

# Impaired Antiatherogenic Functions of High-density Lipoprotein in Patients with Ankylosing Spondylitis

Christina Gkolfinopoulou, Efstratios Stratikos, Dimitris Theofilatos, Dimitris Kardassis, Paraskevi V. Voulgari, Alexandros A. Drosos, and Angeliki Chroni

**ABSTRACT. Objective.** Ankylosing spondylitis (AS) is a chronic inflammatory disease associated with increased risk of cardiovascular disease (CVD). High-density lipoprotein (HDL) exerts a series of antiatherogenic properties and protects from CVD. We evaluated whether HDL antiatherogenic properties are impaired in patients with AS.

**Methods.** HDL (apoB-depleted serum) was isolated from 35 patients with AS and 35 age- and sex-matched controls. We measured the antioxidant capacity of HDL, the ability of HDL to induce cholesterol efflux, the activity of HDL-associated enzymes paraoxonase-1 (PON1) and myeloperoxidase (MPO), as well as the ability of HDL to induce Akt kinase activation.

**Results.** HDL from patients with AS had decreased antioxidant capacity and decreased ability to promote cholesterol efflux from macrophages compared to controls. HDL-associated PON1 activity was lower and HDL-associated MPO activity higher in patients with AS compared to controls. Higher MPO activity correlated positively with lower antioxidant capacity of HDL in patients with AS. In addition, HDL from patients with AS had impaired endothelial Akt kinase activating properties that were inversely correlated with the MPO/PON1 ratio and positively correlated with the cholesterol efflux capacity of HDL.

**Conclusion.** HDL from patients with AS displays impaired antiatherogenic properties. Attenuation of HDL properties may constitute a link between AS and CVD. (First Release August 1 2015; J Rheumatol 2015;42:1652–60; doi:10.3899/jrheum.141532)

## Key Indexing Terms:

ANKYLOSING SPONDYLITIS HIGH-DENSITY LIPOPROTEINS CHOLESTEROL EFFLUX  
ANTIOXIDANT EFFECT CARDIOVASCULAR DISEASE

Ankylosing spondylitis (AS) is a chronic inflammatory disease primarily affecting the skeleton, leading to chronic pain in axial and peripheral joints and to functional impairments<sup>1</sup>. In patients with AS, a higher early mortality of 1.6–1.9 times that of the general population has been reported<sup>2,3</sup>. Studies of the causes of death among patients with AS identified cardiovascular disease (CVD) as the

leading cause<sup>2,3</sup>. Several studies reported that AS was associated with an increased risk for ischemic heart disease, congestive heart failure, peripheral vascular disease, arterial hypertension (HTN), and cerebrovascular disease<sup>4,5</sup>. Some causes of heart disease have been proposed to be direct results of AS, such as aortitis and aortic insufficiency with the possible necessity of cardiac surgery, conduction disturbances of the atrioventricular node with a probable subsequent indication for a pacemaker, and myocardial involvement with a possible compromise of left ventricular function<sup>6</sup>. Analyses of vascular functional and structural variables associated with atheroma development in patients with AS showed increased carotid intima-media thickness (IMT) or flow-mediated dilation, suggesting the presence of increased risk for atherosclerosis<sup>7,8</sup>. A systematic literature review and metaanalysis proposed that patients with AS appear to be at a higher risk of myocardial infarction<sup>2</sup>. Overall, these studies indicate that the excess cardiovascular mortality seen in patients with AS could be related to functional or structural arterial abnormalities. Unraveling the mechanisms, as well as identification of new biomarkers of increased risk for atherosclerosis, in patients with AS is important for both prevention and treatment.

Numerous clinical and epidemiological studies have

From the National Center for Scientific Research Demokritos, Agia Paraskevi, Athens; University of Crete Medical School and Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology of Hellas, Heraklion, Crete; Rheumatology Clinic, Department of Internal Medicine, Medical School, University of Ioannina, Ioannina, Greece.

Funding provided by the General Secretariat of Research and Technology of Greece Grant Synergasia 09SYN-12-897 (to DK and AC); the Ministry of Education of Greece Grant Thalys MIS 377286 (to ES, DK, and AC), and a Hellenic Rheumatology Society grant (to ES, PVV, AAD, and AC).

C. Gkolfinopoulou, MSc; E. Stratikos, PhD, National Center for Scientific Research Demokritos; D. Theofilatos, MSc; D. Kardassis, PhD, University of Crete Medical School and Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology of Hellas; P.V. Voulgari, MD, PhD; A.A. Drosos, MD, FACR, Rheumatology Clinic, Department of Internal Medicine, Medical School, University of Ioannina; A. Chroni, PhD, National Center for Scientific Research Demokritos.

Address correspondence to A. Chroni, Institute of Biosciences and Applications, National Center for Scientific Research "Demokritos," Agia Paraskevi, Athens 15310, Greece. E-mail: achroni@bio.demokritos.gr

Accepted for publication May 29, 2015.

demonstrated an inverse association between high-density lipoprotein cholesterol (HDL-C) levels and the risk of CVD<sup>9,10</sup>. HDL exerts a series of antiatherogenic functions, such as the ability to promote cholesterol efflux from artery wall macrophages, antioxidative activity including the ability to protect low-density lipoprotein (LDL) against oxidation, antiinflammatory effects, and protective effects on the vascular endothelium<sup>11,12</sup>. HDL or its major apolipoprotein, apolipoprotein A-I (apoA-I), can have direct effects on numerous cell types that play roles in cardiovascular and metabolic health including endothelial cells, vascular smooth muscle cells, leukocytes, platelets, adipocytes, skeletal muscle myocytes, and pancreatic  $\beta$  cells<sup>13</sup>. The various effects of HDL/apoA-I include the modulation of intracellular calcium, oxygen-derived free radical production, the activity of numerous kinases and enzymes and expression levels of various genes<sup>13</sup>. Previous studies have shown that during the course of chronic inflammatory rheumatic diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), the antiatherogenic properties of HDL are affected. Specifically, patients with RA and SLE were found to have proinflammatory HDL<sup>14,15</sup>. In addition, the cholesterol efflux capacity of HDL was impaired in RA patients with high disease activity, as well as in SLE patients with the disease under control<sup>16,17</sup>.

Several proteomic studies have shown that a large number of plasma proteins can associate with HDL and this can affect HDL structure and functions<sup>18,19</sup>. Under conditions of acute-phase reaction, as well as in chronic inflammation, the HDL protein composition is altered, resulting in changes in HDL function<sup>20,21</sup>. Among the proteins that associate with HDL and play a role in its atheroprotective functions is paraoxonase-1 (PON1), an enzyme linked to the anti-oxidative, antiinflammatory, and endothelial repair–stimulating effects of HDL<sup>19,22</sup>. Reduced plasma PON1 activity has been proposed to be a risk factor for major adverse cardiac events in humans<sup>23</sup>. Myeloperoxidase (MPO) is another protein that, like PON1, binds to HDL and is linked to oxidative stress and atherosclerosis<sup>24,25</sup>. Site-specific oxidation of apoA-I on HDL by MPO has been linked to impairment of cholesterol efflux capacity and acquisition of proinflammatory functions<sup>26,27</sup>. Further, epidemiological studies have shown an association of plasma MPO with CVD in the general population<sup>25</sup>.

Given the observed impairment of HDL antiatherogenic properties in patients with RA or SLE<sup>14,15,16,17</sup>, it is tempting to generalize to other chronic inflammatory diseases such as AS. Similarly to AS, both RA and SLE have been associated with excess cardiovascular risk<sup>28</sup>. In contrast to AS, however, RA and SLE are characterized by the presence of many autoantibodies that may cause systemic inflammation<sup>29,30</sup>. This pathology is absent in patients with AS<sup>29,30</sup>. It is therefore possible that HDL atheroprotective properties are not affected in AS and the increased predisposition to CVD

for patients with AS is not related to HDL dysfunction. To test this hypothesis, we evaluated the antiatherogenic functions of HDL in patients with AS compared to controls. More specifically, HDL from patients with AS were assessed for their antioxidant properties using a cell-free assay [dichlorofluorescein (DCF) assay]<sup>31,32</sup> that has been used to show the impairment of antioxidant properties of HDL in patients with RA and patients with SLE<sup>14</sup>. Further, we measured the ability of HDL to induce cholesterol efflux from macrophages, considered one of the major antiatherogenic functions of HDL<sup>33,34</sup>. In addition, the activity of HDL-associated enzymes PON1 and MPO was measured, because previous studies have shown that the impairment of atheroprotective properties of HDL may be associated with changes in PON1 and MPO activity<sup>19,26,27,35</sup>. Finally, we examined the ability of HDL to induce Akt kinase activation. HDL contributes to cardiovascular protection, particularly in endothelial cells, by inducing intracellular signaling that leads to the activation of diverse kinase cascades<sup>13</sup>. One of these kinases is Akt, an enzyme that is activated upon phosphorylation.

## MATERIALS AND METHODS

**Human subjects.** Study subjects were recruited among patients diagnosed with AS and/or followed at the outpatient Rheumatology Clinic of the University Hospital of Ioannina, Greece. Forty patients were evaluated. Patients diagnosed with AS should fulfill the 1984 modified New York criteria<sup>36</sup>. AS disease activity was measured by the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)<sup>37</sup>. A BASDAI score < 4 is considered inactive disease. Exclusion criteria were hereditary dyslipidemia, autoimmune conditions not pertinent to AS, active infection at the time of the assessment, liver or renal disease, malignancy, alcohol abuse, pregnancy, and lactation. Five patients were excluded — 2 for liver steatosis, 2 for renal insufficiency, and 1 for alcohol abuse. All patients were under anti-tumor necrosis factor- $\alpha$  (anti-TNF- $\alpha$ ) therapy, while 6 of them were also taking disease-modifying antirheumatic drugs (DMARD) and 1 also received steroids. Controls were recruited who matched patients for age and sex and met the above exclusion criteria. Subjects were defined as having CVD if they had documented history of CVD events, such as myocardial infarction, unstable angina, or cerebrovascular accident. In addition, subjects having a body mass index (BMI) > 30 were classified as obese. The study subjects' informed consent and approval from the institution's ethical committee were obtained.

**Serum lipid and apolipoprotein concentrations.** Serum was obtained from blood samples after an overnight fast and stored at  $-80^{\circ}\text{C}$  until use. Total cholesterol, triglycerides, and apoA-I concentrations were determined using the commercially available reagents Cholesterol LS (Labkit Chemelex SA), Infinity triglycerides (Thermo), and ApoA1 (Labkit Chemelex SA), according to the manufacturer's instructions. HDL-C was determined in apoB-depleted serum using the Cholesterol LS reagent. LDL cholesterol (LDL-C) was calculated using the Friedewald equation<sup>38</sup>.

**HDL preparation.** The HDL-containing supernatant (apoB-depleted serum) from subjects' serum was isolated by the dextran-Mg<sup>2+</sup> method as described<sup>39</sup>.

**DCF assay.** The antioxidant capacity of HDL, prepared by the dextran-Mg<sup>2+</sup> method, was tested in the presence or absence of oxidized (ox) LDL by the DCF assay as described<sup>31</sup> with some modifications<sup>32</sup>. DCF-DA (2,7-dichlorofluorescein diacetate; Molecular Probes/Invitrogen) was dissolved in fresh methanol at 2.0 mg/ml and incubated at room temperature in the dark for 20 min, resulting in the release of DCF. Upon interaction with oxidants, DCF

is oxidized to fluorescent DCF. To compare the HDL antioxidant properties of affected and unaffected subjects, we normalized for differences in HDL-C concentration. HDL (final concentration 50  $\mu$ g cholesterol/ml) in the presence or absence of oxLDL (final concentration 100  $\mu$ g cholesterol/ml) was added into a black 96-well plate in a final volume of 100  $\mu$ l. The plate was incubated at 37°C on a rotator for 1 h in the dark. At the end of this incubation period, 10  $\mu$ l of DCF solution (0.2 mg/ml) was added to each well, mixed, and incubated for an additional 2 h at 37°C with rotation in the dark. Fluorescence was measured with a plate reader (Fluo-Star Galaxy, BMG LabTech) at an excitation wavelength of 465 nm and an emission wavelength of 535 nm. All assays were performed in duplicate.

**Measurement of PON1 activity.** PON1 activity in HDL, prepared by the dextran-Mg<sup>2+</sup> method, was determined using paraoxon as substrate<sup>32,40</sup>. Briefly, the assays were performed in a final volume of 250  $\mu$ l containing 5  $\mu$ l of HDL, 5.61 mmol/l paraoxon (paraoxon-ethyl, Sigma Aldrich), 2 mmol/l CaCl<sub>2</sub>, and 100 mmol/l Tris-HCl, pH 8.0. The rate of p-nitrophenol formed by the hydrolysis of paraoxon was measured by monitoring the increase in absorbance at 405 nm for 15 min at room temperature in a microplate spectrophotometer. PON1 activity was expressed as units per liter of HDL samples. One unit is the activity that catalyzes the formation of 1  $\mu$ mol p-nitrophenol per minute.

**Measurement of MPO activity.** The activity of MPO in HDL, prepared by the dextran-Mg<sup>2+</sup> method, was determined as described<sup>16</sup> using the InnoZyme MPO activity assay kit (EMD Chemicals), according to the manufacturer's instructions. Briefly, 80  $\mu$ l of HDL were added to a 96-well plate with an immobilized polyclonal antibody specific for human MPO. Activity of captured MPO was measured using a detection reagent containing tetramethyl benzidine (TMB) and hydrogen peroxide. Following color development, the reaction was stopped with sulphuric acid and the absorbance of the oxidized TMB detected at 450 nm.

**Cellular cholesterol efflux assay.** The cholesterol efflux capacity of HDL was quantified as described<sup>34,41</sup>. To begin, J774 mouse macrophages plated in 48-well plates were labeled with 0.2 ml of labeling medium [0.25  $\mu$ Ci/ml 4-(<sup>14</sup>C)cholesterol in Dulbecco modified Eagle's medium DMEM (high glucose) supplemented with 0.2% (w/v) BSA]. Following 24 h of labeling and washing, cells were equilibrated for 24 h with 0.3 mM 8-(4-chlorophenylthio)-cyclic adenosine monophosphate (cAMP) in 0.2 ml of DMEM (high glucose) supplemented with 0.2% (w/v) BSA. Subsequently, efflux media containing 2% v/v HDL, prepared by the dextran-Mg<sup>2+</sup> method, in DMEM (high glucose) were added for 4 h. At the end of the incubation, the supernatants were collected and the cells were lysed in 200  $\mu$ l of lysis buffer (PBS containing 1% (v/v) Triton X-100) for 30 min at room temperature by gentle shaking. The radioactivity in 50  $\mu$ l of the supernatant and 100  $\mu$ l of cell lysate was determined by liquid scintillation counting. The percentage of secreted (<sup>14</sup>C)cholesterol was calculated by dividing the medium-derived counts by the sum of the total counts present in the culture medium and the cell lysate. All assays were performed in duplicate. To correct for plate-to-plate and day-to-day variations, HDL samples from the same 3 control subjects were included on each plate.

**Akt kinase activation assay.** The human umbilical vein endothelial cell-derived line EA.hy926 (300,000 cells/well in 6-well plates) was cultured in DMEM (10% fetal bovine serum, 2% pen-strep) to 80% confluence. Cells were starved for 4 h and then treated in the presence or absence of HDL, prepared by the dextran-Mg<sup>2+</sup> method, at a final concentration of 40  $\mu$ g apoA-I/ml for 20 min<sup>42</sup>. Western blot assays were performed using antibodies for phospho-Akt (Ser473) or total Akt (Cell Signaling). Image quantitation was performed using the ChemiDoc XRS+ Gel Imaging System (Bio-Rad) and the Image Lab software (Bio-Rad).

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software) and IBM SPSS Statistics version 22 (IBM Corp.) software. All data presented are mean  $\pm$  SD, unless otherwise indicated. Patient and control groups were compared using Student's t test for continuous variables and the chi-square test of association for categorical variables. Where indicated, p was adjusted for sex, smoking, HTN, obesity,

diabetes mellitus, CVD, and statin use as covariates using general linear model univariate analysis. Analysis of the distribution of the data by the D'Agostino and Pearson omnibus K2 normality test showed that, except for the values obtained from the DCF assay, all other data do not follow a normal distribution. Therefore, correlations between variables were evaluated using the Spearman's correlation coefficient for nonparametric data. P values < 0.05 were considered significant.

## RESULTS

**Study group characteristics.** Thirty-five patients with AS were compared to 35 age- and sex-matched controls for alterations in their HDL atheroprotective properties. On average, patients had longstanding disease with low disease activity (BASDAI < 4; Table 1). All patients received treatment with anti-TNF- $\alpha$  drugs (infliximab), 6 also received DMARD (methotrexate), and 1 also received methylprednisolone. There was no statistical difference for C-reactive protein values between patients with AS and controls. The demographic, lifestyle, and clinical characteristics of patients and controls are summarized in Table 1. BMI and percentage of smokers were similar in patients and controls. There were more subjects using statins among patients, but this did not reach statistical significance. Further, there was no significant difference for the presence of CVD, HTN, diabetes, and obesity between patients with AS and controls. Serum total cholesterol, HDL-C, LDL-C, and apoA-I concentrations did not differ statistically between patients with AS and controls. Serum triglyceride levels were lower in patients with AS compared to controls. Similar lipid profiles between patients with AS with inactive disease and controls have been reported<sup>43</sup>. Disease-related treatment followed by improvement of disease activity has been shown to result in the improvement of lipid profile of patients with AS<sup>44,45,46</sup>.

**Antioxidant capacity of HDL.** HDL samples were assessed for their antioxidant properties using the DCF assay<sup>31,32</sup>. In the absence of oxLDL, HDL (used at equal HDL-C concentration) from both patients and controls produced similar fluorescence signal levels, indicating comparable oxidation status. However, in the presence of oxLDL we saw a marked increase in fluorescence signal when using HDL from patients with AS compared to HDL from the control group, indicating that the capacity of HDL to suppress the oxidative potential of oxLDL is reduced in patients with AS (p < 0.0001; Figure 1). Similar results were obtained when the values for patients with AS and controls who were under statin treatment were excluded (p < 0.0001).

**HDL-mediated cellular cholesterol efflux.** Measurement of cholesterol efflux from macrophages using 2% apoB-depleted serum showed that HDL from patients with AS had decreased capacity to promote cholesterol efflux compared to HDL from controls (p < 0.0001; Figure 2A). The cholesterol efflux capacity of HDL in patients with AS remained decreased compared with controls (p < 0.005), even when cholesterol efflux values were normalized for HDL-C levels (Figure 2B). The cholesterol efflux capacity of HDL in patients with AS



Table 1. Clinical characteristics, lipids, and lipoproteins of patients with AS and controls. Values are mean  $\pm$  SD unless otherwise indicated.

	Patients, n = 35	Controls, n = 35	p*	p (adjusted)**
Age, yrs	46.1 $\pm$ 13.3	45.3 $\pm$ 12.6	0.840	
Men, n (%)	30 (86)	26 (74)	0.232	
Disease duration, yrs	19.0 $\pm$ 11.1	—		
BMI, kg/m <sup>2</sup>	24.9 $\pm$ 4.8	24.7 $\pm$ 2.3	0.806	
CRP, mg/dl	4.9 $\pm$ 3.2	3.7 $\pm$ 3.0	0.110	
BASDAI (0–10)	3.2 $\pm$ 0.7	—		
Anti-TNF- $\alpha$ use, n (%)	35 (100)	—		
DMARD use, n (%)	6 (17)	—		
Steroid use, n (%)	1 (3)	—		
Current smokers, n (%)	13 (37)	12 (34)	0.803	
CVD, n (%)	1 (3)	1 (3)	1.000	
HTN, n (%)	8 (23)	3 (9)	0.101	
DM, n (%)	1 (3)	0	0.314	
Obesity, n (%)	5 (14)	1 (3)	0.088	
Statin use, n (%)	9 (26)	3 (9)	0.057	
Total cholesterol, mg/dl	170.1 $\pm$ 38.9	182.5 $\pm$ 49.6	0.249	0.232
HDL-C, mg/dl	49.9 $\pm$ 17.2	49.3 $\pm$ 13.7	0.855	0.627
LDL-C, mg/dl	105.4 $\pm$ 40.2	110.3 $\pm$ 48.8	0.649	0.491
Triglycerides, mg/dl	89.1 $\pm$ 45.6	114.8 $\pm$ 54.1	0.035	0.005***
apoA-I, mg/dl	144.1 $\pm$ 49.8	165.7 $\pm$ 42.0	0.054	0.202

\*p calculated by Student's t test for continuous variables and chi-square test of association for categorical variables.

\*\*p calculated by general linear model univariate analysis including sex, smoking, HTN, obesity, DM, CVD, and statin use as covariates. \*\*\* Adjustment for each covariant independently of the others resulted in p values between 0.016 and 0.047. AS: ankylosing spondylitis; BMI: body mass index; CRP: C-reactive protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; anti-TNF- $\alpha$ : anti-tumor necrosis factor- $\alpha$ ; DMARD: disease-modifying antirheumatic drugs; CVD: cardiovascular disease; DM: diabetes mellitus; HTN: hypertension; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; apoA-I: apolipoprotein A-I.

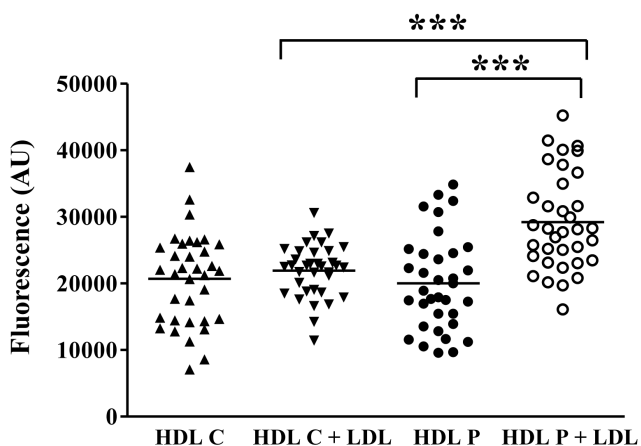
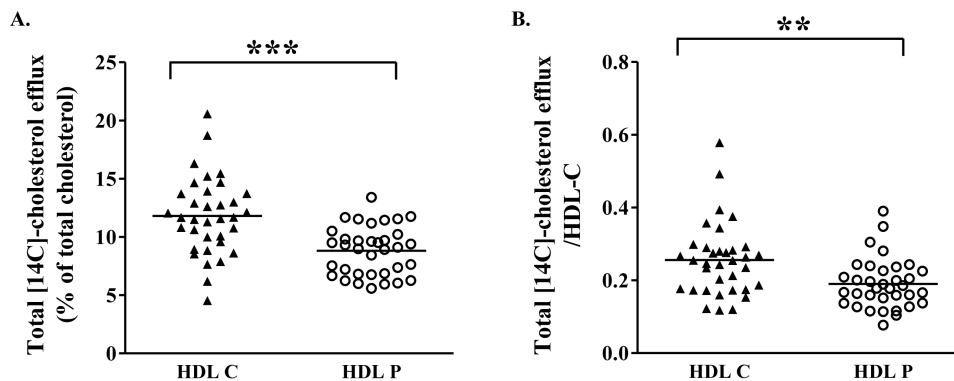


Figure 1. HDL antioxidative capacity in patients with AS and controls. The fluorescence intensity resulting from oxidation of DCFH by test HDL (50  $\mu$ g cholesterol/ml), prepared by the dextran-Mg<sup>2+</sup> method, in the presence or absence of oxLDL (100  $\mu$ g cholesterol/ml) was measured in a spectrofluorometer as described. \*\*\*p < 0.0001. AS: ankylosing spondylitis; C: controls; P: patients; AU: arbitrary units; HDL: high-density lipoprotein; DCF: dichlorofluorescein; oxLDL: oxidized low-density lipoprotein.

was also lower compared to controls when the values for patients with AS and controls using statins were excluded (p < 0.0001 and p < 0.05 without or with normalization for HDL-C levels, respectively).

**PON1 and MPO activities of HDL.** Measurement of PON1 and MPO activity using equal HDL volume for each sample showed that the HDL-associated PON1 activity was significantly lower (p < 0.05) and the HDL-associated MPO activity significantly higher (p < 0.05) in patients with AS compared to controls (Figure 3A, C). Normalization of PON1 and MPO activities for HDL-C levels showed that HDL-associated PON1 activity was also significantly lower (p < 0.05) and MPO activity significantly higher (p < 0.05) in patients with AS compared to controls (Figure 3B, D). Similar results were obtained when the values for patients with AS and controls who were under statin treatment were excluded (p < 0.05 for PON1 activity with or without normalization for HDL-C levels; p < 0.05 for MPO activity without normalization for HDL-C levels, and p < 0.005 for MPO activity with normalization for HDL-C levels).

When all measurements were adjusted for equal HDL-C levels, the relationship between HDL-associated PON1 or MPO activity and the antioxidant or cholesterol efflux capacity of HDL from patients with AS was examined by Spearman's rank correlation. While there was no correlation between PON1 activity and antioxidant or cholesterol efflux capacity, neither between MPO activity and cholesterol efflux capacity, the analysis showed that higher MPO activity correlated positively with worse antioxidant function of HDL from patients with AS (r 0.359, p = 0.020).



**Figure 2.** HDL-mediated cholesterol efflux in patients with AS and controls. The capacity of HDL (2% v/v), prepared by the dextran- $\text{Mg}^{2+}$  method, to promote total cholesterol efflux from J774 mouse macrophages treated with chlorophenylthio-cAMP, was measured as described. All assays were performed in duplicate. A. Values are expressed as % cholesterol efflux of total cell cholesterol. B. Values are expressed as % cholesterol efflux of total cell cholesterol divided by HDL-C concentration in mg/dl. \*\* $p < 0.005$ ; \*\*\* $p < 0.0001$ . AS: ankylosing spondylitis; C: controls; P: patients; HDL: high-density lipoprotein; HDL-C: HDL cholesterol; cAMP: cyclic adenosine monophosphate.

A recent study proposed that the MPO/PON1 ratio could be a useful marker for coronary artery disease (CAD) risk assessment through modulation of HDL properties<sup>47</sup>. As shown in Figure 3E, HDL-associated MPO/PON1 ratio is significantly higher for patients with AS compared to controls ( $p < 0.05$ ). Exclusion of values for patients with AS and controls that were using statins resulted in similar results ( $p < 0.05$ ).

**Akt kinase-activating properties of HDL.** Incubation of endothelial cells with HDL (used at equal HDL-apoA-I concentration) from patients with AS resulted in a reduced ratio of phosphorylated (p) Akt/Akt compared to cells incubated with HDL from controls (Figure 4A), indicating that HDL from patients with AS has reduced capacity ( $p < 0.005$ ) to promote signaling events through Akt activation. Similar results were obtained after normalization for HDL-C levels ( $p < 0.001$ ; Figure 4B) and after exclusion of values for patients with AS and controls that were using statins ( $p < 0.001$  for values without normalization for HDL-C levels and  $p < 0.01$  for values after normalization for HDL-C levels).

The pAkt/Akt ratio in endothelial cells after treatment with HDL from patients with AS showed an inverse correlation with the HDL-associated MPO/PON1 ratio in patients with AS ( $r -0.294$ ,  $p = 0.046$ ). In addition, the pAkt/Akt ratio was positively correlated with the cholesterol efflux capacity of HDL from patients with AS, after normalization for HDL-C levels ( $r 0.354$ ,  $p = 0.020$ ). A positive and stronger correlation between the pAkt/Akt ratio and the cholesterol efflux capacity of HDL was also observed for control subjects ( $r 0.520$ ,  $p = 0.0007$ ).

## DISCUSSION

Autoimmune rheumatic diseases have been associated with atherosclerosis and increased risk of cardiovascular morbidity

and mortality<sup>48</sup>. The impairment of HDL atheroprotective properties as a mechanism that leads to increased atherosclerotic risk has been studied for the rheumatic diseases RA and SLE<sup>14,15,16,17</sup>. Extension of these conclusions to AS should, however, be done with caution because of significant differences in the pathology among these diseases. Specifically, RA and SLE are characterized by the presence of many autoantibodies that may cause systemic inflammation, a pathology that is absent from AS<sup>29,30</sup>. Because systemic inflammation could be the reason behind the impairment of HDL-mediated atheroprotection in RA and SLE, this issue has to be addressed separately in AS. Further, earlier studies reported increased levels of HDL-associated serum amyloid A and reduced plasma PON1 activity in patients with active AS, suggesting impaired HDL antiatherogenic functions<sup>45,49,50</sup>.

In our current study, we show that HDL atheroprotective properties, such as cholesterol efflux ability and antioxidant capacity, are also impaired in patients with AS. Moreover, the atheroprotective properties of HDL in patients with AS are impaired despite the fact that all patients with AS had received disease-related treatment and the disease was clinically under control. This suggests that changes in the functions of HDL can occur even in the absence of marked systemic inflammation and remain persistent even in clinically managed disease.

Differences between patients with AS and controls were, in all cases, unaffected by normalization for HDL-C levels, confirming that our results reflect differences in HDL composition and functionality. Specifically, we find that despite normal serum HDL-cholesterol and apoA-I levels, patients with AS have HDL with (1) reduced capacity to promote cholesterol efflux from macrophages, (2) impaired antioxidant properties, (3) decreased PON1 activity, (4) increased

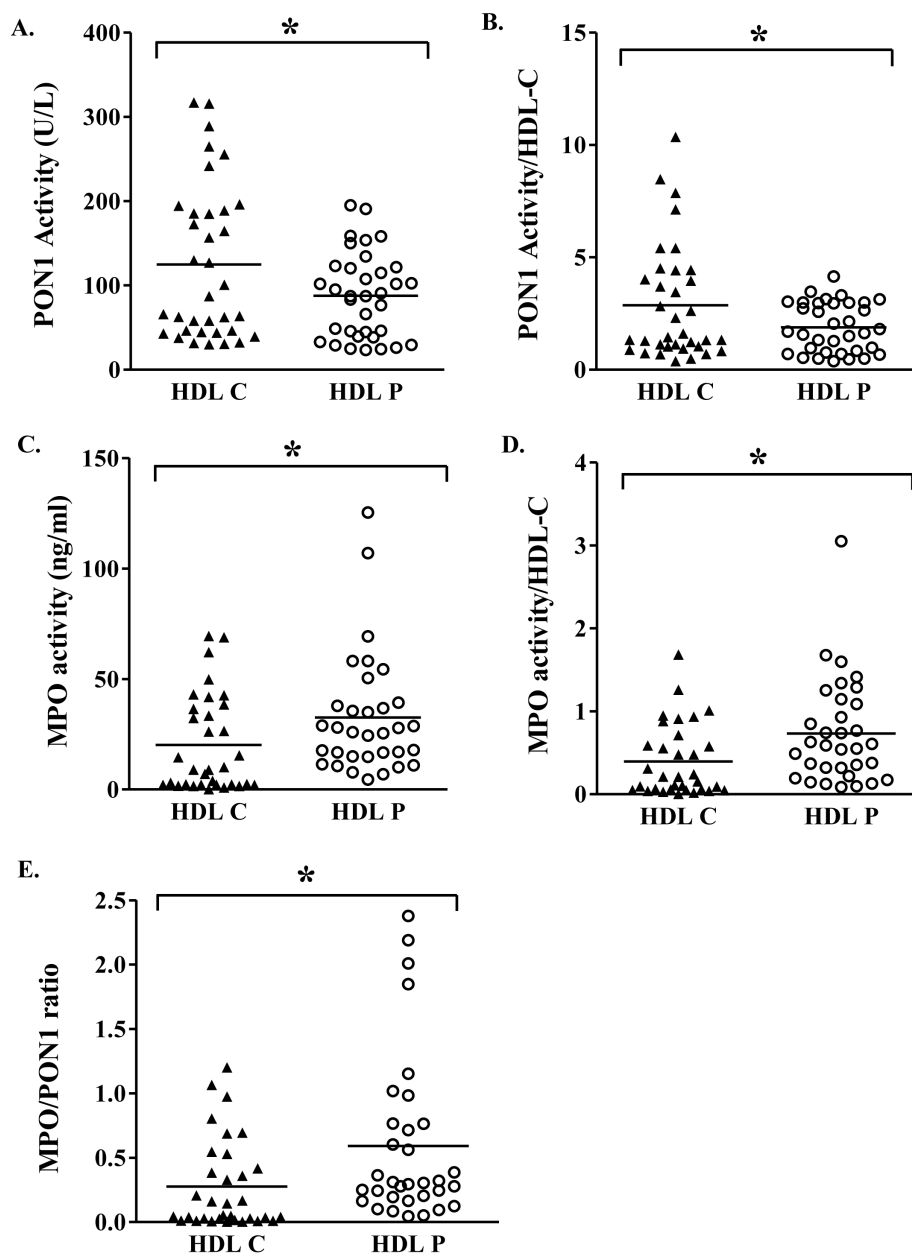
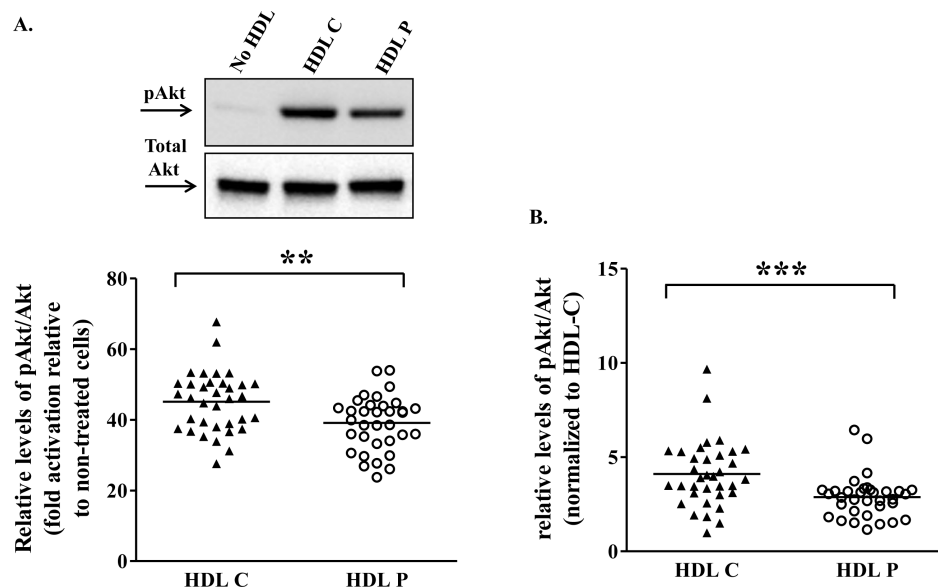


Figure 3. HDL-associated PON1 and MPO activity in patients with AS and controls. HDL-associated PON1 (A, B) and MPO (C, D) activity was measured using 5  $\mu$ l and 80  $\mu$ l of HDL, respectively, prepared by the dextran-Mg<sup>2+</sup> method, as described. A. Values are expressed as HDL-associated PON1 activity in u/l. B. Values are expressed as HDL-associated PON1 activity in u/l divided by HDL-C concentration in mg/dl. C. Values are expressed as HDL-associated MPO activity in ng/ml. D. Values are expressed as HDL-associated MPO activity in ng/ml divided by HDL-C concentration in mg/dl. E. HDL-associated MPO/PON1 ratio. \*p < 0.05. AS: ankylosing spondylitis; C: controls; P: patients; HDL: high-density lipoprotein; HDL-C: HDL cholesterol; PON1: paraoxonase-1; MPO: myeloperoxidase.

MPO activity, and (5) impaired endothelial Akt kinase activating properties. The cholesterol efflux capacity of HDL from macrophages was demonstrated to have a strong inverse association with carotid IMT and the likelihood of angiographic CAD<sup>34</sup> and more recently to be inversely associated with the incidence of cardiovascular events in a popula-

tion-based cohort<sup>35</sup>. The proinflammatory/antiinflammatory properties of HDL were shown to distinguish patients with CVD or CVD equivalents from control subjects better than HDL cholesterol<sup>51</sup> and were associated with an increased prevalence of carotid plaques and with a higher IMT in patients with SLE<sup>52</sup>. Therefore, the impairment of cholesterol



**Figure 4.** Activation of Akt kinase by HDL from patients with AS and controls. **A.** The property of HDL (40  $\mu$ g apoA-I/ml), prepared by the dextran-Mg<sup>2+</sup> method, to activate the Akt kinase in HUVEC-derived line EA.hy926 was measured as described. Cellular pAkt (Ser473) and total Akt levels were measured by immunoblotting (a representative set of images is shown in the upper panel). Western blots were scanned and quantified by Image Lab software (lower panel). The normalized levels of pAkt against total Akt in the treated cells are shown as fold activation relative to the non-treated cells. **B.** Values are expressed as levels of pAkt against total Akt in the treated cells with HDL, calculated as fold activation relative to the non-treated cells, divided by the HDL-C amount in  $\mu$ g for each sample. \*\* $p < 0.005$ ; \*\*\* $p < 0.001$ . C: controls; P: patients; HDL: high-density lipoprotein; HDL-C: HDL cholesterol; AS: ankylosing spondylitis; HUVEC: human umbilical vein endothelial cell; apoA-I: apolipoprotein A-I; pAkt: phosphorylated Akt.

efflux and antioxidant capacity of HDL in patients with AS may in part underlie the increased CVD-associated mortality for this patient group.

HDL can turn from antiinflammatory to proinflammatory during periods of acute or chronic inflammation because of changes in HDL proteomic and lipidomic composition<sup>20,21</sup>. Changes in proteomic composition can lead to decreased PON1 activity and increased MPO activity<sup>19,27</sup>. The latter enzyme also affects HDL cholesterol efflux capacity<sup>26,27</sup>. Both PON1 and MPO activities have been linked to cardiovascular risk<sup>23,25</sup>. In our study, we determined lower HDL-associated PON1 activity and higher HDL-associated MPO activity in patients with AS compared to controls. None of the 2 enzyme activities were correlated with the cholesterol efflux capacity of HDL from patients with AS, suggesting that other changes in HDL composition or other factors are responsible for the reduced cholesterol efflux capacity of HDL in these patients. Regardless, the HDL-associated MPO activity was found to associate with the impairment of the antioxidant capacity of HDL from patients with AS, similarly to what has been previously shown for patients with RA<sup>15</sup>.

A previous study suggested that in patients with coronary artery disease the reduced HDL-associated PON1 activity led to the reduction of endothelial Akt phosphorylation at Ser473, inhibition of eNOS-activation, and subsequent loss of the

endothelial antiinflammatory and endothelial repair-stimulating effects of HDL<sup>22</sup>. Another study demonstrated that MPO-oxidized HDL results in lower Akt phosphorylation in endothelial cells, as well as reduced endothelial repair in mice<sup>53</sup>. HDL from patients with AS showed reduced capacity for endothelial Akt phosphorylation at Ser473 compared to controls. This reduction was not correlated to HDL-associated PON1 or MPO activity when all measurements were adjusted for equal HDL-C levels. However, it was correlated to the HDL-associated MPO/PON1 ratio, indicating a combined effect of changes in HDL composition on HDL-mediated endothelial Akt phosphorylation and subsequent signaling. This is consistent with a recent study that proposed the serum MPO/PON1 ratio as a potential indicator of dysfunctional HDL<sup>47</sup>.

The pAkt/Akt ratio in endothelial cells after treatment with HDL showed a positive correlation with the HDL-mediated cholesterol efflux capacity in controls and a weaker, but statistically significant, correlation in patients with AS. This finding may suggest that in endothelial cells, the efflux of cholesterol can activate signaling pathways and that impairment of HDL cholesterol efflux capacity also affects HDL-mediated signaling. Further studies are needed to elucidate the interactions between HDL-mediated signaling and cholesterol efflux pathways.

A limitation of our present study is that we have no pretreatment data for the subjects. Such data would allow the examination of the relationship between inflammation or autoimmunity and HDL atheroprotective functions in patients with AS and whether treatment improves the HDL atheroprotective functions in these patients. Such analyses will be approached in future studies.

Overall, this is, to our knowledge, the first study to evaluate antioxidant, cholesterol efflux, and signaling capacity of HDL from patients with AS. The attenuation of HDL properties observed in patients with AS suggests a molecular link between AS and CVD. The fact that the disease was substantially controlled by therapy in our patient group suggests that the impairments in HDL atheroprotective properties observed in patients with AS are not dependent on a persistent highly active inflammatory status. It is possible that specific mediators of chronic immune responses or other genetic factors can influence HDL functions in a persistent manner and do not allow easy recovery to normal levels when the disease is under control. Our results provide novel insight into the increased cardiovascular risk observed in AS<sup>2,3,6,7,8</sup>, suggesting that AS affects HDL composition and function and this may result in increased atherosclerosis and cardiovascular risk. Therefore, the functional integrity of HDL may be an important, still unexplored longterm prognostic marker to evaluate risk for atherosclerosis and CVD in patients with AS.

## REFERENCES

- Braun J, Sieper J. Ankylosing spondylitis. *Lancet* 2007;369:1379-90.
- Mathieu S, Gossec L, Dougados M, Soubrier M. Cardiovascular profile in ankylosing spondylitis: a systematic review and meta-analysis. *Arthritis Care Res* 2011;63:557-63.
- Papagoras C, Voulgari PV, Drosos AA. Atherosclerosis and cardiovascular disease in the spondyloarthritides, particularly ankylosing spondylitis and psoriatic arthritis. *Clin Exp Rheumatol* 2013;31:612-20.
- Han C, Robinson DW Jr., Hackett MV, Paramore LC, Fraeman KH, Bala MV. Cardiovascular disease and risk factors in patients with rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis. *J Rheumatol* 2006;33:2167-72.
- Szabo SM, Levy AR, Rao SR, Kirbach SE, Lacaille D, Cifaldi M, et al. Increased risk of cardiovascular and cerebrovascular diseases in individuals with ankylosing spondylitis: a population-based study. *Arthritis Rheum* 2011;63:3294-304.
- Lautermann D, Braun J. Ankylosing spondylitis—cardiac manifestations. *Clin Exp Rheumatol* 2002;20:S11-5.
- Sari I, Okan T, Akar S, Cece H, Altay C, Secil M, et al. Impaired endothelial function in patients with ankylosing spondylitis. *Rheumatology* 2006;45:283-6.
- Peters MJ, van Eijk IC, Smulders YM, Serne E, Dijkman BA, van der Horst-Bruinsma IE, et al. Signs of accelerated preclinical atherosclerosis in patients with ankylosing spondylitis. *J Rheumatol* 2010;37:161-6.
- Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med* 1977;62:707-14.
- Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, et al. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA* 2009;302:1993-2000.
- Navab M, Reddy ST, Van Lenten BJ, Fogelman AM. HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms. *Nat Rev Cardiol* 2011;8:222-32.
- Annema W, von Eckardstein A. High-density lipoproteins. Multifunctional but vulnerable protections from atherosclerosis. *Circ J* 2013;77:2432-48.
- Mineo C, Shaul PW. Regulation of signal transduction by HDL. *J Lipid Res* 2013;54:2315-24.
- McMahon M, Grossman J, FitzGerald J, Dahlin-Lee E, Wallace DJ, Thong BY, et al. Proinflammatory high-density lipoprotein as a biomarker for atherosclerosis in patients with systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Rheum* 2006;54:2541-9.
- Charles-Schoeman C, Watanabe J, Lee YY, Furst DE, Amjadi S, Elashoff D, et al. Abnormal function of high-density lipoprotein is associated with poor disease control and an altered protein cargo in rheumatoid arthritis. *Arthritis Rheum* 2009;60:2870-9.
- Charles-Schoeman C, Lee YY, Grijalva V, Amjadi S, FitzGerald J, Ranganath VK, et al. Cholesterol efflux by high density lipoproteins is impaired in patients with active rheumatoid arthritis. *Ann Rheum Dis* 2012;71:1157-62.
- Ronda N, Favari E, Borghi MO, Ingegnoli F, Gerosa M, Chighizola C, et al. Impaired serum cholesterol efflux capacity in rheumatoid arthritis and systemic lupus erythematosus. *Ann Rheum Dis* 2014;73:609-15.
- Vaisar T, Pennathur S, Green PS, Gharib SA, Hoofnagle AN, Cheung MC, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J Clin Invest* 2007;117:746-56.
- Davidson WS, Silva RA, Chantepie S, Lagor WR, Chapman MJ, Kontush A. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler Thromb Vasc Biol* 2009;29:870-6.
- Rohrer L, Hersberger M, von Eckardstein A. High density lipoproteins in the intersection of diabetes mellitus, inflammation and cardiovascular disease. *Curr Opin Lipidol* 2004;15:269-78.
- Shah AS, Tan L, Long JL, Davidson WS. Proteomic diversity of high density lipoproteins: our emerging understanding of its importance in lipid transport and beyond. *J Lipid Res* 2013;54:2575-85.
- Besler C, Heinrich K, Rohrer L, Doerries C, Riwanto M, Shih DM, et al. Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. *J Clin Invest* 2011;121:2693-708.
- Tang WH, Hartiala J, Fan Y, Wu Y, Stewart AF, Erdmann J, et al. Clinical and genetic association of serum paraoxonase and arylesterase activities with cardiovascular risk. *Arterioscler Thromb Vasc Biol* 2012;32:2803-12.
- McMillen TS, Heinecke JW, LeBoeuf RC. Expression of human myeloperoxidase by macrophages promotes atherosclerosis in mice. *Circulation* 2005;111:2798-804.
- Zhang R, Brennan ML, Fu X, Aviles RJ, Pearce GL, Penn MS, et al. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* 2001;286:2136-42.
- Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, et al. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 2004;114:529-41.
- Undurti A, Huang Y, Lupica JA, Smith JD, DiDonato JA, Hazen SL. Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle. *J Biol Chem* 2009;284:30825-35.
- Haq S, Mirjafari H, Bruce IN. Atherosclerosis in rheumatoid arthritis and systemic lupus erythematosus. *Curr Opin Lipidol* 2008;19:338-43.



29. Appel H, Loddenkemper C, Miossec P. Rheumatoid arthritis and ankylosing spondylitis - pathology of acute inflammation. *Clin Exp Rheumatol* 2009;27:S15-9.
30. Amezcua-Guerra LM. Overlap between systemic lupus erythematosus and rheumatoid arthritis: is it real or just an illusion? *J Rheumatol* 2009;36:4-6.
31. Navab M, Hama SY, Hough GP, Subbanagounder G, Reddy ST, Fogelman AM. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. *J Lipid Res* 2001;42:1308-17.
32. Holleboom AG, Daniil G, Fu X, Zhang R, Hovingh GK, Schimmel AW, et al. Lipid oxidation in carriers of lecithin:cholesterol acyltransferase gene mutations. *Arterioscler Thromb Vasc Biol* 2012;32:3066-75.
33. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, et al. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med* 2014;371:2383-93.
34. Khera AV, Cuchel M, Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* 2011;364:127-35.
35. Rosenblat M, Karry R, Aviram M. Paraoxonase 1 (PON1) is a more potent antioxidant and stimulant of macrophage cholesterol efflux, when present in HDL than in lipoprotein-deficient serum: relevance to diabetes. *Atherosclerosis* 2006;187:74-81.
36. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis Rheum* 1984;27:361-8.
37. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. *J Rheumatol* 1994;21:2286-91.
38. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499-502.
39. Warnick GR, Benderson J, Albers JJ. Dextran sulfate-Mg<sup>2+</sup> precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin Chem* 1982;28:1379-88.
40. Tsimihodimos V, Karabina SA, Tambaki AP, Bairaktari E, Goudevenos JA, Chapman MJ, et al. Atorvastatin preferentially reduces LDL-associated platelet-activating factor acetylhydrolase activity in dyslipidemias of type IIA and type IIB. *Arterioscler Thromb Vasc Biol* 2002;22:306-11.
41. Li XM, Tang WH, Mosior MK, Huang Y, Wu Y, Matter W, et al. Paradoxical association of enhanced cholesterol efflux with increased incident cardiovascular risks. *Arterioscler Thromb Vasc Biol* 2013;33:1696-705.
42. Mineo C, Yuhanna IS, Quon MJ, Shaul PW. High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases. *J Biol Chem* 2003;278:9142-9.
43. Capkin E, Kiris A, Karkucak M, Durmus I, Gokmen F, Cansu A, et al. Investigation of effects of different treatment modalities on structural and functional vessel wall properties in patients with ankylosing spondylitis. *Joint Bone Spine* 2011;78:378-82.
44. Spanakis E, Sidiropoulos P, Papadakis J, Ganotakis E, Katsikas G, Karvounaris S, et al. Modest but sustained increase of serum high density lipoprotein cholesterol levels in patients with inflammatory arthritides treated with infliximab. *J Rheumatol* 2006;33:2440-6.
45. van Eijk IC, de Vries MK, Levels JH, Peters MJ, Huizer EE, Dijkman BA, et al. Improvement of lipid profile is accompanied by atheroprotective alterations in high-density lipoprotein composition upon tumor necrosis factor blockade: a prospective cohort study in ankylosing spondylitis. *Arthritis Rheum* 2009;60:1324-30.
46. Mathieu S, Dubost JJ, Tournadre A, Malochet-Guinamand S, Ristori JM, Soubrier M. Effects of 14 weeks of TNF alpha blockade treatment on lipid profile in ankylosing spondylitis. *Joint Bone Spine* 2010;77:50-2.
47. Haraguchi Y, Toh R, Hasokawa M, Nakajima H, Honjo T, Otsui K, et al. Serum myeloperoxidase/paraoxonase 1 ratio as potential indicator of dysfunctional high-density lipoprotein and risk stratification in coronary artery disease. *Atherosclerosis* 2014;234:288-94.
48. Shoenfeld Y, Gerli R, Doria A, Matsuura E, Cerinic MM, Ronda N, et al. Accelerated atherosclerosis in autoimmune rheumatic diseases. *Circulation* 2005;112:3337-47.
49. Cece H, Yazgan P, Karakas E, Karakas O, Demirkol A, Toru I, et al. Carotid intima-media thickness and paraoxonase activity in patients with ankylosing spondylitis. *Clin Invest Med* 2011;34:E225.
50. Olama SM, Elarman MM. Evaluation of paraoxonase and arylesterase activities in Egyptian patients with ankylosing spondylitis. *Rheumatol Int* 2013;33:1487-94.
51. Ansell BJ, Navab M, Hama S, Kamranpour N, Fonarow G, Hough G, et al. Inflammatory/antiinflammatory properties of high-density lipoprotein distinguish patients from control subjects better than high-density lipoprotein cholesterol levels and are favorably affected by simvastatin treatment. *Circulation* 2003;108:2751-6.
52. McMahon M, Grossman J, Skaggs B, FitzGerald J, Sahakian L, Ragavendra N, et al. Dysfunctional proinflammatory high-density lipoproteins confer increased risk of atherosclerosis in women with systemic lupus erythematosus. *Arthritis Rheum* 2009;60:2428-37.
53. Pan B, Yu B, Ren H, Willard B, Pan L, Zu L, et al. High-density lipoprotein nitration and chlorination catalyzed by myeloperoxidase impair its effect of promoting endothelial repair. *Free Radic Biol Med* 2013;60:272-81.