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Proteomic profiling of oxidative stress in human victims of traffic-related injuries after lower limb revascularization and indication for secondary amputation

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ABSTRACT

Microsurgical replantation and revascularization are frequently performed to salvage devascularized severe lower-extremity fractures in the human victims of road traffic-related injuries. However, some patients require secondary amputation within 1 week of successful revascularization due to tissue necrosis and sepsis. Enhanced efforts to understand the underlying molecular mechanism of such events are needed and should characterized in depth. Thus, functional proteomics were applied in this study to evaluate the role of oxidative stress in acute injury following microsurgery in a set of human subjects surviving serious road traffic accidents. Changes in the levels of protein volume and the accompanying content in protein carbonylation were visualized using two-dimensional electrophoresis (2-DE) and immunoblot analysis. Since oxidation of some acute-phase proteins not only causes them to lose their function as antioxidants but also initiates the intracellular stress signaling pathway that regulates cytokine and chemokine responses, how cytokine expression correlated with oxidative stress was also evaluated via protein array assays. It was observed that the growth-regulated oncogene protein family (GRO), the range of IL-6, IL-8, IL-10 and monocyte chemotactic protein-1 (MCP-1), which are responsible for neutrophil and monocyte aggregation with subsequent cytotoxic effects, were significantly elevated in the plasma of amputees subjects, whilst the level of chemokine recruiting leucocytes into inflammatory sites (RANTES) was diminished in the salvaged group of patients. Our results suggest that severely oxidative injury during revascularization perturbs the normal redox balance and induces carbonylation of specific proteins, thereby activating pro-inflammatory factors leading to severe tissue damage. The dissimilar 2-DE protein and cytokine profiles revealed here might reflect distinct etiologies resulting in oxidative damage to tissues and may serve as pivotal indicators of local necrosis and the subsequent need for secondary amputation of limbs. We believe that the combination of proteomic and cytokine profile results presented in this work offers more reliable information and defines more sophisticated criteria in clinical practice than currently used C-reactive protein levels (CRP) or white blood cells counts (WBC) for predicting secondary amputation requirements in patients requiring limb salvage surgery.

1. Introduction

Mangled lower-extremity injury may present with compromised vascularity requiring either microsurgical replantation or revascularization. The devascularized limb suffers from ischemic damage with resultant tremendous accumulation of metabolic toxins [1]. After revascularization, these toxins can circulate and cause multiple organ failure and death [2]. Furthermore, the restored arterial inflow carrying oxygenated blood to the ischemic tissue will result in reperfusion injury to the ischemic limb, increasing damage and risk of amputation [3]. Alternatively, infection of the lower limb, as the result of necrotic tissue, may not only spread but also generate both reactive-oxygen and -nitrogen species, i.e. nitric oxide and superoxide, that can lead to systemic sepsis and shock [4].

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Oxidative stress, either as a result of ischemia/reperfusion (I/R) injury or infection can damage proteins in variety of ways. Modifications such as cross-linking, peptide fragmentation and alteration of the amino acid sequence can result in loss of protein function [5]. Following reactive-oxygen species (ROS) mediated damage to the particular antioxidant proteins, cells or tissues become more vulnerable to damage in the presence of inflammatory species such as cytokines and free radicals [6].

In the light of systems biology, proteomic tools, including two-dimensional electrophoresis (2-DE) and mass spectrometric techniques combined with sequence database correlation, have enabled speed and accuracy in the identification of specific proteins altered with pathological states. As well as being used as markers for diagnosis, protein signatures can also predict outcome. Therefore they can provide valuable information in this study [7]. Moreover, 2-DE oxyblotting has been widely utilized to study protein modification as a result of oxidative stress. Formation of carbonyl groups, as a result of oxidative damage, reacts with 2,4-dinitrophenylhydrazine (DNP) and can be detected through 2-DE in combination with specific antibodies (anti-DNP) [8]. The presence of carbonylated proteins has been used as a hallmark of ROS-induced alteration of proteins [9,10], which might correlate with the requirement for secondary amputation or revascularization.

A number of studies have indicated that various cytokines and chemokines contribute to the cytotoxic effect of ROS. For instance, interleukin-1 (IL-1) and the tumor necrosis factor (TNF-α) are associated with skeletal muscle ischemia/reperfusion (I/R) injury [11]. TNF-α can significantly affect capillary membrane permeability after septic shock or I/R leading to multiple organ failure. Furthermore, myocardial dysfunction following prolonged I/R is related to neutrophil aggregation provoked by interleukin-6 (IL-6), IL-1 and TNF-α through induction of intercellular adhesion molecule-1 (ICAM-1) [12]. Therefore, the production and regulation of cytokines are critical components and can be used for evaluating the degree of necrosis and responses to ROS after microsurgery.

Our results presented here have highlighted that oxidative stress might cause a reduction in the levels of acute-phase proteins as well as increased generation of highly oxidized proteins, which appear to play a crucial role in regulating differential expression of the cytokines and chemokines during post revascularization or replantation. By means of functional proteome analysis combined with a comprehensive panel of pro- and anti-inflammatory cytokine data, we made a preliminary identification of several possible indicators for evaluating the need for secondary amputation after lower limb revascularization in a representative set of human subjects.

2. Materials and methods

2.1. Patients

The Committee on Research Involving Human Subjects of the Chang Gung Memorial Hospital in Lin-Ko, Taiwan, approved this study. Full informed consent was obtained from all patients involved in the study. In the period of 2006/2007 year, eight adult patients sustained lower limb devascularization due to various road traffic accidents, which occurred in the Tao-Yuan and Lin-Ko urban area of Taiwan. These patients underwent replantation or revascularization in the Chang Gung Memorial Hospital, Taiwan, if their Mangled Extremity Severity Score (MESS) justified attempted limb salvage [13]. Five of the eight patients, however, developed systemic sepsis, resulting in the formation of localized infection or extensive muscle necrosis in the salvaged limbs after 4–7 days, and in consequence, secondary amputation was required. Most of the bacterial flora in these subjects were identified and classified into the *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains. The appropriate antibiotics were administered to these subjects according to the variation of the wound bacterial culture and its sensitivities.

2.2. Handling of plasma samples

The recommended guidelines for standardized specimen collection and reproducible handling of plasma samples in proteomics have been applied as far as possible in this study to avoid ex vivo oxidative changes in the blood-derived samples [14–18]. Thus, human peripheral blood from patients and controls was collected in pre-chilled Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) containing heparin as an anticoagulant, and mixed by gentle inversion five to six times. Each sample vacutainer was kept tightly closed to avoid contact with ambient air and was immediately immersed and maintained in an ice water bath. Then the tubes were centrifuged using a pre-cooled device with a horizontal rotor (accuSpin™ MicroR, Fisher Scientific, Pittsburgh, PA) at $2000 \times g$ for 10 min at 4 °C. The resulting plasma was immediately transferred to pre-labeled, fresh cryovials (Biozym Scientific GmbH, Hess, Germany). Next, to make the plasma samples more time-stable and intact they were immediately frozen and stored at −80 °C with argon addition until analysis. All blood samples were processed with the described protocol within 4 h of the moment of phlebotomy. The aliquot of only the suitable portions of crude plasma sample (50–150 μL) was used to obtain an albumin-depleted fraction (see below) or subsequent proteomic analyses to avoid freeze/thaw cycles. These proteomic analyses were made in a time not exceeding 2 weeks from blood sample collection in order to minimize ex vivo secondary oxidation of albumin, transthyretin and other proteins during plasma storage [19]. In addition, the storage conditions of these plasma samples ensured that protein carbonyls remained stable even under long-term operation and analyses [20]. Peripheral blood plasma samples were collected from 20 age- and sex-matched healthy controls, from five patients with jeopardized limbs immediately before secondary amputation, and from three patients with salvaged limbs at 7 days after revascularization. The plasma samples extracted from 10 patients subjected to cardiovascular disease were considered as ischemia/reperfusion (I/R) reference samples.

2.3. Albumin depletion from plasma samples

The deep frozen crude plasma samples were returned to 4 °C. Using the commercially available ion exchange based Millipore Montage Albumin Deplete Kit (Millipore, Bedford, MA), 50 μL of crude plasma was diluted with 150 μL of equilibration buffer (kit reagent) before application to the column. The protocol was executed as described in the Montage Albumin Deplete Kit user guide. The flow-through fraction contained the albumin-depleted plasma. Albumin was recovered from the column with the 40 mM Tris acetate pH 7.0 and 10.0% sodium dodecyl sulfate (SDS) [14,15,21].

2.4. Two-dimensional polyacrylamide gel electrophoresis (2-DE)

The procedure followed has been described previously [14,15]. Thus, the frozen crude plasma sample or the albumin-depleted plasma sample (150 μL) was precipitated using a 3:1 (v/v) ice-cold acetone-to-sample solution. Protein pellets were collected, dried, and solubilized in a lysis buffer containing 7 M urea, 2 M thiourea, 4.0% CHAPS, 0.1% Triton-100, 2.0% 4–7 non-linear immobilized pH gradient (IPG) buffer (Amersham Bioscience, Uppsala, Sweden), 10 mM DTT, and 10 mM phenylmethanesulfonyl fluoride. Proteins (250 μg) were then applied onto 13 cm Immobiline DryStrip (pH 4–7 linear) on the IPGphor isoelectric focusing (IEF) system

(GE Healthcare Life Science, Pittsburgh, PA). The running conditions of the IEF process were as follows: 30 V, 12 h; 100 V, 1 h; 250 V, 1 h; 500 V, 0.5 h; 1000 V, 0.5 h; 4000 V, 0.5 h; and 8000 V up to 80,000 V h. Before 2D SDS-PAGE, the IPG strips were equilibrated in a solution containing 50 mM Tris–HCl pH 8.8, 6 M urea, 2.0% SDS, 30% glycerol and a trace of bromophenol blue, which included 15 min exposure to 2.0% DTT for the first equilibration, and replacement with 2.5% iodoacetamide for the second equilibration for the next 15 min. Two-dimensional SDS-PAGE was carried out on 12.0% acrylamide gradient gels (Hoefer SE600) at 24 mA/gel until the bromophenol blue dye front reached the end of the gels. After approximately 5 h, all gels were visualized by a silver staining method (Amersham Bioscience) and then scanned using the Image Scanner (Amersham Bioscience). The protein isoelectric point p*I* and molecular weight MW were assigned by, respectively, p*I* calibration markers and MW markers (Bio-Rad, Hercules, CA). Protein spots were quantified using Non-linear Progenesis software (J & H Technology Company, Taiwan).

2.5. Derivatization of protein carbonyls and DNP immunostaining

Following plasma sample rehydration and IEF, the IPG strips were placed in 15 mL tubes and incubated in 2N HCl with 10 mM DNP (2,4-dinitrophenylhydrazine, Sigma, St. Louis, MO) at 25 °C for 20 min. Following the incubation enabling protein-bound carbonyls derivatization, the IPG strips were washed with 2 M Tris–base/30.0% glycerol for 15 min. The IPG strips were then prepared for molecular weight-dependent separation of DNP-derivatized proteins by 2-DE, followed by protein blotting to a PVDF membrane as described previously [22,23]. Next, the PVDF membranes were removed from the ISO-DALT electroblotting apparatus (Amersham Bioscience) and incubated overnight at 4 °C for immunostaining with the primary antibody solution consisting of a 1:16,000 dilution of the anti-DNP IgG antibody (Molecular Probes, Eugene, OR) in the Tris-buffered saline Tween-20 (TBST) containing 5.0% non-fat dry milk. Next, the blots were washed and incubated with the goat anti-rabbit IgG/HRP conjugate (1:6000 dilution in TBST) for 2 h at room temperature. An enhanced chemiluminescence kit (Immobilon Western Chemiluminescent AP substrate, Millipore, Bedford, MA) was used for detection. The gels were also visualized by means of silver staining and scanned with the Amersham Bioscience Image Scanner. The protein spots were quantified using Non-linear Progenesis software (J & H Technology Company, Taiwan).

2.6. In-gel enzymatic digestion and mass spectrometry

Silver-stained spots were excised and in-gel digested with trypsin according to the procedures described previously [14,24]. Briefly, the gels were destained by 1.0% potassium ferricyanide and 1.6% sodium thiosulfate (Sigma). Then the proteins were reduced with 25 mM NH_4HCO_3 containing 10 mM DTT (Amersham Bioscience) at 56 °C for 30 min and alkylated with 55 mM iodoacetamide (Amresco, Solon, OH) at room temperature for 30 min. Then, the proteins were digested with 20 μg/mL trypsin solution (Promega, Madison, WI) at 37 °C overnight. After digestion, the tryptic peptides were acidified with 0.5% TCA and loaded onto an MTP AnchorChip™ 600/384 TF (Bruker-Daltonik). The matrix-assisted laser desorption/ionization-time-of-flight mass spectrometric analyses (MALDI-TOF-MS) were performed on an Ultraflex™ MALDI-TOF mass spectrometer (Bruker-Daltonik, Bremen, Germany). Mono-isotopic peptide masses were assigned and used for database searches with the MASCOT search engine against the NBCInr protein data base using carbamidomethyl and methionine oxidation as variable modifications (Matrix Science, London, UK, http://www.matrixscience.com). Search parameters were set as follows: maximum allowed peptide mass error of 50 ppm, fragment mass tolerance ±0.3 Da, and consideration of the one incomplete cleavage per peptide. For the multistage LIFT-TOF/TOF-MS/MS procedure, employing a potential lift to accelerate fragment ions, the three most intense precursor ions with a signal/noise ratio >25 were selected after exclusion of the common background signal. This multistage MS/MS mode was operated with 1 keV and products of metastable decomposition at elevated laser power were detected. Single stage MALDI-TOF-MS data were acquired with close external calibration and multistage LIFT-TOF/TOF-MS/MS data were acquired using default instrument calibration.

2.7. Human cytokine protein array

The spectrum of cytokines produced by apparently healthy controls, I/R references, Group 1 and Group 2 subjects was tested using an antibody-based protein microarray (RayBio™ Human Cytokine Ab Array I and 1.1 Map, RayBiotech Inc., Norcross, GA) designed to detect 23 growth factors, cytokines of chemokines. The minimum detection limit of the assays was 10 pg/mL and these cytokines/chemokines were expressed constitutively at detectable levels in the plasma of the controls. Experimental procedures were performed as recommended by the manufacturer [25]. The experiments were repeated three times for each group. Proteins were detected via an enhanced chemiluminescence procedure (ELCplus, Amersham Bioscience). The intensity of the chemiluminescent signal for each spot was quantified by GeneTools (Syngene™, Cambridge, UK) image analysis software. By subtracting the background staining and normalizing to the positive controls on the same membrane, we obtained the relative protein concentrations. According to the manufacturer, the imprecision (CV) of the cytokine protein array used here is below 10%.

2.8. Statistical analyses

The study group ($n = 8$) was divided into two subgroups: secondary amputation ($n = 5$, Group 1) and salvage ($n = 3$, Group 2). A Mann–Whitney–Wilcoxon test was performed for independent samples. The significance coefficient $p < 0.05$ was considered as statistically significant. Statistical analyses were performed with SPSS 4.0 (SPPS Inc., Chicago, IL) statistical software. All protein identification scores obtained using the MASCOT search engine were significant ($p < 0.05$).

3. Results

3.1. Clinical characteristics of patients with or without secondary amputation

Of the eight patients who underwent attempted limb salvage, five subjects (three males and two females; mean age 37.8 years, range 17–58) required secondary amputation (Group 1). The limbs of the remaining three patients (two males and one female; mean age 29.0 years, range 21–36) were successfully salvaged (Group 2). The average ischemic time between the injury and replantation (or revascularization) for both groups of subjects was less than 7 h. The Mangled Extremity Severity Score (MESS) values were proposed as five or six for these two cohorts (see Table 1). Here, neither the ischemic time nor the MESS values were able to predict the secondary amputation rate correctly. Blood samples were collected from both groups for biochemical examination, i.e. blood urea nitrogen test (BUN), creatinine, aspartate aminotransferase (AST) which is also called serum glutamic oxalacetic transaminase (SGOT), alanine aminotransferase (ALT), C-reactive protein (CRP) assay, myoglobin and white blood cell count (WBC) as listed in Table 1. The results of the Mann–Whitney–Wilcoxon test showed statistically significant differences in the BUN changes between

Table 1
Characterization of secondary amputation and salvaged groups of patients and their biochemical parameters.

Patients	No.	Age	Gender	MESS	Surgery	Ischemic time (h)	BUN 6.0–21[a] (mg/dL)	Creatinine 0.4–1.4[a] (mg/dL)	AST 5–35[a] (U/L)	ALT 5–40[a] (U/L)	CRP <5[a] (mg/L)	Myoglobin <80[a] (mg/L)	WBC 1000[a] (per μL)
Group 1—amputation, $n = 5$	1	30	M	6	Replantation	4	8	0.8	122	34	196	4290.2	13,700
	2	17	F	5	Replantation	4	9	0.6	71	26	105	1863.3	16,500
	3	45	F	6	Revascularization	5	14	1.0	184	75	137	777.7	8700
	4	58	M	6	Revascularization	6	18	0.9	157	45	187	4145.8	21,300
	5	39	M	6	Revascularization	7	13	0.9	64	18	115	4594.7	16,100
Group 2—salvaged, $n = 3$	6	30	M	5	Replantation	6	4	0.7	419	76	129	8655.4	19,400
	7	36	M	6	Revascularization	5	8	0.9	106	25	125	665.8	6700
	8	21	F	5	Revascularization	4	5	0.9	169	57	101	958.4	6300
p							0.036	0.297	0.874	0.456	0.297	0.297	0.456

MESS—Mangled Extremity Severity Score; BUN—blood urea nitrogen test; AST—aspartate aminotransferase (E.C.2.6.1.1); ALT—alanine aminotransferase (E.C.2.6.1.2); CRP—C-reactive protein; WBC—white blood cell count.
[a] Reference values of healthy subjects; see also http://pathcuric1.swmed.edu/PathDemo/nrrt.htm.

the amputation group and the salvage group ($p = 0.036$). No statistical significant associations were found concerning creatinine, AST, ALT, and myoglobin concentrations. The CRP values and WBC counts were not significantly different between Group 1 and Group 2 ($p > 0.05$), either. Although the number of patients for statistical analysis in this study was relatively small, these results can offer appropriate tools for dealing with similar cases as those described here.

3.2. Analytical 2-DE protein profiles

Since it is difficult to define clear and decisive criteria for secondary amputation from the available biochemical information presented in Table 1, we used 2-DE to analyze the collected plasma samples for different expressions of proteins, and followed this with peptide identification by mass spectrometry. In-depth analysis of the plasma proteome offers a promising tool to yield biomarkers that allow early disease detection and monitor disease progression, regression, and recurrence [18]. Thus, all the crude plasma samples collected here were subjected first to proteomic analysis to characterize the differences in the high-abundant protein fraction in relation to the oxidative stress status of each group of patients.

As expected, albumin and seven other proteins (marked by bold font in Table 2), which constitute approximately 85–90% of the total protein mass of crude human plasma [18,21,26], could be assigned to gel matching spots as confirmed by the performed MALDI-TOF mass spectrometry measurements. However, increases in the number of lower abundant protein spots may be considered as much more powerful diagnostic biomarkers [21,27] and this approach could be implemented after removal of highly abundant proteins such as albumin, which accounts for more than half of the total protein mass in plasma [18,26,27]. As in our previous peptidomic analyses [15,21] we applied the typical, low-cost, high-throughput, and easy-to-use ion exchange albumin removal method, which is characterized by non-perfect performance for depletion of the whole albumin amount from the plasma or serum samples as compared to the antibody-based [21] or affinity adsorption [27] albumin depletion approaches. Thus, after use of this ion exchange method (see Section 2.3) a relatively large amount of albumin was still contained in the albumin-depleted plasma samples, as is visible in the 2-DE gels given in Fig. 1A in this work and presented in numerous earlier papers [14,21,27]. In addition, as the consequence of non-specific retention on the ion exchange cartridges used here, the other highly abundant protein, namely immunoglobulins, α_1-antitrypsin, haptoglobin and transferrin, could not be perfectly removed from the crude plasma samples and would be visible on the 2-DE gel as has recently been proved by study of Chromy et al. [21]. However, the perfect and complete removal of albumin from plasma samples would also cause highly distorted proteomic results such as removing the broad range of other low mass and low-abundant physiologically important regulatory and/or transient proteins which were bound to albumin as the main carrier/transport protein in the blood [18,21,26]. Thus, in view of the demands of our pilot study on the proteomic determination of unknown oxidized proteins related to microsurgery of road traffic accident victims, the albumin depletion method used here was a compromise choice. However, further dedicated and more extensive studies are needed to fully recognize this and other experimentally confounding factors in peptidomic analysis of plasma samples.

In spite of the above-mentioned limitations and challenges, albumin-depleted plasma samples of the healthy controls, the I/R reference patients, Group 1 and Group 2 subjects were prepared to reduce the complexity of proteomic analysis, and these were separated into duplicates on the 2-DE gels with a mass range between 17 and 170 kDa and a p*I* range from 4 to 7. Reproducible gel

Table 2
List of selected principal proteins linked with oxidative stress in microsurgery identified by mass spectrometry analyses.

Spot no.	Protein	Swiss no.	Fold changes	MW (kDa)/p*I*	Peptide matched	MOWSE score*/sequence converage (%)	Biological process/function
1	α_2-Macroglobulin [b]	P01023	7.37	164.06/6.0	69	324/56	Intracellular protein transport
2	α_1-B-glycoprotein[b]	P04217	−3.42	52.48/5.65	18	189/47	Possesses opsonic properties
3	**IgA**[a]	P01876	−7.76	51.96/7.87	9	68/24	Immune response
4	**α_1-B-antitrypsin**[a]	P01009	−6.74	46.86/5.37	34	323/62	Acute-phase response protein activity
5	**Ig κ heavy chain**[a]	P01857	−5.14	51.68/8.91	13	122/36	Immune response
6	**Haptoglobin β chain**[a]	P00738	−3.78	45.86/6.13	15	143/34	Acute-phase response protein activity
7	Leucine-rich-α_2-glycoprotein[b]	P02750	−2.99	40.10/5.43	11	85/50	Acute-phase response protein activity
8	α_2-Glycoprotein (Zn), chain D[b]	P25311	−9.56	30.78/6.03	15	138/59	Acute-phase response protein activity
9	Complement C3 (fragment)	P01024	−2.33	188.59/6.02	38	229/29	Inflammatory response
10	Transthyretin (dimer)[b]	P02766	−22.31	12.84/5.35	12	172/92	Inflammation
11	**Ig κ light chain**[a]	P06309	1.28	28.98/6.09	13	116/52	Immune response
12	Apoliprotein ApoA1[b]	P02647	11.54	28.06/5.27	24	244/73	Cholesterol metabolism
13	**Haptoglobin α chain**[a]	P00738	−3.87	42.13/6.25	12	111/29	Acute-phase response protein
14	**Albumin**[a]	P02768	3.34	70.67/6.09	48	471/74	Body fluid osmoregulation
15	**Transferrin**[a]	P02787	−5.24	78.51/7.14	39	319/53	Iron homeostasis
16	α_2-HS-glycoprotein precursor[b]	P02765	−3.24	40.10/5.43	12	90/32	Possesses opsonic properties

[a] Components of the high-abundant protein fraction of human plasma (bold font).
[b] Components of low-abundant protein fraction of human plasma (normal font).
* Protein scores greater than 75 are significant ($p < 0.05$).

patterns were observed between the replicates. A total of 413 protein spots appeared in the 2-DE maps of albumin-depleted plasma samples under silver staining (see Fig. 1A). Of these low-abundant proteins, 150 proteins were identified by peptide mass fingerprinting (data not shown). For example, the identification of one of the target low-abundant proteins as the complement C3 fragment protein by tandem MS spectra is shown in Fig. 2A. However, in the obtained 2-DE patterns as presented in Fig. 1A, the selected 16 proteins most probably associated with the oxidative modification process related with microsurgery patients were annotated to indicate the identified high- and low-abundant peptide fraction of plasma (marked, respectively, by bold and normal font in Table 2) and were further characterized by performing a post source decay analysis of the mass peptide fragments (see Fig. 2B, for example). In addition, the selected areas in the individual gels containing differentially expressed α_1-antitrypsin and apoliprotein A-1 (ApoA1) from, respectively, crude and albumin-depleted plasma of Group 1 and Group 2 patients, were amplified as presented in Fig. 1B. The protein expression levels of α_1-antitrypsin and ApoA1 in the individual patient samples were in line with those in the pooling samples. The most striking feature of the obtained 2-DE patterns of both the crude and albumin-depleted plasma samples was that there were numerous proteins in the amputation Group 1 that were at significantly lower levels compared to the salvaged Group 2. These included acute-phase proteins, immune response proteins, metabolic proteins and proteins involved in folding or transportation. Oxidative stress in post microsurgery might induce protein oxidation and degradation, leading to functional disruption. In contrast, α_2-macroglobulin, ApoA1 and albumin were significantly elevated in the plasma of the amputated subjects (Group 1), whilst the level of the immunoglobulin κ chain was similar in both groups. Some selected comprehensive results of the mass spectrometric analyses performed here, and assigned protein functions of both high- and low-abundant protein fraction of considered plasma samples are summarized in Table 2.

3.3. Carbonylation of specific proteins

To evaluate the oxidative levels of protein after lower limb revascularization, we used the 2-DE combined with immunoblotting with specific antibodies. The protein carbonyl content recognized by anti-DNP antibodies in the crude plasma samples from the I/R reference patients, Groups 1 and 2, and from healthy controls is reported in Fig. 3. Increases in the number of carbonylated peptides spots were observed in Group 1 as compared with Group 2. The levels of carbonylation in specific proteins increased dramatically in Group 1, including the high-abundant plasma proteins, α_1-antitrypsin, albumin, transferrin, immunoglobulin κ light and heavy chain, haptoglobin β (Hbβ) chain and, as an example of the low-abundant plasma proteins, the complement C3 fragment. The levels of protein carbonylation were quite low in the I/R controls, in which only the carbonylated albumin and transferrin were detected. These proteins were found at physiological pH levels from 4.0 to 7.0. Interestingly, the differences in sensitivity to carbonylation depended on the quantity of proteins present in plasma as well as their susceptibility to oxidation. These results establish that oxidative modification to proteins in post microsurgery is selective, and those proteins with specific sensitivity to oxidation may regulate some cellular events associated with sequential tissue damage. Moreover, both Group 1 and Group 2 showed a significantly higher amount of oxidized proteins in crude and albumin-depleted plasma samples (data not shown) with respect to healthy controls and I/R reference group of subjects, indicating that multiple factors were involved in secondary amputation. Comparison of plasma protein oxidation levels was obtained by matching the 2D-oxyblots and subsequent silver-stained 2D-gel images from the same sample. The anti-DNP immunoreactivity of individual proteins separated by the 2-DE was normalized to their content in plasma as obtained by measuring the intensity of the staining in the healthy subjects group. In Table 3, the comparison of changes in specific carbonyl levels in some MS/MS identified plasma proteins for secondary amputation Group 1, salvage Group 2, and the I/R reference group is presented.

3.4. Overall cytokine profile changes under oxidative stress

Septic shock or ischemia/reperfusion mediated ROS production leads to high levels of protein oxidation, which might activate cytokine cascades and cause increased tissue damage. To investigate global changes in cytokines and growth factors, a simple and cost effective cytokine expression array was used as this approach is more sensitive (at least 100-fold greater for immunoglobulins), specific, requires a small sample volume and offers higher throughput in comparison to the conventional ELISA and Western blotting method [20,28,29]. According to the manufacturer, no cross

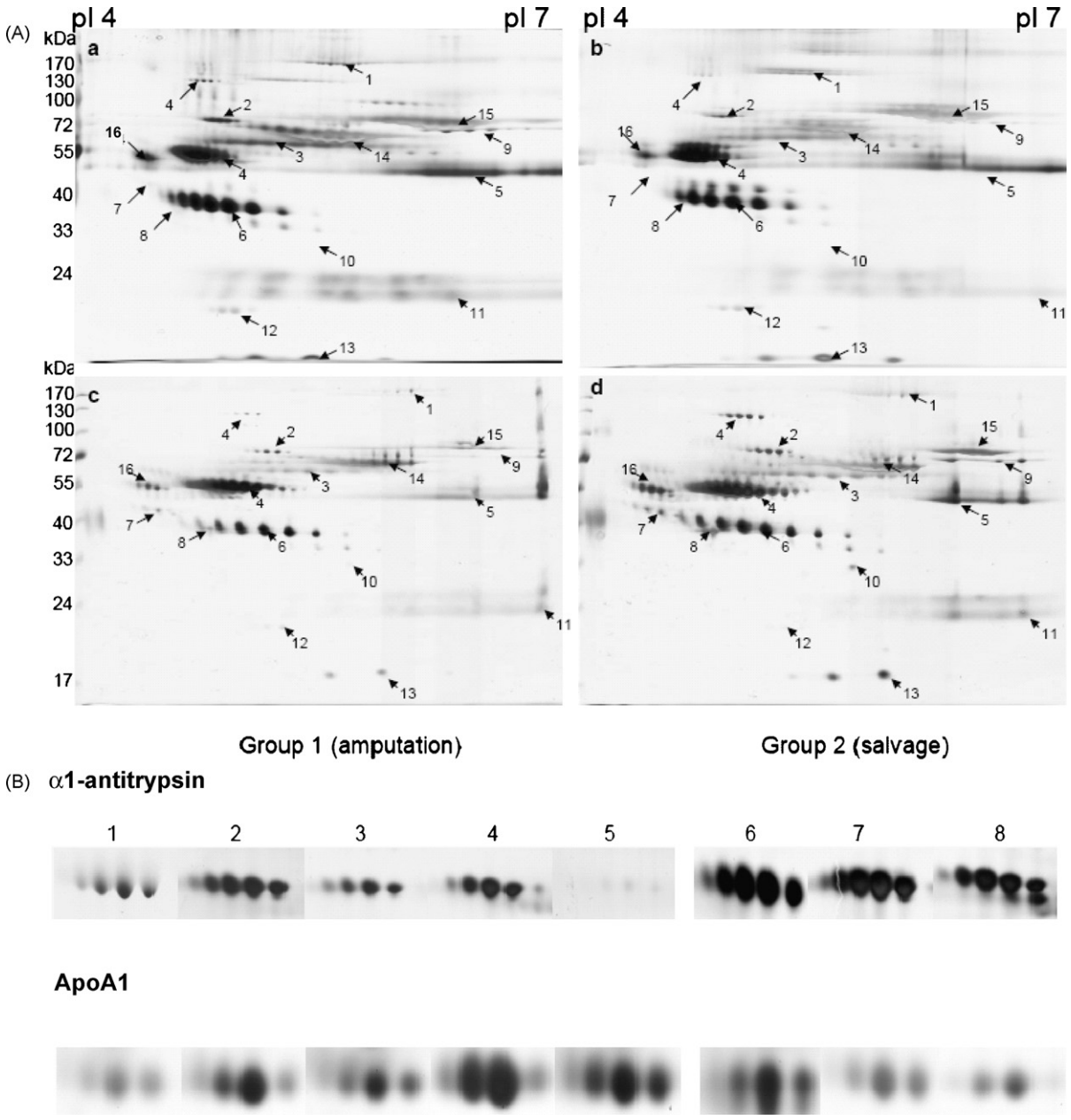

Fig. 1. (A) Two-dimensional electrophoresis patterns of plasma from the healthy controls (a), I/R reference patients (b), amputation Group 1 (c) and salvaged Group 2 (d). The protein lysate (250 μg) was focused on a pH 4–7 linear IPG strip before being separated on a 12% polyacrylamide gel. The identified proteins are annotated and indicated by arrows. 2-DE experiments of individual controls and patients were repeated three times to confirm the reproducibility of the protein profiles. This figure represents a typical result. (B) Comparison of selected protein spots between Group 1 (amputation) and Group 2 (salvage). The individual 2-DE patterns of α_1-antitrypsin and ApoA1 are shown and the patient numbers correspond to those in Table 1.

reactivity has been found for antibodies on the cytokine protein array used here (see Section 2.7). However, the quality of cytokine array measurements can be influenced by many factors including preparation, processing, and standardization of plasma samples. For example, some in-lab optimization of the protein array procedure is often required with regard to the appropriate dilution of the plasma sample. Reproducibility problems with preferential use of the chemiluminescent system currently available in the lab, instead of the one included in the kit supplied by the manufacturer have also been observed, thus leading to the skewed positive control signal on the membrane (see previously reported cytokine array profiles in Refs. [28,29]). Moreover, the internal positive controls on the membrane often give a very high signal compared to the expected levels from analyzed samples. When the expression of a cytokine of interest has diminished, thus requiring an extended exposure time, the internal positive controls become overexposed preventing reliable quantitation, and sometimes they are shadowed, giving the impression of oversaturation, especially if the final view of the membrane is presented in black–white fashion, or overlaps the neighboring cytokine dots. In the past these latter problems have frequently been reported to the manufacturer of the protein array applied here by many users but the positive controls are still high, which would skew the normalized values of determined cytokines, chemokines, and growth factor expression.

As demonstrated in Fig. 4A, undetectable or very low levels of cytokines and chemokines were present in age- and sex-matched control samples. The cytokines secreted in the plasma of patients with cardiovascular disease were considered as the I/R samples and displayed an expression pattern in which the monocyte chemotactic protein (MCP-1), the chemokine recruiting leucocytes

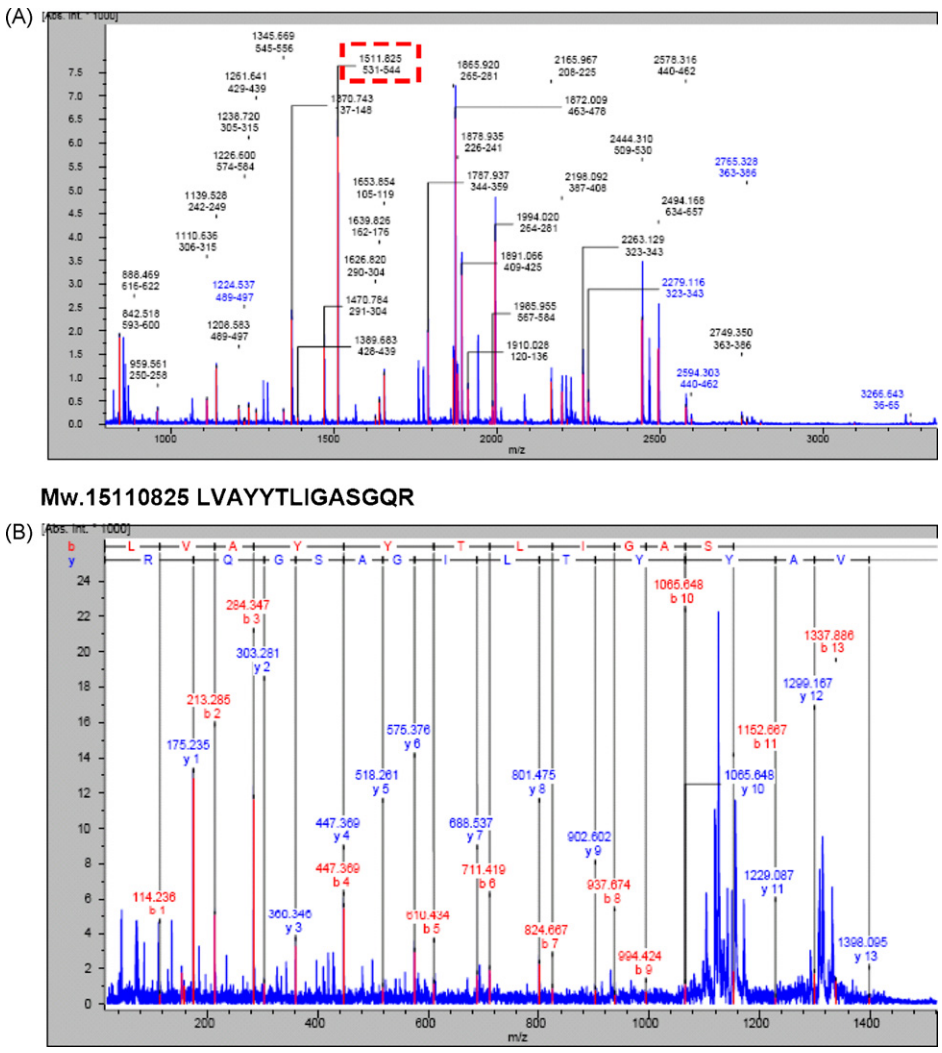

Fig. 2. Identification of target protein and MS/MS spectrum of a complement C3 fragment. (A) MALDI-TOF spectra were obtained by in-gel trypsin digestion of a complement C3 fragment. (B) LIFT-TOF/TOF-MS/MS spectra generated by Ultraflex™ TOF/TOF operated in LIFT mode. The parent ions *m/z* 1511.825 (red dashed (broken) line rectangle) were selected for further analysis by MS/MS. The amino acid sequences LVAYYTLIGASGQR were assigned to a human complement C3 fragment. A sequence was confirmed from the labeled b- and y-ions (marked in red and blue, respectively) in the LIFT-TOF/TOF-MS/MS spectrum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

into inflammatory sites (RANTES) and the growth-regulated oncogene protein (GRO) were significantly increased in respect to the healthy control group. Surprisingly, subjects in Group 1 displayed a distinct profile in which signals corresponding to GRO-α, IL-6, IL-8, and IL-10 were concomitantly increased, but RANTES was slightly decreased in abundance when compared with the I/R samples as positive controls. The cytokine pattern of Group 2 showed low levels of cytokines but these were still much higher than that of the control. The cytokine RANTES and transforming growth factor β_1 (TGF-β_1) did not differ significantly between Group 1 and

Table 3
Relative percentage change in specific carbonyl levels (mole carbonyl/mole protein) in some MS/MS identified proteins in plasma samples for subjects classified into groups with amputation, salvaged and ischemia/reperfusion (I/R) as compared with a healthy subjects group.

Spot no.	Proteins	Group 1—amputation (%)	Group 2—salvage (%)	I/R group (%)
5	**Ig κ heavy chain**[a]	115.08	82.58	6.91
14	**Albumin**[b]	162.66	47.72	11.22
15	**Transferrin**[c]	134.50	0.78	0.20
4	**α_1-Antitrypsin**[d]	24.13	93.28	2.43
11	**Ig κ light chain**[a]	38.18	0.026	2.54
6	**Haptoglobin β (Hpβ) chain**[b]	31.35	39.72	5.07
9	Complement C3 fragment[e]	17.24	19.43	1.95

Components of the high-abundant protein fraction of human plasma are marked by bold font.
[a] Immunoglobulin class of protein fraction in plasma.
[b] Common circulating plasma proteins.
[c] Plasma transport and binding proteins.
[d] Protease inhibitors.
[e] Coagulation and complement factors.

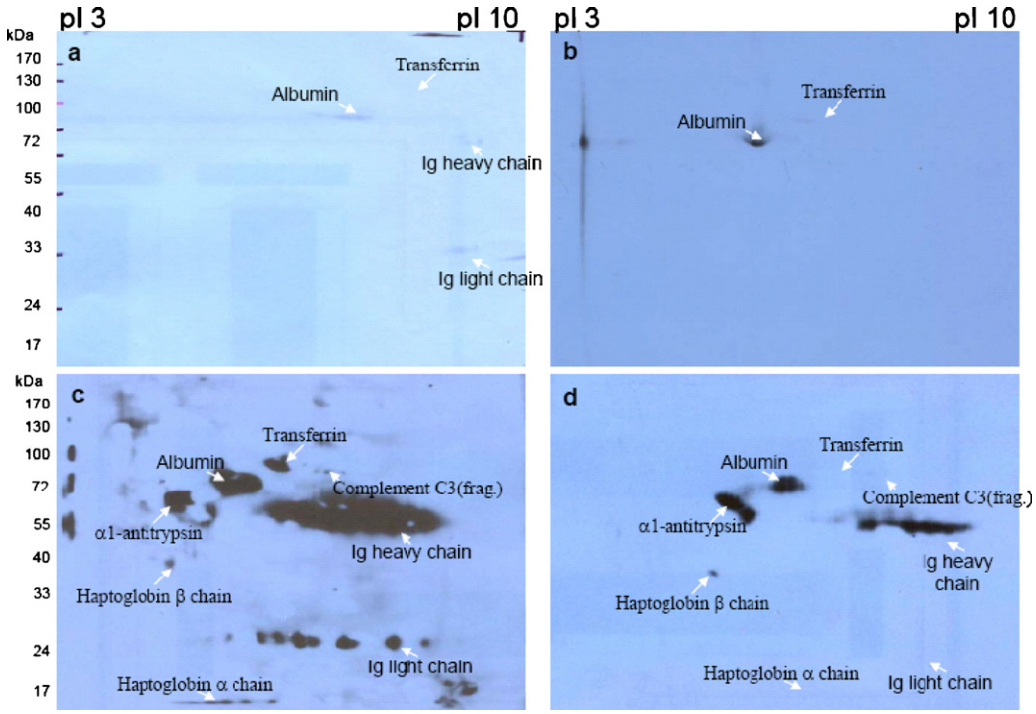

Fig. 3. Immunostaining with an anti-DNP antibody of proteins from the healthy controls (a), I/R reference patients (b), amputation Group 1 (c) and salvaged Group 2 (d) separated by 2-DE and derivatized by DNP. The arrows indicate the carbonylated proteins. We repeated the same experiment three times and the figure represents the results of a typical experiment.

Group 2. Quantitative differences were evaluated using Gene Tools (Syngene^{TM}) software and are shown in Fig. 4B. These data imply that the etiology of secondary amputation could not be a result of severe ischemia/reperfusion related injury but may be due to other factors such as shock and high level of leucocytes described by increased value of WBC.

4. Discussion

Lower limb mutilating injuries are often plagued with complications after limb revascularization. If a jeopardized limb requires secondary amputation, the timing of this decision is critical to prevent wound sepsis from progressing to systemic sepsis. Several scoring systems, such as MESS, are ineffective or misleading indicators for deciding whether a limb should be salvaged or amputated. Clinically, it is difficult to differentiate between an infected and antigen-independent etiology in a subject with symptoms of wound sepsis and extensive muscle necrosis, and traditional laboratory studies are unlikely to provide useful guidance [30,31]. Therefore, we have used proteome methods and cytokine profiles to identify protein alternations and their oxidative state, and these may be applied as useful indicators for limb salvage or secondary amputation.

According to the 2-DE results presented in this work, several target proteins with downgraded modifications in amputees' plasma were identified and defined as acute-phase proteins. A description of the proteins identified here may be crucial in understanding secondary amputation pathogenesis. For instance, the levels of α_1-antitrypsin (α_1 proteinase inhibitors), which serves a protective role against enzymatic digestion during inflammation, were obviously decreased in plasma from the studied subjects in the amputation group. In our previous study upon animal models, visceral organ injury correlated with down-regulated expression of α_1-antitrypsin [32]. Giannoudis et al. [33] reported significantly raised levels of plasma neutrophil elastase–α_1 proteinase inhibitor complex in the plasma of blunt trauma patients with greater degrees of injury and they were susceptible to post-traumatic sepsis development. It was reported earlier that targeted delivery of the α_1-antitrypsin based fusion proteins offered an increased antiproteinase effect on *P. aeruginosa* infected human epithelium cells compared to the native α_1-antitrypsin alone [34]. Therefore, we suggest that α_1-antitrypsin may be considered as a more sensitive marker than C-reactive protein (CRP) for inflammation in the amputee subjects.

Haptoglobin (Hp) is an acute-phase protein induced by infection, tissue injury and malignancy. Hp is the α_2-sialoglycoprotein with hemoglobin binding capacity and consists of two different polypeptides (α- and β-chain) derived from a single gene encoding the Hp precursor. Both the Hpα and Hpβ sub-units were also found in the present study to be decreased in the plasma of patients requiring secondary amputation. Our previous proteomic analysis revealed that Hp down-regulation could be observed in the plasma of human subjects with gouty arthritis [15], but Hp up-regulation of the transcriptional expression level and intracellular localization was found in an animal model of liver transplantation [24]. It has been demonstrated that Hp can prevent hemoglobin-induced oxidative tissue damage after muscle ischemia-reperfusion injury or rhabdomolysis [35]. The potential of recombinant Hp in the onset and progression of oxidative stress related stroke has been suggested recently [36]. Hp is an extremely potent antioxidant and directly protects lipoproteins from copper(II) ion-induced oxidation [37]. In addition, apolipoprotein A-1 (ApoA1), a major component of HDL, binds Hp to prevent hydroxyl radical production [38]. However, the principal biological function of Hp is the capture of free hemoglobin in plasma to allow hepatic recycling of heme iron and to prevent kidney damage during intravascular hemolysis. Down-regulation of plasma Hp might be associated with the release of heme from hemoglobin. Even slight systemic intravascular hemolysis made lead to reduced plasma Hp expression [36]. The risk of hemolysis was therefore heightened in the secondary amputation group of patients in our studies.

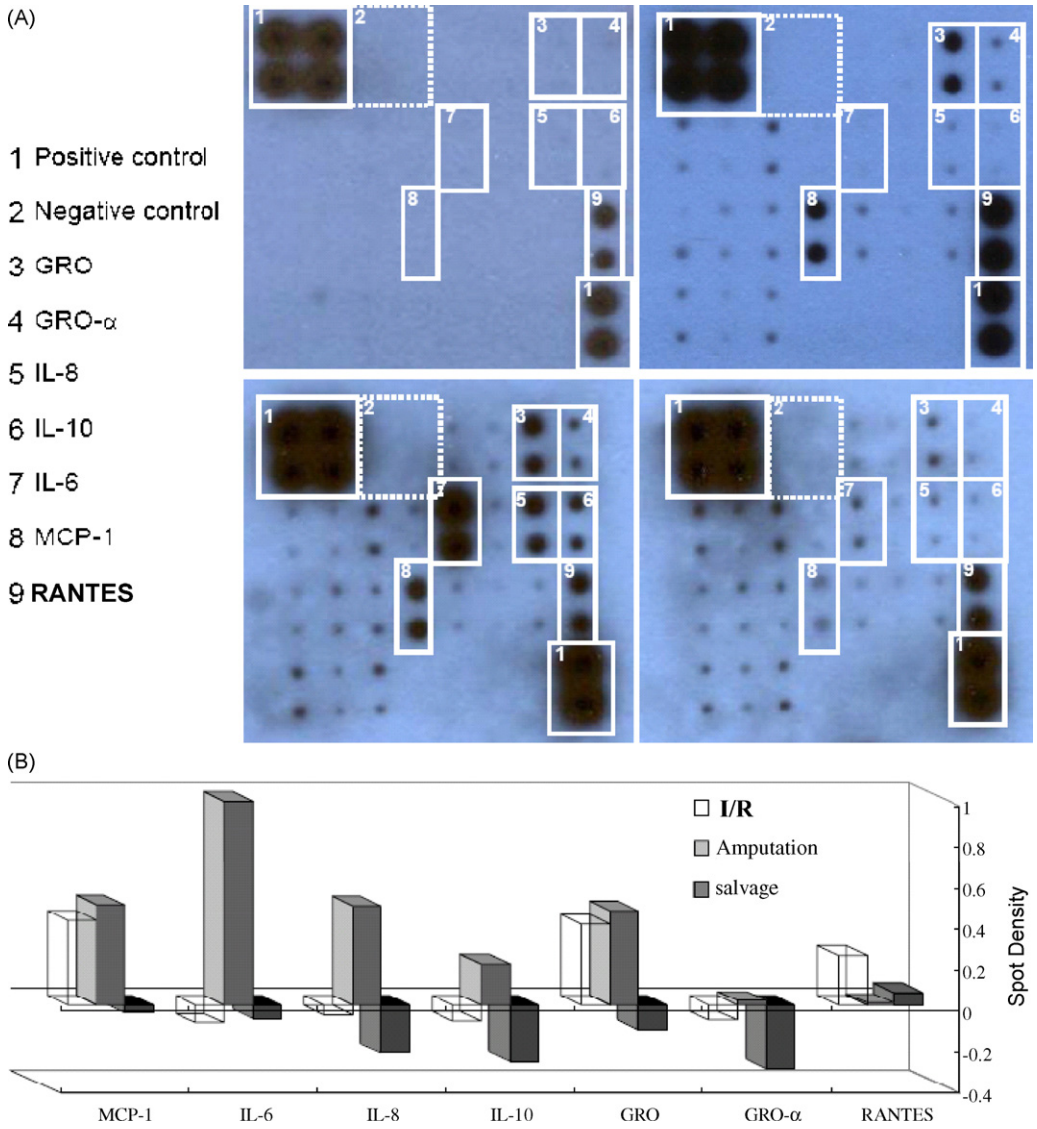

Fig. 4. Cytokine levels were assessed by protein array. (A) Cytokine array of healthy controls (a), I/R reference patients (b), amputation Group 1 (c) and salvaged Group 2 (d) were scanned, and internal positive controls, indicated by the square 1, were compared among array exposures. The means were found to be statistically similar before cross-comparison of sample cytokine levels. Square numbering: 1—positive control; 2—negative control; 3—GRO; 4—GRO-α; 5—IL-8; 6—IL-10; 7—IL-6; 8—MCP-1; 9—RANTES. (B) The intensity of the chemiluminescent signal for each spot was quantificated by GeneTools software. Cytokine/chemokine levels were normalized with respect to positive controls on the array membrane. The quantitative results indicating the different values compared with the control samples were demonstrated as a bar chart.

Transferrin in the form of apotransferrin, an endogenous iron-binding glycoprotein, and ceruloplasmin are similarly involved in the sequestration, transport, and distribution of free circulating, non-protein-bound copper(II) and iron(III) ions to limit free hydroxyl radical-mediated endothelial cell injury at neutral pH [39]. Apotransferrin, in contrast to holotransferrin as the iron-saturated form of transferrin, also inhibits the influx of neutrophils, which mediate ischemia-reperfusion injury of post-transplant organs [40]. Thus, one can suggest that the amputee subjects in Group 1, who indicated low levels of transferrin, would be more susceptible to oxidative stress, inflammation, and loss of visceral organ function.

Depletion of antioxidant levels is frequently reported in certain disease states, such as inflammation or I/R injury, and this oxidant/antioxidant imbalance results in oxidative damage to various tissues [41]. The use of protein carbonyl groups as biomarkers of oxidative stress has some advantages, such as their relatively early formation and stability [10,42]. The ability to identify specific proteins that are most susceptible to oxidative modifications is crucial in the development of methods for early diagnosis, the assessment of new potential therapies, and understanding of the overall disease process. A greater level and number of these protein targets of carbonylation were observed in the amputation group of subjects than in the salvaged group, the I/R group and the healthy controls. This finding may reflect a different pathogenic mechanism associated with amputation with respect to I/R injury. Moreover, the BUN value was significantly different between amputation Group 1 and salvaged Group 2, indicating that the kidney function of patients is critical to the outcome. The different sensitivity of proteins to carbonylation not only depends on the quantity of proteins in the plasma, but also on their susceptibility to oxidation determined by metal-binding sites and specific structures [43]. Proteins such as transferrin and albumin contain iron(III) and copper(II) ion binding sites, respectively. A marked reduction in the expression of transferrin, α_1-antitrypsin and Hpβ sub-units might result from overwhelming degradation of oxidation-modified proteins, mainly through the ubiquitin proteasome pathway, and may increase the risk of cell oxidation [44]. Comparably, there is increased

carbonylation in the plasma albumin, transferrin, and immunoglobulins in subjects belonging to amputation Group 1 (see Table 3). Albumin and immunoglobulin were easily oxidized in amputation Group 1, probably due to their numerous cysteine residues and the high abundance (ca. 85–90%) in plasma [45].

In addition, evidence was observed here that the recruitment of different cytokines/chemokines may reflect some important events in response to severe ROS mediated injury. Our data indicated that GRO, GRO-α, and IL-8, which are potent chemo-attractants for neutrophils, were significantly increased in subjects from amputation Group 1. A complex network of these cytokines, chemokines and other coagulation factors might be involved in phagocytic cell priming during the induction of systemic inflammation and acute sepsis [46]. Cytokine profiles between I/R injury caused by cardiovascular disease and amputated patients were quite different. High levels of inflammatory markers IL-6, IL-8, MCP-1, and IL-10 were observed in our study in the amputation group of subjects as compared with the non-specific healthy control subjects. In marked contrast, with the exception of MCP-1, no increase in levels of IL-6, IL-8, and IL-10 was found in the I/R control group of subjects, suggesting the etiology of secondary amputation might be multi-factorial rather than just due to I/R injury. These data might also highlight the role of MCP-1 in the mediation of local tissue damage.

The α_2-macroglobulin is not only a proteinase inhibitor, but also a specific cytokine carrier that binds pro- and anti-inflammatory cytokines implicated in acute inflammation. Oxidized α_2-macroglobulin binds with greater affinity to acute-phase cytokines such as IL-2 and IL-6, playing an anti-inflammatory role [47]. The α_2-macroglobulin is highly expressed in a number of inflammation diseases such as nephritic syndrome, rheumatoid arthritis and periodontitis. According to our data described here, the α_2-macroglobulin was presented in high concentration in the plasma of amputee subjects; however, we did not find significant levels of carbonylation of the α_2-macroglobulin in these patients as compared to healthy controls. These interesting results implied significantly increased levels of IL-6 in the plasma of amputated patients, highlighting the potential importance of the interaction between oxidized proteins and cytokines [48]. The carboxyl terminal fragment of α_1-antitrypsin was found to bind to both LDL and CD36 scavenger receptors, which are involved in the selective production of pro-inflammatory molecules and activation of the respiratory burst in human monocytes [49]. Monocytes stimulated with oxidized α_1-antitrypsin induced significant elevation in the MCP-1, IL-6, and TNF-α expression and increased the activity of NADPH oxidase, the primary source of production of superoxide in vascular cells. The IL-6, along with IL-10 and TNF-α, induces the synthesis and expression of intercellular adhesion molecule-1 (ICAM-1), which contributes to the adhesion of neutrophils to endothelial and skeletal muscle cells, causing acute inflammation [50,51]. These findings were in line with our hypothesis that the induction of inflammatory factors may result from the oxidation of specific proteins.

5. Concluding remarks

The most common pandemic of the twenty-first century, second only to HIV and AIDS, is road traffic accident (RTA) trauma, since this is the major cause for death and disablement for people below 44 years of age in the worldwide population. Every year, more than 1.2 million people die in RTAs around the world. In addition, more than 10 million people worldwide are crippled, injured, or sustain permanent disabilities each year (i.e. 1000 people every hour) as a result of RTAs [52–54]. As measured in disability adjusted life years, it is estimated that by 2020, road traffic crashes and violence will move from ninth to third place in the world disease burden ranking [53,54]. In other forecasts, the global number of road deaths will rise by 65% between the years 2000 and 2020 [53,54]. A traumatic amputation is the loss of a body part due to an accident, including an RTA, and in many developed countries this type of amputation is the second leading cause of limb loss after vascular disease [55,56]. However, for rationalization, comparison and establishing the severity of RTA injuries, the dominant forms of trauma evaluation systems are based on disintegration of the anatomic structure of organs and tissues, and alternative trauma systems based on pathophysiological values are poorly documented and useless [55,56]. Infection is another major challenge for trauma surgeons, as this is responsible for most secondary amputations and deaths in trauma patients who survive longer than 48 h after trauma [57,58]. The level of such infection-related amputations and deaths could be reduced by more than 50% if the diagnosis were not delayed and if more advanced analytical protocols were used [57]. Thus, there is still an urgent need to provide more effective and optimal trauma care management, especially in the initial assessment procedures at the emergency unit, including establishing more efficient, standardized and reliable clinical diagnosis tools (including optimized plasma proteomic monitoring procedures) in the case of variable road traffic injuries [58], since as many as three-fourths of RTA deaths and disablement cases could be prevented after the elimination of possible undiagnosed injuries and increased treatment completion of such RTA victims [57,58]. The question of how far traumatic and secondary amputations provoked by RTA injuries could be prevented by the use of early diagnostic tools is still a challenge for the trauma care specialist and the modern public health system [58].

Taking the proteomic results described here together, we suggest that differences in protein profiles, their oxidation levels, and cytokine expression in plasma might provide a new insight into pathophysiological events in post revascularization surgery in human subjects. In particular, one can suggest that a combination of proteomic and cytokine results offers more reliable clinical information than CRP and WBC alone for predicting the secondary amputation need in patients with limb salvage surgery following road traffic accidents. Apart from the fact that proteomic analysis is still a time and labor-consuming procedure at the present time, we suggest that further, more systematic and internationally coordinated efforts to determine more detailed plasma proteomic and systemic/local inflammatory response data from a larger cohort of secondary amputation and salvage subjects should be performed to modify and update existing clinical trauma care protocols, management schemes, and training practices to enable improved education and future development of multidisciplinary clinical trauma teams.

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