration of antioxidative potential is tightly associated with the process of aging and is potentially the primary cause of age-related diseases [60]. A well-balanced homeostasis between oxidative and antioxidative mechanisms is critical for enhanced non-enzymatic post-translational oxidative modification and hereby for cellular health [61]. In addition, the progressive deterioration of tissue and organ function during aging is associated with the chronic exposure to high levels of ROS, inducing oxidative damage to DNA, proteins [62] and ultimately promoting cellular senescence, a hallmark of aging [63].

Post-translational protein and lipoprotein **oxidation** is induced either directly by ROS or indirectly by the by-products of oxidative stress [64]. The major contributors of oxidative stress are ROS such as hydrogen peroxide (H_2O_2) and free radicals such as

hydroxyl radical (HO), superoxide anion(O_2^-) (**Figure 2**). Post-translational **carbonylation** as irreversible oxidative modification is associated with permanent ´loss-of-function´ for the protein and are known to accumulate due to aggregation and inhibition of proteosomal activity. Therefore the post-translational carbonylated proteins and lipoproteins serve as biomarkers for prolonged oxidative stress in aging [65] and also play a potential role in CVD [18]. Oxidative stress is associated with arrhythmias causing myocyte apoptosis and necrosis [30], endothelial dysfunction [31], expression of cell adhesion molecules and inflammatory responses, all major risk factors for CVD.

As ROS is difficult to quantify directly in humans due to their instability and low biological half-life [66], oxidized proteins and lipoproteins are used as indirect biomarkers for oxidative stress. Amongst well-known oxidized substances such as oxidized low-density lipoprotein (LDL) with known associations to CVD development, studies have also demonstrated various other oxidized molecules such as isopropstane and myeloperoxidase to be indicative markers for oxidative stress and are associated with the severity of heart failure [32].

nPTMs – CVD risk factor and mediators

Various studies show patients with renal insufficiency, metabolic disorders and advanced age to suffer disproportionately from cardiovascular complications, which cannot be explained solely based on traditional risk factors [28, 43, 45, 67]. With the identification and characterization of a broad spectrum of non-enzymatically modified proteins and lipoproteins, it is becoming increasingly clear, that metabolic stress associated nPTMs are significantly involved in the onset and progression of CVD and represent a major risk factor (**Figure 3**). Notably, cholesterol and numerous highly abundant proteins have been identified to be significant targets of nPTMs.

nPTM of lipoproteins, novel cardiovascular risk factors

Elevated LDL and decreased high-density lipoproteins (HDL) levels are well-known cardiovascular risk factor in the general population [68]. However, **recent studies indicate that total cholesterol content can distort current cardiovascular risk assessment due to neglect of pro-atherosclerotic effects of post-translational modified lipoprotein isoforms** [42, 69-71].

While HDL is widely considered as an atheroprotective mediators, with known anti-oxidative, anti-thrombotic and anti-inflammatory functions [72], non-enzymatic modified HDL isoforms can reverse these effects. Patients with end-stage CKD display a three-fold increased urea plasma level [28], causing a doubling of the levels of **carbamylated HDL**, which is directly proportional to the plasma urea level [42]. Carbamylated HDL suppresses the expression of vascular endothelial growth factor receptor-2 and the scavenger receptor class B type 1 (SRB1) signaling pathway in endothelial cells, decreasing endothelial repair capabilities and consequently increasing the risk for endothelial dysfunction and atherosclerosis [73]. More strikingly, the carbamylation of specific lysine residues per HDL-associated ApoA-I was sufficient to modulate its interaction with SRB1, inducing net cholesterol accumulation and lipid-droplet formation in macrophages. In addition to the loss of its anti-inflammatory and anti-oxidative features, non-enzymatic post-translational carbamylated HDL actively participates in the recruitment of foam cells as a proatherogenic mediator [42].

Similarly to post-translational carbamylated HDL, post-translational glycation also reduces the atheroprotective effect of HDL, in particular its ability to modulate the cholesterol accumulation [74]. HDL isolated from patients with type 2 diabetes mellitus display a 250% higher degree of post-translational glycation in comparison to non-diabetic controls [75]. Post-translational **glycated HDL** trigger oxidative stress, vascular smooth muscle cell proliferation and migration [69] resulting in higher morbidity of cardiovascular disease in diabetic patients. The glycation of ApoA1, a major lipoprotein component of HDL in plasma [76], induces conformational changes that prevents HDL from activating lecithin-cholesterol acyltransferase (LCAT), a key enzyme in the reverse cholesterol transport. The enzymatic activity of LCAT decreases progressively with the increasing post-translational glycation of ApoA1 [77].

Finally, **oxidative and carbonyl stress** in CKD patients leads to post-translational carbonylation of lysines and histines of HDL, modulating its anti-thrombotic function, leading to the inability to inhibit platelet aggregation and thereby increasing the risk for cardiovascular events [78].

In contrast to HDL, LDL is widely known as a pro-atherosclerotic mediator [79]. However, despite the beneficial effects of LDL-lowering therapy, the incidence rate for CVD remains high [80]. According to the “Progression of Early Subclinical Atherosclerosis” (PESA) study, which examines the underlying risk factors of atherosclerosis, approx. 50% of the patients suffering from atherosclerosis do not display abnormal LDL levels, suggesting that LDL plasma level alone is an insufficient indicator for atherosclerosis [81].

Numerous **post-translational modified LDL subsets** with proatherogenic properties have been identified, which contribution could explain the cardiovascular outcome of the PESA study. The most prominent pathological LDL isoform is post-translational oxidized LDL, rendering the innate lipoprotein into a highly potent proatherogenic factor [82]. **Oxidized LDL** activates endothelial cell by inducing cell adhesion molecules, promotes the vascular adhesion and transmigration of monocytes[83], followed by lipid accumulation and foam cell formation [84]. Lesser known LDL isoforms occur under uremic and hyperglycemic stress. Serum levels of carbamylated LDL is significantly elevated in CKD patients with CVD [85] and can reach up to 70 carbamylated lysine residues in end-stage renal disease [86].

Carbamylated LDL has also detrimental effects on the vessels, inducing endothelial dysfunction via activation of LOX-1, increased endothelial ROS production, reduced bioavailability of NO and ultimately the impairment of endothelium-dependent vasodilatation[86]. Additionally, post-translational carbamylated LDL induce dose- and time-dependent endothelial injury and cell death [87], vascular smooth muscle cell proliferation, the acceleration of monocyte adhesion through overexpression of cell adhesion molecules on endothelial cells [88], lipoprotein accumulation, foam cell formation and pro-inflammatory signaling pathways [43]. Consequently, patients with elevated

carbamylated LDL lysine levels have a significant higher all-cause mortality and significantly shorter cardiovascular events-free survival. Even after adjustments for potential confounders, carbamylated LDL lysine remained a predictor for all-cause mortality and cardiovascular events in Cox-proportional hazard models, suggesting an impact in the cardiorenal comorbities [86].

LDL is also highly susceptible to post-translational *glycation*, with three-fold increased serum levels of **glycated LDL** in diabetic patients compared to non-diabetes controls [89]. The glycated isoform of LDL is considered to be a proartherogenic mediator, as it reduces the expression of endothelial nitric oxide synthase, decreases proliferation, trigger apoptosis in vascular endothelial cells [71] and promotes the cellular cholesterol accumulation [90]. Moreover, post-translational glycated LDL has been indicated as both cardiovascular risk factor and predictor[91]. It increases platelet reactivity and their aggregation response, potentially promoting thrombus formation [91]. Clinically studies associated plasma levels of post-translational glycated ApoB as one of the main component of LDL with increased triglyceride levels, suggesting its prognostic value for the development of myocardial infarction in the following five years in both diabetic and non-diabetic individuals [92].

Different modifications of lipoproteins can occur simultaneously and competitively leading to doubled modified lipoproteins [93]. Similar to oxidized or carbamylated LDL, the double modified **oxi-carbamylated LDL** displays proatherogenic features, mediating lipid accumulation and foam cell production via scavenger receptors. The enhanced cytotoxicity towards macrophages indicates that oxi-carbamylated LDL is potentially involved in the inflammatory response [93]. Other nPTMs of LDL besides oxidation, such as carbamylation and glycation, have prognostic value and significant physiological impact on the development and progression of cardiovascular disease. Current diagnostic methods based on oxidation alone are therefore insufficiently accurate to predict CVD.

nPTM of abundant proteins – Cardiovascular risk factors

Serum level of abundant proteins like albumin and hemoglobin are highly suitable markers to quantify nPTM severity and chronic metabolic burdens, i.e. post-translational glycated hemoglobin for glycemic control [94] or post-translational carbamylated albumin for the quantification of the urea load [95]. However, disruption of the physiological functions of abundant proteins by nPTMs have significant impact on CVD onset and progression. For instance, serum albumin is associated with a wide range of cardioprotective functions such as, binding of toxins, antioxidation [96], anticoagulation [97] and regulation of cholesterol transport [98]. As the most abundant protein in plasma, with a blood half-life of 21 days, albumin is highly susceptible to e.g. carbamylation, guanidinylation and glycation [95, 99].

The post-translational **carbamylation rate of albumin** is strongly correlated with blood urea concentrations and is associated with all-cause mortality in CKD patients [95]. Post-translational carbamylation and guanidinylation both induces conformational changes of albumin, significantly inhibiting its physiological function to bind exogenous and endogenous molecules by up to 60% [100]. The reduced ability to scavenge uremic toxins as well as oxygen radicals accelerate the renal deterioration and potentially contributes to CVD progression [23].

In contrast, structural modification due to post-translational **glycation of albumin** elicit endothelial thrombogenic and inflammatory responses [101] and the overexpression of intracellular adhesion molecules such as ICAM-1 and VCAM-1, which are involved in atherosclerotic lesion development [102]. Furthermore, post-translational glycated albumin promotes oxidative stress and inflammation in the vessel wall [103] via its interaction with RAGE [104] and induces the upregulation of platelet activation and aggregation [105], both contributing to a higher cardiovascular risk.

Similar to albumin, hemoglobin with a blood half-life of approximately 120 days is exposed for extended periods of time of metabolic stress, in particular to reducing sugars. Therefore, post-translational **glycated hemoglobin** (HbA1c) is considered to be the gold standard to assess glucose control and therapy efficacy, due to its strong correlation with long-term plasma glucose concentration[106]. Post-translational glycation causes structural modifications leading to hemoglobin aggregation and the de-

crease of its solubility in erythrocytes and therefore increasing the viscosity of the cellular content. The consequent reduction of cellular deformability and flexibility of erythrocytes caused by nPTMs may contribute especially to microvascular diseases [107].

In addition to abundant serum proteins, the extracellular matrix (ECM) such as collagen and elastin displays a high vulnerability towards nPTMs. The chronic exposure of urea induces the progressive accumulation of post-translational **carbamylated ECM** proteins in all major organs such as kidney, heart, liver, aorta, bones and skin, with potentially deleterious effects on the organ architecture and functions [37, 108]. The post-translational carbamylation of collagen-I disturbs its triple-helix structure, leading to a decreased ability to polymerize into normal fibrils and increased susceptibility to collagenases [37]. Especially in blood vessels, these effects promote atherosclerosis, fostering vulnerable atherosclerotic plaques [28] and increase the risk of atherosclerotic plaque rupture, a potentially life threatening thrombotic event [109].

In contrast, post-translational **glycation renders ECM proteins more resistant to proteolysis and ECM remodeling**, thus promoting the accumulation of post-translational glycated elastin and collagen in arterial walls and organs, resulting in increased thickness of tissue and vessel walls [110]. Advanced *glycation* end-products enhance this effect by ECM cross-linking [111], preventing the degradation of post-translational modified ECM, amplifying the ECM deposition and increasing ECM fiber stiffness. This increasing stiffness of ECM alters the mechanical properties of vessels as well as the myocardium, contributing to isolated systolic hypertension and diastolic heart failure in the aging population and is accelerated in young diabetic patients [112].

nPTM and Fibrinolysis

nPTMs of fibrinogen by post-translational glycation [113], **carbamylation** [114], **homocysteinylation** [89] **and guadininylation** [115] leads conformational changes that result in thinner clot fibers, higher clot density, decreased permeability and higher resistance to fibrinolysis. A potential cause for the increased resistance towards fibrinolysis might be the blockade of specific lysine residues of fibrinogen in-

volved in the binding of fibrinolytic proteins [116], such as plasminogen, therefore impairing clot lysis [117]. Fibrinogen is particularly susceptible to **oxidation,** approximately 20-fold higher than albumin, however the oxidation sites are not crucial protein function and therefore have potential anti-oxidant functions [118].

Identification and detection of nPTM – Potential avenue for cardiovascular diagnostics

With increasing reports about the pathological effects of post-translational modified proteins, peptides and lipoproteins in the onset and progression of cardiovascular disease [89], **the reliable detection and qualification of these modified cardiovascular biomarker and mediators is essential for accurate risk stratification and the development of preventive and interventional strategies.**

Besides induction of structural and functional alteration of the native compounds, the presence of covalent PTMs changes their molecular mass as well as their chemical properties, such as charge and hydrophobicity, as described above. These characteristics enables the chromatographic separation of these modified isoforms and their identification via mass spectrometry (MS) by basic MS experiments as well as higher mass-fragment spectra [119] (**Figure 4**).

Using the **untargeted proteomics approach**, any protein or lipoprotein modification can be screened within a complex sample without a priori assumptions about the kind or severity of modifications [120]. The resulting high number of mass signals requires chemometric methods in order to reveal relevant signals and subsequent database searches to identify unknown mass signals and potentially reveal novel post-translational modifications. Despite the high sensitivity of current mass-spectrometric systems, the major drawback of mass-spectrometric approaches is still the detection of low-abundance proteins and lipoproteins, as their mass signals are easily disguised by more abundant native proteins in a complex sample [121].

While the clinical translation of these newly identified post-translational modified proteins and lipoproteins have **immense diagnostic and prognostic value**, currently established diagnostic routine techniques are struggling to detect small molecular

changes in a clinical setting. A number of diagnostic methods are antibody-based detection methods, which rely solely on the availability of antibodies that can detect a modified amino acid residue within a protein or lipid [122]. Such antibodies can be polyclonal or monoclonal and are developed against either the modified peptide/protein or against the modified amino acid; however the spectrum of post-translational modification-specific antibodies is limited in numbers and by their site specificity [123]. In contrast, clinical mass-spectrometry identify specific post-translational modifications with high sensitivity and resolution based on mass changes and can also include a multitude of markers within a certain mass range into a single measurement [124]. Such methods are available and are already supported by e.g. the U.S. Food and Drug Administration (FDA) [125] in the CKD context, but the final translation into clinical practice is still pending.

Anti-nPTM therapy options – Clinical implications

Three therapeutic strategies against pathological nPTMs are available (**Figure 5**): (1) PTM prevention: decrease plasma concentration of nPTM donors, (2) PTM inhibition: scavenging of the reactive metabolites or blocking nPTM sites and (3) PTM clearance: removing the pathogenic end product of PTM.

nPTM prevention

Dietary and lifestyle restriction

Smoking, unhealthy diet, and/or lack of physical activity contribute to the increasing metabolic burden and the development of chronic diseases [126]. While smoking is a known trigger for oxidative stress [127], Wang *et al.* demonstrated that the elevated myeloperoxidase-catalyzed oxidation of thiocyanate, a compound abundant in smokers, facilitates the accelerated formation of cyanate resulting in increased carbamylation of lipoproteins [43].

In addition, high dietary consumption of sugars (i.e. glucose or fructose) represents a substantial source for endogenous post-translational protein glycation and AGE formation [128]. While AGEs are naturally occurring in uncooked animal-derived food products, high temperature and low moisture processing of AGE-containing food

products increase the amount of dietary AGEs by 10-100 fold [129]. Moreover, nutrition can significantly affect serum urea levels. A prospective nutritional study showed that a very low protein diet can significantly decrease serum urea levels and reduce post-translational protein carbamylation [130].

Dietary and lifestyle restriction are considered effective long-term prevention and therapy options and are recommended by organizations such as the ´American Heart Association´ [131], and the ´American Diabetes Association´ [132] to reduce the risk of diabetic and cardiovascular complications. Based on the current state of knowledge, the reduction of reactive metabolites such as free sugars and isocyanate associated with dietary and life style restrictions may be a significant contributing factor to cardiovascular risk reduction [43].

Glucose control

The management of glucose is an efficient way to reduce the ambient levels of reducing sugars in the circulation and hence limit the rate of protein and lipid glycation and the generation of AGEs [133]. Besides dietary restriction, blood glucose levels can be efficiently reduced by medication. Metformin is prescribed as a blood glucose lowering agent in patients with type 2 diabetes, which slows the hepatic glucose production and hereby modulates the glucose plasma level. A meta-analysis of 35 clinical trials demonstrated that Metformin strongly inhibited protein glycation [134] and leads to a significant reduction of cardiovascular mortality [135]. Preclinical studies indicate additional cardioprotective effects of Metformin beyond glucose control, i.e. protection against oxidative stress [67], the inhibition of AGE-induced inflammation [136], and scavenging of glycation dicarbonyl intermediates [137].

Post-translational modification inhibition

A promising therapeutic strategy is to boost the innate defense mechanisms against nPTMs. A limited number of studies already demonstrated the feasibility of nPTM reduction by supplying exogenous scavenger, such as **free amino acids** [138], **short peptides** [139] and **antioxidants** [140] or drugs [141], that either compete with

native protein for reactive metabolites or block vulnerable PTM sites, hence establishing a protective buffer to scavenge circulating reactive metabolites or shield proteins from nPTMs [127].

Free amino acid supplement

nPTMs are in general spontaneous and target non-specific modifications of sterically available amino acid side chains by small reactive compounds, therefore **the supplementation of exogenous nucleophilic scavengers might neutralize nPTMs by competing with serum proteins and lipoproteins for the electrophilic metabolites.** In line with that assumption, studies show that amino acid deficiency, a condition widely present in dialysis patients exacerbates urea-mediated protein carbamylation [95]. While protein malnutrition strongly contributes to amino acid deficiency in ESRD patients, dialysis itself removes up to 12% of the patients daily protein intake [142] and therefore reduce the plasma concentration of natural scavenging agents., Nutritional amino acid supplementation is suggested as a safe and cost-effective method to counterbalance the net loss of amino acids during hemodialysis of CKD patients in this context [143]. Its therapeutic potential to inhibit nPTMs was only described recently in CKD patients [138]. Bolus administration of essential amino acids equal to the recommended daily dose at the end of each thrice-weekly dialysis session over the timespan of 8 weeks resulted in a 15% reduction of carbamylated albumin compared non-treated patients, indicating the effectiveness of free amino acids to suppress protein carbamylation [138]. Exemplary analysis of isocyanate affinity towards various positions of amino groups show an approximately 100-fold increased affinity towards the α–amino group of a free amino acid compared to the ε-amino group of an amino side chain on full length proteins, suggesting free amino acids as efficient scavenger to reduce post-translational carbamylation and carbamylation-associated cardiovascular damage [144].

In a similar manner to the scavenging of isocyanate, **free amino acid therapy has shown to reduce glycemic and oxidative burden** [145, 146]. First discovered to counter diabetic cataract in rats, *in vivo* supplementation of lysine has shown to suppress hyperglycemia induced glycation of lens crystallin and prevent subsequent

protein aggregation and cataract formation. Additional *in vivo* studies show daily administration of low doses of L-arginine to significantly reduce serum glucose and HbA1c levels in diabetic rats [147]. Furthermore, the oral administration of essential amino acids for 120 days in a cohort of elderly subjects resulted in a significant increase of antioxidant capacity and reduction oxidative stress levels. Moreover, amino acid supplementation was associated with the increased plasma level of ´insulin growth factor 1´, a cardioprotective protein with anti-atherosclerotic effects [148].

Peptide scavengers

In a similar manner to free amino acids, **nucleophilic dipeptides and polypeptides** have shown similar protective features against nPTMs by scavenging electrophilic metabolites in preclinical studies [95, 147]. The dipeptide glycylglycine inhibited *in vitro* carbamylation of albumin more efficiently than any free amino acid, resulting in a 64% reduction rate compared 23% reduction by single glycine [149]. However, the potentiated inhibition efficiency remains unclear [149]. Polypeptides have also shown to inhibit post-translational glycation. Linear polyamine peptides such cadaverine, putrescine spermidine and spermine are formed from L-lysine or L-arginine and present multiple nucleophilic amino groups, able to inhibit AGE formation *in vitro [150]*.

Next to a variety of post-amadori inhibitors reviewed by Nenna *et al.* [139], the polyamine aminoguanidine was extensively validated as a treatment option for reactive carbonyls such as Amadori intermediates suppressing its transition to AGE. As a result of reduced AGE formation, aminoguadinine has various atheroprotective effects such as decreasing arterial AGE accumulation [151], improved vascular elasticity [152] and increased LDL clearance [153]. However, its commercialization was terminated due to lack of efficacy and safety concerns due to extensive adverse effects such as, abnormalities in liver functions, vasculitis and flu-like symptoms [154].

Drug repurposing – Potential anti-nPTM drugs

Nonsteroid anti-inflammatory drugs (NSAIDs) are considered anti-cataract agents as they suppress protein *carbamylation* and *glycation* of proteins involved in cataract formation [155]. For instance, aspirin prevents *glycation* and *carbamylation* of

lens proteins by acetylating free amino groups and therefore occupying the binding sites of reducing sugars or isocyanate [141]. Similarly effects have been observed for the NSAIDS ´diclofenac´ and ´ibuprofen´ *in vitro*, where albumin *glycation* was significantly decreased [156]. However, clinical evaluation of NSAIDs as systemic post-translational modification inhibitors is pending.

Most prominently known as a **lipid-lowering drug**, **statins** have both antioxidant and AGE-lowering effects. Via the transcriptional suppression of NADPH oxidase subunits and blockade of NADPH oxidase activation, statins inhibit superoxide generation [157]. In addition to lipid lowering, statins also have AGE-lowering effects, suppressing both AGE formation and RAGE expression in atherosclerotic plaques and promoting the proteolytic shedding of RAGE to soluble receptor for advanced-glycation-end-product (sRAGE). The increase circulating levels of sRAGE scavenge AGE and simultaneously attenuate RAGE-mediated cellular signaling pathways [158].

Antioxidants and ROS scavenger

Antioxidants are produced endogenously as a mechanism to counteract ROS, oxidative stress and maintain cellular homeostasis [61]. As recently reviewed by Ndhlala *et al.* [159] the vitamins A, C and E, flavonoids, carotinoids, glutathione, polyphenols, uric acid, melatonin, bilirubin, albumin, and polyamines are natural antioxidants with free radical scavenging and metal chelating properties. Under pathological conditions, where oxidative stress overwhelms the endogenous antioxidant defense, the exogenous supplementation of antioxidants can be beneficial [160]. However, the efficacy of antioxidant supplementation in a clinical setup remains controversial due to a series of failed or inconclusive antioxidant clinical trials [161] with not yet proven benefit for the cardiovascular outcome [162].

However, several synthetic compounds such as ethylpyruvate or N-acetylcysteine have been demonstrated as efficient ROS inhibitors in acute cardiovascular events, such as myocardial ischemia reperfusion injury or post-operative oxidative stress [163]. N-acetylcysteine is a potent scavenger for nucleophilic metabolites [73].

Additionally, it also serves as the precursor for the synthesis of glutathione, an important component the cellular defense against oxidative damage [164]. Several clinical trials have demonstrated the suitability of N-acetylcysteine to mediate oxidative damage in CVD [140]. These clinical studies show that low-dose of N-acetylcysteine significantly reduce myocardial oxidative stress in coronary bypass surgery [165].

nPTM clearance

Dialysis

Dialysis efficiently remove small water-soluble metabolites from the circulation from CKD patients, thereby temporarily relieving the metabolic pressure generated by reactive metabolites. Perl *et al.* demonstrated that intensification of dialysis through extended duration on a thrice-weekly basis significantly reduces the serum level of carbamylated albumin as a reference for overall protein carbamylation [166]. Similarly, more frequent dialysis (up to 6 times a week for a period of 2h) effectively lowers the mean plasma level of glycation-related compounds [167]. With the reduction of both urea and advanced glycan end products, dialysis reduces protein *carbamylation* and AGE accumulation and can significantly improve cardiovascular outcomes [20, 166]. Due to the non-specific removal of small molecules, dialysis causes a net loss of PTM-protective compounds such as antioxidants and free amino acids, hence weakening the innate defenses against nPTMs [168].

AGE-breakers and RAGE inhibitor

N-phenylacylthiazolium and Alagebrium as so-called “AGE-breaker” have been proposed as therapeutic agents for reversing the AGE-mediated protein cross-linking by cleaving di-carbonyl bonds in cross-linked structures [169]. Counteracting the cross-linking and accumulation of ECM in blood vessels, clinical pilot studies already show Alagebrium to decrease arterial pulse rate and increase vessel compliance in hypertensive patients [170] and improve endothelial functions in patients with systolic hypertension [171]. In an effort to reduce AGE-mediated cellular responses via receptor binding to RAGE, Azeliragon was developed as a RAGE antagonist to reduce AGE-associated tissue damage [172]. Upcoming findings of the two on-going phase III clinical studies (Clinical Trail IDs: NCT03980730; NCT02080364) exploring the short-

and long-term efficacy and safety of Azeliragon may prove the feasibility for the treatment of AGE-associate cardiovascular damage.

Concluding Remarks

Non-enzymatic post-translational modifications are key factors for protein and lipoprotein dysfunction, which is tightly associated with aging, renal deterioration, metabolic disorders and ultimately CVD. With the rapid development of high-resolution analytical tools, it is coming increasingly clear nPTM induced proteins and lipoproteins dysfunctions have major impact on the progression of both renal impairment and metabolic disorders and may simultaneously facilitate the comorbid development of CVD as a non-traditional risk factor.

This review focused on numerous non-enzymatically modified isoforms of protein and lipoprotein with recently identified pathological functions in the onset and progression of CVD in detail. Moreover, in sight of growing numbers of identified pathological post-translational modified protein and lipoprotein isoforms, clinical mass spectrometry will be an emerging tool for clinical analytics, which is able to improve risk stratification for cardiovascular event by including nPTMs.

Currently, several promising novel therapy options are in preclinical and clinical development, which could limit nPTM associated cardiovascular damage. However, as a rather new field of research, the lack of patient data from large cardiovascular outcome studies remains an obstacle (see Outstanding Questions).

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Figure Legends

Key figure: Schematic illustration of multimorbid disease development under different metallic stress factors, such as oxidative stress, renal impairment and metabolic disorder

Figure 1. Schematic illustration of enzyme-catalyzed (left) and non-enzymatic (right) post-translational modification the abundant human serum albumin protein. Enzymatic PTMs occur at specific enzyme recognition sites, while non-enzymatic PTMs occur when electrophilic metabolites such as isocyanate, reducing sugars and oxygen radical come in close proximity with nucleophilic amine side chains (primary amines). Exemplary amino acid distribution for cysteine (red) as the most nucleophilic and lysine (green) as the most frequently modified amino acid are displayed as examples for different amino acid abundancies and especially sterical availability.

Figure 2. Uremia, hyperglycemia and oxidative stress are main causes of non-enzymatic post-translational modification of proteins and lipoproteins. Isocyanate, guanidine and homocysteine are major components of the uremic burden during the onset of renal insufficiency leading to non-enzymatic carbamylation, guanidylation and homocysteinylation. Similarly, the abundance of reducing sugars such as glucose under hyperglycemic conditions induce the formation of Schiff's base and transform to Amadori products over the timespan of weeks and month, releasing secondary reactive Dicarbonyl intermediates and Advanced glycation end products. Finally, oxidative stress in the form of hydrogen peroxide, hydroxyl and superoxide radicals induce non-enzymatic and irreversible modification to protein and lipoprotein structures.

Figure 3: Post-translation modification of abundant lipoproteins and serum proteins leads to the formation and accumulation of modified isoforms with pathological functions.

Figure 4. Schematic illustration of mass spectrometric approach to the detection of post-translational modification. Complex patient samples such as plasma or urine are fractionated chromatographically based on chemical and molecular properties to enrich particular protein and lipoproteins of interest. These fractions are analysed via mass spectrometric methods to generate MS spectra of detected proteins. Database comparisons

identify mass changes of individual modified protein isotypes. The subsequent MS/MS spectra can isolate the specific amino acids modified and identify the mode of modification.

Figure 5. Categorization of preventive and therapeutic strategies in early and late stage PTM-associated effects

Outstanding Questions

What is the added value of non-enzymatically modified proteins and lipoproteins in the context of cardiovascular disease risk stratification?

Which less abundant proteins and lipoproteins are also modified and what pathological phenotype do these isoforms display?

What potential therapeutic options are available to inhibit specific non-enzymatic modifications?

How is the biosynthesis of non-enzymatic post-translational modification regulated?

What is the clinical utility of non-enzymatically modified proteins and lipoproteins for CVD staging?

Clinicians corner

Glossary

Key Figure

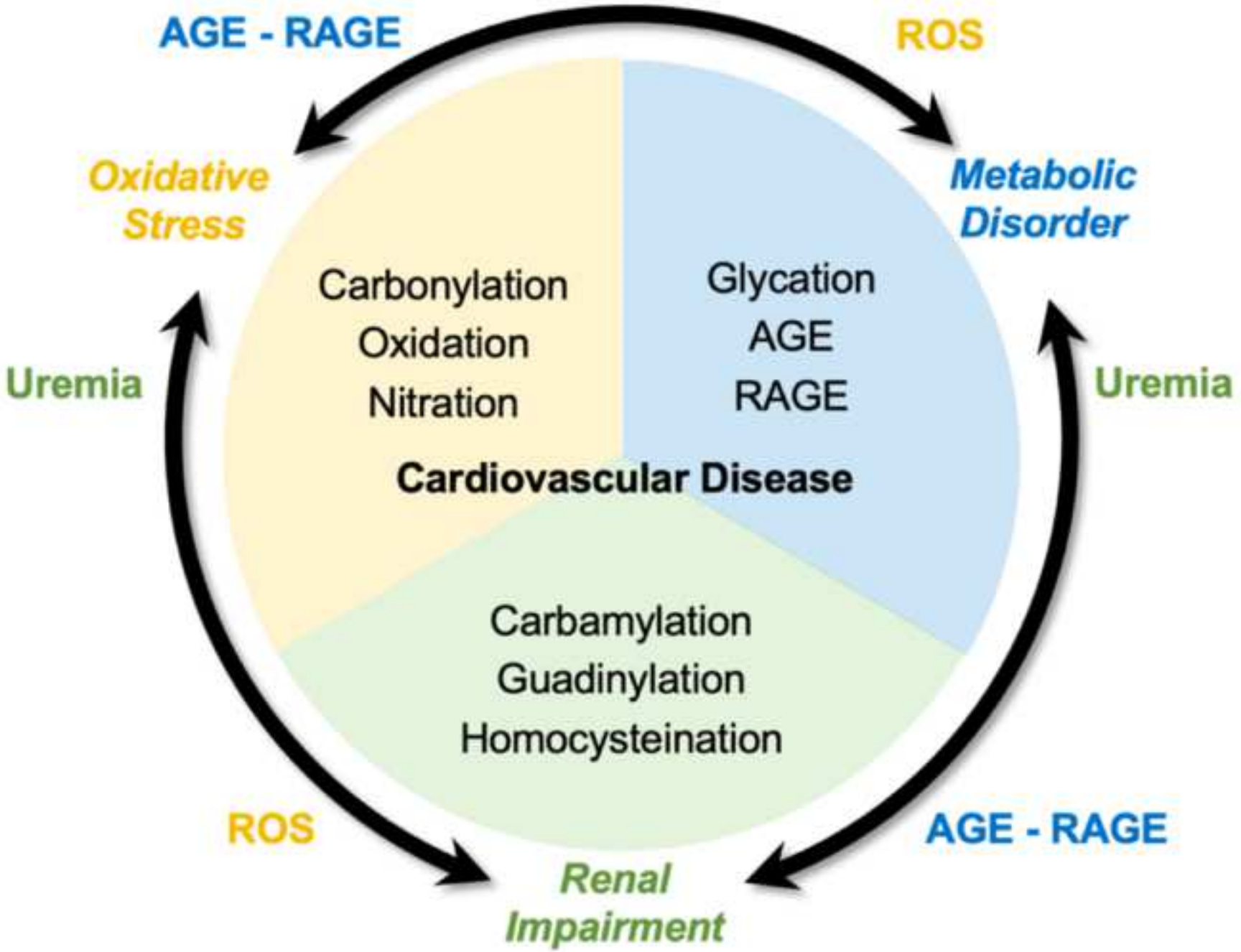

Figure 1

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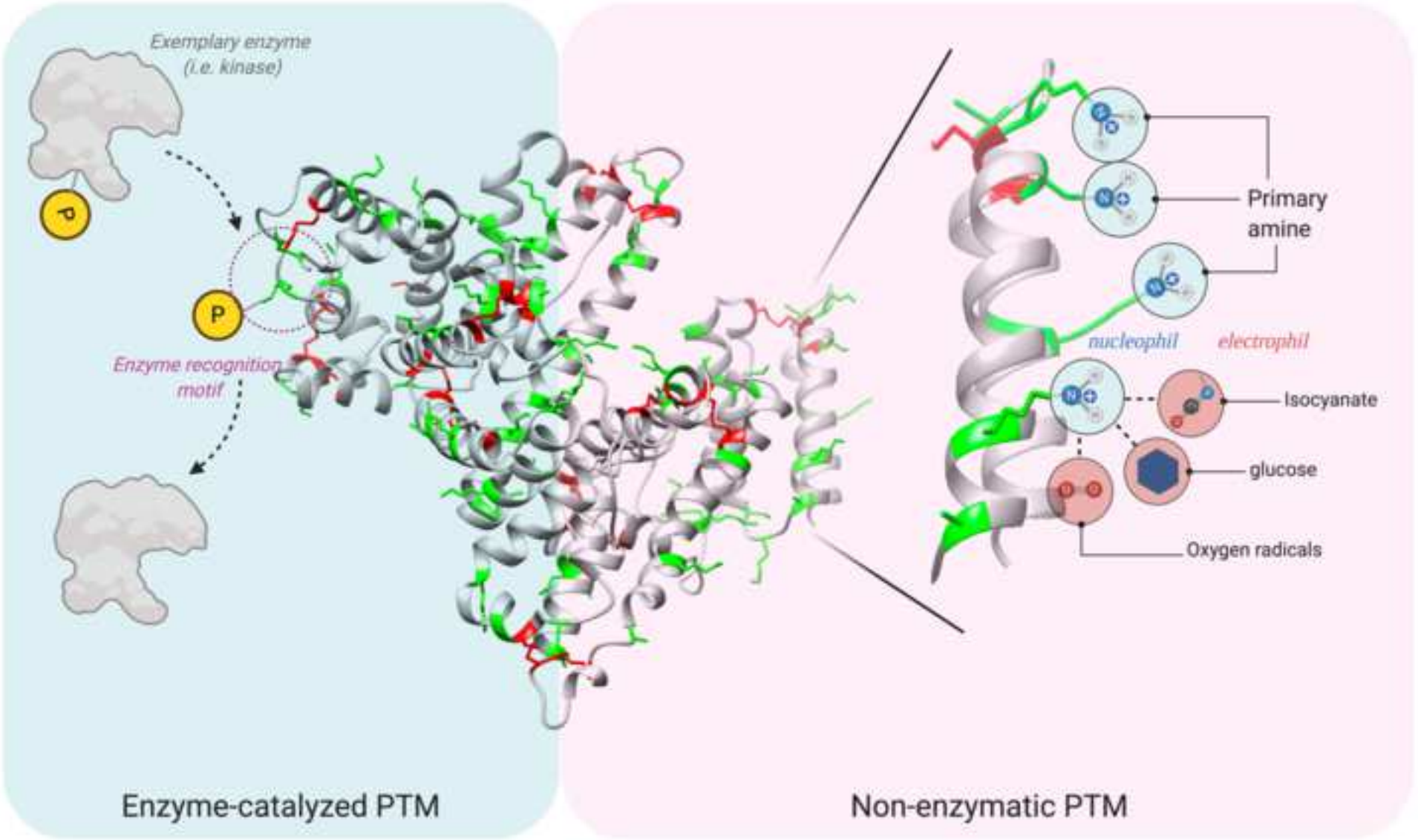

Figure 2

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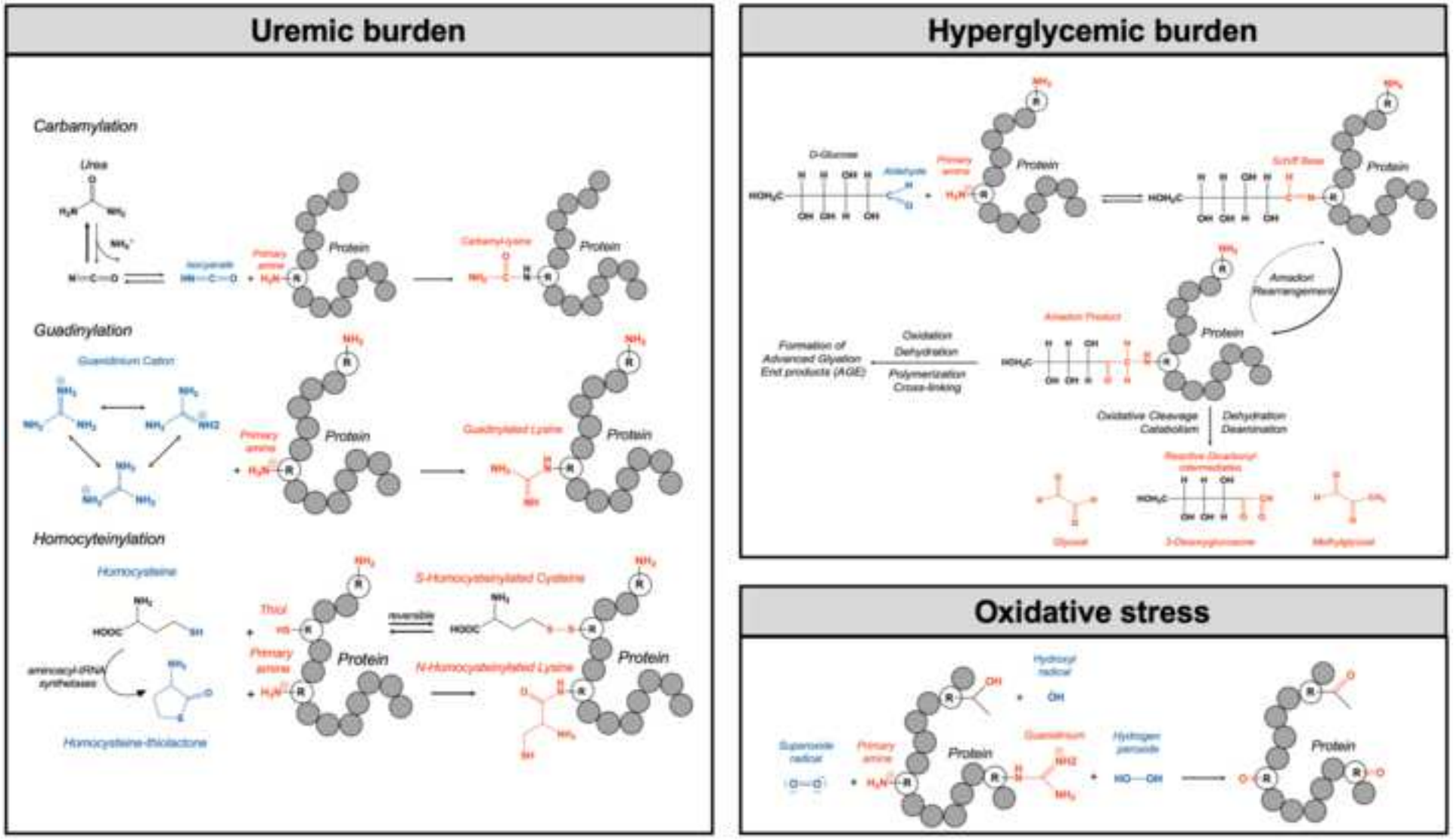

Figure 3

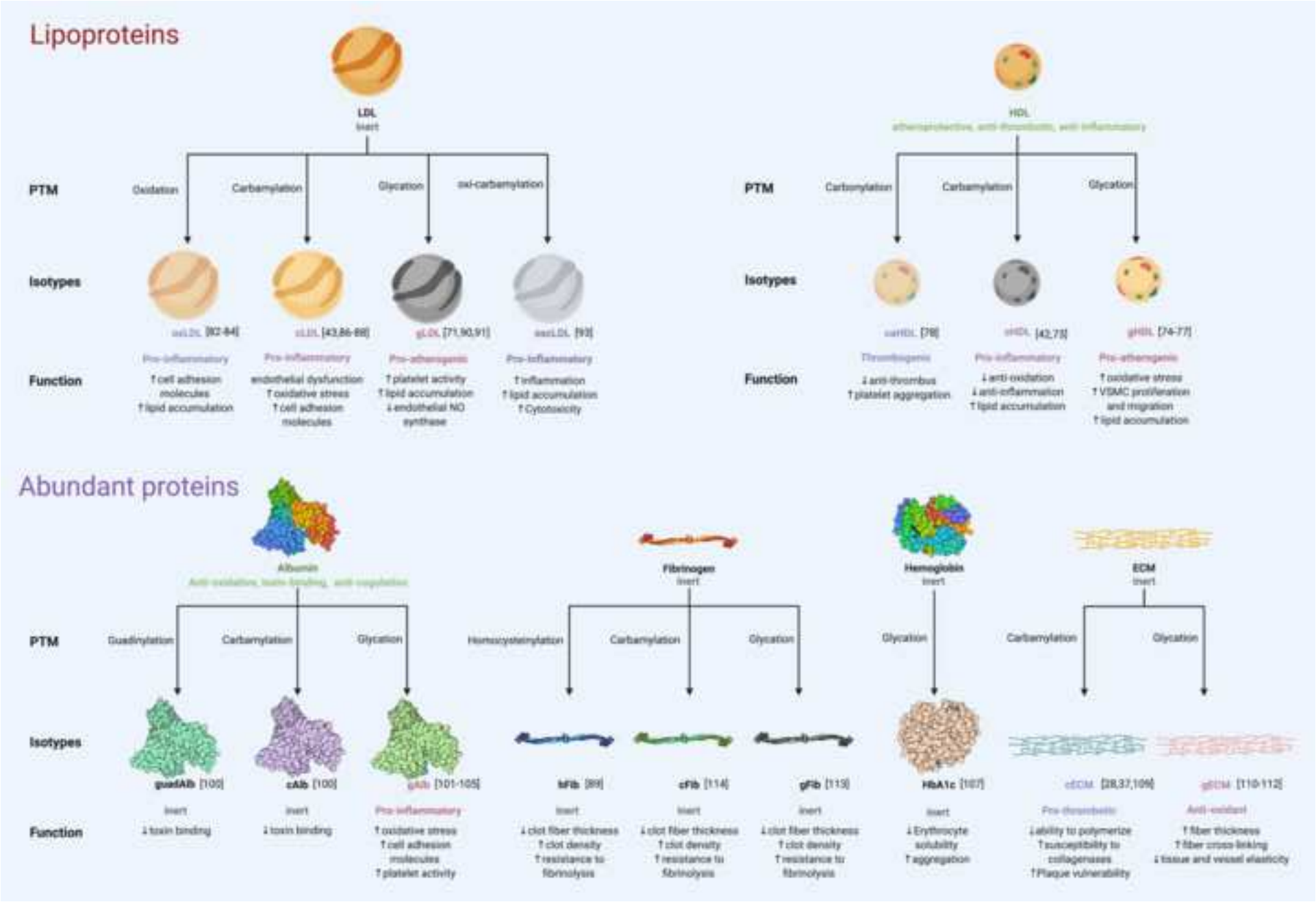

Figure 4

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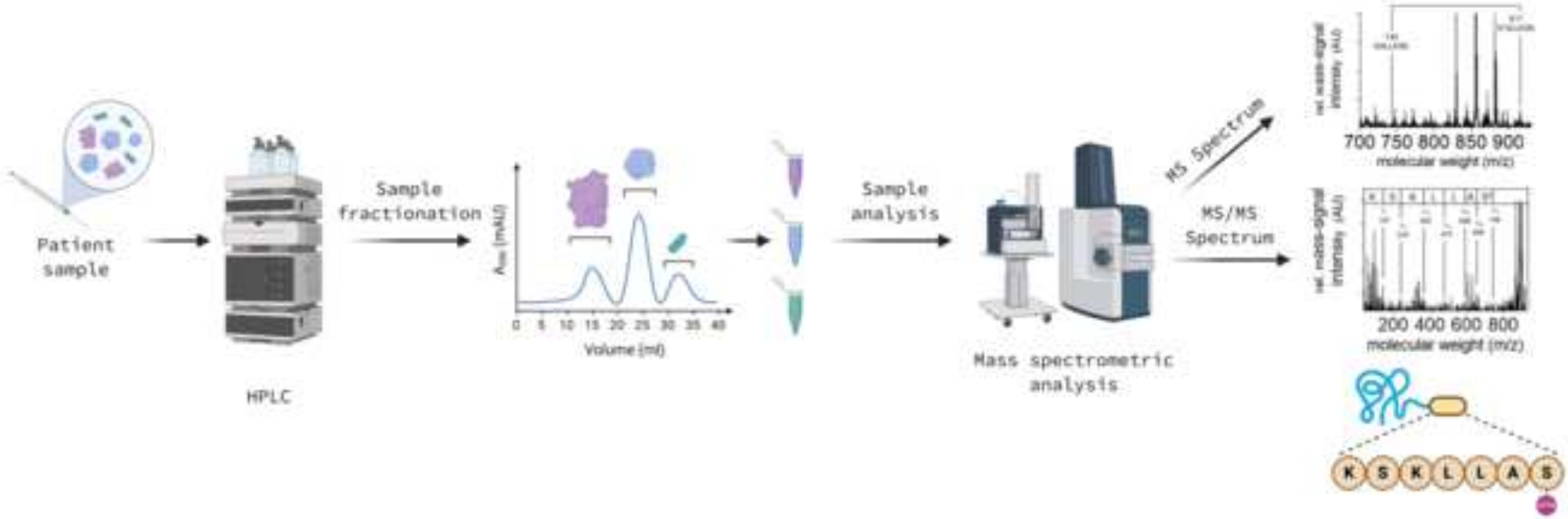

Figure 5