Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: Relevance to antioxidative function

W. Sean Davidson[*†], R.A. Gangani D. Silva [*], Sandrine Chantepie[‡,§,‖], William R. Lagor[¶], M. John Chapman[‡,§,‖] and Anatol Kontush[‡,§,‖]

[*] Department of Pathobiology and Laboratory Medicine, University of Cincinnati, Cincinnati OH, USA; [‡] Université Pierre et Marie Curie-Paris 6, Paris, F-75013 France; [§] AP-HP, Groupe hospitalier Pitié-Salpétrière, Paris, F-75013 France; [‖] INSERM, Dyslipoproteinemia and Atherosclerosis Research Unit 551, Paris F-75013 France. [¶] Institute for Translational Medicine and Therapeutics, University of Pennsylvania, Philadelphia, PA, USA

Supplement Material

Blood sampling. Venous blood from normolipidemic healthy male volunteers was collected into sterile evacuated tubes (Vacutainer) in the presence or absence (to obtain serum) of EDTA (final concentration, 1.8 mg/ml) after an overnight fast. EDTA plasma was used to isolate HDL subfractions for proteomics studies in order to protect HDL from autoxidation by trace amounts of transition metals during experimental procedures.[1] Serum was employed to obtain HDL subfractions with intact paraoxonase (PON) activity for studies of antioxidative activity of HDL as PON is strongly inhibited by EDTA[2] (see below). All subjects gave informed consent; the study was performed in accordance with the Institutional Review Committee. Donors receiving antioxidant vitamin supplementation or drugs known to affect lipoprotein

metabolism were excluded as were smokers and excessive alcohol consumers. After blood collection, EDTA plasma and serum was immediately separated by centrifugation at 4°C. Two independent sets of plasma samples were analyzed in this study. The first sample set was composed of plasmas from 9 individual healthy normolipidemic donors. The second set was comprised of 3 samples, each containing plasma pooled from 20 individual healthy normolipidemic donors.

Isolation of lipoproteins. Plasma lipoproteins were fractionated from serum (in order to preserve the activity of paraoxonase 1 (PON1)) or EDTA plasma by isopycnic density gradient ultracentrifugation as previously described.[3-5] Five major subfractions of HDL were isolated, i.e. light HDL2b (d 1.063–1.087 g/mL) and HDL2a (d 1.088–1.110 g/mL), and dense HDL3a (d 1.110–1.129 g/mL), HDL3b (d 1.129–1.154 g/mL), and HDL3c (d 1.154–1.170 g/mL).[6] The validity and reproducibility of our density fractionation of HDL particle subspecies have been extensively documented.[3-5] The 9 individual plasma samples were directly subjected to a gradient fractionation protocol, whereas the 3 plasma pools were first subjected to sequential ultracentrifugation to isolate the total HDL fraction (d 1.063 – 1.21 g/ml) which was then subjected to our density gradient fractionation protocol in order to isolate HDL subfractions. Before use, KBr (and, in the case of plasma, EDTA) was removed from HDL solutions by exhaustive dialysis for 24 h at 4^{o}C. Lipoproteins were stored at 4ºC and used within 10 days.

Lipoprotein chemical composition. Total protein, total cholesterol (TC), free cholesterol, phospholipid and triglyceride contents of isolated lipoprotein subfractions were determined using commercially available enzymatic assays.[1, 7] Cholesteryl ester (CE) content was calculated by multiplying the difference between total and free cholesterol by 1.67 in order to take into consideration contribution from fatty acid

moiety of CE.[3-5] Total protein was measured using bicinchoninic acid (Pierce/Thermo Fisher Scientific, Brebières, France). Apo A-I and apo A-II were measured using immunonephelometry.[1, 7] PON1 activity of HDL subfractions (100 µg protein/ml) was determined photometrically in the presence of $CaCl_2$ (1 mM) using phenyl acetate as a substrate.[1, 7]

HDL-mediated protection of LDL against oxidation. LDL (10 mg TC/dL) was oxidized at 37 °C in Dulbecco's PBS (pH 7.4) in the presence of 1 mM 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH); HDL subfractions (final concentration, 10 mg total mass/dl) obtained from serum were added to LDL directly before oxidation. Accumulation of conjugated dienes was measured as the increment in absorbance at 234 nm.[7] The kinetics of diene accumulation revealed two characteristic phases, the lag and propagation phases. For each curve, the duration of each phase, average oxidation rates within each phase and the amount of dienes formed at the end of the propagation phase (maximal amount of dienes) were calculated.

Sample preparation for LC-MS analysis. 150 µg of total protein from each HDL subfraction were lyophilized to dryness in borosilicate tubes. The lipids were extracted by adding 1 ml of an ice-cold 2:1 (vol:vol) mixture of chloroform:methanol followed by ultrasonic dispersal of the protein pellet and a 30 min incubation on ice. Then 1 ml of ice-cold methanol was added followed by centrifugation at 4000 x g for 30 min at 4 ºC. The pellet was washed with 2 ml cold methanol and respun. The moist pellet was resuspended in 90 µl of 20% methanol/80% ammonium bicarbonate at pH 7.6. The proteins were reduced by incubation for 30 min at 42 °C in 10 mM DTT and carboxymethylated with additional 30 min incubation at room temperature with 40 mM iodoacetamide in the dark. 20 to 30 µg of the protein were then digested by sequencing grade trypsin at 5% of protein by wt (Promega) and incubated

overnight at 37 °C. The peptides were lyophilized to dryness and stored at -80 °C until analyzed by mass spectrometry. Peptides were resolubilized in 20 µl 0.1% TFA in ddwater and gently vortexed. Any particulates were pelleted in an Eppendorf microfuge set at maximum speed for 10 min. The top 15 µl was placed into an autosampler vial.

LC-MS analysis. 30 pmol (using an average MW for HDL proteins of 25,000) was injected onto a C18 capillary reversed phase column (Vydac, 500 µm X 15 cm) on a capillary HPLC (Agilent 1100) and eluted on an acetonitrile gradient of 0-40% with 0.1%TFA for 120 min at 6.0 µl/min. The eluting peaks were subjected to ESI-MS detection on a Sciex/Applied Biosystems QSTAR XL mass spectrometer equipped with an electrospray ionizer and a quadropole time-of-flight (Q-Tof) dual analyzer in the range 300-1800 m/z. Automated MS/MS sequencing was carried out between 100-2000 m/z in Q2 pulsing mode. The instrument was externally calibrated using a CsI and [Glu1]-Fibrinopeptide B (Sigma, St. Louis, MO) prior to each set of runs.

Analysis of MS data. A typical MS run resulted in approximately 1500 to 2000 MS/MS spectra. These were converted to a Mascot Generic File using Analyst QS software via a script available from Matrix Sciences (www.matrixscience.com). The data was then used to probe the SwissProt human database (update 071807) using three different search engines: MASCOT (Matrix Sciences), Phenyx (GenBio, Geneva Switzerland), and X! Tandem (The Global Proteome Machine, www.theGPM.org). Each algorithm was used with statistical score cut-off options disabled, an allowance of up to 2 missed tryptic cleavages, 0.15 Da MS and MS/MS accuracy, with oxidized methionines and carboxymethylated cysteines enabled. Protein identifications were considered reliable if they met all of the following conditions: 1) were found in any two of the three search algorithms, 2) contained at

least 2 unique peptides from the putative protein sequence, 3) the MS/MS evidence was judged to be plausible for the identification (typically about 50% of expected y- or b-series ions present). Criteria 2 was applied across samples and HDL subfractions.

Western blotting for apoF. HDL subfractions (eight micrograms of protein per lane) were subjected to denaturing SDS-PAGE on pre-cast 12% Bis-Tris gels (Invitrogen). Proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for one hour in blocking solution (5% nonfat dry milk in PBS with 0.05% Tween 20). Rabbit anti-human Apo F antisera raised against virally expressed full length human Apo F was added in a 1: 5,000 dilution in blocking solution and incubated overnight at 4ºC. Membranes were washed and donkey anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (Amersham) was added in a 1:5000 dilution in blocking solution and incubated for 3 hours. Membranes were washed again as before, and proteins were visualized on autoradiography film by chemiluminescence using ECL reagent (Amersham).

Validation of the MS analysis and protein identification strategy. The identification strategy listed in *Methods* was verified using the Universal Proteomics Standard (Sigma) which contains a mixture of 48 purified human proteins. Using the same protocol, including the delipidation procedure, a blinded operator correctly identified 43 of the proteins. Importantly, the stringent identification criteria resulted in 0 false positive identifications. An additional 3 proteins would have been correctly identified, but the presence of only one unique peptide precluded them from the final identification list. Thus, our analysis strategy may exclude some proteins, especially those of extremely low abundance, but is unlikely to indicate the presence of proteins that are not truly present in the samples.

Statistical analysis. All data are shown as means ± 1 sample standard deviation. Pearson's moment-product correlation coefficients were calculated to evaluate relationships between variables. Correlations among HDL proteins across the density subfractions were additionally analyzed using the organic algorithm of the Cytoscape software package. The nodes (HDL-associated proteins) were laid out and modified to cluster highly correlated nodes in close proximity. The nodes were treated like physical objects with mutually repulsive forces connected by metal springs representing the experimentally derived correlations between the nodes. The algorithm arranges the nodes so that the sum of the forces emitted by the nodes and the edges reaches a local minimum.

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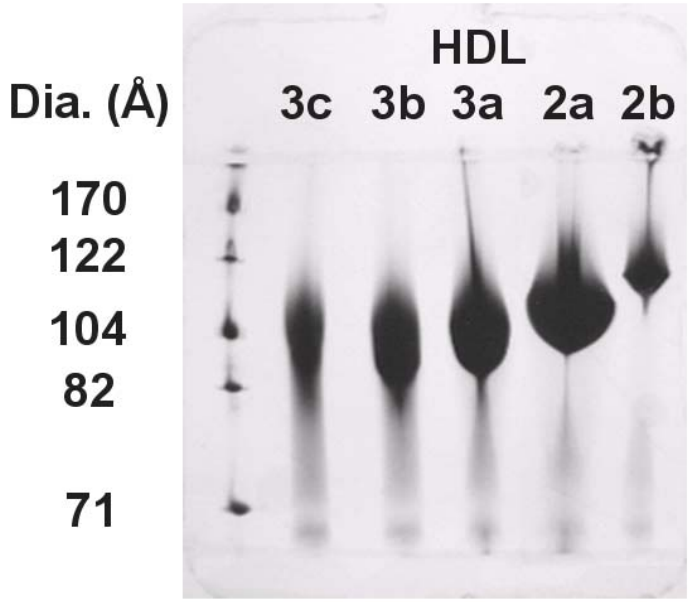
Figures and Tables



Online Figure I. Native polyacrylamide gel analysis of HDL density subclasses. 10 µg of total HDL protein for each subfraction was loaded onto an Amersham Phast 8-25% native gel and run for 264 aVh. The gel was stained with Coomassie blue. Amersham HMW standards were used in lane 1.

Online Table I. Chemical properties and antioxidative activity of HDL subfractions[a]

Subfraction	Density range (g/ml)	Avg. Dia. (nm)	Average chemical composition (%) Protein/PL/FC/CE/TG	Antioxidative capacity of HDL (% decrease in the LDL propagation rate)[b]
HDL2b	1.063 – 1.087	10.4	33/30/4/29/4	-2 ± 7
HDL2a	1.088 – 1.110	10.3	34/33/3/27/4	10 ± 9
HDL3a	1.111 – 1.129	9.9	41/29/2/24/3	17 ± 8
HDL3b	1.130 – 1.154	8.0	50/24/1/23/2	33 ± 7
HDL3c	1.155 – 1.170	7.3	66/16/<1/17/1	70 ± 3

[a] Information reprinted from [1].

[b] Oxidation of total LDL in the presence of individual HDL subfractions was performed *in vitro* in the presence of the azo-initiator, AAPH. Antioxidative capacity of HDL is presented as a decrease in the oxidation rate in the propagation phase of LDL oxidation, during which the inhibitory action of HDL is particularly potent.[1] Data are shown for four healthy normolipidemic donors.

Online Table II. *Peptide count data for HDL subfractions derived from individual normolipidemic donors (n=9).*

		SUM					Average					Standard Deviation				
		3c	**3b**	**3a**	**2a**	**2b**	**3c**	**3b**	**3a**	**2a**	**2b**	**3c**	**3b**	**3a**	**2a**	**2b**
A1AT	alpha1 anti-trypsin	12	0	0	0	0	1.3	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0
APOA	apolipoprotein (a)	1	0	0	17	29	0.1	0.0	0.0	1.9	3.2	0.3	0.0	0.0	5.0	4.2
APOA1	apolipoprotein A-I	442	455	489	444	438	49.1	50.6	54.3	49.3	48.7	13.2	10.0	22.8	16.5	19.4
APOA2	apolipoprotein A-II	89	99	113	107	95	9.9	11.0	12.6	11.9	10.6	4.4	3.2	3.1	3.6	4.5
APOA4	apolipoprotein A-IV	7	0	0	0	2	0.8	0.0	0.0	0.0	0.2	1.3	0.0	0.0	0.0	0.4
APOB	apolipoprotein B	84	11	0	52	686	9.3	1.2	0.0	5.8	76.2	25.1	2.4	0.0	10.3	41.7
APOC1	apolipoprotein C-I	42	45	52	58	51	4.7	5.0	5.8	6.4	5.7	1.4	1.3	1.2	1.2	2.0
APOC2	apolipoprotein C-II	15	17	22	28	34	1.7	1.9	2.4	3.1	3.8	1.4	1.3	1.3	1.8	1.4
APOC3	apolipoprotein C-III	20	21	26	23	26	2.2	2.3	2.9	2.6	2.9	1.2	1.2	1.5	1.7	1.7
APOC4	apolipoprotein C-IV	2	1	1	2	3	0.2	0.1	0.1	0.2	0.3	0.7	0.3	0.3	0.7	1.0
APOD	apolipoprotein D	60	58	44	30	27	6.7	6.4	4.9	3.3	3.0	1.9	1.7	1.3	2.3	2.0
APOE	apolipoprotein E	106	78	63	90	170	11.8	8.7	7.0	10.0	18.9	5.4	5.8	6.7	7.0	8.2
APOF	apolipoprotein F	12	8	2	0	0	1.3	0.9	0.2	0.0	0.0	1.2	0.8	0.4	0.0	0.0
APOL1	apolipoprotein L-I	125	73	8	5	3	13.9	8.1	0.9	0.6	0.3	6.1	6.6	2.3	1.7	1.0
APOM	apolipoprotein M	80	88	65	29	18	8.9	9.8	7.2	3.2	2.0	1.7	1.6	2.6	3.2	3.0
CLUS	apolipoprotein J	13	1	0	0	0	1.4	0.1	0.0	0.0	0.0	1.4	0.3	0.0	0.0	0.0
PLTP	Phospholipid transfer protein	15	13	1	1	0	1.7	1.4	0.1	0.1	0.0	1.4	1.9	0.3	0.3	0.0
PON1	Paraoxonase 1	64	26	3	0	0	7.1	2.9	0.3	0.0	0.0	4.1	3.5	1.0	0.0	0.0
PON3	Paraoxonase 3	12	5	0	0	0	1.3	0.6	0.0	0.0	0.0	1.1	0.9	0.0	0.0	0.0
SAA	Serum amyloid A (1 and 2)	18	27	15	7	7	2.0	3.0	1.7	0.8	0.8	2.4	2.5	1.9	0.7	0.7
SAA4	Serum amyloid A 4	65	75	66	63	57	7.2	8.3	7.3	7.0	6.3	3.2	2.0	2.1	1.9	1.6
LIS1	PAF-AH IB subunit alpha	1	3	3	1	3	0.1	0.3	0.3	0.1	0.3	0.3	0.5	0.5	0.3	0.5
ALBU	Albumin	16	0	0	0	5	1.8	0.0	0.0	0.0	0.6	3.0	0.0	0.0	0.0	1.7
FIBA	Fibrinogen alpha chain	3	0	0	0	0	0.3	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0
HPTR	Haptoglobin related protein	13	0	0	0	0	1.4	0.0	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0
PAFA	Platelet-activating factor acetylhydrolase	0	0	0	0	1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.3
SCYB7	Platelet basic protein	6	6	2	0	0	0.7	0.7	0.2	0.0	0.0	1.7	1.0	0.7	0.0	0.0
THRB	Prothrombin	4	2	0	1	0	0.4	0.2	0.0	0.1	0.0	1.3	0.7	0.0	0.3	0.0
TTHY	Transthyretin	3	0	0	0	0	0.3	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0

Online Table III. *Peptide count data for HDL subfractions derived from three samples, each containing pooled plasma from 20 normolipidemic donors (n=3).*

		SUM					Average					Standard Deviation				
A1AT	alpha1 anti-trypsin	**3c**	**3b**	**3a**	**2a**	**2b**	**3c**	**3b**	**3a**	**2a**	**2b**	**3c**	**3b**	**3a**	**2a**	**2b**
APOA	apolipoprotein (a)	1	0	0	0	0	0.3	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
APOA1	apolipoprotein A-I	0	0	0	0	7	0.0	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0	1.2
APOA2	apolipoprotein A-II	224	238	259	229	210	74.7	79.3	86.3	76.3	70.0	45.1	38.9	37.5	39.8	38.4
APOA4	apolipoprotein A-IV	25	24	30	32	22	8.3	8.0	10.0	10.7	7.3	0.6	0.0	1.0	2.3	1.5
APOB	apolipoprotein B	1	0	0	0	0	0.3	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
APOC1	apolipoprotein C-I	1	0	0	0	254	0.3	0.0	0.0	0.0	84.7	0.6	0.0	0.0	0.0	35.8
APOC2	apolipoprotein C-II	10	10	13	16	17	3.3	3.3	4.3	5.3	5.7	0.6	1.5	1.5	3.1	2.5
APOC3	apolipoprotein C-III	2	3	11	13	13	0.7	1.0	3.7	4.3	4.3	0.6	0.0	1.5	0.6	1.5
APOC4	apolipoprotein C-IV	8	10	13	14	15	2.7	3.3	4.3	4.7	5.0	0.6	0.6	0.6	0.6	1.0
APOD	apolipoprotein D	3	2	1	2	3	1.0	0.7	0.3	0.7	1.0	0.0	0.6	0.6	0.6	1.0
APOE	apolipoprotein E	17	21	13	10	3	5.7	7.0	4.3	3.3	1.0	1.5	1.0	0.6	0.6	1.0
APOF	apolipoprotein F	33	31	22	24	47	11.0	10.3	7.3	8.0	15.7	7.0	3.8	1.2	1.7	5.7
APOL1	apolipoprotein L-I	9	5	2	0	0	3.0	1.7	0.7	0.0	0.0	1.7	0.6	0.6	0.0	0.0
APOM	apolipoprotein M	26	20	0	0	0	8.7	6.7	0.0	0.0	0.0	4.0	1.2	0.0	0.0	0.0
CLUS	apolipoprotein J	25	28	19	6	3	8.3	9.3	6.3	2.0	1.0	2.1	1.2	5.5	2.0	1.0
PLTP	Phospholipid transfer protein	2	6	2	0	0	0.7	2.0	0.7	0.0	0.0	0.6	2.6	1.2	0.0	0.0
PON1	Paraoxonase 1	5	5	1	0	0	1.7	1.7	0.3	0.0	0.0	1.5	2.1	0.6	0.0	0.0
PON3	Paraoxonase 3	10	5	0	0	0	3.3	1.7	0.0	0.0	0.0	3.1	1.5	0.0	0.0	0.0
SAA	Serum amyloid A (1 and 2)	4	0	0	0	0	1.3	0.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0
SAA4	Serum amyloid A 4	5	7	4	3	3	1.7	2.3	1.3	1.0	1.0	1.5	1.5	1.2	0.0	0.0
LIS1	PAF-AH IB subunit alpha	14	16	17	15	19	4.7	5.3	5.7	5.0	6.3	1.2	1.5	2.5	1.0	1.2
ALBU	Albumin	2	4	2	1	1	0.7	1.3	0.7	0.3	0.3	1.2	1.5	0.6	0.6	0.6
FIBA	Fibrinogen alpha chain	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HPTR	Haptoglobin related protein	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PAFA	Platelet-activating factor acetylhydrolase	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SCYB7	Platelet basic protein	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
THRB	Prothrombin	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

TTHY	Transthyretin	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0