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RESEARCH ARTICLE

Mitochondrial Dysfunction In Intensive Care Unit Patients

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Abstract: ***Background:*** In patients admitted to the Intensive Care Unit (ICU), mortality is high due to multiple organ damage. Mitochondrial dysfunction and impaired oxygen consumption, as causative mechanisms, play a significant role in reducing the activity of immune cells in sepsis, resulting in the progression of the multiple organ dysfunction syndromes (MODS). The evaluation of mitochondrial function in critical care patients in the immune cells, especially in lymphocytes, could reveal the target point that determines mitochondrial failure.

Objective: To find the relationship between mitochondrial reactive oxygen species production (mROS), mitochondrial membrane potential (ΔΨm), and mitochondrial oxygen consumption (mVO2) in peripheral plasma lymphocytes collected from ICU patients. We also compared these three characteristic mitochondrial functions with C-reactive protein (CRP), serum lactate, and central venous saturation (SvO_2) that would enable the prediction of the ultimate outcome.

Methods: Isolated lymphocytes from 54 critical care patients with SIRS by sepsis and non-sepsis etiologies were analyzed with flow cytometry by staining with dihydroethidium and JC-1, measuring mROS, ΔΨm, and mVO_2. Clinical variables, such as serum lactate (mmol/L) and C-reactive protein (mg/L) from peripheral blood, were measured in the first 24 hours of admission. A confounding analysis was performed using logistic regression, and a p-value of <0.05 was considered statistically significant.

Results: It has been confirmed that there is a drastic increase in reactive oxygen species (ROS) and mVO2 in critically ill patients immediately after exposure to the insult pathogen-associated molecular pattern /damage-associated molecular pattern (PAMPS/DAMPS) and continued for the first 24 hours thereafter. The results showed no significant alterations in the mitochondrial membrane potential (ΔΨm) compared with the lymphocytes in controls. A significant correlation between CRP and SvO2 and a strong positive relationship between CRP, values above 3 mg/l, and white blood cells were observed.

Conclusion: Lymphocytes from patients with SIRS displayed higher mitochondrial respiratory capacities and reactive oxygen species production compared with controls. Clinical markers of inflammation indirectly evaluate the mitochondrial function, most of which have been validated in a clinical setting.

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1. INTRODUCTION

Multiple organ damage is the principal cause of death in critically ill patients associated with sepsis [1-3]. The initial phase of sepsis is characterized by a prompt increase in circulating pro-inflammatory cytokines triggering the systemic inflammatory response syndrome (SIRS) [4, 5]. To counteract the acute inflammatory response, anti-inflammatory mediators are released, and, therefore, the host defense system is suppressed, which leads to activation of the compensatory anti-inflammatory response syndrome (CARS). Lymphocytes play a central role in modulating the sepsis response and orchestrating the inflammatory response in the innate and adaptive immune system [6]. In the latter stage of CARS, the function of immune cells such as lymphocytes, neutrophils, etc., may downregulate, leading to an anergy (the inability to respond to recall antigens) [7]. Immunoparalysis is suggested to decrease the clearance of septic foci and could leave the septic patient more vulnerable to deleterious secondary infections, as well as reactivation of latent infections [8-10].

In addition, the inflammatory response is also characterized by an increase in cellular immunological activity, generating a high-energy supply, and promoting a catabolic state. An energetic imbalance has a negative impact on the immune response, leading to poor results since the cells of these patients are apparently unable to maintain the immunometabolism and, consequently, develop an

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energy deficit. This energy failure, in turn, can lead to cell death and, ultimately, compromise the patient's life [11].

Mitochondrial dysfunction as a causative mechanism plays a significant role in reducing the activity of immune cells in sepsis, resulting in the progression of multiple organ dysfunction syndrome (MODS) [12]. Therefore, *in vivo* evaluation of mitochondrial function in critical care patients in the immune cells, especially in lymphocytes, could reveal the target point that determines mitochondrial failure. The mitochondrial reactive oxygen species production (mROS) [13], mitochondrial membrane potential ($\Delta\Psi m$) [14], and mitochondrial oxygen consumption (mVO_2) [15] are key factors to evaluate the mitochondrial function in immune cells of ICU patients, especially during the acute phase of inflammatory response.

The objective of this study was to evaluate the three main characteristics of mitochondrial function in peripheral plasma lymphocytes (blood immune cells) collected from ICU patients with SIRS during the first 24 hours. These three characteristics are compared with traditional markers of hypoxia and metabolic stress, such as C-reactive protein (CRP), serum lactate, and central venous saturation. These factors may identify potential survivors and could aid in reducing the heavy economic burden associated with adverse outcomes.

2. MATERIAL AND METHODS

2.1. Study Population

This prospective, observational study was conducted at the San Ignacio University Hospital in Bogotá, Colombia (Pontificia Universidad Javeriana, Bogotá D.C. Carrera 7 No. 40, Colombia) from January 2016 to March 2017. The study protocol was approved by The Human Research Ethics Committee and the Institutional Review Board of Javeriana University, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Informed written consent was obtained from all patients or family members, as well as controls, enrolled in the study.

Fifty-four (54) patients who entered the ICU during the first 24 hours were divided into two cohorts: septic and non-septic patients. The inclusion criteria were as follows: age over 18 years with two or more of the following signs of SIRS: fever (temperature >38ºC or <36ºC), tachycardia (heart rate >90 beats per minute), tachypnea (respiratory rate >20 breaths per minute), partial pressure of carbon dioxide in the blood ($PaCO_2$) at <32 mmHg, white blood cell count above $12x10^9$ cells per liter or below $4x10^9$ cells per liter of immature forms, infectious diagnosis, or positive blood culture.

Patients were excluded who were under 18 years of age, known to be or suspected of being pregnant, had a body mass index (BMI) greater than 25, family history of primary mitochondrial disease, were under treatment with drugs that could affect mitochondrial function (see Table **1**), or had a known chronic disease (hypertension, diabetes, and cancer). Data were collected daily during patient hospitalization from the time of admission until 28 days or death. The control group consisted of 10 healthy volunteers, matched by the age and BMI with septic patients, without known morbidities.

2.2. Data Collection

Medical records provided demographic data, comorbid conditions, source of infection, laboratory and microbiology results, use of vasoactive infusions, mechanical ventilation, antimicrobials administration, length of stay in ICU, and vital status. The criteria that were adopted to define sepsis were based on the Surviving Sepsis Campaign Guidelines [16].

These data were organized using a standard form for analysis. Sequential Organ Failure Acute Physiology and Chronic Health Evaluation (APACHE II) scores were calculated [17-19]. A daily evaluation of organ function was performed according to the sequential organ failure assessment (SOFA) score, and organ failure was defined as a SOFA subscore >2 for the organ dysfunction.

2.3. Clinical Variables and Blood Mitochondrial Function

Clinical variables such as serum lactate (mmol/L) and C-reactive protein (mg/L) from peripheral blood were measured in the first 24 hours of admission. In order to evaluate mitochondrial parameters, a 10-ml blood sample was drawn from the central line catheters as soon as possible after obtaining consent, but not later than 24 hours after admission to the ICU. Analyses for mitochondrial dysfunction were performed on fresh samples immediately upon collection. The variables of mitochondrial function were measured in lymphocytes isolated with the Ficoll-Hipaque technique and labeled with anti-CD45 antibody (Dako Corporation, USA).

2.4. ROS Production Measurement

Flow cytometry and staining with dihydroethidium (DHE) were used to evaluate the production of superoxide anions in the lymphocytes of each patient. The cells of both populations were stained with 2.5 uM DHE for 30 minutes, washed with PBS, and acquired in the Guava-EasyCyte capillary cytometer 6-2L. The analysis of the mean fluorescence intensity for DHE was reported.

2.5. Mitochondrial Membrane Potential Measurement

Flow cytometry was used to evaluate the mitochondrial membrane potential in lymphocytes by J-C1 staining. The cells were isolated from whole blood by Ficoll gradient centrifugation and were stained with JC-1 2.5 µg/ml for 30 minutes, washed with PBS, and acquired in the Guava-EasyCyte 6-2L capillary cytometer. The relationship between the mean fluorescence intensity from red to green was reported.

2.6. Oxygen Consumption

To evaluate oxygen consumption (mVO2) in lymphocytes of each patient, cytometry was used. The cells were isolated from whole blood by Ficoll gradient centrifugation. After phosphate-buffered saline, lymphocytes were transferred to three Eppendorf tubes for patients and controls: Tube 1 contained untreated cells (control); Tube 2 contained cells treated with 100 µM pimonidazole, and Tube 3 contained cells treated with 100 µM pimonidazole and NaCN 5 mM.

The cell suspensions were covered with 500 µl of mineral oil and incubated for 2 hours at 37°C. After incubation, the cells were recovered and transferred to new tubes. After 2 washes with PBS, the cells were fixed with 1.6 % formaldehyde at room temperature for 15 minutes and permeabilized with 100% cold methanol at minus 20°C overnight. The next day, the cells were washed twice with PBS supplemented with 2 percent FBS and incubated with 1:200 anti-pimonidazole antibody at room temperature for 1 h. After 2 washes with PBS, the cells were acquired in the Guava-EasyCyte 6-2 L capillary cytometer. The mean fluorescence intensity was recorded for emission at 525 nm and excitation at 488 nm.

2.7. Statistical Analysis

The statistical analysis was carried out with the IBM® SPSS statistics software version 25 for Windows (Armonk, NY, USA). One-way ANOVA was performed to evaluate whether there were significant differences in the physical variables between the ICU patients and the controls. Student's t-tests were used to evaluate the difference in the distributions of markers of inflammatory and mitochondrial function. Correlations were performed using linear regression. A p-value of <0.05 was considered statistically significant. Logistic regression analysis was used to evaluate the univariate and multivariate association between the septic state (positive versus negative) and the inflammatory and mitochondrial function

Table 1. Physical characteristics of the study group.

Characteristics	Control (n = 10)	ICU Patients		*p*-Value
		Non-Septic (n = 29)	Septic (n = 25)	
	Mean ± SD	Mean ± SD	Mean ± SD	
Age (years)	30.6 ± 13.4*	58.8 ± 18.2	58.6 ± 18.2	**< 0.0001**
Weight (m)	65.2 ± 11.3	64.7 ± 10.3	62.5 ± 13.6	0.752
Height (Kg)	1.68 ± 0.1	1.63 ± 0.1	1.64 ± 0.1	0.369
BMI (Kg/m^2)	22.9 ± 2.1	24.2 ±2.9	23.2 ± 3.9	0.435

SD, Standard Deviation; p < 0.05 was considered significant and are depicted in bold; p - values were calculated by the One-way ANOVA; *, Significance difference with ICU patients.

Table 2. Conventional clinical inflammatory markers distribution in ICU patients.

Characteristics	ICU Patients		*p*-Value
	Non-Septic (n = 29)	Septic (n = 22)	
	Mean ± SD	Mean ± SD	
CRP	13.7 ± 10.9	19.2± 15.3	**0.042**
Leukocytes	13.2 ± 8.6	12.7 ± 8	0.821
Lactate	1.9 ± 1.2	2 ± 1.1	0.642
SvO_2	68.7 ± 11.3	75.7 ± 13.1	**0.048**
SOFA	8 ± 2.5	8.1 ± 4.1	0.991
APACHE II	12 ± 5.6	14.4 ± 7.6	0.212

Key: CRP, C Reactive protein; SD, Standard Deviation; p < 0.05 was considered significant and are depicted in bold; p - values were calculated by Student's t-test.

markers. The odds ratio (OR) was used as an association force index and the 95 percent confidence limits (95% CL) were calculated. Statistics with p<0.05 were considered statistically significant. All statistical tests were bilateral (two-tailed).

3. RESULTS

3.1. Patients Selection Criteria

Fifty-four (54) patients were recruited in ICU: 25 septic patients (46.3%), 29 non-septic patients (53.7%). The patients included were predominantly male (32). The mean age of the study subjects did not vary between the two groups. Ten (10) volunteers composed the control group. All patients (septic and non-septic) had inflammatory or post-operative clinical conditions (abdominal, cardiac or orthopedic surgery), acute respiratory failure, and trauma. In addition, septic patients had abdominal sepsis (acute appendicitis 10 and 5 with acute diverticulitis) or pulmonary sepsis (5 patients with pneumonia). The physical characteristics, such as age, weight, WCI for the study population are shown in Table **1**. Gram-negative microorganisms were isolated in all septic patients. The septic group had a higher rate of in-hospital mortality (37.03%).

3.2. Clinical Conventional Inflammatory Markers

Conventional inflammatory clinical markers in septic and non-septic patients were analyzed. The mean C-reactive protein (CRP) and venous oxygen saturation (SvO_2) were higher in the patients with septic than in the non-septic patients, confirming their usefulness as predictors of the severity of inflammation (p=0.042 and p=0.048). Other inflammatory markers showed no significant differences when comparing septic and non-septic patients. The results of the complete clinical conventional inflammatory markers are presented in Table **2**.

3.3. Mitochondrial Function Variables

Three mitochondrial functions were evaluated: ROS production, mitochondrial membrane potential ($\Delta\Psi m$), and mitochondrial oxygen consumption (mVO_2). A significant increase in superoxide anion production (mROS) (p=0.0001), as well as mitochondrial oxygen consumption (mVO_2) (p=0.011), was found in ICU patients compared to control subjects (Fig. **1A** and **1B**). However, there were no significant differences in mitochondrial membrane potential ($\Delta\Psi m$) (p = 0.336).

3.4. Biomarkers Distribution in Septic *vs*. Non-Septic ICU Patients

Significant correlations were found between sepsis and selected markers: there was a significant correlation between C-reactive protein (CRP) and venous oxygen saturation (SvO_2). There is a strong positive relationship between C-reactive protein (CRP) and leukocytes (Fig. **2**). Other inflammatory markers, such as lactate, leukocytes, SOFA, and APACHE II, as well as markers of mitochondrial function, did not show significant differences (p > 0.05) in the presence of sepsis (Table **2**).

3.5. Biomarkers Distribution in SIRS *vs*. Non-ICU Controls

Taking these clinical and mitochondrial markers together, logistic regression was performed to evaluate their predictive ability. The presence of SIRS in patients increased significantly with CRP (OR = 1.1, p = 0.046). Similarly, the results indicated a significant correlation between SvO_2 and the presence of SIRS (OR = 1.1, p = 0.048).

The univariate and multivariate analyses of the severity of inflammation as predictors showed that the increase in C-reactive protein (CRP) and venous oxygen saturation (SvO_2) maintained a

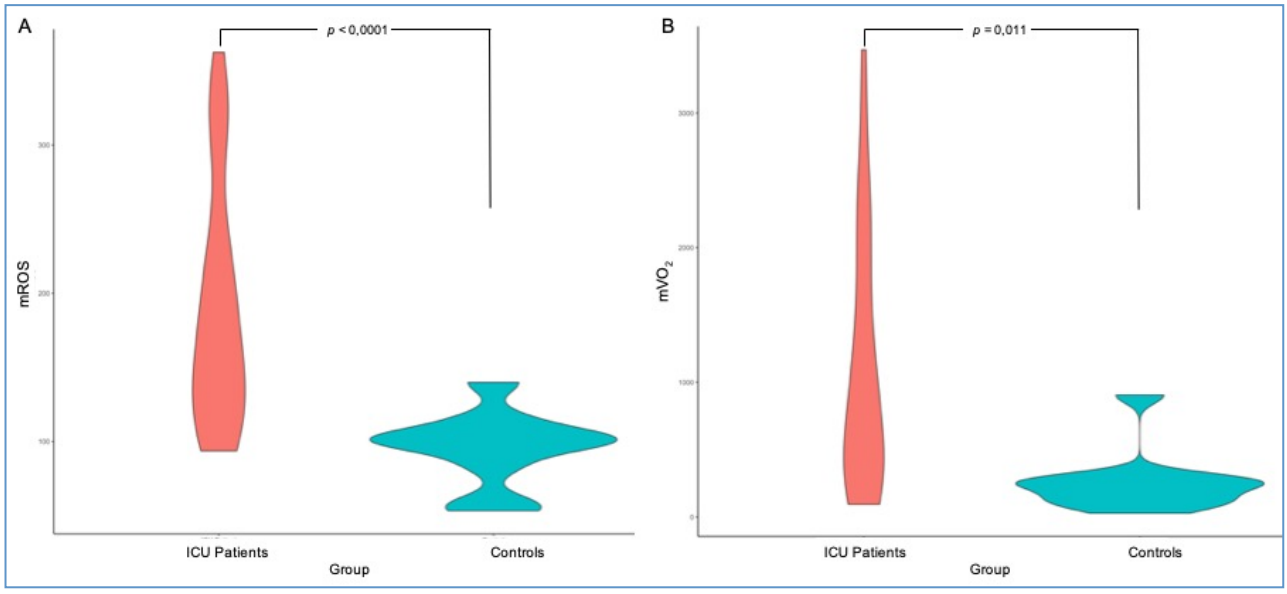

Fig. (1). (A) Median value of mROS in ICU patient's *vs*. control, **(B)** Median value of mVO2 in ICU patient's *vs*. control. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

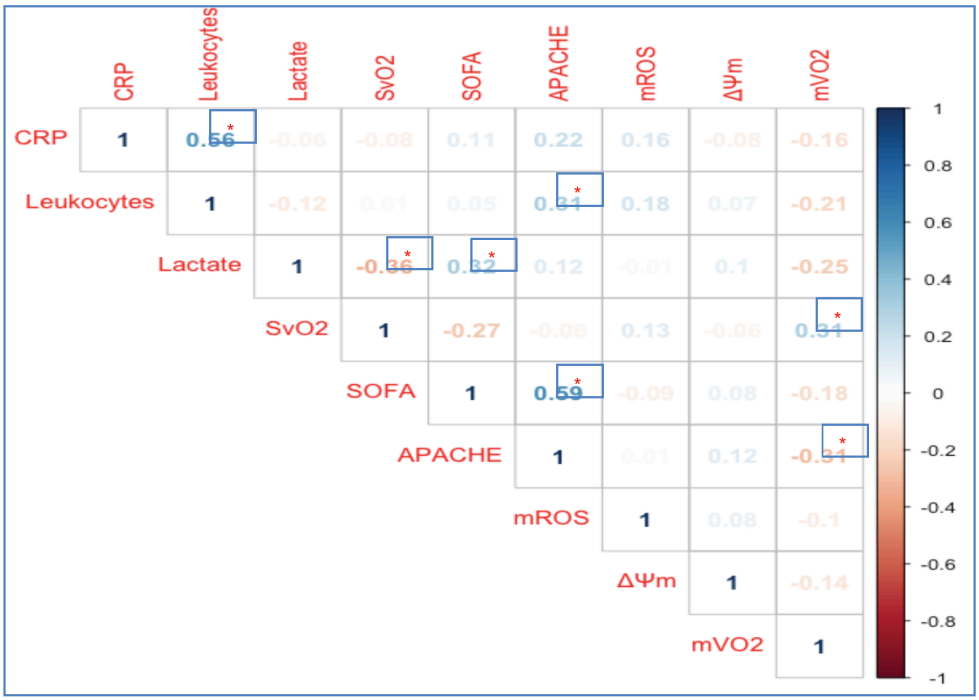

Fig. (2). Correlation coefficients for biochemical and mitochondrial markers. Blue color indicates a positive relationship; red is negative**. ***, Significance difference with ICU patients. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

statistically significant correlation (OR = 1.1, p = 0.036 and OR = 1.1, p = 0.032, respectively).

We also find that the presence of SIRS was not associated with superoxide production (mROS), membrane mitochondrial potential (ΔΨm), or mitochondrial oxygen consumption (mVO_2) (Table **3**).

The area under the curve (AUC) for the CRP and SvO_2 scores was 0.742 (p<0.001), and the cut-off point was associated with the best sensitivity for CRP above 3 mg/l (Fig. **3**).

The results of this study revealed a sensitivity of 59.1 percent (95%, confidence interval 48.8-68.4) and showed a specificity of 70.8 percent (95% CI 56.5-87.3) with respect to CRP and venous oxygen saturation. (SvO_2) is a marker of SIRS (Fig. **3**). The positive predictive value (PPV) for this marker was 77.7 percent (95% CI 57.8-90.2) and the negative predictive value (NPV) was 59.1 percent (95% CI 36.9-78.5).

4. DISCUSSION

In sepsis, mitochondrial dysfunction and deficient oxygen consumption play key roles [20,21]. It has been shown that the magnitude of inflammation correlates with mitochondrial dysfunction both in human and experimental models [22]. Changes in mitochondrial structure and function have been described in several cell types isolated from septic patients, but not in alive patients as in the present study [23].

Table 3. Association analysis between SIRS status in ICU patients and inflammatory and mitochondrial functional markers.

Characteristics	Univariate			Multivariate		
	OR	**95% CI**	***p***	**OR**	**95% CI**	***p***
CRP	1.06	0.99 - 1.12	**0.046**	1.07	1.01 - 1.14	**0.036**
Leukocytes	0.99	0.92 - 1.06	0.817			
Lactate	1.12	0.69 - 1.83	0.636			
SvO_2	1.05	0.99 - 1.11	**0.048**		1.01 - 1.12	**0.032**
SOFA	1.00	0.84 - 1.19	0.990			
APACHE	1.06	0.97 - 1.16	0.189			
mROS	0.99	0.92 - 1.01	0.795			
ΔΨm	1.02	0.58 - 1.77	0.952			
mVO_2	1.00	0.99 - 1.01	0.500			

CRP, C Reactive protein; OR, Odds ratio; CI, Confidence Interval, SD, Standard Deviation; mROS, mitochondrial reactive oxygen species; ΔΨm, mitochondrial membrane potential; mVO_2, mitochondrial oxygen consumption; $p < 0.05$ was considered significant and are depicted in bold; p - values were calculated by Student's t-test.

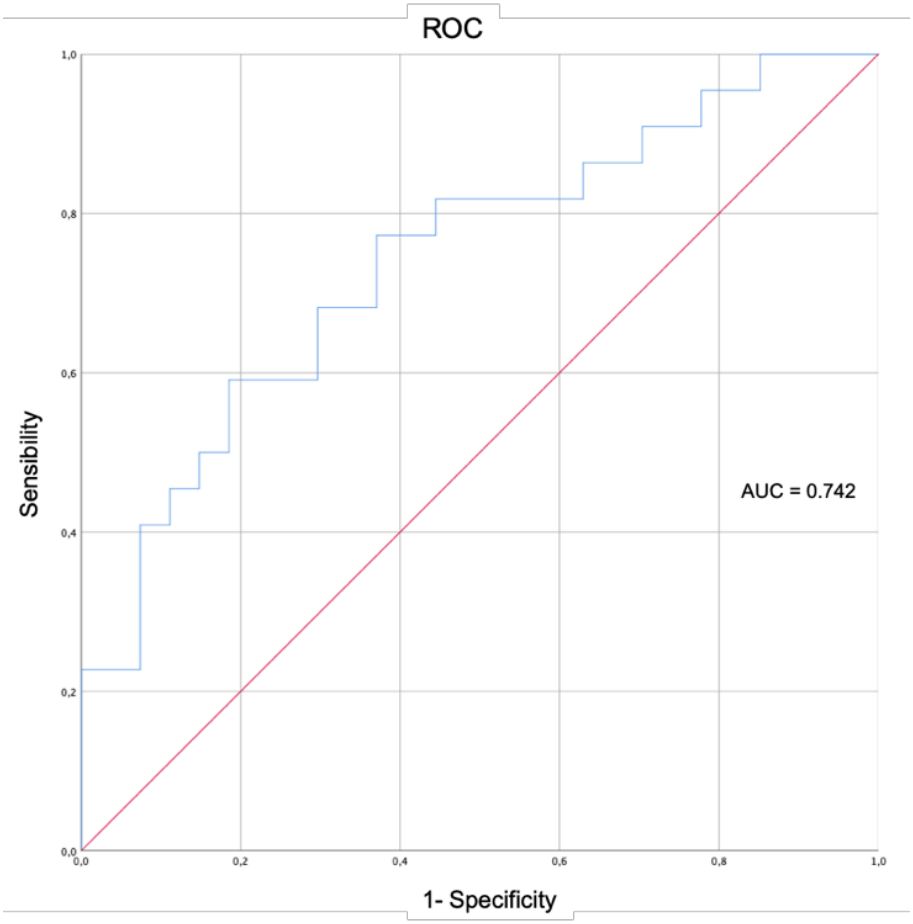

Fig. (3). ROC curves showing AUC of 74% for C-reactive protein and SvO_2 for predicting pattern in sepsis. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

It is known that the mitochondrion is a *good* sensor of warning signals and can trigger an inflammatory response. One of the most powerful warning signs is a change in mitochondrial DNA [15]. During the so-called *"overwhelming sepsis,"* the increased flow of Ca^{2+} in mitochondria leads to mitochondrial dysfunction, concomitantly resulting in the release of cytochrome C and cell death [24]. In this context, mitochondrial lesions most likely release mito-DAMP; DAMP (associated molecular patterns of danger/ damage) through TLR receptors to initiate a protection mechanism through the MyD88/TRADD/NF-kB and JAK1 / STAT3 pathways [25].

Septic plasma samples have shown increased amounts of mtDNA and inflammatory cytokines. A correlation between adverse outcomes in sepsis and levels of extracellular mtDNA has been demonstrated [22]. Since mtDNA can activate the NLRP3 inflammasome, its role in inflammation is evident. In acute inflammation, mitochondria activate, through mROS, important transcription factors involved in inflammation as hypoxia-inducible factor-1a (HIF-1a), NF-κβ, p53, and adenosine monophosphate (AMP)-dependent protein kinase (AMPK) [26].

Another characteristic of inflammation is the positive regulation of factors inducible by hypoxia, vascular endothelial growth factor (VEGF), and glycolytic enzymes to maintain ATP production, and evidence of ROS regulation. [27,28]. Consequently, mROS not only function as signaling molecules but also as powerful initiators of the innate immune response through the activation of NRLP3 [29] (discussed above) and through modulation of the current Toll-like receptor (TLR) downstream in its activation pathways, such as tumor necrosis factor (TNF) mediated by NF-kB activation [30].

In SIRS, one of the cornerstone features is the endothelial dysfunction. The endothelium is not a membrane between body compartments, it is considered an organ endowed with paracrine and autocrine properties. The endothelium is one of its main targets of ROS, and its dysfunction not only favors hemodynamic instability but also enhances inflammatory response and could be considered the end-stage of the process, leading to more morbi-mortality.

The interest in correlating clinical parameters with mitochondrial markers has increased over the last five years. In 2015, a group of international experts with distinguished clinical and scientific backgrounds (23 experts spanning 5 continents) addressed several questions about mitochondrial physiology within the context of acute illness [31]. They concluded that the relationship between acute organ failure and mitochondrial dysfunction had not been elucidated fully. Nevertheless, oxidative stress contributes mainly to mitochondrial dysfunction, making mitochondria an important source of ROS (mROS).

In vivo assessments of mitochondrial function in immune cells provide an excellent opportunity to reveal their behavior in acute inflammatory environments. Inflammatory response implies a compromise of cellular metabolism, with mitochondria being the main cellular entity involved, given its function of producing energy as well as other important functions [32].

To the best of our knowledge, based on a thorough review of the literature, this observational study is the first to evaluate mitochondrial function *in vivo* in humans during the first 24 hours after admission to the ICU. The study used flow cytometry [33], which is a sufficiently validated technique, to evaluate mitochondrial function either from isolated mitochondria or within cells.

Our results showed three main mitochondrial dysfunctions, including ROS production, mitochondrial membrane potential ($\Delta\Psi m$), and mitochondrial O_2 consumption (mVO_2). These markers were assessed in circulating lymphocytes, both septic and non-septic, and correlated with traditional clinical parameters such as lactate, SvO_2, and C-reactive protein. We confirmed ROS as the main increased factor in critically ill patients immediately after exposure to insult (PAMPS / DAMPS) without alterations in ($\Delta\Psi m$) and in mVO_2.

In the first 24 hours of inflammation in otherwise healthy people, without previous mitochondrial dysfunction (*e.g.*, obesity or other metabolic disorders), an acute inflammatory attack (infectious and non-infectious) results in an increase in the production of mitochondrial ROS and an increase in mVO_2 without compromising mitochondrial membrane potential. Our data confirm that the white blood cell count, CRP, and venous oxygen saturation are accurate markers of acute inflammation.

CRP is an acute-phase protein produced by the liver, although it can also be synthesized by other cells like alveolar macrophages. An accumulating number of studies have showed a moderate relationship between the number of organ failures in septic patients with CRP [34-36]. The CRP plasma concentration appears to reflect the magnitude of the inflammatory stimulus and sepsis severity [37].

High levels of SvO2 can be associated with intrinsic failure in cellular respiration and impairment of oxygen consumption, known as cytopathic hypoxia. The cytopathic hypoxia leads to impairment of mitochondrial oxidative phosphorylation and reduces aerobic adenosine triphosphate (ATP) production [38,39] which potentially causes sepsis-induced organ dysfunction. In septic patients with organ failure, high ScvO2 values may be a marker of unfavorable outcomes [40].

ROS production occurs early in SIRS, as has been demonstrated to have a key role in immunity. In this way, the mitochondria are involved in the inflammatory response caused by an infection (in our cases, sepsis) and those caused by injuries (surgery, burns and *sterile or called aseptic* inflammation). This response is through the production of ROS.

Finally, a healthy mitochondrial population maintains its viability in terms of normal $\Delta\Psi m$. Several responses allow the mitochondrial network to adapt to stress and lose membrane potential. These include fission and mitochondrial fusion, mitophagy, and mitochondrial biogenesis [41]. The $\Delta\Psi m$ loss may be a final stage of mitochondrial dysfunction, with irreversible consequences due to an energy failure. Until now, monitoring of mitochondrial health has been limited to experimental work.

These findings corroborate what tissue samples and *in vivo* studies have reported. We confirm that mtROS are the main mitochondrial effectors in the inflammatory response. The magnitude of their production determines the quality of the response in terms of its regulation. The unregulated or exaggerated response is strongly associated with high mortality rates. Following the recommendations of international experts (see above statement), this study is the first to measure, in real-time, *in vivo* three main markers of mitochondrial characteristics. These findings suggest the merit of future investigations of interventions in mitochondrial regulation. For instance, *in vivo* assessment of AMPK [42], or SIRTUIN has shown positive regulation in mitochondrial structural stabilities and function.

CONCLUSION

The mitochondria are the *master* regulators of the immune response both in acute and chronic inflammation scenarios. It initially regulates by mROS, as we have shown in our cohort of critically ill patients. If there are pre-existing mitochondrial impairments (*e.g.*, obesity, diabetes and/or shock), then energetic failure develops earlier in the course of the disease's progression, which is expressed by loss of its membrane potential. This favors early FOM, which, in turn, increases patient's mortality rates. If the damage is beyond recovery, it is mitochondria that determines cellular fate by activating signaling pathways of *"metabolic rescue"* mediated by AMPK and SIRTUINS and/or by activating cellular death pathways (Bcl-xL/Bcl-2-associated death promoter).

Clinical markers of inflammation indirectly evaluate the mitochondrial function, most of which have been validated in the clinical setting. Lactate is likely the most validated; however, physicians generally do not discriminate important characteristics of patients, such as age, SvO_2 and CRP, which could influence its plasma value and not just inflammation. The results obtained from the current study can serve as a basis and/or as an initial point to broaden the knowledge of the key participation of mitochondria in the context of acute critical care.

AUTHOR CONTRIBUTIONS

Juan Carlos Ayala (JCA), Adriana Grismaldo (AG), Andres Felipe Aristizabal-Pachon (AFAP), Ludis Morales (LM)- data acquisition, organization of the database, statistical analysis, JCA, AG, AFAP, LM, Elizaveta V. Mikhaylenko (EVM), Vladimir N. Nikolenko (VNN), Liudmila M. Mikhaleva (LMM), Siva G. Somasundaram (SGS), Cecil E. Kirkland (CEK), and Gjumrakch Aliev (GA) — contributed to the design of the study, conception of the study, and writing the manuscript. SGS, CEK, GA, LM and EVM prepared the preparation and editing of the manuscript. All authors contributed to manuscript revisions and approved the submitted version.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics approval was provided by the Ignacio University Hospital in Bogotá, Colombia (Pontificia Universidad Javeriana, Bogotá D.C. Carrera 7 No. 40, Colombia).

HUMAN AND ANIMAL RIGHTS

Human Study: Ethics approval was provided by the Human Research Ethics Committee and the Institutional Review Board of Javeriana University, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Informed consent was obtained from all patients or family members, as well as controls, enrolled in the study.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

Gjumrakch Aliev was employed by GALLY International Biomedical Research Consulting LLC, San Antonio, Texas, USA. All other authors declare no competing interests.

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Declared none.

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