Ankyrin is the major oxidised protein in erythrocyte membranes from end-stage renal disease patients on chronic haemodialysis and oxidation is decreased by dialysis and vitamin C supplementation

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ABSTRACT

Chronically haemodialysed end-stage renal disease patients are at high risk of morbidity arising from complications of dialysis, the underlying pathology that has led to renal disease and the complex pathology of chronic kidney disease. Anaemia is commonplace and its origins are multifactorial, involving reduced renal erythropoietin production, accumulation of uremic toxins and an increase in erythrocyte fragility. Oxidative damage is a common risk factor in renal disease and its co-morbidities, and is known to cause erythrocyte fragility. Therefore we have investigated the hypothesis that specific erythrocyte membrane proteins are more oxidised in end-stage renal disease patients and that vitamin C supplementation can ameliorate membrane protein oxidation.

Eleven patients and fifteen control subjects were recruited to the study. Patients were supplemented with 2 x 500mg vitamin C per day for four weeks. Erythrocyte membrane proteins were prepared pre- and post-vitamin C supplementation for determination of protein oxidation. Total protein carbonyls were reduced by vitamin C supplementation but not by dialysis when investigated by enzyme linked immunosorbent assay. Using a western blot to detect oxidised proteins, one protein band, later identified as containing ankyrin, was found to be oxidised in patients but not controls and was reduced significantly by 60% in all patients after dialysis and by 20% after vitamin C treatment pre-dialysis. Ankyrin oxidation analysis may be useful in a stratified medicines approach as a possible marker to identify requirements for intervention in dialysis patients.

INTRODUCTION

In the absence of kidney transplantation, chronic haemodialysis is a life-saving treatment for patients with end-stage renal disease (ESRD) irrespective of its aetiology. However, there are many complications in chronically haemodialysed patients. These can be classified into two general categories: a) specific complications which are a result from the haemodialysis treatment itself, and b) complications of the primary disease which ultimately led to its ESRD [1]. The most common complications are the following: anaemia, congestive heart failure, vascular and cardiac calcifications, hypertension, dyslipidaemia, accelerated atherosclerosis, immune deficiency, inflammation, nausea and anorexia, malnutrition, gastrointestinal bleeding, hyperparathyroidism and bone disorders, dementia and infection with blood-borne viruses - mainly hepatitis B and C. These complications, together with additional co-morbidities and the complex pathology contribute to very high morbidity and mortality among chronically haemodialysed patients [2].

Many clinical chemistry biomarkers have been tested for their power to predict morbidity and mortality in chronically haemodialysed patients; serum albumin [3] and high sensitivity C-reactive protein have been recognised as the most appropriate biomarkers for predicting all-cause mortality [4,5], mainly because of their high reliability, stability and relative ease of analysis. There is emerging evidence that biomarkers of plasma (anti)oxidant status could also predict the all-cause mortality in chronically haemodialysed patients [6].

Oxidative damage is a common underlying feature of chronic haemodialysis [7-9] and may contribute to anaemia [10-13] through increasing the extent and nature of oxidation and fragility of erythrocyte membranes. Oxidative damage has been attributed to: a) the uremic syndrome itself [14,15], and b) the bio-incompatibility of

the haemodialysis membrane [16]. To address this, vitamin E is used for both coating of the haemodialysis membrane [17,18], and as an oral supplementation [19] and has been proven to reduce the recombinant human erythropoietin (rHuEPO) requirement in patients to mitigate anaemia. Besides erythropoietin deficiency, and even in the presence of erythropoiesis stimulating agents, the reduced erythrocyte survival time is another important cause for anaemia in chronically haemodialysed patients. It is generally accepted that the toxic uremic environment accounts for the decreased erythrocyte life-span and this is not explained by mechanical damage from the dialysis membrane [20].

Previous studies have shown a high prevalence of ascorbic acid deficiency in chronically haemodialysed patients which is accompanied by increased levels of oxidative stress biomarkers, thus suggesting that ascorbate supplementation may be of value [21]. Therefore, vitamin C as an oral [22] or intravenous [23] supplement is very often used as an antioxidant treatment in chronically haemodialysed patients, alone or in combination with vitamin E [24]. There is also evidence that vitamin C is effective in increasing the haemoglobin concentrations in patients who are hypo-responsive on treatment with erythropoietin, and/or decreasing of the rHuEPO requirements [25,26]. However, there may be adverse effects of vitamin C supplementation, a) especially in relation to the hyperoxalemia [27], and b) its paradoxical pro-oxidant effect in the presence of iron [28].

In summary, the molecular mechanisms involved in the development of anaemia in ESRD patients who are on chronic haemodialysis are not completely clear. Knowing that the erythrocyte membrane plays a key role in its mechanical properties and survival, a recent study investigated the erythrocyte membrane proteome in a) non-dialysed chronic kidney disease patients, b) erythropoietin/haemodialysis-treated

chronic kidney disease patients, and c) healthy individuals. Differentially expressed erythrocyte membrane proteins were observed between the groups of subjects under study, which could be potentially relevant for understanding of anaemia in uremic syndrome and chronic haemodialysis [29].

Based on the close interconnection between oxidative stress, anaemia and decreased rHuEPO responsiveness in chronically haemodialysed patients, we hypothesise that specific erythrocyte membrane protein oxidation contributes to the pathogenesis of anaemia in these patients. Thus, the aim of our study is to determine the total erythrocyte membrane protein carbonyls, to identify specific erythrocyte membrane proteins which are more oxidised in chronically haemodialysed patients, and to understand the effects of short-term oral vitamin C supplementation and dialysis on oxidative damage.

PATIENTS AND METHODS

PATIENTS

All 26 patients attending the two morning shifts of the Department of Haemodialysis at the Clinical Hospital in Stip, Republic of Macedonia, receiving chronic haemodialysis treatment were considered for enrolment in the study. An initial questionnaire revealed that 13 of the 26 patients were taking intravenous vitamin C supplementation of 500mg after each haemodialysis session, and one was taking 1000mg of vitamin C per day as an oral supplementation so these patients were excluded from the study. In addition, one of the patients was on haemodialysis treatment for less than 1 year, and was also excluded. The remaining 11 patients were consented and enrolled in the study. None of them reported use of any antioxidants and supplements.

Blood for analysis was taken immediately before the start of the haemodialysis session, after an overnight fast, in a blood collection tube containing heparin as an anticoagulant. Before haemodialysis, blood was also taken in an additional EDTA blood collection tube. This sample was used for analysis of the full blood count (FBC) on a 3-part differential automatic haematology analyser (Cobas Micros, Roche). The FBC analysis was run in the Laboratory for Biochemistry at the Clinical Hospital in Stip within few hours after the sampling. In addition, blood was taken again in a heparin containing blood collection tube, immediately after the haemodialysis session.

Blood samples from heparin containing collection tubes were processed immediately after the sampling. After the plasma and buffy coat removal, erythrocytes were washed 3 times with 4mL of physiological saline, each time discarding the small

quantity of the erythrocytes and remaining leucocytes from the top layer. Washed erythrocytes from the bottom of the tube were carefully aspirated, transferred in another tube, lysed with 2mL of cold water, and immediately frozen at -80°C.

Starting the next day after the initial sampling, an oral vitamin C supplementation (Pliva, Zagreb, Croatia) was provided to the patients enrolled in the study, in a dose of 2 x 500mg per day, for a period of four weeks. All of the patients enrolled in the study had taken the vitamin C supplementation regularly as assessed by pill return and interview, except of one of them who occasionally missed some of the doses. None of the patients reported any adverse effects. In addition, none of them experienced any acute inflammatory or infectious disease. During the 4 weeks study period all parameters of the haemodialysis treatment were kept constant for each patient.

After four weeks, the sampling was repeated before the haemodialysis session only, and the same protocol for preparation of the samples was followed. On the day of the sampling the patients did not take their dose of the vitamin C. A schematic representation of the study design is given in Figure 1.

A group of 15 healthy volunteers, non-smokers, with normal FBC and without any acute or chronic disease, taking no medications nor any vitamins or supplements, were included in the study as controls. Fasting blood samples (after an overnight fast) were taken for analysis.

All patients and control subjects gave informed written consent for participation in the study. This study was performed in accordance to the ethical principles for medical research involving human subjects (Declaration of Helsinki, 2008). The study was approved by the Institutional Review Board of the Clinical Hospital in Stip, and

conducted under ethical guidance of Dr. Slave Dimitrov, as stated in document No.1/12 from 28.05.2012, Clinical Hospital in Stip, Republic of Macedonia. Erythrocyte lysates were transported under dry ice to Aston University in Birmingham, United Kingdom, and were kept at -80°C. All analyses were performed within 9 months of the blood withdrawal.

METHODS

PREPARATION OF ERYTHROCYTE MEMBRANES

The erythrocyte membranes were prepared in accordance to the previously published protocols [30-32], slightly modified. Namely, 1mL of the lysed erythrocytes, well mixed, was centrifuged for 15 minutes at 16,600g at 4ºC and the supernatant discarded. Erythrocyte membranes were then mixed thoroughly using a pipette and resuspended in a high-salt buffer (1mL; 2.9g NaCl in 100mL lysis buffer. Lysis buffer: 5mM Na_2HPO_4, 1mM Na_2EDTA, 1mM phenylmethanesulfonyl fluoride, pH 8.0). Following vortex, erythrocyte membranes were centrifuged for 15 minutes at 16,600g at 4ºC and supernatant discarded. Erythrocyte membranes were then mixed thoroughly using a pipette and resuspended in 1mL lysis buffer. Following vortex, erythrocyte membranes were centrifuged for 15 minutes at 16,600g at 4ºC and supernatant discarded. To remove any particulate matter, the erythrocyte membranes were mixed well and resuspended in 1mL lysis buffer. Following vortex, erythrocyte membranes were centrifuged at 200g for 30 seconds. Supernatant was removed, further centrifuged for 15 minutes at 16,600g at 4ºC and subsequent supernatant discarded. Erythrocyte membranes were then mixed thoroughly using a pipette and resuspended in 1mL lysis buffer. Following vortex, erythrocyte

membranes were centrifuged for 15 minutes at 16,600g at 4ºC and supernatant discarded. The washing steps were repeated until supernatant was completely clear and a pale yellow pellet visible. Erythrocyte membrane pellets were resuspended with 1% triton X-100 in lysis buffer (100µL), mixed well by vortex and stored at -80ºC. Protein concentration was determined using the bicinchoninic acid (BCA) assay.

MEASUREMENT OF TOTAL PROTEIN CARBONYLS IN ERYTHROCYTE MEMBRANES

Reduced and oxidised bovine serum albumin (BSA) was prepared for carbonyl standards and the enzyme linked immunosorbent assay (ELISA) was performed as described by Carty et al. (2000) [33].

SDS-PAGE ELECTROPHORESIS

Erythrocyte membrane preparations were mixed in a 1:1 ratio with Laemmli buffer (2X, Sigma, UK), boiled for 5 minutes at 95°C and separated by reducing SDS-PAGE using 10% polyacrylamide gels. Each gel included lanes for protein markers (Bio-Rad Precision Plus Protein Standard Kaleidoscope, 10 - 250kDa), an oxidised BSA sample (positive control), and erythrocyte membrane preparations. Electrophoresis was undertaken using a Mini-PROTEAN® Tetra Cell (Bio-Rad) and a constant voltage (115V) was applied to each gel for 1 hour 45 minutes or until the gel front had migrated fully. Gels were electrophoresed in parallel. One gel was stained with Flamingo fluorescent stain (Bio-Rad) according to the manufacturer's protocol, while the other one was used for western blot analysis. The stained gels were scanned on a Pharos FX Plus Molecular Imager (Bio-Rad).

WESTERN BLOT ANALYSIS

Erythrocyte membrane proteins were transferred to Hybond-P® PVDF membranes (GE Healthcare, Amersham, UK).

Briefly, for analysis of protein carbonyls the PVDF membranes were washed six times for 5 minutes in 0.05% Tween 20, in Tris buffered saline (TBS) [34]. The proteins were derivatised using 1mM dinitrophenylhydrazine (DNPH) in 2M hydrochloric acid for one hour. Membranes were washed six times for 5 minutes in 0.05% Tween 20, in TBS and blocked overnight at room temperature with 0.1% Tween 20 and 3% BSA in TBS. Membranes were further washed six times for 5 minutes in 0.05% Tween 20, in TBS and incubated for two hours with monoclonal mouse anti-DNP (Sigma, UK) antibody diluted at 1:1000 with 0.2% BSA in TBS. Membranes were washed a further six times for 5 minutes with the same washing buffer and then incubated for one and a half hour with peroxidase conjugated rat anti-mouse IgE antibody (AbD Serotec) diluted at 1:10000 with 0.2% BSA in TBS. Subsequently, membranes were washed five times for 5 minutes with 0.05% Tween 20, in TBS and once with TBS only. Oxidised erythrocyte membrane proteins were visualised using ECL+ chemiluminescence (GE Healthcare, Amersham, UK), and protein bands scanned using a molecular imager GS-800 Calibrated Densitometer (Bio-Rad).

For anti-ankyrin western blot, membranes were washed six times for 5 minutes in 0.05% Tween 20 in TBS and incubated for two hours with rabbit anti-human ankyrin antibody (Abcam, UK; raised against C-terminal residues1862-1874 of human erythroid ankyrin) diluted at 1:1000 with 0.2% BSA in TBS. Membranes were washed a further six times for 5 minutes with the same washing buffer and then incubated for one and a half hour with peroxidase conjugated mouse anti-rabbit IgG antibody

(Sigma) diluted at 1:10000 with 0.2% BSA in TBS. Subsequently, membranes were washed and visualised for ankyrin as above.

LC-MS/MS

Flamingo fluorescence-stained bands were excised, divided into ~2mm^3 cubes and destained with acetonitrile followed by 100mM ammonium bicarbonate. This cycle was repeated until gel pieces were destained. Gel pieces were dried (vacuum centrifugation; 5 min) and rehydrated in 10mM Dithiothreitol and 100mM ammonium bicarbonate and reduced at 60°C for 15 min. The liquid was removed and replaced with 50mM iodoacetamide and 100mM ammonium bicarbonate. Gel pieces were incubated at room temperature in the dark for 45 min and then washed with 100mM ammonium bicarbonate. After drying the gel (vacuum centrifugation; 5 min) 0.3µg trypsin gold (Promega, WI, USA) was added and shaken at room temperature for 30 min, before dilution with 100mM ammonium bicarbonate. Hydrolysis was allowed to occur overnight (~16 h) at 37°C. Peptides were extracted with the initial solution of 2% acetonitrile and 0.1% formic acid in water was added and shaken for 30 minutes. Supernatant was removed to a clean plate. A second peptide extraction was performed using 40% acetonitrile and 0.1% formic acid in water, shaken for 30 minutes at room temperature. The supernatant was removed, pooled with the previous extracted peptides and dried in an evaporator. The samples were re-suspended in 0.1% formic acid/water.

UltiMate® 3000 HPLC series (Dionex, Sunnyvale, CA USA) was used for peptide concentration and separation. Samples were separated in Nano Series™ Standard Columns 75 µm i.d. x 15 cm, packed with C18 PepMap100, 3 µm, 100Å (Dionex, Sunnyvale, CA USA). The gradient used was from 3.2% to 44% solvent B (0.1%

formic acid in acetonitrile) for 30 min. Peptides were eluted directly (~ 350 nL min^{-1}) via a Triversa Nanomate nanospray source (Advion Biosciences, NY) into a LTQ Orbitrap Velos ETD mass spectrometer (ThermoFisher Scientific, Germany). The data-dependent scanning acquisition was controlled by Xcalibur 2.1 software. The mass spectrometer alternated between a full FT-MS scan (m/z 380 – 1600) and subsequent collision-induced dissociation MS/MS scans of the 7 most abundant ions. Survey scans were acquired in the Orbitrap with a resolution of 60 000 at m/z 400 and automatic gain control $1x10^6$. Precursor ions were isolated and subjected to CID in the linear ion trap with automatic gain control $1x10^5$. Collision activation for the experiment was performed in the linear trap using helium gas at normalised collision energy to precursor m/z of 35% and activation Q 0.25. The width of the precursor isolation window was 2 m/z and only multiply-charged precursor ions were selected for MS/MS.

The MS and MS/MS scans were searched against NCBI database using the Mascot algorithm (Matrix Sciences). Variable modifications were deamidation (N and Q), oxidation (M), and carboxyamidomethylation (C). The precursor mass tolerance was 10 ppm and the MS/MS mass tolerance was 0.8Da. Two missed cleavage was allowed and were accepted as a real hit protein with at least two high confidence peptides.

IMMUNOPRECIPITATION OF ANKYRIN

Isolated membranes (50µg) were mixed with rabbit anti-human ankyrin antibody in solution overnight at 4ºC. Protein G-coupled agarose beads (Invitrogen) were prepared according to the manufacturer's protocol and then incubated with isolated membrane-antibody complexes for four hours at 4ºC. Agarose beads were then

washed twice to remove any non-specifically bound proteins prior to elution with 0.1M glycine buffer (pH 3).

FRAP ASSAY

Measurement of the ferric reducing ability of plasma (FRAP) was undertaken using the assay based on the method of Benzie and Strain [35], slightly modified. The method is based on the principle of reduction of the ferric-tripyridyltriazine complex to the ferrous form, upon which an intense blue colour develops, and the change of absorbance is measured at 593nm (kinetic method). We have performed the measurement in a microplate format, by end-point approach. Briefly, 10μL of sample and 40μL of water were pipetted in microtiter plate in duplicate. After that, 200μL of working reagent were added in each well (a: acetate buffer pH 3.6; b: $FeCl_3$ solution; c: 2,4,6,-tripyridyl-s-triazine solution; 10:1:1), and the reaction mixture was incubated for exactly 8 min at 37ºC [36]. The absorbance was measured on a Bio-Rad microplate reader model 680 XR, at 595nm, against reagent blank. Standards of 500, 1,000 and 2,000μmol/L $FeSO_4$ were used for calibration of the assay.

CLINICAL CHEMISTRY ASSAYS

The plasma concentrations of urea, creatinine, albumin, total cholesterol, triglycerides, HDL-cholesterol, and iron were measured on a standard clinical chemistry analyser (Dimension RxL Max, Siemens) in the routine clinical chemistry laboratory in Stip.

STATISTICAL ANALYSIS

Numerical data were analysed by Microsoft Excel, and expressed as mean ± standard deviation. The normality of distribution was confirmed by the Kolmogorov-Smirnov test, using Statistica 7 software. Statistical significance was assessed by the Student's t-test: a) two-sample equal variance, or b) paired, as appropriate. Differences between groups or data sets were considered as statistically significant if the p value was lower than 0.05.

The statistical significance of the coefficients of correlation was assessed according to the number of subjects within the group, using a statistical table [37].

RESULTS

CLINICAL DATA

Patients who were included in this study (N=11) were on haemodialysis treatment for more than 18 months (mean duration of haemodialysis: 6 ± 3 years; range: from 2 to 12 years), and were treated with a protocol of 3 haemodialysis sessions per week. Two of the patients had diabetes mellitus type 2 with diabetic retinopathy and concomitant cardiomyopathy. Cardiomyopathy was also present in another one patient. One of the patients was diagnosed with systemic lupus erythematosus, one with chronic gastritis, and six with arterial hypertension. A presentation of the co-morbidities in the haemodialysed patients included in our study is given in Table 1. All of the patients were treated with a rHuEPO (Recormon). However, as measured before the haemodialysis session before the vitamin C supplementation, the blood haemoglobin concentrations were above 100g/L in only four patients, and none of them had a haemoglobin concentration above 115g/L. Six of the patients had low plasma iron concentrations. These data indicate a suboptimal erythropoietin/iron treatment. There was also a high and statistically significant correlation between the concentrations of plasma iron and blood haemoglobin ($r = 0.77$; $p<0.01$).

Blood haemoglobin and haematocrit values did not change significantly as a result of the vitamin C supplementation ($p>0.05$ for both parameters; paired Student's t-test).The majority of patients showed an increase in iron concentration after the vitamin C treatment but the effect was not statistically significant ($p>0.05$; paired Student's t-test). However, the strong correlation between haemoglobin and iron was reduced from 0.77 before vitamin C, to 0.02 after vitamin C supplementation, suggesting an interaction between the vitamin C and circulating iron.

In the chronically haemodialysed patients we observed a typical clinical chemistry profile consisting of high concentrations of plasma urea and creatinine, low plasma albumin, and low total and HDL-cholesterol (compared to the reference values). Following vitamin C supplementation none of these parameters changed significantly ($p>0.05$ for all parameters; paired Student's t-test).

The results from the routine haematology and clinical chemistry parameters are summarised in Table 2.

FRAP ASSAY

As a result of the vitamin C supplementation, there was a statistically significant increase in the concentration of total plasma antioxidants in chronically haemodialysed patients, from 1182 ± 241 to 1322 ± 306 μmol/L ($p<0.05$; paired Student's t-test), as measured before the hemodialysis session by the FRAP assay. However, FRAP values were always higher in chronically haemodialysed patients compared to healthy controls (966 ± 113 μmol/L $FeSO_4$), probably due to higher plasma concentration of small antioxidant molecules in chronic kidney disease.

ELISA PROTEIN CARBONYLS

As measured by the ELISA method, in the chronically haemodialysed patients not taking vitamin C the total erythrocyte membrane protein carbonyls were not significantly different before (1.03 ± 0.42 nmol carbonyl / mg protein) and after the single haemodialysis session (0.96 ± 0.73 nmol carbonyl / mg protein), $p>0.05$, paired Student's t-test, and also in comparison with the healthy controls (0.96 ± 0.45 nmol carbonyl / mg protein), $p>0.05$ for both comparisons. However, vitamin C supplementation decreased the content of the total erythrocyte membrane protein

carbonyls in the chronically haemodialysed patients before the single haemodialysis session (0.38 ± 0.19 nmol carbonyl / mg protein) which was statistically significant in comparison to a) the values measured before the single haemodialysis session without vitamin C supplementation ($p<0.025$, paired Student's t-test) and b) the healthy controls ($p<0.025$). ELISA total protein carbonyl measurements in the isolated erythrocyte membranes are summarised in the Table 3, expressed as mean ± standard deviation.

There were no statistically significant correlations between total protein carbonyls in erythrocyte membranes and measures of anaemia (iron, haemoglobin and haematocrit) in patients on haemodialysis. Besides the complex aetiopathogenesis of anaemia in haemodialysis itself, these findings also suggest involvement of more subtle mechanisms of oxidative stress in induction of anaemia in haemodialysis, which we have further investigated.

SDS-PAGE AND WESTERN BLOT ANALYSIS OF OXIDISED PROTEINS

In contrast to the results obtained by the ELISA assay for total protein carbonyls, the western blot analysis demonstrated a well-defined heavily oxidised protein in the erythrocyte membranes isolated from the chronically haemodialysed patients, which was completely absent in the healthy controls. This oxidised protein was present in the erythrocyte membranes of chronically haemodialysed patients irrespective of treatment – haemodialysis session or vitamin C supplementation (arrow; Figure 2B). To investigate whether the difference in intensity of protein oxidation observed in chronically haemodialysed patients reflects differences in expression of the protein in the membranes of patients compared to controls, samples were run in parallel and stained with either Flamingo fluorescent protein stain (Bio-Rad) or underwent

western blot analysis for protein oxidation (Figure 2A and B respectively). The gels demonstrate the presence of the protein(s) of interest in both patients and healthy subjects, confirming the difference in western blot signal is due to protein oxidation rather than protein expression. In addition, this figure illustrates a difference in the distribution and predicted masses of erythrocyte membrane proteins in patients undergoing chronic haemodialysis with a slightly higher MW band present in controls but absent in patients.

Subsequent analysis of integrated protein bands using ImageJ software has shown that intensity of carbonylation of the protein which is most sensitive to variable oxidation was reduced in average by 60% following haemodialysis ($p<0.001$; paired Student's t-test), but ~20% after vitamin C supplementation ($p>0.05$; paired Student's t-test). Samples from all three time points for each patient were analysed at the same time and were obtained for 7 patients along with 8 samples from the healthy control subjects. Data were normalised to healthy controls and show a decrease of carbonylation of the protein of interest as a result of the single haemodialysis session (Figure 3A). Decreased protein carbonyl content of this specific protein as a result of the vitamin C supplementation is depicted in Figure 3B. There was no significant difference in yield of protein recovered from erythrocyte membranes nor was there was no significant difference in % intensity for the band of interest relative to total protein stained (surrogate protein concentration) between patients, pre- and post-treatment and controls (Table 4).

MS ANALYSIS

The major band which was heavily oxidised in patients but not in membrane proteins from control subjects indicated by an arrow in Figure 2 was digested with trypsin,

extracted from the gel and analysed by LC-MS/MS. The most significant identification was attributed to ankyrin, with 65% sequence coverage including both N- and C-terminal residues and with an expected MW of 206kDa. There are many splice variants of ankyrin and it frequently undergoes modifications which can influence its electrophoretic mobility [38].

IMMUNOLOGICAL VALIDATION OF ANKYRIN IDENTITY

To confirm ankyrin's electrophoretic mobility in the SDS-PAGE gel conditions adopted here and its specific oxidation we also performed western blotting for ankyrin using an antibody raised against the regulatory domain, analysis of specific protein oxidation by western blot and immunoprecipitation. Figure 4 confirms that ankyrin migrates to the equivalent of a 75kDa standard on the gel, is a major oxidised band and the extent of oxidation alters with dialysis.

DISCUSSION

We have shown that in ESRD patients, total erythrocyte membrane protein carbonyl concentrations are not different from healthy controls nor are affected by dialysis. However, total protein carbonyls are reduced by vitamin C supplementation. Detailed analysis of the major band which was susceptible to oxidation in ESRD erythrocytes by mass spectrometry, immune-precipitation and western blot confirmed its identity as ankyrin. Our SDS-PAGE gels of erythrocyte membrane proteins confirm findings of others that protein expression differs in ESRD patients [29]; 2D gel studies showed that tropomodulin 1 (47kDa) was elevated in pre-dialysis compared to controls and post-dialysis treatment whereas the 97kDa protein beta-adducin was elevated in patients irrespective of treatment. Changes in band intensity were not evident here by 1D SDS-PAGE at these previously reported molecular masses. Following oxidative damage, proteins are susceptible to both aggregation and fragmentation. Aggregated proteins are frequently excluded from SDS-PAGE gels, and we did observe lower band intensity at >100kDa and higher intensity of bands in membranes from ESRD consistent with the hypothesis of increased protein oxidation in ESRD. Both aggregation and fragmentation of proteins may affect erythrocyte membrane deformability.

Although ESRD patients who are dependent on chronic haemodialysis treatment are widely recognised as having an oxidative stress related condition, the lack of significant difference in overall protein oxidation results are consistent with others [39]. The decrease in total protein oxidation after vitamin C intervention is again in accordance with our previous study; combined vitamin C / vitamin E antioxidant supplementation resulted in significant decrease of the plasma hydroperoxides of nearly 70% in chronically haemodialysed patients [40]. Together these data suggest

either the existence of some compensatory mechanism(s) to cope with the increased oxidative stress in these patients or that oxidation leads to enhanced fragility and lysis with greater chance for clearance of oxidised erythrocytes after haemodialysis. Ankyrin normally enables erythrocytes to resist shear forces experienced in the circulation through anchoring to cytoskeletal components such as spectrin. Moreover, individuals with reduced or defective ankyrin have a form of haemolytic anaemia [41]. While the effect of ankyrin oxidation on its capacity to resist shear forces is unknown, loss of function is a common observation in carbonylated, oxidised proteins. Ankyrin oxidised cells may be more fragile and more susceptible to haemolysis. One interpretation of the present data is that the more oxidised ankyrin bearing red cells are at increased probability of clearance after dialysis, so that overall the proportion of red cells with oxidised ankyrin and the extent of ankyrin oxidation is lower relative to total proteins in post-dialysis samples. In support of this, healthy control erythrocytes do not exhibit any significant oxidation of ankyrin.

Anaemia in ESRD usually develops as a consequence of erythropoietin deficiency, and results in impaired quality of life and increased morbidity and mortality [42]. As a result, erythropoiesis stimulating agents are widely used for both increasing and maintaining of the haemoglobin levels in chronically haemodialysed patients [43,44]. However, there is evidence for an increased incidence of adverse effects in patients with haemoglobin concentrations of more than 130g/L [45], which needs further careful evaluation [46]. The present findings suggest that oxidised ankyrin may be used as both a stratifying medicines biomarker that identifies those patients most likely to require rHuEPO following haemolysis and a surrogate marker of disease for evaluating clinical benefit of intervention.

Increased oxidative damage in patients on chronic haemodialysis treatment has been considered to contribute to their increased cardiovascular risk [47-49], which cannot be completely explained by traditional cardiovascular risk factors and may be ameliorated with antioxidant intervention. Despite evidence for harmful effects in the general population of high doses of some common supplements (β-carotene, vitamin E, vitamin A) [50,51], there is evidence, although limited, that chronically haemodialysed patients could benefit from the use of antioxidants to prevent cardiovascular complications [52,53]. However, there are also some contradictory findings which suggest that the mechanism and consequences of oxidative damage in ESRD need closer examination before antioxidant interventions are evaluated [54]. Previous studies have largely focused on studying plasma biomarkers of oxidation, however, cell function is likely to be particularly important in disease pathogenesis. Here, we have described a methodology that has enabled us to evaluate specific membrane protein oxidation which can be extended to the study of monocyte membrane protein oxidation, since monocyte subset distribution and function are pivotal in balancing the risk of vascular disease [55]. In particular, a closer examination of oxidation in monocyte cell membranes of ESRD for proteins which are uniquely sensitive to oxidation or which may favour cell fragility may provide a useful insight into mechanisms and targets for intervention to mitigate risks of vascular complications.

CONCLUSION

Total erythrocyte membrane protein carbonyls from ESRD patients were reduced by vitamin C supplementation but not by dialysis. Using a western blot to detect oxidised proteins, one protein band, later identified as containing ankyrin, was found to be

oxidised in patients but not controls. Erythrocyte ankyrin oxidation was reduced significantly by 60% in all patients after dialysis and by 20% after 4 weeks vitamin C treatment when measured pre-dialysis. Strategies for preventing ankyrin oxidation may reduce erythocyte clearance, increase survival time and reduce anaemia. These findings are consistent with the observations that vitamin C is effective in increasing the haemoglobin concentrations in patients who are hypo-responsive on treatment with erythropoietin, and/or decreasing of the rHuEPO requirements [25,26].

AUTHOR CONTRIBUTION

TR designed the intervention study in collaboration with SD and NK. TR isolated membrane proteins. HRG designed the analysis of membrane protein oxidation study. TR, SJB and CRB analysed membrane protein oxidation. SJB prepared samples for mass spectrometry and CRB performed immunoprecipitation.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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Table 1. Co-morbidities in chronically haemodialysed patients

Patient	Diabetes mellitus type 2	Retinopathy	Cardiomyopathy	Hypertension	Lupus erythematosus	Gastritis
1			*			
2				*		*
3 (A)	*	*	*	*		
4 (B)						
5 (F)				*	*	
6				*		
7 (G)						
8	*	*	*	*		
9 (C)						
10 (D)				*		
11 (E)						

Table 2. Basic haematology and clinical chemistry profile in chronically haemodialysed patients

	Before HD, before vitamin C supplementation		Before HD, after vitamin C supplementation		
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	**Reference range**
Haemoglobin (g/L)	92 ± 16	66 - 114	90 ± 14	66 - 110	110 – 165
Haematocrit (%)	27.6 ± 4.4	20.0 – 33.0	27.9 ± 3.9	21.3 – 34.1	35.0 – 50.0
Urea (mmol/L)	21.3 ± 6.4	12.4 – 33.2	20.5 ± 5.6	11.9 – 28.2	2.8 – 7.2
Creatinine (µmol/L)	778 ± 225	557 - 1299	776 ± 198	547 - 1218	58 – 110
Albumin (g/L)	34.8 ± 3.4	29.8 – 40.7	35.1 ± 3.4	30.1 – 41.4	35.0 – 52.0
Total cholesterol (mmol/L)	3.7 ± 0.9	1.9 – 4.9	3.6 ± 0.8	1.8 – 4.6	4.1 – 5.2
Triglycerides (mmol/L)	1.9 ± 0.9	0.7 – 3.4	1.7 ± 0.8	0.7 – 3.2	0.3 – 1.7
HDL-cholesterol (mmol/L)	0.75 ± 0.26	0.41 – 1.27	0.76 ± 0.28	0.40 – 1.30	1.00 – 2.00
Iron (µmol/L)	11.5 ± 6.9	1.9 – 22.8	12.1 ± 6.8	4.8 – 24.4	10.7 – 32.2

Table 3. TOTAL PROTEIN CARBONYLS IN ISOLATED ERYTHROCYTE MEMBRANES (nmol carbonyl / mg protein)

A. Chronically haemodialysed patients, without vitamin C supplementation, before single haemodialysis session	**B.** Chronically haemodialysed patients, without vitamin C supplementation, after single haemodialysis session	**C.** Chronically haemodialysed patients, with vitamin C supplementation, before single haemodialysis session	**D.** Healthy controls
1.03 ± 0.42	**0.96 ± 0.73**	**0.38* ± 0.19**	**0.96 ± 0.45**

* Statistically significant ($p<0.025$) in comparison to A and D

Table 4. Membrane protein concentration and % of total protein as ankyrin by densitometry following Flamingo staining of SDS-PAGE

	Protein extracted from erythrocyte membrane (mg/ml)	Protein on parallel gels visualised by Coomassie (AU)	% ankyrin intensity of total protein
Patients, before HD session, without vit. C supplementation	1.68±0.22	1103 ± 205	3.07±0.90
Patients, after HD session, without vit. C supplementation	2.14±0.69	968 ± 172	2.74±0.70
Patients, before HD session, with vit. C supplementation	1.85±0.23	1017 ± 138	3.22±0.65
Healthy subjects	2.29±0.19	960 ± 176	2.40±0.47

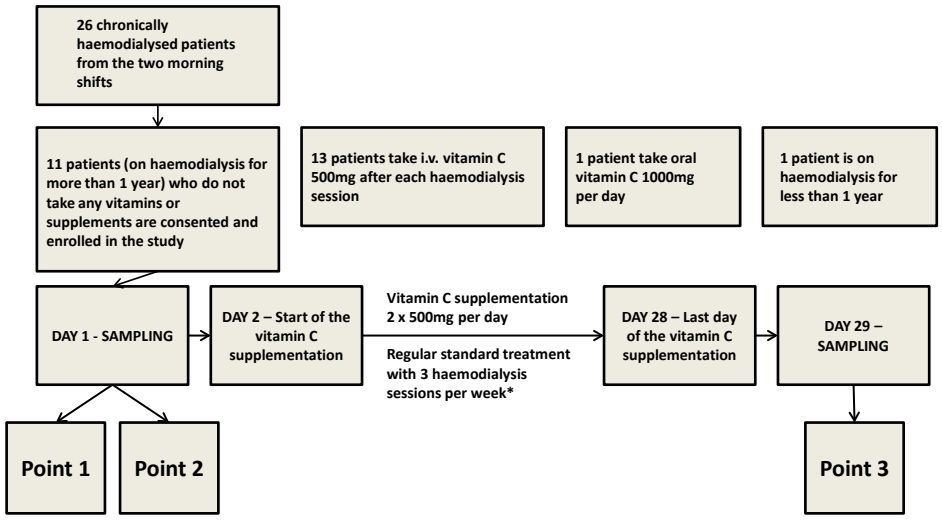


Figure 1. Study design – schematic presentation. Point 1: Day 1, immediately before the start of the haemodialysis session, fasting blood samples. Point 2: Day 1, at the end of the haemodialysis session. Point 3: Day 29, immediately before the start of the haemodialysis session, fasting blood samples.
*During the study period all parameters of the haemodialysis sessions were kept constant. None of the patients had any infectious or inflammatory condition.

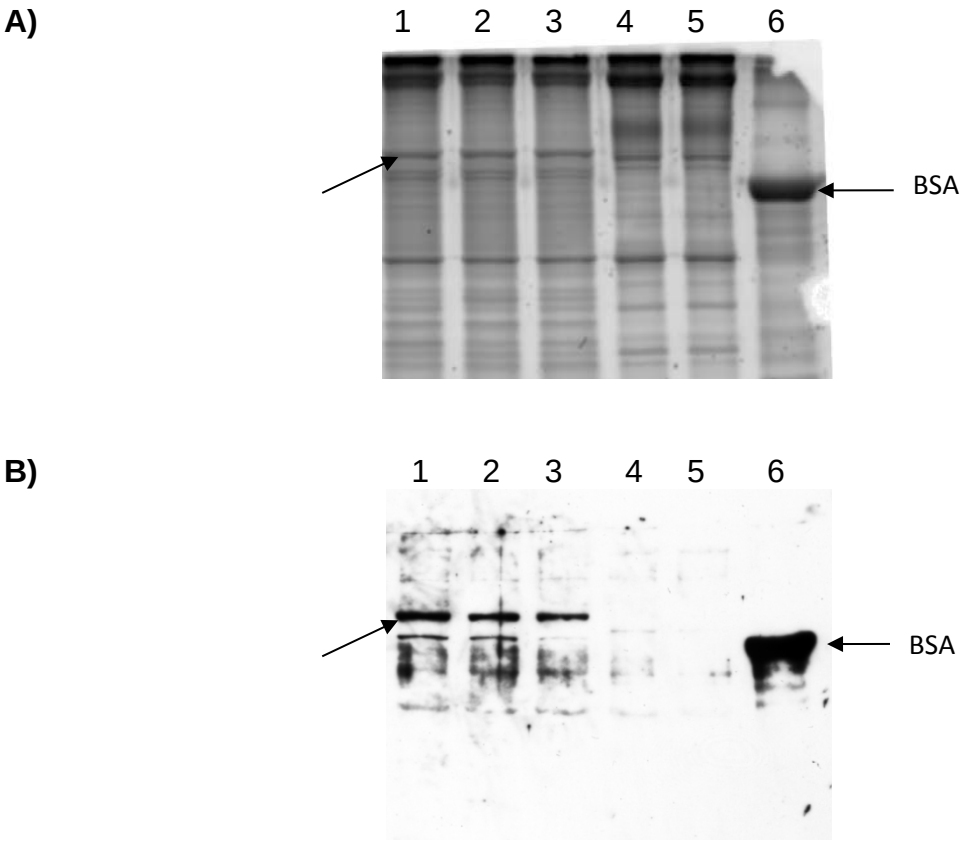
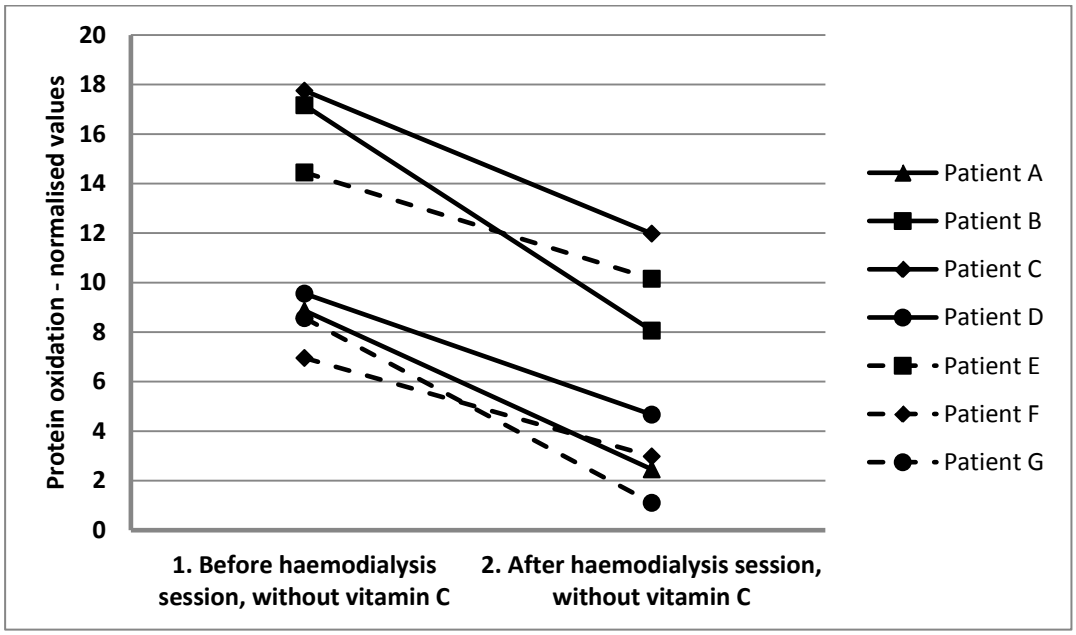


Figure 2. Erythrocyte membrane protein distribution and oxidation are altered in patients who are chronically haemodialysed. A) Erythrocyte membrane proteins stained with Flamingo fluorescent protein stain. B) Western blot analysis of oxidised erythrocyte membrane proteins.
BSA=oxidised bovine serum albumin standard.
Lane 1: Subject 9 (chronically haemodialysed patient) without vitamin C supplementation, before haemodialysis session. Lane 2: Subject 9 without vitamin C, after haemodialysis session. Lane 3: Subject 9 with vitamin C, before haemodialysis session. Lanes 4 and 5 are from 2 healthy subjects and lane 6 is the positive control (oxidised BSA).
The arrow highlights the protein most sensitive to variable oxidation which was extracted for identification by MS.

A)



B)

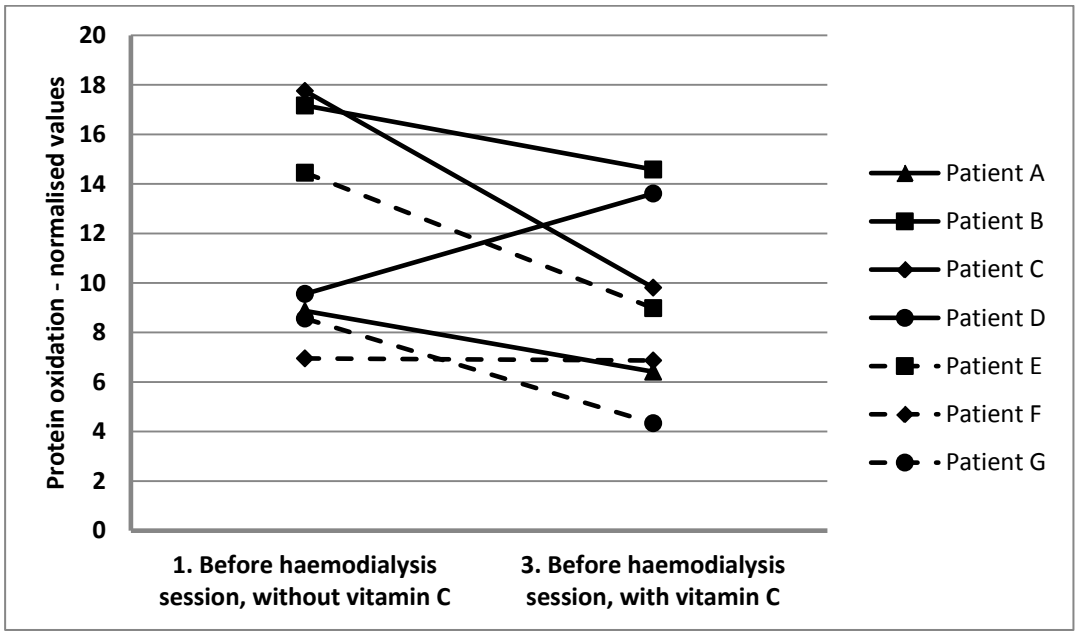


Figure 3. Erythrocyte membrane protein which is most sensitive to variable oxidation is reduced after the single haemodialysis session (A) and vitamin C supplementation (B). Patient protein oxidation was normalised to control membrane protein oxidation.

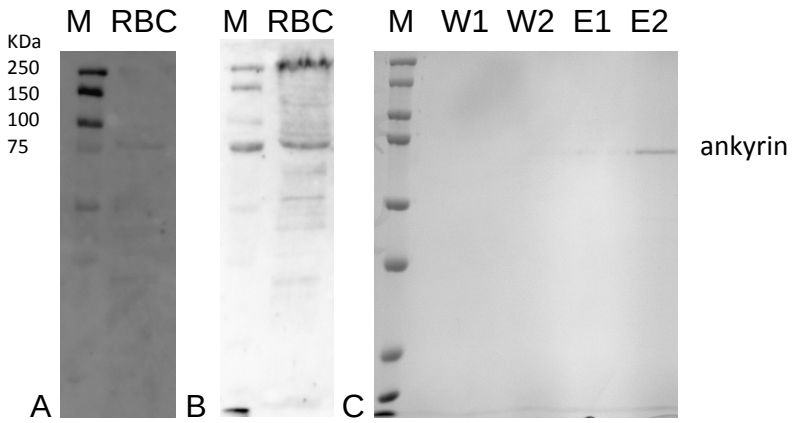


Figure 4. Ankyrin is oxidised in erythrocytes (red blood cells; RBC) from a patient undergoing chronic haemodialysis. A) Western blotting for protein carbonyls of erythrocyte membrane proteins. B) Coomassie stained gel of erythrocyte membrane proteins. C) Coomassie stained gel of ankyrin immunoprecipitation from erythrocyte membranes, where W=wash and E=elution and M=markers.