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ARTICLE

Identification of peripheral vascular function measures and circulating biomarkers of mitochondrial function in patients with mitochondrial disease

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Abstract

The development of pharmacological therapies for mitochondrial diseases is hampered by the lack of tissue-level and circulating biomarkers reflecting effects of compounds on endothelial and mitochondrial function. This phase 0 study aimed to identify biomarkers differentiating between patients with mitochondrial disease and healthy volunteers (HVs). In this cross-sectional case-control study, eight participants with mitochondrial disease and eight HVs matched on age, sex, and body mass index underwent study assessments consisting of blood collection for evaluation of plasma and serum biomarkers, mitochondrial function in peripheral blood mononuclear cells (PBMCs), and an array of imaging methods for assessment of (micro)circulation. Plasma biomarkers GDF-15, IL-6, NT-proBNP, and cTNI were significantly elevated in patients compared to HVs, as were several clinical chemistry and hematology markers. No differences between groups were found for mitochondrial membrane potential, mitochondrial reactive oxygen production, oxygen consumption rate, or extracellular acidification rate in PBMCs. Imaging revealed significantly higher nicotinamide-adenine dinucleotide-hydrogen (NADH) content in skin as well as reduced passive leg movement-induced hyperemia in patients. This study confirmed results of earlier studies regarding plasma biomarkers in mitochondrial disease and identified several imaging techniques that could detect functional differences at the tissue level between participants with mitochondrial disease and HVs. However, assays of mitochondrial function in PBMCs did not show differences between participants with mitochondrial disease and HVs, possibly reflecting compensatory mechanisms and heterogeneity in mutational load. In future clinical trials, using a mix

Trial registration number: NL77982.056.21.

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of imaging and blood-based biomarkers may be advisable, as well as combining these with an in vivo challenge to disturb homeostasis.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Mitochondrial disorders cause significant disease burden, and staging and evaluation of mitochondrial disease relies mainly on clinical evaluation and invasive procedures. Development of treatments for mitochondrial disease is likewise burdened by a lack of non-invasive evaluable end points in early phase research, in addition to the large variability between different mitochondrial diseases, between patients with the same mitochondrial mutation, and between different tissues in a single patient, resulting in few evidence-based treatments being available.

WHAT QUESTION DID THIS STUDY ADDRESS?

Whether a combination of circulating biochemical markers, ex vivo cellular assays, and imaging techniques can differentiate between patients with mitochondrial disease and healthy volunteers (HVs).

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Several imaging techniques and serum biomarkers can distinguish individuals with mitochondrial disease from HVs. Ex vivo cellular assays are less reliable in distinguishing individuals with mitochondrial disease from HVs, possibly due to tissue heterogeneity in mutational load causing cells collected from blood to be less affected by disease than end organs such as the heart.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

This study confirms the need for an integral approach to the development of treatments for mitochondrial disorders, including end points at various tissue levels (e.g., blood, skin, muscle, and blood vessels).

INTRODUCTION

Mitochondrial disorders are a group of diseases caused by defects in the mitochondrial oxidative phosphorylation chain and presenting with a variety of phenotypes. The most common mutation in mitochondrial DNA causing mitochondrial dysfunction is m.3243A>G, also known as the mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) mutation,¹ which causes a combined defect of the oxidative phosphorylation chain proteins encoded in mitochondrial DNA.² The resulting disorders due to mitochondrial dysfunction in these individuals include MELAS, maternally inherited diabetes deafness, hypertrophic cardiomyopathy, macular dystrophy, focal segmental glomerulosclerosis, and myoclonic epilepsy with ragged-red fibers.

Defects in mitochondrial function lead to a disturbance in cellular redox balance and increase in cellular oxidative stress.³ This results in, among other effects, cardiovascular disease, and in particular endothelial dysfunction.⁴ Assessment of functional status in individuals with mitochondrial disease can be done through questionnaires or

evaluation of clinical symptoms, or by in vitro assays of mitochondrial function,⁵ although these have limitations such as high inter-tissue variability, necessitating invasive procedures to acquire affected tissues.⁶ Mitochondrial mutation load, for example, was found to be correlated with functional status in muscle tissue, but not in blood.⁷ Other limitations of in vitro functional assays are high inter-laboratory variability, a low margin between individuals with mitochondrial disease and healthy controls, and the inability to differentiate between primary mitochondrial dysfunction (e.g., due to a mutation in mitochondrial DNA) and mitochondrial dysfunction due to other factors (e.g., sedentary lifestyle).⁶

Recently, the Centre for Human Drug Research has developed and validated a test array for non-invasive evaluation of metabolic and endothelial function in vivo in different tissues. This test battery includes the flow-mediated skin fluorescence (FMSF) technique, which measures nicotinamide-adenine-dinucleotide hydrogen (NADH) fluorescence in the skin and can be used to assess cellular metabolic status and response to ischemia, near-infrared spectroscopy which can be used to measure

skin and muscle tissue oxygenation and has been used previously to evaluate mitochondrial oxidative capacity *in vivo*,⁸ laser speckle contrast imaging (LSCI), which when combined with reactive hyperemia and thermal hyperemia challenges can measure microvascular reactivity in the skin, passive leg movement, used to measure nitric oxide-mediated large vessel vasodilation, and sidestream dark field microscopy to assess sublingual vascular density and perfusion status.

In addition, the Centre for Human Drug Research developed novel cell-based techniques capable of assessing mitochondrial status. These include assessments of mitochondrial reactive oxygen species (ROS) and mitochondrial membrane potential. ROS are important regulators of physiological cell signaling, and excessive mitochondrial ROS production can induce mitochondrial damage and may have a role in the pathogenesis of mitochondrial disorders,⁹ while mitochondrial membrane potential is an essential energy storage component for oxidative phosphorylation and ATP production.¹⁰ These cell-based biomarkers can be combined with serum or plasma biomarkers such as GDF-15, an established systemic biomarker of mitochondrial disease and integrated stress response,¹¹ to assess mitochondrial function on multiple physiological levels.

In current clinical practice there are limited treatments available for patients with mitochondrial disease. Treatments include administering of arginine and citrulline as nitric oxide donors to improve endothelial function¹² and thereby possibly prevent or treat MELAS-related stroke,¹³ exercise to improve mitochondrial function, and administration of vitamins and supplements such as coenzyme Q10, creatine, L-carnitine, dichloroacetate, dimethylglycine, α -lipoic acid, and B-vitamins, although evidence of clinical efficacy of these treatments is very limited and mixed.¹⁴ Moreover, the measures used to evaluate clinical effects in these trials are variable and not all proven to correlate with the functional status of patients.

A phase 0 clinical study was performed to identify biomarkers differentiating between healthy volunteers (HVs) and patients with mitochondrial disease, based on the aforementioned set of imaging and cellular techniques, supported by circulating biochemical biomarkers of inflammation and myocardial damage. Ultimately, these biomarkers could be used as early clinical end points in future phase 1B/phase 2A clinical pharmacology studies in patients with mitochondrial disease.

METHODS

This study was conducted at the Centre for Human Drug Research (Leiden, The Netherlands), in accordance

with the principles of the Declaration of Helsinki, the International Conference on Harmonisation Good Clinical Practice, and ethical principles as referenced in the European Union (EU) Directive 2001/20/EC. The protocol was approved by the Medical Research Ethics Committee of the BEBO foundation (Assen, The Netherlands).

The trial was prospectively registered in toetsingonline.nl (CHDR2111, NL77982.056.21, ABR number 79322).

Participants

Eight participants with mitochondrial disease, with a confirmed m.3243A>G mutation in genetic testing and a Newcastle Mitochondrial Disease Scale score ≥ 11 , and eight matched HVs, all aged between 18 and 75 years and with body mass index (BMI) between 18 and 30 kg/m², were recruited at Radboud University Medical Center, Nijmegen, The Netherlands. For participants with mitochondrial disease, only individuals with current cardiomyopathy defined as evidence of left ventricular hypertrophy, reduced systolic function, or strain or electrocardiographic abnormalities consistent with cardiac involvement of mitochondrial disease were included. HVs were included if no clinically significant abnormal findings were obtained on medical history, physical examination, hematological laboratory tests, or drug and alcohol screening. Pregnant women were excluded from participation, as were participants who received treatment with metformin, cytostatic medication, soluble guanylate cyclase stimulators or activators, or nitrate agents less than 3 months before study day 1. Healthy volunteers were matched to participants with mitochondrial disease on sex, age (± 5 years), and BMI (± 3 kg/m²).

Study design

This was a translational phase 0, non-interventional, cross-sectional case-control study in which all participants with mitochondrial disease and healthy participants underwent all study assessments once. Participants received no investigational treatment.

Study assessments

Safety

Safety evaluation included assessment of adverse events and concomitant medication use and measurement of vital signs.

Plasma and serum biomarkers

Venous blood was collected in K2EDTA tubes for assessment of hematology and glycated hemoglobin (HbA1c), Serum Separator Tubes (SSTs) and Gel and Clot Activator Tubes for assessment of clinical chemistry, and sodium fluoride tubes for assessment of glucose at the Clinical Chemistry Laboratory of Leiden University Medical Center (Leiden, The Netherlands). Additional venous blood was collected in K2EDTA tubes for assessment of plasma biomarkers growth/differentiation factor 15 (GDF-15; ELISA, Quantikine ELISA Human GDF-15, R&D Systems), pentraxin 3 (PTX 3; ELISA, Quantikine ELISA human Pentraxin 3/TGS-4, R&D Systems), interleukin-6 (IL-6; ECLIA, Proinflammatory Panel 1 (human) Kit, Meso Scale Discovery), N-terminal prohormone of brain natriuretic peptide (NT-proBNP; ECLIA, Elecsys proBNP II, Roche Diagnostics), cardiac troponin I (cTNI; CLEIA, Lumipulse® G hs Troponin I, Fujirebio). and high-sensitivity C-reactive protein (hsCRP; Immunoturbidimetric Test Kit, CRP4, Roche Diagnostics) at MLM Medical Labs GmbH (Mönchengladbach, Germany). All blood collection was performed in a fasted state after an overnight fast.

Mitochondrial functional assays

Mitochondrial function was evaluated in fresh peripheral blood mononuclear cells (PBMCs). Venous blood was collected in Cell Preparation Tubes (CPTs) containing sodium-heparin (Becton Dickinson). Blood was centrifuged at 1800g for 30 min and PBMCs were collected by pouring supernatant into a polypropylene tube. PBMCs were assessed by flow cytometry ($\sim 2 \times 10^5$ cells/well) and by plate reader (2.5×10^5 cells/well).

Mitochondrial ROS were quantified by MitoSOX™ Red (Molecular Probes, Invitrogen). PBMCs were incubated with MitoSOX™ at 5 μM for 15 min at 37°C in a humidified atmosphere with 5% CO₂. Mitochondrial mass was assessed by incubation of PBMCs with 25 nM MitoTracker™ Green FM (Molecular Probes, Invitrogen) for 45 min at 37°C in a humidified atmosphere with 5% CO₂. PBMCs were stained with CD14 and CD3 markers for monocyte and T-cell discrimination, respectively. Propidium iodide was used to assess PBMCs viability. After staining, PBMCs were washed twice with phosphate buffer saline and analyzed by flow cytometry (MACSQuant16, Miltenyi Biotec). For flow cytometry data, to ensure proper gate setting, a minimum of 100,000 events (viable leukocytes) were collected. Gating strategy plots can be found in [Figures S2 and S3](#).

In addition, mitochondrial membrane potential was assessed by tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining (JC-1 kit fluorometric, Abcam). PBMCs were

incubated with 0.5 μM of JC-1. A positive control for membrane depolarization was included by incubation of PBMCs with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) at 100 μM. Incubations were done for 30 min at 37°C in a humidified atmosphere with 5% CO₂. Technical duplicates were produced for all mitochondrial membrane potential measurements. A Varioskan Lux plate reader (ThermoFisher) equipped with fluorescence filters was used to measure JC-1 fluorometric signals using excitation filter = 475 ± 20 nm and emission filters: 530 ± 15 and 590 ± 17.5 . Data were collected using SkanIt software for microplate readers RE version 4.1.0.43. Mitochondrial membrane potential was calculated as presented in Equation 1:

$$\text{MMP} = \frac{\frac{\text{red aggregates}_{\text{condition}}}{\text{green aggregates}_{\text{condition}}}}{\frac{\text{red aggregates}_{\text{CCCP or FCCP}}}{\text{green monomers}_{\text{CCCP or FCCP}}}} \quad (1)$$

Bioenergetic profiles of freshly thawed and subsequently cultured PBMCs were evaluated using the Seahorse XF96 platform, measuring oxygen consumption rate at baseline and after oligomycin, FCCP, and antimycin A treatment to evaluate mitochondrial function and extracellular acidification rate at baseline and after oligomycin treatment to assess glycolytic capacity (Bioenergetics LLC).

Imaging assessments

Measurements were conducted in temperature-controlled rooms (20–24°C) at the Centre for Human Drug Research. Study assessments conducted included FMSF, passive leg movement, and sidestream dark field microscopy.

FMSF is a technique used to measure mitochondrial function in vivo based on measuring the intensity of NADH fluorescence in skin tissue on the forearm during a challenge consisting of occluding and then releasing arterial flow. FMSF was used to assess cellular metabolic status by measuring NADH fluorescence during the various stages of the intervention, and vascular responses were assessed by analyzing vasomotion using Fourier transformation. FMSF was measured using the purpose-built Angionica AngioExpert device (Angionica, Łódź, Poland).

Near-infrared spectroscopy is used to measure fractions of oxygenated and deoxygenated hemoglobin in tissues up to 3–4 cm deep with a spectroscopic device placed on the skin (Artinis Portamon, Artinis Medical Systems). Near-infrared spectroscopy was conducted on the forearms of participants and combined with an arterial and venous occlusion challenge in which blood flow is temporarily occluded with a blood pressure cuff inflated above systolic pressure (arterial occlusion) and diastolic pressure (venous occlusion). In combination with the arterial and venous

occlusion, near-infrared spectroscopy allowed the quantification of tissue oxygen consumption, blood flow, and vascular response to influx of blood in the arm.¹⁵ LSCI is a non-invasive imaging method that uses changes in the speckle pattern reflected when illuminating an imaged object with laser light (Pericam PSI NR system, Perimed). Changes in the reflected pattern signify any movement on or inside the imaged object, which when imaging still human tissue reflects the flow of blood cells, which can be used to derive an estimation of blood flow in the imaged tissue. LSCI was performed in combination with post-occlusive reactive hyperemia, where blood flow was temporarily occluded with a blood pressure cuff placed around the upper arm and then released. The subsequent increase in flow was used as a measure of vascular reactivity to shear stress caused by the sudden influx of blood into the arm.¹⁶ LSCI was also combined with the local thermal hyperemia challenge, in which skin is heated to approximately 43°C while continuously measuring blood flow, allowing the assessment of axon- and nitric oxide-dependent vasodilation.¹⁷

Passive leg movement-induced hyperemia is a physiological response in the common femoral artery to passive movement of the lower leg. Passive movement of the lower leg induces peripheral vasodilation, which then induces an increase in arterial blood flow, quantifiable by measuring the flow speed through the common femoral artery with ultrasonography (Sparq Ultrasound System, Philips Medical Systems). Passive leg movement-induced hyperemia is mediated mainly by nitric oxide release in endothelial cells, which makes it a reliable investigation to assess nitric oxide bioavailability.¹⁸

Sidestream dark field microscopy is a technique used to visualize blood vessels *in vivo* using light in a wavelength absorbed by red blood cells emitted by a microscope (MicroScan, MicroVision Medical). Sidestream dark field microscopy assessments were conducted on the mucous membranes of the mouth, which allow penetration of the light and visualization of the underlying blood vessels. Analyzed sidestream dark field microscopy parameters included the number of vessel crossings on an imaginary grid, De Backer density of vessels, and the proportion of perfused vessels in the field of view.¹⁹

Statistical analysis

All parameters were summarized by participant group and listed with mean, standard deviation (SD), coefficient of variation (CV), median, minimum, and maximum. Differences between parameters of all assessments were compared between HVs and participants with mitochondrial disease. Parameters were assessed for normality and log-transformed if necessary to facilitate analysis. Log-transformed end

points were back-transformed after analysis where results could be interpreted as percentage difference.

For imaging assessments, group differences were assessed using a mixed model analysis of covariance with time and group as fixed factors and subject as random factor. Results were reported with the estimated difference, 95% confidence interval, least square mean (LSM) estimates and the *p*-value. Graphs of the LSM estimates by participant group were presented with 95% confidence intervals as error bars.

For biomarkers and mitochondrial function assessments, differences in continuous variables between groups were assessed using non-parametric tests (i.e., Wilcoxon rank-sum and Kruskal–Wallis) and categorical data were analyzed with cross-tables by Fisher's exact test. Data from the Seahorse assessment were analyzed with a Student's *t*-test or a two-way analysis of variance with a Dunnett's multiple comparison test using GraphPad Prism 7.00.

RESULTS

Clinical and demographic characteristics of study participants

A total of 18 participants were screened, nine in the participants with mitochondrial disease group and nine in the HV group. An overview of the flow of participants in the study is shown in [Figure S1](#).

An overview of characteristics and demographics for the participants is provided in [Table S1](#). Participants were all white (100%) and predominantly female (HV 57% vs. MitoD 63%). Temperature and ethnicity did not differ significantly between study groups. No clinically significant medical history or concomitant medication was noted in the HV group. In the mitochondrial disease group, 7/8 participants had a history of diabetes mellitus, treated with long- and short-acting insulin in 5/8 participants and with sulfonylureas in 2/8 participants, in one participant combined with dipeptidyl peptidase-4 inhibition. Other notable medical history included hearing loss in 7/8 participants as well as vision loss in 3/8 participants, myocardial infarction in 2/8 participants, and cardiac arrhythmia and kidney insufficiency, the latter in one different participant each. All participants were prescribed an angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker for their documented cardiomyopathy, in one participant combined with neprilysin inhibition. Six of eight participants used β -blockers and 3/8 loop diuretics. Two participants used acetylsalicylic acid and a platelet aggregation inhibitor for coronary artery disease. Other notable medication use was pancreatic enzymes for chronic pancreatitis in one participant and a vitamin K antagonist for prevention of vascular events in another participant.

Safety data

No safety evaluation was planned for this study since participants did not receive a study intervention. However, adverse events were collected. No participants experienced adverse events during the study.

Biomarkers and mitochondrial functional assays

Clinical chemistry, hematology, and additional plasma biomarkers

Results of clinical chemistry, hematology, and additional plasma biomarkers are summarized in [Table 1](#). Significant clinical chemistry differences between participants with mitochondrial disease and HVs were seen in medians

of glucose, lactate dehydrogenase (LDH), sodium, and HbA1c. Significant median differences between MitoD patients and HVs were also seen for lymphocyte count and monocyte count. Evaluation of additional plasma biomarkers revealed significant median differences for GDF-15, IL-6, NT-proBNP, and cTNI, and a trend towards higher hsCRP in participants with mitochondrial disease versus HVs.

Mitochondrial ROS production, mitochondrial mass, and mitochondrial membrane potential

Results of flow cytometry analyses are summarized in [Table 2](#). No significant differences between participants with mitochondrial disease and HVs were observed in mitochondrial ROS production or mitochondrial membrane potential.

TABLE 1 Comparison between healthy volunteers and mitochondrial disease participants for clinical chemistry, hematology, and biomarker parameters.

Parameter (unit)	HV (median, 95% CI)	MitoD (median, 95% CI)	P-value HV vs. MitoD
Glucose (mmol/L)	5.20 (4.90/5.30)	8.8 (6.3/11.2)	0.002
Creatine kinase (U/L)	75 (64/150)	220 (109/355)	0.054
Lactate dehydrogenase (U/L)	165 (152/245)	250 (187/288)	0.037
Sodium (mmol/L)	142 (141/142)	135 (130/138)	0.001
Triglycerides (mmol/L)	1.14 (0.89/1.64)	2.04 (1.24/3.63)	0.072
Blood urea (mmol/L)	5.00 (4.70/7.10)	8.15 (6.70/8.95)	0.054
Uric acid (mmol/L)	0.270 (0.250/0.320)	0.335 (0.280/0.500)	0.063
HbA1c (mmol/mol Hb)	35.1 (32.4/36.0)	60.5 (57.5/69.7)	0.001
Basophil count (10 ⁹ /L)	0.0400 (0.0300/0.0800)	0.0350 (0.0300/0.0551)	0.637
Eosinophil count (10 ⁹ /L)	0.140 (0.060/0.230)	0.180 (0.120/0.220)	0.601
Lymphocyte count (10 ⁹ /L)	1.58 (1.38/1.67)	1.94 (1.84/2.12)	0.009
Monocyte count (10 ⁹ /L)	0.490 (0.410/0.540)	0.675 (0.560/0.790)	0.004
Neutrophil count (10 ⁹ /L)	4.09 (3.00/4.69)	3.84 (3.63/5.75)	0.955
Platelet count (10 ⁹ /L)	227 (209/336)	204 (192/292)	0.463
GDF-15 (pg/mL)	470 (393/728)	2141 (1755/3927)	0.001
PTX-3 (ng/mL)	0.318 (0.156/0.608)	0.340 (0.156/0.484)	0.720
IL-6 (pg/mL)	0.200 (0.200/0.600)	0.90 (0.40/2.10)	0.026
NT-proBNP (pg/mL)	60.0 (38.0/79.0)	680 (94/2262)	0.021
cTNI (pg/mL)	23.1 (19.3/25.8)	152 (53/497)	0.001
hsCRP (mg/L)	0.60 (0.30/2.10)	1.55 (1.10/3.15)	0.056

Note: P-value based on Wilcoxon-test; *for three subjects with values < BLoQ the value 0.1565 was taken (1/2 of BLoQ); **for four subjects with values < BLoQ the value 0.2 was taken (1/2 of BLoQ). P-values <0.05 in bold type.

Abbreviations: CI, confidence interval; cTNI, cardiac troponin I; GDF-15, growth/differentiation factor 15; HbA1c, glycated hemoglobin; hsCRP, high-sensitivity C-reactive protein; HV, healthy volunteer; IL-6, interleukin-6; max, maximum; min, minimum; MitoD, mitochondrial disease patients; NT-proBNP, N-terminal prohormone of brain natriuretic peptide.

Measure of mitochondrial function	HV (median, 95% CI)	MitoD (median, 95% CI)	P-value HV vs. MitoD
MitoSOX monocytes	1.01 (0.83/1.19)	0.85 (0.78/1.10)	0.4634
MitoTracker monocytes	6.1 (4.1/14.3)	15.4 (4.7/32.4)	0.1206
MitoSOX/MitoTracker ratio (monocytes)	0.152 (0.095/0.241)	0.073 (0.026/0.156)	0.0721
MitoSOX T cells	0.280 (0.270/0.300)	0.280 (0.250/0.330)	1.0000
MitoTracker T cells	2.35 (1.61/4.55)	5.7 (2.0/11.9)	0.0721
MitoSOX/MitoTracker ratio (T cells)	0.119 (0.071/0.161)	0.056 (0.029/0.145)	0.0939
JC-1, aggregates/monomers ratio	4.84 (1.61/6.02)	5.34 (4.54/6.12)	0.2319

Note: P-value based on Wilcoxon-test with adjustment.

Abbreviations: CI, confidence interval; HV, healthy volunteers; max, maximum; min, minimum; MitoD, mitochondrial disease patients; *n*, number of subjects.

Mitochondrial bioenergetic profiles

One participant with mitochondrial disease and one HV were excluded from Seahorse analysis due to insufficient cells, one HV due to insufficient quality of cells and one participant with mitochondrial disease due to a positive tetrahydrocannabinol drug screening. There were no statistically significant differences between HVs and participants with mitochondrial disease detected in any oxygen consumption rate or extracellular acidification rate parameter except for a lower glycolytic compensation in participants with mitochondrial disease when compared to HVs ($p = 0.0417$; Figures 1 and 2).

Imaging assessments

Flow-mediated skin fluorescence

Assessment of skin NADH content showed a baseline NADH (LSM difference $-201,771$, 95% CI: $-352,349$, $-51,193$), end-test NADH (LSM difference $-214,942$, 95% CI: $-367,462$, $-62,423$), maximum NADH (LSM difference $-229,504$, 95% CI: $-400,052$, $59,555$), and minimum (LSM difference $-180,800$, 95% CI: $-298,856$, $-62,743$) were all significantly higher in participants with mitochondrial disease when compared to HVs as shown in Figure 3. No other statistically significant differences were found in FMSF parameters.

Near-infrared spectroscopy

Assessment of skin and muscle oxygenated and deoxygenated hemoglobin did not show statistically significant

differences between HVs and participants with mitochondrial disease in muscle oxygen consumption, muscle blood flow, hyperemic response speed, or hyperemic response duration as assessed with near-infrared spectroscopy.

Laser speckle contrast imaging

The results from LSCI measurements are summarized in Table 3. No statistically significant differences between HVs and participants with mitochondrial disease were seen in basal, maximal, or plateau flow (including change from baseline for maximal and plateau flow) during local thermal hyperemia challenge, although all observed dermal blood flows were lower in participants with mitochondrial disease, specifically local thermal hyperemia-induced plateau blood flow. Similarly, no statistically significant differences in basal, maximal, or mean flow (including change from baseline for maximal flow) during post-occlusive reactive hyperemia challenges were found.

Passive leg movement

Flow increase after passive leg movement was significantly higher in HVs when compared to participants with mitochondrial disease (LSM difference: 224.05 , 95% CI: 12.34 , 435.76) as shown in Figure 4.

Sidestream dark field microscopy

De Backer density, a measure of vessel density, was significantly higher in HVs when compared to participants with mitochondrial disease (LSM difference 0.94 , 95% CI:

TABLE 2 Comparison between healthy volunteers and mitochondrial disease patients for mitochondrial reactive oxygen species and mitochondrial membrane potential parameters.

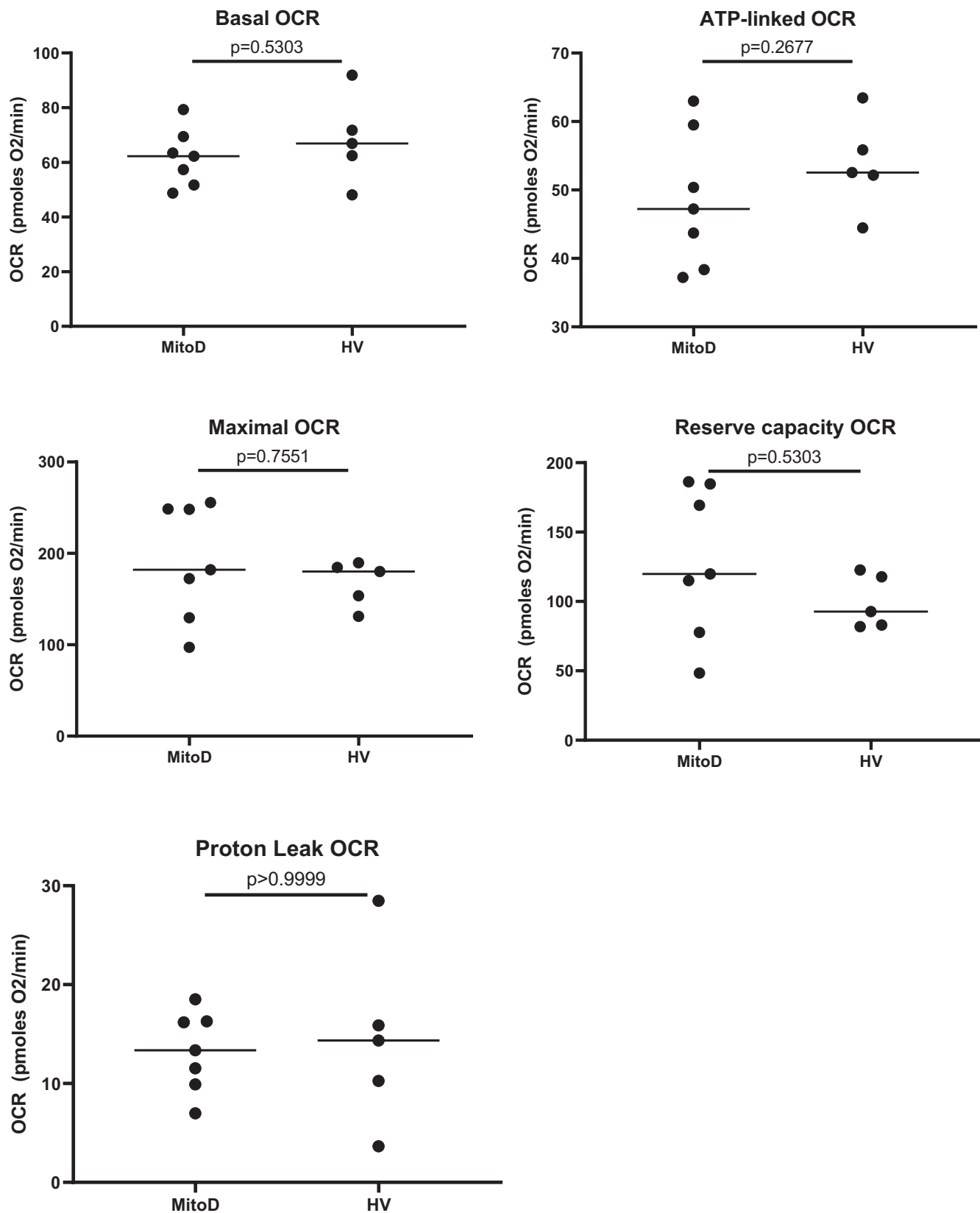


FIGURE 1 Extracellular acidification rate (ECAR) and glycolytic compensation, individual datapoints with medians. HV, healthy volunteers; MitoD, mitochondrial disease.

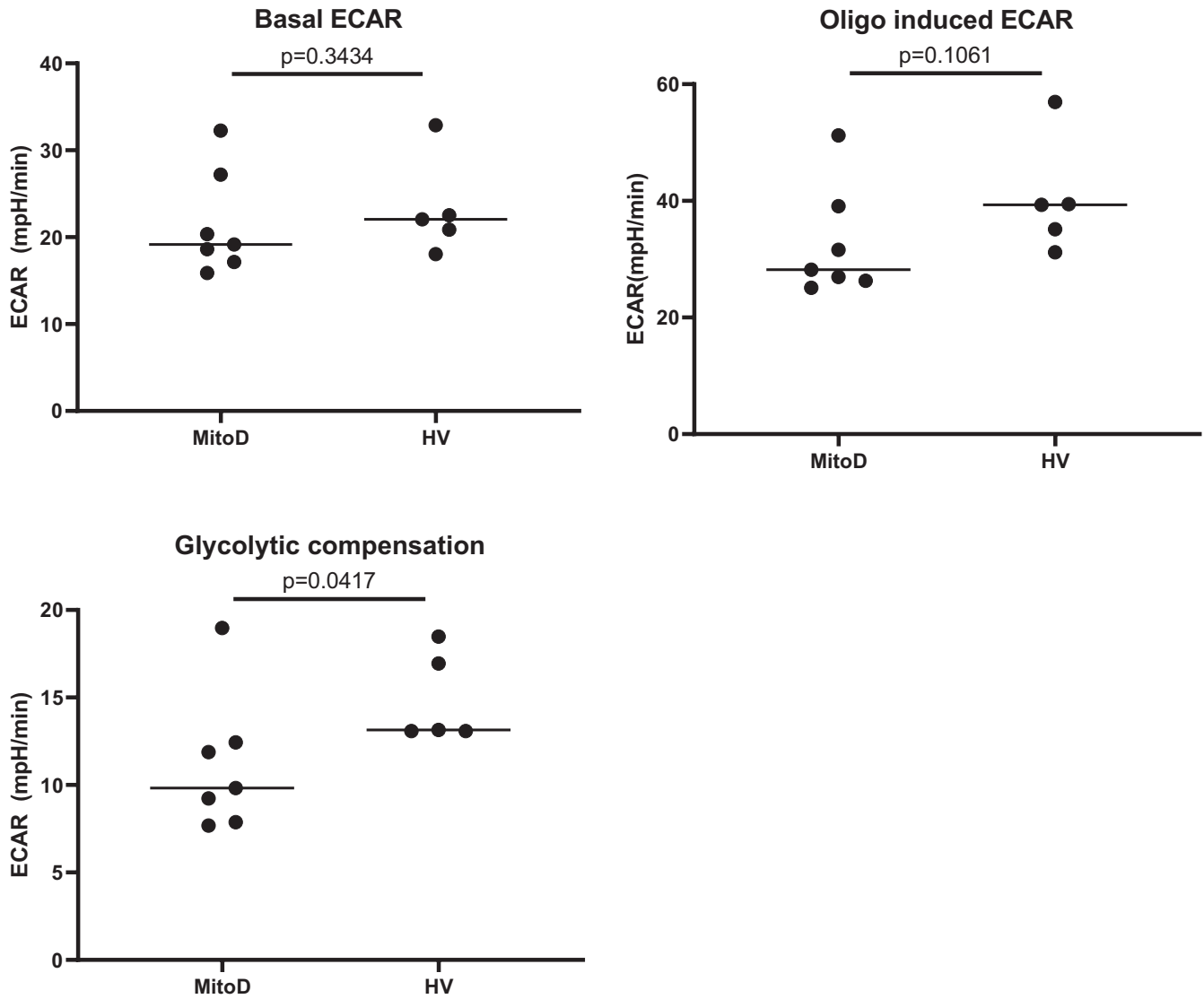


FIGURE 2 Oxygen consumption rate, individual datapoints with medians. HV, healthy volunteers; MitoD, patients with mitochondrial disease; OCR, oxygen consumption rate.

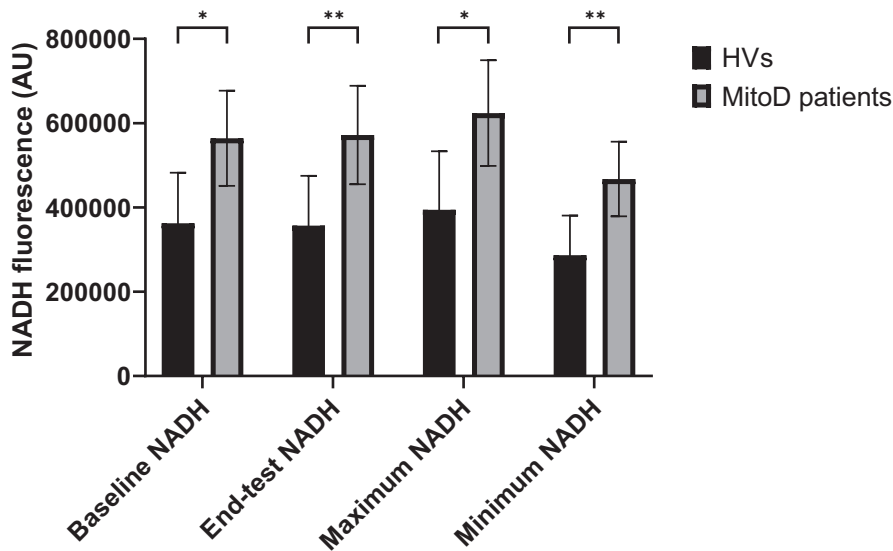


FIGURE 3 Nicotinamide adenine dinucleotide hydrogen (NADH) fluorescence during flow-mediated skin fluorescence, means with 95% confidence intervals. *Significance $p < 0.05$. **Significance $p < 0.01$. AU, arbitrary units; HVs, healthy volunteers; MitoD, mitochondrial disease.

TABLE 3 Comparison between healthy volunteers and mitochondrial disease patients for laser speckle contrast imaging parameters.

Parameter (unit)	P-value	HV (LSM)	MitoD (LSM)	LSM difference	95% CI	
					Lower	Upper
LSCI LTH basal flow (AU)	0.4605	102.394	94.877	7.517	-13.634	28.668
LSCI LTH maximal flow (AU)	0.2165	177.339	156.152	21.187	-13.809	56.182
LSCI LTH maximal flow CFB (AU)	0.2626	74.945	61.276	13.669	-11.361	38.700
LSCI LTH plateau flow (AU)	0.1212	191.409	161.578	29.831	-8.875	68.537
LSCI LTH plateau flow CFB (AU)	0.1431	89.015	66.701	22.314	-8.462	53.090
LSCI PORH basal flow (AU)	0.2635	33.921	30.096	3.826	-3.193	10.845
LSCI PORH maximal flow (AU)	0.2521	70.020	60.258	9.762	-7.708	27.232
LSCI PORH maximal flow CFB (AU)	0.4299	36.099	30.162	5.937	-9.662	21.535
LSCI PORH mean flow (AU)	0.3605	12.640	10.990	1.650	-2.078	5.378
LSCI PORH rest flow (AU)	0.7304	37.664	36.308	1.356	-6.876	9.588

Abbreviations: AU, arbitrary unit; CFB, change from baseline; CI, confidence interval; HV, healthy volunteers; LSCI, laser speckle contrast imaging; LSM, least squares mean(s); LTH, local thermal hyperemia; MitoD, mitochondrial disease patients; PORH, post-occlusive reactive hyperemia.

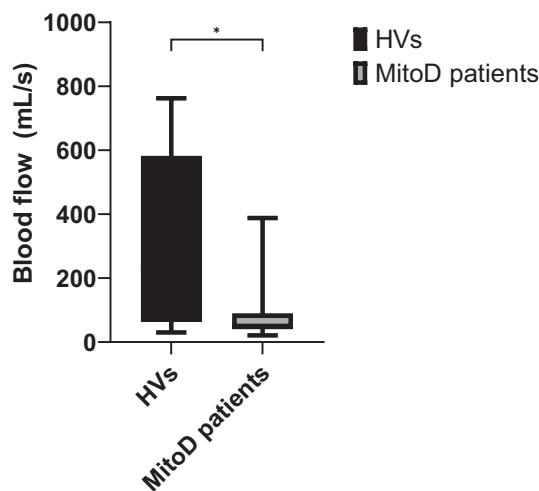


FIGURE 4 Femoral artery blood flow change from baseline after passive leg movement, means with 95% confidence intervals. *Significance $p < 0.05$. HVs, healthy volunteers; MitoD, mitochondrial disease.

0.015, 1.87), as was the number of crossings (LSM difference 9.5, 95% CI: 0.1, 18.9), which is a related parameter. There were no other statistically significant differences between groups in other sidestream dark field microscopy parameters.

DISCUSSION

In this phase 0, observational, translational, and mechanistic study, biomarkers and imaging methods were evaluated for their ability to distinguish between mitochondrial disease participants with a confirmed m.3243A>G mutation and healthy participants matched on sex, age, and

BMI. Significant differences in clinical chemistry, hematology, and markers of inflammation and myocardial damage were identified, unsurprising given the clinical status of the participants with mitochondrial disease, all of whom were diagnosed with diabetes mellitus and cardiomyopathy. This study also confirmed earlier results showing GDF-15 as a biomarker specific for mitochondrial disease.¹¹

No significant differences between study groups were seen in experiments evaluating mitochondrial ROS production or mitochondrial membrane potential in fresh PBMCs, and only one significant difference, a lower glycolytic compensation in participants with mitochondrial disease, was found when assessing oxygen consumption rate and extracellular acidification rate in freshly thawed PBMCs. Higher mitochondrial ROS production, reduced oxygen consumption rate, and impaired glycolysis in PBMCs has been shown in patients with heart failure,²⁰ chronic kidney disease,²¹ and other patient groups,^{22,23} although literature is relatively scarce and heterogeneous, and many studies are conducted in cells other than PBMCs. Mitochondrial function has also previously been evaluated in specific but heterogeneous mitochondrial disease patient populations in small samples, and in various cell or tissue types.¹¹ In a study of children with various defects of the oxidative phosphorylation chain, mitochondrial membrane potential and ATP production was found to be lower in the lymphocytes of affected participants compared to controls.²⁴ For patients with the specific m.3243A>G mutation, higher mitochondrial ROS production, lower ATP production, and lower mitochondrial membrane potential in PBMCs was shown in two previous studies,^{25,26} and higher mitochondrial ROS production and lower mitochondrial membrane potential and ATP production was also shown in m.3243A>G

mutated fibroblasts,²⁷ and in endothelial cells created from induced pluripotent stem cells derived from an individual with high m.3243A>G mutational load.²⁸ Lower mitochondrial oxygen consumption rate has been found in both human myoblasts²⁹ and induced pluripotent stem cells with the m.3243A>G mutation.³⁰ This study did not replicate the findings regarding mitochondrial membrane potential and mitochondrial ROS production seen in previous studies. This might reflect a small sample size with intra-individual variability, especially in the heterogeneous group of participants with mitochondrial disease, or a higher metabolic flexibility of PBMCs compared to other tissues affected in individuals with the m.3243A>G mutation.³¹

This is the first study to evaluate an array of imaging methods for evaluation of metabolic and endothelial function in individuals with the m.3243A>G mutation, although the methods employed in this study have been studied previously in diverse study populations, such as patients with cardiovascular disease, diabetes mellitus, chronic kidney disease, chronic obstructive pulmonary disease, and critical illness.^{17,32–34}

NADH fluorescence was significantly elevated in the skin of participants with the m.3243A>G mutation compared to HVs. Due to dysfunction of mitochondrial complex I of the mitochondrial OXPHOS chain and decrease in activity of NADH reductive pathways in these patients,^{35,36} NADH/NAD⁺ ratio and consequently cellular reductive stress will increase, leading to downstream metabolic changes in these patients and contributing to the disease phenotypes associated with this mutation. NAD⁺ metabolism and NADH/NAD⁺ ratio have previously been the target of interventions aiming to treat mitochondrial disorders,^{37,38} but these have not been proven efficacious in human trials. The FMSF method likely detected the higher NADH levels associated with the pathophysiology of mitochondrial dysfunction in peripheral tissue (skin), suggesting that there is a difference in the effects of the genetic defect in peripheral tissue compared to PBMCs, in which no differences in mitochondrial function were detected.

Hypoxia sensitivity as measured with FMSF and resting muscle tissue oxygen consumption as measured with near-infrared spectroscopy did not differ significantly between participants with mitochondrial disease and HVs, possibly due to the compensatory mechanisms in the former during rest (e.g., hyperoxygenation of muscle³⁹), which might be revealed by applying blood volume corrections in future studies.⁴⁰

Microvascular reactivity to passive leg movement of the lower leg was significantly lower in participants with mitochondrial disease compared to HVs, probably reflecting the higher oxidative stress in the former⁴¹ causing reduced nitric oxide bioavailability. This is supported by

a trend towards lower local thermal hyperemia-induced dermal blood flow in participants with mitochondrial disease versus HVs, another indicator of nitric oxide bioavailability.¹⁷ Last, significantly lower sublingual vessel density was observed in participants with mitochondrial disease versus HVs. This contradicts earlier findings of higher capillary growth induced by poor oxygen utilization in muscles affected by mitochondrial dysfunction,⁴² but in this study vessel density was measured in non-muscular tissue which may be affected more by reduced angiogenic capacity of endothelial cells due to mitochondrial dysfunction.⁴³

In this study, mitochondrial functional assays conducted on PBMCs did not distinguish between individuals with mitochondrial disease and HVs, while several imaging methods testing skin metabolic status (FMSF) or general vascular function (passive leg movement, LSCI, sidestream dark field microscopy) detected differences. This likely reflects the heterogeneity of mutational load within individuals with mitochondrial disease, with some tissues with a higher mutational load than others,⁴⁴ as well as compensatory mechanisms (e.g., metabolic flexibility) in PBMCs.⁴⁵ In addition, the process of purifying selection, by which PBMCs expressing high mutational loads of the pathogenic mitochondrial DNA are filtered out during production or targeted for removal after entering the bloodstream,⁴⁶ thereby resulting in PBMCs with low mutational loads predominating in the bloodstream and therefore in the blood samples taken for analysis, may have reduced the likelihood of finding significantly decreased mitochondrial function in PMBCs of individuals with mitochondrial disease compared to PMBCs of HVs. Moreover, small differences on proximal, cell-level end points might coalesce into detectable effects on tissue-level end points such as imaging and serum biomarkers. In future clinical studies evaluating potential treatments for mitochondrial disease, a mixed approach of cellular, imaging, and serum biomarker end points may be advisable to fully capture pharmacodynamic effects of the studied compound in different tissues and on different physiological levels. Moreover, since basal cellular assays conducted during homeostasis did not discriminate between participants with mitochondrial disease and HVs in this study, *in vivo* stressors, such as intravenous administration of lipopolysaccharide⁴⁷ or oral administration of statins⁴⁸ may challenge the system and thereby reveal drug effects.

Limitations

Since the array of circulating biomarkers evaluated in this study was limited, biomarkers that have since been

identified by proteomics and metabolomics were not tested in this study.³⁶ This study was further limited by variability in disease severity, comorbidity, medication use, lifestyle, and age in the mitochondrial disease group. Some of the medications used by the participants with mitochondrial disease in this study are known to influence inflammation, oxidative stress, and mitochondrial function and may therefore have influenced the outcomes of the assessments of this study.^{49,50} Variation within the mitochondrial disease group was partially compensated for by matching HVs and participants with mitochondrial disease, but due to a small sample size, further stratification in subgroups or other means of controlling for this variation were not possible. However, it is likely that the used medications shifted the measured parameters towards levels observed in HVs, reducing the likelihood that observed differences would disappear when controlling for medication use. A limitation of the mitochondrial ROS assay specifically is that detection of superoxide only does not reflect the complete spectrum of ROS and its effects. A limitation of the FMSF technique is that only skin NADH content can be measured, hence NADH/NAD⁺ ratio cannot be determined, and only partial evaluation of cellular metabolic state is possible.

CONCLUSIONS

In this study several biomarkers and imaging assessments were able to distinguish between healthy participants and participants with mitochondrial disease, indicating a potential for use as end points in clinical trials investigating treatments for mitochondrial dysfunction in addition to traditional methods of assessment, such as clinical evaluation.³⁵ Further avenues for research on these investigative tools could be to combine the different end points with an in vivo medicinal challenge affecting mitochondrial function.

AUTHOR CONTRIBUTIONS

S.J.W.vK., D.R.P., L.S., A.K., J.L., A.B., T.N.G., E.K., R.F., W.H.S., P.G., and M.M. wrote the manuscript. S.J.W.vK., D.R.P., L.S., A.K., J.L., R.F., W.H.S., P.G., and M.M. designed the research. S.J.W.vK., D.R.P., B.S., and P.G. performed the research. A.B., D.R.P., B.S., T.N.G., and E.K. analyzed the data.

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CONFLICT OF INTEREST STATEMENT

L.S., A.K., J.L., and R.F. were OMEICOS Therapeutics GmbH employees at the time of study conduct and may hold or have held shares and/or stock options in the company. W.H.S. and R.F. are co-founders of OMEICOS Therapeutics GmbH. All other authors declared no competing interests for this work.

PRINCIPAL INVESTIGATOR STATEMENT

All authors confirm that the Principal Investigator for this study was Dr. Matthijs Moerland and that he had direct clinical responsibility for participants.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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