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Chronic kidney disease increases carbonylation of HDL and is associated with impaired antiaggregant properties.

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Significance Statement

Chronic kidney disease (CKD) is associated with an increased risk of cardiovascular disease. The failure of statins in hemodialysis patients shed light on the unique phenotype of dyslipidemia in dialysis patients. HDL atheroprotective properties (cholesterol efflux, anti-inflammatory and vasorelaxation) in CKD have been found to be impaired. Little is however known about the anti-thrombotic properties of HDL in CKD. The present study describes the impaired anti-aggregant properties of HDL induced by CKD, and the putative role of carbonylation by 4-HNE adduction on these properties. This study provides important insights into the potential implication of HDL modifications in atherothrombosis and cardiovascular morbidity and mortality among dialysis patients.

Abstract

BACKGROUND: Cardiovascular diseases remain the major cause of death in chronic kidney disease (CKD). As oxidative stress is raised in CKD we aimed to explore the specific role of oxidative modifications of HDL in CKD and their impact on the anti-aggregant properties of HDL.

METHODS: Blood samples were taken from 15 healthy volunteers, 25 hemodialysis (HD) and 20 peritoneal dialysis (PD) patients at the Lyon university hospital. Eight 5/6-nephrectomized rabbits (CKD) were compared with 9 control rabbits (CR). Malondialdehyde (MDA), 4-hydroxy-nonenal (HNE), and 4-hydroxy-2-hexenal (HHE) protein adduct levels were analyzed. Platelet aggregation and activation was assessed by aggregometry, TxB2 assay or FACS. HDL from controls were modified in vitro through an incubation overnight at 37°C with 100µM of HNE.

RESULTS: HNE-adducts were increased in HDL from CKD rabbits and HD patients. Percentage of platelet aggregation/activation induced by collagen was significantly higher when were incubated with HDL from CKD rabbit and HD groups than with HDL from the control group. Platelet aggregation and activation in presence of HNE-modified HDL was also higher than control group either in rabbit or humans ($p < 0.05$ compared to CR or Control patients). Incubation with a blocking antibody directed against CD36 and a pharmacological inhibitor of SRC-kinases restored the anti-aggregative phenotype in CKD rabbit, HD and PD patients and HNE-modified HDL.

CONCLUSIONS: HDL from CKD rabbits and HD patients exhibited an impaired ability to inhibit platelet aggregation suggesting that altered HDL properties could contribute to the increased cardiovascular risk in this population.

Introduction

Chronic kidney disease (CKD) is recognized as a major cardiovascular risk factor.¹⁻³ It is involved in the onset of cardiovascular events such as myocardial infarction, peripheral arterial disease and cerebral ischemia¹. Cardiovascular mortality remains the major cause of death in hemodialysis (HD) and peritoneal dialysis (PD) patients despite the constant improvement of renal replacement therapies.⁴ CKD is associated with an increased oxidative stress that is correlated with the occurrence of cardiovascular events.⁵⁻⁷ This stress is associated with the accumulation of many uremic toxins⁸, some of which have recognized cardiovascular effects.⁹⁻¹² Modifications induced by increased oxidative stress particularly affect circulating lipoproteins such as HDL that exhibit anti-atheromatous and anti-thrombotic properties *in vitro*.

There are many mechanisms that lead to lipoprotein modification, both enzymatic and non-enzymatic, and these affect different lipoprotein sites.¹³ Oxidized and carbamylated LDL are present at higher concentrations in CKD compared to healthy patients^{5,14} and play a major role in the onset and aggravation of atherosclerotic lesions.^{14,15} The concentration of oxidized and carbamylated LDL are, however, lowered by statins.¹⁶⁻¹⁸ HDL is considered to be antiatherogenic as a result of its antiaggregant, anti-inflammatory and antiapoptotic properties¹⁹ as well as its ability to induce cholesterol efflux from macrophages²⁰, a mechanism known as atheroprotective.²¹ HDL is not affected by conventional hypolipidemic strategies, and functional studies in CKD have found impaired biological effects, including decreased capacity of macrophage cholesterol efflux²²⁻²⁴ and anti-oxidation,²⁴ as well as impaired endothelial protection.²⁵ These disorders become more significant with CKD progression.²⁵ In parallel, HDL oxidation has been related to the onset of cardiovascular events in CKD patients.²⁶ These functional modifications could partly explain the failure of statins to reduce cardiovascular risks associated with haemodialysis.^{27,28}

Platelets are the target of anti-aggregant treatments which have been shown to be beneficial in reducing cardiovascular events in at-risk populations. Oxidized LDL of obese and diabetic patients has shown some pro-aggregating properties.^{29,30} In CKD, carbamylated LDL exhibits pro-aggregant proprieties through binding with lectin-like oxidized low density lipoprotein receptor 1 (LOX-1)³¹, and oxidized LDL through binding to the CD36 receptor.³² Oxidation of HDL leads to a loss of antiaggregation properties in some populations,³³ although this observation was not found in type 2 diabetes³⁴ in whom oxidized HDL has shown antiaggregant properties through a SR-B1 -mediated pathway.³⁵ To the best of our knowledge, there is, however, no published data regarding the impact of CKD on the antiaggregant properties of HDL.

In the present study, we therefore aimed to explore the specific role of CKD in the oxidation profile of HDL and the effects of HDL modification on platelet aggregation in a rabbit model of CKD. We then aimed to assess, *ex vivo*, the antiaggregant properties of HDL isolated from HD and PD patients.

Concise Methods

Subjects and ethics statement

Patients were sampled at the Lyon teaching hospitals. Control patients were healthy volunteers for a living kidney donation, hospitalized for their pre-donation laboratory and clinical work-up. HD patients were sampled within the hemodialysis unit of the Edouard Herriot hospital (Lyon) before the mid-week session. PD patients were sampled in the peritoneal dialysis unit of the Association pour l'Utilisation du Rein Artificiel (AURAL, Lyon, France). Inclusion criteria were age ≥ 18 years, undergoing HD or PD for more than 6 months. Exclusion criteria were diabetes mellitus, ongoing inflammatory disease, liver cirrhosis, recent cardiovascular event (< 3 months; myocardial infarction, stroke, acute peripheral artery occlusion), uncontrolled anemia, coagulopathy, and BMI greater than 35 kg.m^{-2} . The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board (*CPP Lyon Sud Est IV*, ref: L16-57). A written informed consent was obtained from all subjects. Blood samples were obtained by venipuncture on EDTA coated tubes. Blood samples were centrifuged at $3500 \times g$ for 10 min to isolate plasma which were stored at -80°C until use.

Animal procedures

All experiments were performed under the authorization no. n°69-266-0501 and agreed with the guidelines laid down by the French Ministry of Agriculture (n° 2013-118) and the European Union Council Directive for the protection of animals used for scientific purposes of September 22, 2010 (2010/63UE). Adult male White New Zealand rabbits (CEGAVssc, Saint Mars d'Egrenne, France) were housed in individual cages at constant ambient temperature ($21\text{-}23^{\circ}\text{C}$) and humidity (45-50%) with a 12h light cycle. All animals had free access to tap water. After a 7-day period of acclimation, rabbits were randomized to either the 5/6 nephrectomy group or in the control group. Nephrectomy was performed as described by

Gotloib *et al.*³⁶ Briefly, rabbits were anaesthetized using an intramuscular injection of ketamine (50mg/kg), xylazine (5mg/kg), and acepromazine (0.5mg/kg, Centravet, Lapalisse, France). The rabbit was placed on the right lateral decubitus. Local anesthesia was performed by a subcutaneous injection of xylocaine 2% (2 ml) at the incision site. The left kidney was externalized and the perirenal adipose tissue was gently dissected. The two poles of the kidney were electrocoagulated using an electric needle to produce a 2/3 reduction of the left kidney mass. One week after surgery, a unilateral right nephrectomy was performed. The kidney pedicle was carefully sectioned between the kidney and the ligature. The control animals underwent the same procedures (general anesthesia, skin, and muscle incisions) followed by a simple kidney mobilization. All rabbits were given buprenorphine (0.05mg/kg sc, 2-times a day) for 2 days to prevent post-surgical pain. Food intake and body weight were measured on a daily and twice-weekly basis, respectively.

Measurement of glomerular filtration rate

The glomerular filtration rate (GFR) in rabbits was measured through the kinetics of decrease of plasma concentration of iohexol as described in Florens *et al.*³⁷ Rabbits were anesthetized with an intravenous injection of 27.5 mg/kg of sodium pentobarbital (Centravet). A PE-50 catheter was introduced in the left jugular vein for the injections and PE-60 catheter was disposed in the left carotid artery for the blood sampling. A 1mL bolus of iohexol (Omnipaque 300[®], GE Healthcare, Chicago, IL, USA) was performed and the timer was started after flushing the residue left in the jugular catheter with a saline solution. Blood was sampled at 5, 15, 30, 45, 60, 120, and 180 min on lithium-heparin coated tubes. The plasma was aliquoted after centrifugation at 5,900 x g for 2 min and stored at -20°C. The serum iohexol concentration was measured by HPLC as previously described³⁸ and GFR was calculated using a bi-compartmental model equation.

Sacrifice, necropsy and renal histology

At the end of the GFR measurement, rabbits were deeply anesthetized with an overdose of sodium pentobarbital (70 mg/kg iv). Blood was removed by cardiac puncture and placed in EDTA-coated tubes. After a centrifugation at $1250 \times g$ for 10 min, plasma was aliquoted and stored at -80°C . Urine was obtained via a direct bladder puncture and stored at -20°C . Kidneys were removed, weighed and stored in buffered formalin 10% (w/v) for histological examination. Kidneys were dehydrated using ethanol, embedded in paraffin and sliced. Haematoxylin Erythrosine Saffron (HES) and Sirius Red stainings were performed.

Isolation of lipoproteins from the plasma

Lipoproteins were separated from plasma by stepwise potassium bromide (KBr) density gradient ultracentrifugation as described by Havel *et al.*³⁹ Briefly, plasma was fractionated in the Beckman ultracentrifuge with a rotor TAL 100.3 (Beckman, Brea, CA, USA). A first centrifugation at $100,000 \times g$ for 3h 30min at 15°C was performed to remove the top layer corresponding to VLDL and chylomicrons. Then, the plasma density was adjusted to 1.063 g.mL^{-1} with KBr ($M=119.01 \text{ g.mol}^{-1}$). After a second centrifugation at $100,000 \times g$ for 5h at 4°C the orange ring, corresponding to LDL, was collected. Finally, plasma density was adjusted to 1.21 g.mL^{-1} with KBr and after centrifugation at $100,000 \times g$ for 6 h 30 at 4°C the orange ring corresponding to HDL, was collected. For the isolation of all the lipoproteins together, a single ultracentrifugation was performed at $100,000 \times g$ for 6h 30min at 4° after an adjustment of plasma density to 1.21 g.mL^{-1} with KBr.

For platelet aggregation and copper-induced HDL oxidation assay freshly isolated HDL samples were used to prevent from HDL ultrastructure modification due to freezing,⁴⁰ after the ultracentrifugation, lipoproteins were extensively dialyzed against phosphate saline buffer

(PBS) with 1mM EDTA for 3h, twice at room temperature and then overnight at 4°C. A last dialysis without EDTA was performed just before the platelet aggregation and copper-induced oxidation. HDL were stored at 4°C maximum 48 hours before the experiments.

Biochemistry

Serum creatinine measurement was performed using the Siemens enzymatic method (on the Dimension Vista System, Siemens Healthcare, Erlangen, Germany). Urea was measured with urease test (Vista 1500). Cholesterol and triacylglycerols levels were measured using enzymatic kits (Biomerieux, Marcy l'Etoile, France). HDL concentration was measured with an enzymatic kit (Abcam, Paris, France). Malondialdehyde (MDA) was measured by HPLC coupled to UV-visible detection (Diode Array detector) as described by Grotto *et al.*⁴¹ Antioxidant activity (AOA) of the plasma was measured as described by Koracevic *et al.*⁴² Proteinuria was measured using the Bradford protein assay (BioRad, Marne-la-Coquette, France) using bovine serum albumin as a standard.

Lipoprotein assays

MDA concentration in HDL was determined by HPLC according to the method described by Therasse and Lemonnier.⁴³ Anti-4-hydroxy-nonenal (HNE)-Michael adduct (ref 393207) and anti-4-hydroxy-2-hexenal (HHE)-Michael adduct (ref NOF-N213730-EX) antibodies were obtained from Calbiochem (San Diego, CA, USA) and Cosmobio (Tokyo, Japan), respectively. Fifty micrograms of HDL were loaded directly on to a nitrocellulose membrane using the Bio-Dot apparatus (BioRad, Marne-la-Coquette, France). Following saturation with 5% BSA, membranes were probed overnight with primary antibodies; anti-HHE-Michael adducts, or anti-HNE-Michael adducts. After incubation with HRP-coupled secondary antibodies, membranes were processed for chemiluminescence (ECL plus, GE Healthcare,

Chicago, IL, USA) and quantitated by densitometry using Image J software (NIH, Bethesda, MD, USA). 8-isoprostane was measured using an immunoassay (Bertin Pharma, Montigny Le Bretonneux, France). 4-HNE was synthesized as described by Soulière *et al.*⁴⁴ and was diluted in dimethylsulfoxide (DMSO). 4-HNE was added to HDL solutions to final concentrations of 1, 10, 50 or 100µM. After a 16 h (overnight) incubation at 37°C in a water bath, HDL were dialyzed three times against PBS to remove the free fraction of 4-HNE.

Platelet aggregation, activation and intra-cellular pathways assays

Blood was collected at the regional blood center from healthy volunteers who had not ingested any aspirin or any other non-steroidal anti-inflammatory drug in the previous 10 days. Platelets were prepared for the assays as described by Lagarde *et al.*⁴⁵ Platelet function test was carried out according to the Born turbidimetric method.⁴⁶ Platelet aggregation was measured in isolated platelets in a dual-channel aggregometer (Chrono-log; Coulter, Margency, France). Platelet suspensions were pre-incubated for 5 min at 37°C in the presence or absence of lipoproteins (0.025 mg of protein/mL for rabbit, 0.050 mg/mL for human) and stimulated with threshold concentrations of collagen (75 ± 9 ng/ml) while being continuously stirred at 1,000 rpm. The threshold concentration of collagen was defined as the concentration of collagen that induced a 50% increase in light transmission. The extent of platelet aggregation was expressed in terms of percentage of change in light transmission 4 min after the addition of collagen. Blocking of CD36 or SRB1 receptor was achieved by the pre-incubation with 10 µL of an anti-CD36 (Ab-CD36) or anti-SRB1 (Ab-SRB1) antibodies (dilution 1:500, Abcam, Paris, France) for 10 min at 37°C before the incubation with HDL. Blocking of SRC-kinases was achieved by the pre-incubation with Naphtyl PP1 (final concentration 1 µM, Santa Cruz Biothechnologies, Dallas, TE, USA) for 10 min at 37°C before the incubation with HDL. Aggregation values from lipoprotein assays were expressed

as a percentage of the maximum aggregation induced by the collagen alone (considered as 100%). Levels of thromboxane B2 (TxB2) was measured using an immunoassay expressed as a percentage of the maximum level induced by the collagen alone (considered as 100%, Cayman Chemical, Ann Arbor, MI, USA). After pre-incubation with HDL and antibodies or pharmacological inhibitor, platelets were activated with collagen then fixed in paraformaldehyde 5% and stained with anti-CD62/P-Selectin (Thermo Fisher Scientific, San Jose, CA, USA) according to the manufacturer protocol and analyzed by flow cytometry.⁴⁷

Copper-induced HDL oxidation

Aliquots of freshly dialyzed HDL (50 µg protein) were oxidized in the presence of 2.5 µM of CuSO₄ within the day following the last dialysis. The oxidation was monitored by measuring the formation of conjugated dienes at 234 nm.⁴⁸

Mass spectrometry

Samples were reduced, alkylated and digested with trypsin at 37°C overnight. They were desalted with spin column C18, dried and then analyzed in triplicate using an Ultimate 3000 nano-RSLC (Thermo Fisher Scientific) coupled on line with a Q-Orbitrap (Q Exactive HF, Thermo Fisher Scientific). Briefly, peptides were separated on a C18 nano-column with a linear gradient of acetonitrile and analyzed in a Top 20 HCD (Higher Collision Dissociation) data-dependent mass spectrometry. Data were processed by database searching using SequestHT (Thermo Fisher Scientific) with Proteome Discoverer 2.2 software (Thermo Fisher Scientific) against a human Swissprot database and quantified with a label free quantitation approach. Precursor and fragment mass tolerance were set at 10 ppm and 0.02 Da respectively. Trypsin was set as enzyme, and up to 2 missed cleavages were allowed. Oxidation (M), acetylation (Protein N-terminus) and HNE (+ 156.115 Da on K or H) were set

as variable modification as fixed modification. Peptides were filtered with false discovery rate (FDR) at 1%. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013301 (DOI: 10.6019/PXD013301; username: reviewer26456@ebi.ac.uk; password: oMD4gyDT).

Molecular modelling

To study the accessibility of residues, the structure of truncated human apolipoprotein A-I (pdb code 1av1) was downloaded from the PDB database⁴⁹. All observations were performed using PyMOL as software by representing the α -helices and histidine and lysine residues (colored in blue) in CPK to facilitate the study. The same method was achieved with the HDL model which was download from the PDB database.⁵⁰

Statistical analysis

Data were expressed as median \pm interquartile range (IR). All analyses were performed using GraphPad Prism version 6.0 (GraphPad software, La Jolla, CA, USA). Simple comparisons were made using Mann-Whitney U-test. Multiple comparisons were made with Kruskal-Wallis test and whenever appropriated Dunn's tests. Differences were considered as significant at the $P < 0.05$ level.

Results

General characteristics of the CKD rabbit model

Rabbits developed a significant renal insufficiency in the CKD group; there was a significant difference ($P < 0.001$) in the median GFR between the control group and the CKD group (-58%, **Table 1**). CKD rabbits exhibited increased levels of lipid peroxidation in the plasma as evidenced by the 10-fold significant increase in MDA concentration ($P < 0.001$, **Table 1**). Median antioxidant activity of the plasma was significantly ($P < 0.05$) lower in the CKD group compared with control (-15%, **Table 1**). Histological examination found a diffuse fibrosis in the parenchyma in the CKD group as well as a glomerulomegaly (**Supplementary Figure 1**).

HDL oxidation level and oxidizability in CKD rabbits

HDL from CKD rabbits had significantly higher levels of HNE-Michael adducts on proteins ($p < 0.05$, **Figure 1A**). By contrast, HHE-adducts on proteins were not significantly higher in the HDL of the CKD group (**Figure 1A**). MDA concentration was significantly higher in the HDL of the CKD group (**Figure 1B**) while the 8-isoprostane concentration was not significantly different (**Figure 1C**). Despite a non-significant difference in the concentration of tocopherols (**Figure 1D**), HDL lipids from CKD rabbits were more prone to oxidize than HDL from control animals; there was a significant difference from 70 min until the end of the experiment ($p < 0.05$, **Figure 1E**).

Platelet aggregation induced by HDL from rabbits

When human platelets were incubated with HDL from the CKD group, the median aggregation was 75% as compared to collagen alone; when incubated with HDL from the control group it was 30% ($p < 0.05$ as compared to when incubated with HDL from the CKD

group, **Figure 2A**). As HNE-adducts were increased in CKD HDL, we incubated control rabbit HDL with 100 μ M HNE; median platelet aggregation in the presence of HNE-modified HDL was 85% (**Figure 2A**, $p < 0.05$ as compared to when incubated with HDL from the control group). Pre-incubation of platelets with an anti-CD36 antibody restored the antiaggregant effect of the HDL from CKD rabbits and HNE-modified HDL (median: 25 and 22%, respectively, **Figure 2A, C**). Pre-incubation of platelets with Ab-CD36 did not change significantly the aggregation induced by collagen (median: 95% vs. 100%, NS).

To investigate the effect of total lipoproteins (HDL, LDL, and triglyceride-rich lipoproteins; LPP) of CKD animals, platelets were incubated with a lipoprotein mix resulting from a single ultracentrifugation. The median aggregation in the CKD LPP group was 98% vs. 21% in the control group ($p < 0.05$, **Figure 2B**). Platelet aggregation in the presence of HNE-modified LPP was 99% ($p < 0.05$ compared to control LPP group). Pre-incubation of platelets with an anti-CD36 antibody restored the antiaggregant effect of the LPP from CKD rabbits and HNE-modified LPP (median: 23 and 11%, respectively, **Figure 2B**). HNE-adduct levels in HNE-HDL and LPP were higher than in control using an immuno-blot (**Figure 2D**).

Carbonylation of HDL from HD patients

Nine controls were compared to 9 non-diabetic HD, the main characteristics of whom are presented in **Supplementary Table 1**. HD patients had significantly lower total cholesterol, LDL-cholesterol, and HDL cholesterol than the controls ($p < 0.05$). There was a higher level of HNE-adducts in HDL from HD patients than in controls (**Figure 3A**). To map sites on proteins susceptible to carbonylation, HDL from control and HD patients were digested with trypsin; digested peptides were separated by liquid chromatography (LC), and peptides were analyzed for carbonylation with tandem mass spectrometry (MS/MS). We then expressed a ratio of amount of the 4-hydroxy-2-nonenal into the HD patient sample versus the mean

amount of control patients. An higher ratio (>1) of 4-HNE Michael adduct was detected onto 48 amino-acids (Lysine or Histidine) from 8 constitutive proteins of HDL from HD patients (**Table 3**). The majority was located onto apolipoprotein A1 and concerned various important functional sites of the protein (**Figure 3B, Supplementary Figure 2**). Lys 250, located in the C terminal part of apolipoprotein A1, was the most frequently modified site in HD patients (5.3-fold increase compared to controls, $p < 0.01$, **Figure 3C**).

In silico modelling of 4-HNE adduction onto apolipoprotein A1

In order to gain insights into the accessibility of histidine and lysine residues of apolipoprotein A1, a molecular modeling study was achieved with the lipid-free structure of the truncated protein (**Figure 4A**).⁴⁹ Histidine and lysine residues mentioned in the primary sequence of amino-acids namely K83, K130, H179, H186, H216, H223, K226, K250 and K262 were examined and were found to be readily accessible and susceptible to react with 4-HNE (not available for H20, K47 and K64 residues in the truncated protein). Therefore, the modification of these residues may occur in a random event on free apolipoprotein A1. Careful examination of the model of HDL proposed by Wu et al (**Figure 4B**)⁵⁰ led to the following observations: some histidine and lysine residues are not involved in interactions with lipids and are located at the external part of HDL. These residues which can react with 4-HNE may not affect the binding of apolipoprotein A-I with lipids but with other proteins involved in HDL metabolism (see **Figure 3B**, residues K47, K83, H216, K250 and K262); in contrary, some histidine and lysine residues (residues K64, K130, H179, H186 and H223) interact tightly with lipids in the internal side of the complex. In this case, it can be proposed that these modifications may alter the ability of apolipoprotein A-I to bind lipids. Overall, this molecular modeling study shows that the reaction of lysine and histidine residues leading to

structural modifications may occur randomly but may be critical for lipids and protein binding depending on the residue which is modified.

Platelet activation and aggregation phenotype of HDL from HD and PD patients and effects of carbonylation

As HD procedure *per se* can lead to an enhanced oxidative and carbonylated stress, we included PD patients to avoid any effects resulting from bio-incompatibility of HD devices. Fifteen healthy controls were compared to 25 non-diabetic HD and 20 PD patients, the main characteristics of whom are presented in **Table 2**. HD patients had significantly lower total cholesterol, LDL-cholesterol, and HDL cholesterol than the controls ($p < 0.05$). Platelet activation was measured with the production of thromboxane B₂ and expressed as a percentage of the amount found in platelet activated with collagen. When human platelets were incubated with HDL from the HD group, the median activation was 147% as compared to collagen alone; when incubated with HDL from the control group it was 75% ($p < 0.05$ as compared to when incubated with HDL from the HD group, **Figure 5A**). When human platelets were incubated with HDL from the PD group, the median activation was 221% as compared to collagen alone ($p < 0.05$ as compared to when incubated with HDL from the control group, $p = \text{NS}$ as compared to when incubated with HDL from the HD group, **Figure 5A**). As HNE-adducts were increased in HD HDL, we incubated 5 healthy donors HDL with 100 μM HNE; median platelet activation in the presence of HNE-modified HDL was 123% as compared to collagen alone; when incubated with HDL from the control group it was 29% (**Figure 5B**, $p < 0.05$ as compared to when incubated with HDL from the healthy donor group).

As we performed aggregation assays in rabbits, we chose 9 control and 9 HD patients (characteristics in **Supplementary Table 1**) and performed platelet aggregation assays. When

human platelets were incubated with HDL from the HD group, the median aggregation was 50% as compared to collagen alone; when incubated with HDL from the control group it was 19% ($p < 0.05$ as compared to when incubated with HDL from the HD group, **Figure 5C**). We found a good correlation between percentage of activation with TxB2 assay and percentage of aggregation ($r^2 = 0.3894$, $p < 0.05$, **Supplementary Figure 3**). As HNE adducts were increased in HD HDL, we incubated healthy donors HDL with 100 μM HNE; median platelet aggregation in the presence of HNE-modified HDL was 170% (**Figure 5C**, $p < 0.05$ as compared to when incubated with HDL from the control group).

As there was an interindividual variability of platelet response to HD-HDL, we tested several concentrations of HNE for the modification of control HDL from healthy donors (1, 10, 50, and 100 μM). A threshold effect was found as only the 100 μM solution of HNE led to a significant alteration of the anti-aggregant properties of HDL (median: 5% at 100 μM vs. 98, 99, and 99% at 1, 10, and 50 μM , respectively, $p < 0.05$, **Figure 5D**). Immunoblotting found a dose-dependent increase of HNE-adducts in HDL incubated with 4-HNE (1-100 μM ; **Supplementary Figure 4**). The median levels of 4-HNE adducts in HNE-modified HDL were similar to those of HD and PD patients (see **Supplementary Figure 5**).

Activation is mediated by the phosphorylation of JNK through a CD36 and SRC kinases pathway.

To get an insight into the potential pathways involved in the effects described herein, the effect of CD36, SRB1 blockade and SRC kinase pharmacologic inhibitor were tested. Pre-incubation of platelets with an anti-CD36 antibody significantly decreased their activation measured by TxB2 levels when incubated with HDL from control, HD, PD and HNE-modified HDL (median: 39%, 41%, 22% and 49% respectively, $p < 0.05$, as compared to when incubated without the antibody, **Figure 6A**). P-selectin expression was also lowered by the pre-incubation of platelets with an anti-CD36 antibody for HDL from control, HD, PD and

HNE-modified HDL (median: -43%, -30%, -42% and -27% respectively, $p < 0.05$, as compared to when incubated without the antibody, **Figure 6B**). Pre-incubation of platelets with an anti-CD36 antibody restored the antiaggregant effect of the HDL from HNE-modified HDL (median: 12%, $p < 0.05$, as compared to when incubated without the antibody, **Supplementary Figure 6**). Pre-incubation of platelets with Ab-CD36 did not change significantly the aggregation induced by collagen (median: 107% vs. 100%, NS, **Supplementary Figure 6**).

Pre-incubation of platelets with an anti-SRB1 antibody significantly increased their activation measured by TxB2 levels when incubated with HDL from control, HD, PD and HNE-modified HDL (median: 385%, 178%, 288% and 758% respectively, $p < 0.05$, as compared to when incubated without the antibody **Figure 6A**). P-selectin expression was also increased by the pre-incubation of platelets with an anti-SRB1 antibody for HDL from control, HD, PD and HNE-modified HDL (median: +35%, +93%, +54% and +88% respectively, $p < 0.05$, as compared to when incubated without the antibody, **Figure 6B**). Pre-incubation of platelets with anti-SRB1 did not significantly alter the aggregation induced by collagen (data not shown).

Pre-incubation of platelets with Naphtyl PP1, a potent pharmacological inhibitor of SRC-kinases, decreased their activation measured by TxB2 levels when incubated with HDL from control, HD, PD and HNE-modified HDL (median: 22%, 46%, 24% and 22% respectively, $p < 0.05$, as compared to when incubated without the inhibitor **Figure 6A**). P-selectin expression was also decreased by the pre-incubation of platelets with Naphtyl PP1 for HDL from control, HD, PD and HNE-modified HDL (median: -48%, -48%, -53% and -14% respectively, $p < 0.05$, as compared to when incubated without the inhibitor, **Figure 6B**).

We confirmed that intra-platelet phosphorylation of JNK was reduced by CD36 and SRC Kinases blocking while SRB1 blocking increased its phosphorylation (data not shown).

Discussion

The present study confirms that HDL isolated from patients with CKD exhibits impaired biological functions that may participate in the onset of cardiovascular disease in this population. The work emphasizes the effect of carbonylation from long-chain n-6 fatty acids (4-HNE adducts) on the antiaggregative properties of HDL. This adduction onto the protein component of the HDL particle was responsible for a deep and significant alteration of the antiaggregant properties of HDL in CKD. Combined with all the others impaired functions of HDL, this could be a potent contributor to the increased cardiovascular morbidity and mortality in CKD³ and the failure of statins in HD patients.^{27,28} Moreover, a significant part of this pathological phenotype was mediated by a CD-36 dependent pathway as its blockade restored normal anti-aggregant properties. Furthermore, CKD *per se* was responsible for a large part of this carbonylation as these adducts were obtained in a rabbit CKD model without other cardiovascular risk factors.

The rabbit model is of interest for cardiovascular research (e.g. atherosclerosis, lipid metabolism) since these animals have a lipoprotein metabolism much closer to humans than mice or rats due to the expression of the cholesterylester transfer protein (CETP) gene. The CKD rabbits, despite having a lower body weight, developed a metabolic disturbance as their plasma cholesterol and glucose levels were significantly higher. The median measured GFR reduction in CKD animals corresponded approximately to stage 3 CKD in humans, and

results of oxidative stress assays (MDA and AOA) were consistent with values observed in a human CKD population,⁵ which supports the appropriateness of the model. However, a natural history of 3-weeks is unlikely to lead to the same modifications as several years of interaction between cardiovascular risk factors and uremia in humans. Nevertheless, we found an increase in 4-HNE adducts and the impaired antiaggregant properties of HDL in HD and PD patients, rendering the animal findings relevant.

The results of the present study suggest that CKD *per se* may trigger a strong oxidation of HDL particles. Interestingly, 4-HNE adducts were both increased in the HDL of CKD rabbits and HD and PD patients. This can be due to a loss of antioxidant capacity of HDL particles in CKD as HDL from CKD rabbits were more sensitive to copper-induced oxidation. This is in accordance with data from Holzer *et al.* who reported in PD and HD patients a profound modification of HDL structure in particular a lower concentration of paraoxonase.²⁴ Moreover, the short natural history of CKD in the model used herein suggests that these modifications occur during the early stages of the disease. These results are consistent with that reported by Shroff *et al.*²⁵ and Kaseda *et al.*⁵¹ in pediatric CKD patients, who can be considered as close to the model used because of a short natural history of CKD and a lower number of CKD-associated comorbidities.

The results regarding platelet activation and aggregation are consistent with the altered properties of HDL in CKD; we observed a significant increase in the activation of platelets incubated with HDL from HD and PD patients and a 2-fold reduction in anti-aggregation with HDL from HD patients and CKD rabbits suggesting a blunted anti-thrombotic profile of uremic HDL. To exclude that the blunted anti-aggregant properties in HD patients did not only result from HD procedure *per se*, we confirmed this profile in PD patients. This

strengthened the role of CKD and especially ESRD in the pathological properties of HDL. This is consistent with the increase in the incidence of thrombotic events in CKD, especially in HD patients^{27,28,52,53} and with a higher platelet reactivity in CKD.⁵⁴⁻⁵⁷ We cannot however totally rule out the role of age as a confounding factor in the different aggregation behavior of HDL since PD patients were older than HD and control ones. The pathophysiological pathways of the platelet aggregation profile in CKD has only been studied for LDL; Holy *et al.* found a pro-aggregant profile of LDL in CKD that was mostly explained by carbamoylation of these proteins.³¹ Moreover, this study highlighted the role of the LOX-1 receptor in the onset of this profile; a receptor mainly involved in LDL-mediated pathophysiological pathways. The pro-aggregant profile of oxidized LDL has been described by Chen *et al.* to be mediated by CD-36³² and this receptor is also known to bind heavily modified-HDL^{58,59} and oxidized albumin.⁶⁰ The results of the present study suggest a strong implication in the blunted anti-aggregant profile of CD-36-mediated pathway as its blocking restored the antiaggregant properties of CKD and HNE-modified HDLs. Furthermore, this study suggests the involvement of SRC-kinases mediated pathways in the platelet activation process in response with CKD and HNE-modified HDLs as the use of a specific pharmacological inhibitor (Naphthyl PP1) also restored the antiaggregant properties of these HDLs. Finally, we confirmed that the binding of HDL onto the CD36 receptor resulted in a significant increase in JNK phosphorylation as demonstrated by Chen *et al.* for oxidized LDL.³² Schematic summary of these pathways is summed up in **Figure 7**.

The study also suggests that there is a threshold effect of 4-HNE adduction on aggregative properties of HDL as the incubation of control HDL with 4-HNE in concentrations lower than 100µM did not modify the anti-aggregant profile of HDL. This could be explained by the multiple receptors that are involved in the aggregant properties of HDL. Indeed, native and mild oxidized HDL binds ApoER2 and SR-B1 scavenger receptor,

inducing an intracellular down regulation of platelet activation pathways. Conversely, heavily oxidized HDL binds CD-36 and platelet activation is upregulated.^{58,61} Thus, we can hypothesize that the adduction induced by the incubation with 4-HNE at a concentration lower than 100 μ M caused light or mild modifications of the protein component of HDL insufficient to bind CD-36. 4-HNE adducts to the protein component of HDL particles were associated with pro-aggregative properties of HDL. Interestingly, mass spectrometry assay specifically identified such 4-HNE adducts on lysines and histidines from 8 constitutive proteins of HDL and especially onto Apolipoprotein A1 of HD patients. This apolipoprotein is widely responsible for the antiaggregant properties of HDL³³ and several modifications were located onto key parts of the protein for cholesterol efflux, platelet aggregation and lipid binding.

Post-translational modifications of proteins represents a wide spectrum of potential toxic modifications such as carbamoylation, generations of advanced-glycation end by-products (AGEs), chlorination or nitration. For example, carbamoylation of LDL particles was responsible for pro-aggregant properties of platelets in CKD patients.³¹ Despite a strong effect of the carbonylation by 4-HNE described herein, we cannot exclude that others post-translational modifications could contribute to the pro-aggregant properties of HDL. Further studies will however be dedicated to explore the specific role of other kinds of post translational modification of lipoproteins. To conclude, we describe herein for the first time the impaired anti-aggregant properties of HDL in CKD. To improve cardiovascular outcomes in CKD, future studies need to focus on HDL quality as well as quantity.

Author Contributions

N.F., P.M., L.J. and C.O.S. conceived and designed the study. N.F., C.C., S.L., M.M.B, C.B., J.R., N.G. and C.O.S. performed the experiments. F.D. and A.P. performed the mass spectrometry analysis. N.F. and C.O.S. analyzed the data. N.F. and C.O.S. wrote the manuscript. F.G.E edited the manuscript. N.F., C.C., S.L., M.M.B, J.M.P., M.L., P.M., F.G.E., L.J. and C.O.S. revised the manuscript. N.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Supplementary Table of Content

Complete Methods

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Supplementary Figure 4: Spearman correlation of the amount of 4-HNE adducts on in-vitro modified HDL

Supplementary Figure 5: Immunoblotting of 4-HNE adducts median levels of HNE-modified HDL, HD, PD and Control HDL

Supplementary Figure 6: Aggregation levels of HNE-modified HDL vs control and CD36 blockade effect

References

1. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu C-Y: Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 351: 1296–1305, 2004
2. Schiffrin EL, Lipman ML, Mann JFE: Chronic kidney disease: effects on the cardiovascular system. *Circulation* 116: 85–97, 2007
3. Tonelli M, Muntner P, Lloyd A, Manns BJ, Klarenbach S: Risk of coronary events in people with chronic kidney disease compared with those with diabetes: a population-level cohort study. *Lancet* 380: 807-14, 2012
4. Maduell F, Moreso F, Pons M, Ramos R, Mora-Macia J, Carreras J, Soler J, Torres F, Campistol JM, Martinez-Castelao A, for the ESHOL Study Group: High-Efficiency Postdilution Online Hemodiafiltration Reduces All-Cause Mortality in Hemodialysis Patients. *J Am Soc Nephrol* 24: 487–497, 2013
5. Kuchta A, Pacanis A, Kortas-Stempak B, Çwiklińska A, Ziętkiewicz M, Renke M, Rutkowski B: Estimation of Oxidative Stress Markers in Chronic Kidney Disease. *Kidney Blood Press Res* 34: 12–19, 2011
6. Dounousi E, Papavasiliou E, Makedou A, Ioannou K, Katopodis KP, Tselepis A, Siamopoulos KC, Tsakiris D: Oxidative stress is progressively enhanced with advancing stages of CKD. *Am J Kidney Dis* 48: 752–760, 2006
7. Aveles PR, Criminácio CR, Gonçalves S, Bignelli AT, Claro LM, Siqueira SS, Nakao LS, Pecoits-Filho R: Association between Biomarkers of Carbonyl Stress with Increased Systemic Inflammatory Response in Different Stages of Chronic Kidney Disease and after Renal Transplantation. *Nephron Clin Pract* 116: c294–c299, 2010
8. Vanholder R, Massy Z, Argiles A, Spasovski G, Verbeke F, Lameire N, European Uremic Toxin Work Group: Chronic kidney disease as cause of cardiovascular morbidity and mortality. *Nephrol Dial Transplant* 20: 1048–1056, 2005
9. Dou L, Sallée M, Cerini C, Poitevin S, Gondouin B, Jourde-Chiche N, Fallague K, Brunet P, Calaf R, Dussol B, Mallet B, Dignat-George F, Burtey S: The Cardiovascular Effect of the Uremic Solute Indole-3 Acetic Acid. *J Am Soc Nephrol* 26: 876-87, 2015
10. Ito S, Osaka M, Edamatsu T, Itoh Y, Yoshida M: Crucial Role of the Aryl Hydrocarbon Receptor (AhR) in Indoxyl Sulfate-Induced Vascular Inflammation. *J. Atheroscler Thromb* 23: 960–975, 2016
11. Lin C-J, Liu H-L, Pan C-F, Chuang C-K, Jayakumar T, Wang T-J, Chen H-H, Wu C-J: Indoxyl sulfate predicts cardiovascular disease and renal function deterioration in advanced chronic kidney disease. *Arch Med Res* 43: 451–456, 2012
12. Velasquez MT, Ramezani A, Manal A, Raj DS: Trimethylamine N-Oxide: The Good, the Bad and the Unknown. *Toxins (Basel)* 8: 326, 2016
13. Levitan I, Volkov S, Subbaiah PV: Oxidized LDL: diversity, patterns of recognition, and pathophysiology. *Antioxid Redox Signal* 13: 39–75, 2010
14. Apostolov EO, Ray D, Savenka AV, Shah SV, Basnakian AG: Chronic Uremia Stimulates LDL Carbamylation and Atherosclerosis. *J Am Soc Nephrol* 21: 1852–1857, 2010

15. Apostolov EO, Ok E, Burns S, Nawaz S, Savenka A, Shah SV, Basnakian AG: Carbamylated-oxidized LDL: proatherosclerotic effects on endothelial cells and macrophages. *J Atheroscler Thromb* 20: 878–892, 2013
16. Van den Akker JM, Bredie SJH, Diepenveen SHA, van Tits LJH, Stalenhoef AFH, van Leusen R: Atorvastatin and simvastatin in patients on hemodialysis: effects on lipoproteins, C-reactive protein and in vivo oxidized LDL. *J Nephrol* 16: 238–244, 2003
17. Tsirpanlis G, Boufidou F, Manganas S, Chantzis K, Bleta A, Stamatelou K, Psimenou E, Nicolaou C: Treatment with Fluvastatin Rapidly Modulates, via Different Pathways, and in Dependence on the Baseline Level, Inflammation in Hemodialysis Patients. *Blood Purif* 22: 518–524, 2004
18. Baigent C, Landray MJ, Reith C, Emberson J, Wheeler DC, Tomson C, Wanner C, Krane V, Cass A, Craig J, Neal B, Jiang L, Hooi LS, Levin A, Agodoa L, Gaziano M, Kasiske B, Walker R, Massy ZA, Feldt-Rasmussen B, Krairitichai U, Ophascharoensuk V, Fellström B, Holdaas H, Tesar V, Wiecek A, Grobbee D, de Zeeuw D, Grönhagen-Riska C, Dasgupta T, Lewis D, Herrington W, Mafham M, Majoni W, Wallendszus K, Grimm R, Pedersen T, Tobert J, Armitage J, Baxter A, Bray C, Chen Y, Chen Z, Hill M, Knott C, Parish S, Simpson D, Sleight P, Young A, Collins R, SHARP Investigators: The effects of lowering LDL cholesterol with simvastatin plus ezetimibe in patients with chronic kidney disease (Study of Heart and Renal Protection): a randomised placebo-controlled trial. *Lancet* 377: 2181–2192, 2011
19. Navab M, Reddy ST, Van Lenten BJ, Fogelman AM: HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms. *Nat Rev Cardiol* 8: 222–232, 2011
20. Khera AV, Cuchel M, la Llera-Moya de M, Rodrigues A, Burke MF, Jafri K, French BC, Phillips JA, Mucksavage ML, Wilensky RL, Mohler ER, Rothblat GH, Rader DJ: Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* 364: 127–135, 2011
21. Rosenson RS, Brewer HB, Davidson WS, Fayad ZA: Cholesterol efflux and atheroprotection advancing the concept of reverse cholesterol transport. *Circulation* 125: 1905–1919, 2012
22. Holzer M, Birner-Gruenberger R, Stojakovic T, El-Gamal D, Binder V, Wadsack C, Heinemann A, Marsche G: Uremia Alters HDL Composition and Function. *J Am Soc Nephrol* 22: 1631–1641, 2011
23. Yamamoto S, Yancey PG, Ikizler TA, Jerome WG, Kaseda R, Cox B, Bian A, Shintani A, Fogo AB, Linton MRF, Fazio S, Kon V: Dysfunctional High-Density Lipoprotein in Patients on Chronic Hemodialysis. *J Am Coll Cardiol* 60: 2372–2379, 2012
24. Holzer M, Schilcher G, Curcic S, Trieb M, Ljubojevic S, Stojakovic T, Scharnagl H, Kopecky CM, Rosenkranz AR, Heinemann A, Marsche G: Dialysis Modalities and HDL Composition and Function. *J Am Soc Nephrol* 1–10, 2015
25. Shroff R, Speer T, Colin S, Charakida M, Zewinger S, Staels B, Chinetti-Gbaguidi G, Hettrich I, Rohrer L, O'Neill F, McLoughlin E, Long D, Shanahan CM, Landmesser U, Fliser D, Deanfield JE: HDL in Children with CKD Promotes Endothelial Dysfunction and an Abnormal Vascular Phenotype. *J Am Soc Nephrol* 25: 2658–2668, 2014
26. Honda H, Ueda M, Kojima S, Mashiba S, Michihata T, Takahashi K, Shishido K, Akizawa T: Oxidized high-density lipoprotein as a risk factor for cardiovascular events in prevalent hemodialysis patients. *Atherosclerosis* 220: 493–501, 2012

27. Wanner C, Krane V, März W, Olschewski M, Mann JFE, Ruf G, Ritz E, German Diabetes and Dialysis Study Investigators: Atorvastatin in patients with type 2 diabetes mellitus undergoing hemodialysis. *N Engl J Med* 353: 238–248, 2005
28. Fellström BC, Jardine AG, Schmieder RE, Holdaas H, Bannister K, Beutler J, Chae D-W, Chevaile A, Cobbe SM, Grönhagen-Riska C, De Lima JJ, Lins R, Mayer G, McMahon AW, Parving H-H, Remuzzi G, Samuelsson O, Sonkodi S, Sci D, Süleymanlar G, Tsakiris D, Tesar V, Todorov V, Wiecek A, Wüthrich RP, Gottlow M, Johnsson E, Zannad F, AURORA Study Group: Rosuvastatin and cardiovascular events in patients undergoing hemodialysis. *N Engl J Med* 360: 1395–1407, 2009
29. Calzada C, Coulon L, Halimi D, Le Coquil E, Pruneta-Deloche V, Moulin P, Ponsin G, Véricel E, Lagarde M: In Vitro Glycooxidized Low-Density Lipoproteins and Low-Density Lipoproteins Isolated from Type 2 Diabetic Patients Activate Platelets via p38 Mitogen-Activated Protein Kinase. *J Clin Endocrinol Metab* 92: 1961–1964, 2007
30. Colas R, Sassolas A, Guichardant M, Cugnet-Anceau C, Moret M, Moulin P, Lagarde M, Calzada C: LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and activate platelets. *Diabetologia* 54: 2931–2940, 2011
31. Holy EW, Akhmedov A, Speer T, Camici GG, Zewinger S, Bonetti N, Beer JH, Lüscher TF, Tanner FC: Carbamylated Low-Density Lipoproteins Induce a Prothrombotic State Via LOX-1: Impact on Arterial Thrombus Formation In Vivo. *J Am Coll Cardiol* 68: 1664–1676, 2016
32. Chen K, Febbraio M, Li W, Silverstein RL: A Specific CD36-Dependent Signaling Pathway Is Required for Platelet Activation by Oxidized Low-Density Lipoprotein. *Circulation Research* 102: 1512–1519, 2008
33. Nofer J-R, Brodde MF, Kehrel BE: High-density lipoproteins, platelets and the pathogenesis of atherosclerosis. *Clin Exp Pharmacol Physiol* 37: 726–735, 2010
34. Calzada C, Vericel E, Colas R, Guillot N, Khoury El G, Draï J, Sassolas A, Peretti N, Ponsin G, Lagarde M, Moulin P: Inhibitory effects of in vivo oxidized high-density lipoproteins on platelet aggregation: evidence from patients with abetalipoproteinemia. *FASEB J* 27: 2855–2861, 2013
35. Valiyaveetil M, Kar N, Ashraf MZ, Byzova TV: Oxidized high-density lipoprotein inhibits platelet activation and aggregation via scavenger receptor BI. *Blood* 15: 1962–71, 2008
36. Gotloib L, Crassweller P, Rodella H, Oreopoulos DG, Zellerman G, Ogilvie R, Husdan H, Brandes L, Vas S: Experimental Model for Studies of Continuous Peritoneal Dialysis in Uremic Rabbits. *Nephron* 31: 254–259, 1982
37. Florens N, Lemoine S, Pelletier CC, Rabeyrin M, Juillard L, Soulage CO: Adenine Rich Diet Is Not a Surrogate of 5/6 Nephrectomy in Rabbits. *Nephron* 135: 307–314, 2017
38. Soman RS, Zahir H, Akhlaghi F: Development and validation of an HPLC-UV method for determination of iohexol in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 816: 339–343, 2005
39. Havel RJ, Eder HA, Bragdon JH: The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 34: 1345–1353, 1955
40. Holzer M, Kern S, Trieb M, Trakaki A, Marsche G: HDL structure and function is profoundly affected when stored frozen in the absence of cryoprotectants. *J Lipid Res* 58: 2220–2228, 2017

41. Grotto D, Santa Maria LD, Boeira S, Valentini J, Charão MF, Moro AM, Nascimento PC, Pomblum VJ, Garcia SC: Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. *J Pharm Biomed Anal* 43: 619–624, 2007
42. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V: Method for the measurement of antioxidant activity in human fluids. *J Clin Path* 54: 356–361, 2001
43. Therasse J, Lemonnier F: Determination of plasma lipoperoxides by high-performance liquid chromatography. *J Chromatogr* 23: 237-41, 1987
44. Soulère L, Queneau Y, Doutheau A: An expeditious synthesis of 4-hydroxy-2E-nonenal (4-HNE), its dimethyl acetal and of related compounds. *Chem Physics Lipids* 150: 239–243, 2007
45. Lagarde M, Bryon PA, Guichardant M, Dechavanne M: A simple and efficient method for platelet isolation from their plasma. *Thromb Res* 17: 581–588, 1980
46. Born GV: Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 194: 927–929, 1962
47. Skinner SC, Diaw M, Pialoux V, Mbaye MN, Mury P, Lopez P, Bousquet D, Gueye F, Diedhiou D, Joly P, Renoux C, Sow D, Diop S, Ranque B, Vinet A, Samb A, Guillot N, Connes P: Increased Prevalence of Type 2 Diabetes-Related Complications in Combined Type 2 Diabetes and Sickle Cell Trait. *Diabetes Care* 41: 2595–2602, 2018
48. Esterbauer H, Gebicki J, Puhl H, Jürgens G: The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med* 13: 341–390, 1992
49. Borhani DW, Rogers DP, Engler JA, Brouillette CG: Crystal structure of truncated human apolipoprotein A-I suggests a lipid-bound conformation. *PNAS* 94: 12291–12296, 1997
50. Wu Z, Gogonea V, Lee X, Wagner MA, Li X-M, Huang Y, Undurti A, May RP, Haertlein M, Moulin M, Gutsche I, Zaccai G, DiDonato JA, Hazen SL: Double superhelix model of high density lipoprotein. *J Biol Chem* 284: 36605–36619, 2009
51. Kaseda R, Jabs K, Hunley TE, Jones D, Bian A, Allen RM, Vickers KC, Yancey PG, Linton MF, Fazio S, Kon V: Dysfunctional high-density lipoproteins in children with chronic kidney disease. *Metabolism* 64: 263–273, 2015
52. Kawamura M, Fijimoto S, Hisanaga S, Yamamoto Y, Eto T: Incidence, outcome, and risk factors of cerebrovascular events in patients undergoing maintenance hemodialysis. *Am J Kidney Dis* 31: 991–996, 1998
53. Konishi A, Shinke T, Otake H, Nakatani D, Nakagawa M, Inoue T, Hariki H, Osue T, Taniguchi Y, Iwasaki M, Nishio R, Hiranuma N, Kinutani H, Kuroda M, Shite J, Hirata K-I: Impact of hemodialysis on local vessel healing and thrombus formation after drug-eluting stent implantation. *J Cardiol* 64: 25–31, 2014
54. Yang K, Du C, Wang X, Li F, Xu Y, Wang S, Chen S, Chen F, Shen M, Chen M, Hu M, He T, Su Y, Wang J, Zhao J: Uremic solute indoxyl sulfate-induced platelet hyperactivity contributes to CKD-associated thrombosis in mice. *Blood* 129:2667–2679, 2017
55. Jalal D, Chonchol M, Targher G: Disorders of Hemostasis Associated with Chronic Kidney Disease. *Semin Thromb Hemost* 36: 034–040, 2010

56. Nishi T, Ariyoshi N, Nakayama T, Fujimoto Y, Sugimoto K, Wakabayashi S, Hanaoka H, Kobayashi Y: Impact of chronic kidney disease on platelet inhibition of clopidogrel and prasugrel in Japanese patients. *J Cardiol* 69: 752–755, 2016
57. Vazzana N, Santilli F, Lattanzio S, Liani M, Giacci L, Del Rosso G, Salvati F, Boccacchia A, Ferroni P, Davì G: Determinants of thromboxane biosynthesis in patients with moderate to severe chronic kidney disease. *Eur J Intern Med* 33: 74–80, 2016
58. Van der Stoep M, Korporaal SJA, Van Eck M: High-density lipoprotein as a modulator of platelet and coagulation responses. *Cardiovasc Res* 103: 362–371, 2014
59. Park YM: CD36, a scavenger receptor implicated in atherosclerosis. *Exp Mol Med* 46: e99–7, 2014
60. Pasterk L, Lemesch S, Leber B, Trieb M, Curcic S, Stadlbauer V, Schuligoi R, Schicho R, Heinemann A, Marsche G: Oxidized plasma albumin promotes platelet-endothelial crosstalk and endothelial tissue factor expression. *Sci Rep* 6: 22104, 2016
61. Assinger A, Koller F, Schmid W, Zellner M, Babeluk R, Koller E, Volf I: Specific binding of hypochlorite-oxidized HDL to platelet CD36 triggers proinflammatory and procoagulant effects. *Atherosclerosis* 1–8, 2010

Table 1.**General characteristics of sham-operated (control) and 5/6 nephrectomized rabbits.**

	Control	CKD	P-value
N	9	8	
Biometry			
Body weight, kg	3.5 (3.2 – 3.9)	3.1 (3.0 – 3.3)	0.040
Body weight gain, kg	1.0 (0.9 – 1.2)	0.7 (0.6 – 0.8)	0.001
Kidney weight, g	19 (16 – 22)	14 (12 – 16)	0.008
Kidney weight, g/kg body weight	5 (4 – 7)	5 (4 – 5)	0.212
Biological features			
Urea, mmol/L	6.8 (5.6 – 8.4)	12.8 (12.6 – 19.2)	<0.001
Creatinine, μ mol/L	57 (52 – 75)	193 (154 – 270)	<0.001
Bicarbonate, mmol/L	20.1 (16.8 – 24.0)	21.0 (14.6 – 22.7)	0.700
Glucose, mmol/L	5.6 (4.8 – 6.9)	7.4 (7.1 – 8.1)	0.042
Total cholesterol, mg/dL	47 (37 – 68)	109 (73 – 198)	<0.001
HDL cholesterol, mg/dL	29 (18 – 37)	56 (35 – 85)	0.043
HDL-C/TC ratio	0.66 (0.33 – 0.85)	0.49 (0.35 – 0.53)	0.269
Triacylglycerols, mg/dL	270 (228 – 677)	316 (165 – 719)	0.842
Protidemia, g/L	59 (48 – 69)	39 (32 – 49)	0.015
Proteinuria, g/mmol creat	617 (60 – 1287)	19 (9 – 56)	0.198
Iohexol clearance			
GFR, mL/min/kg	4.3 (4.1 – 4.8)	2.2 (1.8 – 2.5)	0.008
Oxidative stress markers (plasma)			
MDA, μ mol/L	0.8 (0.5 – 1.2)	2.3 (1.2 – 3.0)	0.009
AOA, mmol/L	1.0 (0.8 – 1.1)	0.8 (0.7 – 0.9)	0.021

Data are expressed as median (IQR). CKD: chronic kidney disease; HDL-C: HDL cholesterol; TC: total cholesterol; GFR: glomerular filtration rate; MDA: malondialdehyde; AOA: anti-oxidant activity (of the plasma). Mann-Whitney test, $p < 0.05$ was considered as significant.

Creatinine: $\times 0.011$ for mg/dl; urea: $\times 2.8$ for mg/dl

Table 2.**General characteristics of hemodialysis, peritoneal dialysis and control patients.**

	Control	Hemodialysis (HD)	Peritoneal dialysis (PD)
N	15	25	20
General characteristics			
Age, years	46 (36 – 58)	56 (43 – 73)	67 (56 – 84)*†
Gender, % male	56	43	56
BMI, kg/m ²	26 (24 – 28)	24 (20 – 26)	25 (23 – 28)
<i>Comorbidities</i>			
HT, n(%)	3(21)	17(74)	19(95)
Stroke, n(%)	0(0)	2(9)	1(5)
CHD, n(%)	0(0)	5(22)	3(15)
Cardiopathy, n(%)	0	9(39)	8(40)
PVD, n(%)	0	4(17)	3(15)
<i>Therapies</i>			
Statins, n(%)	0	6	5
PI, n(%)	0	5	6
RASi, n(%)	1	3	11
β-blockers, n(%)	1	5	4
CCB, n(%)	1	1	6
Biological parameters			
Urea, mmol/L	5.4 (4.4 – 7.5)	17.1 (13.3 – 19.8)*	18.8 (16.1 – 22.6)*
Creatinine, μmol/L	73 (69 – 85)	702 (506 – 862)*	575 (422 – 758)*
mGFR, mL/min/1.73m ²	94 (86 – 96)	-	-
Total cholesterol, mg/dL	208 (189 – 237)	149 (113 – 180)*	209 (155 – 232)†
LDL cholesterol, mg/dL	139 (105 – 158)	71 (54 – 103)*	125 (81 – 159)†
HDL cholesterol, mg/dL	59 (53 – 66)	41 (30 – 47)*	45 (36 – 62)*
Triacylglycerols, mg/dL	91 (64 – 116)	119 (88 – 173)*	149 (118 – 207)*
CRP, mg/L	1.9 (0.5 – 3.5)	3.3 (1.9 – 8.0)	3.7 (1.7 – 5.8)

Data are expressed as median (IQR). BMI: body mass index; HT: hypertension; CHD: coronary heart disease; PVD: peripheral vascular disease; PI: platelet inhibitor; RASi: renin-angiotensin system inhibitor; CCB: calcium-channel blocker; eGFR: estimated glomerular filtration rate by CKD-EPI formula; mGFR: measured GFR by iohexol clearance; CRP: C-reactive protein. *: $p < 0.05$ vs controls, †: $p < 0.05$ vs HD, Mann-Whitney U-test.

Table 3.**List of 4-hydroxy-2-nonenal adducted amino-acid in HDL constitutive proteins from hemodialysis patients.**

Protein name	Protein label	%	Site of adduction	Abundance ratio (HD 1 to 9, respectively)								
Apolipoprotein B100	APOB	0.04	[K3689]	0.293	0.825	0.468	4.099	8.148	1.488	1.626	3.606	0.322
			[K4498]	-	-	-	-	4.421	-	-	2.339	-
Serum albumin	ALB	1.15	[H152]	2.646	5.05	0.373	-	0.036	0.072	1.263	0.874	6.837
			[K160 or H152]	2.956	6.049	0.229	-	0.295	-	0.718	0.405	12.994
			[K347]	-	1.452	-	0.879	-	0.582	1.767	2.442	1.966
			[H170]	1.463	-	-	0.919	-	0.178	0.957	-	-
			[K581]	0.861	1.148	-	-	-	-	0.668	1.944	2.204
			[K198]	1.715	-	-	-	-	2.864	-	-	1.28
Apolipoprotein A1	APOA1	4.50	[H20]	0.516	0.863	1.131	2.298	1.414	1.284	1.133	1.109	0.556
			[H179]	2.448	2.756	5.133	1.117	0.995	1.306	0.316	0.802	0.853
			[H186]	2.646	2.508	4.401	0.613	0.806	0.93	0.168	1.221	1.767
			[H223]	0.456	0.549	1.471	0.101	0.125	0.188	-	0.136	-
			[H217]	1.342	0.939	3.216	0.039	0.175	0.266	-	0.14	-
			[K36]	0.89	1.362	1.181	1.64	0.996	1.36	1.873	1.863	1.128
			[K250]	1.423	3.189	0.548	4.329	31.087	1.035	3.154	30.549	2.522
			[K130]	0.332	1.236	0.856	-	-	-	1.018	-	0.728
			[K47]	0.596	1.345	1.237	2.083	0.861	1.211	2.596	2.217	1.557
			[K83]	0.393	-	0.943	1.004	0.559	0.79	2.181	2.642	2.131
			[K64]	0.571	1.139	1.079	1.643	1.119	1.543	1.403	1.616	0.935
			[K262]	-	10.052	-	0.757	7.534	-	0.442	42.042	-
Apolipoprotein(a)	LPA	0.02	[K215]	-	100	-	-	-	-	-	100	-
Apolipoprotein A2	APOA2	1	[K26]	-	1.983	-	0.134	-	-	0.963	0.29	0.689
Keratin, type I, cytoskeletal 10	KRT10	0.17	[K362]	2.598	3.076	3.419	1.44	0.596	1.462	0.778	2.66	1.595
Trypsin-3	PRSS3	0.32	[H153]	1.292	0.7	1.056	1.332	0.827	5.646	0.972	0.914	0.906
UDP-glucose:glycoprotein glucosyltransferase 2	UGGT2	0.06	[H1388]	1.46	1.385	3.62	3.525	4.433	2.838	5.1	11.999	1.877

H, histidine; K, lysine. %: percentage of potential sites of adduction regarding all the amino-acids of the protein sequence. Abundance ratio was calculated against the mean of the 9 controls. -: not found

Figure legends

Figure 1. HNE-adducts were increased in HDL from CKD rabbits (A) as well as MDA concentration (B). No difference was observed concerning 8-isoprostane (C) and tocopherols (D) concentrations. HDL from CKD rabbits were more prone to oxidation after a copper-induced oxidation (E) as described in concise methods section. n=8 and 9, CKD and control respectively, $p < 0.05$. Mann-Whitney test, $p < 0.05$ was considered as significant. Data are expressed as median and interquartile range.

Figure 2. CKD is responsible for impaired anti-aggregant properties of HDL in rabbits. CKD HDL exhibited blunted anti-aggregative properties compared to control HDL (A). Control HDL modified by an incubation overnight with HNE (HNE HDL) solution exhibited similar blunted properties to CKD HDL compared to Control HDL (A). Pre-incubation with anti-CD36 antibody restored to CKD and HNE HDL anti-aggregant properties like Control HDL (A). Typical aggregation curves obtained with collagen, control HDL and CKD HDL. Collagen aggregation level is considered as 100% (B). Same results were observed with a lipoprotein mix containing triglyceride-rich lipoproteins (VLDL and chylomicrons), LDL and HDL (LPP) from Control and CKD rabbits. CKD LPP exhibited blunted anti-aggregative properties compared to Control LPP. Control LPP modified by an incubation overnight with HNE solution (HNE LPP) exhibited similar properties to CKD LPP (C). Pre-incubation with anti-CD36 antibody restored to CKD and HNE LPP anti-aggregant properties like Control LPP (C). HNE HDL had increased level of HNE-adducts compared to control HDL (D). * n=8 and 9, CKD and control respectively, $p < 0.05$. Mann-Whitney test, $p < 0.05$ was considered as significant. Data are expressed as median and interquartile range.

Figure 3. 4-HNE adducts are increased in HDL from hemodialysis (HD) patients compared to controls. Immunoblotting (dot blot) of 4-hydroxy-nonenal (4-HNE) Michael adducts were performed as described in methods section (A). Identification of increased 4-HNE Michael adduct on lysine and histidine residues of HDL from HD patients by liquid chromatography—mass spectrometry/mass spectrometry assay (LC-MS/MS). HDL were reduced, alkylated and digested with trypsin. Sequence of Apolipoprotein A1 (accession number: P02647-1) indicating the position of the adducts (bold characters) and the sequences of interest (highlighted and framed, B). Typical MS/MS spectra from unmodified peptide (upper spectrum) and modified peptide (lower spectrum). The abundance of 4-HNE adduct of Lys 250 in HD HDL was 5.3-fold higher than in controls ($p < 0.01$, C). $n = 9$ and 9 , HD and control HDL respectively, Mann-Whitney test, * $p < 0.05$, data are expressed as median and interquartile range.

Figure 4. Structure of truncated human apolipoprotein A-I (pdb code 1av1). Four molecules are associated with their hydrophobic faces to form an antiparallel four-helix bundle. Lysine and histidine residues possibly modified by 4-HNE are colored in cyan with CPK representation (A). Model of HDL: Phospholipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, POPC) are colored in yellow and cholesterol in magenta. Lysine and histidine residues possibly modified by 4-HNE are colored in cyan with CPK representation (pdb code 3k2s, B).

Figure 5. HDL from peritoneal (PD) and hemodialysis (HD) patients exhibited impaired anti-aggregant properties. Regarding levels of Thromboxane B2 (TxB2), HD and PD HDL triggered over-activation of platelets compared to control HDL ($n = 15$ for control, $n = 25$ for HD group and $n = 20$ for PD group, A). Same observations were found for healthy volunteers HDL modified by an incubation overnight with HNE (HNE HDL, $n = 5$ for each group, B). HD, PD and HNE HDL exhibited blunted anti-aggregant properties compared to control HDL ($n = 9$ for control and HD groups, $n = 5$ for HNE group). We found a threshold effect of HNE modification on HDL as only a $100 \mu\text{M}$ solution of HNE permitted to significantly alter the anti-aggregant properties of control HDL ($n = 4$ for each group, D).

* $p < 0.05$, Mann-Whitney test and Kruskal-Wallis test (D), data are expressed as median and interquartile range.

Figure 6. The impaired anti-aggregant properties of HDL in peritoneal (PD), hemodialysis (HD) and from healthy volunteers HDL modified by an incubation overnight with HNE (HNE HDL) is mediated by CD36 binding and SRC kinases-mediated pathways. Regarding levels of Thromboxane B2 (TxB2), the pre-incubation of platelets with an antibody against CD36 receptor (Ab anti-CD36) significantly lowered the activation of platelets exposed with control (CTL), HD, PD and HNE compared without the antibody (A). The pre-incubation of platelets with an antibody against SRB1 receptor (Ab anti-SRB1) significantly increased the activation of platelets exposed with CTL, PD and HNE compared without the antibody (A). The pre-incubation of platelets with a pharmacological inhibitor of SRC kinases (Naphthyl PP1) significantly lowered the activation of platelets exposed with CTL, HD, PD and HNE compared without the inhibitor (A). In a fluorescent activated cell sorting assay, activation of platelets were determined with the P-selectin expression. The pre-incubation of platelets with Ab anti-CD36 and Naphthyl PP1 significantly decreased the activation of platelets exposed with CTL, HD, PD and HNE HDL compared to the samples without the antibody/inhibitor (B). The pre-incubation of platelets with Ab anti-SRB1 significantly increased the activation of platelets exposed with CTL, HD, PD and HNE HDL compared to the samples without the antibody/inhibitor (B). $n=5$ for each group. Mann-Whitney test, * $p < 0.05$ compared to Control HDL without blocker; \$ $p < 0.05$ compared to HD HDL without blocker; † $p < 0.05$ compared to PD HDL without blocker; ◇ $p < 0.05$ compared to HNE HDL without blocker. Without blocker: without either Ab anti-CD36, anti-SRB1 or Naphthyl PP1.

Figure 7. HDL from chronic kidney disease patients and rabbit (CKD HDL) exhibited blunted anti-aggregant properties by interaction with CD36 receptor. Carbonylation by 4-hydroxy-2-nonenal (4-HNE) of proteins from HDL was found to be part of this pathological behavior. Activation of the platelet in response of this binding was through a SRC-kinases mediated pathway. SRC kinase activation resulted with an increase of Jun-Kinase phosphorylation (pJNK). Native and CKD HDL apolipoprotein A1 binds SRB1 and inhibits platelet activation.