Lipoprotein profiles in human heterozygote carriers of a functional mutation P297S in scavenger receptor class B1.

Stefan A Ljunggren, Johannes H M Levels, Kees Hovingh, Adriaan G Holleboom, Menno Vergeer, Letta Argyri, Christina Gkolfinopoulou, Angeliki Chroni, Jeroen A Sierts, John J Kastelein, Jan Albert Kuivenhoven, Mats Lindahl and Helen Karlsson

Original Publication:

Stefan A Ljunggren, Johannes H M Levels, Kees Hovingh, Adriaan G Holleboom, Menno Vergeer, Letta Argyri, Christina Gkolfinopoulou, Angeliki Chroni, Jeroen A Sierts, John J Kastelein, Jan Albert Kuivenhoven, Mats Lindahl and Helen Karlsson, Lipoprotein profiles in human heterozygote carriers of a functional mutation P297S in scavenger receptor class B1., 2015, Biochimica et Biophysica Acta, (1851), 12, 1587-1595.
http://dx.doi.org/10.1016/j.bbalip.2015.09.006

Lipoprotein profiles in human heterozygote carriers of a functional mutation P297S in Scavenger Receptor class B1

Stefan A. Ljunggren[a] , Johannes H.M. Levels[b], Kees Hovingh[b], Adriaan G. Holleboom[b], Menno Vergeer[b], Letta Argyri[c], Christina Gkolfinopoulou[c], Angeliki Chroni[c], Jeroen A. Sierts[b], John J. Kastelein[b], Jan Albert Kuivenhoven[d], Mats Lindahl[e], [§]Helen Karlsson[a]

[a]Occupational and Environmental Medicine Center, and Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden, [b]Department of Vascular Medicine, Academic Medical Centre, Amsterdam, The Netherlands, [c]Institute of Biosciences and Applications, National Center for Scientific Research, "Demokritos", Athens, Greece, [d]Department of Pediatrics, section for Molecular Genetics, University of Groningen, University Medical Center Groningen, Groningen, Netherlands, [e]Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

Email addresses:

helen.m.karlsson@liu.se
stefan.ljunggren@liu.se
H.Levels@amc.uva.nl
G.K.Hovingh@amc.uva.nl
A.G.Holleboom@amc.uva.nl
j.a.kuivenhoven@umcg.nl
mats.lindahl@liu.se
mennovergeer@hotmail.com
j.j.kastelein@amc.uva.nl
achroni@bio.demokritos.gr
jasierts@yahoo.com
largyri@bio.demokritos.gr
xristinaki.1987@hotmail.com

[§]Corresponding author

Helen Karlsson
Occupational and Environmental Medicine
Heart Medical Centre
Linkoping University Hospital
SE-581 85 Linköping, SWEDEN
Phone; +46-10-1034414
Email; helen.m.karlsson@liu.se

Abbreviations;
2DE - Two-dimensional gel electrophoresis, ABCA1 - ATP-binding cassette sub-family A member 1, AREase - Serum Paraoxonase 1 arylesterase activity, apo - apolipoprotein, CEC - Cholesterol efflux capacity, CETP - cholesteryl ester transfer protein, CVD - cardiovascular disease, DCF - 2,7-dichlorofluorescein, DCFH - 2,7 dichlorofluorescin, HDL - high density lipoprotein, HDL-C - high density lipoprotein cholesterol, LCAT - Lecithin-cholesterol acyltransferase, LDL/VLDL - low-/very low-density lipoprotein, PLS - Partial least square, SAA - Serum Amyloid A, PON1 - Serum Paraoxonase 1, PONase - Serum Paraoxonase 1 paraoxonase activity, TTR - Transthyretin, SR-B1 - scavenger receptor class B type 1, TBS - Tris-buffered saline, WB - Western blot

Keywords; apoE, apoL-1, HDL, LDL/VLDL, P297S, SR-B1

Highlights

- SR-B1^{P297S} carriers show increased LDL apoE/free apoE compared to family controls
- SR-B1^{P297S} carriers show increased apoL-1 in HDL compared to family controls
- Carriers had equivalent HDL anti-oxidative function and PON1 activities as controls
- Carriers had significantly increased methionine oxidations in HDL-apoA-I
- Carriers HDL had similar cholesterol efflux capacity as controls

Abstract

The scavenger receptor class B type 1 (SR-B1) is an important HDL receptor involved in cholesterol uptake and efflux, but its physiological role in human lipoprotein metabolism is not fully understood. Heterozygous carriers of the SR-B1^{P297S} mutation are characterized by increased HDL cholesterol levels, impaired cholesterol efflux from macrophages and attenuated adrenal function. Here, the composition and function of lipoproteins was studied in SR-B1^{P297S} heterozygotes.

Lipoproteins from six SR-B1^{P297S} carriers and six family controls were investigated. HDL and LDL/VLDL were isolated by ultracentrifugation and proteins were separated by two-dimensional gel electrophoresis and identified by mass spectrometry. HDL antioxidant properties, paraoxonase 1 activities, apoA-I methionine oxidations and HDL cholesterol efflux capacity were assessed.

Multivariate modelling separated carriers from controls based on lipoprotein composition. Protein analyses showed a significant enrichment of apoE in LDL/VLDL and of apoL-1 in HDL from heterozygotes compared to controls. The relative distribution of plasma apo E was increased in LDL and in lipid-free form. There were no significant differences in paraoxonase 1 activities, HDL antioxidant properties or HDL cholesterol efflux capacity but heterozygotes showed a significant increase of oxidized methionines in apoA-I.

The SR-B1^{P297S} mutation affects both HDL and LDL/VLDL protein composition. The increase of apoE in carriers suggests a compensatory mechanism for attenuated SR-B1 mediated cholesterol uptake by HDL. Increased methionine oxidation may affect HDL function by reducing apoA-I binding to its targets. The results illustrate the complexity of lipoprotein metabolism that has to be taken into account in future therapeutic strategies aiming at targeting SR-B1.

1. Introduction

Numerous studies have shown that plasma high-density lipoprotein cholesterol (HDL-C) levels are inversely associated with risk for cardiovascular disease (CVD) [1, 2, 3]. Since there is a need for alternative interventions alongside LDL-cholesterol lowering drugs, these observations have resulted in the exploration of other targets, such as cholesteryl ester transfer protein (CETP) inhibitors, to raise HDL-C levels [4]. In addition, a central mediator of high-density lipoprotein (HDL) metabolism, the scavenger receptor class B type 1 (SR-B1), is being considered as a drug target [5]. SR-B1 is a multi-functional receptor, abundantly expressed in liver and steroidogenic tissues [6], and a wide array of native and modified lipoproteins has been shown to interact with SR-B1 [7]. Regarding the role of SR-B1 in CVD, it has been shown to protect from diet-induced atherosclerosis in apoE-/- mouse models [8, 9] while SR-B1 deficient mice with high HDL-C, showed increased atherosclerosis and increased expression of inflammatory markers [7]. Consistent with this, it has also been shown that a coding variant for SR-B1 (I179N) caused increased atherosclerosis in LDL-receptor knockout mice on a Western-type diet [10]. In humans, variation at the *SCARB1* locus (the gene encoding for SR-B1) has been associated to CVD, albeit in a gender specific way [11, 12, 13]. These findings indicate that a functional SR-B1 receptor may be athero-protective and that high HDL-C levels resulting from reduced hepatic clearance, by a less functional SR-B1 receptor, may not be beneficial. In accordance, a recent review concluded that therapies aimed at adjusting the HDL-C levels have so far failed and that modulation of HDL functions may be a better way to achieve therapies that reduces the risk for CVD [14]. This also raises the question whether HDL derived from carriers of *SCARB1* mutations itself has detrimental properties. Consistent with this, it has been postulated that genetic variation in *SCARB1* may give rise to changes in non-lipid pathways which affects the CVD risk, such as inflammation and endothelial function [12].

In humans, there are three described mutations of SR-B1 that cause increased HDL-C. Heterozygous carriers of the first identified mutation SR-B1^{P297S} were characterized by increased plasma HDL-C mainly in the larger HDL2 fraction while other lipoprotein fractions were consistent with the controls, macrophages with impaired capacity to promote cholesterol efflux to normal HDL, altered platelet function and attenuated adrenal function but without apparent effects on atherosclerosis [15]. The other two mutations, SR-B1^{S112F} and SR-B1^{T175A}, occupying the same extracellular loop as SR-B1^{P297S}, are also suggested to be less

efficient in mediating cholesterol efflux [16]. Interestingly, also in patients with these two mutations, clinically observed accelerated atherosclerosis was not found [17]. In summary, the alterations in SR-B1 of all three mutations caused elevated HDL-C but to our knowledge, data is still limited regarding possible metabolic consequences. Investigating the lipoprotein composition of subjects with functional mutations in the SR-B1 receptor may be a useful tool for improved understanding. Therefore, we have in the present study investigated the protein composition of HDL and LDL/VLDL and the function of HDL from the heterozygous carriers of the SR-BI^{P297S} mutation and from family controls.

2. Materials and Methods

2.1. Plasma measurements

Plasma from SR-B1^{P297S} heterozygotes and matched family controls, previously identified by screening for individuals with high HDL-C values [15], was collected in EDTA-tubes after an overnight fast. Total cholesterol and triglycerides levels were measured using standard assays. ApoA-I concentration was determined using a commercially available assay (Randox, Wako) on a COBAS MIRA analyzer. HDL-C was measured as cholesterol remaining after precipitation of apolipoprotein (apo) B-containing lipoproteins. Plasma was frozen immediately in different aliquots for different analyses and was thawed once before use and was not re-used for other purposes. Informed consent was obtained from all subjects and the study was approved by the ethics committee of the Academic Medical Center in Amsterdam.

2.2. HDL and LDL/VLDL isolation

HDL and LDL/VLDL isolation of six SR-B1^{P297S} heterozygotes (2 males and 4 females, age range 21-67 years) and six family controls (3 males and 3 females, age range 39-78 years) was performed by ultracentrifugation as described [18]. Briefly, plasma was isolated from blood samples collected in EDTA by centrifugation for 10 min in 700*g* at room temperature. EDTA (1mg/mL) and sucrose (5mg/mL) were added to prevent LDL/HDL oxidation and aggregation. Five mL of EDTA-plasma was adjusted to a density of 1.24 g/mL with solid KBr. The plasma samples were layered in the bottom of a centrifuge tube and were gently overlayered with KBr/PBS with a density of 1.063 g/mL. The first ultracentrifugation step was performed at 290 000*g* for 4h at 15°C. HDL, located in the middle of the KBr/PBS solution and LDL/VLDL located at the top of the tube were then collected with a syringe. An additional centrifugation step was performed for 2h in KBr/PBS solution with a density of

1.24 g/mL. The HDL and LDL/VLDL fractions were again collected from the tube with a syringe. All fractions were desalted and protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Samples were lyophilized prior to 2-DE analysis.

2.3. 2-DE

Proteins were separated on 2-D gels as described previously [19]. Briefly, 400 µg HDL or 300 µg LDL/VLDL proteins were isoelectrically focused on immobilized pH gradient strips (pH 3–10) at 46 000 Vh (max 8 000 V), then separated overnight on homogenous (T=14, C=1.5%) gels and fixed and detected by fluorescent SYPRO Ruby protein stain (Bio-Rad). Gel images were evaluated using PDQuest 2-D gel analysis software, v. 7.1.0 (Bio-Rad). Protein abundance was expressed as percent of total 2-D gel fluorescence, multiple isoforms of proteins added together.

2.4. Mass spectrometry

Protein spots detected on 2-D gels were map-matched [18] and identified with mass spectrometry. Spots were excised manually and subjected to in-gel trypsin digestion. Peptides were analysed with MALDI-TOF MS (Voyager DE PRO, Applied Biosystems, Foster City, CA, USA). Peptides were mixed 1:1 with matrix (2,5-dihydroxybenzoic acid) in 70% acetonitrile/0.3% trifluoracetic acid (20 mg/mL). Databases NCBI, Swiss-Prot, and UniProt were searched using MS-Fit as search engine (http://prospector.ucsf.edu). Search criteria were defined as; isoelectric point, molecular weight, human species, mass tolerance <50 ppm, methionine oxidation, and cysteine modification by carbamidomethylation.

2.5. Western blot of apoL-1

For Western blot (WB), 25 µg of HDL proteins were separated with SDS-PAGE and transferred to a PVDF membrane. After blocking 1h (5% milk in tris-buffered saline, TBS) and incubation over night with anti apoL-1 primary antibodies (Abcam, Ab79282, 1:1000 in 2% milk in Tween-TBS), the membranes were incubated for 1h with HRP-conjugated secondary antibodies 1:40 000 (2% milk in Tween-TBS) . Proteins were visualised using ECL plus WB detection system (GE Healthcare, Little Chalfont, UK) and X-ray film. The apoL-1 intensity (optical density/mm^2) was calculated with ImageLab (version 3.0.1, Bio Rad) and the relative quantity of apoL-1 was expressed as percent of the total apoL-1 intensity.

2.6. FPLC-fractionation and Western spot-blot of apoE

Lipoprotein (plasma) fractionations were carried out with equal portions of EDTA plasma from carriers for the SR-BI^{P297S} mutation (n=4) or matched controls (n=4) (total pool of 1 mL respectively). A superose-6 column combined with an ÄKTA explorer 10S system was used for separation of the lipoprotein fractions (GE Healthcare). The buffer flow was 0.3 mL/min using TBS as flow-buffer (50 mM TRIS, 150 mM NaCl, pH 7.4). A total of 35 plasma fractions (1.0 mL each) of plasma from carriers and controls were collected and further processed for apoE Western spot-blot analysis. Spot-blot analysis was carried out using a 96 wells casting system with a mounted PVDF membrane. Of each fraction, 0.9 mL was blotted on the PVDF membrane by vacuum suction. Subsequently the PVDF membrane was blocked with PBS/0.05% tween-20/Milk 5% (pH 7.4) for 30 minutes and washed between each incubation step with PBS/0.05% Tween-20 (pH 7.4). The first incubation for 2 hours with goat anti-human apoE antibodies (1:1000, Bioconnect) as primary antibody was followed by an 1 hour incubation with anti-goat HRP labeled (1:100000, DAKO) as the secondary antibody, all at room temperature. After the final wash-step the blot was incubated with the detection agent using the ECL staining femto kit (Pierce, Rockford, IL, USA). For reading the apoE chemo luminescence intensity the blot was scanned on a ChemiDoc MP imager (Bio Rad). Values for each fraction were expressed as percent of total apoE staining in all fractions.

2.7. Measurements of HDL anti-oxidant properties and paraoxonase 1 antioxidant activities

HDL was prepared by the dextran-Mg^{2+} method [20]. The antioxidant properties of HDL were tested in the presence or absence of oxidized LDL as described [21] with some modifications [22]. In short, 2,7-dichlorofluorescin diacetate (Molecular Probes/Invitrogen, Carlsbad, CA, USA) was dissolved in fresh methanol at 2.0 mg/mL and incubated at room temperature for 20 min in the dark, resulting in the generation of 2,7-dichlorofluorescin (DCFH). Upon interaction with oxidants, DCFH is oxidized to fluorescent 2,7-dichlorofluorescein (DCF). Patient and control HDL-C (final concentration 50 μg cholesterol/mL) in the presence or absence of LDL-C (final concentration 100 μg cholesterol/mL) was added into a black 96-well plate in a final volume of 100 μl. The plate was incubated at 37^{o}C on a rotator for 1 h in the dark. At the end of this incubation period, 10 μl of DCFH solution (0.2 mg/mL) was added to each well, mixed, and incubated for an

additional 2 h at 37°C with rotation in the dark. Fluorescence was measured with a plate reader (Fluo-Star Galaxy, BMG, Ortenberg, Germany) at an excitation wavelength of 465 nm and an emission wavelength of 535 nm.

Serum Paraoxonase 1 (PON1) paraoxonase activity (PONase) in HDL, prepared by the dextran-Mg^{2+} method, was determined using paraoxon as substrate [23]. Briefly, the assay was performed in a final volume of 250 µL containing 5µL of dextran-Mg^{2+}-prepared HDL, 5.5 mmol/L paraoxon (paraoxon-ethyl, Sigma-Aldrich, St. Louis, MO, USA), 2 mmol/L $CaCl_2$ and 100 mmol/L Tris-HCl, pH 8.0. The rate of p-nitrophenol formed by hydrolysis of paraoxon was measured by monitoring the increase in absorbance at 405nm for 25min at room temperature in a microplate spectrophotometer. PON1 activity was expressed as U per L. 1U is defined as the activity that catalyzes the formation of 1 µmol substrate per min.

PON1 arylesterase activity (AREase) was measured in plasma [24]. Briefly, plasma from heterozygotes and wild-type controls were diluted 1:80 with a salt buffer, containing 20 mM Tris–HCl and 1.0 mM $CaCl_2$ in water. A triplicate of 20 µL diluted plasma were added to the wells in an UV-transparent 96-well plate (Sigma-Aldrich). 200 µL of phenyl acetate solution, containing 3.26 mM phenyl acetate in salt buffer, were added to each well and the absorbance of produced phenol was measured at 270 nm with 250 nm as background in a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). The initial period when the reaction was linear were used for calculation of activity, expressed as U/mL, using an extinction coefficient of phenol of 1310 $M^{-1}cm^{1}$.

2.8. Measurement of apoA-I methionine oxidations in HDL by LC-MS/MS

Desalted HDL obtained by ultracentrifugation, was digested with trypsin (1:25 trypsin:protein ratio) in a sonicator bath for 30 min at 37°C. Peptide solution was dried by a vacuum centrifugation system before resuspension in 0.1% formic acid in dH_2O. Peptides were analyzed using a nanoflow liquid chromatography system coupled to an Orbitrap Velos Pro (Thermo Fisher Scientific, Waltham, MA, USA). Separation was performed during a 90 min linear increase from 2 to 40% ACN with 0.1% FA. Peptides were analyzed by a data-dependent acquisition mode in which spectra were obtained using Fourier Transmission MS (Orbitrap) and the top 20 peaks selected for CID using linear ion trap. Obtained data was processed in MaxQuant v1.5.12 (Max Planck Institute of Biochemistry, Martinsried, Germany) to search against human ApoA-I. Search was performed using 5 ppm mass

tolerance for MS and 0.5 Da for MS/MS. Peptides with a false-discovery rate of less than 1% was retained. Variable modifications searched were methionine oxidation and a minimum score of 40 were needed for accepting modified peptides. Results are expressed as average ± SD for the intensity of peptides with methionine oxidations as well as the ratio between modified and unmodified peptides as obtained through the MaxQuant software.

2.9. Cholesterol efflux capacity (CEC) assay

The cholesterol efflux capacity (CEC) of HDL was quantified as previously described [25, 26]. In brief, J774 mouse macrophages plated in 48-well plates were labeled with 0.2 ml of labeling medium (0.25 μCi/ml 4[^{14}C] cholesterol in DMEM (high glucose) supplemented with 0.2% (w/v) BSA). Based on the findings of Li et al, an acyl–coenzyme A:cholesterol acyltransferase inhibitor was not used [26]. Following 24 h of labeling and washing, cells were equilibrated for 24 h with 0.3 mM 8-(4-chlorophenylthio)-cAMP in 0.2 ml of DMEM (high glucose) supplemented with 0.2% (w/v) BSA. Subsequently efflux media containing HDL, prepared by the dextran-Mg^{2+} method, at a final concentration of 10 μg apoA-I/mL (determined in serum) in 0.2% (w/v) BSA/DMEM (high glucose) were added for 4 hours. At the end of the incubation, the supernatants were collected and the cells were lysed in 200 μl of lysis buffer (PBS containing 1 % (v/v) Triton X-100) for 30 min at room temperature by gentle shaking. The radioactivity in 50 μl of the supernatant and 100 μl of cell lysate was determined by liquid scintillation counting. The percentage of secreted [^{14}C]-cholesterol was calculated by dividing the medium-derived counts by the sum of the total counts present in the culture medium and the cell lysate. To calculate the net cholesterol efflux that is promoted by each HDL sample, the percentage of secreted [^{14}C]-cholesterol in the absence of HDL (control sample) was subtracted from the percentage of secreted [^{14}C]-cholesterol in the presence of HDL. HDL samples were prepared freshly and assays were performed in duplicate. All samples were analyzed simultaneously on the same plate.

2.10. Statistical analyses

To identify alterations important for discrimination between heterozygotes and wild-types, multivariate modeling by Partial least squares (PLS) using the NIPALS algorithm was performed on 2-DE, plasma lipid/protein, PON1 activities, apoA-I methionine oxidation and CEC data in Statistica (Statsoft, Tulsa, OK, USA). The same factors were investigated by comparing heterozygotes to wild-type controls using t-test in Statistica. Non-gaussian distributed proteins were log-transformed before t-test. Significant variables were also tested

with age-adjusted main effects ANOVA in Statistica. Pearson correlation analysis of apoL-1 staining in 2-DE and WB was performed in Statistica. Anti-oxidant properties and PON1 activities were investigated by t-test in Graphpad (Graphpad Software, La Jolla, CA, USA).

3. Results

The SR-B1^{P297S} mutation was found in a family in a previous study involving screening of individuals with HDL-C above the 95th percentile [15]. The carriers of the novel mutation showed increased plasma HDL-C and apoA-I as well as slightly decreased apoB compared to their family controls. In the present study, a subgroup of heterozygous SR-B1^{P297S} carriers and controls were analyzed showing similar differences in plasma lipid and protein levels (Table 1). For HDL and LDL/VLDL proteomics, 2-DE and mass spectrometry was used and representative protein patterns are illustrated in Figure 1 and protein identities are presented in Table 2.

To study differences in SR-B1^{P297S} heterozygotes compared to wild-type controls, 2-DE protein intensities, plasma lipid/protein data, HDL CEC, PON1 activities and apoA-I methionine oxidations were investigated by multivariate modelling with PLS. Two components were used and the score plot showed a clear separation of the individuals in the two groups along the predictive x-axis (Figure 2A, x-axis; R^2=0.25, Q^2=0.47). The variables with the greatest score contribution (defined as the average sample contributions in each group), and thereby the most important values for separating the carriers from the controls, were apoE in LDL/VLDL and apoL-1 in HDL (score contribution of 1.05 and 1.17 for the heterozygotes, respectively). As illustrated in figure 2B, these two variables were located close to the heterozygote node in the loading plot. In addition, univariate statistics of the 2-DE data showed a significant increase of apoE in the LDL/VLDL and a significant increase of apoL-1 in the HDL fraction (Table 2, $p<0.05$). Confirming the 2-DE analysis, western blot of apoL-1 in HDL showed a significant increase in the heterozygotes compared to the wild-type controls (Figure 3A-B) and further supporting the data, western blot intensities were positively correlated to the 2-DE intensities (Figure 3C, r=0.75, $p<0.05$). Moreover, plasma was fractionated using FPLC and the relative levels of apoE in different lipoprotein fractions were analyzed by western spot-blot. The analysis showed a pronounced increase of apoE in LDL, while the increase in the less dense HDL fraction and the decrease in the denser HDL fraction of the heterozygotes as compared to the wild-type controls (Figure 4) are in

accordance with the previously described shift towards larger HDL particles in the heterozygotes. Interestingly, an increase in lipid-free apoE was also found.

The in vitro anti-oxidative capacity of HDL in SR-B1^{P297S} carriers and controls were measured by incubating HDL with oxidized LDL and the ability of HDL to inhibit oxidized LDL from forming DCF was measured. There were no significant changes in the anti-oxidative capacity of HDL in SR-B1^{P297S} carriers compared to the controls (Figure 5A). The PONase and AREase activities of PON1 were also measured, and consistent with unchanged anti-oxidative capacity, no significant difference between carriers and controls was observed (Figure 5B and 5C).

Methionine oxidations in HDL apoA-I was investigated using LC-MS/MS. Three positions with methionine oxidations were found; methionine 110, 136 and 172 (including pre- and propeptide). Methionines at positions 136 and 172 showed a significant increase of oxidized peptide intensity as well as the ratio of modified to unmodified peptides for the specific position in SR-B1^{P297S} heterozygotes compared to wild-type controls (Table 3).

The CEC of heterozygote and control HDL was investigated by measuring cholesterol efflux from J774 mouse macrophages. Results showed that HDL from heterozygotes had no significant differences in their CEC, compared to the controls (Figure 6).

4. Discussion

Here, multivariate modeling in the form of PLS was utilized to investigate differences between controls and SR-B1^{P297S} heterozygotes regarding LDL/VLDL and HDL proteomics, plasma lipid/protein data, HDL CEC as well as PON1 activity data (Figure 2). The modelling investigates which factors are responsible for separating the groups. The PLS analyses showed that increased levels of apoE in LDL as well as increased levels of apoL-1 in HDL were important for discriminating between heterozygote carriers for SR-B1^{P297S} and wild-type controls. The model also contained plasma lipid/protein data which reflected the apparent increase of apoA-I and HDL-C as well as the decrease of apoB and LDL-C in carriers compared to controls.

ApoE, being an LDL receptor ligand, mediates the cellular uptake of triglyceride-rich lipoproteins [27] and is regarded to have anti-atherosclerotic and anti-oxidative properties [28]. Interestingly, in the present study, we found an enrichment of apoE in LDL/VLDL from SR-B1^{P297S} heterozygotes, which is consistent with the previously indicated increase of plasma apoE in heterozygotes from the same family [15] and with SR-B1 mutant mice having elevated levels of apoE in LDL [29].To further study the apoE distribution in the carriers, the relative distribution of apo E in different plasma fractions was assessed after FPLC size-exclusion chromatography. More apoE was found in the LDL fraction while less was present in the HDL3 fraction (smaller more dense HDL particles) compared to the HDL2 fraction (cholesterol loaded, larger less dense HDL particles) in carriers compared to controls (Figure 4), which may be explained by the previously found shift from HDL3 towards HDL2 in the heterozygotes [15]. Interestingly, the FPLC-separation also revealed an increase of lipid-free apoE in the heterozygotes compared to the controls. Free circulating apoE is normally found at very low concentrations in the circulation and it has been proposed that tissues with increased need for cholesterol such as liver and adrenal gland may secrete free apoE to facilitate the SR-B1 mediated cholesterol uptake, a process in which apoE directly binds to SR-B1 and contributes to increased cholesteryl ester uptake from both HDL and LDL [30]. Further supporting these findings, overexpression of apoE in mice has been shown to increase the uptake of cholesterol via SR-B1 [31]. Enrichment of apoE in LDL/VLDL combined with increased free apoE, as found in the present study, may therefore reflect compensatory mechanisms for attenuated cholesterol uptake in the SR-B1^{P297S} carriers. Also supporting our data, apoC-I in HDL showed a trend towards lower level in the heterozygotes, as well as being the protein with least association to heterozygotes in the multivariate model (largest negative score contribution with -0.33 in the PLS model). ApoC-1 is a known inhibitor of SR-B1 and CETP action [32, 33] and a reduction of apoC-1 may therefore be a consequence of reduced need for inhibition of SR-B1 pathways in carriers. Considering the increase in apo E, the lower LDL/apoB levels and the absence of reported vascular complications in the heterozygotes, future studies should include investigations of apoB/E receptor mediated cholesterol uptake in subjects with functional SR-B1 mutations.

Interestingly, the multivariate analysis pointed out apoL-1 in HDL as a key component to discriminate heterozygotes from controls regarding lipoprotein patterns. Accordingly, apoL-1 was significantly enriched in HDL in the 2-DE patterns from carriers vs controls, The apo L-1 increase was then further confirmed by western blots. As illustrated in figure 4, two 37

kDa bands of apoL-1 were detected by immunoblotting, probably corresponding to glycosylated and non-glycosylated apoL-1 [34]. The main increase was found to be in the lower, non-glycosylated band. The function of the glycosylation in apoL-1 is not clear, but enzymatic glycosylation in general is known to provide increased solubility, correct folding or increased stability to the protein. The cause for the increase of the non-glycosylated isoform in the SR-B1^{P297S} heterozygotes is not known but may depend on de-glycosylation, possibly due to increased retention time of apoL-1 in the circulation but may also reflect increased synthesis, with disturbed glycosylation. In addition, a challenging finding was that an additional 25 kDa band was detected only in heterozygotes. This band may represent an enzymatically modified or truncated form of apoL-1 but was not included in the quantitative analysis since the abundance was too low for identification by MS. ApoL-1 is a HDL-associated apolipoprotein which has been reported to exert several extra- and intracellular functions in host defence and hemostatic mechanisms [35, 36]. Although previously mostly studied for its role in trypanosomal lysis [37] recent research has shown polymorphisms in apoL-1 associated to an increased risk for CVD [38]. Thus, indicating a possible, but yet to be identified, function for apoL-1 in CVD development. Notably, apoL-1 plasma levels have been found to be significantly increased in patients with primary cholesteryl ester transfer protein (CETP) deficiency [39], a condition that, as the SR-B1 mutation, results in increased HDL-C levels. The apoL-1 containing HDL subpopulation has previously been shown to represent about 10 % of total HDL [36] and apoL-1 has been reported to preferentially associate with the more dense HDL3 fraction [18, 40]. In contrast, the SR-B1^{P297S} heterozygotes in the present study have a shift towards more large cholesterol loaded HDL2 particles [15] but still increased levels of apoL-1. Further studies are warranted to clarify the role of apoL-1 and how enrichment in HDL may affect HDL functionality in SR-BI^{P297S} carriers.

Based on the suggestion that mutations in SR-B1 may increase CVD risk by non-lipid pathways [12], an important aspect to investigate is HDL function where PON1 is a vital component due to its ability to hydrolyze lipid peroxides in LDL [41]. However, in the present study, no differences in anti-oxidative properties of HDL or PONase and AREase activities could be found in SR-B1^{P297S} carriers compared to controls. These results are consistent with a previous study investigating heterozygous carriers of a CETP deficiency mutation, also with increased levels of HDL-C, where carriers did not show any change in anti-oxidant capacity or PON1 activity as compared to controls [42]. Regarding other factors

that may have impact on cholesterol metabolism, we did find significantly increased levels of methionine oxidations at two positions in HDL-apoA-1 of the heterozygotes compared to the controls. Oxidation of methionine 172 have previously been linked to a reduction in Lecithin-cholesterol acyltransferase (LCAT) activation [43] as well as reduced ATP-binding cassette sub-family A member 1 (ABCA1) mediated cholesterol efflux [44]. The increased oxidation found in the SR-B1^{P297S} heterozygotes may result from prolonged retention time of HDL in the circulation, which is a realistic consequence of reduced SR-B1-mediated uptake by the liver and steroidogenic tissues.

The HDL CEC has been shown to be inversely associated to markers of CVD and may be a better tool for CVD prediction than HDL-C [25, 45]. However, the CEC assay showed that HDL from SR-B1^{P297S} heterozygotes had similar capacity to accept cholesterol from J774 mouse macrophages as the controls, despite increased methionine oxidations in apoA-I. The unaffected cholesterol efflux capacity may be related to other factors such as the apparently increased apoE or possibly apo L-1 that may normalize the total cellular cholesterol uptake, by redirection or facilitation. Investigating apo E abundance as well as cholesterol uptake via other cholesterol accepting receptors in families with functional mutations in SR-B1 would be highly interesting in future studies.

Limitations of the study

SR-B1^{P297S} is a rare mutation and a major limitation of the study is the small number of samples analyzed. Therefore, we cannot exclude the possibility of false negative results due to limited statistical power. For example, apo C-1, apoA-II, apoE and transthyretin in HDL showed trends towards alterations but were not statistically different from controls. On the other hand, multiplex techniques such as 2-DE invites the presence of false positive results. However, in the case of apoE and apoL-1 this possibility is highly unlikely since the 2-DE results were confirmed by both immunological assays and multivariate statistics. Another limitation is that 2-DE analysis is not optimal for large, hydrophobic, low abundant proteins. In HDL there are two well-known examples of proteins that are underrepresented; apoA-II which is the second most abundant protein in HDL while relatively low abundant in the 2-DE pattern and PON-1 that most often is not detected in HDL by 2-DE. Finally, this study focus on the protein composition of the lipoprotein particles and additional analyses of the lipid composition may contribute to improved understanding and thereby enhance the possibility to elucidate relevant functional implications.

4. Concluding remarks

The present study indicates how the functional mutation SR-B1^{P297S} affects lipoprotein composition and contributes to methionine oxidation of apoA-I but does not affect PON-1 activities, HDL antioxidant properties or HDL cholesterol efflux capacity. The increase of apoE in carriers may indicate a compensatory mechanism for attenuated SR-B1 mediated cholesterol uptake by HDL but further studies are warranted to fully understand the complexity of altered lipoprotein composition in subjects with functional mutations in SR-B1.

5. Acknowledgements

This work was funded by EU's Sixth Framework Program: FP6-2005 No. 037631 (HDLomics), European Union (TransCard: FP7-603091-2), CardioVascular Research Initiative (CVON2011-16; Genius), the Research Council of South East Sweden (FORSS-3755), County Council of Östergötland (C-ALF) and Faculty of Health Sciences in Linköping. Drs Hovingh and Holleboom are supported by Veni grants (project numbers 91612122 and 91613031, respectively) from NWO.

6. Conflicts of interest statement

All authors have filled in ICMJE disclosure form for Potential Conflicts of Interest.
Dr. Kastelein reports personal fees from Cerenis, The Medicines Company, CSL Behring, Amgen, Regeneron, Eli Lilly, Genzyme, Aegerion, Esperion, AstraZeneca, Omthera, Pronova, Vascular Biogenics, Boehringer Ingelheim, Catabasis, AtheroNova, UniQure, Novartis, Merck, Isis Pharmaceuticals, Kowa, Dezima Pharmaceuticals and Sanofi, not connected to the submitted work.

7. References

[1] Gordon, T., Castelli,W.P., Hjortland, M.C., Kannel, W.B., Dawber ,T.R., High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am. J. Med., 1977; 62:707-714.

[2] Gordon, D., Probstfield, J., Garrison, R., Neaton, J. et al., High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation, 1989;79:8-15.

[3] Emerging Risk Factors Collaboration, Di Angelantonio, E., Sarwar, N., Perry, P. et al., Major lipids, apolipoproteins, and risk of vascular disease. JAMA, 2009;302:1993–2000.

[4] Barter, P., Brewer, B., Chapman, J., Hennekens, C. et al., Cholesteryl Ester Transfer Protein A Novel Target for Raising HDL and Inhibiting Atherosclerosis. Arterioscler. Thromb. Vasc. Biol., 2003;23:160-167.

[5] Masson, D., Koseki, M., Ishibashi, M., Larson, C. et al., Increased HDL Cholesterol and ApoA-I in Humans and Mice Treated With a Novel SR-B1 Inhibitor. Arterioscler. Thromb. Vasc. Biol., 2009;29:2054-2060.

[6] Danilo, C., Gutierrez-Pajares, J., Mainieri, M., Mercier, I. et al., Scavenger receptor class B type I regulates cellular cholesterol metabolism and cell signaling associated with breast cancer development. Breast Cancer Res., 2013;15:R87.

[7] Van Eck, M., Twisk, J., Hoekstra, M., Van Rij, B.T. et al., Differential effects of scavenger receptor B1 deficiency on lipid metabolism in cells of the arterial wall and in the liver. J. Biol. Chem., 2003;278:23699-23705.

[8] Braun, A., Trigatti, B., Post, M., Sato, K. et al., Loss of SR-B1 Expression Leads to the Early Onset of Occlusive Atherosclerotic Coronary Artery Disease, Spontaneous Myocardial Infarctions, Severe Cardiac Dysfunction, and Premature Death in Apolipoprotein E-Deficient Mice. Circ. Res., 2002;90:270-276.

[9] Trigatti, B., Rayburn, H., Vinals, M., Braun, A. et al., Influence of the high density lipoprotein receptor SR-B1 on reproductive and cardiovascular pathophysiology. Proc. Natl. Acad. Sci. U.S.A., 1999;96:9322-9327.

[10] Picataggi, A., Lim, G., Kent, A., Millar, J. et al., A coding variant in SR-B1 (I179N) significantly increases atherosclerosis in mice. Mamm. Genome, 2013;24:257-265.

[11] Grallert, H., Dupuis, J., Bis, J., Deghan, A. et al., Eight genetic loci associated with variationin lipoprotein-associated phospholipase A2 mass and activity and coronary heart disease:meta-analysis of genome-wide association studies from five community-based studies. Eur. Heart J., 2012;33:238-251.

[12] Manichaikul, A., Naj, A., Herrington, D., Post, W. et al., Association of SCARB1 Variants With Subclinical Atherosclerosis and Incident Cardiovascular Disease: The Multi-Ethnic Study of Atherosclerosis. Arterioscler. Thromb. Vasc. Biol., 2012;32:1991-1999.

[13] Naj, A., West, M., Rich, S., Post, W. et al., Association of Scavenger Receptor Class B Type I Polymorphisms With Subclinical Atherosclerosis - The Multi-Ethnic Study of Atherosclerosis. Circ. Cardiovasc. Genet., 2010;3:47-52.

[14] Verdier, C., Martinez, L., Ferrières, J., Elbaz, M. et al., Targeting high-density lipoproteins:Update on a promising therapy. Arch. Cardiovasc. Dis., 2013;106:601-611.

[15] Vergeer, M., Korporaal, S., Franssen R., Meurs I. et al., Genetic Variant of the Scavenger Receptor B1 in Humans. N. Engl. J. Med., 2011;364:136-145.

[16] Chadwick, A., Sahoo, D., Functional Characterization of Newly-Discovered Mutations in Human SR-BI. PLoS ONE, 2012;7:e45660.

[17] Brunham, L., Tietjen, I., Bochjem, A., Singaraja R., Novel mutations in scavenger receptor BI associated with high HDL cholesterol in humans. Clin. Genet., 2011;79:575-581

[18] Karlsson, H., Leandersson, P., Tagesson, C., Lindahl, M., Lipoproteomics II: Mapping of proteins in high-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. Proteomics, 2005;5:1431-1445.

[19] Lindahl, M., Ståhlbom, B., Svartz, J. Tagesson, C., Protein patterns of human nasal and bronchoalveolar lavage fluids analyzed with two-dimensional gel electrophoresis. Electrophoresis, 1998;19:3222-3229.

[20] Warnick, G.R., Benderson, J., Albers, J.J., Dextran sulfate-Mg2+ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. Clin. Chem., 1982;28:1379-1388.

[21] Navab, M., Hama, S.Y., Hough, G.P., Subbanagounder, G. et al., A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. J. Lipid Res., 2001;42:1308-1317.

[22] Daniil, G., Phedonos, A.A., Holleboom, A.G., Motazacker, M.M. et al., Characterization of antioxidant/anti-inflammatory properties and apoA-I-containing subpopulations of HDL from family subjects with monogenic low HDL disorders. Clin. Chim. Acta, 2011;412:1213-1220.

[23] Tsimihodimos, V., Karabina, S.A., Tambaki, A.P., Bairaktari, E. et al., Atorvastatin preferentially reduces LDL-associated platelet-activating factor acetylhydrolase activity in dyslipidemias of type IIA and type IIB. Arterioscler. Thromb. Vasc. Biol., 2002;22:306-311.

[24] Richter, R., Jarvik, G., Furlong, C., Determination of Paraoxonase 1 Status Without the Use of Toxic Organophosphate Substrates. Circ. Cardiovasc. Genet., 2008;1:147-152.

[25] Khera, A., Cuchel, M., de la Llera-Moya, M., Rodrigues A. et al., Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N. Engl. J. Med., 2011;364:127-135.

[26] Li, X., Tang,W., Mosior, M., Huang, Y. et al. Paradoxical association of enhanced cholesterol efflux with increased incident cardiovascular risks. Arterioscler. Thromb. Vasc. Biol. 2013;33:1696-1705.

[27] Kitajima, K., Marchadier, D., Miller, G., Gao, G. et al., Complete Prevention of Atherosclerosis in ApoE-Deficient Mice by Hepatic Human ApoE Gene Transfer With Adeno-Associated Virus Serotypes 7 and 8. Arterioscler. Thromb. Vasc. Biol., 2006;26:1852-1857.

[28] Tangirala, R., Praticó, D., Fitzgerald, G., Chun, S., Reduction of Isoprostanes and Regression of Advanced Atherosclerosis by Apolipoprotein E. J. Biol. Chem., 2001;276:261-266.

[29] Rigotti, A., Trigatti, B., Penman, M., Rayburn, H. et al., A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type 1 reveals its key role in HDL metabolism. Proc. Natl. Acad. Sci. U.S.A., 1997;94:12610-12615.

[30] Bultel-Brienne, S., Lestavel, S., Pilon, A., Laffont, I. et al., Lipid free apoE binds to the class B type I Scavenger Receptor 1 (SR-B1) and enhances cholesteryl ester uptake from lipoproteins. J. Biol. Chem., 2002;277:36092-36099.

[31] Annema, W., Dikkers, A., Freark de Boer, J. et al., ApoE promotes hepatic selective uptake but not RCT due to increased ABCA1-mediated cholesterol efflux to plasma. J. Lipid Res., 2012;5:929-940.

[32] De Barros, J.P., Boualam, A., Gautier, T., Dumont, L. et al., Apolipoprotein C1 is a physiological regulator of cholesteryl ester transfer protein activity in human plasma but not in rabbit plasma. J. Lipid Res., 2009;50:1842-1851.

[33] De Haan, W., Out, R., Berbée, J.F., van der Hoogt, C.C. et al., Apolipoprotein C1 inhibits scavenger receptor B1 and increases plasma HDL levels in vivo. Biochem. Biophys. Res. Commun., 2008;377:1294-1298.

[34] Liu, T., Qian, W., Gritsenko, M., Camp, D. et al., Human plasma N-glycoproteome analysis by immunoaffinity subtraction, hydrazide chemistry, and mass spectrometry. J. Proteome Res., 2005;4:2070-2080.

[35] Vanhollebeke, B., Pays, E., The function of apolipoproteins L. Cell. Mol. Life. Sci., 2006;63:1937-1944.

[36] Duchateau, P.N., Pullinger, C.R., Orellana, R.E., Kunitake, S.T. et al., Apolipoprotein L, a new human high density lipoprotein apolipoprotein expressed by the pancreas.

Identification, cloning, characterization, and plasma distribution of apolipoprotein L. J. Biol. Chem., 1997;272:25576-25582.

[37] Vanhamme, L., Paturiaux-Hanocq, F., Poelvoorde, P., Nolan, D.P. et al., Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature, 2003;422:83-87.

[38] Kaoru, I., Bick, A., Flannick, J., Friedman, D., Increased Burden of Cardiovascular Disease in Carriers of APOL1 Genetic Variants. Circ. Res., 2014;114:845-850.

[39] Duchateau, P.N., Movsesyan, I., Yamashita, S., Sakai, N. et al., Plasma apolipoprotein L concentrations correlate with plasma triglycerides and cholesterol levels in normolipidemic, hyperlipidemic, and diabetic subjects. J. Lipid Res., 2000;41:1231-1236.

[40] Davidson, W.S., Silva, R.A., Chantepie, S., Lagor, W.R. et al., Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. Arterioscler. Thromb. Vasc. Biol., 2009;29:870-876.

[41] Precourt, L.P., Amre, D., Denis, M.C., Lavoie, J.C. et al. The three-gene paraoxonase family: physiologic roles, actions and regulation. Atherosclerosis, 2011;214:20–36.

[42] Chantepie, S., Bochem, A., Chapman, J., Hovingh, K., Kontush, A., High-Density Lipoprotein (HDL) Particle Subpopulations in Heterozygous Cholesteryl Ester Transfer Protein (CETP) Deficiency: Maintenance of Antioxidative Activity. PLoS One, 2012;7:e49336.

[43] Shao, B., Cavigiolio, G., Brot, N., Oda, M. et al., Methionine oxidation impairs reverse cholesterol transport by apolipoprotein A-I. Proc. Natl. Acad. Sci. U S A., 2008;105:12224-12229.

[44] Shao, B., Tang, C., Sinha, A., Mayer, P. et al., Humans With Atherosclerosis Have Impaired ABCA1 Cholesterol Efflux and Enhanced High-Density Lipoprotein Oxidation by Myeloperoxidase. Circ. Res., 2014;114:1733-1742.

[45] Favari, E., Ronda, N., Adorni, M., Zimetti, F. et al., ABCA1-dependent serum cholesterol efflux capacity inversely correlates with pulse wave velocity in healthy subjects. J. Lipid Res. 2013;54:238–243.

Table 1. Plasma lipid and protein data.

	Controls (n=6)	SR-B1^{P297S} Heterozygotes (n=6)
Total Cholesterol (mmol/L)	4.7 ± 1.0	4.2 ± 1.3
HDL-C (mmol/L)	1.1 ± 0.3	1.6 ± 0.6
LDL-C (mmol/L)	3.0 ± 0.7	2.5 ± 1.1
Triglycerides (mmol/L)	1.5 ± 0.6	1.0 ± 0.3
ApoA-I (mg/dL)	146 ± 22	177 ± 36
ApoB (mg/dL)	91± 20	66 ± 20

Lipid and lipoprotein levels in SR-B1^{P297S} heterozygotes (n=6) and family controls (n=6). Values are mean ± SD.

Table 2: Data from protein identification by 2-DE/ MALDI TOF MS. Protein intensities on 2-D gels are normalized by relative quantification.

Protein	Seq. Cov. (%)	No. of pept.	kDa/pI	Controls	SR-BI^{P297S} Heterozygotes
			HDL		
ApoA-I	31.5	14	29/5.4	46.15 ± 7.05	44.16 ± 3.98
ApoA-II	21.0	3	9/6.5	1.08 ± 0.87	0.53 ± 0.84
ApoA-IV	49.7	20	43/5.2	0.27 ± 0.12	0.23 ± 0.11
ApoC-I	65	7	7/7.9	0.13 ± 0.19	0.02 ± 0.03
ApoC-II	50.5	5	9/4.7	0.22 ± 0.29	0.28 ± 0.38
ApoC-III	37.4	4	9/4.7	3.34 ± 2.69	2.82 ± 1.70
ApoE	64	23	34/5.5	1.52 ± 0.95	2.30 ± 1.23
ApoL-1	32	16	41/5.7	0.03 ± 0.04	0.15 ± 0.10*
ApoM	18.1	4	21/5.7	0.17 ± 0.1	0.23 ± 0.17
SAA1	25.4	3	12/5.9	0.13 ± 0.17	0.16 ± 0.18
SAA4	50	10	13/9.2	5.37 ± 2.68	4.26 ± 3.52
TTR	25.2	3	14/5.3	0.20 ± 0.26	0.06 ± 0.07
			LDL/VLDL		
ApoA-I	59.2	20	29/5.4	2.84 ± 2.38	2.88 ± 0.88
ApoC-II	34.7	2	9/4.7	0.06 ± 0.04	0.40 ± 0.52
ApoC-III	37.4	4	9/4.7	2.21 ± 0.54	3.13 ± 0.94
ApoE	64	22	34/5.5	1.59 ± 0.45	8.62 ± 2.91*
ApoJ	18.3	9	50/5.9	0.13 ± 0.06	0.21 ± 0.17
ApoM	23.4	5	21/5.7	1.85 ± 1.39	3.04 ± 0.85
SAA4	32.3	4	13/9.2	1.56 ± 0.43	1.07 ± 0.78

Protein differences in HDL and LDL/VLDL from heterozygotes with an SR-B1^{P297S} mutation compared to family controls. Values are mean ± SD expressed as percent of total 2-D gel fluorescence. * = $p<0.05$ as compared to controls.

Table 3. Apolipoprotein A-I methionine oxidations in SR-B1^{P297S} heterozygotes (Het) and wild-type (WT) controls.

Position	Oxidized Peptide Intensity		Ratio Modified/Unmodified Peptide	
	WT	SRB1 P297S Het	WT	SRB1 P297S Het
Methionine-110	0.9E+7 ± 2.1E+7	2.6E+7 ± 3.2E+7	0.02 ± 0.04	0.03 ± 0.03
Methionine-136	6.4E+7 ± 6.5E+7	17.7E+7 ± 5.5E+7 **	0.06 ± 0.06	0.20 ± 0.07 **
Methionine-172	3.6E+7 ± 2.5E+7	9.9E+7 ± 3.9E+7 **	0.04 ± 0.01	0.19 ± 0.06 ***

Values are mean ± SD. Indicated positions are the amino acid positions of apoA-I, including pre- and propeptide. ** $p<0.01$, *** $p<0.001$ as compared to wild-type controls.

Figure texts

Figure 1. 2-DE pattern of HDL and LDL/VLDL proteins from SR-B1^{P297S} heterozygotes and family controls. The 2-DE patterns illustrate increased abundance of apoL-1 in HDL (A) and increased abundance of apoE in LDL/VLDL (B) in heterozygous carriers of SR-BI^{P297S} mutation compared to family controls. 400 µg and 300 µg of proteins were loaded on HDL and LDL/VLDL gels respectively. Proteins were visualized by Sypro Ruby staining and identified by MALDI-TOF MS.

Figure 2. Partial least squares model of HDL and LDL/VLDL 2-DE results, plasma lipid/protein data, HDL cholesterol efflux capacity (CEC) and Paraoxonase 1 activities in SR-B1^{P297S} carriers and controls.
A. Score plot showing the separation between the two groups in the model. X-axis represents the separation between the two groups and the Y-axis individual variation in the two groups.
B. Loading plot of the variables showing an association between heterozygotes and proximal variables apoL-1 in HDL as well as apoE in LDL/VLDL.
WT-wild-type controls, Het – SR-B1^{P297S} heterozygotes. Apo – apolipoprotein, SAA – serum amyloid A, TTR – transthyretin.

Figure 3. ApoL-1 levels in HDL from SR-B1^{P297S} heterozygotes and family controls.
A. Western blot of apoL-1, 25 µg of HDL loaded in each well. B. Graph representing results from of ApoL-1 Western blot. The values are mean ± SD percent of total apoL-1 intensity, * $p < 0.05$ as compared to controls. C. Correlation between apoL-1 levels determined by 2-DE/Sypro Ruby staining and the Western blot.

Figure 4. ApoE distribution in the main lipoprotein classes.
Fractionation was performed by FPLC of plasma from heterozygous carriers of the SR-BI^{P297S} mutation compared to controls. After spot blot of all fractions (0.9 ml) of carriers (Het) and controls (WT) on PVDF membrane the relative abundance (%) of each apoE fraction of the SR-B1^{P297S} carriers (■) and controls (▲) was determined. The main fractions (VLDL, LDL, HDL and lipid-free) have been indicated in the graph. For comparison, lipoprotein cholesterol profiles are presented in the previous paper of this family (Ref 15; Vergeer et al 2011).

Figure 5. HDL antioxidant properties and PON1 activities of HDL from SR-B1^{P297S} mutation heterozygotes and family controls. A: Antioxidant properties of HDL analyzed by the DCF assay. The fluorescence intensity resulting from oxidation of DCFH by HDL in the presence or absence of LDL was measured in a spectrofluorometer and expressed in arbitrary units (AU). B: HDL-associated PON1 paraoxonase (PONase) activity, expressed as units/L (U/L). C: Plasma PON1 arylesterase (AREase) activity, expressed as units/mL (U/mL).WT = wild-type control; Het = heterozygote.

Figure 6. Cholesterol efflux capacity (CEC) of HDL from SR-B1^{P297S} mutation heterozygotes and family controls. Measurement of the capacity of HDL (10 μg apoA-I/mL) to promote total cholesterol efflux from J774 mouse macrophages. The results are mean of two independent experiments performed in duplicate. Values are expressed as % cholesterol efflux of total cell cholesterol. WT = wild-type control; Het = heterozygote

Figures

Fig 1

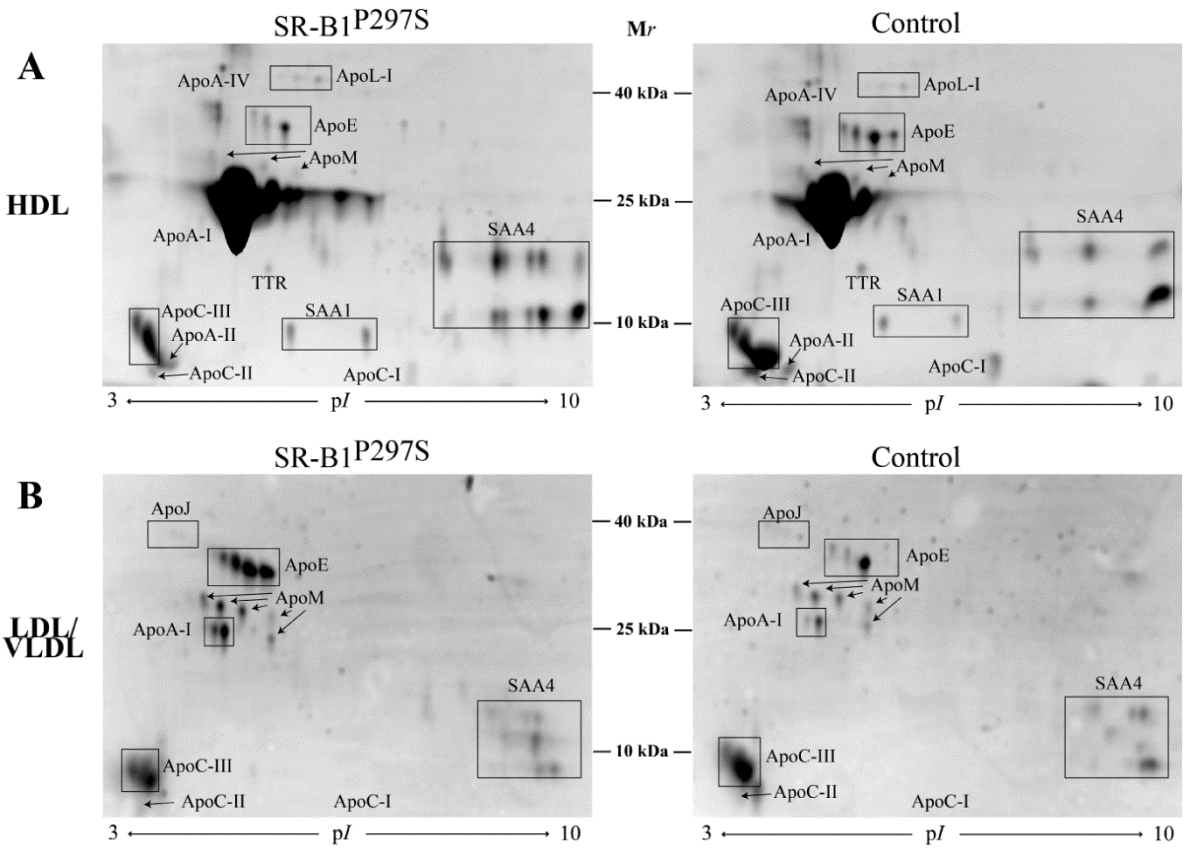


Fig 2

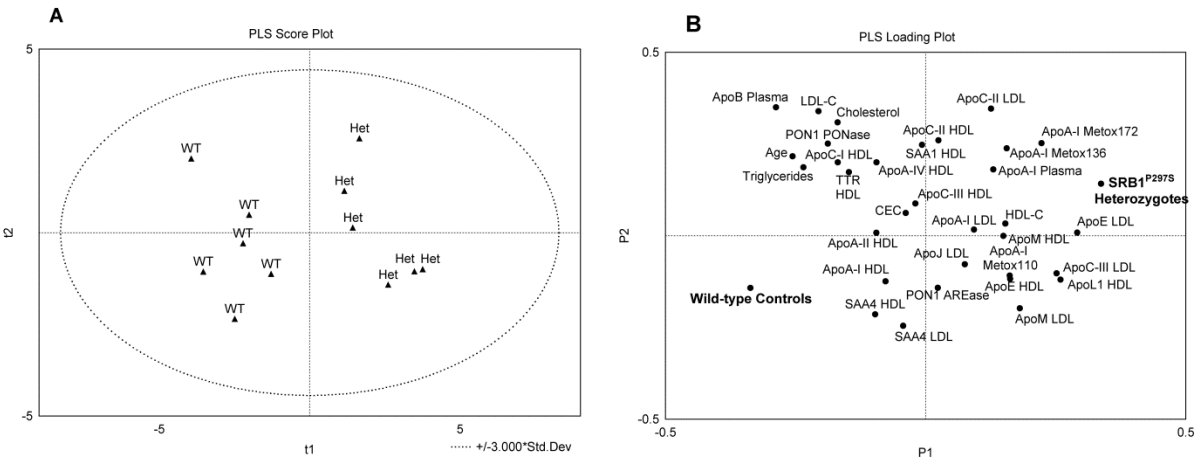
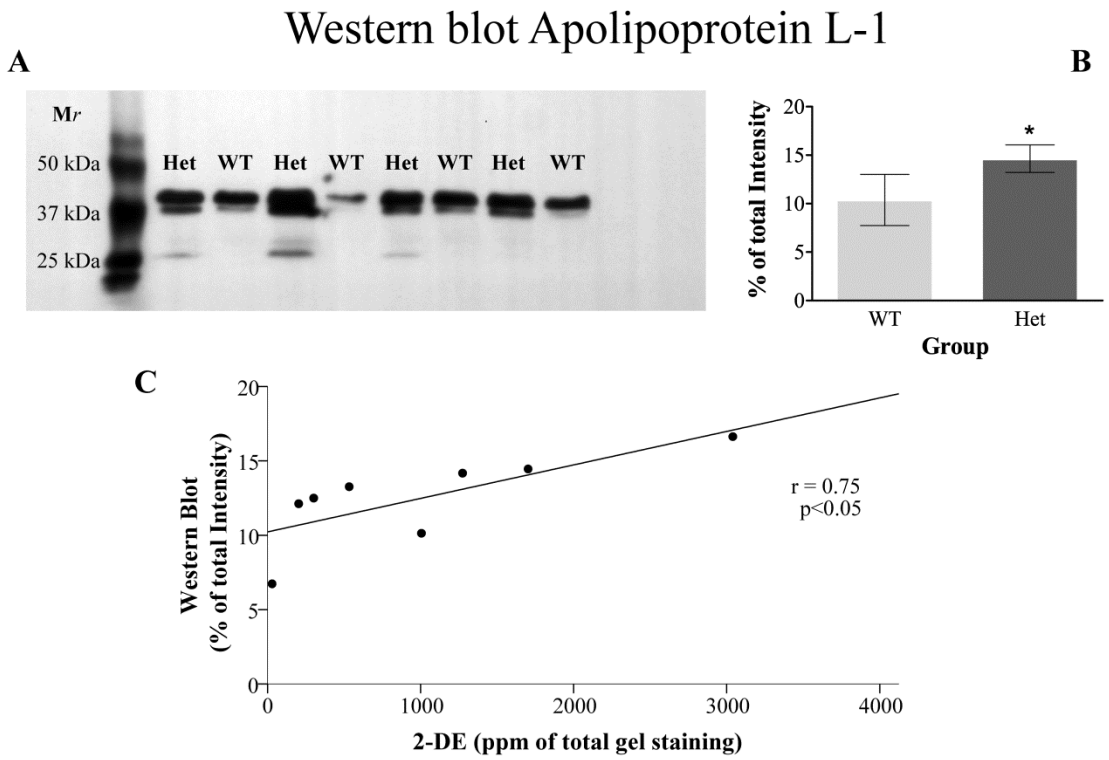


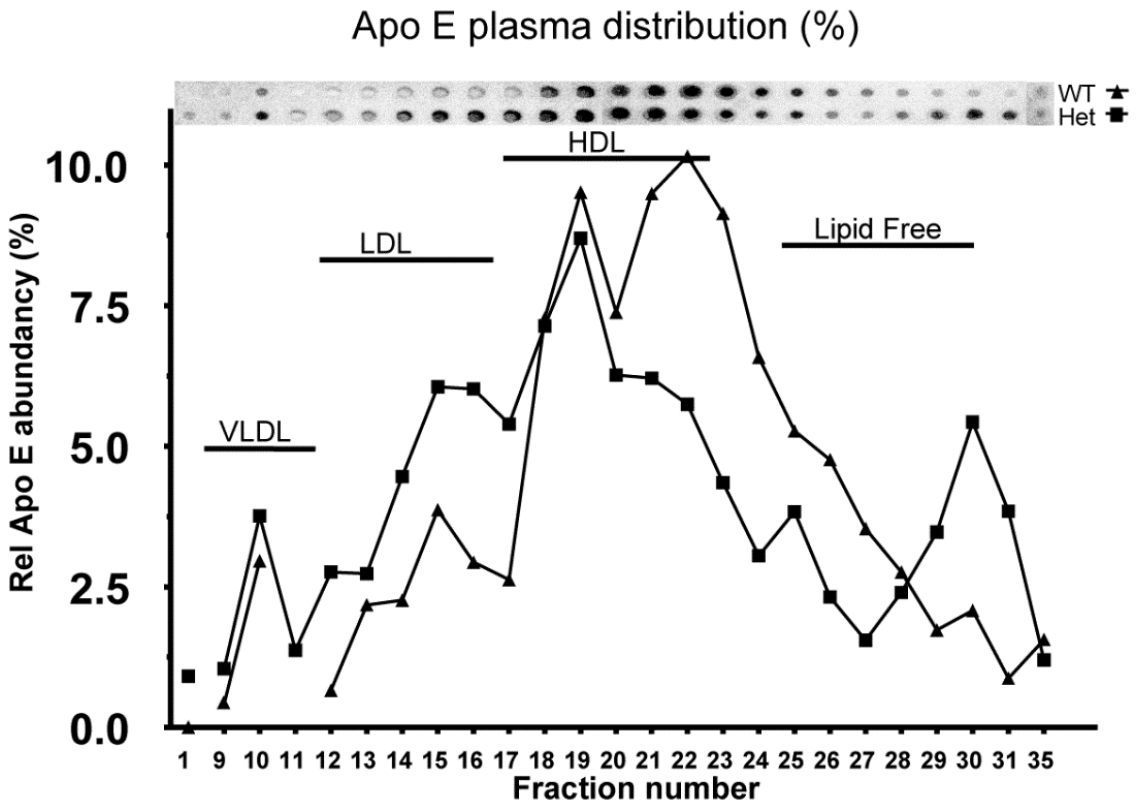
Fig 3



1

2

Fig 4



3

Fig 5

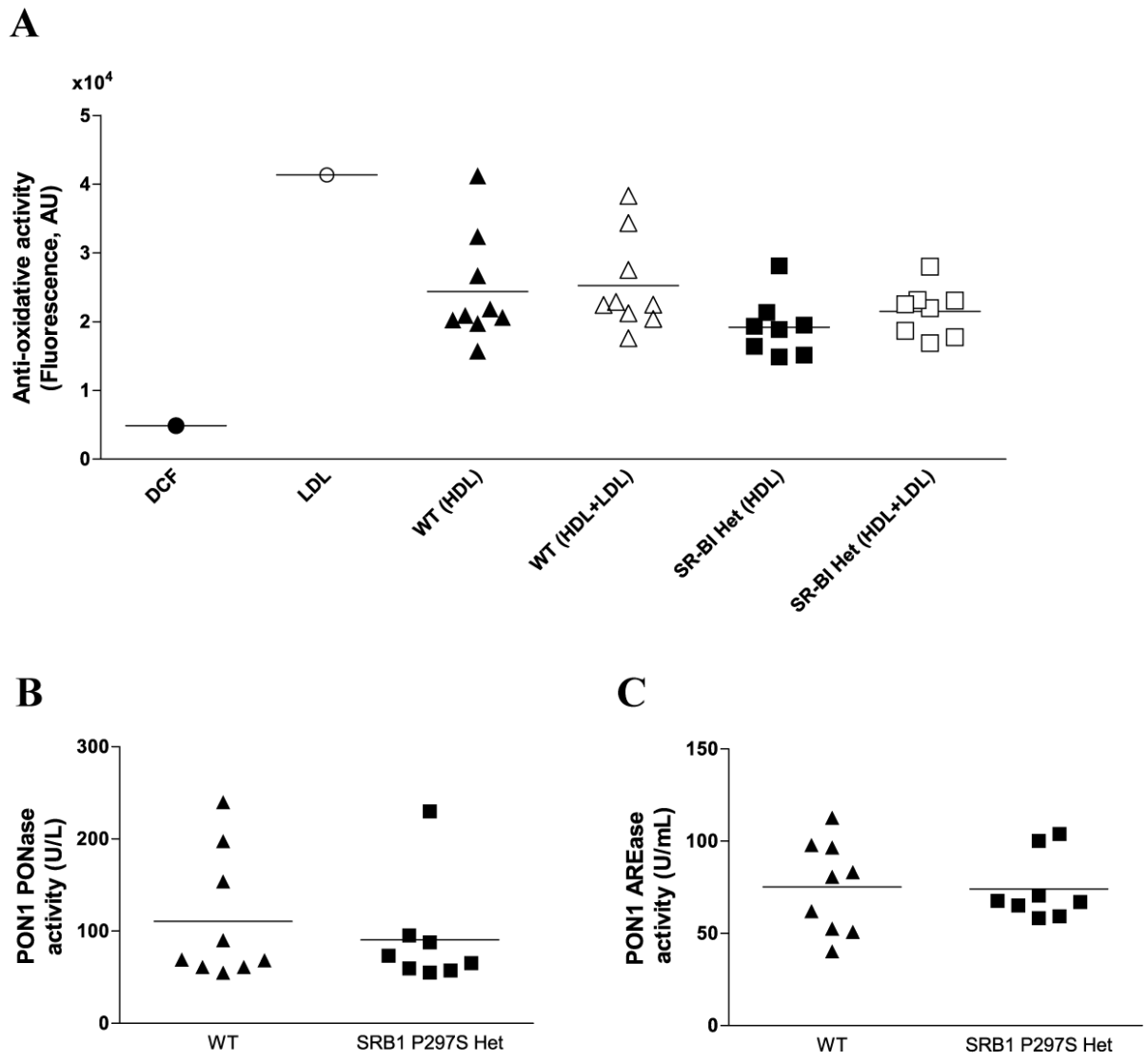
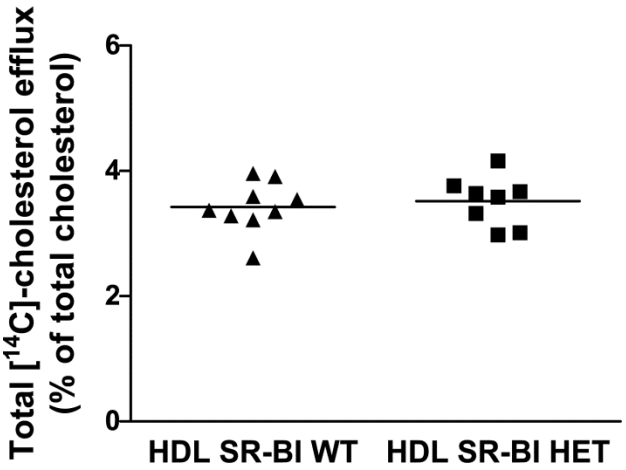

A
x10^4
5
4
3
2
1
0
Anti-oxidative activity (Fluorescence, AU)
DCF
LDL
WT (HDL)
WT (HDL+LDL)
SR-BI Het (HDL)
SR-BI Het (HDL+LDL)
B
300
200
100
0
PON1 PONase activity (U/L)
WT
SRB1 P297S Het
C
150
100
50
0
PON1 AREase activity (U/mL)
WT
SRB1 P297S Het

Fig 6


Total [14C]-cholesterol efflux
(% of total cholesterol)
6
4
2
0
HDL SR-BI WT
HDL SR-BI HET

1