Low Levels of Antibodies Against Oxidized but not Nonoxidized Cardiolipin and Phosphatidylserine Are Associated with Atherosclerotic Plaques in Systemic Lupus Erythematosus

Jun Su, Anna G. Frostegård, Xiang Hua, Thomas Gustafsson, Tomas Jogestrand, Ingiäld Hafström, and Johan Frostegård

ABSTRACT. ***Objective.*** We have reported that the prevalence of atherosclerotic plaques and their echolucency was increased in systemic lupus erythematosus (SLE). We here study antibodies against oxidized cardiolipin (anti-OxCL) and phosphatidylserine (anti-OxPS) in SLE and in relation to atherosclerosis measures.

Methods. Patients with SLE (n = 114) were compared with age- and sex-matched population-based controls (n = 122). Common carotid intima-media thickness and plaque occurrence were determined by B-mode ultrasonography. Plaques were graded according to echogenicity as 1–4, with 1 being echolucent. Antibodies were determined by ELISA.

Results. In the SLE group, the prevalence of low IgM anti-OxPS and low total IgM levels (below 33rd percentile) was increased compared to controls ($p = 0.045$ and $p = 0.0079$, respectively). Among SLE patients with atherosclerotic plaques, the prevalence of low IgM anti-OxPS ($p = 0.0019$) and anti-OxCL ($p = 0.031$) was increased. Only IgM anti-OxPS remained significant ($p = 0.019$) after adjusting for other significant factors. Echolucent plaques (total, or left side) were more prevalent among SLE patients with low IgM anti-OxCL and anti-OxPS when controlled for other significant factors ($p < 0.05$). Low total IgM was independently associated with echolucent plaque on left side ($p < 0.05$), but not other atherosclerosis measures. IgM anticardiolipin antibodies (aCL) and antiphosphatidylserine antibodies (anti-PS) were higher among SLE patients with cardiopulmonary disease, including arterial, valvular, and venous disease ($p < 0.05$). There were no associations between antibodies and other disease manifestations or activity. Both anti-OxCL and anti-OxPS, in contrast to aCL, and anti-PS, were cofactor–β_2-glycoprotein I (β_2-GPI)-independent.

Conclusion. The prevalence of low levels of IgM anti-OxCL and anti-OxPS (both cofactor-β_2-GPI-independent) is associated with the presence of plaques and echolucent plaques in SLE. (J Rheumatol First Release Sept 15 2013; doi:10.3899/jrheum.121173)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS ATHEROSCLEROSIS OXIDATION
VULNERABLE PLAQUE ANTICARDIOLIPIN ANTIBODIES PHOSPHATIDYLSERINE

Atherosclerosis is the leading cause of cardiovascular disease (CVD) and is an inflammatory condition in which activated immune-competent cells, producing mainly pro-inflammatory cytokines, are present in the lesions[1]. The inflammatory nature of atherosclerosis was described in the early 19th century by Rokitansky and Virchow[2]. Development of atherosclerosis can be modulated by immune system, as demonstrated in animal models in which immunization with putative antigens [oxidatively modified

From the Institute of Environmental Medicine, Unit of Immunology and Chronic Disease, Karolinska Institutet, Stockholm; Department of Laboratory Medicine, Division of Clinical Physiology, and Department of Rheumatology, Karolinska Institutet at Karolinska University Hospital, Huddinge, Stockholm, Sweden.

Supported by the regional agreement on medical training and clinical research (ALF) between Stockholm county council and the Karolinska Institutet, the Swedish Science Fund, the King Gustav V 80th year's foundation, The Swedish Heart Lung foundation, The Swedish Rheumatism Association, CIDaT, Vinnova, Torsten Söderberg Foundation, AFA. Sixth Framework Program of the European Union, Priority 1: Life sciences, genomics and biotechnology for health (grant LSHM-CT-2006-037227 CVDIMMUNE), with Dr. Frostegård as coordinator.

J. Su, PhD; A.G. Frostegård, MD, PhD; X. Hua, MD, PhD, Institute of Environmental Medicine, Unit of Immunology and Chronic Disease, Karolinska Institutet; T. Gustafsson, MD, PhD; T. Jogestrand, MD, PhD, Department of Laboratory Medicine, Division of Clinical Physiology, Karolinska Institutet at Karolinska University Hospital; I. Hafström, MD, PhD, Department of Rheumatology, Karolinska Institutet at Karolinska University Hospital; J. Frostegård, MD, PhD, Institute of Environmental Medicine, Unit of Immunology and Chronic Disease, Karolinska Institutet.

Dr. Su and Dr. Frostegård contributed equally to this manuscript.

Address correspondence to Dr. A.G. Frostegård, Unit of Immunology and Chronic Disease, Institute of Environmental Medicine, Karolinska Institutet, S-171 77 Stockholm, Sweden. E-mail: anna.frostegard@ki.se

Accepted for publication June 11, 2013.

forms of low-density lipoprotein (OxLDL)] ameliorates atherosclerosis[3]. A potential protective function of B cells has been suggested in human studies where posttraumatic splenectomy was linked to an increased risk of myocardial infarction[4].

In systemic lupus erythematosus (SLE), the risk of CVD is raised and a combination of traditional and nontraditional risk factors appears to account for this. Increased prevalence of atherosclerotic plaques is associated with CVD in both general and SLE population in several independent case-control studies[5,6,7,8].

Traditional risk factors for CVD in SLE include dyslipidemia and hypertension, while nontraditional inflammation-related factors include raised activity of tumor necrosis factor, levels of platelet-activating factor acetylhydrolase, and C-reactive protein, as well as circulating OxLDL[8,9,10,11,12]. Recently, we have demonstrated that natural IgM antibodies against phosphorylcholine (anti-PC) are protection biomarkers for atherosclerosis and CVD in both general[13] and SLE populations[14,15].

Antiphospholipid antibodies (aPL) and the antiphospholipid antibody syndrome are of special interest in SLE, because aPL in SLE represent a major risk factor for stroke, deep venous thrombosis, and pulmonary embolism[6,8]. Of note, aPL levels measured in such a clinical context are very high. Typically, routinely-used methods define aPL as present with levels 2–3 above SD compared to a general population control group. Prothrombotic aPL properties [anticardiolipin (aCL) in particular] might involve interference with annexin A5 binding to endothelium[16,17]. Importantly, CL and phosphatidylserine (PS) have been described as major aPL antigens, when presented in association with cofactors such as β2-glycoprotein I (β_2-GPI).

Rupture of atherosclerotic plaques is of major importance in CVD, and it is crucial to identify factors promoting this. We reported that prevalence of echolucent plaques, as determined by ultrasound (US) measurements, are increased in SLE[14]. This finding may explain the increased risk of CVD in SLE because plaque echolucency is suggested to indicate increased vulnerability[18,19].

To our knowledge, the clinical appearance or associations with plaque presence of IgM anti-OxCL and anti-OxPS have not been described previously in SLE or other autoimmune conditions.

MATERIALS AND METHODS

Study group. The study group consisted of 114 patients with SLE from the Karolinska University Hospital, Huddinge, and 122 sex-matched and age-matched population-based controls. The details of this SLE Vascular Impact Cohort (SLEVIC) study have been published[14].

All patients fulfilled the 1982 revised criteria of the American College of Rheumatology for SLE. The study was approved by the Karolinska Institutet Research Ethics Committee and performed in accordance with the Helsinki Declaration. All subjects gave informed consent before entering the study.

Study protocol. The investigation included a written questionnaire, an interview, a physical examination by a rheumatologist, laboratory determinations, and an US examination of the carotid arteries in all but 3 patients. SLE activity was determined by the Systemic Lupus Activity Measure and also by the Systemic Lupus Erythematosus Disease Activity Index[20]. Organ damage was determined through the Systemic Lupus International Collaborating Clinics Damage Index[20].

Assays. To determine oxidation of CL and PS, they were purchased as ethanol solution (Sigma GmbH) and stored at –20°C. CL and PS were oxidized in aqueous solutions containing 1.5 mmol/l tert-butylhydroperoxide and CuSO4 in 20 μmol/l. Phospholipids were measured with mass spectrometry (electrospray ionization mass spectrometer, Micromass), to confirm that CL and PS had been oxidized by copper and tert-butylhydroperoxide.

IgM and IgG antibodies to OxCL and OxPS were determined by ELISA. Serum from a healthy donor was used as internal standard and tested on every plate, and set at 100 arbitrary units as a standard to which the SLEVIC cohort sera were compared. The plateau of antibody binding was reached with the antigen concentration of 10 μg/ml. Immulon 1B plates (Thermo Labsystems) were coated with OxCL or OxPS (10 μg/ml) 50 μl/well in ethanol. Coated plates were incubated overnight at 40°C. After 5 washings with phosphate buffered saline (PBS), the plates were blocked with 2% bovine serum albumin (BSA)-PBS for 2 h at room temperature and washed as described. Serum samples were diluted (1:50) in 0.2% BSA-PBS and added at 50 μl/well.

Plates were incubated overnight at 40°C and washed as described. Alkaline phosphatase (ALP) conjugated goat antihuman IgM (diluted 1:7000 in the sample buffer) and IgG (diluted 1:7000 in the sample buffer) were added at 100 μl/well and incubated at 40°C overnight. After 5 washings, color was developed by adding the ALP substrate at 100 μl/well and incubating the plates for 60 min at room temperature in the dark. The plates were read in an ELISA Multiscan Plus spectrophotometer at 405 nm. All samples were measured in duplicates in a single assay, and the coefficient of variation was below 10–15%.

IgM antibodies to CL and PS where measured by a standard ELISA kit (Orgentec Diagnostika GmbH) according to the manufacturer's description, although samples were diluted 10 times less than the instructions indicated, so that not only very high antibody levels were measured.

Carotid B-mode US. The right and left carotid arteries were examined with a duplex scanner (Sequoia, Siemens Acuson) using a 6 MHz linear array transducer, as described[14].

The far wall of the common carotid artery (CCA), 0.5 to 1.0 cm proximal to the beginning of the carotid bulb, was used for measurements of the intima-media thickness (IMT). The IMT was defined as the distance between the leading edge of the lumen-intima echo and the leading edge of the media-adventitia echo. The CCA lumen diameter was defined as the distance between the leading edge of the intima-lumen echo of the near wall and the leading edge of the lumen-intima echo of the far wall. The examinations were digitally stored for subsequent analyses by a computer system[21]. The mean values of the IMT and lumen diameter within the 10 mm section were calculated. When a plaque was observed in the region of the CCA measurements, the IMT was not measured.

Carotid plaque was defined as a localized intima-media thickening > 1 mm and at least a 100% increase in thickness compared with adjacent wall segments and was screened for in the common, internal, and external carotid arteries. Plaque occurrence was scored as the absence of plaque, the presence of unilateral plaque, and the presence of bilateral plaque. Plaque morphology in terms of echogenicity was assessed in a modified version of the classification proposed by Gray-Weale, *et al*[22] and graded from 1 to 4 as echolucent, predominantly echolucent, predominantly echogenic, and echogenic. Echolucency was defined with the arterial lumen as reference and echogenicity with the far wall adventitia as reference.

The US methods used have been described in detail[23,24].

Statistical methods. Determinations were dichotomized or determined as

continuous variables as indicated. We calculated percentiles based on distributions in the whole study group. Age, sex, and geography were matched for by the design of the study. Data are presented as means (with 95% CI) or medians (with interquartile ranges), depending on their distribution. Comparisons between groups were made with the Mann-Whitney U test, median test, or Student t-test. To establish the association between potential risk factors for atherosclerosis and atherosclerotic plaque, logistic regression was applied with adjustment for covariates. Stat View was used for the statistical analyses (release 4.5, SAS Institute Inc.).

RESULTS

The characteristics of patients and controls are presented in Table 1. Some of the data have been published[14], but are presented for background information and clarity. IMT did not differ between patients and controls. As reported[14], there were more atherosclerotic plaques among patients with SLE ($p = 0.029$), and left-sided echolucent plaques were more prevalent in SLE compared to controls ($p < 0.016$).

Cardiovascular/cardiopulmonary disease occurrence was increased in SLE ($p < 0.01$) when defined as a history of cerebrovascular events, acute coronary syndrome, coronary artery bypass graft, heart valve prosthesis/impairment, peripheral arterial surgery, claudication, deep venous thrombosis, and pulmonary embolism.

There were no significant associations between prevalence of echolucent plaques and history of cardiovascular and thrombotic disease (data not shown).

Antibody levels. Antibody levels among patients with SLE and controls are presented in Table 1. Low IgM anti-OxPS

Table 1. Characteristics of the Systemic Lupus Erythematosus Vascular Impact Cohort (SLEVIC) study groups.

Characteristics	Cases	Controls	p
No. subjects	114	122	
Age, yrs, mean ± SD	47.94 ± 13.17	49.11 ± 12.68	0.45
Male sex, % (n)	12.28 (14)	10.65 (13)	0.94
Current smokers, % (n)	14.03 (16)	15.57 (19)	0.47
Presence of diabetes, % (n)	5.26 (6)	2.45 (3)	0.22
Presence of hypertension, % (n)	57.89 (66)	26.22 (32)	< 0.001
LDL > 3 mmol/l, % (n)	27.19 (31)	44.26 (54)	0.006
Current statins, % (n)	10.52 (12)	4.09 (5)	0.048
Total cholesterol, mmol/l, mean ± SD	4.7 ± 1.1	4.8 ± 1.0	0.23
LDL, mmol/l, mean ± SD	2.5 ± 0.88	2.8 ± 0.80	0.026
HDL, mmol/l*	1.6 (1.3–1.8)	1.6 (1.3–1.9)	0.31
Triglycerides, mmol/l*	0.99 (0.7–1.4)	0.78 (0.55–1.10)	0.003
CRP, mg/dl*	4.44 (0.8–4.8)	2.04 (0.5–2.5)	< 0.001
BMI, kg/m^2*	24.89 (20.96–27.85)	24.67 (22.41–27.82)	0.58
Atherosclerotic plaque, % (n)	42.98 (49)	30.32 (37)	0.029
Low-echogenic plaques (grade 1); LCA or RCA	33	24	0.0931
Low-echogenic plaques (grade 1); LCA	25	13	0.016
Low-echogenic plaques (grade 1); RCA	19	18	0.62
CVD**, % (n)	24.8 (29)	4.1 (5)	< 0.001
Total IgG, g/l, median (IQR)	12.4 (4.8)	9.6 (2.4)	< 0.001
Total IgG ≤ 50th percentile (%)	32.4	65.8	< 0.001
Total IgG ≤ 33rd percentile (%)	21.6	44.2	< 0.001
IgM anti-OxPS, AU, mean ± SD	101.5 ± 39.6	108.0 ± 27.0	0.16
IgM anti-OxPS ≤ 50th percentile (%)	55.2	44.3	0.13
IgM anti-OxPS ≤ 33rd percentile (%)	39.7	26.4	0.045
IgM anti-PS, U/ml, median (IQR)	234.0 (398)	206.0 (168.5)	0.0009
IgM anti-OxCL, AU, mean ± SD	97.4 ± 38.0	87.3 ± 26.4	0.0022
IgM anti-OxCL ≤ 50th percentile (%)	46.0	53.8	0.28
IgM anti-OxCL ≤ 33rd percentile (%)	42.2	37.7	0.20
IgM aCL, U/ml, % (n)	151.0 (291.2)	148.0 (113.3)	0.064
Total IgM, g/l, median (IQR)	0.85 (0.99)	1.1 (0.60)	0.26
Total IgM ≤ 50th percentile (%)	58.0	42.5	0.025
Total IgM ≤ 33rd percentile (%)	42.0	25.0	0.0079

Serum from a healthy donor was used as internal standard and tested on every plate, and set at 100 arbitrary units (AU) as a standard to which the SLEVIC cohort sera were compared. Anti-CL and anti-PS were measured by a commercial ELISA kit, with Units/ml as measure. * Median (range). ** History of any of cerebrovascular events, AMI, CABG, heart valve prosthesis/impairment, peripheral arterial surgery, claudication, peripheral vein thrombosis, or pulmonary embolism. LCA/RCA: left/right carotid artery; LDL: low-density lipoprotein; HDL: high-density lipoprotein; CRP: C-reactive protein; BMI: body mass index; CVD: cardiovascular disease; anti-OxPS: antibodies against oxidized phosphatidylserine; anti-OxCL: antibodies against oxidized cardiolipin; AMI: acute myocardial infarction; CABG: coronary artery bypass graft; IQR: interquartile range.

(lowest tertile) and low total IgM were more common among patients with SLE than controls ($p < 0.05$), while low anti-OxCL was not. IgM anti-OxCL, aCL (trendwise), and anti-PS were significantly higher among patients with SLE, although prevalence of low levels did not differ significantly (data not shown). There were no significant independent associations between medication and antibody levels.

Atherosclerotic plaques and antibodies. In a multivariate analysis we included only factors in the model, which were independently associated with prevalence of atherosclerotic plaques (age, hyperlipidemia, hypertension, and glucose levels).

As described in Table 2, SLE patients with atherosclerotic plaques had lower IgM anti-OxPS and anti-OxCL levels ($p = 0.0034$ and 0.076, respectively). For anti-OxPS levels, the difference remained after adjusting for other risk factors (age, hyperlipidemia, hypertension, and glucose levels; $p = 0.029$). IgM aCL, anti-PS, and total IgM were not independently associated with plaques. There was no association between total IgG, IgG anti-OxCL, or IgG anti-OxPS and atherosclerotic plaques (data not shown).

Echolucent plaques and antibodies. In multivariate analyses, we included only factors in the model that were independently associated with prevalence of echolucent plaques, left or right [age, hyperlipidemia (LDL > 3 mmol/l)] and prevalence of echolucent plaques on the left side (age) in preceding univariate analyses.

As described in Table 3, SLE patients with echolucent plaques (left or right side) had lower IgM anti-OxPS and anti-OxCL levels ($p = 0.0078$; $p = 0.043$, respectively) but only IgM anti-OxPS was significant after controlling for age and hyperlipidemia ($p = 0.018$). Further, prevalence of low anti-OxPS and anti-OxCL levels was more prevalent among SLE patients with echolucent plaques than without ($p = 0.030$ and $p = 0.030$, respectively, after controlling for age and hyperlipidemia). Other antibodies measured (IgM aCL and anti-PS and total IgM) were not significantly associated with presence of echolucent plaques. There was no association between total IgG, IgG anti-OxCL, and anti-OxPS and echolucent atherosclerotic plaque measures (data not shown), and low levels of these antibodies were not associated with SLE (data not shown).

Table 4 describes the associations of the measured antibodies with plaque properties on the left side. SLE patients with echolucent plaques (on left side) had lower IgM anti-OxPS levels ($p = 0.0069$) and anti-OxCL levels ($p = 0.016$), which for anti-OxPS remained significant after controlling for age. Prevalence of low anti-OxPS and anti-OxCL levels was more common among SLE patients with echolucent plaques on the left side than without ($p = 0.012$ for anti-OxPS below 33rd percentile and $p = 0.011$ for anti-OxCL IgM below the 50th percentile after controlling for age). Also, low levels of total IgM were associated with echolucent plaques after controlling for age ($p < 0.05$).

Other antibodies measured (IgM aCL and anti-PS) were not significantly associated with the presence of echolucent plaques.

There was no association between total IgG, IgG anti-OxCL, anti-OxPS, and atherosclerotic plaques or echolucent atherosclerotic plaques (data not shown).

Antibodies and cardiovascular/cardiopulmonary history. There were no associations between the antibodies determined and history of CVD (as defined), except that IgM aCL and anti-PS were higher among patients with SLE who had CVD than among those with no history of CVD ($p < 0.05$). If CVD was defined as a history of arterial disease

Table 2. Antiphospholipid antibodies among systemic lupus erythematosus (SLE) patients with and without atherosclerotic plaques.

Antibodies	Patients with SLE Without Plaque, n = 62	Patients with SLE With Plaque, n = 49	P Value, Crude	P Value Adjusted for Age, Hyperlipidemia, Hypertension, and Glucose
IgM anti-OxPS, AU, mean ± SD	111.6 ± 39.5	89.7 ± 36.6	0.0034	0.029
IgM anti-OxPS ≤ 33rd percentile (%)	27.42	57.14	0.0019	0.019
IgM anti-OxPS ≤ 50th percentile (%)	46.8	65.3	0.033	0.26
IgM anti-PS, U/ml, median (IQR)	270.5 (452)	182.5 (424)	0.61	—
IgM anti-OxCL, AU, mean ± SD	104.1 ± 37.3	91.1 ± 37.8	0.076	—
IgM anti-OxCL ≤ 33rd percentile (%)	19.3	39.6	0.031	0.13
IgM anti-OxCL ≤ 50th percentile (%)	37.1	58.3	0.055	—
IgM aCL, U/ml, n (%)	187 (302)	123 (254)	0.55	—
Total IgM, U/ml, g/l	0.91 (0.90)	0.73 (0.99)	0.095	—
IgM ≤ 33rd percentile (%)	33.3	51.0	0.079	—
IgM ≤ 50th percentile (%)	53.3	63.2	0.33	—

Serum from a healthy donor was used as internal standard and tested on every plate, and set at 100 arbitrary units (AU) as a standard to which the SLEVIC cohort sera were compared for anti-OxCL or anti-OxPS. aCL and anti-PS were measured by a commercial ELISA kit, with units/ml as measure. SLEVIC: Systemic Lupus Erythematosus Vascular Impact Cohort; anti-OxPS: antibodies against oxidized phosphatidylserine; anti-OxCL: antibodies against oxidized cardiolipin; IQR: interquartile range.

Table 3. Antiphospholipid antibodies among SLE patients with and without echolucent atherosclerotic plaques (on left or right side).

Antibodies	Without Echolucent Plaque, n = 78	Patients with SLE With Echolucent Plaque; Left or Right Side n = 33	P Value, Crude	P Value Adjusted for Age and Hyperlipidemia
IgM anti-OxPS, U/ml, mean ± SD	108.4 ± 40.3	86.7 ± 33.4	0.0078	0.018
IgM anti-OxPS ≤ 33rd percentile (%)	32.0	60.6	0.0063	0.030
IgM anti-OxPS ≤ 50th percentile (%)	49.3	69.7	0.063	—
IgM anti-PS, U/ml, median (IQR)	251 (452)	179 (340)	0.22	—
IgM anti-OxCL, AU, n (%)	103.1 ± 38.2	87.3 ± 35.3	0.043	0.10
IgM anti-OxCL ≤ 33rd percentile (%)	19.4	45.5	0.009	0.030
IgM anti-OxCL ≤ 50th percentile (%)	37.6	63.6	0.021	0.045
IgM aCL, U/ml, median (IQR)	168 (307)	98 (221)	0.09	—
Total IgM, g/l	0.91 (1.0)	0.70 (0.62)	0.054	—
IgM ≤ 33rd percentile (%)	37.6	54.5	0.0899	—
IgM ≤ 50th percentile (%)	54.5	66.6	0.29	—
IgM anti-PC, U/ml, median (IQR)	71.2 (189.4)	50.1 (58.9)	0.009	0.075
IgM anti-PC ≤ 33rd percentile (%)	35.1	54.5	0.088	—
IgM anti-PC ≤ 50th percentile (%)	50.7	72.7	0.035	—

Serum from a healthy donor was used as internal standard and tested on every plate, and set at 100 arbitrary units (AU) as a standard to which the SLEVIC cohort sera were compared for anti-OxCL or anti-OxPS. Anti-PC, aCL, and anti-PS were measured by a commercial ELISA kit, with units/ml as measure. SLE: systemic lupus erythematosus; anti-OxPS: antibodies against oxidized phosphatidylserine; anti-OxCL: antibodies against oxidized cardiolipin; SLEVIC: SLE Vascular Impact Cohort; IQR: interquartile range; anti-PC: antibodies against phosphorylcholine.

Table 4. Antiphospholipid antibodies among SLE patients with and without echolucent atherosclerotic plaques (on left side).

Antibodies	Without Echolucent Plaque Left Side, n = 86	Patients with SLE With Echolucent Plaque Left Side, n = 25	P Value, Crude	P Value Adjusted for Age
IgM anti-OxPS, AU, mean ± SD	107.5 ± 40.3	82.7 ± 31.0	0.0054	0.037
IgM anti-OxPS ≤ 33rd percentile (%)	29.51	57.45	0.0023	0.012
IgM anti-OxPS ≤ 50th percentile (%)	50.0	76.0	0.024	0.037
IgM anti-PS, U/ml, median (IQR)	275 (454)	182 (408)	0.45	—
IgM anti-OxCL, AU, n (%)	103.1 ± 37.7	82.4	0.016	0.055
IgM anti-OxCL ≤ 33rd percentile (%)	23.2	48.0	0.0023	0.059
IgM anti-OxCL ≤ 50th percentile (%)	36.6	72	0.0023	0.011
IgM aCL, U/ml	188 (310)	123 (177)	0.40	—
Total IgM, g/l, median (IQR)	0.94 (1.0)	0.69 (0.44)	0.017	0.037
IgM ≤ 33rd percentile (%)	35.7	60.0	0.038	0.044
IgM ≤ 50th percentile (%)	52.9	76.0	0.040	0.039
IgM anti-PC, U/ml	70.7 (185.0)	50.0 (42.8)	0.096	—
IgM anti-PC ≤ 33rd percentile (%)	36.6	56.0	0.11	—
IgM anti-PC ≤ 50th percentile (%)	51.2	76.0	0.0376	0.092

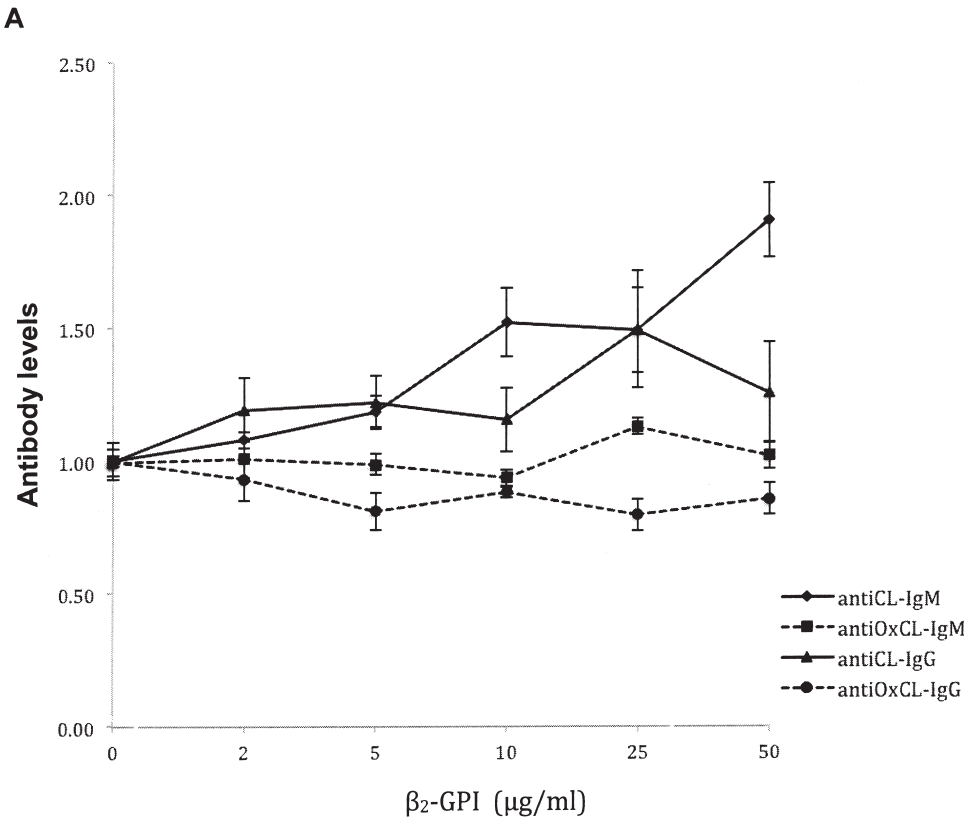
Serum from a healthy donor was used as internal standard and tested on every plate, and set at 100 arbitrary units (AU) as a standard to which the SLEVIC cohort sera were compared for anti-OxCL or anti-OxPS. Anti-PC, aCL, and anti-PS were measured by a commercial ELISA kit, with units/ml as measure. SLE: systemic lupus erythematosus; SLEVIC: SLE Vascular Impact Cohort; anti-OxPS: antibodies against oxidized phosphatidylserine; anti-OxCL: antibodies against oxidized cardiolipin; IQR: interquartile range; anti-PC: antibodies against phosphorylcholine.

including claudication, myocardial infarction, cerebral infarction, coronary artery bypass surgery, or coronary stents, then similar data were obtained with IgM anti-PS and IgM aCL as risk markers ($p < 0.05$), while other antibodies showed no associations.

Antibody specificity. Preincubation with OxCL or OxPS could inhibit up to 50–60% of IgM anti-OxCL and anti-OxPS binding, respectively (data not shown). Binding to OxCL β_2-GPI could induce increased aCL and anti-PS binding to CL and PS but not anti-OxCL or OxPS binding to OxCL (Figures 1A, 1B).

DISCUSSION

Plaque vulnerability and plaque rupture are of major impor-



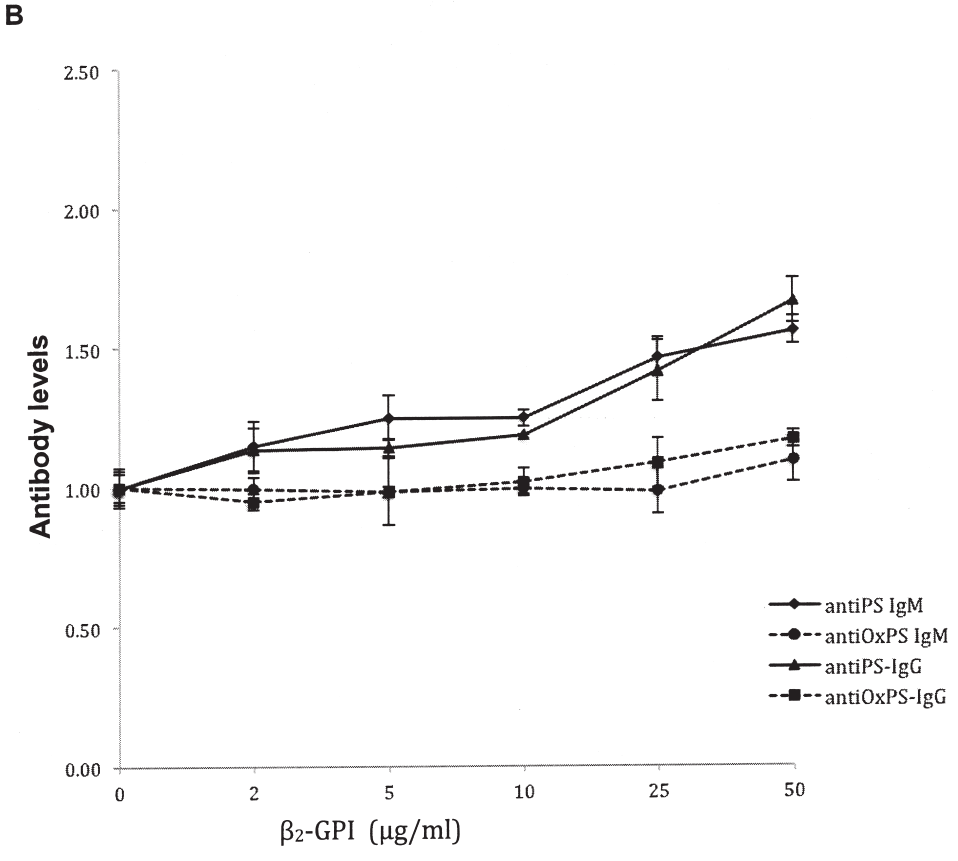


Figure 1. The graphs show the importance of β_2-GPI in antibody binding to antigens. A. ELISA of IgG anti-OxCL, IgG aCL, IgM anti-OxCL, and IgM aCL. B. IgG anti-OxPS, IgG anti-PS, IgM anti-OxPS, and IgM anti-PS, which had been incubated with a different concentration of β_2-GP1 for 1 h at room temperature. β_2-GPI increased aCL binding to CL and anti-PS binding to PS but not anti-OxCL binding to OxCL or anti-OxPS binding to PS. Antibody levels were determined by ELISA, and the optical density value at which no β_2-GPI was added was set at 1 as a standard. The results are expressed as mean ± SD. Anti-OxCL: antibodies against oxidized cardiolipin; anti-OxPS: antibodies against oxidized phosphatidylserine; β_2-GPI: β_2-glycoprotein I.

tance as causes of CVD[25]. Echolucent plaques are considered to represent more vulnerable atherosclerotic lesions[18,19]. One surprising finding in our study was that low levels of IgM anti-OxPS and anti-OxCL were significantly associated with prevalence of vulnerable (echolucent) plaques in general and on the left side, independently of other risk factors.

Further, IgM anti-OxPS, and trendwise, IgM anti-OxCL, were also negatively associated with prevalence of plaques independent of other risk factors, low levels giving rise to high risk. In contrast, antibodies against non-oxidized CL or PS were not significantly associated with the different atherosclerosis measures. Another interesting finding is that low levels of IgM anti-OxPS were more prevalent among patients with SLE compared to controls.

CL and PS are intimately involved in processes such as apoptosis and phagocytosis[26], and antibodies against these compounds are well known as CVD risk factors in SLE. Their mechanisms involve direct effects on endothelium and interference with the coagulation cascade through annexin A5[6,16].

CL is synthesized in the inner mitochondrial membrane of eukaryotic cells and in bacteria[27,28] by CL synthase, an enzyme that is most active in high metabolic tissue such as heart muscle. CL has an unusual, easily oxidized phospholipid structure. Oxidation of CL occurs *in vitro* during association with traditional ELISA methods[29]. *In vivo* CL undergoes oxidation during apoptosis, by cytochrome C. Oxidized CL promotes release of intrinsic proapoptotic factors[30]. OxCL is exposed on apoptotic cells and OxCL is suggested to be one of the pattern of recognition for antibodies[31].

Recognition and clearance of apoptotic cells/debris is a physiological process in which externalization of PS on membrane is an important signal to phagocytes. Without rapid and efficient clearance, remaining apoptotic material might play a role in chronic inflammation and autoimmunity[32].

Oxidized forms of PS may also be important during apoptosis, mediating macrophage recognition and engulfment of apoptotic cells[33]. Extramitochondrial cytochrome C is one factor that could catalyze apoptosis-associated PS oxidation[34]. Further, OxPS is a ligand for the scavenger receptor CD36[35].

Because defective clearance of apoptotic cells and/or bodies is an important feature of SLE[36], our findings may imply that a defective natural immune response against OxCL and OxPS may contribute to SLE manifestations, including plaque vulnerability.

We reported that IgM antibodies against another phospholipid-related epitope, phosphorylcholine (anti-PC), is a protection marker for CVD including stroke and myocardial infarction and atherosclerosis in the general population[13] and in RA[37] and SLE[15]. PC is a component of cell and lipoprotein membranes and is recognized by the humoral immune system when exposed, as in oxidation and during apoptosis[38]. PC is not a component of CL or PS. In our current study, anti-OxPS and anti-OxCL were significantly associated with vulnerable plaques, while anti-PC was only trendwise so. Thus one interesting possibility is that IgM anti-OxCL and anti-OxPS represent a line of defense, like anti-PC, in autoimmune and chronic inflammatory conditions.

To our knowledge, the clinical roles of anti-OxCL or anti-OxPS have not been determined in autoimmune disease, though we recently found a negative association between β_2-GPI–independent IgM anti-OxCL and anti-OxPS with development of CVD in the general population[39].

Though the exact binding properties of aPL to PL have been debated for decades, it appears that plasma protein cofactors such as β_2-GPI are of major importance for traditional aPL such as aCL and anti-PS, though it is still possible that some binding occurs to CL or mildly oxidized versions of CL *per se*[15]. It is therefore interesting that the air-exposed CL (undergoing oxidation) described in a previous publication in fact is a risk marker in a mouse model of atherosclerosis and not a protection marker, as in humans in our current study[29]. Such air-exposed oxidized CL required β_2-GPI as a cofactor for optimal recognition by antibodies[40] in contrast to the OxCL studied herein. It is not clear whether PS, as an antigen in the traditional aPL ELISA, behaves in a similar way as CL, though this would not be unexpected because PS is oxidizable.

In our study, IgM aCL and anti-PS, in contrast to IgM anti-OxCL and anti-OxPS, were positively associated with CV (defined as arterial disease, thrombosis, and valvular engagement), which is not unexpected for these traditional aPL. There were no associations between history of CV or thrombotic disease and presence of echolucent plaques, which may be attributed to the relatively low number of cases in the groups. Further, such plaques may cause future CV events.

Natural IgM in general bind apoptotic cells, increase phagocytosis of apoptotic cells, and have antiinflammatory properties[41]. In our study, low levels of total IgM were associated with vulnerable plaques on the left side, and insignificantly with other atherosclerosis measures, and thus those levels appear to be weaker protection markers than IgM anti-OxPS and anti-OxCL. Interestingly, it has been demonstrated that murine monoclonal IgM-recognizing forms of OxCL distinguish apoptotic cells from healthy cells[31]. Still, this finding suggests that total IgM might have atheroprotective properties in SLE. This notion is in line with a recent report in which natural IgM was required for suppression of experimental inflammatory arthritis induced by apoptotic cells[42]. In addition to anti-PC, anti-OxCL, and anti-OxPS, there may well be other yet unidentified natural

atheroprotective antibodies. In addition to directly inhibiting inflammatory phospholipids[15] and uptake of OxLDL in macrophages[39], there are other potential mechanisms, such as increasing clearance of dying/dead cells within plaques.

There are limitations in our study. One is the size. It would be of interest if the data herein could be confirmed in other larger studies. Whether some of the anti-OxCL or anti-OxPS are present in immune complexes is an interesting possibility and beyond the scope of the present article, which focused on the totality of OxCL/OxPS-binding antibodies. It is also possible that other methods such as magnetic resonance imaging could be developed into better surrogate ways to measure atherosclerotic plaques and their vulnerability than the US methods used here.

Our data indicate that there are negative associations between IgM anti-OxCL and anti-OxPS and atherosclerotic plaques and echolucent plaques in SLE, as determined by US. Further, they are not dependent on plasma cofactor β_2-GPI, in contrast to aCL/antiPS studied. Low levels of these antibodies could predispose to atherosclerotic complications in SLE, and possibly also contribute to the development of SLE itself.

ACKNOWLEDGMENT

Cristina Anania is acknowledged for sample collection.

REFERENCES

1. Frostegård J, Ulfgren AK, Nyberg P, Hedin U, Swedenborg J, Andersson U, et al. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis 1999;145:33-43.
2. Mayerl C, Lukasser M, Sedivy R, Niederegger H, Seiler R, Wick G. Atherosclerosis research from past to present—on the track of two pathologists with opposing views, Carl von Rokitansky and Rudolf Virchow. Virchows Arch 2006;449:96-103.
3. Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. Proc Natl Acad Sci U S A 1995;92:821-5.
4. Robinette CD, Fraumeni JF Jr. Splenectomy and subsequent mortality in veterans of the 1939-45 war. Lancet 1977;2:127-9.
5. Doria A, Shoenfeld Y, Wu R, Gambari PF, Puato M, Ghirardello A, et al. Risk factors for subclinical atherosclerosis in a prospective cohort of patients with systemic lupus erythematosus. Ann Rheum Dis 2003;62:1071-7.
6. Frostegard J. Atherosclerosis in patients with autoimmune disorders. Arterioscler Thromb Vasc Biol 2005;25:1776-85.
7. Roman MJ, Shanker BA, Davis A, Lockshin MD, Sammaritano L, Simantov R, et al. Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus. N Engl J Med 2003;349:2399-406.
8. Svenungsson E, Jensen-Urstad K, Heimburger M, Silveira A, Hamsten A, de Faire U, et al. Risk factors for cardiovascular disease in systemic lupus erythematosus. Circulation 2001;104:1887-93.
9. 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.
10. Manzi S, Selzer F, Sutton-Tyrrell K, Fitzgerald SG, Rairie JE, Tracy RP, et al. Prevalence and risk factors of carotid plaque in women with systemic lupus erythematosus. Arthritis Rheum 1999;42:51-60.
11. Petri M, Roubenoff R, Dallal GE, Nadeau MR, Selhub J, Rosenberg IH. Plasma homocysteine as a risk factor for atherothrombotic events in systemic lupus erythematosus. Lancet 1996;348:1120-4.
12. Svenungsson E, Fei GZ, Jensen-Urstad K, de Faire U, Hamsten A, Frostegard J. TNF-alpha: a link between hypertriglyceridaemia and inflammation in SLE patients with cardiovascular disease. Lupus 2003;12:454-61.
13. Frostegard J. Low level natural antibodies against phosphorylcholine: a novel risk marker and potential mechanism in atherosclerosis and cardiovascular disease. Clin Immunol 2010;134:47-54.
14. Anania C, Gustafsson T, Hua X, Su J, Vikstrom M, de Faire U, et al. Increased prevalence of vulnerable atherosclerotic plaques and low levels of natural IgM antibodies against phosphorylcholine in patients with systemic lupus erythematosus. Arthritis Res Ther 2010;12:R214.
15. Su J, Hua X, Concha H, Svenungsson E, Cederholm A, Frostegard J. Natural antibodies against phosphorylcholine as potential protective factors in SLE. Rheumatology 2008;47:1144-50.
16. Cederholm A, Svenungsson E, Jensen-Urstad K, Trollmo C, Ulfgren AK, Swedenborg J, et al. Decreased binding of annexin v to endothelial cells: a potential mechanism in atherothrombosis of patients with systemic lupus erythematosus. Arterioscler Thromb Vasc Biol 2005;25:198-203.
17. Frostegard AG, Su J, von Landenberg P, Frostegard J. Effects of anti-cardiolipin antibodies and IVIg on annexin A5 binding to endothelial cells: implications for cardiovascular disease. Scand J Rheumatol 2010;39:77-83.
18. Kwee RM, van Oostenbrugge RJ, Hofstra L, Teule GJ, van Engelshoven JM, Mess WH, et al. Identifying vulnerable carotid plaques by noninvasive imaging. Neurology 2008;70 Pt 2:2401-9.
19. Mathiesen EB, Bønaa KH, Joakimsen O. Echolucent plaques are associated with high risk of ischemic cerebrovascular events in carotid stenosis: the tromsø study. Circulation 2001;103:2171-5.
20. Griffiths B, Mosca M, Gordon C. Assessment of patients with systemic lupus erythematosus and the use of lupus disease activity indices. Best Pract Res Clin Rheumatol 2005;19:685-708.
21. Wendelhag I, Liang Q, Gustavsson T, Wikstrand J. A new automated computerized analyzing system simplifies readings and reduces the variability in ultrasound measurement of intima-media thickness. Stroke 1997;28:2195-200.
22. Gray-Weale AC, Graham JC, Burnett JR, Byrne K, Lusby RJ. Carotid artery atheroma: comparison of preoperative B-mode ultrasound appearance with carotid endarterectomy specimen pathology. J Cardiovasc Surg 1988;29:676-81.
23. Lemne C, Jogestrand T, de Faire U. Carotid intima-media thickness and plaque in borderline hypertension. Stroke 1995;26:34-9.
24. Nowak J, Nilsson T, Sylven C, Jogestrand T. Potential of carotid ultrasonography in the diagnosis of coronary artery disease: a comparison with exercise test and variance ECG. Stroke 1998;29:439-46.
25. Packard RR, Lichtman AH, Libby P. Innate and adaptive immunity in atherosclerosis. Semin Immunopathol 2009;31:5-22.
26. Fadeel B, Xue D, Kagan V. Programmed cell clearance: molecular regulation of the elimination of apoptotic cell corpses and its role in the resolution of inflammation. Biochem Biophys Res Commun 2010;396:7-10.
27. Paradies G, Petrosillo G, Paradies V, Ruggiero FM. Oxidative stress, mitochondrial bioenergetics, and cardiolipin in aging. Free Radic Biol Med 2010;48:1286-95.
28. Martin W, Hoffmeister M, Rotte C, Henze K. An overview of

endosymbiotic models for the origins of eukaryotes, their ATP-producing organelles (mitochondria and hydrogenosomes), and their heterotrophic lifestyle. Biol Chem 2001;382:1521-39.
29. Horkko S, Miller E, Dudl E, Reaven P, Curtiss LK, Zvaifler NJ, et al. Antiphospholipid antibodies are directed against epitopes of oxidized phospholipids. Recognition of cardiolipin by monoclonal antibodies to epitopes of oxidized low density lipoprotein. J Clin Invest 1996;98:815-25.
30. Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, Amoscato AA, et al. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat Chem Biol 2005;1:223-32.
31. Tuominen A, Miller YI, Hansen LF, Kesaniemi YA, Witztum JL, Horkko S. A natural antibody to oxidized cardiolipin binds to oxidized low-density lipoprotein, apoptotic cells, and atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2006;26:2096-102.
32. Jitkaew S, Witasp E, Zhang S, Kagan VE, Fadeel B. Induction of caspase- and reactive oxygen species-independent phosphatidylserine externalization in primary human neutrophils: role in macrophage recognition and engulfment. J Leukoc Biol 2009;85:427-37.
33. Arroyo A, Modriansky M, Serinkan FB, Bello RI, Matsura T, Jiang J, et al. NADPH oxidase-dependent oxidation and externalization of phosphatidylserine during apoptosis in Me2SO-differentiated HL-60 cells. Role in phagocytic clearance. J Biol Chem 2002;277:49965-75.
34. Jiang J, Kini V, Belikova N, Serinkan BF, Borisenko GG, Tyurina YY, et al. Cytochrome c release is required for phosphatidylserine peroxidation during Fas-triggered apoptosis in lung epithelial A549 cells. Lipids 2004;39:1133-42.
35. Greenberg ME, Sun M, Zhang R, Febbraio M, Silverstein R, Hazen SL. Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. J Exp Med 2006;203:2613-25.
36. Muñoz LE, Janko C, Schulze C, Schorn C, Sarter K, Schett G, et al. Autoimmunity and chronic inflammation - two clearance-related steps in the etiopathogenesis of SLE. Autoimmun Rev 2010; 10:38-42.
37. Ajeganova S, Ehrnfelt C, Alizadeh R, Rohani M, Jogestrand T, Hafstrom I, et al. Longitudinal levels of apolipoproteins and antibodies against phosphorylcholine are independently associated with carotid artery atherosclerosis 5 years after rheumatoid arthritis onset—a prospective cohort study. Rheumatology 2011;50:1785-93.
38. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, et al. Innate and acquired immunity in atherogenesis. Nat Med 2002;8:1218-26.
39. Su J, Hua X, Vikstrom M, Leander K, Gigante B, Hellenius ML, et al. Low levels of IgM antibodies to oxidized cardiolipin increase and high levels decrease risk of cardiovascular disease among 60-year olds: a prospective study. BMC Cardiovasc Disord 2013;13:1.
40. Horkko S, Miller E, Branch DW, Palinski W, Witztum JL. The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and beta2 glycoprotein 1 (and other proteins). Proc Natl Acad Sci U S A 1997;94:10356-61.
41. Gronwall C, Vas J, Silverman GJ. Protective roles of natural IgM antibodies. Front Immunol 2012;3:66.
42. Notley CA, Brown MA, Wright GP, Ehrenstein MR. Natural IgM is required for suppression of inflammatory arthritis by apoptotic cells. J Immunol 2011;186:4967-72.