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Progression of peripheral blood mononuclear cell mitochondrial function during the early phase of sepsis in Intensive Care Unit patients

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Take home message

The current findings reveal an unexpected association between basal cell functioning and clinical outcomes in sepsis during the first week of ICU admission. Contrary to existing hypotheses, we demonstrated a substantial increase in mitochondrial respiration parameters in septic patients compared to controls, with an association between a progressive increase in basal respiration and 3-month mortality.

LIST OF ABBREVIATIONS

ADP	Adenine diphosphate
ATP	Adenine triphosphate
APACHE II	Acute physiology and chronic health evaluation II
BMI	Body mass index
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
FEV1	First second forced expiration
FVC	Forced vital capacity
ICU	Intensive care unit
HR	Hazard ratio
IQR	Interquartile range
LMR	Lymphocyte-monocyte ratio
LOS	Length of stay
mNUTRIC	Modified nutrition risk in critically ill score
OXPHOS	Oxidative phosphorylation system
PBMC	Peripheral blood mononuclear cells
RCR	Respiratory control ratio
SARS-CoV-2	Severe acute respiratory coronavirus 2
SD	Standard deviation
SOFA	Sequential Organ Failure Assessment score
TCA	Tricarboxylic acid
VIF	Variation inflation factor

WUR	Wageningen University and Research
ZGV	Ziekenhuis Gelderse Vallei (Gelderse Vallei hospital)

Abstract

Sepsis is associated with high rates of multiorgan failure and mortality. Altered mitochondrial function is an essential component of the early sepsis syndrome. However, its progression over time in peripheral blood mononuclear cells (PBMCs) is thus far unclear. Our purpose was to investigate this in the early phase of sepsis in ICU patients.

A single-centre prospective observational cohort study was conducted in sepsis patients and compared with age- and sex-matched controls. Mitochondrial function was measured in PBMCs thrice during the first ICU week. RT-qPCR was used for semi-quantitative analysis of expression of genes involved in oxidative phosphorylation. Secondary endpoints included associations between mitochondrial function and (I) sepsis severity and (II) clinical outcomes, including 3-month mortality.

Basal, ATP-linked, maximal and proton leak associated respiration were increased in sepsis patients (n=25) compared to matched controls (n=24) at all time points. This was associated with increased expression of *SDBH* (complex II) and *ATP5F1A* (complex V). Increased basal respiration was associated with 3-month mortality (HR 3.794, 95%CI 1.018-14.149, p=0.047). No differences were observed in other secondary outcomes.

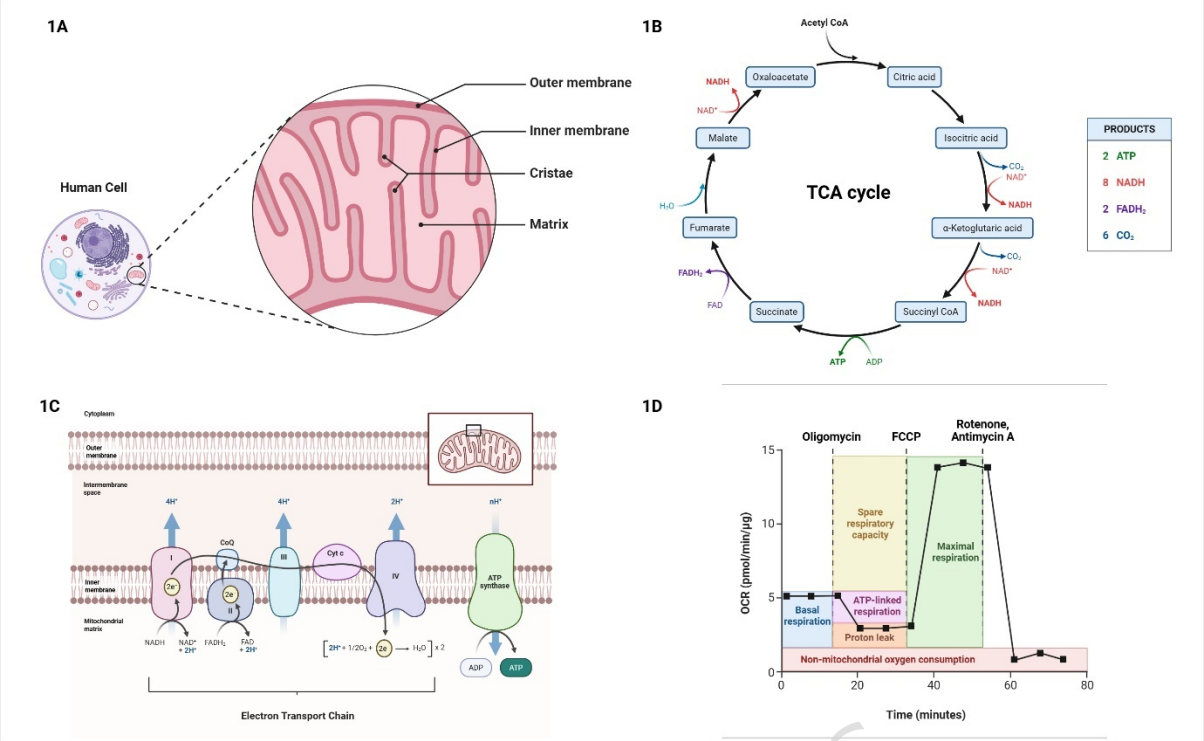
PBMC mitochondria were shown to have an increased respiratory rate during the first week of sepsis. Moreover, a progressive increase was negatively associated with 3-month survival.

Background

Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection, is a primary reason for admission to an Intensive Care Unit (ICU) [1]. Sepsis often contributes to (multi)organ failure and is associated with an average 30-day mortality of up to 35% of septic shock cases, accounting for about 20% of all global deaths in 2017 [2,3]. Sepsis survivors are at an increased risk of post-hospital discharge morbidity, mortality and a markedly reduced quality of life, which may last years after hospital discharge [4,5]. A lack of known therapeutic targets partly explains these poor clinical outcomes.

There is increasing evidence for the role of altered mitochondrial function in the pathogenesis of sepsis-associated multiple organ dysfunction syndrome [6-8]. Mitochondrial respiration is the set of metabolic reactions and processes requiring oxygen at one of the final steps of the oxidative phosphorylation system (OXPHOS) in mitochondria to convert the energy stored in macronutrients to ATP [9-11] (**Figure 1**).

Figure 1. Schematic overview of ATP production in a mitochondrion via the process of the citric acid cycle and oxidative phosphorylation



A. Mitochondria are organelles found in most human cells, the primary function of which is to generate energy in the form of adenosine triphosphate (ATP) through respiration.

B. The tricarboxylic acid cycle, also known as Krebs cycle, consumes acetate (in the form of acetyl-CoA) and water and reduces NAD+ to NADH, releasing carbon dioxide. The NADH generated by the tricarboxylic acid cycle is fed into the oxidative phosphorylation (electron transport) pathway.

C. The electron transport chain (ETC) in the cell is the site of oxidative phosphorylation (OXPHOS). The NADH and succinate previously generated in the tricarboxylic acid cycle are oxidized, releasing the energy of electron transport to power the ATP synthase.

D. Schematic of the contribution of the key parameters of OXPHOS to the mitochondrial oxygen consumption rate over time after addition of mitochondrial inhibitors.

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Mitochondrial respiration can be used as a marker to assess the primary function of mitochondria (**Figure 1D**). A decreased mitochondrial respiration has been demonstrated in various cells in septic ICU patients, including muscle tissue and blood platelets [7-10,12-16]. However, studies that measured mitochondrial function in peripheral blood mononuclear cells (PBMCs), which play an essential role in the initial (hyper)inflammatory response that hallmarks sepsis, have resulted in

conflicting results. Human PBMCs are isolated from peripheral blood and identified as any blood cell with a round nucleus (i.e. lymphocytes, monocytes, natural killer cells and dendritic cells) [17]. Several studies reported a decreased mitochondrial function in PBMCs during sepsis [7,18], while others contrastingly reported an increased mitochondrial function [6,19]. One study even reported an increased mitochondrial respiration, but concomitantly, an increased mitochondrial uncoupling leading to reduced ATP-linked respiration [20].

Methodological differences, such as varying control groups and respiration mediums, might explain the inconsistency in the results of these studies. Furthermore, in two out of four studies, a measurement at only one time point was performed in each patient, which does not create insight into the progression of mitochondrial function in PBMCs during ICU stay [7,18]. This limitation is unfortunate since performing multiple measurements during ICU stay may reveal time-dependent effects of sepsis on mitochondrial function in PBMCs and its association with clinical outcomes. The two studies that did perform two measurements per patient, show conflicting results regarding correlation between mitochondrial respiration and clinical outcomes [7, 19].

Rationale

We set out to fill several knowledge gaps based on previously reported studies. To be able to investigate whether mitochondrial derangements originate from the PBMCs themselves, we opted to resuspend the PBMCs in a standardized medium, not plasma. Secondly, we performed repeated mitochondrial respiration measurements during the first week of ICU stay, as studies assessing the potential time-dependent effects of sepsis on mitochondrial function in PBMCs more than twice are lacking. For mechanistic underpinning, the expression of key genes involved in oxidative phosphorylation was also assessed using RT-qPCR. Lastly, we

calculated correlations between clinical outcomes and mitochondrial function changes to reveal potential time-dependent associations between mitochondrial function and clinical outcomes.

Materials and methods

Study design and setting

A prospective, observational single-centre cohort study with an age- and sex-matched control group was conducted at Gelderse Vallei Hospital (Ede, The Netherlands) between January 1, 2018, and January 27, 2023. Due to closure of the research laboratories during the SARS-CoV-2 pandemic, study inclusions were temporarily halted between March 14, 2020, and October 1, 2020. PBMC measurements were performed at Wageningen University and Research (Wageningen, The Netherlands).

Study participants

Patients (aged ≥18 years) admitted to the ICU with sepsis and/or septic shock were eligible for inclusion. According to the Third International Consensus Definitions, sepsis was defined as a new life-threatening organ dysfunction caused by a dysregulated host response to microbiologically confirmed or clinically suspected (supported by laboratory or radiology findings) infection, as identified by an increase in the sequential organ failure assessment (SOFA) score of ≥2 points. Septic shock was defined as the need for vasopressors to maintain a mean arterial pressure of ≥65 mmHg and serum lactate levels >2 mmol/L (>18 mg/dL) in the absence of hypovolaemia [1]. The control group was recruited from metabolically healthy short-stay hospitalised patients planned to undergo elective surgery for benign non-inflammatory indications, and individuals visiting the outpatient (fracture) clinic, individually matched for age (± 2 years) and sex [21]. Further exclusion criteria can be found in the **Supplemental File**.

Study objectives

The primary study objective was to investigate the progression of mitochondrial respiratory function in PBMCs in septic ICU patients during the first week of ICU admission. Secondary objectives were to investigate the association between mitochondrial respiratory function and (I) sepsis severity and (II) clinical outcomes, including ICU-, hospital and 3-month

mortality, length of ICU and hospital stay (LOS) and duration of mechanical ventilation.

Data collection

This study used PBMCs to measure mitochondrial respiratory function.

Sepsis group

Arterial blood samples were collected at three time points via an indwelling arterial access: day 1-2 (24-48h), day 3-4 (72-96h) and day 5-6 (120-144h) after ICU admission (indicated with T1, T2 and T3), respectively.

Control group

The control group underwent blood sampling by venepuncture once during their visit to the outpatient clinic or short-stay hospitalisation prior to surgery.

PBMC isolation, washing and counting for high resolution respirometry

Blood samples for PBMC isolation to be used for high resolution respirometry were collected in sodium citrate buffered cell preparation tubes containing a ficoll solution and centrifuged at 1000g for 30 minutes at room temperature. Next, PBMCs were resuspended in warm (37°C) 10mL of Hank Balanced Salt Solution (HBSS) without $CaCl_2$ or $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 400g for 10 minutes at room temperature. The supernatant was then removed, and this washing step was repeated twice. After washing, the resulting PBMC pellet was resuspended in 1 mL of warm (37°C) Seahorse XF base medium supplemented with 2 mM glutamine and 25 mM glucose. The PBMCs were counted using the Cellometer auto T4, and cell viability was assessed by mixing 10 µL of cells with 10 µL acridine orange and propidium iodide stain. PBMCs were then immediately used for high-resolution respirometry.

High-resolution respirometry

Two to five million live PBMCs were injected into a chamber of the Oroboros O2K (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria). The chamber volume was set to 2mL and filled with Agilent Seahorse XF Base medium supplemented with 25 mM glucose and two mM glutamate, and the pH was set to 7.4. The temperature within the chamber was set to 37°C and stirring speed to 750 rotations per minute. Oxygen concentration is continuously measured, recorded and used to calculate oxygen flux per one million live PBMCs using DatLab Software 4.3 (Oroboros Instruments, Innsbruck, Austria) (**Figure 1**).

After injection of the PBMCs, the basal respiration was recorded first. Second, the complex V inhibitor, oligomycin, was added (2.5 µM), which induced a state in which respiration is primarily to compensate for proton leakage. Third, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added repeatedly (20 nM) until maximum mitochondrial respiration was reached. Fourth, the complex I inhibitor rotenone and the complex III inhibitor, antimycin A, were added (0.5 µM and 2.5 µM, respectively) to determine non-mitochondrial respiration. Each step of the function profiling test was recorded after respiration had stabilised. Additionally, three parameters were calculated. ATP-linked respiration was calculated by subtracting leak respiration from basal respiration. Coupling efficiency was calculated by dividing ATP-linked respiration by basal respiration. Spare respiratory capacity was calculated by subtracting basal respiration from the maximal respiration. Measurements were considered to be failed when the blood sample yielded too few cells to perform the primary study measurements, if there was no basal respiration signal or if there was no measurable reaction in respiration to the addition of CCCP or one of the inhibitors.

PBMC isolation and lysing for RT-qPCR

After the isolation of PBMCs as previously described, PBMCs were washed three times with 10 mL of cold (4°C) HBSS and centrifuged at 400 g for 10

minutes at 4°C. After washing, the PBMCs were resuspended in 300 µL of RLT buffer (Qiagen, Hilden, Germany) with 1% (v/v) ß-mercaptoethanol (BME) (Sigma-Aldrich, St. Louis, MO, USA). Lysed PBMCs were stored at -80°C.

Real-time quantitative polymerase chain reaction

For gene expression analysis, samples from 10 septic ICU patients and 10 matched controls were used. Samples of other subjects were excluded due to insufficient quality or concentration of RNA in at least one of the three timepoints (T1, T2 or T3). All lysates were homogenized by passing it six times through a blunt 20-gauge needle (0.9 mm diameter). Total RNA was extracted from PBMCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and RNA quantity and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). A total of 500 ng of purified RNA was reverse transcribed into complementary DNA (cDNA) using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA), following the manufacturer's instructions. The reaction mixture included 500 ng RNA, 1 µL of iScript reverse transcriptase, 4 µL of 5x iScript reaction mix, and RNase/DNase-free water to a final volume of 20 µL. Reverse transcription was carried out in a C1000 Touch™ Thermal Cycler (BioRad, Hercules, CA, USA) with the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Pooled cDNA stock was created by mixing 5 µL from each individual cDNA sample to be used for generating calibration curves. Each cDNA sample was then diluted 1:100 before performing real time quantitative reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed to assess the expression of genes involved in mitochondrial OXPHOS system (*NDUFB8*, *SDHB*, *UQCRC2*, *MTCO2*, and *ATP5F1A*). The housekeeping genes *RPS15*, *CSNK2A2*, and *ACTB* were used as internal controls. All primers targeted all known isoforms of the respective genes and are listed in **Supplemental Table 1.** The qRT-PCR reactions were conducted using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA)

with an end volume of 25 µL consisting of 12.5 µL of Supermix, 8.5 µL RNase/DNase-free water, 1.0 µL each of forward and reverse primers (10 pmol/µL), and 2 µL of 100x diluted cDNA. Amplification was performed on a C1000 Touch™ Thermal Cycler under the following conditions: 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds and 58°C for 45 seconds, followed by melt-curve analysis. Relative gene expression levels were normalized with the three reference genes and all mean gene expression values of the patients were compared to the controls.

Additional data sources

Data collection from the electronic medical record systems MetaVision® (iMDsoft, Tel Aviv, Israel) and NeoZIS® (MI Consultancy, Katwijk, The Netherlands) included baseline patient characteristics (including disease severity scores), laboratory values and outcome parameters, such as duration of mechanical ventilation and length of ICU and hospital stay.

Study size

Japiassú *et al.* previously studied mitochondrial oxygen consumption in PBMCs of septic ICU patients (n = 20) and critically ill postoperative control patients (n = 18) [7]. The respiratory control ratio (RCR) was significantly reduced in the septic ICU patient group compared to the control group. The difference in the RCR between day 1 and day 7 was approximately half of the difference between the study and the control group. Assuming the changes in mitochondrial oxygen consumption develop linearly during sepsis, the expected difference (effect size) between the measurements at the three different time points during the first week, is similar to 50% of the observed differences by Japiassú et al. Therefore, to achieve a power of 0.95 with a two-sided significance level of 0.05, 30 subjects per group were needed (as calculated with G*power, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany).

When three valid measurements from an ICU subject could not be obtained due to withdrawal of consent, death, early ICU discharge or technical

problems, patients were not matched with a control subject. Moreover, additional patients were included consecutively until complete measurements were obtained in 30 pairs. Separate measurements from patients who did not have three complete consecutive measurements were excluded from mitochondrial function progression analyses (**Figures 3 and 4**). Patients with valid measurements at T1 and T3 were included in the cox regression models and analyses comparing survivors vs. non-survivors (**Figures 5 and 6**), which served as exploratory analyses.

Statistical analyses

Data verification was conducted manually. Descriptive statistics were performed for demographic and clinical data of all patients and the primary outcome. Normality was assessed numerically and graphically. Continuous values were reported as means with standard deviations (SD; parametric data) or medians with interquartile ranges [IQR; non-parametric data]. Discrete data were presented as proportions (%). A complete-case approach was applied for each analysis, with patients included only when all variables required for that specific analysis were available. Differences in baseline characteristics and clinical outcomes between the sepsis and control group were assessed using the independent samples t-test, Wilcoxon rank sum, Wilcoxon signed rank, chi-squared, or Fisher's exact tests, where appropriate.

Secondary outcomes were evaluated using uni- and multivariable Cox proportional hazards regression models or ANOVA analyses, where appropriate. Multivariable Cox regression analyses were performed using the Enter and Forward Stepwise Wald methods.

Based on literature and clinical relevance, the variables age, sex, body mass index (BMI), acute physiology and chronic health evaluation II (APACHE II), SOFA and modified nutrition risk in critically ill (mNUTRIC) scores were analysed in regression analyses. Variables were dichotomised (using the median) in case of non-linearity, with the outcome parameter assessed by visual inspection of boxplots.

Finally, all samples' PBMC lymphocyte-monocyte ratios (LMR) were calculated. Their changes over time and differences between survivors and non-survivors were evaluated. Moreover, correlation with parameters of mitochondrial function was assessed using Kendall's Tau-b. Multicollinearity was assessed using the variance inflation factor (VIF); a value below two was considered acceptable.

Gene expression analysis was performed using relative expression values calculated via the ΔΔCq method. For statistical analysis, the ΔΔCq values were used as input, and consistency with the raw Bio-Rad qPCR output files was manually verified. Fold changes ($2^{-\Delta\Delta Cq}$) were calculated and used exclusively for graphical representation of the results. Normality was assessed of both gene expression data and mitochondrial respiration parameters using the Shapiro-Wilk test. When data were not normally distributed, logarithmic transformations were applied in an attempt to normalize the distribution. Data that remained non-normally distributed after transformation were analyzed using a non-parametric one-way ANOVA (Kruskal-Wallis test). For data that were normally distributed after transformation, a parametric one-way ANOVA was performed using the transformed data. When significant differences were detected, Tukey's post-hoc tests (in case of normally distributed data) or Dunn's test (in case of not normally distributed data) were applied.

IBM SPSS statistics 27 (IBM Corp, Armonk, NY, USA) or Graphpad Prism 10.4.2 (GraphPad Software, Boston, MA, USA) was used for statistical analyses and figures representing statistics. Only two-sided analyses were used. P-values ≤0.05 were considered statistically significant.

Ethics approval

The study was approved by the Medical Ethical Committee of Wageningen University (METC-WUR, which was incorporated in the METC Oost-Nederland in 2021, dossier no. 2021-13011) and the assessment

Committee for Scientific Research of ZGV (dossier no. 1801-004). The protocol was registered in the Netherlands Trial Register (number NTR6969) and was made available through the International Clinical Trial Registry Platform (NL5918). Patients were enrolled after the informed consent form was signed by the patient or legal representative. All experiments were performed in accordance with relevant regulations and guidelines.

Results

To obtain thirty matched pairs with complete data, informed consent was obtained from 46 septic patients and 30 age- and sex-matched controls (**Figure 2**). The septic patients were predominantly male (n=33, 72%) and had a mean age of 68 (± 13 SD) years, with a mean BMI of 27 (± 6 SD) kg/m^2. Pneumonia was found to be the most common cause of sepsis (n=24, 52%), followed by an abdominal origin (n=17, 37%). At baseline, patients had the following clinical scores: mNUTRIC 5 [IQR 3-6], SOFA 8 [7-10] and APACHE II 18 [14-22]. The control patients were age- and sex matched and were subsequently predominantly male as well (n=22, 73%) and had a mean age of 71 (± 15 SD) years, which was not different from the sepsis group (p=0.3).

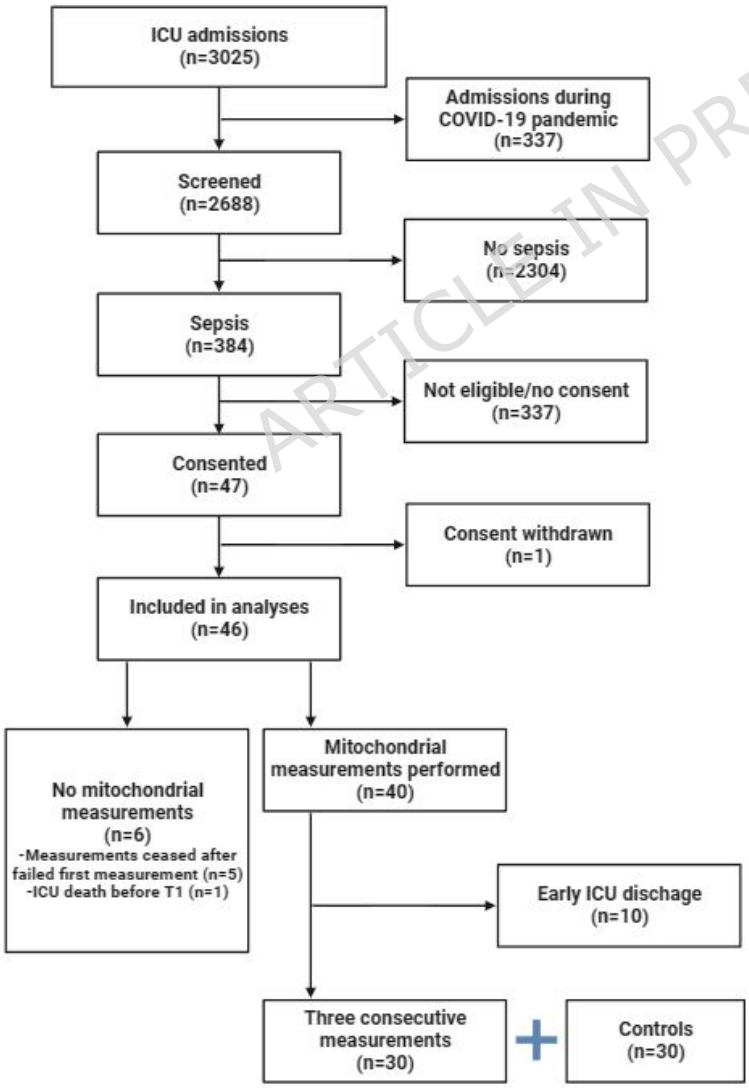

Figure 2. Study flow chart. ICU = intensive care unit. Created with Biorender.com.

After study completion, upon reviewing the obtained data, single respirometry measurements in five patients had to be discarded as they did not meet quality standards (T1 n=1, T2 n=1, T3 n=2 and T1-3 n=1). Measurements were discovered to have failed in four controls (13 %) and partially failed (no reaction to CCCP, rendering basal respiration and proton leak useable) in one patient. As the inclusion phase had already been completed, no further inclusions were performed to compensate for these losses. The clinical patient characteristics at the time of blood samplings are shown in **Table 1**.

[Insert Table 1 here]

Study measurements

Mitochondrial function over time in septic patients and controls

Basal respiration was significantly higher in septic patients compared to controls (2.5±0.3 pmol O_2/(s*ml)) at timepoints 1 (5.3±1.0 pmol O_2/(s*ml), $p<0.05$), 2 and 3 (4.8±0.6 and 6.1±1.0 pmol O_2/(s*ml) respectively, both $p<0.01$, **Figure 3A**). Proton leak was significantly higher in septic patients compared to controls (1.1±0.1 pmol O_2/(s*ml)) at timepoints 2 and 3 (1.6±0.2 and 1.7±0.2 pmol O_2/(s*ml), respectively, both $p<0.05$, **Figure 3B**). Maximal respiration was significantly higher in septic patients (12.4±2.1 pmol O_2/(s*ml)) compared to controls (5.7±0.6 pmol O_2/(s*ml), $p<0.05$, **Figure 3C**) at timepoint 3 only. ATP-linked respiration was significantly higher in septic patients compared to controls (1.4±0.2 pmol O_2/(s*ml)) at timepoints 2 (3.3±0.5 pmol O_2/(s*ml), $p<0.05$) and 3 (4.4±0.7 pmol O_2/(s*ml), $p<0.001$**, Figure 3D**). No differences were detected in spare respiratory capacity **(Figure 3E)**.

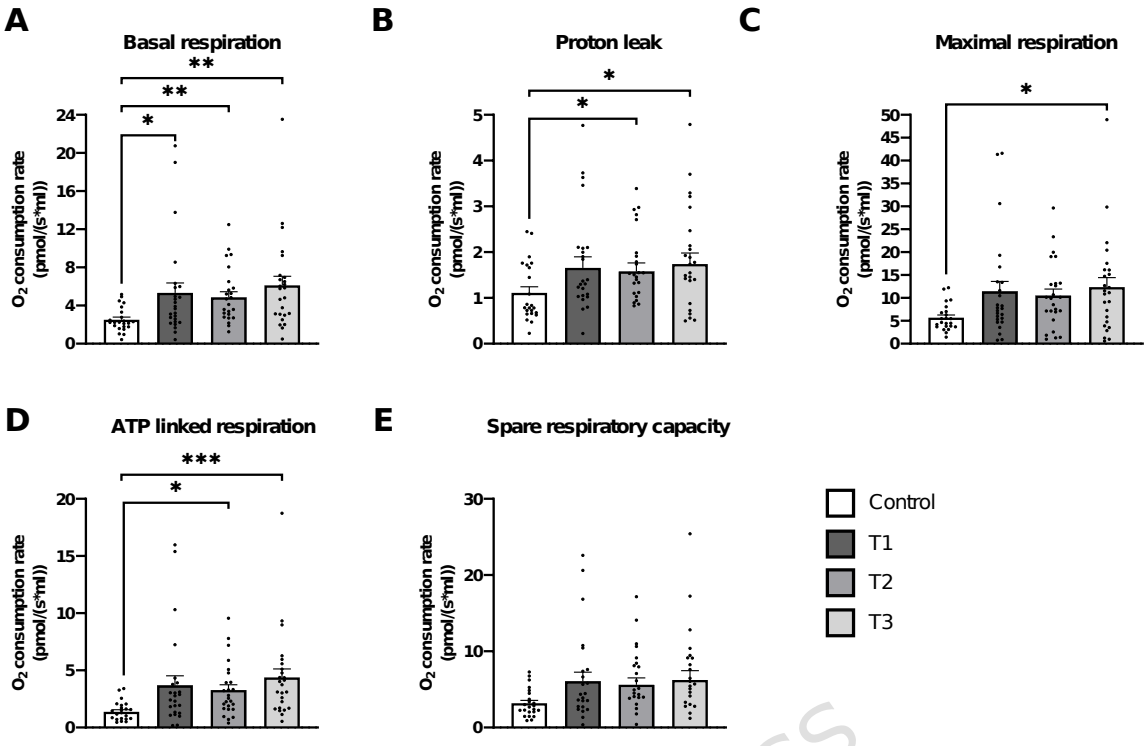

Figure 3. Progression of mitochondrial respiratory parameters during the first week of ICU admission in septic patients and controls. T1, day 1-2 (24-48h); T2, day 3-4 (72-96h); T3, day 5-6 (120-144h) after ICU admission. Values represent mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

Expression of oxidative phosphorylation genes over time

The expression of genes encoding for subunits in complex II (*SDHB*) and complex V (*ATP5F1A*) was significantly higher in septic patients compared to controls at multiple timepoints of the first week of ICU admission (**Figure 4**). At T1, *SDBH* and *ATP5F1A* expression were 43% ($p<0.05$) and 52% ($p<0.01$) higher in septic patients compared to controls, respectively. At T2, *SDBH* was 54% higher in septic patients compared to controls ($p<0.01$). At T3, *SDBH* and *ATP5F1A* expression was 54% ($p<0.01$) and 40% ($p<0.01$) higher in septic patients compared to controls, respectively. In genes encoding other subunits no statistically significant differences were detected.

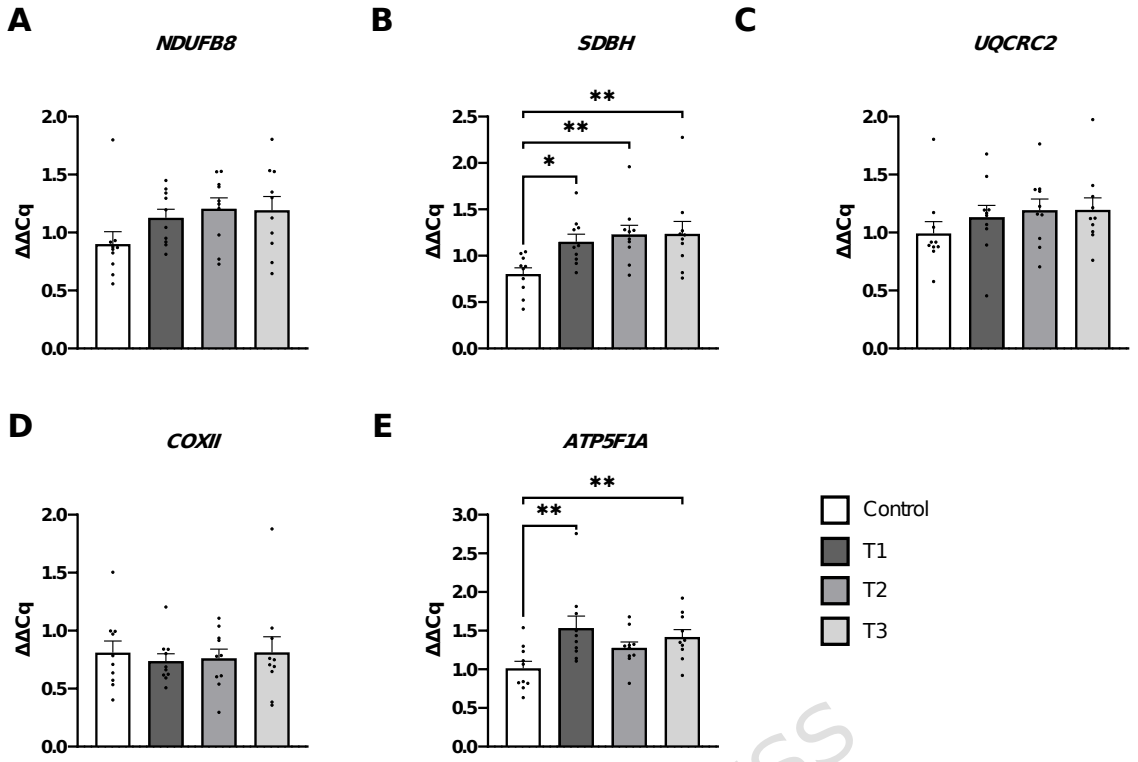

Figure 4. Expression of genes encoding subunits of each of the five OXPHOS complexes; (A) *NDUFB8* (Complex I), (B) *SDHB* (Complex II), (C) *UQCRC2* (Complex III), (D) *COXII* (Complex IV) and (E) *ATP5F1A* (Complex V). Data were obtained from septic patients and matched controls (n=10 per group). T1, day 1-2 (24-48h); T2, day 3-4 (72-96h); T3, day 5-6 (120-144h) after ICU admission. Values represent mean ± SEM. *p < 0.05 and **p < 0.01.

Survivors versus non-survivors

All-cause 3-month mortality in the sepsis cohort (n=40) was 35% (n=14). Five (36%) of the deceased patients died in the ICU, seven (50%) in the ward and two after hospital discharge (14%). Compared to the surviving patients (n=31), the non-survivors (n=15) were older (77 (± 10 SD) versus 63 (± 13 SD) years of age, p<0.001), had higher APACHE II (median 20 [IQR 17-26] versus 15 [12-20], p=0.008] and mNUTRIC scores (6 [IQR 6-7] versus 4 [3-5], p<0.001) at ICU admission. No significant differences were found in other baseline characteristics. Regarding biochemical parameters, only significant differences in serum insulin levels between survivors and non-survivors were found (see **Table 2**).

[Insert Table 2 here]

In high-resolution respirometry, no mitochondrial respiration parameters were significantly different at T1 between survivors and non-survivors (**Figure 5**). A trend for a lower mitochondrial proton leak at T1 in survivors (1.14±0.2 pmol O_2/(s*ml) compared to non-survivors 1.4±0.2 pmol O_2/(s*ml)) (p=0.06, **Figure 5B**) was found.

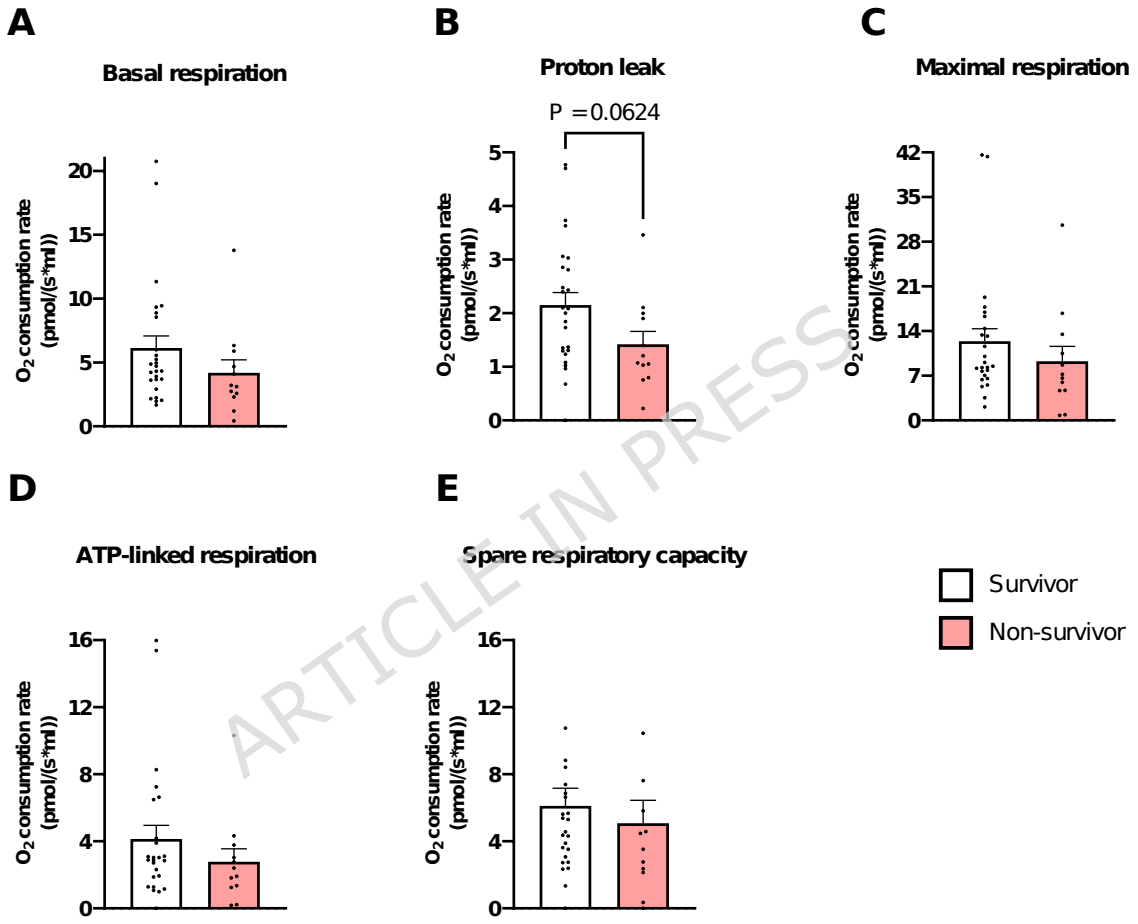

Figure 5. Comparison of mitochondrial respiratory parameters at T1 (24-48h after ICU admission) of sepsis survivors versus non-survivors. Values represent mean ± SEM.

To assess potential differences in the progression of mitochondrial respiratory parameters between non-survivors vs. survivors, delta (Δ) values were calculated by subtracting values at timepoint 1 from those at timepoint 3. This exploratory analysis revealed significant differences between survivors and non-survivors in ΔBasal respiration (-0.8±0.9 vs.

2.7±0.9 pmol O_2/(s*ml), respectively), ΔMaximal respiration (-2.3±2.0 vs. 4.8±2.5 pmol O_2/(s*ml), respectively), ΔATP-linked respiration (-0.5±0.8 vs. 2.3±0.7 pmol O_2/(s*ml), respectively), and ΔSpare respiratory respiration (-1.4±1.2 vs. 2.5±1.4 pmol O_2/(s*ml), respectively), were detected (all $p<0.05$, **Figure 6**). In fact, delta values were generally negative for survivors, indicating a decrease in mitochondrial respiration over-time during ICU stay. Conversely, in non-survivors delta values were positive, indicating an increase in mitochondrial respiration over-time during ICU stay (**Figure 6**).

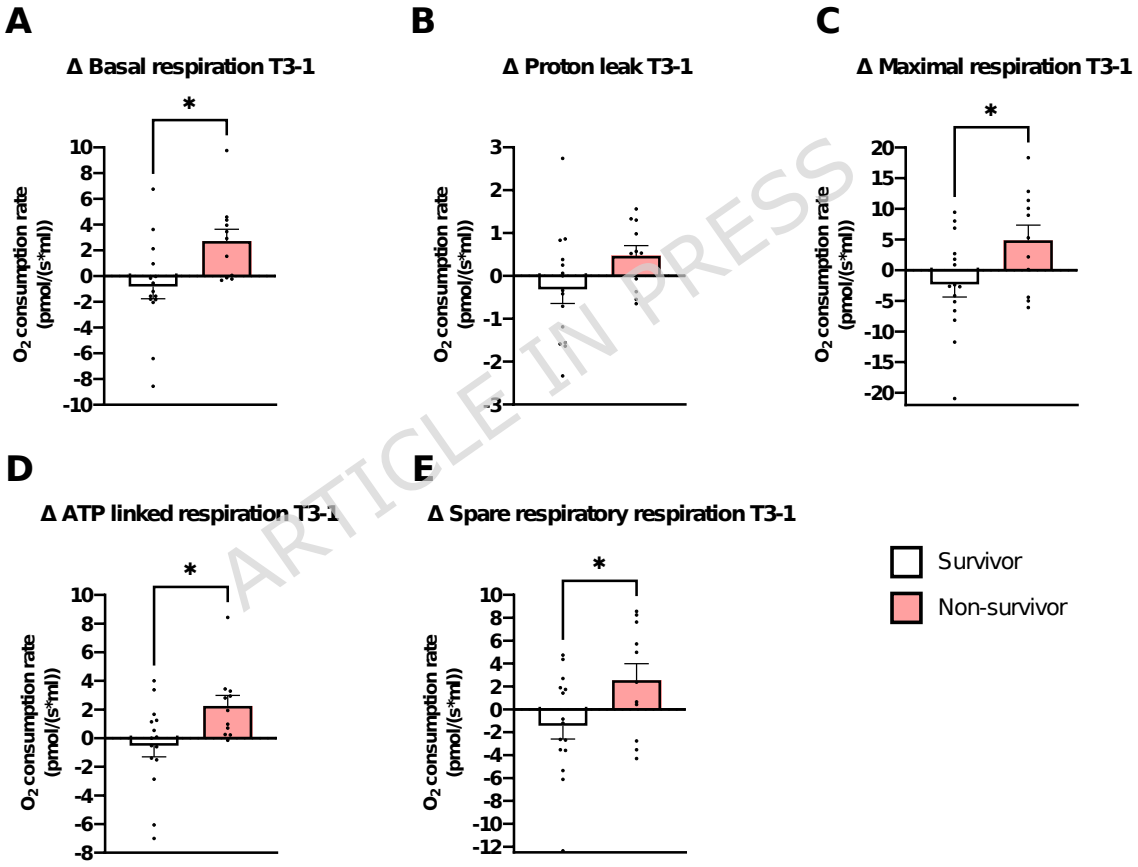

Figure 6. Comparison of the progression of mitochondrial respiratory parameters during the first week of ICU stay of sepsis survivors and non-survivors. Δ = delta (as calculated by subtracting values at timepoint 1 from those at timepoint 3); T1, day 1-2 (24-48h); T3, day 5-6 (120-144h) after ICU admission. Values represent mean ± SEM. *p < 0.05.

Cox regression

The variables age and mNUTRIC were omitted in the final regression models because of their overlap (and visual correlation) with the APACHE II score. Measured mitochondrial respiration parameters were intercorrelated (all $p<0.01$); therefore, only basal respiration was used in the final model as a representative and integrative parameter to minimize overfitting. None of the mitochondrial respiratory parameters were correlated with the SOFA score. The deltas of these two parameters over time were entered into the final model (ΔSOFA and ΔBasal respiration as calculated by T3 minus T1). In the final Cox regression multivariable model, ΔBasal respiration (≥0.07 nmol O_2/min/10^7 from T1 to T3) was associated with the primary endpoint of 3-month mortality (HR 3.8, 95%CI 1.0-14.1, p=0.047) (see **Table 3**). The VIF was <2 for the variables in this final model, however, given the limited number of events and the exploratory nature of this analysis, these findings should be interpreted with caution.

[Insert Table 3 here]

Lymphocyte-monocyte ratio

The PBMC LMR was lower in sepsis patients than in controls. No change over time (T1-T3) was noted, nor were any differences between survivors and non-survivors (**Tables 1-2**).

Discussion

In this prospective observational study, we found a significant increase in basal, ATP-linked, maximal and proton leak associated respiration in sepsis patients compared to controls within the first week of ICU admission. These findings were corroborated by gene expression analysis, showing an increased expression of genes encoding OXPHOS subunits (complex II and V) in septic patients, consistent with the functional respiration data. These observations contrasted our hypothesis, as we did not demonstrate a decrease in PBMC mitochondrial respiration during sepsis. Moreover, a more significant increase in basal, ATP-linked and maximal respiration and spare respiratory capacity was observed during the first week of ICU stay in non-survivors compared to survivors ($p<0.05$), although these respiration parameters were not statistically different between the sepsis and control patients at baseline measurements. This progression of basal respiration was associated with the primary endpoint of 3-month mortality after correction for relevant covariates. Therefore, the current results show that septic patients undergo a progressive upregulation of PBMC mitochondrial respiration, which may be related to adverse outcome of disease. Importantly, this association is based on a limited number of mortality events with wide confidence intervals and should therefore be interpreted as exploratory and hypothesis-generating rather than confirmatory.

Our findings are consistent with those of Sjövall *et al.* [19] and Belikova *et al* [6], who also found that basal mitochondrial respiration in PBMCs was significantly increased within the first 48 hours of ICU admission. In addition, Sjövall *et al.* [19] demonstrated a progressive increase in basal and maximal respiration during the first week of sepsis patients compared to healthy controls. Strikingly, they observed no differences between surviving and non-surviving patients at any point in time. Both their inclusion and mortality rates were lower than in the present study, which may have led the study to be underpowered for differentiation between survivors and non-survivors. However, their article does neither report the

original data nor p-values for the comparisons, so this claim cannot be substantiated.

In contrast with our findings, Jang *et al.* (studying mitochondrial respiration of PBMCs in 10 septic patients measured once shortly after presentation to an emergency department), Japiassú *et al.* (studying mitochondrial respiration of PBMCs in 20 patients during the first 48 hours of septic shock) and Garrabou *et al.* (studying mitochondrial respiration in 19 septic patients, time of measurement not mentioned) observed a significant reduction of ADP-linked respiration in permeabilized PBMCs of septic patients compared to controls [7,8,18]. In addition, in the study of Japiassú *et al.*, a significant reduction was observed in ADP-linked respiration in non-surviving sepsis patients compared with the postoperative controls without sepsis (5.60 versus 9.89 nmol O_2/min/10^7, respectively, $p < 0.01$). Survivors demonstrated a 2.9x increase in ATP-linked respiration after one week [7]. Contrastingly, we did not observe a significant change in respiratory function over time in survivors. Instead, we found a significant increase in basal and ATP-linked respiration in non-survivors during the first week of ICU stay.

Similarly, studies reporting gene expression data of genes encoding OXPHOS subunits are conflicting. We observed an increased expression of OXPHOS genes (*SDBH* and *ATP5F1A*) in septic patients compared to controls, a finding that is in line with those of Li *et al.*, whom also found an increased expression of OXPHOS subunits compared to controls [22]. Similarly, Sjövall *et al.* reported increased mtDNA copies in septic patients compared to controls, indirectly indicating a higher number of mitochondria and OXPHOS subunits present in septic patients [19]. However, Nucci *et al.* reported a downregulation of genes encoding OXPHOS subunits in samples collected from septic patients during admission [23].

These contradictory observations may be due to methodological variety. A clear difference between the abovementioned studies is the composition of the control group, which may influence the outcomes of comparisons between sepsis and control groups. In the current study, we chose to include sex- and age-matched controls since those are two factors known to influence mitochondrial respiratory function, which has not been done in other studies besides the study of Garrabou *et al.* [8]. Moreover, we selected metabolically healthy age- and sex-matched controls. In contrast, Japiassú *et al.* included critically ill postoperative ICU patients, whereas Jang *et al.* chose to use three unmatched control groups of younger, older and infected (but not septic) patients [7,18]. It is intriguing that Sjövall *et al.* and our study still proved an increase in mitochondrial function parameters in PBMCs of septic patients, eventhough healthy controls were included [19]. Although the inclusion of non-septic ICU controls could help disentangle sepsis-specific effects from critical illness, such a group would be inherently heterogeneous with respect to diagnosis, disease severity, and medication exposure, all of which may independently affect mitochondrial function. We therefore intentionally selected metabolically healthy, age- and sex-matched controls to provide a homogeneous physiological reference. Nonetheless, we acknowledge that an optimal design would include both healthy controls and non-septic ICU controls, which should be considered in future studies.

Secondly, exclusion criteria differ between mentioned studies. We excluded many common comorbidities known to affect mitochondrial respiratory function (such as diabetes mellitus and COPD), which allowed us to exclude the potential confounding effect of these comorbidities. Such exclusion criteria were not reported in other studies.

Thirdly, the time at which the PBMCs were collected and measured respiration differed between studies. The timing of blood collection is not described by Garrabou *et al.* [8]. Jang *et al.* and Nucci *et al.* collected blood samples from patients with sepsis or septic shock upon presentation to the

emergency department [18], whereas our measurements and those of Japiassú *et al.* commenced within 48 hours of ICU admission [7]. These may be very different (metabolic) time points in a patient's journey. Furthermore, our cohort was slightly older than those of Jang [18] and Garrabou [8] and their coworkers (68 vs 63, resp. 64 years of age), and although SOFA scores were similar in all studies, it is unknown whether all patients in the Garrabou and Jang cohorts required ICU admission.

Fourthly, the methods of respiration measurement vary in comparison with current literature. The current study resuspended PBMCs in a standardized medium, not plasma. This was similar to Japiassú *et al.* [7]. On the contrary, Sjövall and coworkers used the patient's plasma [19]. However, mitochondrial function in PBMCs is altered by plasma, as was demonstrated by Belikova and co-workers [6]. Consequently, it is difficult to disentangle the effects of sepsis on plasma content from the effect of sepsis on mitochondria in PBMCs per se. Strikingly, in the current study, decreased mitochondrial respiration and decreased expression of OXPHOS genes was, in fact, not visible. This approach revealed that the mitochondria of PBMCs are not dysfunctional and capable of improving respiratory function. In addition, this suggests that if a worsened respiratory function is observed in PBMCs of septic patients, this is perhaps more likely to originate from potential dysregulating components present in plasma. In addition, in some studies, PBMCs were permeabilized, while we used non-permeabilized PBMCs for respiratory measurements [7,8]. It could be hypothesized that this explains the differences with our results. However, since both Sjövall *et al.* [19] and Jang *et al.* [18] have performed their measurements in both permeabilized and non-permeabilized PBMCs and found consistent results between those experiments, this is unlikely to explain the contrasting results with our studies.

Lastly, it can be hypothesized that the differences in respiratory function of PBMCs belonging to the different groups of our study are caused by a

shift in PBMC composition rather than a shift in mitochondrial function per se. In humans, PBMC cell ratios vary across individuals, but typically, lymphocytes are in the range of 70–90 %, monocytes from 10 to 20 %, while dendritic cells are rare, accounting for only 1–2 % [17]. In our cohort, the PBMC lymphocyte-monocyte ratio (LMR) was lower in sepsis patients than in controls. This lower count is to be expected, as a lower LMR is associated with systemic inflammation [24]. However, parameters of mitochondrial function did not correlate with the LMR, nor did the LMR change over time, and also not when survivors versus non-survivors were compared (**Table 2**). However, it could still be of interest to measure mitochondrial function in distinct cell populations in future studies and should perhaps be considered when leukocytes are used as bioenergetic biomarkers [25].

Although we could not identify consistent methodological differences among all the studies mentioned, combining these methodological differences can contribute to the contrasting results.

Previous studies, although few, measuring mitochondrial function in muscle tissue of different origins have consistently reported a lower activity of mitochondrial complexes and a lower ATP content, concomitant with an altered expression of genes involved in regulating mitochondrial dynamics [15,26,27]. In the current study, mitochondrial function was measured in PBMCs. PBMCs are easily and non-invasively obtained. However, PBMCs are important cells during inflammation and systemic infection and may have a different metabolic response to sepsis compared to other tissues directly involved in multiorgan failure, such as the liver and muscles. Based on the results of the current study and in comparison to studies performed using skeletal muscle biopsies, PBMCs do not necessarily reflect the decrease in mitochondrial function, which is reported elsewhere in the body. Indeed, Jeger *et al.* reported that results from previous animal and clinical studies investigating mitochondrial function in several tissue types during sepsis are heterogeneous, reporting

increased and decreased mitochondrial oxygen consumption [28]. Although speculative, these differences in mitochondrial functioning between tissues may reflect differences in their role during sepsis.

Thus, the increased basal, ATP-linked and maximal respiration together with the increased expression of genes encoding for subunits of OXPHOS complexes (in particular complex II and IV) as found in the current study may reflect an increased ATP demand at the bulk level of PBMCs during human sepsis, as a result of an activation of the immune system to combat the underlying infection, although contributions from changes in PBMC composition or cell-type–specific metabolic reprogramming cannot be excluded. The clinical relevance of this increase is suggested by the higher increase in basal, ATP-linked and maximal respiration respiration and spare respiratory capacity over time (i.e., between T1 and T3) in non-survivors, compared to survivors. Our findings may thus contrast speculation that mitochondrial dysfunction is the root cause of immunoparalysis and could be responsible for the onset of organ dysfunction [29]. Still, pathophysiological interpretation of this difference between survivors and non-survivors is precarious. The higher increase over time may, partially, be due to a lower basal and ATP-linked respiration in PBMC's at T1 of non-survivors compared to survivors, although this was not statistically significant. Thus, a delayed up-regulation of PBMC activation in PBMCs of non-survivors compared to survivors, cannot fully be excluded but, in view of the lack of statistical significance, is highly speculative. Still, disregarding possible differences at T1, a higher increase in both respiration parameters over time could reflect differences in the development of the infection, PBMC composition or indicate immune dysfunction. This study does not provide clarity in this respect, especially since immune mechanisms during sepsis are complex, consisting of simultaneous hyperinflammation and immune suppression. Future studies, however, will take these hypotheses into account. These findings should be interpreted in the context of immunometabolic reprogramming during sepsis, where early phases are dominated by increased glycolysis and

Warburg-like metabolism, followed by later shifts toward oxidative metabolism mediated by AMPK- and sirtuin-associated pathways (30). While metabolomic profiling was beyond the scope of this study, the observed respiratory changes are compatible with such dynamic metabolic adaptations.

Strengths

Combining functional measurements of mitochondrial functioning with relevant gene expression patterns, both exhibiting increases in sepsis, provides a more robust and in-depth analysis of the situation. Also, the consecutive measurements at three moments during the first week of ICU admission with fixed intervals are considered a strength of this study, as this enabled us to better understand the progress of mitochondrial respiration during the first week of ICU admission in septic patients. In addition, the extensive list of exclusion criteria based on common comorbidities (e.g., diabetes mellitus and COPD) known to affect metabolism and mitochondrial function is a unique strength of this study, as this allowed us to exclude potential confounding effects of these comorbidities.

Limitations

The current study is limited by its single-centre design. However, as the samples needed to arrive at the laboratory in a fresh state, the hospital's proximity to a university laboratory equipped with an Oroboros was an essential condition for this study. Secondly, the possible effects of administered medication on mitochondrial function may represent an unaccounted confounder. Thirdly, multivariate Cox regression analyses performed on small numbers found an association between the delta basal respiration and ICU and 3-month mortality, not the severity-of-disease score APACHE II. This may be due to additional confounding factors, which were not accounted for (residual confounding) or multicollinearity, although the VIF was low (<2). Fourthly, changes over time were primarily assessed using delta values between T1 and T3, which does not fully

exploit the repeated-measures design and may obscure non-linear temporal patterns. Fifthly, PBMC heterogeneity represents an important limitation, as bulk respiration measurements and stable lymphocyte–monocyte ratios do not exclude cell-type specific metabolic adaptations, highlighting the value of future cell-sorted or single-cell analyses, as there is increasing evidence for critical lymphocyte counts resulting in four discrete sepsis endotypes with markedly divergent 28-day survival (31-33). Finally, the recruitment period covered five years. During this period the standard of care may have changed, weakening the in- and external validity of our findings.

Future directions

Further research is needed to elucidate the role of mitochondria in the sepsis pathophysiology. More extensive multicentre trials are needed to consolidate the current study's findings. It would be interesting to measure mitochondrial respiratory function in various other tissues in parallel to create more insight into the potential role of PBMCs as a proxy marker for mitochondrial respiratory function in other tissues.

Conclusion

This study demonstrated a basal, ATP-linked, maximal and proton leak associated respiration in PBMCs within the first week of ICU admission in sepsis patients compared to their healthy matched controls. Similarly, the expression of genes encoding subunits of complex II and V were increased in septic patients compared to controls. In addition, a progressive increase of mitochondrial respiration in PBMCs during the first week of ICU stay was negatively associated with 3-month mortality, although this observation requires confirmation in larger studies.

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Declarations

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Authors' contributions

HM and RSB contributed equally to data collection, data analysis and interpretation, and writing and revising of the manuscript. AvN, SG, and JdJ contributed to the conception of the study, data collection, analysis, interpretation, and revision of the manuscript. AW contributed to data collection and interpretation. AvZ contributed to the conception of the study, data interpretation and revision of the manuscript.

Availability of data and material

The dataset supporting the conclusions of this article is available upon reasonable request from the corresponding author.

Competing interests

Prof. Dr. Van Zanten reported receiving honoraria for advisory board meetings, lectures, research grants, and travel expenses from Abbott, AOP Pharma, Baxter, Danone-Nutricia, Dutch Medical Food, Fresenius Kabi, GE Healthcare, Medcaptain, Nestle, PAION and Rousselot. The other authors have nothing to declare.

Ethics approval and consent to participate

The study was approved by the Medical Ethical Committee of Wageningen University (METC-WUR, which was incorporated in the METC Oost-

Nederland in 2021, dossier no. 2021-13011) and the assessment Committee for Scientific Research of ZGV (dossier no. 1801-004). The protocol was registered in the Netherlands Trial Register (number NTR6969) and was made available through the International Clinical Trial Registry Platform (NL5918).

Patients were enrolled after signing the informed consent by the patient or legal representative.

Consent for publication

Not applicable.

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Tables

Table 1. Patient and control characteristics at time of the blood samplings.

	Controls (n=26)	Sepsis patients (n=40[a])					
		T1 (n=38)	p-value[b]	T2 (n=35)	p-value[c]	T3 (n=28)	p-value[d]
Clinical parameters							
SOFA score	n.a.	6 [4-8]	n.a.	4 [3-8]	<0.001*	5 [3-7]	<0.001*
Lactate (mmol/L)	n.a.	1.2 [0.8-1.7]	n.a.	0.9 [0.8-1.2]	0.002*	0.8 [0.6-1.2]	<0.001*
Leukocytes (*10^9/L)	6.5 [5.7 – 8.0]	13.6 [10.0-18.9]	<0.001*	12.7 [9.1-16.3]	0.189	12.2 [9.4-19.1]	0.388
PMBC LMR	4.6 [3.3-5.6]	2.0 [1.1-3.0]	0.010*	2.0 [1.5-3.3]	0.465	2.0 [1.3-3.0]	0.670
CRP (mg/L)	0	249 [148-323]	<0.001*	120 [77-214]	<0.001*	109 [58-204]	<0.001*
Ureum (mmol/L)	6.3 [5.5 – 7.1]	10.7 [7.8-19.5] n=26	0.002*	11 [5.5-25] n=20	0.844	10.1 [7.5-15.8] n=18	0.529
Creatinine (umol/L)	74 [62 – 87]	84 [60-145]	0.024*	72 [52-117]	0.002*	60 [46-112]	<0.001*
Cortisol (nmol/L)	395 [311 – 472]	724 [546-1568]	<0.001*	676 [418-1005]	<0.01*	592 [431-725]	<0.001*
Insulin (mmol/L)	19 [9.4 – 18.5]	18 [8.8-30]	0.280	21 [12-35]	0.523	11.5 [7.7-31.5]	0.755
Insulin supplementation (IU)	n.a.	0 [0-19]	n.a.	0 [0-24]	0.449	0 [0-26]	0.234

a. Unless stated otherwise due to missing variables. b. T1 compared to the control group; p-values were calculated using the Wilcoxon signed rank test c. T1 compared to T2, p-values were calculated using the Wilcoxon signed rank test; d. T1 compared to T3, p-values were calculated using the Wilcoxon signed rank test; * p-value <0.05.

Definitions: T1, day 1-2 (24-48h); T2, day 3-4 (72-96h); T3, day 5-6 (120-144h) after ICU admission; PBMC, peripheral blood mononuclear cell; LMR, lymphocyte-monocyte ratio; CRP, C-reactive protein; IU, international units; n.a., not applicable; SOFA, Sequential Organ Failure Assessment. All values are reported as medians with interquartile ranges.

Table 2. Survivors (n=26) [a] versus all-cause 3-month non-survivors (n=14)[a]

	T1			T2			T3		
	survivors (n=26)	**non-survivors (n=12)**	**p-value**	**survivors (n=22)**	**non-survivors (n=13)**	**p-value**	**survivors (n=15)**	**non-survivors (n=13)**	**p-value**
Clinical parameters									
SOFA score	6 [3-8]	7 [5-8]	0.6	3 [2-7]	5 [4-6]	0.3	4 [2-8]	5 [3-5]	0.9
Lactate (mmol/L)	1.3 [0.8-1.725]	1.2 [1.0-1.6]	0.9	0.9 [0.8-1.3]	1.0 [0.7-1.3]	0.9	0.8 [0.5-1.2]	0.9 [0.7-1.2]	0.5
Leukocytes (*10^9/L)	14.3 [8.5-19.0]	12.8 [10.5-16.8]	1.0	12.5 [9.1-16.3]	13.2 [8.2-16.6]	1.0	12.1 [9.5-17.7]	14.9 [9.2-22.6]	0.3
PBMC LMR	2 [1.1-3.1]	1.6 [1.0-3.9] n=9	1.0	2.3 [1.5-3.6] n=21	1.8 [1.2-2.4]	0.3	2.0 [1.7-3.5]	2.0 [1.1-2.5]	0.2
CRP (mg/L)	252 [169-323]	236 [137-318]	0.63	140 [74-214]	112 [87-280]	0.9	109 [70-168]	97 [35-212]	0.8
Ureum (mmol/L)	10.3 [6.5-18.2]	11.4 [9.1-25.2]	0.3	10.8 [5.1-14.4]	16.4 [8.8-32.6]	0.3	10.8 [6.6-24.7]	9.4 [7.9-14.4]	0.5
Creatinine (umol/L)	84 [57-188]	95 [75-132]	0.9	70 [52-114]	66 [52-124]	0.7	64 [46-137]	53 [44-109]	0.4
Cortisol (nmol/L)	674 [560-1397]	1398 [525-1959]	0.2	549 [418-1005]	697 [583-1062]	0.5	617 [368-761]	601 [506-768]	0.6
Insulin (mmol/L)	20.0 [11.4-36.3]	8.4 [4.6-20.5]	0.014*	20 [12-35]	17.5 [7.9-40.3]	0.8	12.5 [9.1-25.8]	13.0 [5.7-35.8]	1.0
Insulin supplementation (IU)	0 [0-0.75]	9 [0-38]	0.066	0 [0-1]	0 [0-45]	0.3	0 [0-0.75]	0 [0-31]	0.3
Insulin supplementation (IU)**	32 [5-55]	36 [28-54]	0.5	41 [8-95]	45 [38-87]	0.6	50 [13-59]	36 [21-57]	0.8

a. Unless stated otherwise due to missing variables. Definitions: T1, day 1-2 (24-48h); T2, day 3-4 (72-96h); T3, day 5-6 (120-144h) after ICU admission; Abbreviations SOFA, Sequential Organ Failure Assessment; PBMC, peripheral blood mononuclear cell; LMR, lymphocyte-monocyte ratio; CRP, C-reactive protein; IU, international units; ATP-linked respiration = basal respiration minus proton leak; SRC = spare respiratory capacity; maximal respiration minus basal respiratory capacity; coupling efficiency = ATP-linked respiration divided by basal respiration. All values are reported as medians with interquartile ranges. P-values were calculated using the Mann-Whitney U test; * p-value <0.05.

Table 3. Univariable and multivariable COX regressions for the association of primary endpoint 3-month mortality, baseline and clinical characteristics and mitochondrial respiratory function (n=40).

	Univariable HR (95%-CI)	**p-value**	**Multivariable HR (95%-CI)**	**p-value**
A. 3-month mortality (n=14)				
Sex (female)	1.060 (0.332-3.385)	0.9	0.707 (0.164-3.051)	0.6
BMI (>25.7)	1.040 (0.364-2.967)	0.9	1.400 (0.381-5.139)	0.6
APACHE II on admission (>18)	2.029 (0.703-5.859)	0.2	1.926 (0.509-7.289)	0.3
delta SOFA T3-1 (≥-2)	0.863 (0.303-2.463)	0.8	1.038 (0.218-3.832)	0.9
delta basal T3-1 (>0.068**)	3.041 (0.886-10.431)	0.08	3.794 (1.018-14.149)	0.047*
B. ICU mortality (n=5)				
Sex (female)	1.633 (0.182-14.639)	0.7	Difficulties with multivariable regression due to low number of deaths	
BMI (>25.7)	1.578 (0.263-9.460)	0.6		
APACHE II on admission (>18)	2.139 (0.356-12.861)	0.4		
delta SOFA T3-1 (≥-2)**	0.223 (0.025-1.998)	0.2		
delta basal T3-1 (>0.068**)	118 (0.007-2.121*10^6)	0.3		
C. Hospital mortality (n=7)				
Sex (female)	1.291 (0.349-4.775)	0.7	0.979 (0.183-5.228)	0.9
BMI (>25.7)	1.028 (0.331-3.192)	1.0	1.554 (0.389-6.207)	0.5
APACHE II on admission (>18)	1.490 (0.480-4.630)	0.5	1.232 (0.288-5.273)	0.8

delta SOFA T3-1 (≥-2)**	0.61 (0.197-1.959)	0.4	0.916 (0.232-3.627)	0.9
delta basal T3-1 (>0.068**)	2.112 (0.565-7.901)	0.3	2.341 (0.577-9.494)	0.2

T1, day 1-2 (24-48h); T2, day 3-4 (72-96h); T3, day 5-6 (120-144h) after ICU admission. Delta was calculated as: (mitochondrial parameter at T3 minus T1). Abbreviations: HR; hazard ratio, 95%-CI; 95%-confidence interval, BMI; body mass index, APACHE II; Acute Physiology And Chronic Health Evaluation, SOFA; sequential organ failure assessment, basal; basal respiration (measured in nmol O2/min/10^7). * p-value <0.05. ** including negative values (=a decrease in SOFA score or basal respiration, respectively, over time). Multivariable Cox regression analyses were performed using the Enter and Forward Stepwise Wald methods.