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Serum protein oxidation and apolipoprotein CIII levels in people with systemic lupus erythematosus with and without nephritis

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Abstract
Increased oxidative stress is a hallmark of the autoimmune disease systemic lupus erythematosus (SLE). This study compares serum protein oxidation levels in SLE patients without and with renal involvement (lupus nephritis); the latter have a significantly poorer prognosis. Similar increases in protein carbonyls and decreases in protein thiols were observed in both SLE groups compared to controls. Protein carbonyl distribution, determined by Western blotting of 2D gels, was similar in both SLE groups, suggesting factors other than oxidation also play a role in SLE complications. 2D electrophoresis examined the serum proteome further. Six proteins were significantly decreased in non-renal SLE patients compared to controls; five were identified by mass spectrometry, including one isoform of pro-atherogenic apoCIII. Total apoCIII levels (assessed by ELISA) in lupus nephritis patients were significantly elevated compared to controls or non-renal SLE patients. Thus, levels of oxidized proteins and apoCIII may be useful biomarkers in SLE studies.

Keywords: *Apolipoprotein CIII, atherosclerosis, lupus nephritis, protein oxidation, protein carbonyls, systemic lupus erythematosus*

Abbreviations: *anti-dsDNA, antibodies to double-stranded DNA; apoCIII, apolipoprotein CIII; BCA, bicinchoninic acid; BSA, bovine serum albumin; CHAPS, 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulphonate; CRP, C-reactive protein; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; Hb, haemoglobin; HSA, human serum albumin; IEF, isoelectric focusing; Ig, immunoglobulin; IPG, immobilized pH gradient; Met(O), methionine sulphoxide; PAGE, polyacrylamide gel electrophoresis; rpm, revolutions per minute; SDS, sodium dodecyl sulphate; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; TFA, trifluoroacetic acid.*

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease in which the immune system loses self-tolerance. As a result autoantibodies are produced, resulting in tissue damage and impaired function. Factors implicated in the development or progression of SLE include a complex genetic contribution [1], alterations in cytokine levels [2], modified sex hormone metabolism [3], an increased rate of apoptosis [4] and increased oxidative stress (reviews include [5–7]).

Previous studies have provided evidence for a role of oxidative stress in SLE, with much of the evidence

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based on assays of lipid oxidation [8,9]. A number of studies have also shown increased levels of oxidative damage to DNA [10,11]. Few studies have quantified protein oxidation in SLE, despite evidence that proteins are likely to be major target for oxidants *in vivo* [12]. We have previously reported elevated levels of protein carbonyls and decreased levels of protein thiols, along with evidence for increased methionine oxidation and 3-nitrotyrosine formation, in SLE patients without renal involvement [13].

Certain manifestations of SLE result in significantly worse clinical outcomes. Up to 60% of adult SLE patients and 80% of children with SLE eventually develop renal complications (lupus nephritis) [14], with the presence of nephritis, or reduced creatinine clearance at disease onset, predictive of death in SLE patients [15]. Long-term studies have found renal damage to be a prognostic factor associated with mortality in SLE [16], whilst renal complications are predictive of increased atherosclerosis risk factors in SLE [17]. Increased lipid peroxidation and decreased antioxidant enzyme activity have been found in lupus nephritis, patients in comparison to healthy controls [18] and it has been suggested that oxidative stress is increased in lupus nephritis compared to non-renal SLE [19]. This enhanced stress may contribute to the poorer outcome in lupus nephritis, with reports of increased oxidized phospholipids on LDL [8] supporting this suggestion. An elevated level of plasma nitric oxide has also been reported [19], though whether this is protective or deleterious is unclear. With regard to protein oxidation, a previous study has reported elevated levels of serum 3-nitrotyrosine levels in some, but not all, SLE patients with active renal disease, compared to both SLE patients with no renal disease and healthy controls [20].

In the study reported here, we have extended our previous studies on protein oxidation in SLE patients without nephritis [13] to examine the levels in SLE patients without and with this complication to determine whether the worse prognosis in patients with nephritis is associated with a further enhancement in oxidative stress, as measured by serum protein thiols and carbonyls. These studies were extended to examine potential changes in total serum protein expression in SLE patients without renal complications and matched controls, by a proteomics approach, with mass spectrometry used to identify proteins of interest; no analogous studies of the entire serum proteome have been previously reported for this disease despite the fact that altered expression of individual proteins and their isoforms might prove useful as markers of disease and help to rationalize the observed complications.

Materials and methods

Materials

Chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise noted. Water used in all experiments was passed through a four-stage Milli Q system.

Ethics approval and patients/controls

Studies were approved by the St George Hospital (Kogarah, NSW, Australia) Human Ethics Committee and the Sydney South West Area Health Service Ethics Review Committee. SLE patients were recruited from the St George Hospital Rheumatology Department. Lupus nephritis patients were outpatients of the Departments of Renal Medicine at St George and Royal Prince Alfred (Camperdown, NSW, Australia) Hospitals. All SLE patients satisfied the American College of Rheumatology 1997 revised criteria [21]; lupus nephritis patients also met World Health Organization criteria [14], with nephritis confirmed by renal biopsy. All non-nephritis patients exhibited normal renal function (serum creatinine < 0.11 mM). Proteomics controls were outpatients of the St George Hospital Rheumatology Department with non-autoimmune complaints. Additional controls were staff of the Rheumatology Department and the Heart Research Institute.

Blood collection

Serum was prepared from venous blood by centrifugation ~ 30 min after collection and subsequently frozen at -80°C. Once thawed for experiments, any excess serum was discarded.

Disease activity assessment

Disease activity was assessed, at the time of blood collection, using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI [22]). This scale examines nine organ systems and gives a weighted score; higher disease activity accrues a higher score. Autoantibodies to double-stranded DNA (anti-dsDNA) were determined by the St George Hospital Rheumatology Department laboratory, using a commercial kit (Diagnostic Products Corporation, Los Angeles, CA). An anti-dsDNA titre > 4.2 IU/mL was classed as positive. Erythrocyte sedimentation rate (ESR), plasma C-reactive protein (CRP) and albumin levels and whole blood haemoglobin were determined by the South East Area Laboratory Service (Sydney, Australia). Elevated ESR and plasma CRP were taken as ≥ 12 mm/h and ≥ 3 mg/L, respectively. Albumin levels of 33–48 g/L and haemoglobin levels of 115–165 g/L were considered normal.

Serum protein, thiol and carbonyl concentration determinations

Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) in triplicate using 96-well plates [13]. Protein thiol concentrations and standards were quantified spectrophotometrically in triplicate using 5,5′-dithionitrobenzoic acid (DTNB) [13]. Protein carbonyl concentrations and standards were determined in duplicate by ELISA using a commercial kit (Zenith Technology, Dunedin, New Zealand) [13]. Different plates were standardized by running repeat samples on every plate.

Two-dimensional electrophoresis of serum proteins

Samples were prepared for isoelectric focusing by combining 12.5 μL of serum with 20 μL of a mixture of 10% (w/v) SDS and 2.3% (w/v) DTT (Amresco); this was heated at 95°C for 5 min. After cooling (< 30°C), a solution containing urea (8 M), CHAPS (4% w/v), Tris base (40 mM), bromophenol blue (0.001%) and DTT (65 mM) was added to a final volume of 1000 μL. Immobilized pH gradient (IPG) strips (either 7 cm pH 3–10 non-linear or 17 cm pH 4–7; Bio-Rad), were rehydrated overnight with 135 μL (7 cm)/330 μL (17 cm) of a solution containing urea (8 M), CHAPS (2% w/v), bromophenol blue (0.001%), pH 3–10 carrier ampholytes (Bio-Rad; 0.2% w/v) and DTT (10 mM). Twenty micrograms (7 cm)/100 μg (17 cm) of protein was loaded per strip by cup loading at the high pH end. Isoelectric focusing was performed in a Bio-Rad Protean IEF cell at 20°C. The initial steps were identical for 7 and 17 cm IPG strips: 100 V for 3 h; 300 V for 2 h; 600 V for 1 h; 1000 V for 1 h; 2000 V for 1 h. The main focusing step was at 3000 V for 24 000 Volt-hours (7 cm) or 5000 V for 80 000 Volt-hours (17 cm).

After focusing, the 7 cm IPG strips were incubated (20 min, 5 mL/strip) with 10 mm DNPH in 2.5 M HCl [23] and subsequently rinsed (5 × 5 min; 2 mL/strip) using a wash solution containing urea (6 M), Tris (375 mM, pH 8.8), SDS (2% w/v) and glycerol (20% v/v). Both 7 and 17 cm strips were then equilibrated (10 min) in wash solution (2 and 5 mL, respectively) with added DTT (2% w/v), followed by 20 min in wash solution with added iodoacetamide (2.5% w/v). Strips were sealed on top of 8–16% gradient gels using 0.6% (w/v) agarose in gel running buffer (6 g/L Tris base, 28.8 g/L glycine and 1 g/L SDS). For the 7 cm IPG strips Ready Gel Tris-HCl 8–16% linear gradient gels (Bio-Rad) were used, with these run on a Bio-Rad Mini-Protean III cell (150 V, ~1 h). The 17 cm IPG strips were run on 18.5 × 20 cm gels, prepared in house, using a Bio-Rad Protean II XL cell (95 V, ~16 h).

Western blotting to determine protein carbonyl distribution

Western blotting was performed using the Invitrogen iBlot™ semi-dry blotting system with PVDF membranes, as per the manufacturer's instructions (7 min transfer). Carbonyls were detected using an Oxy-Blot™ Protein Oxidation Detection Kit (Chemicon, Boronia, VIC, Australia), with spots visualized by chemiluminescence (ECL; Amersham). Gel images were captured using a Bio-Rad ChemiDoc XRS molecular imager (10 min exposure). Duplicate gels were run in parallel for each sample, with one used for carbonyl detection and the other for protein (as below).

Visualization of proteins

Protein spots were visualized following electrophoresis by silver staining [24]. Digitized gel images were acquired using a Umax PowerLook 1120 UDS scanner (Umax Technologies Inc, Dallas, TX) with SilverFast Ai acquisition software (V 6.2.1r1—Mac OSX; LaserSoft Imaging AG, Germany), via Adobe Photoshop CS (ver. 8.0; Adobe Systems, San Jose, CA). Images were acquired in greyscale (14→8 bit) in positive transparency mode at 300 dpi; no level adjustments or filters were utilised.

Proteomics image analysis

Protein spot intensities on large-format gels were determined from images of triplicate gels for each patient and control using a Z3 2D-PAGE Analysis System (ver. 3.0.4; Compugen, Israel). After automatic spot detection, gel edges and molecular weight markers were excluded. Spot quantities were determined as a proportion of the total staining intensity (Q; in parts per million) for all discrete spots [25]. Spots with uneven (doughnut) staining, e.g. albumin [26], were excluded; all spots were checked manually for this effect. The Q values for each patient/control were averaged before statistical analysis.

Isolation and identification of proteins of interest

Protein spots were excised from gels, destained with 15 mm potassium ferricyanide in 50 mm sodium thiosulphate (250 μL), washed in water (4 × 250 μL) and dehydrated, in 12.5 mm ammonium bicarbonate in 50% (v/v) acetonitrile (250 μL), then 100% acetonitrile (2 × 50 μL). After drying under vacuum (SpeedVac; Thermo Savant), spots were reduced with DTT (10 mM, 200 μL, 60 min, 37°C), alkylated with iodoacetamide (25 mM, 200 μL, 60 min, 37°C) and washed with water (3 × 200 μL), then NH_4HCO_3 (10 mM, 200 μL). Following dehydration with 100% acetonitrile (2 × 100 μL), spots were dried under vacuum (SpeedVac). Trypsin (100 ng in 30 μL of 10 mM NH_4HCO_3) was added to each piece and

incubated (37°C, 14 h). Peptides were subsequently extracted with water + 0.1% (w/v) TFA (25 μL), followed by 50% acetonitrile + 0.1% (w/v) TFA (25 μL); the combined washes were dried (SpeedVac), and frozen at −20°C prior to mass spectrometric analysis at the Bioanalytical Mass Spectrometry Facility (UNSW, Sydney, Australia) as previously [27]. The Tof MS survey scan was acquired for 1 s, the two largest multiply charged ions with counts > 20 were sequentially selected by Q1 for MS-MS and tandem mass spectra accumulated for ≤ 6 s. Peak lists were generated by MassLynx (Micromass) using the Mass Measure program and submitted to the database search program Mascot (version 2.1, Matrix Science, London, UK). Search parameters were: precursor and product ion tolerances ± 0.25 and 0.2 Da, respectively; Met(O) and Cys-carboxyamidomethylation specified as variable modification, enzyme specificity was trypsin, one missed cleavage was possible, with the NCBInr database searched.

Serum apolipoprotein CIII determination

Serum levels of apoCIII were determined by an ELISA [28]. Unless stated otherwise, plate washing steps consisted of five washes (200 μL/well) using PBS with added 0.5% (v/v) Tween-20 (PBS-T). Sample buffer consisted of PBS-T with added 0.5% (w/v) BSA and 0.1% (w/v) NaN_3. Incubations were carried out using an orbital shaker set at 60 rpm.

Ninety-six well plates (Nunc MaxiSorp) were coated with 1 μg/well (in 100 μL) of polyclonal goat anti-human apoCIII (Biodesign, Jomar Diagnostics, Stepney, SA, Australia), diluted with PBS + 0.1% NaN_3 just prior to use. Perimeter wells were not used. Plates were incubated at 37°C for 3 h, then overnight at 4°C. After washing (3 × 200 μL/well PBS-T, 21°C) wells were blocked (1 h, 21°C) using 200 μL/well PBS containing 0.5% (w/v) BSA and 0.1% (w/v) NaN_3. Standards (0.05–0.5 ng/well) were run on each plate, using a pooled serum sample standardized for apoCIII concentration by spiking with purified human apoCIII (Biodesign). Serum samples of known apoCIII concentration (high and low) were run on each plate as internal controls. All serum test samples were diluted 1:80 000, incubated with 100 μL/well (2 h, 37°C), followed by washing and addition of secondary antibody (100 μL/well of 1:30 000 dilution polyclonal goat anti-human apoC-III conjugated with biotin; Biodesign). Plates were then incubated (2 h, 37°C), washed, avidin-alkaline phosphatase added (100 μL/well, 1:30 000 dilution), incubated at 21°C (1 h) then washed. Colour development was initiated by the addition of alkaline phosphatase yellow liquid substrate (100 μL/well). After regular shaking for 11 min, the reaction was halted by addition of NaOH (25 μL/well, 3 M) and the absorbance at 405 nm determined. Concentrations were determined by reference to a second-order polynomial curve generated using standards run on each plate. Standards and samples were run in duplicate. Inter-assay variability was assessed by re-running several samples across multiple assays. Based on these samples, a correction factor was calculated to give consistent values between plates on which the samples were run. The between-assay coefficient of variation was 9.3%, whilst the within-assay coefficient of variation was 3.3%.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.03 for Windows, (GraphPad Software, San Diego, CA). Student's *t*-test was used to compare one condition with its corresponding control where numbers of both groups were similar, otherwise a Mann-Whitney non-parametric test was used. For multiple conditions, one-way ANOVA was used, with Newman-Keuls post-hoc test. In all cases, *p*-values of < 0.05 were considered significant.

Results

Patients and controls

The demographics of the controls, non-renal SLE patients and lupus nephritis patients for whom serum protein thiols, protein-bound carbonyls and apoCIII levels were measured are given in Table I. Serum protein carbonyl distribution was determined using a sub-set of the thiol/carbonyl study patients; their demographics are given in Table II, whilst those used for the proteomics experiments are given in Table III. All of the SLE patients used in the proteomics study had well-controlled non-renal SLE and relatively low disease activity. Care was taken to ensure that the non-autoimmune controls were matched according to age (± 5 years), gender (all female) and race.

Serum protein thiol and protein carbonyl levels

Serum protein thiols and carbonyls were measured as generic markers of protein oxidation. Protein thiol levels were significantly decreased in the lupus nephritis patients compared to the controls; however there was no significant change compared to the SLE patients without renal involvement (Figure 1). The serum protein carbonyl levels from the patients with SLE, both with and without nephritis, were significantly increased compared to the controls, but there was no significant difference between the two SLE patient groups (Figure 2).

Serum protein carbonyl distribution

The distribution of protein-bound carbonyls was examined by Western blotting of serum proteins

Table I. Demographics of the patients whose sera were used to determine serum protein oxidation levels (non-bracketed) and apoCIII levels (bracketed). The same cohort of lupus nephritis patients was used in both the oxidation and apoCIII studies. There was no significant difference between the ages of any of the groups analysed in these studies (one-way ANOVA with Newman-Keuls post-hoc test).

Parameter	Controls	SLE	Lupus nephritis
Female (*n*)	8 (17)	19 (22)	11
Male (*n*)	1 (2)	2 (2)	0
Age (y)	32.9±9.0 (38.3±10.7)	41.1±14.0 (43.6±15.0)	35.3±8.8
Race/ethnicity			
Caucasian	100% (100%)	38.1% (54.2%)	36.4%
Asian	0 (0)	42.9% (33.3%)	45.5%
Other races	0 (0)	19.0% (12.5%)	18.1%
Medication			
Immunosuppressives		14.3% (12.5%)	63.6%
Corticosteroids		38.1% (33.3%)	72.7%
Anti-malarials		28.6% (29.2%)	27.3%
COX-2 inhibitors		9.5% (8.3%)	0
Anticoagulants		9.5% (8.3%)	0
ACE inhibitors		0 (0)	9.1%
Autoantibodies%			
Anti-dsDNA positive*		61.9% (66.7%)	100%†
SLEDAI score			
Average		3.4±3.3 (3.8±4.1)	12.6±3.5‡
Median		2.7 (2)	12‡
Range		0–12 (0–14)	9–17‡

*Autoantibodies to double-stranded DNA; patients are considered to be positive for these autoantibodies if the titre is >4.2 IU/mL.
†Anti-dsDNA antibody levels were not available for two lupus nephritis patients from Royal Prince Alfred Hospital.
‡SLEDAI scores were only available for the four lupus nephritis patients regularly assessed at the Rheumatology Department of the St George Hospital.

separated by 2D electrophoresis, in controls and SLE patients with and without nephritis. A representative (control) silver-stained gel, with the major protein classes labelled, is shown in Figure 3A; all patients examined had similar distributions of the major protein classes. Representative Western blots, showing the distribution of protein-bound carbonyls in controls, SLE patients and lupus nephritis patients are given in Figure 3B–D, respectively. Protein carbonylation was widespread across the proteome in all three groups, affecting all major protein classes. A striking and reproducible difference between the control and

Table II. Demographics of the sub-set of patients whose serum was used to examine protein carbonyl distribution.

Sex	Race	Age	Carbonyls (nmol/mg)*	Albumin (mg/mL)†	SLEDAI score‡	Anti-dsDNA (IU/mL)¶	Medication
SLE patients							
F	Caucasian	42	0.2536±0.0036	44	2	2.5	Anti-malarial
F	Asian	49	0.1062±0.0038	37	0	3.9	Nil
F	Caucasian	30	0.1002±0.0094	ND	6	43.1	Prednisolone; Anti-coagulant
F	Asian	22	0.1017±0.0059	ND	10	578	Prednisolone
Lupus nephritis patients							
F	Hispanic	33	0.0530±0.0019	32	ND	66	Prednisolone; Immunosuppressive
F	Asian	40	0.1675±0.0010	36	ND	8.8	Prednisolone; Immunosuppressive
F	Caucasian	32	0.0683±0.0036	28	ND	45	Nil
F	Asian	41	0.1550±0.0061	24	ND	120	Nil
Controls							
F	Caucasian	28	0.0503±0.0029				
F	Caucasian	41	0.0456±0.0005				
F	Caucasian	38	0.0338±0.0015				
F	Caucasian	43	0.0307±0.0010				

ND = not determined.
*Serum protein carbonyls were determined by ELISA. Concentrations are given as nmoles of carbonyl/mg of protein.
†Normal albumin concentration is 33–48 g/L.
‡Systemic lupus erythematosus disease activity index; a score of ≥6 is taken as an indicator of high disease activity.
¶Autoantibodies to double-stranded DNA; patients are considered to be positive for these autoantibodies if the titre is >4.2 IU/mL.

Table III. Demographics of SLE patients and controls used in the proteomics experiments.

Sex	Race	Age	ESR (mm/h)⋆	CRP (mg/L)†	Albumin (mg/mL)‡	Hb (g/L)¶	SLEDAI score§	Anti-dsDNA (IU/mL)‖	Medication
SLE patients									
F	Caucasian	41	8	2	42	139	2	2.5	Anti-malarial
F	Caucasian	54	13	ND	36	123	2	15	Immunosuppressive; Anti-malarial
F	Caucasian	64	18	9	41	139	2	13	Anticoagulant
F	Asian	38	38	1	40	122	3	2.6	Nil
F	Caucasian	38	30	<4	44	136	2	2.2	Prednisolone; COX-2 inhibitor
F	Caucasian	27	ND	ND	38	136	6	2.5	COX-2 inhibitor
F	Arabic	33	10	5	38	141	4	2.8	Nil
Controls									
F	Caucasian	40	4	<1	41				
F	Caucasian	59	6	1	37				
F	Caucasian	59	ND	ND	ND				
F	Asian	40	12	ND	39				
F	Caucasian	36	6	4	ND				
F	Caucasian	30	ND	ND	ND				
F	Arabic	34	ND	ND	ND				

ND = not determined.
⋆Erythrocyte sedimentation rate; a rate of ≥12 mm/h is considered to be elevated.
†C-reactive protein; a concentration of ≥3 mg/L is considered to be elevated.
‡Normal albumin concentration is 33–48 g/L.
¶Haemoglobin; normal range in women is 115–165 g/L.
§Systemic lupus erythematosus disease activity index; a score of ≥6 is taken as an indicator of high disease activity.
‖Autoantibodies to double-stranded DNA; patients are considered to be positive for these autoantibodies if the titre is >4.2 IU/mL.

SLE groups was the enhanced levels of protein carbonyls on the immunoglobulins. Identification of particular proteins that are carbonylated only in SLE or lupus nephritis patients was not possible, owing to variation between patients within each group.

Identification and quantification of proteins on 2D gels

Large-format 2D electrophoresis was used to screen for proteins that may be perturbed in SLE patients compared to controls. Only SLE patients without nephritis were compared to controls, as the majority of lupus nephritis patients showed perturbed serum albumin concentrations; this would skew the proteomics results, making analysis impossible. The potential removal of albumin (using Cibacron-Blue based spin columns) was examined, but this process was also found to alter the levels of several hundred other proteins, which was not acceptable (data not shown).

Serum proteins from seven controls and seven SLE patients were separated by 2-dimensional electrophoresis in triplicate; a typical gel is shown in Figure 4. As immunoglobulins are poorly resolved by 2D electrophoresis, a narrower pH range was separated during the isoelectric focusing step in comparison to

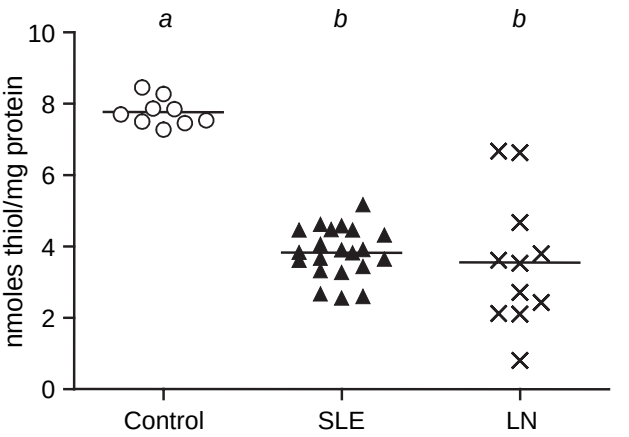

Figure 1. Average levels of serum protein thiols in controls (○) and SLE patients both without (▲) and with (×) renal involvement (lupus nephritis; LN). Statistical analysis was by one-way ANOVA with Newman-Keuls post-hoc test; different letters indicate statistically distinct results at the $p < 0.01$ level.

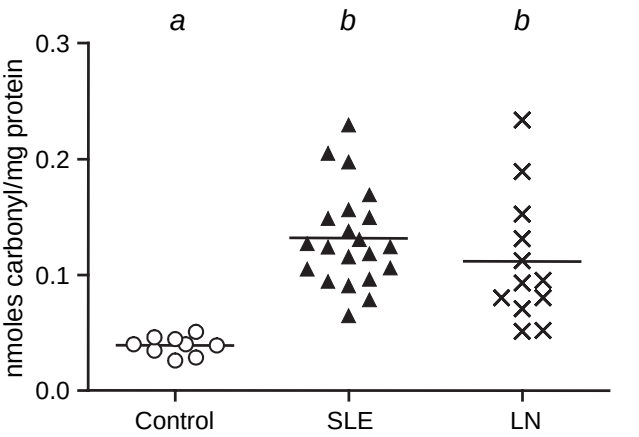

Figure 2. Average levels of serum protein carbonyls in controls (○) and SLE patients both without (▲) and with (×) renal involvement (lupus nephritis; LN). Statistical analysis was by one-way ANOVA with Newman-Keuls post-hoc test; different letters indicate statistically distinct results at the $p < 0.01$ level.

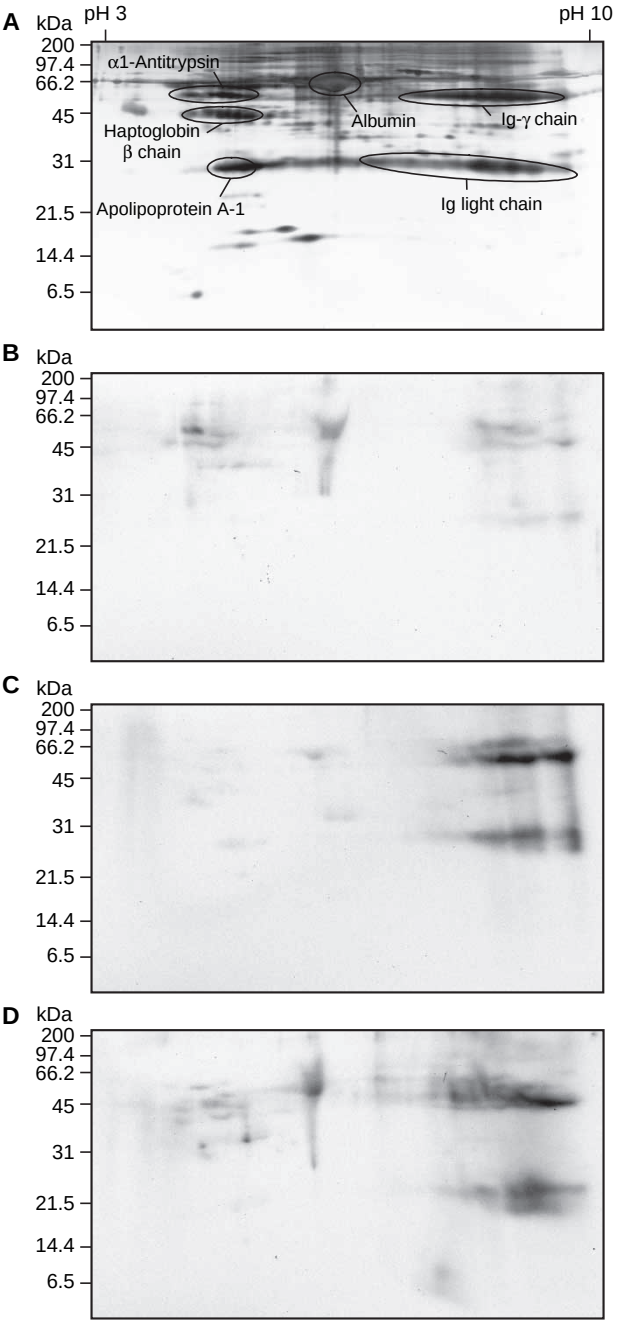

Figure 3. Distribution of protein-bound carbonyls on serum proteins in controls and SLE patients both without and with nephritis. Proteins (20 μg) were separated by 2D electrophoresis, with subsequent detection of DNPH-derivatized protein-bound carbonyl groups by Western blotting, as detailed in the Materials and methods section. (A) Typical gel stained for protein (silver stain), showing the identities and positions of the most abundant serum proteins. (B–D) Western blots showing the typical protein carbonyl distribution in: (B) a control; (C) a SLE patient without nephritis; and (D) a SLE patient with nephritis. The protein-bound carbonyl concentrations, as determined by ELISA, were 0.0456 ± 0.0005, 0.1017 ± 0.0059 and 0.1675 ± 0.0010 nmol/mg protein in (B), (C) and (D), respectively.

the protein carbonyl Western blotting experiments (pH 4–7, rather than the pH 3–10).

No spots were detected that were novel or absent in all of the SLE patients compared to controls, thus only protein spots that were present in all gels were subject to further analysis. Application of the criteria detailed in the Materials and methods section enabled reproducible identification of 68 protein spots on each of the 42 gels analysed from the seven controls and seven SLE patients (circled spots in Figure 4). Six protein spots were identified in which there was a statistically significant change in Q value in the SLE patients compared to the controls (inset graphs in Figure 4). The only statistically significant changes were decreases in the SLE patients compared to controls, however several proteins were increased in the SLE patient group by an appreciable, though not significant, amount (data not shown). The majority of proteins analysed showed little or no change in their levels between the control and SLE patient groups, ruling out systematic errors. The six protein spots of interest were subsequently isolated, digested with trypsin and subjected to LC/MS/MS analysis. The identity of five of the spots is detailed in Table IV. Attempts to characterize the sixth protein spot by mass spectrometry following electroelution were unsuccessful, due to its low abundance.

As no more than two of the seven SLE patients used in the proteomics experiments were taking the same medication (or no medication; see Table III), it was not possible to perform statistical analysis to determine whether medication was modulating protein levels. However, this variation in medication makes it highly unlikely that drug effects are responsible for the observed decreases in spot intensities in the SLE group compared to the controls.

Serum apolipoprotein CIII levels determined by ELISA

The significant decrease in one of the apoCIII isoforms (spot 21, Figure 1 and Table III)—a protein considered to be pro-atherogenic—was examined further by ELISA using a larger cohort of 19 controls and 24 SLE patients (Table I). Eleven lupus nephritis patients were examined, since this method is not perturbed by abnormal albumin concentrations. The control levels of serum apoCIII (Figure 5) agree with previous literature data [29]. The mean apoCIII level in the non-renal SLE patients was lower than in controls (Figure 5), but this did not reach statistical significance. In contrast, the mean serum level of apoCIII in the lupus nephritis patients was significantly increased compared to both the controls and SLE patients with no renal involvement (Figure 5). This increase appears to be derived from a sub-set of the lupus nephritis patients rather that the group as a whole.

Effect of anti-malarials on total apoCIII levels in SLE patients

As the anti-malarial drug hydroxychloroquine has previously been reported to cause a significant

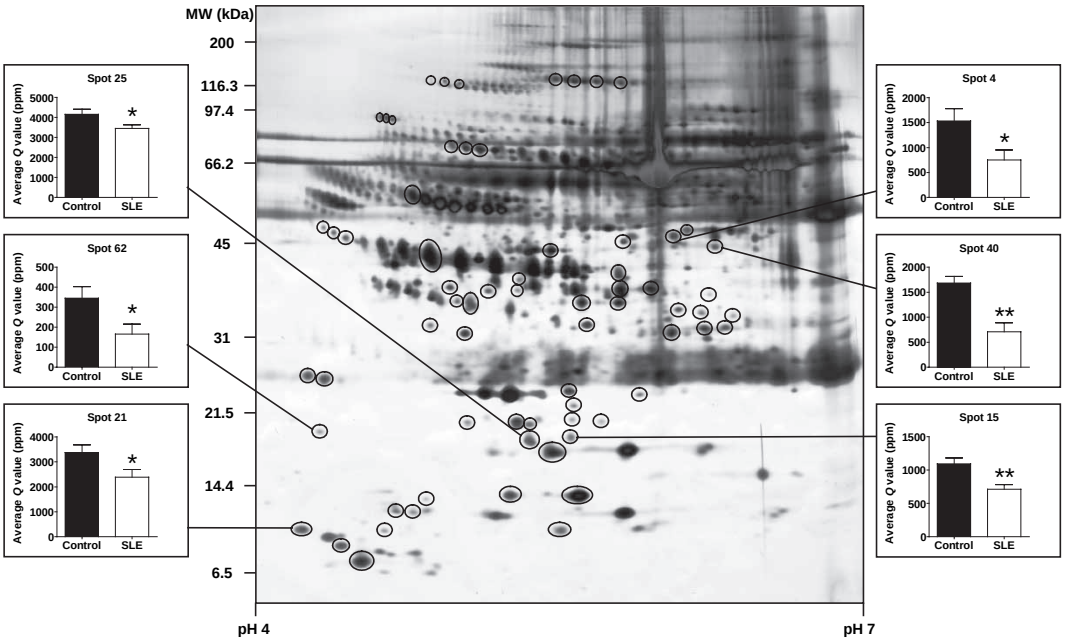

Figure 4. Digitized image of 100 μg of human serum proteins separated by two-dimensional SDS-PAGE, showing the 68 protein spots analysed in this study (circled). First dimensional separation was by isoelectric focusing using 17 cm pH 4–7 IPG strips. Second dimensional separation was by SDS-PAGE using 18.5 × 20 cm 8–16% gradient gels. Digitized gel images were analysed using Z3 image analysis software as detailed in the Materials and methods section. Inset graphs show the averaged spot *Q* values ± SEM (in part per million of total staining intensity) for the protein spots where a significant difference was found between controls and SLE patients, as determined by unpaired two-tailed *t*-test; $^{\star}p < 0.05$; $^{\star\star}p < 0.01$.

decrease in plasma apoCIII levels of SLE patients without renal disease [30], the data for those on such medication was compared to non-users. No significant difference ($p = 0.4022$ by one-tailed *t*-test) was observed in serum apoCIII levels between these two groups (Figure 6). This effect was also examined in the lupus nephritis patients; non-parametric statistical analysis confirmed significantly lower levels of apoCIII in the lupus nephritis patients taking hydroxychloroquine ($p = 0.0121$ by one-tailed Mann-Whitney test,

Table IV. Identification of proteins for which statistically significant differences in expression were detected between controls and SLE patients, and control proteins. Proteins were extracted from gels and digested with trypsin, with subsequent mass spectrometric analysis as detailed in the Materials and methods section. The results denoted by 'Expt. 1' and 'Expt. 2' in the table were obtained in independent blinded experiments to ensure methods were robust.

Spot ID	Protein ID by LC/MS	NCBI accession No.	Protein/fragment mass (kDa)	Score*	Sequence coverage	No. of peptides detected
Unknown proteins						
4	IgM heavy chain	38408	49.4†	159	12.4%	4
15	—	—	~18‡	—	—	—
21	Apolipoprotein CIII	37499463	10.8†	177	27.3%	2
25 (Expt. 1)	HSA fragment	4389275	~18‡	261	ND§	11
25 (Expt. 2)	HSA fragment	4389275	~18‡	333	ND§	7
40	IgM heavy chain	38408	49.4†	90	8.4%	3
62 (Expt. 1)	Haemopexin fragment	386789	23.6¶	95	ND§	2
62 (Expt. 2)	Haemopexin fragment	386789	23.6¶	72	ND§	2
Known proteins						
Transthyretin	Transthyretin	339685	12.8†	154	23.1%	3
BSA	BSA	30794280	69.3†	894	26.4%	18

ND = not determined.

*High scores indicate a likely match.

†Mass as given by the NCBInr protein database.

‡Mass approximated by comparison to molecular weight markers and reference to other known mass proteins in the region of the spot of interest.

¶Mass determined as per matching with SWISS-2DPAGE human plasma protein map (http://ca.expasy.org/cgi-bin/map2/big?PLASMA_HUMAN).

§Sequence coverage was unable to be determined as the protein spot does not correspond to the entire protein sequence.

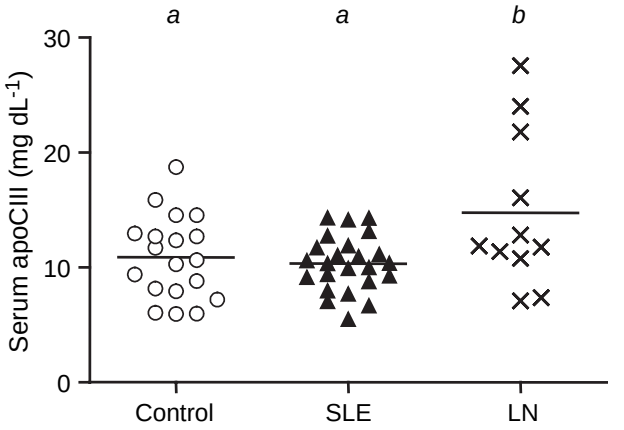

Figure 5. Serum levels of apoCIII in controls (○), and SLE patients with no renal involvement (SLE; ▲) or lupus nephritis (LN; ×). Serum levels of apoCIII were determined by ELISA, as detailed in the Materials and methods section. Statistical analysis was by one-way ANOVA with Newman Keuls post-hoc test; different letters indicate statistically distinct results at the $p < 0.05$ level.

data not shown), but the low numbers ($n = 3$) in the hydroxychloroquine group requires that this result be treated with care.

Discussion

The results of the studies presented here are consistent with elevated levels of serum protein oxidation in SLE patients with nephritis, in a similar manner to that previously seen in SLE patients without nephritis [13], as manifested by decreased levels of serum protein thiols and increased levels of protein-bound carbonyls. The lack of any significant difference between the SLE patients without and with nephritis is surprising, given the markedly worse prognosis of these patients.

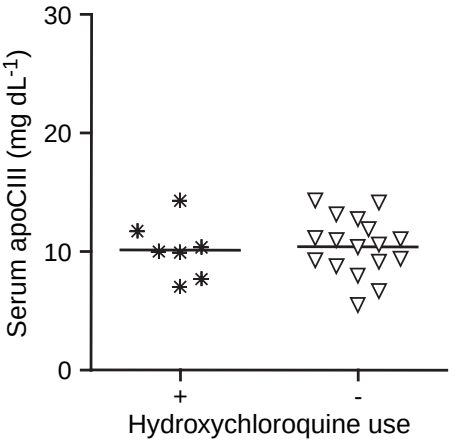

Figure 6. Serum levels of apoCIII in SLE patients without renal disease who were taking (✱) or not taking (▽) the anti-malarial drug hydroxychloroquine. Serum levels of apoCIII were determined by ELISA, as detailed in the Materials and methods section. Statistical analysis was by one-tailed *t*-test, $p > 0.05$.

Decreases in protein thiols and increases in protein carbonyls can result from the presence of multiple oxidants [31–33]. Thus, although the results of this study cannot tell us which specific oxidant(s) cause the observed damage, both thiol loss and carbonyl formation are good indicators that total protein oxidation levels are increased in the SLE and lupus nephritis patients studied here. This suggests that the balance between oxidation and repair has been perturbed in these patients. Other pathologies in which thiol loss is evident include senile cataract, chronic renal failure, various liver diseases and atherosclerosis [34–36]. Similarly, enhanced levels of protein-bound carbonyls have been detected with ageing [37] and in a range of diseases, including rheumatoid arthritis, pulmonary fibrosis, diabetes, Parkinson's disease and Alzheimer's disease [33,38,39].

The complexity and diversity of the symptoms seen in SLE makes the identification and quantification of the oxidants difficult. It is already known that genetic factors play a role, with elevated levels of 3-nitrotyrosine [20], found to correlate more strongly with disease activity in African American patients than Caucasians. We have previously reported that the haem enzyme myeloperoxidase does not appear to play a role [13].

Examination of the distribution of protein-bound carbonyls, by 2D electrophoresis with subsequent Western blotting, has shown that protein carbonyls are formed on all the major protein classes in all three study groups, but that these are particularly prevalent on the immunoglobulins in the SLE patients both with and without nephritis. The pattern of carbonyl distribution between the SLE patients with and without nephritis has been shown to be similar, suggesting that the overall levels of protein carbonyls detected for these two groups are not being skewed by any altered protein profiles present in the nephritis patients. The significance of the increased concentration of carbonyls present on the immunoglobulins remains to be determined, however a similar phenomenon has been observed in rheumatoid arthritis patients [40]. Whether this merely reflects the increased concentration of this class of proteins in patients with these autoimmune diseases or a specific targeting of oxidative damage to these proteins remains to be determined.

These 2D gels also suggest that there are some variations between the expression levels of the minor classes of serum proteins between the controls and SLE patients with and without nephritis. This observation prompted a closer examination of the serum proteome, with mass spectrometric identification of proteins that were significantly altered in comparison to controls. Of the differences detected, the significant decrease of the 10.8 kDa isoform of apoCIII in SLE patients compared to controls (spot

21, Figure 1 and Table IV) is of particular interest. ApoCIII plays an important role in the modulation of triglyceride levels [41]. ApoCIII over-production is associated with hypertriglyceridaemia [42] and increased apoCIII levels have been found to be a better predictor of coronary heart disease risk in non-SLE patients than plasma triglycerides [43]. Furthermore, the generally accepted 'lupus pattern' of increased plasma VLDL cholesterol and triglycerides and decreased HDL cholesterol [44,45] might be expected to result in increased apoCIII levels in SLE patients.

The apoCIII ELISA results, although not showing a significant decrease in apoCIII levels in non-renal SLE patients compared to controls, nonetheless confirm a lack of elevation of serum apoCIII levels in non-renal SLE patients. These results are complimentary to the proteomics results, suggesting a change in isoform distribution in the SLE patients, but no overall change in apoCIII levels. Recent work has identified all three isoforms of apoCIII on 2D gels [46,47], potentially allowing quantitation of individual isoforms once issues of co-migration with other lipoproteins (particularly apoCII) are overcome.

In contrast, however, there is clearly a sub-set of lupus nephritis patients in whom serum apoCIII levels are significantly increased. This may, at least in part, contribute to the increased risk of atherosclerosis in these patients. These results also highlight the importance of stratifying lupus nephritis patients from other SLE patients. Some of the previous studies that have reported a pro-atherogenic lipid profile in SLE patients have included people with compromised renal function [45] or have not assessed this potential confounding factor [48]. As nephritis is a significant independent risk factor for pro-atherogenic lipid profiles in non-SLE patients [49], the inclusion of such patients would be expected to result in misleading data. Our results clearly support this.

To date there have been very few studies of apoCIII levels in SLE patients. One study found that nine patients taking the anti-malarial drug hydroxychloroquine displayed a 43% reduction in whole serum apolipoprotein CIII levels, when compared to nine SLE patients not taking hydroxychloroquine [30]; unfortunately no comparisons to healthy controls were given. In contrast, two of the SLE patients studied here, who were taking hydroxychloroquine, had the highest and fourth highest spot intensities (*Q* values) for the apoCIII isoform examined, whilst the ELISA results showed no significant effect of this drug on apoCIII levels in non-renal SLE patients (Figure 6). This issue warrants further study in the light of the reputed reduction in pro-atherogenic lipid profiles by this drug [50]. It may be that this drug only provides significant benefit in those in whom the lipid/lipoprotein levels are already significantly elevated, e.g. in lupus nephritis patients.

The other proteins/isoforms found to be present, by proteomics, at altered levels may also have important implications for SLE. The decreased levels of (free) haemopexin may arise from its role as a protective agent against oxidative stress by binding free haem that might otherwise catalyse oxidation [51]. Decreased levels as a result of haemolytic anaemia can be ruled out here, as all SLE patients in the proteomics study had blood haem levels within the normal reference range.

The decrease in two IgM heavy chain isoforms is also noteworthy, since increased levels of various Ig isotypes are normally associated with this disease. The significance of this elevation is unknown at this time. The significant decrease in the SLE patient group of the 18 kDa HSA fragment (spot 25) was evident despite the fact that total plasma albumin concentrations for all of the SLE patients in the proteomics study were well within the normal range. If albumin depletion had been carried out, as is often the case in proteomic studies [52,53], this change would have been missed. A potential drawback of not carrying out albumin depletion, however, is that changes in the expression of low abundance proteins, particularly those of similar mass and isoelectric point, may have been missed due to the dominance of this spot on the gels.

In summary, our findings indicate that serum protein oxidation levels, as measured by thiol loss and carbonyl formation, are increased by a similar amount in SLE patients both with and without nephritis. This is in contrast with the worse pathological outcomes observed in lupus nephritis patients. However, the fact that protein oxidation levels are chronically increased in SLE patients in comparison to healthy controls (Morgan, Sturgess and Davies; unpublished results) means that oxidative stress is likely to be involved in the chronic organ damage observed in SLE. It must be remembered that factors such as increased atherosclerosis in SLE patients cannot be explained merely by traditional risk factors [54,55]. Where added complications are also present, it will be more difficult for the already stressed organs to cope. Our finding that serum concentrations of pro-atherogenic apoCIII are significantly higher in a sub-set of lupus nephritis patients further supports the premise that it is the combination of factors in SLE that lead to their worse outcomes, including the increased incidence of atherosclerosis. To this end, serum apoCIII levels should be investigated in future studies, as a possible biomarker for lupus nephritis and increased atherosclerotic risk. Overall, these results confirm the need for continued research into protein perturbations in SLE and highlight the need

for continued examination of the mechanisms of the increased atherosclerosis seen in this disease.

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