Mitochondrial Stress, Brain Imaging, and Epigenetics (MiSBIE)

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Lay Summary of Proposed Research

Age-related physical and cognitive decline, as well as the risk of neurological diseases, are increased by the effects of psychosocial stress. Psychosocial stress triggers neuroendocrine, metabolic, cardiovascular, and inflammatory changes in the body. These changes vary in nature and magnitude between individuals, and are associated with long-term disease risk. However, the biological determinants of the stress response are not well understood. Why do some individuals respond more strongly than others to the same stressor?
Our preclinical studies in mouse models with dysfunctional mitochondria, a cellular organelle which produces energy and signals of adaptation, indicate that mitochondria regulate how different organ systems and major stress response axes are activated during psychological stress. This project aims to translate this discovery in a population of individuals with varying degree of mitochondrial dysfunction, and to test potential neural mechanisms. This study will take place at Columbia University Irving Medical Center (CUIMC) and the Mortimer B. Zuckerman Mind Brain Behavior Institute (ZMBBI) and will recruit patients with genetic mitochondrial disease and healthy controls.

Each participant will be studied over two days. On Day 1, we propose to monitor dynamic changes in in different hormones and molecules in blood (catecholamines, inflammatory cytokine interleukin 6) and saliva (cortisol) in response to a standardized laboratory “stressor”. Participants will also undergo a medical assessment. On Day 2, we propose to examine whether these changes are mediated by inter-individual differences in functional connectivity among different brain regions measured by functional magnetic resonance imaging (fMRI).

Participants will also complete a questionnaire package to evaluate psychosocial functioning and clinical disease severity as well as undergoing a neuropsychological assessment. This interdisciplinary project leverages access to a unique population of patients with genetically

defined primary mitochondrial disease within our clinic and nationally. This translational project will generate a unique combination of complimentary molecular, physiological, and neuroimaging data that will substantially advance our understanding of the mechanisms linking mind and body processes. We expect that the proposed studies will thus begin to explore the role of mitochondria in stress regulation, from organelle to organism, and may reveal clinically and/or behaviorally modifiable pathophysiological mechanisms for mitochondrial disease.

Background, Significance, and Rationale

The cellular mechanisms by which mitochondrial defects lead to disease are not fully understood. Mitochondria are organelles that produce cellular energy required for the normal function of cells, organs and physiological systems. They are known as the cell's "powerhouse". When maternally-transmitted mitochondrial DNA (mtDNA) mutations and other genetic defects were discovered to cause debilitating human disease in 1988, it was naturally assumed that this could be explained by an energy production deficit. Although it is certainly the case that energy production deficiency is a consequence of genetic mitochondrial defects, dysfunctional mitochondria also impact other cellular processes, including the expression of thousands of genes in different cell types (1-4), and possibly cellular aging via premature telomere erosion (5). Evidence indicates that respiratory chain alterations can have systemic, cell non-autonomous effects on the functioning complex physiological systems (6). As a result, the impact of mitochondrial defects may reach beyond the confines of individual cells and affect the regulation of multiple physiological systems, thus contributing to the pathogenesis of inherited and acquired mitochondrial disease.

Physiological systems are normally and regularly activated as part of the normal stress response to physical, psychological and social events that are perceived as stressors (7). This involves the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal- medullary (SAM) axes, and the autonomic nervous system (ANS) which is itself divided into sympathetic and parasympathetic divisions. The HPA axis leads to cortisol secretion, the SAM axis leads to the secretion of adrenaline and noradrenaline, and the ANS exerts direct control over heart rate via vagal input. These neuroendocrine factors in turn regulate the levels of metabolic substrates (glucose, lipids, amino acids) (8), and immune system- derived pro- and anti-

inflammatory cytokines (9). Together, mobilization of these physiological systems aims to maintain a stable (healthy) internal milieu within the body by matching these to environmental demands. Achieving stability of certain vital parameters through change in other parameters (e.g., hormones, metabolites, cytokines) is termed "allostasis" (10).

During chronic stress, the repeated and prolonged activation of stress response systems can lead to imbalance, and "wear-and-tear" among these systems. In this case, certain hormones, metabolites, and cytokines become chronically dysregulated (elevated or reduced), culminating in a multi-systemic state of physiological dysregulation termed "allostatic load" (11). The allostatic load (AL) model underlies the potential adaptive and damaging action of stress mediators (12). AL is an integrative perspective that is more precise than the term "stress", since it includes the physiological effects of health-promoting and health- damaging behaviors as well as stressful experiences (13, 14). These concepts refine our understanding of "stress" used to describe adaptation and maladaptation to stressors.

The chronic cumulative effect of systemic dysregulation can be measured by assessing *allostatic states* or specific dysregulated states of specific systems. There are four postulated physiological profiles that represent allostatic states (12, 15): (1) repeatedly activated responses, (2) non-habituating responses, (3) prolonged responses, and (4) inadequate responses. Detecting allostatic states is possible by investigating repeated measures such as stress hormone dynamics during diurnal collection or in response to laboratory-based stressor. Collecting detailed biological data over time allows researchers to identify prodromal/underlying allostatic states that can then be aggregated into a multi- systemic algorithm to calculate allostatic load, and possibly predict disease risk.

An allostatic load index can be quantified by combining a specific set of biomarkers representing multisystem physiological functions (16-18). Allostatic load measured in large cohorts of individuals can predict later physical function and cognitive decline (16, 19, 20), indicating the prognostic value of physiological perturbations. As a predictor of mortality and decline in physical functioning in the elderly, the allostatic load index outperforms traditionally accepted risk factors such as the metabolic syndrome or individual biomarkers (21). Thus, allostatic load represents a research concept to comprehensively quantify multi-systemic dysregulation, as well as a potentially useful clinical predictor of disease incidence and progression. Allostatic load has never been measured nor studied in the context of mitochondrial medicine. An objective of this project is therefore to determine the influence of mitochondrial defects on allostatic load.

Work from our team and other laboratories have shown that mitochondria may alter the regulation of different allostatic physiological systems, which could promote allostatic load and exacerbate disease through systemic effects (8). Mouse models generated to have the

same nuclear genome (i.e., conplastic) but different mtDNAs showed differential neuroendocrine and behavioral responses to stress (22, 23). A different study with mice containing a whole-body heteroplasmic mixture of two normal mtDNAs also resulted in greater anxiety responses to behavioral testing (24), suggesting that mitochondrial dysfunction causes abnormal stress reactivity.

More recently, we mapped the multisystemic stress response in normal mice compared to four strains of mice with whole-body genetic mitochondrial defects (25). Defects consisted of either a mtDNA point mutation in either the ND6 or COXI genes, deletion of a mitochondrial antioxidant enzyme (NNT, nicotinamide nucleotide transhydrogenase), or deletion of the major transporter for ATP/ADP across the inner mitochondrial membrane (ANT1, adenine nucleotide translocase 1). In response to a single episode of psychological challenge, each mitochondrial defect caused a distinct multi-systemic stress response signature involving modulation of the HPA, SAM, inflammatory, metabolic, and transcriptional responses (25), demonstrating the pervasive effect of mitochondrial functions on multiple physiological systems.

In humans, limited evidence indicates that allostatic systems are likewise influenced by primary mtDNA defects. As in our preclinical study, HPA axis function was found to be blunted in individuals with NNT mutations associated with familial hypocortisolemia. In patients with ANT1 mutations, resting noradrenaline levels were significantly elevated, indicative of hyperactive SAM axis activity (27). In response to exercise, a physical stressor used to discern allostatic states, patients with mtDNA 3243A>G (m.3243A>G) mutation and single large-scale deletions also show increased adrenaline/adrenaline responses (28), and exaggerated cardiovascular responses to exercise (29), indicative of abnormal autonomic regulation. Accordingly, heart rate variability (HRV) may also be altered in patients with the m.3243A>G mutation, indicative of both sympathetic and parasympathetic changes (30). Collectively, this body of evidence is consistent with our pre-clinical data, indicating robust effects of mitochondrial functions on the major stress response systems in mouse and human. However, no study to date has evaluated AL, stress reactivity, and stress recovery to psychological/physical stress in patients with mitochondrial disease.

The reason why this is relevant to health, and to mitochondrial disease in particular, is because psychological and social stressors increase disease risk (31). The underlying mechanisms likely involve stress-induced release of mediators from the nervous, endocrine, immune and metabolic systems (8, 12). In support of this model, research has demonstrated that several psychological and social factors are associated with elevated allostatic load index (17, 32). For example, elevated allostatic load is correlated with adverse social contexts (e.g., social support, social hierarchy, and adverse life events) (33, 34), workplace stress and burnout symptoms (35-37), sleep disturbances (38), and psychiatric symptoms (39, 40), to name a few.

The allostatic load model can be used to link psychosocial contexts to biological mechanisms and health outcomes. For example, mothers caring for children with life- threatening conditions manifest elevated allostatic load that is further associated with psychiatric symptoms (41) and hippocampal atrophy within the brain (42).

Combining psychosocial measures in allostatic load research provides a more global understanding of inter-individual differences in stress (patho)physiology. With the objective to delineate specific profiles of disease trajectories, integrating psychosocial measures (i.e., questionnaires) with biological parameters (blood-, saliva-, hair-, and urine-based biomarkers) and clinical data (i.e., functional capacity, mutation load) enables the triangulation of methods. Scientifically, this combination of converging methods is especially important when investigating the function of stress response axes and hormones such as the HPA axis and cortisol that may exhibit hypo- or hyper-activity in association with diverse psychiatric conditions, disease states, and ultimately allostatic load (43).

Evaluating biological disturbances in the context of psychosocial factors can enhance overall understanding of existing dysregulations of neuroendocrine systems, and to isolate the role of mitochondrial dysfunction independent from potentially confounding variables. A meta-analysis of 62 studies concluded that while the cortisol awakening response is positively associated with workplace stress and general life stress, it is negatively associated with symptoms of burnout, fatigue, and exhaustion (44). Furthermore, hypocortisolism is present in approximately 20-25% of patients suffering from stress-related diseases like burnout, chronic fatigue syndrome, fibromyalgia, post-traumatic stress disorder, and atypical depression (43). Taken together, testing the effects of mitochondrial dysfunction (independent variable) on specific biomarkers/AL (dependent variable) while accounting for continuous measures of depressive, burnout, and other symptoms (covariates) will add strength to our study findings.

The inclusion of questionnaires may provide insights that have yet to be applied in mitochondrial biology. By studying genetic, biochemical, and physiological measurements together, this project will use validated questionnaires for assessing domains of psychological and social functioning previously shown to correlate with allostatic load and specific biomarkers. The goals of these measurements are two- fold: i) To account for potential confounding variables; and ii) To understand the determinants of vulnerability and resilience in patients by exploring psychosocial factors/chronic stressors which may interact with mitochondrial dysfunction to impact physiological dysregulation, and eventually disease progression.

Multi-systemic dysregulation can arise from peripheral alterations of specific organ functions, such as the adrenal glands, the liver, peripheral nerve function, or the cardiovascular system. Alternatively, maladaptive neuroendocrine, metabolic, and epigenetic profiles may be driven by abnormal central nervous system functions, including resting brain connectivity and activation patterns during stressful situations. Thus, the brain is understood to play a central role in stress- related pathogenesis (45). To date, however, neither brain connectivity nor activation has been examined in patients with mitochondrial disease. The proposed study will be the first study to assess the pathways linking mitochondrial dysfunction to stress pathophysiology, and to identify candidate neural regulatory mechanism to explore further. This would provide us with unique insights into the long-standing question of how psychosocial stress 'gets under our skin and skull'.

To study the potential role of the brain as a driver of abnormal stress reactivity, we therefore plan to use functional magnetic resonance imaging (fMRI) and multivariate analyses to test a mediation model where abnormal stress perception caused by mitochondrial dysfunction occurs at the level of the brain and drives abnormal allostatic and epigenetic responses peripherally. This study will thus begin to untangle the role of central nervous system *vs* peripheral physiological processes in mitochondrial disease, allowing us to explore a previously unrecognized sub- cellular mitochondrial pathophysiological mechanism.

Deciphering the role of mitochondrial dysfunction and the mechanism(s) by which it alters stress physiology and is transduced into allostatic load is not only directly relevant to mitochondrial disease, but also to a greater spectrum of neurodegenerative disorders where both life stress (46, 47) and mitochondrial dysfunction (48, 49) have been implicated, including Alzheimer disease, Parkinson disease, schizophrenia, and depression. In addition, this will be the first study to examine the relations between mitochondria, epigenetic changes, and brain activation. At the end of this study, we will have identified candidate molecular, neuroendocrine, and neural mechanisms that can be further investigated in clinical populations and mechanistically explored further in animal models.

Specific Aims and Hypotheses

To translate our preclinical findings and test these hypotheses with the same level of genetic specificity in humans, we leverage our access to a population of patients with molecularly defined m.3243A>G mtDNA mutation and individuals with single large scale mtDNA deletions in our clinical service for mitochondrial disease at CUIMC. To ascertain if the observed associations are linked to symptom types, we will also recruit individuals with the m.3243A>G mutation who have experienced stroke-like episodes or seizures. These symptoms characterize a clinical subtype known as MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes).

Aim 1: Determine the effect mtDNA m.3243A>G and single mtDNA deletion on chronic and reactive stress biomarkers.

Longitudinal studies of stress biomarkers demonstrate that cumulative dysregulation of the HPA (hypothalamic-pituitary-adrenal) and SAM (sympathetic- adrenal- medullary) axes, cardiovascular, metabolic, and pro-inflammatory systems predispose to chronic physical and cognitive decline that overlap with symptoms of mitochondrial diseases. *We hypothesize that patients with mtDNA defects (m.3243A>G) will manifest elevated allostatic load and stress reactivity compared to healthy controls.* This will be assessed on a first hospital visit (Day 1) with repeated measures of blood (catecholamines, inflammatory cytokine IL-6), saliva (cortisol), and cardiovascular monitoring (heart rate variability, blood pressure) in response to a validated laboratory stress protocol, the socially evaluated cold pressor test (SECPT), which elicits strong multi-systemic reactivity. Results from this aim will establish for the first time the effect of inherited mtDNA defects on the nature and magnitude of stress responses in humans, and evaluate their association with patients' symptoms.

Aim 2: Assess DNA methylation signatures and cell aging in patients with the m.3243A>G mutation and single mtDNA deletion.

Mitochondrial signaling involves the production of diffusible signals that alter the epigenetic machinery and the expression of most genes in the human genome; and stress alters DNA methylation in brain remodelling and stress reactivity genes. *We hypothesize that patients with mitochondrial disease will show abnormal baseline DNA methylation signatures, and*

exaggerated epigenetic responses to stress. We will isolate sub-types of peripheral blood mononuclear cells at baseline to map genome- wide cytosine methylation levels. Pre- and post-stress blood samples will also be collected to track stress reactive differences by pyrosequencing of target genes linked to stress regulation and brain remodelling. Results from this aim will complement those of Aim 1 by defining stress reactivity at the sub-cellular, molecular levels.

Aim 3: Map brain-wide neural connectivity patterns in m.3243A>G and single mtDNA deletion patients, and determine if this mediate abnormal peripheral stress reactivity.

Combining measurements of peripheral stress physiology with functional neuroimaging (fMRI-BOLD), our team and others have shown that neuroendocrine, inflammatory, and cardiovascular reactivity to stress can be predicted from discrete

activation patterns among specific brain regions in healthy adults. Furthermore, the brain is an organ primarily affected in mitochondrial diseases and functional connectivity is sensitive to energetic defects, but the nature and impact of these alterations in human mitochondrial disease is unknown. *We hypothesize that patients with mitochondrial defects will exhibit abnormal patterns of resting state and task- elicited neural connectivity, including the executive network, which will statistically mediate alterations in physiological stress reactivity.* On a second visit (Day 2), we will perform structural and functional whole-brain neuroimaging of default mode network and tasks selected to match the stress reactivity on Day 1.

Taken together, this translational study will establish how neural connectivity is altered in mitochondrial disease, and begin to untangle neural pathways that may translate mitochondrial dysfunction into abnormal physiological dysregulation. This study will be the first to test the role of mitochondria in mediating mind-body processes. The resulting papers will inform future work in various fields including clinical mitochondrial medicine and neurology, psycho-neuro-endocrinology, psycho- neuro- immunology, and psychosomatic medicine.

Aim 4: To establish the within-person and technical variability in key study outcomes, two healthy volunteers will “repeat” visits to obtain blood samples up to 10 timepoints over the study period.

To establish the within-person and technical variability in key study outcomes, we will

perform "repeat" visits on two healthy volunteers. This will provide essential quantitative information on some novel mitochondrial parameters that are measured in the MiSBIE study (cell bioenergetics, cell-free mitochondrial DNA, metabolic markers), including how they may vary within a person over time, relative to between- person differences. These difficult to obtain data can be used to quantify intra- individual variation over months-to-years and provide an empirical basis to evaluate the magnitude of differences among the primary study outcomes between the MiSBIE study groups.

Description of Subject Population

Sample #1

Sample subject population:

Subject Population	Number of completers required to accomplish study aims	Projected number of subjects who will be enrolled to obtain required number of completers	Age range of subject population
Individuals with the m.3243A>G mitochondrial DNA mutation	30	33 (accounting for 10% attrition or unusable data)	18 to 60

Description of subject population:

We will investigate individuals carrying the m.3243A>G $tRNA^{Leu(UUR)}$ mutation which is the most frequent mtDNA point mutation, and presents in the most severe case as MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes) (50, 51) as well as non-MELAS carrier relatives. From this cohort and the existing patient registry, individuals who have previously had genetic diagnostic testing confirming the presence of the m.3243A>G will be identified and invited to participate in the study. Maternally related individuals with a first-degree relative who is a known carrier of the mutation are considered "obligate carriers" and will also be included.

Individuals without a confirmed genetic test who are otherwise eligible and who have family members with 3243A>G mitochondrial DNA mutation and/or suspect they have the mutation will be invited to provide a buccal or urine sample to test for mitochondrial mutations. The genetic counselor or physician will review the specific consent form for genetic testing

[Genetic Testing Consent Form MiSBIE], and sample collection instructions remotely with these participants or at the Columbia University Irving Medical Center research site.

Sample #2

Sample subject population:

Subject Population	Number of completers required to accomplish study aims	Projected number of subjects who will be enrolled to obtain required number of completers	Age range of subject population
Individuals with single large-scale mitochondrial DNA deletions	30	33 (accounting for 10% attrition or unusable data)	18 to 60

Description of subject population:

We will investigate individuals carrying a single large-scale mtDNA deletions that causes CPEO (chronic progressive ophthalmoplegia) and Kearns-Sayre syndrome (KSS) (52). Dr. Hirano follows >20 patients with CPEO/KSS, and leads a national consortium (NAMDC – North American mitochondrial disease consortium) where >70 patients with CPEO/KSS have consented to be contacted for research. From this group, patients who have previously had diagnostic testing confirming the presence of mtDNA single deletion will be identified and invited to participate in the study.

Individuals without a confirmed genetic test who are otherwise eligible and who have family members with single large-scale mitochondrial DA deletions and/or suspect they have the deletion will be invited to provide a buccal or urine sample to test for mitochondrial mutations. The genetic counselor or physician will review the specific consent form for genetic testing [Genetic Testing Consent Form MiSBIE], and sample collection instructions remotely with these participants or at the Columbia University Irving Medical Center research site.

Sample #3

Sample subject population:

Subject Population	Number of completers required to accomplish study aims	Projected number of subjects who will be enrolled to obtain required number of completers	Age range of subject population
Controls	100	118 (accounting for 10% attrition or unusable data n=10 and pilot testing n=8)	18 to 60

Description of subject population:

We will study healthy individuals without a diagnosis of mitochondrial disease recruited from the community. These participants will be age-, sex- and physical activity level- matched to participants from Groups 1 and 2.

Sample #4

Sample subject population:

Subject Population	Number of completers required to accomplish study aims	Projected number of subjects who will be enrolled to obtain required number of completers	Age range of subject population
Individuals with the m.3243A>G mitochondrial DNA mutation with MELAS symptoms. This is the same diagnostic group as Group 1, with the exception that patients who have experienced stroke-like episodes or seizures will not be excluded	30	33 (accounting for 10% attrition or unusable data)	18 to 60

Description of subject population:

We will investigate individuals carrying the m.3243A>G $tRNA^{Leu(UUR)}$ mutation who have experienced stroke-like episodes, seizures, or both which characterize MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes) (50, 51). From this cohort and the existing patient registry, individuals who have previously had genetic diagnostic testing confirming the presence of the m.3243A>G will be identified and invited to participate in the study.

Individuals without a confirmed genetic test who are otherwise eligible and who have family members with 3243A>G mitochondrial DNA mutation with MELAS and/or suspect they have the mutation with MELAS will be invited to provide a buccal or urine sample to test for mitochondrial mutations. The genetic counselor or physician will review the specific consent form for genetic testing [Genetic Testing Consent Form MiSBIE], and sample collection instructions remotely with these participants or at the Columbia University Irving Medical Center research site.

Inclusion/Exclusion Criteria

Groups 1 (m.3243A>G) & 2 (single mtDNA deletion)

CRITERION	METHOD OF ASCERTAINMENT
Inclusion:	
1. Patients between the age of 18-60.	Patient clinical records Phone screen or clinical evaluation
2. Willing to provide saliva samples and have venous catheter installed for blood collection during the hospital visit	Phone screen or clinical evaluation
3. Willing to provide informed consent and capacity to consent	Self report
4. Use of effective method of birth control for women of childbearing capacity	Phone screen or clinical evaluation
5. Harbours a mtDNA mutation. Either the m.3243A>G point mutation, or a single large scale mtDNA deletion.	Patient clinical records, or family history involving them as "obligate carrier" of the mtDNA defect
6. English Speaking	Self report Phone screen or clinical evaluation
7. Confirmatory genetic test or willing to undergo genetic testing if one is not available.	
Exclusion:	
1. Patients with cognitive deficit incapable of providing informed consent will not be included	TICS scores $\geq$ 30, administered via phone screen or clinical evaluation
2. Neoplastic disease	Patient clinical records, and self-report

3. Symptoms of flu or other seasonal infection four weeks preceding hospital visit, as this would influence immune system parameters	Phone screen or clinical evaluation
4. Strokes & seizures.	Patient clinical records, and self-report
5. Raynaud's syndrome (Rayneau phenomenon)	Phone screen or clinical evaluation
6. Involvement in any therapeutic trials listed on clinicaltrials.gov, including exercise	Phone screen or clinical evaluation
7. Clinical use of steroid therapy, which would impact the HPA-axis and other physiological systems (e.g., oral dexamethasone, prednisone, or similar)	Patient clinical records Phone screen or clinical evaluation

Group 3 (Controls)

CRITERION	METHOD OF ASCERTAINMENT
Inclusion:	
1. Individuals between the age of 18- 60.	Patient clinical records Phone screen or clinical evaluation
2. Willing to provide saliva samples and have venous catheter installed for blood collection during the hospital visit	Phone screen or clinical evaluation
3. Willing to provide informed consent and capacity to consent	Self report
4. Use of effective method of birth control for women of childbearing capacity	Phone screen or clinical evaluation

5. English Speaking	Self report, Phone screen or clinical evaluation
Exclusion:	
1. Individuals with cognitive deficit incapable of providing informed consent will not be included	TICS scores ≥ 30, administered via phone screen or clinical evaluation
2. Symptoms of flu or other seasonal infection four weeks preceding hospitalvisit, as this would influence immune system parameters	Phone screen or clinical evaluation
3. Raynaud's syndrome (Rayneau phenomenon)	Phone screen or clinical evaluation
4. Involvement in any therapeutic trials listed on clinicaltrials.gov, including exercise	Phone screen or clinical evaluation
5. Metal inside or outside the body or claustrophobia prohibitive to MRI testing	Phone screen or clinical evaluation
6. Diagnosed with mitochondrial disease m.3243A>G, or large scale mtDNA deletion	Phone screen or clinical evaluation

Group 4 (m.3243A>G with MELAS symptoms

CRITERION	METHOD OF ASCERTAINMENT
Inclusion:	
1. Patients between the age of 18- 60.	Patient clinical records Phone screen or clinical evaluation

2.Willing to provide saliva samples and have venous catheter installed for blood collection during the outpatient visit	Phone screen or clinical evaluation
3. Willing to provide informed consent and capacity to consent	Self-report and clinician assessment
4. Use of effective method of birth control for women of childbearing capacity	Phone screen or clinical evaluation
5. Carries the m.3243A>G point mutation and has had at least one stroke-like episode, seizure, or both.	Patient clinical records, or family history involving them as "obligate carrier" of the mtDNA defect
6. English Speaking	Self-report Phone screen or clinical evaluation
7. Confirmatory genetic test or willing to undergo genetic testing if one is not available.	Medical Records Genetic Test
Exclusion:	
1. Patients with cognitive deficit incapable of providing informed consent will not be included	TICS scores $\geq$ 30, administered via phone screen or clinical evaluation
2. Neoplastic disease	Patient clinical records, and self-report
3. Symptoms of flu or other seasonal infection four weeks preceding hospital visit, as this would influence immune system parameters	Phone screen or clinical evaluation
4. Raynaud syndrome (Raynaud phenomenon)	Phone screen or clinical evaluation

5. Involvement in any therapeutic trials listed on clinicaltrials.gov, including exercise	Phone screen or clinical evaluation
6. Clinical use of steroid therapy, which would impact the HPA-axis and other physiological systems (e.g., oral dexamethasone, prednisone, or similar)	Patient clinical records Phone screen or clinical evaluation

Study Procedures

We will only recruit and see one participant per week, as per the standard MiSBIE protocol. This low scheduling frequency eliminates any possible exposure between participants.

The study will unfold in two visits, distributed over two consecutive days. For review purposes, the study "Data Collection Form" (Appendix #5) in addition to a Study diagram summarizing study procedures in chronological order (Figure 1), are uploaded with this protocol. Breaks have been built into the study protocol, and participants are free to request breaks at any time. The study procedures comprise eight parts:

Participants who have family member with 3243A>G mitochondrial DNA mutation or deletion and/or who suspect they have the mutation or deletion will be asked to confirm through genetic testing prior to participation. Kris Engelstad will approach these patients with information regarding genetic testing. These participants will provide a buccal cell or urine sample to test for mitochondrial variants. This genetic testing procedures (consent, sample obtain, genetic consult) will occur online over video chat or at the research site. The

genetic counselor or physician will walk you through taking the at- home or at site sample which we will use for genetic testing. The sample may take up to 5 minutes to collect. Participants will then mail samples using a prepaid label through USPS if they are not on the Columbia University Medical Campus.

Samples will be processed at the Personalized Genome Laboratory (PGL) in pathology at Columbia University – this is a CLIA approved laboratory for genetic analysis. Samples will not be coded and will be stored according to standard CLIA approved mandate. The sample will be destroyed when a verification of genetic mutation or none is complete. Name and date of birth will be collected as mandated for genetic testing and verification of genetic mutation. Only the PGM laboratory will have access to these samples during processing. The results of this verification of genetic mutation will be shared with the genetic counselor, Kris Engelstad, PGM lab, Principal Investigator and Study Coordinator.

The study team will reach out to participants with their genetic results. If they test positive for the m.3243A>G mutation or a single large-scale deletion, they will be eligible to continue in MiSBIE.

Genetic testing results will be reported to the participant in person or via telemedicine by the investigator or the genetic counselor. The post-test consult includes: 1) A review of mitochondrial genetics regarding the mutation, 2) Potential clinical symptoms, 3) Treatment options/or lack of options, and 3) Brief discussion about family planning options (if female). If the participant is upset by the results and requests additional counseling they will be referred to a genetic counselor at Columbia University. Additionally, should they wish genetic counseling near to their home, the study team will provide them with contact for appropriate local genetic counseling services.

DAY 1

(1) Recruitment
(2) Initial Blood Draw and Biological Samples Collection
(3) Medical Examination
(4) Breaks
(5) Stress Reactivity-Recovery Paradigm
(6) Urine Collection

DAY 2

(7) Metabolic Rate Analysis

(8) Questionnaires and Neuropsychological Assessment
(9) Brain Imaging
(10) Take Home Protocol
(11) Study Monitor

DAY 1

(1) Recruitment: Patients with mitochondrial disease and healthy age/sex/race/physical activity level-matched controls will be recruited. As part of existing clinical care, some patients will have undergone (a) neuropsychological assessment, (b) metabolic assessment and (c) structural magnetic resonance imaging (MRI) as part of the diagnostic services of the Clinical Research Center for Muscular Dystrophy and Related Diseases Laboratory. From this patient pool, those molecularly identified to carry mtDNA defects (m.3243A>G, single deletion) will be provided with a study brochure and invited to sign a form agreeing to be contacted for future research. Potential participants will be contacted directly by the clinical coordinator or study coordinator who will provide information concerning the MiSBIE study and assess inclusion/exclusion criteria over the phone or in person. Should potential participants be interested in the study, they will be forwarded consent forms for their review.

We will screen participants for COVID symptoms at the following timepoints: during the initial screening call: the Monday night before the participant arrives, the Tuesday morning of study activities and the Wednesday morning of study activities. We will take the participant's temperature before starting study activities. We have a no-touch thermometer used for other MiSBIE procedures and have the required expertise to perform this measure.

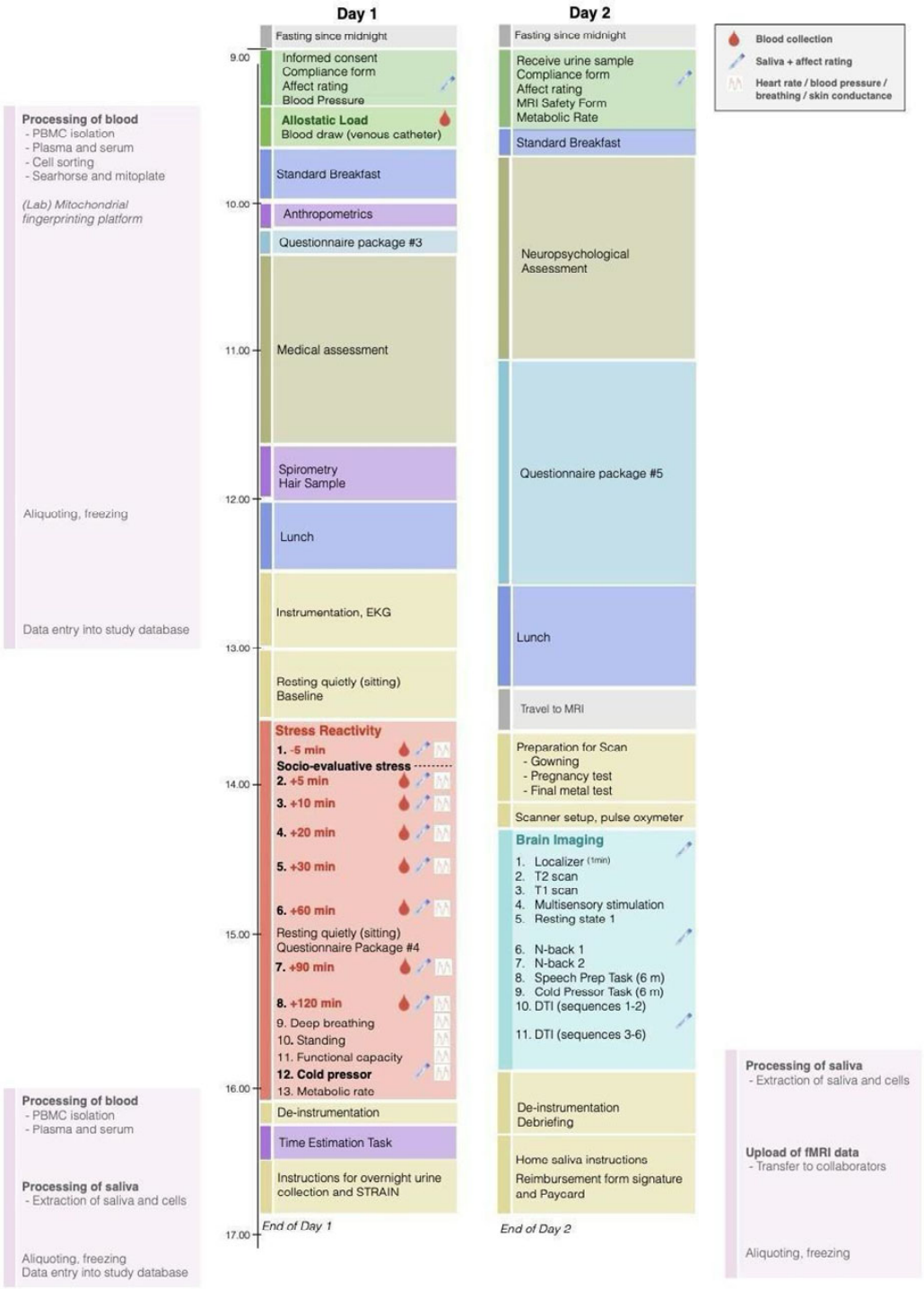

Figure 1. Diagram illustrating study design and workflow.

For participants who agree to participate in the study, the a 2-day visit at the Columbia University Irving Medical Center will be scheduled and transportation/lodging arranged. Outside participants will be housed at the Edge Hotel (Washington Heights, 514 W 168th street), and given detailed instructions to follow prior to their arrival.

(2) Initial Blood Draw and Biological Samples Collection: On the first outpatient visit (Day 1), a fasting blood draw to index allostatic load and cell isolation for mitochondrial function measurements and DNA/RNA analyses. Participants will first arrive at the Division of Behavioral Medicine, Presbyterian Hospital, 15th floor at 9:00am and will be greeted by Catherine Kelly, the MiSBIE study coordinator. Participants will have been instructed not to eat or drink beverages other than water starting at midnight the previous day.

Height, weight, waist circumference, hip circumference, and blood pressure will be collected before blood draw. Weight will be assessed on an electrical impedance scale (TANITA) to simultaneously estimate body composition (% body fat). Two readings will be taken, and the average of both used for analyses.

Participants will next be relocated to the neighboring Psychophysiology Laboratory (Presbyterian Hospital, 16th floor). As per CUIMC's workspace assessment, this space can comfortably accommodate 1 researcher and 1 patient. Furthermore, we will keep a window open to facilitate airflow, and designate one door to the lab as entrance and one as exit to ensure safe traffic flow.

The study nurse will insert a venous catheter in the antecubital vein to draw blood amounting to 100 mL. This includes one EDTA coated tube (2mL), two Serum Separation Tubes (5mL), and five Buffered Sodium Citrate tubes (2.7mL) for complete blood count on whole blood and two for plasma (8mL), two non- coated tubes for serum (8mL) and five Acid Citrate Dextrose tubes (8.5mL) for cells to be used to assess mitochondrial function. Allostatic load biomarker analyses will be performed on plasma and serum.

Cheek swab: A cheek swab (MasterAmp™ Buccal Swab Brush) of the internal mucosa from both cheeks will be performed to collect DNA. This procedure takes approximately 5 seconds and is painless. Participant will pull down their face mask for the duration of this cheek swab. Collected material will enable the measurement of mtDNA copy number, DNA methylation, and mtDNA mutation heteroplasmy in a

somatic tissue.

Breakfast: Following blood draw and anthropometric measurements, participants will break their fast with a standardized continental breakfast in accord with any

dietary restrictions (gluten intolerance, etc.) ascertained beforehand on a standardized menu. Participant will eat alone with face covering removed.

Participants will be suggested to go for a bathroom break before the next part.
Clip of hair: A small clip of hair is collected from the back of the head to measure cumulative levels of steroid hormones (cortisol, sex hormones, endocannabinoids). Two small bundles of approximately 100-200 hairs each (a few millimeters) are clipped near the scalp, just below the cranial bone. The instructions are attached with this protocol (Appendix #1.2). Analyses will be performed by our collaborator Dr. Clemens Kirshbaum (Technische Universität Dresden, Germany).

Spirometry: A spirometry test will be used to assess lung function of the participants. The participant will be asked to inhale as much air as they can and exhale into the spirometer as hard as possible and as long as possible.

(3) Medical Examination:

A medical assessment of clinical status and symptomatology will be performed by Dr. Shufang Li using three validated clinical forms:

Measure	Description	Time (minutes)
Columbia Neurological Score (CNS)	Assessment of central nervous system symptoms	**30**
Newcastle Mitochondrial Disease Assessment Scale (NMDAS)	Semi-quantitative assessment of different domains of functioning Current Function (Section I) Functional capacity and daily living System Specific Involvement (Section II) Current Clinical Assessment (Section III) Clinical assessment of current symptoms	**40**
NAMDC Case Report Form (CRF	Detailed assessment of clinical history, comorbidities, symptoms checklist	**30**

	comorbidities, symptoms checklist	
Karnofsky Performance Scale Index	Classifies functional impairment	**1**
		Total Time: 101

(4) Breaks: Given the lengthy nature of the protocol and that some participants may experience fatigue, breaks have been scheduled at designated times along the protocol:

a. Breakfast
b. After the Medical assessment (Day1
c. Lunch Break on Day 1 (30 minutes)
d. Following the Stress Reactivity–Recovery Paradigm (10 minutes)
e. After the neuropsychological assessment (30 minutes)
f. Lunch Break on Day 2 (30 minutes)
g. After the MRI scan (15 minutes)

The study procedures have been scheduled with ample time to account for unanticipated delays and breaks. During the consent procedures, the study coordinator will explicitly tell participants that he/she can request breaks at any time. The study coordinator will continuously interact with the participant, be available to answer any question, and to organize breaks as needed. During breaks, participant will also have the option of utilizing either a reclining chair to rest in a horizontal position.

(5) Stress Reactivity–Recovery Paradigm: After the lunch, participants will have an IV line placed and be instrumented with physiological data acquisition equipment. Participants will be exposed to a standardized socio-evaluative laboratory challenge (58, 59). Participants will be asked to perform a simulated public speaking task, consisting of 2 min of preparation for a speech defending themselves against an alleged transgression followed by 3 min of videotaped speech delivery, as previously described (58, 59) (please find the protocol attached in Appendix #6). Throughout, we will collect repeated measures of blood (cortisol, ACTH, catecholamines, inflammatory cytokines), saliva (cortisol), and physiological monitoring (blood pressure, heart rate variability, galvanic skin response, and respiration). Research assistant Sophia Tepler and Vincenzo Lauriola will monitor physiological data collection during the session and equip participants. This stress paradigm session consists of a five-part protocol:

(A) Participants will be equipped with:

a. A 3-lead ECG to monitor of heartrate
b. A finger cuff blood pressure sensor to monitor blood pressure
c. Skin conductance surface device to monitor sympathetic nervous system activity (sweating)
d. Two elastic breathing bands on the chest and abdomen.

(B) Participants will rest for 30 minutes in a calm room and asked to refrain from any other medium (e.g., smartphones) that could be a source of distraction. This will avoid to introduce variability in inter-individual responses. This will allow them to acclimate to their surroundings and to the equipment (e.g., ECG electrodes, finger cuff).

(C) Participants will be exposed to a socio-evaluative stress (58, 59) that induces significant psychophysiological responses among multiple systems. Psychosocial stress is induced by simulating videotaping and that these video recordings will be analyzed for speech performance by comparing their performance with those of other participants. Eight (10) mL of blood for plasma (5ml) and serum (5ml) will be collected via the venous catheter at eight defined time-points relative to the onset of the SECPT (-5, +5, +10, +20, +30, +60, +90 and +120 minutes), for a total of 80 mL of blood. Time points are illustrated in Figure 2.

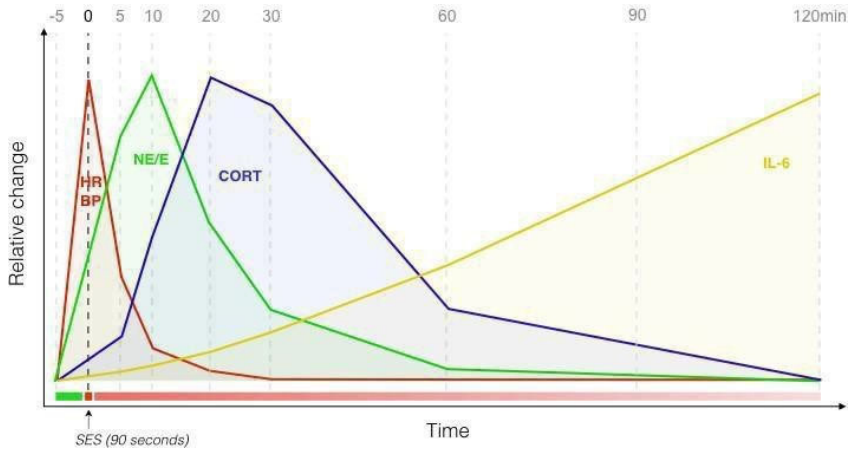

Figure 2. Expected kinetics of stress biomarkers after the socio-evaluative stress. BP = blood pressure; CORT = cortisol; E = epinephrine; HR = heart rate; IL-6 = interleukin-6; NE = norepinephrine.

Affect ratings will be collected at each time point on an iPad to measure subjective feelings of positive and negative affect (calmness and nervousness, see attached in appendix #1.3).

In addition, 1 mL of saliva will be collected with salivettes (Starsted) at each time point. A sterile cotton swab will be introduced in the participant's mouth, soaks with saliva for a period of approximately 2-3 minutes, and is placed back into the tube for subsequent saliva extraction by centrifugation. Oral (sub- lingual) and epidermal (anterior part of neck, back of the dominant hand) temperature will be measured non-invasively at the same time points using a no-touch infrared thermometer (AccuMed AT2102 or HTD8808) to detect thermal changes during stress reactivity and recovery.

At the last time point (+120 minutes), an additional 8.5 mL of blood will be collected to isolate PBMCs to assess effects of stress on epigenetic modifications of DNA and gene expression on sub-populations of cells and one EDTA coated tube (2ml) for complete blood count on whole blood, which totals 10.5ml.

Total blood volume drawn including the fasting morning collection and stress reactivity is approximately 190.5 mL (100 mL baseline for AL, mitochondrial function, and DNA/RNA) + (10 x 8 = 80 mL for stress reactivity biomarkers) + (10.5 mL for DNA/RNA)]. This volume does not exceed the prescribed limit for adult individuals above 110lb of body weight (200mL), and it is less than half the volume of a standard blood donation (approximately 500 mL). After the 120min time point, the venous catheter will be removed.

(D) Deep breathing: Five minutes later, participants will be asked to perform a paced deep breathing exercise with visual feedback lasting two minutes while heart rate is recorded. This will enable assessment of autonomic regulation through breathing.

(E) Standing: Sit-to-stand posture changes elicit changes in heart rate and blood pressure. Participants will be asked to go from the sitting position and stand for 5 minutes. Then sit down again.

(F) Functional capacity: Participants will then be asked to stand as many times as they can, as quickly as possible over the course of 30 seconds. This simple 30 second sit-to-stand test is used to measure functional capacity.

Respiratory rate, heart rate, blood pressure, and skin conductance will be continuously recorded.

If the participant's gait is unsteady or if participants are known to have ataxia or other movement abnormalities, an additional study personnel will be in the room to spot the participant during this task.

(G) Cold pressor test: Participants will be asked to immerse their right hand up to and

including the wrist into a bucket filled with ice cold water (4±0.5°C) for 90 seconds (60). This will be followed by a 10-minute recovery period after which a saliva sample will be collected.

(H) Metabolic Rate Analysis: Participants will then be asked to breathe normally into the ReeVue, a device that measures basal metabolic rate, for 10 minutes. The device analyzes oxygen consumption and uses standard algorithms to convert oxygen consumption to metabolic rate or energy consumption.
The ReeVue breathing hose is specifically designed with a one-way valve such that participants breathe in from the room and out through the device, thus avoiding exposure to any particles from the device. All participants will use a new, disposable breathing tube and nose clip during data collection.

(I) Participants will (a) be de instrumented of equipment.

For most of the afternoon protocol, only 1 researcher will be in the lab space with the participant. The only exception to this is during periods where the nurse will collect a blood sample at the same time as the study coordinator is collecting a saliva sample. This timing is imperative to the study design of this protocol.

The participant will be stationary and instrumented with physiological equipment for the course of the afternoon.

Time estimation task: After being de-instrumented, participants will take part in a time estimation task. For the first part of this task, the coordinator sets a buzzer for a certain number of seconds, and the participant must estimate how much time has passed. For the second part of the task, the participant must say when a certain amount of time has passed. Each of the two parts consists of 10 brief trials of 10, 30, or 60 seconds. In total, this task takes about 20 minutes.

Early MiSBIE participants who did not complete this task during their visit will be recontacted to complete the task over password-protected video call. Calls will take places between 3PM-5PM to replicate timing during normal study procedures.

(6) Urine collection: At the end of the Day 1 visit, participants will be provided with a designated container to collect overnight urine. The container contains a small amount of acetic acid to preserve catecholamines. Participants are provided with a disposable plastic cup(male) or a urine hat(female) and instructed to urinate in the cups, and then poor their

content into the larger container to avoid any potential skin contact with the preservative. The target period will be from 8pm to 8am (12 hours) between Day 1 and 2. The container will be provided into a large sealed "Ziplock" bag and in an opaque tissue bag for transportation. This prevents undesirable odors and minimize psychological discomfort or stigmatization that could be associated with this task. An instructions sheet (attached for review in appendix #1.4) will be provided to all participants along with the container at the end of Day 1. Urine will be processed and biomarkers analyzed on site.

DAY 2

The urine container will be received, and compliance assessed.

(7) Metabolic Rate Analysis: Participants will be asked to breathe normally into the ReeVue for 10 minutes. This will give us information about the participant's resting metabolic rate.

(8) Questionnaires and Neuropsychological Assessment: The participant will be asked to complete a few questionnaires on paper at the hotel both at the day of arrival and at the end of Day 1 (Package #1 and #2). After the breakfast and between the +60 and +120 minute time points of the stress reactivity protocol, participants will complete a small questionnaire package (listed in the "Assessment Instruments" section, Package #3 and #4) on an electronic device (iPad) via an encrypted online system (RedCap) to assess psychosocial variables. Two RedCap projects will be created to manage data: one for screening of potential participants, and one for enrolled participants. RedCap will be used throughout the study to store and export data for analyses.

For participants who have visual processing difficulties, questions can be read aloud to the participant by either the coordinator or caregiver.

On Day 2, participants will also complete another set of questionnaires listed in the "Assessment Instruments" section (Package #5) via RedCap. The participant will be alone while completing the questionnaire package.
Some of the questions included in these questionnaires address suicidal ideation. To assess participant safety, we will monitor participant responses to these questions. If the participant indicates 1 (I have thoughts of killing myself but would not carry them out) on Beck Depression Inventory (BDI) (Day 2 Part 2) plus a moderate BDI score (17 or over) OR Participant rates a 2(I would like to kill myself on BDI) the study coordinator will follow a series of questions to assess risk. If the participant indicates they have a plan, contact Dr. Peter Shapiro, if he is unreachable contact Dr. Luis Periera. If the participant indicates, they have the means and/or a timeframe inmind, this indicates serious risk and the study coordinator will remain with the participant at all times and contact Dr. Peter Shapiro.

In addition, a compliance form will be submitted to the participants at the beginning of Day 1 and Day 2 to ensure they followed the instructions (please see attached in appendix 8 and 9).

Neuropsychological functioning will be assessed by a trained study coordinator using the following instruments:

Measure	Description	Time (minutes)
Wechsler Abbreviated Test of Intelligence (WASI-II) (54)	Estimate of current intellectual function	20
Vocabulary		
Matrix Reasoning		
Neuropsychological Assessment Battery (NAB) (55)		10
Digits Forward and Backward	Working memory	
Shape Learning	Visual learning and memory	
Numbers and Letters	Selective and divided attention	
Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (56)		20
Line Orientation	Visual spatial skills	
List Learning	Verbal learning and memory	
Picture Naming	Word generation	
Coding	Processing speed	
List Recall	Delayed Memory	
List Recognition	Delayed Recognition	
Delis-Kaplan Test of Executive Functions (D- KEFS) (57)		38
Trail Making	Working memory, cognitive flexibility and speed	
Verbal Fluency	Language production	
Color-Word	Inhibition	

Sorting	Mental flexibility and conceptualization	
Test of Premorbid Functioning (TOPF)	Estimate of premorbid intellectual function	2
		90

(9) Brain Imaging: On the second outpatient hospital visit (Day 2) that will take place at the Mortimer B. Zuckerman Mind Brain Behavior Institute (ZMBBI), we will collect fMRI-BOLD data during psychological tasks and rest. ZMBBI now sanitizes the MRI machine before and after each participant. Specifically, we will administer three tasks to assess function of the putative threat/salience network (61, 62), or brain appraisal system (63), a neural network showing stress-related activity that includes the amygdala, anterior cingulate cortex, and hippocampus.

Magnetic resonance imaging will be used with a protocol consisting of one structural scan of high–resolution T1–weighted images, and fMRI blood oxygen level-dependent imaging (BOLD) will be employed over task runs consisting of T2*weighted gradient–echo images. Scanning will take place on a GE MR750 3 Tesla scanner at ZMBBI. These techniques were selected as they are non- invasive and widely used, ideally suited to evaluate functional connectivity, and they will provide measurements built on the previous literature.

Participants will be escorted to ZMBBI on the second day of the study, on the first floor. Upon arrival, participants will be ushered by the study coordinator to a private waiting room in the MRI suite on the basement floor of the ZMBBI building. The urine container will be received, and compliance assessed.

Five minutes after arriving, 1 mL of saliva will be collected with a salivette, as per the previous day. Affect ratings (4 questions) will also be collected, as per the previous day.

For female participants, a pregnancy test will be performed because of potential unknown long-term effects of MRI on fetal development. Participants who refuse to take a pregnancy test prior to the start of the MRI scan for any reason they will forgo their participation in the MRI.

The scanning protocol will last 1.5 hours, including all scanning sequences and set up of the participant in the scanner suite. The participant will be positioned on the MRI table, using a plastic head-holder to decrease head movement during the scan. Participants will be given foam earplugs to reduce the noise of the MRI as well as a MRI-compatible hand held

response pad.

A three-lead ECG will be connected to participants to monitor heart rate responses in conjunction with brain activation.

The task instructions will be projected from a liquid crystal display projector zoomed to a screen inside the MR suite or viewed through dual channel binocular goggles. Functional fMRI-BOLD images will be acquired.

To account for the accessibility needs of patients with visual processing issues or ophthalmoplegia, instructions and text during scanning will be read aloud by the coordinator.

During each pause between acquisitions, the participant is asked to rate their physical discomfort on a scale of 0-10. If the score rises above 5, the coordinator pauses the session and asks if the participant wishes to continue or if any adjustments can be made (i.e. change in position, an additional blanket for warmth, a sitting break).

As on Day 1, at three time points during the MRI imaging session saliva samples will be collected, along with affect ratings (5 questions).

These fMRI tasks will be administered in the following order:

Saliva sample and affect rating #10

(A) Localizer (1 run, 1 min)
Localizing of brain and defining region of interest.

(B) Field map (1 run, 1 min)
Acquisition of field map for multiband correction.

(C) T2 scan (1 run, 12 min)
T2-weighted images for detection of potential lesions and ventricles. This scan will be collected as participants are instructed to “rest and allow their minds to wonder and not to think of anything in particular”. Participants are also instructed not to fall asleep.

(D) T1 structural scan (1 run, 5 min)
T1-weighted images for anatomical co-registration. This scan will be collected as participants are instructed to “rest and allow their minds to wonder and not to think of anything in particular”.

(E) Multisensory (1 run, 5 min)

Participants will be trained and later exposed to the multisensory task. This taskconsists of visual (checker board), auditory stimulation (constant pitch sound), button push on the button box.

(F) Resting State Run #1 (1 run, 11min)

This scan will be collected as participants are instructed to "rest and allow their minds to wonder and not to think of anything in particular". Participants are reminded not to fall asleep.

Saliva sample and affect rating #13

(G) N-back task Run #1 (1 run, 6min)

This working memory task (64) consists of 9 blocks presented in ABBA or BAAB order, interspersed by 25 s of resting fixation. On each of 360 trials, a word is presented for 2 s followed by an inter-stimulus-interval fixation cross for 1 s. Participants are instructed to respond as quickly and accurately as possible to each word with a button press indicating, "yes, this word matches the word presented n-trials ago" (a target response) or "no, this word does not match the one presented n-trials ago" (a nontarget response). The first 3 trials of each block are not included in the analysis; these trials are always nontargets in the 3-back block and therefore did not carry the same task demand as the remaining trials.

(H) N-back task Run #2 (1 run, 6min)

This is the second run of the N-back task. Instructions are the same as in "G".

(I) Socio-evaluative Task (1 run, 6min)

The procedure will be the same as in day 1 except subjects will be told that their "performance on the first speech task was slightly below average when compared with other participants' speeches" and participants will be asked to "try to be more persuasive when delivering this speech". They will be told that the speech will be delivered after the scan session to the expert who evaluated you yesterday. At the end of the task, participants will be told that they were randomly selected not to give their speech, as in previous work. A pulse oximeter will be hooked up to participants, and heart rate responses in conjunction with brain activation will be assessed

(J) Cold Pressor Task (1 run, 6minutes)

This is a modified task from the cold pressor administered on Day 1. Participants will be instrumented with a thermal arm wrap designed specifically for the MRI environment and will be instructed as follows: "We will place this arm wrap around your hand and arm. It is quite cold and may be uncomfortable, but it will not injure you." The arm wrap is a fabric-based foldable unit fitted with Velcro attachments

for detachable gel packs, which are kept in a temperature-controlled freezer prior to the procedure. Five (5) minutes before the procedure, gel packs are removed from the freezer, attached to the arm wrap, and layered with two layers of waterproof film paper to adjust immediate contact temperature to safe but uncomfortable -2±2 degrees Celsius, as previously described (65, 66).

*(K) Diffusion Tensor Imaging – DTI (*Seq 1-2, *6 min)*
This scan will be collected as participants are instructed to "rest, allow their minds to wonder and not to think of anything in particular".

Saliva sample and affect rating #14

(L) Diffusion Tensor Imaging – DTI (Seq 3-6, 15 min
This scan will be collected as participants are instructed to "rest, allow their minds to wonder and not to think of anything in particular".

Participants will then be de-instrumented (chest electrodes removed) and recover their personal belongings that could not be introduced in the MRI room.

We expect all participants to perform all tasks adequately. The total scan time is (1 + 1 + 12 + 5 + 5 + 11 + 5 + 5 + 6 + 6 + 6 + 15 min) 90 minutes. The scanner will be booked for 90 minutes for each participant to accommodate saliva sample #11, instrumentation/de-instrumentation with EKG, time between tasks, and additional setup time.

Finally, participants will be debriefed on the nature of the stress reactivity tasks and the reason for deception. The debriefing script has been uploaded to this protocol (Appendix #7). Specifically, the social-evaluative component is meant to maximize neuroendocrine stress responses (67), and understanding these profiles allows insights into physiological stress functions that are predictive of many health problems. Participants will be reassured that this task was meant to elicit a physiological stress response, not to evaluate them as a person, and that they did very well. Participants will be invited to ask the study staff any question they may have.

(10) Take Home Protocol:

(A) Saliva collection: Starting a week from the study visit, participants will be instructed to provide saliva samples on three non-consecutive weekdays separated by one day. Each participant will receive an iPad with a custom made home logbook

application prompting them to provide four 1 mL saliva samples:

(1) upon awakening, (2) +30 minutes after awakening, (3) +45 minutes after awakening, and (4) before bedtime. Participants will then be given a Medication Event Monitoring System (MEMS 6 TrackCap Monitor, Aardex Ltd., Switzerland) cap container with the salivettes, which time stamps usage of each salivette. In addition, the application will record awakening time, sample collection times, and affect ratings. These measures will insure compliance and document any deviation from the protocol. Instructions for home collection of saliva samples are provided in the Home Logbook application and will be reviewed with the study coordinator at the end of Day 2. The Home Logbook application content is attached with the instruments for review (Appendix #10).

(B) Fecal sample collection: Along with the saliva samples, participants will be provided with a sample collection kit (collection tube, spatula and toilet hat) for fecal collection. The container will be provided into a large sealed "Ziplock" bag. Instruction for fecal collection will be watched with the study coordinator at the end of Day 2 (https://www.youtube.com/watch?v=ytr_hmJdHqM) and an instructions sheet (attached for review in Appendix #11) will be provided to all participants along with the container.

(C) Actigraph: Participant sleep and activity will be monitored over 7 days. Participants will wear a wrist actigraph, a device that detects body movement and weighs about 2 ounces. It is worn comfortably around the wrist, similar to a wristwatch, and will be left in place for the entire 7 days. A pre-stamped express courier box (FedEx) will be provided to participants to return samples upon completion.

(11) Study monitor: Although we do not anticipate any adverse effects of the study procedures, at all times during the study protocol a clinician will be on call in the event that a participant would become distressed. The identified medical doctors are Dr. Li and Dr. Hirano. The Division of Behavioral Medicine, where the laboratory stressor is conducted, has on call clinical psychologists and social workers.

To establish the within-person and technical variability in key study outcomes, we will perform "repeat" visits on two healthy volunteers. This will provide essential information on some novel mitochondrial parameters that are measured in the MiSBIE study (cell bioenergetics, cell-free mitochondrial DNA, metabolic markers), including how they may vary within a person over time, relative to between-person differences. These difficult to obtain data will allow us to quantify intra-individual variation over months-to-years, thus providing an empirical basis upon which to evaluate the magnitude of differences among the primary study outcomes between study groups.

For this purpose, we will obtain blood samples from two individuals at multiple points over the study period. These repeated (MiRep) visits will not be complete study visits, and are therefore not considered as MiSBIE visits for study monitoring purposes. Each visit will take no longer than 15 minutes and will be completed by a trained phlebotomist or by the study nurse. Consent will be completed prior to the blood draw, all study procedures other than the blood draw will be crossed out prior to obtaining a signature by the study coordinator.

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