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Elevated oxidized lipids, anti-lipid autoantibodies and oxidized lipid immune complexes in active SLE

Yujin Ye[b,c], Tianfu Wu[a], Ting Zhang[a], Jie Han[b], Deena Habazi[a], Ramesh Saxena[b], Chandra Mohan[a,*]

[a] Department of Biomedical Engineering, University of Houston, Houston, TX, USA
[b] Department of Internal Medicine and Immunology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA
[c] Department of Rheumatology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China

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ABSTRACT

Background: Here, we explore the serum levels of anti-oxidized lipid autoantibodies as well as immune complexes in patients with SLE and determine their correlation with disease.
Methods: Serum levels of oxidized-LDL immune complexes, autoantibodies to dsDNA, ox-LDL, MDA-LDL, 9-HODE, 13-HODE and POVPC were detected by ELISA in 64 SLE patients and 9 healthy controls.
Results: Active SLE patients exhibited increased serum levels of autoantibodies compared to healthy controls, including anti-MDA-LDL-IgG ($p = .003$), anti-ox-LDL-IgG ($p = .004$), anti-9-HODE-IgG ($p = .001$), anti-13-HODE-IgG ($p = .0003$), anti-POVPC-IgG ($p = .001$) and ox-LDL-IC ($p = .003$). Serum anti-ox-LDL-IgG was positively correlated with SLEDAI ($r = 0.34$; $p = .01$), and negatively with C3 ($r = -0.40$; $p = .01$). Anti-9-HODE-IgG and anti-POVPC-IgG were positively correlated with SLEDAI and negatively with C4.
Conclusions: Active SLE patients exhibit significantly increased serum levels of IgG anti-oxidized-lipid autoantibodies. Coordinated elevation of oxidized lipids, autoantibodies to these lipids, and immune complexes of these lipid-antibody components could potentially serve as pathogenic drivers and serum markers of SLE disease activity.

1. Background

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease characterized by the production of a wide range of autoantibodies against self-antigens such as DNA, proteins, and nucleosomes [1]. Increasing evidence suggests that excess production of reactive oxygen species (ROS) may cause oxidative stress and favor the development of immune-cell dysfunction and the production of autoantigens and autoantibodies. This in turn leads to multi-organ damage, particularly atherosclerosis and renal injury, which act as major causes of eventual SLE-associated morbidity and mortality [2,3]. Among the oxidative species, oxidized low-density-lipoprotein (ox-LDL) plays a key role in the initiation and progression of atherosclerosis and renal damage. Ox-LDL is taken up by macrophages in the intima of arteries, which subsequently become foam cells and form the basis of plaques [4]. Ox-LDL exhibits chemotactic, immune-stimulatory, and toxic properties and acts as a potent inflammatory agents [5]. It triggers a cascade of pro-inflammatory cytokines and stimulates the expression of adhesion molecules (ICAM-1, VCAM-1) on endothelial cells. It also promotes monocyte migration and transfer to macrophages [6]. Similarly, in the kidneys, ox-LDL has been shown to stimulate the proliferation of mesangial cells and promote macrophage infiltration and transformation to foam cells. It also modulates vasoactive mediator secretion and the overproduction of extracellular matrix that contributes to glomerulosclerosis [7,8]. Ox-LDL also plays a pivotal role in the injury of renal tubular epithelial cells and podocytes [9].

In addition to its pro-inflammatory properties, ox-LDL is a prominent autoantigen due to its immunogenicity. It encompasses various oxidation-specific neoepitopes, such as malondialdehyde-modified LDL (MDA-LDL), phosphorylcholine headgroups of oxidized phospholipids, and oxidized metabolites of linoleic acid – 9-HODE (9-hydroxy-10,12-octadecadienoic acid) and 13-HODE (13-hydroxy-10,12-octadecadienoic acid) [10,11]. These neoepitopes promote the production of autoantibodies and, subsequently, the formation of immune complexes, which inflict direct endothelial cell injury and promote the diapedesis of macrophages and inflammatory processes in atherogenesis [10,11].

* Corresponding author at: Science & Engineering Research Center, Department of Biomedical Engineering, 3605 Cullen Blvd. Room 2027, Houston, TX 77204, USA.
E-mail addresses: cmohan@central.uh.edu, twu13@central.uh.edu (C. Mohan).

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Some studies have demonstrated that anti-ox-LDL antibodies are elevated in both non-SLE-associated atherosclerosis and SLE patients, in whom the levels of anti-ox-LDL antibodies appear to correlate with anti-ds-DNA antibody titers, loss of complement, and disease activity scores, although other studies have yielded contradictory or inconclusive results [12,13].

Our previous work has shown increased levels of oxidized lipids, including HODE and MDA, in the sera of SLE patients [14]. Since there has been limited information on the possible role of anti-ox-LDL antibodies in the inflammatory responses underlying SLE, we evaluated the serum levels of autoantibodies against different ox-LDLs in SLE patients and examined the relationship between these autoantibodies and clinical features.

2. Methods

2.1. Reagents

Anti-ox-LDL antibody ELISA kits and anti-MDA-LDL antibody ELISA kits were purchased from Immco Diagnostics and Rocky Mountain Diagnostics, respectively. 9-HODE and 13-HODE were purchased from Cayman Chemicals. POVPC was obtained from Avanti Polar Lipids, Inc. Mouse anti-human C1q antibody and oxidized LDL antibodies were purchased from Abcam. LDL, copper-oxidized LDL and MDA-modified LDL were purchased from Cell Biolabs.

2.2. Patients and sample handling

Serum from a total of 64 patients with lupus and 9 age- and sex-matched healthy volunteers were used for the study. Prior to participation, written informed consent was obtained from all subjects. All studies were performed in accordance with the Declaration of Helsinki. All patients with SLE met the 1997 American College of Rheumatology revised criteria for the classification of SLE. Serum aliquots were prepared and frozen at −80 °C for future use. The clinical data of the SLE patients, including SLEDAI, proteinuria, complements levels, ANA, anti-ds-DNA titers, and lipid panels were obtained for stratification of samples prior to the analysis of the sera.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The antibody levels of ox-LDL and MDA-LDL were assessed using commercial ELISA kits and the procedures were performed according to the provided manuals. Serum autoantibody levels in 64 SLE patients (37 with active SLE, SLEDAI > 9) and 9 healthy controls were assayed using a sandwich ELISA. Immulon 2HB plates were coated overnight with capture antibodies specific for the targeted antigens (ds-DNA, 9-HODE, 13-HODE, POVPC). Plates were subsequently washed in 0.05% TWEEN-20 PBS and blocked for 2 h in 5% bovine serum albumin (BSA) in PBS. Plates were then incubated with serum samples at room temperature for one hour. After an additional wash to remove unbound sample, plates were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies specific for the targeted antigen or, in the case of antibody ELISAs, with secondary antibodies specific for human IgG or IgM. After a final wash, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added for half an hour and quenched for absorbance reading at 450 nm. To measure oxidized-LDL immune complex (ox-LDL-IC), anti-C1q antibody was applied to the ELISA plate as the capture antibody, in order to capture immune complexes. After incubation with serum, ox-LDL-IC were detected using a rabbit anti-human oxLDL antibody.

To verify the binding specificity of the anti-oxLDL antibody used for the immune complex ELISA assay, two different assays were carried out. First, we verified that the commercial anti-ox-LDL did bind to the commercial anti-ox-LDL antibody ELISA kit in a dose dependent manner (Supplementary Fig. S1). Second, in order to obtain independent verification that the anti-ox-LDL is specific for the oxidized version of LDL, three different types of LDL, including LDL, copper-oxidized LDL and MDA-modified LDL were coated onto ELISA plates separately, following which the anti-oxLDL antibody was applied. As shown in Supplementary Fig. S2, the anti-oxLDL antibody exhibited preferential binding to two different versions of ox-LDL, although some binding to total LDL was also seen at higher doses of LDL. Thus, although the assay described in this communication is specifically detecting immune complexes bearing ox-LDL, we cannot exclude the possibility that these immune complexes may also include lower quantities of un-oxidized LDL.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. All values are expressed as mean ± SEM. Student's *t*-test was used to evaluate the difference between two groups. The relationship between autoantibodies and clinical features of SLE was ascertained using Spearman correlation analysis. Values were considered statistically significant at P values < .05.

3. Results

3.1. IgG autoantibodies against oxidized lipids and an oxidized lipid derivative (POVPC) were elevated in active SLE patients versus healthy controls

We tested sera from healthy controls (n = 9), patients with inactive SLE (n = 27), and patients with active SLE (n = 37). Serum levels of autoantibodies against malondialdehyde (MDA), oxidized LDL and immune complexes bearing ox-LDL(ox-LDL and ox-LDL-IC, respectively), and 1-palmitoyl-2-(5- oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) were detected by ELISA. As shown in Fig. 1 and Table 1, the results indicated that IgG autoantibodies against oxidized lipid-antigens, and immune complexes generated from these autoantibodies were elevated in patients with SLE, particularly in patients with high disease activity.

3.2. Active SLE patients showed increased IgG autoantibodies but decreased IgM autoantibodies to oxidized lipid derivatives – 9/13-HODE

As shown in Fig. 2 and Table 2, IgG autoantibodies against 9-hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-10,12-octadecadienoic acid (13-HODE) were significantly elevated in both inactive and active SLE patients versus healthy controls. In the case of 9-HODE, there was also a significant elevation in active SLE patients versus inactive ones. In contrast, IgM autoantibodies against 9-HODE and 13-HODE were each significantly decreased compared to healthy controls. The ratio of IgG to IgM autoantibodies to these same antigens was particularly elevated in patients, compared to the controls.

3.3. Correlation between autoantibodies against ox-LDLs with clinical markers in SLE patients

As shown in Fig. 3, anti-POVPC-IgG, anti-9-HODE-IgG, and anti-ox-LDL-IgG autoantibodies were similarly correlated with markers of SLE activity or severity. Significant positive correlations were found with the patients' SLEDAI scores and a significant inverse correlation was found with complement component C3 and/or C4.

4. Discussion

Autoantibodies targeting specific autoantigens are mechanistic contributors to many autoimmune diseases and are recognized as clinical hallmarks. SLE is a multifactorial disease with varying symptoms and clinical outcomes. Therefore, new biomarkers may be useful

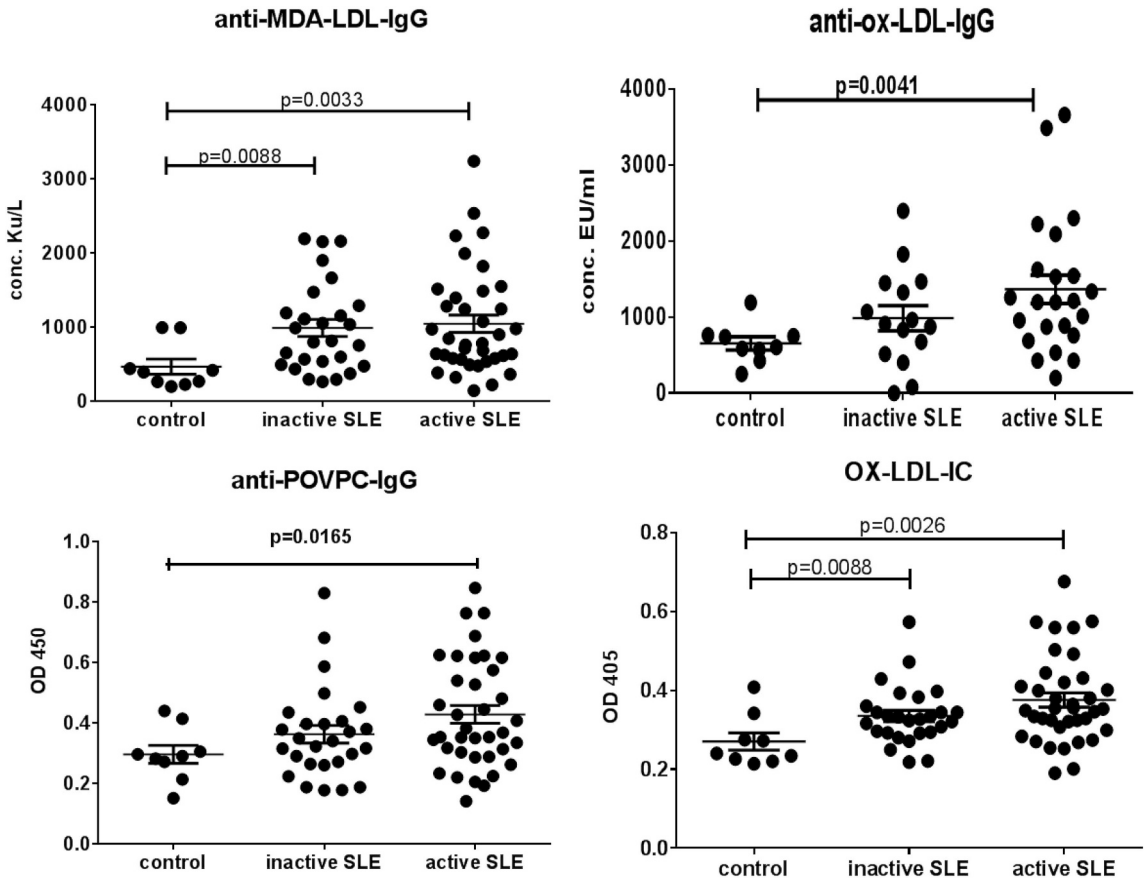

Fig. 1. Sera from control (n = 9), inactive SLE (n = 27), and active SLE (n = 37) patients were tested using ELISA kits for autoantibodies against malondialdehyde (MDA), 1-palmitoyl-2-(5- oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), oxidized LDL (ox-LDL), and oxidized LDL-immune complexes (ox-LDL-IC). Each dot represents an individual subject. IgG autoantibodies were significantly elevated against each oxidized antigen versus healthy controls. *P*-values were calculated using the Student's *t*-test.

in SLE, both as clues for underlying pathogenesis and as clinically measurable indicators of disease state. Current standard markers include indicators such as SLEDAI (Systemic Lupus Erythematosus Disease Activity Index), loss of complement components C3 and/or C4, anti-ds-DNA antibodies, and proteinuria.

The main findings of this study are that serum levels of IgG anti-oxidized LDL antibodies (anti-MDA-LDL, anti-ox-LDL, anti-POVPC, anti-9/13-HODE antibodies) and ox-LDL-immune complexes are elevated in SLE patients compared to healthy controls, while the IgM isotype of autoantibodies for 9,13 HODE are decreased in. This study is the first to investigate the relationship between the autoantibodies to HODE/POVPC and disease activity in SLE. Some IgG anti-ox-LDL antibodies (anti-ox-LDL, POVPC, 9-HODE-antibodies) are positively correlated with SLE disease activity (SLEDAI and complements loss). Hence, these anti-lipid autoantibodies should be examined for their clinical potential in tracking disease activity longitudinally. In addition, IgG anti-9-HODE-antibodies showed a positive correlation with proteinuria, levels of ox-LDL immune complex, and anti-dsDNA antibodies in SLE.

Ox-LDL has been found in both atherosclerotic plaques and glomerulosclerotic lesions. Autoantibodies against ox-LDL were demonstrated as being associated with clinical progression of atherosclerosis, which was supported by experimental models and clinical studies [15]. Anti-ox-LDL antibodies, particularly IgG autoantibodies, have been reported to be associated with increased atherosclerotic risk in patients with higher SLE disease activity [16]. However, the mechanisms underlying their pro-atherogenic contributions in SLE remain unclear. It has also been reported that IgG anti-ox-LDL antibodies facilitate ox-LDL uptake, trigger macrophage activation and inflammatory responses, and ultimately lead to foam cell generation, destabilization, and rupturing of the plaque [17,18].

Ox-LDLs encompass a large group of oxidized lipids. In the current study, we could only detect some antibodies, including antibodies to MDA-modified LDL, 9/13-HODE and 1-palmitoyl-2-(5- oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC). Malondialdehyde (MDA) is an end product of lipid peroxidation and plays a critical role in autoimmune diseases. It covalently binds to proteins and is responsible for oxidative modification of proteins, generation of neoantigens, and formation of autoantibodies [11]. Significant elevation of IgG anti-MDA-LDL

Table 1
Elevated IgG autoantibodies to ox-LDL in SLE patients compared to healthy controls.

	Healthy control	Inactive SLE patients	Active SLE patients	P value
Anti-MDA-LDL-antibody (ku/L)	468.7 ± 103.6	992.2 ± 115.3**	1049.0 ± 116.2*	0.003
Anti-ox-LDL-antibody (EU/ml)	654.0 ± 87.17	1031.0 ± 157.8	1368.0 ± 183.6*	0.004
Anti-POVPC-antibody (OD450)	0.297 ± 0.030	0.363 ± 0.029	0.429 ± 0.030*	0.001
Ox-LDL-IC (OD450)	0.270 ± 0.022	0.335 ± 0.014**	0.375 ± 0.018*	0.003

*/** Compared to healthy control.

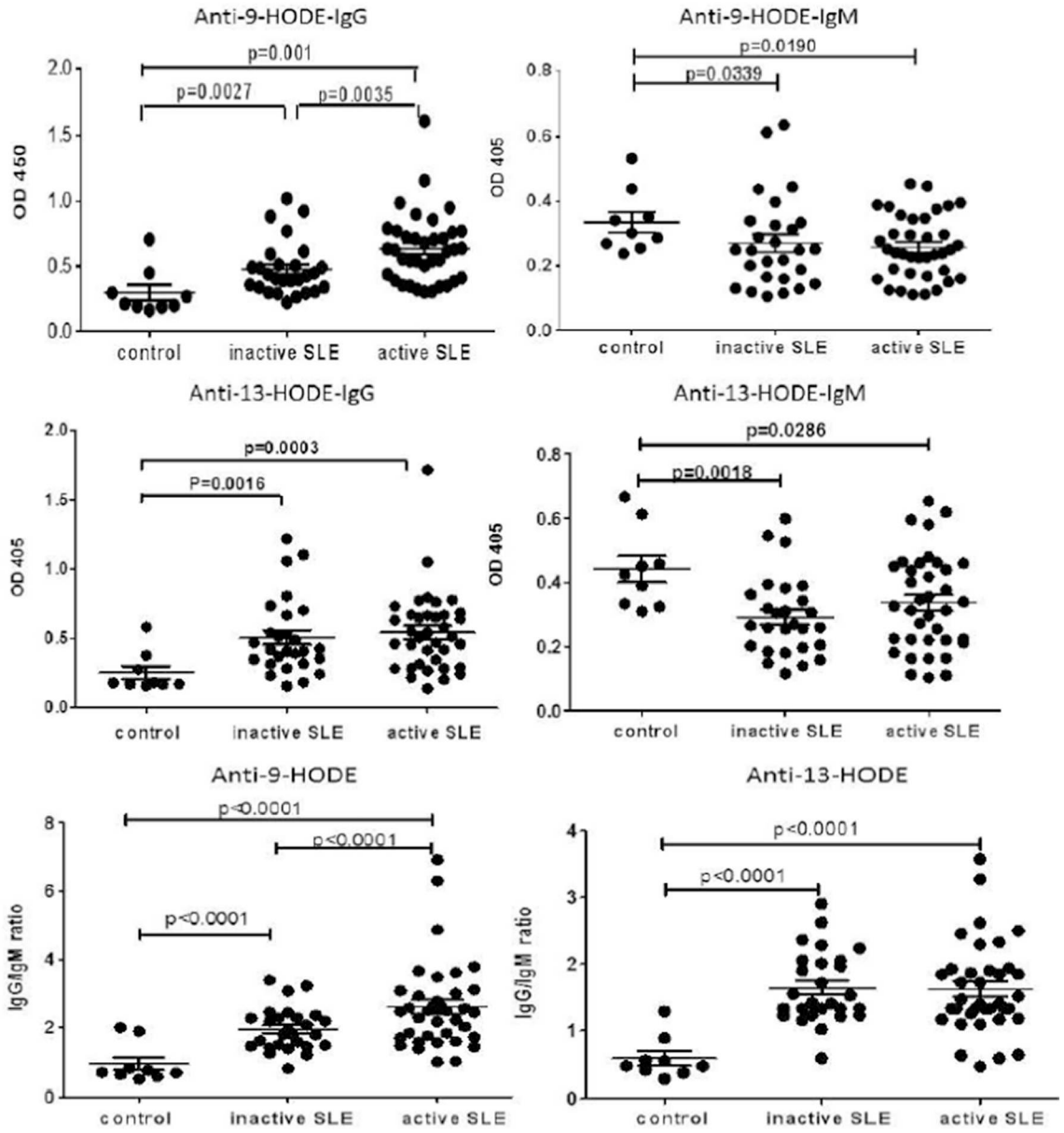

Fig. 2. Sera from control (n = 9), inactive SLE (n = 27), and active SLE (n = 37) patients were tested using ELISA for IgG and IgM autoantibodies against 9-HODE and 13-HODE. Each dot represents an individual subject. IgG autoantibodies were significantly elevated against each oxidized antigen, but IgM autoantibodies were significantly reduced against healthy controls. P-values were calculated using the Student's t-test.

autoantibodies may well be the consequence of increased oxidative stress in SLE and/or underlying atherosclerosis. In our research, IgG MDA-LDL autoantibodies were elevated in SLE patients, which is in accordance with previous studies.

13- and 9-hydroxy-octadecadienoic acid (13-HODE and 9-HODE) are stable oxidation products of linoleic acid – the most abundant fatty acid in atherosclerotic plaques [19]. High levels of HODEs accumulate in low-density lipoproteins (LDLs) and have been demonstrated to contribute to the pathogenesis of atherogenesis by regulating macrophage apoptosis in diseases such as atherosclerosis, diabetes, psoriasis, chronic inflammation, obesity and cancer [20–22]. In addition, both 9-HODE and 13-HODE have been shown to increase vascular epidermal growth factor (VEGF) and are responsible for mesangial cell proliferation and extracellular matrix (ECM) production [10].

Table 2
Elevated IgG and decreased IgM autoantibodies to 9/13-HODE in SLE patients.

	Healthy control	Inactive SLE patients	Active SLE patients	P value
anti- 9-HODE-IgG (OD450)	0.298 ± 0.058	0.478 ± 0.039**	0.632 ± 0.044*	0.0027/0.001
anti-13-HODE-IgG (OD450)	0.251 ± 0.048	0.505 ± 0.053**	0.542 ± 0.047*	0.0016/0.0003
anti- 9-HODE-IgM (OD450)	0.335 ± 0.032	0.270 ± 0.027**	0.257 ± 0.016*	0.0339/0.019
anti-13-HODE-IgM (OD450)	0.441 ± 0.042	0.292 ± 0.024**	0.336 ± 0.025*	0.0018/0.029

*/** Compared to healthy control.

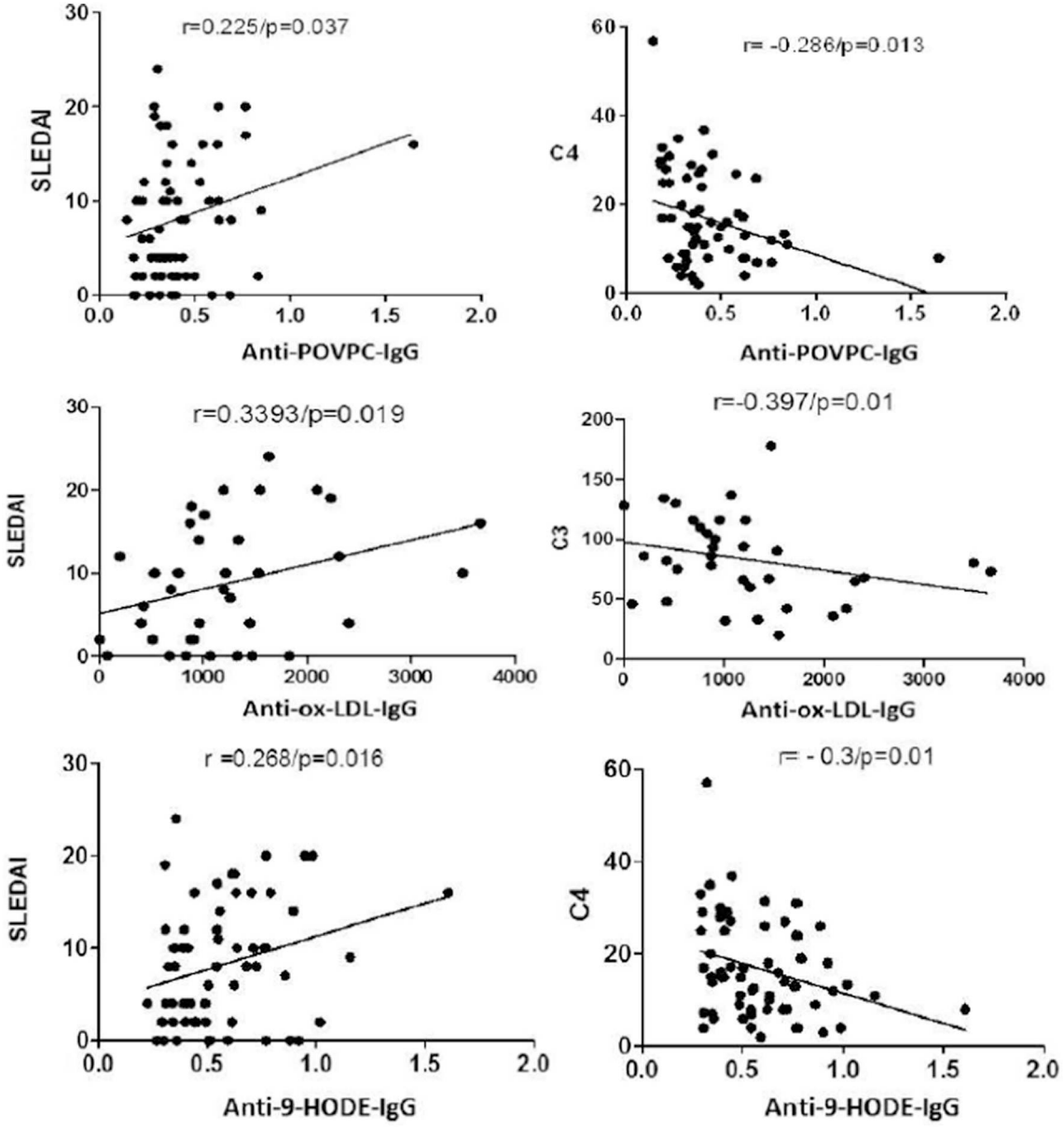

Fig. 3. Anti-POVPC-IgG, anti-ox-LDL-IgG, and anti-9-HODE-IgG correlate well with existing markers of SLE severity in SLE patients. Each dot represents an individual subject. P-values were calculated using the Student's t-test.

Oxidized lipids are responsible for various proinflammatory effects in human and in animal models. Among them, oxidized phospholipids (ox-PL) act as a major group of proinflammatory lipids that participate in atherogenicity [22]. Watson et al. have identified two predominant oxidized phospholipid species – 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) and 1-palmitoyl-2-(5- oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) in atherosclerotic plaques [23]. These lipid species exhibited deleterious properties by inducing apoptosis of cultured VSMC, activating endothelial cells, vascular cells, leukocytes, and platelets [24,25].

In the present study, IgG anti-ox-LDL autoantibodies were elevated in SLE, consistent with previous reports [26], whereas IgM antibodies against 9,13 HODE were decreased. IgM is the first antibody isotype to arise upon immunization and is generally considered to play a protective role against atherosclerosis or autoimmunity, through several means, including blocking ox-LDL uptake, causing foam cell formation, and promoting apoptotic cell clearance. On the other hand, IgG is demonstrated to be pro-atherogenic [27], and more pathogenic in lupus nephritis. Repeated and prolonged exposure to oxidized lipids in SLE patients can be expected to induce Ig class switching and the emerging IgG antibodies to oxidized lipids may promotes atherosclerotic plaque development and lupus nephritis. Interestingly, when the ratio of IgG/IgM anti-9-HODE autoantibody was plotted, inactive and active SLE patients showed increasingly elevated levels, with correlation with disease activity. Hence, the ratio of IgG/IgM antibodies to 9-HODE may also be a potential novel indicator of SLE disease activity.

Finally, we found that ox-LDL-containing immune complex (ox-LDL-IC) was elevated in SLE patients compared to healthy controls and showed a positive correlation with IgG anti 9-HODE-antibodies. Ox-LDL-IC could potentially engage the higher avidity Fcγ receptor (FcγRI) on monocytes and macrophages [28,29]. This would activate the classic pathway of the complement system, resulting in cell activation, release of pro-inflammatory cytokines, proliferation of mesangial cells, and expansion of the extracellular matrix in nephropathy [30]. Thus, heightened ox-LDL-IC formation arising from elevated levels of oxidized lipids and the autoantibodies they elicit may play a critical role in driving disease pathogenesis, particularly effector macrophages, both within atherosclerotic blood vessels as well as within the kidneys.

Besides their potential pathogenic role, ox-LDL-IC also has the potential to be a novel biomarker of atherogenesis, particularly in the context of systemic rheumatic diseases. Both these predictions warrant experimental validation using larger cohorts of SLE patients with or without atherosclerosis.

5. Conclusions

In conclusion, patients with active SLE exhibit significantly increased serum levels of oxidized lipids, anti-oxidized lipid autoantibodies, and immune complexes. Coordinated elevation of all three of these components could serve as potential serum markers of disease activity in SLE. Furthermore, the link between oxidized lipid neoantigens may help explain some of the pathological association between SLE and atherosclerosis. Further work is needed to help substantiate this hypothesis and elucidate the underlying pathogenic pathways.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.05.004.

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