Clinica Chimica Acta 495 (2019) 422–428

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Serum albumin saturation test based on non-esterified fatty acids imbalance for clinical employment

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ARTICLE INFO

Keywords:
Albumin
Non-esterified fatty acids
Lipotoxicity

ABSTRACT

Fatty acids are fundamental as energy and structural source to the human cells. They are not usually found free in human circulation. Alteration in fatty acids metabolism is linked to diseases such as diabetes, preeclampsia, heart disease, and some infectious diseases. Increased levels of non-esterified fatty acids (NEFA) may cause cell dysfunction and lipotoxicity. Since physiologically fatty acids are transported bound to albumin, we propose here a simple and cheap test that consists of albumin isoelectric focusing determination to measure the potential systemic NEFA cytotoxicity. For validation of this method, albumin isoelectric focusing in 51 serum samples from 40 critically ill patients and 11 controls was compared with NEFA/albumin ratios measured by HPLC. We called this approach an albumin saturation test. This test may indicate to physicians the potential NEFA lipotoxicity guiding them throughout better patient management. The albumin saturation test can point out serum albumin-NEFA saturation through a cheap assay that could be performed by any care facility.

1. Introduction

Fatty acids (FA) are gathered from human diet and stored in adipose tissue. Changing dietary habits and the modern lifestyle have an impact on lipid metabolism in the worldwide population. Obese patients have elevated plasma levels of non-esterified fatty acids (NEFA) [1] and NEFA alterations are found in pathological conditions such as metabolic syndrome [2], type II diabetes [3], cardiovascular diseases [4], liver diseases [5], renal diseases [6], pancreatitis [6] and neoplasms [7]. Plasma FA (99%) are transported in blood bound to albumin. Each albumin molecule is capable of binding up to 7 FA molecules under optimal conditions [8,9].

High serum levels of NEFA surpass the albumin binding capacity leading to free fatty acid in serum, which may result in tissue damage. Therefore, serum FA/albumin ratio could be used to predict FA toxicity [5]. FA accumulation in tissues may cause cellular disorders called lipotoxicity [10,11]. Lipotoxicity appears in diverse pathological conditions such as trauma [12], sepsis [13], pancreatitis [13], embolism [13], neoplasia [14], vasculopathy [15] and diabetes [6] leading to severe cell dysfunction, apoptosis [16,17] and necrosis [18]. Various mechanisms were attributed to NEFA lipotoxicity including inhibition of Na, K-ATPase pump causing lung injury [19–21]. We showed that in vitro inhibition of Na, K-ATPase pump activity by FA is reversible with albumin addition [22], and this albumin protective effect was also confirmed in cell cultures were albumin prevented toxic effects of FA overloading [23].

NEFA impacts immune system function such as inhibition of lymphocyte activation with immunosuppressive effect [24] and macrophage function [25]. Saturated fatty acid but not unsaturated fatty acid triggers TLR4 signaling [26]. Curiously, a single dose of oleic acid downregulates systemic NEFA in healthy animals [27] and septic animals [28]. Thus, not all fatty acid is toxic, but free saturated fatty acids

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https://doi.org/10.1016/j.cca.2019.05.003
Received 12 June 2018; Received in revised form 15 April 2019; Accepted 4 May 2019
Available online 10 May 2019
0009-8981/

seem to be damaging to cell function.

NEFA lipotoxicity can be acute, such as in severe leptospirosis patients [29], in which we showed a correlation between high levels of NEFA organ dysfunction [21]. Also, critically ill patients usually evolve with low levels of albumin, thus favoring the elevation of free serum NEFA [30], which may contribute to the immune response imbalance. Chronic lipotoxicity takes place in metabolic diseases such as diabetes, metabolic syndrome and obesity because high levels of NEFA interferes with insulin signaling, are toxic to pancreatic cells and may trigger systemic, sustained inflammatory response [31,32].

Herein we propose a simple and low-cost test of albumin isoelectric focusing on measuring albumin-NEFA saturation revealed by specific albumin staining. This assay could be included as a functional test to access the levels of NEFA and its toxic effects in critically ill patients.

2. Material and methods

2.1. Ethical statement

All procedure was followed according to institutional ethical committee of the Hospital Universitário Pedro Ernesto of Universidade do Estado do Rio de Janeiro under the number 14017313.4.0000.5259. We used in our experiments the total of 58 patients and 26 controls.

2.2. Patients and inclusion criteria

All patients are in the intensive care unit and had confirmed sepsis with SOFA score > 2 according to Sepsis-3 [33]. Duplicate serum samples were sequentially collected and kept frozen at −70 °C until the analyses. We studied a total of eighty-four samples taken from: a) 58 patients (39 men and 17 women with age average 56.6 years old) and b) 26 healthy donors (18 men and 8 women with age average 60.4 years old) for NEFA and albumin measurement. For albumin isoelectric focusing we collected 51 serum samples from 40 critically ill patients and 11 controls. All patients received antibiotic therapy and careful crystalloid fluid replacement.

2.3. Schematic protocol

The process is depicted in Fig. 1. The overall process takes 6 h, including two electrophoresis run and sample staining. In the next day after overnight incubation, the pH is checked and matched with samples (Fig. 1 steps 1 to 7).

2.4. Albumin dosage

For the dosage of albumin, we used the green bromocresol reagent, according to the method described by Doumas et al. [34]. For this purpose, we prepared stock solutions of 0.10 M succinate buffer, pH 4.0 adjusted with NaOH, 0.6 mM bromocresol green with 10% final volume in 0.1 N NaOH and 30% Brij-35. The working solution is the mixing of these three solutions in the ratio of 30: 10: 0.16 (v/*v*/v). The standard solutions of bovine albumin (Sigma) were 2.0; 2.5; 4.0; 5.0 and 6.0 g/dL. The dosage was made by adding 1 mL working solution to 5 μL of serum or standard albumin solution. After 10 min at room temperature, the reading was done at 628 nm in a spectrophotometer.

2.5. Albumin isoelectric focusing

The isoelectric focusing method (IF) was chosen in this work because albumin molecules, depending on the number of fatty acids bound, can acquire a new effective electric charge (final load of fatty acids with protein) and also new conformation [35]. Then, depending on its saturation with fatty acids, the albumin acquires new isoelectric points that can be analyzed by the IF.

2.6. Isoelectric focusing methodology

We based on Basu et al., 1978 [36]; briefly gel preparation used 2.0 mL 7% polyacrylamide (bisacrylamide + acrylamide), 3.0 μL TEMED (N, N, N ‘, *N*'-Tetramethylethylenediamine), 2.0 mL distilled water, 4.0 mL of ammonium persulfate 0.14%, 0.2 mL ampholyte (Ampholyte High Resolution pH 4–6/Sigma). Two buffers were prepared, the cathode buffer was 0.4% triethanolamine (TEA), and the anode buffer was 0.2% sulfuric acid (H_2SO_4). After polymerization, the gel was placed in the electrophoretic cell without the samples, and subjected to 200 V, amperage 1.5 mA for 30 min, to form the pH gradient on the gel. For the electrophoretic run, a source of the brand Incibras was used, that develops a voltage of up to 1000 V. After 30 min of this initial run, samples from control sera and from patients were placed in their respective wells. Some wells were left without samples so that the pH of the gel was measured, and thus, the pH gradient could be traced. The second run, after placement of the samples, lasted 210 min at a voltage of 300 V and amperage of 1.5 mA. After the second run the gel was cut and the part containing the samples was stained with a high-affinity albumin dye, bromocresol green (according to modification of the albumin dosage method of Doumas, et al. 1971 [34]), usually used for the determination of serum albumin or plasma). Such dye adapted for the staining of albumin in the gels contained 59.4% 0.1 M succinate buffer/pH 4.0; 0.95% Brij-35 (detergent) 10%; 39.65% bromocresol green 0.6 mM. The final working solution should have a pH of 4.2 ± 0.5. The gel remained in contact with the dye for 1 h. The part of the gel without samples was cut into 12 pieces of 0.5 cm in length each. Each piece was placed in a glass tube, and 2.0 mL of distilled water was added, and finally, the tubes were stored at 4 °C, and the pH was measured the next day in pH meter, in order to define the pH gradient formed.

2.7. Serum samples

About 5 mL of blood was used. After coagulation, the samples were centrifuged at 2000 x *g* for 15 min at 25 °C, and the supernatants (sera) were aliquoted into Eppendorf tubes. Patient and control samples were stored in a freezer at −70 °C.

2.8. Lipids serum extraction

For each 100 μL of serum sample or 16-C:18 long chain fatty acid standards (palmitoleic, oleic, linoleic, palmitic and stearic - which make up about 80 to 90% of total serum fatty acids) was added 10 μL of the internal standard margaric acid (0.5 mg/mL of isopropanol). Then, the mixtures were gentle stirred and 500 μL isopropanol/n-heptane/phosphoric acid mixture (40:10:1 *v*/v) was added. After stirring and 10 min incubation at room temperature, 200 μL n-heptane and 300 μL distilled water were added. It was vortexed and centrifuged at 500 x*g* for 5 min at 25 °C. 200 μL of the upper layer was removed, and this volume was evaporated in 1.3 × 10 cm tubes with a nitrogen cap screw.

2.9. Derivatization of fatty acids

We modified the Dole and Meinertz 1960 protocol [37] according to Puttmann et al., 1993 [38]. To the extracted lipids after drying, 6 μL of the derivatizing catalytic reagent (50 mmol/L bromophenacyl bromide and 5 mmol/L 18-crown-6 in acetonitrile), 500 μL acetonitrile and 1 mg $KHCO_3$ were added. Magnetic bars were inserted and passed the nitrogen stream before the sealing of the tubes with teflon caps. The tubes were kept under stirring for 45 min at 85 °C. The tubes were weighed before and after this shaking period at 85 °C to allow for any loss of volume. In cases of loss by evaporation, the sample was discarded. After stirring, the magnetic bar was removed and the derivatized mixture centrifuged at 15,000 x *g* for 20 min at 25 °C. The supernatant was removed and used for application on the high-performance liquid

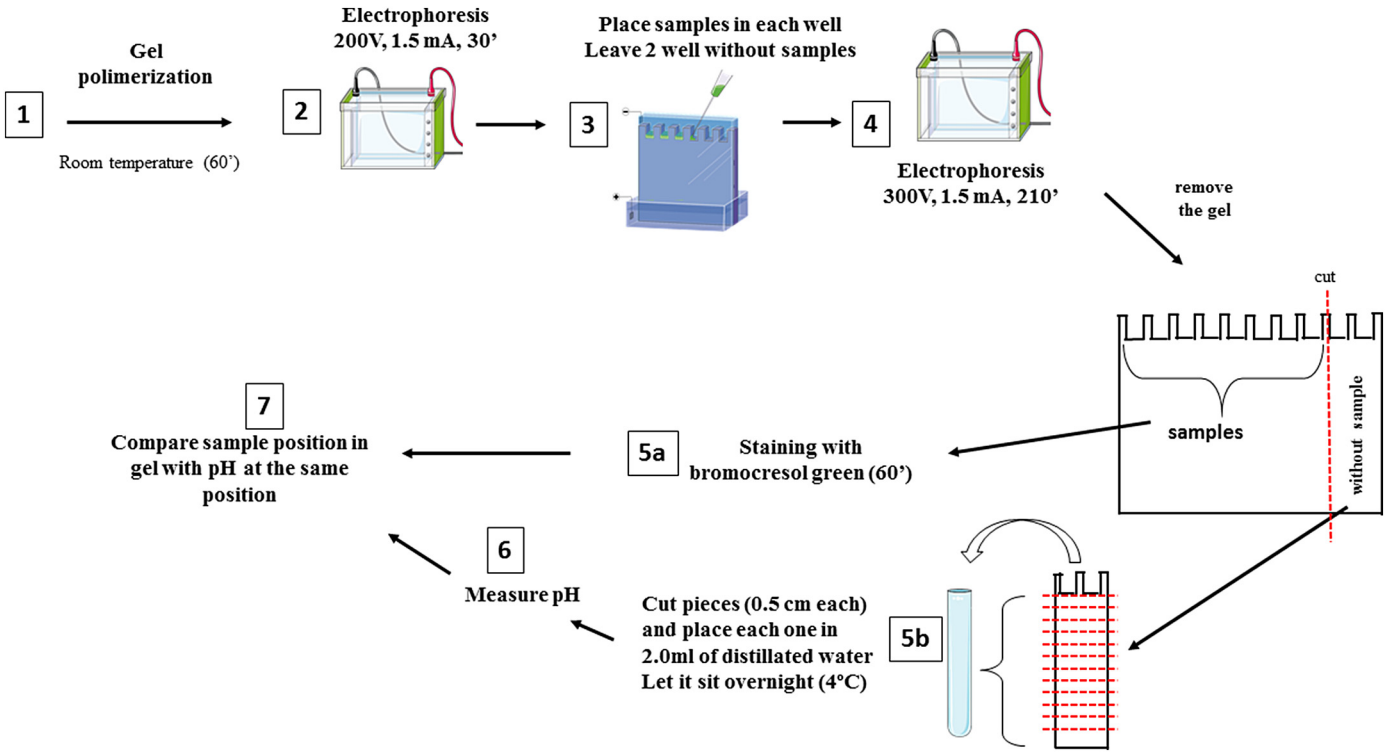

Fig. 1. Experimental schematic drawing. The first step is gel polymerization with the first electrophoresis (1 and 2). Next step is sample application and second electrophoresis (3 and 4). Cut the gel in two pieces, one with two lanes without samples and cut it in pieces and place them in distilled water (5a) and stain the samples in the second gel piece (5b). After measuring pH (6) compare the stained samples position with pH at the same position (7).

chromatography (HPLC) column.

2.10. Plasma NEFA quantification

Plasma concentrations of the predominant NEFA-palmitic, oleic, linoleic, palmitoleic, and stearic acids were determined by HPLC as described by Puttman et al. 1993 [38]. Methodological details were delineated in a prior publication [21].

2.11. Statistical analysis

The results were presented as mean and standard error for quantitative variables with normal distribution. When comparing two groups, the test applied was the Student *t*-test. The Chi-square, Wilcoxon test, Mann Whitney test and linear regression tests were used to compare qualitative and quantitative variables. Differences were considered significant when $P < .05$. The program used for statistical analysis and graphing was Graph Pad Prism 5.1.

3. Results

3.1. Albumin isoelectric focusing on serum from healthy donors and critically ill patients and saturated or not with bilirubin

Albumin plays a protective role for lipotoxicity through the adsorption of NEFA in the blood. Depending on the amount of FA bound to the protein, it acquires a new effective electric charge and also a new molecular conformation. As bilirubin can compete with NEFA for albumin binding sites [39], it was assessed whether the albumin isoelectric focusing profile could be altered at high concentrations of bilirubin. In infectious and noninfectious diseases, the concentration of this pigment may be quite high [21,40]. Healthy donor serum albumin isoelectric focusing without the addition of bilirubin (Fig. 2A) and previously saturated with bilirubin (Fig. 2B). Critically ill patient serum albumin isoelectric focusing without bilirubin (Fig. 2C) and previously saturated with bilirubin (Fig. 2D). No appreciable changes were detected in healthy donors and patient (Fig. 2).

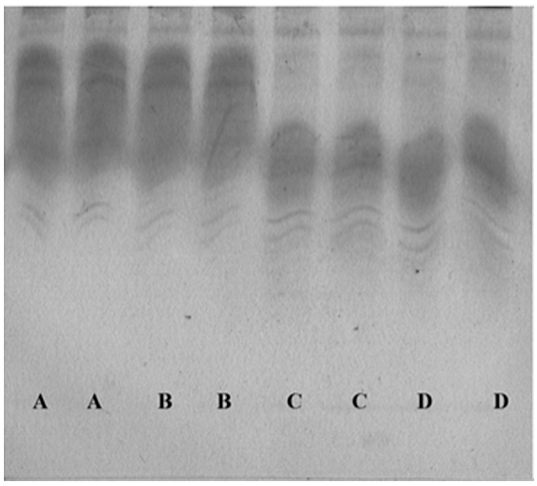

Fig. 2. Albumin isoelectric focusing on health donors and critically ill patients. Healthy donor serum albumin isoelectric focusing without the addition of bilirubin (A) and previously saturated with bilirubin (B). Critical ill patient serum albumin isoelectric focusing without bilirubin (C) and previously saturated with bilirubin (D). The samples were processed according to MM.

3.2. Albumin isoelectric focusing on healthy and critically ill patients

When we compared albumin isoelectric focusing of controls to critically ill patients, the pI changed probably because of high NEFA concentration. In our results, the pI of albumin ranged from 4.8 to 5.66 (from the most saturated to the less saturated with a fatty acid) similar

Table 1
NEFA and albumin serum levels in controls and critically ill patients.

	Media	± Standard deviation
Control total NEFA	485 μmol/L	133 (n = 26)
Critical ill patients NEFA	*1262 μmol/L	243 (n = 58)
Control albumin	860 μmol/L	72 (n = 26)
Critical ill patients albumin	*297 μmol/L	50 (n = 58)

* $P < 0.05$.

to the data from literature [36]. We measured NEFA serum levels from critically ill patients and healthy donors. NEFA were lower in healthy donors compared to critically ill patients (Table 1).

3.3. Correlation of NEFA and albumin isoelectric focusing on controls and septic patients

Absolute values of NEFA from critically ill patients were very high compared to the control group (Table 1), so we evaluated the ratio between NEFA and albumin isoelectric focusing. As NEFA concentrations increase, the pI decreases with a correlation of $R = 0.65$ (Fig. 2a). Most patients present high levels of NEFA, which correlates to lower pI albumin isoelectric focusing. Controls present lower levels of NEFA with higher pH albumin isoelectric focusing, and they are grouped near the Y-axis in the graph (Fig. 3a).

3.4. Correlations of NEFA and albumin molar ratio and albumin isoelectric focusing on control and septic patients

Since albumin binds to NEFA in the blood, we measured the correlation between NEFA/albumin and albumin isoelectric focusing.

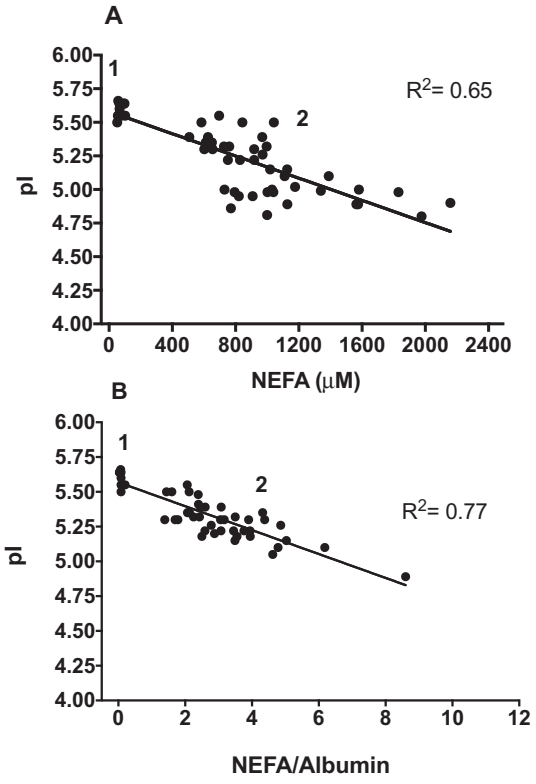

Fig. 3. The pI values of albumin isoelectric focusing correlating to NEFA and to NEFA/albumin ration on septic patients. Each dot represents a person. The controls focused near to Y-axis, and septic patients are scattered along the X-axis. The number of patients is 40 and healthy controls 11. Correlation to NEFA $R^2 = 0.66$ and correlation to NEFA/albumin $R^2 = 0.77$.

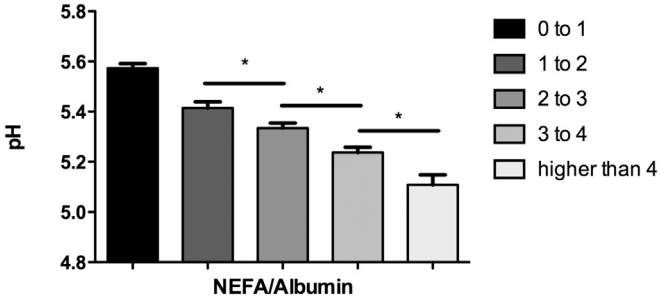

Fig. 4. NEFA/albumin ratio determines the pI values. All samples were analyzed and distributed according to NEFA/albumin ration versus pI values.

Septic patients have higher levels of NEFA and lower molar levels of albumin compared to controls. Our group developed a test called "protection factor" which is an experimental test based on the Na, K-ATPase inhibitory property of NEFA to test the capacity of serum albumin to prevent the cytotoxic effects of NEFA in vitro [21]. So, higher levels of NEFA surpass the ability of albumin to avoid its deleterious toxic effects. Therefore, high levels of NEFA combined with low levels of serum albumin indicate a lower protection factor against NEFA toxic effects. Herein we showed a strong correlation between NEFA/albumin ratio and albumin isoelectric focusing (Fig. 3b) with high NEFA/albumin ratio correlating to lower pI.

3.5. Determination of pI ranges according to the molar ratio NEFA/albumin

Through the obtained values of pI in the isoelectric focusing we scale NEFA/albumin molar ratio in four different grades (Fig. 4). Values of the NEFA/albumin molar ratio between 0 and 1, the average pI found was 5.57 ± 0.01; between 1 and 2, pH = 5.41 ± 0.24; between 2 and 3, pI = 5.35 ± 0.02; between 3 and 4, pI = 5.23 ± 0.02 and above 4, pI = 5.10 ± 0.03. Using the Mann Whitney test the p values between the successive ranges of NEFA/albumin were: ($p < 0.0001$, $p = 0.02$, $p = 0.001$, $p = 0.01$ respectively).

4. Discussion

Literature shows that besides the physiological role, NEFA also have pathophysiological effects implicated on different diseases. The first association described was between elevated NEFA and impaired sensitivity to insulin. The pivotal pathology in diabetes and obesity is the chronic inflammatory syndrome leading to chronically up-regulated immune responses with poor [41] microbial killing efficiency [42]. The mechanism involves a complex interplay between genetic and environmental factors that predispose to insulin resistance and higher circulating levels of blood glucose and NEFA [43,44]. Increased levels of NEFA (derived from excessive dietary intake and increased lipolysis secondary to insulin resistance) stimulate the production of inflammatory mediators and reactive oxygen species (ROS) [45–47]. Likewise, NEFA are increased in pathological conditions such as sepsis [48], thromboembolism [49], leptospirosis [21] and chronic kidney disease [50]. Thus, NEFA seems to be a central pathological mechanism to different pathologies, and they interfere with immune system functions, enzyme inhibition and gene transcription [51,52].

Isoelectric focusing relies on the fact that the molecules are separated according to their effective electric charge [34]. Albumin isoelectric focusing in human serum is influenced by NEFA and electrophoresis time [36]. Depending on the amount of FA bound to the protein, it acquires a new effective electric charge and also a new molecular conformation. Previous work verified that the acidic shift in the isoelectric point of plasma albumin in preeclamptic women is due to increased NEFA binding in these subjects, and albumin avoided NEFA toxic effect on the endothelial cells [53]. Herein we confirmed this shift

because albumin bound to few NEFA focused on higher pH, whereas NEFA-saturated albumin focused on lower pH.

We presented a modification on Basu [36] methodology in which after running the samples we stained with bromocresol green, prepared by modification of Doumas [34]. This dye primarily targets albumin, although some serum alpha-proteins may be interfering.

We could address to potential risks, limitations, and pitfalls, for instance, lack of reproducibility of the IF of albumin can occur due to variations of the experimental conditions. Such variations may change the charge and shape of albumin. Temperature, solvent ionic strength, drugs or endogenous compounds transported by albumin, besides the ampholytes used in the technique, are factors that can lead to such changes, which interferes in the rate of migration and isoelectric point [54]. The addition of denaturing agents, such as urea, promotes the decrease of the isoelectric point of albumin. The time and voltage of the experimental run also interfere with the focused albumin. A long time to run the IF could promote the release of the fatty acids bound albumin. Higher voltage also influences on band sharpness and band distribution [36,55]. Besides, bilirubin ligation to albumin confers the pI to 4.7 [56]. In the pathological situations which albumin saturation occurs due to the large increase in NEFA, all these disturbing factors probably will be minimized. If the experimental conditions are preserved, the results should remain reliable and reproducible.

Potential limitations include critically ill patients usually receive sedation drugs such as the alpha-2 agonist dexmedetomidine, propofol, fentanyl, benzodiazepines, opioids, barbiturics, and antibiotics such as carbapenems, vancomycin, and cephalosporin [57–62]. Large numbers of these drugs are carried by albumin and under physiologic condition may have a charge so interfering with albumin pI. Unfortunately, we have not measured the influence of those drugs in our experiments, although in our results we have got similar albumin pI from patients with different treatment scheme.

Both NEFA and bilirubin bind to albumin and high levels of serum bilirubin can be found in patients with severe leptospirosis [21] and septic patients [40]. As bilirubin can compete with NEFA for albumin binding sites [39], it was assessed whether the albumin isoelectric focusing profile could be altered at high concentrations of bilirubin. We showed that bilirubin serum saturation did not alter the albumin migration pattern in controls and critically ill patient's serum, suggesting that NEFA affinity for albumin is not modified by high bilirubin levels.

Hence, we can conclude that albumin isoelectric focusing was dependent on NEFA saturation. NEFA quantification from critically ill patients confirmed that healthy donors serum have lower levels of NEFA while the patients presented higher levels. Higher albumin saturation directly correlates to lower pH albumin focused. As albumin isoelectric focusing from controls showed higher pH it denotes a negative charge lower than the charge in critically ill patient's NEFA-albumin. Therefore, albumin from healthy subjects will remain at higher pH and reinforces the notion that the lower albumin isoelectric focusing is related to high molar albumin saturation by NEFA in the critically ill patient.

We have to consider that a substantial change in NEFA levels can take part during daytime [63–65], but there is a difference between healthy individual and critically ill patients. In patients where NEFA is higher, the albumin levels are lower. Thus, levels of NEFA are important, but the ration albumin/NEFA is what really matters because when albumin is low NEFA will be potentially lipotoxic (includes chronic adipose tissue inflammation, mitochondrial dysfunction, insulin resistance, lipoapoptosis) [66–71]. In our experience NEFA/albumin ration 3 to 4 means that albumin administration is strongly considered, and at ratio higher than 4 that albumin should be administrated.

Many tests are available for NEFA measurement as NEFA kit assay (from Wako, Zen-Bio, Perkin Elmer, Elab Science, Randox, Abcam, Pacific Biomarkers). All of them are colorimetric assays used for quantification of NEFA. Our method quantifies the NEFA saturation of albumin determined by isoelectric focusing of albumin. It would vary less than the daytime variation of absolute NEFA values, allowing a more reliable use as an indicator of NEFA toxicity.

Determination of serum NEFA and especially the serum NEFA/albumin ratio could impact on clinical practice. Alongside other clinical markers, it might guide physician's decisions on treatment of the critically ill patients, including venous albumin administration. Our suggested novel test should be used as a preliminary assessment. Future tests are still needed, for instance albumin serum measurement because low albumin levels are linked in high mortality risk in hospitalized patients [72]. Herein we suggest albumin supplementation to reach physiological levels. We are in agreement with other authors that support the use of albumin in patients with septic shock in certain conditions [73,74]. Currently, the techniques employed to quantify NEFA profile are not available in clinical practice. NEFA quantification requires whether highly specialized personnel trained in extraction and performing HPLC analysis or expensive NEFA kit assay, which also requires proper equipment to read out the results.We propose this assay to indirectly measure NEFA on critically ill patients promptly available in healthcare facilities.

5. Conclusions and considerations

In this work, we improved and developed a cheap test to indirectly estimate levels of serum NEFA. It consists of serum albumin isoelectric focusing with selective staining. This test also provides information about the albumin-NEFA saturation so it could be categorized as an “albumin saturation test”. In general practice, such a test could be used to evaluate albumin-NEFA saturation, identifying patients at risk for lipotoxicity. Thus this assay could be an accessible alternative to current methodology for serum NEFA evaluation which could be performed by most care facilities and become a useful biomarker for clinical practice.

Competing interests

The authors declare that they have no competing interests.

Funding statement

The funders had no role in study design, data collection and analysis, decisions to publish, or preparation of the manuscript. This work was supported by grants from: Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa Estratégico de Apoio à Pesquisa em Saúde (PAPES) - FIOCRUZ, Universidade Federal do Estado do Rio de Janeiro (Unirio), FIOCRUZ, Universidade Federal Fluminense - UFF and Universidade do Estado do Rio de Janeiro (UERJ). We acknowledge graduate programs: Programa de Pós-graduação em Ciências e Biotecnologia (PPBI) from UFF, Programa de Pós-Graduação em Fisiopatologia Clínica e Experimental – (FISCLINEX) from Faculdade de Ciências Médicas from UERJ, Programa de Biologia Molecular e Celular from Unirio and Programa de Biologia Celular e Molecular from FIOCRUZ. We also acknowledge financial support by the European Community's Seventh Framework Programme (FP7-2007-2013) under grant agreement HEALTH-F4-2011-282095 (TARKINAID), and Programa de Produtividade Científica da Universidade Estácio de Sá.

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