



Anti-C1Q Antibodies Concentrations by Elisa in Systemic Lupus Erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is an inflammatory disorder in which autoantibodies contribute to impaired apoptosis and clearance of cell debris. Anti dsDNA and anti C1q antibodies have been implicated, as well as complement protein C1q itself. IgG autoantibodies reacting with the collagen-like region of C1q protein (α C1q ab) were quantitated in serum of 56 patients diagnosed with SLE and undergoing treatment for variable periods, together with 33 age/sex-matched controls. Analysis of the results showed optimal sensitivity and specificity of 57% and 91% respectively at a cut-off concentration for positivity of 20 U/ml. The assay is a potentially useful confirmatory test for SLE, but is not suitable as a screening test for SLE with the probability of a positive test and SLE in an individual within a random population of only $\leq 1\%$. α C1q ab concentrations were detectable in all samples tested with concentrations manifesting no correlation with age and serum C1q levels in SLE patients and a negative correlation with age in controls. The α C1q ab detected by this assay do not react therefore with native C1q. In SLE patients, α C1q ab concentrations correlated with the concentrations of dsDNA antibodies, ($p=0.0001$) and C-reactive protein and inversely with complement component C4 (C4) concentrations ($p=0.041$). α C1q ab concentrations were not associated with individual therapeutic regimens, but were higher in those patients receiving a combination of three drug therapies and with the presence of renal disease. The diagnostic relevance of this complex autoantibody will require further definition of its antigenic specificities.

Introduction

There is good evidence that impaired apoptosis and clearance of cell debris induces autoimmune responses associated with aberrant inflammation [1]. Systemic lupus erythematosus (SLE) is an important systemic autoimmune disease characterised by the presence of autoantibodies that bind double stranded DNA (henceforth dsDNA ab) and often decreased levels of serum complement components C3 and C4 [2]. Complement component C1q is one of three proteins comprising the first component (C1) of the classical pathway of complement activation. C1q molecules can activate the complement system by recognizing different structures on microorganisms and apoptotic bodies, or indirectly by means of binding to immunoglobulins and classical acute phase proteins such as C-reactive protein, CRP resulting in clearance of immune complexes and apoptotic material [2,3]. The avidity of binding of C1q to the Fc domain of IgG antibodies is augmented by the presence of hexameric IgG/antigen complexes on cell surfaces or polyvalent antigens [4]. A consequence of high affinity binding of C1q to Fc receptors is the formation of a neo-antigen on the collagen-like tails of the C1q molecule subunits [5]. Production of auto-antibodies reacting with this neo-antigen has been observed following C1q binding with immune complexes or nonspecific binding to chromatin [6]. The role of these autoantibodies in the pathogenesis of specific disease, for example SLE, is yet to be clearly defined [2]. Uwatoko and colleagues [7] devised an assay capable of quantifying anti-C1q antibodies (henceforth α C1q ab) by their interaction with C1q bound to a plastic surface in the presence of a high salt concentration (1M NaCl) to preclude any interaction with soluble C1q present in the test sample, usually serum.

In the last decade, the relationship between α C1q ab and clinical profiles in SLE patients, as well as relationships with other laboratory parameters has been investigated. It has been reported by several groups [6,8-10] that the levels of α C1q ab titers are usually significantly higher in SLE patients than healthy controls or non-SLE autoimmune patients. The concentrations of α C1q ab correlate with disease activity and/or severity [6,8-19] as well as with other laboratory parameters including complement protein C3 and/or C4 concentrations, dsDNA ab, proteinuria and the presence of immune complexes [6,8,11,12,14-19]. Whether serum concentration of α C1q ab is a useful tool to predict up-coming flares in patients with nephritis remains to be confirmed. A strong association between α C1q ab and active renal disease has been demonstrated [18]. Only nine studies have longitudinal data permitting this specific assessment (including 10,14), although other reports have supported its potential for this use [9,10,12,17]. The present study measured the levels of α C1q ab in a cohort of diagnosed and treated Western Australian SLE patients using a commercial ELISA kit.

Methods

Cohorts and ethics

Human ethics approval: Curtin University, Sir Charles Gairdner Hospital and Royal Perth Hospital Human Ethics Committees approved the human studies (approval numbers HR 202/2013, HREC 2013-174). Written and informed consent was obtained from all study participants.

Fifty-six (56) treated systemic lupus erythematosus patients aged 47 ± 15 years were recruited from Sir Charles Gairdner (SCGH) and

Royal Perth Hospitals (RPH-Perth, Western Australia) between March 2014 and December 2015. The diagnoses were based on the American College of Rheumatology (ACR) classification criteria. In addition to the SLE diagnosis, patients had co-morbidities including renal disease, liver pathologies as well as Sjögren and Raynaud's syndromes. These are summarised in (Table 1). SLE patients in this cohort were treated with various therapeutic regimens including steroids (low dose

prednisolone ($\leq 5\text{mg/day}$), immunosuppressant therapies (azathioprine, cyclosporine, cyclophosphamide, methotrexate, mycophenolate, mofetil) and antimalarial therapies (hydroxychloroquine). Thirty-three age and sex-matched healthy control subjects (47 ± 15 years) were recruited from the Perth metropolitan area.

	Steroids (1)	Anti-malarial (2)	Immuno-suppressants (3)	1+2	1+3	2+3	Combination of 1, 2 & 3	Other therapeutics
Renal symptoms (N=11)	8 (72.7%)	7 (63.6%)	8 (72.70%)	1 (9.1%)	3 (27.3%)	0 (0%)	4 (36.4%)	8 (72.7%)
Hepatic involvement (N=4)	2 (50%)	4 (100%)	2 (50%)	1 (25%)	0 (0%)	1 (25%)	1 (25%)	3 (75%)
Anti-phospholipid (N=17)	13 (76.5%)	9 (52.9%)	7 (41.20%)	3 (17.60%)	3 (17.6%)	0 (0%)	4 (23.5%)	16 (94.1%)
Raynaud's phenomenon (N=4)	2 (50%)	2 (50%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	4 (100%)
Sjögren's disease (N=4)	0 (0%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (75%)
Cutaneous/discoid symptoms (N=12)	5 (41.7%)	6 (50%)	7 (58.30%)	1 (8.30%)	0 (0%)	0 (0%)	3 (25%)	9 (75%)
Neuronal symptoms (N=1)	1 (100%)	1 (100%)	0 (0%)	1(100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
T1DM (N=3)	3 (100%)	2 (66.7%)	2 (66.70%)	1(33.30%)	1 (33.3%)	0 (0%)	1 (33.3%)	3 (100%)
Other symptoms (N=13)	9 (69.2%)	11 (84.6%)	7(53.80%)	2(15.40%)	2 (15.4%)	1 (7.7%)	4 (30.8%)	13 (100%)

Table 1: The table indicates the number and percentage of patients in each category of lupus manifestation under specific types of treatment (Patients may fit into multiple lupus manifestations as well as treatments under review with other type of medications).

Sample collection

Peripheral blood from SLE patients was collected into ethylenediaminetetraacetic acid (EDTA), heparin and serum tubes at PathWest collection centres. Serum samples were separated at PathWest laboratories within one hour of collection using centrifugation and frozen to between -70°C and -80°C . Samples from healthy human controls were collected into EDTA, heparin and serum vacutainer tubes (Beckton Dickinson (BD), San Jose, USA) and processed in the same way at Curtin University laboratory (Perth, WA).

C1q ab concentrations

A solid-phase ELISA kit (Bulmann Laboratories, Schönenbuch Switzerland) was used to quantify IgG αC1q ab. Stored patient sera were diluted in high salt buffer (0.5M NaCl) and incubated in microtiter wells coated with human C1q. Horseradish peroxidase (HRP) labeled anti-human IgG was then added, followed by tetramethylbenzidine (TMB) substrate. A washing step was included between each incubation. The substrate reaction was terminated through the addition 0.25M sulphuric acid and the absorbance measured at 450 nm using a microtiter plate reader (Bio-Rad). Test sample concentrations were interpolated using a four-parameter logistic calibration curve fitted to the standards provided by the manufacturer (5, 25, 100 and 400 U/mL based on an international reference standard). The manufacturer's suggested value for positivity was 15 units/ml (Figure 1).

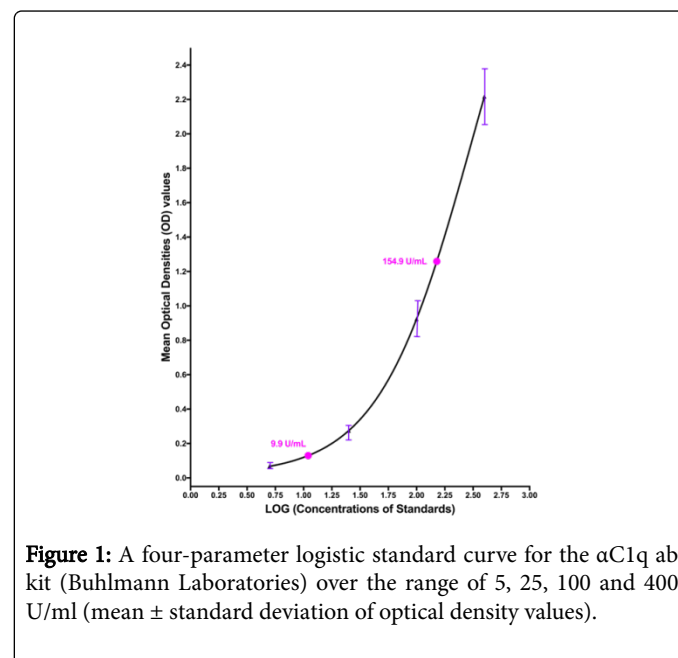


Figure 1: A four-parameter logistic standard curve for the αC1q ab kit (Bulmann Laboratories) over the range of 5, 25, 100 and 400 U/ml (mean \pm standard deviation of optical density values).

Complement C1q, C3, C4 concentrations, CRP, double stranded DNA antibodies (dsDNA ab) assays

Patients data for dsDNA ab, CRP, C4 and C3 were collected from the patients' files and corresponded to the time-point at which they

were recruited and blood samples obtained for the study. All the above parameters were assayed in the PathWest Diagnostic Laboratories at Fiona Stanley Hospital, Fremantle and Sir Charles Gairdner Hospital. Samples tested at PathWest for antibodies to double stranded DNA: dsDNA ab were assayed using a radioimmunoassay dsDNA ab kit (Trinity Biotech; NY, USA) as per the manufacturer's directions. C-reactive protein (CRP) concentrations in serum or plasma samples were assessed using the turbidimetric anti CRP antibody coated latex particle assay in an Abbott Architect C16000 instrument (Abbott Laboratories, Abbott Park, Illinois USA). Complement protein C3 and C4 concentrations in serum or plasma samples were also measured by turbidimetry assay using the Abbott Architect C16000 instrument. Complement C1q levels were assayed using a nephelometry method on a Siemens BN2 nephelometer.

Data analysis

Statistical significance was calculated using Mann-Whitney ranking U-test with the program GraphPad PRISM 7 (GraphPad Software Inc, California, USA). P values of <0.05 were considered statistically significant.

Results

Quantitation of anti-C1q antibody in treated SLE patients and controls

Multiple assays (including repeated samples) were performed in accordance with the manufacturers specifications using a range of international calibrator concentration standards (5, 25, 100 and 400 U/ml) provided with the kit. Two quality control samples provided by the manufacturer were included in each assay. These comprised a negative control with expected values between 4.0-7.9 U/mL and a positive control sample between 118-233 U/mL. The positive control was always within the expected concentration range. The negative control sample was marginally higher in our analysis (average of 9.9 U/mL) than that stipulated by Buhlmann Ltd, but still lower than the 20 U/mL cut-off point for positivity used in this analysis.

The non-Gaussian distributions of the concentration of the α C1q ab in patients and controls are shown in (Mann Whitney rank test analysis $p \leq 0.0001$) (Figures 2A-C).

The optimal specificity and sensitivity of the assay for inclusion or exclusion of SLE were estimated from the proportions of true positive and true negative results as a function of variable "cut-off concentrations of α C1q ab. From these data, a receiver-operator characteristic curve (ROC: plot of sensitivity (%) vs. 1-specificity (%), see Figure 3) was generated with an area under the curve of 0.78 (0.68-0.88, 95% CI, $p=0.0001$).

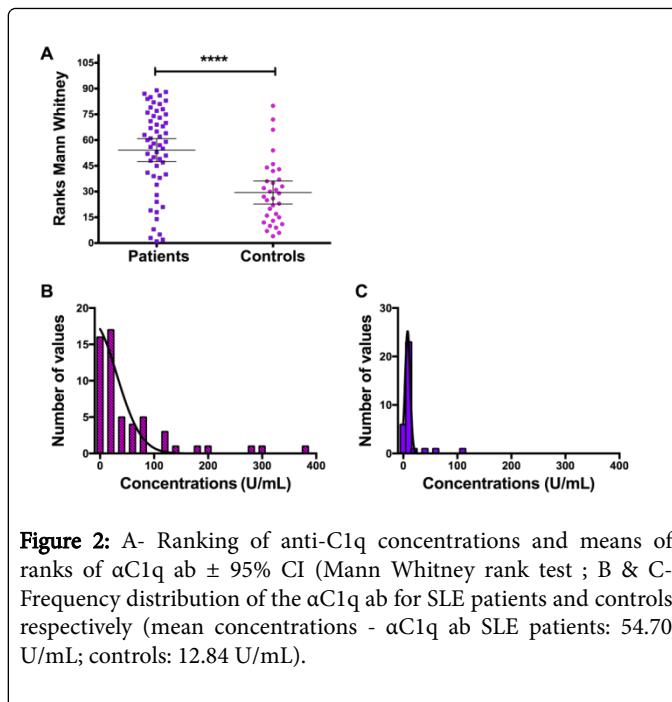


Figure 2: A- Ranking of anti-C1q concentrations and means of ranks of α C1q ab \pm 95% CI (Mann Whitney rank test ; B & C- Frequency distribution of the α C1q ab for SLE patients and controls respectively (mean concentrations - α C1q ab SLE patients: 54.70 U/mL; controls: 12.84 U/mL).

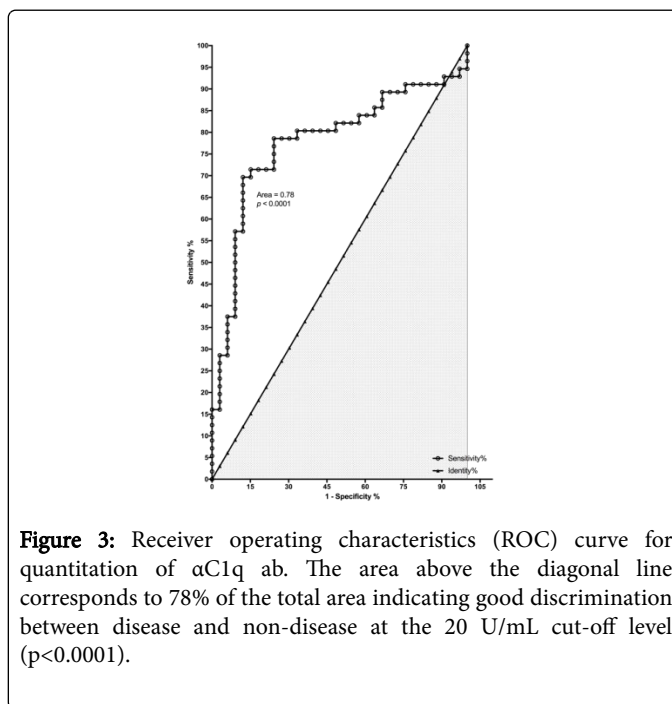


Figure 3: Receiver operating characteristics (ROC) curve for quantitation of α C1q ab. The area above the diagonal line corresponds to 78% of the total area indicating good discrimination between disease and non-disease at the 20 U/mL cut-off level ($p < 0.0001$).

From this graph the 20 U/mL cut-off concentration provided a likelihood ratio of 6.3 (i.e. probability of a positive test in patients with SLE/probability of a positive test in persons without SLE). This compares with the likelihood ratio of 5.0 for the 15 U/mL cut-off value recommended by the manufacturer, and often quoted by other groups using the same international control samples (6). Henceforth the 20 U/mL discrimination value has been used throughout this analysis. The sensitivity and specificity of the assay for the diagnosis of SLE were 57% and 91% respectively. Assuming an average prevalence of SLE of $\approx 0.1\%$ in the WA population [20], the estimated predictive value of a positive and negative α C1q ab test for SLE was 91% and 56%

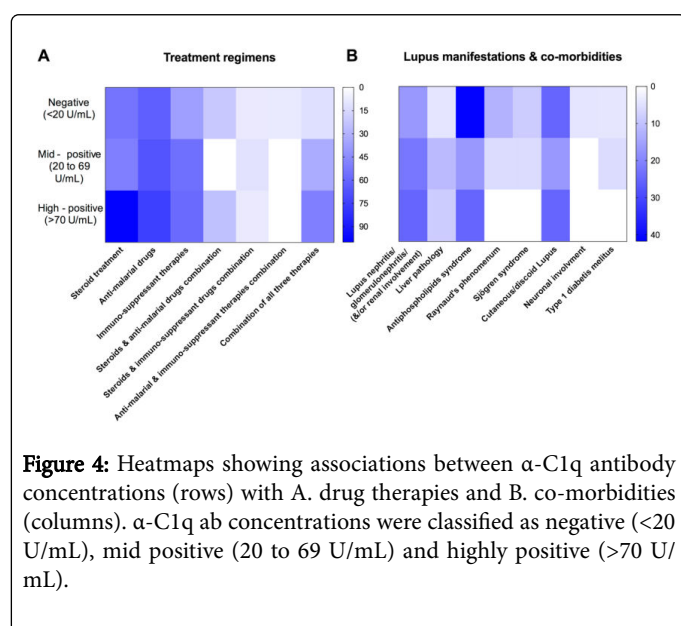
respectively. Bayesian analysis results in the probability of a positive test and SLE in an individual within a random population of the order of 1-2%. There was a higher percentage of positive α C1q ab concentrations in SLE patients than in controls (57% vs. 9% respectively, ($p=0.0001$); Mann Whitney rank test), and it was noted that all patients and control samples tested manifested some level of α C1q ab.

Anti-C1q antibody concentrations correlate in SLE patients with dsDNA ab concentrations and inversely with complement C4 concentrations

Correlation coefficients were estimated for α C1q ab concentrations and those of several other serological analytes usually measured in SLE

	Age	(C4)	(C1q)	(C3)	(CRP)	(Ds-DNA ab)	(DNASE-I)	(Anti-C1q ab)
Age		-0.073	0.07	0.063	0.014	-0.275 (P=0.042)	0.034	-0.146
(C4)	-0.073		0.085	≤ 0.0001	0.203	-0.087	-0.045	-0.274 (P=0.041)
(C1q)	0.07	0.085		0.248	0.325 (P=0.024)	0.241	0.263 (P=0.050)	0.088
(C3)	0.006	0.116	-0.02		0.239	-0.118	0.017	-0.131
(CRP)						0.406 (P=0.005)	-0.063	0.136
(Ds-DNA ab)							0.059	($p \leq 0.0001$)
(DNASE I)	0.14	-0.141	0.23	0.225				0.038
(Anti-C1q ab)	-0.566 (P=0.001)	-0.246	-0.024	0.057			-0.368 (P=0.035)	

Table 2: Correlation matrix between α C1q ab and other serological parameters and age. The values above the diagonal are the Spearman (r) correlation values and when significant the significance levels in brackets ($p \leq 0.05$). The same comparisons, when available, are shown for the control population below the diagonal line.



testing. These are shown in Table 2. Significant correlations were observed between α C1q ab and dsDNA ab ($p=0.0001$); an inverse correlation between α C1q ab concentrations and complement component C4 concentrations was noted ($p=0.041$). The correlation matrix also revealed associations in SLE patients between C-Reactive Protein (CRP) and both dsDNA ab ($p=0.005$) and C1q complement protein levels ($p=0.024$). However, C1q protein levels in SLE patients were not significantly correlated with anti-dsDNA antibodies. There was a weak negative correlation (not statistically significant), between the age at time of testing of individual SLE patients and the concentration in serum of α C1q ab, whereas in the control cohort the concentration of α C1q ab was negatively correlated with age at time of collection ($p=0.001$).

Anti-C1q ab concentrations are associated with treatments and renal co-morbidities in patients

Heatmaps were used to show any patterns in the patient group linking concentrations of α C1q ab and therapeutic treatments, as well as co-morbidities. Figure 4A shows the association of the α C1q ab with the drug treatments offered to SLE patients over the course of their disease. It was observed that patients who received all three treatments of steroids, anti-malarial and immune-suppressants (7th lane) exhibited higher levels of C1q abs. Figure 4B shows associations of α C1q abs with the co-morbidities of the patients. The first lane shows an association of higher concentrations of α C1q abs in patients with diagnosed renal disease. In contrast, patients with hepatic involvement (3rd lane) exhibit lower levels of α C1q abs.

Discussion

C1q molecules can activate the complement system by recognizing different structures on microorganisms and apoptotic bodies, or indirectly by means of bound immunoglobulins and classical acute phase proteins such as C-reactive protein, (CRP) [3]. Following these processes the generation of a neo antigen on the α C1q ab concentrations were assayed in the serum of a cohort of SLE patients undergoing clinical management for varying durations, and a panel of 33 healthy controls using a commercially available ELISA (Buhlmann Laboratories). The ROC graph analysis of the test result data showed that 20 U/mL was a more discriminatory cut-off concentration than

the value of 15 U/mL recommended by the manufacturer and some other groups [8]. The relatively low sensitivity of the assay (57%) restricts the utility of the test for exclusion of SLE, however the high specificity of the assay (91%) provides a useful confirmatory test. Further, the probability of a positive test AND SLE in an individual in a random population is only of the order of 1-2%, thereby ruling out the assay as a screening test for SLE. It is noted that three of the 33 serum samples from the healthy control cohort exhibited high levels of α C1q ab; we cannot explain this anomaly. It is clear the presence of α C1q ab as detected by the ELISA assay used herein is not pathognomonic for SLE.

This study reports a point-in-time estimate of α C1q ab concentrations in the serum of a cohort of SLE patients under clinical management for varying durations. Hence, it is not possible in this study to relate α C1q ab levels to events of clinical significance such as flare of renal disease. α C1q ab levels were inversely correlated with serum C3 and C4 concentrations in SLE patients (albeit C3 not significantly) as would be expected if classical pathway complement activation was contributing to the disease process [8]. These observations may be confounded by treatments (such as immunosuppressant therapies) received by the SLE patients over the course of their disease. Both dsDNA ab and CRP levels were significantly positively correlated with α C1q ab concentrations suggesting that in SLE patients, some of these analytes contributed directly to the disease mechanism [2]. An important cognate study [21] demonstrated two human monoclonal anti-DNA antibodies (R4A and G11) that cross-reacted with a globular region (as opposed to the collagen-like region) antigenic determinant in mouse and human C1q proteins. Both antibodies bound both soluble C1q as well as solid phase C1q. Franchin et al., [21] postulated that anti-C1q antibodies could contribute to the development of immune complex mediated glomerulonephritis by direct binding to glomeruli or by removal of soluble C1q. In our study α C1q ab concentrations were not correlated with serum concentrations of C1q in either the patient or control cohorts. This implies that most of the α C1q ab detected in this assay are specific for non-globular antigenic epitopes on α C1q. The significant correlation of α C1q ab concentration with anti-dsDNA antibodies is consistent with the cross-reactivity reported by Franchin et al., [21], but may also just reflect two separate but related processes. It is also of interest that Vanhecke et al., [22] described α C1q ab reacting with peptide epitopes on the A and B collagen-like tails of human C1q that were not present on the C chain. These epitopes were only present on bound C1q and not on soluble C1q and mapped to different regions of the collagen-like region of C1q. This raises the possibility that the α C1q ab detected in this study may include a proportion of antibodies directed against the peptide epitopes described by Vanhecke [22]. It is clear the specificities of the α C1q ab observed in the serum of SLE patients need to be characterised in more detail, especially if different antibody specificities reflect distinct attributes of SLE pathology eg presence of nephritis. Further, as C1q is synthesised mainly by macrophages and immature dendritic cells, serum C1q levels may be relatively insensitive to C1q consumption within tissues [23,24].

During a primary immune response C1q is activated by binding to pentavalent Fc structures in IgM antibodies. In secondary immune responses, high avidity binding and activation of C1q is achieved by binding to Fc domains of antigen bound IgG molecules associated in hexameric clusters located in membranes of cells [4]. In both instances, it is known that a neo-antigen(s) is generated on the C1q molecule that elicits synthesis of α C1q ab [25]. Thus, it would be expected that the

sequelae of humoral immune responses to diverse antigens may result in neo C1q antigen formation and subsequent autoantibody synthesis. Clearly, these autoantibodies would not be expected to be specific for SLE and hence constitute a more general epiphenomenon. This statement is consistent with the presence of α C1q ab reported in the serum of individuals with a variety of non-SLE autoimmune disorders and infectious diseases, as well as in a proportion of healthy individuals (reviewed by [5]). The enigmatic role of C1q in SLE is emphasized further by the recent review (by international survey) of C1q deficiency in humans [26] in which the authors reported a high prevalence (\approx 80%) of SLE, together with decreased survival times and increased susceptibility to infections. Furthermore, C1q has been linked to activation of a tumour suppressor locus with prostatic hyperplasia and carcinoma [27].

It seems there are at least two distinct mechanisms in the predisposition to SLE explained by C1q. SLE occurs with high frequency in C1q protein deficient persons [26,28] and SLE can also occur in the presence of normal C1q protein levels and relatively high concentrations of α C1q ab that contribute to inflammatory processes [16]. SLE patients who received all three classes of drug therapies tended to have higher levels of α C1q ab. This suggests that patients with a more severe disease phenotype requiring multiple therapeutic treatments to reduce organ damage also produce higher levels of α C1q ab. In contrast, patients with less severe disease, requiring fewer therapeutic agents and manifesting lower levels of α C1q ab. This observation supports, but does not confirm, reports that relate high α C1q ab levels to renal symptoms and/or renal flares [18]. There have been many studies over the past two decades of α C1q ab levels in disease, including SLE [17,18]. These, together with this report, suggest that quantitation and standardization of anti-C1q antibodies has a useful role in the classification of subtypes of SLE and as a potential confirmatory diagnostic and monitoring assay [17,18].

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