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REVIEW ARTICLE

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Novel insights about albumin in cardiovascular diseases: Focus on heart failure

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Abstract

The Human Plasma Proteome has always been the most investigated compartment in proteomics-based biomarker discovery, and is considered the largest and deepest version of the human proteome, reflecting the state of the body in health and disease. Even if efforts have been always dedicated to the refinement of proteomic approaches to investigate more deeply the plasma proteome, it should not be forgotten that also highly abundant plasma proteins, like human serum albumin (HSA), often neglected in these studies, might provide fundamental physiological functions in plasma, and should be better considered. This review summarizes the important roles of HSA in the context of cardiovascular diseases (CVD), and in particular in heart failure. Notwithstanding much attention has been historically directed toward the association of HSA levels and CVD risk, the advances in the field of mass spectrometry research allow also a better characterization of the effects of oxidative modifications that could alter not only the structure but also the function of HSA.

KEYWORDS
albumin, heart failure, oxidative stress

1 | INTRODUCTION

The Human Plasma Proteome has always been the most investigated compartment in proteomics-based biomarker discovery and is considered the largest and deepest version of the human proteome, reflecting the state of the body in health and disease (Anderson & Anderson, 2002). Despite its biological and diagnostic relevance, the study of plasma proteome is challenging because of the large number of proteins and the high

Abbreviations: 3-NT, 3-nitrotyrosine; ACB, albumin cobalt-binding; ACR, acrolein; AGE, advanced glycation end-product; CAD, coronary artery disease; CBP, cardiopulmonary by-pass; CEL, carboxy-ethyl lysine; CML, carboxy-methyl lysine; CVD, cardiovascular diseases; cys-HSA, cysteinylated albumin; DAABD-Cl, 4-(2-(dimethylamino)ethylaminosulfonyl)−7-chloro-2,1,3-benzoxadiazole; DNPH, 2,4-dinitrophenylhydrazine; ESI, electrospray ionization; GA, glycated albumin; GO, glyoxal; GSH, glutathione; HSA, human serum albumin; Hcy, homocysteine; HF, heart failure; HNA, human non-mercaptalbumin; HNE, 4-hydroxynonenal; HSA-SH, mercaptalbumin; HUVEC, human umbilical vein endothelial cells; IMA, ischemia modified albumin; LC, liquid chromatography; LMW, low molecular weight; LPO, lipid peroxidation; MALDI, matrix assisted lase desorption ionization; Malpeg, polyethylene glycol maleimide; MDA, malondialdehyde; MGO, methylglyoxal; MS, mass spectrometry; NEM, N-ethylmaleimide; ONE, 4-oxo-nonenal; PAF, platelet-activating factor; PGI2, prostacyclin; q-ToF, quadrupole time of flight; RCS, reactive carbonyl species; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNO-HSA, S-nitrosylated human serum albumin; TCEP, tris(2-carboxyethyl)phosphine.

Equal contribution

dynamic range of protein concentrations. Specifically, highly abundant proteins limit the detection of potential biomarkers that are usually less concentrated, resulting in the identification of only a few hundred of the most abundant proteins.

For many decades, protein research has been limited by the unavailability of reproducible and sensitive analytical platforms capable of detecting specific molecular species in complex biological systems. Only nowadays, with the recent advance in modern analytical techniques for proteomics, especially in mass spectrometry (MS), those limitations have been overcome. Because of its high sensitivity and specificity, MS is widely used for proteomic analysis in many research areas, including plasma biomarker discovery (Aebersold & Mann, 2003; Cominetti et al., 2016). In characterizing plasma proteome, the so-called MS-based bottom-up workflow is mainly followed to identify and quantify proteins (Cominetti et al., 2016).

Bottom-up proteome analysis is based on the measurement of peptides instead of full-length proteins, which are the focus of top-down approaches. Proteins derived from a plasmatic sample are firstly digested with trypsin to obtain peptides, which are then separated by liquid chromatography (LC) and subjected to MS analysis (Figure 1). According to the experimental need, different enrichments may be performed at different steps of this workflow. In the case of very complex samples like plasma, the depletion of highly abundant proteins by immunoaffinity may be required to enhance the protein coverage and the dynamic range. Enrichment strategies at the peptide level, which are often useful for the identification of modified peptides, may be further employed.

By means of MS-based bottom-up proteomic approaches, it is possible to measure the accurate mass and fragmentation spectra of peptides and, thanks to the uniqueness of the masses and the primary sequences of peptides, this approach allows the detection and quantification of hundreds of proteins in a biological sample (Aebersold & Mann, 2003).

Even if efforts have been always dedicated to the refinement of proteomic approaches to investigate more deeply the plasma proteome, it should not be forgotten that also highly abundant plasma proteins like human serum albumin (HSA), often neglected in these studies, might provide fundamental physiological functions in plasma and should be better considered.

2 | STRUCTURE AND FUNCTIONS OF HUMAN ALBUMIN

HSA is the most abundant and versatile protein in human blood, accounting for 50% of the plasma protein and providing ligand binding, transport and antioxidant properties (Rozga et al., 2013). HSA is a small globular protein of 585 amino acids forming a single polypeptide chain of molecular weight of 66.5 kDa, abundant in charged residues such as lysine, arginine, aspartate, and glutamate. The chain is arranged to create three homologous domains (I–III) each containing two subdomains (A and B) held by 17 disulphide bonds, which confer flexibility to the structure of the protein, and enable it to adapt to a variety of endogenous and exogenous ligands (Fasano et al., 2005). At physiological pH, HSA is negatively charged and in plasma assumes an ellipsoid configuration, which is different from the heart-shaped molecule obtained by crystallography (Figure 2). Of note, the molecule also contains one free thiol group (Cys34), highly reactive against oxidant species, which accounts for 80% of free thiols in plasma and gives HSA crucial

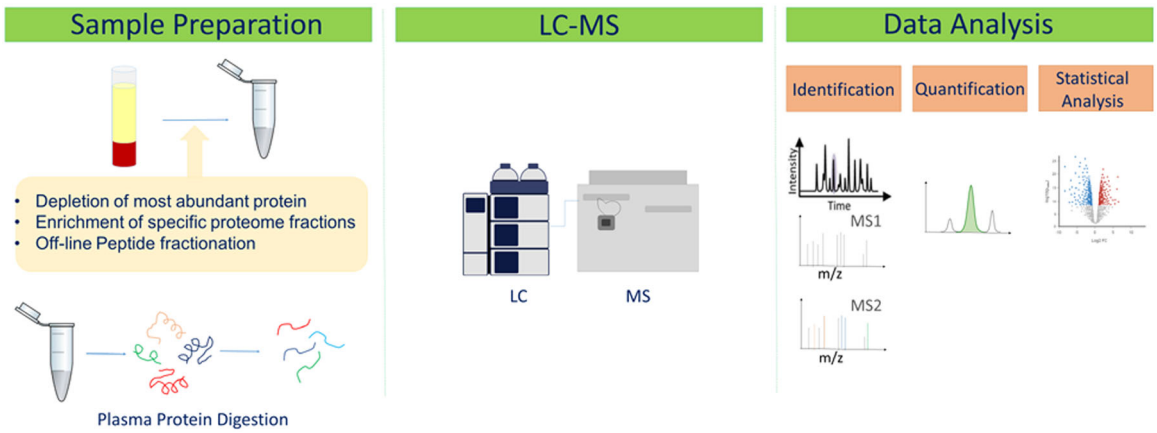

FIGURE 1 Schematic representation for a classical bottom-up proteomic approach applied to the study of plasma proteome [Color figure can be viewed at wileyonlinelibrary.com]

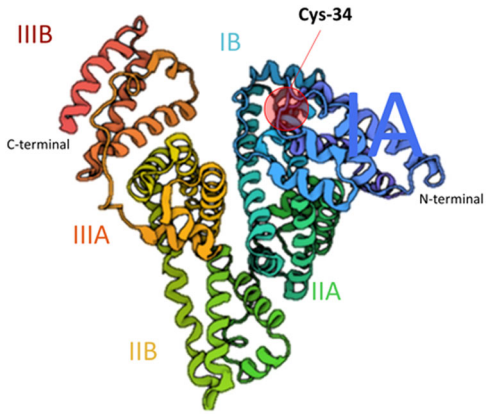

FIGURE 2 The three-dimensional structure of the human serum albumin. HSA consists of three homologous domains (I–III), each of which contains two subdomains (A and B). Cys34 is indicated by a red circle [Color figure can be viewed at wileyonlinelibrary.com]

antioxidant properties. The biological functions carried out by the protein are due to multiple binding sites located in the three domains.

Albumin is synthesized by the liver as preproalbumin, which is converted to proalbumin in the lumen of the endoplasmic reticulum of hepatocytes. The mature protein is then formed in the Golgi apparatus, and its majority is immediately released into the vascular space (Quinlan et al., 2005). Its synthesis is a constant process, which can be affected by several stimuli (both external and endogenous), such as high oncotic pressure, systemic inflammation, malnutrition, changes in hormonal response and critical illnesses (Nicholson et al., 2000). Albumin concentration depends on its rates of synthesis, degradation, and distribution between the intravascular and extravascular compartments. The total albumin content is around 250–300 g in healthy 70 kg adults of which 40% is held in the plasma compartment, being present at concentration ranges of 35–50 g/L, while the other is in the extravascular compartment (Nicholson et al., 2000). Daily degradation is around 14 g, of which 40%–60% occurs in muscle and skin.

HSA covers a variety of physiological functions. It is a relatively small but extremely abundant plasma protein responsible for 80% of total plasma colloid oncotic pressure, which ensures the maintenance of fluid balance across the capillary wall. The contribution depends on both its osmotic effect and the retention of water due to sodium ions attraction. Albumin opposes to fluid extravasation outside the intravascular compartment and maintains capillary membrane integrity. In the presence of hypoalbuminemia, which is common in many clinical settings, capillary membrane permeability increases, thus leading to interstitial edema and sodium retention through baroreceptor activation (Demling, 1986).

HSA exerts anticoagulant and antiplatelet aggregation activity. Lam et al. (2013) demonstrated that albumin can inhibit histone-induced aggregation in a dose- and charge-dependent manner, by binding circulating histones, which are released during inflammation and known to promote platelet aggregation. Moreover, the binding of prostacyclin (PGI_2) to albumin prolonged PGI_2 bioavailability, and so its antiaggregatory activity (Mikhailidis et al., 1982). The inhibitory effect on platelet aggregation could be also attributed to albumin ability to inhibit platelet agonists, such as thromboxane A2 and platelet-activating factor, through their direct binding (Grigoriadis & Stewart, 1992; Maclouf et al., 1980). Biological pieces of evidence show that albumin exerts anticoagulant action by interacting with fibrinogen (Galanakis, 1992). A heparin-like activity has also been suggested (Joorgensen & Stoffersen, 1979).

HSA is a negative-phase protein whose synthesis may be inhibited in response to inflammation by proinflammatory mediators, such as interleukin-6, interleukin-1, and tumor necrosis factor (Gabay & Kushner, 1999). At the physiological level, however, it acts as anti-inflammatory agent by selectively inhibiting cytokine-induced endothelial activation, which is known to be a critical initiating pathway in inflammation (Zhang & Frei, 2002).

Of note, HSA provides a significant antioxidant effect in the plasma compartment, which is constantly exposed to oxidative stress caused by reactive oxygen and nitrogen species (ROS and RNS). Oxidative stress damages molecules, leading to cellular dysfunction and has been implicated in the pathogenesis of inflammatory diseases. HSA is involved in scavenging more than 70% of free radicals, and its antioxidant effect is mainly due to the presence of the free thiol group on Cys34. Cys34 constitutes the largest pool of free thiols in circulation and works as a radical scavenger thanks to its peculiar acidity ($pKa = 8.1$) and spatial accessibility.

The presence of binding sites and its net charge give HSA the ability to bind and transport many different substances. Among endogenous ligands, HSA is known to bind metal ions, such as copper, at the N-terminal tripeptide Asp-Ala-His (Laussac & Sarkar, 1984), and hydrophobic organic anions, including long-chain fatty acids, polyunsaturated fatty acids, and bilirubin (Quinlan et al., 2005). Albumin is also a carrier of vitamins, hormones and steroids (Chien et al., 2017) (Figure 3).

Drug binding to albumin can be useful for therapeutic purposes, because its alteration may change the distribution volume, clearance, and elimination of drugs, thus modulating their therapeutic effects (Ishtikhar et al., 2014). Drugs that bind to albumin have been classified according to two binding sites, Sudlow's site I

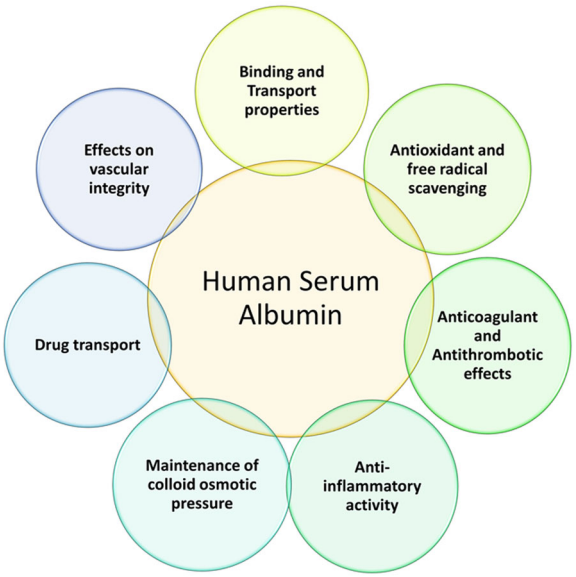

FIGURE 3 Summary of albumin functions [Color figure can be viewed at wileyonlinelibrary.com]

and site II, which are located in the subdomain IIA and subdomain IIIA, respectively (Rabbani & Ahn, 2019; Sudlow et al., 1975). Some drugs, like warfarin, salicylates, furosemide and phenytoin, bind to HSA at site I, while Sudlow's site II having a smaller size binding pocket and hydrophobic property accommodates the binding of hydrophobic drugs such as diazepam and ibuprofen (Ghuman et al., 2005). The binding capacities may be influenced by several factors, such as HSA concentration and the presence of compounds that compete for the same binding sites.

3 | THE PROGNOSTIC VALUE OF HSA IN THE CARDIOVASCULAR FIELD

Numerous clinical studies have established that hypoalbuminemia is a powerful prognostic marker in the general population as well as in many diseases, including CVD (Lyons et al., 2010). Serum HSA concentration serves as a reliable prognostic factor for CVD since a serendipitous finding in 1989 (Philips, 1989). Indeed, low serum albumin is independently and inversely correlated with the occurrence of various CVD conditions such as coronary artery disease (CAD), heart failure (HF), atrial fibrillation, stroke and venous thromboembolism, independently from causative confounders such as malnutrition and inflammation (Table 1).

Generally, hypoalbuminemia results from a decrease in liver synthesis, increased catabolism, extravasation to the interstitial area, volume overload and enteral or renal loss (Levitt & Levitt, 2016); however, the respective contribution of these different mechanisms has not been specifically studied in patients with CVD. Nevertheless, the ability of hypoalbuminemia to predict the onset and progression of various CVD (Figure 4) suggests that it might represent a potentially modifiable risk factor (Chien et al., 2017).

From a mechanistic point of view, the potential involvement of hypoalbuminemia in the physiopathology of CVD likely refers primarily to the anti-inflammatory, antioxidant, anticoagulant, and antiaggregating capabilities of serum albumin. Albumin is an acute-phase reactive protein with antioxidant and antiplatelet activity in vitro, and its levels inversely correlate with markers of platelet activation with mechanisms related to its binding capacity and antioxidant properties. By binding arachidonic acid released during phospholipase A2 (PLA_2) activation, for example, albumin affects the clinical efficacy of antiplatelet drugs such as aspirin in patients at high risk of CVD (i.e., patients with type 2 diabetes mellitus). An observational cohort study conducted on a total of 612 aspirin-treated type 2 diabetes mellitus patients with a follow-up of approximately 54 months, showed that patients who had serum albumin <3.5 g/dl (41% of the study population) presented an impaired cyclooxygenase 1 (COX-1) inhibition as demonstrated by the higher level of serum thromboxane B2, and an increased incidence of long term cardiovascular events, in particular coronary events (Sciacqua et al., 2021).

The most studied cardiovascular condition, according to a recent review, is HF, a systemic syndrome characterized by a complex interplay of cardiocirculatory failure, neurohormonal and biochemical disorders and organ injuries (Arques, 2020; Arques & Ambrosi, 2011).

Hypoalbuminemia is common in HF and occurs in HF patients with reduced systolic function and preserved systolic function, as well as in stable chronic disease and acute HF. In all these conditions, hypoalbuminemia worsens the patient survival and represents an independent predictor of mortality at 1 year after hospital discharge (Liu et al., 2012; Uthamalingam et al., 2010).

It is known that hypoalbuminemia occurs in at least one-quarter of HF patients and is a powerful predictor of adverse outcomes, particularly in HF with reduced ejection fraction (HFrEF). Indeed, once HF is manifest, it has been demonstrated that low albumin levels are independently associated with an increased risk of death in HF (Horwich et al., 2008). Lower albumin has been also shown to predict incident HF among adults aged (≥65 years) patients without CVD (Filippatos et al., 2011). Unfortunately, most studies on the prognostic role of albumin in HF either excluded patients with HF with preserved ejection fraction (HFpEF) or did not

TABLE 1 Oxidative modifications in cardiovascular diseases

Modification	Disease	Method	Reference
Nitration and nitrosylation			
Protein-bound tyrosine	CAD	stable isotope dilution LC-MS/MS	Shishehbor et al. (2003)
Nitrotyrosine	CAD	ELISA	Bencsik et al. (2015)
3-Nitrotyrosine	HF	ELISA	Eleuteri et al. (2009)
Protein nitration	HF	Immunoprecipitation and immunoblotting	Cabassi et al. (2014)
Nitrosylated proteins	CV outcome in end-stage renal disease	Fluorometric method	Massy et al. (2004)
Thiolation			
Circulating and plaque-filtered albumin (mercaptoalbumin and LMW bound thiols)	Stable and unstable angina	Maleimide labelling and capillary Zone Electrophoresis Analysis	Lepedda et al. (2013)
Circulating and plaque-filtered albumin (mercaptoalbumin and LMW bound thiols)	Stable and unstable angina	Maleimide labeling and capillary electrophoresis laser-induced fluorescence	Lepedda et al. (2014)
HNA and mercaptoalbumin	CAD patients	HPLC	Kadota et al. (1991)
HNA and mercaptoalbumin	CV outcome in hemodialyzed patients	HPLC	Lim et al. (2013)
Thiolated albumin	hyperlipidemic subjects	HPLC and MALDI-TOF/TOF; LMW thiol identification by LC-MRM-MS	Nakashima et al. (2018)
Thiolated albumin	HF	ESI-MS; LMW thiol identification by LC-MRM-MS	Brioschi et al. (2020)
Ischemia modified albumin (IMA)			
IMA	myocardial ischemia in patients after percutaneous coronary intervention	albumin cobalt-binding assay	Sinha et al. (2003)
IMA	CV outcome in dialyzed patients	albumin cobalt-binding assay	Su et al. (2013)
Glycation			
GA	Coronary heart disease	Enzymatic method	Mihara et al. (2020)
GA	HF	MALDI-TOF analysis	Paradela-Dobarro et al. (2019)
GA	HF	ESI-MS	Martinez Fernandez et al. (2019)
Lipid perodixation products addducts			
RCS adducts	Ischemia-reperfusion injury during hepatectomy	Affinity chromatography and MS analysis by precursor ion scanning	Witort et al. (2016)
RCS adducts	HF	Affinity chromatography, MS analysis by precursor ion scanning and semi-quantitative targeted MS	Altomare, Baron, Balbinot, et al. (2021)

Abbreviations: CAD, coronary artery disease; CV, cardiovascular; ELISA, enzyme linked immunosorbent assay; ESI, electrospray ionization; HF, heart failure; HPLC, high performance liquid chromatography; LMW, low molecular weight; MALDI-TOF, matrix assisted laser desorption ionization- time of light; MRM, multiple reaction monitoring; MS, mass spectrometry; RCS, reactive carbonyl species.

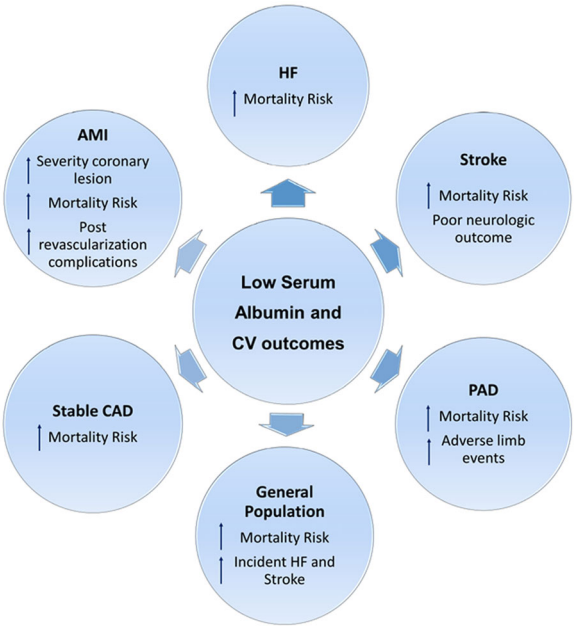

FIGURE 4 Hypoalbuminemia in cardiovascular diseases. Low levels of serum albumin have been associated with poor outcomes both in the general population and in patients with cardiovascular diseases. HF, heart failure; AMI, Acute myocardial infarction; CAD, coronary artery disease; PAD, peripheral artery diseases [Color figure can be viewed at wileyonlinelibrary.com]

distinguish the type of HF. Prenner et al. (2020) demonstrated the independent prognostic value of albumin in HFpEF. This study revealed that lower albumin was associated with increased levels of various inflammatory markers, correlating with incident HFpEF. Systemic inflammation may be one of the primary drivers of myocardial disease in HFpEF (Paulus & Tschope, 2013). These studies demonstrated that albumin was an integrated marker of systemic inflammation, renal and liver dysfunction, and more advanced diastolic dysfunction, and arterial stiffness. Thus, in this population, albumin was a strong independent clinical predictor of adverse outcomes beyond other traditional risk factors, even within normal ranges (>3.5 g/dl) (Prenner et al., 2020).

Further, several studies have reported the prognostic value of HSA level independently of B-type natriuretic peptides, which have been recommended as the gold standard biomarkers for the diagnosis and prognosis of HF according to the current clinical guidelines.

Thus, emerging data suggest that the quantitation of HSA levels should be part of the CVD risk assessment, being a simple, inexpensive laboratory test, which provides essential prognostic information in CVD. However, there is currently no evidence that prevention or correction of hypoalbuminemia improves the prognosis of patients with CVD. Despite some evidence suggests that albumin transfusion improves the functional prognosis of patients with severe hypoalbuminemia, prospective studies are needed to determine the impact of the correction of hypoalbuminemia in patients with or at risk for CVD (Arques, 2020).

4 | ALBUMIN OXIDATIVE MODIFICATIONS AND CVD: THE CONTRIBUTION OF MS

Oxidative stress has been increasingly related to the onset and/or progression of several diseases including CVD (van der Pol et al., 2019), and there is a considerable research interest in the identification of oxidative stress biomarkers for earlier prediction of disease onset, monitoring of the progression, and outcome prediction.

In particular, the role of oxidative stress in HF has been extensively demonstrated, both in the development and progression of the disease. Under pathophysiological conditions, the production of ROS exceeds the buffering capacity of the antioxidant defense system, causing cell and tissue damage. Despite several studies have tried to target oxidative stress in HF with very limited success, one plausible reason might be that currently employed strategies target oxidative stress by exogenous inhibition of ROS production or supplementation of exogenous antioxidants while activating the endogenous antioxidant capacity might be more efficient (van der Pol et al., 2019).

Thus, in addition to the important role of albumin concentration as a diagnostic or prognostic biomarker in CVD, particular attention should be paid to albumin modifications, and in particular oxidative modifications, which could impair its function with possible effects on the development and progression of CVD.

Albumin is indeed susceptible to different oxidative modifications, in particular, thiol oxidation and formation of adducts with lipid peroxidation (LPO) products. In this review, we will focus our attention mainly on oxidative modifications, whose role in CVD has been evaluated.

4.1 | Nitration and nitrosylation

Protein damage caused by free radicals can result in several modifications of specific amino acid residues, cross-linking of side chains or fragmentation. In particular, peroxynitrite and other reactive nitrogen species (i.e., nitrogen dioxide, nitrous acid, nitryl chloride) are prone to attack free L-tyrosine and protein-bound tyrosine leading to the formation of 3-nitrotyrosine (3-NT), by the addition of a NO_2 group (in ortho position to the

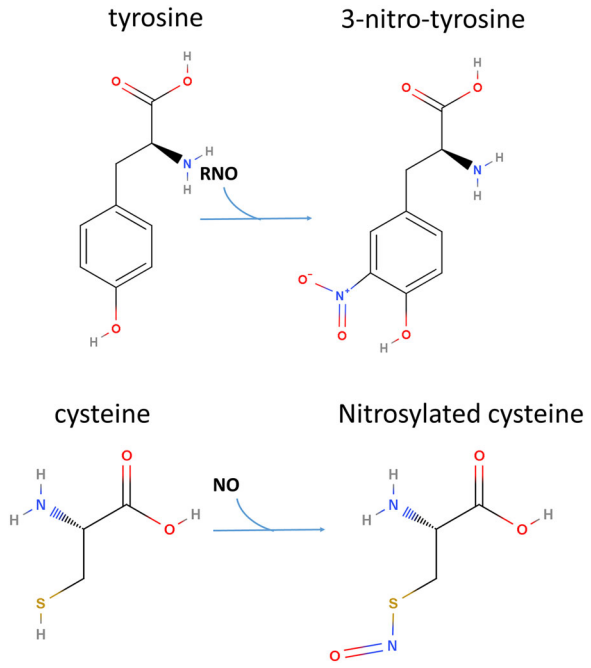

FIGURE 5 Reaction of reactive nitrogen species with aminoacids. Mechanism of the formation of 3-nitro-tyrosine (upper panel) and cysteine nitrosylation (lower panel) [Color figure can be viewed at wileyonlinelibrary.com]

phenolic hydroxyl group) (Figure 5). In addition, 3-NT formation could be also catalyzed by a class of peroxidases derived from inflammatory cells utilizing nitrite and hydrogen peroxide (Ahsan, 2013). Several methods have been developed so far to detect this protein modification, ranging from immunoenzimatic assays to MS-based methods coupled with gel electrophoresis, LC or gas chromatography (Abello et al., 2009; Upmacis, 2008).

Nitration of tyrosine residues can profoundly alter not only the protein structure but also the functions, suggesting that protein nitration could be predictive of oxidative cell injury. Indeed, several studies have demonstrated the presence of 3-NT in pathological conditions, including CVD (Ahsan, 2013; Souza et al., 2008).

Measurement of protein-bound nitrotyrosine, by means of a stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS)-based method, showed a strong correlation between 3-NT plasma levels and the prevalence of CAD, revealing also the protective role of statins against this deleterious process (Shishehbor et al., 2003). Moreover, nitrotyrosine correlates with serum lipids, MMP activity and cardiac function in CAD patients selected for percutaneous coronary intervention (Bencsik et al., 2015). In the context of HF, data demonstrate the presence of an increased 3-NT plasma level in patients with moderate/severe HF in association with markers of systemic inflammation (Eleuteri et al., 2009), and in particular ceruloplasmin nitration has been evidenced and associated bad prognosis in patients with HF (Cabassi et al., 2014). Under inflammatory conditions, such as in CVD, only 1–10 residues of tyrosine every 100,000 have been shown to be nitrated in plasma proteins, while these levels increase approximately 10 times in tissues, such as atherosclerotic plaques (Souza et al., 2008).

Of note, HSA, despite being the most abundant plasma protein, is less extensively nitrated than other plasma proteins (Ahsan, 2013). In vitro study of peroxynitrite-mediated nitration of HSA demonstrated that only two tyrosine residues among the total 18 tyrosine residues of HSA could be susceptible to nitration (Jiao et al., 2001).

In addition to protein nitration, reactive nitrogen species could induce the formation of protein *S*-nitrosylation by the covalent ligand of the nitrosyl group to the sulfur atom of cysteines (Figure 5). Of note, the S-nitrosothiol fraction in plasma is largely composed of S-nitrosylated human serum albumin (SNO-HSA), and that is why it has been tested whether this albumin form could be used as a NO traffic protein (Xie et al., 2018).

Elevated concentrations of plasma SNO-proteins have been associated with adverse cardiovascular outcomes in patients with end-stage renal disease, and correlate with elevated blood pressures (Foster et al., 2005; Massy et al., 2004). However, experiments performed on an isolated erythrocyte-perfused working heart model demonstrated that SNO-HSA attenuates ischemia/reperfusion injury by supplementing NO (Semsroth et al., 2005). Similarly, intravenous SNO-HSA infusion showed positive effects on overall hemodynamics and oxidative stress in a chronic left-to-right shunt-induced pulmonary arterial hypertension model with right ventricle failure (Rungatscher et al., 2015).

4.2 | Thiolation

Thiol content in HSA has a relevant role, especially for its antioxidant activity. Indeed, in plasma samples, the main HSA fraction is the so-called mercaptoalbumin, HSA-SH (accounting for 70% of total HSA), which contains a reduced free thiol at Cys34 that is able to efficiently scavenge several oxidants, including hydroxyl and peroxyl radicals, peroxynitrite and hydrogen peroxide (Regazzoni et al., 2013; Roche et al., 2008). Considering that albumin is the predominant protein in most body fluids, its Cys34 represents the largest fraction of free thiols within body fluids (Colombo et al., 2012). Cys34 resides in a unique microenvironment in HSA that is responsible for the unusually low pKa of Cys34, which justify the predominance of its highly nucleophilic thiolate form at physiological pH

(Colombo et al., 2012; Stewart et al., 2005). An important fraction of non-mercaptoalbumin contains mixed disulfides due to the interaction of albumin free thiols, mainly Cys34 in its sulfenic form (Alb-SOH), with low molecular weight (LMW) thiols, such as cysteine, cysteinyl-glycine (Cys-Gly), homocysteine (Hcys), γ-glutamylcysteine or glutathione (GSH) (Carballal et al., 2007). About 20%–30% of the Cys34 forms these reversible mixed disulfides with low-molecular-weight thiols, generating S-thiolated albumin isoforms, also called human non-mercaptoalbumin 1 (HNA-1), among which the predominant modification is cysteinylated albumin (cys-HSA). Only a smaller fraction of the Cys34 (~2%–5%) is subjected to irreversible oxidation to the sulfinic (Alb-SO_2H) or sulfonic acid (Alb-SO_3H) form (human non-mercaptoalbumin 2 or HNA-2) (Figure 6). Of note, these oxidized isoforms are not present in albumin immediately after secretion from hepatocytes, and thus they may represent potential biomarkers of oxidative stress in human diseases (Bonanata et al., 2017; Colombo et al., 2012).

Recent data revealed that the reactions between sulfenic acids and thiolates, leading to the formation of mixed disulfide, show a correlation with thiolate basicity as expected for SN_2 reactions and that the values of the rate constants could be modulated by electrostatic effects (Turell et al., 2021).

Moreover, fatty acids binding to HSA could induce conformational changes at the thiol site increasing the thiol reactivity toward disulfides (Colombo et al., 2012; Torres et al., 2012).

Several studies have shown an unbalance between the reduced and oxidized forms of albumin in relation to age (Era et al., 1995), or other pathological conditions associated with increased oxidative stress (Colombo et al., 2012), using different biochemical approaches.

A quantification of Cys34 free thiol of albumin has been performed in plasma of patients in hemodialysis in comparison with normal subjects, and in patients with focal segmental glomerulosclerosis by "in gel" labelling with maleimide-PEO2-biotin (biotinyl-3-maleimidopropionamidyl-3,6-dioxactanediamine), which binds to free thiols (Bruschi et al., 2008). A similar approach has been also recently employed by Lim et al., who used polyethylene glycol maleimide (malpeg) to tag free thiols before gel separation. In addition, to measure the levels of reversibly thiolated HSA, they performed a thiol exchange reaction using L-cysteine at pH 3 before binding with malpeg, and they obtained comparable data with LC-MS methods (Lim et al., 2020). Maleimide-labelling has been successfully applied to compare the extent of protein-SH oxidative modification in the plasma and human carotid plaques extract, where HSA infiltrates, in relation to plaque stability. Even if no differences were detected in plasma, unstable plaques showed a lower level of HSA-SH and a concomitant higher concentration of protein-bound LMW thiols measured by capillary electrophoresis after release from HSA (Lepedda et al., 2013). A further comparison of the HSA thiolation in circulating and unstable plaque-filtered HSA, performed with the same methodologies, revealed higher levels of oxidation in the plaque suggesting that Cys34 modifications could be a consequence of the strong pro-oxidant environment present in the plaque (Lepedda & Formato, 2020; Lepedda et al., 2014). Moreover, circulating and plaque–filtered albumin displayed a different pattern of LMW thiols binding; once filtered, albumin released significant amounts of Hcy and Cys-Gly in the plaque (Lepedda et al., 2014).

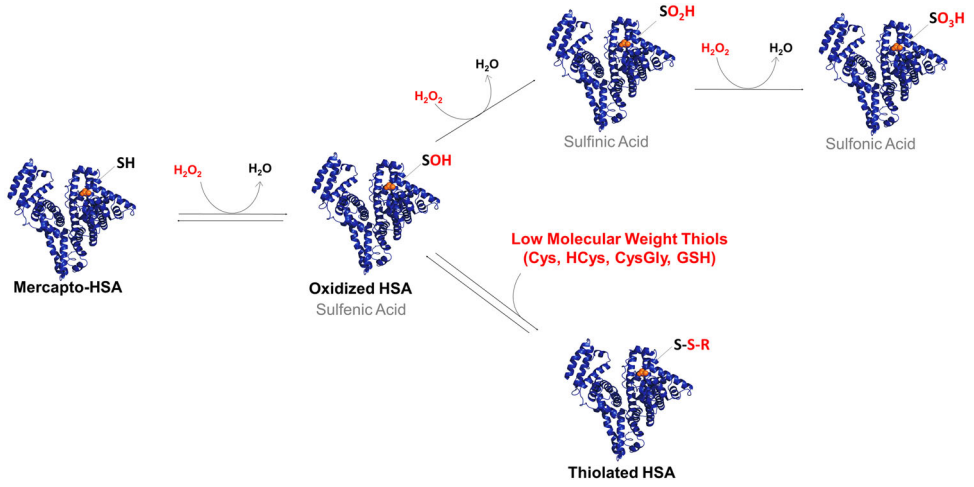

FIGURE 6 Mechanism of the reaction of HSA free thiol at Cys34 leading to the formation of thiolated isoforms of albumin (HNA-1) from the interaction of sulfenic acid on HSA with circulating low molecular weight thiols. Cys, cysteine; CysGly, cysteinyl glycine; GSH, glutathione; HCys, homocysteine [Color figure can be viewed at wileyonlinelibrary.com]

Evidence of albumin oxidation in vivo, and in particular in CVD, have been reported only recently, thanks to the advancement in the field of LC/MS, producing instruments with high resolution, useful for an in-depth characterization of HSA isoforms.

Kadota et al. performed one of the first studies on albumin thiolation in patients with CAD, including patients with stable angina, unstable angina or acute myocardial infarction, in comparison to subjects with normal coronary arteriograms and normal cardiac functions. They measured, with a spectrophotometric detector, albumin isoforms corresponding to HNA-1 or mercaptoalbumin separated by LC, and they compared these results with the measurement of total SH evaluated with the Ellman's reagent. They evidenced a positive correlation between HSA and the total SH content. Moreover, HSA-SH was significantly lower in CAD patients in respect to control with lower levels in patients with a higher level of severity. In addition, they demonstrated that mercaptoalbumin rapidly decreased from admission to the first 6 h after myocardial infarction, while it increased to the levels measured in the chronic stage after 24 h (Kadota et al., 1991).

More recently, Lim et al. (2013) in a cohort of normoalbuminemic hemodialyzed patients identified serum levels of HNA as a positive predictor of cardiovascular mortality, suggesting that changes in oxidative damage to HSA, impairing its scavenging activity, could contribute to the acceleration of atherosclerosis, and to the development of CVD in this population.

A better structural characterization has been obtained with the employment of MS. Beck et al. obtained high-quality electrospray ionization (ESI) mass spectra, in terms of signal to noise and resolution, using a quadrupole time of flight (q-ToF) mass spectrometer by direct infusion, without any enrichment procedure, and showed a clear signal due to the addition of cysteine to HSA (+119 Da), corresponding to 20%–30% of total albumin (Beck et al., 2004). Naldi et al. (2013) obtained better resolved mass spectra of albumin structural modifications purifying HSA by reverse-phase chromatography on a C8 column before MS analysis on an ESI-q-Tof instrument leading to the identification of the sulfinic-HSA, cys-HSA, nitrosylated HSA, glycated isoforms, and also the N-terminal truncated isoform.

An alternative approach has been applied by Regazzoni et al. to study HSA isoforms in end-stage renal diseases to evidence the effects of hemodialysis. They isolated HSA from plasma samples by affinity chromatography, and after denaturation in nonreducing conditions to maintain the mixed disulfides, they acquired MS spectra by a triple quadrupole mass spectrometer to detect and quantitate the relative amounts of the redox isoforms after spectra deconvolution (Regazzoni et al., 2013).

Nakashima et al. analyzed serum samples from normal and hyperlipidemic subjects by HPLC with an anion-exchange column, and evidenced the presence of two main peaks attributable to HSA isoforms with different abundance in the two groups of patients. MALDI-TOF/TOF analysis of the HPLC fractions revealed that the HSA isoform more abundant in hyperlipidemic patients corresponds to a protein with a molecular weight increase of approximately 200 Da, suggesting an adduct with LMW thiols but not enough resolved to properly identify them. To further characterize this oxidized isoform, whose abundance is reduced by treatment with reducing agents, they developed an MS-based method to quantify LMW thiols bound to HSA. They treated human sera with tris(2-carboxyethyl)phosphine, to release LMW thiols, and after HSA elimination they performed derivatization with 4-(2-(dimethylamino)ethylamin osulfonyl)-7-chloro-2,1,3-benzoxadiazole (DAABD-Cl). LC-MS/MS analysis of the oxidized isoform performing a parent ion scan acquisition for a specific fragment generated DAABD-labeled thiols (m/z 72.2) allowed them to specifically identify two precursors (m/z 390.1 and 404.1) corresponding to DAABD derivatives of cysteine and homocysteine, undetectable in the reduced form. Based on this observation, they set up an MRM approach to specifically quantify the two LMW thiols bound to HSA. They also characterized the putative binding sites for these LMW thiols by MALDI-TOF/TOF analysis of HSA peptides obtained by tryptic digestion in nonreducing conditions, identifying other cysteines in addition to Cys34 that might be involved in LMW thiols binding. Altogether, these data suggest that, in hyperlipidemic patients, oxidized HSA mainly exists as a mixed disulphide with cysteine and homocysteine, with cysteine being the most prevalent.

In addition, Nakashima et al. (2018, 2020) elucidated, using multiplexed fluorescence probes, that S-homocysteinylation might induce an increase in both ligand binding activity and hydrophobicity of HSA.

Of note, an elegant method, developed by Fu et al., allowed the simultaneous quantitation of free and protein-bound thiols in human plasma after blocking free thiols with *N*-ethylmaleimide (NEM) and precipitating proteins. Disulfides and thiol-NEM adducts were measured by LC-MS/MS using an MRM approach in comparison to isotopically labelled internal standards, revealing that protein-bound Cys was the most abundant thiol in plasma (Fu et al., 2019).

The importance of thiolation of albumin in HF has been recently described by Brioschi et al. (2020). They measured the relative amount of HSA isoforms in plasma from HF patients in respect to control samples using an MS approach similar to that described by Regazzoni

et al., without any affinity purification step. They demonstrated that HF patients, in addition to hypoalbuminemia, had lower levels of mercaptoalbumin and increased quotes of thiolated albumin in respect to control subjects; thiolated isoforms were further characterized with the method proposed by Nakashima et al. (2018) demonstrating a clear prevalence of cysteinylated HSA in respect to homocysteinylated HSA. In addition, they clearly showed that cysteinylated HSA increase was higher in patients with a higher degree of severity, characterized by worse cardiopulmonary function and that cys-HSA levels correlate with an impairment of the plasma antioxidant activity, suggesting an impact of this modification on HSA structure and function (Brioschi et al., 2020).

Several methods have been also proposed to improve the analysis of adducts with Cys34 after an enrichment step, even if they still have never been applied in the clinical setting. An antibody against the largest peptide of HSA, including Cys34, was used to enrich tryptic digest before analysis by high-resolution MS (Chung et al., 2014). Similarly, enrichment of cysteinyl adducts has been obtained treating HSA with a thiol affinity resin, able to remove mercaptoalbumin (Funk et al., 2010).

4.3 | Ischemia modified albumin (IMA)

The importance of albumin modifications induced by oxidative stress in the cardiovascular field is also evidenced by the increasing body of evidence regarding IMA. IMA is a form of serum albumin, first discovered in the 1990s, characterized by its reduced cobalt-binding affinity, which can be measured indirectly by the Food and Drug Administration-approved albumin cobalt-binding assay (Coverdale et al., 2018). In IMA, the N-terminal amino acids are unable to exert their scavenger activity for transition metals. In ischemic conditions, the generation of free radicals and the release of copper and iron initiate IMA generation. Thus, IMA has been proposed as a sensitive marker of myocardial ischemia in patients after percutaneous coronary intervention (Sinha et al., 2003; Su et al., 2013), and as a biomarker for early detection of myocardial ischemia before the onset of irreversible cardiac injury (Coverdale et al., 2018; Sbarouni et al., 2011).

In addition, in a prospective study with two years of follow-up Su et al. (2013) evidenced that continuous ambulatory peritoneal dialyzed patients with high levels of IMA showed a higher incidence of major adverse cardiovascular events. In patients with chronic HF, an increase of IMA and IMA corrected for serum albumin was evidence, even if IMA levels did not differ among patients with different disease severity, suggesting that IMA is not affected by HF per se, and no correlation was detected with oxidative stress markers (Ellidag et al., 2014).

Evaluating the effects of ventilation in patients undergoing cardiopulmonary bypass (CBP) Ozgunay et al. showed an increase of IMA, both in ventilated or not-ventilated patients, starting intraoperatively, with a concomitant reduction of total thiols and albumin. Of note, continuous ventilation led to lower levels of IMA 24 h after CBP suggesting that lung ventilation during the operation could reduce ischemia and lung damage reducing the drop of blood flow (Ozgunay et al., 2019). In a recent paper, IMA has been described, in hemodialysis patients as a good predictor of arterial stiffness, which is strongly associated with cardiovascular risk in this type of patients (Jiao et al., 2020).

4.4 | Glycation and advanced glycation end-products (AGEs)

Nonenzymatic glycation of plasma proteins occurs in normal conditions, but HSA is typically 2–3 times more glycated than the rest of the serum proteins in hyperglycemic conditions (Rondeau & Bourdon, 2011). Albumin glycation occurs through the well-known Maillard reaction, where the free amine groups of albumin react with glucose to reversibly form a Schiff base product, followed by the formation of a stable fructosamine residue by Amadori rearrangement. The Schiff's base and fructosamine are named early glycation adducts to be distinguished by the AGEs, which can be formed through two main pathways: the first, involving irreversible rearrangements of the Amadori products, following both oxidative and nonoxidative pathways; the second, based on the condensation between the side-chain of lysine, cysteine and arginine residues with dicarbonyls, including glyoxal (GO), methylglyoxal (MGO), and deoxyglucosone, which can be generated by enolization of Amadori products, as well as by the direct degradation of aldoses and ketoses (Vistoli et al., 2013). GA is considered a potential clinical biomarker of glycemic control, which could be utilized to monitor glycemic status in diabetic patients in those clinical conditions in which the measurement of glycated hemoglobin, which is the recommended biomarker, does not reflect glycemic status (i.e. chronic renal failure, recent transfusion, pregnancy), and/or to monitor short-term glycemic fluctuations in patients with poor metabolic control (American Diabetes Association, 2021; Giglio et al., 2020). Indeed, GA reflects mean glycemia over approximately 2–3 weeks and, compared with glycated

hemoglobin by more rapid and greater changes, not influenced by red blood cell lifespan (Li et al., 2021). Interestingly, Chen et al. (2017) demonstrated that, in diabetic patients on hemodialysis, high GA measurements are significantly associated with increased mortality, in contrast to an inconsistent association between higher glycated hemoglobin values and mortality risk, thus highlighting the need to monitor and optimize glycemic levels especially in the patient population with disorders affecting erythrocyte life span. Further, when compared to glycated hemoglobin, the association of GA and fructosamine to clinical cardiovascular outcomes and deaths is still poorly characterized. Only recently, in a clinical study conducted on 11.104 participants with and without diabetes, Selvin et al. (2015) showed that elevated baseline concentrations of these short-term biomarkers were significantly associated with CAD, Ischemic Stroke, HF and mortality in the community, even after adjustment for traditional cardiovascular risk factors. The sites of albumin glycation have been studied by different MS methods including the $^{16}O/^{18}O$ ratio and MALDI-TOF-MS and the major sites are represented by Lys525, Lys199, and Lys439 (Barnaby et al., 2011). The extent of albumin glycation in healthy human subjects is 11%–16%, increasing 2–3-fold in patients with diabetes (Rabbani & Thornalley, 2021). More recently, a relationship between serum levels of GA and the severity of coronary heart disease in a nondiabetic population has been found. In particular, Mihara et al. (2020) recently reported that higher serum GA levels are significantly associated with the development of CVD and its subtypes, even among subjects without diabetes or those with normal hemoglobin A1c levels, in a general Japanese population. By using a MALDI-TOF analysis, Paradela-Dobarro et al. (2019) reported differences in the glycation mass of albumin isolated from HF patients in comparison with healthy subjects. Several new glycation sites on in vivo GA of HF patients were reported, such as Lys4, Arg222, Arg257, Arg336, Arg348, and Arg445. These glycated albumins were used to analyze the effect of in vivo glycation in endothelial dysfunction, and were found to induce an increase in adhesion molecules expression on human umbilical vein endothelial cells, supporting an increase in peripheral blood mononuclear cells adhesion to endothelial cells.

An increased GA content in HF patients in respect to age matched control subjects, as measured by ESI-MS intact protein analysis, has been reported by Alma Martinez Fernandez et al. with GA being highest in subjects with the most severe HF. Moreover, by using quantitative proteomic analysis, GA was found to exert proinflammatory and pro-oxidant effects on murine HL-1 cardiomyocytes, highlighting a causal role in the etiopathogenesis of HF (Martinez Fernandez et al., 2019).

Based on these recent findings, it is quite evident that GA is now widely studied not only as a potential biomarker, but also as a functional mediator, exerting, among others, proinflammatory and pro-oxidant effects. At clinical level, GA is measured by a standardized enzymatic methodology, easy and fast to perform (Freitas et al., 2017) while MS-based approach, by MALDI-TOF and ESI-MS in intact and bottom-up modes, are widely applied at research level to investigate the sites of glycation and to measure the relative abundances of albumin isoforms.

4.5 | LPO products adducts

LPO of polyunsaturated fatty acids leads to the formation of break-down products among which the reactive carbonyl species (RCS), which, from a chemical point of view, can be classified into three groups: α,β-unsaturated aldehydes, such as 4-hydroxynonenal (HNE) and acrolein (ACR), di-aldehydes such as malondialdehyde and GO, and keto-aldehydes, including 4-oxo-nonenal (ONE) and isoketals (Altomare, Baron, Gianazza, et al., 2021).

RCS are electrophilic compounds and, besides undergoing a metabolic deactivation involving phase I and II enzymes leading to the oxidation/reduction of the carbonyl moieties and GSH adducts, they can also react with the nucleophilic sites of proteins (Cys, Lys, His, and Arg) leading to the formation of covalent protein adducts (Altomare, Baron, Gianazza, et al., 2021). Albumin is the main protein in plasma and extracellular space, and it is characterized by many reactive and accessible nucleophilic residues, which react with RCS. Many in vitro studies have reported the ability of albumin to quench RCS, and identified the sites and the adducted moieties (Aldini et al., 2007; Aldini, Vistoli, et al., 2008) and based on such reactivity, albumin has been considered as a line of defense against extracellular RCS (Aldini, Regazzoni, et al., 2008). LPO is a hallmark of the development and progression of atherosclerosis-based diseases, such as HF and other CVD (Gianazza et al., 2021), and hence albumin-RCS adducts can be expected to occur in the plasma samples of such patients. From an analytical point of view, albumin-RCS can be analyzed by a general approach aimed to measure the total content of carbonyl moieties attached to the protein (index of albumin carbonylation). Carbonyl moieties can be detected following derivatization with 2,4-dinitrophenylhydrazine and the reaction products determined by using antibodies upon proteins isolation by 1D or better 2D gel electrophoresis, followed by western blotting analysis (Rogowska-Wrzesinska et al., 2014; Weber et al., 2015). Another approach consists in isolating albumin by affinity chromatography followed by the analysis of the adducted carbonyls by in-solution derivatization methods

(Acimovic et al., 2013). By using such approaches, carbonylated albumin was found to be significantly increased in several oxidative based-diseases including acute ischemic brain injury (Moon et al., 2011), chronic kidney disease (Colombo et al., 2020) and others, as reviewed in (Colombo et al., 2012). Regarding CVD, several studies have found an increased plasma carbonylation in respect to control subjects (Banfi et al., 2008; Lavall et al., 2016; Pirinccioglu et al., 2012), while, to the best of our knowledge, very few investigations have taken into consideration the contribution of albumin on the overall plasma carbonylation.

Another approach used to measure albumin carbonylation consists of isolating albumin by affinity chromatography followed by a bottom-up MS analysis for the identification of the adducted moieties and sites of adduction. The adducted moieties are searched by setting as variable modifications for the identification software those adducts already identified in in vitro studies. The clear advantage of such an approach is that the RCS leading to albumin modification are identified, information that permits to better understand the oxidative pathway, and, in line of principle, to design the most suitable selective RCS sequestering agents. By using such an approach, implemented with a precursor ion scanning method to identify unknown Cys34 adducts, ACR resulted to be the main RCS adducted to albumin Cys34 formed by the ischemia-reperfusion injury that occurs during liver hepatectomy (Witort et al., 2016). The method has been extensively applied, very recently, to map the main RCS albumin adducts which occur in patients with HF. A set of RCS adducts were searched as variable modifications in albumin isolated from HF patients and age-matched control subjects. Several LPO and glycolysis derived RCS were identified in both groups, including the Michael adducts HNE, ACR and ONE and carboxy-methyl (CML) and carboxy-ethyl lysine (CEL), which are the reaction products of GO and MGO with Lys, respectively. Once the adducts have been identified, a semiquantitative targeted approach was then set up to identify those adducts which are significantly increased in HF patients. The analyses showed that four CML derivatives on Lys residues (i.e., CML-Lys12, CML-Lys378, CML-Lys402), and one deoxy-fructosyl derivative on the Lys 389 (DFK-Ly-389) were significantly increased in HF patients (Altomare, Baron, Balbinot, et al., 2021).

5 | CONCLUSION

In conclusion, this review highlights the importance of studying albumin, instead of eliminating or ignoring it, as done in the past in favor of low abundant plasma protein analysis. In particular, due to the high abundance, but also the long half-time in blood, oxidized forms of HSA might be more informative than those of other proteins for the characterization of oxidative stress in pathophysiological conditions (Nakashima et al., 2020).

Nevertheless, there is still a strong need for standardized methods for albumin modifications evaluation for a broader application in clinical practice. It is clear from the data summarized in this review that massive albumin oxidation occurs in vivo in different biological fluids in correlation with organ dysfunction, but current methods for the detailed measurement of oxidative damage to proteins, based on redox proteomics and MS, still are likely to be validated in clinical cohorts (Colombo et al., 2012).

In particular, based on the literature revision of the last years, we can observe that only very few data have been so far reported on albumin RCS adducts in patients affected by CVD. Such limited application can be explained by difficulties in their detection/quantitation not only due to their heterogeneity and low abundances, which require sophisticated MS analyzers, but also considering their metabolic and chemical instability. Indeed, RCS adducts are characterized by the free carbonyl groups, which can undergo an endogenous metabolic process, as well as a chemical transformation during the storage. Hence novel analytical protocols are required for sample preparation, to stabilize albumin-RCS adducts, and for sensitive and specific analyses.

Thus, in the context of CVD, the focus should be also moved from albumin levels to albumin quality, and in particular oxidative modifications, provided that further studies are required to elucidate the exact relationship between albumin oxidation and pathology. In particular, further work is still needed to determine whether albumin and its oxidative modifications could be used to monitor the progression of organ dysfunction and/or systemic inflammation in both HFpEF and HFrEF.

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"It is indeed a great pleasure to be one of the authors of a special issue of the Mass Spectrometry Review dedicated to Pietro Traldi. First of all the great appreciation (since 1981 when I attended a mass spectrometry course in Padua held by him) and then the sincere affection grew over the years, together with his huge expertise in the field. My memories of the many hours spent in his laboratories at CNR are many, but I can try

to list them to better delineate his personality: his helpfulness to all, his ability to passing on knowledge to younger people and to explore new strategies, his allow anyone to use his instrumentation, his openness in understanding how to best use mass spectrometers. This in particular led him to blaze new trails for new applications of mass spectrometry (an attitude seldom shared in those years)! For all these reasons (and I think I have left out many), I am more than happy to honor my friend Pietro with this special issue of Mass Spectrometry Review.

Marina Carini."

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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