Anti-atherogenic and anti-inflammatory properties of high-density lipoprotein are affected by specific antibodies in systemic lupus erythematosus

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Objective. To determine whether antibodies against high-density lipoprotein (aHDL) and apolipoprotein A-I (aApo A-I) interfere with the anti-atherogenic functions of high-density lipoprotein (HDL) and relate to disease activity and damage in SLE.

Methods. Seventy-seven SLE patients were compared with an age- and sex-frequency matched control group. Immunoglobulin G (IgG) aHDL, IgG aApoA-I, soluble vascular cell and intracellular cell adhesion molecules (VCAM-1 and ICAM-1, respectively) were measured by ELISA, paraoxonase (PON) activity by spectrophotometry, nitric oxide (NOx) metabolites by the Griess reaction, and total anti-oxidant capacity (TAC) by chemiluminescence.

Results. Compared with controls, SLE patients showed higher titres of IgG aHDL (P < 0.0001) and IgG aApo A-I (P < 0.0001), lower PON activity (P < 0.0001), increased NOx (P < 0.0001), VCAM-1 (P < 0.0001) and ICAM-1 (P = 0.0008) and lower TAC (P = 0.0006). Titres of IgG aHDL positively correlated with IgG aApo A-I (r = 0.64, P < 0.0001), NOx (r = 0.32, P = 0.007), inversely correlated with PON activity (r = -0.34, P = 0.002) and TAC (r = -0.43, P = 0.0004) and were independently associated with ICAM-1 (t = 3.509, P = 0.001). IgG aApo A-I titres correlated positively with NO (r = 0.37, P = 0.007), inversely with PON activity (r = -0.31, P = 0.006), TAC (r = -0.47, P < 0.0001) and were independently associated with HDL (t = -2.747, P = 0.008) and VCAM-1 (t = 3.311, P = 0.002), the latter alongside NOx (T = 2.271, P = 0.02). Elevated titres of IgG aHDL and IgG aApo A-I and reduced PON activity related to increased disease score (BILAG) and damage index (SLICC/ACR DI).

Conclusion. In SLE, IgG aHDL and aApo A-I associate with disease activity and damage and interfere with the anti-oxidant and anti-inflammatory functions of HDL favouring atherogenesis.

Key words: Systemic lupus, Antibodies against high-density lipoprotein, Antibodies against Apolipoprotein A-I, Paraoxonase, Nitric oxide, Endothelial dysfunction.

Introduction

SLE is a chronic autoimmune disease with a wide spectrum of clinical manifestations. It is characterized by an intense production of autoantibodies to a large variety of antigens [1].

Over the past 50 yrs, patient survival in SLE significantly improved allowing for the emergence of cardiovascular disease (CVD) as a major cause of morbidity and mortality [2]. However, the high prevalence of traditional cardiovascular risk factors found in SLE does not explain the increased incidence of CVD in this disorder, and therefore it has been suggested that disease-specific factors, such as prolonged steroid treatment, chronic inflammation and renal disease, could play additional roles [3, 4].

Nevertheless, very high very-low-density lipoprotein (VLDL) and triglyceride (TG) levels, and particularly low levels of high-density lipoprotein (HDL) and its main protein component apolipoprotein A-I (Apo A-I) are relevant to atherogenesis in SLE [5–8]. HDL inhibits cytokine-induced production of adhesion molecules, an early event in atherogenesis [9], it promotes reverse cholesterol transport [10], and inhibits the oxidative modification of LDL [11] and its consequent uptake by monocytes, preventing thus the formation of foam cells.

The anti-oxidant activity of HDL is particularly important as enhanced lipid peroxidation does occur in SLE [12]. Paraoxonase (PON), the enzyme responsible for most of the anti-oxidant effect of HDL [13], is under the control of a gene family coding for three main enzymatic subtypes whereas polymorphisms at positions 55 (methionine/leucine) and 192 (arginine/glutamine) confer varying activities to the enzyme [14]. PON1 is highly effective in preventing lipid peroxidation of LDL [15, 16], and PON1-deficient mice are highly susceptible to atherosclerosis [17]. Its activity increases with the intake of lipid-lowering drugs [18, 19], and decreases with age [20]. Within HDL, PON is stabilized by Apo A-I [21] that also bears anti-inflammatory properties by blocking contact-mediated activation of monocytes by T lymphocytes [22].

Another important aspect of atherogenesis is endothelial dysfunction. Nitric oxide (NO) and adhesion molecules are markers of endothelial cell activation in response to different stimuli. NO is a biological messenger that mediates many physiological functions as well as pathological processes. It plays a vital role in host defence and immunity by modulating the inflammatory response [23]. Increased expression of adhesion molecules and overproduction of NO could contribute to tissue injury, given its capacity to increase vascular permeability, generate toxic free radicals, such as peroxynitrite, and induce cytotoxicity [24, 25].

The occurrence of autoantibodies against plasma lipoproteins and their constituents has been addressed in SLE but with major emphasis on anti-LDL or oxidized LDL (oxLDL) antibodies [26].

Few recent studies have addressed the importance of the humoral response towards HDL, as Dinu et al. [27] have specifically shown the presence of aApo A-I antibodies in SLE patients. In previous work, we found IgG aHDL and IgG aApo A-I in a small cohort of SLE patients in relation to decreased PON activity [28, 29].

This study aims to confirm the humoral response towards HDL and to assess whether Apo A-I could be a specific target. We also explore the relationship between aHDL and aApo A-I and the anti-oxidant and anti-inflammatory functions of HDL, and investigate a possible relationship with disease activity and damage.
Materials and methods

Patients

SLE patients attending the Lupus Clinic and controls of similar age and sex, recruited amongst healthy staff members and students attending the rheumatology department, were invited to participate in a study assessing the relevance of new markers of atherosclerosis in SLE. Exclusion criteria for SLE patients were secondary APS, a positive anti-cardiolipin and/or lupus anti-coagulant test in the absence of thrombosis or miscarriage and use of lipid-lowering drugs. Ninety-eight consecutive patients fulfilling at least four of the ACR revised criteria [30, 31] for the classification of SLE were considered, of whom 21 were excluded: 16 had at least one of the exclusion criteria and 5 declined to participate. Exclusion criteria for controls were diabetes, hyperlipidaemia, hypertension, kidney, liver, heart or lung disease, and intake of lipid-lowering drugs. A total of 64 healthy subjects were invited to participate but 14 were not considered: 8 met one or more of the exclusion criteria and 5 declined to participate. Exclusion criteria for controls were diabetes, hyperlipidaemia, hypertension, kidney, liver, heart or lung disease, and intake of lipid-lowering drugs. A total of 64 healthy subjects were invited to participate but 14 were not considered: 8 met one or more of the exclusion criteria and 5 declined to participate. Exclusion criteria for controls were diabetes, hyperlipidaemia, hypertension, kidney, liver, heart or lung disease, and intake of lipid-lowering drugs. A total of 64 healthy subjects were invited to participate but 14 were not considered: 8 met one or more of the exclusion criteria and 5 declined to participate. Exclusion criteria for controls were diabetes, hyperlipidaemia, hypertension, kidney, liver, heart or lung disease, and intake of lipid-lowering drugs. A total of 64 healthy subjects were invited to participate but 14 were not considered: 8 met one or more of the exclusion criteria and 5 declined to participate.

Measurement of IgG anti-Apo A-I antibodies

IgG anti-Apo A-I (aApo A-I) antibodies were measured as previously described [29]. Briefly, 96-well plates (PolySorp) were half-coated for 1 h at 37°C with 10 μg/ml human Apo A-I (Sigma-Aldrich) in 70% ethanol. Blocking was performed using PBS containing 1% BSA for 1 h at 37°C. A hundred microlitres of serum samples (1 : 300 dilutions in blocking agent) and positive control were added to duplicate wells in both halves of the plate. A hundred microlitres of samples (1 : 300 dilutions in blocking agent) and positive control were added to duplicate wells in both halves of the plate. A hundred microlitres of samples (1 : 300 dilutions in blocking agent) and positive control were added to duplicate wells in both halves of the plate. A hundred microlitres of samples (1 : 300 dilutions in blocking agent) and positive control were added to duplicate wells in both halves of the plate.

Measurement of IgG anti-HDL antibodies

IgG anti-HDL (aHDL) antibodies were measured as previously described [29]. Briefly, microtitre plates (PolySorp, Nunc, VWR, Portugal) were half-coated for 1 h at 37°C with 20 μg/ml human HDL (Sigma-Aldrich, Sintra, Portugal) in 70% ethanol. Blocking was performed using PBS containing 1% BSA for 1 h at 37°C. Plates were then washed three times using PBS. Samples were diluted 1 : 100 in blocking agent. A hundred microlitres of samples and positive control were added to duplicate wells in both halves of the plate for 1 h at 37°C. After three washes, alkaline phosphatase-conjugated anti-human IgG (1 : 1000 in the blocking agent) was added for 1 h. Then, p-Nitrophenyl phosphate in bicarbonate (BIC) buffer (pH 9.8) was added and incubated at 37°C for colour development and the absorbance read at 405 nm after 1 h. All assays were validated by the inclusion of internal quality control samples of known activity. The results were expressed as the percentage of the positive control present in each plate after subtraction from the background in the uncoated half of the plate. Inter-/intra-plate coefficients of variation were <10%.

Measurement of PON activity

Serum PON activity was measured according to Eckerson with some modifications [34]. Briefly, paraaxon (1.0 mM) (Sigma-Aldrich) freshly prepared in 290 μl of 50 mM glycine buffer containing 1mM calcium chloride (pH 10.5) was incubated at 37°C with 10 μl of serum for 10 min in 96-well plates (PolySorp). p-Nitrophenol formation was monitored at 412 nm. Enzyme activity was calculated with a molar extinction coefficient of 18,290 M cm⁻¹ and expressed as micromoles of p-nitrophenol per millilitre serum per minute.

Determination of nitrite and nitrate levels

Nitric oxide metabolites (NOx), nitrite and nitrate, were divided into halves for, were determined by a modified Griess reaction, following the reduction of nitrate to nitrite using nitrate reductase and nicotine adenine dinucleotide phosphate (NADPH) as previously reported [35]. Briefly, serum was diluted 1:4 with phosphate buffer (pH 7.4). The assay was performed in a standard flat-bottomed 96-well microtitre plate, divided into halves for the simultaneous measurement of nitrite and nitrate concentrations. To each well was added 50 μl of standard or sample. The assay was blanked against phosphate buffer. In half plates, 4 μl of nitrate reductase (Sigma-Aldrich) and 10 μl of NADPH (Sigma-Aldrich) were added to each well giving a final concentration of 6.3 U/l and 550 μmol/l, respectively. The plate was incubated at room temperature for 2 h. The Griess reaction was initiated by the addition to each well of equal volumes of 2% sulphanilamide (Sigma-Aldrich) in 5% H3PO4, 5% and 0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich) in water, mixed just before use. After 10 min incubation at room temperature, the absorbance of the reaction mixture was measured at 540 nm and the NOx levels were expressed as μM.

Total anti-oxidant capacity of plasma

Total anti-oxidant capacity (TAC) of plasma was assessed by the capacity of a sample to scavenge peroxynitrite formed by the reaction between superoxide and nitric oxide released from SIN-1, using an ABEL-41M2 anti-oxidant test kit with Pholasin using peroxynitrite (Knight Scientific, Plymouth, UK), according to the manufacturer’s instructions. For the peroxynitrite assays we used an Anthos Zenyth 1100/3100 multimode detector: this assay is based on the time at which the maximum peak of light occurred, and the results were expressed in vitamin E analogue (VEA)
equivalent units (micromoles) derived from a set of standards run at the same time.

**Soluble vascular cell adhesion molecules and soluble intracellular cell adhesion molecules**

Serum levels of soluble vascular cell adhesion molecule (VCAM-1) and soluble intracellular cell adhesion molecule (ICAM-1) were determined by commercially available ELISA (R&D Systems, Abingdon, UK), according to the manufacturer’s instructions.

**Lipid profile**

Plasma lipid profile (total cholesterol, HDL, LDL and TG) was determined by standard enzymatic techniques.

**Statistical analysis**

Data are shown as means ± s.d.: exceptions are noted. Normally distributed variables were analysed using the unpaired Student’s t-test while non-normally distributed variables were compared using the Mann-Whitney U-test. Correlations between variables were performed using the Spearman rank correlation test. The clinical (SLICC/ACR DI and BILAG) and laboratory (presence of antibodies towards HDL or their components) data were analysed using the Kruskal–Wallis test analysis of variance. All of antibodies towards HDL or their components) data were analysed using the unpaired Student’s t-test. Correlations between variables were determined using the Spearman rank correlation test. The possible independent effect of age, smoking, current steroid dose, current immune suppressor dose, IgG aHDL, IgG aApo A-I, SLICC/ACR and BILAG as independent variables, IgG aApo A-I titre was independently associated with HDL (t = −2.747, P = 0.008).

**Nitric oxide and TAC of plasma in study groups**

Mean NOx was higher in SLE than in healthy controls (Table 2) and positively correlated to IgG aHDL (r = 0.32, P = 0.007) and IgG aApo A-I (r = 0.37, P = 0.007). Mean plasma TAC was lower in SLE than in healthy controls (Table 2) and inversely correlated to the titres of IgG aHDL (r = −0.43, P = 0.0004) and IgG aApo A-I (r = −0.47, P < 0.0001) in SLE (Fig. 3A and B). PON activity correlated with TAC in SLE (r = 0.32, P = 0.008) (Fig. 3C).

**Soluble VCAM-1 and soluble ICAM-1**

Mean VCAM-1 and ICAM-1 were higher in SLE than in healthy controls (Table 2). The possible independent effect of age, smoking, current steroid dose, current immune-suppressors, HDL, LDL, IgG aApo A-I, IgG aHDL, NOx, PON activity, TAC, SLICC/ACR and BILAG on VCAM and ICAM was assessed in a regression model that revealed IgG aHDL as being independently associated with ICAM-1 (β = 3.509, P = 0.001) and IgG Apo A-I and NOx as being independently associated with VCAM-1 (β = 3.311, P = 0.002 and β = 2.717, P = 0.02, respectively). An inverse correlation was also found between PON activity and VCAM-1 (r = −0.39, P = 0.004) levels but no relationship was observed between VCAM-1 or ICAM-1 and TAC.

**Results**

**aHDL and aApo A-I antibodies, PON activity and plasma lipids in study groups**

Patients with SLE showed lower HDL and higher TG, LDL and total cholesterol than controls (Table 1). Mean IgG aHDL and IgG aApo A-I were higher in SLE patients than healthy controls (Fig. 1A and B) and positively correlated (r = 0.64, P < 0.0001) in the SLE population. Mean PON activity was lower in SLE than controls (P < 0.0001) (Table 2) and negatively correlated to IgG aHDL (r = −0.34, P = 0.002) and IgG aApo A-I titres (r = −0.31, P = 0.006) (Fig. 2A and B).

In a regression model including HDL as the dependent variable and age, smoking, current steroid dose, current immune suppressor dose, IgG aHDL, IgG aApo A-I, SLICC/ACR and BILAG as independent variables, IgG aApo A-I titre was independently associated with HDL (t = −2.747, P = 0.008).

**Table 2. Biological variables (oxidation and inflammation markers) measured in controls (CTRL) and patients with SLE**

<table>
<thead>
<tr>
<th></th>
<th>CTRL (n=50)</th>
<th>SLE (n=77)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON activity, mean ± s.d., µmol/min/ml</td>
<td>97.40 ± 19.08</td>
<td>56.72 ± 25.92</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TAC, VEA, mean ± s.d., equivalent units, µM</td>
<td>6773 ± 1343</td>
<td>5448 ± 2519</td>
<td>0.001</td>
</tr>
<tr>
<td>Nox, mean ± s.d., µM</td>
<td>32.71 ± 15.60</td>
<td>62.94 ± 38.38</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VCAM-1, mean ± s.d., ng/ml</td>
<td>2519 ± 0.001</td>
<td>357.90 ± 0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ICAM-1, mean ± s.d., ng/ml</td>
<td>2519 ± 0.001</td>
<td>357.90 ± 0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>aHDL, % positive control</td>
<td>19.08 ± 56.72</td>
<td>91.67 ± 816.80</td>
<td>0.0001</td>
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**Fig. 1. Levels of aHDL (A) and aApo A-I antibodies (B) in healthy controls (CTRL) and patients with SLE. Bars show the means.**
Relationship between serological and clinical data

A heightened damage index related to elevated IgG aHDL and IgG aApo A-I titres (Fig. 4A and B) and to decreased PON activity (Fig. 4C). SLE patients with active disease (BILAG) showed higher titres of IgG aHDL, IgG aApo A-I and lower PON activity than patients with inactive disease ($P = 0.03$, $P = 0.03$ and $P = 0.002$, respectively) (Fig. 4A–C). Mean NOx was associated with an increase in the SLICC/ACR DI ($P = 0.02$), but no association was found with disease activity (data not shown).

TAC was not associated with either disease activity or damage. VCAM-1 levels were associated with an increased SLICC/ACR DI ($P = 0.006$) and disease activity ($P = 0.03$), but no associations were found between ICAM-1 levels and clinical data. Current treatment did not affect the titres of aHDL and aApo A-I antibodies or PON activity in the SLE group.

**Discussion**

This study confirms the presence of IgG antibodies towards HDL and its main protein component Apo A-I in patients with SLE. They are associated with a decrease in PON activity, endothelial activation (elevated NO, VCAM-1 and ICAM-1), reduced TAC (suggesting a lower resistance to oxidation of the plasma), and with an increase in damage and disease activity. Altogether, these findings suggest that IgG aHDL antibodies increase the risk of oxidative stress, therefore contributing to the accelerated atherogenesis present in SLE.

The immune system is now recognized as a key factor in atherogenesis. However, the humoral response and its relationship with plasma lipids has been seldom considered. In fact, the importance of HDL [9, 21, 36], and how the humoral response towards its components may impair its functions, has only recently been addressed [27, 28]. In our cohort, titres of aHDL and aApo A-I antibodies strongly correlate, suggesting that Apo A-I might be one of the key antigens for aHDL antibodies. This does not exclude the possibility of other potential targets within the HDL complex.

Patients with SLE have increased levels of TG, LDL and total cholesterol and lower HDL levels when compared with controls. In our patients, aApo A-I antibody titres were independently associated with HDL levels, suggesting that quantity as well as quality of plasma HDL may be under their influence.
patients with SLE (Kruskal–Wallis test—ANOVA; error bar indicates S.D.). However, free radicals such as peroxynitrite, when proliferation, platelet aggregation and monocyte adhesion to the plasma NO concentration in patients when compared with healthy including nitrite and nitrate, are widely used as indicators of the active disease. NO has a short half-life and its metabolites, reported overexpression of inducible NOS (iNOS) in periods of stress. Indeed our previous work showed that aHDL and aApo A-I may tilt the oxidant/anti-oxidant balance towards oxidative oxidation of LDL, and consequent reduction of foam cell formation. Diminished bioavailability of NO is a key role in the early pathogenesis of atherosclerosis. Another important anti-inflammatory property of HDL is favouring the production of NO by up-regulating endothelial NO synthase (eNOS) expression via the high-affinity HDL receptor scavenger receptor class B type I (SR-BI) in a process that requires Apo A-I [38].

The presence of aHDL and aApo A-I antibodies may have clinical implications as their relationship with the SLICC/ACR DI indicates. This finding is further highlighted by the lower PON activity in patients with higher disease activity, PON is the enzyme that accounts for most of the capacity of HDL to prevent oxidation of LDL, and consequent reduction of foam cell formation. By interfering with PON function, aHDL and aApo A-I may tilt the oxidant/anti-oxidant balance towards oxidative stress. Indeed our previous work showed that aHDL and aApo A-I interfere with HDL disrupting the normal activity of PON [29]. This enzyme has already been associated with particular SLE-related features as Tripi et al. [37] have highlighted by showing an association between single nucleotide polymorphism in PON and lupus nephritis.

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NO plays a central role in regulating vascular tone, but its overproduction may contribute to tissue injury, by increasing the vascular permeability, generating toxic-free radicals such as peroxynitrite, and inducing cytotoxicity [23]. Belmont et al. [25] reported overexpression of inducible NOS (iNOS) in periods of active disease. NO has a short half-life and its metabolites, including nitrite and nitrate, are widely used as indicators of the effective availability of NO [39]. Our study confirms elevated plasma NO concentration in patients when compared with healthy controls [25, 40].

Under normal conditions, NO inhibits vascular muscle cell proliferation, platelet aggregation and monocyte adhesion to the endothelium. However, free radicals such as peroxynitrite, when excessively generated by the reaction of NO with superoxide, induce modifications in proteins, lipids and DNA that may increase the immunogenicity of intracellular antigens, leading to a break in immune tolerance.

Furthermore, the overproduction of free radicals and the excess NO concentration primes the vascular endothelium for subsequent injury. Here we report for the first time that NO is in a positive relationship with IgG aHDL and in a negative one with PON activity, highlighting how these changes may reflect clinical damage (SLICC/ACR DI).

Oxidative stress plays a vital role in the pathogenesis of atherosclerosis and its complications. TAC reflects the capacity of plasma to resist oxidation and has been defined as a global measure that takes into account the overall anti-oxidant defence of plasma.

In the present study, TAC of patients with SLE was significantly lower than the control group and in negative association with aHDL and aApo A-I antibody titres. Moreover, the positive correlation between TAC and PON activity suggests that aHDL and aApo A-I may decrease TAC by an inhibitor effect on PON. Since PON inhibits oxidation of phospholipids, thus preventing peroxynitrite-mediated lipid peroxidation, the reduction of PON activity, as seen in SLE patients, would further increase the overall oxidative state showed by the reduction of TAC.

The interaction of leucocytes with the vascular endothelium is pivotal to the inflammatory process, and is mediated amongst others by adhesion molecules, such as VCAM-1 and ICAM-1, which participate in atherogenesis by promoting monocyte adhesion on the endothelium and subsequent accumulation in the arterial intima. In the above process, HDL inhibits the expression of cell surface adhesion molecules by activated endothelial cells [9]. Levels of soluble adhesion molecules have been shown to correlate with various cardiovascular risk factors including low-HDL cholesterol [41].

Our study shows that soluble VCAM-1 levels correlate with low HDL levels and with disease activity, being significantly higher during active disease. Soluble ICAM-1 levels did not reflect disease activity. Whilst the variations in VCAM-1 are in agreement with previous studies [42–44], ICAM-1 levels have generated contradictory results, with studies reporting a positive correlation between s-ICAM-1 levels and disease activity [45], and others failing to find significant differences when compared with healthy controls, or even an association with disease activity [42, 46]. This difference was addressed by Cybulsky et al. [47], who showed that VCAM-1 deficiency significantly diminishes early foam cell lesion formation in the aorta of Ldlr /C0 mice, suggesting that VCAM-1 may play a role in preventing the initiation of atherosclerosis. However, ICAM-1 deficiency did not influence early foam cell lesion formation, either alone or when combined with VCAM-1 deficiency. Despite the up-regulation of both adhesion molecules in atherosclerotic lesions, VCAM-1 plays a greater role in the initiation of atherosclerosis [47].

In conclusion, by interfering with the anti-atherogenic properties of HDL, aHDL and aApo A-I enhance oxidative stress and the consequent SLE-related atherosclerotic risk. Given the cross-sectional nature of the study, disease activity and damage cannot be causally linked with the markers under study: their prognostic value with regards to SLE-related vascular disease should be assessed prospectively in further studies.

**Rheumatology key messages**

- Anti-HDL and aApo A-I antibodies are present in patients with SLE.
- They are related to an enhanced pro-oxidant and pro-inflammatory status.
- They are associated with disease activity and damage.
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