Low non-carbonic buffer power amplifies acute respiratory acid-base disorders in septic patients: an *in-vitro* study

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TL conceived the study, collected, interpreted and analyzed data, searched literature, and wrote the manuscript; SB collected, interpreted and analyzed data, searched literature, and contributed to manuscript drafting; EC interpreted and analyzed data, searched literature, and contributed to manuscript drafting; FZ, PB and CFF collected data and critically revised the manuscript; MF, ML, TA performed biomolecular analyzes, contributed to manuscript drafting and critically revised the manuscript; FD, PC, LG, AZ, AP and GG contributed to study design, data interpretation and critically revised the manuscript. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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Abstract

Rationale: Septic patients have typically reduced concentrations of hemoglobin and albumin, the major components of non-carbonic buffer power (β). This could expose patients to high pH variations during acid-base disorders.

Objectives: To compare, *in-vitro*, non-carbonic β of septic patients with that of healthy volunteers, and evaluate its distinct components.

Methods: Whole blood and isolated plasma of 18 septic patients and 18 controls were equilibrated with different CO_2 mixtures. Blood gases, pH and electrolytes were measured. Non-carbonic β and non-carbonic β due to variations in Strong Ion Difference (β_{SID}) were calculated for whole blood. Non-carbonic β and non-carbonic β normalized for albumin concentrations (β_{NORM}) were calculated for isolated plasma. Representative values at pH=7.40 were compared. Albumin proteoforms were evaluated via two-dimensional electrophoresis.

Measurements and Main Results: Hemoglobin and albumin concentrations were significantly lower in septic patients. Septic patients had lower non-carbonic β both of whole blood (22.0±1.9 *vs.* 31.6±2.1 mmol/L, $p<0.01$) and plasma (0.5±1.0 *vs.* 3.7±0.8 mmol/L, $p<0.01$). Non-carbonic β_{SID} was lower in patients (16.8±1.9 *vs.* 24.4±1.9 mmol/L, $p<0.01$) and strongly correlated with hemoglobin concentration ($r=0.94$, $p<0.01$). Non-carbonic β_{NORM} was lower in patients (0.01 [-0.01 – 0.04] *vs*. 0.08 [0.06 – 0.09] mmol/g, $p<0.01$). Septic patients and controls showed different amounts of albumin proteoforms.

Conclusions: Septic patients are exposed to higher pH variations for any given change in CO_2 due to lower concentrations of non-carbonic buffers and, possibly, an altered buffering function of albumin. In both septic patients and healthy controls, electrolyte shifts are the major buffering mechanism during respiratory acid-base disorders.

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New & Noteworthy (67/75)

Septic patients are poorly protected against acute respiratory acid-base derangements due to a lower non-carbonic buffer power, which is caused both by a reduction in the major non-carbonic buffers, *i.e.* hemoglobin and albumin, and by a reduced buffering capacity of albumin. Electrolyte shifts from and to the red blood cells determining acute variations in Strong Ion Difference are the major buffering mechanism during acute respiratory acid-base disorders.

Introduction

Sepsis is a life-threatening disease leading worldwide to 5 million yearly deaths (1, 2). Metabolic acidosis is common in sepsis and is a marker of severity (3, 4). Furthermore, about 40% of septic patients develop secondary respiratory failure and are therefore at risk of respiratory acidosis (5, 6).

In human physiology, two systems limit pH changes in acute acid-base perturbations: the carbonic (carbonic acid/bicarbonate) and non-carbonic buffers (mainly albumin and phosphates in plasma, with the addition of hemoglobin in whole blood) (7).

Both systems contribute to the acute compensation of metabolic derangements, *e.g.* in case of lactic acidosis both bicarbonate ions (HCO_3^-) and the dissociated part of non-carbonic buffers (A^-) will be reduced. On the contrary, during acute respiratory acid-base derangements only non-carbonic buffers minimize pH variations, as the change in carbonic acid/bicarbonate is the primary disorder (8). In this context, the ability of non-carbonic buffers to limit pH excursions has been termed as non-carbonic buffer power (β) and defined as the negative value of the variation in HCO_3^- divided by the corresponding variation in pH, induced by acute changes in partial pressure of carbon dioxide (P_{CO_2}) (7, 9-11). Simplifying, this means that, for any given variation in P_{CO_2}, a patient with a lower non-carbonic β will have greater pH variations as compared to a patient with higher values of non-carbonic β.

In order to effectively buffer respiratory acid-base derangements, and limit pH variations, non-carbonic buffers have to favor changes in HCO_3^- consensual to the variations in P_{CO_2}. According to the electrical neutrality principle, the total concentration of cations has to be equal to the total concentration of anions (12, 13). As a direct consequence, any increase in HCO_3^- observed during acute hypercapnia must be accompanied by a reduction in negative charges and/or an increase in positive charges. This can be achieved in two ways. First, through a reduction in the dissociated part of total non-carbonic weak acids (A^-), such as proteins and phosphates, therefore

operating in the buffer base domain (14-16). Second, through a reduction in strong anions, mainly chloride (Cl^-) or an increase in strong cations, mainly sodium (Na^+), therefore operating in the Strong Ion Difference (SID) domain (12, 17, 18). The first mechanism is considered the main in isolated plasma. On the other hand, when dealing with a complex solution, such as whole blood, red blood cells gain a key role as they allow electrolytes shifts across their cellular membrane, leading to SID changes consensual to P_{CO_2} variations (8, 19, 20).

During sepsis, patients are typically anemic (21) and hypoalbuminemic (22, 23), and have therefore reduced concentrations of non-carbonic buffers. Moreover, albumin is a macromolecule whose structure might vary significantly (24). Indeed, pathological conditions such as inflammation might favor the transition from reduced albumin to "oxidized" albumin (25). If and how these changes of protein structure might affect its buffering function is currently unknown.

As data regarding the non-carbonic β of septic patients are currently lacking, we decided to conduct this prospective, experimental *in-vitro* acid-base study. We hypothesized that septic patients' non-carbonic β of whole blood and plasma would be significantly lower as compared to healthy volunteers, thus exposing patients to greater pH variations.

Materials and Methods

Study population

The study was approved by the ethical committee and registered at ClinicalTrials.gov (NCT03503214). Patients admitted to the intensive care unit for sepsis/septic shock (3) were enrolled. Age < 18 years and pregnancy were exclusion criteria. Healthy, age-matched volunteers were recruited for comparison. Informed or deferred informed consent was obtained.

Blood and plasma sample

In both groups, 25 milliliters of venous blood were collected (26, 27). Complete blood count, concentrations of magnesium, phosphate and albumin (Cobas c-702, Roche, Switzerland) were measured. Six milliliters were placed in anti-foam syringes (T310 Syringes, RNA Medical, USA), *i.e.* plastic syringes pre-treated with an anti-foam material in order to prevent excessive foaming during tonometry. The remaining blood was centrifuged for 10 minutes at 4°C at 3000 rpm to isolate plasma. A sample was frozen at -85°C for subsequent biomolecular analyses.

Tonometry of blood and plasma

The whole blood sample was divided in four aliquots and immediately equilibrated at different CO_2 concentrations through tonometry (Equilibrator, RNA Medical, USA) (27-29). Four gas mixtures containing 2, 5, 12 or 20% of CO_2 with 21% of oxygen and nitrogen for the remaining percentage were used (15 minutes at 37°C). Thereafter, samples were analyzed for blood gases, pH and concentrations of sodium, potassium, calcium, chloride and lactate (ABL 800 FLEX Radiometer, Denmark). Samples in which lactate increased more than 1 mmol/L as compared to baseline values, and samples in which hemoglobin saturation for oxygen was below 94% were discarded. The same procedure was performed on isolated plasma.

143 *Definitions and calculations*

144 For each whole blood sample the actual SID obtained at every CO_2 concentration was calculated as:

145 $$[SID] = [Na^+] + [K^+] + 2 \times [Ca^{2+}] - [Cl^-] - [lactate^-] \quad [1]$$

146 where Na^+, K^+, Ca^{2+}, Cl^- and $Lactate^-$ refer to plasma sodium, potassium, ionized calcium, chloride
147 and lactate concentrations (expressed in mmol/L) measured with the point-of-care blood gas
148 analyzer. Magnesium was not used for the calculation, as it was measured only in the central
149 laboratory on baseline venous blood. The actual HCO_3^- concentration of each sample was calculated
150 by applying known values for the solubility of CO_2 in plasma (S = 0.0307 mmol/(L·mm Hg)) (30)
151 and the negative logarithm of the first apparent equilibrium dissociation constant of carbonic acid
152 (pK'_1 = 6.095 for whole blood and pK'_1 = 6.105 for isolated plasma) (31):

153 $$[HCO_3^-] = S \cdot P_{CO_2} \cdot 10^{(pH - pK'1)} \quad [2]$$

154 where P_{CO_2} = partial pressure of carbon dioxide (expressed in mm Hg) and measured with the
155 point-of-care blood gas analyzer and pH = pH measured with the point-of-care blood gas analyzer.

156 Variations of HCO_3^- and SID over pH in whole blood and plasma were modeled according to a
157 polynomial multilevel model (32) in order to obtain overall HCO_3^-/pH and SID/pH curves for the
158 two study groups and individual best fits for all subjects/patients.

159 Non-carbonic β was defined as $-\Delta HCO_3^-/\Delta pH$ resulting from a variation in P_{CO_2} (7). Non-carbonic
160 β is therefore the opposite of the first derivative of the HCO_3^-/pH curve:

161 $$\text{Non-carbonic } \beta = -d[HCO_3^-]/dpH \quad [3]$$

162 Individual non-carbonic β curves were used to obtain representative non-carbonic β values
163 at a pH of 7.40 ($\beta_{7.40}$).

164 The same analysis was performed substituting SID to HCO_3^- in order to quantify the contribution of
165 the electrolyte shifts to total non-carbonic β in whole blood:

$$\beta_{SID} = -\, dSID/dpH \quad [4]$$

Individual values of non-carbonic β of isolated plasma obtained at pH=7.40 were divided by the albumin concentration in order to normalize for different albumin concentrations and investigate the molecular buffering function:

$$\beta_{NORM} = \beta_{7.40}/\text{Albumin [g/L]} \quad [5]$$

Polynomial multilevel model

Variations of dependent variables over pH were modeled according to a polynomial multilevel model (general linear mixed models) with random intercept at subject level and random slope at pH level. Statistical analyses were performed as previously described (29) and carried out by SAS 9.4 statistical package. The exponential power of pH was decided according to model-based likelihood ratio tests (cut-off P-value $\leq$ 0.100). Interaction between the independent variables, pH and subjects' group (controls and patients), was also included according to model-based likelihood ratio tests.

Two-dimensional electrophoresis (2-DE)

Total proteins in plasma samples were quantified and separated by two-dimensional electrophoresis (2-DE). The total protein content in plasma samples was assessed by the bicinchoninic acid (BCA) protein assay (EuroClone). After normalization based on albumin concentration (assessed by monodimensional gel electrophoresis), the same amount (10 µg) of proteins per sample was separated by 2-DE (33). Briefly, plasma samples were diluted in 2.5% dithiothreitol (DTT) /5% sodium dodecyl sulfate (SDS) and denatured at 95°C for 5 minutes. Then, they were diluted (250 µl) in UTC buffer (7M urea, 2M thiourea, 4% CHAPS) added with 0.04% ASB-14, 5% glycerol, 0.2% Tween-20, 100 mM DTT and 2% IPG buffer pH 4-7 (GE Healthcare). Samples were loaded on 13 cm Immobiline™ DryStrip pH 4-7 (GE Healthcare) and isoelectric focusing was performed

with an Ettan™ IPGphor II system (Amersham Biosciences). Strips were equilibrated in 50 mM Tris-HCl pH 8.8, 36% urea w/v, 2% SDS w/v, 67% glycerol v/v, bromophenol blue added with 1% DTT (30 minutes incubation) and then with 2.5% IAA (30 minutes). Second dimension (based on MW) was then performed on 12.5% polyacrylamide gels by SDS-PAGE. Gels were stained with ProteinStain Fluo-R (SERVA) and acquired with a GelDoc-It™310 Imaging System (UVP), at four different apertures (4.0, 5.6, 8.0 and 11).

Albumin proteoforms were identified, aligned and quantified using the ImageJ software. Briefly, a linear background correction was applied (10 pixels rolling ball, sliding paraboloids) and all images were aligned based on the main albumin protein spot (Registration plugin, alignment by line ROI). Then, signals intensity was calculated for all albumin spots. The integrated density values of each albumin proteoforms were normalized based on the sum of the intensities of all albumin spots per sample. Results were expressed as mean±SEM in the two groups (Controls vs. Patients).

Statistical analysis

Data are expressed as mean±SD unless otherwise specified. Baseline data from patients and controls were compared via t test or Mann-Whitney rank sum test, as appropriate. Different albumin proteoforms were compared via two-tailed t-test, applying Benjamini-Hochberg correction for multiple testing (FDR<0.05 as threshold). Pearson's correlation coefficient was employed to assess the degree of linear relationship between two variables. Analysis was performed with SAS 9.4 (SAS Institute Inc., USA). A P value <0.05 was considered statistically significant.

Results

Eighteen healthy controls and 18 septic patients were enrolled. Age (52 [47 – 60] *vs.* 58 [47 – 74] years p=0.32), body mass index (23.4 [20.9 – 25.1] *vs.* 23.5 [20.8 – 26.0] kg/m^2, p=0.54) and gender (8 (44%) *vs*. 5 (28%) n (%) of females, p=0.49) of healthy volunteers did not differ from those of patients with sepsis. Septic patients had a SOFA score of 9 ± 2 points; eleven (61%) had septic shock and were on vasopressors at the time of study. Infection sites were the lung in 9 (50%), abdomen in 5 (28%), genitourinary tract in 3 (17%) and "other" in 1 (5%) case. Twelve patients (67%) where mechanically ventilated, 3 (17%) where supported also by extracorporeal membrane oxygenation and 2 (11%) where undergoing continuous renal replacement therapy. Overall mortality of the studied population was 6 (33%). Patients received a median amount of 100 [0 – 300] ml of commercial 20% albumin (Albital, Kedrion, Italy) before the study time.

Baseline laboratory data and results of blood gas analysis performed on whole blood at 5% of CO_2 are reported in **Table 1**. Of note, among non-carbonic buffers, hemoglobin and albumin concentrations were significantly lower in septic patients (p<0.01), while phosphate concentrations were similar in the two groups. Values of SID, Base Excess (BE) and HCO_3^- were significantly lower in septic patients, while lactate concentration had a significantly higher value in this population.

Non-carbonic β of whole blood

Tonometry of whole blood with 2, 5, 12 or 20% of CO_2 was performed to study *in-vitro* the capacity of whole blood to resist to respiratory acid-base disorders. The experiments allowed to explore P_{CO_2} values between 13 and 138 mm Hg, with a resulting pH range of 6.83 to 7.81. As expected, the increase in P_{CO_2} caused a decrease in pH and an increase in HCO_3^- (**Figure E1, Panel A** and **B,** https://doi.org/10.6084/m9.figshare.14398211.v1). Consequently, there was a negative relationship between HCO_3^- and pH. Individual pH and HCO_3^- values resulting from the equilibration process in

whole blood of healthy controls and septic patients are reported in **Figure 1A** and **1B**. Overall HCO_3^-/pH curves for whole blood of the two study populations obtained by the polynomial multilevel model are reported in **Figure E1, Panel C**. The resulting non-carbonic β/pH curves of the populations, for the explored pH range, are two straight lines with equal slope and different intercept ($p<0.01$, see **Table E1**, https://doi.org/10.6084/m9.figshare.14398250.v1), resulting in lower absolute β values in septic patients (**Figure E1, Panel D**).

Non-carbonic β of isolated plasma

Subsequently, to exclude red blood cells from the system, tonometry with 2, 5, 12 or 20% of CO_2 was performed on isolated plasma. This allowed to explore P_{CO_2} values between 16 and 130 mm Hg, with a resulting pH range of 6.73 to 7.90. Individual experimental points of P_{CO_2} and the resulting pH and HCO_3^- values for isolated plasma are reported in **Figure E2, Panels A** and **B** (https://doi.org/10.6084/m9.figshare.14398226.v1). Absolute pH and HCO_3^- values of the study populations resulting from the equilibration process in isolated plasma are reported in **Figure 2A** and **2B**. Overall HCO_3^-/pH curves for isolated plasma of the two study populations obtained by the polynomial multilevel model, are reported in **Figure E2, Panel C**. As for whole blood, the resulting non-carbonic β/pH curves of the study populations, for the explored pH range, are two straight lines with equal slope and different intercept ($p<0.01$, see **Table E2**, https://doi.org/10.6084/m9.figshare.14398247.v1), resulting in lower absolute non-carbonic β values in septic patients (**Figure E2, Panel D**).

Non-carbonic β of whole blood and isolated plasma at pH of 7.40

To describe the capacity to resist to acid-base variations in normal conditions, absolute values of non-carbonic β of both whole blood and isolated plasma were calculated at a pH value of 7.40. Values of healthy volunteers were significantly higher than those of septic patients both for whole

blood (31.6±2.1 *vs.* 22.0±1.9 mmol/L, p<0.01) and isolated plasma (3.7±0.8 *vs.* 0.5±1.0 mmol/L, p<0.01), as shown in **Figure 3**. Of note, for both patients and controls, isolated plasma had significantly lower values of non-carbonic β as compared to whole blood (p<0.01, for both).

Buffering mechanisms

a. Whole blood

The mechanisms underlying the buffering capacity of whole blood where then investigated and changes in sodium, chloride and SID consequent to the applied P_{CO_2} were assessed. When passing from the lowest (around 20 mm Hg) to the highest P_{CO_2} (around 120 mm Hg), a significant increase in sodium concentration (5±1 mmol/L *vs.* 4±1 mmol/L, p<0.01, in healthy volunteers and septic patients, respectively) and reduction in chloride concentration (7±1 mmol/L and 5±1 mmol/L, p<0.01, in healthy volunteers and septic patients, respectively) was observed (**Figure E3**, **Panel A** and **B**, https://doi.org/10.6084/m9.figshare.14398244.v1). On the contrary, no significant variations in potassium were observed. Changes in sodium and chloride in the two populations, according to the pH changes resulting from CO_2-tonometry are provided in **Figure E4** (https://doi.org/10.6084/m9.figshare.14398238.v2). As a result, a significant increase in SID (13.5±1.2 mEq/L and 9.8±1.4 mEq/L, p<0.01, in healthy volunteers and septic patients, respectively) was observed with the maximum P_{CO_2} variation (**Figure 4A** and **4B**).

The contribution of electrolyte shifts to total non-carbonic β of blood (non-carbonic β_{SID}) was then computed. Overall SID/pH curves for the two study groups are reported in **Figure E3, Panel C**. The resulting function describing non-carbonic β_{SID} in the explored pH range was a horizontal line (**Figure E3, Panel D**). According to this model (**Table E3**, https://doi.org/10.6084/m9.figshare.14398223.v1), β_{SID} of whole blood is therefore independent from pH values. Furthermore, significantly lower values of β_{SID} were observed in septic patients as compared to controls (16.8±1.9 *vs.* 24.4±1.9 mmol/L, p<0.01, **Figure 5)**. A strong correlation

287 ($r=0.94$, $p<0.01$) between hemoglobin concentration and β_{SID} was found when pooling data from
288 both groups (**Figure 6**).

289 *b. Isolated plasma*

290 A possible correlation between albumin concentration and non-carbonic β of isolated plasma was
291 investigated to understand the mechanisms underlying the buffering capacity of isolated plasma.

292 A strong correlation ($r=0.93$, $p<0.01$) was observed when pooling data from both groups (**Figure**
293 **E5**, https://doi.org/10.6084/m9.figshare.14720898.v1). In addition, the representative values
294 obtained at pH = 7.40 of non-carbonic β of isolated plasma normalized for albumin concentration
295 (β_{NORM}) were compared in order to assess possible differences of albumin buffering capacity. Septic
296 patients had significantly lower values of β_{NORM} (0.01 [-0.01 – 0.04] *vs*. 0.08 [0.06 – 0.09] mmol/g, p
297 <0.01) as compared to controls (**Figure 7**). In addition, a different amount of specific albumin
298 proteoforms was observed by two-dimensional electrophoretic separation of plasma samples
299 (**Figure 8A**). In particular, amongst the nine detected albumin proteoforms, in septic patients a
300 significant increase (FDR<0.05) in the amount of the acidic ones was observed (spots 7 and 9),
301 accompanied by a downward trend of the alkaline ones (spots 1, 2 and 3) (**Figure 8B**).

302

Discussion

In the present study, respiratory acid-base derangements were induced *in-vitro.* We simulated hypoventilation/hypercapnia by equilibrating our samples with gases containing high CO_2 concentrations. Moreover, hyperventilation/hypocapnia was simulated using gases containing a low CO_2 concentration. This allowed to measure the *in-vitro* non-carbonic β, *i.e.* the capacity of blood/plasma to limit pH changes secondary to acute P_{CO_2} variations, in septic patients and healthy volunteers. Our main finding is that septic patients' capacity to buffer respiratory acid-base disorders is markedly reduced (approximately by 30%) as compared to healthy volunteers. This, of course, exposes this category of patients to more pronounced pH variations for any given respiratory and/or metabolic acid-base derangement. The reduced non-carbonic β of septic patients is due to two major factors. First, and more importantly, this category of patients has lower concentrations of non-carbonic buffers. Indeed, both hemoglobin and albumin concentrations were significantly lower (**Table 1**). Second, it appears that septic patients' albumin has a reduced buffering capacity. Indeed, when normalizing the non-carbonic β of isolated plasma by the measured albumin concentration, we found significantly lower buffering capacity for every gram of plasma albumin (**Figure 7**). This finding suggests that the molar buffering capacity of the protein macromolecule might be altered by critical illness and/or by the administration of exogenous albumin. The difference observed in albumin proteoforms (Figure 8) supports this finding. In other words, these results suggest that the protein buffering system of septic patients might have different acid dissociation constants. In addition, it has been demonstrated that commercial albumin, frequently administered to our patients, has an altered red-ox state (41). However, how this red-ox state alteration affects albumin buffering capacity and its acid dissociation constant needs to be determined in further studies, properly designed to address this question.

In addition, the present study shed light on the physiologic and pathophysiologic mechanisms of buffering during acute respiratory acid-base derangements. In line with previous studies performed in the setting of metabolic acid-base disorders (34, 35), our data show, overall, that whole blood has a significantly higher buffering capacity as compared to isolated plasma (**Figure 3**). To understand the underlying mechanisms, we partitioned and quantified the two components of non-carbonic buffer power, *i.e.* i) changes in electrolytes induced by P_{CO_2} variations, resulting in variations in SID and ii) variations in the dissociation/association of plasma proteins.

We observed that electrolytes vary remarkably, when P_{CO_2} is changed acutely in whole blood. Indeed, when the P_{CO_2} of whole blood is increased through tonometry, chloride decreases significantly (**Figure E3, Panel B**). Moreover, a concomitant significant increase in sodium concentration was observed. Interestingly, similar results were reported by Giebisch in *in-vivo* experiments performed in dogs (8). When analyzing intracellular fluid, the authors did not find a reduced sodium concentration, suggesting that the increase in sodium observed during respiratory acidosis was not caused by a shift from the red blood cell. This difference between our results and the finding of Giebisch and colleagues could be explained by known differences between canine and human red blood cell physiology (36). Another factor potentially at play is the salt-type binding/unbinding of electrolytes to large proteins, which appears to be influenced by P_{CO_2} and pH (27, 37-39).

The result of the observed electrolyte shifts secondary to a primary increase in P_{CO_2} was an increase in SID up to 15 mmol/L (**Figure 4**). As an increase in SID favors an increase in HCO_3^-, and shifts the system towards alkalosis, it is clear that this mechanism of interdependence between P_{CO_2} and SID during respiratory acid-base disorders limits the resulting pH changes of whole blood (19). Of note, in the present study hemoglobin was always completely saturated with oxygen. The contribution of the Haldane effect, with the related chloride shift due to the transition of hemoglobin

352 from the deoxygenated tense (T-state) to the oxygenated relaxed (R-state), could thus be excluded
353 as contributing factor to the observed electrolyte shifts (40, 41).

354 We therefore quantified the contribution of SID variations to the non-carbonic β and called
355 this term β_{SID}. The aim of this analysis was to quantify the changes in HCO_3^- secondary to CO_2
356 variations determined by electrolyte shifts. It is worth underlining that the SID changes observed in
357 whole blood samples were secondary to electrolyte shifts from and to the red blood cells, as no
358 electrolytes were added or removed from the sample. Moreover, it is important to state that the β_{SID}
359 concept used in the present study differs significantly from other studies, in which P_{CO_2} was kept
360 constant, SID was changed through the addition of strong acids or bases, and the resulting pH
361 variation was assessed (42).

362

363 In both healthy volunteers and septic patients, we found that the electrolyte shift and
364 therefore β_{SID} is undoubtedly the most important buffering mechanism of blood, accounting for
365 approximately 80% of the total non-carbonic buffering capacity (**Figure 5**).

366 Interestingly, the value of β_{SID} was found to be, in the explored pH range, independent from
367 pH (**Figure E3, Panel D**), *i.e.* in our model the "hemoglobin-red blood cell" buffering system does
368 not seem to behave as a weak acid, but seems to have a fixed, pH-independent buffering effect.
369 Finally, we were able to demonstrate that the interindividual differences in P_{CO_2}-induced SID
370 variations (**Figure 4**) and therefore the differences in β_{SID} were strongly correlated with hemoglobin
371 concentration, a reasonable proxy of red blood cell intracellular volume (**Figure 6**).

372 When studying isolated plasma, we were able to exclude the buffering function of the
373 "hemoglobin-red blood cell" system, and therefore focused our attention on a second physiologic
374 mechanism, *i.e.* the variations in dissociation/association of plasma proteins (15, 16). Also in the
375 experiments performed on isolated plasma, we found significant differences in the buffering
376 capacities of healthy volunteers and septic patients (**Figure 3**). This finding is certainly explained,

at least in part, by a lower albumin concentration in septic patients (**Table 1**). Nevertheless, also when normalizing the non-carbonic β for measured albumin (the major non-carbonic buffer of plasma) we found lower values in patients. This finding suggests that the molar buffering capacity of the protein macromolecule might be altered by critical illness and/or by the administration of exogenous albumin. The difference observed in albumin proteoforms (**Figure 8**) supports this finding. In other words, these results suggest that the protein buffering system of septic patients might have different acid dissociation constants. In addition, it has been demonstrated that commercial albumin, frequently administered to our patients, has an altered red-ox state (43). However, how this red-ox state alteration affects albumin buffering capacity and its acid dissociation constant needs to be determined in further studies, properly designed to address this question.

Clinical implications

Our study suggests that septic patients, due to lower buffer concentration and, possibly, an altered buffering function of albumin, are more exposed to acute acid-base derangements. In line with previous studies (44, 45), we clearly observed that the "hemoglobin-red blood cell" system is by far the most important buffering mechanism. It is thus clear that the red blood cell transfusion strategy (46, 47) will have a significant impact on the ability of blood to resist to acid-base perturbations, with a more liberal strategy being associated with higher non-carbonic buffer power and a more restrictive strategy with a lower capacity to resist to respiratory acid-base derangements. In addition, the *in-vitro* carbon dioxide titration curve that we performed clearly underlines the importance of tailoring nomograms to the specific clinical condition. Indeed, rules frequently applied in critical care to predict changes in HCO_3^- following an acute hypercapnia (48) or hypocapnia (49) are derived from healthy volunteers or from healthy surgical patients, *i.e.* not from critically ill patients. In addition, the studies that derived these rules hardly discussed the buffering

role of the "hemoglobin-red blood cell" system, which, as previously observed (44, 45) and confirmed by our data, is by far the major non-carbonic buffer. Finally, it is important to mention that non-carbonic buffer power is employed in all equations used to calculate base excess (50-52). Many equations estimate the non-carbonic buffer power according to the hemoglobin concentration, other equations, such as the "Van Slyke equation" introduced by Siggaard-Andersen and suggested by the Clinical and Laboratory Standards Institute (CLSI) (31) assume a constant value of 16.2 mmol/L for the extracellular fluid (50). This value was derived from *in-vivo* experiments performed in 8 healthy subjects undergoing either voluntary hyperventilation or inhalation of CO_2 (53). The author thus derived the following equation: $\beta = 2.3 \cdot \text{ctHb (Ecf)} - 7.7$ mmol/L, which yields a value of -16.2 mmol/L for a blood hemoglobin concentration of 16 g/dL and a total protein concentration of 7 g/dL (53, 54). While changes in the non-carbonic buffer power have a small effect in the computation of Base Excess (50), our study describes the variability of whole blood non-carbonic buffer power in septic patients (range between 20 and 28 mmol/L), caused both by the variability of hemoglobin and albumin concentration, and by different acid-base characteristics (β_{NORM}) of albumin. These findings hence underline the importance of using personalized values in order to compute accurate base excess values.

Limitations

We need to mention some limitation of our study. The *in-vitro* nature of the study, while allowing to exclude confounding factors, such as intravenous fluid therapy (55-58), did exclude the interaction with the interstitium which is certainly clinically relevant. Brackett et al (48) suggest that the *in-vivo* non-carbonic β is lower than the equivalent *in-vitro* determination due to a higher volume of distribution (59). This aspect, which needs to be clarified in future clinical studies, might be exacerbated in critically ill patients, frequently characterized by an expanded extracellular volume. Another limitation of our study relies in the fact that we analyzed whole blood, *i.e.* the

427 "plasma-red blood cell system". Our data therefore do not allow to draw conclusions on the
428 "buffering" role of free hemoglobin. Moreover, we have no information on the intracellular effects
429 of CO_2- tonometry. Finally, our data suggest that plasma proteins of septic patients might have a
430 lower acid dissociation constant. However, in our experimental design we explored only 4 different
431 partial pressures of CO_2 which did not allow us to determine experimentally the dissociation
432 constant (27). Future studies are warranted to determine the acid dissociation constant of septic
433 patients' plasma proteins.
434

435 **Conclusions**

436 Septic patients, as compared to healthy volunteers, have a reduced non-carbonic β, which exposes
437 this category of patients to more pronounced pH shifts for any given respiratory acid-base
438 derangement. Moreover, the reduced non-carbonic β likely exposes these patients also to greater pH
439 shifts during metabolic acid-base derangements. The "hemoglobin-red blood cell" system is by far
440 the most important buffering mechanism and seems to be pH-independent, *i.e.* it does not behave as
441 a classic buffer in the explored pH range. Indeed, this system limits pH changes through electrolyte
442 shifts determining variations in SID, proportional to the variation in P_{CO_2}. This finding, besides
443 underlining the interdependence of P_{CO_2} and SID, suggests that the pathophysiology of acid-base
444 equilibrium is far from being fully understood.
445

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Availability of data and materials

The complete dataset is available at the following link:

https://doi.org/10.6084/m9.figshare.14709609.v2

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615

616

617

618 **FIGURE AND TABLE LEGEND**

619

620 **Figure 1. pH to bicarbonate relationship in whole blood.**

621 Experimental points of pH and HCO_3^- concentration obtained through equilibration of whole blood
622 at different P_{CO_2} values in healthy controls (Panel **A**) and septic patients (Panel **B**). Each
623 subject/patient is identified by a different symbol.

624

625 **Figure 2. pH to bicarbonate relationship in isolated plasma**

626 Experimental points of pH and HCO_3^- concentration obtained through equilibration of isolated
627 plasma at different P_{CO_2} values in healthy controls (Panel **A**) and septic patients (Panel **B**). Each
628 subject/patient is identified by a different symbol.

629

630 **Figure 3. Non-carbonic β at pH 7.40.**

631 Representative values at pH = 7.40 for non-carbonic buffer power (β) in healthy controls and septic
632 patients for whole blood (left side of the graph) and isolated plasma (righ side of the graph). Dots
633 represent 5^{th} and 95^{th} percentiles.

634

635 **Figure 4. P_{CO_2} to SID relationship in whole blood**

636 Experimental points of P_{CO_2} and Strong Ion Difference (SID) obtained through equilibration of
637 whole blood at different P_{CO_2} values in healthy controls (Panel **A**) and septic patients (Panel **B**).
638 Each subject/patient is identified by a different symbol.

639

640 **Figure 5. Non-carbonic β due to SID variations in whole blood**

641 Values of the buffer component due to SID variation (β_{SID}) in whole blood of healty controls and
642 septic patients. This values are independnt of the applied pH, as β_{SID} did not change with pH in the
643 explored P_{CO_2} range. Dots represent 5^{th} and 95^{th} percentiles.

644

645 **Figure 6. Hemoglobin to β_{SID} variation relationship**

646 Relationship between hemoglobin and β_{SID} in the whole study population. Black circles represent
647 data from control subjects, while white cricles represent data from septic patients. A strong
648 correlation was found for the overall population. A similar finding was observed, when analyzing
649 Patients ($r = 0.74$, $p < 0.01$) and Controls ($r = 0.61$, $p = < 0.01$) separatedly.

650

651 **Figure 7. Normalized non-carbonic β of plasma at pH = 7.40**

652 Representative values of non-carbonic β of isolated plasma at pH = 7.40 normalized for albumin
653 concentrations (β_{NORM}). Dots represent 5^{th} and 95^{th} percentiles.

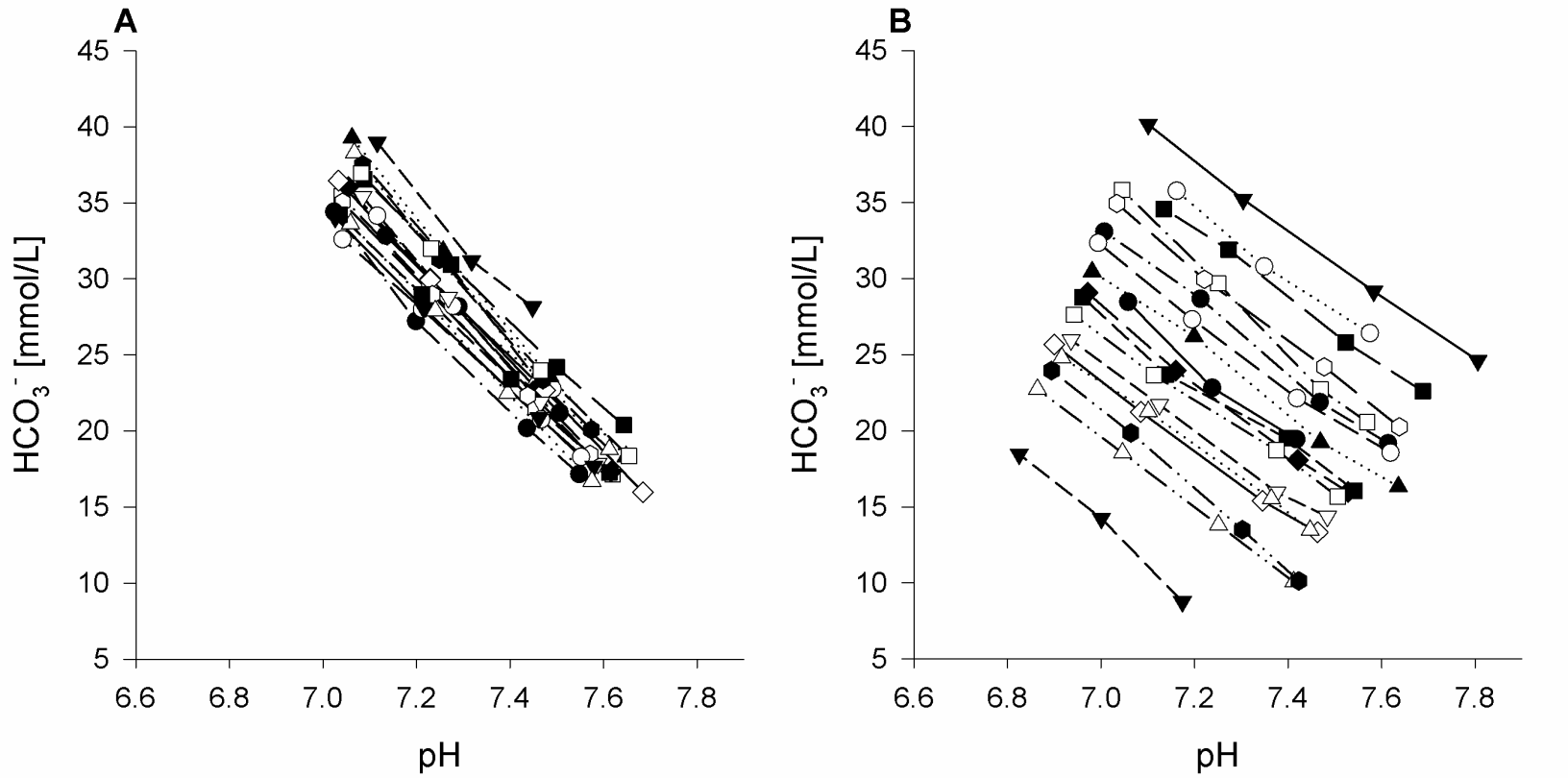
654

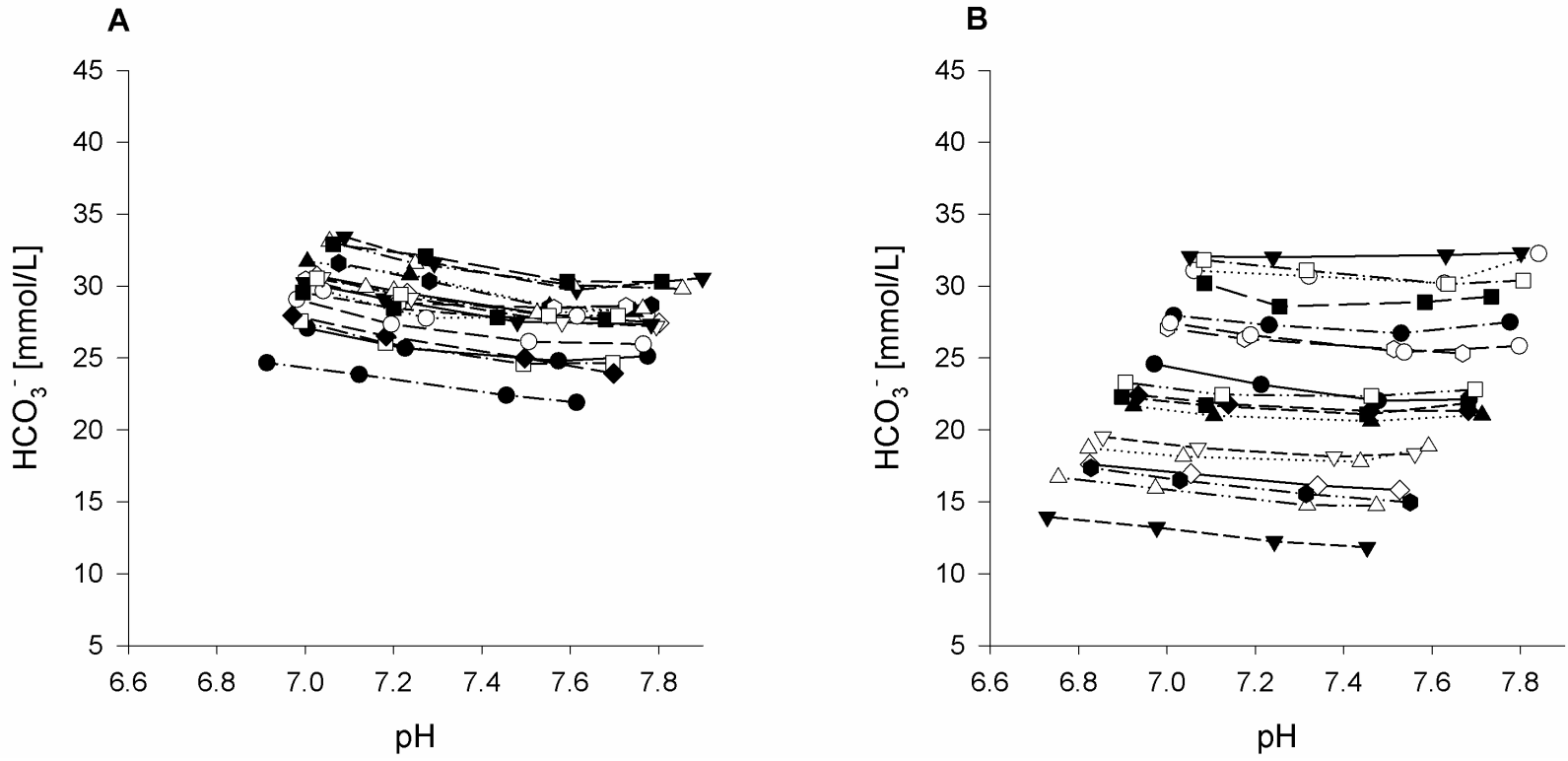
655 **Figure 8. Albumin proteoforms.**

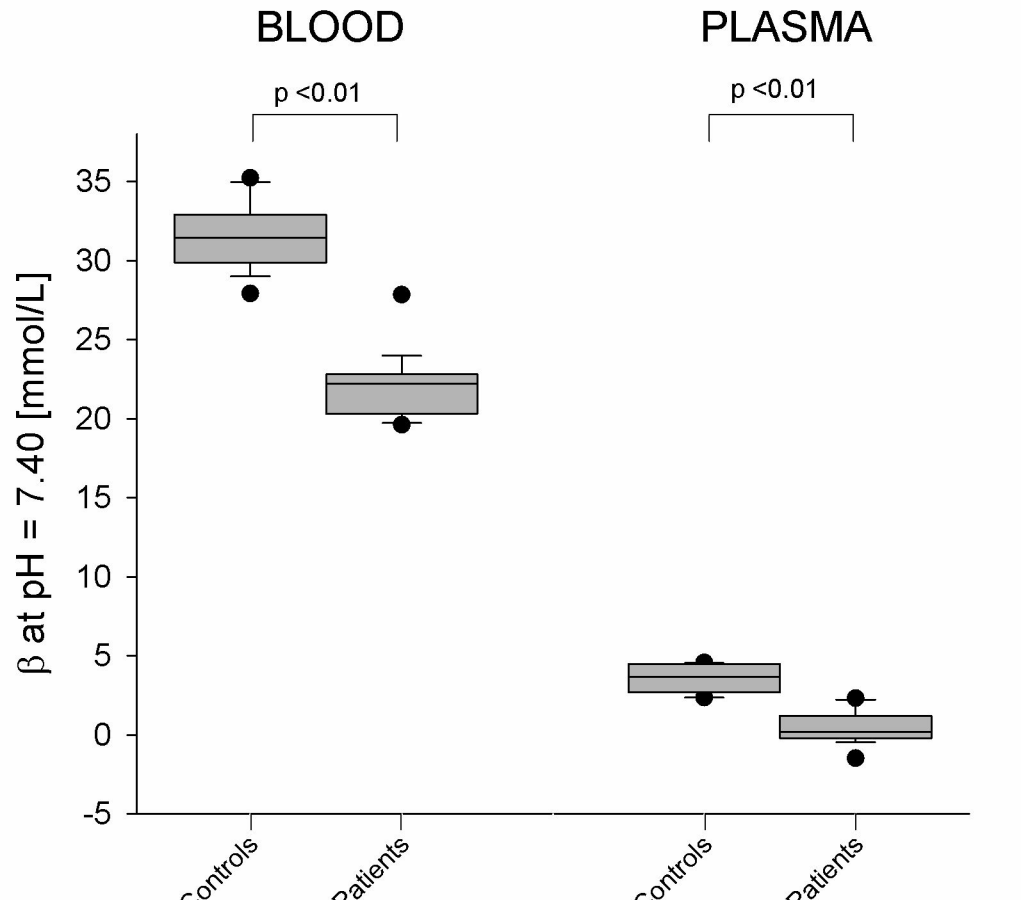
656 **A)** Representative map of plasma proteins after two-dimensional electrophoresis (2-DE). Proteins
657 were separated horizontally by their isoelectric point (plus end: acidic; minus end: alkaline) and
658 then vertically by their molecular weight (MW). Lane 1: protein marker. Lane 2: loading control
659 (10 μg). Inset image: magnification of albumin proteoforms, identified by nine adjacent spots. **B)**
660 Quantification of albumin proteoforms after 2-DE. *FDR<0.05.

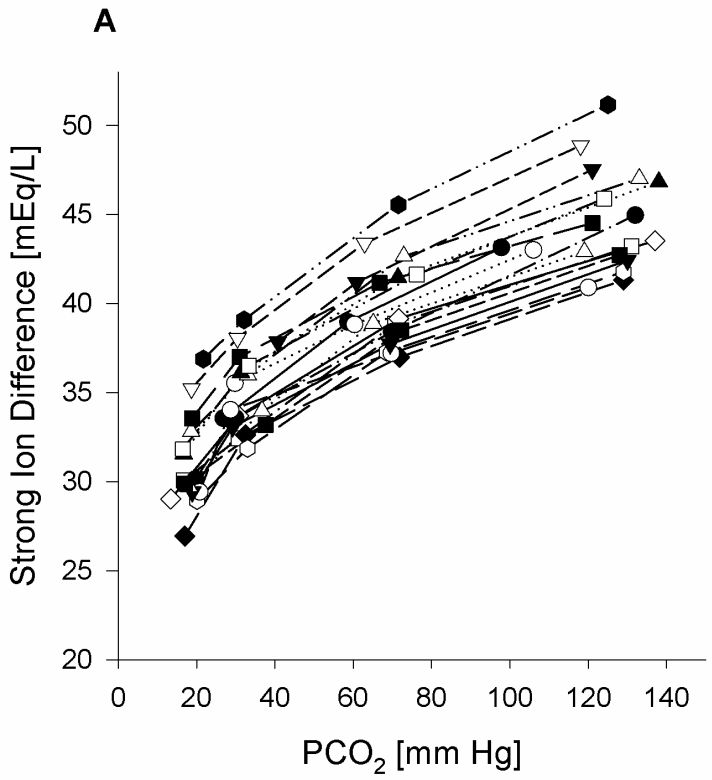
661

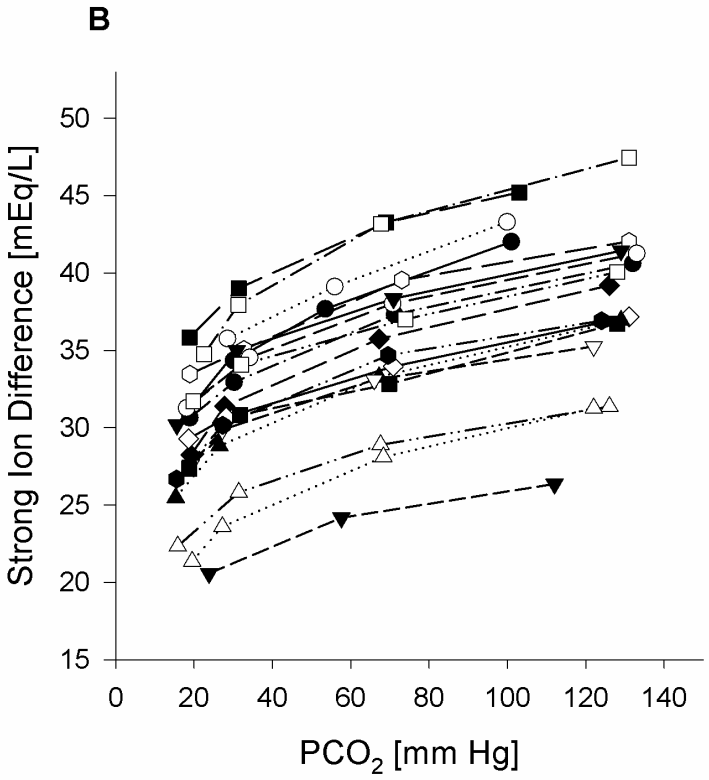
662 **Table 1. Baseline laboratory values and blood gases and electrolytes obtained at 5% of CO_2.**
663 Baseline laboratory data for albumin, total proteins, phosphates and hemoglobin concentrations, and
664 results of gas analysis performed on whole blood at 5% of CO_2. P values refer to t-test or Mann-
665 Whitney rank sum test, as appropriate.

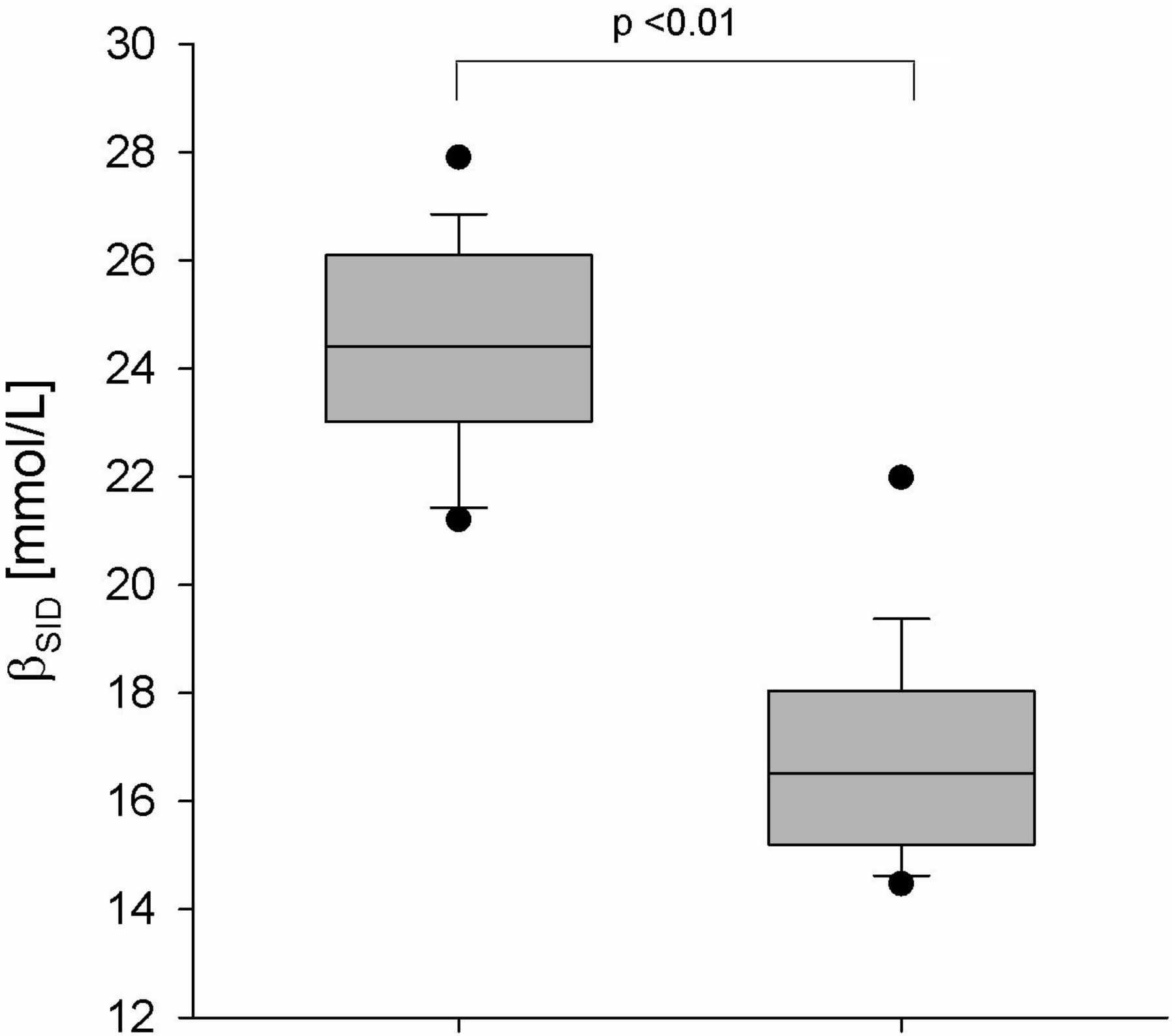


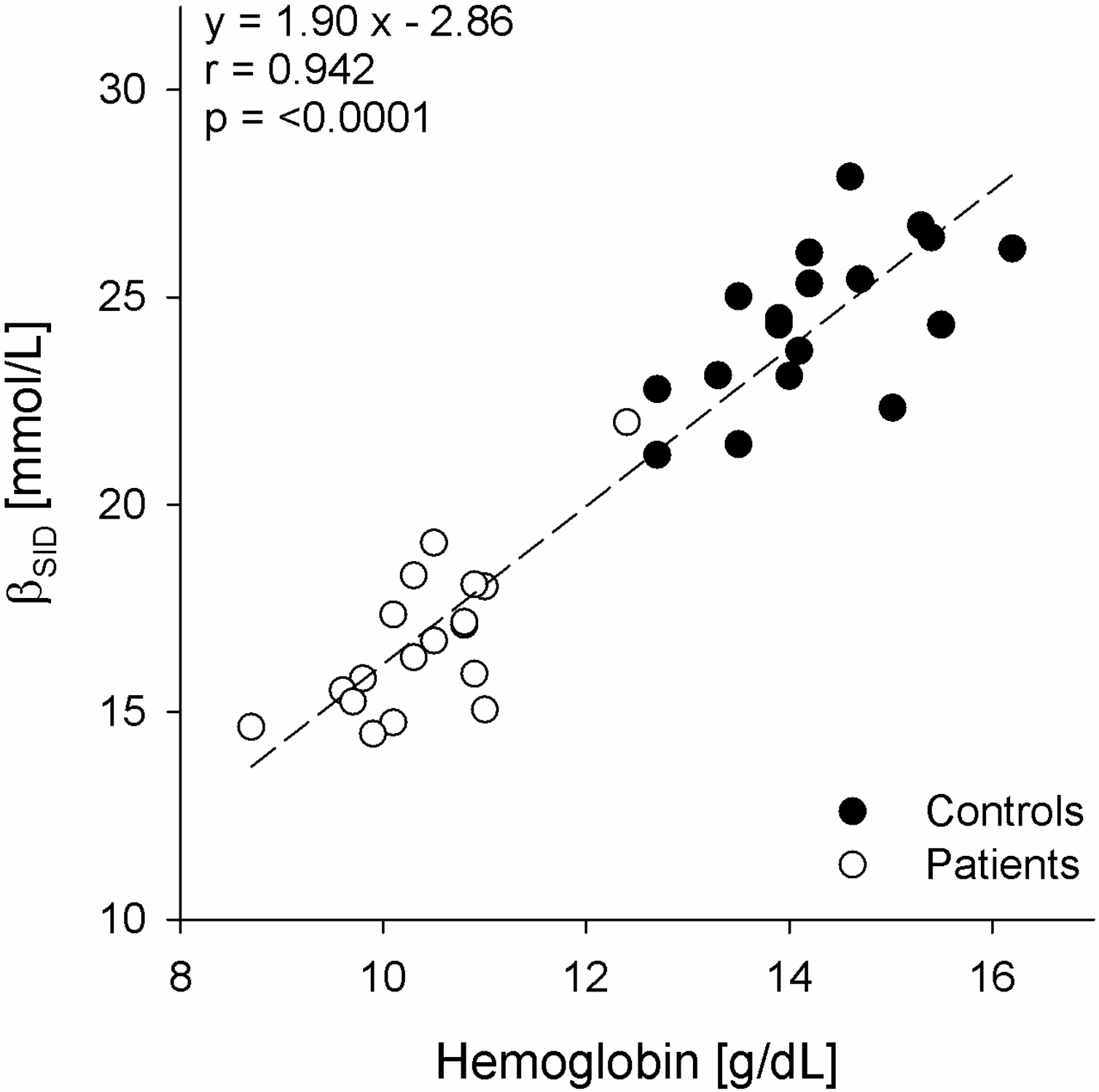


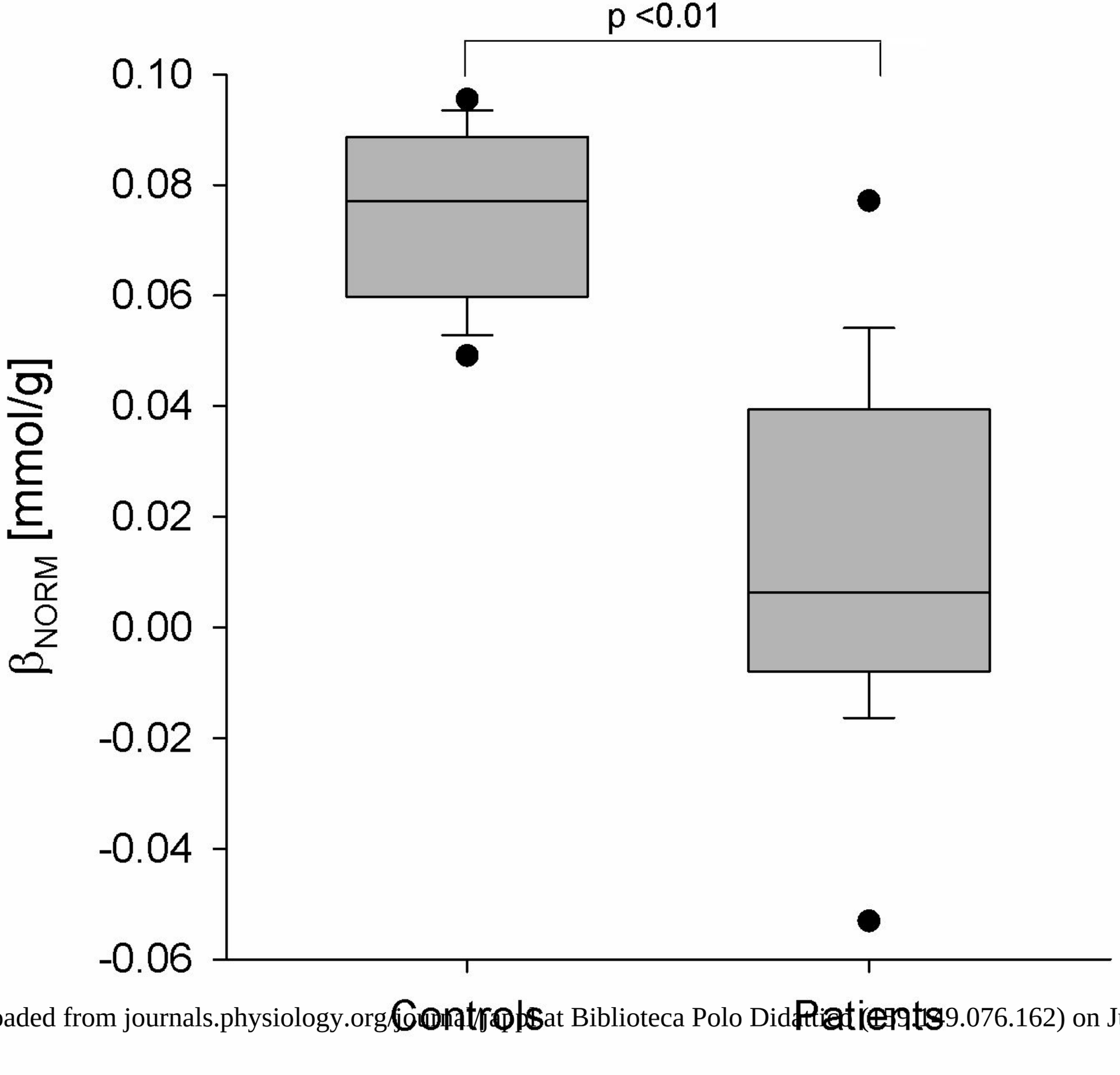




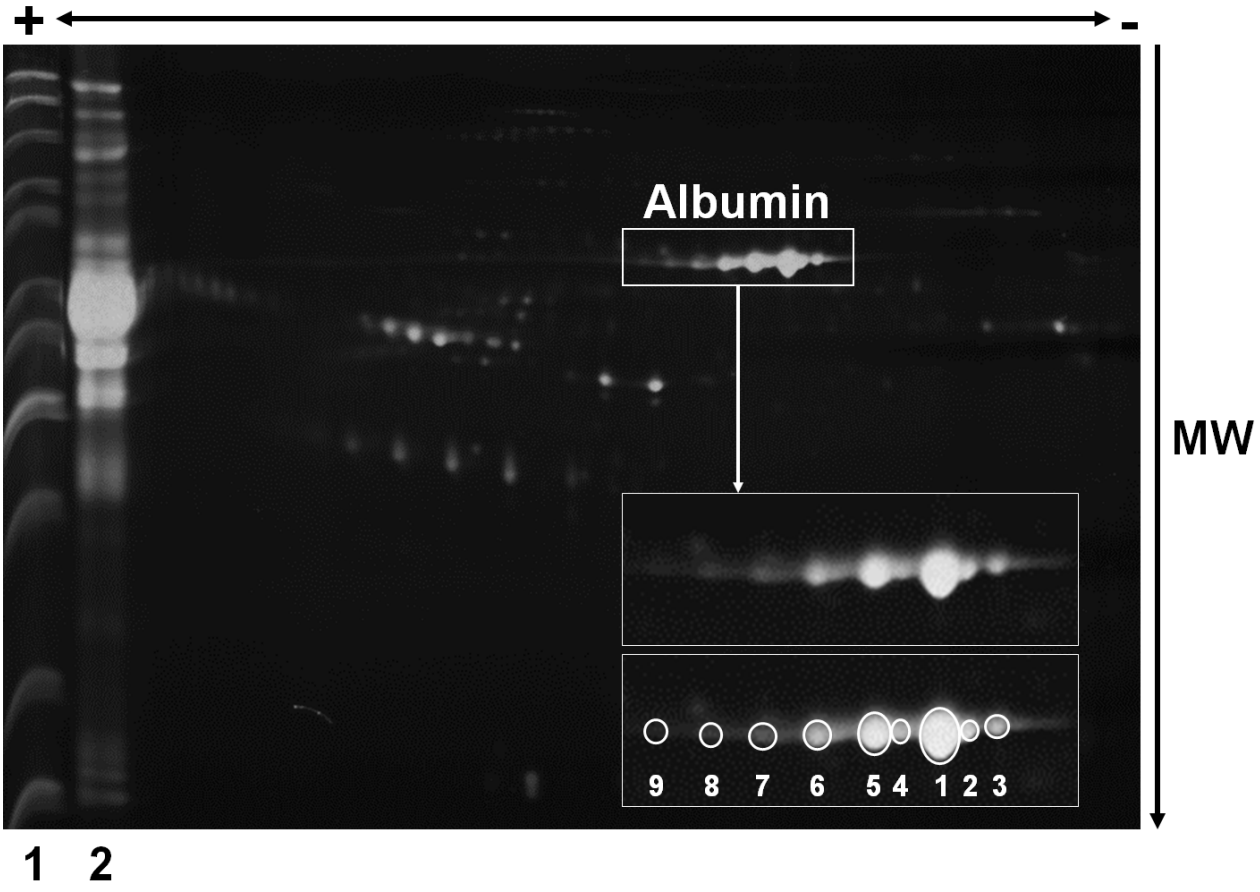








A



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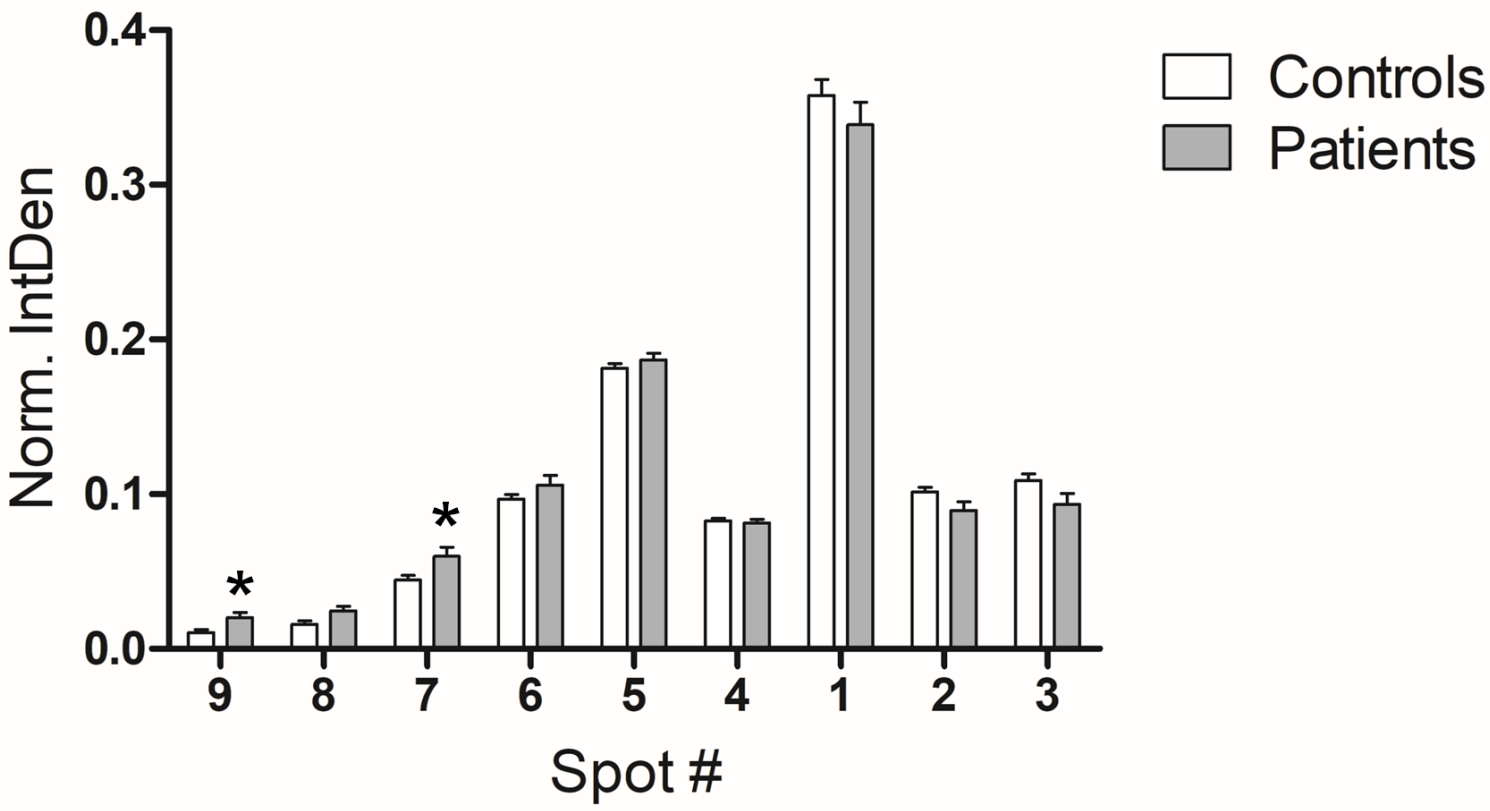

Table 1

	Controls (n=18)	Patients (n=18)	P
Albumin [g/dL]	4.7 [4.6 – 4.9]	3.0 [2.8 – 3.2]	<0.01
Hemoglobin [g/dL]	14.3 ± 1.0	10.4 ± 0.8	<0.01
Hematocrit [%]	41 [40 – 44]	30 [29 – 32]	<0.01
Phosphate [mg/dL]	3.4 [2.9 – 3.5]	4.4 [2.8 – 5.3]	0.12
pH	7.463 [7.446 – 7.477]	7.418 [7.359 – 7.471]	0.11
P_{CO_2} [mm Hg]	31.2 [30.0 – 33.2]	30.2 [27.3 – 31.5]	0.05
HCO_3^- [mmol/L]	20.9 [19.9 – 21.7]	17.9 [14.3 – 21.4]	0.03
Na^+ [mmol/L]	139 [137 - 140]	139 [135 - 144]	0.78
K^+ [mmol/L]	4.3 ± 0.4	4.3 ± 0.6	0.65
Ionized Ca^{2+} [mmol/L]	1.16 [1.13 – 1.20]	1.08 [1.06 – 1.14]	<0.01
Total Calcium [mg/dL]	9.5 ± 0.3	7.8 ± 0.2	<0.01
Magnesium [mg/dL]	2.1 [2.0 – 2.2]	2.0 [1.9 – 2.3]	0.40
Cl^- [mEq/L]	108 [108 – 110]	111 [107 – 113]	0.31
$Lactate^-$ [mmol/L]	1.7 [1.1 – 2.1]	2.0 [1.7 – 5.2]	0.01
SID [mEq/L]	34.0 [33.2 – 36.6]	32.2 [29.6 – 35.0]	0.04
BE [mmol/L]	-1.2 [-2.0 – -0.1]	-4.8 [-9.2 – -0.5]	0.02