Bedside Monitoring of Mitochondrial Function in Post-COVID Patients

A Feasibility Study

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BEDSIDE MONITORING OF MITOCHONDRIAL FUNCTION IN POST-COVID PATIENTS

-A Feasibility Study-

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Abstract

Background: Post-COVID (PC) is characterized by prolonged symptoms following a COVID-19 infection, with Post-Exertional Malaise (PEM) being a feature with a large negative impact on quality of life. Mitochondrial dysfunction has previously been related to PEM-related symtpoms. This study non-invasively investigates the in-vivo mitochondrial oxygenation (mitoPO$_2$) and mitochondrial oxygen consumption (mitoVO$_2$) in PC patients experiencing PEM compared to convalescent controls using the COMET device.

Methods: We conducted a comparative, non-randomized, observational study with 24 PC patients and 20 convalescent controls. MitoPO$_2$ and mitoVO$_2$ were measured at baseline, after short exercise, and after 10 minutes of recovery. Linear mixed models assessed the effects of exercise and recovery on mitoPO$_2$ and mitoVO$_2$. PROMs related to tiredness, PEM, and function were evaluated for explainability of mitoPO$_2$ heterogeneity in PC participants using Spearman correlations.

Results: No significant differences were found between the PC and control groups in mitoPO$_2$ and mitoVO$_2$ ($p>0.05$). Exercise led to a significant decrease in mitoPO$_2$ ($p < 0.001$), which recovered after 10 minutes. No significant differences were observed between groups regarding the effects of exercise and recovery ($p>0.05$). A large variance in mitoPO$_2$ was noted in the PC group, suggesting heterogeneity within this population. Additionally, mitoVO$_2$ increased significantly after recovery in both groups, with no significant group differences.

Conclusion: The study implies that while mitoPO$_2$ decreases significantly after exercise and mitoVO$_2$ increases significantly after recovery, both do not differ significantly between PC patients and controls. However, the observed variance in mitoPO$_2$ after exercise and recovery among PC patients suggests varying disease mechanisms related to oxygen supply. Future research should focus on longitudinal assessments, home-based measurements, and exploring alternative methods to assess mitochondrial and microcircular function to better understand these findings and address the limitations of the current study.

Keywords: Post-COVID, mitoPO$_2$, mitoVO$_2$, COMET, mitochondrial function, Post-Exertional Malaise

Contents

1

Introduction

During the COVID-19 pandemic, more than 650 million people were infected with the SARS-CoV-2 virus [1]. The infection had varying acute effects on individuals, ranging from 20-40.5% of patients remaining asymptomatic to people developing severe pneumonia, acute respiratory distress syndrome (ARDS), and even sepsis, necessitating urgent care [2, 3, 4]. Fortunately, widespread vaccination in combination with the mutation of the SARS-CoV-2 virus has reduced the acute effects. However, the subacute and chronic period after the onset of the first symptoms has posed a new challenge, as part of COVID-19 patients remain symptomatic for 4–12 weeks or even longer [5, 6].

This lingering effect of the COVID-19 infection has been named post-COVID (PC), long-COVID or Post-Acute Sequelae of SARS-CoV-2 (PASC). The World Health Organisation (WHO) consensus diagnosis of PC is "Post COVID-19 condition occurs in individuals with a history of probable or confirmed SARS CoV-2 infection, usually 3 months from the onset of COVID-19 with symptoms that last for at least 2 months and cannot be explained by an alternative diagnosis. Symptoms occur after initial recovery from an acute COVID-19 episode or persist from the initial illness. Symptoms can also fluctuate or relapse over time" [7]. The incidence of PC after COVID-19 infection is estimated at 10-30% to 50-70% in non-hospitalized and hospitalized patients, respectively [8, 9]. Over 200 symptoms have been described in the subacute and chronic phase of PC [10]. These symptoms range from respiratory (dyspnea, cough) to neurologic (dysautonomia, dizziness) and can persist for months to years after the initial infection [1, 6, 11]. The number of existing symptoms and their impact on quality of life evolve during the course of PC, i.e. cardiovascular symptoms mainly have an impact in the first two years of PC, whereas neurological, gastrointestinal and pulmonary problems are more likely to remain even after three years [11].

In the Netherlands, it is estimated that over 100.000 people are experiencing the effects of PC, limiting them in their daily activities and working life [12]. Consequently, The average cost of sick leave in the Netherlands is 3.284 euros per PC patient per month [13]. On top of this, annually, PC patients spend an average of 1.125 euros on healthcare [14]. Despite the large individual and societal impact of PC, the exact disease mechanism remains unclear. Multiple theories have been suggested and are being researched, such as immune dysregulation, autoimmunity, microclotting or dysfunctional neurological signalling [10]. However, research remains inconclusive. This is the main reason why the diagnosis of PC is based on the exclusion of other possible illnesses, which may lead to late acknowledgement of symptoms by physicians, patients, and patient's relatives. This late acknowledgement can harm the mental status of PC patients [15]. Furthermore, challenges arise in obtaining adequate compensation for the inability to work, since government institutions do not always acknowledge (the severity of) the impact of PC [16]. Therefore, a tool to objectively diagnose PC would positively affect both individuals and society. Several tools are currently in the trial phase, all focussing on parts of the possible mechanisms between PC, such as microclot detection and corneal microscopy for small fibre neuropathy [17, 18].

A group of PC patients that is significantly impaired in daily life is the group that experiences post-exertional malaise (PEM). PEM is defined as the worsening or appearance of new symptoms in the 12-to-72-hour period following physical or mental activity [19, 20]. This patient population has not only experienced the struggle of late diagnosis, but they also experienced the negative effects of faulty recovery strategies. Initially, most

of these patients were advised to improve their fitness and physical condition through exercise training. Unfortunately, studies have showed that symptoms in 75% of people with PEM worsened following exercise, indicating the opposite outcome [21]. That is why there is an urgent need for a tool that can help with both diagnosis and recovery strategy.

Changes in cardiopulmonary function could seem like a logical explanation in patients experiencing PEM. However, various research has shown no significant change in cardiopulmonary function, despite the presence of decreased exercise capacity [22, 23]. On the contrary, differences in peak oxygen consumption have been found [24]. Unfortunately, it remains difficult to adjust these results for reduced fitness due to prolonged periods of bedrest [24]. Research in myalgic encephalomyelitis/chronic fatigue syndrome patients who experience PEM has suggested that two-day cardiopulmonary exercise testing (CPET) may be a solution [25]. Using this methodology, patients undergo CPET testing in two subsequent days. Outcomes between the two days of testing are compared to objectify recovery capacity [26]. A significantly decreased ventilatory threshold workload on the second day has been found in patients experiencing PEM, whereas healthy controls showed no difference. The ventilatory threshold is defined as the point where ventilation increases faster than oxygen consumption, which suggests a transition from aerobic to anaerobic respiration [25]. An earlier shift towards anaerobic respiration fits studies performed in PC patients with PEM, where lower fatty acid oxidation was identified [27].

A recent study by Appelman et al. further elucidated the mechanisms behind the changes in oxidative respiration in PEM [28]. In their research, they observed that the mitochondrial function of muscle cells was reduced in PC patients compared to healthy individuals, using ex-vivo respirometry. Furthermore, the function was significantly lowered 24 hours after an incremental exercise test. This suggests that PEM can be explained by impaired recovery of mitochondrial function. These observations fit the hypotheses that the SARS-CoV-2 virus multiplies inside mitochondria, possibly damaging the repair function in the process [29, 30].

In current studies, mitochondrial function is mainly assessed using indirect biomarkers or ex-vivo cellular testing following invasive biopsies [31, 32, 33, 34]. These testing methods do not enable real-time, in-vivo measurement of the mitochondrial oxygenation. Thus, it is not possible to obtain a direct insight into the patient's physiology at the patient's bedside. Furthermore, invasive blood sampling or muscle biopsies are required to perform the tests. Fortunately, a solution to these challenges might have been found in the development of the COMET (monitor for Cellular Oxygen METabolism). Assessment of the mitochondrial oxygen pressure (mitoPO_2) and consumption (mitoVO_2) using the COMET enables non-invasive and continuous monitoring of mitochondrial function. Furthermore, the adequacy of oxygen supply towards the mitochondria can be assessed [35]. Long-term inadequate mitochondrial oxygen supply could explain the changed mitochondrial function in PC [36].

In summary, the key to objectifying long-term PC diagnosis and providing recovery strategies could be found in the assessment of mitochondrial oxygenation. The COMET enables non-invasive, in-vivo and continuous measurement of the oxygen pressure and consumption inside the mitochondria. In this thesis, the COMET will be used to non-invasively examine changes in mitochondrial oxygenation in PC patients experiencing PEM compared to convalescent controls. It is hypothesized that mitochondrial oxygenation (mitoPO_2 and mitoVO_2) is impaired in PC patients compared to healthy controls. This difference could be even more evident after physical effort, explaining the increased symptom burden after exercise. Furthermore, differences within the PC group will be analysed to identify possible recovery strategies and improve exercise recommendations. By understanding these variations, more tailored and effective interventions can be developed to enhance the quality of life for PC patients.

2

Methods

2.1. Study design

This study was a comparative, non-randomized, observational study performed at the Erasmus Medical Center, Rotterdam. The study is registered under trial number NL 85146.078.23. Measurements and questionnaire data corresponded to the second study visit for all participants.

2.2. Study objectives and hypotheses

The study objectives were the following:

1. To determine the differences in mitochondrial oxygen pressure (mitoPO_2) and consumption (mitoVO_2), measured by the COMET, between participants with PC experiencing PEM and convalescent controls.
    a Determine the impact of exercise and recovery on these values.
2. To explain the mitoPO_2 differences within the PC group be explained by exercise intensity or the Patient Related Outcome Measures (PROMs) regarding level of PEM, general tiredness or level of functioning.

It was hypothesised that mitoPO_2 and mitoVO_2 values were lower in the PC group, especially after exercise and recovery. This hypothesis was based on the study by Appelman et al [28]. A positive correlation between mitochondrial parameters and exercise capability and level of functioning was hypothesized. A negative correlation between mitochondrial parameters and level of exhaustion and level of PEM were hypothesized.

2.3. Participants

2.3.1. Recruitment

All participants were included more than 6 months before our measurements for the first study visit of study NL 85146.078.23. Doctor-diagnosed PC patients who met the in- and exclusion criteria were included. Healthy relatives of the PC patients, who recovered from COVID-19 were included as convalescent controls.

2.3.2. In- and exclusion criteria

Post-COVID patients

Inclusion criteria

- Age > 18 years, < 65 years
- Past COVID-19 diagnosis, based on
    - Positive PCR
    - Positive Sars-CoV2 serology
    - Positive rapid antigen test
    - Typical clinical syndrome during first pandemic wave, when testing was not available.
- PC diagnosis based on World Health Organisation consensus diagnosis [7]
- Overal functioning <70% compared to functioning prior to onset of PC/ COVID-19 infection

- PC duration > 6 months
- Presence of post-exertional malaise (sf-DSQ-PEM) [37]
- Provided written informed consent

Exclusion criteria

- Unable or not willing to provide written informed consent
- Unable to complete written questionnaires in Dutch
- Unable to draw blood for study purposes
- Diagnosis of dementia
- Active treatment with hyperbaric oxygen treatment during study start
- Alternative diagnosis that may explain clinical symptoms
- Suffering from any pre-existing immune-driven disease or use of anti-inflammatory therapy of any kind (including NSAIDs and steroids) during the last 3 months
- Suffering from diabetes mellitus, hypertension, severe mental conditions or use of anticoagulant treatment in the past 4 weeks.
- Re-infection with COVID-19 in the past 3 months

Convalescent controls
Inclusion criteria

- Age > 18 years, <65 years
- Past COVID-19 diagnosis, based on
  - Positive PCR
  - Positive Sars-Cov2 serology
  - Positive rapid antigen test
  - Typical clinical syndrome during the first pandemic wave, when testing was not available
- No diagnosis of PC, good recovery. Overall functioning >95% compared to functioning prior COVID-19 infection
- Self-reported general good wellbeing
- Provided written informed consent

Exclusion criteria

- Unable or not willing to provide written informed consent
- Unable to complete written questionnaires in Dutch
- Unable to draw blood for study purposes
- Diagnosis of dementia
- Genetically related to participating patients (e.g. brother/sister/parent)
- Suffering from any immune-driven disease or use of anti-inflammatory therapy of any kind (including NSAIDs and steroids), including during the last 3 months
- Suffering from diabetes mellitus, hypertension, severe mental conditions or use of anticoagulant treatment in the past 4 weeks.
- Re-infection with SARS-CoV-2 in the past 3 months.

2.4. Measurement protocol

Measurements using the COMET were performed in an outpatient clinic setting. The measurements were performed at three point in time during the visit: Baseline, post-exercise and post-recovery. All COMET measurements were performed at a frequency of 1 Hz. After arrival, participants were asked to lie down on an exam bank. A baseline mitoPO$_2$ was measured until at least 60 subsequent stable mitoPO$_2$ were measured. Subsequently, mitoVO$_2$ measurements were performed in threefold to obtain a baseline mitoVO$_2$. Next, participants were asked to sit down on a chair and asked to perform as many sit-to-stands as they could in one minute [38]. Immediately afterwards, participants were asked to lie down again. MitoPO$_2$ measurements were again performed, followed by three mitoVO$_2$ measurements, these parameters were named mitoPO$_2$ and mitoVO$_2$ exercise, respectively. Finally, 10 minutes after the first successful mitoVO$_2$ measurement, a mitoPO$_2$ and three mitoVO$_2$ were performed. These were named mitoPO$_2$ and mitoVO$_2$ recovery. Times of exercise and recovery measurements were written down for data-extraction purposes (see section 2.7).

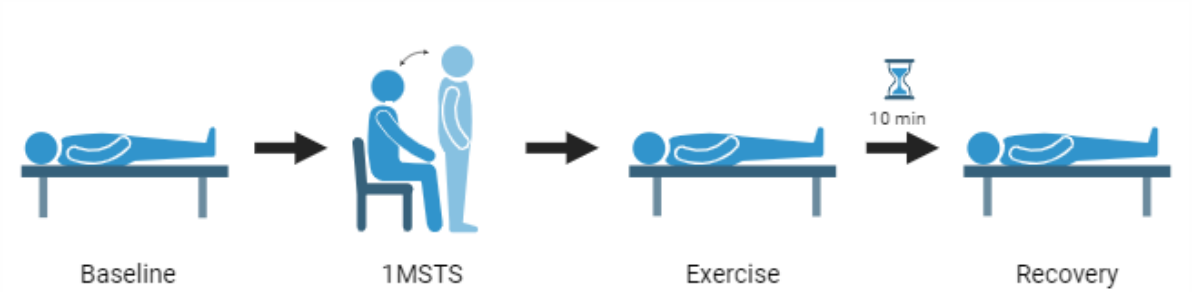

Figure 2.1: Measurement protocol. Measurements were performed at time points Baseline, Exercise and Recovery. 1MSTS: 1-minute sit-to-stand test.

2.5. Questionnaires

Participants were asked to complete a series of online questionnaires to gather information about their medical history, current health status, and other patient-related Outcome Measures (PROMs). The PROMs included perceived level of functioning (percentage compared to pre-infection), duration of PEM symptoms after infection (<1h, 2-3h, 4-10h, 11-13h, 14-23h, >24h) and the Fatigue Assessment Scale (FAS) [39]. The FAS consists of 10 items on a 5-scale Likert scale ranging from 'Never' to 'Always'. Items assess both the physical and mental component of fatigue. The final outcome of the FAS-score is based on the sum of all items. Scores are interpreted as follows:

- 10-21: No or mild fatigue
- 21-34: Moderate fatigue
- 34-50: Severe fatigue

2.6. COMET

The COMET uses a protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT) to measure mitochondrial oxygen tension (mitoPO2) [40]. PpIX is a precursor of heme and is naturally present inside mitochondria. The amount of PpIX inside mitochondria is elevated by the local application of 5-aminolevulinic acid (ALA) using a 2x2cm plaster from Alacare (Photonamic, GmbH & Co. KG, Pinneberg, Germany). The ALA diffuses through the skin and is converted to PpIX in the mitochondria (see fig. 2.2). To ensure enough PpIX has accumulated, the plaster has to be placed at the measurement site at least 8 hours before the measurement.

The measurement is then performed by placing the measurement probe on the skin. In this probe, a lightsource emits a green light pulse at wavelength 510 μm. As a result, part of the PpIX gets excited into the first-singlet state. Part of this population then reaches the triplet state through intersystem crossing. This triplet state protoporphyrin population has two main options: 1) Fall back into the relaxed state, thereby emitting green light with wavelength 670-700 μm, this is called delayed fluorescence or 2) lose the extra energy by colliding with oxygen, and thereby not emitting any light (see fig. 2.2) [41].

The chance that PpIX spontaneously relaxes into the relaxed state is denoted by rate constant (k_s), the chance triplet state PpIX collides with oxygen depends on the amount of oxygen present in the mitochondria

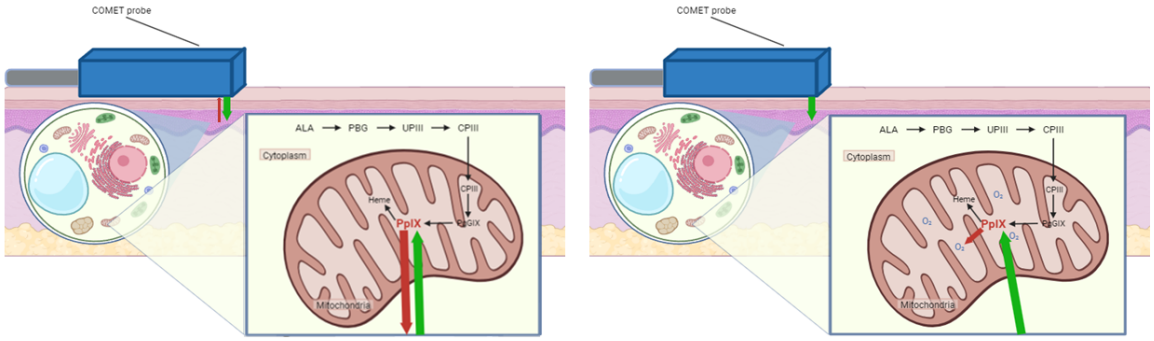

Figure 2.2: Schematic image showing the uptake of 5-aminolevulinic acid (ALA) into the skin and its conversion to protoporphorin IX (PpIX). If a green light pulse is directed at the PpIX, it can get excited into the triplet state. From that state, it can fall back to the relaxed state, emitting red light, or react with oxygen.

and is denoted by the quenching constant (k_q). The decay rate of PpIX that is in the excited triplet state can mathematically be described as eq. (2.1).

$$\frac{d[T_1](t)}{dt} = -k_s[T_1](t) - k_q PO_2[T_1](t) \tag{2.1}$$

In which $[T_1](t)$ is the amount of PpIX in the triplet state at time point t. The solution to this differential equation can be written as eq. (2.2).

$$[T_1](t) = [T_1]_0 e^{-t/\tau} \tag{2.2}$$

In which $[T_1]_0$ is the amount of PpIX molecules in the triplet state at t=0 and τ is the decay lifetime, which can be written as $\tau = 1/\tau_0 + k_q PO_2$, according to the Stern-Volmer equation [42]. τ_0 is time constant of the decay when no oxygen is present ($1/k_s$). The measurement probe measures the intensity of the delayed fluorescence, which is proportional to the amount of PpIX in the triplet state. Therefore, his exponentially decaying intensity curve can be mathematically described as eq. (2.3) [41].

$$I(t) = I_0 e^{-t/\tau} \tag{2.3}$$

In which I(t) is the intensity, I_0 is the intensity directly after the light pulse.
The COMET fits a rectangular distribution based on eq. (2.3) and an adjustment for the background noise on the measured decay curve (more information see appendix A). The mitoPO$_2$ can then be calculated based on the value of τ (see eq. (2.4)). An example of two decay curves with high and low mitoPO$_2$ can be found in fig. 2.3.

$$mitoPO_2 = (\frac{1}{\tau} - \frac{1}{\tau_0})/k_q \tag{2.4}$$

MitoVO$_2$

During mitoVO$_2$ measurements, pressure is exerted on the measurement probe. As a result, the blood flow towards the cells in the measurement frame is stopped. Since the mitochondria still consume oxygen, a time-dependent reduction of mitoPO$_2$ can be observed fig. 2.4. The pressure on the probe is maintained until the mitoPO$_2$ has been stable and low for at least 10 seconds. The pressure is then released. The rate at which the mitoPO$_2$ declines can be described by eq. (2.5).

$$\frac{dPO_2}{dt} = VO_2(t) - OCM(t) + DOI(t) \tag{2.5}$$

Where dPO$_2$/dt is the rate of the mitoPO$_2$ disappearance, VO$_2$(t) is the tissue oxygen consumption, OCM(t) is the oxygen consumption measured and DOI(t) is the diffusive oxygen influx into the tissue [43].

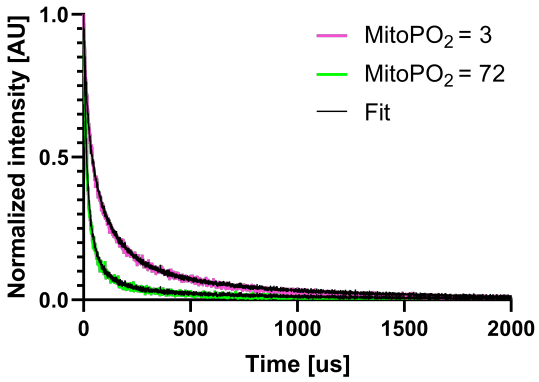

Figure 2.3: Two decay curves with corresponding fits based on eq. (2.3). MitoPO$_2$ is then calculated based on eq. (2.4).

Combining this formula with the Michaelis-Menten kinetics yields the following formula for determining the mitoVO$_2$ (eq. (2.6)

$$\frac{dPO_2}{dt} = -\frac{V_0 \cdot P_n}{P_{50} + P_n} + Z(P_0 - P_n) \tag{2.6}$$

As stated by Harms et al., V_0 is the slope of the curve determined from the linear part of the curve directly after the beginning of tissue compression, P_n is the measured PO_2 after n seconds, P_{50} is the oxygen consumption PO_2 at $0.5 \cdot$ Vmax and P_0 is the mean PO_2 before the pressure is applied to the tissue [43].

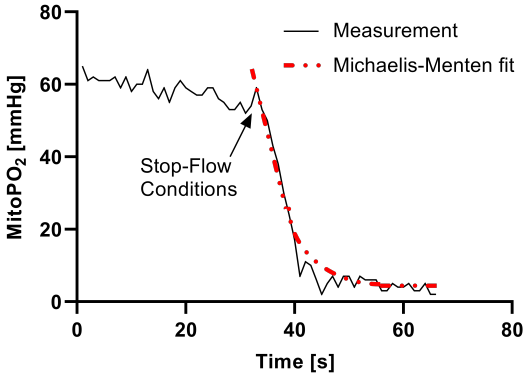

Figure 2.4: A mitoVO$_2$ measurement. Stop-Flow conditions are achieved by applying pressure on the measurement probe. A Michaelis-Menten curve is fitted to determine the mitoVO$_2$.

2.7. Data extraction

MitoPO$_2$

MitoPO$_2$ values for all analyses should have resulted from decay curves that were fitted adequately. The requirements for a valid mitoPO$_2$ that were determined are described below. An in-depth explanation can be found in appendix A.

1. The maximal intensity (at the start of the measurement) should be at least 10.000.
2. The value of fit a from the raw COMET data should be > 0.

Baseline mitoPO$_2$ was defined as the mean of at least 60 mitoPO$_2$ measurements at the start of the measurement protocol. The included measurements were based on the visual annotation of the mitoPO$_2$ data. Exercise and recovery mitoPO$_2$ were calculated by averaging the 10 mitoPO$_2$ values post-exercise and -recovery, respectively. MitoPO$_2$ that met the data quality requirements were automatically extracted from raw COMET data using a script created in Python 3.11.3 [44].

MitoVO$_2$

The mitoVO$_2$ curves are automatically analysed using a self-designed software program, created in MatLab 2022b [45]. This program uses a long-short term memory neural network to automatically determine start points of the mitoVO$_2$ measurements. Subsequently, the Michaelis-Menten fit is performed. To make sure that only accurate mitoVO$_2$ curves were analysed, a researcher verified each curve using the recorded measurement times. A semi-automatic Labview program was used to analyse MitoVO$_2$ curves that the automated software was unable to identify [43, 46].

2.8. Statistical Analysis

The statistical analysis for this study was conducted using R version 4.2.2 [47]. Baseline characteristics between groups were compared using a χ^2-test for contingency data. A Wilcoxon rank-sum test was used for continuous data.

Further analysis was divided into primary and secondary objectives (see section 2.2). An α-level of 0.05 was set for all statistical tests. Prior to formal analysis, a visual inspection of the mitoPO$_2$ and mitoVO$_2$ data distributions was conducted to assess normality using histograms and Q-Q plots. Normally distributed data is presented as mean ± standard deviation (SD). Non-normally distributed data is presented as median ± interquartile range (IQR; 25th and 75th percentiles).

2.8.1. Primary objective

Linear Mixed Models (LMM) were used to determine the effect of group and the time points (baseline, exercise, recovery) on the mitoPO$_2$ and mitoVO$_2$. The LMM included random effects for participants to account for within-subject variability and fixed effects for group (post-COVID vs. convalescent controls) and time (baseline, exercise and recovery), as well as their interaction. This model allowed for the assessment of changes over time within each group and the differences between groups at each time point. The assumptions of homoscedasticity and normality of the model were visually inspected using fitted versus residuals plots, Q-Q plots and histograms of residuals. The data is visually presented using boxplots showing the mitoPO$_2$ and mitoVO$_2$ data for both groups at all three measurement points.

2.8.2. Secondary objective

Spearman correlation coefficients were calculated to assess the relationships between mitoPO$_2$ and mitoVO$_2$ and secondary outcome variables, including the FAS questionnaire scores, percentage of functioning after infection, and duration of post-exertional malaise (PEM) after exercise. Strengths of significant correlation were interpreted based on the ρ^2-value: Very weak (0-0.19), weak (0.20-0.39), moderate (0.40-0.59), strong (0.60-0.79), very strong (0.80-1.00).

The results of the 1-minute sit-to-stand test (1MSTS) were normalized based on age and gender prior to calculating the correlation coefficients [38].

3

Results

3.1. Baseline characteristics

Two participants from the PC group had to be excluded, as the ALA-plaster was not (timely) placed. This yielded a total of 44 participants in this study, 24 and 20 in the PC and convalescent control group, respectively. Baseline characteristics can be found in table 3.1. No significant differences were found between groups based on baseline characteristics.

	PC	**CC**	**p-value**
N	24	20	
Man/Woman	13/11	11/9	0.8
Age [years]	41.5 (35.0-47.2)	38.5 (33.8-51.2)	0.8
BMI [kg/m^2]	25.1 (22.4 - 26.9)	23.8 (21.4 - 26.2)	0.3
Duration of infection [years]	3.2 (2.5 - 4.2)	N.A.	N.A.
Smoking status			0.1
Never smoked	21	12	
Used to smoke	2	6	
Not daily	0	0	
Daily	1	1	
Not available	0	1	
Tested positive			0.06
PCR test	15	12	
Selftest	3	7	
Serology/blood test	3	0	
No possibility (first wave)	3	0	
Not available	0	1	
Medical history			0.17
Hypertension	0	1	
Cardiovascular illness	1	0	
Asthma	7	3	

Table 3.1: Baseline characteristics of study participants. p-values for contingency tables are based on χ^2-test. For continuous values, median, 25th and 75 percentile are given, p-values are based on the Wilcoxon rank-sum test. PC: Post-COVID, CC: Convalescent Controls, BMI: Body Mass Index.

3.2. Primary outcome

$MitoPO_2$

The $mitoPO_2$ values at the three time points for the two groups are shown in fig. 3.1. Median $mitoPO_2$ values in the convalescent control group at baseline, exercise and recovery were 116.0 (IQR 87.6 - 131.0), 55.3 (IQR: 40.6 - 70.9) and 99.8 (IQR 62.9 - 118) mmHg. Median $mitoPO_2$ values in the PC group at baseline, exercise, and recovery were 88.6 (IQR 71.5 - 98.9), 18.8 (IQR 2.6 - 79.7), and 66.2 (IQR 0 - 110) mmHg, respectively.

The LMM revealed a significant effect of time point exercise ($p < 0.001$). No significant effects of group, recovery or interactions were found ($p = 0.095$, 0.117 and >0.05, respectively, fig. 3.1). Estimates can be found in appendix C.

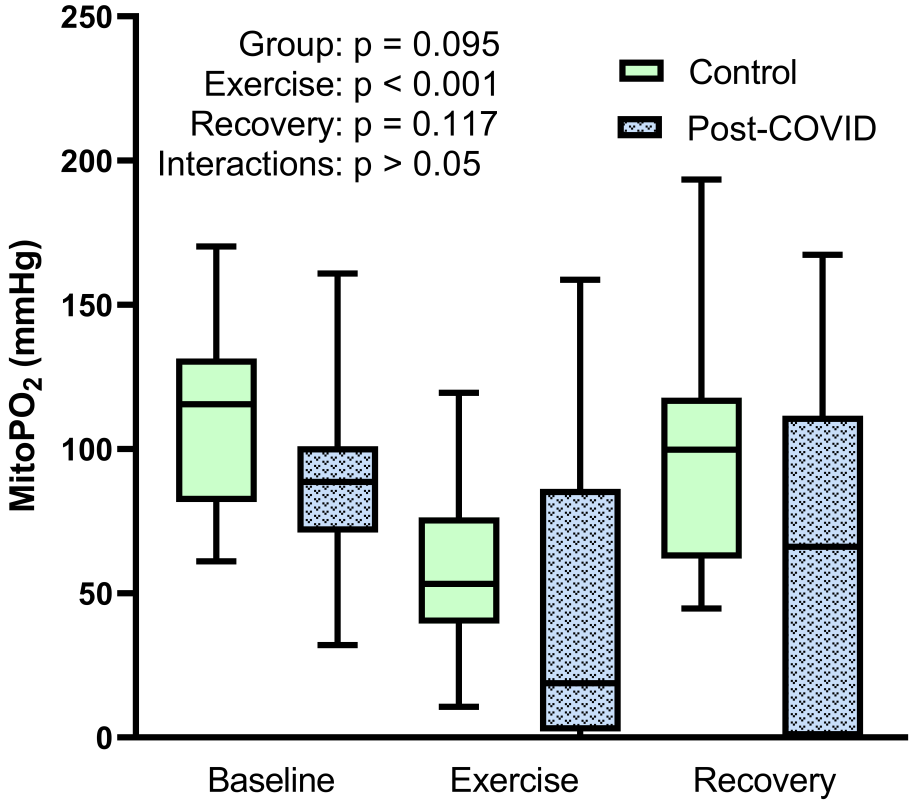

Figure 3.1: Boxplots of $mitoPO_2$ measurements of post-COVID (blue dotted) and Convalescent Control (green) groups at time points Baseline, Exercise and Recovery. P-values of the linear mixed model are shown in the top left.

MitoVO2

The $mitoVO_2$ values at the three time points for the two groups are shown in fig. 3.1. Median $mitoVO_2$ values in the convalescent control group at baseline, exercise, and recovery were 8.34 (IQR 6.35 - 10.1), 8.70 (IQR 7.17 - 9.76), and 9.14 (IQR 7.81 - 10.4) mmHg s^{-1}. Median $mitoVO_2$ values in the PC group at baseline, exercise, and recovery were 8.02 (IQR 6.99 - 9.23), 7.16 (IQR 6.48 - 8.58), and 8.25 (IQR 6.28 - 9.57) mmHg s^{-1}. The revealed a significant effect of time points recovery ($p = 0.049$). No significant effects of group, exercise or interactions were found ($p > 0.05$). Estimates can be found in appendix C.

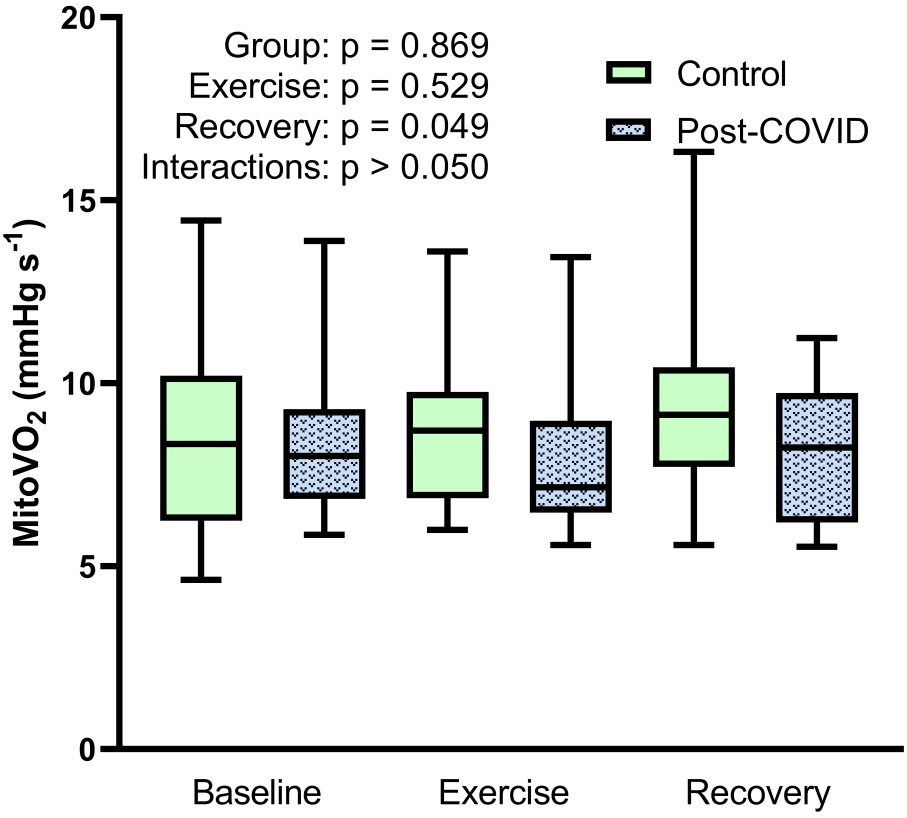

Figure 3.2: Boxplots of mitoVO$_2$ measurements of post-COVID (blue dotted) and Convalescent Control (green) groups at time points Baseline, Exercise and Recovery. P-values of the linear mixed model are shown in the top left.

3.3. Secondary outcome

Exercise mitoPO2

No significant correlations were found in the secondary analysis. Scatterplots of the mitoPO$_2$ against the parameters with corresponding correlation values are shown in appendix D.

Recovery mitoPO2

No significant correlations were found in the secondary analysis. Scatterplots of the mitoPO$_2$ against the parameters with corresponding correlation values are shown in appendix D.

4

Discussion

The results of this study imply that mitoPO$_2$ as measured by the COMET in post-COVID patients with PEM is not significantly lower than in healthy controls. Furthermore, exercise significantly lowers mitoPO$_2$ in the entire study population, whereas mitoPO$_2$ recovers after 10 minutes of recovery. The effects of exercise and recovery did not significantly differ between groups. Large variance in mitoPO$_2$ values is prominent in the PC group, especially after exercise and recovery. This possibly implies that different disease mechanisms are present within our PC study population. The mitoVO$_2$ after recovery for both groups was significantly higher compared to baseline. The mitoVO$_2$ did not show a significant difference between the two study groups.
PROM related to level of functioning, level of PEM and tiredness or normalized 1-minute sit-to-stand-test did not seem suitable for identifying different groups based on mitoPO$_2$, as no significant correlations were found.

The lower mitoPO$_2$ in part of the PC group might be caused by poor delivery of oxygen to cells. Several explaining mechanisms in PEM have been described in literature. Endothelial damage seems to play a key role in PEM [20]. This damage can cause an inadequate response of the endothelium to different flow speeds. This impaired flow mediated dilation (FMD) might be worsened by the inadequate nitric oxide production, which usually functions as a vasodilator [48]. As a result, (micro-)circulation might be more heterogenic and dysfunctional [49, 50]. These alterations have been seen in different ilnesses, causing both venous pooling, thereby reducing venous return, as well as arteriovenous shunting [51, 52]. Other factors influencing the microcirculation in PC might be linked to autonomous dysfunction [53]. Distal small fibre neuropathy has been found in the skin of ME/CFS patients experiencing PEM [53]. The resulting abnormal innervation of the arterial myocytes could further worsen microcirculation. Finally, the thromboinflammatory state identified in PC might further hamper microcirculation through the formation of microclots [54]. Mitochondria could be damaged by both the low mitoPO$_2$, as well as the following reperfusion [55]. Any of these mechanisms, or a combination of these mechanisms could be underlying to the hypoxic situations (mitoPO$_2$ = 0 mmHg) found in part of the PC participants.

Conversely to the study of Appelman et al, no differences in mitoVO$_2$ between study groups were found. However, the study might not have been powered to find a statistically significant difference in mitoVO$_2$. Furthermore, mitoVO$_2$ was not directly measurable after exercise in participants who had a mitoPO$_2$ of 0 mmHg. These participants, therefore, had more time to recover, which might have raised mitoVO$_2$ in this group. Nevertheless, a non-significant decrease in median mitoVO$_2$ is found after exercise in the PC group, whereas the control group showed a non-significant increase in median mitoVO$_2$ after exercise. Furthermore, the mitoVO$_2$ in Appelman's study was measured 24 hours after exercise, focussing more on the time at which PEM typically occurs [19, 20].

The result that mitoPO$_2$ decreases after exercise has previously been described by Baumbach et al [56]. Conversely to this study, they found mitoPO$_2$ reduces 10 minutes after exercise. Various factors might have influenced these results. In their study, a more demanding exercise protocol was followed, which might have a larger impact on mitoPO$_2$. Furthermore, both studies have only included a small number of participants. Focussing on mitoVO$_2$, they found a reduced mitoVO$_2$ directly after exercise. This contrasting result can be explained by their calculation method for the mitoVO$_2$, as they use a sigmoid fit. In this study, the Michaelis-Menten fit was used because it represents biological processes more adequately [57].

Limitations

The results of this research should be interpreted with caution, as several limitations are present. The first and foremost limitation concerns the small number of participants included in this study. This may have resulted in the study being underpowered for the small difference in mitoVO_2 and correlation calculation. Next to the small number of participants, heterogeneity due to patient-specific factors cannot be excluded. The in- and exclusion criteria described in section 2.3.2 were applied when participants were included for the first visit, 6 months prior to the visit at which COMET measurements were performed. Participants may no longer meet these strict criteria. One participant, for example, reported a level of functioning of 100% compared to pre-infection. As the original inclusion criteria required participants to be below 70%, this highlights the possibly increased heterogeneity. Furthermore, no data is available about re-infections or treatments in these six months, which might have an impact on exercise capacity and/or mitochondrial function. For instance, difference in mitochondrial oxygenation could occur in participants who underwent hyperbaric treatment [58].

Another possible source of heterogeneity is the exercise intensity. The 1MSTS-test explanation by the researchers was not described in the original protocol. This led to a difference in word choice and external motivation, which might have caused different exercise intensity levels between participants. As mitoVO_2 is known to be affected by exercise, this may have influenced the results. [56]. Furthermore, it can be questioned whether the different exercise intensities caused PEM in all PC participants. On the other hand, it cannot be excluded that PEM might, in some participants, have been caused by the effort required to reach the hospital, thereby affecting the validity of the baseline measurements.

The PROM data poses a different challenge, as the results of the questionnaires are based on subjective assessments of the participants' own status. Several participants noted that they struggled with filling in the questionnaires, especially since they had already filled them in once six months earlier. This subjectivity results in a questionable comparability of some of the PROM results. The level of functioning compared to pre-infection is expected to be most affected, as this measure tries to capture all the participant's subjective experiences into one number. The FAS-scores can be interpreted with more certainty, as it is a validated question set [39].

5

Conclusion

The mitoPO$_2$ and mitoVO$_2$ measurements using the COMET did not show significant differences between the PC and convalescent control groups. However, in the overal group, mitoPO$_2$ was significantly lowered after exercise, whereas mitoVO$_2$ was significantly higher after recovery. No significant differences were observed between groups regarding the effects of exercise and recovery on mitoPO$_2$ and -VO$_2$. Notably, a large decline in mitoPO$_2$ occurs in some PC patients after short exercise, which does not recover in 10 minutes. This can possibly be explained by heterogeneity of microcirculation or autonomic dysfunction. The variance of mitoPO$_2$ in the PC population after exercise and recovery could not be explained by PROMs related to tiredness, PEM or level of functioning, nor by exercise intensity.

5.1. Future directions

Adjustments in the study set-up should be made in future research to overcome the limitations of the current study. Firstly, participants should be measured at multiple points in time. At least two moments with a 24-48 hour interval should be considered to capture the recovery capacity of participants. A 1MSTS-test at the second measurement moment could be performed, creating research comparable to the two-day CPET [25]. To reduce the influence of other PEM-causing factors, home measurements can be performed. The COMET is relatively easily transportable, so this should only cause minor logistical problems. This would also allow the inclusion of the PC population that is unable to go to the hospital for research participation.

Exercise-free methods to in-vivo test the maximum mitochondrial respiration could also be explored. This would reduce the impact of different exercise intensities and levels of external motivation by researchers. Mitochondrial uncouplers such as dinitrophenol, BAM-15 or niclosamide could be promising in this aspect [59, 60, 61]. These substances act as protonophores to the mitochondrial membrane, which allows protons to freely cross the membrane. As a result, oxidative respiration is uncoupled from ATP production. Mitochondria will maximally upregulate oxidative regulation in an attempt to create ATP. Topical application of these uncouplers could therefore enable measurement of maximal mitoVO$_2$ [62]. However, none of these substances is currently in clinical practice with this goal, nor would it provide direct insight into recovery capacity.

Finally, the disturbed microcirculation hypothesis could be further elucidated by adjusting the COMET software and adding microcirculation parameters to the research. The COMET should not only give a mean mitoPO$_2$ for the entire measurement region but should also provide some insight into the heterogeneity (e.g. by giving a hypoxic fraction). Microcirculation parameters could be added using the CytoCam [63]. If these challenges are addressed, the COMET might have future place in PC diagnosis and prognosis.

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Joris

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A

Technical supplement (Confidential)

The Confidential Appendix is accessible only to the graduation committee, so this page is intentionally left blank.

B

Matlab code (Confidential)

The Confidential Appendix is accessible only to the graduation committee, so this page is intentionally left blank.

C

Supplementary primary outcomes

C.1. MitoPO$_2$

Description

$$mitoPO_2 \sim Group \cdot timepoint + timepoint + (1|Participant) \tag{C.1}$$

Effect estimates

	Estimate	SE	df	t-value	p-value
(Intercept)	109.59	8.76	77.3	12.509	<2e-16
Group	-20.08	11.86	77.3	-1.693	0.095
Exercise	-52.40	8.32	80.4	-6.302	1.5e-08
Recover	-13.16	8.32	80.4	-1.583	0.117
Group:Exercise	7.00	11.34	80.9	0.617	0.539
Group:Recovery	-9.66	11.51	81.4	-0.839	0.404

Table C.1: Fixed effect estimates of the linear mixed model with mitoPO2 as dependent variable. Group, time points and their interaction were set as independent variables. SE: Standard Error, df: Degrees of Freedom.

Random effects		Variance	SD
Participants		844	29.0
Scaled residuals			
Min	Median	IQR	Max
-1.5342	-0.0552	-0.5907 -0.5330	2.2142

Table C.2: Random effects and residuals of linear mixed model with mitoPO2 as dependent variable. SD: standard deviation. IQR: interquartile range (1st - 3rd quartile).

C.2. MitoVO$_2$

Description

$$mitoVO_2 \sim Group \cdot timepoint + timepoint + (1|Participant) \tag{C.2}$$

Effect estimates

	Estimate	SE	df	t-value	p-value
(Intercept)	8.420	0.493	75.7	17.07	<2e-16
Group	-0.111	0.668	75.7	-0.17	0.869
Exercise	0.298	0.472	78.7	0.63	0.529
Recover	0.945	0.472	78.7	2.00	0.049
Group:Exercise	-0.547	0.644	80.9	-0.85	0.398
Group:Recovery	-1.065	0.649	79.5	-1.64	0.104

Table C.3: Fixed effect estimates of the linear mixed model with mitoVO\textsubscript{2} as dependent variable. Group, time points and their interaction were set as independent variables. SE: Standard Error, df: Degrees of Freedom.

Random effects		**Variance**	**SD**
Participants		2.64	1.62
Scaled residuals			
Min	Median	IQR	Max
-1.740	-0.071	-0.573 - 0.451	3.178

Table C.4: Random effects and residuals of linear mixed model with mitoPO2 as dependent variable. SD: standard deviation. IQR: interquartile range (1st - 3rd quartile).

D

Supplementary secondary outcomes

D.1. Exercise mitoPO$_2$

Parameter	Rho^2	p-value
Normalized 1-MSTS	0.0274	0.451
Duration of PEM	0.0284	0.442
FAS score	0.0284	0.442
Level of functioning	0.0064	0.717

Table D.1: Results of Spearman's correlation of exercise mitoPO$_2$ vs parameters above. 1-MSTS: 1-minute sit to stand. PEM: Post exertional malaise. FAS: Fatigue assessment scale.

D.2. Recovery mitoPO$_2$

Parameter	Rho^2	p-value
Normalized 1-MSTS	0.0131	0.622
Duration of PEM	0.00208	0.844
FAS score	0.028	0.469
Level of functioning	0.000429	0.929

Table D.2: Results of Spearman's correlation of recovery mitoPO2 vs parameters above. 1-MSTS: 1-minute sit to stand. PEM: Post exertional malaise. FAS: Fatigue assessment scale.

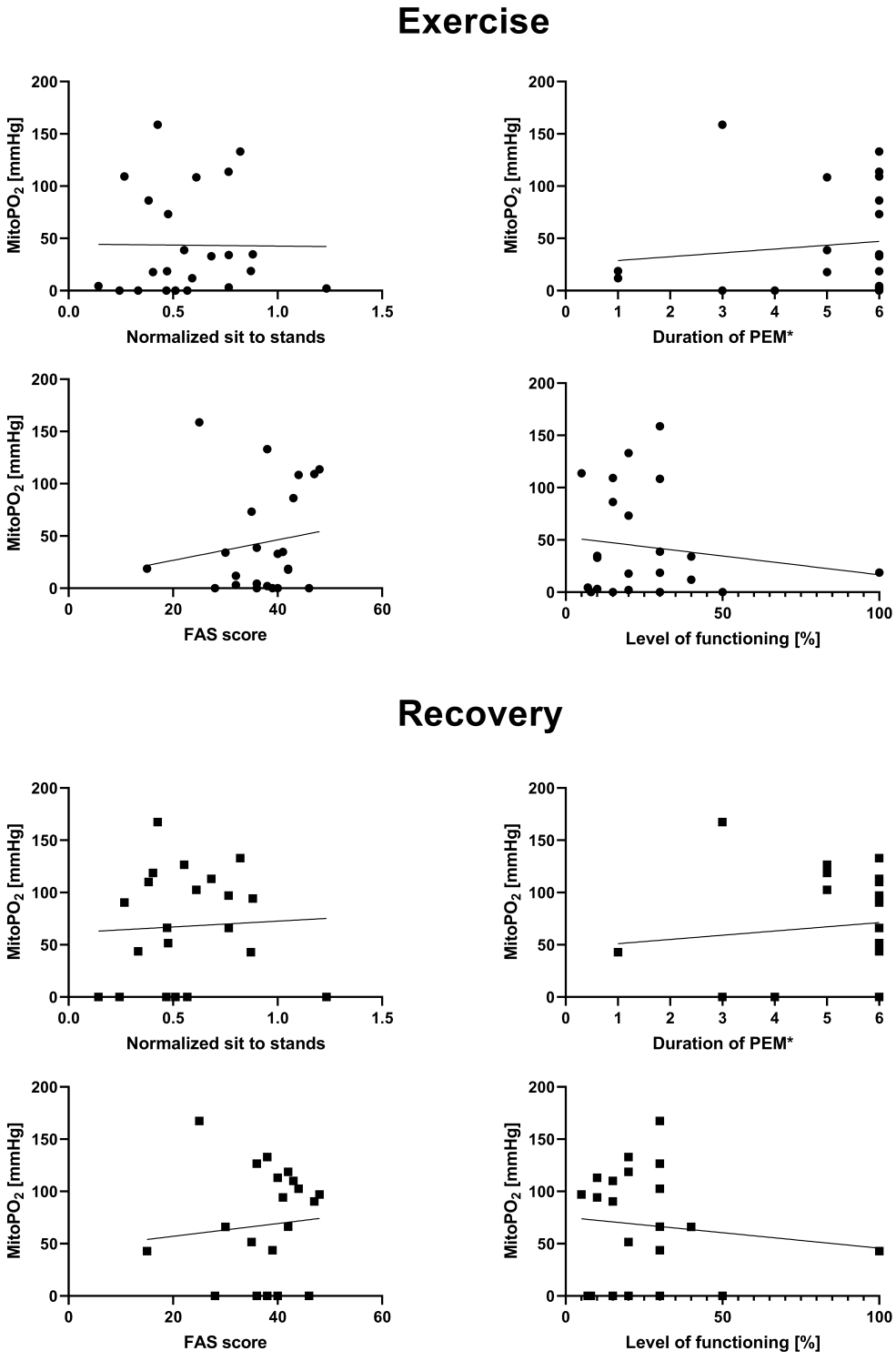

Figure D.1: Correlation plots of the different Patient-related outcomes, normalized one minute sit to stands and exercise mitoPO$_2$ (above) and recovery mitoPO$_2$. Post-exertional malaise duration scores correspond to <1, 2-3, 4-10, 11-13, 14-23, >24h. Sit to stands were normalized by adjusting for gender and age. FAS: Fatigue assessment score.

E

Python code

```
import os
import pandas as pd
import numpy as np

IDX_MITOPO2 = 5
IDX_SQ = 6

# Define the path to the main directory containing all subfolders and files
main_directory = "dir"

# Load the "mitoVO2_metingen.xlsx" file
mito_data_path = os.path.join(
    "dir",
)
mito_data = pd.read_excel(mito_data_path, sheet_name="Meetpunten")

# Create a DataFrame to store the results
results_columns = ["Participant", "Baseline_mitoPO2"]
# Add columns for start and end points
for i in range(1, 10):
    results_columns.append(f"mitoPO2_start_{i}")
    results_columns.append(f"mitoPO2_end_{i}")
results_columns += ["Exercise_mitoPO2", "Recovery_mitoPO2"]

results_df = pd.DataFrame(columns=results_columns)


# Function to calculate the mean of specified rows in a given column
def calculate_mean(data, row_indices, col_index):
    return np.mean([data.iloc[row, col_index] for row in row_indices])


# Iterate through all subdirectories and files in the main directory
for root, dirs, files in os.walk(main_directory):
    for file in files:
        if file.endswith(".xlsx") and "LC" in file:

            # Extract participant number from the file name
            participant_number_str = file.split("LC")[1][:3]
```

```
participant_number = int(participant_number_str)
print(f"calculating␣parameters␣for␣participant␣{participant_number}")
# Load the measurement data for the current participant
file_path = os.path.join(root, file)
try:
    measurement_data = pd.read_excel(file_path, header=0)
except:
    print(f"cannot␣open␣file␣{file_path}")
    continue
# Remove the first column
measurement_data = measurement_data.iloc[:, 1:]

# Convert all columns to numerical values
measurement_data = measurement_data.apply(pd.to_numeric, errors="coerce")
# Find the corresponding participant data in mito_data
participant_data = mito_data[mito_data["participant"] == participant_number]
if not participant_data.empty:
    # Extract relevant measurement numbers and indices
    baseline_start = participant_data.iloc[0, 2]
    baseline_end = participant_data.iloc[0, 3]

    exercise_point = participant_data.iloc[0, 22].astype(int)
    recovery_point = participant_data.iloc[0, 23].astype(int)

    # Calculate Baseline mitoPO2
    baseline_mitoPO2 = np.mean(
        measurement_data.iloc[IDX_MITOPO2, baseline_start:baseline_end]
    )  # Row 7 is index 6

    # Calculate mean mitoPO2 after exercise and recovery if the peak intensity is hi
    exercise_mitoPO2_values = []
    recovery_mitoPO2_values = []

    for idx in range(exercise_point, exercise_point + 100):
        relevant_intensity = measurement_data.iloc[50:61, idx - 1].astype(
            int
        )  # Rows 51-61 are indices 50-60

        if (
            relevant_intensity.max() > 10000
            and measurement_data.iloc[23, idx - 1].astype(float) > 0
        ):
            exercise_mitoPO2_values.append(
                float(measurement_data.iloc[IDX_MITOPO2, idx - 1])
            )

            if len(exercise_mitoPO2_values) == 10:
                break

    try:
        for idx in range(recovery_point, recovery_point + 100):
            relevant_intensity = measurement_data.iloc[
                50:61, idx - 1
            ].astype(
                int
            )  # Rows 51-61 are indices 50-60
```

```
                    if (
                        relevant_intensity.max() > 10000
                        and measurement_data.iloc[23, idx - 1].astype(float) > 0
                    ):
                        recovery_mitoPO2_values.append(
                            float(measurement_data.iloc[IDX_MITOPO2, idx - 1])
                        )
                        if len(recovery_mitoPO2_values) == 10:
                            break
            except:
                print(
                    f"Recovery parameters could not be calculated for participant {participant_num
                )
                recovery_mitoPO2_values = [0]
            # If less than 10 valid points are found, fill the remaining with NaN
            while len(exercise_mitoPO2_values) < 10:
                exercise_mitoPO2_values.append(np.nan)
            while len(recovery_mitoPO2_values) < 10:
                recovery_mitoPO2_values.append(np.nan)
            # Store the results
            participant_results = {
                "Participant": participant_number,
                "Baseline_mitoPO2": baseline_mitoPO2,
                "Exercise_mitoPO2": np.mean(exercise_mitoPO2_values),
                "Recovery_mitoPO2": np.mean(recovery_mitoPO2_values),
            }

            for i, (start_mean, end_mean) in enumerate(
                zip(mitoPO2_start_means, mitoPO2_end_means), start=1
            ):
                participant_results[f"mitoPO2_start_{i}"] = start_mean
                participant_results[f"mitoPO2_end_{i}"] = end_mean

            results_df = results_df.append(participant_results, ignore_index=True)

# Save the results to a new Excel file
results_df.to_excel(os.path.join(main_directory, "processed_results.xlsx"), index=False)
```