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# Paraoxonase-3 is depleted from the high density lipoproteins of autoimmune disease patients with subclinical atherosclerosis

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## Abstract

Patients with autoimmune diseases have a significantly increased risk of developing cardiovascular disease. In disease, high density lipoprotein (HDL) particles lose their anti-inflammatory and antioxidant properties, becoming dysfunctional. The purpose of this study was to test the hypothesis that alterations in the HDL proteomic profile are associated with subclinical atherosclerosis and HDL dysfunction in patients with autoimmune diseases such as systemic lupus erythematosus (SLE) and type 1 diabetes. Targeted proteomics was used to quantify the relative abundance of 18 proteins in HDL from SLE patients with and without atherosclerotic plaque detectable by carotid ultrasound. Changes in the proteomic profile were compared against the *in vitro* ability of HDL to protect against lipid oxidation. The same proteins were quantified in HDL

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ASSOCIATED CONTENT

Supporting Information for publication

This material is available free of charge via the Internet at http://pubs.acs.org:

(1) A file that includes: Peptides monitored for HDL proteomics (Table S1); Relative concentrations of proteins in different groups in the SLE cohort (Table S2); Precision and linearity of the measurement of 18 proteins in HDL (Table S3); Univariate analysis of proteins with piHDL in SLE patients (Table S4); Relative concentrations of proteins in different groups in the diabetic cohort (Table S5); PON3 and apoA-I immunoblots of HDL particles isolated from SLE patients (Figure S1); and Representative immunoblots.
(2) A spreadsheet with all of the raw mass spectrometric peak area ratios for each peptide, calculated relative concentrations for each protein, and the clinical metadata associated with each sample.

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

The other authors have nothing to disclose.

from patients with type 1 diabetes with or without coronary artery calcification as determined by computed tomography. In each population, paraoxonase-3 (PON3), a potent antioxidant protein, was depleted from the HDL of patients with subclinical atherosclerosis. PON3 expression in HDL was positively correlated with HDL antioxidant function. These results suggest that PON3 may be an important protein in preventing atherosclerosis and highlights the importance of antioxidant proteins in the prevention of atherosclerosis *in vivo*.

**Keywords**

high density lipoproteins; pro-inflammatory; dysfunctional; carotid plaque; coronary artery calcification; systemic lupus erythematosus; type 1 diabetes; mass spectrometry; paraoxonase-3

## INTRODUCTION

Patients with autoimmune disease are often at greatly increased risk of cardiovascular disease when compared with the general population.[1, 2] It has been hypothesized that humoral and cellular responses to specific antigens lead to localized and systemic inflammation that activate the endothelium and lead to atherosclerotic plaques.[3] While the clinical manifestations of different autoimmune diseases can be quite disparate, the influence of those diseases on the development of preclinical atherosclerosis may closely parallel one another. For example, young patients (35–44 years of age) with systemic lupus erythematosus (SLE) have 7-fold higher prevalence of carotid artery atherosclerotic plaque by ultrasound compared with age and gender matched controls.[4] Similarly, patients with type 1 diabetes have an increased prevalence of coronary artery calcification (CAC) when compared with non-diabetic controls.[5] While disruptions in glucose homeostasis partially contribute to the increased risk of atherosclerosis in patients with type 1 diabetes, it is well known that patients with type 1 diabetes have other autoimmune features as well.[6]

Elevated concentrations of plasma high density lipoprotein-cholesterol (HDL-C) are associated with a reduced risk of atherosclerosis in large epidemiological studies,[7] studies in animals have demonstrated the cardioprotective effects of HDL-associated proteins *in vivo*,[8] and small intervention trials of the infusion of apolipoprotein A-I (apoA-I) and mutant forms of apoA-I have led to plaque reduction in humans.[9] The proposed cardioprotective functions of HDL particles include 1) reverse cholesterol transport of lipids from the periphery, including lipid-laden macrophages in atherosclerotic plaques, to the liver for excretion, 2) anti-inflammatory blockade of the complement cascade and granulocyte and endothelial cell activation, and 3) prevention of low density lipoprotein (LDL) oxidization. Importantly, an expert panel convened by the National Lipid Association recently concluded that HDL-cholesterol should not be considered a target for therapeutic intervention, based on the convincing evidence from recent randomized intervention trials that demonstrated the futility of increasing HDL-cholesterol via pharmacological means in conjunction with LDL-cholesterol-lowering therapies.[10] It is currently unclear whether and how therapeutically raising the concentration of HDL-cholesterol affects the structure and function of HDL particles, with one preliminary report suggesting that HDL function *in vitro* may remain mostly unchanged.[11]

The proteome of HDL is altered in patients with cardiovascular disease[12] and in obese and insulin resistant patients.[13] Additionally, the HDL proteome may be modified by statin and niacin therapy.[12] It has subsequently been hypothesized that the HDL proteome may be an effective biomarker of disease risk and activity.[14] However, the relationship between HDL structure and function remains unknown. Laboratory assays have been developed to evaluate HDL function in population studies. Three of those assays evaluate the ability of HDL to mediate lipid efflux from lipid-loaded macrophages, to prevent activation of macrophages, or to prevent oxidation of LDL particles. In one case-control study, lipid efflux activity was more strongly associated with cardiovascular disease than HDL-cholesterol.[15] HDL particles purified from patients with cardiovascular disease are less efficient at preventing activation of endothelial cells by TNFα.[16] Similarly, HDL particles from patients with end-stage renal disease[17] or SLE[18] and preclinical atherosclerosis are either unable to reduce oxidation of co-incubated LDL particles or, in many cases, actually cause the oxidation of these LDL particles, which has been termed dysfunctional or pro-inflammatory HDL. Taken together, these studies provide ample support for the hypothesis that HDL can become dysfunctional in patients with cardiovascular disease. If HDL proteins are indeed responsible for these functions, it follows that alteration or post-translational modification of the protein cargo of HDL particles in these affected patients could explain the reduction in HDL activity.

We hypothesized that the protein cargo of HDL would be altered in autoimmune disease patients with subclinical cardiovascular disease and that changes in the protein cargo would correlate with HDL function. To test these hypotheses, we used a targeted proteomics assay[19] to measure the relative concentration of proteins in the HDL fraction of plasma in patients with SLE who did or did not have plaque detectable by carotid ultrasound. Previously, the protective function of HDL was shown to be diminished in SLE patients with plaque in this cohort.[18] We also evaluated HDL particles from patients with type 1 diabetes who did or did not have significant CAC (defined as CAC >100) by computed tomography. Clinical measures of autoimmune disease status and other known cardiovascular disease risk factors were included in the statistical analysis of the association between relative protein concentrations in HDL particles and the presence of subclinical cardiovascular disease.

## MATERIALS AND METHODS

### Study participants

Female lupus and control subjects were drawn from the Institutional Review Board-approved prospective Biomarkers of Atherosclerosis in SLE cohort study conducted at the Rheumatology Practices at the University of California, Los Angeles (February 2004 to February 2008). Details of this study protocol have been reported elsewhere.[20] Patients with SLE (n = 54) fulfilled at least four of the 1997 revised American College of Rheumatology classification SLE.[21] Controls (n = 25) were healthy by self-report with no clinical manifestations of SLE on CSQ questionnaires.[22] Exclusion criteria included statin use in the prior three months[23] and renal failure (defined as creatinine > 2.0).[24] Blood samples were obtained, disease activity was assessed using Safety of Estrogens in Lupus Erythematosus-SLE Disease Activity Index (SELENA-SLEDAI) scores,[25] and organ damage was

determined using the Systemic Lupus International Collaborating Clinics/ACR damage index (SDI)[26] on the same day or within 2 weeks of their carotid ultrasound.[20] Carotid ultrasound was performed to investigate the thickness of their carotid arteries. Brightness-mode grey-scale color and spectral Doppler techniques were used. The detailed methodology used has been described elsewhere.[20] One sample from the SLE cohort without carotid plaque had very little protein after the HDL purification (likely a failure of the membrane during buffer exchange) and is therefore not included in the analysis. Subjects with type 1 diabetes were participants in the Epidemiology of Diabetes Interventions and Complications study (EDIC), an observational study following the Diabetes Control and Complications Trial (DCCT).[27] Using a case-control design, we selected EDIC study participants with and without CAC. CAC was assessed using computed tomography. The methods have been described elsewhere.[28] The degree of calcification was measured by the Agatston score, which is the product of the density of calcification in a given coronary artery times the area of calcification. A cutoff of CAC Agatston score >100 was used as the preclinical marker of atherosclerosis. Age-matched control subjects had Agatston scores of 0. Cases and controls (n = 56) were both limited to non-smoking males who were not using lipid-lowering medications, and one control subject was matched to each case subject based on age (within two years). For each subject, plasma samples and concurrent clinical characteristics were obtained as close as possible to the date of CAC measurement (from EDIC year 7 or 8, calendar year 2000–2002).

### HDL function assay

The cell free assay of HDL function is a modification of a previously published method.[29] To determine the functional properties of HDL in preventing lipid peroxidation, the change in fluorescence intensity resulting from oxidation of dichlorofluorescin (DCFH) by LDL in the presence or absence of test HDL was measured. The LDL particles were prepared from normal plasma as previously described.[20] An aliquot of 20 μL of the normal LDL solution (final concentration of 50 μg/mL) and 90 μL of test HDL (at a final concentration of 10 μg/mL cholesterol) were incubated in quadruplicate in 96-well plates for 1 h. Ten μL of DCFH solution (0.2 mg/mL) were added to each well and incubated for 2 h. Fluorescence was determined with a plate reader (Spectra Max, Gemini XS; Molecular Devices, Sunnyvale, CA). Values of DCFH activated by LDL alone were normalized to 1.0. Values equal to or greater than 1.0 after the addition of test HDL indicated pro-inflammatory HDL; values less than 1.0 indicated anti-inflammatory HDL.

### Purification of high density lipoproteins and tryptic digestion of proteins

High density lipoproteins (d = 1.063–1.210 g/mL) were purified from EDTA-anticoagulated human plasma for proteomic analysis using sequential density gradient ultracentrifugation with KBr following a protocol similar to that previously described.[30] In the current protocol, 335 μL of plasma was added to 108.9 mg KBr that had been dried down on the bottom of the tube using centrifugal evaporation. After transferring 350 μL of plasma (d = 1.210 g/mL) to centrifuge tubes (Beckman, Cat No. 343776), the samples were overlaid with 150 μL of KBr (d = 1.210 g/mL). All lipoproteins were isolated by ultracentrifugation (4.5 h, 120,000 rpm, 5°C, Beckman TLA-120.1 rotor). The density was then lowered to 1.063 g/mL by combining the top 120 μL with 239 μL normal saline, 0.5 mM EDTA. This was transferred

to a fresh ultracentrifuge tube, overlaid with 140.8 μL KBr (d = 1.063), and HDL was isolated from other lipoproteins (16 h, 120,000 rpm, 5°C). The bottom 125 μL were removed from the ultracentrifuge tubes and dialyzed against 20 mM potassium phosphate, 100 μM diethylenetriaminepentaacetic acid using microdialysis cups (3.5 kDa MWCO, Cat No. 69552, Thermo Fisher Scientific Inc., Waltham, MA) with three buffer exchanges at 4°C. Protein concentration was determined using the Bradford method with bovine serum albumin as the calibrant (Coomassie Plus, Thermo Fisher Scientific Inc.). The variability in total protein yield of the purification of HDL proteins from human plasma is 10.6–13.4% CV across preps (N=9). After dialysis, HDL (10 μg protein) was reduced in 100 μL of buffer B [0.1% Rapigest (Waters Corp., Milford, MA), 100 mM ammonium bicarbonate] supplemented with 5 mM dithiothreitol at 37°C for 1 h with constant agitation and alkylated with 15 mM iodoacetamide. Alkylated proteins were digested at 37°C for 2 h with 0.5 μg of sequencing-grade modified trypsin (Promega, Madison, WI) and for another 16 h following the addition of another 0.5 μg of trypsin. Rapigest was cleaved with 0.5% (v/v) TFA, and the peptides were lyophilized and suspended in 5% (v/v) acetonitrile, 0.1% (v/v) formic acid.

## Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Tryptic digests were mixed with an equal volume of buffer C (100 mM ammonium bicarbonate) containing one isotopically labeled peptide (synthesized with 15N-labeled and 13C-labeled arginine [R*, 13C6H14O215N4] or lysine [K*, 13C6H14O215N2]) for each peptide that was quantified. Internal standard peptides had been synthesized using standard solid phase chemistry. Purity was estimated using LC-*uv* (>80%), which was confirmed using LC-MS, and accurate mass was confirmed by MALDI-TOF-MS. Peptides were not quantified by amino acid analysis and would therefore be considered crude internal standard peptides appropriate for the relative quantification of peptides rather than the quantification of the absolute concentration in each sample. Each peptide was spiked in at 0.625 pmol/μg HDL protein for the lupus population and 3.75 pmol/μg for the DCCT/EDIC population. See Table S1 (Supporting Information) for the complete details of these peptides and their endogenous counterparts. Peptides were desalted on a C18 trapping column (Dionex, Acclaim PepMap100 C18, 100Å, 5μm, 5×1.0 mm i.d.), using a Tempo 1D Plus autosampler-liquid chromatography system (Applied Biosystems-Life Technologies, Carlsbad, CA), eluted onto a C18 analytical column (Michrom, Magic C18, 200Å, 5μm, 150 × 0.15 mm i.d.), separated with a linear gradient of acetonitrile (5%–40% over 8 min), and ionized with a Microionspray II ion source (1 μL/min flow rate). Precursor-product ion transitions (Table S1, Supporting Information) were monitored simultaneously on multiple channels by selected reaction monitoring with an API 4000 QTRAP mass spectrometer (AB Sciex, Framingham, MA). Chromatographic peak areas were determined using Analyst 1.4.2 Software (AB Sciex).

## Quantitative PON3 Western blot

Selected HDL samples from SLE patients with diverse PON3 concentrations as determined by mass spectrometry were delipidated in a methanol-diethyl ether solvent system. Fifty μg of HDL from each patient were deposited on top of 0.6 mL of methanol and 1.4 mL of ice-cold diethyl ether. The glass tubes were incubated on ice for 10 min and centrifuged for 5 min at 1000 rpm at 4°C. After removing the supernatant from the white precipitate, 2 mL of

ice-cold diethyl ether was added to each tube. After repeating the incubation on ice and the centrifugation, the white protein pellets were evaporated and dissolved in 30 μL of SDS-DTT (2% sodium dodecyl sulfate, 40 mM dithiothreitol). The delipidated HDL of each patient was loaded in duplicates (20 μg of protein per lane) in NuPAGE 4–12% pre-cast gels (Life Technologies). Gel electrophoresis was run under denaturing conditions. Proteins were transferred to PVDF membranes using the iBlot 7-Minute Blotting System (Life Technologies). The membranes were then blocked for 1h shaking at RT with 4% blocking agent (ECL Prime Blocking Agent, GE Healthcare, Pittsburg, PA) in TBST (Tris-buffered saline containing 0.1% Tween-20). The polyclonal PON3 antibodies used were kindly provided by Drs. Bharti and Michael Mackness, and were obtained as previously described.[31] The primary antibody was used at a 1:1,000 dilution and was probed at 4°C overnight. The secondary antibody used was a polyclonal goat anti-rabbit IgG-HRP (1:80,000 dilution) from Sigma-Aldrich (St. Louis, MO), incubated for 1 h at RT. Both antibodies were diluted in 2% blocking agent in TBST. A chemiluminescent substrate (ECL2, Thermo Fisher Scientific Inc.) was used for the detection of the PON3 bands. The blots were then exposed to X-ray film (CL-XPosure film, Thermo Fisher Scientific Inc.). The intensity of the bands was quantified by densitometry using the ImageJ software (National Institutes of Health, Bethesda, MA).

### ApoA-I Western blot

ApoA-I blotting was used as an internal loading control. Following PON3 Western blot, membranes were stripped and reprobed for ApoA-I. Membranes were incubated twice in mild stripping buffer containing 200 mM glycine, 0.1% (w/v) SDS and 1% (v/v) Tween 20 pH 2.2 for 10 min shaking at RT. The stripping buffer was washed away by shaking the membranes in TBS twice for 10 min and TBST twice for 5 min. Then, membranes were blocked as described for PON3 and probed for 1 h at RT in affinity purified polyclonal ApoA-I antibody (1:800,000 dilution in 2% blocking agent in TBST) generated in rabbits by Cocalico Biologicals Inc. [Reamstown, PA, USA (this company maintains a current USDA research license and a current Animal Welfare Assurance from the NIH's Office of Laboratory Animal Welfare)]. The same secondary antibody was used as described for PON3 but incubated for 30 min at RT. Bands were detected using the same chemiluminescent substrate specified for PON3 and exposed to X-ray film.

### Statistical analysis

Chromatographic peak areas for each endogenous peptide were divided by the peak area of the corresponding isotope-labeled internal standard peptide. Because the signal was not calibrated using an external calibration curve, the peak area ratio for each peptide was then divided by the average of the peak area ratios for each peptide across the population (SLE or type 1 diabetes). This permitted the comparison of results across both populations by removing the variability in the amount of internal standard peptide that was added in each experiment. These normalized peak area ratios were used as the relative concentration of each peptide in HDL for these analyses. Distributions were compared by Student's t-test or by Wilcoxon tests. Pearson or Spearman correlation coefficients were calculated for normally distributed and non-normally distributed data, respectively. No correction was made for multiple comparisons because two completely distinct populations were studied

using the same methods at different times (separated by 14 months). For illustrative purposes, HDL pro-inflammatory activity measurements were logarithmically transformed due to their skew. Logarithmically transformed body mass index (BMI) data were used in logistic regression models due to their skew. Proportions were compared using Fisher's exact test. Statistical calculations, including logistic and linear regression, were performed in the R statistical computing environment (version 2.15.2). Statistical analyses were considered significant when $p < 0.05$. The raw data used in the calculations described above are included as a spreadsheet available in Supporting Information.

## RESULTS

### HDL proteomics in lupus subjects with carotid artery plaque

To test the hypothesis that specific proteins are enriched or depleted from HDL in patients with lupus who have carotid artery plaque, a preclinical marker of atherosclerosis, a set of 18 proteins were quantified in HDL using targeted LC-MS/MS.[13, 19, 30] The method provides the relative concentration of proteins in HDL, not the absolute concentration, but the precision of the approach is adequate for comparisons of groups.[13,19] This set of 18 proteins (detailed in Table S2 and Figure S2, Supporting Information) included the HDL proteins with good analytical precision as determined in experiments similar to those previously described (Table S3, Supporting Information).[19] While we found that none of these proteins were significantly different between SLE and control subjects (Table S2, Supporting Information), one protein, paraoxonase-3 (PON3), was depleted from the HDL of patients with SLE who also had carotid plaque ($p = 0.014$, Figure 1) compared with SLE patients without plaque. The carotid intimal media thickness (cIMT) and SELENA-SLEDAI scores (a widely-used validated measure of disease activity in SLE patients)[25] were significantly different between SLE patients with plaque and those without plaque (Table 1). There were no statistically significant associations between PON3 in HDL and either cIMT or SELESA-SLEDAI score. In a logistic model, lower PON3 in HDL was still associated with the presence of plaque when controlling for cIMT and SELENA-SLEDAI score ($p = 0.029$).

### Association of PON3 concentration with the pro-inflammatory activity of HDL

The relative effect size of PON3 in HDL on pre-clinical atherosclerosis was small and would not have been significant after correcting for multiple comparisons. However, when testing the second hypothesis of this study: that proteins in HDL would be associated with the pro-inflammatory activity of HDL in SLE patients (which as previously described,[18] was higher in SLE patients with subclinical atherosclerosis compared with SLE patients without atherosclerosis, $p<0.001$), a statistically significant inverse correlation between PON3 expression in HDL and HDL pro-inflammatory activity was observed (Figure 2, $p = 0.0041$). The pro-inflammatory activity of HDL is a quantitative measure of the ability of HDL to prevent lipid oxidation. HDL with a higher score is less capable of preventing oxidation and HDL with a score >1 actually causes lipid oxidation. The relative concentration of apoA-I in HDL (per mg total protein) was also associated with the pro-inflammatory activity of HDL (Table S4, Supporting Information). In a multiple linear regression model of apoA-I and PON3 in HDL vs. pro-inflammatory activity, relative apoA-

I concentration was not significant (p = 0.17), but PON3 was (p = 0.0041). Similarly, in a logistic model adjusted for apoA-I in HDL, each standard deviation increment of PON3 in HDL was associated with 53% reduced risk of plaque in SLE patients (OR = 0.47, 95% CI [0.22–0.89], p = 0.031).

### Immunochemical measurement of PON3 in HDL

To further support the observed association of PON3 in HDL with carotid plaque and pro-inflammatory activity using mass spectrometric measurements, the level of PON3 in HDL determined by immunoblotting (Figure S1, Supporting Information)[31, 32] was compared with mass spectrometric measurements for selected HDL samples with the highest and the lowest relative PON3 concentrations. The results confirmed that the multiplexed targeted mass spectrometric measurement of PON3 in HDL provides results comparable to classical biochemical methods (Figure 3). Analysis of apoA-I by immunoblotting confirmed that the lower PON3 in HDL is not due to lower HDL particle number in plasma (Figure S1, Supporting Information).

### PON3 is depleted from HDL in type 1 diabetic subjects with coronary artery calcification

To validate the findings from the lupus population, targeted proteomics was used to investigate the HDL proteome in another autoimmune disease (type 1 diabetes), with a different measure of subclinical atherosclerosis, CAC (Table 2).[28] The subjects with CAC >100 had higher plasma triglycerides and higher BMI, consistent with a previous report.[33] Similar to the lupus population, we observed a statistically significant decrease in the relative amount of PON3 in HDL in patients with subclinical atherosclerosis (Figure 4). Furthermore, using a logistic model adjusted for plasma triglycerides and BMI, each standard deviation increment in PON3 in HDL was associated with 70% reduced risk of CAC >100 (OR = 0.30, 95% CI [0.12–0.64], p = 0.0047). Moreover, in this cohort, there were significant decreases in the relative amounts of apoE, phospholipid transfer protein (PLTP), and clusterin (apoJ) in HDL in subjects with CAC >100 (Table S4, Supporting Information).

### PON3 in HDL is associated with other clinical characteristics

In the lupus population, PON3 was also associated with age and BMI (Table 3). When adjusting for age and BMI in a logistic regression model in the SLE population, the association of PON3 with carotid artery plaque was no longer statistically significant (p = 0.060). However, when adjusting for age and BMI in a linear regression model, the association with pro-inflammatory activity of HDL remained significant (p = 0.020). In the type 1 diabetic population, PON3 was not associated with age and BMI, perhaps due to the narrower distribution of age and BMI in this population. When considering the presence of subclinical atherosclerosis in the two populations combined (as determined by carotid plaque or CAC >100, respectively), each standard deviation increment of PON3 in HDL was associated with 51% reduced risk (OR = 0.49, 95% CI [0.31–0.76], p = 0.0021) in a logistic model that adjusted for age and BMI.

## DISCUSSION

In this article we applied a sensitive targeted proteomics method for studying changes in the HDL proteome, with PON1 and PON3 among the proteins that were measured.[19] PON1 and PON3 are calcium-dependent esterases/lactonases that possess antioxidant and anti-inflammatory properties.[34] This was first demonstrated in PON1 by Mackness and colleagues, when they reported that PON1 could retard lipid peroxidation of low-density lipoproteins.[35] PON3 plays also an important role attenuating atherosclerosis.[36] A variety of *in vitro* and *in vivo* studies have confirmed that PON1 and PON3 are able to prevent oxidative modification of lipoproteins and cell membranes, and to promote cholesterol efflux.[37–41] In this manuscript, the populations studied represent two different autoimmune diseases, SLE and type 1 diabetes, with or without subclinical atherosclerosis. Surprisingly in both populations, from the 18 proteins analyzed, only PON3 depletion from HDL was associated with the presence of atherosclerosis, a finding that was consistent across the two cohorts. In each population, an increase in the amount of PON3 in HDL was statistically significantly associated with protection from subclinical atherosclerosis after controlling for relevant covariates. In addition, the amount of PON3 in HDL was associated with the function of HDL. More specifically, depletion of PON3 was correlated with the pro-inflammatory activity of HDL, a measure of HDL dysfunction. PON1 was not associated with preclinical atherosclerosis (Tables S2, S4, and S5, Supporting Information).

Previous studies have failed to find a link between genotypic polymorphisms in PON3 and cardiovascular disease or plasma concentrations of PON3, apoA-I, or plasma HDL cholesterol, which suggests that environmental factors may play a more important role in plasma concentrations of PON3 and its activity therein.[42–45] Additionally, the finding that lower PON3 in HDL correlates with subclinical atherosclerosis contradicts previous studies reporting increased PON3 protein concentrations (as measured by ELISA) in atherosclerosis vs. healthy subjects.[44, 46, 47] One explanation for this apparent discrepancy could be the sample used to determine PON3 concentration, serum in the previous studies and isolated HDL from plasma in the current study. These results are consistent with the proposal that certain HDL particles (*e.g.*, particles containing PON3) acquire or shed lipids in the presence of preclinical atherosclerosis and thus possess a density outside the HDL purified in this study (*i.e.*, 1.063–1.210 g/mL). The different methodologies used, ELISA vs. mass spectrometry/Western blot, could also influence the results. However, it should be noted that the PON3 antibody used in the ELISA from the previous studies and in the Western blot from this study comes from the same source. Mass spectrometry is increasingly used in clinical settings and could potentially replace traditional and sometimes problematic immunoassays.[48] In addition, the mass spectrometry and Western blot analyses of PON3 showed a strong correlation, helping to validate the quantitative results obtained.

Increased lipid peroxidation and inflammation, and a decrease of specific antioxidant enzymes have been reported in SLE patients, which could lead to increased oxidative stress and cardiovascular events.[49, 50] Increased oxidative stress has been described in patients with type 1 diabetes as well, which could also result in accelerated progression of atherosclerosis.[51] In this study, HDL from SLE patients with subclinical atherosclerosis (as determined by carotid plaque) possessed increased pro-inflammatory activity *in vitro*, which

was accompanied by depletion of PON3. Since PON3 has a higher antioxidant capacity than PON1,[36, 38] it is expected that decreases in the content of just PON3 would result in a dysfunctional HDL with more pro-inflammatory and less antioxidant properties. This confirms our hypothesis that changes in protein cargo affect HDL function. Additionally, PON3 is not only associated with atherosclerosis but may also play a role in protecting against obesity.[52] In this regard, we found that PON3 was inversely related to BMI in SLE patients, supporting an inverse relationship between adiposity and PON3 expression.

In this study we did not find significant changes in PON1 protein concentrations in HDL from SLE and type 1 diabetes patients. This is not surprising, since PON1 and PON3 have different expression profiles and protein localization, with the suggestion that they may act in an independent, but synergistic way.[53] Their enzymatic activities are also different, with organophosphates being exclusively hydrolyzed by PON1 and PON3 hydrolyzing bulky drug lactones.[34] Altogether, PON1 and PON3 may have different physiological functions with similar effects, which would include the hydrolysis of oxidized lipids and the promotion of macrophage cholesterol efflux.

There are important limitations of our study. First, the two cohorts are very different. SLE and type 1 diabetes are similar in that they are both autoimmune diseases that predispose patients to increased risk of renal and cardiovascular disease. However, for the most part, other disease manifestations are disparate. Second, the lupus cohort was all female and the diabetic cohort was all male. This likely explains why the HDL cholesterol was significantly higher in the lupus cohort. Third, our study was cross-sectional in nature, and as a result, it is unclear whether the association of HDL proteomic profile with *in vitro* HDL oxidative dysfunction and subclinical atherosclerosis are due to a causal interaction, or not. For example, it is unclear if HDL proteomic changes precede HDL dysfunction or develop simultaneously. Similarly, it is not possible to determine if proteomic changes in HDL cause subclinical atherosclerosis or vice-versa. Lastly, there is no established mechanism for how PON3 depletion could lead to the formation of dysfunctional HDL and atherosclerosis. More studies are needed to generalize our results, determine causal relationships, and identify mechanisms that lead to disease.

Despite these limitations, we were able to apply targeted proteomic technologies in two distinct populations and identify a single protein (PON3) whose concentration in HDL particles declines in association with subclinical atherosclerosis. Additionally, the concentration of PON3 in HDL correlated with the antioxidant function of HDL *in vitro*. These results support the hypothesis that proteins in HDL are integral to preventing oxidation of atherogenic particles *in vivo* and demonstrate the importance of HDL proteomics in potentially discovering novel mechanisms of atherosclerosis in at-risk populations.

In conclusion, we have demonstrated that the newest member of the serum esterase/lactonase family of enzymes, PON3, is depleted from HDL particles in autoimmune disease patients with atherosclerosis, and that this depletion is correlated with HDL particle dysfunction. Relatively little is known about PON3, compared to PON1 or PON2. Our data

shed light on a potential mechanism of HDL particle dysfunction in disease states and highlight the importance of antioxidant proteins in the prevention of atherosclerosis *in vivo*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

| | |
|---|---|
| **apoA-I** | apolipoprotein A-I |
| **BMI** | body mass index |
| **CAC** | coronary artery calcification |
| **HDL** | high density lipoprotein |
| **HDL-C** | high-density lipoprotein cholesterol |
| **LC-MS/MS** | liquid chromatography-tandem mass spectrometry |
| **LDL** | low-density lipoprotein |
| **PLTP** | phospholipid transfer protein |
| **PON3** | paraoxonase-3 |

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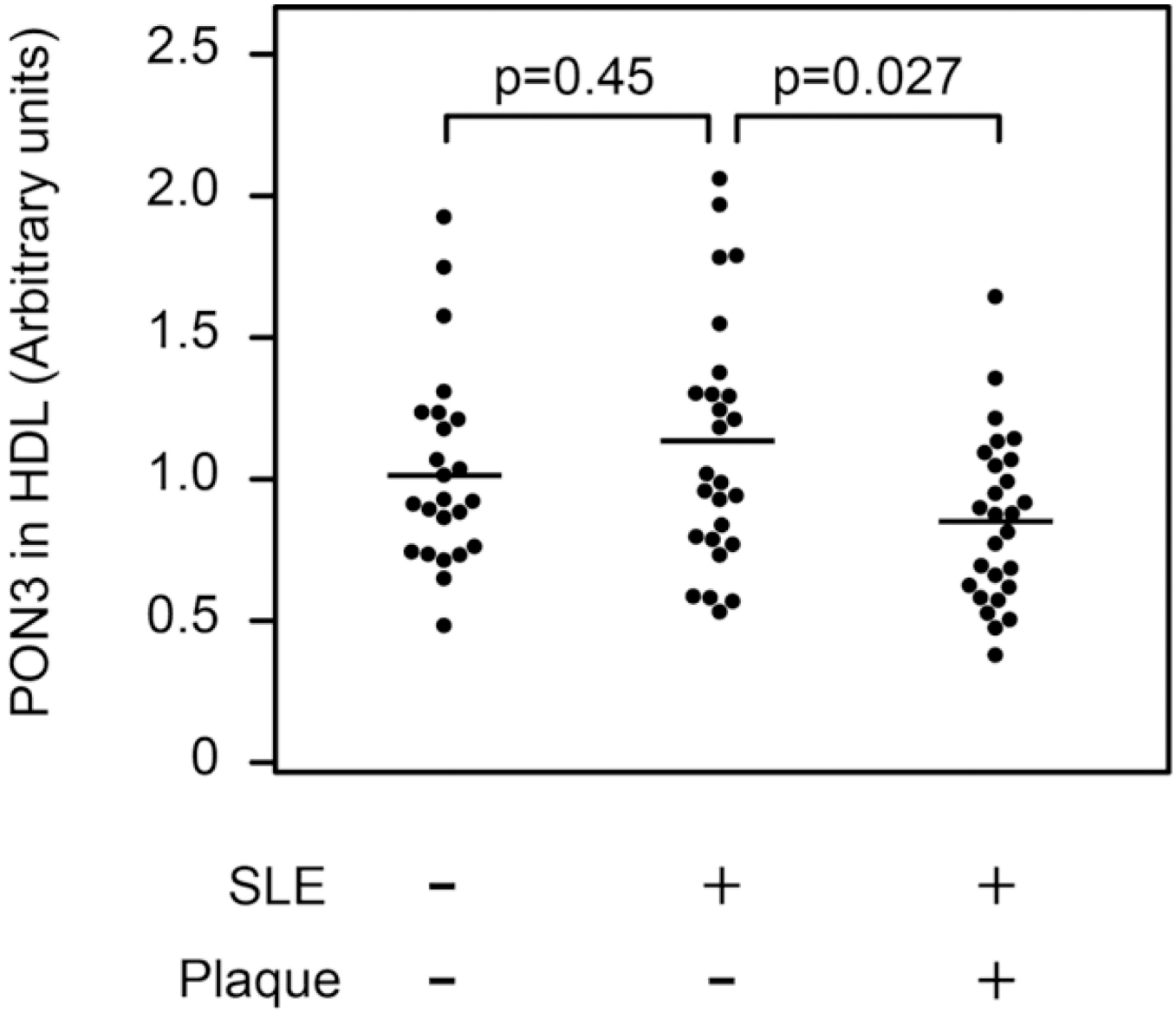


**Figure 1.**

PON3 in HDL from SLE subjects with carotid artery plaque. The relative concentrations of PON3 in the HDL fraction of plasma is shown for patients with or without SLE (without carotid plaque) and for patients with SLE and carotid plaque. The median of each group is represented with a horizontal bar. Comparison of patients with and without SLE revealed no statistically significant difference by Student's t-test. Within patients with SLE, there was a relative depletion of PON3 from HDL in patients with plaque, which was slightly statistically significant.

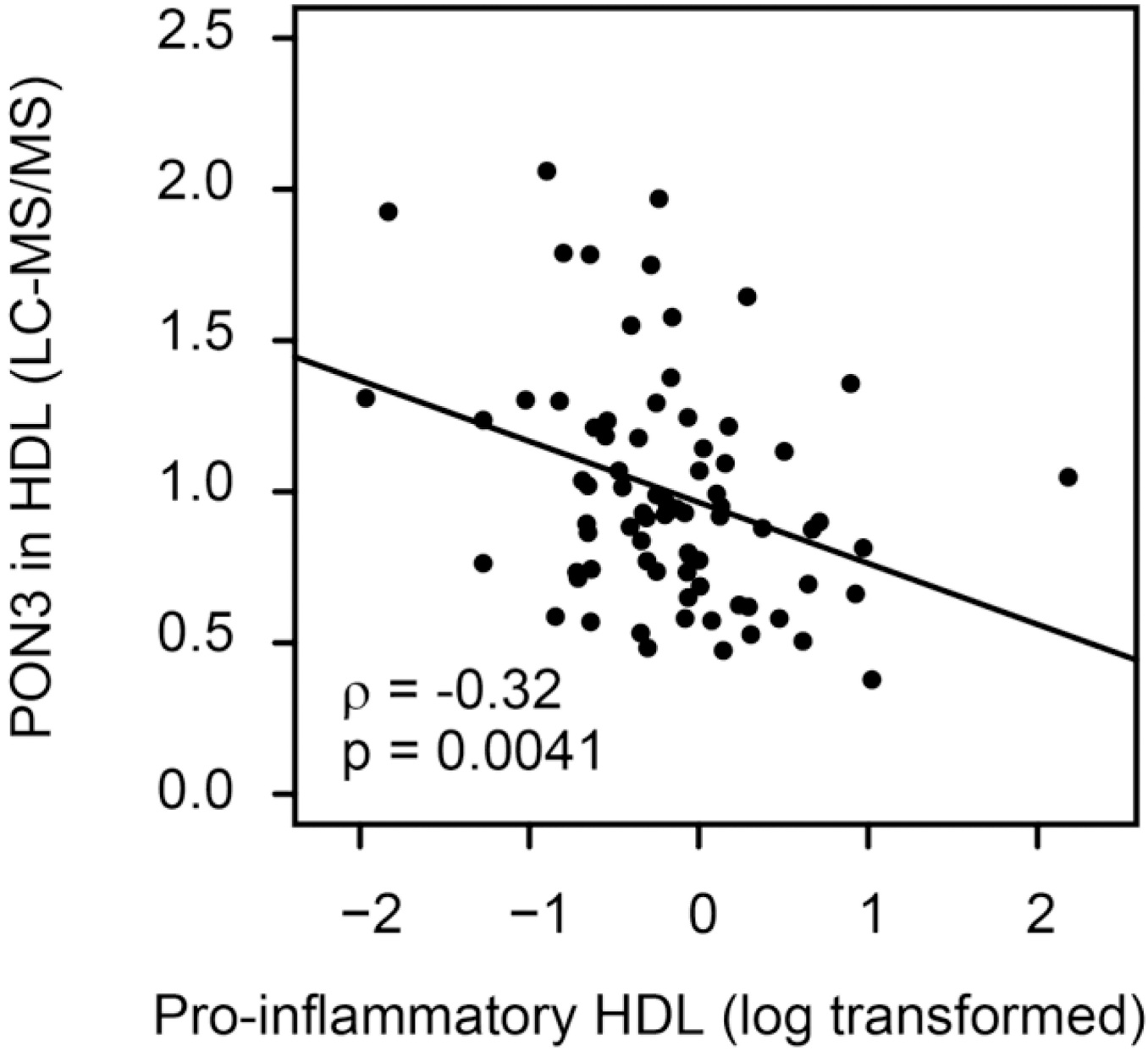


**Figure 2.**

Correlation of PON3 in HDL with pro-inflammatory activity of HDL. The relative concentrations of PON3 in HDL as measured by LC-MS/MS are plotted against the pro-inflammatory activity of HDL, which can be considered the inverse of the antioxidant activity of HDL. The pro-inflammatory activity of HDL was log transformed and linear regression was performed for illustrative purposes. The Spearman rank-order correlation coefficient (ρ) is listed, which was statistically significant.

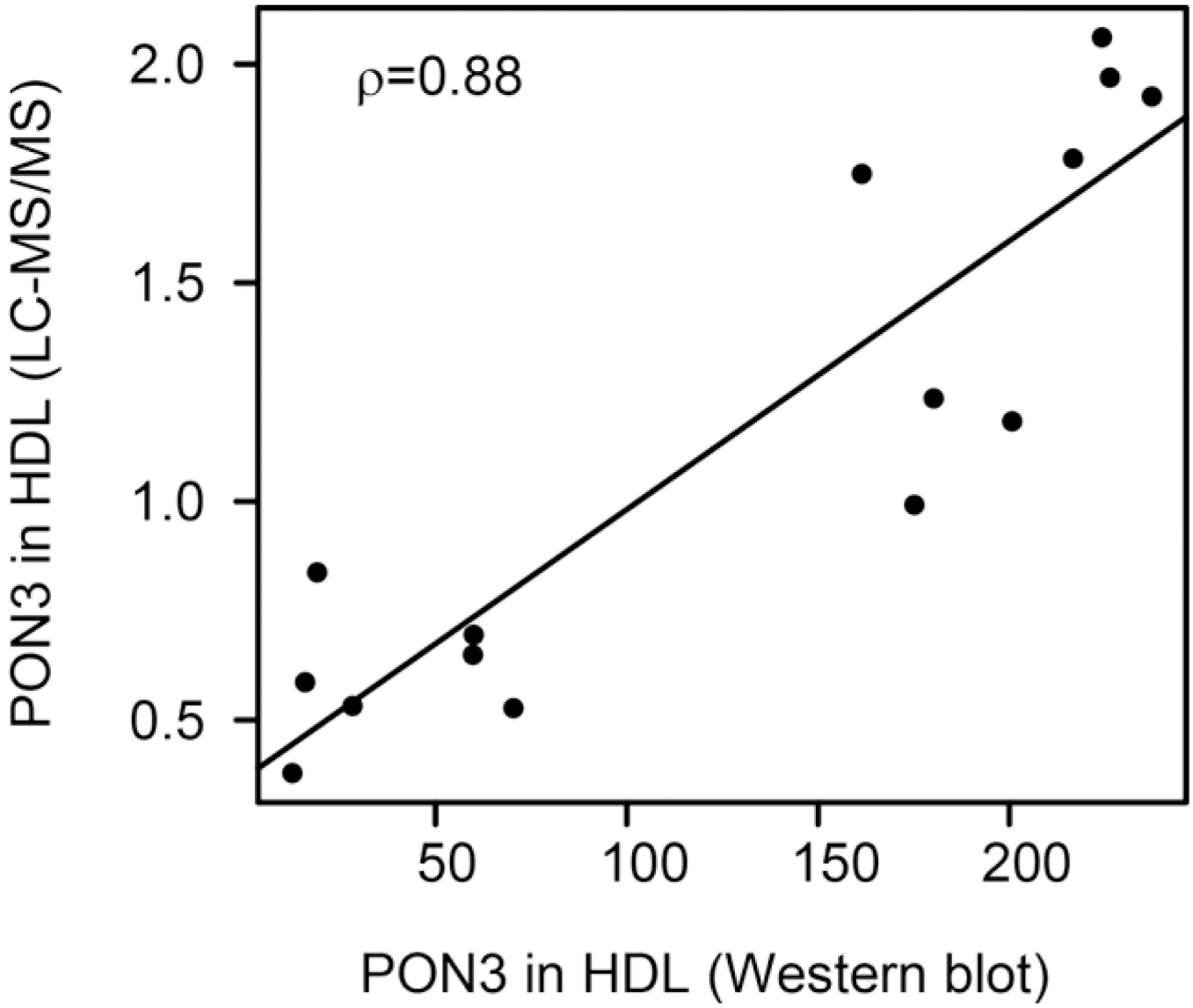


**Figure 3.**

Correlation of LC-MS/MS measurements of PON3 in HDL with immunochemical measurements. The relative concentrations of PON3 in HDL as measured by LC-MS/MS are plotted against the relative concentrations of PON3 in HDL as measured by Western blot (n = 15). The line of standard linear regression is shown, along with the Spearman correlation coefficient (ρ).

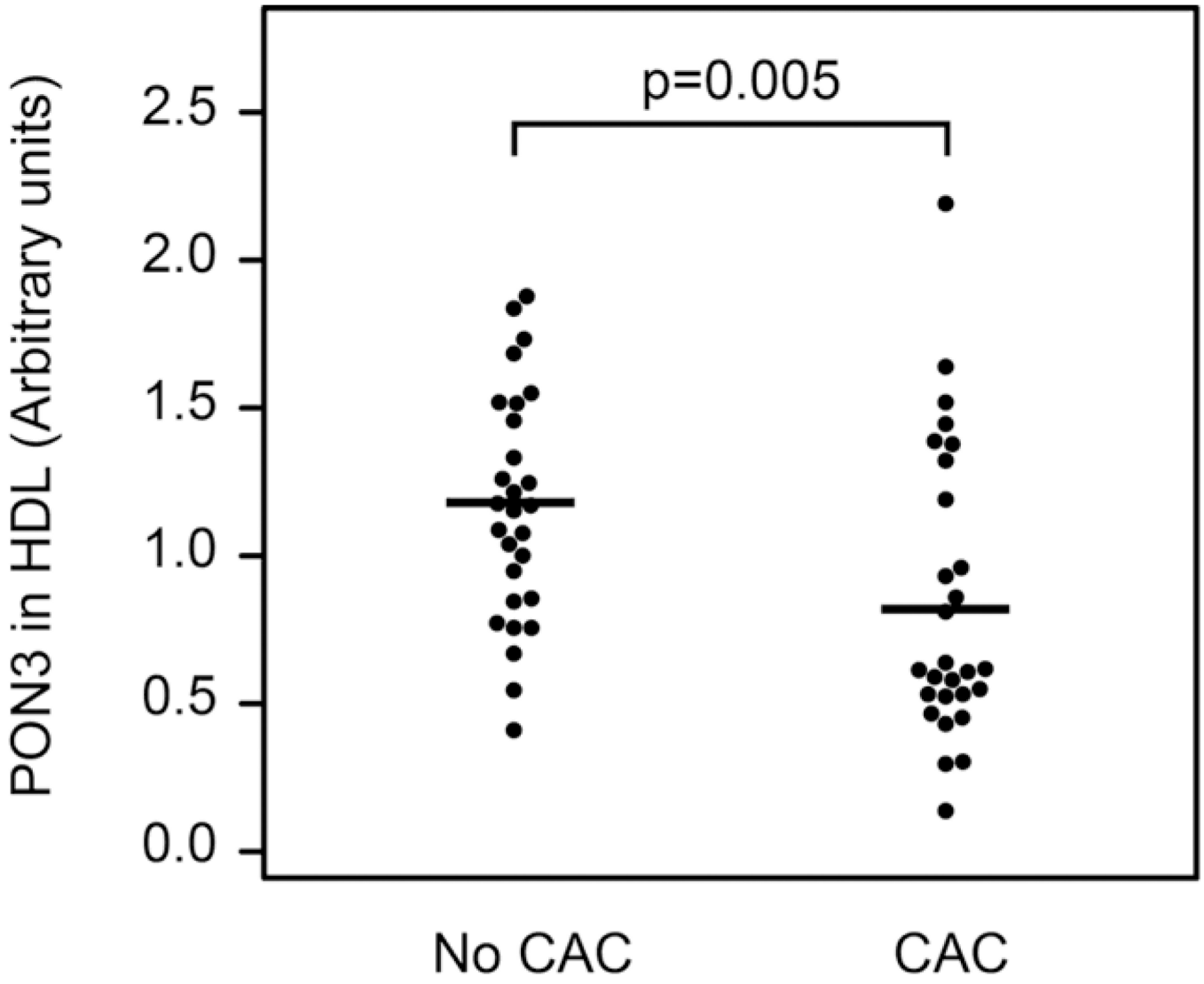


**Figure 4.**

PON3 in HDL from diabetic subjects with coronary artery calcification >100. The relative concentration of PON3 in the HDL fraction of plasma for type 1 diabetic subjects with and without coronary artery calcification (CAC) >100 is shown. The median of each group is represented with a horizontal bar. The results were statistically different by Wilcoxon rank sum test.

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**Table 1**

Clinical characteristics of the female SLE and control subjects[a]

| | Healthy | p-value (healthy vs. SLE)[b] | SLE no plaque | SLE plaque | p-value (plaque vs. no plaque)[b] |
|---|---|---|---|---|---|
| ***N*** | 25 | - | 26 | 27 | - |
| **Age (yr)** | **45.2 (7.6)** | **0.0031** | 51.3 (6.6) | 54.6 (9.6) | 0.24 |
| **Cholesterol (mg/dL)** | 198.8 (43.4) | 0.60 | 199.4 (45.3) | 210.1 (43.0) | 0.29 |
| **LDL-C (mg/dL)** | 117.5 (32.5) | 0.61 | 116.5 (32.6) | 127 (35.7) | 0.28 |
| **HDL-C (mg/dL)** | 64.8 (16.1) | 0.55 | 61 (17.2) | 58.7 (18.2) | 0.48 |
| **Triglycerides (mg/dL)** | 99.9 (42.0) | 0.99 | 109.4 (68.9) | 122.1 (62.1) | 0.28 |
| **Non-HDL-C (mg/dL)** | 136 (38.0) | 0.90 | 138 (39.8) | 150.6 (39.6) | 0.19 |
| **BMI (kg/m$^2$)** | 23.6 (3.1) | 0.38 | 26.1 (6.8) | 29.2 (7.4) | 0.08 |
| **SELENA-SLEDAI score** | - | - | **4.7 (3.1)** | **2.0 (2.0)** | **<0.001** |
| **SDI score** | - | - | 1.3 (1.4) | 1.7 (1.8) | 0.68 |
| **CRP (mg/L)** | 1.5 (1.3) | 0.68 | 1.9 (2.8) | 3.7 (8.1) | 0.34 |
| **% smokers, ever (n)**[c] | 24.0% (6) | 1.0 | 26.9% (7) | 33.3% (9) | 0.78 |
| **cIMT** | 0.6 (0.1) | 0.93 | **0.5 (0.1)** | **0.7 (0.1)** | **<0.001** |
| **Prednisone dose (mg)** | - | - | 3.4 (3.7) | 2.6 (6.3) | 0.91 |
| **%Hydroxychlorquine use (n)**[c] | - | - | 61.5% (16) | 63.0% (17) | 1.00 |
| **% Mycophenolate mofetil use (n)**[c] | - | - | 3.8% (1) | 18.5% (5) | 0.19 |
| **% Azathioprine use (n)**[c] | - | - | 15.4% (4) | 3.7% (1) | 0.19 |
| **% Other DMARD (n)**[c] | - | - | 19.2% (5) | 11.2% (3) | 0.72 |

[a] Data are presented as mean (SD) for clinical characteristics in the SLE cohort.

[b] Distributions were compared with the Wilcoxon signed-rank test.

[c] For smoking and the use of medications (hydroxychloroquine, mycophenolate mofetil, azathioprine and other DMARD), the percent of ever-smokers or those taking medication are presented as percentages (number) in each group. Proportions were compared using the Fisher's exact test.

SELENA-SLEDAI, Safety of Estrogens in Lupus Erythematosus-SLE Disease Activity Index; SDI, Systemic Lupus International Collaborating Clinics / ACR damage index; CRP, C-reactive protein; cIMT, carotid intimal media thickness; DMARD, disease modifying antirheumatic drugs.

## Table 2

Clinical characteristics of the male type 1 diabetic subjects[a]

| | CAC = 0[b] | CAC > 100[b] | p-value[c] |
|---|---|---|---|
| ***N*** | 28 | 28 | - |
| **Age (yr)** | 46.6 (5.6) | 46.6 (5.5) | - |
| **Cholesterol (mg/dL)** | 185.6 (32.3) | 189.1 (30.7) | 0.33 |
| **LDL-C (mg/dL)** | 117.3 (26.5) | 116.1 (28) | 0.52 |
| **HDL-C (mg/dL)** | 53.6 (14.5) | 48.3 (12.1) | 0.18 |
| **Triglycerides (mg/dL)** | 73.2 (40.8) | 123.4 (80.2) | **0.003** |
| **Non-HDL-C (mg/dL)** | 132.0 (32.9) | 140.8 (29.5) | 0.07 |
| **BMI (kg/m$^2$)** | 26.5 (4.4) | 29.2 (4) | **0.02** |
| **DM1 Duration (yr)** | 19.6 (4.8) | 19.9 (5.3) | 0.99 |
| **Creatinine (mg/dL)** | 1.0 (0.1) | 1.1 (0.2) | 0.10 |
| **HgbA1c (%)** | 7.5 (0.7) | 7.9 (1.1) | 0.06 |

[a] Data are presented as mean (SD) for clinical characteristics in the diabetes cohort.

[b] Coronary artery calcification was measured by computed tomography.

[c] Distributions were compared with the Wilcoxon signed-rank test.

CAC, coronary artery calcification; HgbA1c, hemoglobin A1c.

## Table 3

Univariate regression of PON3 vs. other clinical data[a]

| | SLE | | DCCT | |
|---|---|---|---|---|
| | $r^2$ | p-value | $r^2$ | p-value |
| **piHDL**[b] | −0.32 | **0.0041** | - | - |
| **Age** | 0.05 | **0.047** | 0.00 | 0.60 |
| **Cholesterol** | 0.00 | 0.55 | 0.00 | 0.82 |
| **HDL-C** | 0.02 | 0.18 | 0.00 | 0.89 |
| **LDL-C** | 0.02 | 0.24 | 0.00 | 0.72 |
| **Triglycerides** | 0.02 | 0.26 | 0.00 | 0.71 |
| **Non-HDL** | 0.02 | 0.23 | 0.01 | 0.87 |
| **BMI**[b] | −0.19 | **<0.001** | 0.06 | 0.66 |
| **Creatinine** | - | - | 0.00 | 0.78 |
| **HgbA1c** | - | - | 0.00 | 0.98 |

[a] Pearson correlation coefficients are expressed as $r^2$.

[b] Correlations between the relative concentration of PON3 in HDL and piHDL or BMI are expressed by the Spearman correlation coefficient (ρ) due to the non-normal distribution of piHDL and BMI.

piHDL, pro-inflammatory HDL score; HgbA1c, hemoglobin A1c.