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### ► **To cite this version:**

Maria Klaude, Katarina Fredriksson, Inga Tjäder, Folke Hammarqvist, Bo Ahlman, et al.. Proteasome proteolytic activity in skeletal muscle is increased in patients with sepsis. *Clinical Science*, 2006, 112 (9), pp.499-506. 10.1042/CS20060265 . hal-00479345

**HAL Id: hal-00479345**

**<https://hal.science/hal-00479345>**

Submitted on 30 Apr 2010

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## **Proteasome proteolytic activity in skeletal muscle is increased in patients with sepsis**

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**Short title:** Proteasome activity in human skeletal muscle

**Key words:** Protein degradation, Muscle wasting, Sepsis, Multiple organ dysfunction

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## Abstract

Patients with sepsis in the intensive care unit (ICU) are characterised by skeletal muscle wasting. This leads to muscle dysfunction that also influences the respiratory capacity leading to prolonged mechanical ventilation. Catabolic conditions are associated with a general activation of the ubiquitin proteasome pathway in skeletal muscle. In the present study, the aim was to measure the proteasome proteolytic activity in both respiratory and leg muscles from ICU patients with sepsis and in addition to assess the variation of proteasome activity between individuals and between duplicate leg muscle biopsy specimens. As compared to a control group (n=10), patients with sepsis (n=10) had a 30 % ( $p<0.05$ ) and 45 % ( $p<0.05$ ) higher proteasome activity in the respiratory and leg muscle respectively. In a second experiment ICU patients with sepsis (n=17) showed a 55 % ( $p<0.01$ ) higher proteasome activity in the leg muscle as compared to a control group (n=10). The inter-individual scatter of proteasome activity was larger between the patients with sepsis than between the controls. We also observed a substantial intra-individual difference in activity between duplicate biopsies in several of the subjects. In conclusion, the proteolytic activity of the proteasome was higher in skeletal muscle from patients with sepsis and multiple organ failure as compared to healthy controls. It was shown for the first time that respiratory and leg muscles were affected similarly. Furthermore, the variation of proteasome activity between individuals was more pronounced in the ICU patients for both muscle types, while the intra-individual variation between biopsies was similar for ICU patients and controls.

## Introduction

Patients with septic shock in the intensive care unit are prone to develop muscle wasting, a condition that is mainly manifested in skeletal muscle. The net loss of muscle proteins could either depend on decreased protein synthesis or an increase in protein degradation or a combination of both. Despite the progressive loss of muscle proteins during critical illness, fractional protein synthesis rate in skeletal muscle is on average within the range of healthy subjects [1-3]. This indicates that muscle wasting in these patients is mainly caused by an increase in protein degradation.

The majority of intracellular proteins, including myofibrillar proteins in muscle, are degraded via the ubiquitin proteasome pathway [4]. Most proteins degraded by this pathway are first marked with a polyubiquitin chain in order to be degraded by the 26S proteasome. The 26S proteasome consists of two 19S regulatory domains and a central 20S protease core. The proteolytic activity comprises two chymotrypsin-like, two trypsin-like and two caspase-like active sites. The chymotrypsin-like activity is the rate-limiting step and initially cleaves the protein thereby stimulating the caspase-like activity [5]. Catabolic conditions have been associated with an increase in mRNA expression of several subunits of this pathway as well as an increase in the proteasome proteolytic activity. Increased levels of mRNA encoding ubiquitin and subunits of the 20S proteasome have been found in animal models for muscle wasting caused by fasting and denervation [6], sepsis [7], diabetes [8] and glucocorticoid treatment [9]. Also studies of patients with different catabolic diseases such as trauma [10-11], cancer [12-13] and sepsis [14] have revealed increased mRNA levels of ubiquitin and proteasome subunits. The proteolytic activity of the proteasome can be studied *in vitro* in muscle extracts incubated with fluorogenic peptide substrates. In animal experiments increased activities were found after induction of sepsis or burn injury [7,15]. Also in humans increased proteasome proteolytic activities were found in abdominal muscle from cancer patients [16] and by our group in leg muscle from patients with sepsis and multiple organ failure [17].

In septic shock with a prolonged critical illness, the depletion of skeletal muscle is of increasing importance for morbidity and mortality. The majority of patients with septic shock treated in the ICU suffer from respiratory dysfunction and need mechanical ventilation. Many of these patients have difficulties in being weaned from the ventilator, thus prolonging the ICU treatment and increasing the incidence of pneumonia [18]. The underlying causes of

weaning difficulties might be attributed to muscle weakness due to atrophy. Today there are no efficient prophylactics or treatment for the muscle depletion although a number of strategies have been suggested over the years. The understanding of the underlying biochemical and molecular mechanisms for muscle protein degradation in catabolic patients is still limited. To be able to better predict and evaluate the outcome of therapeutic efforts for critically ill patients, it is therefore necessary to develop experimental approaches that increase our understanding of how the proteolytic machinery is regulated in the cell.

The aim of the present study was to measure the proteasome proteolytic activity simultaneously in respiratory and leg muscles in patients with septic shock requiring mechanical ventilation in comparison to healthy controls. In earlier studies we observed a large scatter of both protein synthesis rates and proteasome activity in leg skeletal muscle in septic patients as compared to control subjects [1-3, 17]. So a second aim of the present study was to elucidate the variation of proteasome activity in between duplicate muscle biopsy specimens and in between the individual subjects.

## Materials and methods

*Patients:* Two different experiments were performed in this study, both consisting of one group of patients with septic shock admitted to the general intensive care unit at Karolinska University Hospital Huddinge and another group of metabolically healthy, age and sex matched control patients undergoing elective surgery. In experiment II the control subjects were recruited at Ersta Hospital Stockholm. The study protocols conformed to the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Ethical Committee of Karolinska Institutet, Stockholm, Sweden. All patients, or for the ICU patients close relatives, gave informed consent to participate in the studies after receiving both oral and a standardised written information approved by the Ethical Committee.

Experiment I included ICU patients (n=10) with septic shock at admittance requiring mechanical ventilation and control patients (n=10) undergoing elective surgery. Muscle biopsies were taken from leg muscle and external respiratory muscle. Pre-existing neuromuscular disease, COPD, long-term pre-ICU corticosteroid treatment or severe coagulopathies not enabling muscle biopsies were exclusion criteria. All ICU patients suffered from respiratory dysfunction and multiple organ failure as indicated by the SOFA scores (Sepsis-related Organ Failure Assessment) [19] at the day of study (Table 1). All patients had sepsis according to the Bone criteria [20]. All patients were sedated with propofol together with intermittent doses of analgesics. Mechanical ventilation was pressure support in 8 and pressure control in 2 patients. All patients were circulatory stable at the time of study and received low rate infusion of vasopressor drugs, if any. Three of the patients did not receive any glucocorticoid treatment, 4 of them received a substitution dose and 3 a therapeutic dose of short duration. Nutrition was given according to the routines of the unit with a target of 20-25 kcal/kg/24 h. Six patients received enteral nutrition, 3 received combined enteral and parenteral nutrition and one received parenteral nutrition only. Both enteral and parenteral nutrition contained glutamine. Three of the patients did not survive. Patient No 5 died after one week and patients No 4 and 8 two months after the study. The control group consisted of 3 women and 7 men with a median age of 67 years (range 45-87) undergoing elective surgery for hernia repair, ileostomy closure, recurrent diverticulitis, or colorectal resection. One control patient had a malignant disease without evident spread.

Experiment II included ICU patients with septic shock at admittance (n=17) and control patients (n=10) undergoing elective surgery. A muscle biopsy from vastus lateralis was taken

from both the left and right leg. Patients with severe liver failure or undergoing dialysis or with impaired coagulation, not enabling muscle biopsies, were excluded from the study. None of the patients had a long-term, pre-ICU systemic corticosteroid treatment. All patients had sepsis according to the Bone criteria [20]. Characteristics of the ICU patients are given in table 1. According to the SOFA scores all patients except one had multiple organ failure. All patients were sedated with propofol together with intermittent doses of analgesics and required ventilator treatment. At the time of study all patients were circulatory stabilised, although all but three patients required vaso-pressor and/or inotropic support. Intravenous short-term corticosteroid treatment was given to four patients and another six patients were given corticosteroid substitution therapy. Antibiotics were given to all patients and nine patients were treated with more than one antibiotic. Anti fungal therapy was given to four patients. At the time of study, three of the patients had an indwelling thoracic epidural catheter with continuous infusion of local anaesthetics and short acting opioids. Total parental nutrition, enteral nutrition or combinations were given to 15 of the patients and the two remaining patients received 50 mg/ml and 100 mg/ml of glucose respectively. All patients received intravenous insulin to keep blood glucose between 4-8 mmol/L. Four of the patients did not survive (patient No 3, 12, 13, and 17) but died 40 days, 3 months, 2 weeks and 1 week respectively after the study. The control group was entirely separate from that in experiment I and consisted of 9 males and one female patient with a median age of 70 years (range 48-76). They were all undergoing elective surgery for ventral hernia repair except for one male patient undergoing laparoscopic cholecystectomy.

*Muscle biopsies:* Leg muscle biopsies were taken percutaneously from the vastus lateralis using a Bergstrom needle. The biopsies were taken using local anaesthesia confined to the skin and fascia only. In the control patients, biopsies were taken just after induction of anaesthesia, but before surgery was started. In experiment I, the muscle covering the rib, mainly the anterior serratus muscle, was identified and used for sampling in order to prevent complications like pneumothorax and bleeding. The anterior serratus is considered as an accessory muscle for respiration [21]. Biopsies were taken by a surgeon through a 3-5 cm long incision at the level of the 6<sup>th</sup> rib in the anterior axillar line. The biopsy was taken using local anaesthesia confined to the skin and subcutaneous tissue without infiltration in the muscle. Local anaesthesia was used in all patients, both the ICU patients with sedation and in the control patients preoperatively but after induction of general anaesthesia. A surgical biopsy of 50-200 mg of wet weight was taken. The biopsy site was sutured keeping attention

to prevent bleeding. The incision was finally closed using intracutaneous technique. No complications were seen with regard to this technique. All biopsies were weighed within three minutes, frozen in liquid nitrogen and thereafter stored at  $-80^{\circ}\text{C}$  until analysis.

Muscle biopsies were homogenised in a buffer (50 mM Tris-HCl pH 7.2, 1 mM EDTA, 100 mM KCl, 5 mM  $\text{MgCl}_2$  1.8 mM ATP) to obtain a 5 % homogenate and then centrifuged at  $700 \times g$  for 10 min to remove cell debris. The resulting supernatant was centrifuged for 10 min at  $15,000 \times g$ . Glycerol was added to this supernatant (final concentration 10 % v/v) which was thereafter frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis.

Haemoglobin concentrations in the homogenates were estimated by a Haemometer (Zeiss).

*Assay of proteasome activity:* Proteasome chymotrypsin-like activity was measured using the fluorogenic peptide substrate succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (LLVY) (Sigma, St Louis, MO, USA). Triplicates of the  $15,000 \times g$  supernatant (10  $\mu\text{l}$ , 10-20  $\mu\text{g}$  of protein) were incubated in 50  $\mu\text{l}$  of buffer (50 mM Tris-HCl pH 7.5, 1 mM ATP, 5 mM  $\text{MgCl}_2$ , 1 mM DTT and 150  $\mu\text{M}$  LLVY). In parallel incubations leupeptin or MG115 were added to the incubation system to a final concentration of 100  $\mu\text{M}$  respectively (Both from Sigma, St Louis, MO, USA). As blank, LLVY was incubated in the absence of muscle extract. After incubation at  $37^{\circ}\text{C}$  for 45 min the reaction was stopped by adding 1 ml of 100 mM sodium acetate buffer, pH 4.3. Standard curves were prepared using 7-amino-4-methylcoumarin (AMC) (Sigma, St Louis, MO, USA). In experiment I the fluorescence was measured at 342 nm excitation and 442 nm emission wavelengths with a Perkin-Elmer LS 50B and in experiment II at 380 nm excitation and 460 nm emission with a FLUOstar OPTIMA (BMG Labtechnologies) spectrofluorometer. The proteolytic activity was expressed as pmol of released AMC per  $\mu\text{g}$  of protein and min. In all experiments we chose to measure the chymotryptic activity since this is the rate limiting step of the protease activities in the proteasome [5] and also the peptide substrate used has been found to be the most proteasome specific substrate [22].

*Statistical methods:* Students t-test was used to compare the proteasome activity between the ICU patients and their respective control groups. Values are given as means  $\pm$  SD.



## Results

In experiment I the proteolytic activity of proteasomes was measured in respiratory and leg muscles from patients with sepsis and control patients. In the respiratory muscle the proteasome activity was 30 % higher in the septic patients as compared to the activity in the control patients ( $0.24 \pm 0.07$  versus  $0.18 \pm 0.04$  pmol/ $\mu$ g protein  $\times$  min,  $P=0.04$ ) (Fig 1). Also the leg muscle showed a similar pattern with a 45 % higher activity in the septic patients ( $0.29 \pm 0.10$  versus  $0.20 \pm 0.05$  pmol/ $\mu$ g protein  $\times$  min,  $P=0.02$ ) (Fig 1).

In experiment II the variability of proteasome activity between individuals and between biopsy specimens within an individual was assessed in one group of patients with sepsis and one group of control patients. In accordance with experiment I, the scatter of proteasome activity between individuals was larger in the group of septic patients than in the controls (Fig 2). Biopsies were taken from the left and right leg of each subject to avoid interference from the 1<sup>st</sup> biopsy taken and be able to take the muscle biopsies from the same position in the leg. Compared to the control group the mean proteasome activity in the patients with sepsis was  $0.16 \pm 0.06$  versus  $0.10 \pm 0.04$  pmol/ $\mu$ g protein  $\times$  min ( $P=0.003$ ) in the left leg and  $0.16 \pm 0.06$  versus  $0.11 \pm 0.02$  pmol/ $\mu$ g protein  $\times$  min ( $P=0.005$ ) in the right leg (Fig 2). The intra individual variation in proteasome activity between the two biopsies taken from the legs was quite high in several of the subjects in both septic and control patients (Fig 3). Each individual muscle extract was assayed for proteasome activity in triplicates. The mean difference in activity between duplicate biopsies was 44 % in the septic patients and 52 % in the controls whereas the mean difference between the highest and lowest activity values in the triplicates was 17 % and 24 % respectively. This means that the difference in activity between biopsies cannot be explained by the variation in the method. The presence of blood in the muscle extracts differed between biopsies as judged by haemometer measurements which might contribute to the variation between the duplicate biopsies since proteasome activity was expressed per protein. In the septic patients haemoglobin concentration correlated negatively with the proteasome activity ( $r=-0.476$ ,  $P<0.01$ ,  $n=34$ ) whereas no such correlation was found in the controls ( $r=0.250$ , NS,  $n=20$ ).

All determinations of the proteasome proteolytic activity were performed in three parallel incubations: in the presence of the substrate LLVY only, with addition of the lysosomal inhibitor leupeptin or with addition of the proteasome inhibitor MG115. Leupeptin did not

influence the proteasome activity whereas MG115 almost completely inhibited the activity during incubation, a pattern that was the same for all biopsies from both patients with sepsis and controls (Fig 4). This shows that the proteolytic activity measured in the assay represents the proteasome activity only.

## Discussion

Catabolic conditions including sepsis, trauma, and cancer are associated with loss of skeletal muscle tissue [23]. In the present study two groups of ICU patients with sepsis and multiple organ failure were found to have increased proteasome proteolytic activity in both leg and respiratory muscles. Compared to control groups the mean proteasome activity of ICU patients was 30 % higher in the respiratory and 45-55 % higher in the leg muscle.

From the results in the present study it is not possible to ascertain whether the increased activity is a result of an increased number of proteasomes in the muscle cell or of an increased intrinsic proteolytic activity of the proteasomes. In a recent study of cancer patients with weight loss concomitant increases in mRNA levels and protein levels for the proteasome subunits C2 and C5 were found [13]. Yet, other studies have failed to show a correlation between mRNA expression and translation products. After mitogen-induced proliferation of human T-lymphocytes, increased levels of mRNA for proteasome subunits C2, C3 and C5 were detected but no increase in the cellular content of proteasomes could be seen [24]. Also in a rat model of sepsis no correlation between mRNA expression and proteasome content in skeletal muscle was seen [7]. In mammalian cells the 20S proteasomes exist in several different forms which exhibit different proteolytic activities. Six different forms of proteasome subtypes have been separated from rat skeletal muscle [25]. After induction of diabetes mellitus a redistribution of proteasome subtypes was found in rat muscle [26]. So the increase of proteasome activity seen in the ICU patients could be an effect of an increased number of proteasomes in the muscle cells or also be due to a redistribution of proteasome subtypes exhibiting a higher intrinsic proteolytic activity or maybe a combination of both.

Patients with sepsis and multiple organ failure usually suffer from respiratory impairment and therefore need mechanical ventilation. As many as 20 % of these patients, experience difficulties in weaning from the ventilator [18]. Atrophy of respiratory muscles is thought to be one of the underlying causes of the development of respiratory difficulties. In a group of patients with mild to moderate chronic obstructive lung disease a reduction in diaphragm myosin heavy chain content together with an increase in ubiquitin-protein conjugation in diaphragm muscle homogenates indicated an enhanced protein degradation [27]. On the other hand mechanical ventilation alone seems to induce respiratory muscle atrophy and thus may contribute to prolonged ventilation treatment. Studies of the diaphragm from ventilated rats show a decrease in myofibrillar protein and an increase in proteasome activity [28-29]. Here

we show that also patients with sepsis-induced multiple organ failure and needing mechanical ventilation, in addition to general muscle wasting, also had an increased proteasome activity in respiratory muscles. However, the muscle used in this study, serratus anterior, is an accessory respiratory muscle and results might be different in the main respiratory muscles i.e. diaphragm and intercostal muscles.

In experiment II the inter- and intra-individual variation of proteasome activity was investigated in a group of septic patients and a control group. The greater variation of proteasome activity in between septic patients, as compared to controls, may be dependent on the size of muscle mass by the onset of disease. One theory is that muscle wasting might be beneficial during sepsis supplying splanchnic organs and immune cells with increased amounts of amino acids released from skeletal muscle. If muscle mass is small a proportionally larger amount of muscle might have to be degraded in order to provide sufficient amounts of amino acids for other organs. It is known that lean body mass decrease by age, however, no correlation was found between age of the ICU patients and proteasome activity ( $r=-0.045$ , NS). Nor was there any correlation between proteasome activity and SOFA scores at study day ( $r=-0.286$ , NS). When looking upon length of ICU stay a correlation was found ( $r=0.621$ ,  $P<0.01$ ), but two patients that stayed 42 and 35 days respectively was the determining factor with their high activity. This correlation was not found in the rest of the 15 patients that had stayed between 1 to 7 days in the ICU ( $r=0.276$ , NS). Some of the patients received short-term glucocorticoid treatment at different doses and glucocorticoids are known mediators of muscle proteolysis (9). However, we found no correlation between proteasome activity and glucocorticoid dose when comparing patients with no treatment, low-dose substitution therapy and high-dose treatment ( $r=-0.132$ , NS). Of course ICU patients with sepsis have a great variety of underlying diseases that probably contributed to the large variation.

Fractional protein synthesis of total protein in leg muscle varies 6 % between duplicate biopsy specimens [30]. The variation of proteasome activity between the two biopsy specimens in experiment II was significantly larger. It can probably not be explained by skewed distribution of fibre type between biopsies, since human leg muscle consists of a mixture of slow and fast fibre types that are evenly distributed within vastus lateralis [31-32]. The presence of blood in the biopsies can to some extent explain the result, at least in the septic patients, where high haemoglobin concentration in the homogenates correlated negatively

with proteasome activity. However, no such correlation was found in the control patients. In both experiments, in the present study, we did not have access to large enough biopsy specimens and therefore decided to measure the proteasome activity in crude homogenate fractions ( $15,000 \times g$  supernatants). Probably the variation between duplicate biopsies would decrease if activity is measured in fractions of proteasomes isolated by sequential ultra centrifugation as we did in an earlier study [17] but this method requires muscle biopsies of at least 70 mg. Nevertheless, in spite of the large variation between duplicate biopsies in both groups of study subjects in the present study, it was still possible to detect a statistically significant difference in proteasome activity between the septic patients and their controls. Thus, proteasome activity measurements in crude extracts still could be an option in studies with limited size of biopsy specimens.

In conclusion, our results showed that proteasome activity was significantly increased in skeletal muscle during sepsis and multiple organ failure and we showed for the first time that both respiratory and leg muscles were affected. The increased proteasome activity in respiratory muscle indicates an accelerated protein degradation that may contribute to the respiratory dysfunction in patients that are treated in the intensive care unit. Since protein synthesis rate in skeletal muscle of these patients is not changed, the increased proteasome activity supports the hypothesis that muscle wasting during severe catabolic conditions like sepsis and multiple organ failure is the result of increased protein degradation.

### **Acknowledgements**

The authors would like to thank Viveka Gustavsson at Karolinska University Hospital Huddinge, Ann-Sofie Andersson and Nina Johansson at Ersta Hospital for excellent nursing assistance.

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## Figure legends

### Figure 1:

Proteasome proteolytic activity in muscle extracts from patients with sepsis (n=10) and controls (n=10). Biopsies were taken from respiratory and leg muscles. Each circle represents an individual subject. Horizontal lines indicate mean values. \* Significantly different from mean control value  $P < 0.05$ , Students t-test.

### Figure 2:

Proteasome proteolytic activity in muscle extracts from patients with sepsis (n=17) and controls (n=10). Muscle biopsies were taken from the left and right leg. Each circle represents an individual subject. Horizontal lines indicate mean values. \* Significantly different from mean control value  $P < 0.01$ , Students t-test.

### Figure 3:

Proteasome proteolytic activity in muscle extracts from the left and right leg in individual septic patients (1-17) and controls (1-10). Black bars represent the left leg and grey bars represent the right leg.

### Figure 4:

Proteasome proteolytic activity in muscle extracts in the absence (LLVY) and presence of lysosomal (Leup) or proteasome (MG115) inhibitors expressed as percentage of LLVY value. Calculations were made from mean values of all subjects in Experiments I and II.

Figure 1

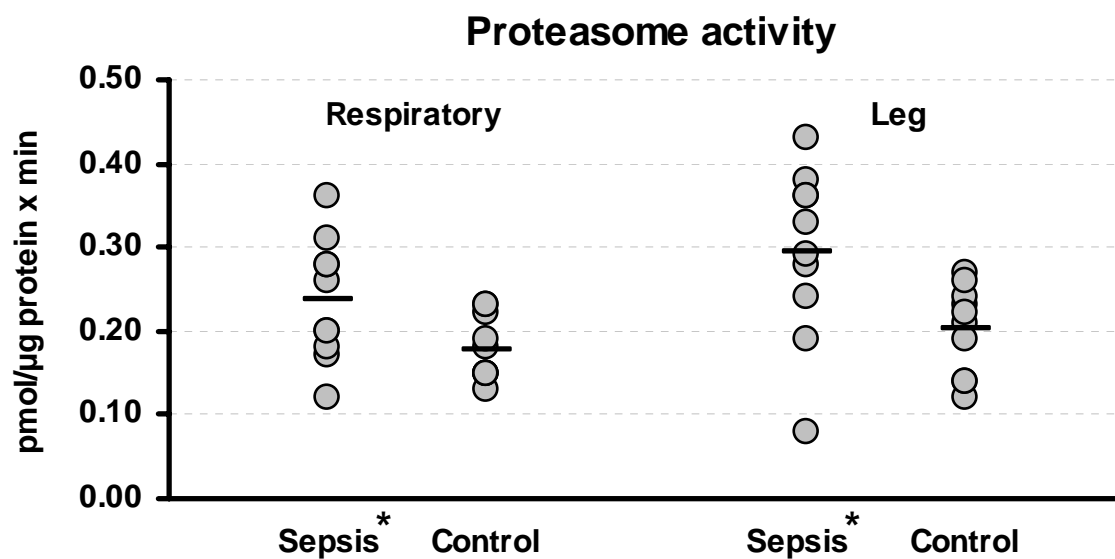


Figure 2

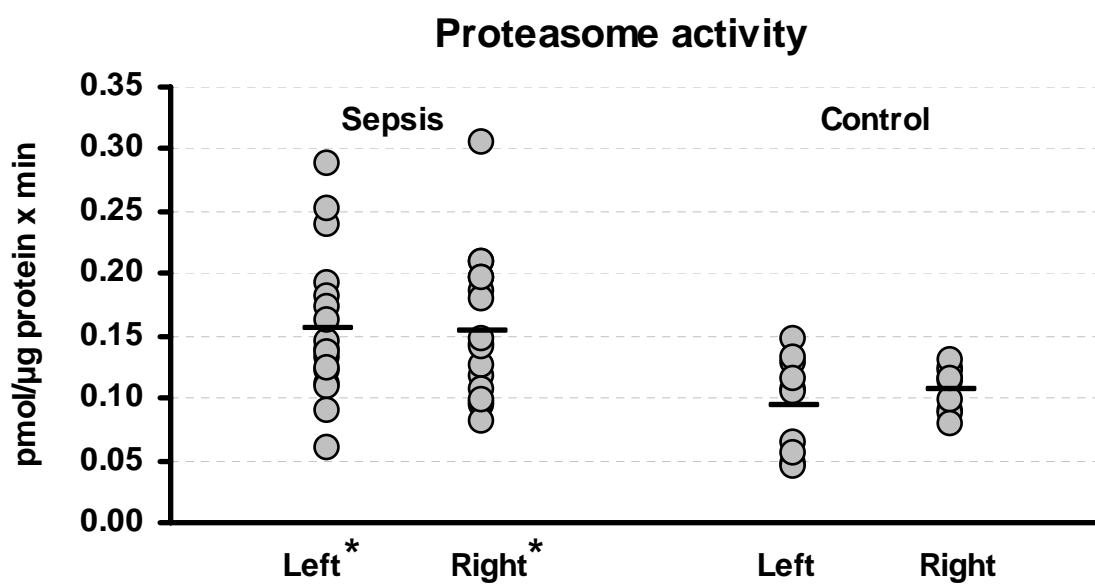


Figure 3

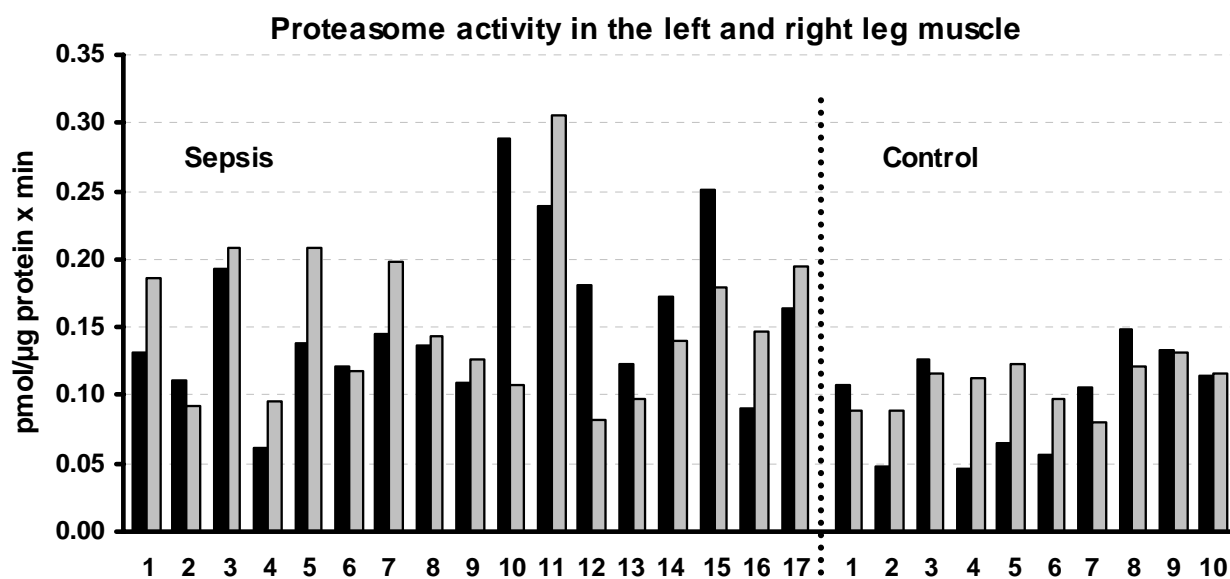
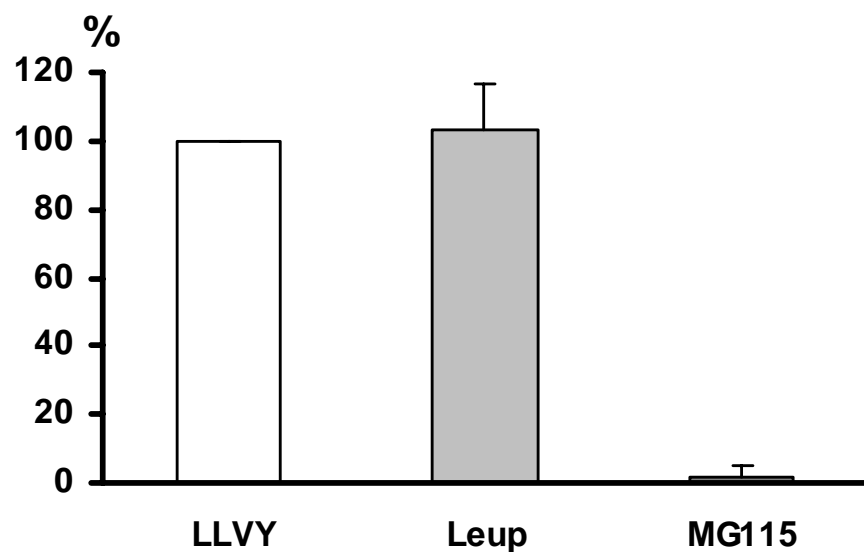


Figure 4



**Table 1.** Patient characteristics

Patient No	Diagnosis	Age	Gender	Days in ICU	SOFA
<b>Experiment I</b>					
1	Throat abscess/pneumonia	67	F	2	7
2	Liver transplant/quadruplegia	62	F	9	11
3	Bacterial meningitis	51	F	7	8
4	Pneumonia	51	M	9	4
5	Surgical complications	74	M	22	7
6	Surgical complications/pneumonia	76	M	4	5
7	Keto acidosis/pneumonia/quadruplegia	40	M	8	5
8	Peritoneal abscess	78	M	6	5
9	ARDS	80	M	2	6
10	Surgical complications/ARDS	67	M	5	10
	<b>Median values</b>	<b>67</b>		<b>8</b>	<b>6</b>
<b>Experiment II</b>					
1	Abdominal aortic aneurysm/pneumonia	60	F	2	7
2	Pneumonia	47	M	1	9
3	Stroke/pneumonia/cardiac arrest	63	M	42	8
4	Respiratory failure (surg)	72	F	1	5
5	Sepsis	77	M	2	1
6	Respiratory failure/COPD	57	F	1	6
7	Respiratory failure (surg)	68	M	2	2
8	Oesophageal resection/COPD	77	M	7	5
9	Respiratory failure/COPD	69	F	1	6
10	Pneumonia/COPD	77	M	3	5
11	Abdominal aortic aneurysm	73	F	35	4
12	Multiple rib fractures/respiratory failure	74	M	6	9
13	Respiratory failure/AMI	66	F	2	4
14	Pneumonia	69	M	2	7
15	Respiratory failure	48	M	2	7
16	Abdominal sepsis (surg)/COPD	71	F	1	12
17	Abdominal sepsis (surg)/COPD	25	F	6	3
	<b>Median values</b>	<b>69</b>		<b>2</b>	<b>6</b>

*Abbreviations:* SOFA = sepsis-related organ failure assessment score; ARDS = acute respiratory distress syndrome; COPD = chronic obstructive pulmonary disease; AMI = acute myocardial infarction.