The proteomic signature of dysfunctional uremic HDL identifies SAA as proinflammatory component

Thomas Weichhart[*], Chantal Kopecky[*], Markus Kubicek[†], Michael Haidinger[*], Dominik Döller[*], Karl Katholnig[*], Cacang Suarna[‡], Philipp Eller[§], Markus Tölle[¶], Christopher Gerner [**], Gerhard J Zlabinger[||], Markus van der Giet[¶], Walter H. Hörl[*], Roland Stocker[‡] and Marcus D Säemann[*]

[*] Department of Internal Medicine III, Division of Nephrology and Dialysis, Medical University of Vienna, Vienna, Austria

[†] Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Vienna, Austria

[‡] Centre for Vascular Research, School of Medical Sciences (Pathology) and Bosch Institute, Sydney Medical School, University of Sydney, Camperdown, Australia

[§] Department of Internal Medicine I, Graz Medical University, Graz, Austria

[¶] Charité - Universitätsmedizin Berlin, Campus Benjamin Franklin, Med. Klinik mit Schwerpunkt Nephrologie, Berlin, Germany

[**] Department of Medicine I, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria

[||] Institute of Immunology, Medical University of Vienna, Vienna, Austria

Running title: Proteomics of uremic HDL

Correspondence: Dr. Thomas Weichhart or Dr. Marcus D Säemann, Department of Internal Medicine III, Division of Nephrology and Dialysis, Medical University Vienna, Währinger Gürtel 18-20, Vienna, Austria. Phone: +43(1)40400 5593; Fax:

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+43(1)40400 7790; E-mail: thomas.weichhart@meduniwien.ac.at or marcus.saemann@meduniwien.ac.at

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ABSTRACT

Cardiovascular events in chronic kidney disease (CKD) and in end-stage renal disease (ESRD) are driven by risk factors including inflammation and oxidative stress. The atheroprotective properties of high-density lipoprotein (HDL) are impaired in uremia, however, the reasons for this defect remain unresolved. Here we demonstrate a novel anti-inflammatory property of HDL on monocytes along with a profound defect of this function in HDL from ESRD patients. We therefore employed mass spectrometry and identified 49 HDL-associated proteins in an uremia-specific pattern. Several of these proteins were previously not known to reside in HDL and were highly enriched in ESRD-HDL including surfactant-protein B (SP-B), apolipoprotein C-II, serum amyloid A (SAA) or α-1-microglobulin/bikunin precursor. Importantly, some of these ESRD-enriched proteins could already be detected in earlier CKD stages. Of note, assessing HDL oxidation and also oxidation susceptibility, we could not detect an altered oxidation status of uremic HDL compared to healthy controls. At the functional level, SAA but not the other uremia-specific proteins mimicked ESRD-HDL by promoting inflammatory cytokine production and reverting the anti-inflammatory properties of healthy HDL. Furthermore, SAA levels in ESRD-HDL correlated with the defective anti-inflammatory potency indicating that the presence of SAA induces proinflammatory properties within HDL. In conclusion, we demonstrate a novel anti-inflammatory role of HDL that is defective in uremic patients due to specific alterations of its molecular composition. These data may have broad implications for the regulation of immune responses *in vivo* and provide a molecular explanation for the excessive inflammation and cardiovascular mortality in uremia.

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End-stage renal disease (ESRD) or stage 5 chronic kidney disease (CKD) represents a major health problem and requires renal replacement therapy such as maintenance dialysis.[1,2] Mortality remains above 20 percent per year in the United States with the use of dialysis, with more than half of the deaths related to cardiovascular disease (CVD).[3-5] Atherosclerosis as underlying cause for cardiovascular morbidity and mortality is increased up to 30-fold in patients with ESRD, as well as in milder degrees of renal dysfunction such as stage 3 and 4 CKD, which have a moderate and severe reduced glomerular filtration rate (GFR), respectively.[4,6-9] Several factors including inflammation, oxidative stress, and dyslipidemia are considered decisive for the progression of atherosclerosis in ESRD.[10,11] Dyslipidemia in ESRD patients is characterized by a dysregulation of the synthesis and activity of high-density lipoprotein (HDL) leading to decreased plasma levels of HDL cholesterol (HDL-C).[10] Many epidemiological studies have documented an inverse relationship between HDL-C levels and the progression of atherosclerosis and increased risk of CVD in the general population.[12] Proposed mechanisms for the atheroprotective function of HDL include reverse cholesterol transport (RCT), reduction of oxidative stress, and potent anti-inflammatory effects.[13-17] On the other hand, HDL might lose its anti-atherogenic properties by chemical modifications such as oxidation, which negatively affects RCT and other events associated with the development of atherosclerosis.[18-22] Hence, oxidized HDL can be detected in lesions and plasma of individuals at increased atherosclerotic risk.[23-26] It has been suggested that malnutrition and inflammation induce HDL oxidation in maintenance hemodialysis patients,[27] which in turn is responsible for the increased risk of cardiovascular morbidity and mortality in ESRD patients.[28-30]

Despite reduced serum HDL-C concentrations in ESRD patients, a clear association of HDL-C with survival has not been demonstrated.[5,31] However, anti-

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inflammatory functions of HDL such as its abilities to inhibit low density lipoprotein (LDL) oxidation[32] and monocyte chemotaxis[33] are defective in ESRD patients and this correlates with overall survival.[32] The conversion of anti-inflammatory to proinflammatory HDL has also been proposed to represent a novel risk factor for the progression of CKD to ESRD.[34,35] Qualitative differences in the protein and lipid composition of HDL rather than their mere concentration appear to be critical for the anti-atherogenic and anti-inflammatory effects in CKD and ESRD.[14,36,37] Recent studies that elucidated the proteome of HDL from healthy individuals and patients with coronary artery disease by mass spectrometry (MS) revealed that the protein cargo is a major determinant of the anti-atherogenic and anti-inflammatory function of HDL.[38-44] For example, approximately 50% of the proteins associated with HDL are implicated in the acute-phase response or innate immunity.[40]

As qualitative alterations of HDL are directly linked with increased cardiovascular complications, we hypothesized that HDL from ESRD patients on maintenance hemodialysis might display defective anti-inflammatory potency, protein cargo and/or oxidative status. In this study we describe a loss of anti-inflammatory efficiency along with an altered HDL protein composition in ESRD patients compared with HDL from healthy controls. Surprisingly, the HDL of ESRD is not oxidized or more vulnerable to oxidation. After pinpointing the molecular composition of HDL, we link the molecular changes with the proinflammatory function of uremic HDL. The potential clinical relevance of this novel immunomodulatory activity of HDL and its impaired function during uremia is discussed.

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RESULTS

HDL from ESRD patients display defective anti-inflammatory properties

HDL was recently identified as major endogenous inhibitor of inflammatory responses.[45] We speculated that the chronic inflammatory milieu observed in ESRD patients might be linked with a defective anti-inflammatory potency of HDL. Therefore, we isolated HDL from ESRD patients and healthy individuals by sequential ultracentrifugation (Supplementary Figure 1).[46] Next, we stimulated peripheral human monocytes with the Toll-like receptor 2 (TLR2) agonist *Staphylococcus aureus* (SAC) in the presence or absence of 10 or 100 μg/mL HDL and measured the inflammatory response. We observed that HDL from healthy individuals potently inhibited the production of the inflammatory cytokines IL-12p40, TNF-α, and IL-10 after stimulation with SAC (Figure 1, A-C). Strikingly, in the majority of cases HDL from ESRD patients did not show these potent anti-inflammatory effects but, in contrast, many ESRD-HDL samples promoted inflammatory cytokine production (Figure 1, A-C). Of note, the apoA-I mimetic peptide 4F did not modulate the expression of these cytokines (data not shown). Moreover, dendritic cells stimulated with lipopolysaccharide (LPS) showed a reduced expression of the costimulatory molecules CD40, CD83, and CD86 in the presence of HDL isolated from healthy controls (Figure 1D). In contrast, the HDL from ESRD patients showed a diminished ability to inhibit the surface expression of these molecules (Figure 1D). These results demonstrate that uremic HDL from ESRD patients display defective anti-inflammatory properties with regard to innate immune responses.

Assessment of the oxidation status of HDL in ESRD patients

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To investigate potential causes for the defective anti-inflammatory potential of ESRD-HDL, we analyzed the oxidation status of ESRD-HDL as oxidized HDL promotes inflammation and atherosclerosis. Accordingly, we measured the levels of oxidized apoA-I and apoA-II in HDL from ESRD patients by high performance liquid chromatography using an assay that detects oxidized methionine residues, which is an early event during HDL oxidation.[25,26,47-49] We detected a decrease in the relative content of total apoA-I and apoA-II in freshly isolated HDL from ESRD patients compared with controls (Figure 2A). Surprisingly, we were unable to detect oxidized apoA-I or apoA-II in HDL either in ESRD patients or in healthy controls (Figure 2B). To determine potential differences in susceptibility to oxidation of HDL between the two groups, we subjected isolated HDL stored for three days at 4°C to *in vitro* oxidation with a peroxyl radical generator. Interestingly, storing HDL samples alone significantly increased the content of oxidized apoA-I/II in both groups (Figure 2B). *In vitro* oxidation for 2h further augmented the extent of apoA-I/II oxidation (Figure 2B). Oxidation was associated with the loss of non-oxidized apoA-I/II (Figure 2C). When expressed as relative to the total amount of apoA-I/II, there was no difference in oxidized apoA-I/II between HDL from controls and ESRD patients (Figure 2D). These results suggest that HDL in plasma from ESRD patients is not significantly oxidized *in vivo* or more vulnerable to peroxyl radical-mediated oxidation than HDL from healthy controls.

Identification of HDL-associated proteins by shotgun proteomics

As the oxidation status was similar, we speculated that the protein cargo of uremic HDL might differ compared to HDL isolated from healthy individuals. To identify HDL-associated proteins, we performed electrospray ionization (ESI)-Ion Trap MS of purified HDL from 10 patients on maintenance hemodialysis and from 10 healthy

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controls. Table 1 describes the clinical characteristics of the subjects studied. The following criteria were used to identify proteins: a high peptide identification score (see *Methods*) and at least 3 peptides corresponding to a protein of interest had to be detected in at least 3 subjects. Using these criteria, we identified 49 proteins that were HDL-associated in controls or ESRD patients (Table 2). Of these, 44 proteins have been described previously, and 5 represent novel HDL-associated proteins. The newly identified HDL-associated proteins are α-1-acid glycoprotein 1, zinc-α-2-glycoprotein, surfactant-associated protein B (SP-B), c-src, and complement factor D (Table 2). Importantly, we confirmed our results by performing MS of HDL from an independent cohort of healthy individuals and ESRD patients (Supplementary Table 2 and Table 1).

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Gene ontology analysis identifies iron and heme proteins linked to HDL

Previously, HDL-associated proteins have been linked to lipid metabolism, acute-phase response and innate immunity.[40] Therefore, we performed gene ontology analysis to ascertain which of our identified proteins were linked to these clusters and to look for additional functional categories. As anticipated, many of the identified proteins (21 of 49) were linked to cholesterol and lipoprotein metabolism (Figure 3). Some proteins were identified as regulators of complement activation, the acute-phase response, and also linked to protein breakdown processes (Figure 3). Unexpectedly, we identified 7 proteins, which directly regulate heme/iron metabolism such as hemopexin, transferrin, or α-1-microglobulin/bikunin precursor (AMBP) (Figure 3). Although heme/iron metabolism has been linked to atherogenesis, a critical regulatory role of HDL for this process is unknown at present.[50,51]

The proteomic fingerprint of HDL from ESRD patients

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We then grouped the MS-identified proteins (Table2) and compared the proteomic composition of ESRD-HDL with HDL from individuals with normal kidney function. To quantify differences between the two groups, we used the previously described "peptide index", an empiric test based on peptide abundance to measure the relative protein abundance in different groups of subjects.[40] We identified 4 proteins that were significantly enriched in HDL from ESRD patients according to the peptide index: SP-B, apoC-II, SAA, and AMBP (Figure 4). Indeed, SP-B was exclusively detected in HDL from ESRD patients but not in healthy individuals (Figure 4 and Table 2). These results were supported by immunoblotting the enriched proteins in HDL from the replication cohort (Figure 5). Moreover, we observed that various proteins, which were enhanced in the ESRD group but did not reach statistical significance according to the peptide index, were still highly enriched in the independent patient cohort such as transferrin or PEDF (Figure 5). These results imply that distinct HDL-associated proteins are strongly enriched or are exclusively present in ESRD patients.

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SP-B and PEDF are gradually enriched in HDL from CKD3 to CKD4 to ESRD

Next we asked whether the presence of the proteins enriched in ESRD-HDL merely reflects their occurrence in the plasma. SP-B and PEDF were present in the plasma of control and ESRD subjects to a similar amount (Figure 6A) suggesting that HDL-associated SP-B and PEDF but not SAA and transferrin are exclusively incorporated into the HDL of ESRD patients by an unknown mechanism. Then we sought to determine if the identified HDL-enriched proteins from ESRD patients can already be detected in earlier stages of CKD. While in CKD3 patients HDL-associated SP-B was detectably only in one subject, SP-B was already present in 5 out of 11 HDL samples of CKD4 patients (Figure 6B). In contrast, HDL-associated SAA was already present in healthy controls, but noticeably increased in CKD4 patients (Figure 6C). PEDF was

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not found in the HDL of CKD3 patients, whereas 4 CKD4 patients displayed PEDF-enriched HDL (Figure 6D). These results suggest that the incorporation of SP-B, SAA or PEDF into HDL precedes ESRD, can already be detected in many CKD4 patients, and thus may be evaluated as biomarkers for subsequent kidney failure, disease progression, or cardiovascular morbidity and mortality.

SAA induces inflammatory reactions in human monocytes and reverts the anti-inflammatory properties of HDL

Finally, we wanted to functionally assess the importance of the enriched ESRD-HDL proteins for the defective anti-inflammatory effects of HDL that we observed in ESRD patients (Figure 1). Therefore, we tested if SP-B, apoC-II, SAA, and AMBP might be able to induce or modulate inflammatory cytokine expression. We stimulated human monocytes with increasing doses of the proteins and observed that SAA but not the other proteins potently induced the expression of IL-12p40, IL-10, TNF-α, and IL-6 (Figure 7A-D). Moreover, only SAA significantly enhanced the production of these inflammatory cytokines induced by SAC or LPS (Figure 7E-H and data not shown). Next, we directly tested whether SAA can revert the anti-inflammatory effects of HDL. We incubated plasma from a healthy individual with SAA or PBS and afterwards isolated HDL (SAA-HDL and Ctrl-HDL, respectively). We observed that SAA readily incorporated into the SAA-HDL particle (Figure 8A). Functionally, Ctrl-HDL potently inhibited IL-12 and TNF-α production in LPS-stimulated monocytes, whereas the incorporation of SAA abrogated the anti-inflammatory action of HDL (Figure 8 B and C). Finally, we assessed whether the amount of SAA in the HDL of ESRD patients correlated with their defective anti-inflammatory potential (as shown in Figure 1). Notably, SAA significantly correlated with the amount of IL-12 production (Figure 9A), whereas the levels of IL-10 and TNF-α did not correlate with SAA (Figure 9B and C).

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These results suggest that the incorporation of SAA into HDL contributes to the reduced anti-inflammatory or sometimes even proinflammatory potency of HDL in ESRD patients.

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Discussion

The molecular causes for the excessive cardiovascular morbidity and mortality of uremic patients are only incompletely understood. Recently, it has been documented that HDL from patients with ESRD is dysfunctional and unable to inhibit oxidation of LDL, a cardinal attribute of the anti-atherogenic property of HDL.[32] The molecular basis for the dysfunctionality of ESRD-HDL is unresolved. We now show that HDL from ESRD patients is also defective in a recently described anti-inflammatory function, i.e. the direct inhibition of inflammatory cytokine production in innate immune cells.[45] To identify possible features that might distinguish healthy from uremic HDL, we analyzed the molecular composition of the HDL proteome in ESRD patients by shotgun proteomics. Our analysis identified many proteins that have been previously assigned to uremia and CKD such as SAA, hemoglobin, transferrin, LCAT, ceruloplasmin, antithrombin-III, or apoA-IV, which is also a novel independent predictor of CKD progression.[52,53] Moreover, these data revealed that a specific set of proteins including SP-B, apoC-II, SAA, AMBP, transferrin and PEDF define the molecular organization of HDL from ESRD patients. Notably, SP-B, SAA, and PEDF were already broadly detectable in HDL from CKD4 patients. The early appearance of these proteins demonstrates that the observed compositional changes are caused by uremia *per se* and not merely by bioincompatibility or the mechanical-induced trauma due to hemodialysis, however, the functional relevance of the identified proteins for HDL biology needs to be determined in further studies. Interestingly, the set of proteins most strongly enriched in HDL from ESRD patients does not overlap with proteins reported to be enriched in HDL from patients with coronary artery disease, namely apoC-IV, PON1, C3, apoA-IV, and apoE.[40] These results support a

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novel concept that distinct diseases with atherosclerotic and cardiovascular risk are associated with a characteristic “disease-specific” HDL proteome.

Notably, SP-B was selectively found in HDL of ESRD and CKD4 patients, but did not exist at all in HDL from healthy subjects. The groups had comparable plasma concentrations of SP-B demonstrating that the mechanism responsible for SP-B incorporation into HDL is restricted to uremia. Plasma SP-B has recently been identified as a novel biomarker in chronic heart failure (CHF).[54] As pulmonary edema and pleural effusion are common in CHF and in ESRD, we hypothesize that fluid overload, retention and/or cardiac dysfunction characterized by diastolic dysfunction along with increased pulmonary resistance,[55] results in the release of SP-B with distinct physicochemical properties into plasma that may become incorporated into the HDL. Consequently, HDL-associated SP-B in CKD4 and ESRD patients might be an early indicator for fluid overload, cardiovascular events, and/or the progression of CKD. Further studies are urgently warranted to assess the potential of SP-B as novel biomarker for cardiovascular morbidity and mortality in uremia.

By investigating the functional properties of the enriched proteins to modulate inflammatory responses, we observed that SAA but not SP-B, AMBP or ApoC-II was able to induce the production of IL-12, IL-10, TNF-α, and IL-6 from human monocytes. In addition, SAA acted in concert with SAC or LPS (Fig. 7 and data not shown) to augment inflammatory cytokine production. Strikingly, incorporation of SAA into healthy HDL reverted its anti-inflammatory effects. Moreover, the levels of SAA in ESRD-HDL significantly correlated with the inability to inhibit IL-12 production by ESRD-HDL. In line, SAA is known to bind to HDL[56] and SAA is systemically increased in uremic patients.[37,57] SAA was present in ESRD-HDL at a mean concentration of 7.07 µg/mg HDL. Hence, in ESRD serum that contains ~40 mg/dL HDL, the SAA levels in HDL should be around 2.8 µg/mL. This is in agreement with

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the total SAA levels measured in ESRD serum (3.7 µg/mL)[58] and indicate that the majority of SAA is bound to HDL. These results suggest that SAA is a critical component of ESRD-HDL responsible for its defective anti-inflammatory potency. This is in line with recent evidence suggesting that SAA modulates innate immune responses[59] by inducing the secretion of IL-8 in neutrophils,[60,61] and activating the inflammasome to produce IL-1β.[62] These effects might be mediated via TLR2 or TLR4.[63,64] Interestingly, it was also suggested that SAA may potentiate prothrombotic and proinflammatory events in acute coronary syndromes.[65] Therefore, SAA might be a critical functional modifier of HDL that contributes to its anti- versus proinflammatory properties. This does not exclude the possibility that other proteins or lipids might be directly important for different functional aspects of the altered ESRD-HDL physiology.

Evidence for the presence of oxidized HDL in lesions and in plasma is abundant in atherosclerosis.[23-26,66,67] Oxidized HDL not only loses critical atheroprotective functions, but even acquires proinflammatory and pro-thrombotic properties. It has been suggested that oxidized HDL in ESRD patients may be causally linked to cardiovascular complications of uremia but this has not been rigorously tested yet.[23,27-29] However, we were unable to detect oxidized HDL in ESRD *in vivo*, and there was no difference in the susceptibility of HDL from control and ESRD patients to *ex vivo* oxidation induced by peroxyl radicals, as assessed by the extent of oxidized apoA-I/II containing methionine sulfoxide(s). We did not determine the extent of lipid (per)oxidation in these experiments. HDL's lipids containing bisallylic hydrogen atoms are more susceptible to peroxyl radical mediated oxidation than apoA-I/II's methionine residues.[47] Therefore, we cannot exclude the possibility that there are differences in the oxidation state and/or oxidation resistance of the lipid moiety of HDL from control and ESRD patients. We consider such a

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possibility as unlikely, however, because the primary and major lipid oxidation products formed during the early stages of HDL oxidation, *i.e.* hydroperoxides of phospholipids and cholesterylesters, react with and convert methionine residues of apoA-I/II to the corresponding methionine sulfoxides[47]. Thus, if there were substantial differences in the extent of lipid oxidation between HDL from control and ESRD patients, we would have expected this to translate into differences in the extent of apoA-I/II methionine oxidation, which we did not observe.

In conclusion, our systematic analysis of the HDL proteome identified a set of novel proteins that are either unique to or greatly enriched in HDL from patients with ESRD. These molecules may be assessed as novel biomarkers to improve our ability to monitor therapeutic responses and predict meaningful future events including cardiovascular complications. Moreover, HDL appears to be a critical part of the innate immune system and can be either proinflammatory or anti-inflammatory influenced by the presence or absence of SAA, respectively. Finally, our data provide a framework for evaluating novel proteins and pathways such as heme/iron metabolism for a direct involvement in the pathogenesis of ESRD to study the functional consequences of the complex alterations in HDL of ESRD patients.

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Concise Methods

Subjects

The study was approved by the ethics committee of the General Hospital Vienna according to the declaration of Helsinki (EK 407/2007 and EK 584/2009). Informed consent was obtained from all subjects. For the proteomics analysis, a total of 10 stable patients with ESRD undergoing maintenance hemodialysis for a minimum of 3 months were recruited for the study. Patients with decompensated heart failure were excluded. Patients were in a stable condition and free from intercurrent illness and infection for at least 3 months. As confirmed by clinical examination, patients were in a good state of health, notably without signs of malnutrition or wasting. All the patients were dialyzed on standard bicarbonate basis for 4–5 h three times weekly using biocompatible polysulfone hemodialysis membranes (Fresenius, Germany). Dialysis adequacy was estimated using Kt/V values > 1.2 in all patients. None of the patients had residual renal function (diuresis over 24 h below 100 mL). The venous blood sample was drawn before the dialysis session. Blood was immediately placed on ice and centrifuged at 4,000×g for 5 min at 4°C to obtain plasma. A group of 10 subjects with normal kidney function were used as controls. The clinical and biochemical characteristics of the patients and control subjects are given in Table 1. We used a replica cohort, which consisted of 14 ESRD patients and 12 controls (Table 1) to confirm our proteomics data. The 11 CKD3 patients (Table 2) had an estimated mean glomerular filtration rate (GFR) of 44.1(SD±6.9). The estimated GFR of the 11 CKD4 patients was 22.3(SD±5.5).

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Isolation of High-Density Lipoproteins

HDL was isolated from fresh human plasma by sequential ultracentrifugation at a density d of 1.063<d<1.210 kg/L, respectively.[46] In brief, the density of plasma was raised by addition of KBr (Merck, Darmstadt, Germany) to 1.063 kg/L, and ultracentrifugation was carried out for 12 h at 20°C in a 50.4 Ti rotor at 50,000 rpm using an Optima L-90-K ultracentrifuge (Beckman, Fullerton, CA). After removing the supernatant with the larger lipoproteins, the density of the infranate fraction was raised to 1.210 kg/L by further addition of KBr. HDL was then collected from the top of each polycarbonate thick wall centrifuge tube (Beckman) after another centrifugation step in the 50.4 Ti rotor at 50,000 rpm for 12 h. To prepare SAA-HDL, we dissolved 50 µg SAA in PBS, which was added to 8 mL of plasma from a healthy individual and incubated the mix for 3 h at 4° C. As control PBS was added to 8 mL plasma from the same individual. Afterwards, HDL was isolated from the plasma samples.

Monocyte stimulation assay

Human peripheral blood mononuclear cells (PBMC) were isolated as described.[68] Monocytes were isolated from PBMCs by MACS using CD14 Microbeads (Miltenyi Biotec) and cultured in Macrophage-SFM medium (Invitrogen). $5x10^5$ monocytes were pretreated for 90 min with 10 or 100 µg/mL HDL or medium and then stimulated with 20 µg/mL SAC (PANSORBIN; Calbiochem). Alternatively, monocytes were stimulated with the indicated concentrations of SAA (Sigma), SP-B (a kind gift of Andreas Günther and Clemens Ruppert[69]), AMBP (My-Bio-Source), or apo-CII (My-Bio-Source) with or without SAC. Cell-free supernatants were collected after 20 h and measured by ELISA to determine IL-12p40, IL-10, TNF-α, IL-6 levels (all R&D Systems).

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Dendritic cell stimulation assay

Monocyte-derived dendritic cells were cultured as described.[70] Cells were pretreated with 100 µg/mL HDL and then stimulated with 100 ng/mL LPS for 20 h. The expression of surface markers was evaluated by flow cytometry as described.[70]

1D-PAGE for subsequent shotgun analysis

The different protein fractions were loaded on 12% polyacrylamide gels, electrophoresis was performed until complete separation of a pre-stained molecular marker was visible. Gels were fixed with 50% methanol/10% acetic acid and subsequently silver stained as described below. The entire gel lanes were cut into 6 pieces and digested with trypsin as described below.

Tryptic digest

Gel pieces were destained with 15 mM $K_3Fe(CN)_6$/50 mM $Na_2S_2O_3$ and intensively washed with 50% methanol/10% acetic acid. The pH was adjusted with 50 mM NH_4HCO_3, and proteins were reduced with 10 mM DTT/50 mM NH_4HCO_3 for 30 min at 56°C and alkylated with 50 mM iodacetamide/50 mM NH_4HCO_3 20 min in the dark. Afterwards the gel pieces were treated with acetonitrile and dried in a vacuum centrifuge. Between each step, the tubes were shaken 5-10 min. Dry gel pieces were treated with trypsin 0.1 mg/mL (Trypsin sequencing grade, Roche Diagnostics)/50 mM NH_4HCO_3, in a ratio of 1:8 for 20 min on ice, afterwards covered with 25 mM NH_4HCO_3 and were subsequently incubated overnight at 37°C. The digested peptides were eluted by adding 50 mM NH_4HCO_3, the supernatant was transferred into silicon-coated tubes, and this procedure was repeated two times with 5% formic acid/50% acetonitril. Between each elution step the gel pieces were ultrasonicated

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for 10 min. Finally the peptide solution was concentrated in a vacuum centrifuge to an appropriate volume.

Mass spectrometry analysis

Mass spectrometry was performed as described previously.[71] Peptides were separated by nano-flow LC (1100 Series LC system, Agilent) using the HPLC-Chip technology (Agilent) equipped with a 40 nl Zorbax 300SB-C18 trapping column and a 75 µm x 150 mm Zorbax 300SB-C18 separation column at a flow rate of 400 nL/min, using a gradient from 0.2% formic acid and 3% ACN to 0.2% formic acid and 50% ACN over 60 min. Peptide identification was accomplished by MS/MS fragmentation analysis with an iontrap mass spectrometer (XCT-Ultra, Agilent) equipped with an orthogonal nanospray ion source. The MS/MS data were interpreted by the Spectrum Mill MS Proteomics Workbench software (Agilent; version A.03.03.081), including peak list-generation and search engine, allowing for two missed cleavages and searched against the SwissProt Database for human proteins (Version 14.3 containing 20,328 protein entries) allowing for precursor mass deviation of 1.5 Da, a product mass tolerance of 0.7 Da and a minimum matched peak intensity (%SPI) of 70%. Due to previous chemical modification, carbamidomethylation of cysteine was set as fixed modification, and methionine oxidation was allowed as variable modification.

Estimated error rates (mean of 1.3% ±0.8) were calculated from reversed database searches as previously described.[72] From this we automatically assigned a protein valid if it was identified with at least one specific peptide scoring above 13.0, which does not occur in other proteins identified in same samples. Protein identifications not fulfilling that requirement were selected manually as previously described[73] whereby selection of protein isoforms was essentially performed as

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described by Zhang et al.[74] Only peptides scoring above 9.0 were entered into the database. Novel HDL-associated proteins were only considered when they were also detected in the MS analysis of the replication cohort.

Peptide index

For quantitative spectral counting[74] we summed the number of specific spectral peptide counts assigned to one protein across all fractions of a sample and normalized it against the sum of all identified specific peptide counts in that sample. Normalized spectral counts were multiplied with the mean value of total specific spectral peptide counts per patient. Finally, these normalized spectral counts were used to calculate the peptide index for each protein identified by MS/MS, as follows: [(peptides in ESRD subjects/total peptides) × (% of ESRD subjects with ≥1 peptide)] – [(peptides in control subjects/total peptides) × (% of control subjects with ≥1 peptide)]. We chose a peptide index of less than –0.4 or greater than 0.4, respectively, to identify selective enrichment of proteins in control subjects or ESRD subjects as described previously.[40]

Immunoblotting

Reduced HDL or plasma preparations (10 µg per lane) separated by 10% SDS-PAGE were transferred to a nitrocellulose membrane and probed overnight at 4° C with primary antibodies as follows: SP-B and SAA (Santa Cruz Biotechnology), apoC-II (GenScript), apoA-I (Cell signaling), PEDF (R&D Biosystems) and transferrin (Biodesign International). Blots were washed, incubated with appropriate secondary horseradish peroxidase–conjugated antibodies, and developed using ECL Western blotting detection reagents (Amersham).

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Oxidation of HDL and HPLC analysis

Oxidation of HDL and analysis of oxidized HDL by HPLC was essentially performed as described.[47,75] Briefly, fresh human blood was collected into heparinized vacutainers directly on ice, and plasma was immediately obtained by centrifugation at 1,000 g for 15 min at 4°C and stored until use at -80° C. HDL was isolated from the plasma by sequential ultracentrifugation. Native HDL (1–1.5 mg protein/mL) was oxidized in 20 mM PBS containing 100 μM diethylene triamine pentaacetic acid under air and at 37°C by exposure to the peroxyl radical generator AAPH (2 mM) for 2 h. The reaction was terminated by addition of butylated hydroxytoluene (100 μM). The reaction mixture (1 mL) was then passed through a gel filtration column (3 mL, NAP-10, GE Healthcare) eluted with 1.5 mL of PBS. The oxidized HDL was analyzed within 24 h. For HPLC analysis, freshly isolated HDL (0.06 mg protein) or differently oxidized HDL (1 mg protein) was subjected to a C18 column (250 × 4.6 mm, 5 μm, Vydac) with guard (5 μm, 4.6 ID, Vydac) eluted at 50°C and 0.5 mL/min, with the eluant monitored at 214 nm as described.[75]

Determination of SAA in HDL

The levels of SAA in the HDL from ESRD patients were determined by the N LATEX SAA Kit (Siemens).

Statistics

For proteins that appeared enriched by the peptide index, Student's unpaired 2-tailed t test was used to compare the number of unique peptides identified in ESRD patients versus control subjects. For proteins found in only 1 group of subjects, a 1-sample t test was used to compare the number of unique peptides to a theoretical mean of 0. HPLC analysis of (oxidized) apoA-I/II is expressed as means ± SEM and

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was compared using Student's t test. Cytokine expression was compared using Student's t test. For all statistical analyses, $P < 0.05$ was considered significant. The Pearson's correlation coefficient r was used as measures for the correlation analysis. Calculations were carried out using GraphPad Prism (GraphPad Software).

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DISCLOSURES

None.

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FIGURE LEGENDS

Figure 1. Defective anti-inflammatory potency of HDL from ESRD patients. (**A-C**) Human monocytes were pretreated with HDL from ESRD patients (ESRD-HDL; n=27) or HDL from healthy controls (control-HDL; n=7) and then stimulated with SAC for 20 h. The amount of (**A**) IL-12p40, (**B**) TNF-α, and (**C**) IL-10 was determined in the supernatant and is expressed relative to cells stimulated with SAC only. The individual values and the median is shown in scatter plots. (**D**) Monocyte-derived dendritic cells were treated with 100 µg/mL HDL from 4 healthy controls (control-HDL) or 10 ESRD patients (ESRD-HDL) and then stimulated with LPS for 20 h. The expression of the indicated surface markers was evaluated by flow cytometry and is expressed relative to cells stimulated with LPS only. * <0.05, ** <0.01, *** <0.001.

Figure 2. Amount of oxidized apoA-I and apoA-II in HDL and its susceptibility to oxidation of ESRD patients and control subjects. HDL was tested from the replica cohort consisting of 14 ESRD patients and 12 controls. The amounts of (**A**) total apoA-I and apoA-II, (**B**) oxidized apoA-I and oxidized apoA-II, (**C**) total non-oxidized apoA-I/II and oxidized apoA-I/II, and (**D**) the ratio of oxidized apoA-I/total apoA-I and oxidized apoA-II/total apoA-II were determined by HPLC analysis from freshly isolated HDL (Fresh), HDL that was stored for 3 days at 4° C (3d), and HDL that was stored for 3 days at 4° C and then chemically oxidized by 2,2'-azo*bis*-2-methyl-propanimidamide (3d+AAPH) for 2 h. *P<0.05.

Figure 3. Gene ontology functional associations of HDL proteins. Total identified HDL proteins from 10 healthy controls and 10 ESRD patients were associated with

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biological functions using Gene Ontology process annotations. HRP, Haptoglobin-related protein; TTR, Transthyretin; IGHG1, Ig gamma-1 chain C region; PLTP, Phospholipid transfer protein; A1BG, α-1B-glycoprotein; DBP, Vitamin D-binding protein; AHSG, α-2-HS-glycoprotein; VTN, Vitronectin; AGP 2, α-1-acid glycoprotein 2; PEDF, pigment epithelium-derived factor; Ugrp2, secretoglobin family 3A member 1; PCYOX1, prenylcysteine oxidase 1; CP, Ceruloplasmin; PBP, platelet basic protein; AGP 1, α-1-acid glycoprotein 1; AZGP1, zinc-α-2-glycoprotein; SP-B, surfactant protein B; CFD, complement factor D.

Figure 4. Relative abundance of proteins identified by MS from HDL of ESRD patients and healthy controls. Data are from 10 subjects with ESRD and 10 controls. The relative abundance of the HDL-associated proteins was assessed by the peptide index as described in Methods. *$P < 0.05$.

Figure 5. Immunodetection of HDL-associated proteins in both ESRD patients and healthy subjects. Shown are immunoblots of the indicated MS-identified HDL proteins in 10 µg of HDL from an independent cohort consisting of 14 ESRD and 12 healthy controls (see Table 1).

Figure 6. Immunodetection of MS-identified proteins in the plasma or HDL of healthy subjects and patients with CKD3, CKD4 or ESRD. (**A**) Shown are immunoblots for SP-B, PEDF, SAA, and transferrin in 10 µg whole plasma of 6 ESRD and 5 healthy controls. (**B-D**) Immunodetection of HDL-associated proteins in healthy subjects and patients with CKD3 or CKD4. Immunoblot of (**B**) SP-B, (**C**) SAA, and (**D**) PEDF in 10 µg HDL samples.

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Figure 7. SAA but not SP-B, apoC-II or AMBP mediates the proinflammatory effects of ESRD-HDL. Human monocytes from three individual donors were treated with the indicated concentrations of SAA, SP-B, AMBP, or apoC-II (in ng/mL) without or together with 20 µg/mL SAC for 20 h. The amount of (**A,E**) IL-12p40, (**B,F**) TNF-α, and (**C,G**) IL-10, and (**D,H**) IL-6 was determined in the supernatant and is expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the respective SAC controls.

Figure 8. SAA reverses the anti-inflammatory effects of HDL. (**A**) Immunoblot of 3.5 µg HDL isolated from plasma of a healthy individual incubated with PBS (Ctrl-HDL) or SAA (SAA-HDL) before. As control 5 ng SAA was loaded. (**B,C**) Human monocytes were pretreated with Ctrl-HDL or SAA-HDL (in µg/mL) and then stimulated with 100 ng/mL LPS for 20 h. The amount of (**B**) IL-12p40 , and (**C**) TNF-α was determined in the supernatant and is expressed relative to cells stimulated with LPS only (n=3). *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 9. SAA in ESRD-HDL correlates with IL-12p40 expression. The expression values of human monocytes stimulated with 20 µg/mL SAC together with 10 µg/mL ESRD-HDL (Figure 1A-C) were used to correlate SAA levels and the expression of (**A**) IL-12p40, (**B**) IL-10, and (**C**) TNF-α. Pearson's correlation coefficient r was used as measure for the correlation analysis.

Tables

Table 1. Clinical characteristics of the study subjects used in the study. Data are given as means (SD). NA: not available.

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Table 2. Proteins detected by ESI-Ion trap MS in HDL from 10 ESRD patients and 10 healthy control subjects.

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Table 1

	Proteomics cohort		Replica cohort		CKD cohort	
	ESRD	Control	ESRD	Control	CKD3	CKD4
n	10	10	14	12	11	11
Age (years)	62.5 (12.4)	55.5 (11.5)	56.1 (18.8)	37.0 (11.5)	65 (16.6)	59 (18.7)
Weight (kg)	77.8 (15.5)	84.4 (11.2)	NA	NA	NA	NA
Gender (male/female)	6/4	5/5	7/7	10/2	7/4	7/4
Cholesterol (mg/dL)	198.4 (59.5)	138.5 (30.4)	165.8 (40.6)	198.8 (27.2)	188.5 (27.1)	182.2 (36.2)
Triglycerides (mg/dL)	178.8 (45.3)	98.8 (15.8)	145.4 (64.4)	92.8 (54.6)	142 (73.1)	176.3 (45.8)
HDL-cholesterol (mg/dL)	36.5 (12.8)	45.1 (6.7)	47.6 (12.6)	64.5 (14.0)	56,3 (13.5)	44.5 (10.4)
LDL-cholesterol (mg/dL)	143.3 (38.3)	88.5 (28.6)	94.9 (37.8)	115.8 (23.0)	104.1 (27.7)	102.4 (29.0)
Albumin (g/L)	34 (7.1)	49.5 (5.1)	36.4 (4.5)	47.1 (2.8)	42.8 (3.2)	38.6 (3.9)
Blood urea nitrogen (mg/dL)	60.1 (18.3)	18.9 (3.7)	57.4 (18.6)	13.6 (2.3)	30.2 (7.6)	52.6 (13.3)
Creatinine (mg/dL)	7.8 (2.9)	0.89 (0.2)	8.4 (2.4)	1.0 (0.1)	1.6 (0.3)	2.8 (0.9)

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Table 2

AccNr	Name	No. of peptides		Subjects with detectable proteins		References
		ESRD	Control	ESRD	Control	
Previously identified HDL-associated proteins						
P02647	ApoA-I	416	375	10	10	39,40,43,76
P02768	Serum albumin	252	222	10	9	39,40,76
P06727	ApoA-IV	206	125	10	9	39,40,43,76
P02649	ApoE	154	111	10	10	39,40,43,76
P05090	ApoD	96	75	10	10	39,40,76
P02652	ApoA-II	85	75	10	10	39,40,43,76
P27169	PON1	62	62	10	10	39,40,43,76
P02656	ApoC-III	63	43	10	10	39,40,43,76
P35542	SAA4	54	49	10	10	40
P02735	SAA	58	25	10	7	39,40,76
P02654	ApoC-I	37	38	10	9	40,43
O95445	ApoM	41	32	10	10	39,40,43,76
O14791	ApoL	36	34	10	10	39,40,43,76
P02787	Transferrin	35	23	6	5	39,40
P00738	Haptoglobin	30	25	6	6	39,51
P00739	Haptoglobin-related protein (HRP)	26	29	7	7	40,76
P10909	ApoJ (Clusterin)	26	22	9	7	40,43
P02655	ApoC-II	37	10	10	5	39,40,76
P02766	Transthyretin (TTR)	22	19	6	5	39,40,43,76
P68871	Hemoglobin (subunit beta)	16	12	7	4	51
Q15166	PON3	14	14	8	7	40,76
P01857	Ig gamma-1 chain C region (IGHG1)	15	13	5	5	43
Q13790	ApoF	15	11	9	9	40,76
P02760	AMBP	23	1	5	1	40
P55058	Phospholipid transfer protein (PLTP)	13	10	8	6	40,76
P04217	Alpha-1B-glycoprotein (A1BG)	12	10	5	5	39,40
P02749	ApoH (Beta-2-glycoprotein 1)	18	3	5	1	40
P04180	LCAT	13	8	6	3	40
P02774	Vitamin D-binding protein (DBP)	13	7	6	5	40,43
P04114	ApoB-100	7	13	5	3	40,43
P02765	Alpha-2-HS-glycoprotein (AHSG)	8	9	6	5	40,43
P04004	Vitronectin (VTN)	5	9	4	4	40,43
P01024	Complement C3	7	5	4	5	39,40,43
P0C0L4	Complement C4-A	5	6	4	5	40
P08519	Apo(a)	2	9	2	3	76,77
P19652	Alpha-1-acid glycoprotein 2 (AGP 2)	6	4	4	3	40
P36955	Pigment epithelium-derived factor (PEDF)	7	3	2	1	40,43
P69905	Hemoglobin (subunit alpha)	6	3	4	2	51
Q96QR1	Secretoglobin family 3A member 1 (Ugrp2)	2	5	1	4	78
Q9UHG3	Prenylcysteine oxidase 1 (PCYOX1)	3	3	3	3	40
P02790	Hemopexin	3	3	2	3	40,43
P00450	Ceruloplasmin (CP)	1	5	1	2	39
P02775	Platelet basic protein (PBP)	1	5	1	3	76
P01008	Antithrombin-III	0	4	0	3	43
P22614	SAA3P	4	0	3	0	79
Proteins identified in this study as HDL associated						
P02763	Alpha-1-acid glycoprotein 1 (AGP 1)	15	12	6	5	
P25311	Zinc-alpha-2-glycoprotein (AZGP1)	9	6	3	2	
P07988	Surfactant protein B (SP-B)	11	0	7	0	
P41240	c-src tyrosine kinase CSK	6	4	6	4	
P00746	Complement factor D (CFD)	7	1	3	1	

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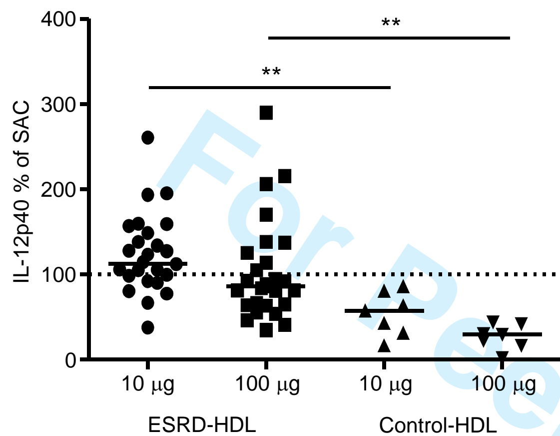

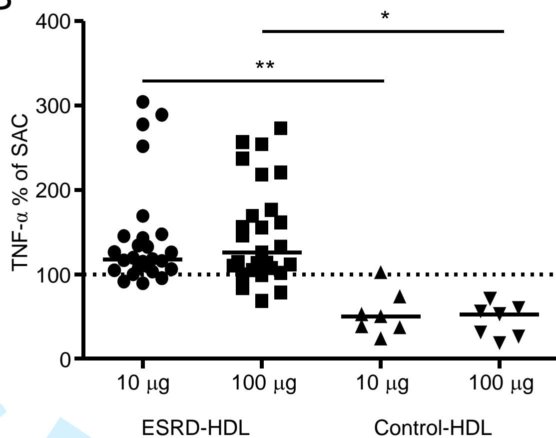

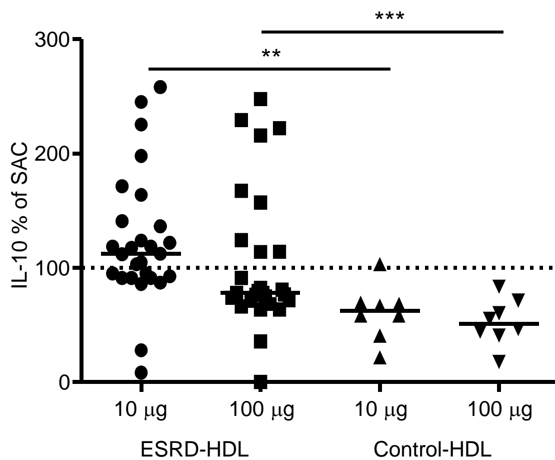

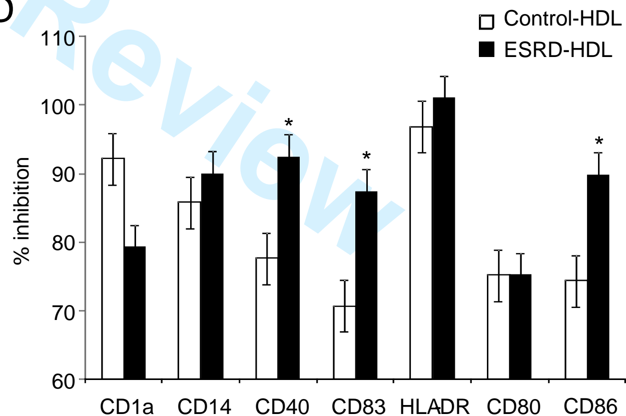

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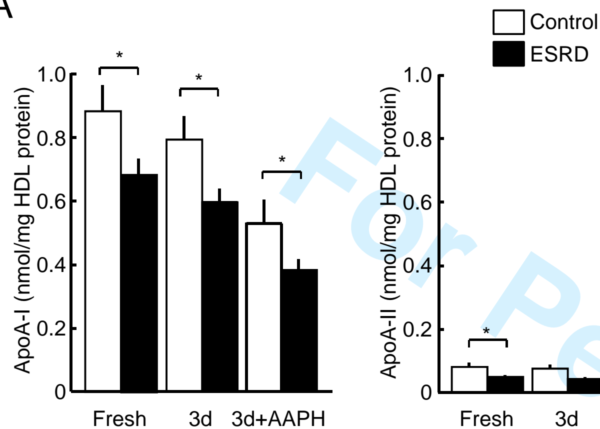

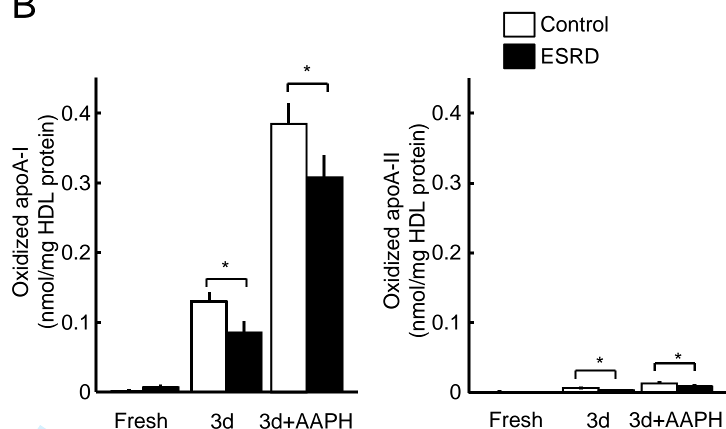

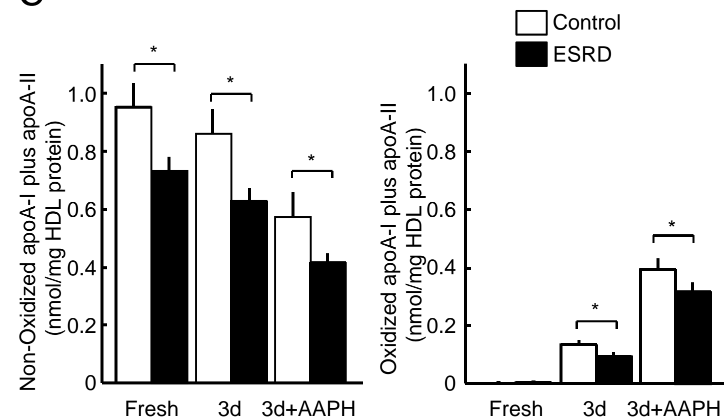

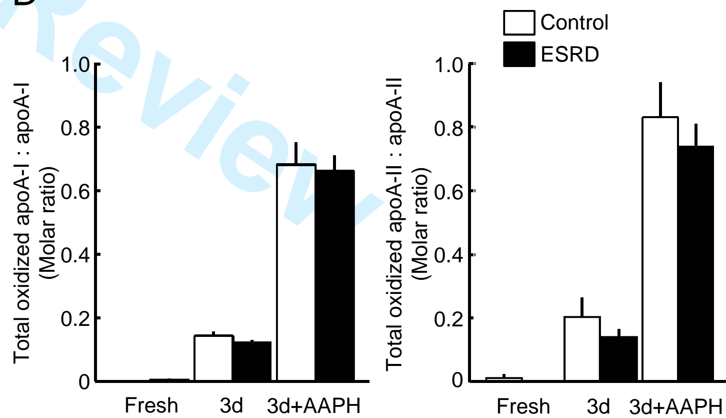

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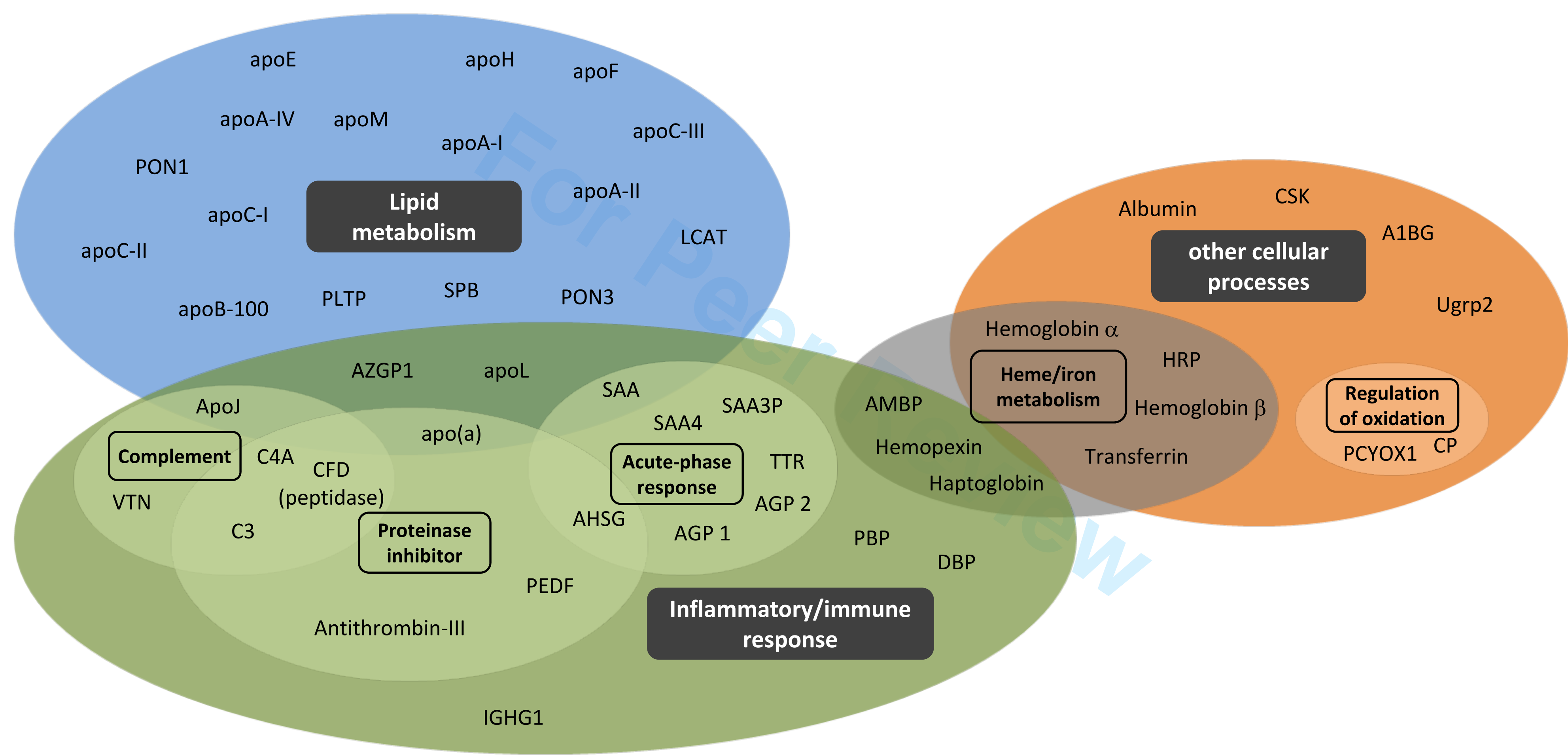

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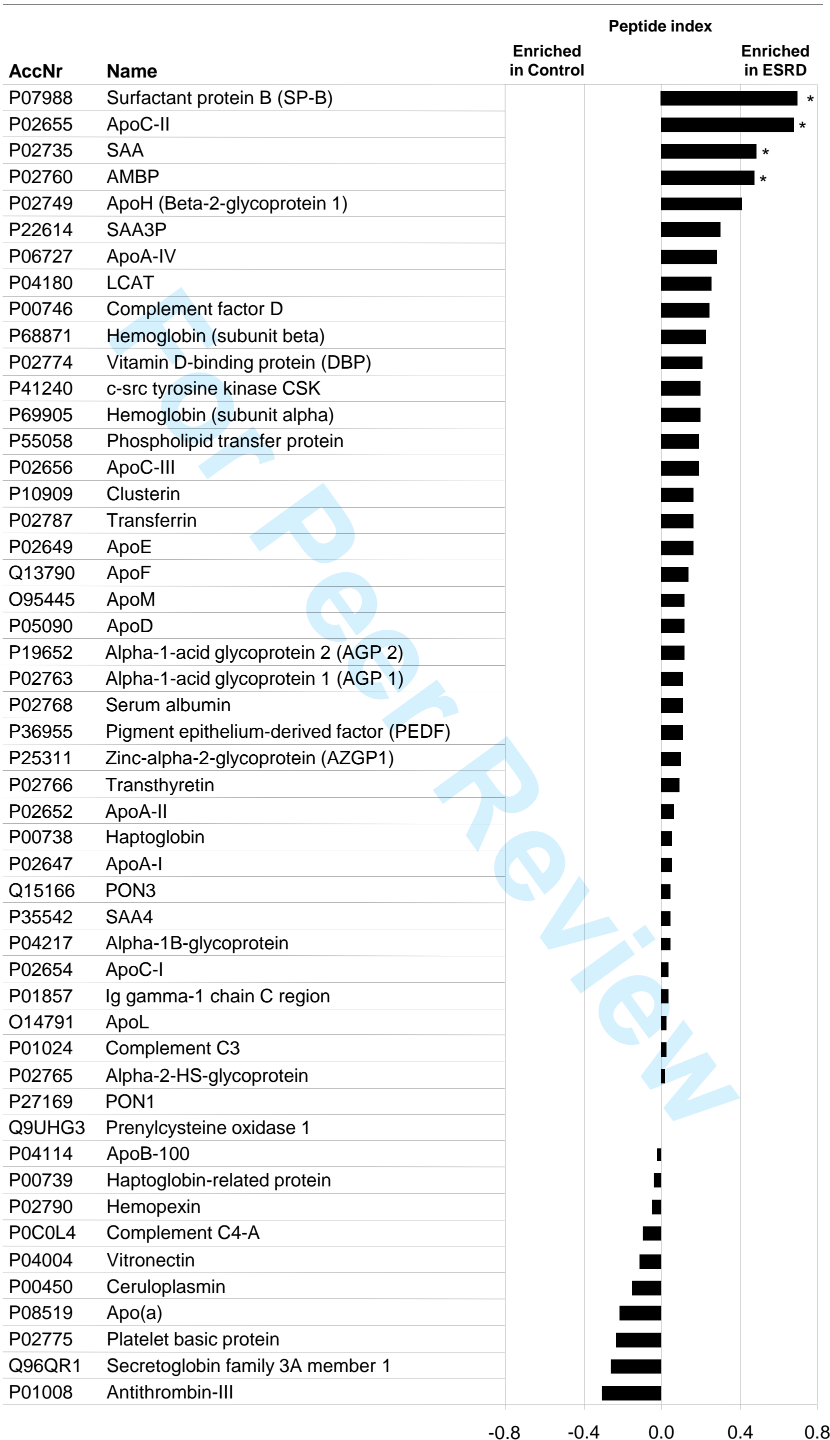

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ESRD | Control | ESRD | Control

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Transferrin

SP-B

PEDF

SAA

Apo-CII

ApoA-I

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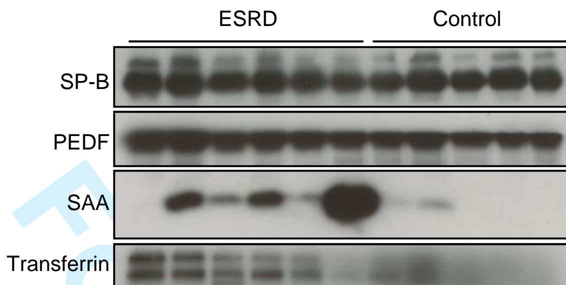

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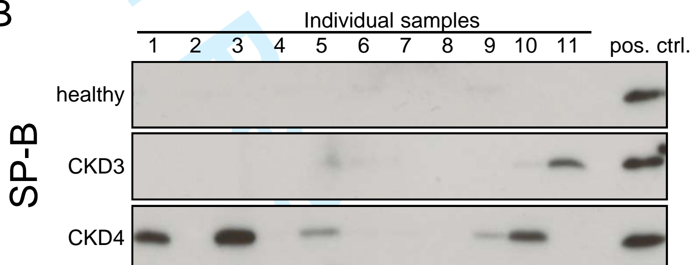

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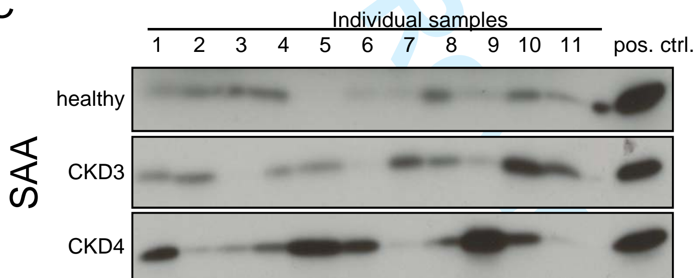

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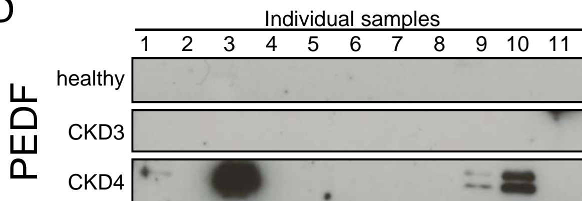

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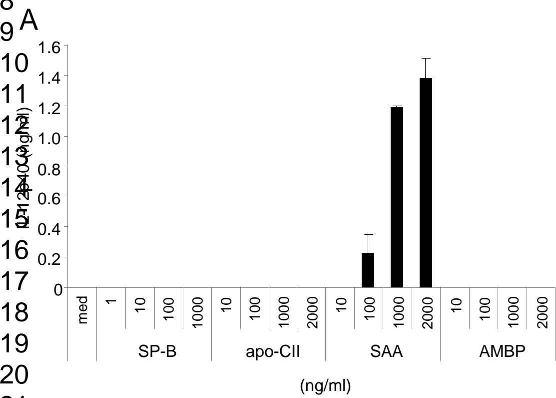

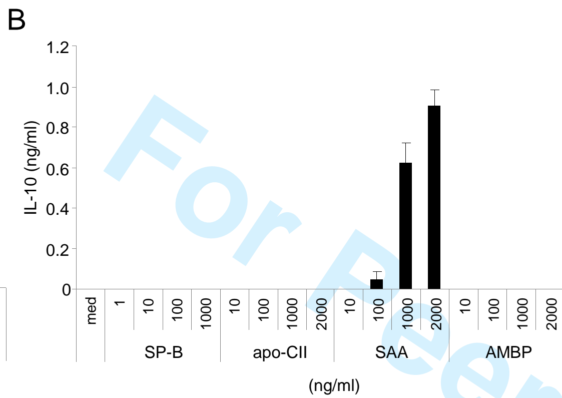

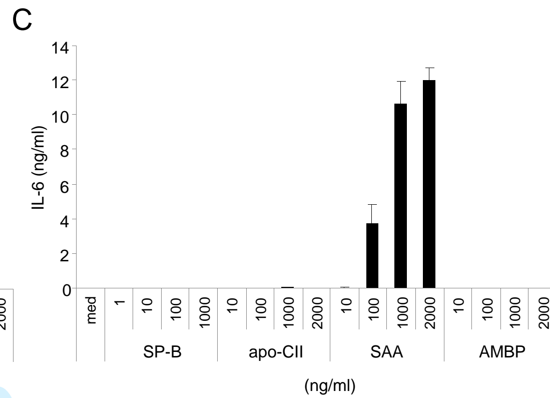

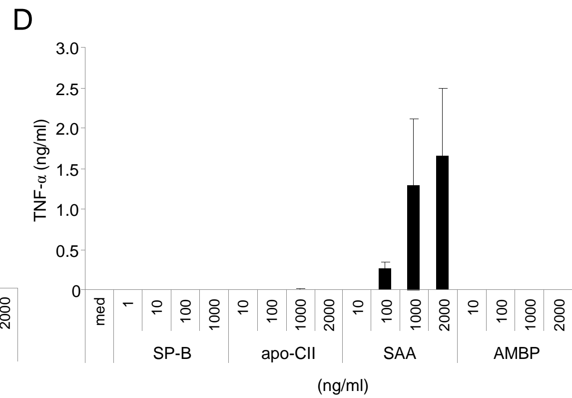

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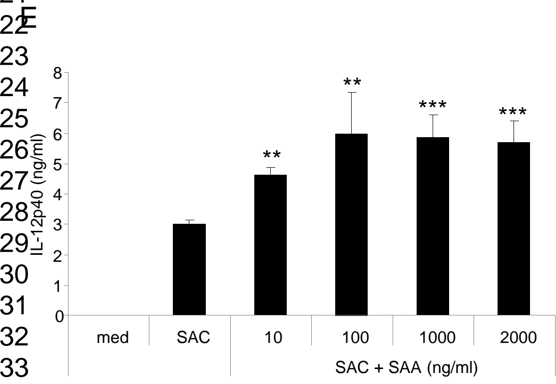

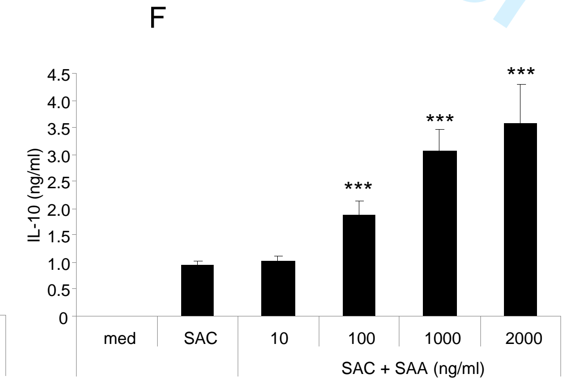

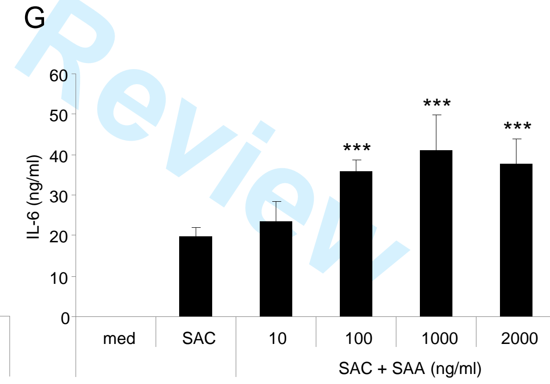

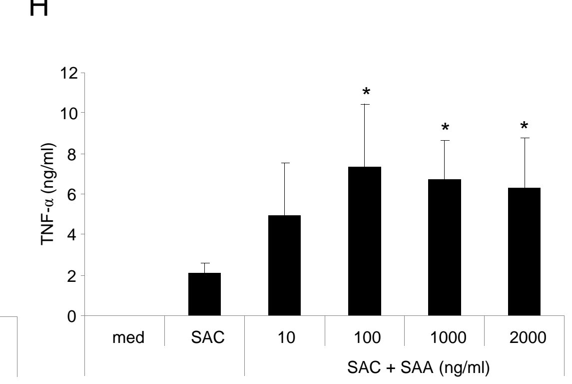

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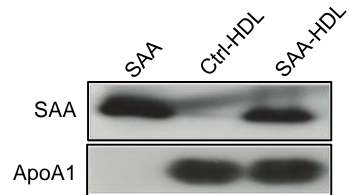

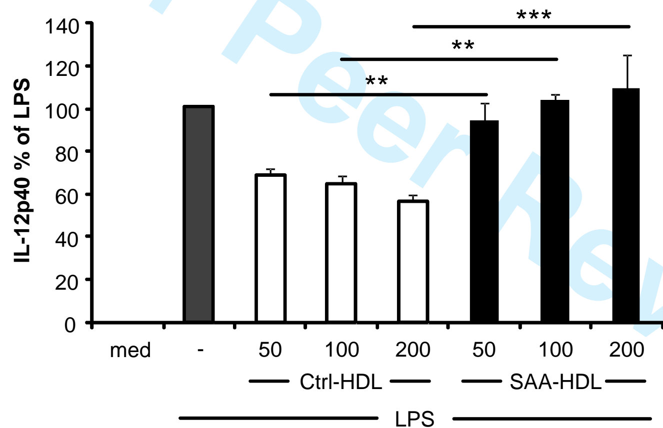

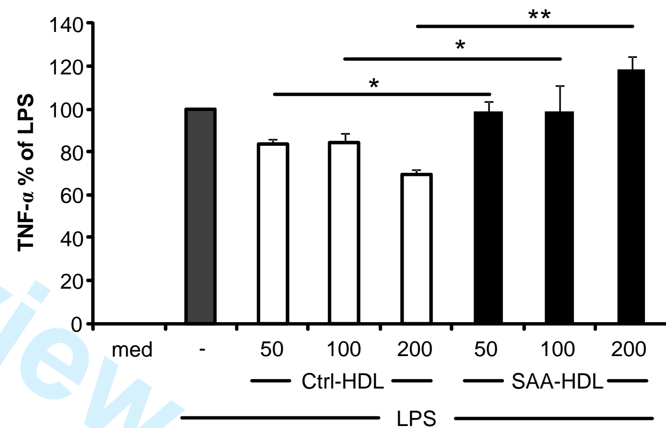

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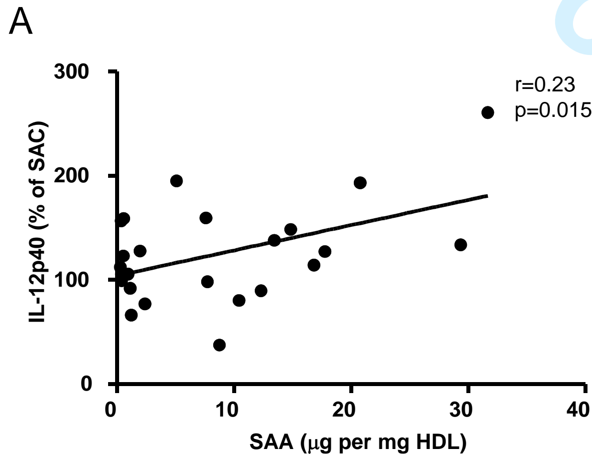
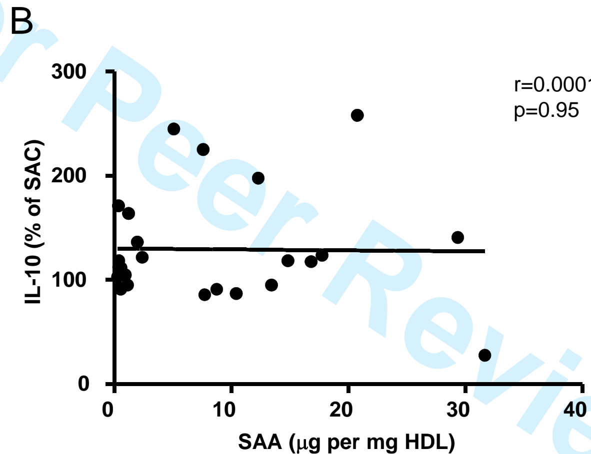
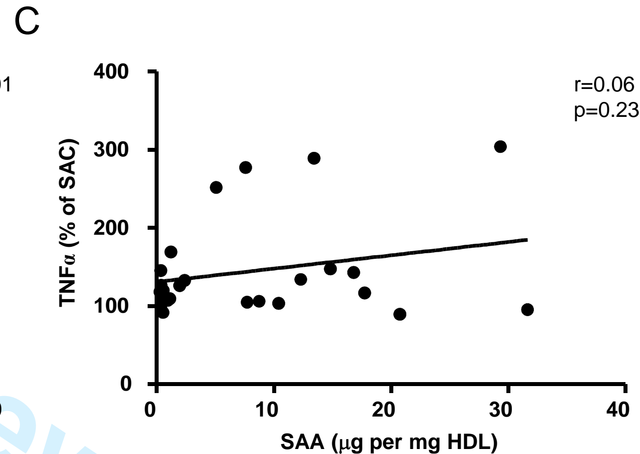
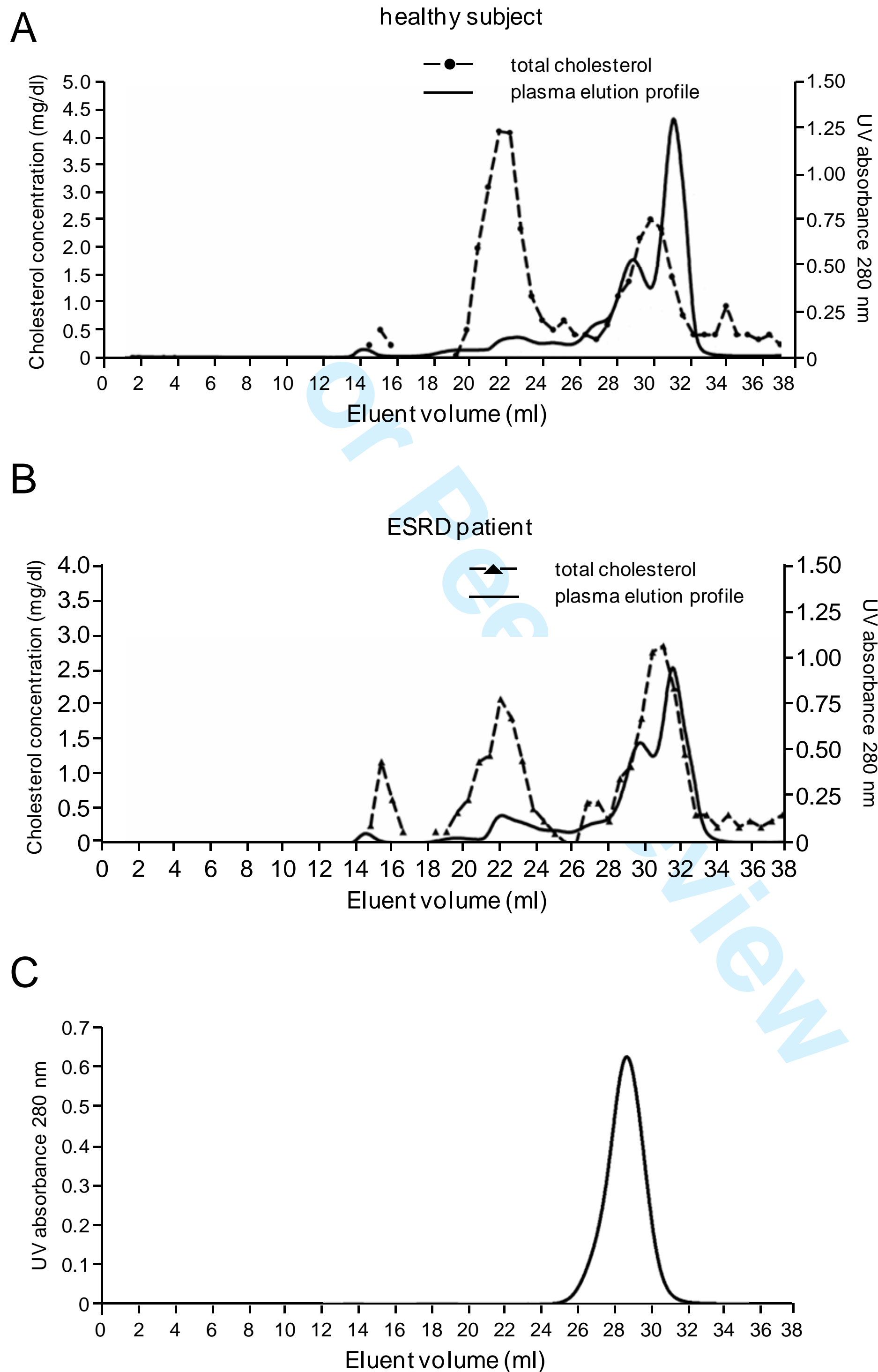






Weichhart et. al.: The proteomic signature of dysfunctional HDL in human uremia identifies serum amyloid A as proinflammatory component

Supplementary Figure 1



Supplementary Figure 1 | Cholesterol and lipoprotein profiles of plasma and isolated HDL. Representative FPLC (black lines) and cholesterol profiles (dashed lines) of plasma samples from a (a) healthy and an (b) ESRD subject. Total cholesterol concentrations were analyzed photometrically. (c) Cholesterol elution profile of healthy HDL isolated by sequential ultracentrifugation.

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Supplementary Table 1

AccNr	Name	Number of individual peptides	ESRD Subjects with detectable proteins	emPAI	Number of individual peptides	Control Subjects with detectable proteins	emPAI
P02647	ApoA-I	65	10/10	12.373	69	8/8	14.489
P02652	ApoA-II	15	10/10	5.135	15	8/8	6.295
P05090	ApoD	18	10/10	4.546	17	8/8	3.897
P68871	Hemoglobin (subunit beta)	13	5/10	2.249	5	2/8	0.833
P02656	ApoC-III	8	10/10	2.117	7	8/8	1.695
P35542	SAA4	13	10/10	2.037	12	8/8	2.232
P69905	Hemoglobin (subunit alpha)	9	4/10	1.917	2	3/8	0.348
P06727	Apolipoprotein A-IV (Apo-AIV)	47	10/10	1.828	26	7/8	0.741
P02768	Serum albumin	74	10/10	1.570	49	8/8	0.899
P02735	SAA	11	9/10	1.523	6	7/8	0.885
P27169	PON1	21	10/10	1.405	19	8/8	1.294
P01009	Alpha-1-antitrypsin	30	9/10	1.368	24	6/8	1.109
P02655	ApoC-II	8	8/10	1.339	7	7/8	0.507
O95445	ApoM	14	10/10	1.126	7	8/8	1.009
P02649	ApoE	29	10/10	1.096	29	8/8	1.440
P01034	Cystatin C	6	7/10	0.718	0	0/8	0.000
O14791	ApoL	18	10/10	0.677	17	8/8	0.579
P02654	ApoC-I	6	9/10	0.473	6	7/8	0.644
Q96QR1	Secretoglobin family 3A member 1 (Ugrp2)	1	2/10	0.468	2	2/8	0.468
P01834	Ig kappa chain C region	4	4/10	0.465	0	0/8	0.000
P55058	Phospholipid transfer protein (PLTP)	16	10/10	0.431	13	6/8	0.462
Q9UHG3	Prenylcysteine oxidase 1 (PCYOX1)	20	9/10	0.394	17	7/8	0.485
P10909	ApoJ	14	10/10	0.334	13	7/8	0.369
P02766	Transthyretin (TTR)	2	3/10	0.329	2	2/8	0.233
Q15166	Serum paraoxonase/lactonase 3	9	9/10	0.316	10	8/8	0.254
Q13790	ApoF	6	10/10	0.277	3	8/8	0.194
P00746	Complement factor D (CFD)	3	7/10	0.261	0	0/8	0.000
P04180	LCAT	10	8/10	0.260	5	5/8	0.209
P02775	Platelet basic protein (PBP)	2	5/10	0.243	2	2/8	0.166
P61769	Beta-2-microglobulin	3	5/10	0.243	1	1/8	0.166
P11597	CETP	6	5/10	0.242	8	6/8	0.325
P15144	Aminopeptidase N	11	4/10	0.223	0	0/8	0.000
P55056	ApoC-IV	2	2/10	0.214	3	4/8	0.219
P00739	Haptoglobin-related protein	7	6/10	0.213	3	3/8	0.177
P02760	AMBP	7	6/10	0.206	0	0/8	0.000
P02763	Alpha-1-acid glycoprotein 1 (AGP 1)	3	7/10	0.200	2	1/8	0.259
P00738	Haptoglobin	9	6/10	0.195	3	2/8	0.125
Q8TDL5	VEMSGP	5	3/10	0.165	0	0/8	0.000
P19652	Alpha-1-acid glycoprotein 2 (AGP 2)	2	3/10	0.159	2	1/8	0.245
P01019	Angiotensinogen (Serpin A8)	4	4/10	0.153	4	3/8	0.098
P02765	Alpha-2-HS-glycoprotein	4	6/10	0.145	5	4/8	0.201
P18428	Lipopolysaccharide-binding protein (LBP)	7	8/10	0.131	9	5/8	0.297
P05155	Plasma protease C1 inhibitor	4	3/10	0.122	1	1/8	0.059
P02787	Transferrin	6	2/10	0.120	0	0/8	0.000
Q14623	Indian hedgehog protein (IHH) (HHG-2)	2	1/10	0.116	3	2/8	0.116
P02748	Complement component C9	5	3/10	0.114	4	3/8	0.055
P60709	Actin, cytoplasmic 1	2	2/10	0.111	3	2/8	0.233
P02749	ApoH	2	5/10	0.107	0	0/8	0.000
P07988	Surfactant protein B (SP-B)	3	4/10	0.101	1	1/8	0.101
P25311	Zinc-alpha-2-glycoprotein (AZGP1)	3	4/10	0.101	0	0/8	0.000
P04114	ApoB-100	63	8/10	0.097	26	6/8	0.053
P05556	Integrin beta-1	7	5/10	0.094	2	2/8	0.049
Q13093	Platelet-activating factor (PAF)	3	3/10	0.092	1	1/8	0.054
Q9BUN1	Uncharacterized protein C1orf56	3	4/10	0.092	2	3/8	0.098
P13688	Biliary glycoprotein 1 (BGP-1)	2	3/10	0.083	0	0/8	0.000
P07602	Proactivator polypeptide	2	2/10	0.082	1	1/8	0.054
P58335	Anthrax toxin receptor 2	3	2/10	0.080	2	2/8	0.108
Q16762	Thiosulfate sulfurtransferase	1	9/10	0.080	1	4/8	0.080
P01024	Complement C3	21	9/10	0.071	7	7/8	0.020
P02774	Vitamin D-binding protein (DBP)	4	4/10	0.061	0	0/8	0.000
Q9H6X2	Anthrax toxin receptor 1	4	4/10	0.058	0	0/8	0.000
P02671	Fibrinogen alpha chain	4	4/10	0.057	2	2/8	0.057
P04004	Vitronectin (VTN)	3	4/10	0.055	2	5/8	0.078
P0C0L4	Complement C4-A	12	8/10	0.052	2	1/8	0.030
Q6PEZ8	Podocan-like protein 1	1	3/10	0.050	1	1/8	0.050
P80108	GPI-specific phospholipase D (GPLD1)	4	3/10	0.049	1	1/8	0.037
P41240	c-src tyrosine kinase CSK	1	5/10	0.044	1	3/8	0.044
P08514	Integrin alpha-IIb	2	2/10	0.037	6	2/8	0.139
Q99741	Cell division control protein 6 homolog	1	5/10	0.033	1	4/8	0.033
P08519	Apo(a)	1	2/10	0.009	0	0/8	0.000

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