

Influence of Immune Cell Subtypes on Mitochondrial Measurements in Peripheral Blood Mononuclear Cells From Children with Sepsis

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ABSTRACT

Introduction: Peripheral blood mononuclear cells (PBMCs) are commonly used to compare mitochondrial function in patients with versus without sepsis, but how these measurements in this mixed cell population vary by composition of immune cell subtypes is not known, especially in children. We determined the effect of changing immune cell composition on PBMC mitochondrial respiration and content in children with and without sepsis.

Methods: PBMC mitochondrial respiration and citrate synthase (CS) activity, a marker of mitochondrial content, were measured in 167 children with sepsis at three timepoints (day 1-2, 3-5, and 8-14) and once in 19 non-septic controls. The proportion of lymphocytes and monocytes and T, B, and NK cells was measured using flow cytometry. More specific CD4+ and CD8+ T cell subsets were measured from 13 sepsis patients and 6 controls. Spearman's correlation and simple and mixed effects linear regression were used to determine the association of PBMC mitochondrial measures with proportion of immune cell subtypes.

Results: PBMC mitochondrial respiration and CS activity were correlated with proportion of monocytes, lymphocytes, T, B, and NK cells in controls, but not in sepsis patients. PBMC mitochondrial respiration was correlated with CD4+ and CD8+ T cell subsets in both groups. After controlling for differences in immune cell composition between groups using linear

regression models, PBMC respiration and CS activity remained lower in sepsis patients than controls.

Conclusions: Mitochondrial measurements from PBMCs varied with changes in immune cell composition in children with and without sepsis. However, differences in PBMC mitochondrial measurements between sepsis patients and controls were at least partially attributable to the effects of sepsis rather than solely an epiphenomena of variable immune cell composition.

Key Words: mitochondria; metabolism; human immunology; immunometabolism; sepsis; pediatrics

INTRODUCTION

Mitochondria play a central role in cellular metabolism in all organ systems. Alterations in mitochondrial function have been implicated in the initiation, propagation, and resolution of organ dysfunction and immune dysregulation in critical illness, such as sepsis (1-4). When mitochondria are unable to use oxygen and other substrates to sustain adenine triphosphate production through oxidative phosphorylation (OXPHOS), a resulting energy deficit can impair cell function, particularly when delivery of metabolic substrates is limited, as in septic shock (5, 6). The resulting bioenergetic crisis may explain the paradox of organ injury despite limited cell death in sepsis (7, 8).

Indirect measures of OXPHOS, such as blood lactate and venous oxygen saturation, are not specific to mitochondria and do not specify a mechanism of metabolic impairment (9). As such, there is considerable interest in more direct measures of mitochondrial dysfunction. Peripheral blood mononuclear cells (PBMCs) have often been used for this purpose (10-14), as they provide an accessible source of mitochondria, have demonstrated moderate correlation with mitochondrial changes in other organ systems (15), and are directly involved in the immuno-inflammatory response characteristic of sepsis and other illnesses (3, 16, 17). However, PBMCs are a heterogeneous mixture of lymphocyte and monocyte subtypes with variable metabolic profiles. Most studies relating PBMC mitochondrial respiration and content to clinical outcomes have largely ignored the potential confounding introduced by differences in the relative abundances of different cell types across individuals and over time (18, 19).

A recent study of healthy adults demonstrated that mitochondrial phenotypes measured in PBMCs are confounded by variable composition of immune cell subtypes and urged caution in using PBMCs to assess mitochondrial changes within and between individuals (20). However, there are no data about the influence of immune cell subtypes on mitochondrial phenotypes measured in PBMCs from children. It is also unclear if an inflammatory disease state, such as sepsis, might overwhelm metabolic influences

attributable to different immune cell subtypes, such that PBMCs might still be useful as a pragmatic source to measure mitochondrial function during illness. Thus, we sought to compare the effect of immune cell subtype variation on PBMC mitochondrial respiration and content in children with and without sepsis using data from a prior study which demonstrated that low PBMC respiration in children with sepsis, relative to non-sepsis controls, was associated with slower recovery from organ dysfunction and sustained inflammation (17, 21). We hypothesized that PBMC mitochondrial respiration and content would vary with changes in the proportions of immune cell subtypes. We further hypothesized that this confounding influence would not fully explain differences in PBMC mitochondrial measures between children with versus without sepsis. A better understanding of how PBMC mitochondrial measures vary as the immune subsets comprising this group of cells is necessary to ascertain whether PBMCs offer a useful source to assess mitochondrial dysfunction in children.

MATERIALS AND METHODS

Study Design and Population: We performed a secondary analysis of a prospective observational study of patients <18 years treated for sepsis or septic shock in a single academic pediatric intensive care unit between May 2014 and June 2018. Sepsis and septic shock were defined using consensus pediatric criteria (see Supplemental Digital Content, <http://links.lww.com/SHK/B390>) (22). Exclusion criteria were weight <7.5 kg (due to limited blood collection), white blood cells <0.5x10³/μL, mitochondriopathy, unrepaired cyanotic heart disease, and prior enrollment. A convenience sample of PICU patients, matched to sepsis by age and sex, without evidence for infection or organ dysfunction were enrolled as controls. The study was approved by the Children's Hospital of Philadelphia Institutional Review Board and written informed consent was obtained. Additional details of the primary study have been previously published (17, 21).

Blood Collection: An initial blood sample of 7-9 mL was collected as soon as possible after consent from sepsis patients and non-septic PICU controls for measurement of PBMC mitochondrial respiration and content and basic flow cytometry to define immune cell subtypes. For sepsis patients, this initial sample was collected within 48 hours of sepsis recognition (day 1-2), and additional blood was collected between study days 3-5 (at least two days after first sample) and again between days 8-14. A complete blood count ordered by the treating team for clinical purposes was measured as close as possible to the study blood draw in the main clinical hematology laboratory. For a subset of patients with sepsis and non-septic PICU controls who weighed ≥20 kg and provided supplementary consent, an additional 5.4 mL of blood was collected to define T- and B-lymphocyte subtype composition.

Mitochondrial Respiration: PBMCs were isolated from citrated whole blood within 60 minutes of collection by density gradient centrifugation report (see Supplemental Digital Content, <http://links.lww.com/SHK/B390>), as previously reported (21). The median viability of the isolated PBMCs was 88% (interquartile range 76-95%) using trypan blue exclusion (Countess, Life Technologies, Grand Island, NY). Immediately following isolation, the rate

of oxygen consumption was measured in $2-4 \times 10^6$ intact PBMCs at 37°C using a high-resolution oxygraph (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), as previously described (21). Respirometry within intact cells preserves the cellular microenvironment such that oxygen consumption relies on endogenous substrates and best approximates *in vivo* metabolic conditions (23). We directly measured basal respiration, proton leak after inhibition of ATP synthase (leak), and maximal uncoupled respiration through the electron transport system (ETS_{max}), with subtraction of non-mitochondrial respiration from all parameters. Respiration supporting mitochondrial ATP synthesis (ATP-linked respiration) was calculated as basal minus leak. Spare respiratory capacity (SRC), calculated as ETS_{max} minus basal respiration, is the mitochondrial bioenergetic reserve available for cells to produce ATP in response to a stress-induced increase in metabolic demand (23).

Mitochondrial Content: Mitochondrial content was estimated as citrate synthase (CS) activity using spectrophotometry and normalized to total cellular protein, as previously described (21). Specific activity of the CS enzyme, part of the tricarboxylic acid cycle, is commonly used as a marker of mitochondrial content within cells (24, 25).

Immune Cell Subtypes: The percentage of circulating white blood cells that were neutrophils, lymphocytes, and monocytes was obtained from a clinical complete blood count (Sysmex 3100 analyzer, Lincolnshire, IL) sampled at the same time of PBMC collection (or closest measurement within 24 hours if not measured concurrently). The percentage of total lymphocytes that were T cells, B cells, and NK cells was determined using flow cytometry from blood sampled at the same time as PBMC collection. A $50 \mu\text{L}$ sample of whole blood collected on EDTA was incubated with $5 \mu\text{L}$ of the CD45-Alexa Fluor 647 antibody (Biolegend, San Diego, CA) for 15 minutes in a dark room at room temperature. The sample was then diluted 1:1,000 in Hank balanced salt solution and further stained using standard cell surface markers for T cells (CD3+), B cells (CD20+), and NK cells (CD56+). Percentages of total CD45+ lymphocytes that were T, B, and NK cells were measured using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo (Treestar, Ashland, OR).

For a subgroup of patients who weighed ≥ 20 kg and consented to additional blood collection, a portion of isolated PBMCs were cryopreserved as $8-10 \times 10^6$ cells/mL in 90% fetal bovine serum and 10% dimethylsulfoxide. Samples were then stored at -80°C for batched analysis. Cryopreserved PBMCs were rapidly thawed in a 37°C water bath. Thawing media (RPMI, 10% fetal bovine serum, penicillin, streptomycin and glutamine) was added to the cell suspension, which was then spun down. After removal of the supernatant, the cell pellet was re-suspended in media and standard cell surface markers for T and B cell subsets were added. Flow data were gated using FlowJo to determine the percentages of total CD4+ and CD8+ T cells that were naïve (CD27+CD45RA+), central memory (CM; CD27+CD45RA-), effector memory (EM; CD27-CD45RA-), and effector memory T cells re-expressing CD45RA (TEMRA; CD27-CD45RA+) subsets.

Statistical Analysis: Analyses were performed using STATA Version 16.1 (College Station, TX). Data are presented as means (\pm SD) or median (interquartile range, IQR) for continuous variables and percentages for categorical variables. The relationship of mitochondrial respiration and CS activity with the composition of immune cell subtypes is presented with Spearman's correlation coefficient. We used simple linear regression for non-septic controls and mixed effects linear regression with patient as a random effect and timepoint as a random slope to determine the association of PBMC respiration and CS activity with the percentage of each immune cell subtype. While sepsis patients could contribute data from up to three timepoints, our aim was to determine the association of PBMC mitochondrial measurements with concurrent variation in immune cell subtype composition rather than how changes in these two parameters may interact over time. Mixed effects regression allowed inclusion of sepsis patients with missing data at some timepoints and accounted for repeated measures within patients. To differentiate the effects of sepsis from variability in immune cell subtype on mitochondrial measurements, we calculated the predicted values for PBMC mitochondrial respiration and CS activity for controls and sepsis patients with an average composition of immune cell subtypes using the linear regression and mixed effects regression models, respectively. We then compared, using unpaired t-tests, the predicted PBMC mitochondrial respiration and CS activity between a) controls with the average composition of immune cell subtypes observed in controls, b) sepsis patients with the same average composition of immune cell subtypes observed in controls, and c) sepsis patients with the average composition of immune cell subtypes observed in sepsis. Statistical significance was defined as p-value ≤ 0.05 without correction for multiple comparisons due to the exploratory nature of these analyses.

RESULTS

Of the 167 patients with sepsis enrolled in the parent study, mitochondrial respirometry data were able to be paired with immune cell subtypes from a concurrently measured complete blood count at 286 timepoints (from 151 patients) and with flow cytometry at 50 timepoints (from 40 patients; Figure 1A). Citrate synthase activity was able to be paired with immune cell subtypes from a concurrently measured complete blood count at 269 timepoints (from 143 patients) and with flow cytometry at 49 timepoints (from 39 patients). Of the 19 non-septic PICU controls (each enrolled in the parent study with only a single blood collection), mitochondrial respirometry data were able to be paired with immune cell subtypes from a concurrently measured complete blood count from 9 patients and with flow cytometry from 11 patients (Figure 1B). From controls, citrate synthase activity was able to be paired with a complete blood count from 7 patients and with flow cytometry from 10 patients. Patient characteristics of sepsis and control patients are summarized in Table 1, as more detailed clinical data about these groups have been previously published (17, 21).

Variation in PBMC mitochondrial measurements by proportion of circulating white blood cell subtypes: PBMC mitochondrial measures correlated with the proportion of major circulating white blood cell subtypes in controls, but not in sepsis patients (Figure 2). Among controls, all measures of mitochondrial respiration from intact PBMCs were moderately to

highly correlated with the composition of immune cell subtypes, most notably the proportion of monocytes and T, B, and NK cells (Supplemental Figure 1, <http://links.lww.com/SHK/B390>). Although few of these associations met statistical significance due to the small number of controls, the Spearman's correlations were generally in the moderate (>0.4 or <-0.4) to high (>0.7 or <-0.7) range. There were also moderate-high correlations between PBMC CS activity and proportion of monocytes ($\rho=0.43$), T cells ($\rho=-0.50$), and NK ($\rho=0.52$) cells in controls (Supplemental Figure 1, <http://links.lww.com/SHK/B390>). In contrast, among sepsis patients, there was minimal correlation between mitochondrial respiration from intact PBMCs or CS activity with composition of these immune cell subtypes (Figure 2, Supplemental Figure 2, <http://links.lww.com/SHK/B390>), with the most prominent Spearman's correlation being only $\rho=-0.28$ (CS activity and proportion of B cells).

Variation in PBMC mitochondrial measurements by proportion of T cell subsets:

Data from T cell subsets were available from 13 sepsis patients that yielded 32 measurements of PBMC mitochondrial respirometry and 29 measurements of CS activity that could be paired with concurrently measured proportions of CD4+ and CD8+ cells (Figure 1A). Six control patients yielded six paired measurements of PBMC mitochondrial respirometry and four paired measurements of CS activity with T cell subsets (Figure 1B). Among controls, PBMC mitochondrial respiration was moderately correlated with proportion of CD4+ EM and TEMRA cells (Figure 3, Supplemental Figure 3, <http://links.lww.com/SHK/B390>). In patients with sepsis, PBMC mitochondrial respiration was also moderately to highly correlated with proportion of CD4+ EM and TEMRA cells (Figure 3, Supplemental Figure 4, <http://links.lww.com/SHK/B390>), although the direction of association differed between sepsis and control patients. PBMC respiration was not correlated with CD4+ EM and TEMRA cells in controls but these parameters were generally negatively correlated in sepsis. CS activity did not demonstrate moderate/high correlation with varying proportions of T cell subsets in controls or sepsis patients (Figure 3, Supplemental Figures 3, 4, <http://links.lww.com/SHK/B390>). The proportions of CD8+ T cell subsets generally mirrored the findings with CD4+ T cell subsets, except that CD8+ EM cells demonstrated moderate negative correlations with all PBMC respiration measures, except leak, in controls (Figure 3, Supplemental Figures 3, 4, <http://links.lww.com/SHK/B390>).

Differences in circulating immune cell subtypes in sepsis versus controls: The composition of immune cell subtypes in sepsis patients and controls is shown in Figure 4. Within the PBMC population, approximately two-thirds of the cells were lymphocytes and one-third monocytes (Figure 4A). Sepsis patients had a numerically higher median ratio of lymphocytes to monocytes than controls, though this difference was not significant (sepsis day 1-2: 2.3, IQR 1.5-4.3 on sepsis day 1-2 versus controls: 1.5, IQR 1.3-4.8; $p=0.86$, Supplemental Figure 5, <http://links.lww.com/SHK/B390>). T cells comprised a mean \pm SD of $61 \pm 10\%$ of total lymphocytes in controls and $57 \pm 20\%$ on day 1-2, $50 \pm 19\%$ on day 3-4, and $69 \pm 22\%$ on day 8-14 in sepsis patients (Figure 4B), with significantly lower T cells on day 3-5 compared to controls ($p=0.05$). The NK proportion decreased over time in sepsis and was significantly lower than the NK proportion in controls by day 8-14 ($p=0.003$). Among

CD4⁺ T cells subsets, CM cells comprised a lower proportion, while TEMRA cells comprised higher proportions, in sepsis compared to controls (Figure 4C-D).

Attribution of PBMC mitochondrial measurements to sepsis versus variation in immune cell composition: Because circulating immune cell subtypes differed between sepsis patients and controls and PBMC mitochondrial measurements varied with the composition of immune cells, we next sought to differentiate the effects of sepsis from variability in immune cell subtype on PBMC mitochondrial measurements. We used regression models to compare predicted PBMC mitochondrial respiration and CS activity in sepsis patients and controls with the same composition of immune cell subtypes under modeled conditions (Figure 5, Supplemental Figure 6, <http://links.lww.com/SHK/B390>). If the observed differences in PBMC mitochondrial measurements between sepsis patients and controls were only attributable to variation in immune cell composition, then sepsis patients and controls should exhibit similar predicted values for respiration and CS activity when the immune cell profile is held constant. For example, among controls, the mean proportions of lymphocytes and monocytes were 20% and 8%, respectively. A control patient with these average values of lymphocytes and monocytes would have a predicted PBMC SRC of 8.96 pmole/sec/10⁶ cells using the linear regression model comparing PBMC mitochondrial respiration to lymphocyte and monocyte counts among controls (Supplemental Figure 6A, <http://links.lww.com/SHK/B390>). A sepsis patient with the same lymphocyte and monocyte profile would be predicted to have a lower PBMC SRC of 6.64 pmol/sec/10⁶ cells using the mixed effects linear regression model comparing PBMC mitochondrial respiration to lymphocyte and monocyte counts in sepsis. This difference (8.96 versus 6.64 pmol/sec/10⁶ cells) is the estimated effect of sepsis on PBMC SRC, since the lymphocyte-monocyte composition is held constant. However, the observed mean profile of 17% lymphocytes and 7% monocytes in sepsis would be predicted to yield PBMC SRC of 6.49 pmol/sec/10⁶ cells. This difference (6.64 versus 6.49 pmol/sec/10⁶ cells) is the estimated effect of the variability in immune cell subtype. Therefore, the difference in PBMC SRC between sepsis and control patients was largely attributable to factors other than a change in the abundance of lymphocytes and monocytes, which is consistent with the low correlation between PBMC mitochondrial respiration/CS activity and lymphocytes or monocytes in sepsis.

In contrast, PBMC SRC was predicted to be significantly lower in sepsis patients than controls with the same CD4⁺ T cell composition, as well as an additional significant decrease attributable to the variability in CD4⁺ T cell subtypes (Figure 5, Supplemental Figure 6B, <http://links.lww.com/SHK/B390>). This latter finding is consistent with the moderate-high correlation of PBMC mitochondrial respiration with proportion of CD4⁺ cells and suggests that PBMC mitochondrial measurements are attributable to both sepsis and variation in T cell subset composition.

DISCUSSION

In this analysis, we demonstrated that PBMC mitochondrial respiration and CS activity correlate with the abundance of specific immune cell subtypes in children with and

without sepsis. In non-sepsis controls, PBMC mitochondrial measurements correlated with the proportions of lymphocytes, monocytes, T, B, and NK cells but these correlations were not evident in sepsis patients. However, PBMC mitochondrial respiration (but not content) did correlate with variation in the proportion of T cell subsets in both children with and without sepsis. Although these data indicate that PBMC mitochondrial measurements can be confounded by variable composition of immune cell subtypes, differences in PBMC mitochondrial respiration and content between sepsis patients and controls when immune cell composition was held constant under modeled conditions indicate that sepsis itself contributes to alterations in PBMC mitochondrial function beyond changes in immune cell composition.

Interpretation of mitochondrial function measured using PBMCs can follow two different, albeit related, paths. First, PBMC mitochondrial respiration and content could be viewed solely as a diagnostic, prognostic, or predictive biomarker. In this case, such measures must be associated with a clinical diagnosis, outcome, or response to therapy, respectively, but not necessarily through a specific mechanistic link. For example, even if PBMC mitochondrial respiration changes in a predictable way only because of an unmeasured—and perhaps even unknown—biologic phenomenon (e.g., change in the predominant immune cell subtypes), so long as the measurements are strongly associated with an event it will not matter whether it is the PBMC mitochondrial biomarker or the underlying biologic phenomenon that is the cause of event. This scenario was evident in a prior study from our group in which persistence of low PBMC mitochondrial SRC was associated with prolonged organ dysfunction in children with sepsis (21). Similarly, decreased PBMC mitochondrial respiration has been associated with degree of organ failure in adult sepsis (10), while mitochondrial biogenesis within PBMCs is predictive of early ICU discharge (11).

A second interpretation of PBMC mitochondrial function may be more explanatory, in which metabolic alterations could be ascribed as directly contributing to immune activation or dysregulation. Indeed, functionally distinct T cell subsets are known to utilize different bioenergetic pathways and lymphocyte activation is linked to a metabolic switch from OXPHOS to glycolysis (26, 27). Such a mechanistic attribution implies that therapeutic intervention guided by alterations in PBMC mitochondrial function could regulate the immune response. However, if PBMC mitochondrial alterations were solely an epiphenomenon of an underlying biologic pathway, such as shifting immune cell subtypes, this interpretation would be misguided since measurements obtained using bulk PBMCs would reflect only different, rather than metabolically deficient, immune cell subtypes.

Our study suggests that the reality lays somewhere in between. Clearly, PBMC mitochondrial measurements do reflect the metabolic variability of the immune cell subtypes that comprise this heterogeneous cell population. Many of the correlations between PBMC mitochondrial respiration and CS activity we observed in non-septic children parallel the findings recently reported from 21 healthy adults, especially across varying proportions of B cells and CD4⁺ T cell subsets (20). However, we also demonstrate that sepsis itself has an

impact on PBMC mitochondrial measurements beyond what would be predicted solely from the immune cell composition. This latter finding supports the potential utility of PBMC mitochondrial function as a diagnostic, prognostic, or predictive biomarker in children with sepsis, so long as the “signal” from sepsis outweighs the “noise” from shifting immune cell composition. And while measures of PBMC mitochondrial function may be more limited as a window to assess mechanisms of specific immune cell activation or dysregulation, our findings that PBMC mitochondrial alterations are not solely an epiphenomenon of evolving immune cell subtypes in sepsis are consistent with previously identified metabolic defects in these cells (3) and suggest that PBMCs could offer a pragmatic method to guide metabolic therapies in the future.

Challenges in interpreting mitochondrial respiration measured from PBMCs, as a mixed cell population, must be balanced by the opportunities and limitations of mitochondrial assays from other human sources. Blood is clearly a more accessible biological compartment than skeletal muscle or other tissues that could be biopsied, but isolation of homogenous cell populations requires larger blood volume collection and risks excluding important cell subtypes (28). Circulating levels of cell-free mitochondrial DNA is another blood-based biomarker of mitochondrial dysfunction, but current measurement techniques are prone to misleading results and do not offer information about OXPHOS (29). Non-invasive monitors of mitochondrial respirometry could eventually offer an alternative to blood or tissue sampling (30, 31). Other methods to measure mitochondrial function, such as single-cell imaging or transcriptomics and NADH fluorescence lifetime imaging microscopy (32-34), that require fewer cell numbers may be applicable to purified immune cell subsets from small volume blood samples, but none currently offer the relative ease and historical confidence of respirometry.

This study has several limitations. First, since only select mitochondrial assays of respiration and content were performed, it is not clear if other mitochondrial measures could be more or less confounded by variability in immune cell subtypes. In addition, CS activity, though commonly used, has recently been challenged as a valid marker of mitochondrial content in some organs (35). Second, our control group was relatively small and sampled from children hospitalized in a PICU for monitoring, largely following neurosurgery. It is possible that PBMC mitochondrial measurements in a truly healthy control group may compare differently to sepsis and that effect sizes could be over-estimated given the small control group sample size. Moreover, without a control group with comparable illness severity to those with sepsis, differences in the variation of PBMC mitochondrial measures with immune cell subtypes from controls cannot be attributed to sepsis versus general critical illness. Third, we were not able to directly measure mitochondrial respiration or content within specific immune cell subpopulations due to limited blood volumes from children. Measurement of mitochondrial respiration and content in purified immune cell subtypes would confirm the extent to which each cell subpopulation is represented or masked within bulk PBMC mitochondrial measurements. Fourth, while T, B, and NK cells and T cell subsets were always measured from the sample blood sample used to isolate PBMCs, the complete blood count that was not always performed at the exact time of PBMC isolation.

This could have led to measurement error if the proportion of lymphocytes, monocytes, and neutrophils changed in this time. Fifth, we were not able to ascertain which aspects of sepsis, including inflammatory mediators or therapeutic agents, contributed to alterations in PBMC mitochondrial function. Finally, we did not account for the possibility of residual platelet contamination of PBMCs in our study. Although routine surveillance of PBMC processing for this study demonstrated >80% PBMC purity (data not shown), platelets contain large amounts of mitochondria by volume and, even at relatively low amounts, have been shown to influence PBMC mitochondrial measures (20).

CONCLUSION

PBMC mitochondrial respiration and content varied with the abundance of immune cell subtypes in children with and without sepsis. However, because differences in PBMC mitochondrial measurements between sepsis and controls were at least partially attributable to the effects of sepsis beyond changes in immune cell composition, PBMCs may offer a useful source from which to assess mitochondrial alterations in critically ill children.

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FIGURE LEGENDS

Figure 1: Patient Flow Diagram. Inclusion of patients with sepsis (A) and non-sepsis controls (B) for comparison of PBMC mitochondrial respiration and citrate synthase activity with proportion of total white blood cells that were neutrophils, monocytes, and lymphocytes obtained from a concurrent clinical complete blood count (CBC), proportion of lymphocytes that were T cells, B cells, and NK cells measured using flow cytometry, and proportion of total CD4+ and CD8+ T cells that were naïve, central memory, effector memory, and effector memory T cells re-expressing CD45RA (TEMRA subsets measured using flow cytometry). Sepsis patients could contribute data from up to three timepoints measure on days 1-2, 3-5, and 8-14 of illness. Control patients contributed data from one timepoint measured as close as possible to study enrollment.

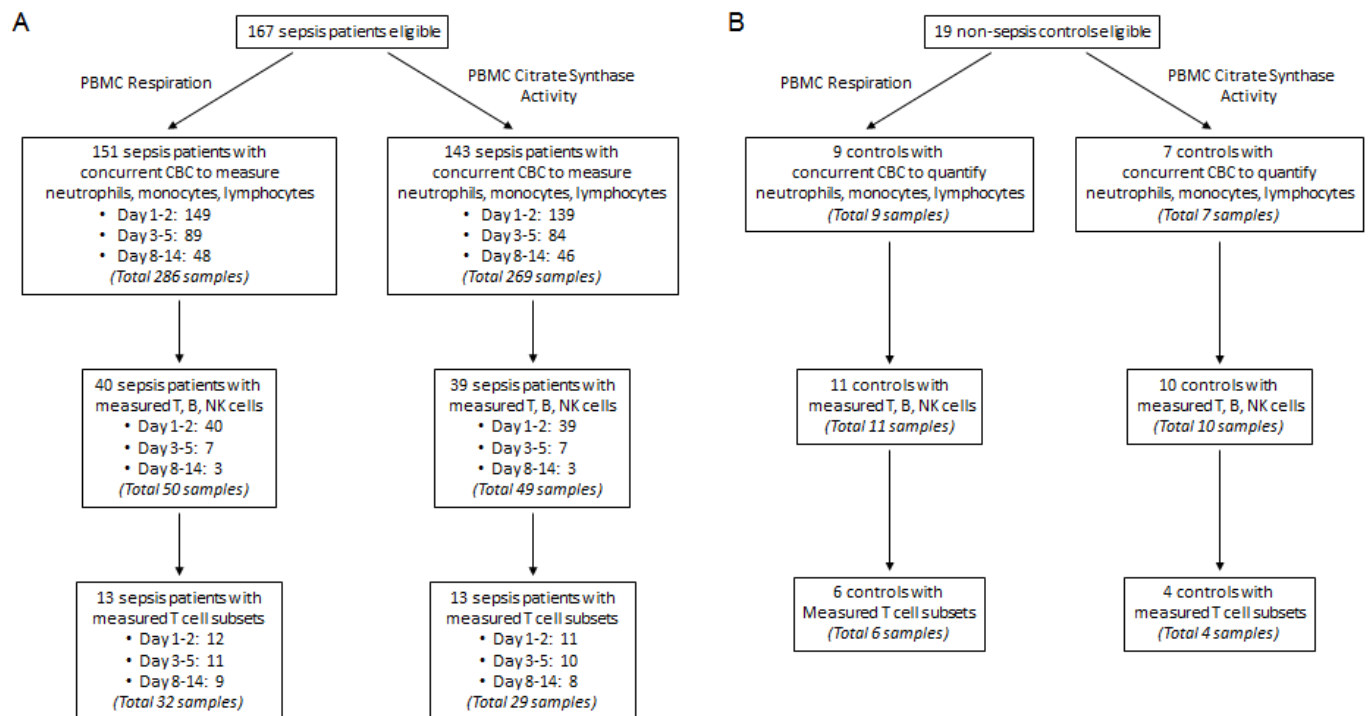


Figure 2: Correlation Heatmap of PBMC Mitochondrial Respiration and Citrate Synthase Activity with Percentage of Immune Cell Subtypes. Correlation heatmap reporting Spearman correlation coefficients for peripheral blood mononuclear cell (PBMC) mitochondrial measures with proportion of total white blood cells that were neutrophils, monocytes, and lymphocytes and proportion of total lymphocytes that were T cells, B cells, and NK cells. PBMC mitochondrial respiration was measured in intact cells as basal, ATP-linked, leak, and maximal respiration through the electron transport system (ETS_{max}). Spare respiratory capacity (SRC), an index of bioenergetic capacity linked to cell health, was calculated as ETS_{max} minus basal respiration. Citrate synthase (CS) activity is a measure of mitochondrial content. The bar to the right side of each heatmap indicates the color legend of the Spearman correlation coefficients. PBMC mitochondrial measures were more strongly correlated with immune cell subtypes in controls than sepsis patients. * p-value ≤0.05

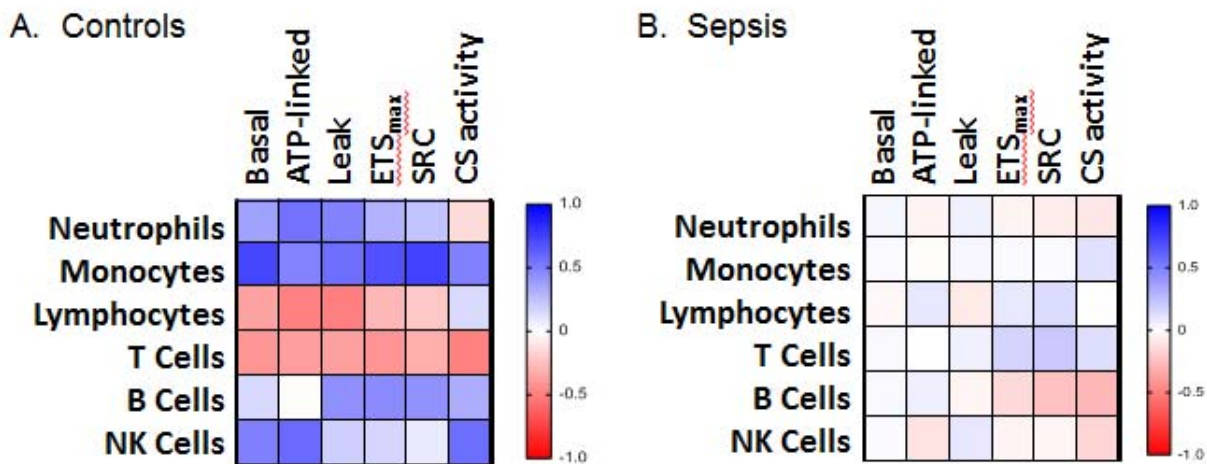


Figure 3: Correlation Heatmap of PBMC Mitochondrial Respiration and Citrate Synthase Activity with Percentage of CD4+ and CD8+ T Cell Subtypes. Correlation heatmap reporting Spearman correlation coefficients for peripheral blood mononuclear cell (PBMC) mitochondrial measures with percentages of total CD4+ and CD8+ T cells that were naïve, central memory CM, effector memory (EM), and effector memory T cells re-expresses CD45RA (TEMRA) subsets. PBMC mitochondrial respiration was measured in intact cells as basal, ATP-linked, leak, and maximal respiration through the electron transport system (ETS_{max}). Spare respiratory capacity (SRC), an index of bioenergetic capacity linked to cell health, was calculated as ETS_{max} minus basal respiration. Citrate synthase (CS) activity is a measure of mitochondrial content. The bar on the left side of the map indicates the color legend of the Spearman correlation coefficients. PBMC mitochondrial measures were more strongly correlated with immune cell subtypes in controls than sepsis patients.

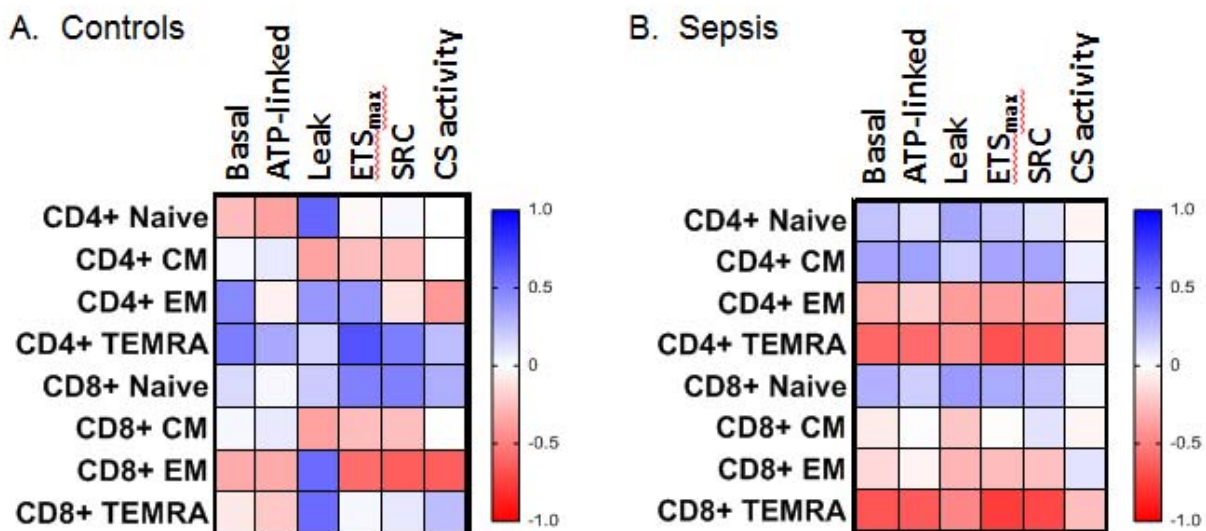


Figure 4: Composition of Immune Cell Subtypes in Control and Sepsis Patients. Bar graphs showing (a) percentage of total white blood cells composed on neutrophils, monocytes, lymphocytes, and other cell types, (b) percentage of total lymphocytes composed of T, B, and NK cells, (c) percentage of total CD4+ T cells composed of naïve, central memory (CM), effector memory (EM), and effector memory T cells re-expresses CD45RA (TEMRA) subsets, and (d) percentage of total CD8+ T cells composed of naïve, CM, EM, and TEMRA subsets in controls and sepsis patients at each timepoint. T cells comprised a lower percentage of total lymphocytes on sepsis day 3-5 compared to controls ($p=0.05$). The NK proportion decreased over time in sepsis and were significantly lower than the NK proportion in controls by day 8-14 ($p=0.003$). CM cells comprised a lower proportion of total CD4+ T cells in sepsis at all timepoints than controls, while TEMRA cells comprised higher proportions of total CD4+ T cells on days 1-2 and 3-5 in sepsis compared to controls. * p -value ≤ 0.05 compared to controls

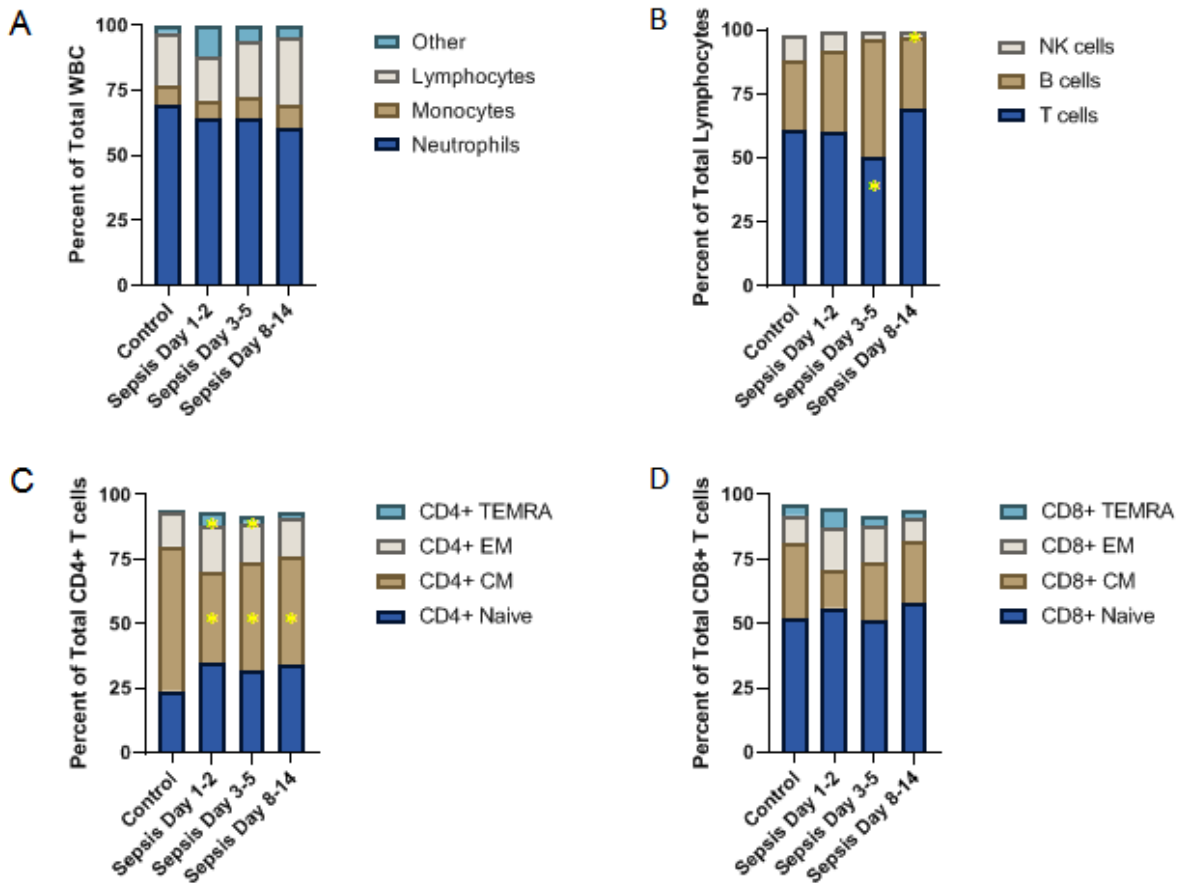


Figure 5: Predicted PBMC Mitochondrial Respiration and Citrate Synthase Activity.

Bar graphs of predicted peripheral blood mononuclear (PBMC) mitochondrial respiration parameters (basal, ATP-linked, leak, maximal respiration through the electron transport system [ETS_{max}], spare respiratory capacity [SRC], and citrate synthase [CS] activity) for a control patient at the average composition of immune cell subtypes observed among all measured controls (blue), a sepsis patient with the same average composition of immune cell subtypes observed among all measured controls (red), and a sepsis patient with the average composition of immune cell subtypes observed among all measured sepsis patients (green). Predicted values for controls were generated using simple linear regression. Predicted values for sepsis were generated using mixed effects linear regression. The change in values from controls with control immune composition (blue bars) to sepsis with control immune composition (red bars) indicates the impact of sepsis of PBMC mitochondrial respiration or CS activity, since immune cell composition is held constant. The change from in values from sepsis with control immune composition (red bars) to sepsis with sepsis immune composition (green bars) indicates the impact of variable immune cell composition on PBMC mitochondrial respiration or CS activity. Error bars indicated 95% confidence interval of predicted values. * p-value ≤0.05 **p-value ≤0.01

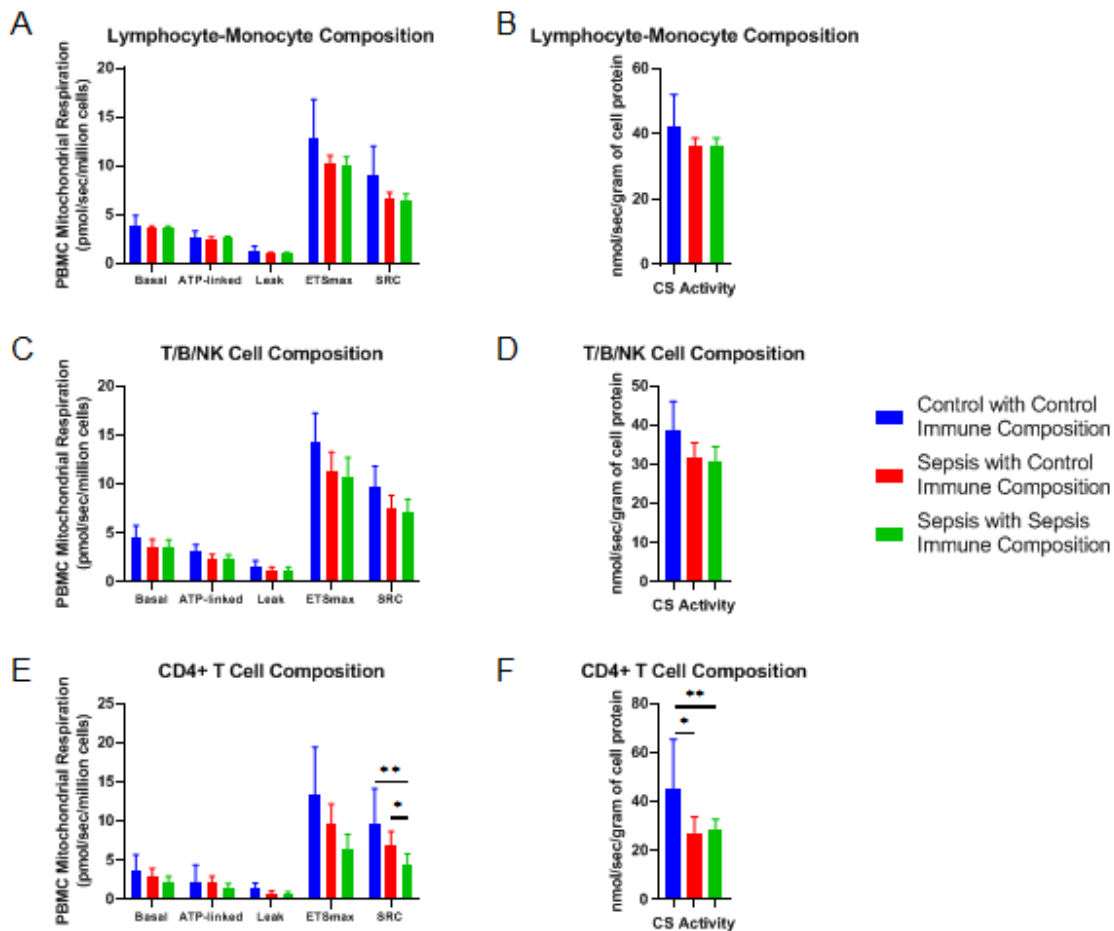


Table 1: Patient Characteristics^a

Variable ^b	Controls	Sepsis
N	19	167
Age, years	9.7 (1.2-13.4)	8.1 (3.4-13.7)
Sex, male	9 (47)	88 (53)
Race		
White	13 (68)	83 (50)
Black	3 (16)	47 (28)
Other or not reported	3 (16)	37 (22)
Previously healthy	2 (11)	20 (12)
Cancer or HSCT	2 (11)	27 (16)
PRISM-III score	0 (0-3)	11 (6-18)
No. of organ dysfunctions at study enrollment	0 (0-0)	2 (2-3)
Reason for PICU admission		
Sepsis		167 (100)
Neurosurgery	16 (84)	
Intracranial hemorrhage	2 (11)	
Deep vein thrombosis	1 (5)	
Mortality, hospital discharge	0	12 (7)

HSCT, hematopoietic stem cell transplant; PRISM, pediatric risk of mortality; PICU, pediatric intensive care unit

^aSelect patient characteristics adapted from Weiss SL, Zhang D, Bush J, Graham K, Starr J, Henrickson S, Kilbaugh T, Deutschman CS, Murdock D, McGowan FX, et al.: Persistent mitochondrial dysfunction linked to prolonged organ dysfunction in pediatric sepsis. *Crit Care Med* 47(10):1433-1441, 2019 and Weiss SL, Zhang D, Bush J, Graham K, Starr J, Murray J, Tuluc F, Henrickson S, Deutschman CS, Becker L, et al.: Mitochondrial Dysfunction is Associated With an Immune Paralysis Phenotype in Pediatric Sepsis. *Shock* 54(3):285-293, 2020.

^bData presented as median (interquartile range) or n (%)