Free Radical Research, February 2009; 43(2): 117–127

informa
healthcare

Evidence for chronically elevated serum protein oxidation in systemic lupus erythematosus patients

PHILIP E. MORGAN[1], ALLAN D. STURGESS[2], & MICHAEL J. DAVIES[1,3]

[1]*Free Radical Group, The Heart Research Institute, Camperdown, Sydney, NSW, Australia,* [2]*Rheumatology Department, The St George Hospital, Kogarah, Sydney, NSW, Australia, and* [3]*Faculty of Medicine, University of Sydney, Sydney, NSW, Australia*

(Received 21 August 2008; revised 26 October 2008)

Abstract
Serum protein oxidation levels in people with the autoimmune disease systemic lupus erythematosus (SLE) have previously been shown to (a) be elevated at a single time point and (b) correlate with disease activity. This study investigates whether this elevation is a chronic phenomenon, by analysis of multiple serum samples collected from 21 SLE patients and nine controls over a period of up to 38 months. Protein thiols were chronically decreased in SLE patients with stable or variable disease activity compared to controls, whilst protein-bound carbonyls and glycine were chronically increased. 2D-gel analysis of carbonyl distribution showed albumin and immunoglobulins to be particularly affected. In SLE patients with stable disease activity, higher long-term protein oxidation correlated with higher long-term disease activity. SLE patients with variable disease activity exhibited varying correlations between protein oxidation and disease activity markers. These results further support a role for oxidative stress in the pathogenesis of SLE.

Keywords: *Systemic lupus erythematosus, protein oxidation, oxidative stress, protein thiols, protein carbonyls, chronic disease*

Abbreviations: *anti-dsDNA, antibodies to double-stranded DNA; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; GSH, reduced glutathione; HPLC, high performance liquid chromatography; HSA, human serum albumin; IEF, isoelectric focusing; Ig, immunoglobulin; IPG, immobilized pH gradient; MetSO, methionine sulphoxide; $MetSO_2$, methionine sulphone; OPA, o-phthaldialdehyde; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; THF, tetrahydrofuran.*

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease of unknown aetiology. The immune system of people with SLE loses self-tolerance, resulting in the production of autoantibodies. The range of autoantibodies produced is broad, and as a consequence the manifestations of the disease are diverse. Certain long-term complications are quite common; these include renal, cardiovascular, neuropsychiatric and musculoskeletal damage [1].

Despite the diverse manifestations of SLE, it appears that elevated levels of oxidative stress are a consistent and universal factor. Previous studies have demonstrated a role of oxidative stress in the atherosclerosis in people with SLE [2,3], as well as a more general correlation between oxidation and patient-reported symptoms of SLE, including fatigue and

Correspondence: Dr Philip Morgan, The Heart Research Institute, 114 Pyrmont Bridge Road, Camperdown, Sydney, NSW 2050, Australia. Fax: +61 2 9565 5584. Email: morganp@hri.org.au

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802623896

lower quality of life [4]. It has been shown that some autoantibodies exhibit a preference for oxidized substrates [5–7], suggesting that oxidation could be an important factor in antigenicity. Much of the evidence for increased oxidative stress in SLE has arisen from studies on lipid oxidation [2,8–10], but it is unclear whether lipids are exclusive or major targets of oxidation in the absence of quantitative data for modification of other materials (e.g. proteins or DNA) and, hence, which processes may be targets for potential therapies or protective strategies. A small number of studies have examined DNA damage (e.g. by quantification of the marker compound 8-hydroxydeoxyguanosine [11,12]), but little is known regarding the role of protein oxidation in SLE.

Proteins are likely to be a major target for oxidative damage due to their abundance in cells, plasma and most tissues and their rapid rates of reaction with many oxidants [13]. Elevated levels of protein oxidation have been associated with a number of chronic diseases [14], especially those that include inflammatory components, such as asthma, multiple sclerosis, arthritis, kidney disease, Alzheimer's disease, diabetes and atherosclerosis [15–18]. Most of the limited studies undertaken to date on protein oxidation in SLE have examined only a single marker of damage (e.g. protein thiols [19,20] or the tyrosine oxidation product 3-nitrotyrosine [21]). As different sources of oxidative stress can result in the formation of a wide range of different oxidation products [14,22, 23], reliance on a single marker may be misleading. Furthermore, it is unclear whether the detected oxidation arises from acute events or is a chronic feature of the disease. Thus, there is a lack of definitive information as to the time course of oxidation events relative to disease activity, whether these are elevated in synchrony and whether oxidation levels are predictive of disease severity.

In previous studies we have examined serum protein oxidation levels in SLE patients vs healthy controls [24,25], with loss of protein-bound amino acids, as well as increases in a range of well-characterized specific protein side-chain oxidation products (methionine sulphoxide (MetSO), 3,4-dihydroxyphenylalanine, di-tyrosine, 3-chlorotyrosine, 3-nitrotyrosine and *o*-tyrosine) quantified at a single time point [24]. Protein-bound carbonyls and thiols were also measured as generic markers of protein oxidation. A significant elevation of most of these products, as well as a marked decrease in protein thiols, was detected. In a follow-up study it was shown that serum protein thiol and carbonyl levels in patients with lupus nephritis are significantly elevated compared to controls; however, these levels did not vary significantly from those from SLE patients with normal renal function [25]. In the current study this work has been extended to examine whether enhanced protein oxidation is a chronic feature of SLE or a response to acute events. This has been accomplished by repeated quantification (≥ 3 assessments in all cases) of multiple protein oxidation markers in a cohort of non-renal SLE patients and controls over a period of up to 38 months. We have also examined whether average levels of these markers correlate with multiple clinical measures of disease status, including SLEDAI score (systemic lupus erythematosus disease activity index), levels of antibodies to double-stranded DNA (anti-dsDNA; an autoantibody specific for SLE), erythrocyte sedimentation rate (ESR) and human serum albumin (HSA) concentrations. This approach has allowed us to address whether protein oxidation levels are chronically increased in SLE patients compared to a healthy population and whether levels of protein oxidation correlate with common disease assessment measures.

Materials and methods

Materials

Chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise noted. HPLC solvents were purchased from EMD Chemicals (Merck, Kilsyth, Vic, Australia) and filtered before use through VacuCap 90 filter units with 0.2 μm Supor membranes (Pall Corporation, Cheltenham, Vic, Australia). Water used in all experiments was passed through a four-stage Milli Q system. Phosphate buffer solutions were pre-treated with Chelex 100 resin (Bio-Rad, Hercules, CA) to remove trace metal ions.

Patients/controls

This study was approved by the Human Ethics Committee of The St. George Hospital, Kogarah, NSW, Australia. All SLE patients were outpatients of the hospital Rheumatology Department with normal renal function (serum creatinine concentrations < 0.11 moles/L) and no history of lupus nephritis at the time of recruitment. All SLE patients satisfied the American College of Rheumatology (ACR) 1997 revised criteria [26]. Controls were staff of the Heart Research Institute with no family history of autoimmunity. The demographics of the controls and SLE patients are given in Table I. There were no statistically significant differences in subject ages between the control and SLE patient groups (one-way ANOVA).

Blood collection and serum preparation

Serum was prepared from venous blood collected into plain clot tubes (Vacutainer, Becton Dickinson) by centrifugation at 3500 rpm for 10–15 min $\sim$ 30 min after blood collection. The time between sequential sample collection from patients and controls

Table I. Demographics of the SLE patients and controls.

Parameter	Controls	SLE patients characterized by disease activity[a]	
		Stable	Variable
Female (*n*)	8	14	5
Male (*n*)	1	2	0
Age at 1st sample (y)	32.9±9.0	42.1±13.2	38.0±17.5
Observation period (m)	1.9±0.1	14.3±5.9	20.0±12.7
Race/ethnicity (%)			
Caucasian	100%	43.75%	20%
Asian	0	37.5%	60%
Other races	0	18.75%	20%
Medication (%)			
Immunosuppressives		12.5%	20%
Corticosteroids		31.3%	60%
Anti-malarials		25%	40%
COX-2 inhibitors		12.5%	0
Anticoagulants		12.5%	0
Autoantibodies (%)			
Anti-dsDNA positive[b]		56.3%	80%
Anti-cardiolipin positive[c]		18.8%	20%
SLEDAI score[d]			
Average		2.6±2.9	8.2±4.3
Median		2	8
Range		0–10	2–16

[a]Disease activity as assessed by SLEDAI score at the time that each blood sample was collected. Stable SLEDAI scores varied by ≤ 3 over the period of observation, whilst variable SLEDAI scores varied by >3 after the guidelines in Gladman et al. [28].
[b]Patients positive for antibodies to double-stranded DNA.
[c]Patients positive for anti-cardiolipin antibodies of either IgG or IgM isotype.
[d]SLEDAI scores were assessed at the time of blood collection ($n=49$ from 16 patients with stable disease activity; $n=19$ from five patients with variable disease activity).

varied from 19 days to 19 months, with a total observation time of up to 38 months.

Disease activity measurement

Disease activity was assessed on blood collection, using the SLEDAI scoring system; higher scores indicate higher disease activity [27]. All patients were assessed by the same clinician with extensive experience of this scale. Patients with SLEDAI scores varying by >3 over the time of observation were classified as having variable disease activity, whilst those with scores varying by ≤ 3 were classified as having stable disease, as previously [28].

Anti-dsDNA antibody measurement

Autoantibodies to double-stranded DNA (anti-dsDNA) were determined for all SLE patients using a commercially-available anti-dsDNA radioassay kit (Diagnostic Products Corporation, Los Angeles, CA); titres above 4.2 IU/mL were considered positive for anti-dsDNA antibodies. Logarithm values of the anti-dsDNA titre of antibodies were used during analysis, to eliminate bias from extremely high titre results.

Acute phase reactants

Whole blood ESR and HSA levels were determined for all patient samples during routine patient assessment, by the South East Area Laboratory Service (SEALS; South East Health, Sydney, Australia). Albumin determination used bromocresol purple; a bichromatic method was used to avoid over-estimation. Under the conditions used, ESR ≥ 12 mm/h was considered to be elevated, whilst albumin levels between 33–48 g/L were considered normal.

Protein concentration determination

Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL) using 96-well plates, with incubation at 60°C for 30 min. Absorbance was measured at 562 nm using a 96-well plate reader (Tecan, Grôdig, Austria) and converted to absolute values using BSA standards (0-0.5 mg/mL)

Serum protein thiol determination

Protein thiols were quantified spectrophotometrically using 5,5'-dithionitrobenzoic acid (DTNB) at 412 nm [29] as described previously [24]. Freshly thawed serum samples, and reduced glutathione (GSH) standards, were analysed in duplicate. Under these

conditions only protein-bound thiols were assumed to be present; low molecular mass thiols are known to be unstable under the storage conditions used [30].

Determination of serum protein carbonyls

Carbonyl concentrations were determined by ELISA using a commercial kit (Zenith Technology, Dunedin, New Zealand) as described previously [24]. Samples and standards were assayed in duplicate. Different plates were standardized by running repeat samples on every plate.

Two-dimensional electrophoresis of serum proteins

Serum samples were prepared for isoelectric focusing using 7 cm IPG strips as described previously [25]. A protein load of 20 or 40 μg of protein was used, as determined by BCA assay of serum prior to any treatment. After focusing, protein carbonyl groups were derivatized by incubation with 2,4-dinitrophenylhydrazine (DNPH) and separated in the second dimension by SDS-PAGE using 8–16% linear gradient Ready Gel Tris-HCl gels (Bio-Rad) in a Mini-PROTEAN III electrophoresis cell (Bio-Rad); also as described previously [25]. Reduced broad range molecular markers (Bio-Rad) were added to gels used for protein staining, whilst prestained standards (Bio-Rad) were included on those for Western blotting.

Western blotting to determine protein carbonyl distribution

Western blotting was performed using a Bio-Rad Mini Trans-Blot transfer cell with PVDF membranes, Briefly, Hybond-P PVDF (Amersham Biosciences, GE Healthcare, Rydalmere, NSW, Australia) was prepared by pre-wetting in 100% methanol (10 s) and washing in distilled water (5 min). Both the membranes and gels were equilibrated in transfer buffer (10 min) prior to assembly of the transfer apparatus; the transfer buffer consisted of Tris base (1.45 g/L) and glycine (7.20 g/L) in 20% (v/v) methanol, pH 8.3. Protein transfer was achieved by applying 10 V overnight at ca. 4°C.

Protein carbonyls were detected using an OxyBlot™ Protein Oxidation Detection Kit (Chemicon, Boronia, VIC, Australia), with spots visualized by chemiluminescence (ECL; Amersham). Carbonyl distribution was captured by exposure to Hyperfilm ECL (Amersham). Films were developed manually. When membranes were directly compared, all steps were run in parallel. Control experiments where proteins were not treated with DNPH showed no signal. Duplicate gels were run in parallel for each sample, with one used for carbonyl detection and the other for protein.

Protein detection and identification

Following second dimension separation, serum proteins were detected by silver staining [31]. The most abundant serum proteins were identified by comparison to human plasma protein maps [32,33] and mass spectrometric identification [25].

Protein hydrolysis with methanesulphonic acid and amino acid analysis

Reduced and delipidated protein samples (free of free amino acids) were prepared and subsequently hydrolysed in 4 M methanesulphonic acid exactly as described previously [24]. Samples were then analysed by HPLC, as previously [24], except that the elution gradient consisted of: 5% buffer B for 12 min; 5–17% B over 1 min; 17–25% B over 4 min; 25–45% B over 2 min; 45–50% B over 8 min; 50–58% B over 8 min; 58–100% B over 5 min; 100% B for 5 min; 100–5% B over 1 min; re-equilibration at 5% buffer B for 9 min. Buffer A consisted of 20% methanol and 2.5% tetrahydrofuran (THF) in 50 mm sodium acetate (BDH, Merck), pH 5.4. Buffer B consisted of 80% methanol and 2.5% THF in 50 mm sodium acetate, pH 5.4. Buffers were degassed with a vacuum degasser unit (Shimadzu) before and during use.

Statistical analysis

Analyses were performed using GraphPad Prism v. 4.0.3 for Windows (GraphPad Software, San Diego, CA). For multiple conditions at a single time point, a one-way ANOVA with Newman-Keuls post-hoc test was employed. Comparison of multiple conditions over time used a repeated measures two-way ANOVA, with Bonferroni post-tests to compare different groups at each time point. Correlation analyses were performed using two-tailed Spearman correlation. In all cases significance was assumed where $p < 0.05$.

Results

Protein oxidation levels over time in controls and SLE patients

Serum protein oxidation levels, as assessed by the concentrations of protein thiols, protein carbonyls, individual protein-bound amino acids and the side-chain oxidation product MetSO, were determined for each of three or more serum samples collected from 21 SLE patients and nine controls over a period of up to 38 months. The demographics of the patients and controls are given in Table I. As the collection time points were, of necessity, different for each subject, when presenting the data the samples are notated by sample numbers, rather than specific times. Due to the incomplete nature of the data set if >3 samples were analysed, only the first three samples collected were used for analysis in Figures 1 and 2; this also

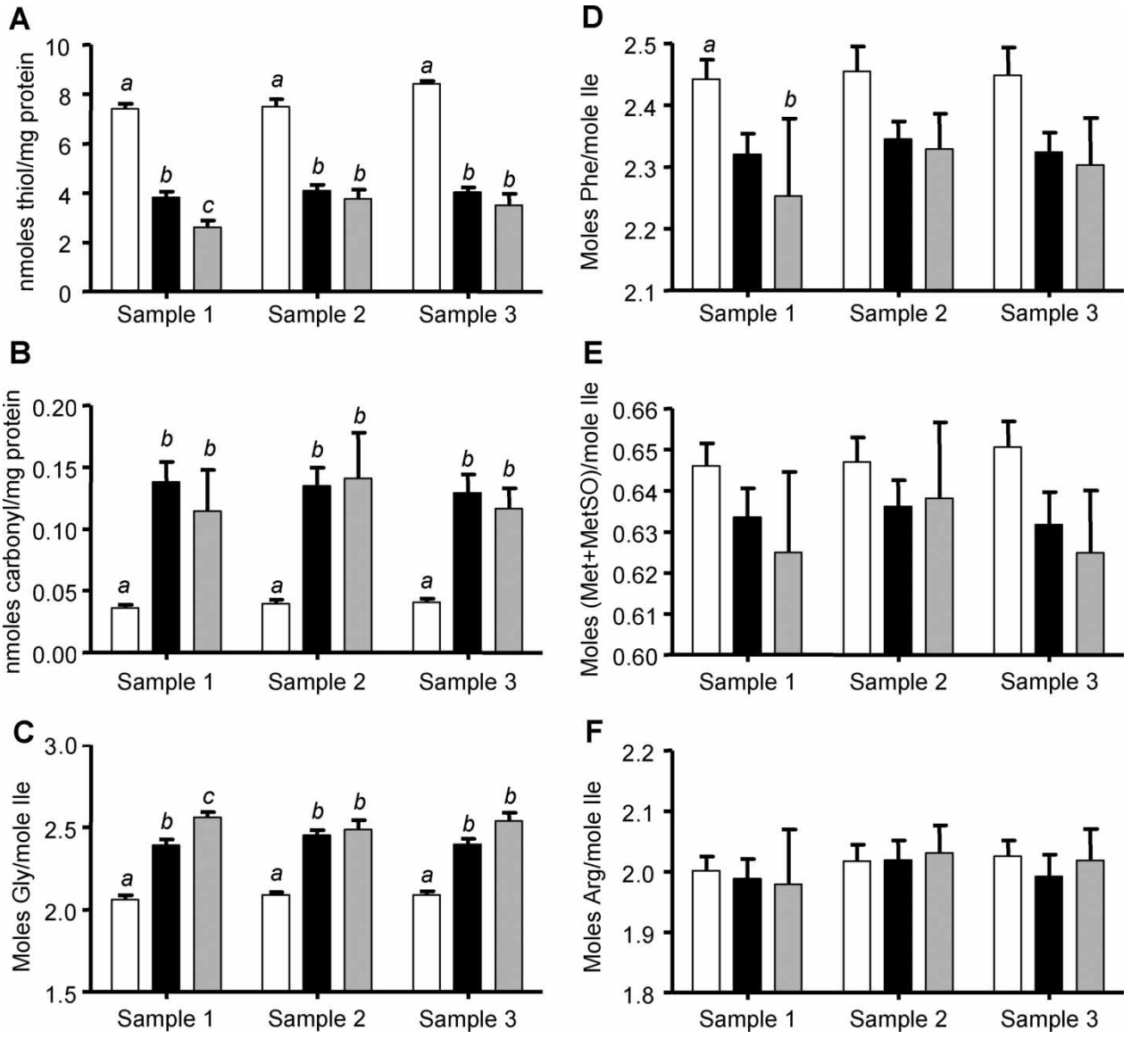

Figure 1. Averaged data (±SEM) at each sample time of protein oxidation markers measured in controls (white bars) and SLE patients with stable (black bars) or variable (grey bars) disease activity. (A) protein thiols; (B) protein carbonyls; (C) protein-bound Gly; (D) protein-bound Phe; (E) protein-bound (Met+MetSO); and (F) protein-bound Arg. Three longitudinal serum samples, collected from 19 days to 19 months apart, were analysed for each control and SLE patient. Details of the experimental methods used to determine each marker are given in the Materials and methods section. Statistical analyses were by two-way ANOVA with Bonferroni post-tests; different letters where present indicate significant changes at the $p < 0.05$ level at each sample time; the absence of letters indicates no significant differences. The statistical analysis also confirmed that there was no significant difference in levels of each protein oxidation marker at each sampling point in the control or stable SLE groups ($p < 0.001$; not shown).

avoids skewing of the data, as generally the patients with more samples available had more severe disease. The (healthy) controls were observed for a shorter period of time than the SLE patients, as their variation in protein oxidation levels over time was relatively small; this was expected based on results from single time point studies, where oxidation levels in controls show little variation from person to person [24].

Protein oxidation levels at each sampling number were averaged for the control cohort and SLE patients with stable or variable disease activity in order to assess whether the levels of each of the oxidation markers were chronically modulated; these data are given in Figure 1. Protein thiols were chronically decreased in both groups of SLE patients (Figure 1A), whilst there was a chronic elevation in protein carbonyls (Figure 1B) and protein-bound Gly (Figure 1C) in each SLE patient group in comparison to controls. Statistical analysis confirmed that these changes were sustained at each sampling point. There is little or no overlap between the levels of protein thiols and carbonyls or protein-bound Gly detected for the SLE patients and controls when these data are plotted longitudinally for each control and SLE patient (Figure 2; protein-bound Gly data not

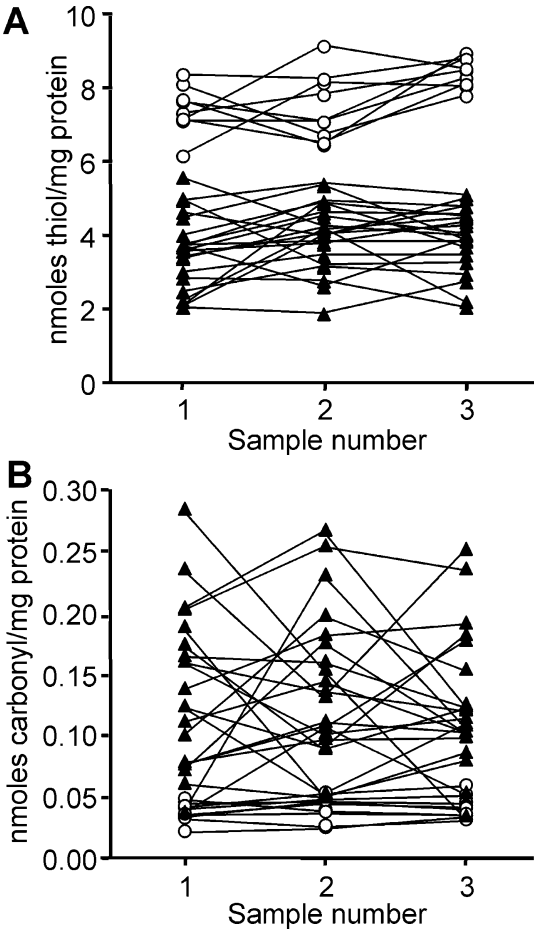

Figure 2. The chronic nature of the increased protein oxidation observed in 21 SLE patients in comparison to nine controls, as shown by levels of (A) serum protein thiols and (B) serum protein carbonyls. Three longitudinal protein thiol levels determinations were carried out for all controls (○) and SLE patients (▲); lines connect the thiol or carbonyl levels for each sample in an individual control/patient. Protein thiols were determined by reaction with 5,5'-dithionitrobenzoic acid and protein carbonyls were quantified by ELISA, as detailed in the Materials and methods section; both were standardized to serum protein concentrations.

shown). Protein-bound Phe (Figure 1D) and (Met + MetSO) (Figure 1E) showed a trend towards a decrease in both SLE patient groups, in comparison to controls at each sampling point, but this only reached statistical significance for Phe in the variable SLE patients at one of the three sampling points. In contrast to previous work [24], levels of protein-bound Arg levels showed no consistent trends between groups (Figure 1F).

Correlations between protein oxidation and disease status in patients with stable SLE

In the majority of SLE patients with stable disease activity (variation in SLEDAI scores of ≤ 3 over the period of observation) the variation in protein oxidation levels between samples collected at different time points was minimal, i.e. the increase in protein oxidation in these patients was chronic rather than acute. The only exception was protein-bound carbonyls (see Figure 2B). In order to determine whether higher long-term disease activity was associated with increased protein oxidation, Spearman correlation analysis was carried out in the SLE patients with stable disease activity. For each patient, the mean value of the three determinations for each protein oxidation marker studied over the period of analysis were compared with their corresponding average disease status measures (SLEDAI score, anti-dsDNA antibody levels, ESR and albumin); using one data point per patient avoids skewing of the data by any particular patient, possibly resulting in spurious correlations [34]. The results of these analyses are given in Table II. Increasing ESR was a particularly strong predictor of increasing protein oxidation levels (decreased thiols, Phe and Met + MetSO, and increased carbonyls and Gly). Decreasing albumin levels were also generally associated with increases in serum protein oxidation; carbonyl levels were a notable exception.

Protein oxidation and disease status in patients with variable SLE

Comparison of oxidation levels and disease status data for the five SLE patients with variable disease activity (variation in SLEDAI scores of > 3 over the period of observation) was not performed in the same way as for the SLE patients with stable disease activity, as the variation in their averaged data would be too great to give meaningful results. This is clearly illustrated when levels of serum protein oxidation (decreasing protein-bound thiols and [Met + MetSO] and increasing carbonyls) and disease status (SLEDAI score and anti-dsDNA levels) are plotted longitudinally for the two SLE patients with variable disease activity where seven samples were available (Figure 3). The data obtained from the other three SLE patients with variable disease activity were difficult to interpret, owing to only three samples being available for each of these patients (not shown). Even so, the available data show that changes in the levels of different protein oxidation markers, as well as the well-established disease activity markers SLEDAI score and anti-dsDNA antibodies, vary considerably from patient to patient. For example, in patient L5 (Figure 3A), the three markers of protein oxidation varied in unison and this was also reflected in their SLEDAI score and anti-dsDNA antibody levels. Conversely, patient L10 (Figure 3B) showed relatively poor correlation between levels of serum protein thiols, carbonyls and (Met + MetSO); this was also reflected in a poorer correlation between SLEDAI scores and anti-dsDNA levels. Clearly further investigation is warranted, as more samples become available, to determine

Table II. Correlations between protein oxidation levels and disease activity measures in SLE patients with stable disease activity (variation in SLEDAI of ≤ 3 over the observation period). For the three blood samples collected from each patient, the average for each patient of serum protein thiols and carbonyls and protein-bound Gly, Phe and (Met+MetSO) was compared, where available, to the corresponding average SLEDAI score ($n=16$ patients), Log of anti-dsDNA antibody ($n=16$ patients), ESR ($n=15$ patients) and serum albumin concentration ($n=15$ patients). Thiols and carbonyls were standardized per mg of serum protein; all amino acid concentrations were standardized per mole of protein-bound Ile. Spearman correlation was used to determine correlations, with two-tailed *p*-values.

	SLEDAI score	Log[anti-dsDNA]	ESR	Albumin
Thiols	−0.1723	−0.1442	−0.6130*	0.5188*
Carbonyls	−0.1543	−0.2428	0.0125	0.2809
Gly	0.2592	0.2472	0.5827*	−0.6547**
Phe	−0.1184	−0.4489	−0.7435**	0.3792
Met+MetSO	−0.1199	−0.3988	−0.7989***	0.3077

* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

whether the differences are related to the differences in autoantibodies and other manifestations in individual patients.

Serum protein carbonyl distribution

In order to determine whether the observed protein oxidation was present on specific serum proteins or a general phenomenon, the serum proteins were separated by two-dimensional electrophoresis, then subjected to Western blotting for the presence of protein carbonyls. Initial experiments showed that loading of 20–40 μg serum protein gave reproducible and readily detectable spots on the gels. Silver staining of two-dimensional gels allowed the detection of a large number of proteins, with albumin and several classes of immunoglobulins giving distinct spots (Figures 4D–F). As with our previous work examining protein carbonyl levels at a single time point [25], immunodetection of protein-bound carbonyls showed that a large number of proteins contribute to the overall protein carbonyl yields and that damage is not localized to a small number of, or a single, protein. In controls (Figure 4A) carbonyls were detected on a large number of proteins including the Ig-γ and Ig light chains and albumin; control experiments confirmed that in the absence of DNPH no false-positive carbonyl detection occurred (data not shown). In SLE patients a similar broad distribution of protein carbonyls over the serum proteome was evident, though at elevated levels (Figures 4B and C).

In order to examine the distribution of protein carbonyls as the total levels changed, multiple serum samples from three SLE patients were subjected to immunoblot analysis. Two of the SLE patients studied had markedly different carbonyl concentrations in the serum samples analysed (as determined by ELISA); the other had stable protein carbonyl concentrations and thus served as a control. Figure 4B shows the carbonyl distribution in one SLE patient sample with a high carbonyl concentration (0.276 ± 0.017 nmol/mg protein), whilst Figure 4C shows a sample from the same patient with a relatively low carbonyl concentration and 0.067 ± 0.001 nmol/mg protein. Changes in carbonyl levels on albumin and Igs, as well as other proteins, were clearly apparent in both SLE patients with variable carbonyl levels; these changes were not parallelled by alterations in the protein distribution, as shown by silver staining of gels run in parallel, which remained constant (Figure 4E and F). The SLE patient with stable protein carbonyl levels exhibited a similar distribution and intensity of staining for protein carbonyls in both blots (data not shown).

Discussion

The results presented here are consistent with sustained, elevated levels of protein oxidation in patients with SLE compared to healthy controls. Correlation analysis confirmed that in patients with stable disease activity, patients with higher average protein oxidation levels also exhibited higher average acute-phase response markers. When combined with our previous studies showing increased serum protein oxidation in SLE patients at a single time point [24,25], as well other evidence for protein-bound 3-nitrotyrosine levels correlating with disease activity [21], this study adds to the evidence that protein oxidation plays an important role in the pathogenesis of this disease. It should also be noted that all of the SLEDAI and anti-dsDNA correlations (Table II), although not always statistically significant, exhibited correlation coefficients consistent with greater serum protein oxidation in patients with greater disease activity; the low occurrence of significance is not surprising in these patients considering their relatively mild disease activity (c.f. the SLEDAI scores in Table I).

Serum albumin is considered to be a negative acute-phase protein [35] and would be expected to be a major target for oxidants due to its abundance in serum/plasma. This is supported by the data for the SLE patient group with stable SLEDAI scores, where lower albumin concentrations correlated significantly with lower protein thiols and higher protein-bound

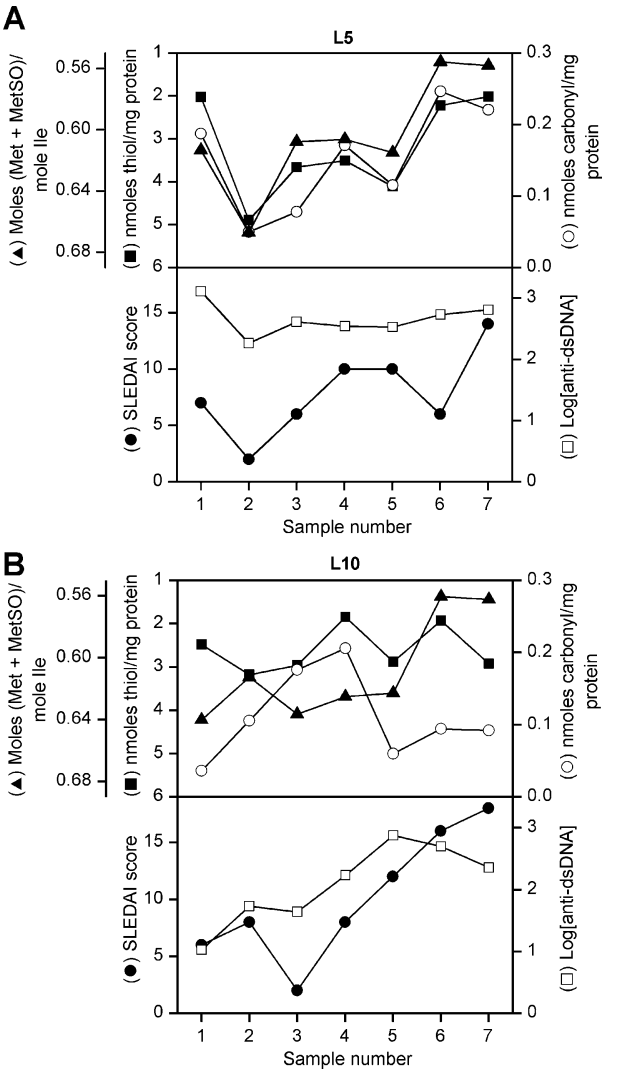

Figure 3. Longitudinal protein oxidation levels and disease activity for two SLE patients with variable disease activity (variation in SLEDAI scores of >3 over the period of observation). (A) patient L5, (B) patient L10. For each patient, the upper panel shows levels of serum protein thiols (■), protein carbonyls (○) and protein bound (Met+MetSO) (▲) at each sampling time. The lower panel shows the corresponding SLEDAI score (●) and anti-dsDNA antibody titre (□) determined at the time of sample collection. The *y*-axis values have been kept constant for both patients for each oxidation and disease activity marker.

Gly (Table II). Up to 80% of the thiol groups present in serum are accounted for by the free thiol (Cys-34) on HSA [35]. This thiol is readily oxidized *in vitro* by hydrogen peroxide, hypochlorous acid, nitric oxide and peroxynitrite, amongst others [36,37]. Increased extents of thiol oxidation have been detected in a range of pathologies including senile cataract, chronic renal failure, various liver diseases and atherosclerosis [38–40]. The significant correlation observed in the present study between decreasing albumin concentrations and decreasing serum protein thiol levels in the pool of SLE patients with stable disease activity (Table II) is consistent with the free thiol in albumin being a major target of oxidative damage in SLE.

It is apparent that the levels of other oxidation markers are related to factors not limited to albumin concentrations, otherwise all of the oxidation markers measured would correlate significantly with this parameter. The heterogenous nature of protein oxidation levels and disease activity markers in the SLE patients with variable disease activity (Figure 3) support this assertion. The Western blots of the protein carbonyl distribution clearly indicate that many proteins, in addition to albumin, are subject to oxidation and protein carbonyl formation and that the oxidation of the Ig chains increases in parallel with the oxidation of albumin, as judged by the chemiluminescence intensity of the various proteins with attached carbonyl groups (Figure 4). Irrespective of the exact nature of the proteins on which the carbonyls are formed, the observed chronic increase in protein carbonyl levels in all of the SLE patients compared to controls suggests that this may be pathologically important. The further increase seen previously in lupus nephritis patients [25], who have significantly worse outcomes, supports this hypothesis. Enhanced levels of protein-bound carbonyls have also been detected with ageing [15], as well as with development of a range of diseases, including rheumatoid arthritis, pulmonary fibrosis, diabetes, Parkinson's disease and Alzheimer's disease [41–43].

ESR, though not of particular use in diagnosing SLE, can be used to monitor systemic inflammation in SLE patients [44]. Thus, the significant correlations observed between this parameter and all the protein oxidation markers studied here, except protein-bound carbonyls, point to protein oxidation being intimately involved with inflammation in SLE. The fact that ESR does not correlate with the serum protein carbonyl levels in the SLE patients may reflect a differing rate of turnover (formation and/or removal) of oxidized serum proteins compared to the variation in ESR. ESR is known to be slow to increase, and persist after, causative inflammation has subsided [45]; in contrast the half-life of HSA is typically 15–20 days [46]. Furthermore, even amongst the serum protein pool there are likely to be major differences in the rates of turnover between proteins and as a result of the modulation of this parameter resulting from cellular damage (c.f. data for both enhanced and decreased rates of turnover of oxidised proteins in cells [47]). Although a number of enzymatic repair systems are known for oxidized/modified proteins within cells (e.g. protein disulphide isomerases and methionine sulphoxide reductases, which reduce disulphides and methionine sulphoxide, respectively), these do not appear to be present in plasma/serum [48]. Thus, the major route of removal of oxidized plasma/serum proteins is likely to be cellular uptake, catabolism and excretion of the oxidized material in urine, rather than repair [49].

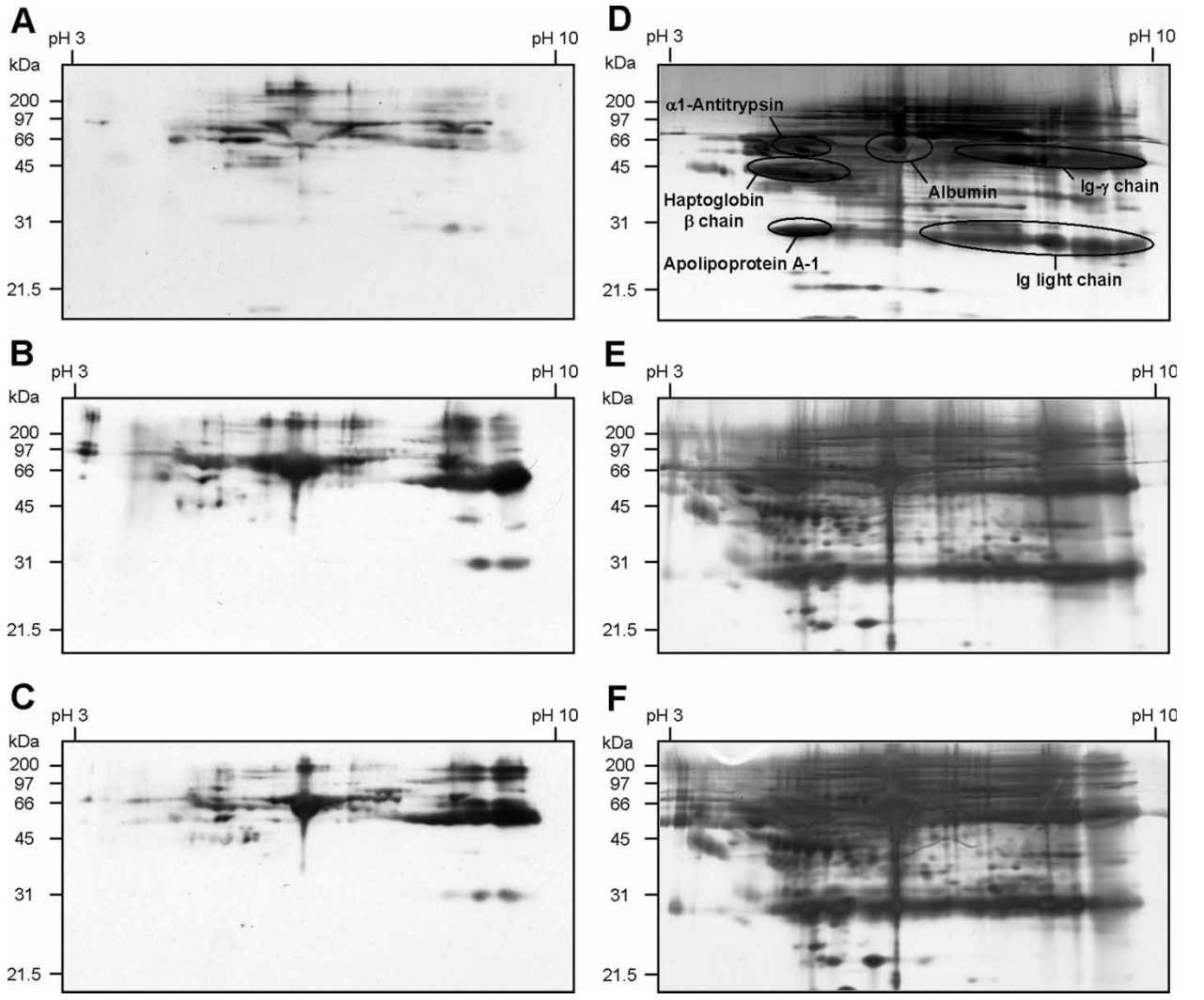

Figure 4. Distribution of protein-bound carbonyls on serum proteins. Proteins were separated by 2-dimensional electrophoresis with subsequent detection of DNPH-derivatized protein-bound carbonyl groups by Western blotting (A–C) or silver staining of gels to visualize proteins (D–F), as detailed in the Materials and methods section. (A) and (D) show representative control data (C29; 20 μg protein loaded; blot visualised for 10 min), with the identities of the most abundant serum proteins labelled (D). (B/E) and (C/F) show data for two serum samples (40 μg protein; 2 min blot visualization) taken from one SLE patient (L10), where the protein-bound carbonyl concentrations changed markedly (0.275 ± 0.017 nmol/mg protein in B/E and 0.067 ± 0.001 nmol/mg protein in C/F) despite insignificant changes in albumin concentrations (34 and 31 mg/mL, in B/E and C/F, respectively). Processing of samples for (B/E) and (C/F) was undertaken in parallel to minimize inter-experimental errors.

Although individual Met and MetSO levels were not determined in this study (c.f. previous work where these were quantified individually and the ratio observed to change with disease status [24]), the quantification of combined (Met + MetSO) levels, as carried out in the current study, is still informative. In isolated systems it has been shown that treatment of proteins with oxidants causes significant losses of (Met + MetSO), consistent with the further oxidation of Met to the sulphone ($MetSO_2$) [50,51]. It has also been shown in Alzheimer's and Parkinson's diseases that increased methionine oxidation occurs, with both MetSO and $MetSO_2$ formed [52], even though MetSO formation *in vivo* can be reversed enzymatically [53]. In the studies reported here, there was a significant correlation in SLE patients with stable disease activity between decreased levels of total (Met + MetSO) and increased ESR (Table II) and a striking correlation with other oxidation markers and disease activity status in at least one of the SLE patients with variable disease activity (Figure 3, patient L5). This correlation indicates that patients with higher disease activity tend to have more extensive serum Met oxidation. This suggests that Met can be oxidized irreversibly to other products *in vivo*, including $MetSO_2$ and materials that result from one-electron oxidation of Met [54].

We have previously suggested that the elevated protein oxidation observed in SLE patients, compared to controls, is indicative of either continuously elevated oxidation levels or acute oxidation with inefficient repair or removal of damaged materials

once formed [24]. The data obtained in the current study strongly supports the case for chronically elevated oxidation levels in SLE patients, as shown by the consistently increased levels of serum protein oxidation products in SLE patients with both stable and variable disease activity. Being a heterogeneous disease, it is not surprising that there are differing correlations between protein oxidation and disease assessment parameters in patients with variable SLE; as more (relatively rare) patients with poorly controlled disease activity are recruited this phenomenon could be further examined. Regular assessment of these patients could potentially answer the question as to whether the observed oxidation is a cause or effect of varying disease status. Regardless, the results presented here clearly show that increases in a range of markers of protein oxidation correlate with worsening disease status, thus further strengthening the case for protein oxidation being intimately involved with the chronic organ damage observed in SLE.

Acknowledgements

The authors wish to thank the staff and patients of the Rheumatology Department laboratory, The St George Hospital, for their assistance, and Dr David Pattison, for useful discussions. This work was supported in part by the Frank. G. Spurway Scholarship from Arthritis Australia, a University Postgraduate Award from the University of Sydney, and the Australian Research Council through the ARC Centres of Excellence programme.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Gordon C. Long-term complications of systemic lupus erythematosus. Rheumatology 2002;41:1095–1100.
[2] Frostegard J, Svenungsson E, Wu R, Gunnarsson I, Lundberg IE, Klareskog L, Horkko S, Witztum JL. Lipid peroxidation is enhanced in patients with systemic lupus erythematosus and is associated with arterial and renal disease manifestations. Arthritis Rheum 2005;52:192–200.
[3] Alves JD, Ames PRJ. Atherosclerosis, oxidative stress and auto-antibodies in systemic lupus erythematosus and primary antiphospholipid syndrome. Immunobiology 2003;207: 23–28.
[4] Avalos I, Chung CP, Oeser A, Milne GL, Morrow JD, Gebretsadik T, Shintani A, Yu C, Stein CM. Oxidative stress in systemic lupus erythematosus: relationship to disease activity and symptoms. Lupus 2007;16:195–200.
[5] de Carvalho JF, Bonfa E, Borba EF. Systemic lupus erythematosus and 'lupus dyslipoproteinemia'. Autoimmunity Rev 2008;7:246–250.
[6] Sheikh Z, Ahmad R, Sheikh N, Ali R. Enhanced recognition of reactive oxygen species damaged human serum albumin by circulating systemic lupus erythematosus autoantibodies. Autoimmunity 2007;40:512–520.
[7] Horkko S, Miller E, Dudl E, Reaven P, Curtiss LK, Zvaifler NJ, Terkeltaub R, Pierangeli SS, Branch DW, Palinski W, Witztum JL. Antiphospholipid antibodies are directed against epitopes of oxidized phospholipids. Recognition of cardiolipin by monoclonal antibodies to epitopes of low density lipoprotein. J Clin Invest 1996;98:815–825.
[8] Tam LS, Li EK, Leung VYF, Griffith JF, Benzie IFF, Lim PL, Whitney B, Lee VWY, Lee KKC, Thomas GN, Tomlinson B. Effects of vitamins C and E on oxidative stress markers and endothelial function in patients with systemic lupus erythematosus: a double blind, placebo controlled pilot study. J Rheumatol 2004;32:275–282.
[9] Nuttall SL, Heaton S, Piper MK, Martin U, Gordon C. Cardiovascular risk in systemic lupus erythematosus—evidence of increased oxidative stress and dyslipidaemia. Rheumatology 2003;42:758–762.
[10] Ames PRJ, Alves J, Murat I, Isenberg DA, Nourooz-Zadeh J. Oxidative stress in systemic lupus erythematosus and allied conditions with vascular involvement. Rheumatology 1999;38:529–534.
[11] Evans MD, Cooke MS, Akil M, Samanta A, Lunec J. Aberrant processing of oxidative DNA damage in systemic lupus erythematosus. Biochem Biophys Res Commun 2000;273:894–898.
[12] Lunec J, Herbert K, Blount S, Griffiths HR, Emery P. 8-Hydroxydeoxyguanosine. A marker of oxidative damage in systemic lupus erythematosus. FEBS Lett 1994;348: 131–138.
[13] Davies MJ. The oxidative environment and protein damage. Biochim Biophys Acta 2005;1703:93–109.
[14] Davies MJ, Fu S, Wang H, Dean RT. Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Radic Biol Med 1999;27:1151–1163.
[15] Beal MF. Oxidative modified proteins in aging and disease. Free Radic Biol Med 2002;32:797–803.
[16] Fu S, Davies MJ, Stocker R, Dean RT. Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. Biochem J 1998;333:519–525.
[17] Martin-Gallan P, Carrascosa A, Gussinye M, Dominguez C. Biomarkers of disease-associated oxidative stress and antioxidant status in young diabetic patients with or without subclinical complications. Free Radic Biol Med 2003;34: 1563–1574.
[18] Heinecke JW. Mechanisms of oxidative damage by myeloperoxidase in atherosclerosis and other inflammatory disorders. J Lab Clin Med 1999;133:321–325.
[19] Lorber A, Pearson CM, Meredith WL, Gantz-Mandell LE. Serum sulfhydryl determinations and significance in connective tissue diseases. Ann Intern Med 1964;61:423–434.
[20] Lorber A, Bovy RA, Chang CC. Sulfhydryl deficiency in connective tissue disorders: correlation with disease activity and protein alterations. Metabolism 1971;20:446–455.
[21] Oates JC, Christensen EF, Reilly CM, Self SE, Gilkeson GS. Prospective measure of serum 3-nitrotyrosine levels in systemic lupus erythematosus: correlation with disease activity. P Assoc Am Physician 1999;111:611–621.
[22] Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 1997;324:1–18.
[23] Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997;272:20313–20316.
[24] Morgan PE, Sturgess AD, Davies MJ. Increased levels of serum protein oxidation and correlation with disease activity in systemic lupus erythematosus. Arthritis Rheum 2005;52: 2069–2079.
[25] Morgan PE, Sturgess AD, Hennessy A, Davies MJ. Serum protein oxidation and apolipoprotein CIII levels in people

with systemic lupus erythematosus with and without nephritis. Free Radic Res 2007;41:1301–1312.
[26] Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus (letter). Arthritis Rheum 1997;40:1725.
[27] Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum 1992;35:630–640.
[28] Gladman DD, Urowitz MB, Kagal A, Hallett D. Accurately describing changes in disease activity in systemic lupus erythematosus. J Rheumatol 2000;27:377–379.
[29] Hu ML. Measurement of protein thiol groups and glutathione in plasma. Method Enzymol 1994;233:380–385.
[30] Kleinman WA, Richie JPJ. Status of glutathione and other thiols and disulfides in human plasma. Biochem Pharmacol 2000;60:19–29.
[31] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 1996;68:850–858.
[32] Anderson NL, Anderson NG. A two-dimensional gel database of human plasma proteins. Electrophoresis 1991;12: 883–906.
[33] Hughes GJ, Frutiger S, Paquet N, Ravier F, Pasquali C, Sanchez JC, James R, Tissot JD, Bjellqvist B, Hochstrasser DF. Plasma protein map: an update by microsequencing. Electrophoresis 1992;13:707–714.
[34] Ward MM, Leigh JP. Pooled time series regression analysis in longitudinal studies. J Clin Epidemiol 1993;46:645–659.
[35] Quinlan GJ, Martin GS, Evans TW. Albumin: biochemical properties and therapeutic potential. Hepatology 2005;41: 1211–1219.
[36] Carballal S, Radi R, Kirk MC, Barnes S, Freeman BA, Alvarez B. Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. Biochemistry-US 2003;42:9906–9914.
[37] DeMaster EG, Quast BJ, Redfern B, Nagasawa HT. Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. Biochemistry-US 1995;34:11494–11499.
[38] Hayashi T, Era S, Kawai K, Imai H, Nakamura K, Onda E, Yoh M. Observation for redox state of human serum and aqueous humor albumin from patients with senile cataract. Pathophysiology 2000;6:237–243.
[39] Sogami M, Era S, Nagoka S, Kuwata K, Kida K, Shigemi J, Miura K, Suzuki E, Muto Y, Tomita E, Hayano S, Sawada S, Noguchi K, Miyata S. High-performance liquid chromatographic studies on non-mercapt in equilibrium with mercapt conversion of human serum albumin. II. J Chromatogr 1985;332:19–27.
[40] Kadota K, Yui Y, Hattori R, Murohara Y, Kawai C. Decreased sulfhydryl groups of serum albumin in coronary artery disease. Jpn Circ J 1991;55:937–941.
[41] Rottoli P, Magi B, Cianti R, Bargagli E, Vagaggini C, Nikiforakis N, Pallini V, Bini L. Carbonylated proteins in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systemic sclerosis and idiopathic pulmonary fibrosis. Proteomics 2005;5:2612–2618.
[42] Chapman ML, Rubin BR, Gracy RW. Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis. J Rheumatol 1989;16:15–18.
[43] Chevion M, Berenshtein E, Stadtman ER. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Free Radic Res 2000;33:S99–S108.
[44] Shmerling RH. Diagnostic tests for rheumatic disease: clinical utility revisited. Southern Med J 2005;98:704–711.
[45] van Leeuwen MA, van Rijswijk MH. Acute phase proteins in the monitoring of inflammatory disorders. Bailliere Clin Rheum 1994;8:531–552.
[46] Jones IR, Owens DR, Williams S, Ryder RE, Birtwell AJ, Jones MK, Gicheru K, Hayes TM. Glycosylated serum albumin: an intermediate index of diabetic control. Diabetes Care 1983;6:501–503.
[47] Dean RT, Armstrong SG, Fu S, Jessup W. Oxidised proteins and their enzymatic proteolysis in eukaryotic cells: a critical appraisal. In: H Nohl, H Esterbauer, C Rice-Evans, editors. Free radicals in the environment, medicine and toxicology. London: Richelieu Press; 1994. p 47–79.
[48] Glaser CB, Karic L, Parmelee S, Premachandra BR, Hinkston D, Abrams WR. Studies on the turnover of methionine oxidized alpha-1-protease inhibitor in rats. Am Rev Respir Dis 1987;136:857–861.
[49] Grune T, Jung T, Merker K, Davies KJA. Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging and disease. Int J Biochem Cell B 2004;36: 2519–2530.
[50] Hawkins CL, Davies MJ. The role of aromatic amino acid oxidation, protein unfolding, and aggregation in the hypobromous acid-induced inactivation of trypsin inhibitor and lysozyme. Chem Res Toxicol 2005;18:1669–1677.
[51] Hawkins CL, Davies MJ. Inactivation of protease inhibitors and lysozyme by hypochlorous acid: role of side-chain oxidation and protein unfolding in loss of biological function. Chem Res Toxicol 2005;18:1600–1610.
[52] Choi J, Sullards MC, Olzmann JA, Rees HD, Weintraub ST, Bostwick DE, Gearing M, Levey AI, Chin LS, Li L. Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. J Biol Chem 2006;281:10816–10824.
[53] Stadtman ER. Cyclic oxidation and reduction of methionine residues of proteins in antioxidant defense and cellular regulation. Arch Biochem Biophys 2004;423:2–5.
[54] Schoneich C. Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. Biochim Biophys Acta 2005;1703:111–119.

This paper was first published online on iFirst on 18 December 2008.