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OXIDATIVE DAMAGE TO PLASMA PROTEINS IN ADULT RESPIRATORY DISTRESS SYNDROME

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There is evidence that patients with adult respiratory distress syndrome are under severe oxidative stress that leads to molecular damage. Oxidative stress appears to be inherent in the disease process as well as an unfortunate complication of essential treatment with oxygen. Eight critically ill patients with an established diagnosis of adult respiratory distress syndrome requiring high inspired oxygen concentrations administered by ultra high frequency jet ventilation, were studied. Three patients survived (38%). For the group as a whole, there was evidence of increased protein damage, measured on serial plasma samples as an increase in protein carbonyls (mean ± SEM, 1.41 ± 0.09 nmol/mg protein), compared with Intensive Care Unit (ICU) controls (1.24 ± 0.09 nmol/mg protein), and normal healthy controls (0.940 ± 0.04 nmol/mg protein). Protein thiol groups were decreased in the ARDS group (4.56 ± 0.50 nmol/mg protein) compared with ICU controls (5.5 ± 0.27 nmol/mg protein), and the normal healthy controls (6.55 ± 0.52 nmol/mg protein). However, when ARDS patients were grouped as survivors and non-survivors, total plasma protein levels were lower in survivors (53.9 ± 2.15 mg/ml) compared with non-survivors (78.2 ± 4.68 mg/ml); but the protein thiol content was significantly higher ($p = < 0.001$) in survivors (6.24 ± 0.09 nmol/mg protein) compared with non-survivors (3.56 ± 0.16 nmol/mg protein). Serial plasma measurements of protein damage indicated two different patterns. Survivors had higher total plasma thiol values (protein corrected), which increased as the lung injury resolved, and failing protein carbonyl values. By contrast, non-survivors had low and failing protein thiols often accompanying rising carbonyls.

We suggest that patients with adult respiratory distress syndrome undergo intermittent phases of severe oxidative stress during intensive care which can lead to protein damage, and that the protein thiol pattern may be a useful indicator of patients more likely to survive, the disease.

KEY WORDS: Adult respiratory distress syndrome, oxygen free radicals, protein thiols, protein carbonyls, oxidative stress, acute lung injury.

1. INTRODUCTION

The adult respiratory distress syndrome (ARDS) is characterised by refractory hypoxaemia secondary to non-hydrostatic pulmonary oedema and is associated with a wide variety of precipitating factors, often not directly involving the lung.[1,2] Thus, ARDS can result from such diverse clinical conditions as sepsis, gastric aspiration, polytrauma, pancreatitis, haemorrhagic shock, severe burns, oxygen toxicity and cardiopulmonary bypass.[2] In spite of the increasing complexity and scientific basis of medical support, ARDS still carries a mortality rate of around 50%, little changed from when it was first described.[3]

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When patients die within the first three days of the onset of ARDS, their primary illness is usually thought to be responsible, but late deaths are more commonly attributable to infection.[4] Patients in whom the lung is the primary site of infection appear to have the worst prognosis, often responding poorly to antibiotic therapy.[5] When ARDS is triggered by sepsis bacterial lipopolysaccharides are thought to elicit the expression of a number of cytokines, activating the complement and coagulation cascades. These changes have important effects upon several cellular systems including circulating and resident phagocytic cells in the lung.[6,7] Activation of neutrophils, sequestered within the lung, causes the release of reactive oxygen species such as superoxide, hydrogen peroxide, and hypochlorous acid as well as other damaging agents including eicosanoids and proteases.[8] The unavoidable use of high inspired oxygen concentrations in such patients would be expected to greatly potentiate the oxidative stress resulting from neutrophil recruitment and activation.[9]

Much recent evidence suggests that patients with ARDS are exposed to extreme oxidative stress. In support of this concept, myeloperoxidase and oxidised alpha 1-anti proteinase have been recovered from bronchoalveolar lavage fluid obtained in such patients;[10,11] hydrogen peroxide has been detected in expired breath,[12,13] antioxidants are depleted in body fluids,[14] and products of peroxidised lipids have been detected in bronchoalveolar lavage fluid and plasma (reviewed in 15).

In the study described here, we monitored eight patients with established ARDS for oxidative changes in their circulatory plasma proteins. Our results suggest that oxidative protein damage is greater in the ARDS group when compared with non-ARDS ICU patients and normal healthy controls, and that patients ultimately surviving ARDS have and maintain normal levels of total plasma thiols when corrected to protein content. Furthermore, the changing relationship between total plasma thiols and protein carbonyls may help to predict those patients most likely to survive the disease when subjected to the supportive therapy used in our intensive care unit.

2. MATERIALS AND METHODS

2.1 Diagnostic Criteria for ARDS

In our intensive care unit, ARDS is diagnosed clinically in patients with refractory hypoxaemia (ratio of arterial oxygen tension to fractional inspired oxygen concentration of less than 20 in SI units); evidence of diffuse bilateral pulmonary infiltrates on chest radiograph and a pulmonary artery occlusion pressure less than 18 mm Hg. In the current study, the diagnosis was also confirmed using the lung injury score (LIS) of Murray described in detail elsewhere.[16] Briefly, lung compliance, the fractional inspired oxygen concentration, the degree of positive end expiratory pressure utilised and a chest radiograph were scored numerically. In addition to the clinical criteria described above, a LIS score greater than 2.5 was required before ARDS was diagnosed.[16]

All ARDS patients were intubated, sedated, paralysed and mechanically ventilated with a high frequency jet throughout the study with radial artery and pulmonary artery catheters in situ. An inspired oxygen concentration of 0.6–1.0 was required in all cases. All patients were treated with inotropic agents (dobutamine, dopamine) in doses sufficient to maintain oxygen delivery (DO_2) at or above 660 ml/min/m^2. Some constituents of parenteral feeds might afford additional protection from oxidant stress to plasma proteins. For clinical reasons, patients were provided with

enteral nutrition wherever possible, but enteral and total parenteral feeds were selected from one of 3 standardised regimens used in our Unit.

Institutional Review Board approval is not required in our Hospital for studies on blood samples, but permission to perform the study was obtained from relatives where possible.

2.2 *Collection of Blood Samples*

10 ml of mixed venous and arterial blood was withdrawn simultaneously through the pulmonary artery and radial artery catheters and placed into lithium heparin tubes. The blood was immediately centrifuged at 3000 rpm for 6 minutes to separate the plasma which was removed and analysed within the shortest possible time. Aliquots were also stored frozen at −20°C. Venous blood samples were also obtained from patients requiring mechanical ventilation for conditions predisposing them to ARDS (cardiopulmonary bypass, major vascular and thoracic surgery), but none of whom reached the diagnostic criteria outlined for ARDS. All control patients were discharged from the Intensive Care Unit alive (age range 14–59 mean 43 years) and from normal healthy controls (age range 19–36 mean 32 years). No patient was receiving any treatment regimens designed to protect against oxidative damage, and all were studied sequentially upon admission to intensive care. All the results shown on ARDS patients represent the mean of duplicate assays performed on venous and arterial plasma, which gave essentially the same information (see Figure 2, patient G).

2.3 *Measurement of Protein Carbonyls*

The oxidation of certain amino acid residues in proteins to a carbonyl function was measured using the method of Oliver *et al.*[17] with the following minor modifications. Into new clean glass tubes was added 200 μl of plasma and 1.0 ml of 2,4-dinitrophenyl hydrazine 10 mM in 2 M hydrochloric acid. Controls containing 200 μl of plasma and 1.0 ml of 2 molar hydrochloric acid were set up in parallel. The tube contents were incubated at 37°C for 90 minutes in a shaking waterbath. Proteins were precipitated by adding 1.0 ml of trichloroacetic acid 28% w/v. All tubes were centrifuged at 10,000 g for 5 minutes and the supernatant fluid discarded. The remaining pellet of protein was vigorously washed with ethanol: ethyl acetate 1:1 v/v on three separate occasions to removed lipid derived material and unreacted 2,4-dinitrophenyl hydrazine, centrifuging between each wash. The final pellet was resuspended in 1.0 ml of guanidinium chloride 6 M dissolved in 2M HCl, and left for one hour at room temperature to effect solution. Resulting test and blank solutions were read at 360 nm. To calculate carbonyl values, blank readings were subtracted from test readings and the results expressed in nmol of carbonyl groups/mg of protein using a molar absorption coefficient of 21,000 litre.mol^{-1}.cm^{-1} for the carbonyl-dinitrophenylhydrazine derivatives.[17]

2.4 *Measurements of Total Plasma Thiol Groups*

Total plasma thiol groups were determined using Ellman's reagent[18] using the following reaction conditions; 5,5'-dithio-bis (2-nitrobenzoic acid) 10 mM dissolved in sodium phosphate buffer 0.1 M, pH 7.4. The following reagents were added to new clean plastic tubes in the order stated: 50 μl of plasma, 0.55 ml of distilled water, 200 μl of phosphate/saline buffer pH 7.4 (0.1 M sodium phosphate in 0.15 M NaCl)

and 200 μl of Ellman's reagent. Blanks were established for each sample containing distilled water instead of Ellman's reagent and distilled water instead of plasma. The reaction was allowed to proceed for 15 minutes at room temperature and the resulting chromogen was measured at 412 nm. Values for total plasma thiols were expressed in nmol/mg protein using a molar absorption coefficient of 13,600 litre.mol^{-1}.cm^{-1} for the thiol-5,5'-dithio-bis (2-nitrobenzoic acid) complex.[19] A 0.1 mM cysteine solution, (freshly prepared) was used with each assay as an internal standard. Thiol levels (mean $\pm$ SEM) in survivors and non-survivors were compared by Student's t test.

2.5 Ultrafiltration of Plasma

Plasma at 25°C was passed through a 'Centricon' 10,000 Da exclusion membrane (Amicon Corporation) by centrifugation at 5000 g for 30 minutes. The protein-free ultrafiltrate and the protein-rich concentrate, retained on the filter, were immediately assayed for thiol content using Ellman's reaction described above.

2.6 Total Protein Measurements

Plasma total protein values were measured using the Lowry technique according to the assay kit instructions (Sigma, Poole, Dorset, UK).

2.7 Reagents

5,5'-dithio-bis (2-nitrobenzoic acid) and 2,4-dinitrophenyl-hydrazine were obtained from the Sigma Chemical Company, Poole, Dorset, UK. All other chemicals were of the highest purity available from BDH-Merck, Poole, Dorset.

3. RESULTS

All the patients studied were clinically diagnosed as having ARDS. Three (patients F, G, H) were diagnosed and treated elsewhere for varying periods of time before being transferred to our care (see Table 1 in reference 20, this volume). In all cases, a diagnosis of ARDS was established, or confirmed, on admission to our Unit based on the diagnostic criteria described, and Murray scores in excess of 2.5. No patient had clinical or biochemical evidence of multiple organ dysfunction, but 2 (patients I and J) developed biochemical renal failure on admission and 4 days prior to death respectively (18 and 12 days respectively after the diagnosis of ARDS was made).

Plasma thiols Measurement of total plasma thiols (sulphydryl groups) includes both high molecular mass protein thiols and low molecular mass non-protein thiols such as reduced glutathione, although the plasma level of reduced glutathione is probably less than 1 μM.[21] Ultrafiltration of plasma through a 10,000 Da exclusion membrane confirmed that almost all the thiols detected in our assay were protein-associated. Protein-corrected plasma thiol levels in the three patients surviving ARDS were maintained at almost normal levels throughout the disease. The mean value $\pm$ SEM for survivors was 6.24 ± 0.09 nmol/mg protein whilst that for non-survivors was 3.56 ± 0.16 nmol/mg protein ($p = < 0.001$) (Table 1). However, the mean total

TABLE 1
Plasma protein values in surviving and non-surviving ARDS patients

Patient Code	Number of Samples	Total Protein mg/ml	Total Thiols nmol/mg protein	Carbonyls nmol/mg protein
B	9	49.9 ± 2.10	6.26 ± 0.67	1.649 ± 0.14
C	12	57.4 ± 2.63	6.40 ± 0.24	1.610 ± 0.14
A	9	57.3 ± 3.80	6.07 ± 0.32	1.350 ± 0.08
I	11	74.3 ± 2.59	3.68 ± 0.24	1.135 ± 0.07
J	15	85.7 ± 1.45	3.10 ± 0.15	1.077 ± 0.04
H	15	67.4 ± 1.47	4.04 ± 0.29	1.460 ± 0.04
G	14	71.2 ± 2.10	3.57 ± 0.13	1.750 ± 0.06
F	19	92.4 ± 1.60	3.39 ± 0.17	1.270 ± 0.04
ARDS Survivors ($n = 3$)		53.9 ± 2.15	6.24 ± 0.09	1.536 ± 0.09
ARDS non-survivors ($n = 5$)		78.2 ± 4.68	3.56 ± 0.16	1.338 ± 0.12
ARDS Total ($n = 8$)		69.5 ± 5.17	4.56 ± 0.50	1.413 ± 0.09
Normal controls ($n = 12$)		83.6 ± 1.82	6.55 ± 0.52	0.940 ± 0.04
Non-ARDS ICU controls (10)		60.5 ± 3.00	5.50 ± 0.27	1.240 ± 0.09

Patients A, B, C survived ARDS, patients F, G, H, I, J did not. Values shown for ARDS patients are the mean ± SEM of venous and arterial plasma samples assayed in duplicate.

plasma protein value for survivors was considerably lower (53.9 ± 2.15 mg/ml) than that of non-survivors (78.2 ± 4.68 mg/ml), which was closer to the control value of healthy subjects (83.63 ± 1.82 mg/ml) (Table 1). Ignoring differences in total plasma protein content, mean thiol values for both groups of ARDS patients (expressed on a molar basis) were low (317 μM) when compared with normal healthy controls (548 μM), but similar to our non-ARDS ICU patients (333 μM).

Plasma carbonyls The assay for protein carbonyls measures the oxidative formation of aldehydic functions on certain amino acids and has been shown to accompany metal catalysed free radical oxidation of proteins.[21] Both groups of ARDS patients (survivors and non-survivors) showed higher mean protein-corrected carbonyl values (1.413 ± 0.09 nmol/mg protein) than the normal controls (0.940 ± 0.04 nmol/mg protein) and non-ARDS ICU patients (1.24 ± 0.09 nmol/mg protein). None of the ARDS patients were receiving albumin infusions which could have conceivably influenced protein carbonyl values[23] as well as thiols.[24]

Protein damage patterns When plasma values for thiols and carbonyls were sequentially examined in each patient, two different patterns were seen for ARDS patients. Survivors had higher total protein-corrected plasma thiol levels, which increased as the lung injury resolved (Figures IA-IC). At the same time points, protein carbonyl values usually fell. In non-surviving ARDS patients, however, low and falling thiols were often, but not always, seen to accompany rising protein carbonyl values (Figures 2F–2J).

4. DISCUSSION

Adult respiratory distress syndrome is a blanket term used to describe acute respiratory failure due to high permeability pulmonary oedema arising from a multiplicity of primary insults, many originating at a distant (non pulmonary)

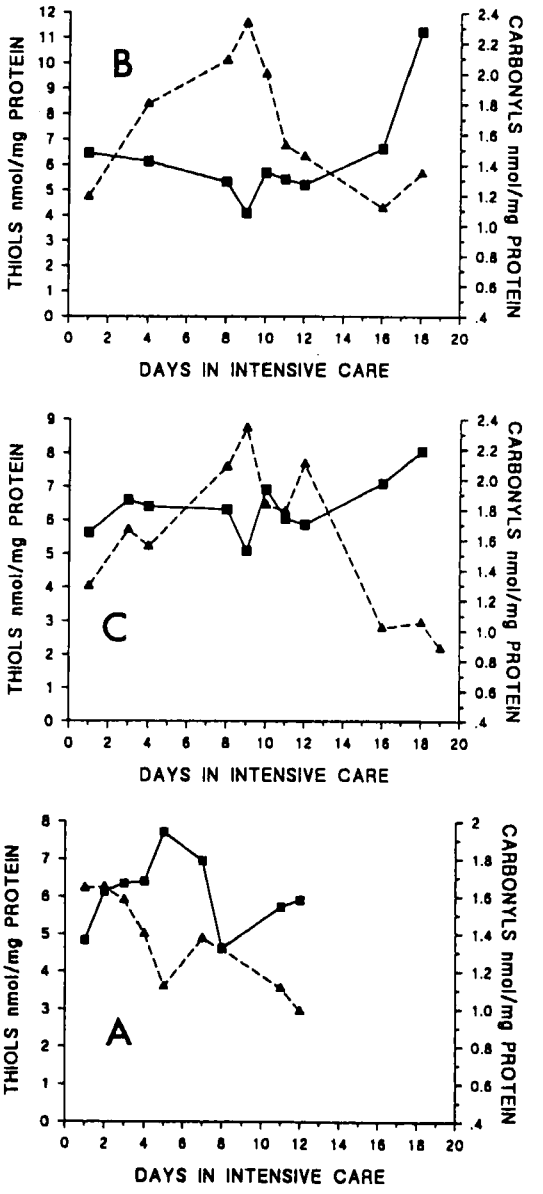

FIGURE 1 Sequential plasma protein carbonyl (▲) values (nmol/mg protein), and total plasma thiol (■) values (nmol/mg protein) in the patients surviving ARDS (A,B,C).

site.[2,3] The accumulation and activation of neutrophils leads to the generation of reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). When suitable transition metal complexes are present, particularly those of iron, aggressive and highly damaging species such as the hydroxyl radical are likely to be formed.[25] Recent studies, however, suggest that the hydroxyl radical may also be generated by the interaction of two endogenous free

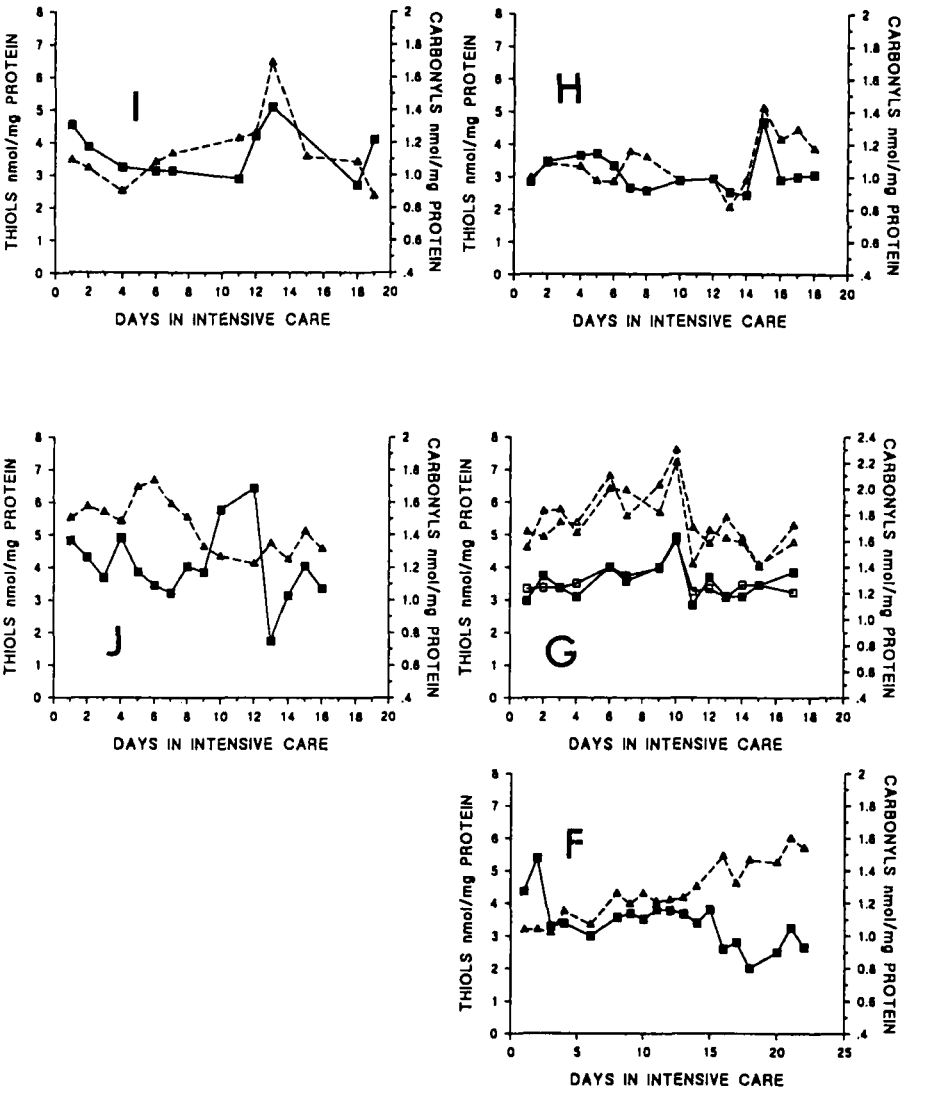

FIGURE 2 Sequential plasma protein carbonyl values (nmol/mg protein), and total plasma thiol values (nmol/mg protein) in the non-surviving group of ARDS patients (F,G,H,I,J). In (G) carbonyl values are shown for both (▲) arterial and (△) venous plasma, and for thiol values (■) arterial and (□) venous plasma.

radicals without transition metal involvement. When superoxide and nitric oxide ($NO^{\cdot}$), (which is indistinguishable from endothelium-derived relaxing factor), are produced by the vascular endothelium, macrophages, neutrophils and other cells, they can react to form peroxynitrite ($ONOO^{-}$). Peroxynitrite has the potential to directly damage biological molecules[26, 27] or possibly decompose to release hydroxyl radicals,[26, 28] both mechanisms result in molecular damage. Few neutrophils are normally present in the lung, but large numbers can dramatically appear during the

onset of ARDS. Neutrophil accumulation, mediated by activation of the complement system, can lead to the production of reactive oxygen intermediates that substantially contribute to the pathology of ARDS.

Oxidative stress, leading to oxidative damage, however, can arise in ARDS patients for reasons other than the participation of activated neutrophils. Indeed, ARDS has been observed in neutropaenic patients.[29] Refractory hypoxaemia is a characteristic feature of ARDS and patients require treatment with high fractional inspired oxygen concentrations. Thus, ventilatory support can itself almost certainly facilitate the formation of reactive oxygen species.[30,31] ARDS patients have been reported to have increased plasma xanthine oxidase activities[32] which could also lead to increased reactive oxygen formation from enzyme-catalysed substrate oxidations. It is well-known that organisms respond to hyperoxia by elevating protective enzymes, such as superoxide dismutase and glutathione peroxidase as well as glutathione.[33] Interestingly, our patients surviving ARDS appeared to be able to maintain normal plasma protein thiol levels, throughout their illness, whereas non-survivors did not. *In vitro* experiments have shown that the generation of reactive forms of oxygen in plasma leads to a significant loss of plasma protein thiols, and that the loss of thiols is a more sensitive indication of oxidative damage than modification of lipid.[34]

Protective antioxidant vitamins such as vitamin C (ascorbic acid)[35] and vitamin E (alpha-tocopherol)[36] are reported to be decreased in ARDS patients although the latter finding has not been confirmed in all studies.[35] Further evidence to support increased oxidative molecular damage occurring in ARDS patients is based on findings that enzymes[10,11] and lipids[15] are damaged, and that increased hydrogen peroxide is expired.[12,13] Our sequential studies in a small number of ARDS patients, show that as a group, there is evidence of increased oxidative damage to their plasma proteins. This is seen as an increase in carbonyl formation, and as a loss of protein thiol groups even when corrected to plasma protein content. More importantly, ARDS patients that survive appear to be more likely to maintain normal levels of thiols on their proteins throughout the disease. A normal plasma total thiol value of 500 μM is known to represent sulphydryl groups mainly associated with albumin, and to a lesser extent other plasma proteins.[34] All of our ARDS patients showed evidence of increased protein damage, and oxidative damage to proteins has been shown to render them highly susceptible to proteolytic degradation[22,37,38] thereby markedly increasing enzyme and protein turnover. Whether more efficient proteolysis of oxidatively modified protein contributes to the low protein values seen in survivors is at present under consideration.

By plotting serial changes in plasma protein carbonyls and total thiols of ARDS patients during their time in intensive care, different patterns for surviving and non-surviving patients were observed. The survivors appeared to display an inverse relationship between loss of thiols and formation of carbonyls, and changes in these values suggested that intermittent bursts of oxidative stress were experienced over the course of their admission. However, patterns of protein damage in non-survivors were less distinct, with low thiol values often continuing to fall as carbonyl formation increased.

By monitoring oxidative changes to the plasma proteins of ARDS patients during intensive care we observed fluctuations that may reflect phases of severe oxidative stress occurring during the course of the disease. Interestingly, the different patterns of protein damage may help to identify those patients most likely to survive the disease using the supportive therapies adopted in our intensive care unit.

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