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SUPPORTING INFORMATION

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Abnormal composition and function of high-density lipoproteins in atopic dermatitis patients

To the Editor,

High-density lipoprotein (HDL) is conserved and present in most species, suggesting an important biological role from an evolutionary standpoint. HDL suppresses the activation of immune cells,¹ including eosinophils.² Despite the evidence indicating that HDL is an important modulator of the immune response, not much is known about the role of HDL in human atopic diseases. Moreover, there is no information available whether allergy alters HDL composition and function.

In the present study, we isolated HDL from atopic dermatitis (AD) patients and control subjects and used mass spectrometry and biochemical analysis as well as cell-based assays for detailed compositional and functional characterization. Methods and clinical characteristics of study subjects can be found in this article's Data S1.

Strikingly, we identified complex alterations in the composition of AD-HDL when compared to control HDL (Figure 1). As expected, the most abundant HDL protein was apolipoprotein (apo) A-I (Figure 1A). AD-HDL was enriched in apoA-II (+12%, $P = 0.0484$) and the acute-phase protein serum amyloid A (SAA) (+114%, $P = 0.0250$) when compared to control HDL, suggesting low-grade inflammation (Figure 1B,F). However, median C-reactive protein levels of AD patients were in the normal range (Table S1), suggesting only minimal inflammation. Of particular interest, levels of AD-HDL-associated apoC-III (−70%, $P < 0.0001$) and apoE (−39%, $P = 0.0014$) were markedly decreased (Figure 1D,E), which appears

to be unique, and were not reported in other disease states including psoriasis, an inflammatory skin disease.³ In good agreement with our results, a previous study demonstrated that the proteomic blood signature of AD is largely different from psoriasis.⁴ HDL-associated apoA-I and apoC-II contents were not altered in AD patients (Figure 1A,C).

In addition, we observed striking low contents of HDL-associated cholesteryl esters (−42%, $P < 0.0001$) (Figure 1G), free cholesterol (−45%, $P = 0.0032$) (Figure 1H), and lysophosphatidylcholine (LPC) (−39%, $P < 0.0001$) (Figure 1K). Moreover, AD-HDL-associated low abundant lipid species including phosphatidylethanolamine (−12%, $P = 0.0093$) (Figure 1M) and phosphatidylinositol differed (+36%, $P = 0.0196$) (Figure 1N), whereas contents of phosphatidylcholine, triglycerides, and sphingomyelin were not altered (Figure 1I,J,L). A detailed analysis of all analyzed lipid subspecies can be found in the Data S1 (Figure S1). Interestingly, this profoundly altered lipid composition of AD-HDL is not seen in other disease states.³

Eosinophils are end-stage effector cells inducing tissue damage in the inflammatory infiltrate within the dermis of AD patients.⁵ Isolated human eosinophils are suitable to conduct reproducible functional assays to test effector responses to HDL from AD patients and controls. We had to re-isolate HDL from serum of AD patients and controls, given that storage damages HDL structure within 2 weeks.⁶ Sufficient serum to re-isolate HDL was available from 16 patients and 8 controls. We pretreated eosinophils with HDL

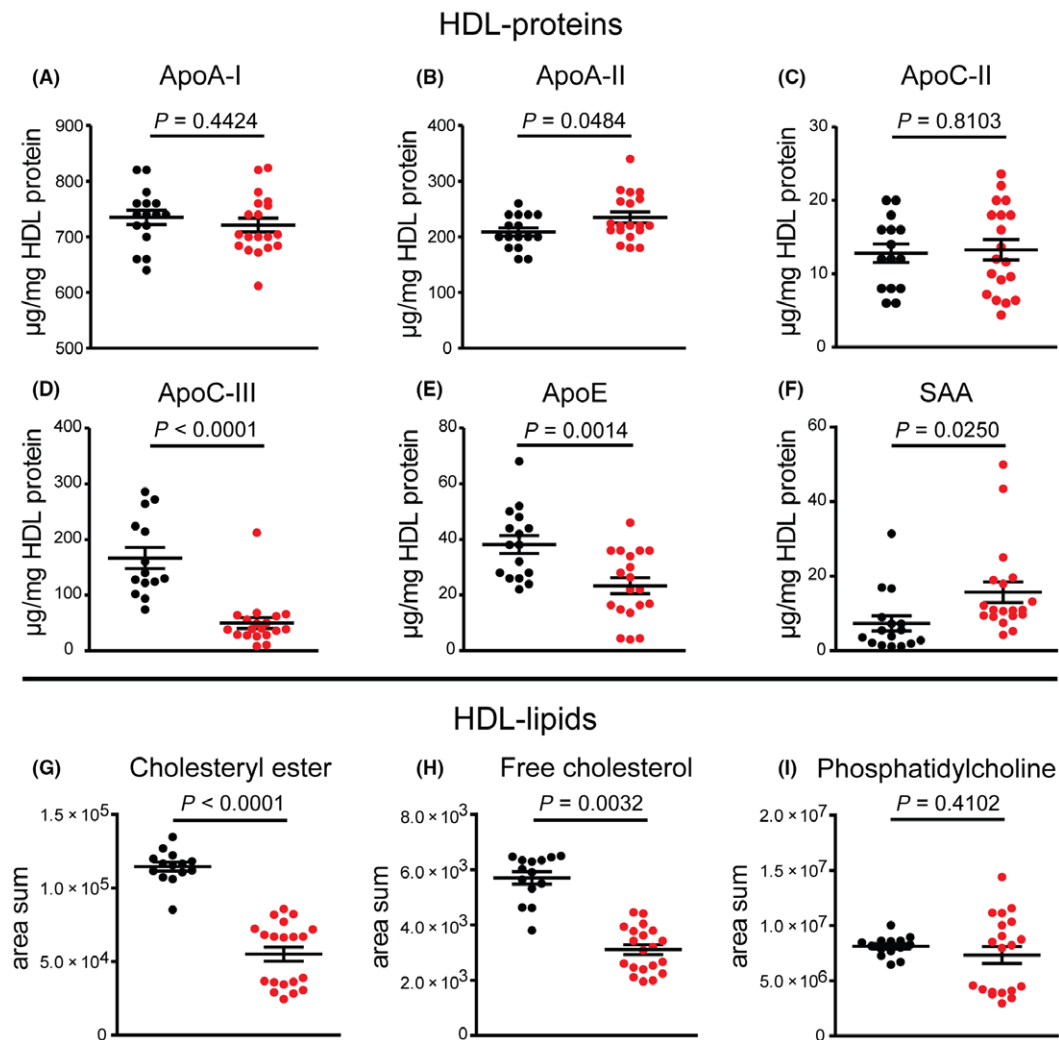


FIGURE 1 Protein and lipid pattern of high-density lipoprotein (HDL). HDL was isolated from healthy subjects (control, $n = 19$) and patients with atopic dermatitis ($n = 20$) by ultracentrifugation and analyzed for its apolipoprotein (apo) A-I (A), apoA-II (B), apoC-II (C), apoC-III (D), and apoE (E) content by immunoturbidimetry. Serum amyloid A (SAA) content was measured using a commercially available ELISA (F). HDL contents of cholesteryl ester (G), free cholesterol (H), phosphatidylcholine (I), and triglycerides (J) were measured by mass spectrometry and are expressed as area sum. Relative amounts of lysophosphatidylcholine (K), sphingomyelin (L), phosphatidylethanolamine (M), and phosphatidylinositol (N) are expressed as percent to the total amount of PC. Levels of significance were calculated using Student's *t* test. Bars indicate mean and standard error of mean of the different test groups [Colour figure can be viewed at wileyonlinelibrary.com]

preparations, followed by stimulation with eotaxin-2/CCL24 and monitored morphological changes (by flow cytometry) or performed chemotaxis assays using transwell plates. Strikingly, in contrast to HDL of controls, the majority of AD-HDL samples promoted agonist induced chemotaxis (Figure 2A-C) and did not suppress agonist induced shape change (Figure 2G-I). A representative dot plot of eosinophil shape change is shown in Figure S2. An important HDL-associated anti-inflammatory enzyme is paraoxonase.⁷ Paraoxonase activity of most AD-HDL samples was decreased when compared to control HDL (Figure 2N). Interestingly, we observed no change in the capacity of AD-HDL to mobilize cholesterol from cells when compared to control HDL (Figure 2O).

Prompted by the profound alterations in AD-HDL composition and function, we performed a detailed correlation analysis to

determine which proteins and lipids in AD-HDL are associated with a loss of function. We observed multiple and complex associations (Table S2). Interestingly, the HDL-triglyceride content correlated with the inhibitory activity of HDL toward agonist-induced eosinophil shape change ($P = 0.039$) and migration ($P = 0.008$). HDL-associated phospholipids and cholesteryl ester showed similar but weaker associations. SAA, an HDL-associated pro-inflammatory acute-phase protein,³ correlated inversely with the ability to suppress agonist-induced shape change of eosinophils ($P = 0.001$). However, agonist-induced migration of eosinophils was not associated with SAA, suggesting that shape change and migration are affected by different components carried by HDL particles.

Other altered apolipoprotein contents in AD-HDL could play a role in allergic diseases, given that apoE is an endogenous negative regulator

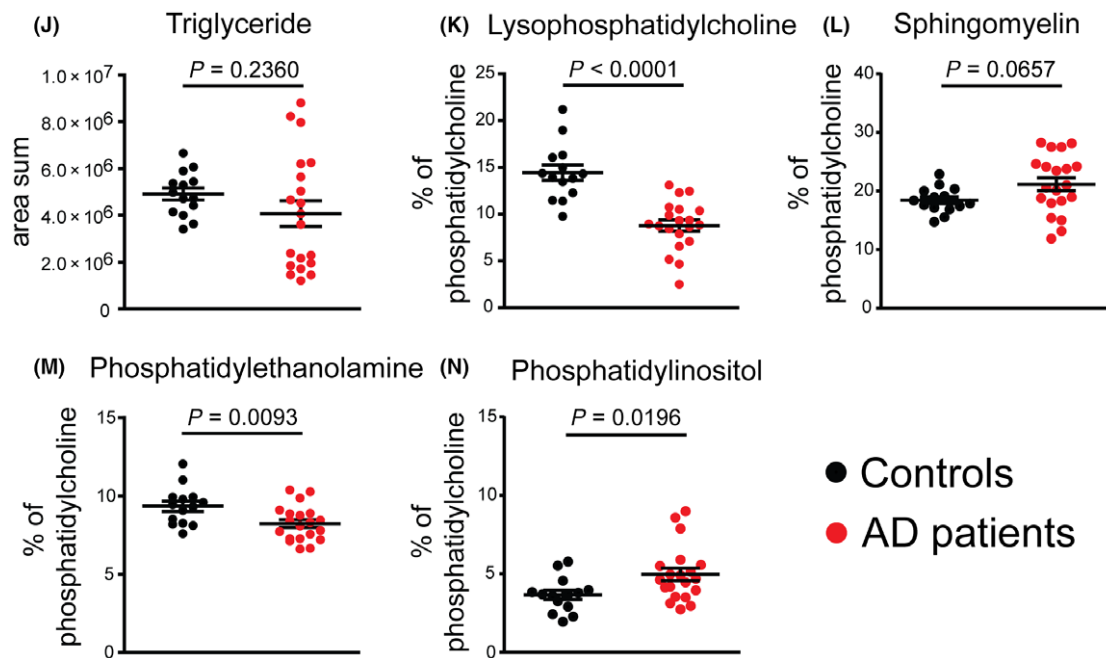


FIGURE 1 (Continued)

of house dust mite-induced asthma in mice⁸ and apoC-III affects catabolism of HDL.³ In line with that notion, we observed a strong correlation between the apoC-III content of HDL and the HDL-cholesterol content (HDL-apoC-III vs HDL-cholesterol: $r = 0.541$, $P = 0.003$; HDL-apoC-III vs HDL-free cholesterol: $r = 0.612$, $P < 0.001$).

Moreover, AD-HDL showed reduced levels of LPC (Figure 1K), especially LPC 16:0 (Figure S1, Data S1). We could recently demonstrate that HDL enriched with LPC 16:0 potently suppresses neutrophil effector responses.⁹ In agreement with our previous result, we observed that LPC 16:0 and LPC 18:0 dose-dependently suppressed eosinophil shape change and migration (Figure 2D,E,J,K), while LPC 18:1 was able to reduce eosinophil shape change but had no inhibitory effect on eosinophil migration (Figure 2F,L). ApoC-III levels were markedly decreased in AD-HDL (Fig. 1D). Importantly, addition of recombinant apoC-III reduced shape change of eosinophils (Fig. 2M), suggesting that the low contents of LPC and apoC-III in AD-HDL contribute to functional impairment. Further studies are needed to test whether other HDL-associated proteins and lipids also affect eosinophil function.

Of note, we did not observe an impact of current treatment on HDL composition (Table S3) in our study.

We acknowledge limitations to this study. Due to the laborious experiments and analyses, we had to keep the patient number rather small. Therefore, additional studies with larger cohorts are required to confirm our results.

In conclusion, we show that AD is associated with profound alterations in HDL composition linked to the formation of dysfunctional HDL. Our results suggest a novel link between HDL dysfunction and allergy and may lead to new diagnostic and therapeutic approaches.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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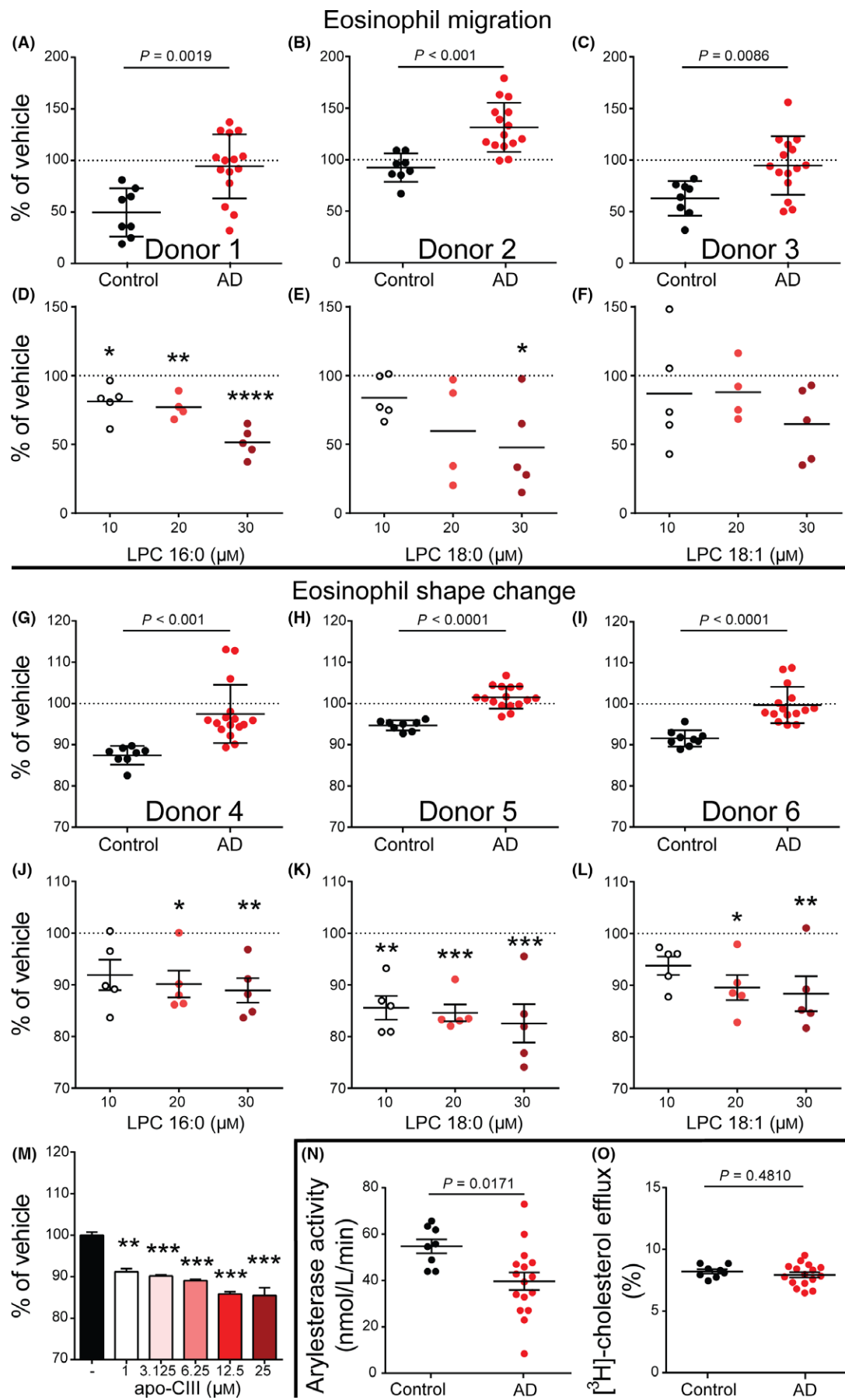


FIGURE 2 Metrics of high-density lipoprotein (HDL) function. Isolated eosinophils of healthy, non-atopic volunteers were pretreated with HDL (50 µg/mL) of control subjects (n = 8) and atopic dermatitis (AD) patients (n = 16) (A-C) or indicated concentrations of lysophosphatidylcholine (LPC) 16:0 (D), LPC 18:0 (E) or LPC 18:1 (F). Samples were placed into transwell plate inserts with 5 µm pore size and allowed to migrate toward eotaxin-2/CCL24 (30 nM) into the bottom well for 1 h at 37°C. Bars indicate mean and SEM of duplicate measurements of the different test groups (A-C). Data are shown as mean and SEM from 5 individual donors (D-F). Isolated eosinophils of healthy, non-atopic volunteers were pretreated with HDL (50 µg/mL) of control subjects (n = 8) or AD patients (n = 16) (G-I), indicated concentrations of LPC 16:0 (J), LPC 18:0 (K), LPC 18:1 (L) or indicated concentrations of apolipoprotein (apo)-CIII (M) for 30 min and subsequently stimulated with eotaxin-2/CCL24 (10 nM) for 4 mins at 37°C. Shape change was analyzed by flow cytometry as increase of forward scatter. Bars indicate mean and SEM of duplicate measurements of the different test groups (G-I). Data are shown as mean and SEM of 5 individual donors (J-L). Values shown as mean and SEM are representative of two independent experiments measured in duplicates (M). Responses were expressed as percent of vehicle (eotaxin-2/CCL24 in the absence of HDL, apoC-III or LPC) treated cells (A-M). Arylesterase activity of HDL-associated paraoxonase 1 of control subjects (n = 8) and AD patients (n = 16) was measured using phenylacetate as substrate (N). [³H]-cholesterol-labeled J774 macrophages were incubated with HDL (50 µg/mL) of control subjects (n = 8) and AD patients (n = 16) for 4 h (O). Cholesterol efflux is expressed as radioactivity in the supernatant relative to total radioactivity (in supernatant and cells). Values shown represent means of two individual experiments measured in duplicates (N, O). Bars indicate mean and SEM of the different test groups. Levels of significance were calculated using Student's t-test or one-way ANOVA and Dunnett post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 [Colour figure can be viewed at wileyonlinelibrary.com]

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Milk and egg intervention during pregnancy and allergic disease in offspring up to 30 years of age

To the Editor,

The role of maternal ingestion of allergens during pregnancy for allergy development in the child is debated. In the 1980s, it was

hypothesized that avoidance of potent food allergens during pregnancy might prevent allergy-related disease in the child. In 2012, a Cochrane review concluded that there was no evidence in favor of

Methods and extended results

Ethical approvals

All procedures involving human subjects were approved by the Institutional Review Board of the Medical University of Graz and carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all subjects.

Study subjects

The clinical and laboratory characteristics of 20 study subjects with AD and 19 age- and sex-matched controls are given in Table E1. At study enrollment with consecutive recruitment (between March and June 2013), the majority of patients ($n = 16$) had long-lasting and widespread AD with affection of more than 10% of body surface area, high SCORAD values and very high total (> 1000 IU/ml) immunoglobulin E levels ($n=13$). Serum samples were stored at -70°C until analysis. The primary treatment modalities of the patients with AD included UVB-311nm phototherapy ($n=5$), antihistamines ($n=2$), oral steroids ($n=1$) and cyclosporin ($n=3$). Additional topical treatment with steroids ($n=7$), tacrolimus ($n=2$) and pimecrolimus ($n=1$) was given. The patients had decreased total and LDL-cholesterol levels, being in line with previous studies in German adults and Finnish children ^(1, 2). There was no relation of levels of total cholesterol, LDL-cholesterol and triglycerides to current treatment and none of the patients was taking lipid-lowering medication, such as statins.

Determination of serum lipid composition

Levels of total cholesterol and triglycerides (Diasys, Holzheim, Germany) were measured enzymatically. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation using HDL cholesterol values measured in the supernatant of the phosphotungstic acid/ MgCl_2 precipitation.

Isolation of HDL

Serum density was adjusted with potassium bromide (Sigma, Vienna, Austria) to 1.24 g/ml, and a two-step density gradient was generated in centrifuge tubes (16×76 mm, Beckman) by layering the density-adjusted serum underneath a KBr density solution (1.063 g/ml) as described ⁽³⁾. Tubes were sealed and centrifuged at $65,000$ rpm for 6 h in a 90Ti fixed angle rotor (Beckman Instruments, Krefeld, Germany). After centrifugation, the HDL-containing band was collected, desalted via PD10 columns (GE Healthcare, Vienna, Austria) and immediately used for experiments or stored at -70°C in the presence of 10% glycerol as described ⁽⁴⁾.

Biochemical quantification of HDL-associated proteins

ApoA-I, apoA-II, apoC-II, apoC-III and apoE (Greiner, Fracht, Germany) were determined by immunoturbidimetry as described ⁽⁵⁾. All lipoprotein analyses were performed on an Olympus AU640 analyzer (Olympus Diagnostika, Hamburg, Germany).

SAA was determined by using an enzyme-linked immunosorbent assays (Life Technologies, Vienna, Austria) as described ⁽⁶⁾.

Mass spectrometry analysis of HDL lipid composition

Total lipids of HDL preparations (300 μg protein) were extracted twice according to Folch et al. ⁽⁷⁾ using chloroform/methanol/water ($2/1/0.6$, v/v/v) containing 500 pmol butylated hydroxytoluene, 1% acetic acid, and 100 pmol of internal standards ($17:0$ - $17:0$ phosphatidylcholine, $17:0$ - $17:0$ - $17:0$ triglyceride, $17:0$ lysophosphatidylcholine (LPC), Avanti Polar Lipids, Alabama, AL) per sample. Extraction was performed under constant shaking for 60 min at

room temperature. After centrifugation at 1,000 x g for 15 min at room temperature the lower organic phase was collected. 2.5 mL chloroform were added to the remaining aqueous phase and the second extraction was performed as described above. Combined organic phases of the double-extraction were dried under a stream of nitrogen and resolved in 450 µl 2-propanol/chloroform/methanol (7/2/1, v/v/v) for UPLC-qTOF analysis. Chromatographic separation was performed as described⁽⁸⁾ with modifications, using an AQUITY-UPLC system (Waters Corporation), equipped with a HSS T3 column (2.1x100 mm, 1.8 µm; Waters Corporation). A SYNAPTTMG1 qTOF HD mass spectrometer (Waters Corporation) equipped with an ESI source was used for detection. Data acquisition was done by the MassLynx 4.1 software (Waters Corporation) and lipid classes were analyzed with the "Lipid Data Analyzer 1.6.2" software⁽⁹⁾. Data were normalized for recovery and extraction- and ionization efficacy using internal standards.

Preparation of human eosinophils

Blood was taken from healthy, non-atopic volunteers not taking any medication, according to a protocol approved by the Institutional Review Board of the Medical University of Graz.

Human peripheral blood eosinophils were isolated immediately from serum as described before⁽¹⁰⁾ and were used for experiments within 4 hours.

Shape change assay

Isolated eosinophils (25,000 maintained in PBS with Ca²⁺ and Mg²⁺, HEPES 10 mmol/L, glucose 10 mmol/L and bovine serum albumin 0.1%, pH 7.4) were pretreated with HDL (50 µg/mL) for 30 minutes and subsequently stimulated with eotaxin-2/CCL24 (10 nM) (Immunotools, Friesoythe, Germany) for 4 minutes at 37°C. Afterwards, samples were fixed with BD CellFIX solution (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by flow cytometry. Shape change was estimated as increase of forward scatter⁽¹¹⁾.

Chemotaxis assay

Isolated eosinophils (50,000 maintained in PBS with Ca²⁺ and Mg²⁺, HEPES 10 mmol/L, glucose 10 mmol/L and bovine serum albumin 0.1%, pH 7.4) were pretreated with HDL (50 µg/mL) for 30 minutes. Then, cells were placed into Transwell inserts with 5 µm pore size filters (Corning, Acton, MA, USA). Cells were allowed to migrate toward eotaxin-2/CCL24 (30 nM) (Immunotools, Friesoythe, Germany) into the bottom well for 1 hour at 37°C. Afterwards, migrated cells were fixed with BD CellFIX solution (BD Biosciences, Franklin Lakes, NJ, USA) and counted by flow cytometry.

Arylesterase activity assay

Ca²⁺-dependent arylesterase activity was determined with a photometric assay using phenylacetate as substrate as described⁽¹²⁾.

HDL cholesterol efflux capability

Cholesterol efflux capacity was assessed using a validated assay⁽⁴⁾.

Statistical analysis

Differences between two groups were analyzed with the Student's t-test (unpaired, two-tailed). Comparison of normal distributed groups was performed with One-Way ANOVA and Bonferroni post-hoc test. Correlations were determined using the Pearson product-moment estimates. Group differences were considered statistically significant for **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and **** *p* < 0.0001. Statistical analyses were performed using GraphPad Prism (Version 4.0, GraphPad Software) or SPSS Statistics Version 22.

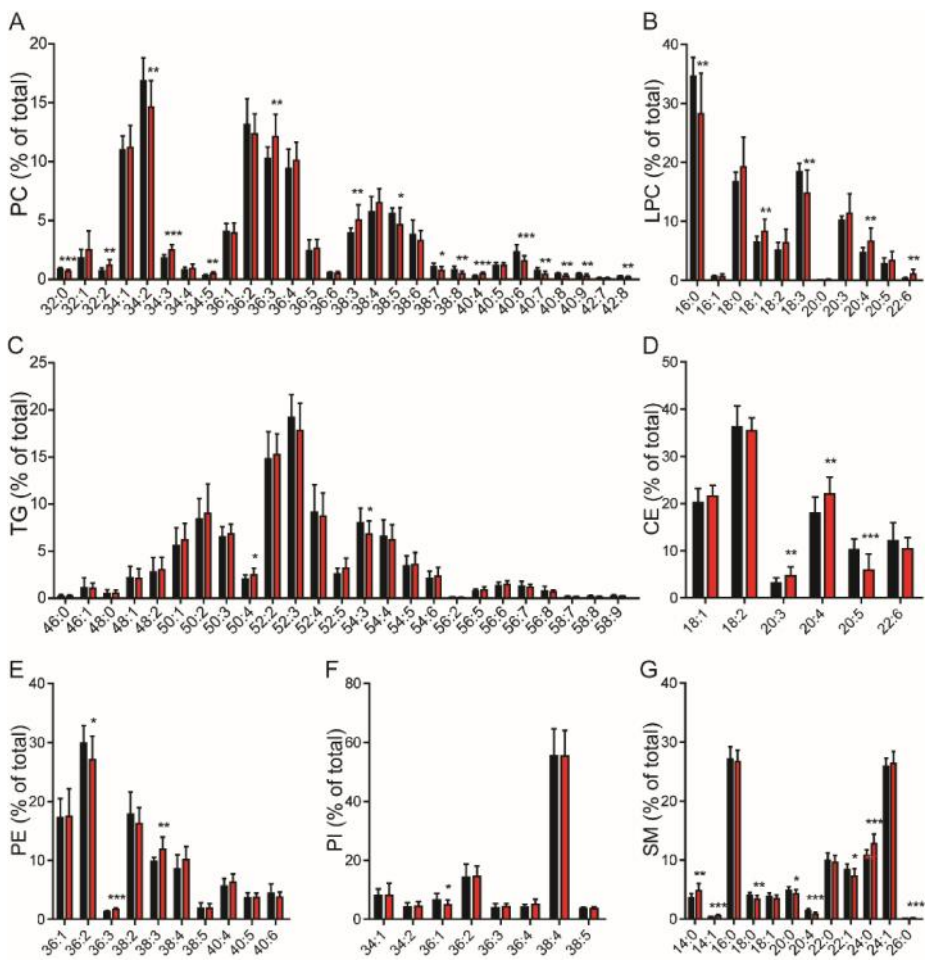


Figure E1: Detailed analysis of lipid species of HDL of healthy controls (black bars) and patients with atopic dermatitis (red bars). Analysis showing the prevalence of chain length variants for phosphatidylcholine (PC), triglycerides (TG), lysophosphatidylcholine (LPC), cholesteryl ester (CE), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and sphingomyelin (SM) (A – G). **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

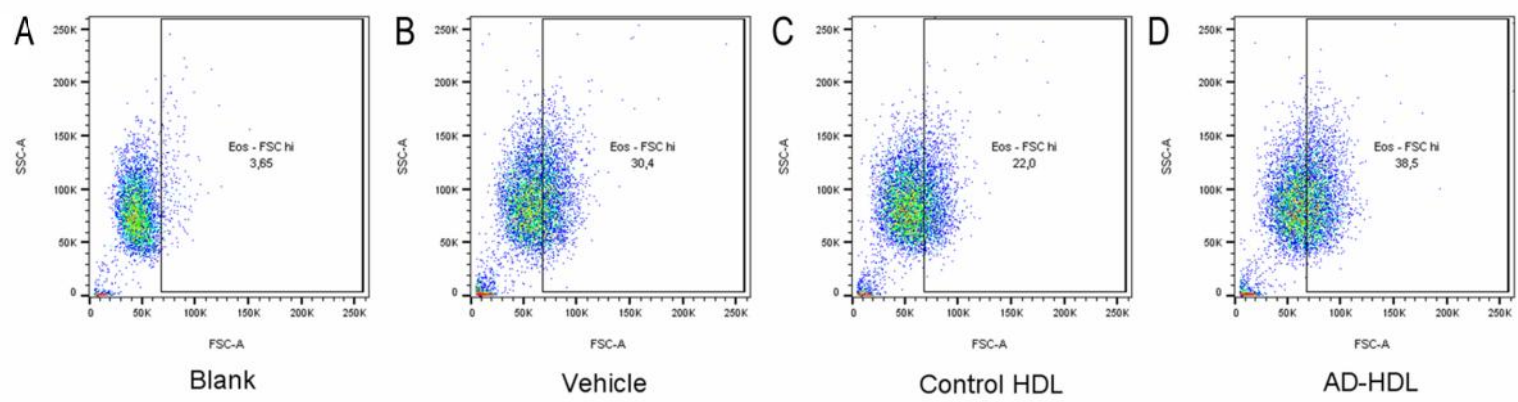


Figure E2: Shape change of isolated eosinophils from a healthy, non-atopic volunteer. Forward scatter (FSC) versus sideward scatter (SSC) dot plots of (A) unstimulated eosinophils or (B-D) eosinophils stimulated with eotaxin-2/CCL24. (C) Eosinophils pretreated with control HDL. (D) Eosinophils pretreated with HDL of patients with atopic dermatitis (AD-HDL). Representative values are shown.

Tables

Table E1. Clinical characteristics of the study subjects

	Controls	Atopic dermatitis	<i>p</i>
n	19	20	
Age (y)	31 (26-38)	33 (19-50)	0.2598
Male/female	7 / 12	7 / 13	0.9080
Total cholesterol (mg/dL)	174 (151-199)	144 (137-179)	0.0087
Triglycerides (mg/dL)	78 (56-123)	61 (40-95)	0.0876
HDL-cholesterol (mg/dL)	51 (44-70)	55 (45-74)	0.4279
LDL-cholesterol (mg/dL)	107 (78-121)	73 (62-109)	0.0099
Creatinin (mg/dL)	n.d.	1.4 (1-7)	-
SCORAD	n.d.	48 (39-61)	-
CRP (mg/L)	n.d.	1.3 (0.6-6.9)	-
IgE (U/mL)	n.d.	3453 (198-5000)	-

Values are given as medians with the interquartile range. n.d.: not determined; SCORAD, scoring atopic dermatitis; CRP, C-reactive protein; IgE, immunoglobulin E.

Table E2. Correlation of HDL composition with HDL function of AD patients (n=16)

	Shape change (mean value)		Chemotaxis (mean value)		Paraoxonase activity (mean value)	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
HDL-ApoA-I (μg/mg protein)	-0.048	0.860	-0.291	0.292	0.222	0.408
HDL-ApoA-II (μg/mg protein)	-0.128	0.637	-0.282	0.308	-0.400	0.125
HDL-ApoC-II (μg/mg protein)	0.374	0.154	0.331	0.229	0.013	0.963
HDL-ApoC-III (μg/mg protein)	0.454	0.077	-0.050	0.858	-0.231	0.389
HDL-ApoE (μg/mg protein)	0.458	0.074	0.289	0.296	0.210	0.435
HDL-SAA (μg/mg protein)	0.731	0.001 [#]	0.238	0.394	0.113	0.677
HDL-PC (area sum)	-0.509	0.044 [#]	-0.584	0.022 [#]	-0.046	0.865
HDL-TG (area sum)	-0.521	0.039 [#]	-0.654	0.008 [#]	-0.174	0.520
HDL-CE (area sum)	-0.545	0.029 [#]	-0.414	0.125	-0.201	0.456
HDL-FC (area sum)	-0.457	0.075	-0.450	0.092	0.019	0.943
HDL-LPC (% of PC)	0.095	0.728	-0.066	0.814	-0.003	0.992
HDL-SM (% of PC)	-0.601	0.014 [#]	-0.400	0.140	-0.036	0.895
HDL-PE (% of PC)	-0.282	0.290	0.079	0.779	0.409	0.116
HDL-PI (% of PC)	-0.267	0.318	-0.094	0.740	0.084	0.757

Shape change and chemotaxis of eosinophils were each analyzed in 3 individual donors measured in duplicates. Paraoxonase was measured in two individual experiments measured in duplicates. Apo, apolipoprotein; SAA, serum amyloid A; PC, phosphatidylcholine; TG, triglyceride; CE, cholesteryl ester; FC, free cholesterol; LPC, lysophosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PI, phosphatidylinositol. [#] significant correlation.

Table E3. Impact of treatment on HDL composition

	Current treatment		<i>p</i>
	no (n=13)	yes (n=7)	
HDL-SAA (μg/mg protein)	11.5 (7.9-19.5)	10.8 (9.7-17.9)	0.56
HDL-PC (area sum)	8.2×10 ⁶ (4.1×10 ⁶ -1.1×10 ⁷)	8.0×10 ⁶ (3.9×10 ⁶ -9.0×10 ⁶)	0.79
HDL-TG (area sum)	4.1×10 ⁶ (1.8×10 ⁶ -5.6×10 ⁶)	3.6×10 ⁶ (2.0×10 ⁶ -6.3×10 ⁶)	0.85
HDL-CE (area sum)	6.7×10 ⁴ (3.3×10 ⁴ -7.2×10 ⁴)	6.7×10 ⁴ (3.5×10 ⁴ -7.7×10 ⁴)	0.90
HDL-SM (% of PC)	21.0 (18.1-24.4)	20.1 (15.4-28.1)	0.91

Values are given as medians with the interquartile range. SAA, serum amyloid A; PC, phosphatidylcholine; TG, triglyceride; CE, cholesteryl ester; SM, sphingomyelin. The primary treatment modalities of patients with atopic dermatitis included UVB 311nm phototherapy (n=2), oral cyclosporine (n=1) and topical steroids (n=4). One patient was additionally treated with topical tacrolimus.

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