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SPB and RAGE increases in the plasma during cardiopulmonary by-pass: a pilot study.

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ABSTRACT

Surfactant derived protein B (SPB) and plasma receptor for advanced glycation end products (RAGE) have been proposed as markers of lung injury. The former is produced specifically by pneumocytes while RAGE production is present in several body tissues. Cardiopulmonary by-pass (CPB) generates a transient lung injury. We measured SPB and RAGE in plasma before surgery, after CPB, as well as 24h and 48h later.

We analyzed plasma samples of 20 subjects scheduled for elective CABG. We performed a quantitative analysis of plasma levels of RAGE and SPB mature form (8 kDa) by enzyme-linked immunosorbent assays and a semiquantitative analysis of SPB immature form (~40 kDa) by Western blotting.

Surgery procedures were uneventful. After CPB RAGE increased from 633 (median) pg/ml (539, 75^{th} - 25^{th} interquartile difference) to 1362 (557)$^{\$}$, while mature SPB increased from 5587 ng/ml (3089) to 20307 (19873)$^{\$}$, $^{\$}$ = $p<0.01$. RAGE and mature SPB returned to normal values within 48h. This behaviour was confirmed when RAGE and SPB were normalized for protein content. Parallel changes were observed for immature SPB.

Plasma RAGE and SPBs are sensible and rapid markers of lung distress.

Abstract words count: 185

Key words: gas diffusion, heart failure, lung injury, surfactant.

INTRODUCTION

Pulmonary surfactant is a membrane-based lipid-protein complex with surface-active characteristics [1, 2]. The protein component, accounting for ~10% of the total pulmonary surfactant, is synthesized only by type II pneumocytes of the alveolar epithelium. Surfactant proteins are responsible for several innate defense mechanisms and have a key role in stabilizing the surfactant itself, contributing to maintain normal pulmonary biophysics [3-5]. The surfactant protein type B (SPB) is one of the four surfactant proteins and its specific function is to stabilize the alveolar surfactant [6, 7]. SPB is produced in the alveolar cell as an immature ~40 kDa form which undergoes, inside the type II pneumocytes, complex proteolytic processes which lead, through intermediate SPB precursors weighing ~24 and ~17 kDa, to the ~8 kDa SPB mature and active form [8, 9]. The latter is secreted into the alveolar space [9]. SPB has a relevant gradient across the alveolar-capillary membrane so that, under physiological conditions, only a low concentration of SPB is found in the blood. *Viceversa,* in case of alveolar-capillary membrane damage a higher level of SPB is detected in the blood stream. Indeed, a high level of plasma SPB, either the mature or the immature forms, has been reported in acute pulmonary edema [10], Acute Respiratory Distress Syndrome [11, 12] and chronic heart failure (HF) [13, 14].

Lung function abnormalities are part of the chronic HF syndrome, being both lung mechanics and gas exchange impaired [15-17]. Recently, we and others observed increased circulating plasma SPB values in patients with chronic HF [13, 14]. We specifically showed a correlation between SPB and lung diffusion abnormalities, suggesting this protein as a possible biological marker of alveolar-capillary barrier damage [13]. Moreover, other researchers hypothesized that an acute increase in pulmonary vascular pressure, as that consequent to exercise-induced myocardial dysfunction, may result in an augmented SPB leakage from the alveoli into the circulation due to integrity loss of the alveolar-capillary barrier to protein [10, 14, 18]. Notably, in chronic HF patients, lung diffusion capacity is reduced after exercise [19].

Plasma receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily that acts as a progression factor amplifying the immune and inflammatory response in several pathophysiological conditions including lung disease [20]. During lung injury RAGE may be secreted in the alveolar space and in the blood [21] and, albeit RAGE may be secreted by several tissues, it has been proposed as a strong prognostic marker of lung disease [22].

In patients undergoing cardiac surgery, cardiopulmonary by-pass (CPB) may generate lung dysfunction which is an important factor of morbidity and mortality [23-25]. It has been suggested that neutrophil activation during extracorporeal circulation and during lung reperfusion leads to a significant release of inflammatory mediators [24-26] with a consequent increase in pulmonary vascular permeability and endothelial cells damage [27, 28], both possibly reflected in an increase in plasma SPB and RAGE levels. Furthermore, the required mechanical ventilation, although time-limited, might represent *per se* a cause of acute lung injury [12]. We thought to use CPB and mechanical ventilation, needed during coronary surgery (CABG), as a tool to investigate the correlation between acute lung distress, RAGE and SPB into the blood stream. To our knowledge, no attempts have been made to evaluate the possible relationship between plasma RAGE and SPB levels and the CPB-induced lung distress. Indeed SPB, being uniquely produced in the type II pneumocytes might become a useful tool to predict post-surgical respiratory outcome or, more in general, SPB may become an index of lung injury.

This pilot study was, therefore, designed to compare the behavior of circulating plasma SPB levels with RAGE in patients undergoing CPB and mechanical ventilation during CABG. We measured SPB both as large precursor of ~40 kDa, the heaviest of the so-called immature forms, and as mature and active form weighing ~8 kDa. We did so to be able to compare, as much as possible, our results with previous reported data. Moreover we measured SPB mature (~8kDA) form to have a quantitative SPB measurement and SPB immature form (~40kDA), which, at present, can be measured only semiquantitatively by Western blotting analysis, to evaluate the

possibility that lung abnormalities consist only in an exaggerated production of mature SPB with an abnormal SPB absorption. Indeed, presence in the blood of the immature SPB, which physiologically is only located inside alveolar type II cells, suggests alveolar cell damage.

METHODS

- Study Population

Twenty subjects scheduled for elective CABG procedure were consecutively enrolled in the study. Study inclusion criteria were stable clinical conditions for at least 2 months, absence of clinical history and/or documentation of HF, pulmonary embolism or primary valvular heart disease, pericardial disease, chronic bronchitis, primitive or occupational lung disease, anemia (haemoglobin < 11 g/dL), renal insufficiency (serum creatinine > 2.0 mg/dL), significant peripheral vascular disease. Perioperatory risk was assessed by EuroSCORE [29]. All patients underwent standard echocardiographic pre-surgical evaluation. During the surgical procedure all patients went through routine hemodynamic, ventilatory and blood gases surveillance. Red blood cells count, serum creatinine, and lactate dehydrogenase (LDH) were also measured before surgery and 24h and 48h later. Blood samples for SPBs determination were taken before surgery, after CPB, as well as 24h and 48h later.

The investigation was approved by the local ethics committee and subjects signed a written informed consent before participating in the study. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

- Specimen Handling and Assays.

Fresh blood (5 mL) was drawn into Vacutainer tubes containing citrate 0.129 mol/L as anticoagulant. Plasma was immediately prepared by means of centrifugation at 1.500 x *g* for 10 minutes at 4°C, divided into aliquots, and frozen at -80°C until assayed.

The analysis of the immature form of SPB (~40 kDa) was performed by Western blotting on plasma samples, as previously described [13]. Briefly, in order to precisely resolve low molecular weight proteins, equal amounts of plasma proteins (50 μg) were separated by one dimensional SDS-PAGE on 15% polyacrylamide gels using a Tris-Tricine buffer system in non reducing conditions [30]. The protein concentration was evaluated by Bradford method as previously described [31]. Gels were electrophoretically transferred to nitrocellulose at 60 V for 2h. Immunoblotting on transferred samples was performed as follow: blocking in 5% w/v non-fat milk in Tris-buffered saline (100 mmol/L TrisHCl, pH 7.5, 150 mmol/L NaCl) containing 0.1% Tween 20 (TBS-T) for 1h at room temperature; overnight incubation at 4°C with primary antibody against SPB (rabbit anti human SPB H300, Santa Cruz Biotecnology, Santa Cruz, CA 95060-5706, United States) diluted at 1:200 in 5% w/v non-fat milk in TBS-T; incubation with secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad, Milan, Italy) at 1:1000 for 1h. Bands were visualized by enhanced chemiluminescence using the ECL kit (GE Heathcare, Milan, Italy) and acquired by a densitometer (GS800 Bio-Rad, Milan, Italy). Bands at 40 kDa detected by ECL were quantified by densitometry of exposed film using image analysis software QuantityOne (version 4.5.2) from Bio-Rad (Milan, Italy). Membranes following transfer were stained with MemCode™ reversible protein stain (Pierce Biotechnology, Cramlington, Northumberland, UK) according to manufacturer's instructions to ensure equivalent loading of protein. For each subject data are reported as ratio of bands volume at after CPB, 24h after, and 48h after versus the volume of the sample before surgery after local background subtraction and are expressed as arbitrary units (au). Inter-assay coefficient of variation was 12.1 ± 2.9%. The quantitative analysis of the levels of the mature form (8 kDa) of SPB was performed by an enzyme-linked immunosorbent assay purchased from Uscn Life Science Inc. (Wuhan, China). Briefly, the microtiter plate provided in this kit is pre-coated with an antibody specific to the mature SPB. Standards or samples are added to the microtiter plate wells followed by the incubation with a biotin-conjugated polyclonal antibody specific for SPB. Avidin conjugated to Horseradish Peroxidase (HRP) is then added to each

microplate well. Next, a tetramethylbenzide substrate solution is added to each well and the enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution. The absorbance (O.D.) is measured spectrophotometrically at a wavelength of 450 nm on a Microplate Reader (Mithras LB 940, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The concentration of SPB in the samples is then determined by comparing the O.D. of the samples to the standard curve and expressed as ng SPB/ml or ng SP-B/mg protein. Limit of sensitivity was 1.95 ng/mL. Inter-assay coefficient of variation was 11.6 ± 2.1%. Intra-assay coefficient of variation was 7.9 ± 1.5%.

Plasma RAGE levels were determined using a commercially available enzyme-linked immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Measurements were performed in duplicate and the results were averaged. The intra-assay and interassay coefficients of variation were <6 and <8%, respectively.

- Perioperative management

Patients received routine cardiac medication except for antiplatelets drugs until day of surgery. Premedication consisted of morphine (0.1 mg/kg, i.m.) and atropine (0.05 mg/kg, i.m.), 1h before surgery. A prophylactic intravenous antibiotic (cefazoline, 2 g/8h during the first 24 hours) was given after the positioning of a venous access. Anesthesia was induced with thiopental (4-6 mg/kg), sufentanil (1 μcg/kg), and succinilcholine (1 mg/kg), and maintained with inhaled sevorane and incremental doses of pancuronium to facilitate muscle relaxation. Patients were monitored with 5-lead electrocardiogram, central venous catheter, radial artery line, Foley catheter, and naso-pharingeal and rectal temperature probes. Traditional surgical procedures were used to position the CPB circuits. Non pulsatile CPB was conducted in moderate hypothermia (34°C), using roller pump with a membrane oxygenator and a micron arterial filter. The circuit was primed with 1 L of Normosol R®, 500 mL of Glucose 5%, 100 mL of 18% sodium bicarbonate solution, and 100 ml bolus of mannitol 18% was given just before the opening of aortic cross clamp. The flow rate, always > 2.4 L/min/m^2, was titrated to ensure a mean arterial pressure between 55 and 80 mmHg

and noreprinephine was injected when necessary. If needed additional Ringer's lactate solution was added into the venous reservoir. Hemodilution up to a minimum hemoglobin concentration of 7 g/dL was allowed. Myocardial protection was achieved by mean of intermittent anterograde and retrograde cold blood cardioplegia. After weaning from CPB, the entire content of the circuit was collected and slowly returned to the patient.

During surgery patients were ventilated with intermittent positive pressure ventilation at PEEP = 0 cm H_2O with 8 ml/kg of tidal volume, a respiratory frequency of 13 breath/m and an inspiratory/expiratory ratio of 1:2. The fraction of inspiratory oxygen (FiO_2) started with 1 and was thereafter reduced to the lowest value which maintains arterial oxygen saturation (SaO_2) higher than 95%. During surgery tidal volume, respiratory frequency and FiO_2 were adjusted to maintain a PCO_2 around 35 mmHg. After weaning from CPB, mechanical ventilation was resumed with the same parameters but with 5 cmH_2O of PEEP. After surgery, patients were transferred to the ICU, still sedated and intubated; propofol was used for sedation until complete rewarming. Patients were ventilated in intermittent positive pressure ventilation with tidal volume, respiratory frequency and FiO_2 adjusted to maintain normocapnia and arterial SaO_2 greater > 95% till the beginning of weaning protocol. Weaning started when the following conditions were reached: hemodynamic stability (low doses of inotropic drugs allowed), no major recurrent cardiac arrhythmia, no surgical bleeding, patients awaked and fully rewarmed. Patients were weaned from mechanical ventilation through a cycle of pressure support ventilation. In intensive care, following the on duty physician decision, temporarily intravenous vasodilating agents (nitroglycerine) in 3 cases, vasocostrictors (noradrenaline) in 2 cases and positive inotropic agents in 5 cases (dopamine/dobutamine) were used. Hemodinamic and gas exchange parameters recorded during surgery and in the 2 following days are reported in table 1.

- Statistical Analysis

Normally distributed variables are expressed as means ± SD and the differences between groups were analysed by ANOVA followed by paired t-tests where necessary. Categorical variables

were analyzed with the χ^2 test. Data with skewed distribution are given as median and interquartile range difference (75^{th} - 25^{th}). RAGE measurements, albeit normally distributed, are given as median and interquartile range difference (75^{th} - 25^{th}) to provide a more immediate comparison with SPB values. Wilcoxon test was used to compare the not normally distributed data. Moreover, because plasma SPB values showed a non linear distribution, Spearman correlation was used to disclose possible correlations between these proteins and clinical, echocardiographic, and laboratory data. P values of < 0.05 were considered statistically significant. All tests were 2-sided. All data were evaluated with the database SPSS-PC+ (SPSS-PC+ Inc, Chicago, Illinois).

RESULTS

General characteristics of the study population, including CPB and mechanical ventilation time, are reported in table 2. Seven patients had diabetes mellitus and 10 systemic hypertension. A previous cardiac surgery had been done in 4 cases. Treatment included ACE inhibitors in 10 cases, diuretics in 7 cases, beta-blockers in 14 cases, calcium channel blockers in 3 cases and nitrates in 7 cases. CPB lengthened always < 3 hours (range 71 to 169 minutes). Patients were free from HF as showed by history and average normal left ventricular ejection fraction; only 2 subjects had a left ventricular ejection fraction between 35 and 40%. EuroSCORE was also low (3.5 ± 1.9). In no case a further surgical procedure was needed for bleeding or other reason. CPB was associated to a significant haemoglobin reduction due to blood loss and/or blood dilution (table 3). Twenty four hours after surgery white blood cells count and LDH were increased (table 3).

Mature SPB (~8 kDa) plasma concentration before surgery, immediately after CPB, 24h and 48h later is reported in table 3 and figure 1 both as absolute values (ng/mL, upper panel) and as normalized values corrected for total protein concentration (ng/mg total proteins, lower panel). The latter was needed because during CPB total plasma protein concentration dramatically decreased (table 3). A 4 to 5-fold increase of mature SPB was observed immediately after CPB which was almost recovered in 24h and totally normalized within 48h.

The immature SPB form (~40 kDa) cannot be precisely measured because Western blotting allows only a semiquantitative analysis and, moreover, only arbitrary units can be used. The ratios from pre-surgery increased similarly to mature SPB (table 3 and figure 2).

RAGE plasma concentration before surgery, immediately after CPB, 24h and 48h later is reported in table 3 and figure 3 both as absolute values (pg/mL, upper panel) and as normalized values corrected for total protein concentration (pg/mg, lower panel).

No correlation was found between CPB duration, PaO_2/FiO_2 ratio and absolute LDH values and SPB and RAGE absolute values or changes. A weak linear correlation was found between highest LDH increase and the highest increase of mature SPB normalized for dilution ($r= 0.436$, $p< 0.05$). This correlation did not reach statistical significance when absolute mature SPB values were considered ($r= 0.377$, $p= 0.06$) and was totally absent if immature SPB was considered. No correlation was found between RAGE and SPB mature form before surgery. Differently a strong correlation ($r = 0.66$, $p < 0.001$ and $r = 0.75$, $p < 0.000$) was found between SPB mature form and RAGE increases from before surgery to immediately after CPB, both as absolute and protein normalized values, respectively.

DISCUSSION

This study shows that CPB is associated to an acute 4 to 5-folds increase of the mature SPB, a 1.5 fold increase of SPB immature form and a 2 fold increase of RAGE. At 24h RAGE and both SPB forms are significantly reduced and return to normal values within 48h.

RAGE has been shown to be a marker of lung injury and specifically of type I pneumocytes injury [32]. Moreover RAGE has been recently proposed as a plasma marker of poor prognosis in patients ventilated with high tidal volume [22] or post lung transplantation [33]. However RAGE is expressed on multiple cell types, from vascular to inflammatory cells, such as smooth muscle cells, monocytes/macrophages, T lymphocytes, dendritic cells, glomerular epithelial cells or podocytes, cardiomyocytes, and neurons (central and peripheral nervous systems), as examples [34, 35]. Thus,

it is not surprising that the biology of RAGE impacts several biological and pathological settings. Differently SPB is selectively produced by the pneumocites type II.

Previously immature and mature forms of SPB have been measured, usually in fluids obtained by broncho-alveolar lavage, by different methods and have been reported with different units [11, 12, 14, 18, 36]. Very few data are available for plasma levels of immature SPB [14, 18]. We analyzed immature SPB by Western blotting analysis which allows only a semi-quantitative evaluation. Therefore, we cannot compare our results with those of previous studies. Mature SPB form has been measured by ELISA which allows a quantitative analysis. Our results are comparable with those reported by Doyle et al [12] and De Pasquale et al [18].

Our population consisted of patients undergoing CABG in the absence of relevant comorbidities and, particularly, of known lung disease. Therefore, we cannot say if the SPB or RAGE changes would have been the same in the presence of previous lung diseases. We utilized CPB and mechanical ventilation, both needed because of CABG, as a tool to induce some acute lung distress if not injury. It is recognized, however, that previous studies have demonstrated, in children undergoing CPB, an increase in endotracheal SPB [36], while others did not [37]. Indeed, clinically relevant lung impairment is rarely observed after CABG if surgery is performed in subjects free of relevant lung and cardiac comorbidities as our patients were [23, 25]. We cannot say, however, if CPB, mechanical ventilation or their combination, was needed for the observed SPB changes to take place. We did not identify any correlation between CPB or mechanical ventilation duration and SPB changes. However, our population was small and we had no patient with prolonged surgical procedures or need of long lasting mechanical ventilation.

The parallel behavior of RAGE and SPB and the strong correlation between RAGE and SPB mature form changes are impressive and reinforce the concepts that SPB can be used as a plasma marker of lung injury. Indeed albeit RAGE has been suggested as marker of lung injury [21] its almost ubiquitary production made the association between elevated RAGE = lung injury potentially questionable. Differently SPB is produced only in the lung.

SPB has been proposed as a marker of lung damage by several groups evaluating patients with both lung and cardiac disease [10-14, 36, 38, 39]. De Pasquale et al [18] showed that an acute pulmonary hemodynamic derangement, which usually develops in patients with exercise-induced cardiac ischemia, induces a SPB increase; unfortunately, post exercise SPB recovery was not assessed. Our study is the first in which mature and immature SPB changes were separately evaluated after a lung insult. We showed a rapid SPB increase after CPB (means CPB duration < 2h) and a return to normal values almost in 24h. Therefore SPB can be seen as a rapid and sensible marker of lung distress. However, because the present is a pilot study done in subjects free of previous lung disease and we have had no complication during surgery, we have no way to relate SPB changes in relationship to severity of lung damage. Furthermore we and others [10-14, 36, 38, 39] have proposed SPB as a marker of lung injury and not as its cause, albeit the administration of exogenous surfactant has been proposed [40-42]. It should be noticed that we have no established biological marker in the blood for acute lung injury. Indeed, only LDH has been occasionally used for this purpose but it is highly aspecific and characterized by a slow kinetics [43, 44]. Regardless we observed a weak correlation between greatest LDH and SPB changes. The increase of both immature and mature SPB forms after CPB, as well as the correlation of LDH and SBP changes, suggest that the observed increase of SPB forms in the blood is due to type II pneumocytes cells damage and not to a pure SPB diffusion across the alveolar-capillary membrane. Indeed, the SPB immature form is usually intracellular and not found in the alveolar space. Both the mature and the immature form of SPB returned to normal values within 48 hours. However, by comparing the time behaviour of the two SPB forms we studied (figure 1 and 2), the immature SPB, which is a progenitor of the mature form, seems to remain high in the blood for a longer time than the mature form. It is possible, but totally unproved, that these differences are due to differences in clearance time.

Further studies are needed to confirm our observations in a larger population, to assess SPB changes after CABG in patients with previous lung and cardiac diseases, and to evaluate if SPB

changes shortly after the beginning of CPB can predict the post surgical clinical outcome of patients and, therefore, the need of post surgical aggressive cardiorespiratory assistance. Moreover, the relation of SPB changes during CABG with other surfactant derived proteins such as SPD, the factors modifying the production of surfactant by alveolar type 2 cells or the role of alveolar macrophages in the regulation of surfactant, all need to be evaluated. The present is the first study where mature SPB form was measured in the plasma. We showed that SPB forms in the plasma, as well as RAGE, all are sensible and rapid markers of lung distress whose clinical relevance needs further investigation.

FIGURE LEGEND

Figure 1: ELISA measurement of mature SPB isoform in the plasma before surgery, immediately after CPB, 24h, and 48h after CABG. **Upper panel,** data are expressed as ng/ml of SPB at different time points. **Lower panel,** data are expressed as ng/mg proteins after correction for total protein concentration. Data are median, 25^{th} and 75^{th} percentile range and minimum and maximal values. $^{\$}$ = $p < 0.01$ after CPB *vs* before surgery. * = $p < 0.01$ after 24h *vs* before surgery. □ = $p < 0.01$ after 24h *vs* after CPB. † = $p < 0.01$ after 48h *vs* after CPB. $^{\#}$ $p<0.01$ after 48h *vs* after 24h.

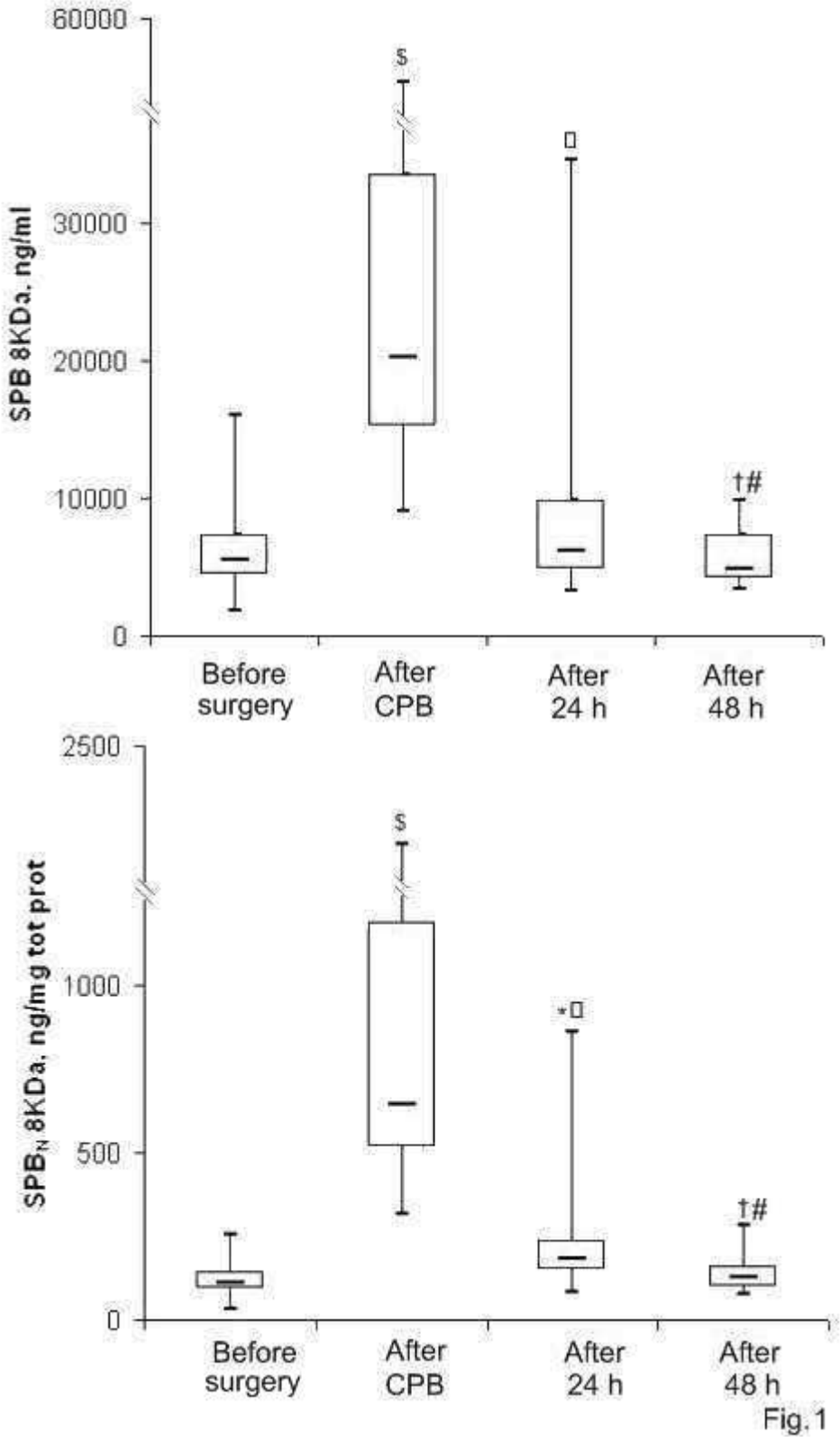


Figure 2: Immature SPB levels in the plasma before surgery, immediately after CPB, 24h, and 48h after CABG. **Upper panel**, representative Western blotting of immature SPB; **Lower panel,** densitometric analysis of immature SPB expressed as the ratio of the SPB level at each time point

over before surgery. Data are median, 25th and 75th percentile range, and minimum and maximal values. $ = p < 0.01 after CPB *vs* before surgery. * = p < 0.01 after 24h *vs* before surgery. † = p < 0.01 after 48h *vs* after CPB. # p<0.01 after 48h *vs* after 24h.

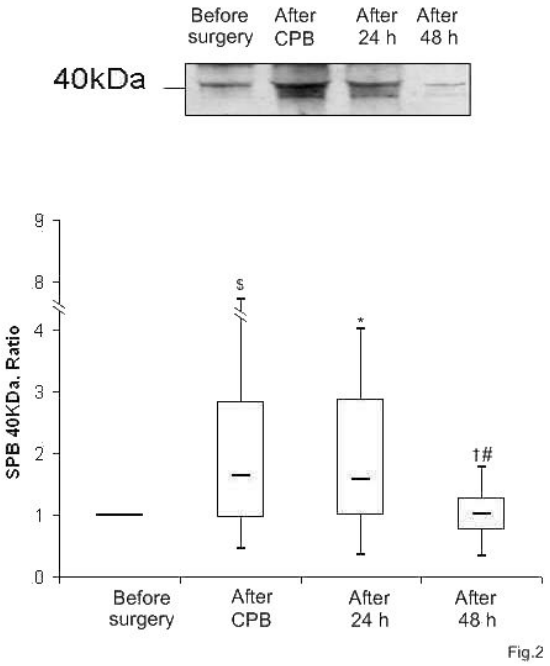
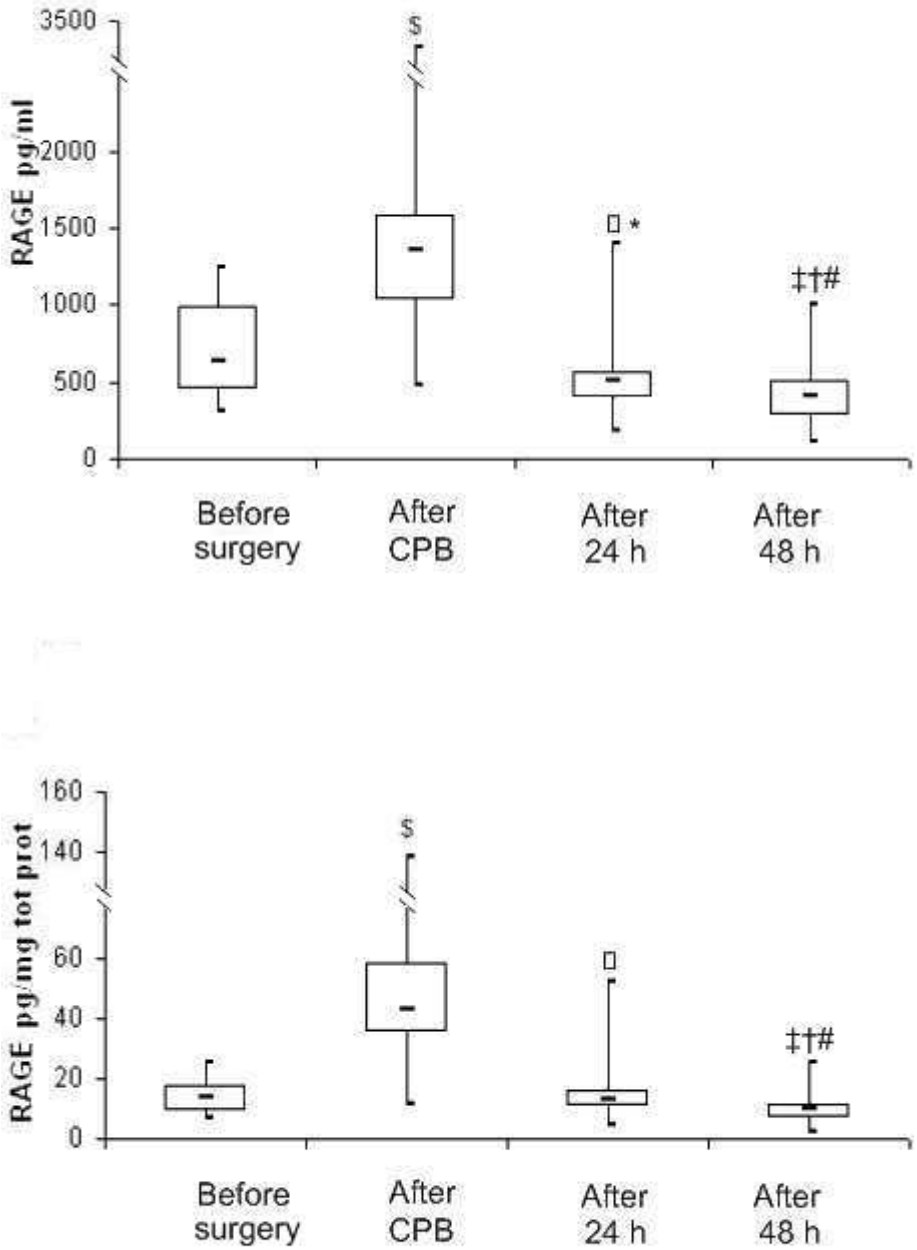


Figure 3: ELISA measurement of RAGE in the plasma before surgery, immediately after CPB, 24h, and 48h after CABG. **Upper panel,** data are expressed as ng/ml of RAGE at different time points. **Lower panel,** data are expressed as ng/mg proteins after correction for total protein concentration. Data are median, 25th and 75th percentile range and minimum and maximal values. $ = p < 0.01 after CPB *vs* before surgery. * = p < 0.01 after 24h *vs* before surgery. □ = p < 0.01 after 24h *vs* after CPB. ‡ p< 0.01 after 48h *vs* before surgery. † = p < 0.01 after 48h *vs* after CPB. # p<0.01 after 48h *vs* after 24h.


RAGE pg/ml
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Before surgery
After CPB
After 24 h
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Before surgery
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After 24 h
After 48 h

Fig 3

Table 1. Hemodynamic variables at SPB and RAGE measurements

Variables	before surgery	after CPB	after 24h	after 48h	**ANOVA**
HR b/min	77.9±10.9	80.7±12	87.6±14.7	83.9±15.6	NS
PAS mmHg	157±28	94±22$^{\$}$	119±23$^{*\square}$	117±18‡†	<0.001
PAD mmHg	77±9	62±16$^{\$}$	54±10*	55±9‡	<0.001
PaO_2 mmHg	-	242±26	92±6$^{\square}$	82±7†	<0.001
FiO_2	Room Air	1	Room Air	Room Air	

All data are expressed as mean±SD; CPB: cardiopulmonary by-pass, RAGE: plasma receptor for advanced glycation end products, HR: Heart Rate, PAS: Systolic Pressure, PAD: Diastolic Pressure; PaO_2: Alveolar Pressure of O_2; FiO_2: Inspired Fraction of O_2.

$^{\$}$ p<0.01 after CPB *vs* before surgery; * p< 0.01 after 24h *vs* before surgery; $^{\square}$ p< 0.01 after 24h *vs* after CPB; ‡ p< 0.01 after 48h *vs* before surgery; † p< 0.01 after 48h *vs* after CPB; $^{\#}$ p<0.01 after 48h *vs* after 24h.

Table 2. General characteristic of the study sample.

Variables	
Age, years	65 ± 9
Gender, male (%)	17 (85)
BMI, kg/m^2	27 ± 4
LVEF, %	52 ± 10
Smoke habit, a/p/n	4 / 7 / 9
Serum Creatinine, mg/dL	1.0 ± 0.3
CPB time, minutes	103 ± 28
Intubation time, minutes	582 ± 106

Data are expressed as mean ± SD or as (percentage). BMI: body mass index; LVEF: left ventricular ejection fraction; CPB: cardiopulmonary by-pass. a = actual smoker, p = previous smoker, n = never smoker.

Table 3. Laboratoristic, SPB and RAGE data modification in the study sample.

Variables	before surgery	after CPB	after 24h	after 48h	**ANOVA**
Hb, mg/dL	13.8 ± 1.6	-	9.8 ± 1.6*	9.5 ± 1.3‡	<0.001
Hct, %	39 ± 5	-	27 ± 5*	27 ± 4‡	<0.001
WBC, u*10^{9}	7.3 ± 1.4	-	10.9 ± 2.8*	11.9 ± 3.7‡	<0.001
Neutrophil, %	63.1 ± 8.4	-	81.5 ± 5.0*	79.3 ± 5.5$^{‡\ \#}$	<0.001
Total proteins, g/dL	5.27 ± 0.62	3.05 ± 0.59$^{\$}$	3.72 ± 0.55$^{*□}$	4.12 ± 0.7$^{‡†\#}$	<0.001
LDH, mg/dL	232 ± 130	-	364 ± 171*	347 ± 175‡	<0.001
SPB 40 kDa, ratio	1	1.63 (2.4)$^{\$}$	1.58 (2.2)*	1.01 (0.66) $^{†\#}$	
SPB 8 kDa, ng/mL	5587 (3089)	20307 (19873)$^{\$}$	6154 (5788)$^{□}$	4810 (3340) $^{†\#}$	
SPB$_N$ 8 kDa, ng/mg	112 (62)	644 (758)$^{\$}$	184 (103)$^{*□}$	125 (66) $^{†\#}$	
RAGE, pg/mL	633 (575)	1361 (216) $^{\$}$	508 (216) $^{*□}$	417 (247) $^{‡†\#}$	
RAGE$_N$, pg/mg	13.5 (9.2)	42.6 (24.4) $^{\$}$	13.0 (6.8) $^{□}$	10.2 (6.2) $^{‡†\#}$	

All data are expressed as mean±SD or as median and between brackets 75th - 25th interquartile difference. Hb: haemoglobin; Hct: haematocrit; WBC: white blood cell; LDH: lactate dehydrogenase; SPB: surfactant protein B; SPB$_N$: SPB normalized for total protein amount; RAGE: plasma receptor for advanced glycation end products; RAGE$_N$: RAGE normalized for total protein amount.

$^{\$}$ p<0.01 after CPB *vs* before surgery; * p< 0.01 after 24h *vs* before surgery; $^{□}$ p< 0.01 after 24h *vs* after CPB; ‡ p< 0.01 after 48h *vs* before surgery; † p< 0.01 after 48h *vs* after CPB; $^{\#}$ p<0.01 after 48h *vs* after 24h.

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