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Altered lipidome and antioxidative activity of small, dense HDL in normolipidemic rheumatoid arthritis: Relevance of inflammation

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ABSTRACT

Objective: High-density lipoprotein (HDL) particles exert potent antiatherogenic activities, including antioxidative actions, which are relevant to attenuation of atherosclerosis progression. Such activities are enriched in small, dense HDL and can be compromised under conditions of chronic inflammation like rheumatoid arthritis (RA). However, structure–function relationships of HDL largely remain indeterminate. **Methods**: The relationships between HDL structure and function were evaluated in normolipidemic patients with active RA (DAS28 > 3.2; $n = 12$) and in normolipidemic age-matched controls ($n = 10$). Small, dense HDL3b and 3c particles were isolated from plasma or serum by density gradient ultracentrifugation and their physicochemical characteristics, lipidome (by LC/MS/MS) and antioxidative function (as protection of normolipidemic LDL from free radical-induced oxidation) were evaluated. **Results**: As expected, active RA patients featured significantly elevated plasma levels of high-sensitivity C-reactive protein (hsCRP; $p < 0.001$) and serum amyloid A (SAA; $p < 0.01$) relative to controls. Antioxidative activity and weight % chemical composition of small, dense HDL did not differ between RA patients and controls ($p > 0.05$), whereas HDL phosphosphingolipidome was significantly altered in RA. Subgroup analyses revealed that RA patients featuring high levels of inflammation (hsCRP>10 mg/l) possessed small, dense HDL with reduced antioxidative activities ($p < 0.01$). Furthermore, antioxidative activity of HDL was inversely correlated with plasma hsCRP ($p < 0.01$). **Conclusions**: These data revealed that (i) despite normolipidemic state, the lipidome of small, dense HDL was altered in RA and (ii) high levels of inflammation can be responsible for the functional deficiency of small, dense HDL in RA.

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1. Introduction

Rheumatoid Arthritis (RA), a chronic inflammatory disease that affects up to 1% of the general population, is associated with increased mortality and reduced life expectancy, as a result of accelerated cardiovascular (CV) disease [1,2]. Strikingly, CV events occur approximately a decade earlier in RA patients, who are in turn twice more likely to present a myocardial infarction than healthy subjects [3–5]. Traditional CV risk factors, such as dyslipidemia, hypertension and obesity, do not account for the elevated CV risk in RA [6]. Indeed, classic dyslipidemia, consisting of increased low-density lipoprotein (LDL) levels and subnormal concentrations of anti-atherogenic high-density lipoprotein (HDL), is scarcely observed in RA [7,8].

As a corollary, qualitative (functional), rather than quantitative, lipoprotein alterations have been proposed to contribute to the increased CV risk in RA [7,9]. In this context, atheroprotective activities exerted by HDL may constitute a primary target in this pathology. HDL particles exhibit marked compositional, structural and functional heterogeneity in healthy normolipidemic subjects [10]. Among HDLs, the small, dense HDL3c subpopulation features a distinct proteome [11] and lipidome [12], which confer potent antiatherogenic properties to this subfraction [10].

Antiatherogenic functions of HDL primarily include capacity to mediate cellular cholesterol efflux, protection of LDL against oxidative stress, anti-inflammatory actions on arterial wall cells and vasculoprotective properties [13]. Such biological functions directly rely on the composition and structure of HDL. However, the impacts of structural particularities of each HDL subpopulation on their cardioprotective actions remain largely indeterminate. Although proteins are typically considered to be the major functional components of HDL, lipids may play key roles in anti-atherogenic HDL functionality. Using a lipidomic LC/MS/MS approach, we have recently shown that several components of the HDL phosphosphingolipidome are associated with metrics of HDL function in healthy normolipidemic subjects [14].

To provide insights into structure–function relationships of HDL in a chronic inflammatory condition such as RA, we performed a phosphosphingolipidomic analysis of small, dense HDL3c particles and evaluated their antioxidative activity in normolipidemic active RA patients and in control subjects. Our data reveal that RA HDL3c displayed an altered lipidome and that its antioxidative activity was reduced according to the degree of inflammation.

2. Materials and methods

2.1. Subjects

Twelve post-menopausal female patients with clinical manifestations of RA were consecutively recruited at the Hospital Italiano de Buenos Aires and at the Hospital de Clínicas José de San Martín, University of Buenos Aires (Buenos Aires, Argentina), from April 2009 to January 2010. All patients met the 1987 revised RA criteria of the American College of Rheumatology [15]. RA patients who presented a low disease activity (inactive RA) measured by a disease activity score using 28 joint count (DAS28) <3.2 were excluded from the study, resulting in the values of DAS28 of 4.0 ± 1.1 and erythrosedimentation rate/1st hour of 34.7 ± 13.4.

Additional exclusion criteria were dyslipidemia (plasma triglyceride (TG) >1.7 mmol/l and HDL-cholesterol (HDL-C) <1.29 mmol/l), diagnosis of diabetes (according to the World Health Organization criteria) [16], hypo- or hyperthyroidism, renal or hepatic alterations, presence of infectious diseases, acute myocardial infarction or stroke during the last six months, and use of antioxidant vitamins or lipid-lowering drugs during the last

month. Dyslipidemia was selected as an exclusion criterion in order to distinguish the contribution of RA from that of dyslipidemia to the HDL composition and function. Indeed, atherogenic dyslipidemias of Metabolic Syndrome, Type 2 diabetes and normocholesterolemic low HDL-C phenotype, all feature altered HDL composition and function [17–19]. Special care was taken to avoid including subjects with secondary causes of dyslipidemia such as excessive tobacco smoking (>10 cigarettes/day) or ethanol consumption (>30 g/day).

All patients were receiving disease-modifying antirheumatic drugs (DMARDs): 11 (92%) patients were receiving metothrexate (MTX; eight as monotherapy and three in combination with other traditional DMARDs, notably two with hydroxychloroquine and one with sulfazalacine). One patient was receiving leflunamide as monotherapy. Three patients (24%) were receiving tumour necrosis factor (TNF) inhibitors (two in combination with MTX and one as monotherapy). For further analyses, RA patients were divided into two subgroups according to their inflammatory status. Thus, patients were classified as low-grade inflammation (high-sensitivity C-reactive protein (hsCRP) <10 mg/l [LI-RA subgroup]) and high-grade inflammation (hsCRP >10 mg/l [HI-RA subgroup]). The selection of this cut-off was based on the following considerations. Given the lack of prognostic information on CV risk for patients displaying high levels of hsCRP, a cut-off of hsCRP >10 mg/l was established in the Women's Health Study to identify patients at a very high risk of CV disease [20]. This value is highly relevant in RA patients whose serum levels of hsCRP are usually far above 3 mg/l. In addition, the usefulness of the hsCRP >10 mg/l cut-off was confirmed in other studies evaluating CV risk specifically in RA patients. Thus, the CARRE, a cohort study, reported that associations between lipid measures and CV disease were more evident in RA patients featuring hsCRP levels beyond 10 mg/l [21]. Moreover, Graf et al. [22], also using this cut-off point determined that even among RA patients whose disease was judged to be controlled by joint counts or standardized disease scores, a substantial proportion displayed hsCRP levels that were associated with very high risk for future CV events.

Ten female healthy non-smoking normolipidemic age-matched postmenopausal controls were recruited to form a normolipidemic control group. Written informed consent was obtained from all patients and control subjects and the project was approved by the Ethics Committees of the Hospital Italiano de Buenos Aires and the Hospital de Clinicas José de San Martín, University of Buenos Aires in accordance with local institutional guidelines conformed to the Declaration of Helsinki.

2.2. Blood samples

Venous blood samples were withdrawn using sterile evacuated tubes (Vacutainer) after a 12-h overnight fast. Serum and EDTA plasma were each mixed with sucrose (final concentration, 0.6%) as a cryoprotectant for lipoproteins [23], aliquoted and frozen; each aliquot was thawed only once directly before analyses. To evaluate paraoxonase (PON)1 activity and antioxidative activity of HDL, venous blood was collected in the absence of EDTA, since PON1 is strongly inhibited by EDTA.

2.3. Clinical and biological parameters

Plasma levels of total cholesterol (TC), TG, LDL Cholesterol (LDL-C) and HDL-C were measured using commercially available enzymatic kits. Plasma apolipoprotein (apo) A-I, apoA-II and apoB were quantitated by immunoturbidimetry (Diasys, France). hsCRP was measured by immunoassay and serum amyloid A (SAA) and glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD)

by ELISAs (KHA0011, Invitrogen, USA and Cusabio, Wuhan, China, respectively).

2.4. Activities of lipoprotein-associated enzymes

2.4.1. Paraoxonase (PON1)

PON1 activity was measured in serum samples following the method of Furlong et al. [24]. Measurements were all carried out within the same assay. Within-run precision was 4.6%.

2.4.2. Lipoprotein-associated phospholipase A₂ (Lp-PLA₂)

Lp-PLA$_2$ activity was measured following the radiometric assay described by Blank et al. [25]. Measurements were all carried out within the same assay. Within-run precision for Lp-LPA$_2$ activity was 5.1%.

2.5. Isolation of lipoproteins

Lipoproteins were isolated from serum and plasma by single step, isopycnic non-denaturing density gradient ultracentrifugation in a Beckman SW41 Ti rotor at 40,000 rpm for 44 h in a Beckman XL70 ultracentrifuge at 15 °C by a slight modification of the method of Chapman et al. [26] as previously described [27]. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards into 11 fractions corresponding to very low-density lipoprotein (VLDL) + intermediate-density lipoprotein (IDL) (d < 1.019 g/ml), 5 subfractions of LDL (1.019−1.063 g/ml) and 5 subfractions of HDL (HDL2b, d 1.063−1.091 g/ml; HDL2a, d 1.091−1.110 g/ml; HDL3a, d 1.110−1.133 g/ml; HDL3b, d 1.133−1.156 g/ml and HDL3c, d 1.156−1.179 g/ml). This procedure facilitates fractionation of HDL particle subspecies in a non-oxidised, native state [26,28]. Lipoproteins were extensively dialysed against phosphate-buffered saline (PBS; pH 7.4) at 4 °C in the dark, stored at 4 °C and used within 10 days.

2.6. Total chemical composition of lipoproteins

Total protein (TP), TC, free cholesterol (FC), phospholipid (PL) and TG contents of isolated lipoprotein subfractions were determined using commercially available assays. cholesteryl ester (CE) was calculated by multiplying the difference between TC and FC concentrations by 1.67 [26]. Total lipoprotein mass was calculated as the sum of TP, CE, FC, PL and TG. ApoA-I and apoA-II content in HDL were quantitated by immunoturbidimetry (Diasys, France).

2.7. Antioxidative activity of HDL particles

Antioxidative activity of serum-derived HDL3b and 3c subfractions (final concentration of each, 10 mg total mass/dl), and of total HDL (final concentration, 40 mg total mass/dl), was assessed towards reference LDL (10 mg TC/dl) obtained from EDTA plasma from one healthy normolipidemic control subject. Before use, EDTA was removed from LDL solution by exhaustive dialysis for 24 h at 4 °C [17,18]. Total HDL from each donor was prepared by mixing all five HDL subfractions at their equivalent serum concentrations. LDL oxidation was performed at 37 °C in PBS (pH 7.4) in the presence of a water-soluble azo-initiator 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH; 1 mM). HDL subfractions were added to LDL directly before oxidation. Serum was used as a source of HDL for this assay to ensure authentic PON1 activity, which is inhibited by EDTA [29]. Accumulation of conjugated dienes was measured as the increment in absorbance at 234 nm [17,18,30]. Absorbance kinetics were corrected for the absorbance of AAPH itself assayed in parallel as a blank. The kinetics of diene accumulation revealed two characteristic phases, the initiation (lag) and propagation phases. For each curve, the duration of each phase, average oxidation rates within each phase and amount of dienes formed at the end of the propagation phase (maximal amount of dienes) were calculated [17,18,30].

2.8. HDL3c lipidomic analysis

Ten PL and sphingolipid (SL) subclasses, specifically phosphatidylcholine (PC), sphingomyelin (SM), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), PC-based plasmalogen (PC-pl), ceramide (Cer), phosphatidic acid (PA) and phosphatidylglycerol (PG), comprising together >160 individual lipid species were assayed by LC/MS/MS. The PL subclasses were divided into major (those whose content was >1% of total PL, i.e. PC, SM, LPC, PE and PI) and minor (those whose content was <1% of total PL, i.e. PS, PC-pl, Cer, PA and PG).

2.8.1. Extraction

The HDL3c subpopulation (30 μg of enzymatically-quantified PLs) was extracted with 4 ml of cold $CHCl_3$/acidified CH_3OH (5/2, v/v) containing 4 μg of PC d9, 400 ng of LPC 15:0, 100 ng of PI 25:0, 80 ng of PE 25:0, 80 ng of PA 25:0, 40 ng of PS 25:0, 20 ng of PG 25:0 and 20 ng of Cer 17:0. Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). A blank and a control samples were extracted in parallel with each batch to ensure quality control; each sample was corrected for blank readings. K_4EDTA (200 mM) solution was added (1:5 v/v) and the mixture was vortexed for 1 min and centrifuged at 3600 g for 10 min at 4 °C. The organic phase was transferred into 5 ml Chromacol glass tubes and dried under nitrogen. PLs were reconstituted into 150 μl isopropanol/hexane/water (10/5/2, v/v), transferred into LC/MS amber vials with inserts and dried under nitrogen. Lipids were reconstituted in 40 μl of isopropanol/hexane/water (10/5/2, v/v) and assayed by LC/MS/MS.

2.8.2. LC/MS analysis

Ten PL and SL subclasses were quantified by LC-ESI/MS/MS using a QTrap 4000 mass spectrometer (AB Sciex, Framingham, MA, USA). Lipid species were detected using multiple reaction monitoring reflecting the headgroup fragmentation of each lipid class. PC, LPC and SM species were detected as product ions of m/z 184, PE, PS, PG and PA as neutral losses of respectively m/z 141, 185, 189 and 115, PI molecular species as product ions of m/z −241. N_2 was used as a collision gas. PE, PS, PG, PI, PA and Cer species were monitored for 18 ms, and PC, LPC and SM species were monitored for 30 ms at a unit resolution (0.7 atomic mass unit at half peak height).

2.8.3. Quantification

Lipids were quantified using calibration curves specific for the nine individual lipid classes and up to 12 fatty acid moieties. Twenty-three calibration curves were generated in non-diluted and 10-fold diluted matrices to correct for matrix-induced ion suppression effects. More abundant lipid species which displayed non-linear response in non-diluted extracts were quantified from a 10- or 100-fold diluted sample. An in-house developed Excel Macro was used to compile the data from the three injections.

Limits of detection (LOD) and limits of quantitation (LOQ) were assessed on at least 2 lipid species of the 9 lipid classes quantified and was 1.5 pg for ceramides, 0.5 pg for LPC, between 140 and 550 pg for PA, between 0.2 and 0.8 pg for PC, between 15 and 30 pg for PE, 3 pg for PG, 35 pg for PI, between 3 and 7 pg for PS, 0.7 pg for SM.

Repeatability of lipidomic analysis was tested on 9 quality controls extracted on three different days. The average coefficient of variations (%CV) for lipid species was 11.6% with 90% of total

species below 20% of CV. Lipid species with %CV greater than 20% were minor species with abundances very close to LOD.

2.9. HDL enrichment in PL subclasses

To enrich HDL in PC, PI, or PG, each of these lipids was incubated with normolipidemic plasma. Briefly, egg yolk PC (3 mg), bovine liver PI (0.5 mg) or egg yolk PG (50 μg) was placed as a chloroformic solution in an empty tube, chloroform evaporated and plasma (3 ml) added. The amounts of the lipids were chosen to reflect their relative content in HDL. The suspension was gently mixed for 1 min and incubated overnight at 4 °C under constant stirring. Plasma from the same subject was incubated in parallel in the absence of exogenous lipid as a control. Lipid-enriched and control HDL3c was isolated by density gradient ultracentrifugation and its anti-oxidative activity was measured as described above.

To enrich HDL in PA, total HDL fraction (1500 μg PL of a mixture of HDL2b, 2a, 3a, 3b and 3c subfractions at their equivalent plasma concentrations) was incubated with egg yolk PA overnight at 4 °C under constant stirring. PA (50 μg) was placed as a chloroformic solution in an empty tube, chloroform evaporated and PBS (500 μl) added. The amount of PA was chosen to reflect its content in RA HDL. The suspension was vigorously vortexed for 1 min and soni-cated for 20 min at a maximal power immediately before incuba-tion with HDL. HDL from the same subject incubated in parallel with PBS in the absence of exogenous lipid was used as a control. Subsequently, PA-enriched and control HDL3c was reisolated and antioxidative activity measured. Each enrichment experiment was repeated twice.

2.10. Statistical analysis

Power analysis using G*Power software (Dusseldorf University, Dusseldorf, Germany) was performed to estimate the sample size required to detect differences between groups in accordance with previous studies performed in patients with Metabolic Syndrome and Type 2 diabetes [18,31]. The main outcome was defined as impaired HDL3c antioxidative activity. The number of patients with a defined power of 0.80 and alpha error level of 0.05 was 10 sub-jects in each group. Power analysis was performed by a statistician taking into account mean values, standard deviations and between groups differences previously observed in antioxidative activity of HDL across several populations [17–19].

Distributions of all variables were analysed for normality using the Shapiro–Wilks test. Normally distributed variables are expressed as means ± standard deviation (SD); non-Gaussian distributed variables are expressed as median (interquartile range). Between-group differences in normally-distributed vari-ables were analysed using Student's *t*-test. For non-Gaussian distributed variables, the Mann–Whitney *U*-test was used, or they were log transformed to ensure normality before statistical analysis. Differences in dichotomous variables were analysed by Fisher's exact test. Spearman's correlation coefficients were calcu-lated to evaluate relationships between variables. Differences were considered significant at $p < 0.05$ using two-tailed statistics. The statistical software INFOSTAT (National University of Córdoba, Argentina) and SPSS 17.0 (Chicago, Ill, USA) were used.

3. Results

3.1. Clinical and biochemical parameters

As expected, the RA group featured significantly elevated plasma levels of hsCRP and SAA, two acute-phase proteins reflecting the inflammatory nature of this pathology (Table 1). SAA

has been extensively documented to displace apoA-I and apoA-II from circulating HDL. Consistent with this notion, in the present study, RA patients displayed reduced plasma levels of apoA-II (Table 1). Age, body mass index (BMI), and prevalence of hyper-tension, as well as lipoprotein profile were not significantly different between RA patients and control subjects (Table 1), thereby attesting to the normolipidemic condition of the RA group. In addition, activities of both lipoprotein-associated enzymes, PON1 and Lp-PLA2, which may be potentially involved in the inactivation of oxidised lipids, were similar in both groups (Supplement Fig. 1). Finally, plasma levels of GPI-PLD tended to be elevated in RA patients (+15%; $p = 0.10$) (Table 1).

3.2. Plasma levels and total chemical composition of HDL subfractions

Similar circulating concentrations and chemical composition (weight %) of HDL subfractions were observed in RA patients and controls. Indeed, contents of FC, CE, TG, PL, TP (Supplement Table 1), apoA-I and apoA-II (data not shown) in each HDL subfraction did not differ between RA patients and control subjects.

3.3. Antioxidative activity of HDL

Both small, dense HDL3b and 3c, and total HDL displayed similar antioxidative activities towards LDL oxidation in RA patients rela-tive to controls (Fig. 1). Nevertheless, correlation analyses evi-denced an association between the degree of inflammation and defective HDL antioxidative properties. Thus, plasma hsCRP levels were positively correlated with the LDL propagation rate measured in the presence of total HDL ($r = 0.64$, $p < 0.05$). SAA levels were positively correlated with the LDL propagation rate ($r = 0.60$, $p < 0.05$) and negatively with the duration of the propagation phase ($r = -0.66$, $p < 0.05$), in the presence of HDL 3b.

3.4. Influence of inflammation on HDL composition and antioxidative activity

In order to assess the potential impact of inflammation on compositional and functional features of HDL, RA patients were

Table 1
Clinical and biochemical characteristics of RA patients and control subjects.

	RA patients (*n* = 12)	Control subjects (*n* = 10)
Age (years)	62 ± 9	59 ± 9
Sex (M/F)	0/12	0/10
Hypertension (Y/N)	2/10	1/9
Body mass index (kg/m²)	27.4 ± 4.9	24.1 ± 2.9
TC (mmol/l)	5.2 ± 0.6	5.3 ± 0.4
TG (mmol/l)	1.1 (0.6–1.5)	1.0 (0.6–1.6)
LDL-C (mmol/l)	3.2 ± 0.5	3.2 ± 0.5
HDL-C (mmol/l)	1.5 ± 0.2	1.6 ± 0.3
VLDL-C (mmol/l)	0.5 ± 0.2	0.5 ± 0.2
Non-HDL-C (mmol/l)	4.0 ± 1.0	3.8 ± 0.5
ApoB (g/l)	0.89 ± 0.20	0.87 ± 0.14
ApoA-I (g/l)	1.46 ± 0.15	1.52 ± 0.16
ApoA-II (g/l)	0.31 ± 0.05*	0.36 ± 0.04
hsCRP (mg/l)	8.4 (0.2–28.3)**	0.9 (0.2–1.4)
SAA (mg/l)	128 (34–1312)***	25 (9–26)
GPI-PLD (mg/l)	42 (36–47)	36 (33–41)

RA, Rheumatoid Arthritis; TC, total cholesterol; TG, triglycerides; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; apo, apolipoprotein; hsCRP, high sensitive C-reactive protein; SAA, serum amyloid A; GPI-PLD: glycosylphosphatidylinositol-specific phospholipase D. Data are expressed as mean ± S.D. or median (interquartile range), depending on data distribution. *$p < 0.05$, **$p < 0.0005$, ***$p < 0.005$ *vs.* control subjects.
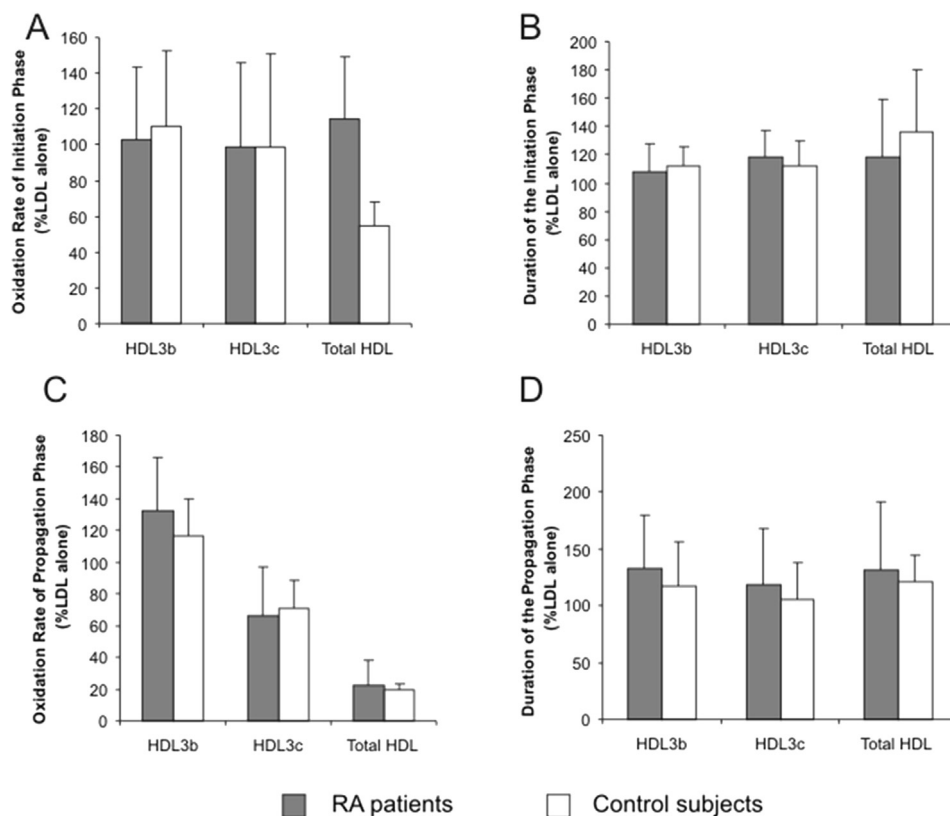
Fig. 1. Influence of small, dense HDL3b and HDL3c subpopulations (10 mg total mass/dl) and total HDL (40 mg total mass/dl) on AAPH-induced oxidation of reference LDL (LDL, 10 mg TC/dl; AAPH, 1 mM) in RA patients ($n = 12$) and control subjects ($n = 10$). Data are shown as % of values measured in reference LDL incubated without added HDL. Initiation phase (A), duration of the initiation phase (B), oxidation rate in the propagation phase (C) and duration of the propagation phase (D) of LDL oxidation are shown.

separated into two subgroups according to their inflammatory status. Specifically, patients were classified as those displaying low-grade inflammation (hsCRP <10 mg/l [LI-RA subgroup]) and high-grade inflammation (hsCRP >10 mg/l [HI-RA subgroup]). Importantly, no difference in clinical and biochemical parameters was observed between the two subgroups, except increases in hsCRP and SAA in the HI-RA group (Supplement Table 2). FC content was reduced in HI-RA HDL3b (1.9 ± 0.3 *vs.* $2.4 \pm 0.2\%$, $p = 0.009$, in HI-RA and LI-RA patients, respectively) and tended to be reduced in HI-RA HDL3c (1.5 ± 0.7 *vs.* 2.1 ± 0.4, $p = 0.09$, in HI-RA and LI-RA patients, respectively). Furthermore, the ratio of surface (FC, PL) to core (CE, TG) lipids was significantly reduced in HDL particles from the HI-RA compared to LI-RA group (HDL2b: 1.34 ± 0.15 *vs.* 1.57 ± 0.16, HDL3a: 1.32 ± 0.13 *vs.* 1.49 ± 0.14, and HDL3c: 1.26 ± 0.09 *vs.* 1.37 ± 0.04, respectively, all $p < 0.05$). Finally, the content of apoA-I tended to decrease in small, dense HDL3c in HI-RA *vs.* LI-RA patients (37 ± 2 *vs.* 46 ± 6 wt% respectively, $p = 0.06$). Interestingly, HDL3c apoA-I was inversely correlated with plasma levels of SAA ($r = -0.65$, $p = 0.05$).

It is noteworthy that LDL lipid peroxidation *in vitro* typically occurs in three consecutive phases, the initiation (lag), propagation and decomposition phases. Evaluation of the oxidation rates in these phases and their duration therefore allows characterizing both LDL protection by endogenous antioxidants (initiation phase) and oxidability of LDL lipids in the absence of antioxidants (propagation phase). Whereas LDL oxidation in the both phases is sensitive to the amount of oxidable lipid substrate, only oxidation in the initiation phase reflects the antioxidant content of the lipoprotein. In the presence of HDL, endogenous lipophilic antioxidants of the latter act in concert with endogenous lipophilic antioxidants of LDL to inhibit oxidation in the initiation phase. By contrast,

amount of oxidable lipid substrate present in HDL contributes to accelerate both the initiation and propagation phases of oxidation.

In the present work, we observed that HDL3c from HI-RA patients exhibited a significantly shorter initiation-phase of LDL oxidation as compared to LI-RA patients (Fig. 2B). Moreover, during the propagation phase of LDL oxidation, HDL3c particles from the HI-RA group were significantly less efficient in protecting LDL as evidenced by higher rate of conjugated diene formation in this phase and by shorter phase duration relative to LI-RA HDL3c (Fig. 2C and D). In addition, an impaired antioxidative activity was also observed in small, dense HDL3b and total HDL from HI-RA *vs.* LI-RA patients (Fig. 2B and C). Overall, we observed that small, dense HDL3c was the most altered HDL subfraction.

3.5. Lipidome of small dense HDL3c

Ten PL and SL subclasses (PC, LPC, SM, PE, PI, PS, PC-pl, Cer, PG and PA) were quantitatively assayed in small, dense HDL3c by LC/MS/MS and compared between RA patients and controls. Among major HDL PL (defined as those present at >1 wt% of total PL), RA patients and controls showed similar percent composition of PC, SM, LPC and PE (Fig. 3A). Conversely, PI was significantly decreased in HDL3c from RA patients. Furthermore, analysis of minor PL (defined as those present at ≤1 wt% of total PL) percent composition revealed a significant enrichment in PA and a depletion in PG contents in HDL3c from RA patients relative to controls (Fig. 3B). In accordance, elevated plasma concentrations of HDL3c-PA (0.10 (0.03–0.10) *vs.* 0.02 (0.01–0.04) mg/l, $p < 0.05$, for patients and controls respectively) were observed in RA patients. By contrast, no difference in the HDL3c content of PL and SL subclasses was found between HI-RA and LI-RA patients (Supplement Fig. 2). When HDL
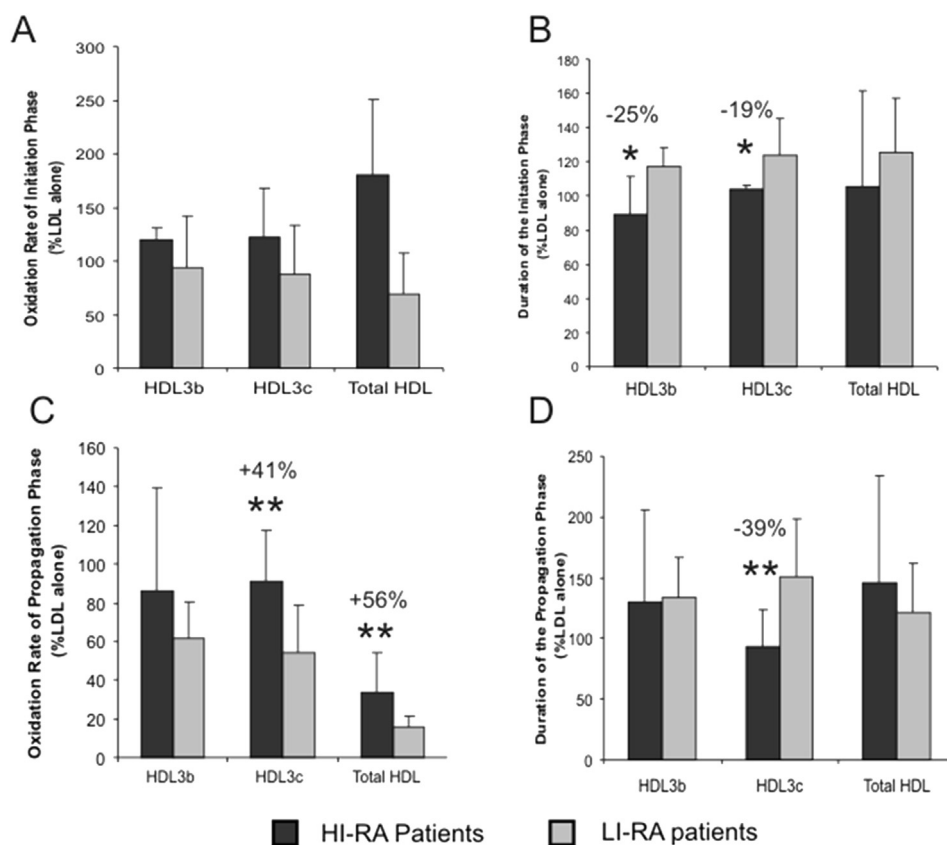
Fig. 2. Influence of small, dense HDL3b and HDL3c subpopulations (10 mg total mass/dl) and total HDL (40 mg total mass/dl) on AAPH-induced oxidation of reference LDL (LDL, 10 mg TC/dl; AAPH, 1 mM) in RA patients presenting with high-grade (hsCRP >10 mg/l [HI-RA subgroup]; $n = 4$) and low-grade (hsCRP <10 mg/l [LI-RA subgroup]; $n = 8$) inflammation. Data are shown as % of values measured in reference LDL incubated without added HDL. Initiation phase (A), duration of the initiation phase (B), oxidation rate in the propagation phase (C) and duration of the propagation phase (D) of LDL oxidation are shown.

lipidome was analysed in terms of its fatty acid composition, the only difference observed between the groups was that in ceramides containing monounsaturated fatty acids, which were significantly reduced in HDL3c from HI-RA vs. LI-RA patients ($0.013 \pm 0.003\%$ vs. 0.021 ± 0.005 of total PL + SL, $p = 0.045$ respectively). Finally, no difference in the HDL content of PC-pl was detected between the groups (data not shown).

Correlation analyses revealed that HDL3c-PC, the major carrier of polyunsaturated fatty acids (PUFAs) in the HDL surface, was correlated to oxidation metrics of the propagation phase (oxidation rate: $r = 0.56$, $p < 0.05$ and phase duration: $r = -0.60$, $p < 0.01$). In addition, HDL3c-PG content, which was reduced in RA patients, was inversely correlated with SAA ($r = -0.57$, $p < 0.05$) and hsCRP levels ($r = -0.56$, $p < 0.05$).

To directly evaluate the impact of HDL lipidomic alterations observed in RA patients on HDL functionality, HDL was in vitro enriched with PC, PI, PA and PG. Enrichment in both PC and PA significantly reduced antioxidative activity of small, dense HDL. Indeed, propagation rate of LDL oxidation measured in the presence of HDL3c was elevated by +115% (from 0.46 to 0.98 μM dienes/min; $n = 2$) and +18% (from 0.15 to 0.18 μM dienes/min; $n = 2$) by the enrichment in PC and PA, respectively, as compared to control HDL3c. By contrast, HDL3c enrichment in PI and PG was ineffective (data not shown).

4. Discussion

It has been well demonstrated that patients with RA present an increased atherosclerotic risk, which is not fully explained by traditional coronary risk factors [32]. Noteworthy, systemic inflammation appears to account for a large proportion of this unexplained risk. In this context, previous studies showed that chronic inflammation in RA patients alters lipoproteins structure and function, impairing HDL antiatherogenic properties [33]. Strikingly, guidelines on the reduction of cardiovascular risk in patients with RA are lacking, highlighting the need for a better understanding of the role of chronic inflammation in the development of CV disease [34].

The present study shows for the first time that normolipidemic patients with active RA display an altered HDL phosphosphingolipidome. Specifically, we observed that small, dense HDL3c particles from RA patients were enriched in PA, and depleted in PI and PG compared to sex and age matched controls.

PA is a negatively charged PL which may significantly impact on the net surface charge of HDL, thereby modulating charged-dependent interactions with lipases and lipid transfer proteins [14]. To evaluate whether enrichment in PA observed in RA HDL may impact HDL functionality, we measured the capacity of HDL to inhibit LDL oxidation following PA enrichment in vitro and observed that PA displayed deleterious effects on this activity. HDL enrichment in PA can be mediated by GPI-PLD, since this enzyme was reported to hydrolyse PC and PI to form PA [35]. GPI-PLD is secreted by hepatic and inflammatory cells and circulates at low concentration exclusively bound to HDL [35,36]. Importantly, GPI-PLD plasma levels were found to increase 20- to 30-fold in response to inflammation [37]. In our study however, plasma levels of GPI-PLD only tended to be elevated in RA patients (+15%; $p = 0.10$), potentially reflecting insufficient sample size. Altogether,
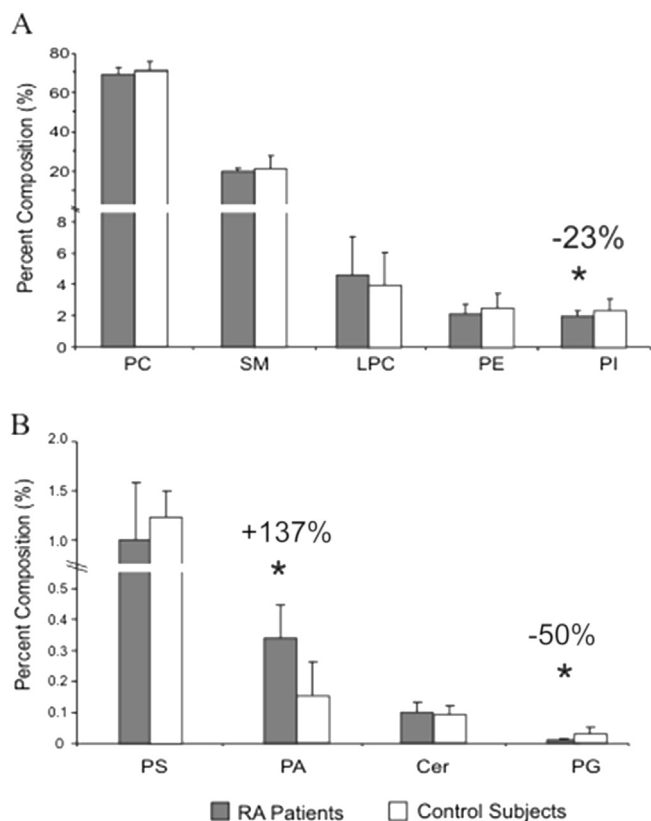
Fig. 3. Lipidome of small, dense HDL3c in RA patients ($n = 12$) and control subjects ($n = 10$). A, Major PL species: PC, SM, LPC, PE and PI; and B, minor PL species: PG, PA, PS, Cer. *$p < 0.01$.

these results suggest that GPI-PLD can play a role in HDL dysmetabolism in RA but other sources of PA accumulation are also plausible.

Depletion of PI and PG in the HDL3c could constitute another main alteration in RA HDL. Indeed, PI is another negatively charged PL regulating intracellular signalling cascades that was previously reported to be enriched in small, dense HDL3 and to enhance cholesterol efflux capacity of HDL via ABCA1 [14].

In addition, we found that altered HDL capacity to inhibit LDL oxidation was directly correlated with HDL3c-PC content, consistent with the role of lipidomic alterations for HDL functional deficiency. In human HDL, PC is the most abundant PL and the major carrier of highly oxidable PUFAs. Thus, enrichment in PC-PUFAs can increase oxidation rates and lower HDL antioxidative capacity. In accordance with the functional role of PC, deleterious effects of PC enrichment on HDL antioxidative activity were also confirmed using *in vitro* experiments. By contrast, Fournier et al. observed that cholesterol efflux capacity of serum was positively correlated with the concentration of HDL-PC [38]. Of note, this study found that HDL2 was more efficient than HDL3 as a cholesterol acceptor. Previous studies also reported that PC enrichment increased cholesterol efflux to HDL2 particles [39]. These results suggest that PC can exert distinct roles in different HDL subclasses, enhancing cholesterol efflux properties of HDL2 while reducing antioxidative properties of HDL3. This apparent discrepancy highlights the importance of the assessment of PL subclass heterogeneity across HDL particle spectrum.

Atheroprotective activities of HDL can be compromised under multiple proatherogenic conditions characterised by acute or chronic inflammatory processes. Accordingly, antiatherogenic properties of HDL were reported to be impaired in chronic inflammatory diseases, including RA and systemic lupus erythematosus [7,40,41]. These activities are efficiently carried out by the small, dense HDL3c subfraction which potently promotes cellular cholesterol efflux and protects LDL from oxidative damage [10,30]. In the present study, antioxidative capacity of small, dense HDL3b and 3c subfractions, and of total HDL, was not substantially altered in RA patients relative to controls. However, patients with high grade inflammation (hsCRP>10 mg/l) exhibited deficient HDL antioxidative capacity. It is worthy to note that this cutoff was proposed for hsCRP by the CARRE study, which recently reported that associations between lipid measures and CV disease were more evident in RA patients featuring CRP levels beyond 10 mg/l [21].

Interestingly, diminished cholesterol efflux capacity has been recently reported in HDL from RA patients with high disease activity [42]. In this work, Charles-Schoeman et al. did not find differences in the cholesterol efflux capacity when comparing RA patients with controls. However, significant differences were noted between RA patients subdivided in low disease activity/clinical remission (DAS28 < 2.6) compared to RA patients with high disease activity (DAS28 > 5.1). Similarly, McGillicuddy and co-workers [43] also reported that HDL functionality in humans was significantly decreased in high-grade inflammation. Indeed, impaired cholesterol efflux was only evidenced in subjects featuring hsCRP levels above 40 mg/l. Overall, these data suggest that high-grade inflammation is required to evidence deficient HDL functionality.

CRP is an acute phase protein that is synthesized by hepatocytes in response to proinflammatory cytokines. Plasma CRP levels have been shown to represent a valuable inflammatory biomarker in RA and have been suggested to account for the link between RA and CV disease in a 10-year follow-up study [44]. Interestingly, this study also reported that other measures of disease activity, such as tender or swollen joint counts or health assessment questionnaire (HAQ) score, were not independently predictive of subsequent death from CV causes after adjustment for CRP levels. Moreover, a recent study of a large cohort of 651 RA patients confirmed that CRP levels were significantly associated with the risk of heart failure and mortality after adjusting for age, sex, and year of RA diagnosis [45]. Thus, hsCRP levels appear to be an independent marker of CV disease in RA patients. In accordance, in the present study, small dense HDL from RA patients who displayed high levels of hsCRP, were inefficient to prevent LDL from oxidation. Furthermore, correlation analyses evidenced an association between the hsCRP levels and defective antioxidative properties of HDL.

In accordance with earlier studies, SAA was elevated in RA patients [7,40,46]. The replacement of apoA-I by SAA in HDL could have been a determinant factor for the decreased antioxidative activity described herein. Indeed, apoA-I provides the major contribution to HDL-mediated protection of LDL from free-radical-induced oxidation [47]. Consistent with this conclusion, metrics of antioxidative activity and content of apoA-I in small, dense HDLs were inversely correlated with plasma concentrations of SAA, potentially reflecting preferential association of SAA with these HDL subpopulations [48,49]. In addition, plasma levels of apoA-II were decreased in RA patients, indicating that apoA-II can also be removed from HDL by SAA. By contrast, as enzymatic activities of PON1 and Lp-PLA$_2$ were not altered in our RA patients, the present results discount the role of hydrolytic inactivation of oxidised lipids in the antioxidative deficiency of small, dense HDL in RA, consistent with previous data [47].

HDL proteome is a key feature known to determine HDL anti-atherogenic properties, which can be affected in inflammatory states and whose importance for HDL function can exceed that of HDL lipidome. Content of several HDL-associated proteins have been reported to be altered in RA. Indeed, Charles-Schoeman et al.

reported that RA patients with pro-inflammatory HDL displayed elevated content of haptoglobin, hemoglobin, apoA-I and myeloperoxidase as compared to RA patients with anti-inflammatory HDL [46]. Moreover, Watanabe et al. identified 78 different proteins in RA HDL, twelve of which, including acute phase proteins such us SAA, were significantly increased in RA patients with pro-inflammatory HDL compared to RA patients with anti-inflammatory HDL [50]. These data suggest a mechanism by which systemic inflammation alters protein cargo of HDL with subsequent effects on its atheroprotective capacity. Large-scale prospective studies are necessary to determine whether such alterations contribute to increased risk of CV disease in RA.

The number of RA patients evaluated can be considered as a limitation of the present study. Nevertheless, it is important to note that power calculation analysis revealed that the sample size was adequate to detect differences between the groups in the functional parameters studied. Furthermore, strict inclusion and exclusion criteria were applied to the recruitment in order to avoid bias related to diseases or drug consumption that could have affected lipid metabolism.

5. Conclusions

The present study adds new elements to our overall understanding of functional HDL deficiency. We show that chronic inflammation leads to modifications in the phosphosphingolipidome of small, dense HDL3 particles and to impairment of HDL capacity to protect LDL from oxidative stress. Noteworthy, these pathophysiological features are linked to the degree of inflammation and might contribute to the high risk of CV disease characteristic of RA.

Conflict of interest statement

The authors have no affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. All financial and material support for this research and work are clearly identified in the manuscript. Moreover, the authors have no relevant financial interests in this manuscript.

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Abbreviations

AAPH	Azo-Initiator 2,2′-Azobis-(2-Amidinopropane) Hydrochloride
Apo	Apolipoprotein
BMI	Body Mass Index
CE	Cholesteryl Ester
CV	Cardiovascular
DAS	Disease Activity Score
EDTA	Ethylene Diamine Tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FC	Free Cholesterol
GPI-PLD	glycosylphosphatidylinositol-specific phospholipase D
HDL	High-Density Lipoprotein
HDL-C	HDL-Cholesterol
HI	High Inflammation
HsCRP	High-sensitivity C-reactive protein
IDL	Intermediate-Density Lipoprotein
LDL	Low-Density Lipoprotein
LDL-C	LDL-Cholesterol
LI	Low Inflammation
Lp-PLA$_2$	Lipoprotein-Associated Phospholipase A$_2$
PBS	Phosphate-Buffered Saline
PL	Phospholipid
PON	Paraoxonase
RA	Rheumathoid Arthritis
SAA	Serum Amyloid A
TC	Total Cholesterol
TG	Triglyceride
TP	Total Protein
VLDL	Very Low-Density Lipoprotein
VLDL-C	Very Low-Density Lipoprotein-Cholesterol.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2014.09.034.

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