

Immune Cell Mitochondrial Phenotypes Are Largely Preserved in Mitochondrial Diseases and Do Not Reflect Disease Severity

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Abstract

Background and Objectives

The aim of this study was to profile immune cell mitochondrial phenotypes in mitochondrial diseases (MitoD) and evaluate how these phenotypes relate to disease manifestations or biomarkers.

Methods

We profiled mitochondrial content and oxidative phosphorylation (OxPhos) enzymatic activities in isolated monocytes, lymphocytes, neutrophils, platelets, and mixed peripheral blood mononuclear cells (PBMCs) from 37 individuals with MitoD (m.3243A > G, n = 23; single, large-scale mitochondrial DNA (mtDNA) deletions, n = 14) and 68 healthy women and men from the Mitochondrial Stress, Brain Imaging, and Epigenetics study.

Results

We first confirmed and quantified robust cell type differences in mitochondrial content; activities of OxPhos complexes I, II, and IV; and the mitochondrial respiratory capacity (MRC) index. In relation to MitoD, neither mitochondrial content nor OxPhos capacity was consistently affected, other than a mild monocyte-specific reduction in complex I (partially mtDNA encoded) relative to complex II (entirely nDNA encoded), consistent with the mtDNA defects examined. Relative to the large differences in cell type-specific mitochondrial phenotypes, differences in MitoD relative to controls were generally small (<25%) across mitochondrial measures. MitoD biomarkers growth differentiation factor 15 and fibroblast growth factor 21, as well as clinical disease severity measures, were most strongly related to mitochondrial abnormalities in platelets, and most weakly related to mitochondrial OxPhos capacity in lymphocytes, which are known to eliminate mtDNA defects. Finally, comparing PBMCs collected in the morning/fasted state with those in the afternoon/fed state after a stressful experience, we report significant time-dependent changes in mitochondrial biology over hours.

Conclusions

Overall, these results demonstrate that the dynamic and cell type-specific mitochondrial phenotypes are preserved in MitoD and are generally unrelated to symptom severity.

Introduction

There is growing interest in probing mitochondrial health profiles using blood-based bioenergetic measures.¹⁻³ Mitochondrial oxidative phosphorylation (OxPhos) capacity deficits and changes in blood immune cells have been identified in neurodegenerative diseases such as Alzheimer disease, metabolic diseases such as diabetes and sepsis, and psychiatric disorders

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Supplementary Material

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Glossary

BCA = bicinchoninic acid; **CBC** = complete blood count; **CS** = citrate synthase; **IQR** = interquartile range; **MiSBIE** = Mitochondrial Stress, Brain Imaging, and Epigenetics study; **MitoD** = mitochondrial disease; **MRC** = mitochondrial respiratory capacity; **mtDNA** = mitochondrial DNA; **OxPhos** = oxidative phosphorylation; **PBMC** = peripheral blood mononuclear cell.

such as major depressive disorder⁴⁻⁶ and are also related to chronic psychosocial stress.⁷ Whether immune cell OxPhos enzyme activities reflect whole-body OxPhos activity is unclear. Existing studies show inconsistent results regarding whether immune and muscle mitochondrial OxPhos capacities are correlated.^{8,9} Moreover, low coherence in mitochondrial gene expression has been found between different organs and tissues within the human body,¹⁰ calling into question the reliability of using immune system mitochondria as a proxy for the rest of the body. Thus, it is important to understand whether immune cell mitochondrial phenotyping can reflect an individual's mitochondrial OxPhos capacity, including those with mitochondrial diseases (MitoD).

Current work using blood-based measures of OxPhos complex enzyme activities in mitochondrial disease has been limited. Some studies report reduced OxPhos complex activities in peripheral blood mononuclear cells (PBMCs), platelets, and lymphocytes in patients with various mitochondrial diseases,¹¹⁻¹⁵ whereas others report no significant differences¹⁶ between patients with MitoD and healthy controls. In general, assessments of immune cell OxPhos complex activities offer poor sensitivity and specificity for MitoD.¹⁷ This is likely due to the elimination of mitochondrial DNA (mtDNA) pathogenic variant within the immune lineage,¹⁸⁻²¹ in contrast to postmitotic tissues, where mtDNA pathogenic variant load can increase over time.²²

In addition, how OxPhos profiles in specific immune cell subtypes are affected in MitoD is not well understood. Immune cell subtypes possess distinct OxPhos profiles,²³⁻²⁷ but how these profiles are affected in MitoD has yet to be investigated. Purifying selection of MitoD mtDNA pathogenic variants also occurs in immune cell types, decreasing pathogenic variant load as these cells mature. This is particularly evident in lymphoid cells (as opposed to myeloid lineage cells) in both human patients with the m.3243A>G pathogenic variant and mouse models with the m.5024C>T variant.¹⁸⁻²¹ Consequently, immune cells' OxPhos enzyme activities may not be affected as severely as in somatic tissues, and OxPhos in immune cell subtypes may be differentially affected by MitoD-causing pathogenic variants. MitoD may also cause changes in relative immune cell subtype abundance,²⁸ further complicating the understanding of the immune OxPhos profile in MitoD and calling for a better understanding of OxPhos activities across different immune cell types. Besides OxPhos capacity, findings around mtDNA copy number (mtDNAcn) changes in MitoD are also mixed.^{17,21,29} Cell type-specific immune studies also show

that mtDNAcn is also tissue and immune cell type specific.^{24,30,31}

Finally, the timing for mitochondrial biology assessment may also be important to understand how immune cells are affected in MitoD. The immune system exhibits diurnal variation in the abundance of circulating cell types,³²⁻³⁴ and there may be natural within-person variation in immune cell subtype abundance and mitochondrial enzyme activity (for example, from week-to-week²⁴). Whether time-dependent changes in immune mitochondrial biology are altered in MitoD has not previously been examined.

In this study, we profiled OxPhos enzyme activities (complexes I, II, and IV) together with the mitochondrial content markers citrate synthase (CS) activity and mtDNAcn, across isolated monocytes, lymphocytes, neutrophils, platelets, and mixed PBMCs in patients with MitoD and age-matched controls. We quantified differences in mitochondrial phenotypes in a cell type-specific manner and systematically examined how immune mitochondrial profiles relate to MitoD severity and disease biomarkers. We also explored how PBMCs OxPhos enzyme activities and mitochondrial content change from morning to afternoon, expanding our understanding of immune mitochondrial biology in relation to mitochondrial diseases.

Methods

The complete methods can be found in eMethods 1, including all details related to participant recruitment and inclusion/exclusion criteria, sample collection, enzymatic measurements, qPCR and protein assays, data cleaning, processing, and statistical analyses. We provide a summary with key design and methodological elements further.

Standard Protocol Approvals, Registrations, and Patient Consents

Participants were recruited for the Mitochondrial Stress, Brain Imaging, and Epigenetics (MiSBIE) study in adherence to the directives outlined by the New York State Psychiatric Institute IRB protocol #7424, Columbia University Medical Center IRB protocol #AAAU9470, and ClinicalTrials.gov NCT04831424³⁵ from whom ethical approval was obtained. All enrolled participants provided written informed consent, authorizing their participation in the investigative procedures and the dissemination of findings.

Participant Recruitment

A total of 110 participants, with 70 healthy controls ($n = 48$ women, 22 men) and 40 individuals with genetically defined mitochondrial diseases (MitoD, $n = 28$ women, 12 men), were recruited. In this study, 68 ($n = 46$ women, $n = 22$ men) healthy controls and 37 ($n = 25$ women, $n = 12$ men) individuals with MitoD were included because of sample collection limitations.

Mitochondrial disease severity quantification and symptom profiling were performed by a qualified physician, and all participants also completed a socioevaluative speech task as described previously.³⁵

Sample Collection

Blood was collected via a central venous catheter both at around 10 AM while participants were in a fasted state and, in the afternoon, around 4 PM 120 minutes after the onset of the socioevaluative speech task. Five 8.5-mL ACD-A tubes (BD-364606) were collected for the morning PBMC samples, platelets, and specific immune cell subtypes. For the afternoon PBMC sample, blood was collected via an IV catheter in one 8.5-mL tube ACD-A (BD-364606).

Immune Cell Isolation

Morning fasting tubes were processed for platelet isolation, using 3 mL of plasma from 5 ACD-A tubes to yield 15 mL of plasma, via differential centrifugation. PBMCs were isolated from morning fasting and afternoon poststress blood samples via differential centrifugation. Monocytes, neutrophils, and lymphocytes were isolated using magnetic bead-tagged antibodies and MAC separator columns (Miltenyi Biotech, #130042401).

Mitochondrial Enzymatic Assays

Homogenization

Cell pellets were homogenized for enzymatic activities as previously described.²⁴ Briefly, 500 μ L of homogenization buffer (1 mM EDTA, 50 mM Triethanolamine) with 2 tungsten beads (Qiagen Cat#69997) were added to cell pellets before homogenization in a Tissue Lyser (Qiagen, Cat# 85300).

Enzyme Activity Assays

Colorimetric enzyme activity assays for complex I (NADH-ubiquinone oxidoreductase, CI), complex II (succinate-ubiquinone oxidoreductase, SDH), complex IV (cytochrome c oxidase, COX), and CS were measured spectrophotometrically as previously described,²⁴ with minor modifications. Assays were performed in 96-well plates and recorded on Spectramax M2 (Spectramax Pro 6, Molecular Devices), using the following volumes of homogenate: complex I: 15 μ L, COX and SDH: 20 μ L, and CS: 10 μ L.

qPCR Assay

mtDNA copy number (mtDNAcn) and nuclear DNA (nDNA) were measured via qPCR as previously described,²⁴

with minor adjustments. A total of 20 μ L of the enzyme activity homogenate was lysed in 180 μ L of lysis buffer (6% Tween20 [Sigma #P1379], 114 mM Tris-HCl pH 8.5 [Sigma #T3253], and 200 μ g/mL of proteinase K [Thermo Fisher #AM2548] for 16 hours at 55°C), followed by proteinase K inactivation at 95°C for 10 minutes. qPCRs were performed in triplicates on two plates using a QuantStudio 7 Flex qPCR instrument (Applied Biosystems, Cat# 4485701) with the following cycling conditions: 50°C for 2 minutes and 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 minutes (total run time: 40 minutes), using TaqMan chemistry for 2 pairs of primers, ND1/B2M and COX1/RNaseP. mtDNAcn was calculated using the Δ Ct between the nDNA and mtDNA Cts. Reported platelet mtDNA content was normalized to protein concentration derived from the linearized mtDNA Ct divided by protein concentration of each sample.

BCA Protein Assay

A bicinchoninic acid (BCA) assay was run to measure total protein content for normalization of the enzymatic activities to protein concentration in platelets. A BCA assay kit (Bio-world #20831001) was used, with reagents A and B mixed at 1:50 ratio. Bovine serum albumin (BSA) protein standards were prepared using a stock 50 mg/mL BSA (Sigma A3733) solution.

Normalization of Enzyme Activity and Data Processing

To account for differences in cell number and protein concentrations in each sample, enzyme activities were normalized to linearized nDNA Ct in PBMCs, monocytes, lymphocytes, and neutrophils while they were normalized to protein concentration in platelets.

Outliers were identified as samples more than 3 times the interquartile range (IQR) higher than the 3rd quartile and 3 times the IQR lower than the 1st quartile and removed from the data. All data processing code for analyses is available in our GitHub repository.³⁶

The mitochondrial respiratory capacity (MRC) considers the dimensional scaling of each variable. MRC is calculated from the average of the square root of RC enzyme activities divided by the average of the cube root of mitochondrial content measures:

$$\text{MRC} = \frac{\text{mean}(\sqrt[3]{z\text{-CI}} + \sqrt[3]{z\text{-SDH}} + \sqrt[3]{z\text{-COX}})}{\text{mean}(\sqrt[3]{z\text{-mtDNAcn}} + \sqrt[3]{z\text{-CS}})} * 100$$

where each variable was mean-centered (divided by the mean) to harmonize the effects of each component on the final metric.

Effect of Time

In previous studies, samples collected more recently (i.e., stored for shorter duration) had higher activities than those collected earlier in the study when stored at -80°C . This

is likely due to the time-dependent degradation of enzyme activities during the storage period. In this MiSBIE data set, however, with liquid nitrogen sample storage, we did not observe a consistent significant decrease in enzyme activity over time across cell types (eFigure 1).

We also tested differences in mitochondrial variables between men and women. We did not observe consistent significant differences between men and women across variables and cell types (eFigure 2).

CBC With Differential

Complete blood count (CBC) with differential was evaluated from blood collected in the fasting state at 10 AM and in the afternoon 120 minutes after the onset of the socioevaluative speech task and used to determine immune cell percentages between the 2 time points in this study. CBC with differential was performed with Sysmex XN in an FDA-approved and CLIA-certified laboratory. Additional information about the assay can be found on the laboratory website.³⁷

Statistical Analyses

All statistical analyses were conducted using GraphPad Prism (version 10.2.0). Effect sizes were computed as Hedge *g* (*g*). Mann-Whitney *t* tests were used to compare differences in mitochondrial features at different time points. Spearman rank (*r*) correlations were used to compute associations between cell types, between mitochondrial features, between mitochondrial features and disease severity measures, and between mitochondrial features and white blood cell (WBC) proportions. One-way nonparametric analysis of variance (ANOVA) Kruskal-Wallis tests were used to compare mitochondrial features between cell types and disease groups.

Data Availability

Anonymized data not published in the article will be shared on reasonable request.

Results

Immune Cell Subtypes Have Distinct OxPhos Capacities and Mitochondrial Content

To first establish how mitochondrial biology differs between immune cell types, we profiled multiple features of mitochondrial content and OxPhos capacity among monocytes, lymphocytes, neutrophils, and mixed peripheral blood mononuclear cells (PBMCs) from 37 individuals with mitochondrial diseases (2 groups – m.3243A>G, *n* = 23; and single, large-scale mtDNA deletions, *n* = 14) and 68 healthy women and men from the Mitochondrial Stress, Brain Imaging, and Epigenetics (MiSBIE) study (aged 18–60 years, 69% women).³⁵ In line with our previous findings in a smaller study,²⁴ healthy controls showed significant differences in OxPhos enzyme activities (complex I (CI), complex II (CII), complex IV (CIV)), CS activity, mtDNAcn, and MRC between immune cell subtypes (Figure 1). Platelet enzyme activities were also measured; however, although immune cell

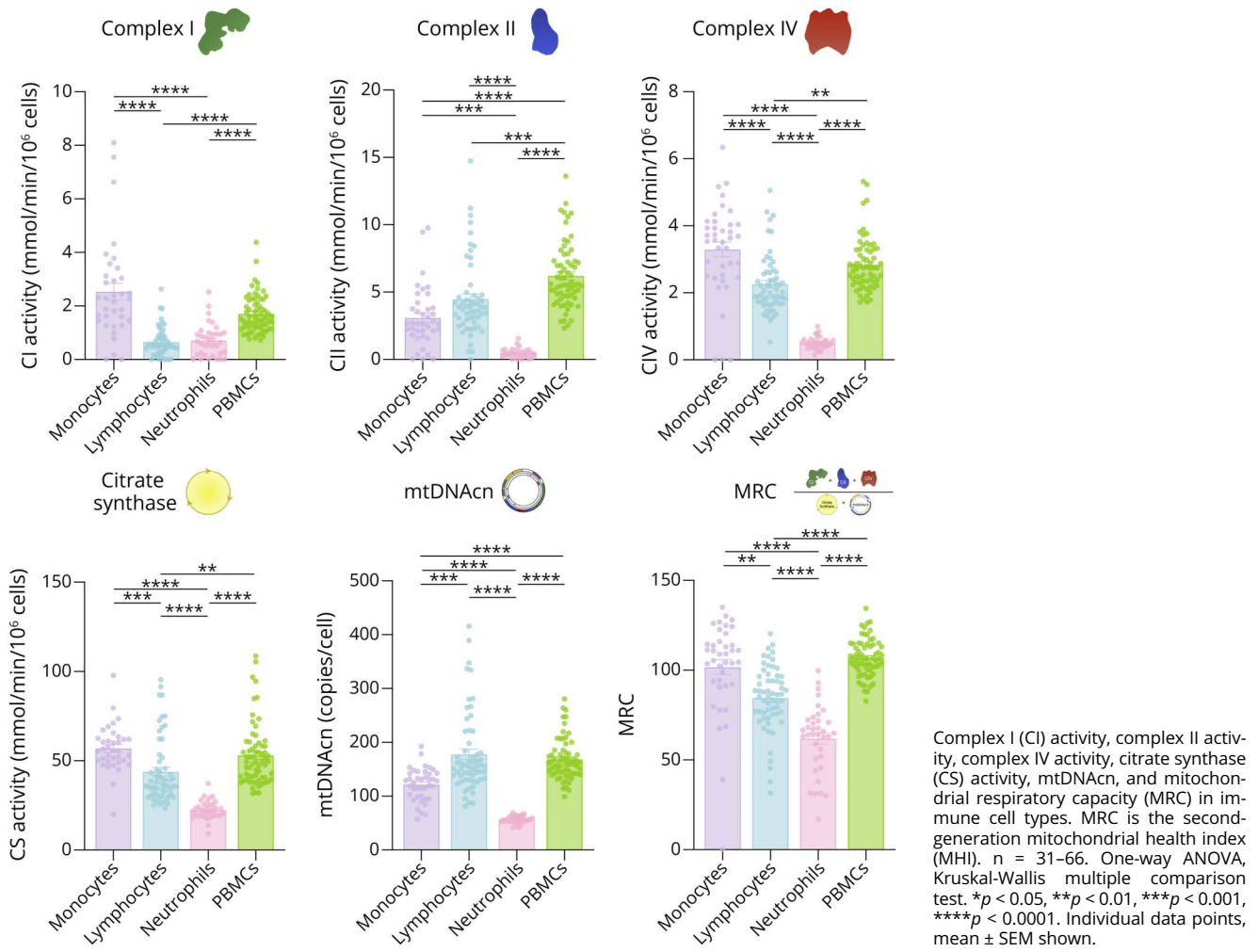
activities can be expressed per nucleated cell, platelet enzyme activities are normalized per protein content and, therefore, not directly comparable to immune cell populations. Results for platelets are included further.

Of all cell types assessed, in healthy controls, neutrophils had the lowest mitochondrial content (indexed by both CS activity and mtDNAcn) and OxPhos enzymatic activities, consistently less than half that of monocytes and PBMCs, which exhibited high activities across all enzymes. Neutrophils also displayed the lowest mtDNAcn (average 56.6 copies/cell), whereas lymphocytes showed the highest mtDNAcn (average 177.7 copies/cell). The integrative measure of mitochondrial respiratory chain capacity on a per-mitochondrion basis, the MRC,³⁸ was highest in PBMCs (MRC = 107, Figure 1), likely because of residual platelet contamination that is typical of PBMC preparations.^{24,39} PBMC MRC was 5.2% higher than in monocytes (101.7), 26.5% higher than in lymphocytes (84.6), and 72.9% higher than in neutrophils (61.9), establishing the spectrum of mitochondrial OxPhos specialization across major immune cell types.

We next assessed whether different mitochondrial features correlated within cell types. As expected, given that most of the cells in PBMCs are lymphocytes, mitochondrial features tended to correlate well within PBMCs and lymphocytes (average *r*'s = 0.59 and 0.56, respectively). Monocytes, neutrophils, and platelets did not display strong correlations with mitochondrial features (average *r*'s = 0.31, 0.06, and 0.06, respectively, eFigure 3A), suggesting that these features may be regulated relatively independently of each other in these cell types. Between cell types of the same individual, we did not find evidence of an association between mitochondrial features, with the exception of CS activity and MRC, which were weakly correlated between neutrophils and PBMCs (*r* = 0.36, *p* = 0.045) and between neutrophils and lymphocytes (*r* = 0.36, *p* = 0.04), respectively (eFigure 3, B, D–G). However, after correction for multiple comparisons, these correlations were no longer significant (Bonferroni-corrected *p*'s = 0.68 and 0.60, respectively). Thus, individuals with high mitochondrial content or OxPhos activity in one cell type do not necessarily have similarly high mitochondrial features in other cell types, consistent with previous findings.²⁴

For each participant, in addition to the PBMCs collected in the morning/fasted state (PBMCs 1), PBMCs were also collected in the afternoon/fed state after a psychologically stressful laboratory task involving a defense against an alleged shoplifting (PBMCs 2), and the mitochondrial profiling was repeated for samples collected at that time point. As expected, mitochondrial features were significantly correlated between the 2 PBMC samples (*r*'s = 0.34–0.49, *p*'s < 0.05), for all measures except CS activity (*r* = 0.36, *p* = 0.081, eFigure 3, B and C). The overall low correlation between different immune cell types within the same person, along with presence of correlation between PBMCs, suggests that mitochondrial respiratory capacity varies by cell type and is not a stable trait of an individual.

Figure 1 Differences Between Measures of Mitochondrial Biology in Immune Cell Subtypes of Healthy Controls



Complex I (CI) activity, complex II activity, complex IV activity, citrate synthase (CS) activity, mtDNAcn, and mitochondrial respiratory capacity (MRC) in immune cell types. MRC is the second-generation mitochondrial health index (MHI). n = 31–66. One-way ANOVA, Kruskal-Wallis multiple comparison test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Individual data points, mean ± SEM shown.

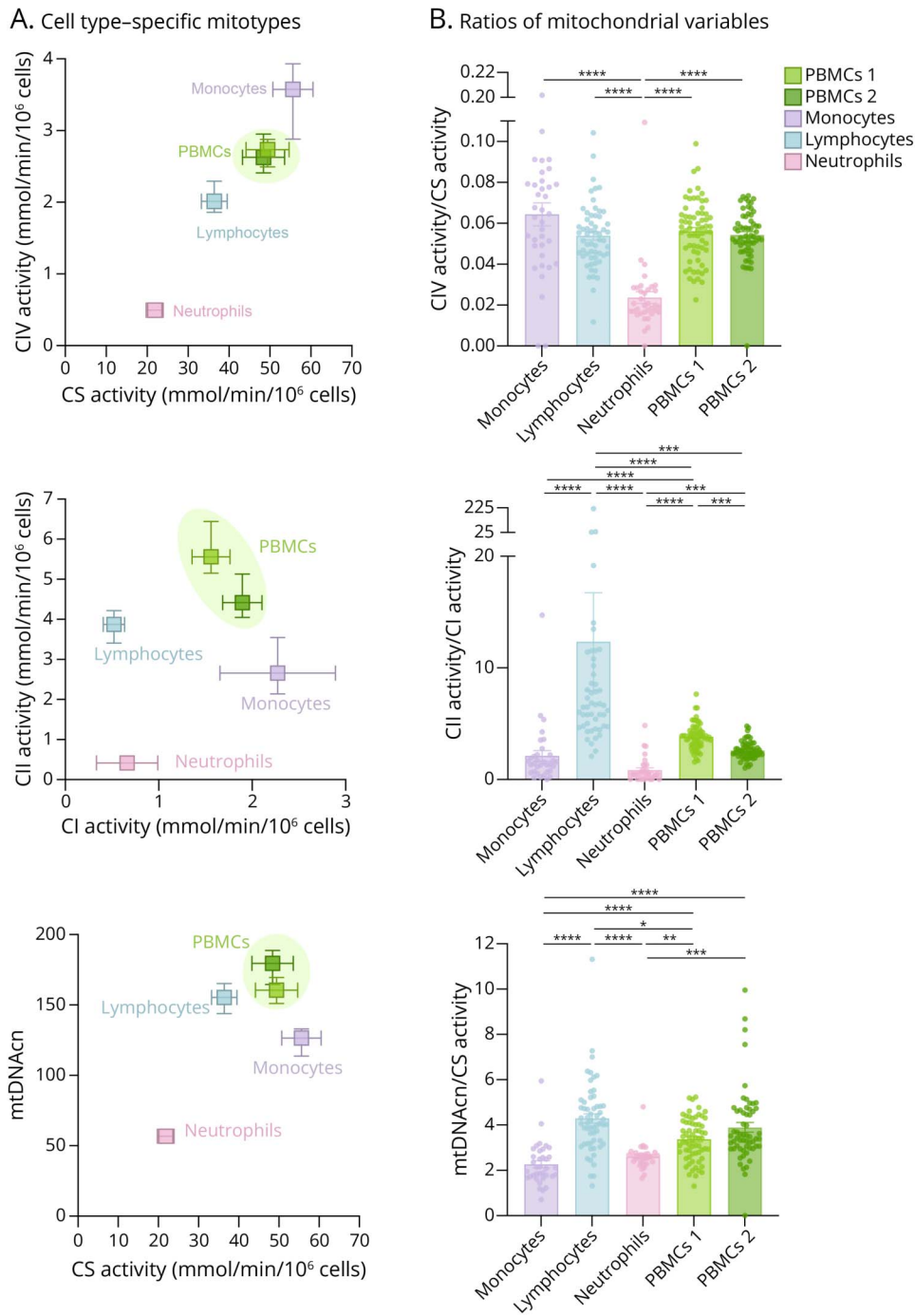
Immune Cell Types Display Distinct Mitotypes

To further examine the differences in mitochondrial respiratory capacity and content between immune cell types and PBMCs, we created bivariate plots of pairs of mitochondrial features. These plots distinguish distinct mitochondrial phenotypes, or *mitotypes*,²⁴ and allow us to situate different cell types relative to each other in a simple bivariate mitotype space. These mitotypes are alternatively presented as ratios, where cell types with the same ratios line up along the diagonal, and allow the quantification and statistical comparison of the mitotype profiles of cell types to each other.

We first examined complex IV activity relative to citrate synthase (CIV/CS ratio). Most cell types showed similar CIV/CS ratios, as shown by their alignment along the same y/x line in the CS vs CIV activity bivariate plot (Figure 2A, upper) and their similar CIV/CS activity ratios (between 0.053 and 0.064) across most cell types (Figure 2B, upper). Other bivariate mitotype plots revealed significantly larger distinctions between cell types (Figure 2, eFigure 4, A–K).

For example, for the CII/CI mitotype reflecting the activity of OxPhos complex II (encoded entirely by nDNA genes) relative to complex I (encoded by both the mtDNA and nDNA), lymphocytes displayed low CI activity (0.52 mmol/min/10⁶ cells) but high CII activity (3.87 mmol/min/10⁶ cells), leading to a large CII/CI ratio of 12.4. This positioned lymphocytes in the upper left quadrant, whereas monocytes displayed the opposite pattern, with lower CII activity (2.27 mmol/min/10⁶ cells) relative to a higher CI activity (2.66 mmol/min/10⁶ cells), yielding a ratio of 2.1 and a position in the lower right quadrant of the mitotype plot. Neutrophils showed low CI activity (0.66 mmol/min/10⁶ cells) and low CII activity (0.42 mmol/min/10⁶ cells), with a low average CII/CI ratio of 0.86 and a mitotype position in the lower left quadrant (Figure 2, A and B, middle). Immune cell types also differed considerably in their CS/mtDNAcn mitotype, reflecting the mtDNA content per unit of CS or Krebs cycle–defined mitochondria (Figure 2, A and B, lower). Together, these results reinforce the notion that distinct immune cell types have unique mitochondrial properties and respiratory capacities.

Figure 2 Relationships Between Different Measures of Mitochondrial Biology by Cell Type



(A) Bivariate plots and (B) ratios showing relationship between different pairs of mitochondrial enzyme measures and mtDNAcn in different cell types of healthy controls. PBMCs 1 are PBMCs collected in the morning/ fasted state; PBMCs 2 are PBMCs collected in the afternoon/fed state after a psychologically stressful laboratory task. $n = 31-66$. One-way ANOVA, Kruskal-Wallis multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Individual data points, mean \pm SEM shown. PBMCs = peripheral blood mononuclear cells.

Mitochondrial OxPhos Capacity and Content Were Not Affected in Mitochondrial Disease

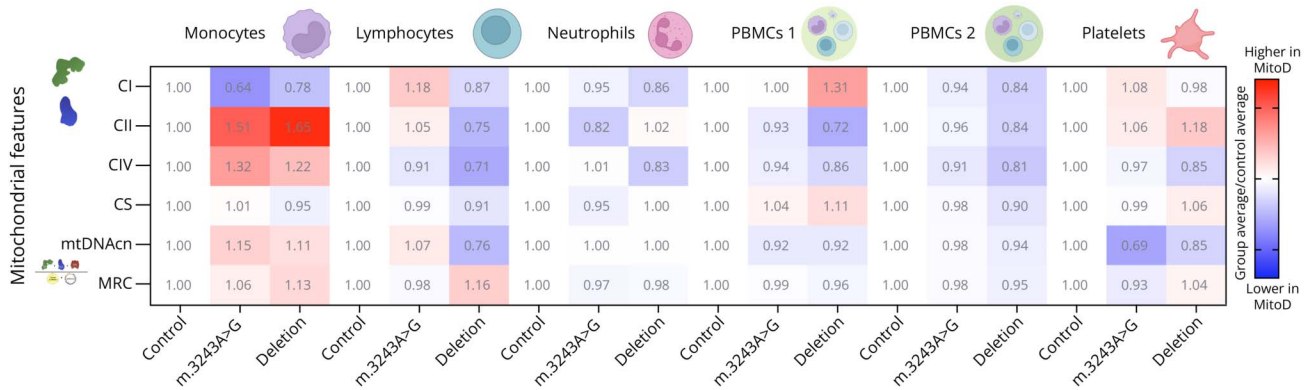
We next compared mitochondrial features between the MitoD group and controls in each of the specific immune cell types. Across all cell types and mitochondrial respiratory capacity measurements, most differences were small (average change 11.7%) and nonsignificant. The only significant difference between control and MitoD groups was a 32% higher CIV activity in monocytes of the m.3243A>G group ($p = 0.02$, Hedge $g = 0.82$, Figure 3A, eFigure 5A). The nDNA-encoded

CII activity in monocytes also tended to be 51% and 65% higher in the MitoD group than in controls, consistent with a compensatory upregulation in both MitoD subgroups. MRC did not differ between patients with MitoD and controls across cell types (Figure 3B).

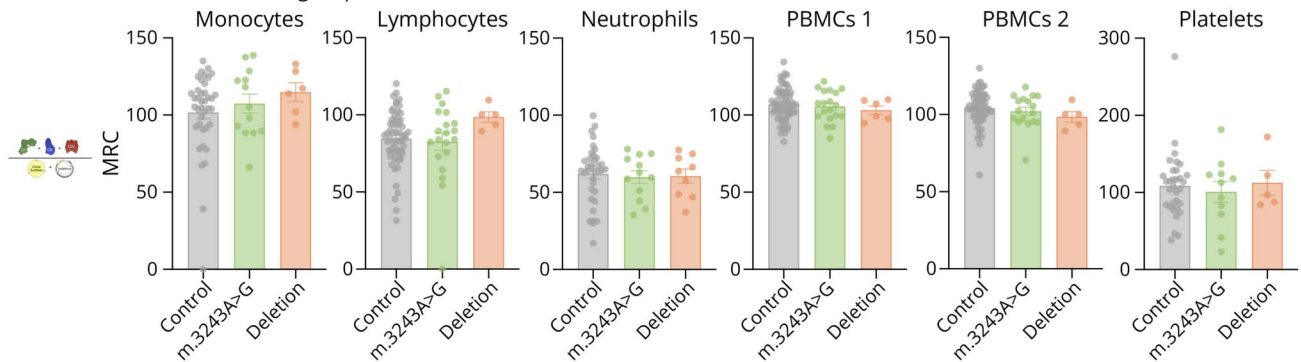
Consistent with the biology of mitochondrial disease, mtDNA-encoded CI activity in monocytes tended to be 22%–36% lower in patients with MitoD and nuclear DNA-encoded CII activity was higher by 51%–65%. These differences

Figure 3 Differences in RC Enzyme Activity, CS Activity, mtDNA, and MRC Between Control and MitoD Groups Across Immune Cell Types, PBMCs, and Platelets

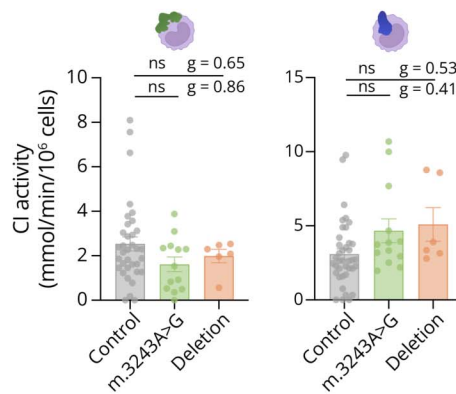
A. MitoD mitochondrial features relative to the control group



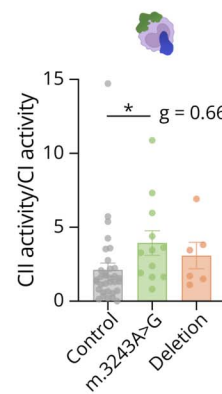
B. MRC in control and MitoD groups



C. Monocyte CI, CII activities



D. Monocyte CI/CII

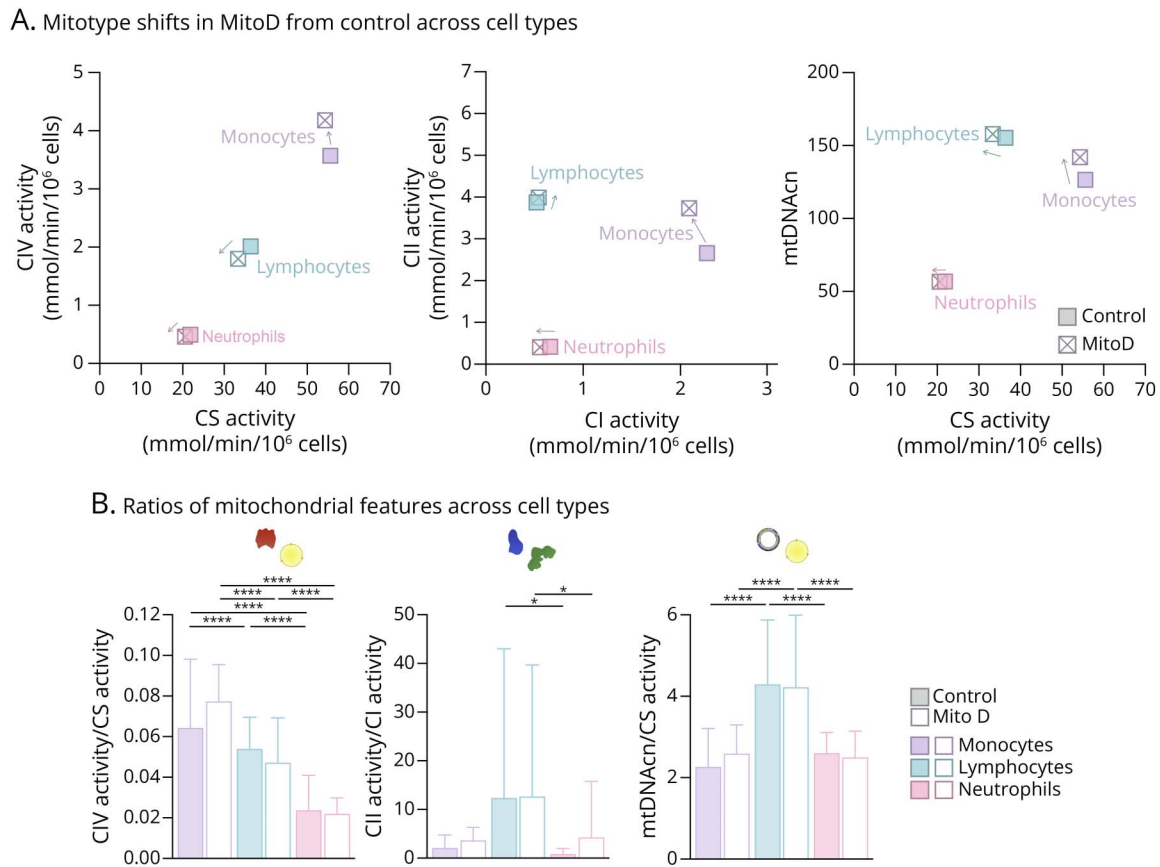


(A) Heatmap of mean mitochondrial measures (CI, CII, CIV, CS activity, mtDNAcn, MRC) in the MitoD group relative to controls in immune cell types, PBMCs, and platelets. (B) MRC activity across different cell types. (C) CI and CII activities and (D) CI/CII activity ratio in monocytes in healthy control and MitoD groups. Individual data points, mean \pm SEM shown. One-way ANOVA, Kruskal-Wallis multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Effect sizes as Hedge g (g). Control $n = 31-66$, m.3243A>G $n = 11-21$, deletions $n = 5-13$. MitoD = mitochondrial disease; MRC = mitochondrial respiratory capacity; PBMCs = peripheral blood mononuclear cells.

showed medium to large effect sizes (Hedge $g = 0.41-0.86$, Figure 3C), and the CII/CI activity ratio was 86% higher in the MitoD group than in controls ($p = 0.017$, Hedge $g = 0.66$, Figure 3D). These trends were not found in other cell types (Figure 3A), and neither of the single-enzyme measures differed significantly between groups, owing mainly to large interindividual variance (Figure 3C, eFigure 5 for all results).

Overall, the differences in mitochondrial features between control and MitoD groups were small compared with those between immune cell subtypes (Figure 4A, eFigure 5D), and the significant differences in the ratios seen between cell types in controls were preserved in the MitoD group (Figure 4B). Differences between cell types were also preserved in MitoD (eFigure 5E), demonstrating that cell

Figure 4 Relationships Between Different Measures of Mitochondrial Biology and MRC in Controls and Patients With MitoD Across Immune Cell Types



(A) Bivariate plots and (B) ratios showing shift in the relationship between different pairs of mitochondrial enzyme measures and mtDNAcn in immune cell types between healthy control and MitoD groups. One-way ANOVA, Kruskal-Wallis multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Control $n = 31-66$, m.3243A > G $n = 11-21$, deletion $n = 5-13$. MitoD = mitochondrial diseases; MRC = mitochondrial respiratory capacity.

type-specific differences in mitochondrial phenotypes are largely unaffected by mitochondrial diseases.

Mitochondrial OxPhos Capacity Does Not Correlate With Mitochondrial Disease Severity

We next asked whether leukocyte mitochondrial respiratory capacity is related to MitoD biomarkers and symptom severity. Figure 5A shows all correlations between immune mitochondrial features and clinical and biomarker metrics, and Figure 5B illustrates some of these associations using scatterplots. Overall, mitochondrial features were weakly correlated with circulating biomarkers of mitochondrial disease such as growth differentiation factor 15⁴⁰ and fibroblast growth factor 21.⁴¹ A similar pattern showing only weak and nonsignificant correlations was observed with clinical measures of symptom severity and functional capacity (absolute value of r 's = 0.10–0.17, Figure 5C, details in *Methods*).

Of all cell types and platelets, platelet mitochondria showed the strongest correlations with clinical measures and lymphocytes showed the weakest correlations (Figure 5C, left). Correlations between mitochondrial features and disease

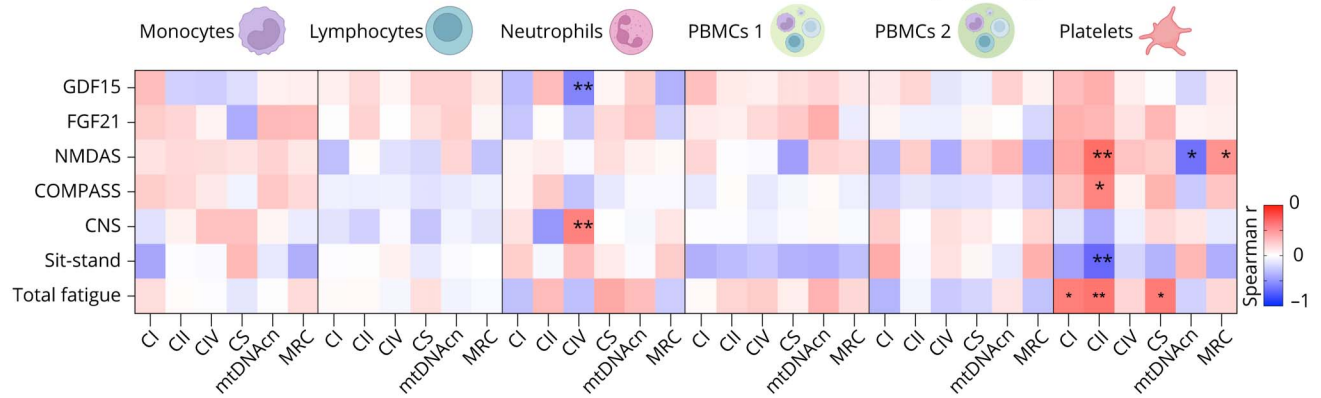
severity measures were strongest with NMDAS, but weak overall across disease severity measures (average absolute values of r 's = 0.13–0.23, Figure 5C, middle). Among mitochondrial features, correlations were strongest with CI activity, consistent with MitoD affecting mtDNA-encoded complexes, but also weak overall (average absolute values of r 's = 0.17–0.21, Figure 5C, right). This lack of correlation between MRC and disease severity was consistent with the lack of difference in immune cell MRC between MitoD and control groups.

Changes in Mitochondrial Biology Over Time in the MitoD Group and Controls

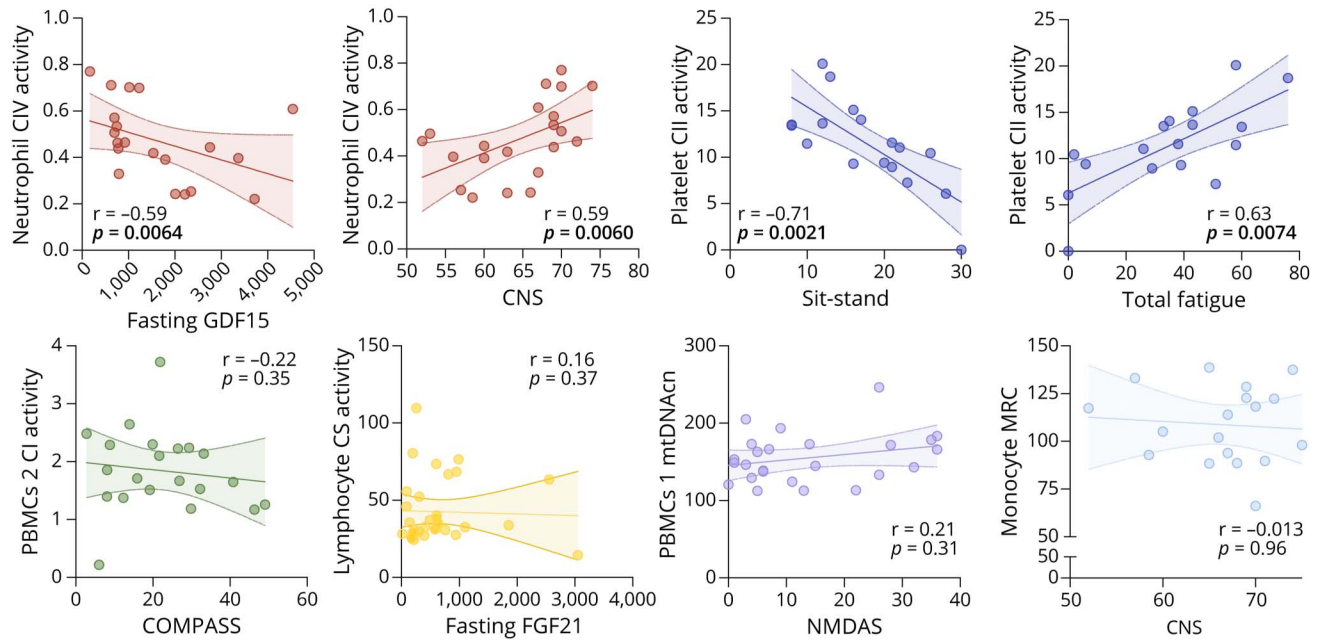
To evaluate the stability of mitochondrial features over different states and time of day, we finally assessed the change in immune mitochondrial biology between the morning/fasted state (Time 1) and poststress afternoon/fed state (Time 2) (Figure 6A). In healthy controls, CI and CII activities, mtDNAcn, and MRC changed significantly from Time 1 to Time 2 (4%–25% change, p 's < 0.048). Similar changes were observed in the MitoD group, although only the increase in mtDNAcn from Time 1 to Time 2 was significant (16%

Figure 5 Correlation Between Clinical Biomarkers and Mitochondrial Disease Severity

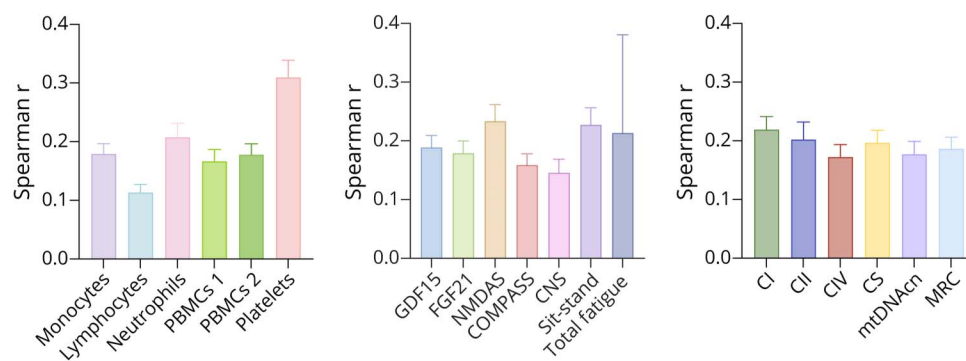
A. Correlations of mitochondrial features with mitochondrial disease biomarkers and severity by cell type



B. Example correlations of mitochondrial features with mitochondrial disease biomarkers and severity scores



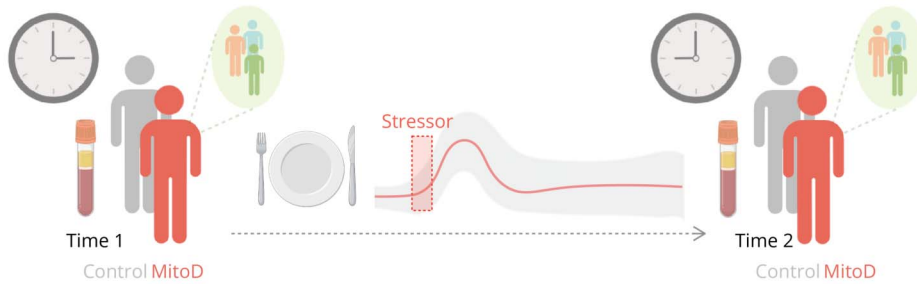
C. Average correlation strengths between mitochondrial features and clinical disease severity



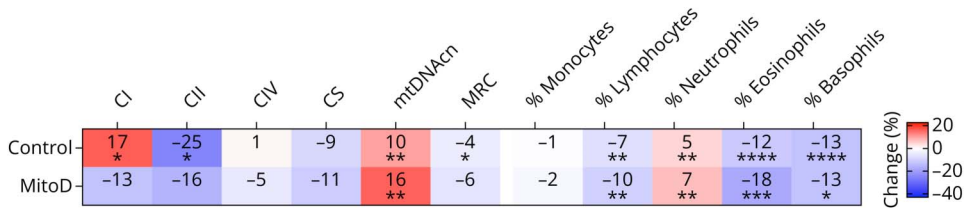
Correlations between mitochondrial features (CI, CII, CIV, and CS activity, mtDNA, and MRC) and measures of mitochondrial disease biomarkers and severity in immune cell types, PBMCs and platelets of mitochondrial disease patients as (A) a heatmap, and scatterplots (B) showing significant correlations between Neutrophil COX activity and Fasting GDF15, CNS, and between Platelet CII Activity and Sit-stand, total fatigue scores (upper) and lack of correlation between CI, CII, CIV, and CS activity, mtDNA and MRC in immune cell types, PBMCs and platelets and clinical biomarkers and mitochondrial disease severity in mitochondrial disease patients (lower). (D) Average Spearman correlations (absolute value of *r* value) between mitochondrial disease biomarkers/severity and mitochondrial features across disease biomarker/severity measures by cell type (left), cell types by disease biomarker/severity measure (middle), and disease biomarker/severity measures by mitochondrial feature measure (right). For example, the first bar in (D, left) represents average correlations between all mitochondrial disease biomarkers/severity and mitochondrial features in monocytes, the first bar in (D, middle) represents correlations between all mitochondrial features and GDF15 across all cell types, and the first bar in (D, right) represents correlations between all mitochondrial disease biomarkers/severity and CI activity across all cell types. *n* = 13–33. Spearman *r* labeled on correlation matrix, with significant correlations marked with asterisks in the matrices. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Histograms show mean ± SEM. GDF15 = growth differentiation factor 15; MRC = mitochondrial respiratory capacity; PBMCs = peripheral blood mononuclear cells.

Figure 6 Shift in Mitochondrial Measures and WBC Composition Between Different Time Points

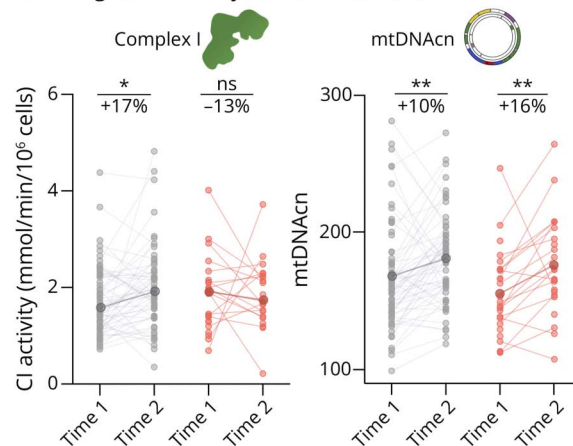
A. Sample collection times: Morning/fasting (1), afternoon/fed/poststress (2)



B. Change in mitochondrial features and WBC proportion between Time 1 and Time 2



C. Change in CI activity and mtDNAcn (%)



(A) Schematic of sampling time. Time 1 refers to a morning, fasted sample before stress exposure. Time 2 refers to an afternoon, fed sample, after socioevaluative stress exposure. Healthy controls are compared with patients with MitoD, which include m.3243A>G/MELAS and deletion subgroups. (B) Median % change in CI, CII, CIV, and CS activities; mtDNAcn; MRC in PBMCs; and % composition of WBCs between Time 1 and Time 2 in healthy controls and patients with MitoD. (C) Individual and percent changes in CI activity and mtDNAcn in healthy controls (gray) and patients with MitoD (red). Raw CI activity and mtDNAcn plotted, median % change (change between Time 1 and Time 2, relative to Time 1 value) labeled. Darker dot represents average group values at each time point. Mitochondrial features: control n = 58–59, MitoD n = 18–19. WBC proportions: control n = 60, MitoD n = 32. Significant changes in heatmap marked with asterisks; the Wilcoxon test for paired time point measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. MitoD = mitochondrial diseases; MRC = mitochondrial respiratory capacity; PBMCs = peripheral blood mononuclear cells.

increase, $p = 0.0039$, Figure 6, B and C, eFigure 6, A and B). CI activity, however, changed in the opposite direction in the MitoD group, exhibiting a 13% morning-to-afternoon decline in patients, whereas healthy controls exhibited a significant 17% elevation ($p = 0.028$, Figure 6, B and C).

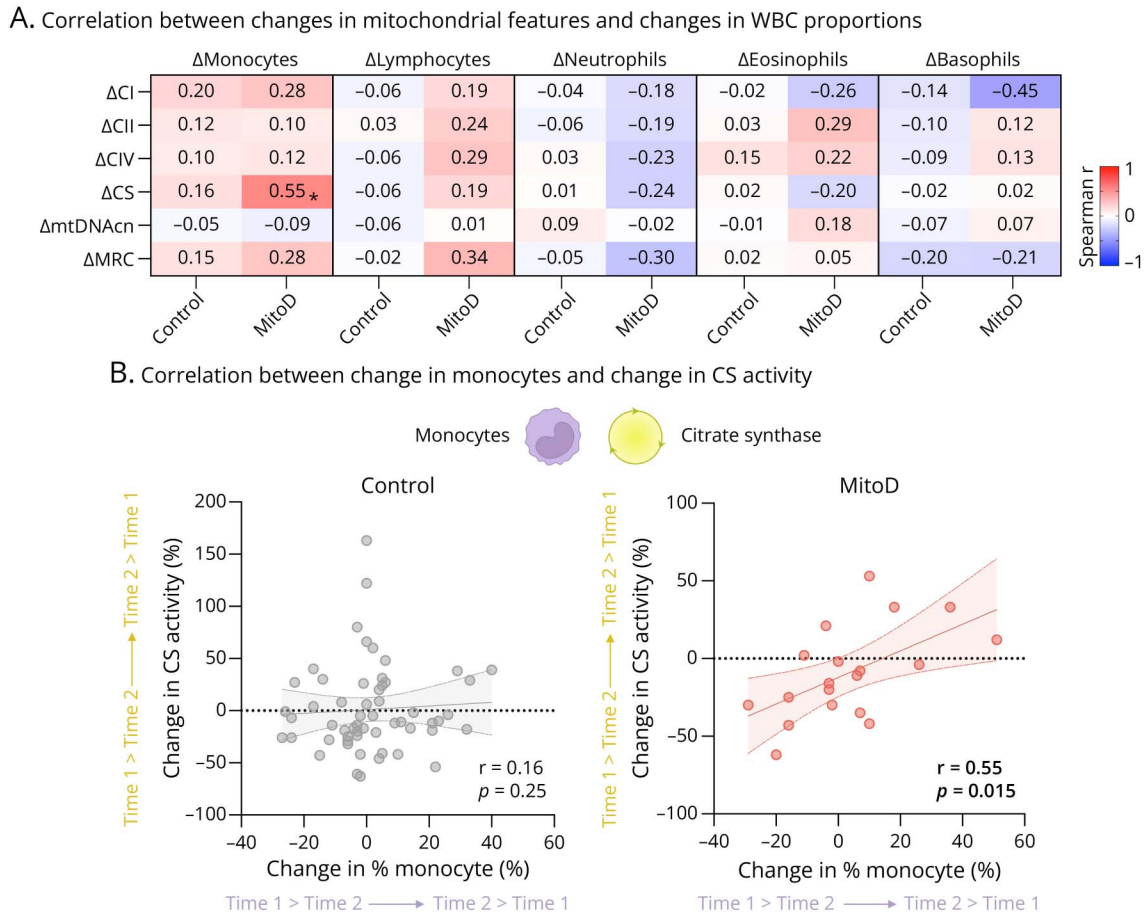
We also examined whether these changes in PBMC mitochondrial features were driven by dynamic changes in cell type composition. The proportion of leukocyte types (lymphocytes, monocytes, neutrophils, eosinophils, and basophils) showed several significant changes from Time 1 to Time 2 (changes ranged from 5% to 13%, p 's = < 0.0061) in all cell types but not in monocytes of healthy controls. Patients with MitoD displayed similar changes (changes of 2%–18%, significant p 's = 0.0003–0.02, Figure 6B, eFigure 6, C and D), suggesting that the gross temporal/diurnal changes in immune biology are not affected in MitoD.

In both control and MitoD groups, changes in cell type abundance largely did not account for the changes in mitochondrial biology observed (average $r = 0.04$, Figure 7A). One exception was a significant correlation between the change in % monocytes and the change in monocyte CS activity in the MitoD group ($r = 0.55$, $p = 0.015$; Bonferroni-corrected $p = 0.45$), which was not observed in healthy controls (Figure 7B). Further work is required to understand the factors that may explain the temporal changes in mitochondrial OxPhos enzyme activities and mtDNAcn levels and their potential relevance to the immune aspects of mitochondrial diseases.

Discussion

In this study, we profiled OxPhos enzyme activities and markers of mitochondrial content in immune cell subtypes, PBMCs, and platelets from dozens of individuals with

Figure 7 Correlations Between Shifts in Mitochondrial Measures and WBC Composition



(A) Correlation between % change in measures of mitochondrial biology and % change in proportion blood cells between Time 1 and Time 2 in healthy controls and patients with MitoD. (B) Correlation between change in CS activity and change in % monocytes in controls and patients with MitoD. Mitochondrial features: control n = 58–59, MitoD n = 18–19. WBC proportions: control n = 60, MitoD n = 32. Spearman r labeled on correlation matrix and correlation plots, with significant correlations marked with asterisks in the matrices; the Mann-Whitney test for group comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. MitoD = mitochondrial diseases.

molecularly defined mtDNA defects causing MitoD. Our results confirm previous work showing distinct mitochondrial profiles of immune cell subtypes^{5,24,26,27} and demonstrate that these profiles are minimally affected by mtDNA defects in MitoD. Accordingly, the immune cell type-specific mitochondrial phenotypes were preserved in MitoD but did not consistently correlate with MitoD biomarkers or symptom severity, and their temporal variation in mitochondrial phenotypes was only minimally affected, if at all, in MitoD. Together, this work emphasizes the notion that immune mitochondrial biology, although conveniently accessible through minimally invasive blood collection, does not reflect the severity of systemic mitochondrial OxPhos deficiency responsible for clinical signs and symptoms in MitoD.

The distinct cell type-specific mitochondrial phenotypes reported here are similar to previous reports.²⁴ Neutrophil mitochondrial features were consistently low across all measures, consistent with previous work demonstrating that

their primary role is not ATP synthesis,²⁷ and monocytes and PBMCs also displayed consistently higher measures across variables.

Despite the fact that both m.3243A>G and single large-scale deletions are expected to influence OxPhos complex gene expression and protein synthesis, we note a lack of differences between the mitochondrial OxPhos profiles of participants with MitoD and healthy controls. This result is consistent with the presence of purifying selection in immune cells,¹⁸⁻²¹ which dramatically reduces heteroplasmic levels of these mitochondrial disease-causing variants, making single, large-scale mtDNA deletions generally undetectable.⁴² The elimination of mtDNA defects in (some) immune cells would minimize OxPhos biochemical deficiency. Although we suspect based on a large body of literature that immune cells carry no mtDNA deletion and variable-to-low levels of the m.3243A>G pathogenic variant in our population, it was unfortunately not possible in this study to quantify heteroplasmic levels.

In alignment with the lack of group differences in leukocyte OxPhos capacity, MRC was not consistently related to mitochondrial disease biomarkers or symptoms. These correlations were weakest in lymphocytes, consistent with lymphoid cells displaying purifying selection, leading to the lowest pathogenic variant load across immune cells.¹⁸ While not possible in this study, further purification of lymphocyte subtypes would also be useful to confirm the finding of greatest purifying selection in T lymphocytes,¹⁸ as well as to assess mitotypes of cell types such as NK cells, which have distinct mitotypes compared with adaptive cells.²⁴ By contrast, platelets displayed the strongest and most significant correlations with mitochondrial disease markers. Purifying selection has yet to be studied extensively in platelets,¹⁸⁻²⁰ but given the results here, it could be that megakaryocytes, from which platelets are derived, experience less purifying selection than other immune cell types.

While not significant, monocytes also demonstrated changes in CI and CII activities that are consistent with mitochondrial disease etiology seen in the skeletal muscle and brain, with a trend toward lower CI and higher CII average activities.^{43,44} These monocyte-specific changes support the lower level of purifying selection in monocytes and dendritic cells relative to lymphoid cells.¹⁸ However, further investigation is needed to establish whether these changes are significant and why they are restricted to monocytes. Overall, given that immune cell OxPhos is minimally affected by mitochondrial diseases and not correlated with disease severity, we might expect that immune cell mitochondrial profiles do not provide an accurate representation of somatic (skeletal muscles, brain, etc.) nor whole-body OxPhos capacity in MitoD.

In addition to cell type–dependent differences in mitochondrial features, our results reveal potential time-dependent changes in PBMC mitochondrial features. These changes were not explained by changes in immune cell proportions, which was somewhat surprising given the previously established association between the proportion of circulating cell types and PBMC mitochondrial features.²⁴ However, additional factors here could have affected mitochondrial phenotypes between these 2 samples in addition to the time of day—including fasting status and the performance of a stressful socioevaluative speech task. In previous work, we found that up to 10%–15% of variation in mitochondrial OxPhos capacities was associated with subjective reports of positive and negative mood (e.g., feeling energetic and loving).⁴⁵ Thus, the brief (5-minute) psychosocial stressor that occurs approximately 2 hours before the afternoon (Time point 2) PBMC sample, which tends to elicit stress and negative mood, could have contributed to some of the differences observed in OxPhos profiles between the morning/prestress and afternoon poststress time points. The simultaneous change in multiple variables across these 2 PBMC samples unfortunately limits the extent to which any differences in their mitochondrial features across the morning and

afternoon time points can be interpreted. To tease apart these potential factors, future work would need to assess mitochondrial parameters from morning and afternoon samples, with and without controlled stress exposure. Whether the causes and consequences of mitochondrial respiratory capacity changes in immune cells reflect those of other organs will also remain an important question to examine. Potential time-dependent changes in mitochondrial biology should also be considered in future studies, both at the timescale of hours/minutes and with more repeated measures in longitudinal studies throughout disease progression.

Limitations of this study include the variable sample sizes across cell types. Owing to sample collection restrictions, sample size and cell count are limited for certain cell subtypes. This limited the sample size of some correlation analyses, particularly with our limited MitoD population, as well as introduced some noise into our enzyme activity assays. Given the variance observed, increasing the sample sizes for all groups would naturally strengthen this study. Furthermore, while we demonstrate that immune cell mitochondria are minimally affected in MitoD, the molecular basis for this effect remains unclear. Purifying selection for low heteroplasmy cells in the bone marrow may play a role. A major limitation of this study is the lack of heteroplasmy measurements of each immune cell type, which, alongside the functional assays, would be valuable to establish the extent to which this effect is attributable to pathogenic variant load or other nongenetic, humoral factors. It would also be valuable to compare these immune results with mitochondrial features in another tissue that is less subject to purifying selection. However, even in the presence of mtDNA heteroplasmy, compensatory mechanisms in specific (sub)populations of immune cells of patients with MitoD could, to some extent, mitigate the influence of mtDNA pathogenic variants on OxPhos enzyme activities and markers of mitochondrial content. Finally, we note that our population of patients with MitoD are limited to those with the m.3243A>G pathogenic variant and single large-scale deletion pathogenic variants. These immune cell mitochondrial phenotypes may not be applicable to all mitochondrial diseases, particularly nuclear DNA variants where no mtDNA purifying selection is possible, and further work would be required to define immune cell mitotypes across the spectrum of mitochondrial diseases.

Overall, this study provides insights into the immune cell–specific and time-dependent PBMC mitochondrial OxPhos profiles of patients with MitoD and healthy controls. Together, the multivariate enzymatic and molecular profiling in multiple leukocyte cell types expands our understanding of immune mitochondrial biology in MitoD. While immune mitochondria are minimally affected by mtDNA pathogenic variants and are unlikely to reflect the severity of MitoD or offer reliable diagnostic and/or prognostic information, immune mitochondria could still offer a window into how disease-modifying factors affect mitochondrial biology across organ systems.

Author Contributions

C.C. Liu: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data. M. Kurade: major role in the acquisition of data. A.S. Monzel: major role in the acquisition of data. C. Kelly: major role in the acquisition of data. R.-P. Juster: study concept or design. C Trumpff: study concept or design. M. Hirano: study concept or design. M. Picard: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data.

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Disclosure

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