Dissertation

Uremic Albumin blocks Reverse Cholesterol Transfer: The Role of Lysine Modifications

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Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all those individuals and organizations that have contributed to the research of this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, 20.06.2013

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Though this work represents first of all my scientific effort it would not be the same, without the ideas and contributions of various people. Therefore I would like to express my gratitude to everyone whose scientific advice or personal support has been inspiring or helpful at some point of my way.

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At this point it should be stated that the PhD program Molecular Medicine provided me perfect surroundings for fruitful and goal-oriented research as well as for pleasurable learning in an international environment.

I am thankful that my family and my friends were always on my side helping me through hard times and celebrating successful moments together. Their company always brightens the colors of my life.

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List of Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
AF488	Alexa Fluor488 carboxylic acid-2,3,5,6-tetrafluorophenyl ester
AGE	Advanced glycation end product
AOPP	Advanced oxidation protein product
apoA-I	Apolipoprotein A-I
BLT-1	Block lipid transport-1
BSA	Bovine serum albumin
CE	Cholesterol ester
CETP	Cholesterol ester transfer protein
CHO	Chinese hamster ovary
CD36	Cluster of differentiation 36
CKD	Chronic kidney disease
CML	N^{ε}(carboxymethyl)lysine
3-CT	3-chlorotyrosine
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
(e)NOS	(Endothelial) Nitric oxide synthase
ESRD	End stage renal disease
FBS	Fetal bovine serum
HCit	Homocitrulline
HD	Hemodialysis
HDL	High-density lipoprotein
HMA	Human mercaptalbumin
HNA1	Human nonmercaptalbumin 1
HNA2	Human nonmercaptalbumin 2
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HSA	Human serum albumin
KBr	Potassium bromide
KOCN	Potassium cyanate
LCAT	Lecithin cholesterol acyltransferase
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-density lipoprotein
LDLR	LDL receptor
LPDS	Lipoprotein-deficient serum
Lyso-PC	Lysophosphatidylcholine
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
O_2^-	Superoxide anion
OH•	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PL	Phospholipid
PTC	Proximal tubular cells
RCT	Reverse cholesterol transport
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SR-BI	Scavenger receptor class B type I

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I. Introduction

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Summary

Neutrophils are the first line of defense against invading microorganisms and thus accumulate in high numbers at sites of tissue injury. Neutrophils contain myeloperoxidase (MPO), which is released after activation by intraluminal degranulation. MPO catalyzes the production of reactive chlorinating species like the powerful oxidant hypochlorous acid (HOCl). HOCl modifies proteins by various ways, including conversion of cysteine residues to disulfides and higher oxidation products, conversion of methionine residues to methionine sulfoxides, oxidation of tryptophan and chlorination of amino groups and tyrosine.

Reaction of chlorinated oxidants with plasma albumin, leads to formation of advanced oxidation protein products (AOPPs). Recent findings revealed that AOPPs are inhibitors of the major high-density lipoprotein (HDL) receptor, scavenger receptor class B type I (SR-BI), blocking HDL association and reverse cholesterol transport (RCT).

Dramatically elevated plasma levels of AOPPs are observed in patients suffering from renal failure, a disease associated with persistent inflammation. Accordingly, albumin isolated from end stage renal disease (ESRD) patients on hemodialysis (HD), but not albumin isolated from healthy controls, markedly inhibits HDL association to SR-BI, indicating that an oxidized fraction of albumin present in uremic blood is recognized by the receptor.

In this thesis project, we demonstrate that the myeloperoxidase product HOCl generates SR-BI inhibitors that irreversibly block the receptor. Our data show that already mild oxidation of albumin generates high affinity ligands to SR-BI that effectively displace HDL from SR-BI and block SR-BI mediated selective lipid uptake. We observed that oxidation/modification of lysine residues is essential for binding of albumin to SR-BI since masking lysine residues prior to oxidation as well as regeneration of lysine oxidation products completely averted binding. In this regard, alterations in the specific charge distribution caused by neutralization of positively charged ε-amino groups of lysine residues through oxidation or carbamylation are most probably decisive for the transformation of albumin into an SR-BI ligand.

We found striking evidence that HOCl-albumin mediated blockade of SR-BI and impairment of RCT are a consequence of irreversible association of HOCl-albumin to the receptor which is promoted by *N*-chloramines within the oxidized protein.

Displacement of HDL from its major receptor results in decreased hepatic cholesterol uptake, depressed HDL metabolism and abnormal HDL composition and function.
We observed that the SR-BI inhibitory activity of albumin isolated from chronic kidney disease (CKD) patients correlated with the content of the myeloperoxidase-specific oxidation product 3-chlorotyrosine (3-CT) and was associated with alterations in the composition of HDL.

The present results raise the possibility that MPO-catalyzed oxidative protein damage is an important component in the pathophysiology of uremia. Impaired reverse cholesterol transfer disables HDL metabolism and directly entails compositional and functional alterations of HDL.

Zusammenfassung

Neutrophile Granulozyten sind Teil der angeborenen Immunabwehr; bei akuten Entzündungen, Sepsis, Nekrosen und Immunerkrankungen steigt der Bedarf an Neutrophilen und es kommt zu einer verstärkten Freisetzung aus dem Knochenmark und zur Akkumulation an den Entzündungsherden.

Neutrophile enthalten das Enzym Myeloperoxidase (MPO) das nach Aktivierung aus deren azurophilen Granula freigesetzt wird und in Gegenwart von Chlorid-Ionen die Umwandlung von Wasserstoff-Peroxid zu Hypochlorit (HOCl) katalysiert. Innerhalb des Phagolysosoms ist HOCl für die Zerstörung von Mikroorganismen und Krankheitserregern verantwortlich, wird es aber freigesetzt kommt es zur Schädigung von umliegenden Zellen und Geweben.

Hypochlorit reagiert mit einer Reihe von Aminosäuren. Oxidation von Cystein, Methionin und Tryptophan und Chlorierung von Aminogruppen und Tyrosin sind die am häufigsten auftretenden Modifikationen und führen zu strukturellen Veränderungen und funktioneller Beeinträchtigung der betroffenen Plasmaproteine.

Durch Oxidation von Plasmaproteinen mit chlorhältigen Oxidationsmitteln entstehen Advanced Oxidation Protein Products (AOPPs). Aus biochemischer Sicht handelt es sich bei AOPPs zum Großteil um Albuminaggregate mit hohem Molekulargewicht, die einen hohen Gehalt an Carbonylgruppen aufweisen und sich durch die Anwesenheit von Dityrosin spektroskopisch von nativem Albumin unterscheiden.

Eine vorausgehende Studie unserer Arbeitsgruppe hat gezeigt, dass AOPPs mit hoher Affinität an den HDL Rezeptor, Scavenger Receptor Class B Type I (SR-BI) binden und dadurch die Bindung des endogenen Liganden blockieren und den reversen Cholesterintransport hemmen.

AOPPs akkumulieren mit dem Fortschreiten von entzündlichen Erkrankungen im Plasma. Plasmaalbumin von Patienten mit Nierenerkrankungen inhibiert signifikant die Bindung von HDL an den SR-BI, was darauf hindeutet, dass der Rezeptor den oxidierten Anteil von Albumin im Plasma erkennt.

Die Expression von SR-BI auf der Leber ist Voraussetzung dafür, dass das von sphärischen HDL Partikeln aus der Peripherie zur Leber transportierte Cholesterin aufgenommen und metabolisiert werden kann. Daher wird angenommen, dass die athero-protektive Wirkung von SR-BI in direktem Zusammenhang mit seiner Rolle im reversen Cholesterintransport steht.

Natives Albumin bindet nicht an den HDL-Rezeptor. Unsere Ergebnisse zeigen, dass nach Oxidation von Albumin modifizierte Lysinreste die Bindung an den HDL-Rezeptor vermitteln. Nachdem oxidiertes Polylysin nicht an den SR-BI bindet, entsteht vermutlich durch eine Veränderung der Ladungsverteilung am modifizierten Albumin ein strukturelles Motiv das vom Rezeptor erkannt wird.
Im Fall von HOCl-oxidiertem Albumin sind die Blockade der HDL-Assoziation und der Cholesterinaufnahme über den SR-BI auf die irreversible Bindung des modifizierten Proteins an den Rezeptor zurückzuführen. Dabei hängt die besondere Bindungscharakteristik von oxidiertem Albumin mit der Anwesenheit von proteingebundenen *N*-Chloraminen zusammen; Abbauprodukte von Chloraminen vermögen nur mehr eine reversible Bindung an den SR-BI zu vermitteln.

Die Bindung von oxidierten Proteinen an den HDL Rezeptor kann mit dem physiologischen HDL Metabolismus interferieren und führt möglicherweise zu einer verlängerten Verweilzeit von HDL-Partikeln im Blutkreislauf, wo sie post-translational modifiziert oder vermehrt enzymatisch hydrolysiert werden können. Unseren Beobachtungen zufolge korreliert die den Rezeptor inhibierende Wirkung von Albumin aus dem Plasma von Dialysepatienten mit dessen Gehalt an 3-Chlortyrosin, einem spezifischen Marker für Myeloperoxidase katalysierte Oxidationsreaktionen und mit Veränderungen in der Zusammensetzung von HDL.

Unsere Ergebnisse weisen darauf hin, dass im Zusammenhang mit oxidativem Stress, eine irreversible Blockade des HDL-Rezeptors durch oxidiertes Plasmaalbumin den reversen Cholesterintransfer beeinträchtigen kann. Folglich kann Cholesterin nicht von HDL an die Leber abgegeben werden sondern zirkuliert an HDL gebunden im Plasma. Im Fall von Dialysepatienten steht die Affinität von Albumin zum HDL-Rezeptor in direktem Zusammenhang mit veränderter Zusammensetzung und funktioneller Einschränkung von HDL. Folglich kann man davon ausgehen, dass die irreversible Blockade des HDL-Rezeptors zu der extrem hohen kardiovaskulären Mortalität von Dialysepatienten beiträgt.

I. Introduction

1. Albumin – the major Plasma Antioxidant

Reactive oxygen species (ROS) develop as byproducts of normal cellular oxygen metabolism and act in low concentrations as physiological mediators of cellular responses (1). Their tremendously reactive nature is attributed to the presence of unpaired valence electrons and if excessively generated or unintentionally released they can inflict damage upon surrounding tissues (1-5). To minimize deterioration of vital cellular components, efficient antioxidant machinery is in place consisting of enzyme systems and small molecule scavengers (1-4).

Oxidative stress is defined as tissue damage occurring as a consequence of massive oxidant generation in the face of deficient antioxidant defense mechanisms resulting in an imbalance between oxidant production and inactivation. Oxidant production is not restricted to the intracellular compartment although it provides much greater protection. Regardless of its inferior antioxidant capacity, plasma is steadily exposed to considerable oxidant activities involving plasma proteins as the most important components of redox-control (2,5).

1.1 Structure and Functions

Albumin is the most abundant protein in plasma amounting to 60% of the total extracellular protein pool. Its primary structure consists of 585 amino acids forming a single polypeptide chain devoid of prosthetic groups, glycans or lipid (6,7). The sequence of albumin contains 35 cysteine residues of which 34 are involved in disulfide bonds stabilizing the characteristic heart-shaped, three-dimensional structure (2,7,8). The last cysteine residue of albumin in position 34 (Cys34) is in the reduced state accounting for the major part of its antioxidant potential (2,8).

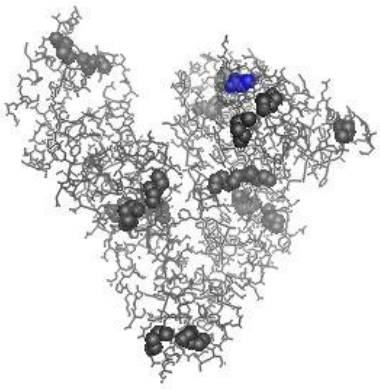

Figure 1: Three-dimensional Structure of Human Serum Albumin (HSA). *Cysteine residues are shown in spherical mode. Discrimination between disulfides and sulfhydryl was accomplished by representing Cys34 in blue color. Atomic coordinates were downloaded from the Protein Data Bank (PDB accession code 2BXF). The figure was prepared using PyMOL.*

Albumin is synthesized in the liver and secreted into the blood stream where under normal conditions its concentration ranges between 35 and 50 g/L representing equilibrium between hepatic synthesis and catabolic rate (5,7,8). In the course of its physiological life-span of more than 20 days, albumin passes approximately 15 000 times through the circulation exchanging between extravascular space and plasma compartment. Previous findings demonstrated that under inflammatory conditions activated neutrophils release chemicals that increase vascular permeability favoring albumin translocation to sites of inflammation where its antioxidant activities are in dire need (5,8).

The flexibility of its three domain design enables albumin to provide binding sites for different ligands and many antioxidant qualities of albumin are connected to its binding properties (5,7). Albumin acts as a transporter for metal ions, fatty acids, cholesterol, bile pigments, drugs and hormones and is an important regulator of osmotic pressure (5).

Free bivalent copper and iron ions readily react with hydrogen peroxide (H_2O_2) giving rise to formation of hydroxyl radicals (OH•). Protein sequestration decreases the likelihood of cationic transition metals to take part in the deleterious Fenton reaction and therefore prevents excessive ROS generation.

The ability of albumin to bind lipid-phase antioxidants like bilirubin and nitric oxide and polyunsaturated fatty acids at the same time protects polyunsaturated fatty acids from oxidation-induced damage (5,9).

Lipidation of albumin seems to entail structural alterations aiming to increase exposure of Cys34. This possibly provides increased antioxidant protection under disease conditions where elevated amounts of albumin-associated fatty acids were found coexisting with high

levels of oxidative stress such as diabetes mellitus. In accordance thereto, oxidized forms of albumin have been detected with increased prevalence under diabetic conditions (10). Due to interactions of NO with the reduced sulfhydryl group of Cys34, albumin acts as a carrier of NO, a function that is of great importance for the regulation of vascular tone (2). In addition albumin has a binding site for homocysteine, a sulfur containing metabolite from methionine break-down. Increased plasma concentrations of homocysteine constitute an established risk factor for atherosclerosis (5).

1.2 Redox-Regulation

Based on the large quantity of albumin in plasma, a single reduced thiol group per molecule can effectually account for 80% of total plasma antioxidant capacity, setting the stage for efficient scavenging of oxidants and radicals (5,6). In healthy subjects, more than three-quarter of albumin is present in the reduced state (human mercaptalbumin, HMA) with the free sulfhydryl group of Cys34 available for antioxidant combat. The remaining fraction is largely involved in formation of disulfide-bridges with low-molecular-weight thiols like cysteine, homocysteine or glutathione (human nonmercaptalbumin 1, HNA1) (5,6). In as little as 1% of albumin the thiol is irreversibly oxidized to higher oxidation states including sulfinic and sulfonic acid (human nonmercaptalbumin 2, HNA2) (8).

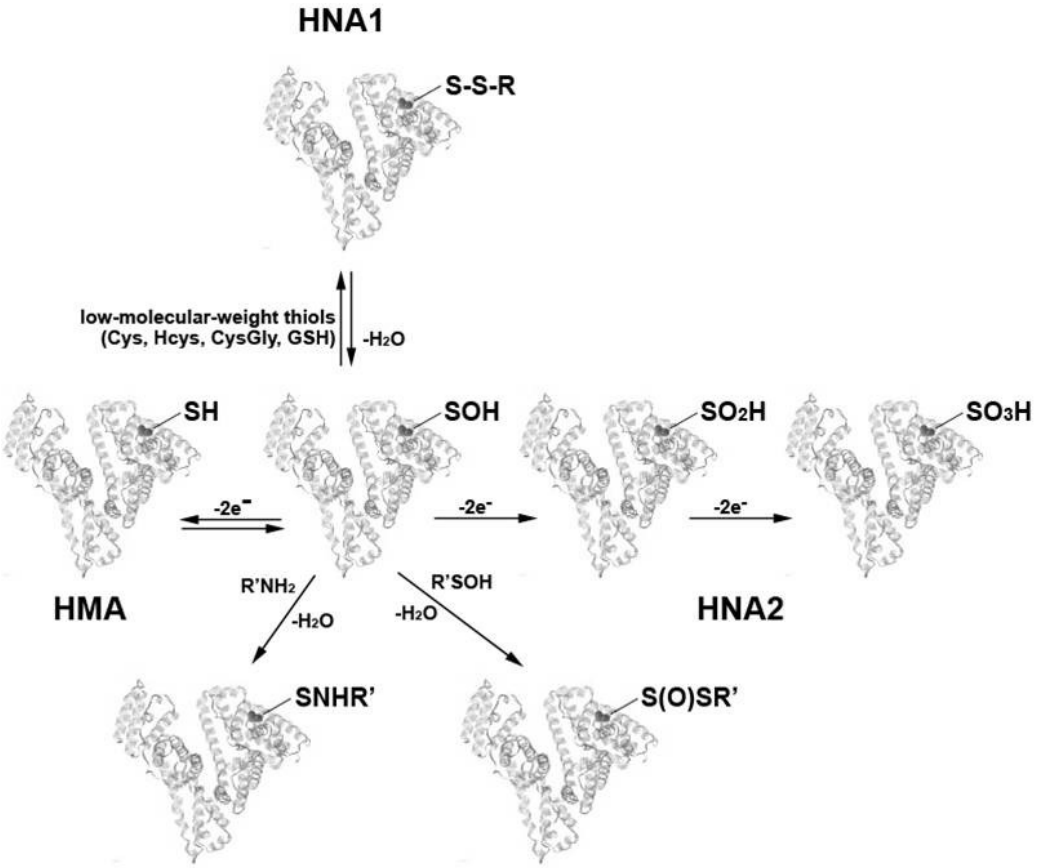

Figure 2: Formation of Sulfenic Acid and its Reactions. *Oxidation of the free thiol group of albumin Cys34 can yield sulfenic acid (HSA-SOH) which can be further converted into the irreversible higher oxidation products sulfinic (HSA-SO_2H) or sulfonic acid (HSA-SO_3H). S-thiolated (HSA-SSR) albumin results from reaction of sulfenic acid with low-molecular-weight thiols, primarily with cysteine, and can be reduced to the initial free thiol form of albumin (HSA-SH). Alternatively, sulfenic acid can react with another sulfenic acid yielding thiosulfinate (HSA-S(O)SR') or with an amine giving a sulfonamide (HSA-SNHR'). (HMA, human mercaptalbumin; HNA1, human nonmercaptalbumin 1; HNA2, human nonmercaptalbumin 2).*

Oxidative reactions of the thiol group (R-SH) with hydroxyl radicals or peroxynitrite ($ONOO^-$) yield in the first step sulfenic acid (R-SOH) representing a key intermediate that can be either reduced back to the thiol (via a mixed disulfide) or transformed into higher oxidation products depending on the reaction conditions (5,6,9,11,12). Thus Cys34 of HSA can perform repeated redox-cycles and thereby regulate the redox-state in the extracellular compartment (5,11). In contrast thereto, stable oxidation to sulfinic ($R\text{-}SO_2H$) or sulfonic acid ($R\text{-}SO_3H$), resulting from exposure to further oxidizing equivalents entails loss of function and break-down of the modified protein (5).

In the course of ageing healthy people exhibit a decrease in HMA in favour of S-thiolated albumin indicating that the antioxidant potency of albumin declines over the years making protein sulfhydryl groups increasingly susceptible to irreversible oxidation (10).
Oxygen free radicals are supposed to be involved in exercise-induced tissue damage and physical exercise modulates the state of plasma protein sulfhydryl. In this regard it was reported that intensive physical exercise significantly increases the nonmercaptalbumin fraction of albumin in extracellular fluids (6,8,13).
Increased concentrations of HNA1/HNA2 paralleled by a decrease in HMA were found related to diverse disease conditions including liver cirrhosis, acute-on-chronic liver failure, Alzheimer's disease and diabetes mellitus. In nephrotic syndrome and focal segmental glomerular sclerosis increased levels of albumin-sulfonic acid were detected, differing from HMA by an increased net negative charge and a different electrophoretic motility. In chronic kidney disease patients the fraction of oxidized forms of albumin correlates with the degree of renal dysfunction suggesting that the redox-state of albumin is an indicator of the current oxidative burden encountered (10).

Besides cysteine, methionine can be seen as a second redox regulator of albumin greatly enhancing its antioxidant potential (5). It was reported that oxidation of exposed methionine residues did not affect biological functions of enzymes like glutamine synthase, proposing that repeated oxidation and reduction of methionine in biological systems represents a ROS scavenging mechanism that contributes to the protection of other proteins from detrimental modifications (5).

1.3 Oxidation-induced Damage

Sustained high oxidant concentrations lead to depletion of reduced thiol of albumin promoting oxidative and advanced glycation end product (AGE) mediated protein modifications. Under these conditions, albumin turns from the major plasma antioxidant to the prime protein target of oxidative stress (5,14). This is evidenced by experimental findings showing that the major part of carbonyls detected in plasma of CKD-patients is located on albumin with minor oxidative modification of fibrinogen, transferrin and immunoglobulin (14).

Under diabetic conditions functional impairment of albumin is largely brought about by increased glycation (5). The hyperglycaemic state of diabetes leads to non-enzymatic attach of glucose molecules to free primary amino groups of albumin yielding after Amadori rearrangements AGE-modified albumin.

Carbonylated albumin and AGE-albumin are functionally distinct from native albumin featuring impaired ligand binding properties and compromised antioxidant function (5,15).

In 30-40% of the cases, diabetes is accompanied by declining renal function including the requirement for dialysis-treatment. Plasma albumin from HD-patients carries considerable amounts of 3-chlorotyrosine, a known biomarker of myeloperoxidase-catalyzed oxidative reactions (14,16). Moreover, dityrosine and carbonyl groups indicating HOCl-induced damage were found with increased prevalence in HD-albumin (5,17). Decreased availability of albumin thiol groups for antioxidant defence enhances the risk for oxidative damage of other proteins and tissues and promotes the development of cardiovascular complications. Accordingly hypoalbuminemia is considered as a cardiovascular risk factor in the dialysis population (14).

Altogether pathological conditions involve changes in redox-state, structure and function of albumin with adverse effects on its appreciated antioxidant properties. Oxidized albumin is a suitable biomarker of oxidative stress and potentially involved in harmful reactions (5).

2. AOPPs – Oxidative Stress Markers and Inflammatory Mediators in Chronic Kidney Disease

Traditional cardiovascular risk factors as those defined in the Framingham heart study have been proven inadequate to explain the increased cardiovascular risk observed in CKD-patients (18). Consequently estimation of the cardiovascular risk accompanying CKD needs to make account of a number of non-traditional uremia-related factors including sub-clinical inflammation and oxidative stress (19,20) involving increased concentrations of modified biomolecules as oxidative stress markers (21-24).

Oxidants can be generated by a variety of mechanisms at multiple cellular sites (25). In the setting of CKD the most important source of oxidants is afforded by circulating polymorphonuclear neutrophils and monocytes when they are activated by uremic toxins or bioincompatible dialysis membranes (25).

2.1 Phagocytic Oxidant Generation

Prompted by appropriate stimulation, polymorphonuclear neutrophils and monocytes increase oxygen consumption to produce large amounts of oxidants for antimicrobial defence (3,4,26,27). This protective mechanism referred to as oxidative burst of phagocytes knows four enzymes, namely nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD), nitric oxide synthase (NOS) and myeloperoxidase acting in concert to generate ROS and chlorinated oxidants to defend the host from invading pathogens (3,4,26).

At the molecular level ROS generation by phagocytes consists of an electron transport chain starting at the four subunit NADPH oxidase complex (25,27) which is inactive in resting phagocytes. However, activation and subsequent assembly of its distinct subunits which are located partly in the cytosol (p47PHOX, p67PHOX and p40PHOX) and partly in the cell membrane (cytochrome b558) render it capable of performing the univalent reduction of molecular oxygen to superoxide anion (O_2^-) (3,4,28). The unstable superoxide anion is converted, either spontaneously or by SOD, into hydrogen peroxide (3,4,27). Both reactive intermediates, O_2^- and H_2O_2, serve as precursors for synthesis of more potent oxidants with respect to host defence (3,4,27,29). Interaction of O_2^- with nitric oxide yields peroxynitrite (nitrosative stress) increasing platelet aggregation and vasoconstriction (3,4,27,30). Reaction of H_2O_2 with intracellular iron results via the Haber Weiss cycle in formation of hydroxyl radicals responsible for degradation of membrane lipids, for protein aggregation, and DNA damage and/or cleavage (3,4,31-33).

Owing to their myeloperoxidase system, phagocytic cells retain the unique capacity to generate chlorinating oxidants (3). MPO is a heme oxygenase enzyme contained in neutrophiles and monocytes amounting to 5% of total neutrophil and 1% of total monocyte protein (26). In the presence of chloride ions (Cl^-), MPO converts H_2O_2 into hypochlorous acid (3,4,34). In the same manner it can produce other hypohalous acids but Cl^- remains the first substrate due to its superior availability under physiologiacal conditions compared to bromide (Br^-) and iodide (I^-) (3). HOCl is essential for host defence participating in destruction of harmful microorganisms within the phagolysosome (26). However if excess HOCl is released it can contribute to tissue injury by oxidative modification of lipids, proteoglycans, proteins, DNA and other functionally important intracellular components (3,4).

Additionally, HOCl and MPO are involved in the conversion of nitrite (NO_2^-) into nitryl chloride (NO_2Cl), a strong promoter of tyrosine chlorination adversely affecting vital cellular functions due to alterations of protein structures (35).

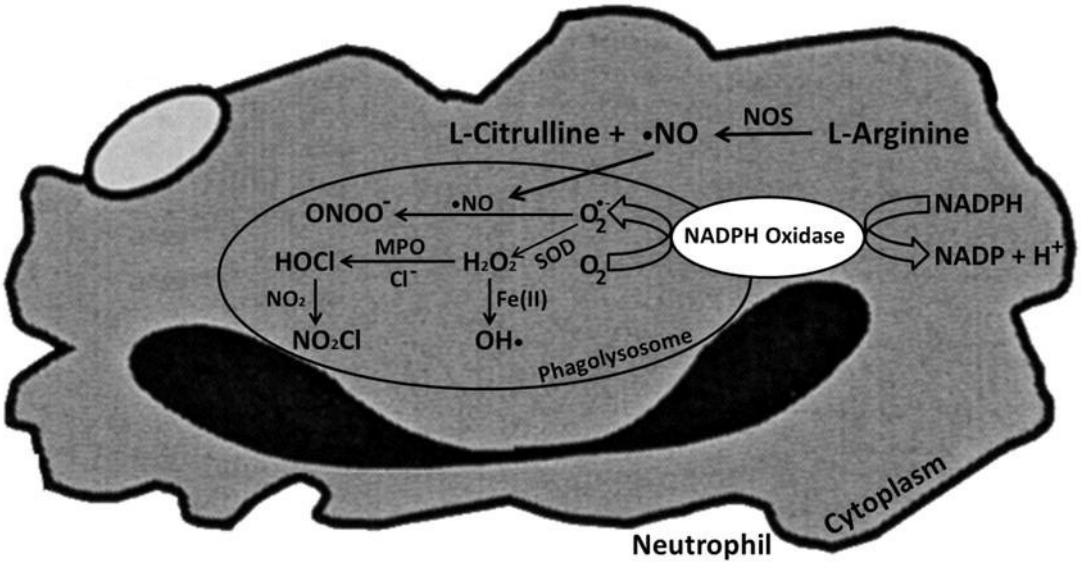

Figure 3: Oxidant Generation during Phagocyte Oxidative Burst. *NOS, nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phoasphate; $O_2^{\bullet-}$, superoxide anion; SOD, superoxide dismutase; NO•, nitric oxide; $ONOO^-$, peroxynitrite; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; NO_2Cl, nitryl chloride; MPO, myeloperoxidase; OH•, hydroxyl radical.*

MPO was detected in human atheromateous tissue supporting the idea that generation of chlorinated oxidants through MPO-catalyzed pathways of activated phagocytes might be involved in the CKD-associated accelerated atherosclerosis (36,37). This is in line with a recent report documenting a decreased prevalence of CVD in a cohort of hemodialysis patients carrying a single nucleotide polymorphism in the promoter region of the MPO

gene (37,38). Accordingly histological analyses of atherosclerotic tissue revealed co-localization of active MPO with macrophages (36,39). Moreover the MPO-specific tyrosine oxidation markers, 3-chlorotyrosine and dityrosine were found in oxidatively modified low-density lipoprotein (LDL) as well as in human atherosclerotic lesions (39-41).

2.2 Proteins as Oxidative Stress Markers

Until the findings of Witko-Sarsat et al. more than ten years ago (22,42,43) evaluation of oxidative stress in CKD-patients largely depended on the measurement of lipid peroxidation products such as malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS). The importance of modified proteins as oxidative stress markers was for a long time neglected even though their vulnerability to ROS was a well-documented observation (24).

Biochemical modifications of proteins caused by oxidative attack may induce structural and functional alterations leading to progressive loss of their metabolic, enzymatic or immunologic properties. Thereby the degree of modification can range from oxidation of a single amino acid residue to complete denaturation/fragmentation and determines the fate of the protein involved. While mild oxidation increases hydrophobicity and facilitates protein degradation by the multicatalytic proteasome complex, severe oxidative damage yields insoluble proteins, resistant to proteolysis (25,28,44,45). Consequently proteolysis of oxidatively modified proteins can be considered as contribution to antioxidant defence eliminating potential precursors for formation of more oxidized cross-linking or aggregation products (3).

2.3 The Chemical Nature of AOPPs

Reaction of plasma proteins, predominantly of albumin, with chlorinated oxidants gives rise to formation of an oxidized protein fraction designated advanced oxidation protein products by Witko-Sarsat et al. who identified AOPPs as a marker for protein oxidation in CKD (42). Besides the basic amino acids lysine and arginine, tyrosine was found to be particularly vulnerable to oxidative attack resulting in formation of dityrosine and dityrosine mediated protein aggregates (3,21).

Biochemically total plasma AOPP consists of two distinct albumin subclasses differing in molecular weight. The high-molecular-weight fraction with an approximate size of 600 kDa most probably corresponds to albumin aggregates originating from formation of disulfide-bridges or dityrosine cross-links. The low-molecular-weight fraction has a size of around 80 kDa and is likely to represent albumin in its monomeric form (3).

Spectral differences between plasma of CKD-patients and control subjects led to the identification of dityrosine as the major chromophore of AOPPs with absorption at 315 nm

and an emission band at 410 nm. In view of that, AOPPs were defined as final dityrosine-containing protein cross-linking products in the plasma of CKD-patients that can reflect dialysis-associated oxidative stress (24).

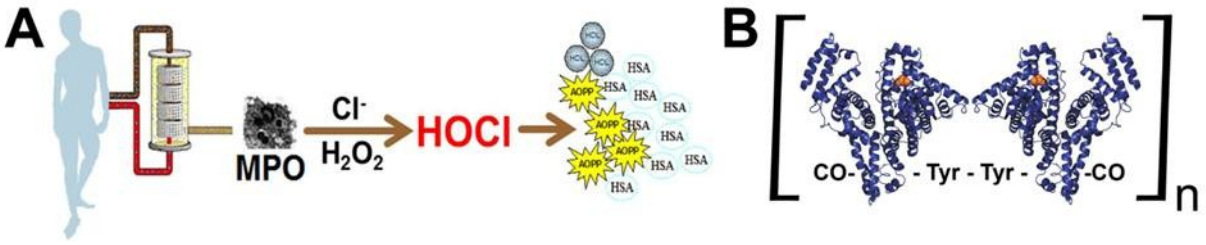

Figure 4: AOPP Formation and Structure. (A) *Neutrophil activation during dialysis sessions leads to HOCl production and AOPP formation.* ***(B)*** *Schematic representation of Advanced Oxidation Protein Products. AOPPs contain highly oxidized proteins, characterized by carbonylation (CO), dityrosine cross-linking (-Tyr-Tyr-) and high molecular weight.*

2.4 The Dual Role of AOPPs in CKD

Plasma AOPP levels are increased throughout all stages of CKD. The fact that AOPP levels are already elevated in CKD-patients not yet on renal replacement therapies indicates that uremia per se constitutes a state of increased oxidative stress (25).

AOPPs successively accumulate with declining renal function and exhibit a strong negative correlation with renal clearance making them an excellent marker for CKD progression (1,3,21,25). It was demonstrated that in CKD-patients plasma AOPPs were closely related to levels of neopterin and tumor necrosis factor (TNF)-α providing evidence that CKD is a state of sustained neutrophil activation (1,3,23). Correlation of AOPPs with the oxidative activity of circulating neutrophils underlines the importance of phagocytic oxidants for AOPP formation (22).

In addition to their suitability as oxidative stress markers, it was experimentally demonstrated that AOPPs are biologically active. *In vitro* generated AOPP-albumin (by treating albumin with HOCl) as well as AOPP-albumin isolated from CKD-patients triggered an oxidative burst in cultured monocytes proportional to the extent of albumin modification (3,23,24). Through their ability to activate monocytes, AOPPs potentially play an important role in the uremia-associated dysregulation of the immune system and in dialysis-related inflammatory complications including accelerated atherosclerosis (25).

However, one should keep in mind that *in vivo* generated AOPPs are probably not identical to the modified proteins resulting from treatment of normal plasma with HOCl since endogenous generation pathways are by far more complex and cannot be completely mimicked *in vitro* (24).

2.5 Identified Receptors of post-translationally modified Albumin

The involvement of AOPPs in the clinical picture of diverse disorders in which high levels of oxidative stress favor protein oxidation gives reasons for their wide acceptance as oxidative stress markers. At the same time it raises interest in identification of the mechanisms that underlie the modulation of cellular functions by AOPPs (46).

Several lines of evidence indicate that AOPPs are functional ligands of the receptor for advanced glycation end products (RAGE) on endothelial cells (46). RAGE belongs to the superfamily of cell surface immunoglobulin receptors and interacts with a diversity of ligands whose common characteristic of a net negative charge seems to be related to the kationic nature of the receptors extracellular domain (46,47). Accordingly modification of albumin with physiologically relevant HOCl concentrations, increasing the net negative charge, induced binding of albumin to RAGE (47). Binding of HOCl-albumin to RAGE activates independent of AGE-structures downstream signaling via the MAP-kinase pathway leading to expression of the chemotactic cytokine monocyte chemoattractant protein-1 (MCP-1) which participates in the recruitment of leukocytes to sites of inflammation (47).
Moreover, studies reported that binding of AOPP-albumin to RAGE forms the molecular basis for the impairment of proliferation and osteogenic differentiation of mesenchymal stem cells implicating a role of AOPPs in the development of osteoporosis (48).

It is supposed that plasma AOPPs can access proximal tubular cells (PTC) across the glomerulus thereby causing PTC injury (49). In this context it was demonstrated that the scavenger receptor CD36 mediates endocytic uptake of AOPPs followed by lysosomal degradation in PTC and human kidney cells and that AOPP-induced PTC damage involves CD36 signaling (49).
In addition it was recently described that AOPP-albumin activates the intrarenal renin-angiotensin system via a CD36 mediated redox-dependent pathway (50).

The most important finding in view of the present investigations is that AOPP-albumin was found to be a high affinity ligand of the HDL receptor SR-BI. Binding of AOPP-albumin to SR-BI involves displacement of HDL and inhibition of selective HDL-lipid uptake both of which adversely affect HDL metabolism and vascular integrity (51).

3. Scavenger Receptor Class B Type I

The recently identified AOPP receptor SR-BI is a 509 amino acid cell surface protein that belongs to the CD36 superfamily of proteins. As the majority of the CD36 superfamily members, SR-BI has a horseshoe-like structure featuring a large extracellular loop confined by two hydrophobic trans-membrane regions with short cytoplasmic extensions anchoring the whole structure to the plasma membrane at the C- and the N-terminal site (52-54). The C-terminal half of the extracellular region is rich in cysteine residues, including two fatty acylated cysteines, one within in the C-terminal membrane-spanning domain and the second one as part of the C-terminal cytoplasmic tail (52-54). SR-BI is *N*-glycosylated at all possible glycosylation sites resulting in a molecular weight of 82 kDa (52-54). Within the plasma membrane SR-BI is supposed to be located in small cholesterol- and sphingolipid-enriched vesicles termed caveolae being the putative site of the first steps of SR-BI mediated selective cholesterol uptake (53-57). However, conflicting data exist, showing that caveolar localization of SR-BI is not required to mediate efficient selective cholesterol ester uptake and that SR-BI is expressed in microvillar channels *in vivo* (53).

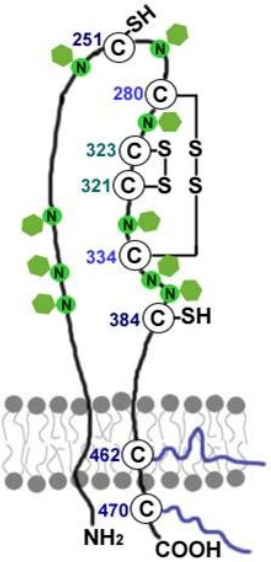

Figure 5: Expected Structure of SR-BI as determined by Yu et al (58). *The extracellular domain contains six cysteine residues and nine putative N-glycosylation sites (indicated by green hexagons). The cysteine residues located in the trans-membrane region and in the C-terminal tail are fatty acylated bearing palmitoyl or myristoyl residues (shown in blue).*
According to mass spectroscopic studies of Yu et al. four of the extracellular cysteine residues are involved in the formation of two intramolecular disulfide-bridges and the residual two are present in the reduced state. Actually, the definite organization of the cysteine residues within SR-BI could not be resolved to date.

3.1 Expression Pattern

SR-BI expression is widespread among mammalian tissues and cell types including the liver, the intestine, the lung, the nervous system, the adrenal gland as well as endothelial cells, smooth muscle cells, macrophages, platelets, adipocytes and keratinocytes (53,59). The highest expression levels are found in the liver and in steroidogenic tissues (adrenal gland, ovary and testis), known to depend on HDL free cholesterol for synthesis of bile acids and steroid hormones (53,59). Importantly, high expression levels in the liver are consistent with the major role of hepatic SR-BI in selective HDL-cholesterol uptake (53). *In vivo* SR-BI expression levels are subject to regulation by trophic hormones, cholesterol and unsaturated fatty acids (53).

3.2 Ligands

The ability of SR-BI to mediate binding of acetylated LDL in transfected cells initially led to its identification as a scavenger receptor (53,57). In fact SR-BI is a multiligand receptor that possesses binding affinity for a variety of apparently unrelated ligands such as native and chemically modified lipoproteins (VLDL, LDL, HDL and AcLDL, OxLDL, OxHDL, AGE-LDL, AGE-HDL respectively), maleylated and AGE-modified bovine serum albumin (BSA; but not its unmodified counterpart), anionic phospholipids, lipopolysaccharide (bacterial cell surface component) and phosphatidylserine potentially contributing to recognition and clearance of senescent, damaged and apoptotic cells (53,57,60).

Most of the SR-BI ligands including native lipoproteins are also recognized by CD36 but the ability to mediate efficient selective uptake of lipids from HDL is a unique characteristic of SR-BI that seems to be associated with its extracellular domain (57,61). Reports about a conserved SR-BI binding motif are still lacking but evidence exists that SR-BI recognizes the consensus conformation of class A amphiphatic helices, like those present in apolipoprotein A-I (apoA-I) explaining the diverse nature of its ligands (57,59,62).

According to a recent study published by Yu et al. the six exoplasmic cysteine residues of SR-BI are organized as two disulphide bonds (Cys^{321}-Cys^{323} and Cys^{280}-Cys^{334}) and two sulfhydryl groups (Cys^{251} and Cys^{384}). Mass spectroscopic analyses revealed that above all structural integrity at positions 323 and 384 appears to be crucial for HDL binding and selective HDL-lipid uptake (58).

The particularity to mediate selective cholesterol ester uptake from HDL, the broad tissue distribution with highest expression levels in liver and steroidogenic tissues which are the principal sites of selective lipid uptake *in vivo*, together with the regulation of SR-BI expression in these tissues clearly demonstrates that SR-BI is a physiologically important HDL receptor (53,57). Moreover the variety of its ligands suggests that SR-BI might be involved in multiple physiological processes aside from lipoprotein cholesterol uptake (57).

3.3 Reverse Cholesterol Transport

There is a consensus that SR-BI expression and function critically contribute to atheroprotection. This quality is essentially linked to the key position of SR-BI and HDL in reverse cholesterol transport describing the process of cholesterol transfer from extrahepatic tissues to the liver for metabolism and final excretion into the bile (52). Thereby HDL takes up excess unesterified cholesterol from peripheral cells (including those in the arterial wall) and macrophages which is subsequently converted into cholesterol esters (CEs) by the HDL-associated lecitihin cholesterol acyltransferase (LCAT) (54,63-65) and transported to the liver for catabolism to bile acids or direct biliary excretion (61).

The first step in RCT is represented by the stepwise lipidation of apoA-I which is mediated by the ATP-binding cassette transporter A1 (ABCA1). Thus the lipid acceptor activity of HDL enables net removal of cholesterol from peripheral cells generating large, lipid-rich spherical HDL particles representing the preferred SR-BI ligand (60). Among the spherical α-HDL particles that bind to SR-BI binding affinity was observed to increase with increasing size and lipid-content (53,60).

Hepatic lipase, lipoprotein lipase and cholesterol ester transfer protein (CETP) decide on efficiency of selective lipid uptake through HDL remodelling generating HDL particles of different composition with different binding properties (53,57,61).

The main route of RCT is built up by the direct transfer of CEs from HDL to the liver where they are internalized through SR-BI mediated selective lipid uptake. Unlike the LDL receptor (LDLR) using receptor mediated endocytosis for LDL-lipid uptake, it is widely assumed that SR-BI takes up cholesterol from HDL without internalization and subsequent degradation of the whole HDL particle (57,66). Following this theory, SR-BI mediated selective lipid uptake is considered to be a two-step mechanism composed of formation of a functional lipoprotein/receptor complex followed by effective lipid transfer across the plasma membrane into intracellular compartments (54,62,67,68). Lipid-depleted HDL subsequently dissociates from the cell surface and re-enters the circulation ready to accept more cholesterol esters completing repeated cycles of RCT (54). Alternatively after delivering its lipid cargo, HDL can be filtered by the kidney, taken up by cubilin mediated endocytosis and finally degraded (61). However, about the definite progression of selective lipid uptake controversial opinions remain. The alternative endocytosis-dependent model gains support from recent reports showing that in physiologically relevant cell lines selective lipid uptake involves internalization of the SR-BI/HDL complex with subsequent re-secretion of lipid-depleted HDL (HDL-retroendocytosis) (53,59,69).

Indirect transport to the liver comprises transfer of the cholesterol esters from HDL to apo-B containing lipoproteins (LDL, VLDL) for further metabolism via LDLR involving receptor mediated endocytosis of the whole lipoprotein particle via clathrin-coated pits followed by its hydrolysis within the lysosomes (57). This metabolic route requires the presence of CETP and is absent in mice lacking the genetic preconditions for CETP expression (54,61,62).

In accordance with the selective uptake of HDL cholesterol esters, SR-BI can mediate the uptake of unesterified (free) cholesterol, phospholipids, triglycerides, α-tocopherol and DiI from HDL (53).

Importantly SR-BI takes part in both ends of RCT mediating hepatic selective uptake of HDL-lipids as well as efflux of free cholesterol from peripheral cells and macrophages to HDL. Thus RCT may represent one mechanism by which SR-BI essentially contributes to the prevention of atherosclerotic plaque formation (59,60).

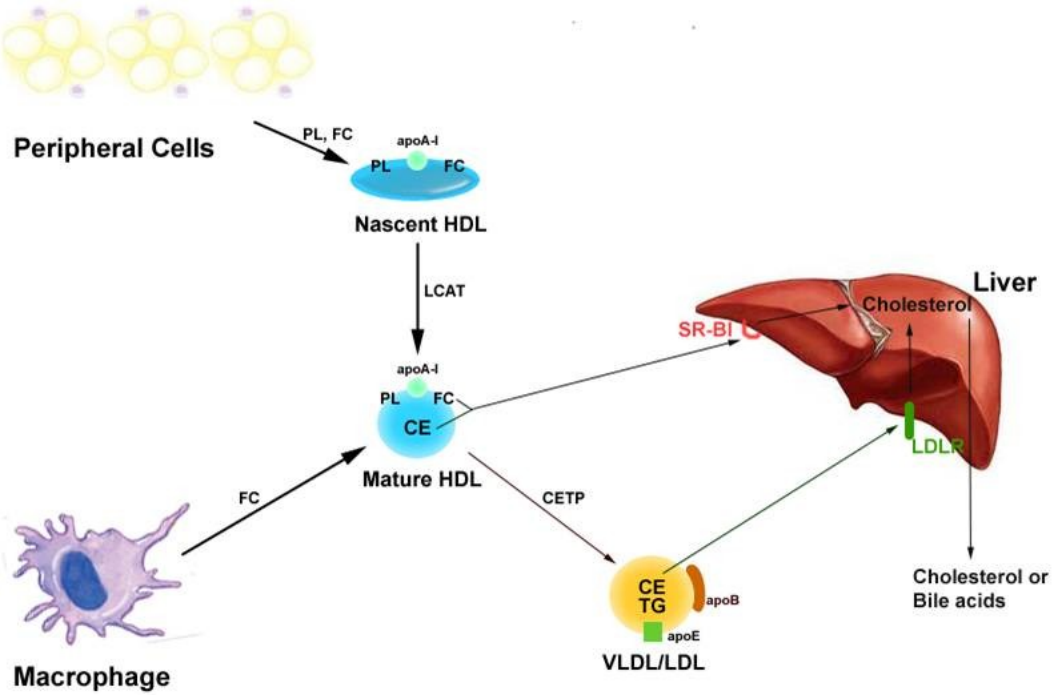

Figure 6: Schematic Representation of the Role of SR-BI in RCT. *Nascent HDL acquires cholesterol from peripheral tissues which is esterified by LCAT generating mature HDL. Efflux of free cholesterol from macrophages to mature HDL is mediated by macrophage SR-BI. CE and free cholesterol can be directly transferred from HDL to the liver and selectively taken up by hepatic SR-BI. Alternatively, CETP can mediate transfer of CE from HDL to apoB-containing lipoproteins for further metabolism via the LDLR. In the hepatocyte CE are hydrolyzed to free cholesterol which is either directly excreted into the bile or after conversion into bile acids.*

PL, phospholipids; FC, free cholesterol; LCAT, lecithin cholesterol acyltransferase; CETP, cholesterol ester transfer protein; TG, triglycerides; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; LDLR, LDL receptor.

Direct evidence, for the importance of SR-BI as a physiological HDL receptor results from manipulations of hepatic SR-BI expression in mouse models of cardiovascular disease (59). Thereby overexpression of SR-BI decreased plasma HDL-cholesterol whereas SR-BI knockout resulted in increased total plasma cholesterol levels due to increased HDL-cholesterol and in formation of heterogeneous, enlarged and apoE-enriched HDL particles compared to wild type animals (53,57,59). These *in vivo* studies provided striking evidence that SR-BI is important for regulation of plasma HDL-cholesterol levels and modulation of HDL structure. Increased HDL-cholesterol levels and HDL particle size seem to be a consequence of compromised selective HDL-cholesterol uptake by the liver as indicated by reduced biliary cholesterol secretion in SR-BI knockout animals (53,57,60,61).

The fact that peripheral cells lack the ability to degrade cholesterol independently emphasizes the relevance of RCT for cholesterol homeostasis and indicates that the functions of hepatic SR-BI in RCT are responsible for a large part of its antiatherogenic activity (53).

Similarities between human and murine SR-BI concerning selective HDL cholesterol ester uptake activity and expression pattern are suggestive of a central role of SR-BI in HDL metabolism in humans (53). Accordingly, plasma HDL-cholesterol levels in humans negatively correlate with the risk of atherosclerotic cardiovascular disease. Therefore directed control of hepatic SR-BI expression could potentially provide the basis for development of new strategies for prevention and treatment of atherosclerotic cardiovascular disease (61).

A very recent study demonstrated expression and physiological importance of SR-BI on lymphatic endothelial cells. Lymphatic vessels potentially participate in the initial step of RCT by mediating the transport of cholesterol from peripheral cells and macrophages to the blood stream through an active process that depends at least in part on SR-BI (70,71). Impaired lymphatic drainage was shown to be associated with lipid accumulation in peripheral tissues pointing to an important role for SR-BI on lymphatic endothelial cells in lipid related disorders including atherosclerosis, diabetes and familial hypercholesterolemia.

3.4 Non RCT Functions of SR-BI relevant to Atheroprotection

The atheroprotective action of SR-BI is not exclusively attributed to its role in RCT (53). In addition SR-BI mediates HDL-dependent activation of signalling pathways in vascular cells that are believed to be of importance for atheroprotection (60).

Nitric oxide is fundamental for endothelial integrity. Endothelium derived NO is largely responsible for acetylcholine mediated vasodilatation and offers a number of beneficial effects with respect to the vasculature (60). Deterioration of endothelial function is considered as one of the first steps in the development of atherosclerosis and decreased NO bioavailability fundamentally participates in the development of endothelial dysfunction (37).

NO synthesis by endothelial nitric oxide synthase (eNOS) is activated by HDL. Studies showing that this HDL-dependent effect was abolished by SR-BI blocking antibodies indicate that HDL-induced eNOS activation relies on HDL binding to SR-BI. Thus the presence of SR-BI affords the essential molecular basis for HDL to exert its protective effect on the endothelial cell layer (53,59,60).

Oxidized lipoproteins and inflammatory mediators like TNF-α and homocysteine can trigger endothelial cell apoptosis. HDL interaction with SR-BI is essential for activation of anti-apoptotic pathways in endothelial cells thereby counteracting destruction of the endothelial cell layer (60).

It is presumed that both damage to anchored endothelial cells and missing repair of this damage by bone marrow-derived endothelial precursor cells play a role in the development of atherosclerosis (72,73). Accordingly, reduced numbers of endothelial precursor cells found in CKD-patients potentially accelerate the development of atherosclerosis in these patients (72,74). In this regard the HDL/SR-BI tandem supports endothelial repair by stimulating the recruitment of endothelial progenitor cells into the arterial intima (60,75).

The ability to mediate selective lipid uptake further permits SR-BI to regulate intracellular α-tocopherol levels providing for protection of the cells against oxidative stress and oxidation-induced damage (53).

It was found that mice with liver-specific SR-BI deletion but normal expression in macrophages developed lower levels of diet-induced atherosclerosis with reduced number of macrophages in atherosclerotic lesions compared to overall knockout animals. Therefrom it can be assumed that macrophage SR-BI accounts at least partially for the differences in lesion size and composition between liver-specific and whole body knockout of SR-BI pointing to a significant role of extrahepatic SR-BI for atheroprotection (52,60).

Ex vivo work revealed that platelet SR-BI binds native HDL and negatively charged phospholipids with high affinity suppressing thrombin-induced platelet aggregation,

fibrinogen binding, P-selectin expression and the mobilization of intracellular Ca^{2+} stores which is a characteristic of platelet activation. Hence binding of HDL or phospholipids to SR-BI on platelets entails antithrombotic functions. By contrast, platelet SR-BI was shown to favour thrombosis in dyslipidemic mice *in vivo* (75).

Apparently, SR-BI owns various activities beyond cholesterol metabolism that are important to safeguard endothelial integrity and counteract the onset of atherosclerotic vascular disease under oxidative stress conditions (59). Association of HDL to SR-BI mediates activation of several atheroprotective pathways such as the initiation of NO production by eNOS, the maintenance of endothelial function and the promotion of endothelial precursor cell migration, the supply of α-tocopherol to the cells and the mediation of cholesterol efflux from macrophages (60).

4. Inhibitors of SR-BI activity

4.1 Chemical Inhibitors

Aiming at resolving the mechanism of SR-BI mediated selective lipid uptake, Nieland et al. identified five small chemical compounds that potently blocked lipid transport (BLTs) in intact cells in the low nanomolar to micromolar range (68). All of the small molecule inhibitors designated BLT-1 to BLT-5 left SR-BI surface expression unaltered, increased HDL binding to SR-BI due to reduced dissociation rates and interfered with selective lipid uptake and cholesterol efflux (68). Among the tested compounds BLT-1 showed the most striking inhibition with an IC_{50} of approximately 50 nM (68,76).

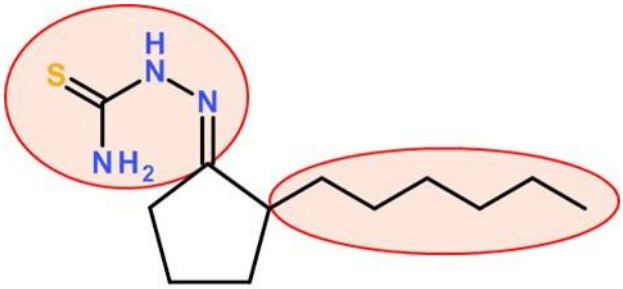

Figure 7: Structure of the Small Molecule Thiosemicarbazone Inhibitor BLT-1. *The semicarbazone moiety and the hydrophobic tail are highlighted in red.*

Blocking SR-BI resulted in a coordinated increase in binding affinity and decrease in lipid transport leading to the conclusion that the two steps of selective lipid uptake are mechanistically interrelated. This concept of a mechanistic coupling of HDL binding to SR-BI and SR-BI mediated cholesterol ester uptake is in good agreement with the established idea that productive binding forms the basis for efficient lipid transfer. Thereby structure and composition of HDL determine the efficiency of cholesterol ester transport between HDL and cells relative to lipoprotein binding (68). Moreover, the finding that CD36 sharing most structural features of SR-BI and apoA-I-deficient reconstituted HDL particles bind HDL with high affinity but do not mediate selective lipid uptake demonstrates that distinct structural features are essential for productive binding and lipid transport (53).

The observation that a C384S mutation in SR-BI resulted in a 60% reduction of intrinsic lipid uptake activity, the irreversible nature of SR-BI blockade by BLT-1 and the knowledge that free thiol groups can interact with thiosemicarbazones collectively suggest that inhibition of SR-BI by BLT-1 is based on interactions between Cys384 of SR-BI and the sulfur of BLT-1 (58). This is highlighted by the finding that conversion of Cys384 to a

serine resulted in complete insensitivity to BLT-1 while the same mutation at Cys251 showed no effect (76).

Altogether the results from cell experiments with BLT-1 support the theory of productive binding describing a mechanistic link between HDL association to SR-BI and SR-BI mediated lipid transport. Moreover, it was found that the intrinsic activity of SR-BI depends on the reduced state of Cys384 and that the presence of this residue is required for BLT-1 to mediate its inhibitory activity (68).

4.2 Endogenous SR-BI Inhibitors

4.2.1 Carbamylated Proteins

Carbamylation describes the post-translational modification of proteins induced by urea-derived cyanate that is under normal conditions present in low concentrations in plasma but significantly increased in CKD. The decomposition of urea to cyanate (OCN^-) and ammonia (NH_3) is inversely associated with renal function resulting in the presence of excess cyanate in plasma potentially acting as a uremic toxin (77). Specifically the active form of cyanate, isocyanic acid (HOCN), is responsible for the irreversible conversion of ε-NH_2 groups of protein lysine residues into ε-carbamyllysine (homocitrulline, HCit) entailing conformational changes followed by loss of protein function as the final consequence (77,78).

Alternatively carbamylated proteins can be generated via an MPO-dependent pathway, involving thiocyanate (SCN^-) and H_2O_2 as co-substrates, which is believed to be of superior relevance in the absence of renal impairment (77). Demonstration of significantly increased carbamyllysine levels in HDL isolated from human atherosclerotic tissue of subjects with normal renal function underlines the contribution of a uremia-independent pathway to protein carbamylation (79).

Evidence suggests that lipoproteins are frequently carbamylated at sites of atherosclerotic lesions suggesting that carbamylation may represent a novel mechanistic pathway for atherogenesis via the formation of dysfunctional LDL and HDL (77). This was substantiated by experimental findings showing that carbamylation of HDL resulted in increased binding affinity and capacity to SR-BI in a macrophage cell line where expression of SR-BI was induced. Furthermore carbamylated HDL caused cholesterol accumulation in cultured macrophages due to decreased SR-BI mediated cholesterol efflux providing evidence that after carbamylation the equilibrium between HDL-lipid uptake and efflux is disrupted resulting in net uptake of cholesterol into macrophages potentially leading to foam cell formation (79).

4.2.2 AGE-modified Albumin

Prolonged exposure of proteins to glucose yields after a series of consecutive reactions, including production of the reactive intermediates glyoxal, methylglyoxal or glycoaldehde advanced glycation end products. AGE-proteins are recognized by specific receptors such as the receptor for advanced glycation end products that recognizes the AGE-structure N^{ε}(carboxymethyl)lysine (CML) (80).

Cell experiments showed that AGE-BSA binds to SR-BI and that binding is followed by endocytosis and lysosomal degradation. Notably these events did not interfere with binding of the endogenous SR-BI ligand HDL suggesting that AGE-BSA and HDL occupy distinct binding sites on SR-BI (62). Importantly, it has been shown that the affinity of AGE-albumin to SR-BI depends on the degree of AGE-modification. Only extensively AGE-modified albumin exhibited significant ligand activity whereas mildly, more physiologically modified AGE-albumin was not recognized by SR-BI irrespective of the presence of CML (54, 84).

Therefore it is unlikely that AGE-albumin interferes with SR-BI mediated activities *in vivo.*

4.2.3 Advanced Oxidation Protein Products

AOPPs result from reaction of plasma proteins with oxidants and accumulate in the course of several chronic inflammatory diseases including chronic renal insufficiency, rheumatoid arthritis and atherosclerosis. One of the major in vivo oxidants is HOCl, generated by the MPO/H_2O_2/Cl^- system of activated phagocytes. (Lipo)proteins modified by HOCl were identified to be high affinity ligands for the HDL receptor SR-BI (51).

In this regard already equimolar concentrations of AOPP-albumin to HDL were sufficient to efficiently block HDL association to SR-BI and SR-BI mediated selective cholesterol ester uptake *in vitro* (51). Albumin isolated from the plasma of HD-patients but not from control subjects inhibited SR-BI mediated HDL-CE transfer in cultured cells depending on the AOPP-content of albumin. Furthermore in the presence of AOPP-albumin plasma half-life of [^{3}H]CE-HDL was found to be extended in animal experiments (51). In addition it was shown that HOCl-modification increases the affinity of HDL to SR-BI up to 10-fold (51).

Thus, when oxidative stress is present, AOPP-albumin and oxidized lipoproteins may potentially block SR-BI and inhibit RCT. An impaired plasma clearance of HDL through AOPP-albumin-induced SR-BI blockade may impair HDL metabolism and lead to abnormal HDL composition thereby probably contributing to the high cardiovascular risk observed in patients suffering from chronic kidney disease.

5. Aims

The present work was intended to identify post-translationally modified amino acid residues that convert albumin into a high affinity SR-BI ligand.

Previous studies have shown that lysine residues are prime targets of reactive species such as hypochlorite (OCl^-) or cyanante (OCN^-) giving rise to formation of chlorolysine and homocitrulline, respectively. We hypothesized that elimination of the positive charge at the nitrogen of the ε-amino groups through oxidation or carbamylation of albumin lysine residues and/or oxidation-induced structural alterations are required for high affinity binding of albumin to SR-BI.

Accordingly, the role of lysine oxidation products for the binding of albumin to SR-BI was investigated using a repertoire of *in vivo* relevant post-translational modifications.

II. Material and Methods

1. Material

1.1 Cell Culture

Substance	Company
Accutase Bovine Serum Albumin (BSA), Fraction V, pH 7.0 CryoMaxx S DMEM, high glucose (4.5 g/L) with Stable Glutamine Dulbecco's PBS (DPBS), 1x, without Ca&Mg Fetal Bovine Serum (FBS) "Gold" (EU approved) G-418 Sulphate Solution Ham's F12 with L-Glutamine Hanks' BSS (HBSS), 1x, with Ca&Mg, without Phenol Red Penicillin/Streptomycin Plastic labware for cell culture	PAA: The Cell Culture Company

1.2 Flow Cytometry

Aqua Bidest.	Fresenius Kabi
BD CellFIX, 10x concentrate	BD Bioscience
BD FACSFlow Sheath Fluid	BD Bioscience
COULTER CLENZ, cleaning reagent	Beckman Coulter
Flow-Check Fluorospheres	Beckman Coulter
ISOTON II Diluent	Beckman Coulter
Paraformaldehyde, reagent grade	Sigma
Single/Loose MicroTubes, 1.1 mL	Bioquote Ltd.
Sodium hypochlorite solution, 12% Cl	Roth

Fixative solution was prepared by mixing 30 mL FACS Flow with 10 mL aqua bidest. and 1 mL CellFIX. Samples were centrifuged in an Eppendorf 5810R bench-top centrifuge (Eppendorf, Vienna, Austria). All flow cytometry experiments were performed on a Beckman Coulter Cytomics FC500 (Werfen, Austria).

1.3 Western Blot

Bromphenol Blue	Sigma
Calcium chloride dehydrate, p.a.	Merck Millipore
Capillary tips, 200 µL	Biozym
Ethylenediaminetetraacetic acid (EDTA) ≥ 99%, p.a., ACS	Roth
Glycerol ≥ 99%	Sigma
Glycin ≥ 99.9%, p.a.	Roth
HRP-conjugated goat anti-rabbit IgG	Sigma
Hydrochloric acid (HCl) ≥ 25%, p.a., ISO	Roth

Immun-Blot PVDF Membrane	Bio-Rad
Immun-Star Chemiluminescent Kit	Bio-Rad
Kodak Biomax light	Sigma
Kodak GBX developer/replenisher	Sigma
Kodak GBX fixer/replenisher	Sigma
2-mercaptoethanol 99% (GC/titration)	Sigma
Methanol ≥ 99.9%, p.a., ACS, ISO	Roth
Non-fat dried milk (Fixmilch Instant Trockenmilch)	Maresi Austria GmbH
Novex 4-20% Tris-Glycine Gels, 1.5 mm, 15 Well	Life Technologies
Ponceau S, practical grade	Sigma
Ponceau S Solution 0.1% (w/v) in 5% acetic acid	Sigma
Potassium chloride (KCl), p.a.	Merck Millipore
Precision Plus Protein All Blue Standard for gel electrophoresis	Bio-Rad
Protease Inhibitor Cocktail (DMSO solution)	Sigma
Rabbit anti-SRBI + SRBII antibody, polyclonal	Abcam
SDS ultrapure ≥ 99.5%	Roth
Sodium azide (NaN_3)	Merck Millipore
Sodium chloride (NaCl) ≥ 99.5%, p.a., ACS, ISO	Roth
Thick Blot Paper 9.5 x 12.5 cm	Bio-Rad
TRIS, Tris-(hydroxymethyl)-aminomethan ≥ 99.9%, p.a	Roth
Triton X-100.	Sigma
Tween 20	Sigma

To assure low contaminant levels, water for preparation of Western Blot buffers was filtered using a Synergy UV Pure to Pure Ultrapure water purification system (100V-240V/50-60 Hz) with remote dispenser (MilliQ H_2O) (Merck Millipore, Darmstadt, Germany).
SDS gel electrophoresis and protein transfer onto PVDF membranes were conducted on Bio-Rad devices (Bio-Rad, Vienna, Austria).
All incubations were carried out on a Duomax 1030 lab shaker (Heidolph, Schwabach, Germany).
Immun-Star Chemiluminescent Kit includes Immun-Star HRP luminol enhancer and Immun-Star HRP peroxide buffer which are mixed in a 1:1 ratio for chemiluminescent detection of protein bands.
Developer and fixer were diluted 1:5 with tab water prior to utilization.

Buffer composition (all reagents dissolved in MilliQ H_2O):
10 x Run Buffer: 2M Glycine, 250 mM TRIS, 35 mM SDS, pH 8.9;
10 x Blot Buffer: 0.4M Glycine, 0.1M TRIS, 3.5mM EDTA, 15mM NaN_3;
1 x Blot Buffer contains 20% Methanol
10 x Wash Buffer: 0.65M NaCl, 20 mM Tween 20, 10% (v/v) Tris/HCl pH 7.4;
Block Buffer: 5% non-fat dried milk in wash buffer
Lysis Buffer: 150mM NaCl, 25mM KCl, 10mM Tris, 1mM $CaCl_2$, 0.1% Triton X-100, 10% Protease Inhibitor Cocktail;
6 x Sample Buffer: 0.5M Tris/HCl pH 6.8, 4% SDS, 15% glycerol, some crumbs of bromphenol blue; addition of 15% mercaptoethanol shortly before usage;

1.4 (Lipo)protein Isolation and Modification

Acetic acid 25%	Roth
Alexa Fluor 488 Carboxylic Acid, 2,3,5,6-Tetrafluorophenyl Ester	Life Technologies
Butylated hydroxyltoluene (BHT) ≥ 99%, FCC	Sigma
Dimethylsulfoxide (DMSO) ≥ 99.5%	Roth
1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	Sigma
1,4-Dioxane, anhydrous, ≥ 99.8%	Sigma
Disodium hydrogen phosphate (Na_2HPO_4) ≥ 99.5%, p.a.	Roth
Fluorescamine ≥ 98% (TLC)	Sigma
Glyoxylic acid solution 50 wt. % in H_2O	Sigma
HiTrap Blue HP	GE Healthcare
^{125}Iodine	Hartmann Analytic
Iodoacetamide ≥ 99% (HPLC)	Sigma
JBS Methylation Kit	Jena Bioscience
Lysophosphatidylcholine (Lyso-PC) 16:0, in CCl_4/MeOH	Avanti Polar Lipids
L-methionine ≥ 98% (TLC)	Sigma
Oleic acid (18:1), powder ≥ 99% (GC)	Sigma
OxyBlot Protein Oxidation Detection Kit	Chemicon (Millipore)
PageBlue Protein Staining Solution	Fermentas
Palmitic acid (16:0), powder ≥ 99%	Sigma
PD-10 Desalting Columns	GE Healthcare
PD MiniTrap G-25	GE Healthcare
PD SpinTrap G-25	GE Healthcare
Poly-L-lysine hydrochloride mol wt. 15.000-30.000	Sigma
Potassium bromide (KBr) ≥ 99%, p.a., ACS	Roth
Potassium cyanate (KOCN), purum, crystallized, ≥ 97.0% (AT)	Sigma
Potassium dihydrogen phosphate (KH_2PO_4), p.a.	Merck Millipore
Quick Seal Polyallomer tubes, 13.5 mL, 16 x 76 mm;	Beckman Coulter
Sodium carbonate (Na_2CO_3), p.a.	Merck Millipore
Sodium cyanoborohydride ($NaBH_3CN$), reagent grade 95%	Sigma
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck Millipore
Sodium hydrogencarbonate ($NaHCO_3$), p.a.	Merck Millipore
Sodium hypochlorite solution (NaOCl), 4.00-4.99% chlorine	Sigma
Thiol Detection Assay Kit	Cayman Europe
Trichloroacetic acid ≥ 99.0% (titration)	Sigma
(*S*)-Trolox methyl ether ≥ 98.0%	Sigma
Vivaspin 4 centrifugal concentrators 5.000 MWCO	Sartorius
Vivaspin 4 centrifugal concentrators 10.000 MWCO	Sartorius
Vivaspin 15 centrifugal concentrators 10.000 MWCO	Sartorius

Samples were concentrated in a Megafuge 1.0R from Kendro (Kendro Laboratory Products GmbH, Langenselbold, Germany)

Lipoproteins were isolated in an Optima L-90K Preparative Ultracentrifuge equipped with a 90Ti fixed angle rotor (Titanium, 8 x 13.5 mL, 90.000 rpm, 694.000 x g;) (Beckman Coulter, Milano, Italy).

Spin Trap filtration was performed in a MiniSpin microcentrifuge (Eppendorf, Vienna, Austria).

(Lipo)protein concentrations were measured on a NanoDrop 1000 spectrophotometer (Thermo Scientific via PEQLAB Biotechnologie GmbH, Polling, Austria).

Fluorescence measurements were performed on a FlexStation II (Molecular Devices, Biberach an der Riss, Germany).

Buffer composition:
Sodium phosphate buffer: 0.2M NaH_2PO_4, 0.2M Na_2HPO_4, pH 7.8;
Sodium carbonate buffer: 0.5M Na_2CO_3, 0.5M $NaHCO_3$, pH 9;

Buffer for albumin isolation:
Binding buffer: 50mM KH_2PO_4, pH 7;
Elution buffer: 50mM KH_2PO_4, 1.5M KCl, pH 7;

1.5 AOPP-Assay

Chloramine-T hydrate 98%	Sigma
Glacial acetic acid ≥ 99%	Roth
Potassium iodide (KI) ≥ 99.0%, p.a.	Sigma

1.6 Immunofluorescence

Glass Bottom Dishes	Willco Wells
Texas Red Goat Anti-Rabbit IgG	Life Technologies

AF 488-labeled native and HOCl-albumin and Texas-Red labeled SR-BI were visualized using a confocal imaging system (Zeiss LSM 510 Meta) with a Plan Neofluar 40x/1.3 N.A. oil-immersion objective (Carl Zeiss, Vienna, Austria).

1.7 Amino Acid Analysis and LC-MS/MS

Amber glass 12 x 32 mm Screw Neck Qsert Vials	Waters
Ammonium formiate (CH_5NO_2) for HPLC, ≥ 99.0%	Sigma
Carboxymethyllysine	PolyPeptide Labs
$^{13}C_6$-homocitrulline	Ascent Scientific
$^{13}C_6$-3-chlorotyrosine	PolyPeptide Labs
$^{13}C_6$-lysine	Cambridge Isotope Laboratories
EZ:faast protein analysis kit	Phenomenex
Hydrobromic acid (HBr) ≥ 48%, p.a.	Sigma
Lithium citrate buffer	Biochrom
Ninhydrin	Sigma
Phenol ≥ 99.5% (GC)	Sigma

Amino acid analysis was performed on a Biochrom 20 amino acid analyzer (Pharmacia Biotech, Stockholm, Sweden).
For HPLC an AAA-MS HPLC column (250 mm x 4 mm) was used (Phenomenex, Aschaffenburg, Germany).

2. Methods

2.1 Blood Collection

Blood was taken from ESRD-patients on hemodialysis (HD-patients) prior to dialysis sessions and from age-matched control subjects at the time of routine laboratory investigations in agreement with the Institutional Review Board of the Medical University of Graz. Blood samples were collected in standard sterile polystyrene vacuum tubes containing 5 mM EDTA. Plasma was separated from the cellular components by centrifugation (600 x g, 10 minutes), divided into 500 µL aliquots and stored at -70°C until further analysis.

2.2 Albumin Isolation

Albumin from ESRD-patients (n=12) and control subjects (n=10) was separated from other plasma proteins by affinity chromatography using HiTrap Blue HP, 1 mL columns according to the instructions of the manufacturer. Briefly, after equilibration with 5-10 column volumes of binding buffer (50 mM KH_2PO_4, pH 7) lipoprotein-deficient serum (LPDS) samples (0.5 mL) were applied at a flow rate of 0.5-1 mL/min using a syringe fitted to the luer connector of the column. Columns were washed with 5-10 column volumes of binding buffer before albumin samples were eluted with 5-10 column volumes of elution buffer (50 mM KH_2PO_4, 1.5 M KCl, pH 7). Elution buffer was exchanged for phosphate buffered saline (PBS) (-/-) (pH 7.4) by passing the purified samples over PD-10 desalting columns.

The purity of the albumin preparations was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie staining.

2.3 AOPP Assay

Isolated albumin from patients and controls was diluted with PBS to give a final protein concentration of 10 mg/mL in a volume of 20 µL. After addition of 2 µL of glacial acetic acid, samples were incubated at room temperature for 10 minutes. Subsequently absorbance (340 nm) and optical density (595 nm) as a measure of light scattering due to sample decomposition were measured on a Nano Drop 1000 spectrophotometer. For conversion of the absorbance into the respective AOPP concentrations, a standard curve was generated, ranging from 1 to 100 µM chloramine-T.

AOPP concentrations were normalized to the protein concentrations in the individual samples and expressed as nmol AOPP per mg protein.

2.4 Amino Acid Analysis

Amino acid analysis was performed by Günther Rathspieler, Institute of Molecular Biology and Biochemistry, Medical University of Graz. Albumin samples were hydrolyzed as described (81). Birefly, protein samples were placed into Qsert vials, hydrobromic acid (containing 0.25% phenol) was added to a final concentration of 6 N, vials were flushed with argon, sealed and hydrolyzed at 160°C for 5 minutes. Thereafter hydrobromic acid was evaporated, hydrolysates were resuspended in 100 µL 0.2 M Li-citrate buffer (pH 2.2) and derivatized with the EZ:faast Kit according to the instructions of the manufacturer.

Total amino acid analysis was performed on a Biochrom 20 amino acid analyzer equipped with a LiHR column (200 mm x 4.6 mm) following standard procedures. Calibration of the instrument was performed with an external amino acid calibration standard. The single amino acids were separated by means of ion exchange chromatography, mixing the eluate continuously with ninhydrine reagent at a coil temperature of 135 °C. The coloured complexes generated by reaction of amino acids with ninhydrine (post-column derivatization) were analyzed using a flow photometer. Primary amino acids were detected at 570 nm and secondary amino acids at 440 nm.

2.5 Homocitrulline (HCit), carboxymethyllysine (CML) and 3-chlorotyrosine (3-CT) quantification

MS-analysis of HCit, CML and 3-CT was performed by Dr. Michael Holzer, Institute of Experimental and Clinical Pharmacology, Medical University of Graz. Isolated albumin from HD-patients and control subjects was hydrolyzed with a fast, low-volume hydrolysis method and derivatized as described (81). Internal standard (10 µL) contained 10 ng $^{13}C_6$-HCit, 10 ng $^{13}C_6$-3-CT, 0.3 µg $^{13}C_6$-tyrosine and 1 µg $^{13}C_6$-lysine.

Electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) was used for quantification of HCit, CML and 3-CT as previously described (79).

LC-MS/MS calibration curves were prepared by varying amino acid, HCit, CML and 3-CT concentrations with fixed amounts of internal standards. The calibration curves had a linearity range from 50 pg – 100 ng for HCit, CML and 3-CT (R^2: 0.998, R^2: 0.989 and R^2: 0.999) and from 100 ng – 3 µg for lysine and tyrosine (R^2: 0.997 and R^2: 0.998).

The HPLC column was equilibrated with 100% solvent A (10 mM ammonium formiate in water) for 15 minutes at 35°C. After equilibration the sample (10 µL) was injected onto the HPLC column at a flow rate of 0.25 mL/min. For elution of the single compounds a discontinuous gradient was applied starting with 83% solvent B (10 mM ammonium formiate in methanol) for 13 minutes followed by 68% of solvent B for 4 minutes. The HPLC column effluent was introduced into an API 200 triple quadrupole mass

spectrometer. Ions were generated by electrospay ionization in the positive-ion mode with multiple reactions monitoring of parent and characteristic daughter ions.

The following transitions were recorded indicated by their mass-to-charge ratio (m/z): m/z 318→127 for HCit; m/z 324→132 for $^{13}C_6$-HCit; m/z 461→170 for CML; m/z 361→170 for lysine; m/z 367→175 for $^{13}C_6$-lysine; m/z 430→170 for 3-CT; m/z 436→176 for $^{13}C_6$-3-CT; m/z 396→136 for tyrosine and m/z 402→142 for $^{13}C_6$-tyrosine. Following mass spectrometric analysis the generated calibration curves were used to quantify HCit, CML, 3-CT, lysine and tyrosine.

2.6 Thiol Detection

Redox-state of cysteine residues was analyzed using the Thiol Detection Assay Kit (Cayman, Tallinn, Estonia). Therefore albumin samples of HD-patients and controls were diluted 1:20 with thiol assay buffer making a final protein concentration of approximately 0.5 mg/mL. Samples were mixed with the fluorometric detector in a 1:1 ratio in a 384 well plate. After 5 minutes incubation avoiding light exposure fluorescence, emerging from reaction of thiol groups with the fluorometric detector, was read on a Flex Station II using excitation and emission wavelengths of 385 nm and 515 nm respectively.

Based on the fluorescence readings of the respective standards a cysteine standard curve was generated ranging from 15.6 nM to 2 µM cysteine. Reduced thiol content in the samples was calculated by means of the equation obtained from the standard curve and normalized to mol cysteine per mol albumin.

As controls native albumin containing one mol SH per mol albumin and HOCl-oxidized albumin (albumin:HOCl molar ratio 1:100) which is devoid of free thiols were used.

2.7 Cell Culture

CHO cells overexpressing murine SR-BI (LdlA(SR-BI)) and control CHO cells expressing background levels of SR-BI (LdlA7) were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. Medium for SR-BI overexpressing cells additionally contained 1% Geniticin to assure continuation of receptor expression. Cells were maintained at 37°C in humidified 5% CO_2.

Cells were passaged when they reached confluence and used for experiments until flow cytometry showed a clear decline in receptor expression. Both cell lines were kindly provided by Dr. Monty Krieger (Massachusetts Institute of Technology, Boston, USA).

2.8 HDL Isolation

HDL (density range 1.125 to 1.21 g/mL) was prepared by discontinuous density ultracentrifugation of plasma obtained from normolipidemic blood donors. Plasma was separated from blood cells by centrifugation at 400 x g for 15 minutes at 8°C and plasma density was adjusted with potassium bromide (KBr) to 1.24 g/mL. To generate a two-layer density gradient the density-adjusted plasma (1.24 g/mL) was layered underneath a KBr-density solution (1.063 g/mL). Centrifugation tubes were sealed and centrifuged at 90.000 x g for 4 hours at 8°C in a 90Ti fixed angle rotor. After centrifugation the clearly separated HDL-containing band was isolated by puncturing the tube with a syringe. Isolated HDL was desalted using PD-10 columns and stored at 4°C until further use.

2.9 HDL Labeling with $Na^{125}I$

Iodination of HDL was performed by Dr. Gunther Marsche, Institute of Experimental and Clinical Pharmacology, Medical University of Graz as described using *N*-Br-succinimide as the coupling reagent (82). Routinely 1 mCi of $Na^{125}I$ was used to label 5 mg of lipoprotein. This procedure resulted in specific activities between 300 and 500 dpm/ng protein.

2.10 Radioactive Competition Experiment

Radioactive Competition Experiments were performed by Dr. Gunther Marsche, Institute of Experimental and Clinical Pharmacology, Medical University of Graz. For determination of ^{125}I-HDL association to SR-BI, LdlA(SR-BI) and control LdlA7 cells were incubated in serum-free DMEM for 2 hours at 37°C with ^{125}I-labeled HDL (10 µg/mL) in the presence of albumin (1 mg/mL) isolated from HD-patients and control subjects. Subsequently the cells were washed and lysed to determine the amount of associated label by scintillation counting and the cellular protein content. Specific association of ^{125}I-labeled HDL to SR-BI was assessed by subtracting values obtained in LdlA7 cells from those obtained in LdlA(SR-BI) cells.

2.11 (Lipo)protein Modifications

2.11.1 HOCl-induced Modification

(Lipo)proteins were incubated with HOCl ((lipo)protein:oxidant molar ratio of 1:12.5 up to 1:100) in PBS (pH 7.4) for 30 minutes at 4°C in absence of free amino acids/carbohydrates/lipids to exclude formation of AGE-like structures (47). HOCl-oxidized BSA preparations were stored at -20°C and HOCl-modified HDL preparations at 4°C until further use.

Human plasma was HOCl-modified (HSA:HOCl molar ratios of 1:25 up to 1:100) assuming an albumin concentration of 50 mg/mL. Plasma was oxidized for 30 minutes at 4°C. Thereafter oxidized HSA was isolated using HiTrap Blue HP, 1 mL columns as described and elution buffer (50 mM KH_2PO_4, 1.5 M KCl, pH 7) was exchanged for PBS. For comparison albumin was isolated from non-oxidized plasma. HSA preparations were used for competition experiments (DiI-HDL uptake).

2.11.2 Reductive Methylation/Regeneration of Lysine Chloramines

Reductive methylation using formaldehyde as alkylating reagent and dimethylamine borane complex for reduction was carried out using the JBS methylation kit according to the instructions of the manufacturer. Therefore all reagents were prepared immediately before use and kept on ice throughout the whole procedure. All reactions were performed at 4°C. 1 mL of protein solution (in PBS) with a concentration between 1 and 10 mg/mL was subjected to methylation. Solution A was prepared by dissolving the 6 mg aliquot of dimethylamine borane complex included in the kit in 100 µL reagent grade water. 20 µL of the resultant solution was added to 1 mL of the protein solution followed by addition of 40 µL of 1 M formaldehyde (solution B) and gentle mixing. The reaction mixture was incubated at 4°C for 2 hours. Subsequently the procedure was repeated, 20 µL of solution A and 40 µL of solution B were added and the mixture was incubated for another 2 hours at 4°C. Thereafter a final aliquot of 10 µL of solution A was added and the reaction mixture was left at 4°C overnight. If precipitation was observed the precipitate was removed by centrifugation before continuing preparation. The reaction was stopped by addition of 125 µL of 1 M Tris (pH 7.5) (solution C). Methylated proteins were separated from the reaction mixture by size exclusion chromatography using PD-10 columns and stabilized by addition of 50 mM dithiothreitol (DTT, solution D) reaching a final DTT concentration in the sample between 1 and 5 mM.

To reconvert the lysine chloramines that were generated upon HOCl-treatment to the parent lysines a 5-fold molar excess of L-methionine over HOCl was added 30 minutes

after oxidation. After 1 hour incubation at 4°C, samples were passed over PD-Mini Trap G-25 columns to remove excess free L-methionine.

To investigate the impact of *N*-chloramines on HDL-lipid delivery, HOCl-albumin was incubated for 5 days at 37°C to allow complete decomposition of *N*-chloramines. Possibly leftover chloramines were tried to recover by addition of L-methionine for 1 hour at 4°C and subsequent filtration using PD-Mini Trap G-25 columns. The resulting “aged” albumin preparations were directly used for competition experiments or first AF488-labeled and then used for binding experiments.

2.12 Fluorescamine Assay

200 µL fluorescamine (0.3 mg/mL in dioxane) were added to 600 µL of (lipo)protein yielding a final (lipo)protein concentration of 200 µg/mL. Upon reaction with primary amines, fluorescamine forms fluorescent conjugates. Fluorescence measurements were performed on a Flex Station II using the fluorescamine excitation and emission wavelengths of 390 and 460 nm, respectively.

2.13 Detection of Carbonylated Albumin

Immunoblot detection of carbonyl groups introduced into proteins by oxidative reactions was accomplished using the chemical and immunologic reagents of the OxyBlot oxidized protein detection kit.

Albumin preparations (5 µL) were denatured by addition of an equal amount of 12% SDS making a final SDS-concentration of 6%.

Carbonyl groups in the protein side chains were converted into the respective 2,4-dinitrophenylhydrazone-derivatives (DNP-hydrazone) by incubation with 10 µL 1x 2,4-dinitrophenylhydrazine (DNPH) solution for 15 minutes at room temperature. Negative controls (one negative control per sample) contained 10 µL Derivatization-Control Solution instead of DNPH-solution. Derivatization was completed by addition of 7.5 µL Neutralization solution to all tubes.

5-10 µg of the DNP-derivatized samples were loaded into Tris/Glycine gels (4-20%), separated by polyacrylamide gel electrophoresis (20 mA) and transferred onto polyvinylidene difluoride membranes (100 V, 4°C, stirring). Membranes were incubated with a rabbit anti-DNP primary antibody (1:150) at 4°C overnight followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:300) as secondary antibody for 2 hours at room temperature. Membranes were treated with chemiluminescent reagents (peroxide buffer and luminol enhancer in a 1:1 mixture for 5 minutes) and the light resulting from conversion of luminol in its light-emitting form by the

antigen/primary antibody/secondary antibody complex was detected by short exposure (30 seconds) to autoradiography (blue-light sensitive) films.

2.14 Carbamylation of Albumin

10 mg BSA (in PBS, pH 7.4) were incubated with 1–40 mg potassium cyanate (KOCN) per mg albumin for 4 hours at 37°C. Carbamylated albumin was passed over PD-Mini Trap G-25 columns to remove excess KOCN and stored at -20°C until further use.

2.15 N^{ε}(carboxymethyl)lysine Modification of Albumin

10 mg BSA in 0.2 M sodium phosphate buffer (0.2 M Na_2HPO_4/0.2 M NaH_2PO_4, pH 7.8) were mixed with 25 µmol sodium cyanoborohydride (1 M stock in sodium phosphate buffer) and 10.8 µmol glyoxylic acid (0.5 M stock in H_2O). The resulting mixture was incubated for 45 minutes at 37°C. CML-modified albumin preparations were passed over PD-Mini Trap G-25 columns to remove excess reactants and stored at -20°C until further use.

2.16 SDS-PAGE

Native, HOCl-oxidized, carbamylated and CML-modified albumin (3 µg) were loaded into tris/glycine gradient gels (4-20%) and separated by polyacrylamide gel electrophoresis (20 mA).

Subsequently Coomassie staining was performed according to the conventional protocol for Page Blue Protein Staining Solution from Fermentas (#R0571). The gel was washed 3 times for 10 minutes in 100 mL deionized water followed by protein fixation in 12% trichloroacetic acid for 30 minutes. After 10 minutes washing in deionized H_2O the gel was incubated for 1 hour in 50 mL Page Blue Protein Staining Solution with gentle agitation. Thereafter the gel was washed thoroughly with deionized H_2O and left overnight in H_2O on the lab shaker at 4°C. The gel was stored in 5% acetic acid in H_2O at 4°C.

2.17 Preparation of Lysophosphatidylcholine (Lyso-PC) and Fatty Acid Conjugates

Saturation of BSA requires a 6-fold molar excess of lyso-PC and an 8-fold molar excess of free fatty acids.

0.54 µmol lyso-PC (4.78 mg/mL in CCl_4/MeOH) were dried under a stream of nitrogen to dispose of chloroform which is harmful for cells. The residue was dissolved in 50 µL PBS and mixed with 0.09 µmol fatty acid free BSA (15 mg/mL stock solution in PBS).

0.72 µmol of palmitic acid (16:0) and oleic acid (18:1) (18 mM stock solutions in methanol) were added drop wise to 0.09 µmol fatty acid free BSA (15 mg/mL stock solution in PBS). All reaction mixtures were incubated for 15 minutes at room temperature and subsequently filtered using PD-Mini Trap G-25 columns. As lyso-PC is not stable in PBS conjugates with lyso-PC need to be prepared on the day of the experiment.

2.18 Cysteine Protection

Albumin was incubated with a 20-fold molar excess of iodoacetamide in PBS for 1 hour at room temperature in the dark. Cysteine-protected albumin was then passed over PD-Mini Trap G-25 columns to remove unreacted iodoacetamide. Cysteine protection was verified using the Thiol Detection Assay Kit.

2.19 Fluorescent Labeling of Albumin and HDL

500 µL of albumin or HDL (2 mg/mL in PBS) were combined with 100 µL sodium carbonate buffer (0.5 M Na_2CO_3, 0.5 M $NaHCO_3$, pH 9) and 3.5 µL Alexa Fluor 488 carboxylic acid 2,3,5,6-tetrafluorophenyl ester (AF488, dissolved in DMSO). Mixtures were incubated at 24°C for 1 hour with vigorous shaking. Subsequently unincorporated dye was separated from the labeled samples by gel filtration using PD-Mini Trap G-25 columns. Preparations were stored at -20°C (albumin) and at 4°C (HDL) respectively.

For 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) labeling, 300 µg DiI were added to 1 mg HDL in PBS. The resulting mixture was incubated for 18 hours at 37°C. The labeled HDL preparations were filtered over PD-Mini Trap G-25 columns to remove low molecular weight compounds, sterile filtered and stored at 4°C until further use.

2.20 SR-BI specific AF488-HDL Association and DiI-HDL Uptake (Competition Experiments)

LdlA(SR-BI) and LdlA7 cells were seeded in 48-well plates and allowed to grow at 37°C overnight. On the next day, cells were washed with PBS and preincubated with modified albumin preparations (1 mg/mL native, oxidized, carbamylated or CML-modified albumin) for 30 minutes at 37°C. After addition of AF488-HDL or DiI-labeled HDL (50 µg/mL) incubation was continued for another 2 hours (AF488-HDL association) and 4 hours (DiI-lipid uptake) respectively. Cells were washed with PBS and detached by application of 200 µL accutase per well for 10 minutes at 37°C. The resuspended cells were transferred into tubes containing 200 µL 1% paraformaldehyde and left for 15 minutes on ice. After centrifugation (600 x g, 7 minutes, room temperature) cells were washed with PBS and

resuspended in a fixative solution to assess AF488-HDL association or DiI-HDL uptake by flow cytometry (FL-1 for AF488-HDL and FL-4 for DiI-HDL). SR-BI specific AF488-HDL association or DiI-uptake was calculated by subtracting values obtained in LdlA7 cells from those obtained in LdlA(SR-BI) cells.

2.21 Determination of Binding and Dissociation Kinetics of Different Albumin Modifications

2.21.1 Time-dependent Binding and Dissociation

LdlA(SR-BI) and control LdlA7 cells were seeded in 48-well plates and left to grow for 24 hours at 37°C. To determine time-dependent AF488-albumin association, cells were washed with HBSS and incubated at 4°C with oxidized or carbamylated AF488-albumin (100 µg/mL) for different time periods (5, 10, 15, 60, 120 minutes). All incubations were performed in presence of native albumin (5 mg/mL) to minimize unspecific binding. After incubation cells were washed, detached with accutase and cell-associated AF488-albumin was measured by flow cytometry (FL-1). SR-BI specific AF488-albumin association was calculated by subtracting values obtained in LdlA7 cells from those obtained in LdlA(SR-BI) cells.

Dissociation was monitored 3, 6 and 24 hours after removal of AF488-albumin. Therefore cells were incubated for the respective durations in serum free Ham's F12 medium containing 5 mg/mL native BSA, washed, detached and remaining AF488-albumin association was detected by flow cytometry.

2.21.2 Concentration-dependent Binding

For assessment of concentration-dependent binding, cells were incubated at 4°C with AF488-albumin concentrations ranging from 1.6 to 200 µg/mL. After an incubation period of 2 hours, AF488-albumin association to SR-BI was determined by flow cytometry.
Cell preparation, flow cytometry and calculation of SR-BI specific AF488-albumin association were conducted as described above.

2.22 SR-BI specific AF488-albumin Association (Binding Experiments)

LdlA(SR-BI) and control LdlA7 cells were seeded in 48-well plates and allowed to grow for 24 hours at 37°C. On the next day, cells were cooled to 4°C, washed with cold HBSS and incubated with different modified AF488-albumin preparations (100 µg/mL native, HOCl- or HCit-albumin) for 2 hours at 4°C. To minimize unspecific binding all incubations were performed in presence of native albumin (5 mg/mL). After incubation cells were harvested to measure AF488-albumin association or incubated with either native albumin (3 mg/mL) to measure AF488-albumin dissociation or with unlabeled oxidized/carbamylated albumin (3 mg/mL) as competitors. After 2 hours incubation at 4°C, cells were washed, detached and fixed for measurements. AF488-albumin association was assessed by flow cytometry and SR-BI specific binding was determined as described.

2.23 Immunofluorescence

Immunofluorescence experiments were performed in collaboration with Dr. Senka Ljubojevic, Division of Cardiology, Medical University of Graz. LdlA(SR-BI) and control LdlA7 cells were seeded in glass bottom dishes. Cells were rinsed with HBSS and incubated with 100 µg/mL AF488-labeled native or HOCl-albumin in the presence of 5 mg/mL native albumin for 2 hours at 37°C. Cells were washed with HBSS, fixed with 2% paraformaldehyde for 30 minutes, washed again and stored in PBS at 4°C.

For antibody staining cells were permeabilized with 0.5% Triton-X 100 in PBS for 15 minutes, rinsed and unspecific binding was prevented by incubation with 5% BSA in PBS for 1 hour. Anti-SR-BI antibody was applied in a 1:300 dilution in PBS/5% BSA without prior washing. After 3 hours, cells were washed and incubated with Texas Red-coupled goat anti-rabbit IgG (1:500 in PBS/5% BSA) for 1 hour at room temperature. Cells were washed and stored in PBS until microscopic analysis.

AF488-labeled native and HOCl-albumin and Texas Red-labeled SR-BI were visualized using a confocal imaging system (Zeiss LSM 510 Meta) with a Plan Neofluar 40x/1.3 N.A. oil-immersion objective. The excitation and emission wavelengths for AF488 were 488 nm and 510-530 nm, respectively. For Texas Red excitation at 543 nm and emission at > 560 nm were used. The optical slice thickness was set to 1.0 µm. 2D images at a central depth of the cell were collected.

2.24 Western Blot

Confluent LdlA(SR-BI) and control LdlA7 cells were incubated with native or HOCl-albumin for 2 hours at 37°C. Subsequently cells were rinsed and detached with lysis buffer (150 mM NaCl, 25 mM KCl, 10 mM TRIS, 1 mM $CaCl_2$ and 0.1% Triton X-100) containing a protease inhibitor cocktail.

20 µL aliquots of the individual samples were loaded into tris/glycine gradient gels (4-20%) under reducing (15% mercaptoethanol) and not-reducing conditions. Proteins were separated by SDS gel electrophoresis and transferred to polyvinylidene difluoride membranes (200 mA for 2 hours at 4°C). Membranes were washed, blocked for 1 hour in 5% fat-free dry milk and incubated with a polyclonal anti-SR-BI primary antibody (1:2000 in blocking buffer) overnight at 4°C. Membranes were washed and horseradish peroxidsae-conjugated goat anti-rabbit IgG (1:10000 in blocking buffer) was applied as the secondary antibody for 2 hours at room temperature. For detection membranes were incubated in a 1:1 mixture of Immune-Star luminol enhancer and peroxide solution for 5 minutes in the dark. Autoradiography films were exposed for different time periods to visualize SR-BI protein bands.

2.25 Statistical Analyses

Results are represented as mean ± SEM for the number of performed experiments (n). One-way analysis of variance (ANOVA) was used for multiple comparison and unpaired t-tests for comparison between two groups. Correlations were determined using Pearson product-moment estimates. Significance was accepted at $P<0.05$. All statistical analyses were performed using GraphPad Prism Version 4.03.

III. Results

1. *N*-chloramine Formation transforms Albumin into an SR-BI Inhibitor

Plasma albumin is oxidized by the myeloperoxidase product hypochlorous acid. Thereby positively charged ε-amino groups of lysine residues are among the prime targets resulting in formation of lysine chloramines and loss of the positive charge.

Native albumin does not bind to the major HDL receptor SR-BI, but by oxidation it is converted into a high affinity SR-BI ligand. Consequently the increased net negative charge after oxidation together with resulting structural alterations might alter interactions of albumin with SR-BI.

To test this hypothesis, albumin was oxidized using HOCl leading to oxidative modification of lysine, tyrosine, histidine, arginine and cysteine **(Table 1)**. To specifically elucidate the role of lysine residues, lysine chlorination was either prevented by reductive methylation or reversed by addition of free methionine to HOCl-treated albumin. After HOCl-modification albumin effectively inhibited binding of Alexa Fluor 488 labeled HDL (AF488-HDL) to SR-BI in competition experiments **(Figure 1A)**. After reductive methylation HOCl-treatment was without effect with respect to the binding properties of albumin **(Figure 1A)**. Methionine-treatment successfully regenerated albumin lysine residues **(Figure 1B)** and completely averted AF488-HDL displacement by oxidized albumin in competition experiments **(Figure 1A)**.

Table 1: Amino Acid Analysis and AOPP-content of native and HOCl-albumin

		Albumin : HOCl molar ratio			
	Native Albumin	**1:12.5**	**1:25**	**1:50**	**1:100**
Tyrosine	22.6	21.0	18.1	14.9	7.0
Lysine	59.0	57.4	55.7	53.1	45.0
Histidine	17.8	18.0	18.1	17.1	14.1
Arginine	26.8	26.5	26.3	24.5	21.1
AOPP [nmol/mg protein]	0.4 ± 0.03	1.2 ± 0.04	1.5 ± 0.03	3.4 ± 0.3	12.0 ± 0.7

Results of the AOPP-measurement are given as mean ± standard deviation.

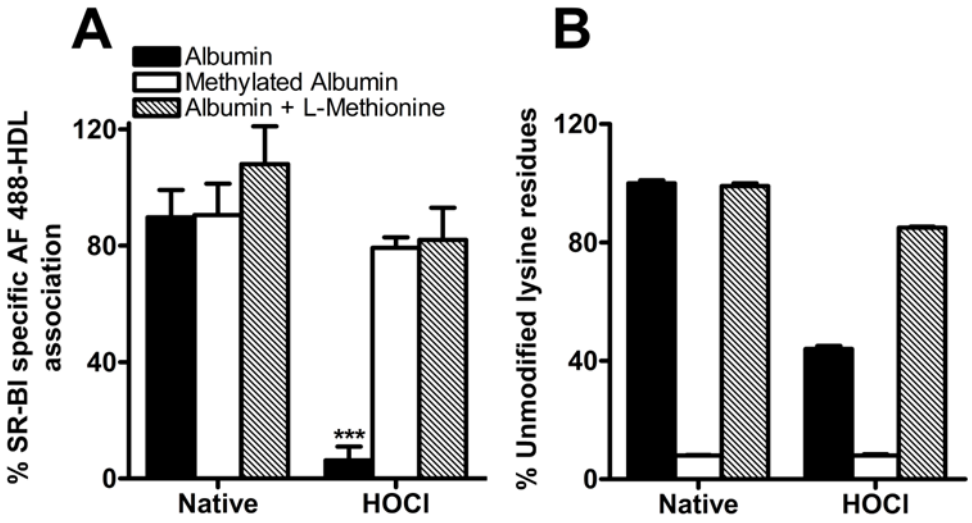

Figure 1: HOCl-induced Lysine Modification transforms Albumin into an SR-BI Inhibitor. (A) *SR-BI overexpressing CHO cells (LdlA(SR-BI)) and control CHO cells (LdlA7) were incubated at 37°C for 2 hours with AF488-HDL (50 µg/mL) in the presence of either 1 mg/mL native albumin or HOCl-albumin (physiological excess of albumin over HDL in plasma is 50-125-fold). To protect lysine residues from oxidation, albumin was methylated prior to oxidation (Methylated Albumin). HOCl-induced lysine chloramine formation was reversed by addition of excess free L-methionine 30 minutes after HOCl-treatment of albumin (Albumin + L-Methionine). AF488-HDL association to SR-BI was assessed by flow cytometry. AF488-HDL association to SR-BI in absence of any competitor were set to 100%. Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to calculate SR-BI specific HDL association. Data are represented as mean ± SEM (n=3-5). ***P < 0.001 versus native albumin.*

(B) *Residual unmodified lysine residues of the different albumin modifications were assessed using the fluorescamine assay.*

2. HOCl-albumin impairs SR-BI mediated HDL-lipid Uptake

SR-BI mediated lipid uptake is widely believed to be a two-step process composed of formation of a functional lipoprotein/receptor complex followed by effective lipid transfer across the plasma membrane into intracellular compartments.

Therefore it can be supposed that albumin containing *N*-chloramines interferes with SR-BI mediated selective lipid uptake.

To address this question, HDL was labeled with the lipid moiety label DiI and used in competition experiments in presence of native and HOCl-modified albumin.

As expected, HOCl-albumin clearly attenuated SR-BI mediated HDL-lipid (DiI) uptake, which was not observed when methionine was added to reverse HOCl-albumin-associated *N*-chloramine formation **(Figure 2A)**.

Reaction of ε-amino groups with HOCl leads to formation of monochloramines in a first step which then lose the NHCl group to finally yield a more stable aldehyde (83). The reactivity of aldehydes with nucleophilic residues of proteins, lipids and DNA molecules suggests that they play a role in HOCl-induced tissue damage at sites of inflammation (83). To investigate the role of *N*-chloramines for the interference of oxidized albumin with HDL-lipid delivery, HOCl-albumin was incubated for 5 days at 37°C to allow complete decomposition of *N*-chloramines. The resulting "aged" HOCl-albumin preparations were used in competition experiments. Aged HOCl-albumin reduced SR-BI mediated DiI-HDL uptake but in contrast to freshly oxidized albumin, addition of L-methionine did not reverse the inhibitory effect **(Figure III-2B)**. Based on these results it can be assumed that *N*-chloramines are degraded during "aging" of albumin and that both, *N*-chloramines and their decomposition products transform albumin into an SR-BI inhibitor.

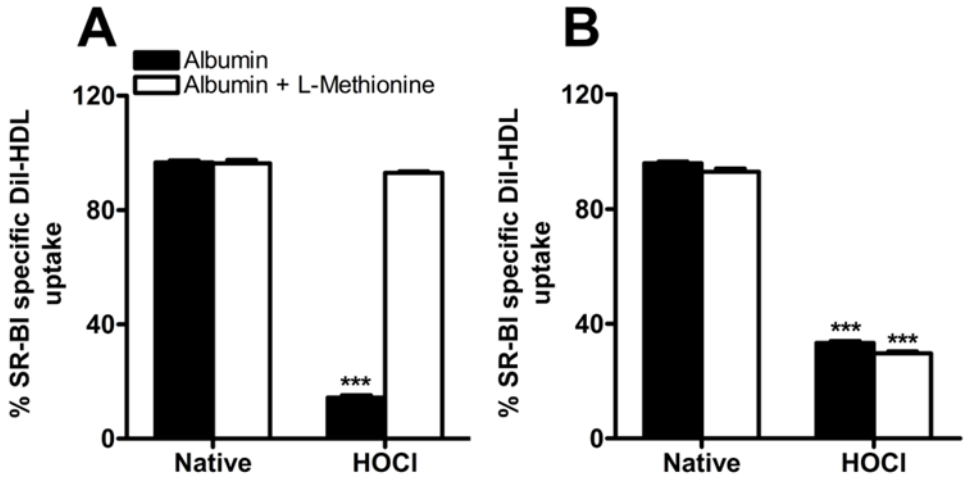

Figure 2: Albumin containing N-chloramines or decomposition products of N-chloramines attenuates SR-BI mediated HDL-lipid Uptake. *LdlA(SR-BI) and LdlA7 cells were incubated at 37°C for 4 hours with DiI-HDL (50 µg/mL) in the presence of 1 mg/mL native albumin or HOCl-albumin. Albumin was oxidized either for 30 minutes at 4°C* ***(A)*** *or for 5 days at 37°C to allow N-chloramine decomposition* ***(B)****. To reverse HOCl-induced lysine-chloramine formation, L-methionine was added in excess 30 minutes after the incubation period (Albumin + L-Methionine). SR-BI mediated DiI-HDL uptake was assessed by flow cytometry. SR-BI mediated DiI-HDL uptake in absence of any competitor was set to 100%. Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to calculate SR-BI specific DiI-HDL uptake. Data are represented as mean ± SEM (n=3). ***P < 0.001 versus native albumin of the respective set of samples.*

3. Diverse Lysine Modifications of Albumin differently affect Albumin/SR-BI Interactions

Atherosclerosis represents a state of persistent increased oxidative stress evidenced by the presence of modified lipids and proteins in the vascular wall.

Among the different post-translational modifications, protein carbamylation is of particular interest as it is known to render proteins dysfunctional. Carbamylated proteins are considered to be independent risk factors for the development of coronary artery disease, myocardial infarction and stroke (79). Protein carbamylation is caused by cyanate leading to irreversible conversion of lysine residues to ε-amino-carbamyllysine (homocitrulline). Cyanate is generated enzymatically by MPO catalyzed oxidation of thiocyanate (HOSCN) and arises from decomposition of urea (79). This is of importance in renal disease where plasma concentrations of urea are known to be elevated and increased concentrations of carbamylated proteins have been detected (79).

Glucose, an α-hydroxy aldehyde, is abundant in the body and in its open-chain conformation it is able to modify proteins. Reaction of glucose with lysine amine groups yields a Schiff-base adduct which is converted first into the Amadori product 1-(deoxyfructose)lysine and finally, after a complex series of reactions, into the major advanced glycation end product N^{ε}(carboxymethyl)lysine. An alternative pathway for formation of AGE-products is the reaction of MPO generated HOCl with serine yielding glycoaldehyde which is involved in CML formation at sites of inflammation. Increased modification of proteins by glucose is observed in diabetic hyperglycemia which is prevalent amongst CKD-patients (84).

Prompted by these findings, we were interested to test whether carbamylation or CML-modification would modulate interactions of albumin with SR-BI.

To address this point, albumin lysine residues were carbamylated with cyanate yielding homocitrulline and carboxymethylated to form N^{ε}(carboxymethyl)lysine and the resulting albumin preparations were used in competition experiments. Thereby HOCl-albumin was used as positive control. To confirm that the degree of lysine modification was comparable throughout the different albumin preparations, lysine modification was determined using amino acid analysis and LC-MS/MS.

In competition experiments, HCit-albumin effectively displaced HDL from SR-BI **(Figure 3A)** and attenuated SR-BI mediated DiI-HDL delivery **(Figure 3B)**, whereas CML-albumin failed to interfere with HDL binding to SR-BI as well as with SR-BI mediated DiI-HDL uptake. This result suggests that the negative charge of the carboxyl group of CML and/or structural differences of CML prevent binding to SR-BI **(Figures 3A, 3B, 3C)**.

Already a minimal modification of 1 to 2 of 59 lysine residues in HOCl-albumin was sufficient to generate SR-BI inhibitors, whereas HCit-albumin was less effective with regard to the number of modified lysine residues **(Figure 3D)**.

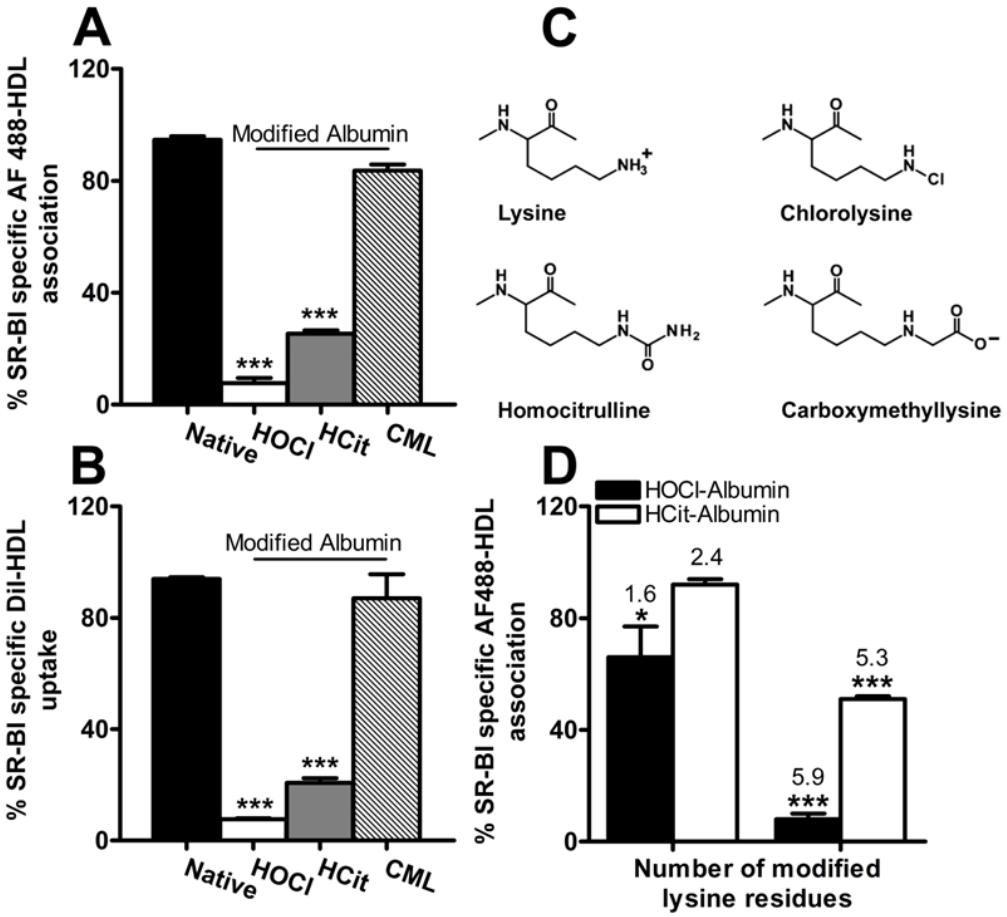

Figure 3: Impact of different Lysine Modifications on Interactions of Albumin with SR-BI. *LdlA(SR-BI) and LdlA7 cells were incubated at 37°C for 2 hours with 50 µg/mL AF488-HDL* ***(A)*** *or for 4 hours with 50 µg/mL DiI-HDL* ***(B)*** *in the presence of 1 mg/mL native albumin, HOCl-albumin, carbamylated albumin (HCit-albumin) or carboxymethylated albumin (CML-albumin). The extent of modified lysine residues was comparable throughout the different albumin preparations (≈ 6 modified lysine residues of 59). Data are represented as mean ± SEM (n=3). ***P < 0.001 versus native albumin.* ***(C)*** *Structures of lysine, homocitrulline, chlorolysine and carboxymethyllysine.* ***(D)*** *LdlA(SR-BI) and LdlA7 cells were incubated at 37°C for 2 hours with 50 µg/mL AF488-HDL in the presence of 1 mg/mL HOCl-albumin or HCit-albumin preparations containing indicated modified lysine residues per albumin. Data are shown from one typical experiment performed in triplicates.*

**P < 0.5, ***P < 0.001 versus 100% AF488-HDL association.*

(A,B,D) *AF488-HDL association to SR-BI or DiI-HDL uptake by SR-BI was assessed by flow cytometry. AF488-HDL association to SR-BI and DiI-HDL uptake by SR-BI, in absence of any competitor, were set to 100%. Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to calculate SR-BI specific HDL association and uptake.*

4. Coupling of Albumin to Lysophosphatidylcholine or free Fatty Acids has no Impact on Binding Affinity to SR-BI

Elevated phospholipase activity in dialysis patients leads to increased plasma concentrations of lyso-PC and free fatty acids associating with albumin (85).

To investigate the influence of albumin-associated lyso-PC and fatty acids on binding affinity of albumin to SR-BI, albumin was saturated with palmitic acid, oleic acid and lyso-PC respectively. The resultant conjugates were tested with respect to their ability to compete binding of AF488-TFP HDL to SR-BI.

According to our *in vitro* studies, coupling of albumin to lyso-PC or fatty acids did not result in conversion of albumin into an SR-BI ligand **(Figure 4)**.

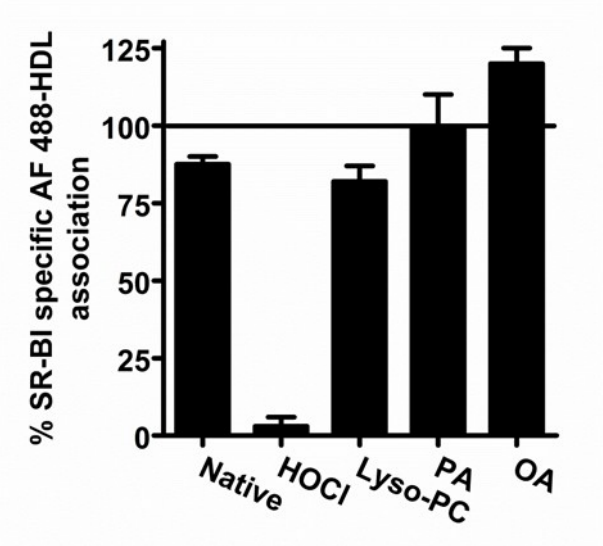

Figure 4: Coupling of Albumin to Lyso-PC and Fatty Acids does not generate SR-BI Inhibitors. *Albumin was saturated with lyso-PC, palmitic acid and oleic acid respectively. The conjugates (1-2 mg/mL in case of fatty acid-conjugates and 5 mg/mL for the lyso-PC-conjugate) were used to compete AF488-HDL (50 µg/mL) binding to SR-BI. LdlA(SR-BI) and control LdlA7 cells were incubated at 37°C for 2 hours with AF488-HDL in the presence of the indicated concentrations of the albumin conjugates. AF488-HDL association to SR-BI was assessed by flow cytometry. AF488-HDL association to SR-BI in absence of any competitor was set to 100%. Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to calculate SR-BI specific HDL association. Data are represented as mean ± SEM (n=2-4).*

5. Oxidation and Carbamylation do not affect Binding of Poly-L-lysine to SR-BI

To assess whether modified lysine residues of albumin are directly recognized by SR-BI, poly-L-lysine was HOCl-oxidized and carbamylated with KOCN. Poly-L-lysine is a cationic homopolymer containing in aqueous milieu a number of positively charged hydrophilic ε-amino groups which are neutralized upon exposure to HOCl or KOCN. The absence of other oxidizable amino acids should exclude any potentially interfering effect of additional amino acid modifications.

SR-BI overexpressing cells were exposed to the modified poly-L-lysine preparations in the presence of DiI-labeled HDL and SR-BI specific DiI-lipid uptake was assessed. In our competition experiments, none of the poly-L-lysine modifications showed any interference with SR-BI mediated HDL-lipid delivery **(Figure 5)**. This indicates that chlorinated lysine residues are not directly interacting with SR-BI and raises the possibility that neutralization of positively charged lysine residues entails alterations in the particular charge distribution in albumin that are critical for the transformation of a protein into an SR-BI ligand.

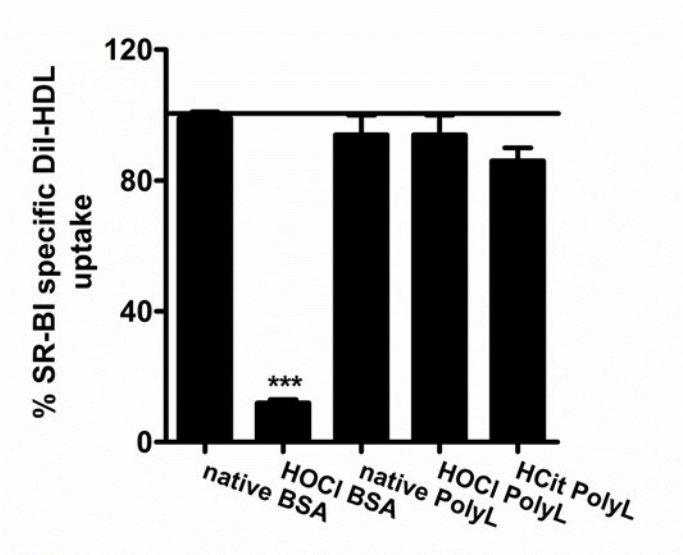

Figure 5: Modified Poly-L-lysine does not impair SR-BI mediated DiI-HDL Uptake. *LdlA(SR-BI) and control LdlA7 cells were incubated at 37°C for 4 hours with DiI-HDL (50 µg/mL) in the presence of native or modified poly-L-lysine (1 mg/mL). DiI-HDL uptake by SR-BI was assessed by flow cytometry. DiI-uptake in absence of any competitor was set to 100%. HOCl-BSA was used as positive control (1 mg/mL). Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to calculate SR-BI specific DiI-HDL uptake. Data are shown from one representative experiment including triplicate measurements.***P < 0.001 versus native BSA.*

6. A small Fraction of oxidized Albumin leads to Impairment of SR-BI mediated HDL-lipid Uptake

Earlier studies reported that albumin isolated from end stage renal disease patients on hemodialysis showed less pronounced SR-BI inhibitory activity compared to *in vitro* oxidized albumin in spite of similar AOPP-contents. This apparent discrepancy can be explained by the fact that, unlike *in vitro* oxidized albumin, HD-albumin is not uniformly oxidized but always exists as a mixture of oxidized and reduced forms with only a small fraction being highly oxidized (6). Based on the assumption that only a fraction of albumin is extensively modified, we were interested to determine the proportion of oxidized albumin in serum necessary to block SR-BI. Notably, already as little as 0.1% oxidized albumin in plasma clearly inhibited SR-BI mediated DiI-HDL uptake **(Figure 6)**.

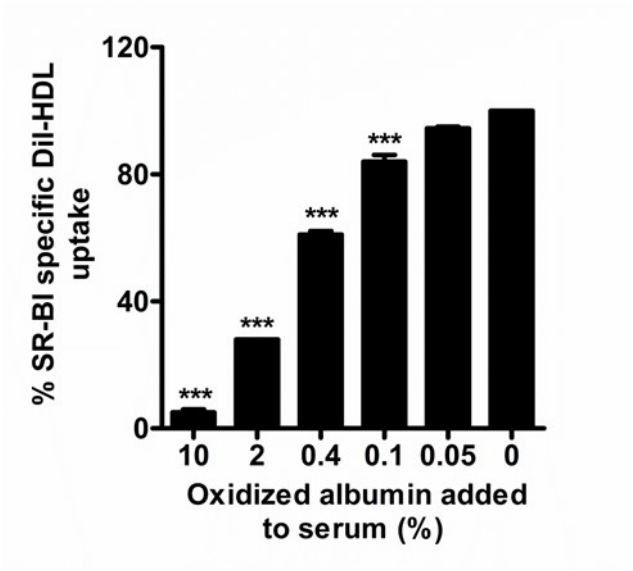

Figure 6: A minimal Fraction of oxidized Albumin in Plasma is sufficient to block DiI-HDL Uptake. *LdlA(SR-BI) and LdlA7 cells were incubated at 37°C for 4 hours with DiI-HDL (50 µg/mL) in presence of plasma which was spiked with indicated concentrations of HOCl-albumin. SR-BI specific DiI-HDL uptake was assessed by flow cytometry. DiI-HDL uptake by SR-BI in absence of any competitor was set to 100%. Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to obtain SR-BI specific DiI-HDL uptake. Data are represented from one typical experiment performed in triplicates. ***P < 0.001 versus plasma without oxidized albumin.*

7. AOPP-albumin blocks SR-BI mediated Cholesterol Efflux

A number of former studies has unraveled SR-BI as a key figure in RCT from cholesterol efflux from peripheral cells to the selective hepatic uptake of HDL-CE for excretion of cholesterol as bile acids (86). In addition surface expression of SR-BI enables macrophages to efflux cholesterol to mature HDL (87). Although the main routes for cholesterol efflux from macrophages proceed via ABCA1 (apoA-I) and ABCG1 (mature HDL) there is some indication resulting from animal experiments that SR-BI on macrophages has an atheroprotective function (87).

These observations brought us to analyze the impact of oxidized albumin on the efflux of free cholesterol from macrophages to HDL. Therefore THP-1 macrophages were labeled with [^{3}H] cholesterol and pre-incubated with native or oxidized albumin before cholesterol efflux to HDL was assessed. As expected we found a significant decrease in SR-BI mediated free cholesterol efflux in the presence of oxidized albumin compared to its unmodified counterpart **(Figure 7)**.

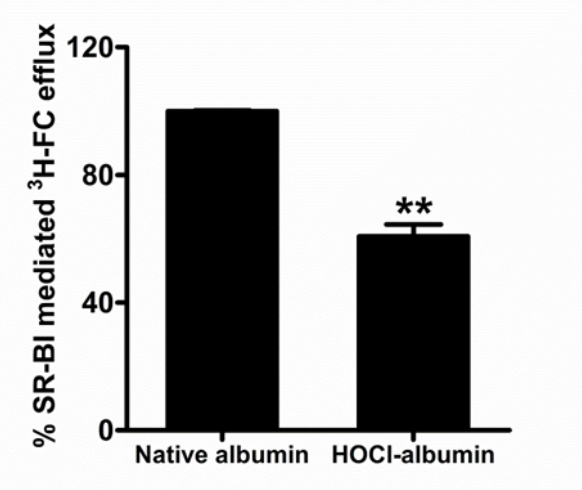

Figure 7: HOCl-albumin compromises SR-BI mediated Cholesterol Efflux from Macrophages to HDL. *THP-1 macrophages were labeled with [^{3}H] cholesterol (1 µCi/mL) for 48 hours and preincubated with native or HOCl-albumin (0.2 mg/mL) for 30 minutes at 37°C. After washing efflux was initiated by addition of HDL (50 µg/mL) and assessed after 3 hours by radioactivity measurements. Efflux of the radioactive label into the medium was calculated as percentage of radioactivity associated with the cells prior to addition of the cholesterol acceptor. Data are represented as mean ± SEM of one experiment measured in duplicates. **P < 0.01 versus Native albumin.*

8. Oxidized and carbamylated Albumin exhibit different Binding Characteristics with Respect to SR-BI

Prompted by the observation that HOCl-albumin was a more effective SR-BI inhibitor than HCit-albumin, binding affinities of modified, AF488-labeled albumin preparations to SR-BI were assessed. Experiments were performed at 4°C in order to prevent receptor internalization and in presence of excess native albumin to block unspecific binding sites.

Binding affinities of HOCl-albumin and HCit-albumin to SR-BI were similar as shown by the respective Kd-values of 47.1 ± 6.3 µg/mL for HOCl-albumin and 34.2 ± 5.2 µg/mL for HCit-albumin. In contrast, dissociation characteristics were clearly different **(Figure 8A and 8B)**. While a major fraction of cell-associated HCit-albumin readily dissociated, about 90% of chlorinated albumin remained associated to SR-BI even after more than 20 hours **(Figure 8B)**. These findings indicate that binding of HOCl-albumin to SR-BI is permanent whereas binding of HCit-albumin to SR-BI is reversible.

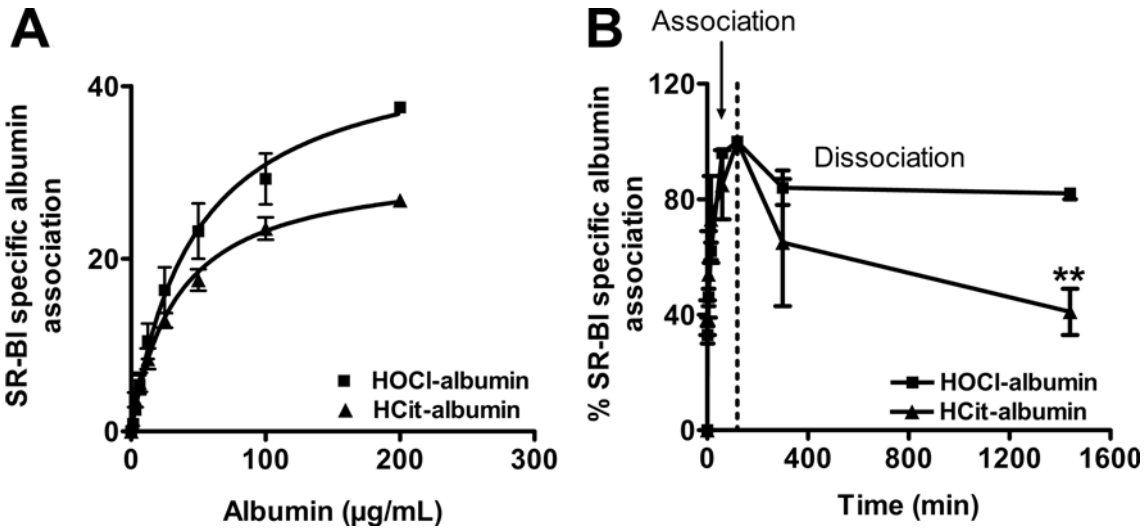

Figure 8: Concentration-dependent Association of modified Albumin to SR-BI. (A) *LdlA(SR-BI) and LdlA7 cells were incubated with the indicated concentrations of oxidized or carbamylated AF488-albumin for 2 hours at 4°C and AF488-albumin association was assessed by flow cytometry. Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to obtain SR-BI specific AF488-albumin association. Data are represented as mean ± SEM of two independent experiments performed in triplicates. Native AF488-labeled albumin showed no binding to SR-BI (data not shown).* ***Dissociation of modified Albumin. (B)*** *LdlA(SR-BI) and LdlA7 cells were incubated with 100 µg/mL oxidized or carbamylated AF488-albumin for 2 hours at 4°C. Subsequently, cells were washed and incubated with medium containing native albumin (3 mg/mL) for up to 22 hours. The remaining cell-associated AF488-albumin was assessed by flow cytometry and SR-BI specific AF488-albumin binding was assessed as described in (A). Data are shown as mean ± SEM of two independent experiments performed in triplicates. **P = 0.0085 versus HCit-albumin.*

9. HOCl-albumin irreversibly binds to SR-BI

To gain more insight into the different interactions of HOCl- and HCit-albumin with SR-BI, further experiments were performed employing excess modified, unlabeled albumin as competitor. Thereby we observed that a 30-fold molar excess of HOCl-albumin was not able to displace AF488 HOCl-albumin from SR-BI **(Figure 9A)**. By contrast, labeled HCit-albumin was effectively displaced by either HCit-albumin or HOCl-albumin **(Figure 9B)**. These results are in agreement with the experiments shown in Figure 8 and are a further indication of the irreversible HOCl-albumin/SR-BI interaction.

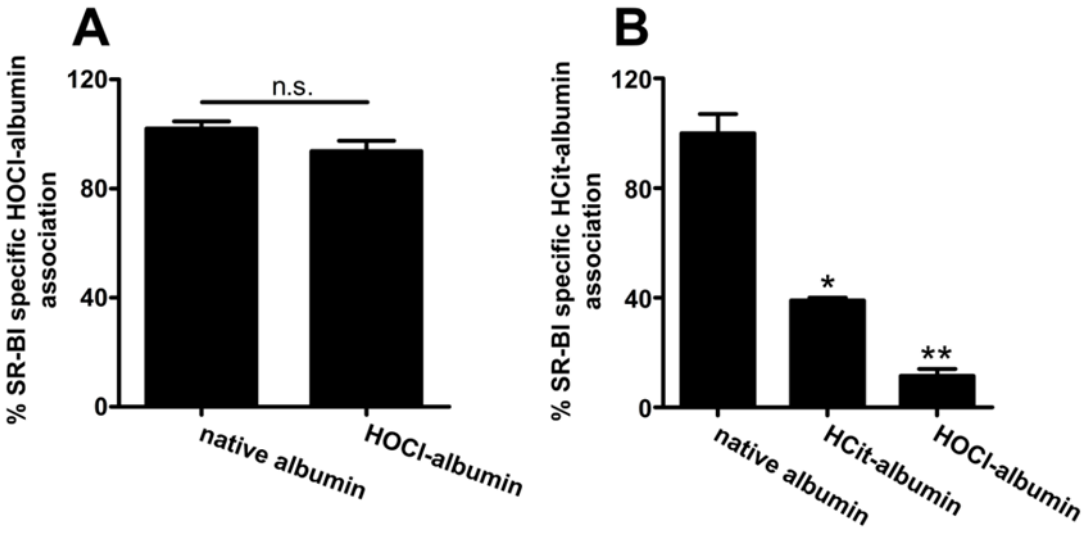

Figure 9: HOCl-oxidation converts Albumin into a permanent SR-BI Ligand. (A) *LdlA(SR-BI) and LdlA7 cells were incubated with 100 µg/mL AF488 HOCl-albumin for 2 hours at 4°C. After washing, cells were incubated for another 2 hours with a 30-fold molar excess of native (control) or HOCl-albumin (competitor). Data are represented as mean ± SEM (n=3).* ***(B)*** *LdlA(SR-BI) and LdlA7 cells were incubated with 100 µg/mL AF488 HCit-albumin for 2 hours at 4°C. After washing, cells were incubated for another 2 hours with a 30-fold molar excess of native, HCit- or HOCl-albumin. Data are represented as mean ± SEM (n=2). *P < 0.05; **P < 0.01 versus native albumin.*
(A, B) *Association of AF488-labeled albumin was measured by flow cytometry and SR-BI specific binding was assessed by subtraction of fluorescence values measured in LdlA7 cells from those measured in LdlA(SR-BI) cells.*

10. *N*-chloramines mediate irreversible Binding of HOCl-albumin to SR-BI

HOCl-oxidation of amino groups generates *N*-chloramines that preferentially target reduced cysteine residues of plasma proteins (88). Hence it can be supposed that long-lived *N*-chloramine derivatives promote irreversible binding of HOCl-albumin to SR-BI by reaction with Cys251 and/or Cys384 of SR-BI forming labile intermolecular sulfenamides or more oxidized sulfinamides / sulfonamides (88).

To explore the relevance of *N*-chloramines for irreversible binding, HOCl-albumin was incubated for 5 days at 37°C ("aged albumin") to allow complete decomposition of *N*-chloramines. Notably, aged HOCl-albumin was displaced from SR-BI by excess HOCl-albumin, indicating that albumin chloramines mediate irreversible receptor association **(Figure 10A)**.

Due to its susceptibility to oxidation, it is conceivable that oxidative modification of the free cysteine residue (Cys34) of albumin is involved in intermolecular cross-link formation with SR-BI.

To address this notion, albumin cysteine residues were alkylated using iodoacetamide prior to HOCl-treatment (89). However, competition experiments revealed that alkylating the free cysteine residue of albumin did not inhibit irreversible binding, suggesting that Cys34 oxidation is not required for irreversible binding **(Figure 10B)**.

The thiol detection assay kit (Cayman) was used to verify cysteine protection after iodoacetamide-treatment **(Figure 10C)**.

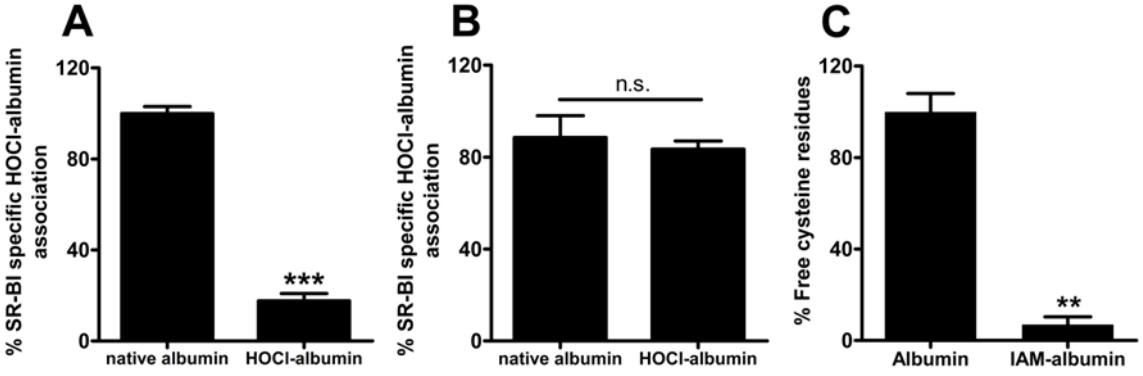

Figure 10: N-chloramines of HOCl-albumin are required for irreversible Binding. (A) *Albumin was oxidized and incubated for 5 days at 37°C to allow N-chloramine decomposition (aged HOCl-albumin). Subsequently, LdlA(SR-BI) and LdlA7 cells were incubated with 100 µg/mL of the labeled albumin preparation for 2 hours at 4°C. After washing, the cells were incubated for another 2 hours with a 30-fold molar excess of native or HOCl-albumin. Results are represented as mean ± SEM (n=3). ***P < 0.001 versus native albumin.*

(B) Cys34 Oxidation is not involved in Cross-link Formation. (B) *Free Cys34 residue of albumin was alkylated (protected) using iodoacetamide prior to HOCl-oxidation. LdlA(SR-BI) and LdlA7 cells were incubated with Cys34 alkylated AF488-HOCl-albumin*

(100 µg/mL) for 2 hours at 4°C. After washing, cells were incubated for another 2 hours with a 30-fold molar excess of native or HOCl-albumin. Data are shown as mean ± SEM (n=2).
***(A, B)** Association of AF488-labeled albumin was measured by flow cytometry and SR-BI specific binding was assessed by subtraction of fluorescence values measured in LdlA7 cells from those measured in LdlA(SR-BI) cells.*
***(C)** The free cysteine residue on albumin (Cys34) was alkylated using a 20-fold molar excess of iodoacetamide (IAM-albumin) and SH groups of cysteine were determined. Results are expressed as percentage free cysteine residues relating to native albumin (100%). **P < 0.01 versus albumin.*

11. HOCl-albumin forms covalent Cross-links with SR-BI

To test whether oxidized albumin covalently cross-links with SR-BI, HOCl-albumin treated SR-BI overexpressing cells and control cells were lysed and Western blot analysis was performed. Immunochemical detection of SR-BI on blotted membranes revealed a broad and diffuse high molecular weight band, indicating SR-BI/HOCl-albumin cross-links **(Figure 11)**. Importantly, the high molecular weight band was not detected when control LdlA7 cells were treated with HOCl-albumin or when SR-BI overexpressing cells were treated with native albumin. Given that the high molecular weight band of SR-BI was much less intense when SDS-PAGE was performed under reducing conditions, disulfide bonds or labile intermolecular sulfenamides or more oxidized sulfinamides / sulfonamides might, at least in part, mediate the irreversible binding of HOCl-albumin to SR-BI.

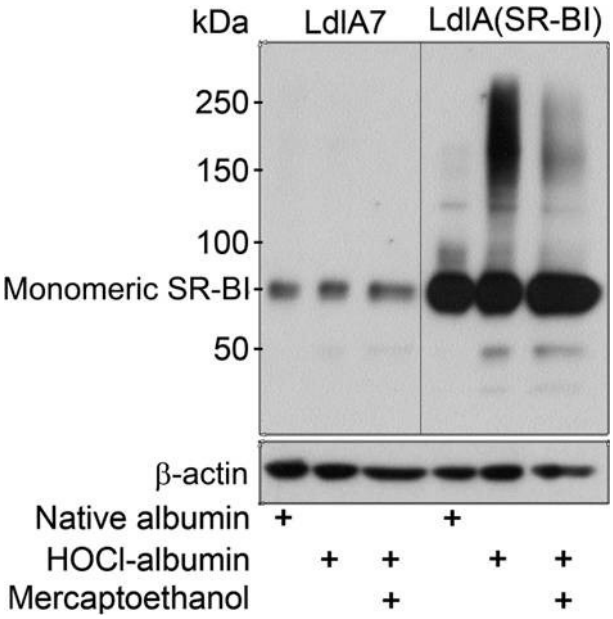

Figure 11: HOCl-treatment of Albumin leads to Cross-link Formation. *LdlA(SR-BI) and LdlA7 cells were incubated with 1 mg/mL native or HOCl-albumin for 2 hours at 37°C. Cells were rinsed, lysed and cell lysates were subjected to SDS-PAGE (4-20%) under reducing and not-reducing conditions. Separated proteins were transferred onto polyvinylidene difluoride membranes for subsequent SR-BI detection.*

12. Cys384 of SR-BI is not involved in Cross-link Formation

Given that *N*-chloramines of HOCl-albumin are likely to react with thiols giving rise to formation of sulfenic acid derivatives and stable higher oxidation products (88) an attempt was made to investigate the role of Cys384 of SR-BI for irreversible binding of HOCl-albumin to SR-BI. In order to selectively address the role of Cys384 in cross-link formation, the small thiosemicarbazone blocker BLT-1 **(Introduction, Figure 7)** was used from which it is known that it covalently interacts with Cys384 of SR-BI (76).

Cells were incubated with BLT-1 for one hour before binding properties of AF488 HOCl-albumin to SR-BI were investigated. We found that BLT-1 slightly increased binding of AF488 HOCl-albumin to SR-BI **(Figure 12A)** which seems feasible considering that former studies reported a common binding site of AOPP-albumin and HDL as well as increased binding of HDL to SR-BI in the presence of BLT-1 (68,76). Moreover our preliminary data provides evidence that the free thiol of Cys384 of SR-BI is not involved in cross-link formation since application of BLT-1 did not influence irreversible binding of HOCl-albumin to SR-BI **(Figure 12B)**. Therefore, further investigations are needed to unequivocally define the molecular structure(s) involved in cross-link formation.

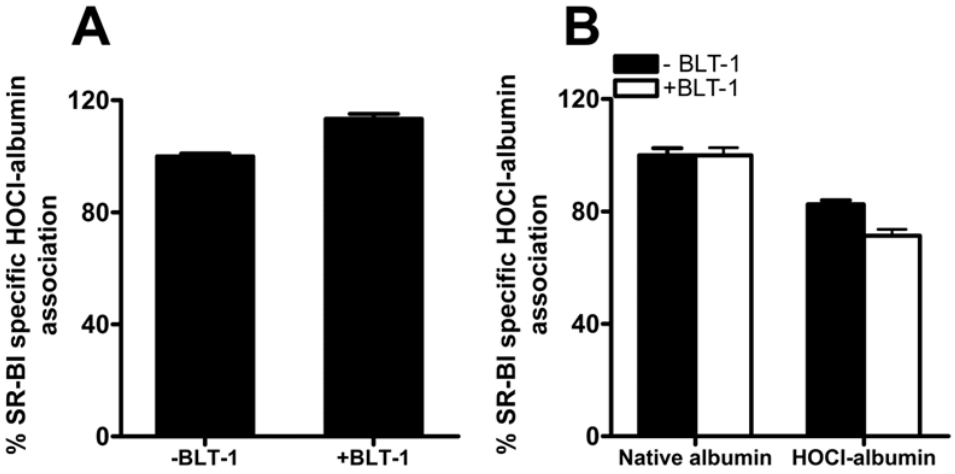

Figure 12: Cys384 of SR-BI does not participate in Cross-link Formation of HOCl-albumin with SR-BI. *LdlA(SR-BI) and LdlA7 control cells were incubated +/- 10 µM BLT-1 in serum free Ham's F12 for one hour at 37°C. After thoroughly washing, cells were brought to 4°C and incubated with 100 µg/mL AF488-HOCl-albumin in HBSS for 2 hours. Thereafter cells for assessment of HOCl-albumin association were harvested and analyzed by flow cytometry **(A)**. Remaining cells were incubated with 3 mg/mL native or HOCl-modified albumin in serumfree Ham's F12 for 2 more hours at 4°C before they were equally harvested and analyzed **(B)**.*

SR-BI specific binding was assessed by subtraction of fluorescence values measured in LdlA7 cells from those measured in LdlA(SR-BI) cells. Data are represented as mean ± SEM from one experiment performed in triplicate.

13. HOCl-albumin colocalizes with SR-BI in LdlA(SR-BI) Cells

The role of receptor ligand endocytosis in SR-BI mediated cholesterol ester uptake is still an area of controversy. In the past several studies provided evidence that SR-BI specific lipid uptake occurs on the cell surface and is independent of receptor or lipoprotein particle endocytosis (66). The proposed, alternative endocytosis-dependent pathway is supported by recent reports showing that in physiologically relevant cell lines selective lipid uptake involves HDL internalization and re-secretion and that endocytosis of SR-BI parallels endocytosis of HDL (69).

To assess whether cells internalize SR-BI/HOCl-albumin complexes, LdlA(SR-BI) and control LdlA7 cells were incubated with AF488-labeled HOCl-albumin for immunohistochemical analysis. Immunohistochemical staining showed that AF488-labeled HOCl-albumin exhibited intracellular colocalization with SR-BI in LdlA(SR-BI) cells, suggesting receptor internalization and efficient uptake of HOCl-albumin **(Figure 13)**. SR-BI association of native AF488-albumin was not detected under these experimental conditions. Control LdlA7 cells showed very low uptake of HOCl-albumin **(Figure 13)**.

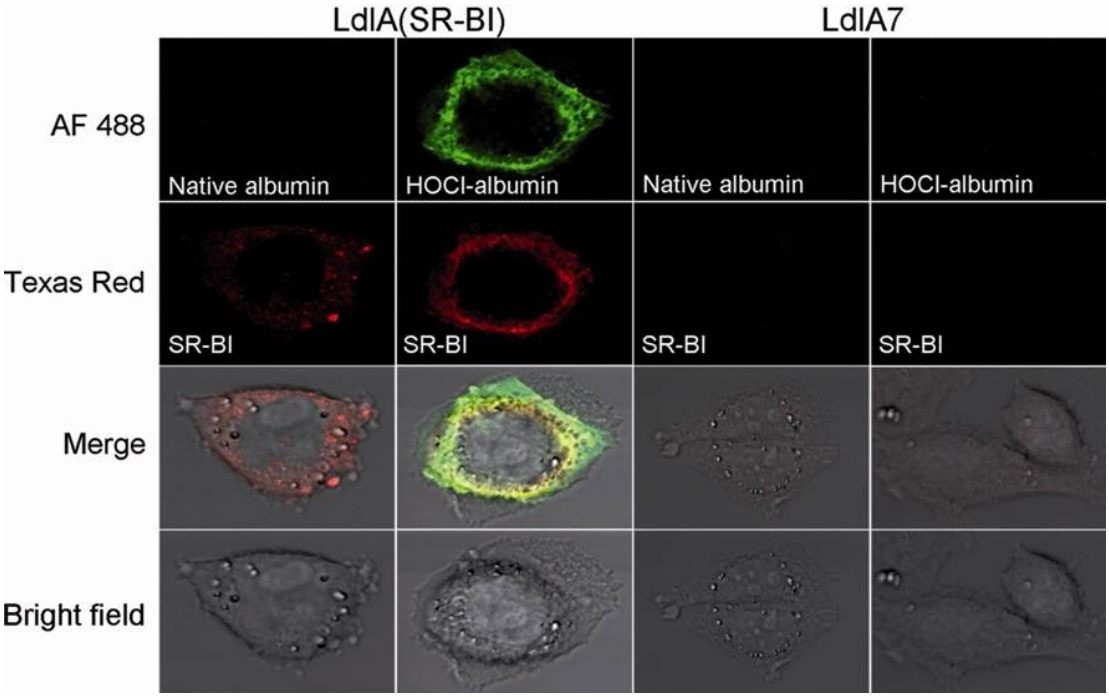

Figure 13: AF488-labeled HOCl-albumin colocalizes with SR-BI in LdlA(SR-BI) Cells. *LdlA(SR-BI) and LdlA7 cells were incubated with 100 µg/mL AF488-labeled native or HOCl-albumin for 2 hours at 37°C. Cells were rinsed, fixed with 2% paraformaldehyde for 30 minutes and permeabilized with Triton X 100 in PBS for 15 minutes. Anti-SR-BI antibody (1:300 in PBS/5% BSA) was applied at room temperature under light exclusion and without*
prior washing. Texas Red-coupled goat anti-rabbit IgG (1:500 in PBS/5% BSA) was used as secondary antibody.

14. The SR-BI inhibitory Activity of Albumin isolated from ESRD-patients correlates with the Content of 3-chlorotyrosine and the Composition of HDL

AOPPs such as oxidized albumin accumulate in the plasma of renal disease patients. AOPPs show a negative correlation with residual renal function (25,42) and are an indicator of increased cardiovascular disease risk (51). MPO levels correlate with plasma AOPPs in CKD and albumin isolated from the plasma of CKD-patients shows increased levels of 3-CT which is a specific marker for MPO-catalyzed reactions (79).

Albumin was isolated from plasma of HD-patients and controls and levels of AOPPs and 3-CT were determined. In line with previous findings, we observed that AOPP levels are significantly increased in HD-albumin **(Figure 14A)** and that HD-albumin interferes with ^{125}I-HDL binding to SR-BI **(Figure 14B)**. In addition we demonstrate that the SR-BI inhibitory activity of HD-albumin, measured as the efficacy to attenuate ^{125}I-HDL association to SR-BI, significantly correlated with the MPO-specific oxidation product 3-chlorotyrosine **(Figure 14C)**. It was previously shown that SR-BI deficiency in mice resulted in the accumulation of CE-enriched and phospholipid (PL)-depleted HDL particles (90). Consequently, blockade of SR-BI through modified albumin may modulate HDL-lipid composition in HD-patients.

To address this question, HDL was isolated from plasma of ESRD-patients (n=12) and controls (n=10). Clinical characteristics of control subjects and HD-patients and a detailed characterization of control and HD-albumin are given in **Tables 2** and **3**.

CE and PL content of isolated HDL was determined and the relationship between the SR-BI inhibitory activity of HD-albumin and the HDL-CE/PL ratio was examined. Interestingly we observed that the SR-BI inhibitory activity of HD-albumin significantly correlates with the CE/PL composition of isolated HDL **(Figure 14D)**.

Table 2: Clinical Chemistry of ESRD-patients and Control Subjects

	Control	ESRD
n	10	12
age (yr)	52 (45 - 67)	69 (48 - 78)
male/female	4/6	5/7
plasma parameter		
Cholesterol (mg/dL)	197 (180 - 228)	168 (137 - 215)
HDL (mg/dL)	69 (47 - 81)	45 (40 - 48)
LDL (mg/dL)	110 (85 - 133)	107 (64 - 132)
Triglycerides (mg/dL)	112 (69 - 199)	144 (86 - 193)
Urea (mg/dL)	28 (25 - 29)	119 (91 - 171)
C-reactive protein (mg/L)	1.0 (0.5 - 5.5)	8.1 (2.9 - 22.4)
Fibrinogen (mg/dL)	303 (217 - 433)	468 (408 - 594)
Creatinine (mg/dL)	0.93 (0.76 - 1.14)	7.08 (5.91 - 10.18)
Uric acid (mg/dL)	4.8 (4.2 - 5.1)	5.6 (5.0 - 6.7)

Results are given as median and interquartile range.

Table 3: Characterization of Control and HD-albumin

	Control	ESRD
AOPP [nmol/mg protein]	0.9 ± 0.4	3.2*** ± 0.60
CML [µmol/mol lysine]	37.7 ± 8.9	96.5*** ± 34.0
HCit [µmol/mol lysine]	129.7 ± 24.1	218.4*** ± 46.2
3-CT [µmol/mol tyrosine]	2.3 ± 1.8	4.4* ± 2.7
Cys [mol/mol albumin]	0.9 ± 0.2	0.5** ± 0.2

Results are given as mean ± standard deviation.

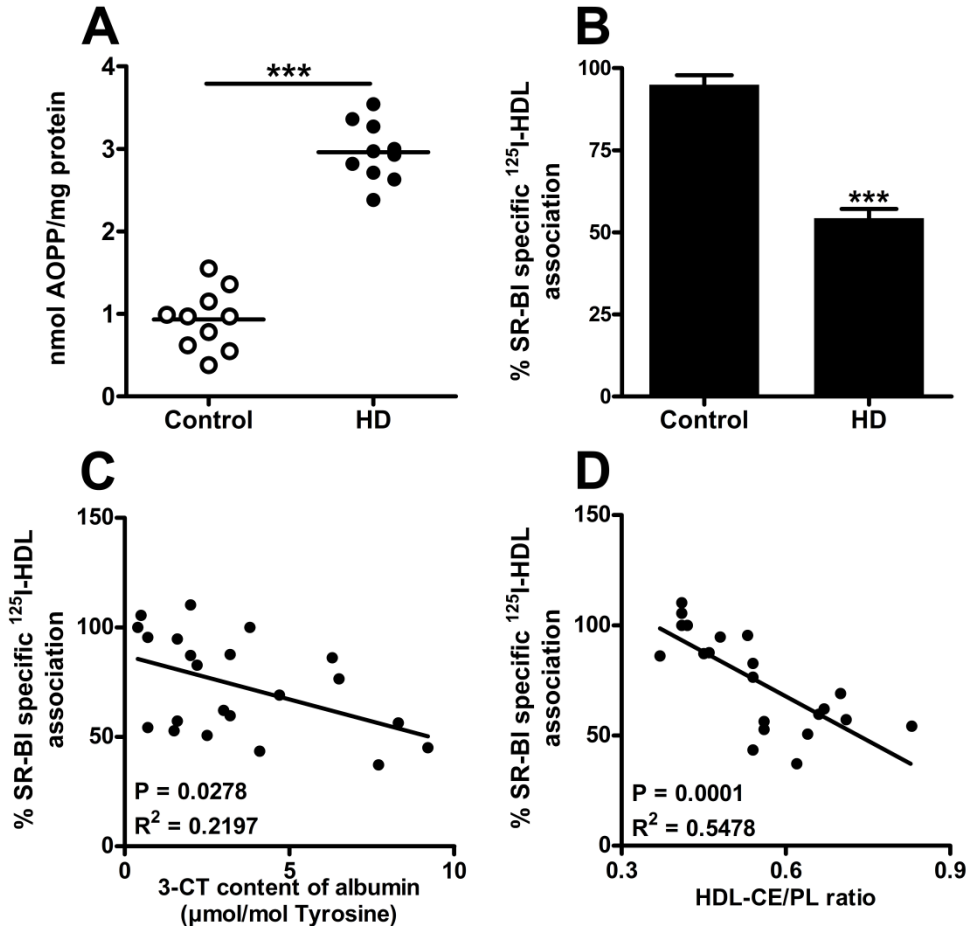

Figure 14: AOPP Content of HD-albumin is significantly increased. (A) *Albumin was isolated from the plasma of HD-patients (HD) and control subjects (Control) by affinity chromatography and AOPP content of the isolated albumin was measured at 340 nm under acidic conditions. AOPP values are expressed as chloramine-T equivalents.*
Albumin isolated from ESRD-patients interferes with HDL Binding to SR-BI. (B) *LdlA(SR-BI) cells and control LdlA7 cells were incubated in DMEM for 2 hours at 37°C with ^{125}I-HDL (50 µg of protein/mL) in the presence of 1 mg/mL albumin isolated from plasma of ESRD-patients on hemodialysis (HD) (n=12) and control subjects (Control) (n=10). Values measured in LdlA7 cells were subtracted from those obtained in LdlA(SR-BI) cells to calculate SR-BI specific binding. Data are represented as mean ± SEM of two independent experiments performed in triplicates. ***P < 0.001 versus Control.* ***(C)*** *3-CT content of the individual albumin samples was plotted against their respective SR-BI inhibitory activities (expressed as SR-BI specific ^{125}I-HDL-association).* ***(D)*** *CE/PL ratio of HDL isolated from HD-patients and control subjects was plotted against SR-BI inhibitory activity of the respective albumin samples (expressed as SR-BI specific ^{125}I-HDL-association).*

15. Lysine Oxidation Products contribute to the increased Affinity of HOCl-HDL to SR-BI

Under conditions of increased oxidative stress lipoproteins are increasingly affronted and modified by reactive oxidants. Previous studies have shown that HOCl-oxidation not only converts HDL into a proatherogenic particle but also increases binding affinity to its physiological receptor proportional to the degree of HOCl-modification (91). HOCl-HDL binds to the same or overlapping domains on SR-BI as does native HDL but exhibits nonproductive binding characteristics indicated by stepwise decrease of SR-BI mediated lipid-tracer uptake with increasing extent of HOCl-modification of HDL. Increased affinity in combination with nonproductive binding results in blockage of association of native HDL and reduced clearance rates of HDL cholesterol esters (91).

Along the lines of the initial investigations of the structural alterations required for binding of oxidized albumin to SR-BI it seemed worth attempting to define the importance of lysine oxidation products for high affinity binding of HOCl-HDL to SR-BI (91).

We observed that HOCl-modified HDL effectively displaced labeled native HDL from SR-BI highlighting its increased affinity. In contrast to albumin, the effect was only partially circumvented by reductive methylation of lysine as well as regeneration of lysine chloramines through addition of L-methionine **(Figure 15A and 15B)**. This indicates that the presence of lysine oxidation products is involved in altering the binding properties of HDL but concurrently that *N*-chloramines are not the sole cause of increased affinity to SR-BI.

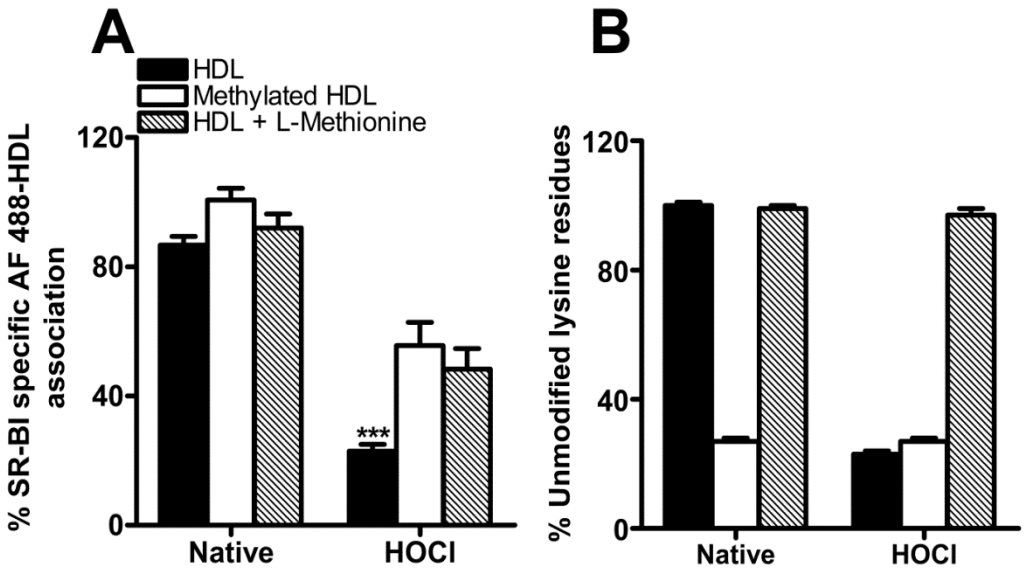

Figure 15: Lysine Oxidation increases the Affinity of HDL to SR-BI. (A) *LdlA(SR-BI) and control LdlA7 cells were incubated at 37°C for 2 hours with AF488-HDL (50 µg/mL) in the presence of either 15 µg/mL native HDL or HOCl-HDL. HOCl-induced lysine*

*chloramine formation was either prevented by precedent reductive methylation (Methylated HDL) or reversed by addition of excess free L-methionine 30 minutes after HOCl-treatment (HDL + L-Methionine). AF488-HDL association to SR-BI was assessed by flow cytometry. AF488-HDL association to SR-BI in absence of any competitor was set to 100%. Average fluorescence values measured in LdlA7 cells were subtracted from those measured in LdlA(SR-BI) cells to calculate SR-BI specific HDL association. Data are represented as mean ± SEM (n=8). ***P < 0.001 versus native HDL.*
(B) *Residual unmodified lysine residues of the different HDL preparations were assessed using the fluorescamine assay.*

16. HOCl-HDL Association to SR-BI is reversible

Based on the observation that HOCl-oxidation of albumin implicates irreversible association to SR-BI we were interested to assess whether binding characteristics of HOCl-HDL would resemble those of HOCl-albumin.

Our results show that, association of HOCl-HDL to SR-BI proceeds in a comparable manner to association of oxidized and carbamylated albumin to SR-BI. Binding affinity of all modified ligands was superior to that of native HDL and binding capacity of HOCl-HDL was doubled compared to its unmodified counterpart.

However, in sharp contrast to results obtained with HOCl-albumin (about 80% of HOCl-albumin remained permanently associated to SR-BI) **(Figure 8)**, HOCl-modified HDL dissociated almost completely **(Figure 16)**.

These findings indicate that solely the presence of *N*-chloramines within an SR-BI ligand does not necessarily entail irreversible binding. The specific arrangement of the chloramines within the structure of albumin seems to critically determine its binding characteristics.

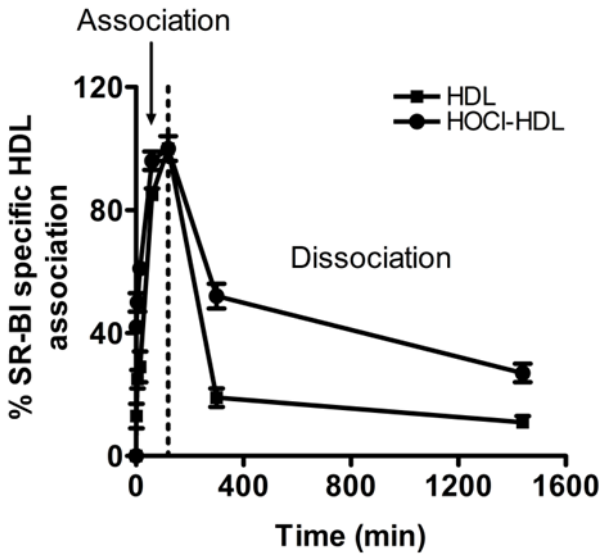

Figure 16: Dissociation of native and HOCl-modified HDL. *LdlA(SR-BI) and LdlA7 cells were incubated with 100 µg/mL native or oxidized AF488-HDL for 2 hours at 4°C. Subsequently, cells were washed and incubated with medium containing native albumin (3 mg/mL) for up to 22 hours. The remaining cell-associated AF488-HDL was assessed by flow cytometry and SR-BI specific AF488-HDL binding was assessed by subtraction of average fluorescence values measured in LdlA7 cells from those measured in LdlA(SR-BI) cells.*

Data are represented as mean ± SEM of three independent experiments performed in triplicates for HDL and one representative experiment performed in triplicates for HOCl-HDL.

IV. Discussion

The heme enzyme myeloperoxidase is released by degranulation of activated human neutrophils at sites of inflammation and catalyzes the reaction of hydrogen peroxide with chloride ions to form hypochlorous acid. HOCl is critically involved in the inflammatory response indicating that MPO represents a key component of immune defense. However, oxidative damage of host tissues has been described as a common consequence of a number of inflammation-associated pathologies and high plasma levels of MPO are considered as a strong independent risk factor for the development of cardiovascular diseases (92).

MPO avidly binds to cell membrane glycosaminoglycans, therefore plasma protein oxidation in close vicinity to cells potentially has important implications for the regulation of cellular function during inflammation (93). Of particular interest, MPO may not only locally oxidize proteins, as shown by increased levels of circulating AOPPs described in a number of chronic inflammatory diseases (10,94).

Several studies have been conducted aiming at analyzing end-products of HOCl-induced damage in plasma proteins including the extent of antioxidant or thiol consumption, protein cross-linking or aggregation and the conversion of amino groups into chloramines (92).

HOCl mediated oxidation of the major plasma protein albumin was previously investigated, predicting that plasma proteins consume the majority of HOCl with limited damage to other materials (92). Ascorbate or α-tocopherol, even at levels achieved in human supplementation studies, could not attenuate these reactions. Amino acid analysis indicated that cysteine, methionine, lysine, tyrosine, histidine and arginine residues are among the prime targets when proteins are exposed to HOCl *in vitro* (51,95).

Reaction rates of HOCl in plasma were found to be the highest with thiols while conversion of amino groups to *N*-chloramines was not observed until complete thiol depletion (88,96). However, given that plasma contains approximately a 100-fold excess of free amines over thiols, a considerable number of protein-associated *N*-chloramines is expected to be formed (88).

Elevated thiocyanate levels, as detected in heavy smokers, can modulate HOCl mediated reactions resulting in formation of hypothiocyanous acid and cyanate, which causes protein carbamylation and oxidation of thiol groups (79,97-99). In addition, MPO was shown to promote formation of glycolaldehyde and other reactive aldehydes and may therefore play a pathogenic role by generating advanced glycation end products like CML

(84,100). Mass spectrometry analyses have shown that HCit- and CML-content of albumin isolated from the plasma of ESRD-patients are significantly increased compared to control albumin indicating that carbamylation and AGE-modification are relevant *in vivo*.

Native albumin is not recognized by the major HDL receptor SR-BI but oxidation and carbamylation convert albumin into an SR-BI ligand. In the present study we discovered that certain lysine modifications are responsible for high affinity binding of albumin to SR-BI.

Modification/oxidation of lysine residues alters structural properties of proteins through elimination of positive charges leading to the assumption that neutralization of the positively charged ε-amino groups of lysine residues critically controls recognition of albumin by SR-BI. While oxidation and/or carbamylation neutralize the positive charge on the amino group of lysine, reductive methylation selectively modifies lysine residues of albumin without altering the positive charge. Interestingly we observed that when reductively-methylated albumin was oxidized with HOCl, it did not bind to SR-BI, despite oxidation of cysteine, methionine, tyrosine, histidine, and arginine residues (95). Furthermore, regeneration of lysine chloramines by addition of free L-methionine (101), restoring the positive charge on the nitrogen, completely averted HDL displacement by oxidized albumin in competition experiments.

CML-modification of albumin did not lead to formation of recognition motifs for SR-BI, suggesting that the negative charge of the carboxyl group and/or structural differences of CML prevent binding of CML-albumin to SR-BI. This is in line with earlier findings referring to AGE-BSA preparations devoid of ligand activity for scavenger receptors irrespective of the presence of CML, known to be a major immunological epitope of AGE (86). Consequently binding to scavenger receptors is most probably mediated by AGE-structures different from CML although it is established that CML serves as recognition motif for the receptor of advanced glycation end products (86). Other investigators found that AGE-albumin was an SR-BI ligand but unlike HOCl-albumin and HCit-albumin does not share the HDL binding domain (51). Therefore AGE-albumin probably interferes with SR-BI mediated lipid transfer by other indirect mechanisms.

Interestingly, we observed that extensively HOCl-oxidized poly-L-lysine failed to displace HDL from SR-BI. These results indicate that chlorinated lysine residues are not directly recognized by SR-BI and strengthen the hypothesis that alterations in the particular charge distribution caused by neutralization of charged lysine residues are a likely determinant for the transformation of albumin into an SR-BI ligand.

HOCl-albumin not only prevented HDL binding to SR-BI but also significantly reduced SR-BI mediated HDL-CE delivery. Thereby a minimal fraction of 0.1% of oxidized albumin in serum was sufficient to selectively inhibit SR-BI mediated lipid uptake.

Due to the long life-span of albumin in circulation complete decomposition of albumin-located unstable *N*-chloramines is expected. We observed that HOCl-albumin, which was stored at 37°C to induce decomposition of chloramines, still blocked SR-BI mediated HDL-lipid uptake in cell experiments. However, addition of free L-methionine did not reverse the inhibitory effect mediated by aged HOCl-albumin. These findings indicate that after incubation *N*-chloramines were no longer present on HOCl-albumin and that both *N*-chloramines and their decomposition products similarly transform albumin into an SR-BI antagonist.

Prompted by the observation that HOCl-albumin was a more efficient SR-BI inhibitor than HCit-albumin, we conducted binding studies of the different albumin modifications.
Based on the finding that the major part of HOCl-albumin remained associated to SR-BI even in the presence of excess unlabeled HOCl-albumin as competitor, our study provides the novel evidence that HOCl-induced oxidation of plasma albumin generated irreversible HDL receptor inhibitors.
Compared to α-monochloroamines, ε-chloramines are more stable and potentially react with cysteine residues in plasma and isolated proteins (88). It is of interest to note that protein thiols are decreased in a number of inflammatory diseases (10,94), which could be due to generation of long-lived, thiol-specific *N*-chloramine derivatives of HOCl (91).
Previous studies referred to cysteine oxidation as a trigger of HOCl-induced intramolecular and intermolecular sulfenamide-, sulfinamide-, and sulfonamide-generation (102,103). Our results suggest that oxidation of Cys34 in albumin is unlikely to be involved in cross-link formation with SR-BI, since methylation of Cys34 prior to oxidation did not inhibit irreversible binding to the receptor. However, given that two of the six exoplasmic cysteines of SR-BI are free thiol groups (Cys251 and Cys384) (76), it is tempting to speculate that albumin-chloramines may oxidize SR-BI Cys251 and/or Cys384 thereby forming labile intermolecular sulfenamides or more oxidized sulfinamides / sulfonamides.
When SR-BI overexpressing cells were incubated with HOCl-albumin, we detected high-molecular-weight complexes containing SR-BI by Western blot analysis. Addition of mercaptoethanol markedly reduced the high-molecular-weight bands, favouring the assumption that free Cys251 and/or Cys384 residues of SR-BI may contribute to cross-link formation.

Preliminary experiments employing the small thiosemicarbazone blocker of lipid transport BLT-1, known to irreversibly interact with Cys384 of SR-BI (76) revealed that despite inaccessibility of Cys384, irreversible binding of HOCl-albumin to SR-BI was not prohibited. This implies that if thiol/*N*-chloramine interactions between SR-BI and HOCl-albumin actually represent the molecular basis of cross-link formation the only target remaining would be Cys251 of SR-BI. But for all that, additional experiments are essential to unambiguously define the molecular structures involved.

A previous study reported that HOCl may induce protein aggregation due to strong, non-covalent interactions between oxidized protein chains (104). Other studies have proposed cross-linking via dityrosine formation or Schiff base formation between proteins through chloramine-derived aldehydes and lysine residues (105). In the present study, we observed that HOCl-albumin, which was incubated at 37°C to induce break-down of chloramines, reversibly binds to SR-BI. Given that *N*-chloramines decompose into the carbonyl aminoadipic semialdehyde, Schiff base formation may not significantly contribute to albumin/SR-BI cross-link formation.

Even though the atheroprotective function of SR-BI is mostly imputed to its key position in reverse cholesterol transport, the HDL/SR-BI tandem confers additional atheroprotective actions. For instance, HDL binding to SR-BI protects endothelial cells from apoptosis (75). Moreover SR-BI is highly expressed in lipid-laden macrophages in atherosclerotic tissue (106) where it represents one pathway for efflux of free cholesterol to mature HDL and thus potentially participates in the prevention of foam cell formation (87).
We discovered that HOCl-albumin significantly reduced cholesterol efflux from macrophages to HDL *in vitro* which permits us to assume that blockade of SR-BI mediated cholesterol efflux by oxidized/modified albumin might contribute to cholesterol accumulation in macrophages and the development of foam cells in the vessel wall. According to previous findings treatment of hypercholesterolemic rabbits with repeated intravenous injections of HOCl-albumin significantly increased macrophage infiltration and smooth muscle cell proliferation in atherosclerotic plaques. Interestingly, this was associated with significantly increased plasma total cholesterol, which is mainly carried by HDL in rabbits (107).

The observation that SR-BI can be irreversibly blocked by oxidized albumin may be of particular relevance, since SR-BI expressed on hepatocytes functions as a positive regulator of cholesterol efflux from macrophages (108). Therefore, SR-BI blockade by oxidized/modified albumin might contribute to alterations in the composition of HDL, as

observed in the present study. This is in good agreement with recent investigations reporting that compositional alterations in HDL may significantly impair HDL functionality in ESRD (109,110).

A recent study demonstrated active participation of lymphatic vessels in RCT by promoting return of cholesterol from macrophages and peripheral tissues to the circulation through a mechanism that involves SR-BI (70,71). Therefore, irreversible blockade of SR-BI on lymphatic endothelial cells by HOCl-albumin might make a pivotal contribution to lipid accumulation, foam cell formation and finally to the accelerated onset of CKD-associated atherocsclerosis.

Intravenous injections of HOCl-albumin in unilateral nephrectomy rats activated the intrarenal renin-angiotensin system via a CD36 mediated pathway and induced podocyte apoptosis in mice via the receptor of advanced glycation end products (50,111). Given that HOCl-albumin is recognized by the receptor for advanced glycation end products and CD36 (47,50), our data raise the possibility that HOCl-modified albumin also irreversibly cross-links with these receptors.

It is generally accepted that oxidized lipoproteins play a role in the genesis of atherosclerosis (112). Evidence indicates that HDL is more susceptible to oxidation and thereby protects LDL from oxidative damage (113). However, *in vitro* studies showed that HOCl-modification can adversely affect beneficial properties of HDL resulting in increased uptake by macrophages, decreased ability to remove cellular cholesterol (112) as well as in increased affinity but non-productive binding to SR-BI (91).
In addition it was described that exposure of LDL to HOCl results in almost immediate amino acid oxidation, carbonyl formation and protein aggregation and converts LDL into a high uptake form for macrophages (105). Notably, protection of lysine residues by reductive methylation inhibited all oxidation-induced proatherogenic activities of LDL described above indicating that lysine oxidation products are crucially involved in rendering LDL a high-uptake form for macrophages (105).
We obtained experimental evidence that oxidative modification of HDL increased binding affinity to SR-BI, but compared to the results obtained with HOCl-modified albumin, protection of lysine residues or regeneration of lysine chloramines only partially reversed the oxidation-induced increase in binding affinity. Therefore in addition to modification of lysine residues through HOCl, changes in the lipid moiety of HDL may have contributed to the increased binding affinity of HOCl-HDL to SR-BI.

These results are in agreement with former studies reporting that in contrast to LDL, where the lipid part appears to be protected from oxidation until comparatively high oxidant to protein ratios, detectable oxidation of HDL fatty acids already occurs at physiological NaOCl concentrations (113).
Interestingly we made the observation that in contrast to HOCl-albumin association of HOCl-HDL to SR-BI was reversible indicating that chloramines within specific structural motifs are required to induce cross-link formation.

One of the most important observations of the present study was that albumin isolated from the plasma of end stage renal disease patients blocked HDL association to SR-BI in cell experiments and that the SR-BI inhibitory activity significantly correlated with levels of the MPO-specific oxidation product 3-CT. We observed that blockade of SR-BI through HD-albumin was associated with alterations in the lipid composition of HDL isolated from the plasma of uremic patients as evidenced by a significant correlation between the SR-BI inhibitory activity of uremic albumin and the CE/PL ratio of isolated HDL. Our findings are in good agreement with a previous study reporting that SR-BI deficiency in mice resulted in the accumulation of cholesterol ester-enriched and phospholipid-depleted HDL particles (93).

In conclusion, the present study demonstrates that modified lysine residues mediate albumin/SR-BI interaction and provides the novel observation that *N*-chloramines transform plasma albumin into an irreversible SR-BI inhibitor. Moreover, blockade of SR-BI through an oxidized fraction of plasma albumin may alter HDL composition and probably HDL functionality. Given that several potential atheroprotective activities of HDL are mediated by SR-BI, a permanent SR-BI blockade may therefore contribute to the pathophysiology of cardiovascular disease.

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