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Mitochondrial Dysfunction in Peripheral Blood Mononuclear Cells in Pediatric Septic Shock

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Abstract

Objective—Mitochondrial dysfunction in peripheral blood mononuclear cells (PBMCs) has been linked to immune dysregulation and organ failure in adult sepsis but pediatric data are limited. We hypothesized that pediatric septic shock patients exhibit mitochondrial dysfunction within PBMCs which in turn correlates with global organ injury.

Design—Prospective observational study.

Setting—Academic pediatric intensive care unit (PICU).

Patients—Thirteen pediatric patients with septic shock and ≥2 organ failures and 11 PICU controls without sepsis or organ failure.

Interventions—*Ex vivo* measurements of mitochondrial oxygen consumption and membrane potential ($\Delta\Psi m$) were performed in intact PBMCs on day 1–2 and day 5–7 of septic illness and in controls. The Pediatric Logistic Organ Dysfunction (PELOD) score, inotrope score, and organ failure-free days were determined from medical records.

Measurements and Main Results—Spare respiratory capacity (SRC), an index of bioenergetic reserve, was lower in septic PBMCs on day 1–2 (median 1.81, IQR 0.52–2.09 pmol O_2/s/10^6 cells) compared to controls (5.55, 2.80–7.21; p=0.03). SRC normalized by day 5–7.

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Septic patients on day 1–2 exhibited a higher ratio of LEAK to maximal respiration than controls (17% versus <1%, p=0.047) with normalization by day 5–7 (1%, p=0.008), suggesting mitochondrial uncoupling early in sepsis. However, septic PBMCs exhibited no differences in basal or ATP-linked oxygen consumption or ΔΨm. Oxygen consumption did not correlate with PELOD, inotrope score, or organ failure-free days (all p>0.05). While there was a weak overall association between ΔΨm on day 1–2 and organ failure-free days (Spearman's ρ=0.56, p=0.06), septic patients with normal organ function by day 7 exhibited higher ΔΨm on day 1–2 compared to patients with organ failure for >7 days (p=0.04).

Conclusions—Mitochondrial dysfunction was present in PBMCs in pediatric sepsis, evidenced by decreased bioenergetic reserve and increased uncoupling. Mitochondrial membrane potential, but not respiration, was associated with duration of organ injury.

Keywords

mitochondria; bioenergetic reserve; energy failure; sepsis; critically ill children

INTRODUCTION

The sepsis syndrome is a leading cause of morbidity and mortality in pediatric patients (1). Despite antibiotics and early goal-directed therapy, 20–40% of children with sepsis develop organ injury requiring hospitalization in an intensive care unit (2). For critically ill children with sepsis-induced organ injury, therapy remains limited to supportive care and mortality is between 15–50% (2–4). In the current era, most of these deaths are related to multiple organ dysfunction syndrome (MODS) (2) or secondary infection (5).

Mitochondrial dysfunction has been implicated in the pathogenesis of organ injury and immune suppresion in adult sepsis but direct mitochondrial measurements in pediatric sepsis are limited (6). Under normal conditions, oxygen consumption through the mitochondrial electron transport system (ETS) is used to drive ATP production. Failure of mitochondria to efficiently use oxygen to sustain ATP production results in an energy deficit that can impair cell, and ultimately, organ function (7). Mitochondrial dysfunction can be detected by measuring oxygen consumption and mitochondrial membrane potential (ΔΨm). Oxygen consumption reflects mitochondrial oxidative respiration and measures using inhibitors and uncouplers are well standardized (8, 9). The spare respiratory capacity (SRC), in particular, reflects the ability of the ETS to keep pace with an increase in energy demand (8, 10, 11). ΔΨm is the difference in electrical potential across the inner mitochondrial membrane and reflects the ability of the ETS to maintain the proton gradient that drives ATP production.

Mitochondrial abnormalities are evident in heart, kidney, and liver following septic insults in animal models (12–14). However, direct evidence for mitochondrial dysfunction in human studies is limited, largely due to the impracticality of tissue biopsies in critically ill patients (15, 16). Several studies have shown that alterations in mitochondrial respiration in peripheral blood mononuclear cells (PBMCs)—monocytes and lymphocytes--are associated with disease severity, immune suppression, and mortality (17–21). Under quiescent conditions, PBMCs rely more on mitochondrial respiration than glycolysis to meet metabolic demands and exhibit a substantial SRC (22–24). These data suggest promise for

using PBMCs to gauge systemic mitochondrial derangements in vital organs and to impart insight into a possible mechanism of sepsis-induced immunosuppression (25–27). At present, there are no data on mitochondrial dysfunction in PBMCs from children with sepsis-induced MODS. Maturational differences in the response to sepsis (28) and age-related changes in mitochondrial biology preclude direct extrapolation of adult data to younger patients (29).

We sought to determine if measurable defects in mitochondrial dysfunction occur within PBMCs and correlate with severity and duration of organ injury in pediatric septic shock. We hypothesized that mitochondrial oxygen consumption and $\Delta\Psi m$ are decreased in PBMCs from septic pediatric patients and correlate with severity and duration of organ injury.

METHODS

This prospective, observational study was performed in the pediatric intensive care unit (PICU) at The Children's Hospital of Philadelphia from July 2012 to September 2013. The study was approved by the Institutional Review Board and informed consent was obtained.

Study Population

All patients ≤ 18 years-old admitted to the PICU who met criteria for septic shock and MODS as defined by the International Pediatric Sepsis Consensus Conference (30) were screened for eligibility. Consensus criteria define septic shock as 1) ≥ 2 age-based systemic inflammatory response syndrome criteria, 2) confirmed or suspected invasive infection, and 3) cardiovascular dysfunction (hypotension, need for vasoactive agent, or impaired perfusion despite 40 mL/kg intravenous fluid). MODS, defined as at least two concurrent dysfunctional organ systems (30), was required to be present for at least 12 hours. To reduce the likelihood that organ dysfunction was solely a consequence of inadequate resuscitation, only patients who achieved an age-appropriate minimum systolic blood pressure within 24 hours of sepsis recognition for at least four consecutive hours were enrolled. PICU patients without evidence for sepsis, infection, or organ dysfunction for at least 48 hours were enrolled as controls. Exclusion criteria were weight <5 kg (due to limitations of blood collection), white blood cell count $<0.5\times10^3/\mu L$, a known mitochondrial disorder, pre-existing chronic kidney disease or liver failure, cyanotic heart disease, surgery in the preceding 24 hours (other than for infectious source control), support with hemodialysis, hemofiltration, and/or extracorporal membrane oxygenation, and treatment with coenzyme Q, arginine, carnitine, or cyclosporine (due to direct mitochondrial effects).

Data Collection

Data were abstracted from the medical record onto a standardized form. Variables included demographics, comorbid conditions, source of infection, laboratory and microbiology results, use of vasoactive infusions, mechanical ventilation, antimicrobials, corticosteroids, and insulin, length of stay, and vital status. Organ dysfunction was monitored for 28 days after sepsis recognition (30). Duration of organ dysfunction was measured by the number of organ failure-free days from study enrollment to day 28, with death corresponding to zero

organ failure-free days (30). We compared patients with rapid recovery (within 7 days) versus prolonged duration (greater than 7 days) of organ dysfunction based on previous reports of the timing of maximal organ dysfunction (31) and the median time to complete resolution of organ dysfunction in pediatric septic shock (3). Severity of illness was determined by the Pediatric Risk of Mortality (PRISM)-III (32), Pediatric Logistic Organ Dysfunction (PELOD) (33), and inotrope scores (34).

Blood collection

A 4.5 mL (patients ≤ 10 kg) or 6 mL (>10 kg) blood specimen was collected as soon as possible after consent was obtained but no later than 48 hours after sepsis recognition (or study enrollment for controls; referred to as day 1–2 specimen). For patients with sepsis, a second blood sample was collected on study day 5–7. Assays for mitochondrial dysfunction were performed on fresh samples immediately after blood collection.

Mitochondrial Oxygen Consumption

PBMCs were isolated from citrated whole blood by density gradient centrifugation. Blood samples were diluted 1:1 using a balanced salt solution (anhydrous D-glucose 5.5mM, $CaCl_2$ 5 mM, $MgCl_2$ 0.98 mM, KCl 5.4 mM, TRIS 145 mM, and NaCl 140 mM with pH adjusted to 7.6) and layered on top of Ficoll-Paque PLUS (density 1.077 g/mL; Amersham Biosciences, Piscataway, NJ). The sample was centrifuged at 400g for 40 minutes at 20–25°C. The lymphocyte/monocyte layer at the interface was gently aspirated and centrifuged again at 1800g for five minutes. The PBMC pellet was then resuspended in Hank's balanced salt solution (pH 7.40) containing 5.5 mM glucose, 1mM pyruvate, and 10 mM HEPES. Cell counts were performed using trypan blue exclusion (Countess, Life Technologies, Grand Island, NY) with >90% viability.

Intact PBMCs were placed in a 2 mL chamber at a final concentration of 1–2 × 10^6 cells/mL. Measurements of oxygen consumption were performed at 37°C in a high-resolution oxygraph (Oxygraph-2k Oroborus Instruments, Innsbruck, Austria). Oxygen flux (in pmol O^2/s/10^6 cells), which is directly proportional to oxygen consumption, was recorded continuously using DatLab software 4.3 (Oroborus Instruments, Innsbruck, Austria) as shown in Figure 1 (9). After basal oxygen consumption was recorded for 10–20 minutes, the ATP-synthase inhibitor oligomycin (1 μg/mL) was added to induce a state 4-like respiration independent of mitochondrial ATP production in which oxygen consumption was primarily due to leakage of protons across the inner mitochondrial membrane (LEAK, abbreviated as L). In this standard protocol, it is assumed that the presence of glucose in the polarographic assay buffer provides ample substrate to support increased cellular flux through glycolysis and, thus, prevents critical ATP deprivation during the short-term incubation (~10–15 minutes) with oligomycin. Maximal oxygen consumption through the electron transport system was obtained by stepwise titration (1–2 μM) of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) until no further increase in oxygen consumption was detected (ETS_{max}, abbreviated as E). Mitochondrial respiration was then inhibited by adding the ETS complex IV inhibitor, sodium azide, in 5–10 mM increments until no further decrease in oxygen consumption was observed. The residual oxygen flux following optimal azide inhibition reflects non-mitochondrial oxygen consumption and was

subtracted from other respiration values. Oxygen consumption supporting mitochondrial ATP synthesis (ATP-linked respiration) was calculated as basal minus LEAK respiration. Spare respiratory capacity (SRC), a measure of mitochondrial reserve available for cells to produce energy in response to a stress-induced increase in metabolic demand, was calculated as ETS_{max} minus basal respiration. SRC indicates how close to its bionenergetic limit a cell is operating (8). The ratio of LEAK to ETS_{max} (L/E or LEAK flux control ratio) is an index for uncoupling of mitochondrial oxygen consumption from ATP synthesis (9).

Mitochondrial Membrane Potential (ΔΨm)

ΔΨm was measured using MitoTracker Red CMXRos (MTR; Molecular Probes, Oregon). MTR is oxidized to a fluorescent state in live cells and is sequestered within the mitochondria in a manner directly related to ΔΨm. MitoTracker Green FM dye (MTG; Molecular Probes, Oregon) was used to normalize ΔΨm to mitochondrial mass (35). A 50 μL sample of whole blood collected on EDTA was incubated with 5 μL of the CD45-Alexa Fluor 647 antibody for 15 minutes in a dark room at room temperature. Separate samples were then diluted 1:1000 in Hank's balanced salt solution and incubated with MTR (100 nM for 45 minutes) or MTG (100 nM for 30 minutes) at 37°C. Using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA), the fluorescence emitted by MTR and MTG was measured in the FL-3 channel (670 nm longpass) and FL-1 channel (530/30 nm), respectively. The 488 nm blue laser was used for excitation and an acquisition threshold was set to exclude Alexa Fluor 647-negative events (i.e., red blood cells and platelets). The ratio of median fluorescence intensities of MTR and MTG was calculated using FlowJo (Treestar, Ashland, OR).

Statistical Analysis

Analyses were performed using STATA (Version 12.1, College Station, TX). Data are presented as medians with interquartile ranges (IQR) for continuous variables and frequencies with percentages for categorical variables. Comparisons between septic and control patients were performed using the Wilcoxon rank sum test and changes in mitochondrial dysfunction over time were analyzed using the Wilcoxon sign rank test. Categorical variables were compared using Fisher's exact test. The association of mitochondrial dysfunction with organ failure-free days and severity of illness scores were analyzed with Spearman's rank-correlation test. Statistical significance was defined as a p-value < 0.05.

RESULTS

Of 175 patients screened, 52 (30%) met criteria for septic shock and MODS. Fifteen (54%) of those approached consented to study participation (Figure 2). Two patients were excluded due to laboratory processing errors that precluded measurement of mitochondrial dysfunction, leaving 13 septic patients for analysis. Eleven control patients were also enrolled. Septic and control patients were similar in age (median, IQR 6.4, 2.2–12.9 versus 8.0, 3.7–15.6 years; p=0.67), sex (male 31% versus 55%; p=0.41), and race (White 38% versus 46%, Black 31% versus 36%; p=0.77). PRISM-III scores were higher in septic (14, 7–18) than control (2, 0–2) patients (p=0.002). Table 1 shows the characteristics of the

septic and control patients. Table 2 shows characteristics and severity of illness of the patients at the time of blood sampling. The normal values for lactate and central venous oxygen saturation indicate that septic patients were appropriately resuscitated prior to study enrollment.

As shown in Figure 3, PBMCs from septic patients on day 1–2 exhibited no differences from controls in basal (2.53, 2.03–2.95 versus 1.81, 0.9–3.07 pmol O_2/s/10^6 cells; p=0.62), LEAK (0.56, 0.15–0.75 versus 0, 0–0.03 pmol O_2/s/10^6 cells; p=0.10), or ATP-linked respiration (2.04, 1.05–3.49 versus 2.52, 1.61, 3.13 pmol O_2/s/10^6 cells; p=0.74). ETS_{max} oxygen consumption trended lower in sepsis but this was not significant (4.30, 2.99–5.27 versus 7.50, 4.07–11.21 pmol O_2/s/10^6 cells; p=0.10). SRC, however, was significantly lower in PBMCs from septic patients on day 1–2 compared to controls (1.81, 0.52–2.09 versus 5.55, 2.80–7.21 pmol O_2/s/10^6 cells; p=0.03). In addition, the ratio of LEAK to ETS_{max} (L/E) was significantly higher in septic patients on day 1–2 (17%) versus controls (<1%, p=0.047) and returned toward baseline by days 5–7 (p=0.008), suggesting partial mitochondrial uncoupling in early sepsis (Figure 4). Although we excluded some pharmacologic agents that strongly target mitochondria, three septic patients were treated with the mitochondrial inhibitor valproic acid. A sensitivity analysis excluding these three subjects paralleled the overall results with SRC trending lower in sepsis patients on day 1–2 versus controls (1.90, 0.76–2.53 versus 5.55, 2.80–7.21 pmol O_2/s/10^6 cells; p=0.06) and L/E trending higher in sepsis patients on day 1–2 versus controls (12% versus <1%, p=0.07). Given the range of medications with potential effects on mitochondrial function, we list all medications prescribed on each study day in Supplemental Table 1.

Septic PBMCs exhibited no change in basal, ATP-linked, or ETS_{max} oxygen consumption over time (Figure 3). However, SRC increased as septic patients recovered organ function (decrease in PELOD in Table 2), showing no difference from control values by day 5–7.

Measures of PBMC mitochondrial respiration, including SRC and LEAK, did not correlate with organ failure-free days, inotrope score, or PELOD (p>0.05 for all pair-wise correlations). However, SRC was inversely associated with $ScvO_2$ in septic patients on both days 1–2 ($\rho = -0.71$, p=0.07) and 5–7 ($\rho = -0.67$, p=0.049), suggesting lower systemic oxygen consumption in patients with diminished capacity for PBMC mitochondrial respiration.

There were no differences in $\Delta\Psi m$, normalized to mitochondrial mass, between controls and septic patients on day 1–2 or day 5–7 (Figure 5A). Within the septic group, there was a weak linear association between $\Delta\Psi m$ and organ failure-free days (ρ=0.56, p=0.06). Septic patients with recovery of organ function by day seven exhibited a higher $\Delta\Psi m$ on day 1–2 (MTR/MTG ratio 0.62, IQR0.47, 0.82) compared to septic patients with organ dysfunction for more than seven days (0.32, IQR 0.14–0.35; p=0.04) and controls (0.19, IQR 0.15–0.37; p=0.02; Figure 5B).

DISCUSSION

Our data is the first to demonstrate that mitochondrial dysfunction is evident in PBMCs from critically ill children with septic shock and MODS. We found that mitochondrial bioenergetic reserve, indicated by spare respiratory capacity, was significantly diminished in PBMCs early in pediatric sepsis with a possible increase in mitochondrial uncoupling. Although mitochondrial respiration was not associated with the severity or duration of organ dysfunction in this small sample, septic patients with recovery from organ dysfunction by day seven exhibited a higher PBMC $\Delta\Psi m$ relative to mitochondrial mass compared to septic patients with prolonged organ dysfunction.

Decreased SRC indicates mitochondrial dysfunction that may not be apparent under basal conditions but becomes manifest as stress and ATP demand increases (8). SRC is a critical determinant of cell survival and function in a variety of cell types (10), including immune cells (11). If SRC is not sufficient to meet a stress-induced increase in metabolic demand (as is present in sepsis), cells may experience an energetic exhaustion that can lead to organ dysfunction. In the case of PBMCs, this may present clinically as immune dysregulation or suppression. Prior studies have shown that reduced mitochondrial respiration in PBMCs is associated with immunosuppression in adult sepsis (18) and in hemorrhagic shock (26). The combined observations in this study that basal oxygen consumption was maintained while SRC was diminished suggests that PBMCs are respiring near maximal levels during early sepsis in order to maintain ATP-linked oxygen consumption and other mitochondrial functions. Although a metabolic transition from oxidative phosphorylation to glycolysis upon PBMC activation (particularly in lymphocytes) could help to preserve overall cellular ATP production, oxidative phosphorylation remains critical to PBMC survival (23, 24) and these immune cells appear to be dangerously close to the point of mitochondrial bioenergetic failure early in pediatric sepsis.

Our pediatric data agree with several prior studies in adult sepsis that have reported decreased mitochondrial oxygen consumption in PBMCs from septic adults (18–20). For example, Belikova et al. found that maximal oxygen consumption in the presence of uncoupler was decreased (though SRC was not explicitly calculated) and the fraction of LEAK respiration was increased compared to healthy controls (18). In contrast to these findings, however, Sjovall et al. demonstrated a progressive increase in basal and maximal respiration in adult PBMCs on day 1–2 through day 6–7 of sepsis compared to healthy controls (21). These contradictory findings may stem from methodological variability between studies. For example, we isolated PBMCs in respiration buffer solution while Sjovall et al. resuspended cells in plasma (21). If intracellular mitochondrial substrates (e.g., fatty acids, NADPH) are limited in sepsis, the lack of available plasma-derived substrates could account for the decrease in maximal oxygen consumption we observed in septic PBMCs compared to the increase observed in the study by Sjovall et al. Alternatively, prior studies have shown that septic plasma increases mitochondrial uncoupling, which should decrease SRC (36, 37). Because this effect appears reversible (18), resuspension of PBMCs in a standard buffer provides a more direct measure of intrinsic PBMC mitochondrial function as well as reduces variability in plasma factors between patients (e.g, different medications). However, as PBMCs function in their plasma environment, it is not clear if

resuspension in plasma more accurately approximates *in vivo* PBMC mitochondrial function.

We also found that the proportion of maximal mitochondrial respiration attributable to LEAK increased from <1% in controls to 17% during early sepsis. An increase in LEAK respiration suggests that mitochondrial oxygen consumption has become uncoupled from ATP synthesis, rendering oxidative phosphorylation less efficient. However, less well-coupled mitochondrial also produce less reactive oxygen species and thus could be a protective mechanism to limit oxidative damage at the expense of a small loss of energy (8). While this finding is suggestive of an increase in mitochondrial uncoupling, it should be noted that the L/E ratio may also increase in normally coupled mitochondria if the ETS_{max} is diminished as was the trend in our study. Furthermore, prior studies using PBMCs from healthy animals and human adults have reported L/E ratios ranging 1–10% (18, 21, 26, 38). The L/E ratio of ~1% observed in our PICU controls was at the lower end of this range, which could reflect either tightly coupled PBMC mitochondria in non-septic PICU patients or the challenge of accurately measuring LEAK in cases where these values approach residual oxygen consumption.

We were surprised to find no difference in ΔΨm between septic and control patients since reduced ΔΨm has been reported in PBMCs from adult patients with sepsis (17, 19). Several possible explanations may account these divergent findings. First, as the one nonsurvivor had the lowest MTR/MTG ratio in our cohort, differences in ΔΨm may have been more apparent if mortality was higher. Second, it has been proposed that MTG itself may be affected by changes in ΔΨm leading to inaccurate measures of ΔΨm using the MTR/MTG ratio (39). While we did not see large changes in MTG with purposeful dissipation of ΔΨm in preliminary work (data not shown), we cannot rule out this possibility. Third, relying on the median MTR/MTG value may fail to discern important changes in ΔΨm in subgroups of septic patients. In support of this explanation, we found that patients with full recovery of organ dysfunction within seven days exhibited the highest MTR/MTG ratio. A rise in MTR/MTG may reflect either an increase in ΔΨm or a decrease in mitochondrial mass. Mitochondrial hyperpolarizaton (increased ΔΨm) protects against formation of the mitochondrial transition pore and thus reduces apoptosis. Insufficient mitophagy of damaged mitochondria has been shown to contribute to organ injury in critical illness (40). Thus, either mitochondrial hyperpolarization (higher MTR) or reduced mitochondrial mass (lower MTG) could potentially underlie the rapid recovery in the subset of patients with highest MTR/MTG ratio.

We did not find an association between PBMC mitochondrial respiration and severity or duration of organ failure in our pediatric patients. Two prior studies using PBMCs in adult sepsis reported an association between reduced mitochondrial respiration and degree of organ injury (19, 20) while a third did not find an association with mortality (21). Given evidence for mitochondrial injury in pre-clinical and adult studies, it is unlikely that vital organ mitochondria remain unaffected in pediatric sepsis. Rather, PBMCs may be an inadequate biomarker of mitochondrial dysfunction in other organs (22). We previously reported strong correlation between mitochondrial dysfunction measured in PBMCs and heart, liver, and kidney cells early in a hemorrhagic shock model, but noted substantial

variability in mitochondrial respiration across organs after resuscitation (27). Moreover, use of a relatively crude mix of PBMCs provides only a weighted average of bioenergetic activity across diverse cell types with known differences in both glycolytic and mitochondrial function. More research is needed to determine if specific leukocyte subsets could provide a stronger clinical biomarker for mitochondrial dysfunction in other organ systems.

There are several limitations to this study. First, minor but significant changes in mitochondrial function would not have been detected in this small study. Our selection of PICU controls versus healthy children may also have biased results toward the null if PBMC mitochondrial function is similarly inhibited by non-septic illness. Second, although we excluded some known pharmacological agents that target mitochondria, given the small sample size, we were not able to control for other potential medication effects on mitochondrial function. However, a sensitivity analysis excluding patients receiving valproic acid yielded similar results to the overall analysis. Second, we normalized measures of oxygen consumption to cell number but not mitochondrial content. A recent study demonstrated that changes in mitochondrial content may be an important cause of altered cellular respiration (21). Third, because we were interested in the occurrence of mitochondrial dysfunction in patients with persistent MODS despite adequate resuscitation, we did not determine if peak mitochondrial injury may be antecedent to or concurrent with shock onset. In addition, although we excluded patients with prolonged hypotension in order to limit the impact of inadequate resuscitation on mitochondrial measurements, this may have also excluded the most severely ill patients. Finally, because we considered PBMCs all together, we do not have information about differences in mitochondrial dysfunction within subpopulations of immune cells (e.g., monocytes, T- and B-lymphocytes). Further insight into the complete bioenergetic profile of distinct leuckocyte subests may also be elucidated by studying these cells under both resting and immunostimulated conditions, similar to provocative assessments of immune function (5).

CONCLUSIONS

Mitochondrial dysfunction, indicated by a decreased spare respiratory capacity and increased uncoupling, occurs in PBMCs from critically ill children with septic shock and MODS. PBMCs may be operating at or near their maximum oxidative respiratory capacity early in pediatric septic shock, leaving these cells vulnerable to stress, bioenergetic exhaustion, and dysfunction. Changes in PBMC mitochondrial membrane potential ($\Delta\Psi m$) relative to mitochondrial mass, but not respiration, were associated with duration of organ failure. Future studies using PBMCs could provide insight into the linkages between mitochondrial dysfunction, immune dysregulation, and organ injury in pediatric sepsis.

Supplemental Table 1: Medications prescribed to patients at time of blood sampling

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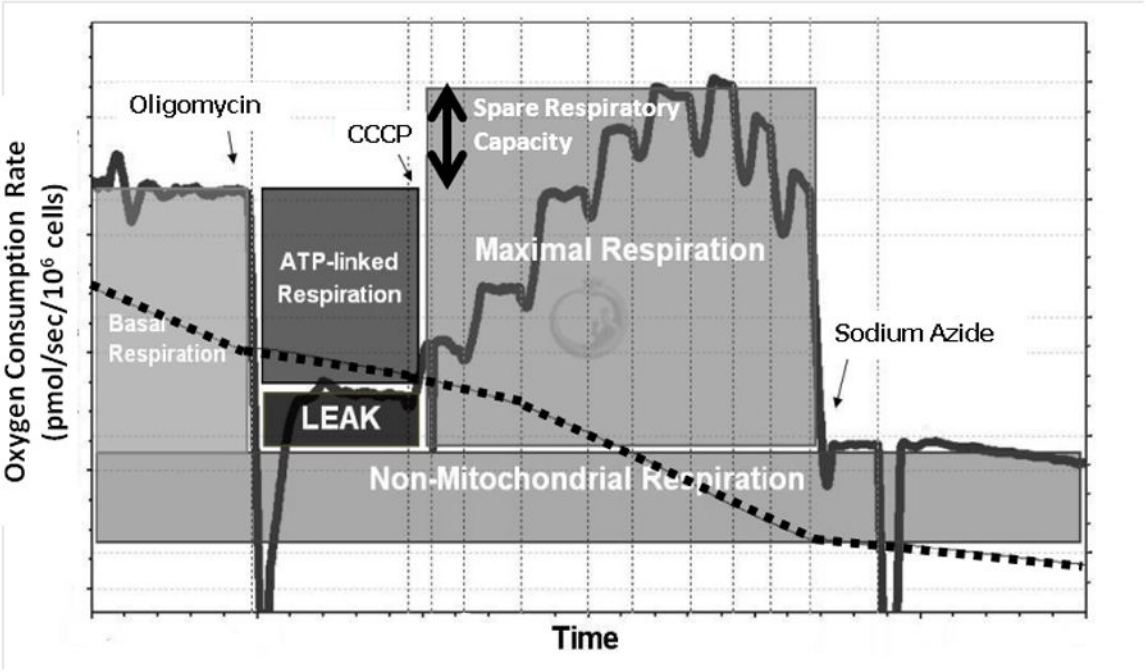

Figure 1.

Representative tracing of oxygen consumption measured in intact PBMCs. The *solid black* line shows the *amount* of oxygen present in the chamber over time. The *dashed black* line shows the *rate* of oxygen consumption (oxygen flux). After measuring basal oxygen consumption, the ATP-synthase inhibitor oligomycin is added to obtain LEAK. Then, to measure maximal oxygen consumption, the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) is repeatedly added until no further increase in oxygen consumption is detected. Residual, non-mitochondrial respiration is obtained by adding the ETS complex IV inhibitor, sodium azide, which is then subtracted from the other measurements. Finally, ATP-linked oxygen consumption is calculated as basal minus LEAK and spare respiratory capacity (SRC, *double-headed arrow*) is calculated as maximal minus basal oxygen consumption.

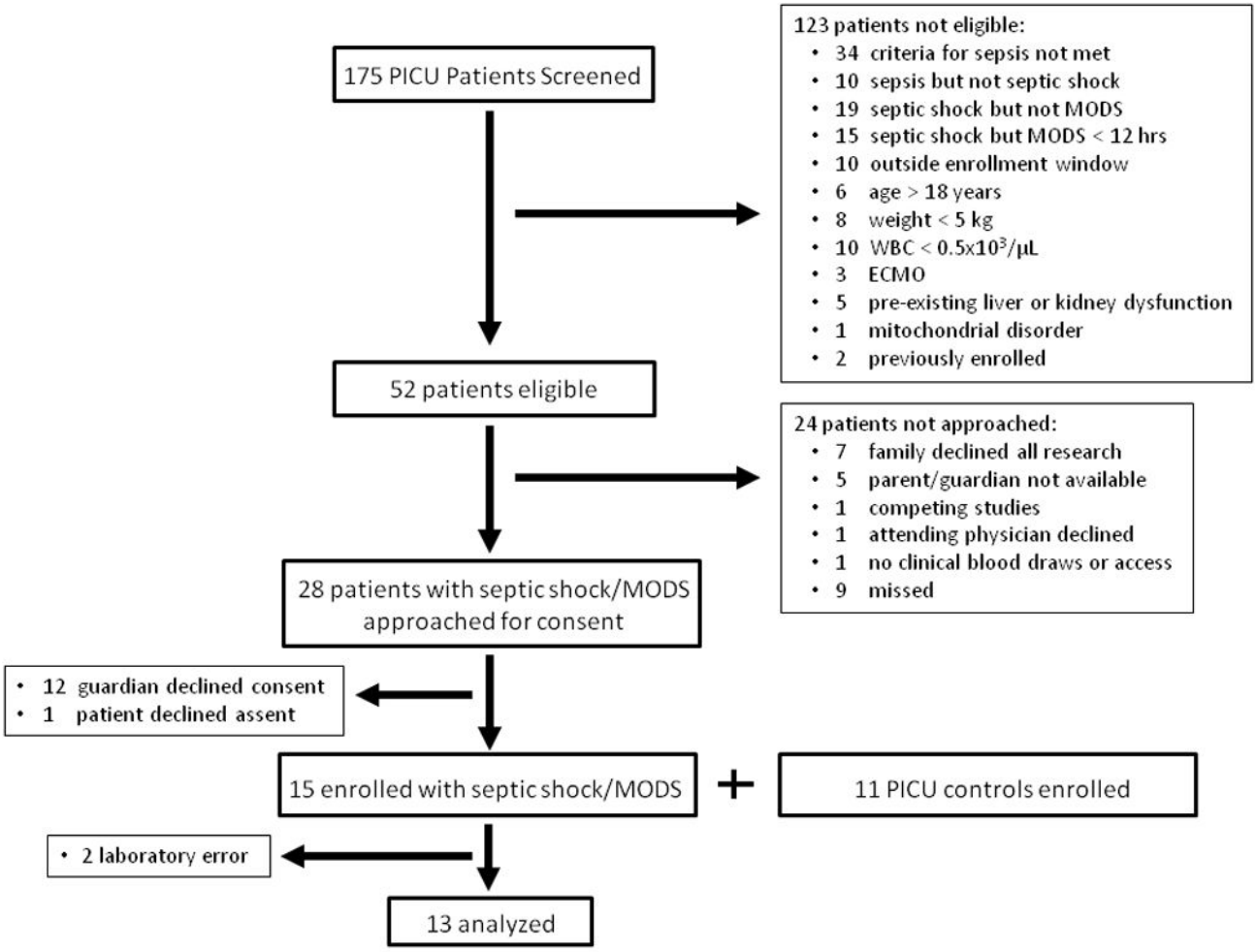

Figure 2.
Patient screening and study enrollment.

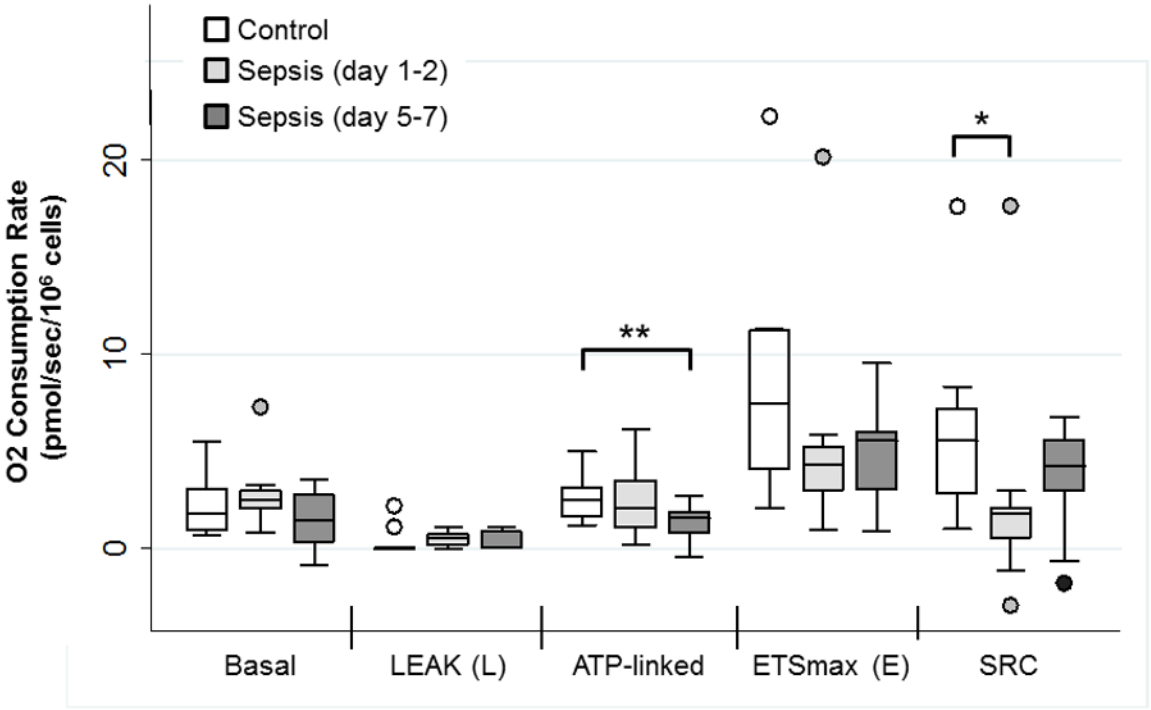

Figure 3.

Mitochondrial oxygen consumption was measured in PBMCs. Septic shock reduces spare respiratory capacity (SRC) but not basal, LEAK, or ATP-linked oxygen consumption in PBMCs on day 1–2. By day 5–7, SRC normalized but ATP-linked oxygen consumption was decreased below control values. Data are presented in box plot analysis with the *central line* indicating the median and the *white* (control) and *gray* (sepsis) boxes indicating the interquartile range. *p=0.03, **p=0.02

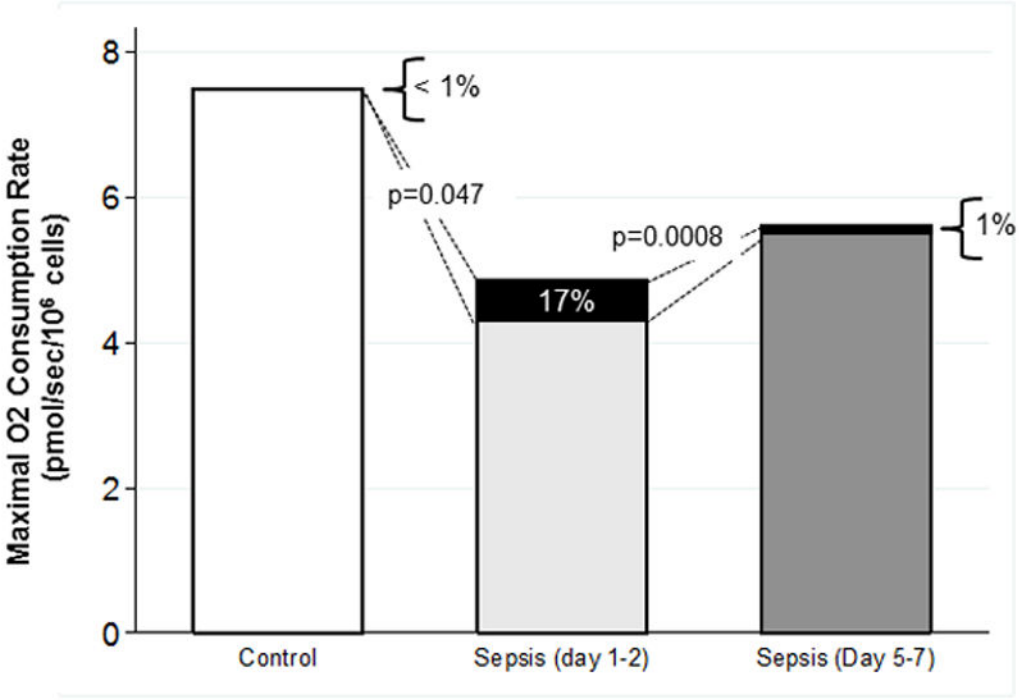

Figure 4.

LEAK flux control ratio. The ratio of LEAK to ETS_{max} respiration (L/E) is an index for uncoupling of mitochondrial oxygen consumption from ATP synthesis. Each bar represents the median maximal oxygen consumption through the ETS ($ETS_{max)}$ obtained by stepwise titration of uncoupler. The *black portion of each bar* is the L/E ratio, or proportion of maximal respiration not inhibited by oligomycin (called LEAK respiration). The L/E ratio was highest in septic patients on day 1–2, suggesting partial mitochondrial uncoupling early in pediatric sepsis.

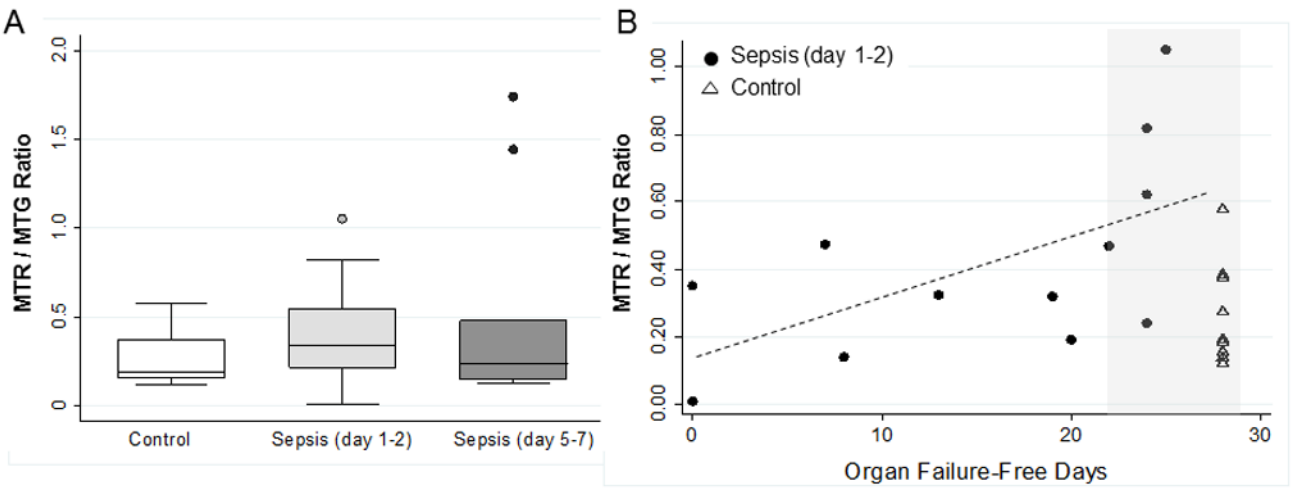

Figure 5.

Assessment of mitochondrial membrane potential (ΔΨm) in PBMCs. MitoTracker Red (MTR) indicates ΔΨm and MitoTracker Green (MTG) indicates mitochondrial mass. *A*, ΔΨm, normalized to mitochondrial mass, was not different between controls and septic patients on day 1–2 or day 5–7. Data are presented in box plot analysis with the *central line* indicating the median and the *white* (control) and *gray* (sepsis) boxes indicating the interquartile range. B, In septic patients, MTR/MTG on day 1–2 weakly correlated with organ failure-free days (*dashed line*, ρ=0.56, p=0.06). Control patients cluster at 28 organ failure-free days because none had organ dysfunction during the study period. MTR/MTG was higher in septic patients with a short duration of organ failure ≤7 days (i.e., more organ failure-free days, *shaded region*) compared to septic patients with organ failure duration >7 days (p=0.04) and controls (p=0.02).

Table 1

Patient characteristics

	Group	Age (yrs)	Comorbid Conditions	Indication for PICU Admission	Type of Infection	Micro-organism	No. of Organ Failures[a]	PRISM-III[b]	Day 28 Outcome[c]
1	Sepsis	16	Previously healthy	Septic shock	Bacteremia	*H. influenza*	4	22	A
2	Sepsis	2	Cerebral palsy	Hypoxia	Pneumonia	*H. influenza* + *M. catarrhalis*	2	0	A
3	Sepsis	13	Previously healthy	Septic shock	Toxic shock syndrome	*S. aureus*	2	14	A
4	Sepsis	12	Epilepsy	Septic shock	Pneumonia	Parainfluenza	3	15	A
5	Sepsis	6	Cerebral palsy	Septic shock	Pneumonia	Not found	2	6	A
6	Sepsis	7	Pilocytic astrocytoma	Septic shock	Pneumonia	Not found	5	11	A
7	Sepsis	3	Cerebral palsy	Septic shock	Pneumonia	RSV[d]	3	7	A
8	Sepsis	6	Crouzon's syndrome	Septic shock	Meningitis	*S. pneumoniae*	4	14	A
9	Sepsis	8	Schizencephaly	Septic shock	Pneumonia	*P. aeruginosa*	4	9	A
10	Sepsis	16	Cerebral palsy	Status epilepticus	Peritonitis	Not found	3	31	D
11	Sepsis	2	VACTERL	Septic shock	Bacteremia	*E. coli* + *K. pneumoniae*	5	18	A
12	Sepsis	1	Neuroblastoma	Septic shock	Colitis	*C. difficile*	2	30	A
13	Sepsis	10 mos	Prematurity	Respiratory distress	Pneumonia	HMPV[e]	2	2	A
14	Control	15	Eating disorder	Bradycardia			0	6	A
15	Control	16	Goiter	Hypertension			0	0	A
16	Control	12	Neuroenteric cyst	Post-op monitoring			0	0	A
17	Control	6	Nephrotic syndrome	Pulmonary embolism			0	0	A
18	Control	9	Subglottic stenosis	Tracheal dilitation			0	0	A
19	Control	3	Spina bifida, scoliosis	Post-op monitoring			0	3	A
20	Control	17	Previously healthy	Ingestion			0	12	A
21	Control	8	Diabetes mellitus	Electrolyte disorders			0	2	A
22	Control	9 mos	Spina bifida, Chiari II	Post-op monitoring			0	0	A
23	Control	3	Previously healthy	Epidural hematoma			0	5	A
24	Control	1	Tracheo-esophageal fistula	Tracheostomy removal			0	3	A

[a] Number of organ failures at time of informed consent/assent

[b] PRISM-III score on day of PICU admission

[c] A, alive; D, deceased

[d] Respiratory syncytial virus

[e] Human metapneumovirus

Table 2

Clinical features of patients at time of blood sampling

	Controls	Sepsis (Day 1–2)	Sepsis (Day 5–7)
PELOD score	0 (0–0)	11 (10–11)	1 (0–10)
Inotrope score	0 (0–0)	9 (5–13)	7 (3–11)
WBC (thous/μL)	9.4 (7.9–10.7)[b]	9.8 (8.6–11.5)	9.8 (8.1–11.4)
PBMCs (per μL)[a]	1905 (1208–2765)[b]	1615 (1372–2700)	2958 (2187–3990)
Lymphocytes (per μL)	1270 (900–2370)[b]	1131 (801–1914)	2156 (1768–3306)
Monocytes (per μL)	434 (395–635)[b]	448 (285–696)	696 (486–1048)
Lactate (mmol/L)	n/a	0.9 (0.7–1.3)	0.9 (0.9–1.6)
ScvO2 (%)	n/a	75 (71–83)	76 (70–80)
Corticosteroids, n (%)	2 (18)	5 (38)	4 (40)
Insulin, n (%)	1 (9)	0	0
Aminoglycoside, n(%)	0	0	0

Values are median (interquartile range), unless otherwise noted

PELOD; Pediatric Logistic Organ Dysfunction; WBC, white blood cell count; PBMCs; peripheral blood mononuclear cells; ScvO2; central venous oxygen saturation; n/a, data not available

[a] Absolute number of lymphocytes and monocytes on complete blood count

[b] Complete blood count data available from only 9 of the 11 control patients