Autoantibodies to ox-LDL in Sjögren's syndrome: are they atheroprotective?

I. Cinoku[1, 2], C.P. Mavragani[1, 3], C.C. Tellis[2], A. Nezos[3], A.D. Tselepis[2], H.M. Moutsopoulos[1, 4]

[1]Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens; [2]Atherothrombosis Research Centre/ Laboratory of Biochemistry, Department of Chemistry, University of Ioannina; [3]Department of Physiology, School of Medicine, National and Kapodistrian University of Athens; [4]Chair Medicine/ Immunology, Academy of Athens, Greece.

Ilir Cinoku, MD*
Clio P. Mavragani, MD*
Costantinos C. Tellis, PhD
Adrianos Nezos, PhD
*Alexandros D. Tselepis**, MD, PhD*
*Haralampos M. Moutsopoulos**, MD, FACP, FRCP (hc), Master ACR*

**Equally contributed as first author.*
***Equally contributed as last author.*

Please address correspondence to:
Dr Adrianos Nezos,
Department of Physiology, School of Medicine, National and Kapodistrian University of Athens,
M. Asias 75,
11527 Athens, Greece.
E-mail: anezos@med.uoa.gr

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ABSTRACT

Objective. *The higher incidence of atherosclerosis and cardiovascular disease (CVD) in patients with systemic autoimmune diseases cannot be attributed exclusively to traditional risk factors for CVD. Antibodies to oxidised Low Density Lipoprotein (ox-LDL) seem to have a crucial role in atherogenesis.*

Methods. *Sera from 63 consecutive patients with primary Sjögren's syndrome (pSS), 121 with systemic lupus erythematosus (SLE), 79 with rheumatoid arthritis (RA) and 26 apparently healthy individuals were evaluated for the presence of antibodies to ox-LDL by ELISA. The femoral and/or carotid intima media thickness (IMT) and plaque formation as well as traditional CVD risk factors and disease related features were recorded for all study participants.*

Results. *Anti-ox-LDL antibody levels were significantly reduced in SS and RA patients, but not in SLE patients, compared to their healthy counterparts. Subsequently, SS patients were divided into two groups according to antibody levels to ox-LDL, using as cut off the median of each group studied. SS patients with high titres of antibodies to ox-LDL displayed higher rates of autoantibodies to Ro/SSA and La/SSB antigens, purpura, low complement levels and increased SS activity index. On the other hand, the high anti-ox-LDL group was characterised by reduced rates of carotid and/or femoral plaque after adjusting for potential confounders (OR [95%CI]: 0.14 [0.03-0.72]). Such associations were not shown in all other groups included in the study.*

Conclusion. *These findings suggest that antibodies to ox-LDL, possibly resulting from B cell hyperactivity, might exert a protective role in the development of atherosclerosis among primary SS patients.*

Introduction

It is well established that patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and primary Sjögren's syndrome (SS), exhibit higher rates of subclinical atherosclerosis, compared to age- and sex-matched individuals (1-4). In both SS and SLE patients, traditional risk factors for cardiovascular disease (CVD) do not fully explain the heightened rates of subclinical atherosclerosis seen in these patients. Impaired endothelial function, autoantibodies to endothelial cells, type I Interferon (IFN) activity and neutrophil extracellular traps have been all proposed as potential culprits in SLE-related atherosclerosis (2, 5).

While oxidized-Low Density Lipoprotein (ox-LDL), is a well-established contributor in the pathogenesis of atherosclerosis (6, 7) the role of autoantibodies against ox-LDL in the setting of autoimmune related atherogenic disease, though previously investigated in RA and SLE, is rather conflicting (8-12). Given that data on these autoantibodies in the context of SS is scarce, in the present report we sought to explore the prevalence of autoantibodies against ox-LDL in patients with SS, RA and SLE and study whether they associate with distinct disease related manifestations as well as with markers of subclinical atherosclerosis.

Material and methods

Study subjects

Sixty-three consecutive patients with SS, 121 SLE patients and 79 RA patients fulfilling the corresponding classification criteria (13-15) as well as 26 apparently healthy individuals were included in the study. All patients were followed at the Department of Pathophysiology, School of Medicine, University of Athens and the Department

of Rheumatology, General Hospital of Athens G. Gennimatas, Greece. Pregnant women, patients younger than 18 years and older than 70 years and patients with renal dysfunction (serum creatinine levels >3mg/dl, creatinine clearance <30 ml/min) were excluded from the study. Sera were collected from all patients and healthy individuals and were stored at -80°C. The Ethics Committee of the National and Kapodistrian University of Athens (approved No. 6337) approved this study and all participants provided informed consent prior to their entry in the study.

Clinical assessment

Demographic data, including age, sex and body mass index (BMI), haematological, biochemical and immunological profiles and the classical risk factors for atherosclerosis were recorded in all participants, as previously described (2). For patients with pSS, glandular and extraglandular features, SS disease activity index (16) and histopathological characteristics were recorded, as previously published (2). In SLE patients, rash, photosensitivity, oral ulcers, arthritis, serositis, neurological disorders and Systemic Lupus International Collaborating Clinics/ American College of Rheumatology (SLICC/ACR) Damage Index Activity Index (SLEDAI) (17) were recorded. In patients with RA, recorded clinical features included morning stiffness, arthritis of specific joints, rheumatoid nodules and Disease Activity Score (DAS-28) (18).

Finally, the assessment of subclinical atherosclerosis was achieved via defining the presence of plaque and/or arterial wall thickening (defined as intima media thickness (IMT) score >0.90 mm) in carotid and femoral arteries as determined by ultrasound as previously described (2).

Determination of autoantibody titres against ox-LDL

Low density liporpotein (LDL) (d=1.019-1.063 g/ml) was isolated by sequential ultracentrifugation from pooled fresh plasma of normolipidaemic volunteers, as we previously described (19). LDL, at a final concentration of 100 μg protein/ml, was oxidised in the presence of 5 μmol/l CuSO4, for 3 h at 37ºC, under continuous measurement of the absorbance at 234 nm. Oxidation of LDL was terminated by the addition of 0.01% (v/v) EDTA during the decomposition phase. Ox-LDL preparations were stored 4°C under nitrogen and used within 4 weeks (20).

The serum autoantibody titres of IgG class against ox-LDL were determined by an ELISA method, using an HRP-conjugated rabbit anti-human IgG monoclonal antibody (Dako Cytomation, Denmark) (diluted 1:6000, v/v), as we have previously described (20-22). The results of anti-oxLDL titres are expressed as optical density (OD) values, after subtraction of the non-specific binding values (the binding of serum samples to gelatin-coated wells) which were remarkably low, at 0.021±0.005. All measurements were performed at 492 nm, in a multifunctional microplate reader (Infinite^{M} 200 Pro, TECAN, Italy). The OD values in all samples (including the nonspecific binding values) ranged from 0.036 to 0.435. Within-run and between-run coefficients of variation for the assay were 10.2% and 5.4%, respectively. Individuals were classified into 2 subgroups (high and low anti-oxLDL titres), using as cut off the median titre of anti-ox-LDL antibodies of each group studied.

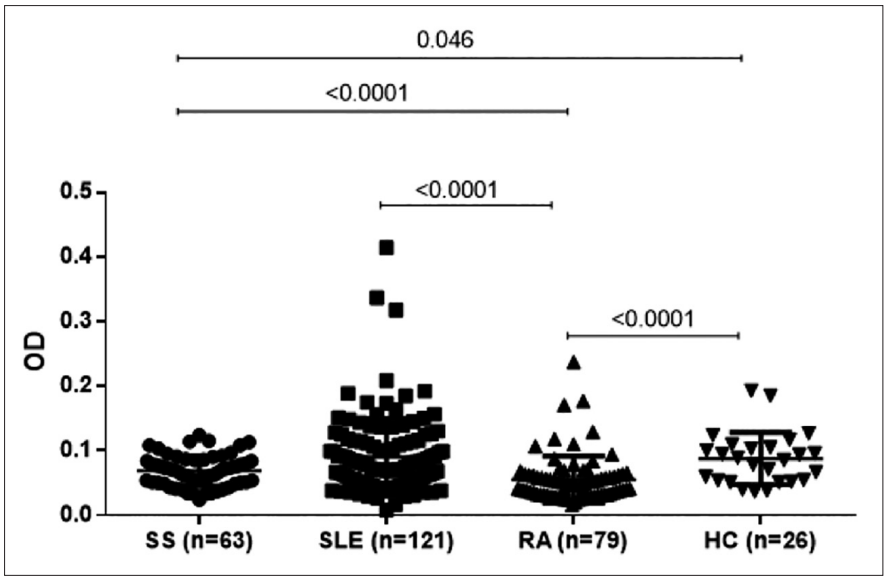

Fig. 1. Autoantibody titres of IgG class against ox-LDL in SS, SLE, RA and healthy individuals expressed as Optical Density (OD). Lower levels of antibodies to ox-LDL in SS and RA patients, but not in SLE patients, compared to their healthy counterparts (0.07±0.02 and 0.06±0.04 *vs*. 0.09±0.04, p=0.046 and p<0.0001, respectively). Also, lower serum anti-ox-LDL antibody levels in RA patients compared to both SLE and SS (0.06±0.04 *vs*. 0.09±0.06 and 0.07±0.02, respectively, p<0.0001 for all comparisons). No significant differences between SLE *vs*. SS and healthy individuals were detected. SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; SS: Sjögren's syndrome; HC: healthy controls.

Statistical analysis

Two-sided Fisher's exact/chi-square and Mann-Whitney tests were employed to compare qualitative and quantitative characteristics, respectively (GraphPad Prism 5.00, GraphPad Software, San Diego, CA, USA). In order to investigate the independent contribution of anti-ox-LDL antibodies in the development of plaque, a multivariate model was applied including as potential confounders age and lymphocytic count, previously shown to be independent determinants for plaque formation in our SS cohort (2) as well as features shown to be related to high anti-ox-LDL status in the current univariate analysis. For the multivariate analysis SPSS 23.0 statistical package has been implemented. The continuous variables in each group of subjects are expressed as mean values ± standard deviation (SD), while the distinct variables are expressed as percentages.

Results

Autoantibodies to ox-LDL in all studied groups

Clinical and laboratory parameters of all participants are presented in Supplementary Table I for all study participants. As shown in Figure 1, anti-ox-LDL antibody levels were significantly lower in SS and RA patients, but not in SLE patients, compared to their healthy counterparts (0.07±0.02 and 0.06±0.04 *vs*. 0.09±0.04, p=0.046 and p<0.0001, respectively). Moreover, RA patients exhibited lower serum anti-ox-LDL antibody levels compared to both SLE and SS (0.06±0.04 *vs*. 0.09±0.06 and 0.07±0.02, respectively, p<0.0001, for all comparisons). No significant differences were detected in the anti-ox-LDL antibody levels between SLE vs SS and healthy volunteers.

Antibodies to ox-LDL and disease-related characteristics in SS, SLE and RA patients

Regarding clinical/serological manifestations in SS, the high anti-ox-LDL group was characterised by increased SS disease activity index (3.4±2.1 *vs*. 1.5±1.3, p=0.02), higher rates of palpable purpura (23.3% *vs*. 0.0%, p=0.01), and anti-Ro/SSA and anti-La/SSB autoantibodies (87.1% *vs*. 56.3%, p=0.01 and 61.3% *vs*. 25.0%, p=0.005, respectively). Furthermore, lower complement C3 and C4 levels (1.06±0.26 *vs*. 1.17±0.23 mg/ml, p=0.04 and 0.17±0.06 *vs*. 0.23±0.08 mg/ml, p=0.002) were detected compared to their counterparts with lower anti-ox-LDL antibody levels, as shown in Table I. No statistically significant differences were detected between anti-ox-LDL positive and negative SS patients in the rates of hydroxychloroquine use (46.4% *vs*. 46.7%, respectively, p=0.98), in mean ±SD values of current (2.2±9.4mg *vs*. 1.2±2.4mg, respectively, p=0.43) or total steroid dose (3.3±5.4gr *vs*. 6.1 ±10.6gr, p=0.83) (data not shown).

As shown in Table II, SLE patients with high titres of anti-ox-LDL antibodies displayed higher serum complement C4 (0.30±0.126 *vs*. 0.18±0.21 mg/ml, p=0.006) and anti-cardiolipin-IgG (18.4±30.7 *vs*. 13.6±27.3 units, p=0.002), but lower high-density lipoprotein cholesterol (HDL-C) levels (48.7±16.5 *vs*. 52.9±14.0 mg/dl, p=0.04).

In RA patients (Table III), male preponderance (Female:Male prevalence: 71.8% *vs*. 95.0 %, p=0.005) and lower total cholesterol levels (195±36 *vs*. 212±38 mg/dl, p=0.05) were the main distinguishing features in the high versus the low anti-ox-LDL antibody

Table I. Disease related characteristics and markers of subclinical atherosclerosis according to antibodies to ox-LDL status (high *vs*. low) in SS patients.

	Anti-ox-LDL Antibodies		
	Low (n=32)	High (n=31)	*p*-value
Disease-related features			
SS Disease Activity Index (mean±SD)	1.5 ± 1.3	3.4 ± 2.1	0.02
Palpable purpura, %	0.0	23.3	0.01
Anti-Ro/SSA, %	56.3	87.1	0.01
Anti-La/SSB, %	25.0	61.3	0.005
C3 (mean±SD), mg/ml	1.17 ± 0.23	1.06 ± 0.26	0.04
C 4 (mean±SD), mg/ml	0.23 ± 0.08	0.17 ± 0.06	0.002
Markers of subclinical atherosclerosis			
High IMT (>0.90 mm), %	63.3	64.5	ns
IMT levels (mean±SD), mm	0.6 ± 0.5	0.7 ± 0.5	ns
Presence of plaque, %	83.3	50.0	0.01

SS: Sjögren's syndrome; IMT: intima media thickness.

Table II. Disease related characteristics and markers of subclinical atherosclerosis associated with antibodies to ox-LDL in SLE patients.

	Anti-ox-LDL Antibodies		
	Low (n=61)	High (n=60)	*p*-value
Disease-related features			
HDL-C (mean±SD), mg/dl	52.9 ± 14.0	48.7 ± 16.5	0.04
Complement 4 (mean±SD), mg/ml	0.18 ± 0.21	0.30 ± 0.126	0.006
ACL-IgG (mean±SD), units	13.6 ± 27.3	18.4 ± 30.7	0.002
Markers of subclinical atherosclerosis			
High IMT (>0.90 mm), %	40.7	32.7	ns
IMT levels (mean±SD), mm	0.9 ± 0.3	0.9 ± 0.3	ns
Presence of plaque, %	61.7	54.1	ns

HDL-C: high density lipoprotein cholesterol; ACL: anticardiolipin antibodies; IMT: intima media thickness.

Table III. Demographic characteristics and markers of subclinical atherosclerosis associated with antibodies to ox-LDL in RA patients.

	Anti-ox-LDL Antibodies		
	Low (n=40)	High (n=39)	*p*-value
Disease-related features			
Female, %	95.0	71.8	0.005
Total cholesterol levels (mean±SD), md/dl	212 ± 38	195 ± 36	0.05
Markers of subclinical atherosclerosis			
High IMT (>0.90 mm), %	76.3	68.6	ns
IMT levels (mean±SD), mm	1.2 ± 0.4	1.1 ± 0.5	ns
Presence of plaque, %	82.5	76.9	ns

IMT: intima media thickness.

group. Of interest, RA individuals found to display the lowest titres of anti-ox-LDL antibodies in their serum, had also increased rates of both plaque formation and arterial wall thickening (IMT>0.90mm) compared to healthy controls (79.8% and 73% in RA *vs*. 52% and 38.5% in healthy controls, p=0.0001 and 0.004, respectively, Supplementary Table I).

Association of antibodies to ox-LDL with markers of subclinical atherosclerosis

We next investigated whether anti-ox-LDL antibody levels were associated with markers of subclinical atherosclerosis in distinct patient groups. In the SS group (Table I, Fig. 2), patients with high titres of anti-ox-LDL antibodies were characterised by lower rates of plaque formation compared to those with low titres (50.0% *vs*. 83.3%, p=0.01). This association remained significant, when potential cofounders -previously shown to be independently associated with plaque formation in our SS cohort (age, lymphocytic number) (2)- together with features shown to be related to high anti-ox-LDL status (purpura, antibodies against Ro/SSA and La/SSB, low complement levels) were included in a multivariate regression model (OR [95%CI]: 0.14 [0.03-0.72]). No significant associations between autoantibody titres with plaque formation or carotid/femoral IMT scores were detected in SLE, RA and healthy individuals groups, when the medians of the oxLDL antibody distributions were used as cut-off (Tables II–IV). Of interest, the 3 SLE patients with the highest titres of anti-oxLDL antibodies (OD>0.3) displayed significantly lower IMT levels compared to those with lower titres (0.90±0.28 *vs*. 0.62±0.16, p=0.04) as well as decreased rates of plaque formation, though this difference was not significant (33.3% *vs*. 58.5%, p=0.57). Additionally, no significant differences were detected when IMT scores and rates of plaque formation were compared between RA patients with the highest anti-ox-LDL titres (OD>0.17) compared to the rest of the population tested (1.20±0.46 *vs*. 1.15±0.44, p=0.91 and 100% *vs*. 78.9%, p=0.1, respectively).

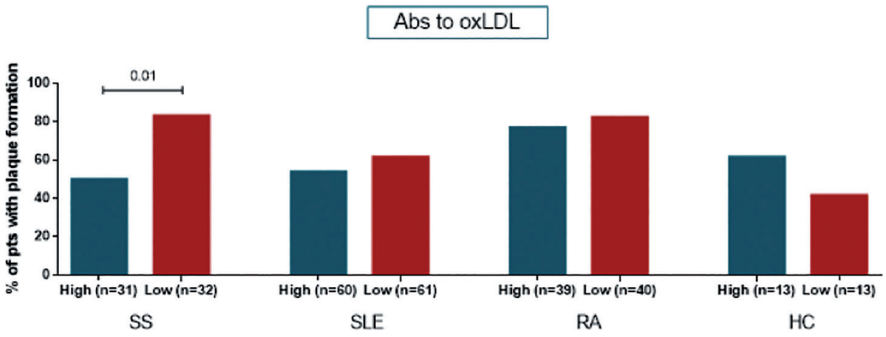

Fig. 2. Presence of plaque in SS, SLE, RA patients and healthy individuals according to antibody titres to ox-LDL. SS patients with high anti-ox-LDL antibody titres are characterised by lower rates of plaque formation compared to those with low titres (50.0% *vs*. 83.3%, p=0.01). No significant differences observed in the others groups compared. For the classification into high and low anti-oxLDL titres, the median titre of anti-ox-LDL antibodies of each group studied was implemented as cut-off value.
SS: Sjögren's syndrome; SLE: systemic lupus erythematosus; RA: rheumatoid athritis; HC: healthy controls.

Table IV. Demographic characteristics and markers of subclinical atherosclerosis associated with antibodies to ox-LDL in healthy individuals.

	Anti-ox-LDL Antibodies		
	Low (n=13)	High (n=13)	*p*-value
Disease related features			
Uric acid (mean±SD), mg/dl	3.3 ± 1.3	4.3 ± 1.3	0.043
Markers of subclinical atherosclerosis			
High IMT (>0.90 mm), %	23.1	53.8	ns
IMT levels (mean±SD), mm	0.8 ± 0.2	0.9 ± 0.2	0.037
Presence of plaque, %	41.7	61.5	ns

IMT: intima media thickness.

In the healthy control group (Table IV), the presence of high anti-ox-LDL titres was found to be associated with higher IMT and uric acid levels (0.9±0.2 *vs*. 0.8±0.2, p=0.037 and 4.3±1.3 *vs*. 3.3±1.3 mg/dl, p=0.043, respectively). CRP or ESR levels as indicators of inflammation did not differ between the two groups. However, this association did not remain significant in a multivariate model, taking into account variables which turned to be significant in the univariate model (data not shown).

Discussion

Though subclinical atherosclerosis is a well-established feature in many autoimmune diseases including SLE, RA and SS – even in the absence of traditional cardiovascular risk factors (1, 2) – the exact aetiopathogenesis remains to be elucidated**.** In the current study, we sought to investigate whether anti-ox-LDL antibodies could contribute to the generation of subclinical atherosclerosis in these patients. While no association between anti-ox-LDL antibodies and arterial plaque formation or arterial wall thickening were detected in RA or SLE groups, these autoantibodies were shown to confer an independent protective role against arterial plaque formation in the setting of SS, after correction for potential confounders including age, lymphocytic count, autoantibodies and disease activity indices. While both SS and RA patients exhibited lower autoantibody titres compared to HC groups, the potential atheroprotective properties were only confined in the SS group, for reasons not easily understood. Nevertheless, SLE patients with the highest anti-ox-LDL serum titres displayed lower median values of IMT scores together with lower rates of plaque formation (though non statistically significant different). A potential explanation could be that compared to

SS syndrome, SLE and RA patients are more likely to receive intensive immunosuppressant therapies, and therefore the association between autoantibodies against ox-LDL and subclinical atherosclerosis might be attenuated by the effect of medications.

A protective role of anti-ox-LDL autoantibodies for the development of atherosclerosis has been previously shown in healthy populations as well as in end stage renal disease (ESRD) patients given the negatively reported association between anti ox-LDL antibodies levels and carotid IMT levels in those populations (23, 24). Other investigators, studying experimental animal models, have also supported a protective role of both antibodies to ox-LDL and cellular immune system components, against the development of atherosclerosis (25). Indeed, the suppression of cellular immune components in rabbits seems to lead to increased development of atherosclerotic lesions (26). Additionally, immunisation of hypercholesterolaemic rabbits with copper oxidised LDL triggered the immune system to produce antibodies against ox-LDL leading to reduced formation of the atherosclerotic plaques. Taken together these data support the notion that the presence of antibodies against ox-LDL may display a protective role against the development of atherosclerosis (27).

The underlying mechanism behind this association remains obscure. One potential explanation could be provided by the fact that anti-ox-LDL antibodies promote clearance of ox-LDL from the circulation, as a result of immunocomplexes formation (28). Another suggested mechanism contributing to the lower levels of IgM anti-ox-LDL antibodies in patients with more extensive disease is the consumption of antibodies at the level of atherosclerotic plaque (29). Finally, it has been proposed that ox-LDL specific IgM monoclonal antibodies have biological properties, such as being able to block ox-LDL uptake by macrophage scavenger receptors and the formation of foam cells, which are crucial for atherosclerosis development (30). An atheroprotective role of IgG anti-ox-LDL antibodies as well in both animal models and human populations including healthy and RA individuals has been also proposed (11, 23, 31).

Of interest, high anti-ox-LDL levels in SS were found to be associated with several features denoting B cell hyperactivity, including higher SS disease activity index, palpable purpura, lower complement C3 and C4 levels, higher rates of anti-Ro/SSA and anti-La/SSB autoantibodies. These findings imply that anti-ox-LDL antibody development results from a hyperactive immune system. In line with these findings, anti-La/SSB positivity was more frequent in patients with normal pulse wave velocity (PWV), an index of arterial stiffness, compared with patients with increased PWV (32) and patients with anti-Ro/SSA and anti-La/SSB antibodies exhibited a lower prevalence of hypertension and hypercholesterolaemia (33). Those associations may support a putative protective role of specific autoantibodies in atherosclerosis development in SS patients, though data from Vaudo *et al*. revealed opposite results, with a positive association between the presence of anti-Ro/SSA and heightened carotid IMT levels (34). However, we cannot exclude the possibility of interaction between anti-ox-LDL antibodies and specific autoantibodies. Since previous studies revealed heightened oxidative stress levels in patients with SS, we postulate that the availability of radical oxygen species as a result of chronic inflammation lead to higher oxidation levels of LDL in the setting of active SS (35, 36).

Autoantibodies to ox-LDL were first identified in lupus patients at a prevalence ranging from 30-80%, often cross reacting with antiphospholipid antibodies and related to active lupus disease as a result of increased oxidative stress (37-40). In the present report, we also confirm the correlation of antiphospholipid antibodies, particularly of IgG anticardiolipin type, with the presence of anti-ox-LDL antibodies. Since, higher complement levels may reflect intense inflammatory activity, heightened oxidative stress might result in higher oxidation levels of LDL and consequently higher titres of anti-ox-LDL antibodies. While using as cut-off value the median of anti-ox-LDL distribution, no clear association between markers of subclinical atherosclerosis and the presence of antibodies against ox-LDL has been depicted in our SLE patients in accord with previous observations (9, 10), patients with the highest titres of anti-ox-LDL antibodies demonstrated lower IMT scores. In RA patients, previously published data suggested a prevalence of 10-35% (41, 42), in association with inflammatory markers or disease severity (42, 43). Increased inflammation in the setting of RA has been previously associated with low cholesterol levels through hypercatabolism of LDL particles (44). In this context, higher inflammatory activity leading to increased availability of radical oxygen species and increased oxidation of circulating LDL might provide a plausible explanation for the lower cholesterol levels in the high anti-ox-LDL RA group. While several investigators supported a potential link between anti-ox-LDL and carotid atherosclerosis (45), IMT (43) and the presence of plaque (8) in these patients, we and others could not confirm such associations (12). Of note and in accord with the currently reported data on SS patients, a negative correlation between the mean carotid IMT and the serum concentration of anti-ox-LDL antibodies has been previously mentioned in an RA population (11). Larger multi-ethnic cohorts are warranted to confirm the above findings taking into account the limitations of the study, the relatively small number of SS patients and the retrospective nature of the study.

In conclusion, autoantibodies to ox-LDL were found to be less prevalent in primary SS patients and particularly in those with evidence of subclinical atherosclerosis. These findings provide a newly suggested mechanism for the pronounced atherosclerotic risk seen in these patients.

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