Evaluating the microcirculation in early phase clinical trials: novel methodologies and interventions

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CHAPTER III

IDENTIFICATION OF PERIPHERAL VASCULAR FUNCTION MEASURES AND CIRCULATING BIOMARKERS OF MITOCHONDRIAL FUNCTION IN PATIENTS WITH MITOCHONDRIAL DISEASE

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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 endpoints 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 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.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Several imaging techniques and serum biomarkers can distinguish individuals with mitochondrial disease from healthy volunteers.
Ex vivo cellular assays are less reliable in distinguishing individuals with mitochondrial disease from healthy volunteers, 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 endpoints at various tissue levels, e.g. blood, skin, muscle and blood vessels

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.

In this cross-sectional case-control study, 8 participants with mitochondrial disease and 8 healthy volunteers (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 content in skin as well as reduced passive leg movement-induced hyperaemia 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 on 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 of imaging and blood-based biomarkers may be advisable, as well as combining these with an *in vivo* challenge to disturb homeostasis.

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 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, which when combined with reactive hyperaemia and thermal hyperaemia 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 and mitochondrial membrane potential. Reactive oxygen species are important regulators of physiological cell signalling, and excessive mitochondrial reactive oxygen species 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 function[12] and thereby possibly prevent or treat MELAS-related stroke,[13] exercise to improve mitochondrial function, and administration of vitamins and supplements such as coenzyme Q_{10}, 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 functional status of patients.

A phase 0 clinical study was performed to identify biomarkers differentiating between healthy volunteers 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 endpoints in future phase 1B/phase 2A clinical pharmacology studies in patients with mitochondrial disease.

Materials & 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 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 healthy matched volunteers (HVS), all aged between 18 and 75 years and with body mass index 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 guanylyl cyclase stimulators or activators, or nitrate agents less than 3 months before study day 1. HVS were matched to participants with mitochondrial disease on sex, age (+/- 5 years), and body mass index (+/- 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), SST 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 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 containing sodium-heparin (Becton Dickinson, San Jose, A, USA). Blood was centrifuged at 1800x g for 30 minutes and PBMCS were collected by pouring supernatant into a polypropylene tube. PBMCS were assessed by flow cytometry (~ 2 x 10^5cells/well) and by plate reader (2.5 x 10^5 cells/well).

Mitochondrial reactive oxygen species were quantified by MitoSOX™ Red (Molecular Probes, Invitrogen). PBMCS were incubated with MitoSOX™ at 5 µM for 15 minutes at 37 °C in a humidified atmosphere with 5% CO_2. Mitochondrial mass was assessed by incubation of PBMCS with 25 nM MitoTracker™ Green FM (Molecular Probes, Invitrogen) for 45 minutes at 37 °C in a humidified atmosphere with 5% CO_2. 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 supplementary 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 FCCP at 100 µM. Incubations were done for 30 minutes 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 nm and 590 ±17.5 nm. Data was collected using SkanIt software for microplate readers RE version 4.1.0.43. Mitochondrial membrane potential was calculated as presented in Equation 1:

$$MMP = \frac{\text{red aggregates}_{\text{condition}} / \text{green aggregates}_{\text{condition}}}{\text{red aggregates}_{\text{CCCP or FCCP}} / \text{green monomers}_{\text{CCCP or FCCP}}}$$

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 flow-mediated skin fluorescence, near-infrared spectroscopy, laser speckle contrast imaging, passive leg movement and sidestream dark field microscopy.

Flow-mediated skin fluorescence 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. Flow-mediated skin fluorescence was used to assess cellular metabolic status by measuring NADH fluorescence during the various stages of the intervention, and vascular responses were assessed by analysing vasomotion using Fourier transformation. Flow-mediated skin fluorescence 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, Elst, the Netherlands). 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]

Laser speckle contrast imaging 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, Järfälla, Sweden). 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. Laser speckle contrast imaging imaging was performed in combination with post-occlusive reactive hyperaemia, 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] Laser speckle contrast imaging was also combined with the local thermal hyperaemia 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 hyperaemia 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, Best, The Netherlands). Passive leg movement-induced hyperaemia 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, Amsterdam, the Netherlands). Sidestream dark field microscopy assessments were conducted on the mucous membranes of the mouth, which allow penetration of the light and visualisation of the underlying blood vessels. Analysed 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, SD, 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 endpoints 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 factor 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 analysed with cross-tables by Fisher's exact test. Data from the Seahorse assessment were analysed 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, 9 in the participants with mitochondrial disease group and 9 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 1 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 arrythmia 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 1 participant combined with neprilysin inhibition. Six out of 8 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 1 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, 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.

TABLE 1 Comparison between HV and MitoD participants for clinical chemistry, haematology 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**

(Continuation Table 1)

Parameter [Unit]	HV (median, 95% CI)	MitoD (median, 95% CI)	p-value HV vs. MitoD
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

*CI=confidence interval, CTNI=cardiac troponin I, GDF-15=growth/differentiation factor 15, HBA1C=glycated hemoglobin, hsCRP=high sensitivity C-reactive protein, HV=healthy volunteers, IL-6=interleukin-6, MitoD=mitochondrial disease patients, MIN=minimum, MAX=maximum, N=number of subjects, NT-PROBNP=N-terminal prohormone of brain natriuretic peptide. p-value based on Wilcoxon-test, * for 3 subjects with values < BLOQ the value 0.1565 was taken (½ of BLOQ), ** for 4 subjects with values < BLOQ the value 0.2 was taken (½of BLOQ). P-values <0.05 bolded.*

MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION, MITOCHONDRIAL MASS AND MITOCHONDRIAL MEMBRANE POTENTIAL

Results of flow cytometry analyses are summarised in Table 2. No significant differences between participants with mitochondrial disease and HVS were observed in mitochondrial reactive oxygen species production or mitochondrial membrane potential.

TABLE 2 Comparison between HV and MitoD participants for MTROS and MMP parameters.

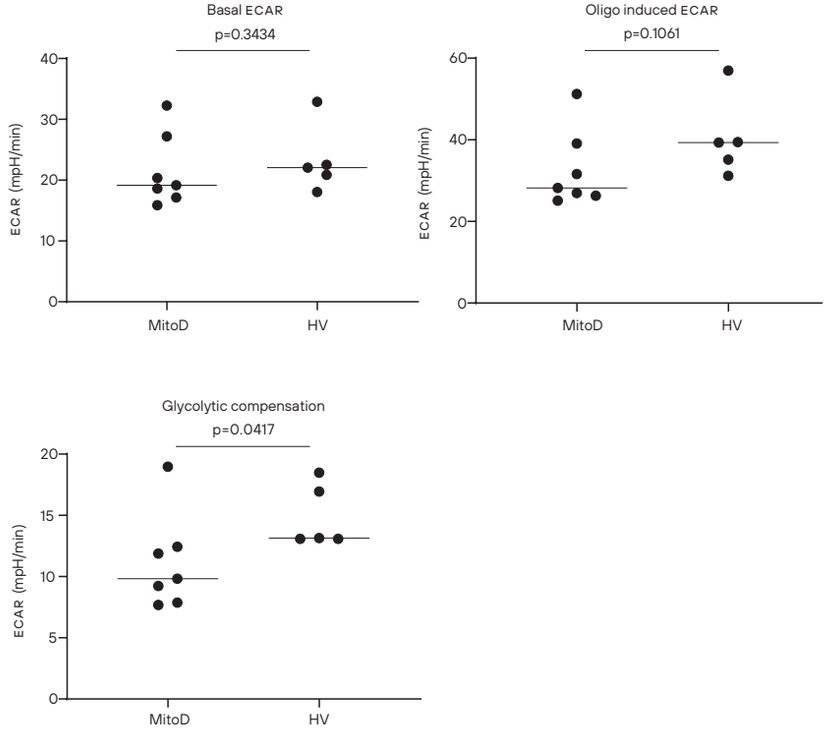
Measure of Mitochondrial Function	HV (median, 95% CI)	MitoD (median, 95% CI)	p-value HV vs MitoD
MitoSOX monocytes	1.01 (0.83 / 1.19)	0.85 (0.78 / 1.10)	0.4634
MitoTracker monocytes	6.1 (4.1 / 14.3)	15.4 (4.7 / 32.4)	0.1206
MitoSOX/MitoTracker Ratio (monocytes)	0.152 (0.095 / 0.241)	0.073 (0.026 / 0.156)	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

HV=healthy volunteers; JC-1=tetraethylbenzimidazolylcarbocyanine iodide; MitoD=mitochondrial disease patients; N=number of subjects; MMP=mitochondrial membrane potential; MTROS=mitochondrial reactive oxygen species; NA=not applicable; min=minimum; max=maximum; CI=confidence interval; p-value based on Wilcoxon-test with adjustment. P-values <0.05 bolded.

MITOCHONDRIAL BIOENERGETIC PROFILES

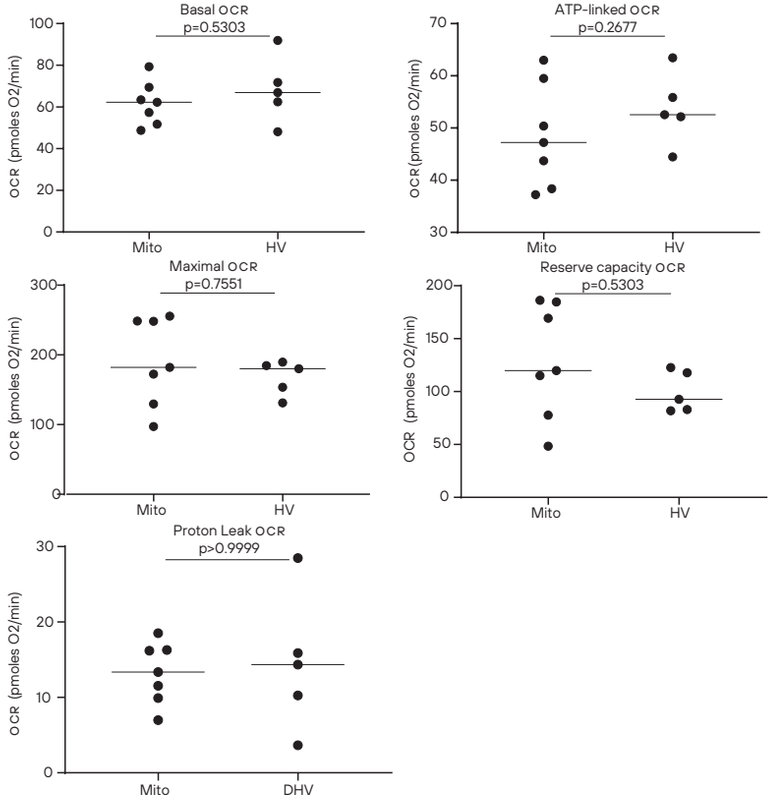
One participant with mitochondrial disease and 1 HV were excluded from Seahorse analysis due to insufficient cells, 1 HV due to insufficient quality of cells and 1 participant with mitochondrial disease due to a positive tetrahydrocannabinol drug screening. There were no statistically significant differences 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) (Figure 1 and 2).

FIGURE 1 Extracellular acidification rate and glycolytic compensation, individual datapoints with medians.



ECAR=extracellular acidification rate; HVs=healthy volunteers; MitoD=mitochondrial disease.

FIGURE 2 Oxygen consumption rate, individual datapoints with medians.



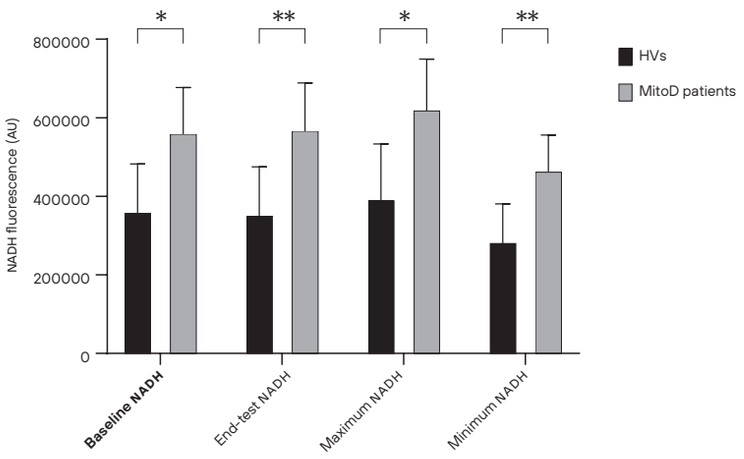
ATP=adenosine triphosphate; HVS=healthy volunteers; MitoD=mitochondrial disease; OCR=oxygen consumption rate.

IMAGING ASSESSMENTS

FLOW-MEDIATED SKIN FLUORESCENCE

Assessment of skin NADH content showed a baseline NADH (LSM difference -201771, 95% CI: -352349, -51193), end-test NADH (LSM difference -214942, 95% CI: -367462, -62423), maximum NADH (LSM difference -229504, 95% CI: -400052, 59555) and minimum (LSM difference -180800, 95% CI: -298856, -62743) 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 flow-mediated skin fluorescence parameters.

FIGURE 3 NADH fluorescence during FMSF, means with 95% CI.



** indicates significance p<0.05, ** indicates significance p<0.01. AU=arbitrary units; FMSF=flow-mediated skin fluorescence; HVS=healthy volunteers; MitoD=mitochondrial disease; NADH=nicotinamide adenine dinucleotide hydrogen.*

NEAR-INFRARED SPECTROSCOPY

Assessment of skin and muscle oxy- and deoxygenated haemoglobin did not show statistically significant differences between HVS and participants with mitochondrial disease in muscle oxygen consumption, muscle blood flow, hyperaemic response speed or hyperaemic response duration as assessed with near-infrared spectroscopy.

LASER SPECKLE CONTRAST IMAGING

The results from laser speckle contrast imaging measurements are summarised 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 hyperaemia challenge, although all observed dermal blood flows were lower in participants with mitochondrial disease, specifically local thermal hyperaemia-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 hyperaemia challenges were found.

TABLE 3 Comparison between HV and MitoD participants for LSCI parameters.

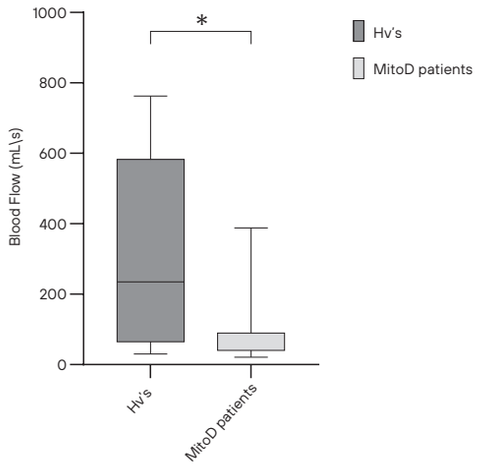
				95% CI		
Parameter [Unit]	**P-value**	**HV (LSM)**	**MitoD (LSM)**	**LSM difference**	**Lower**	**Upper**
LSCI LTH Basal Flow [AU]	0.4605	102.394	94.877	7.517	-13.634	28.668
LSCI LTH Maximal Flow [AU]	0.2165	177.339	156.152	21.187	-13.809	56.182
LSCI LTH Maximal Flow CFB [AU]	0.2626	74.945	61.276	13.669	-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

AU=arbitrary units; CI=confidence interval; HV=healthy volunteers; LSM=least squares mean(s); LSCI=laser speckle contrast imaging; LTH=local thermal hyperaemia; MitoD=mitochondrial disease patients; PORH=post occlusive reactive hypereamia. P-values <0.05 bolded.

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.

FIGURE 4 Femoral artery blood flow CFB after PLM, means with 95% CI.



** indicates significance p<0.05. CFB=change from baseline; HVS=healthy volunteers; MitoD=mitochondrial disease; PLM=passive leg movement.*

SIDESTREAM DARK FIELD MICROSCOPY

De Backer density, a measure of vessel density, was significantly higher HVS when compared to participants with mitochondrial disease (LSM difference 0.94, 95% CI: 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 body mass index. 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 reactive oxygen species 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 reactive oxygen species 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 heterogenous, and many studies are conducted in cells other than PBMCS. Mitochondrial function has also previously been evaluated in specific, but heterogenous 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 lower in lymphocytes of affected participants compared to controls.[24] For patients with the specific m.3243A>G mutation, higher mitochondrial reactive oxygen species production, lower ATP production, and lower mitochondrial membrane potential in PBMCS was shown in 2 previous studies,[25–26] and higher mitochondrial reactive oxygen species 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 myoblasts[29] 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 reactive oxygen species 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 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 flow-mediated skin fluorescence 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 flow-mediated skin fluorescence 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 muscle,[39] 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 former[41] causing reduced nitric oxide bioavailability. This is supported by a trend towards lower local thermal hyperaemia-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 healthy volunteers, while several imaging methods testing skin metabolic status

(flow mediated skin fluorescence) or general vascular function (passive leg movement, laser speckle contrast imaging, sidestream darkfield 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 healthy volunteers. Moreover, small differences on proximal, cell-level endpoints might coalesce into detectable effects tissue-level endpoints 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 endpoints 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 lipopolysaccharide[47] or oral administration of statins[48] 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

REFERENCES

1 Gorman, G. S.; Schaefer, A. M.; Ng, Y.; Gomez, N.; Blakely, E. L.; Alston, C. L.; Feeney, C.; Horvath, R.; Yu-Wai-Man, P.; Chinnery, P. F.; Taylor, R. W.; Turnbull, D. M.; McFarland, R., Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. *Annals of neurology* **2015,** *77* (5), 753-9.

2 de Laat, P.; Koene, S.; van den Heuvel, L. P. W. J.; Rodenburg, R. J. T.; Janssen, M. C. H.; Smeitink, J. A. M., Clinical features and heteroplasmy in blood, urine and saliva in 34 Dutch families carrying the m.3243A > G mutation. *Journal of Inherited Metabolic Disease* **2012,** *35* (6), 1059-1069.

3 Peoples, J. N.; Saraf, A.; Ghazal, N.; Pham, T. T.; Kwong, J. Q., Mitochondrial dysfunction and oxidative stress in heart disease. *Experimental & Molecular Medicine* **2019,** *51* (12), 1-13.

4 Davidson, S. M.; Duchen, M. R., Endothelial Mitochondria. *Circulation Research* **2007,** *100* (8), 1128-1141.

5 Pesta, D.; Gnaiger, E., High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle. In *Mitochondrial Bioenergetics: Methods and Protocols*, Palmeira, C. M.; Moreno, A. J., Eds. Humana Press: Totowa, NJ, 2012; pp 25-58.

6 Parikh, S.; Goldstein, A.; Koenig, M. K.; Scaglia, F.; Enns, G. M.; Saneto, R.; Anselm, I.; Cohen, B. H.; Falk, M. J.; Greene, C.; Gropman, A. L.; Haas, R.; Hirano, M.; Morgan, P.; Sims, K.; Tarnopolsky, M.; Van Hove, J. L.; Wolfe, L.; DiMauro, S., Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genetics in medicine : official journal of the American College of Medical Genetics* **2015,** *17* (9), 689-701.

7 Mehrazin, M.; Shanske, S.; Kaufmann, P.; Wei, Y.; Coku, J.; Engelstad, K.; Naini, A.; De Vivo, D. C.; DiMauro, S., Longitudinal changes of mtDNA A3243G mutation load and level of functioning in MELAS. *American Journal of Medical Genetics Part A* **2009,** *149A* (4), 584-587.

8 Willingham, T. B.; McCully, K. K., In Vivo Assessment of Mitochondrial Dysfunction in Clinical Populations Using Near-Infrared Spectroscopy. *Frontiers in Physiology* **2017,** *8*, 689.

9 Sena, L. A.; Chandel, N. S., Physiological roles of mitochondrial reactive oxygen species. *Molecular cell* **2012,** *48* (2), 158-67.

10 Gorman, G. S.; Chinnery, P. F.; DiMauro, S.; Hirano, M.; Koga, Y.; McFarland, R.; Suomalainen, A.; Thorburn, D. R.; Zeviani, M.; Turnbull, D. M., Mitochondrial diseases. *Nature reviews. Disease primers* **2016,** *2*, 16080.

11 Hubens, W. H. G.; Vallbona-Garcia, A.; de Coo, I. F. M.; van Tienen, F. H. J.; Webers, C. A. B.; Smeets, H. J. M.; Gorgels, T. G. M. F., Blood biomarkers for assessment of mitochondrial dysfunction: An expert review. *Mitochondrion* **2022,** *62*, 187-204.

12 Gambardella, J.; Khondkar, W.; Morelli, M. B.; Wang, X.; Santulli, G.; Trimarco, V., Arginine and Endothelial Function. *Biomedicines* **2020,** *8* (8), 277.

13 El-Hattab, A. W.; Hsu, J. W.; Emrick, L. T.; Wong, L.-J. C.; Craigen, W. J.; Jahoor, F.; Scaglia, F., Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. *Molecular genetics and metabolism* **2012,** *105* (4), 607-614.

14 Kerr, D. S., Review of clinical trials for mitochondrial disorders: 1997-2012. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* **2013,** *10* (2), 307-19.

15 Dennis, J. J.; Wiggins, C. C.; Smith, J. R.; Isautier, J. M. J.; Johnson, B. D.; Joyner, M. J.; Cross, T. J., Measurement of muscle blood flow and O2 uptake via near-infrared spectroscopy using a n ovel occlusion protocol. *Scientific Reports* **2021,** *11* (1), 918.

16 Vuilleumier, P.; Decosterd, D.; Maillard, M.; Burnier, M.; Hayoz, D., Postischemic forearm skin reactive hyperemia is related to cardovascular risk factors in a healthy female population. *Journal of hypertension* **2002,** *20* (9), 1753-7.

17 Cracowski, J. L.; Roustit, M., Local Thermal Hyperemia as a Tool to Investigate Human Skin Microcirculation. *Microcirculation* **2010,** *17* (2), 79-80.

18 Groot, H. J.; Trinity, J. D.; Layec, G.; Rossman, M. J.; Ives, S. J.; Morgan, D. E.; Bledsoe, A.; Richardson, R. S., The role of nitric oxide in passive leg movement-induced vasodilatation with age: insight from alterations in femoral perfusion pressure. *J Physiol* **2015,** *593* (17), 3917-28.

19 Treu, C. M.; Lupi, O.; Bottino, D. A.; Bouskela, E., Sidestream dark field imaging: the evolution of real-time visualization of cutaneous microcirculation and its potential application in dermatology. *Archives of dermatological research* **2011,** *303* (2), 69-78.

20 Zegelbone, P. M.; BAYBAYON-GRANDGEORGE, A.; Stauffer, B.; Sucharov, C.; Miyamoto, S.; Garcia, A. M., Abstract 8963: Mitochondrial Dysfunction in Peripheral Blood Mononuclear Cells is Associated With Heart Failure in Patients With Single Ventricle Congenital Heart Disease. *Circulation* **2021,** *144* (Suppl_1), A8963-A8963.

21 Altintas, M. M.; DiBartolo, S.; Tadros, L.; Samelko, B.; Wasse, H., Metabolic Changes in Peripheral Blood Mononuclear Cells Isolated From Patients With End Stage Renal Disease. *Front Endocrinol (Lausanne)* **2021,** *12*, 629239.

22 Ederlé, C.; Charles, A.-L.; Khayath, N.; Poirot, A.; Meyer, A.; Clere-Jehl, R.; Andres, E.; De Blay, F.; Geny, B., Mitochondrial Function in Peripheral Blood Mononuclear Cells (PBMC) Is Enhanced, Together with Increased Reactive Oxygen Species, in Severe Asthmatic Patients in Exacerbation. *Journal of Clinical Medicine* **2019,** *8* (10), 1613.

23 Lee, H.-T.; Lin, C.-S.; Pan, S.-C.; Wu, T.-H.; Lee, C.-S.; Chang, D.-M.; Tsai, C.-Y.; Wei, Y.-H., Alterations of oxygen consumption and extracellular acidification rates by glutamine in PBMCs of SLE patients. *Mitochondrion* **2019,** *44*, 65-74.

24 Pecina, P.; Houšťková, H.; Mráček, T.; Pecinová, A.; Nůsková, H.; Tesařová, M.; Hansíková, H.; Janota, J.; Zeman, J.; Houštěk, J., Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry. *BBA Clinical* **2014,** *2*, 62-71.

25 Geng, X.; Zhang, Y.; Yan, J.; Chu, C.; Gao, F.; Jiang, Z.; Zhang, X.; Chen, Y.; Wei, X.; Feng, Y.; Lu, H.; Wang, C.; Zeng, F.; Jia, W., Mitochondrial DNA mutation m.3243A>G is associated with altered mitochondrial function in peripheral blood mononuclear cells, with heteroplasmy levels and with clinical phenotypes. *Diabetic Medicine* **2019,** *36* (6), 776-783.

26 Jiang, Z.; Zhang, Y.; Yan, J.; Li, F.; Geng, X.; Lu, H.; Wei, X.; Feng, Y.; Wang, C.; Jia, W., De Novo Mutation of m.3243A>G together with m.16093T>C Associated with Atypical Clinical Features in a Pedigree with MIDD Syndrome. *Journal of Diabetes Research* **2019,** *2019*, 5184647.

27 Shepherd, R. K.; Checcarelli, N.; Naini, A.; De Vivo, D. C.; DiMauro, S.; Sue, C. M., Measurement of ATP production in mitochondrial disorders. *J Inherit Metab Dis* **2006,** *29* (1), 86-91.

28 Pek, N. M. Q.; Phua, Q. H.; Ho, B. X.; Pang, J. K. S.; Hor, J.-H.; An, O.; Yang, H. H.; Yu, Y.; Fan, Y.; Ng, S.-Y.; Soh, B.-S., Mitochondrial 3243A>G mutation confers pro-atherogenic and pro-inflammatory properties in MELAS iPS derived endothelial cells. *Cell Death & Disease* **2019,** *10* (11), 802.

29 Motlagh Scholle, L.; Schieffers, H.; Al-Robaiy, S.; Thaele, A.; Dehghani, F.; Lehmann Urban, D.; Zierz, S., The Effect of Resveratrol on Mitochondrial Function in Myoblasts of Patients with the Common m.3243A>G Mutation. *Biomolecules* **2020,** *10* (8), 1103.

30 Klein Gunnewiek, T. M.; Van Hugte, E. J. H.; Frega, M.; Guardia, G. S.; Foreman, K.; Panneman, D.; Mossink, B.; Linda, K.; Keller, J. M.; Schubert, D.; Cassiman, D.; Rodenburg, R.; Vidal Folch, N.; Oglesbee, D.; Perales-Clemente, E.; Nelson, T. J.; Morava, E.; Nadif Kasri, N.; Kozicz, T., m.3243A > G-Induced Mitochondrial Dysfunction Impairs Human Neuronal Development and Reduces Neuronal Network Activity and Synchronicity. *Cell Reports* **2020,** *31* (3), 107538.

31 Mancuso, M.; Orsucci, D.; Angelini, C.; Bertini, E.; Carelli, V.; Comi, G. P.; Donati, A.; Minetti, C.; Moggio, M.; Mongini, T.; Servidei, S.; Tonin, P.; Toscano, A.; Uziel, G.; Bruno, C.; Ienco, E. C.; Filosto, M.; Lamperti, C.; Catteruccia, M.; Moroni, I.; Musumeci, O.; Pegoraro, E.; Ronchi, D.; Santorelli, F. M.; Sauchelli, D.; Scarpelli, M.; Sciacco, M.; Valentino, M. L.; Vercelli, L.; Zeviani, M.; Siciliano, G., The m.3243A>G mitochondrial DNA mutation and related phenotypes. A matter of gender? *Journal of neurology* **2014,** *261* (3), 504-10.

32 Ives, S. J.; Layec, G.; Hart, C. R.; Trinity, J. D.; Gifford, J. R.; Garten, R. S.; Witman, M. A. H.; Sorensen, J. R.; Richardson, R. S., Passive leg movement in chronic obstructive pulmonary disease: evidence of locomotor muscle vascular dysfunction. *J Appl Physiol (1985)* **2020,** *128* (5), 1402-1411.

33 Katulka, E. K.; Hirt, A. E.; Kirkman, D. L.; Edwards, D. G.; Witman, M. A. H., Altered vascular function in chronic kidney disease: evidence from passive leg movement. *Physiol Rep* **2019,** *7* (8), e14075.

34 Souza, E. G.; De Lorenzo, A.; Huguenin, G.; Oliveira, G. M.; Tibirica, E., Impairment of systemic microvascular endothelial and smooth muscle function in individuals with early-onset coronary artery disease: studies with laser speckle contrast imaging. *Coron Artery Dis* **2014,** *25* (1), 23-8.

35 Fujii, T.; Nozaki, F.; Saito, K.; Hayashi, A.; Nishigaki, Y.; Murayama, K.; Tanaka, M.; Koga, Y.; Hiejima, I.; Kumada, T., Efficacy of pyruvate therapy in patients with mitochondrial disease: a semi-quantitative clinical evaluation study. *Molecular genetics and metabolism* **2014,** *112* (2), 133-8.

36 Sharma, R.; Reinstadler, B.; Engelstad, K.; Skinner, O. S.; Stackowitz, E.; Haller, R. G.; Clish, C. B.; Pierce, K.; Walker, M. A.; Fryer, R.; Oglesbee, D.; Mao, X.; Shungu, D. C.; Khatri, A.; Hirano, M.; De Vivo, D. C.; Mootha, V. K., Circulating markers of NADH-reductive stress correlate with mitochondrial disease severity. *J Clin Invest* **2021,** *131* (2), e136055.

37 Lee, C. F.; Caudal, A.; Abell, L.; Nagana Gowda, G. A.; Tian, R., Targeting NAD+ Metabolism as Interventions for Mitochondrial Disease. *Scientific Reports* **2019,** *9* (1), 3073.

38 Khan, N. A.; Auranen, M.; Paetau, I.; Pirinen, E.; Euro, L.; Forsström, S.; Pasila, L.; Velagapudi, V.; Carroll, C. J.; Auwerx, J.; Suomalainen, A., Effective treatment of mitochondrial myopathy by nicotinamide riboside, a vitamin B3. *EMBO molecular medicine* **2014,** *6* (6), 721-31.

39 Chance, B.; Bank, W., Genetic disease of mitochondrial function evaluated by NMR and NIR spectroscopy of skeletal tissue. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1995,** *1271* (1), 7-14.

40 Ryan, T. E.; Erickson, M. L.; Brizendine, J. T.; Young, H.-J.; McCully, K. K., Noninvasive evaluation of skeletal muscle mitochondrial capacity with near-infrared spectroscopy: correcting for blood volume changes. *Journal of Applied Physiology* **2012,** *113* (2), 175-183.

41 Hayashi, G.; Cortopassi, G., Oxidative stress in inherited mitochondrial diseases. *Free Radical Biology and Medicine* **2015,** *88*, 10-17.
42 Taivassalo, T.; Ayyad, K.; Haller, R. G., Increased capillaries in mitochondrial myopathy: implications for the regulation of oxygen delivery. *Brain : a journal of neurology* **2012,** *135* (Pt 1), 53-61.
43 Yu, B.-B.; Zhi, H.; Zhang, X.-Y.; Liang, J.-W.; He, J.; Su, C.; Xia, W.-H.; Zhang, G.-X.; Tao, J., Mitochondrial dysfunction-mediated decline in angiogenic capacity of endothelial progenitor cells is associated with capillary rarefaction in patients with hypertension via downregulation of CXCR4/JAK2/SIRT5 signaling. *eBioMedicine* **2019,** *42*, 64-75.
44 Grady, J. P.; Pickett, S. J.; Ng, Y. S.; Alston, C. L.; Blakely, E. L.; Hardy, S. A.; Feeney, C. L.; Bright, A. A.; Schaefer, A. M.; Gorman, G. S.; McNally, R. J.; Taylor, R. W.; Turnbull, D. M.; McFarland, R., mtDNA heteroplasmy level and copy number indicate disease burden in m.3243A>G mitochondrial disease. *EMBO molecular medicine* **2018,** *10* (6), e8262.
45 Zeng, Y.; David, J.; Rémond, D.; Dardevet, D.; Savary-Auzeloux, I.; Polakof, S., Peripheral Blood Mononuclear Cell Metabolism Acutely Adapted to Postprandial Transition and Mainly Reflected Metabolic Adipose Tissue Adaptations to a High-Fat Diet in Minipigs. *Nutrients* **2018,** *10* (11), 1816.
46 Walker, M. A.; Lareau, C. A.; Ludwig, L. S.; Karaa, A.; Sankaran, V. G.; Regev, A.; Mootha, V. K., Purifying Selection against Pathogenic Mitochondrial DNA in Human T Cells. *The New England journal of medicine* **2020,** *383* (16), 1556-1563.
47 van Poelgeest, E. P.; Dillingh, M. R.; de Kam, M.; Malone, K. E.; Kemper, M.; Stroes, E. S. G.; Burggraaf, J.; Moerland, M., Characterization of immune cell, endothelial, and renal responses upon experimental human endotoxemia. *J Pharmacol Toxicol Methods* **2018,** *89*, 39-46.
48 van Diemen, M. P. J.; Berends, C. L.; Akram, N.; Wezel, J.; Teeuwisse, W. M.; Mik, B. G.; Kan, H. E.; Webb, A.; Beenakker, J. W. M.; Groeneveld, G. J., Validation of a pharmacological model for mitochondrial dysfunction in healthy subjects using simvastatin: A randomized placebo-controlled proof-of-pharmacology study. *Eur J Pharmacol* **2017,** *815*, 290-297.
49 Rizzo, M. R.; Barbieri, M.; Marfella, R.; Paolisso, G., Reduction of oxidative stress and inflammation by blunting daily acute glucose fluctuations in patients with type 2 diabetes: role of dipeptidyl peptidase-IV inhibition. *Diabetes care* **2012,** *35* (10), 2076-82.
50 Yang, J.; Guo, Q.; Feng, X.; Liu, Y.; Zhou, Y., Mitochondrial Dysfunction in Cardiovascular Diseases: Potential Targets for Treatment. *Frontiers in cell and developmental biology* **2022,** *10*, 841523.

CHAPTER IV

A PHASE 1 RANDOMIZED, OPEN-LABEL CLINICAL TRIAL TO EVALUATE THE EFFECT OF A FAR-INFRARED EMITTING PATCH ON LOCAL SKIN PERFUSION, MICROCIRCULATION, AND OXYGENATION

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