Study Protocol & Statistical Analysis Plan

Cover Page

Study Title:
Mitochondrial DNA Signatures of Poor Aerobic Exercise Trainability in Young Adults Born Preterm

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STUDY PROTOCOL

1. Specific Aims

Specific Aim 1 (SA1): Compare maternal and VPTB young adult mitochondrial genomic characteristics and assess for unique mtDNA variants.

Specific Aim 2 (SA2): Determine the effect of Aerobic Exercise Training (AET) on mitochondrial oxidative capacity and mtDNA heteroplasmy, copy number, and lesions in isolated PBMCs in VPTB and NTB young adults.

2. Study Design and Procedures

Specific Aim 1 Design and Procedures

This aim involves collecting identifiable data, including subject weight, sex, race, and gestational age, from medical records. Blood will be collected via venipuncture from VPTB participants. Saliva samples will be collected from birth mothers, either via a mailed collection tube with instructions or during a facility visit. The birth mothers of the VPTB young adults serve as the control group for this aim. Ultra-deep sequencing of the full-length mitochondrial DNA (mtDNA) will be performed using an established laboratory and bioinformatic pipeline. The sequencing depth will be at least 1,000X, enabling accurate assessment of heteroplasmic sites and frequency. The assessment will identify *de novo* mtDNA variants (i.e., variants not present in the birth mother) in VPTB adults.

Specific Aim 2 Design and Procedures

This study will comprise a **16-week aerobic training intervention** (walking/running) in young adults born with very preterm birth (VPTB ≤32 weeks) and normal-term birth (NTB). Age- and sex-matched NTB young adults will serve as controls. Saliva and venous blood samples will be collected for PBMC isolation to compare mitochondrial genomic parameters (sequence, copy number, and heteroplasmy) at baseline and after the training intervention.

Aerobic Exercise Training (AET) Program: The AET program will last 16 weeks, with sessions occurring 4–5 days per week for 40–60 minutes. The target intensity will be between 60–85% of the participant's predetermined maximal heart rate (HR). To prevent injury and limit dropouts among sedentary participants, the frequency, duration, and intensity will slowly progress.

• **Week 1:** Light intensity (60% HRmax) with flexibility for 4–5 days per week and 40–60 minutes duration.

• **Week 2:** Intensity increases to 65–75% HR max.

• **After 8 weeks:** Intensity increases to 75–85%, and the duration must be 60 minutes.

• Subject progression (VO2max via graded exercise test) and exercise programming will be reassessed and altered at 4 and 8 weeks.

Graded Treadmill Exercise Test: Participants in Aim 2 will complete a modified Balke maximal treadmill exercise test. After a 2-minute warmup at 2.5 mph, the speed will be adjusted to elicit 70–75% of the age-predicted maximum heart rate (Tanaka equation: [208-(0.7xage)]). The incline will increase by 2.5% every 2 minutes until volitional fatigue. Maximal effort is established using three criteria: a heart rate within 10 bpm of the age-predicted maximum, a respiratory exchange ratio ≥1.1, and a rating of perceived exertion of at least 18 (using the 6-20 Borg scale).

Laboratory Assessments:

• **Sample Collection:** Blood samples will be collected via standard venipuncture, and PBMCs will be isolated via differential centrifugation.

• **Mitochondrial Function:** Mitochondrial respiration in isolated PBMCs will be measured using the XFe24 Extracellular Flux Analyzer. PBMC Reactive Oxygen Species (ROS) production will be assessed via a fluorescent assay.

• **mtDNA Analysis (Aim 2):** Heteroplasmy, lesions, and copy numbers will be assessed using PBMCs from VPTB and NTB participants. mtDNA lesions and copy numbers will be determined using a gene-specific quantitative PCR-based assay.

3. Study Population and Recruitment

Inclusion Criteria:

• **Age:** 18–35 years old.

• **VPTB Group:** Males and females born preterm with a gestational age of **≤32 weeks**. Must be inactive (reported exercise < 150 minutes/week).

• **NTB Group (Controls):** Age- and sex-matched subjects born at term (37 wks gestational age).

Exclusion Criteria:

• Diagnosed bronchopulmonary hyperplasia.

• Diagnosed cardiovascular, metabolic, or renal disease, or signs or symptoms of these conditions.

• Participants (and their birth mothers) with significant cardiometabolic events that might induce mtDNA variants are excluded.

Recruitment: Recruitment will target individuals from Texas Tech University (TTU) and the Lubbock County community. Methods include email, local newspaper, radio, and social media advertisements. Informed consent will be obtained for VPTB adults to verify gestational age via medical records. Birth mothers will also be recruited to provide a saliva sample for mitochondrial genome comparisons.

4. Sample Size (Power Analysis)

A power analysis based on prior published data suggests a sample size of **25 subjects per group** is needed to achieve 80% power using a two-way ANOVA with an alpha level of 0.05. To account for potential retention challenges, the study aims to recruit **30 subjects per group**. This sample size is supported by other research that investigated mitochondrial respiration in VPTB young adults.

STATISTICAL ANALYSIS PLAN (SAP)

1. Results Analysis and Interpretation

Specific Aim 1 Analysis

The full-length mtDNA sequence will be compared between the VPTB young adult and their birth mother. Sites will be assessed for variants. A site is deemed an **'informative' mtDNA variant** if it represents a change in amino acid sequence from the birth mother.

• **Expected Outcome (SA1):** The study expects to identify informative mtDNA variants in VPTB young adults, likely in genes regulating oxidative phosphorylation subcomplexes and/or translating essential subunits of the respiratory chain complexes.

Specific Aim 2 Analysis

A **Two-Way ANOVA** will be employed to assess pre- to post-AET changes in key outcomes in VPTB and NTB young adults:

1. PBMC mitochondrial respiration.
2. mtDNA lesions and copies.
3. VO2max.

For AET-induced changes in the mean percent heteroplasmy frequency across heteroplasmic loci and the mean total number of heteroplasmic sites, a **nonparametric statistical test** such as the **Wilcoxon rank test** may be performed due to anticipated variability based on prior findings.

Additionally, the study will assess differences in sites, genes, and regions where heteroplasmy is differentially affected by AET in VPTB and NTB young adults, specifically analyzing whether these align with a specific VPTB phenotype.

• **Expected Outcomes (SA2):**

◦ At baseline, VPTB adults are expected to have abnormally higher PBMC mitochondrial respiration compared to NTB adults.

◦ Mitochondrial respiration is expected to decrease after AET in VPTB adults, suggesting a decline in function due to the exercise stressor overwhelming mitochondrial oxidant capacity.

◦ AET is expected to decrease VPTB heteroplasmy frequency, while an increase is expected in NTB adults.