CELLULAR STRESS & EFFECTS ON MITOCHONDRIAL DNA: AN ASSESSMENT OF STRESS REDUCTION & PROTECTIVE FACTORS

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Abstract

Introduction: Psychological stress results in energy demands and changes in the mitochondria that produce 90% of bodily energy as adenosine triphosphate (ATP). Cell-free mitochondrial DNA (cf-mtDNA) is released into the bloodstream under stress which was induced with the International Affective Picture System (IAPS). We hypothesized that participants exposed to mindfulness meditation (MM) instruction would demonstrate lesser increases in cf-mtDNA levels and negative mood states compared to controls.

Methods: Thirty-five females (18-30 years) were randomized to an experimental group (MM) or a control group (educational podcast). Both groups viewed IAPS images, completed questionnaires and two blood draws.

Results: A paired samples t-test revealed no significant differences in cf-mtDNA levels from pre- to post-IAPS stress exposure between groups.

Conclusion: This randomized controlled trial is the first study to explore the potentially protective effects of MM on cf-mtDNA levels and mood after an acute lab stressor. No statistically significant results were found.

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1. Introduction

1.1. Rationale

Psychological stress affects multiple bodily systems resulting in increases in energy demand and mitochondrial changes (Picard et al., 2018; Trumpff et al., 2019). Mitochondria are subcellular organelles that are central to energy production and contain their own genome represented as mitochondrial DNA or mtDNA (Trumpff et al., 2019). Current knowledge suggests that during adaptation crises, the mtDNA is released from the cell, thereafter referred to as circulating cell-free mitochondrial DNA (ccf-mtDNA). Studies focused on ccf-mtDNA have shown reliable measurements in the blood and other biofluids (Trumpff et al., 2019; Trumpff et al., 2022). For example, recent studies have shown that experimental lab stressors effectively evoke the stress levels that result in a 2-3 fold increase in serum ccf-mtDNA within 30 minutes (Trumpff et al., 2019). In similar studies, a 1.7-fold increase was observed in plasma ccf-mtDNA (Hummel et al., 2018). Similarly, two different cross-sectional studies have found increased levels of plasma ccf-mtDNA in suicide attempters (Lindqvist et al., 2016) and in individuals with major depressive disorder (Lindqvist et al., 2018), suggesting a relationship between negative and stressful psychological states and ccf-mtDNA (Trumpff et al., 2019).

In previous studies, the dominant methods of generating acute (lab) stress involved asking participants to perform a public speaking task as part of the Trier Social Stress Test (TSST). Altogether, the TSST consists of preparing a 2-minute speech during which participants defend themselves against an alleged accusation followed by a 3-minute speech that is videotaped while the participant faces an observer (who is actually a lab staff confederate) dressed in a white lab coat (Carroll et al., 2011; Prather et al., 2009; Trumpff et al., 2019). Besides the current study, one other study employed a lab stressor using the International Affective Picture System (IAPS), by displaying a series of 60 negative emotional images reflecting fear, sadness, anger, and/or frustration. The IAPS differs from the TSST because it requires no deception of participants and evokes multiple emotional stress responses that include but also differ from

social anxiety. Additionally, no studies have used a randomized controlled trial (RCT) to investigate associations between ccf-mtDNA migration (outside the cell) and acute psychological stress exposures where mindfulness meditation was employed as a protective (stress-reducing, stress inoculating) practice in comparison with a control condition consisting of an audio educational podcast.

Additionally, there is discrepancy in the literature regarding whether blood serum or blood plasma is the optimal way to obtain and measure ccf-mtDNA (Trumpff et al., 2021). Some studies have investigated plasma ccf-mtDNA concentrations in patients with psychiatric disorders (Lindqvist et al., 2016; Lindqvist et al., 2018), while other studies have examined serum ccf-mtDNA concentrations after lab stress exposures (Trumpff et al., 2019). According to Trumpff et al. (2021), platelets, found in blood plasma, contain an abundance of mitochondria and mtDNA which may influence ccf-mtDNA preparations by augmenting ccf-mtDNA levels when the speed of centrifugation is low. Similarly, serum has some limitations as the clotting process activates platelets that release additional mtDNA into the sample (Trumpff et al., 2021). Although various methods are aimed at rapidly processing plasma, this study uses serum in ways that are consistent with previous study protocols (Mirzadeh, 2023, Trumpff, et al., 2019).

Since ccf-mtDNA is measurable in the blood of research participants, lab-based assays assist comparisons of ccf-mtDNA levels with mtDNA levels, the latter reflecting mitochondrial DNA still contained in the cell. While multiple studies have examined the role of ccf-mtDNA in male subjects, fewer studies have investigated the role of ccf-mtDNA in female subjects. Thus, the present study evokes stress solely in female subjects (using IAPS photos) to test the hypothesis that the group randomized to receive the pre-stress mindfulness meditation (vs. the pre-stress audio history podcast) will demonstrate lesser increases in ccf-mtDNA levels and negative mood effects.

1.2. Objectives

The objectives of this randomized controlled trial (RCT) are to assess levels of ccf-mtDNA in young, healthy female university students after inducing a lab stressor using the IAPS. The study

specifically aims to compare post-stressor ccf-mtDNA levels in two different groups: in the experimental group that receives meditation instruction, and in the control group that listens to a history podcast.

2. Literature Review

2.1. Mitochondria

Historically, mitochondria have been known as "the powerhouse of the cell" although recent literature additionally emphasize their signaling functions in information exchanges with other mitochondria via proteins and metabolites (Ono et al., 2001; Eisner et al., 2014; Picard et al., 2015). Mitochondria have a double membrane structure, consisting of inner and outer membranes that surround the mitochondrial matrix (Picard & McEwen, 2018). The mitochondrial matrix is an important site for the tricarboxylic acid (TCA) cycle which involves the breakdown of ingested sugars and the β-oxidation pathway where energy is derived from the breakdown of ingested fats (Picard & McEwen, 2018). As mentioned previously, mitochondria contain their own genome called mitochondrial DNA (mtDNA), which contains genes relevant to maintaining a balanced energy flow (Picard & McEwen, 2018). Adenosine triphosphate (ATP) is the biofuel produced from the combination of food molecules and oxygen that fuels multiple cellular activities (Picard & McEwen, 2018).

When the body is psychologically stressed, the structure and function of mitochondria change in ways that, over the long term, can result in an accumulated mtDNA damage (Picard & McEwen, 2018). Notably, balanced energy flows (normally maintained in mitochondria) can be disrupted and lead to mitochondrial dysfunction (Picard et al., 2018). As a result, the influence of psychological stress on mitochondria merits further evaluation.

2.1.1. ATP energy production

The role of mitochondria in ATP production, termed oxidative phosphorylation (OXPHOS) involves the inner mitochondrial membrane, where different proteins and enzymes of the electron

transport chain (ETC) combine to produce ATP. The chain consists of four large complexes (labeled I, II, III and IV) (Chaban et al., 2014) where Complex I is the largest in the ETC and accepts electrons from Nicotinamide Adenine Dinucleotide Hydrogen (NADH) and delivers them to ubiquinone (Chaban et al., 2014). Complex II accepts electrons from Flavin Adenine Dinucleotide Dihydrogen ($FADH_2$) and similarly passes them on to ubiquinone (although complex II differs as it does not directly pump protons) (Chaban et al., 2014). Complex III accepts electrons from ubiquinol and passes them on to another biochemical carrier called cytochrome c (Chaban et al., 2014). Finally, complex IV accepts electrons from cytochrome c and delivers them to a single oxygen molecule. In this process, two water molecules are produced (H_2O) while four protons are pumped into the intermembrane space (IMS) (Chaban et al., 2014). Ultimately, an electrochemical proton gradient is formed across the inner mitochondrial membrane due to the electrons traveling from complexes I to IV (Chaban et al., 2014). The ATP synthase, also known as complex V, uses the energy accumulated in the proton gradient to undertake the synthesis of ATP from ADP (Chaban et al., 2014).

Humans use energy in the form of ATP for neural activity, muscle contraction, food digestion and several other critical cellular activities that occur during resting and stress-evoked conditions (Picard & McEwen, 2018). Stress, a brain and body response when an organism is faced by threats, generates energy output (Picard et al., 2018). Thus stress and energy are interconnected, and mitochondria play important roles in the regulation of stress reactions.

2.2. The Definition of Stress

Claude Bernard, Walter Cannon, and Hans Selye were key developers of stress concepts. Bernard introduced the term the “milieu intérieur” while observing that an organism regulates its internal environment (Goldstein & Kopin, 2007). Cannon built upon the work of Bernard and derived the word “homeostasis”, to describe the body’s self-regulating process of adapting to external change while maintaining the internal environment. He further extended the concept of homeostasis by including

psychosocial threats that lead to "fight or flight" responses (Goldstein & Kopin, 2007). For instance, external threats that activate the sympathoadrenal system (SAS) which is then mobilized to preserve the internal environment with adjustments that ensure survival (Goldstein & Kopin, 2007).

The stress concept was redefined by Hans Selye based on beliefs that stress was the body's response to any and all demands (Selye, 1974). Selye derived a three-stage process termed the "General Adaptation Syndrome" which describes the changes a human body undergoes when exposed to stress (Goldstein & Kopin, 2007). The initial "alarm reaction" stage consists of the "fight or flight" response, followed by an "adaptation" stage where the body counteracts the stressor effects (Goldstein & Kopin, 2007). Finally, the "exhaustion" stage occurs in response to chronic stress and, if appropriate adaptations do not occur, can lead to death (Goldstein & Kopin, 2007).

Lazarus and Folkman have asserted that a transactional perspective is helpful in studying stress and individual coping (1987). They emphasize the importance of viewing stress as a subset of the broader concept of emotion because stress is largely an emotional response, often reflecting fear, anger, guilt, and shame (Lazarus & Folkman, 1987). Positive emotions like joy, happiness, pride, love, and relief can also emerge, depending on how a situation is perceived (Lazarus & Folkman, 1987).

According to Lazarus and Folkman, three sets of ideas are key to understandings of emotion: 1) transaction-relationship, 2) process, and 3) emotion as a system. In reference to transaction-relationship, one must consider person-environment interactions as different people experience threats in particular environments (Lazarus & Folkman, 1987). Secondly, stress and negative or positive emotions change over time as do situations, depending on personal coping processes (Lazarus & Folkman, 1987). As a result, Lazarus and Folkman believe coping must be studied as a process where one observes actual coping via thoughts and actions. Second, coping must be observed in particular contexts, and third, it's important to observe coping responses across particular contexts (Lazarus & Folkman, 1987). Finally, emotion must be viewed as a system that depends on different factors. A systems 'table' (see Table 1, page 7) is useful

in examining: a) person-related variables (e.g. values, commitments, beliefs), b) environmental variables (demands, resources, constraints), c) mediating processes (appraisal and coping), d) immediate effects (e.g. physiological changes), and e) long-term effects (e.g. psychological well-being) (Lazarus & Folkman, 1987).

As a result of the theoretical constructs proposed by Bernard, Cannon, and Selye, and the transactional constructs proposed by Lazarus and Folkman, stress has become an interdisciplinary topic that merits psycho-physiological study. Recent evidence has emphasized the mitochondria responses to psychological stress (Picard et al., 2018). For instance, energy demand increases occur in humans when they encounter psychological stress (Picard et al., 2018). Psychological stress ultimately leads to physical changes such as increases in heart rate and blood pressure (Schubert et al., 2009). Cognitive processing stress (Picard et al., 2018) leads to increases in the brain's energy demands, and the brain's mitochondrial activity (Magistretti & Allaman, 2015; Picard et al., 2018).

Table 1. Illustrative System Variables for The Emotion Process

Causal antecedents ⟶	**Mediating processes** ⟶	**Immediate effects** ⟶	**Long-term effects**
Personal variables: Values, commitments, and goals General beliefs, e.g. Self-esteem Mastery Sense of control Interpersonal trust Existential beliefs Environmental variables: Demands Resources, e.g. social support network Constraints Temporal aspects	Primary appraisal (stakes) Secondary appraisal (coping options) Coping (including use of social support): Problem-focused forms Emotion-focused forms	Affect Physiological changes Quality of encounter outcome	Psychological well-being Somatic health/illness Social functioning

2.2.1. Different Types of Stress

Picard and colleagues have distinguished between three different types of stress: good, tolerable, and toxic. “Good stress” is the type experienced when facing challenges such as job interviews or large audience presentations. These challenges are often linked to related, achievable rewards (Picard et al., 2018). During ‘good stress’, the biochemical stress mediators of cortisol and adrenalin are released and activated during challenge conditions and become inactive when the challenge has been met (Picard et al., 2018). On the other hand, “tolerable stress” refers to experiences that may be severe and traumatic such as the death of a loved one, job loss, or the end of a longtime relationship (Picard et al., 2018). In tolerable stress, the individual has resources and support systems from which they can derive help with recovery. An individual undergoing ‘tolerable stress’ will have stress mediators that may activate or become inactive repeatedly (Picard et al., 2018). The prolonged activation of the stress mediators may result in various pathophysiological consequences such as cardiovascular disease and functional problems. The individual

retains internal and external resources that help them cope with challenges and, in doing so, limit negative health consequences (Picard et al., 2018). Lastly, "toxic stress" refers to negative experiences that resemble those of "tolerable stress", but the individual does not have enough of the internal and external resources that enable stressor transcendence (Picard et al., 2018). As a result, this person may feel they don't have enough control in their life which can lead to chronic pathophysiological consequences (Picard et al., 2018). Ultimately, stress differs from person to person depending on duration, severity, and control leading to the differentiating terms of "allostatic load" or "overload" (Picard et al., 2018).

2.3. Allostatic Load/Overload

Allostatic load refers to the cumulative effects of stress mediators when overused and dysregulated for lengthy periods (Picard et al., 2018). In other words, when the mediators cannot help the body adapt to stress and become overused, pathophysiology may be the result (Picard et al., 2018). Consequences of stress-induced allostatic load can include health reducing behaviors such as alcohol, smoking, lack of sleep, and lack of exercise (Picard et al., 2018). Similarly, *allostatic overload* is an intense form of allostatic load where cumulative changes in the body are due to the *chronic* overuse of the stress mediators and poor health behaviors leading to disease (Picard & McEwen, 2018; Picard et al., 2018). An example is high blood pressure leading to the blockage of the coronary artery that may cause insulin resistance in individuals with type 2 diabetes (Després & Lemieux, 2006; Picard et al., 2018).

2.3.1. Mitochondrial Allostatic Load

Mitochondria have their own form of allostatic load called Mitochondrial Allostatic Load (MAL) (Picard & McEwen, 2018). MAL contributes to the allostatic load and overload of individuals via three functions, notably 1) sensing, 2) integrating, and 3) signaling information about the external environment (see Figure 1, page 10) (Picard & McEwen, 2018). During the *sensing* stage, different psychosocial factors and stressors such as adverse childhood experiences, gender discrimination, low socioeconomic status, social isolation, and caregiving burdens can negatively affect the individual's life (Picard & McEwen,

2018). If the individual does not have the internal and external resources required to overcome the stressor, the mitochondria sense stress mediators that then affect adaptations of mitochondrial structure and function (Picard & McEwen, 2018).

During the *integration* process, mitochondria communicate with one another as well as with stress mediators (Picard & McEwen, 2018). Some stress mediators involved are physiological such as glucocorticoids, catecholamines, sex hormones, pro-inflammatory and anti-inflammatory cytokines, parasympathetic and sympathetic activity, oxidative stress, and brain atrophy (Picard & McEwen, 2018). Conversely, other stress mediators take behavioral forms such as insufficient exercise, poor diet, and sleep disturbance (Picard & McEwen, 2018). The chronic activation of these stress mediators can change the structure and function of mitochondria. Some mitochondrial recalibrations include excess production of reactive oxygen species (ROS), mitochondrial DNA (mtDNA) damage (including mutations and deletions), lower levels of energy production, and the release of pro-inflammatory signals such as ccf-mtDNA (Picard & McEwen, 2018).

The final stage occurs when mitochondria produce *signaling* molecules that influence pathophysiological processes at the cellular and systemic levels. These cellular-systemic changes include the shortening of telomeres, alterations in gene expression, increases in mtDNA copy number, cellular stress, vulnerability, fatigue, and chronic inflammation (Picard & McEwen, 2018). Overall, chronic exposure to psychosocial factors and stressors can lead to MAL which may then result in negative health outcomes like accelerated aging and elevated disease risk (Picard & McEwen, 2018).

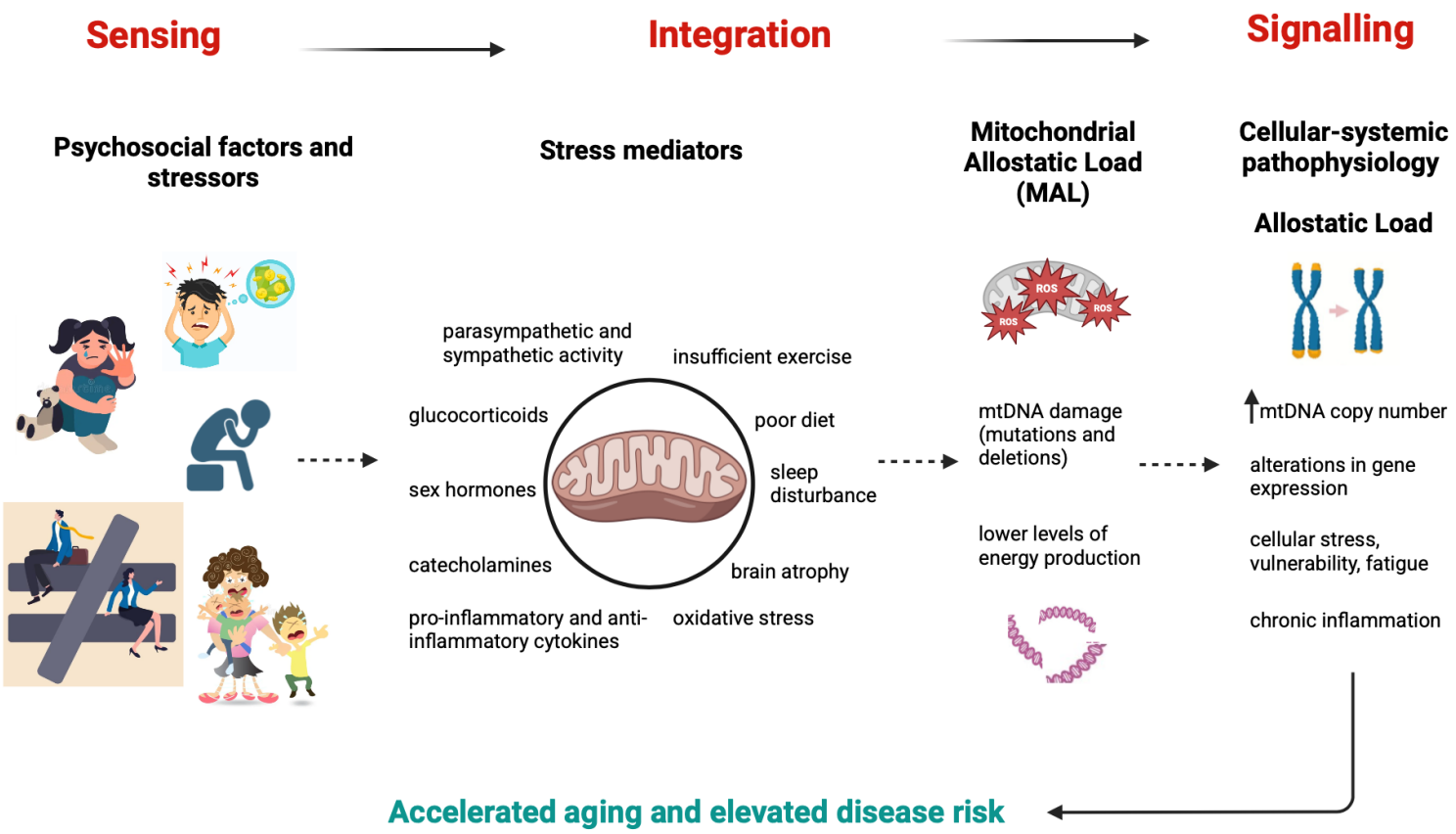

Figure 1. Visual Representation of The Mitochondrial Stress Model

2.4. Mitochondrial DNA vs Nuclear DNA

Each cell in the body contains multiple mitochondria with each mitochondrion containing its own genome (i.e. mitochondrial DNA (mtDNA)) (Gambardella et al., 2019). Human mtDNA replicates independently of nuclear DNA and differs from the nuclear DNA in many ways (Gambardella et al., 2019). For example, mtDNA is comprised of circular molecules containing 16,569 DNA base pairs, while the nuclear DNA is made up of linear molecules containing 3.2 billion DNA base pairs (Gambardella et al., 2019). Also mtDNA contains only 37 genes, 13 of which are protein coding genes that provide instructions for producing the enzymes involved in oxidative phosphorylation (Gambardella et al., 2019).

Furthermore, mtDNA is more vulnerable to oxidative stress than nuclear DNA because it lacks protective proteins, such as histones, and DNA repair mechanisms (Gambardella et al., 2019). Since mtDNA is not compacted around histones, it is more susceptible to DNA damage because of the high levels of reactive oxygen species (ROS) generated during oxidative phosphorylation that are located near the mtDNA (Gambardella et al., 2019). Within high oxidative stress environments, mtDNA is 10- to 200-times more prone to genetic mutations (known as mutagenesis) than nuclear DNA (Gambardella et al., 2019). As a result, neurons and myocytes are more vulnerable to damage mediated by ROS (Gambardella et al., 2019).

2.5. Mitochondrial ROS Production and Antioxidant Defense Systems

ROS are generated during mitochondrial oxidative phosphorylation as 0.2% of the oxygen consumed is converted into a relatively *unstable* molecule termed superoxide (Addabbo et al., 2009). If the superoxide is not detoxified from the system by a neuroprotective enzyme termed superoxide dismutase (SOD), mitochondrial functions are likely to decline over time (Addabbo et al., 2009). The prime sites of ROS generation in the ETC are mitochondrial enzyme complexes I and III as electrons leak from the ETC and interact with oxygen to produce superoxide (Addabbo et al., 2009).

Mitochondria also have ample enzymatic and nonenzymatic antioxidant defense systems that detoxify ROS production. The nonenzymatic antioxidant defense systems include the biochemicals cytochrome c, α-tocopherol, ascorbic acid, reduced coenzyme Q10, and glutathione, while the enzymatic antioxidant defense systems include manganese SOD, catalase, glutathione peroxidase, and phospholipid hydroperoxide glutathione peroxidase (Addabbo et al., 2009). When there is a balance between ROS production and the cellular antioxidant defense capacity, the mitochondrial structures and functions are more likely to remain intact. However, reduced antioxidant defense capacities can lead to more cellular ROS production and more oxidative stress, which can further damage cellular proteins and lipids (Addabbo et al., 2009).

2.6. Mitochondrial DNA Release and the Mitochondrial Permeability Transition Pore

The mitochondrial permeability transition pore (mPTP) is a non-specific channel found in the inner mitochondrial membrane that plays an important role in releasing mitochondrial DNA (Baines, 2009). The pore opens up more frequently under conditions of stress, such as when there are increases in ROS production or Ca^{2+} overload (Baines, 2009). Conversely, when mitochondria are healthy and functioning well, the mPTP opens less frequently as a result of the actions of inhibitors such as ATP and ADP (Baines, 2009). Furthermore, various lifestyle factors, such as exercise, can influence mPTP opening. Gonçalves et al. (2016) have shown that endurance training decreases the frequency of mPTP opening and therefore, the result is less mtDNA release. As a result, one can conclude that regular exercise limits pore opening and improves the functional quality of mitochondria.

2.7. ccf-mtDNA

Circulating cell-free mitochondrial DNA (ccf-mtDNA) appears in blood plasma as a result of the release of mitochondrial DNA fragments outside the cell (Gambardella et al., 2019). Previous studies have found elevated plasma ccf-mtDNA levels in critically ill hospitalized patients who were at 4- to 8-times elevated risks of mortality (Nakahira et al., 2013; Trumpff et al., 2021).

Accumulations of oxidative damage can result in selective degradations of mitochondria through a process called macroautophagy (Moya et al., 2021). During macroautophagy, mtDNA is released into the cytosol and then lysosomes digest various cellular contents via the autophagosome (Moya et al., 2021), a double membrane-bound vesicle consisting of endoplasmic reticulum membrane components and multiple protein complexes (Moya et al., 2021). The autophagosome can tightly encapsulate mitochondria and then fuse with the lysosomal membrane, ultimately forming an autolysosome (Moya et al., 2021). DNase II is an important autophagocytic enzyme that degrades oxidized mtDNA (Moya et al., 2021) by a cleavage (hydrolysis) of the phosphodiester bonds in the DNA backbone. High levels of ROS formation in the mitochondrial matrix can result in the nuclease activity of DNase II becoming saturated in ways that

ultimately prevent the degradation of mtDNA. Thus mtDNA is released into the bloodstream as ccf-mtDNA (Moya et al., 2021). Additionally, mtDNA is released from the cell due to an increase in the permeability of the inner mitochondrial membrane (Moya et al., 2021). This process occurs because increased oxidative stress leads to an increased Ca^{2+} concentration within the matrix which results in the opening of the mPTP (Moya et al., 2021). During normal homeostatic conditions, the mPTP opens transiently to regulate ion levels, small molecules, and mitochondrial matrix proteins (Moya et al., 2021). However, when the mPTP remains open for a lengthy period as a result of high oxidative stress conditions, mtDNA escapes into the bloodstream as ccf-mtDNA (Moya et al., 2021).

Lastly, mtDNA can further be released through the process of cell death termed apoptosis (Moya et al., 2021). While this specific process is still being intensively studied, a current hypothesis is that apoptosis results in the degradation of the cell membrane which ultimately leads to mtDNA being released into the blood circulation as ccf-mtDNA (Moya et al., 2021).

2.7.1. ccf-mtDNA and Psychological Distress

Many studies have indicated a link between ccf-mtDNA and psychological distress. Lindqvist and colleagues found elevated levels of ccf-mtDNA in patients with major depressive disorder (MDD) when compared to healthy controls (2018). A subsample of the MDD subjects were treated with a selective serotonin reuptake inhibitor (SSRI) antidepressant to evaluate whether this treatment would alter ccf-mtDNA levels (Lindqvist et al., 2018). The results showed that non-responders to the SSRI treatment had a further increase in ccf-mtDNA levels from baseline to week 8 of the treatment, while the SSRI responders did *not* show significant changes in ccf-mtDNA levels at week 8 (Lindqvist et al., 2018). These results suggest that SSRI treatment may have therapeutic effects, evident in mitochondria activities in individuals with MDD. Furthermore, elevated levels of plasma ccf-mtDNA were found in suicide attempters who had undergone a dexamethasone suppression test (DST) versus healthy controls (Lindqvist et al., 2016). The results showed that high plasma ccf-mtDNA levels were associated with high levels of

cortisol after completing the DST (Lindqvist et al., 2016). Multiple publications have also demonstrated increased levels of ccf-mtDNA in patients with diabetes, cancer, trauma, myocardial infarction, and sepsis (as reviewed by (Lindqvist et al., 2016)), providing further support for a relationship between ccf-mtDNA and psychological distress.

2.7.2. ccf-mtDNA and SARS-CoV-2

Mitochondria can also be negatively impacted by viral infections such as the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Recent studies suggest that ccf-mtDNA may play a role in the onset of SARS-CoV-2 as seen in the body's inflammatory responses to the infection (Mahmoodpoor et al., 2023). Recent evidence also suggests that signals, such as the toll-like receptor 9 (TLR9) and the cyclic guanosine monophosphate-adenosine monophosphate synthase stimulators of interferon genes (cGAS-STING), play an important role in the immune responses to SARS-CoV-2 (Mahmoodpoor et al., 2023). One study examined the ccf-mtDNA levels in 102 patients hospitalized with SARS-CoV-2 (COVID-19) (Mahmoodpoor et al., 2023). The results showed that there was a significant association between ccf-mtDNA levels and COVID-19 severity (determined by intensive care unit (ICU) admission, intubation, or mortality) (Mahmoodpoor et al., 2023). Furthermore, this study provides evidence that mitochondrial function is disrupted after a viral infection leading to the release of ccf-mtDNA into the blood circulation (Mahmoodpoor et al., 2023). This release then puts the body in a pro-inflammatory state which can worsen existing disease (Mahmoodpoor et al., 2023). As a result, one can conclude that higher ccf-mtDNA levels may be an early prognostic indicator of SARS-CoV-2 (Mahmoodpoor et al., 2023). Future studies will be aimed at determining potential therapeutic interventions in patients diagnosed with COVID-19.

2.7.3. ccf-mtDNA and Exercise

Exercise is a physical stressor type that can promote oxidative damage and inflammation, especially in individuals who engage in prolonged or novel exercise (Shockett et al., 2016). Shockett and

colleagues examined the effects of 90 minutes of prolonged moderate aerobic exercise on ccf-mtDNA responses in seven, moderately trained male youth who ran on a treadmill (at 60% of VO_2 max) versus a resting control group (same subjects used in both groups) (2016). Blood samples were taken at five different time points: at baseline (0 min), during exercise (18 min and 54 min), immediately after exercise (90 min), and after recovery (Shockett et al., 2016). The results showed that plasma ccf-mtDNA levels significantly reduced during exercise (54 min) and immediately after exercise (90 min) in the exercise group (Shockett et al., 2016). This study indicates that different exercise types and intensities, such as prolonged treadmill exercise, can reduce the release of ccf-mtDNA into the blood. Another study found that professional male volleyball players had lower plasma ccf-mtDNA levels during two consecutive seasons than the non-athlete male volunteers (Nasi et al., 2016). This study suggests that individuals who engage in regular exercise training may have reduced baseline levels of ccf-mtDNA, implying that exercise can have protective anti-inflammatory effects (Nasi et al., 2016).

Contrary findings, however, were observed in a study done by Stawski and colleagues which examined increases in ccf-mtDNA after three repeated bouts of *exhaustive* treadmill exercise (at 70% of VO_2 max) in 11 healthy men (2017). There was a 72- hour rest period between each of the three treadmill exercise tests, and blood samples were taken immediately before and after each exercise bout (Stawski et al., 2017). The results showed that plasma ccf-mtDNA levels increased after each bout of exhaustive exercise (separated by 3 days) in healthy, moderately trained men (Stawski et al., 2017). The results of this study suggest that engaging in short, high intensity exercises may induce stress (as indicated by the increases in ccf-mtDNA levels) in individuals who are not active on a regular basis.

All of the above-mentioned studies explored the effects of acute exercise in male subjects, but not female subjects. Thus, it's important for future studies to assess the effects of acute exercise in females since there are sex differences in ccf-mtDNA levels. Furthermore, most of the current literature on acute exercise shows a mixture of results on ccf-mtDNA levels (Trumpff et al., 2021). These mixed ccf-mtDNA

outcomes could be due to small sample sizes, different timings of the blood sampling, differing study protocols, and different instances of ROS formation. Although regular exercise promotes the normal functioning of skeletal muscle, engaging in exhaustive exercise (as seen in the study by Stawksi et al) can lead to increased and excess ROS formation (Wang et al., 2021). As mentioned previously, an increase in ROS formation can result in an imbalance between ROS production and the cellular antioxidant defense capacity, leading to cellular damage.

2.8. Mindfulness Meditation

Mindfulness meditation is a mental training technique that has evoked interest in psychophysiological and mental health researchers. Mindfulness emphasizes the importance of self-regulating attention to conscious awareness of experiences within a prevailing attitude of acceptance (Bishop et al., 2004). Mindfulness can therefore be conveyed and personally applied as a discrete skill that is practiced when attention is focused on the present time-frame, i.e. 'being in the moment' (Bishop et al., 2004). Engaging in mindfulness meditation often promotes a relaxation effect as when attention is directed to breathing experiences in the approach termed 'mindfulness of breath' (Azam et al., 2015). Many studies have shown that mindfulness meditation practices have positive effects on cognitive function and emotion regulation (as well as healthy effects on the brain regions associated with attention) and has a reductive effect on chronic stress (Azam et al., 2015). As a result, the current study aims to explore mitochondrial responses when mindfulness meditation is practiced (prior to exposures to a lab stressor) versus a control condition of listening to a history podcast.

2.9. Hypotheses

1. Participants in the mindfulness meditation (MM) instruction group, compared to the participants in the control group (educational podcast) will demonstrate lower levels of serum ccf-mtDNA after exposure to the IAPS lab stressor.

2. Participants in the mindfulness meditation (MM) instruction group, compared to the control group participants (educational podcast), will demonstrate lower scores on the POMS-2 Anger-Hostility, Confusion-Bewilderment, Depression-Dejection, Tension-Anxiety, and Fatigue-Inertia subscales, after exposure to the IAPS lab stressor.

3. Methods

3.1. Study Design

This is a clinical trial study which adheres to the 2010 CONSORT Guideline. This study was registered in ISRCTN with the registration number ISRCTN16624353 on July 18, 2025. The study design (see Figure 2, page 26), consent form and the recruitment poster were reviewed and approved by the Human Participants Review Committee (certificate #: 2025-130) at York University.

3.2. Participants and Recruitment

A group of 35 female participants between 18-30 years were approached for recruitment purposes through posters and emails at York University. During recruitment discussions, nine participants declined to participate and were excluded prior to randomization. All remaining eligible (n = 26) participants completed baseline questionnaires and after questionnaire were randomized into two groups using a computer random number generator in Microsoft Excel: 1) participants randomized to the experimental group received a mindfulness meditation instruction (via audio) prior to the lab stress induction and; 2) participants randomized to the control group received an educational podcast (via audio) prior to the lab stressor. The computer random number generator based in Microsoft Excel is a well-accepted, reliable and appropriate technique for randomization tasks in RCTs (Baghbaninaghadehi et al., 2016; Bi et al., 2024). Both of the participant groups had two blood draws taken: one at baseline (before the audio interventions) and a second after an interval of 70-80 minutes. Four participants were excluded after randomization due to difficulties with baseline blood draws due to exceptionally small veins. During the interval period between blood draws, the experimental group listened to a 30-minute meditation audio while the control group listened to a 30-minute educational podcast. Both groups next viewed a series of stress inducing pictures from the IAPS database (see Figure 5, page 34). One participant was excluded post-study because additional data indicated a previous exposure to the IAPS photographs in a prior study.

3.2.1. Randomization and Blinding

Computer-generated random numbers were derived by Jasmin Tiwana (JT) in Microsoft Excel using the RAND ( ) function to ensure an even and random distribution of participants to the experimental and control groups (Bi et al., 2024). The following randomization method was used: 1) Column A listed the participants from P01 to P30; 2) In column B2, the formula "=RAND ( )" was entered to generate a random number between 0 and 1, and the formula was applied, i.e. dragged down, for all participants. The data in column B were sorted by smallest to largest values which randomized the participant list; 3) In column C2, the formula "=IF(ROW()-1<=15, "Experimental", "Control")" was entered and applied (dragged down) to include all participants. Due to insufficient staff, the principal investigator (JT) and co-investigator Paul Ritvo (PR) were not blinded, although all other staff were blinded to participant allocation (i.e. staff conducting the blood draw, and the lab stress protocol).

3.2.2. Inclusion Criteria

1. Females between 18-30 years of age; 2. Maintain residency in Canada; 3. General good health as defined by no history of asthma, current cancer diagnosis or treatment, history of myocardial infarction, and/or systematic immune diseases; 4. Non-smokers

3.2.3. Exclusion Criteria

1. Pregnant and/or lactating women; 2. Current or previous (during the last three months) diagnosable or self-reported mental health problems; 3. Chronic or acute physical disabilities or injuries; 4. Current intake of prescribed medications that may interfere with endocrine, nervous and immune system operations (the only exceptions are oral contraceptives); 5. No antibiotic use, known infections and vaccination symptoms; 6. No new tattoos within 2 weeks of the blood withdraw

3.3. Psychometric Assessments

3.3.1. Self-Administered Comorbidity Questionnaire (SCQ) (Appendix A)

The Self-Administered Comorbidity Questionnaire (SCQ) is a self-report tool used in clinical settings that assesses the severity of comorbid conditions in individuals (Sangha et al., 2003). The SCQ is useful in research settings because it's readily understood and can be completed by individuals without help from a medical professional (Sangha et al., 2003). The SCQ also has an excellent test-retest reliability coefficient of 0.94 (n = 26 patients who received surgery at the Brigham and Women's Hospital) (Sangha et al., 2003).

3.3.2. International Physical Activity Questionnaire (IPAQ-short form) (Appendix B)

The International Physical Activity Questionnaire (IPAQ) is a self-administered measure of the time durations spent in low, moderate, or vigorous physical activity per week in young and middle-aged adults (Craig et al., 2017). The IPAQ contains 7 items that assess the intensity of physical activity that individuals engage in during daily living in order to estimate their quantities of metabolic equivalent (MET) minutes per week (Craig et al., 2017). Additionally, the IPAQ has a good test-retest reliability coefficient of 0.80 and has shown acceptable levels of predictive, concurrent, convergent, criterion, and discriminant validity (Craig et al., 2017).

3.3.3. The Profile of Mood States, Second Edition (POMS-2) (Appendix C)

The Profile of Mood States, Second Edition (POMS-2), is a psychological assessment tool that measures the 6 different mood state dimensions: tension, depression, anger, vigor, fatigue, and confusion (Heuchert & McNair, 2012). The POMS-2 contains 65 statements to which participants respond based on a 5-point Likert scale: 0 (not at all); 1 (a little); 2 (moderately); 3 (quite a bit); 4 (extremely) (Heuchert & McNair, 2012). The POMS-2 has been used in various populations such as hospital patients, university students, athletes, and older adults (Heuchert & McNair, 2012). It has been shown to have good internal

consistency (measured with Cronbach's alpha) with alpha coefficients that range from 0.76-0.95 for normative samples and from 0.83-0.97 for clinical samples (Heuchert & McNair, 2012). Similarly, the POMS-2 has good test-retest reliability, with test-retest coefficients ranging from 0.48-0.72 (Heuchert & McNair, 2012).

3.3.4. International Affective Picture System (IAPS)

The International Affective Picture System (IAPS), first introduced by Lang and colleagues in 1997, displays a diverse set of emotion-provoking pictures that are designed to induce emotions of fear, anger, sadness, frustration or pleasure, in research participants. The images depict a variety of life events related to negative human experiences such as wars and disasters, and mutilation imagery of multiple bodies (Bradley et al., 2020). There are also positive images of loving families.

Multiple participant groups have viewed the images and then rated them according to inquiries about feelings of pleasure, arousal, and dominance (Bradley et al., 2020). The IAPS has been shown to be a reliable and valid assessment tool with a good level of consistency in research areas investigating mental disorders and more recently, psychophysiological differences (as reviewed by (Dufey et al., 2011)). During the stress protocol in the current study, participants viewed 60 emotion-evoking images selected from the IAPS database. The images were presented on a laptop computer using Microsoft PowerPoint, with each image displayed for 10 seconds. All selected photos from the IAPS database were carefully screened and approved by the clinical research psychologist, Paul Ritvo (PR), who supervised this study. The 60 photographs correspond to the following sequential ID numbers: 1670, 7016, 2352.1, 2352.2, 2770, 1620, 3001, 1920, 3000, 1617, 1750, 2900, 3030, 3051, 1610, 9043, 6250, 3101, 5600, 9400, 9405, 1560, 3266, 1590, 3050, 3060, 1630, 6530, 3103, 9041, 1710, 1999, 7521, 9163, 1274, 9427, 5781, 3195, 3110, 6838, 3170, 3019, 5825, 2880, 3022, 9910, 3010, 1410, 1525, 1120, 3071, 6837, 3100, 1111, 2655, 9187, 9570, 6520, 5001, 5665.

3.3.5. Menstrual Distress Questionnaire (MEDI-Q) (Appendix D)

The Menstrual Distress Questionnaire (MEDI-Q) is a 25-item assessment tool that evaluates various menstrual-related symptoms, such as pain, bloating, emotional and physical changes, headaches, fatigue, and gastrointestinal distress (Cassioli et al., 2023). It examines four general indices: 1) MEDI-Q Total Score; 2) MEDI-Q Menstrual Symptoms (MS); 3) MEDI-Q Menstrual Symptoms Distress (MSD); and 4) MEDI-Q Menstrual Specificity Index (MESI) (Cassioli et al., 2023). The MEDI-Q has also been demonstrated to have a good intraclass correlation coefficient (ICC) of 0.95 among the responses of English-speaking participants that were measured seven days apart (Cassioli et al., 2023). This high ICC indicates good consistency and confirms the test-retest reliability of the MEDI-Q. Additionally, the MEDI-Q has shown good internal consistency (measured with Cronbach's alpha) where alpha coefficients range from 0.81-0.86 (Cassioli et al., 2023). The MEDI-Q was included to assess whether mood and stress responses could be influenced by the menstrual cycle.

3.3.6. COVID-19 Screening (Appendix E)

A brief COVID-19 screening questionnaire was administered to ensure that potential health risks were minimized for all participants. Individuals who reported COVID-like symptoms were either excluded from the study or rescheduled for another date.

3.3.7. Body Mass Index (BMI) (Appendix F)

Body Mass Index (BMI) was calculated based on the participant's self-reported height and weight. The formula to calculate BMI is kg/m^2, where kg represents the person's body weight in kilograms and m^2 their height in meters squared.

3.3.8. Demographic Self-Report Questionnaire (Appendix F)

The demographic self-report questionnaire includes questions regarding: 1) country of birth; 2) years of residence in Canada; 3) race identification (White, Black, South Asian, Chinese, & Other); and

4) whether the individual experienced what they consider a highly stressful event in the last 12 months. This self-assessment was used in three randomized controlled trials involving university students (Ahmad et al., 2020).

3.3.9. Pre and Post Blood Draw Questionnaires (Appendices G)

Pre and post blood draw questionnaires were administered to assess how participants felt during and after the blood draw procedure. The pre-blood draw questionnaire included items related to: previous experiences with blood draws, current feelings about the upcoming blood draw (based on a calm-distress 7-point scale), specific blood draw concerns (such as pain or discomfort, bruising, becoming dizzy or faint, fear of needles), and a possible history of fainting during previous blood draws. The post-blood draw questionnaire contained information on current feelings after having the blood drawn (based on a calm-distress 7-point scale) and whether specific blood draw concerns were experienced.

3.4. Procedures

3.4.1. Prior to the study:

This study was conducted on weekdays from Monday to Friday with one to two participants assessed each day. This study took place in the mornings from 9AM-12PM so that the stress protocol was performed when the individual is at a lower risk of stress and diet related factors (Trumpff et al., 2019). Additionally, participants were asked to refrain from consuming food and caffeinated drinks (except water) 12 hours before the study (Trumpff et al., 2019). They were also instructed to abstain from non-prescription medications and strenuous physical activities 24 hours before the test, and from alcoholic beverages 48 hours before the experiment (Trumpff et al., 2019). Prior to the study date, participants received emailed instructions to meet at the lab office (Chemistry Building 138) on the scheduled day for 9:30AM.

3.4.2. Baseline Questionnaire Administration

Once participants arrived at room 138 in the Chemistry Building, they completed the informed consent form approved by the Human Participant Research Committee of York University. Afterwards, they completed the following questionnaires: 1) COVID-19 screening; 2) Self-Administered Comorbidity Questionnaire (SCQ); 3) International Physical Activity Questionnaire (IPAQ-short form); 4) Menstrual Distress Questionnaire (MEDI-Q); 5) BMI and Demographic Self-Report Questionnaire.

3.4.3. Randomization and Intervention Assignment

Once the informed consent form and baseline questionnaires were completed, participants then completed the first Profile of Mood States (POMS) questionnaire for an evaluation of baseline mood states (prior to stress exposure). Following questionnaire completion, participants were randomly allocated to the experimental condition (mindfulness meditation) or the control condition (educational podcast) using the computer random number generator in Microsoft Excel.

3.4.4. Baseline (1st blood draw)

After randomization, participants were escorted to Norman Bethune 123 for the baseline blood draw. Participants then completed the Pre-Blood Draw Questionnaire to assess feelings about the upcoming procedure. Next, about 6 ml of blood was collected from the antecubital fossa of one arm (front part of elbow) using a standard venipuncture technique by a qualified person. Participants were then escorted back to 138 Chemistry Building where they were exposed to the assigned intervention (mindfulness meditation or educational podcast) followed by the stress induction protocol (exposure to IAPS photographs).

3.4.5. Experimental Arm

Experimental group participants listened to a 30-minute meditation audio while wearing headphones provided by the lab. Participants were subsequently exposed to the International Affective

Picture System (IAPS) (stress-inducing photographs) designed to reflect fear, sadness, anger, and/or frustration. Participants viewed the slideshow of images on a laptop computer. Once the images on the slideshow ended, participants completed the POMS questionnaire for a second and final time to assess changes in mood states following lab stress induction. Following completion of the follow-up POMS questionnaire, participants rested quietly for 20 minutes (resting phase) and were closely observed to confirm their abstinence from distracting activities. This 20-minute resting period was deemed sufficient to capture immediate post-stress changes.

3.4.6. Control Arm

Control group participants listened to a 30-minute educational podcast while wearing headphones (identical to those worn by the intervention group) provided by the lab. Then, participants were exposed to the International Affective Picture System (IAPS) (i.e. the same artificial stressor photographs as intervention participants) reflecting fear, sadness, anger, and/or frustration. Participants were instructed to view the slideshow of images on a laptop computer. Once the images on the slideshow ended, participants completed the POMS questionnaire for a second and final time to assess changes in mood states following lab stress exposure. Following completion of the follow-up POMS questionnaire, participants rested quietly for 20 minutes (resting phase) while abstaining from distracting activities. This 20-minute resting period was deemed sufficient to capture immediate post-stress changes.

3.4.7. 2nd Blood draw

After the resting phase, participants were escorted back to 123 Norman Bethune building where they had a final 6 ml of blood collected from the antecubital fossa of one arm (front part of elbow) using the standard venipuncture technique by a qualified person. After this blood draw, participants completed the Post-Blood Draw Questionnaire to assess responses following the procedure. All blood draws and artificial stressor exposure took place during mornings between 10:00-11:30AM to control for possible differences in metabolism and blood composition.

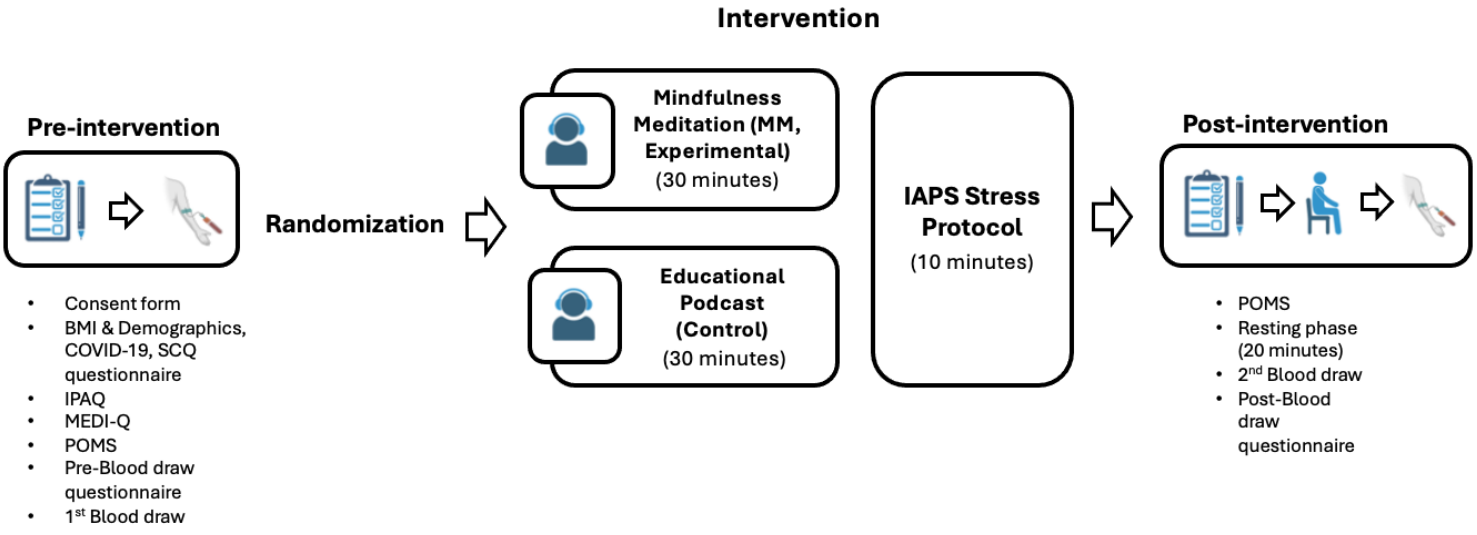

Figure 2. Visual Representation of Study Procedures

3.5. Plasma Versus Serum Collection

The main difference between plasma and serum is that plasma is the liquid component of blood collected by using an anticoagulant to prevent clotting, whereas serum is the liquid part collected *after* the blood has clotted (without an anticoagulant) (Trumpff et al., 2021). As mentioned previously, platelets found in blood plasma contain high amounts of mitochondria and mtDNA which may alter ccf-mtDNA measurements, especially if the centrifugation speed is low (Trumpff et al., 2021). Additionally, serum has its drawbacks as platelets become activated during the clotting process which may release additional mtDNA into the blood sample, thus increasing cf-mtDNA levels (Trumpff et al., 2021). This study used serum to maintain consistency with previous study protocols for measuring ccf-mtDNA levels (Mirzadeh, 2023).

3.6. Serum Isolation Protocol for ccf-mtDNA Measurements

In this study, blood was first collected from participants via serum clot activator tubes. The inner walls of these tubes contain a gel-formed clot activator which activates the blood coagulation process once blood enters into the tube (Michelson et al., 2023). The blood samples were then left alone to clot at room temperature in serum clot activator tubes for ~30 minutes. After the 30 minutes of clotting, a 3 spin centrifugation of blood took place. The first centrifugation of the serum tubes occurred immediately (1,000 X G for 10 min at room temperature) after the 30-minute clotting period (Michelson et al., 2023). After the first spin, 80% of the serum from the tube was transferred to a new 15 ml conical polypropylene centrifuge tube to separate the serum from the red blood cells (Michelson et al., 2023). The remaining 20% of the serum was transferred to a 1.5 ml disposable microcentrifuge tube (red). Then, a second spin occurred at 2,000 X G for 10 minutes at room temperature (Michelson et al., 2023). After, 90% of the serum was transferred to a new 15 ml conical polypropylene centrifuge tube while the remainder 10% was again transferred to the same 1.5 ml disposable microcentrifuge tube. Finally, serum was spun again for the third time at 2,000 X G for 10 minutes at room temperature where 90% of the serum was equally transferred to two new 1.5 ml disposable microcentrifuge tubes (Michelson et al., 2023). The remaining 10% of serum was transferred to a 1.5 ml disposable microcentrifuge tube (same tube as the one used during the first and second spin). All microcentrifuge tubes were mixed and aliquoted, and stored at -80°C (Michelson et al., 2023). The purpose of the second and third spin, as well as the transfer of 90% of serum into new tubes is to get the 'purest form' of the blood serum sample and to reduce risks of cellular contamination (Michelson et al., 2023). Transferring only the top layer of serum (which is cleaner and more pure) to a new tube prevents the unwanted substances at the bottom from mixing. The Mitochondrial Psychobiology Laboratory at Columbia University directed by Martin Picard, performed the blood assay quantification and analysis.

3.7. Quantification of Serum ccf-mtDNA Levels Using qPCR

According to Michelson and colleagues, MitoQuicLy (Mitochondrial DNA Quantification in Cell-Free Samples via Lysis) is an effective method to measure the amount of cf-mtDNA in human biofluids such as plasma, serum, and saliva (2023). Once the 'purest form' of blood serum samples are obtained, 10 µL of serum is mixed with a 190 µL of lysis buffer in a 96-well polymerase chain reaction (PCR) plate sealed with strip caps (Michelson et al., 2023) (see Figure 4, page 31). This lysis buffer contains 6% Tween-20, 114 mM Tris-HCL (pH 8.5), and 200 µg/mL Proteinase K (Michelson et al., 2023). After combining the serum and lysis buffer, the samples are vortexed and centrifuged to ensure they have been mixed properly (Michelson et al., 2023). The serum-lysis buffer mixture is then incubated overnight at 55°C for 16 hours, and then heated at 95°C for 10 minutes (Michelson et al., 2023). The heated mixture, termed the lysate, can either be stored at 4°C or directly used for quantitative polymerase chain reaction (qPCR) (Michelson et al., 2023). The qPCR targets ND1 (a mitochondrial gene to measure mtDNA) using TaqMan primers and probes (Michelson et al., 2023). A pipette is used to transfer 12 µL of TaqMan mastermix (qPCR buffer) to each well in a 384-well plate, followed by 8 µL of lysate for a total reaction volume of 20 µL per well (Michelson et al., 2023). Each reaction is repeated three times and the plates are sealed with a film and centrifuged at 1,000 X G for 10 seconds (Michelson et al., 2023). Additionally, the plates are placed into a QuantStudio 7 Flex Real-Time PCR System and heated at: 1) 50°C for 2 minutes; 2) 95°C for 20 seconds; 3) followed by 40 cycles of 95°C for 1 second, and 60°C for 20 seconds (Michelson et al., 2023). The qPCR detects how many cycles it takes for a light signal to become visible and cross a fixed cycle threshold (Ct) - with fewer cycles indicating more DNA present (Michelson et al., 2023). Finally, the smallest amount of DNA present (called the detection limit) is tested using a series of 16 serial dilutions of DNA standards (Michelson et al., 2023).

3.7.1. mtDNA and nDNA Quantities

According to past protocols outlined by Michelson et al. (2023) and Trumpff et al. (2019), the qPCR results were interpreted using two key genes: ND1 and B2M. The ND1 gene is natural log-transformed into LN ND1 (copies/µL) which is used to measure how much mitochondrial DNA (mtDNA) is found in the serum per unit volume. Similarly, the B2M gene is natural log-transformed into LN B2M (copies/µL) and it measures how much nuclear DNA (nDNA) is found in the serum. LN B2M is used as a reference to compare mtDNA with nDNA levels (Michelson et al. 2023; Trumpff et al., 2019). The ratio of LN ND1 to LN B2M (a unitless ratio) is used to represent, relatively, ccf-mtDNA levels (Michelson et al. 2023; Trumpff et al., 2019). Alternatively, ccf-mtDNA levels can be interpreted by solely examining the changes in LN ND1 from pre to post stress (Trumpff et al., 2019). This study included both comparison of the LN ND1 to LN B2M ratio, and a comparison of the LN ND1 values to assess changes in ccf-mtDNA levels.

3.8. Sample Size Estimation

The required sample size was estimated using G*Power (version 3.1.9.7) (Faul et al., 2007). The required sample size was based on a moderate effect size (f= 0.25), an alpha level of 0.05, and a beta level of 0.80, and a pre-post correlation of 0.50. The G*Power estimated that a total sample size of 34 participants was required to detect a moderate effect size. A repeated measures ANOVA (within-between interaction) is mathematically equivalent to a Linear Mixed Model (LMM) with fixed factors.

3.9. Statistical Analysis

SPSS (version 22.0) was used for all statistical analyses. Numeric variables are presented as means and standard deviations, and categorical variables as frequencies and associated percentages (IBM, 2013). To evaluate possible baseline differences between the mindfulness meditation (MM) and control podcast (CTRL) groups, an independent samples t-test was used for numeric variables and a chi-square test of

independence for categorical variables. Subsequently, a LMM, using restricted maximum likelihood estimation and an unstructured covariance matrix, was used to evaluate the fixed effect of group (MM vs. CTRL), time (pre-stress vs. post-stress), and their interaction (Group x Time). All statistically significant main effects or interactions were designated for follow up with evaluations of Bonferroni-adjusted simple main effects. Additionally, Hedges' g effect sizes, based on both between-group and within-group comparisons, were calculated according to procedures described by Lakens (2013).

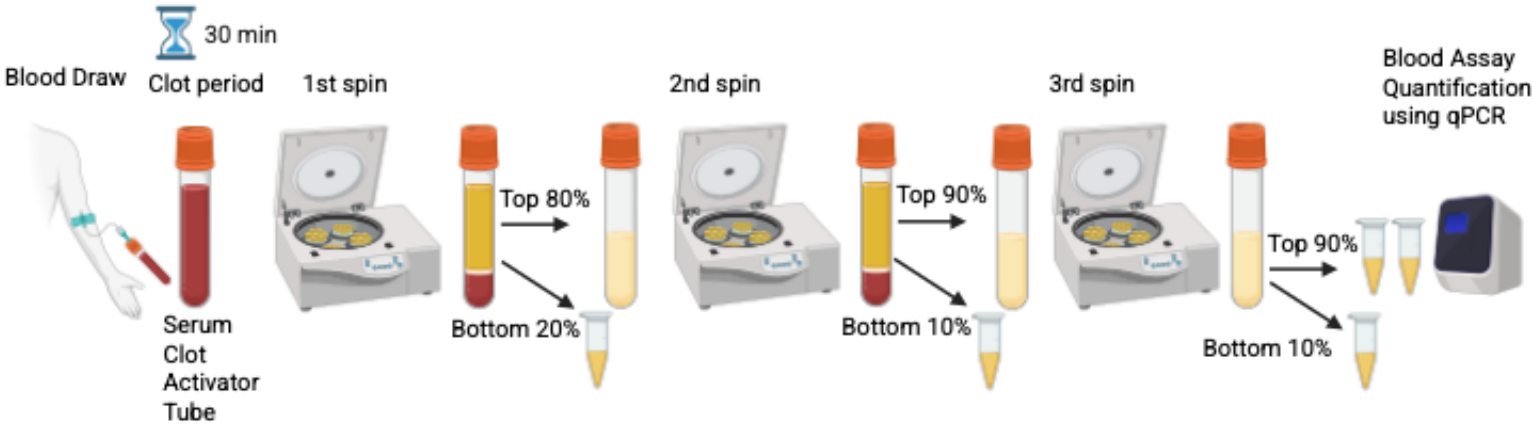

Figure 3. Visual Representaion of Serum Isolation Protocol

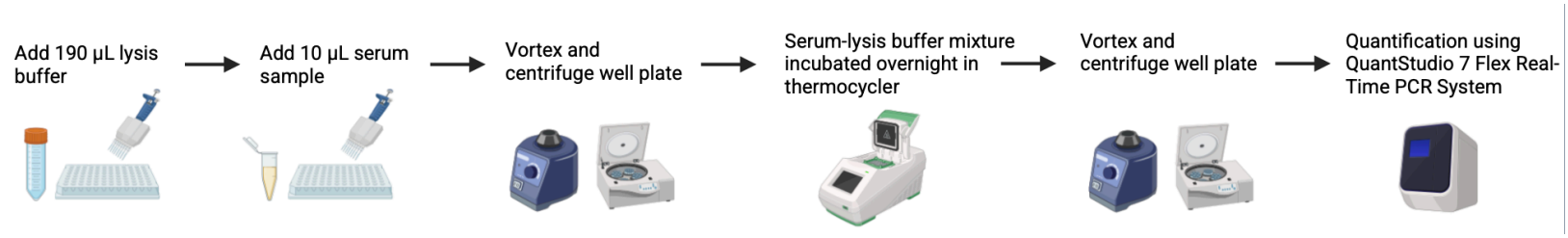

Figure 4. Visual Representation of Quantification of Serum ccf-mtDNA Levels

4. Results

4.1. Participant Characteristics

Between May 2025 and July 2025, n = 35 female participants were recruited through posters and emails at the York University campus. Nine participants declined to participate and were excluded prior to randomization. Another four participants were excluded after randomization due to difficulties with their baseline blood draws due to exceptionally small veins. One more participant was excluded post-study because they were identified as a previous participant in a study during which they viewed IAPS photographs (see Figure 5, page 34).

As a result, n = 21 female participants were assessed with 10 participants in the mindfulness meditation (MM) instruction group and 11 participants in the control podcast (CTRL) group. The mean (SD) age was similar across groups, as was body mass index (BMI), with means of 24.80 (5.98) in the MM group and 22.74 (3.24) in the CTRL group, with no significant differences between groups ($p = 0.33$). Participant self-identified ethnicities (n, %) in the MM group were: White (n = 1, 10.0%), Black (n = 2, 20.0%), South Asian (n = 5, 50.0%), Chinese (n = 0, 0.0%), or Other (n = 2, 20.0%). In the CTRL group, participants ethnically identified as: White (n =2, 18.2%), Black (n = 1, 9.1%), South Asian (n = 6, 54.5%), Chinese (n = 1, 9.1%), or Other (n = 1, 9.1%). There were no significant differences in the ethnicity distribution between groups ($p = 0.73$).

Prior to their baseline blood draw, the Pre-Blood Draw Questionnaire was administered to all participants. The Pre-Blood Draw Questionnaire Calm-Distress continuum scores showed a mean (SD) of 2.40 (1.78) in the MM group and 2.00 (1.00) in the CTRL group, with no significant differences between groups ($p = 0.53$). Baseline mood (prior to the lab stressor) was measured using the Profile of Mood States, Second Edition (POMS-2), subscales. There were no significant differences between groups on the Anger-Hostility ($p = 0.28$), Confusion-Bewilderment ($p = 0.08$), Depression-Dejection ($p = 0.38$), and Fatigue-

Inertia (p = 0.30) subscales. However, participants in the MM group had significantly lower outcome scores at baseline on the Tension-Anxiety subscale than the CTRL group participants (p = 0.05).

The menstrual frequency termed as Last Menstrual Period (LMP) (n, %) in the MM group was reported as: within the last week (n = 3, 30.0%), within the last 2 weeks (n = 2, 20.0%), within the last 3 weeks (n = 4, 40.0%), or difficult to estimate due to irregular cycles (n = 1, 10.0%). In the CTRL group, the LMP was reported as: within the last week (n = 3, 27.3%), within the last 2 weeks (n = 4, 36.4%), within the last 3 weeks (n = 2, 18.2%), or difficult to estimate due to irregular cycles (n = 2, 18.2%). There were no significant differences in the LMP between groups (p = 0.65). Also, the Menstrual Distress Questionnaire (MEDI-Q) revealed no significant differences in the menstrual symptoms (p = 0.65), symptoms distress (p = 0.98), and specificity index (p = 0.99) between groups. Lastly, the International Physical Activity Questionnaire (IPAQ) results revealed that participants were categorized into low, moderate, or high activity levels with no significant differences in activity levels between groups (p = 0.87). These baseline characteristics are reported in Table 2 (see page 35).

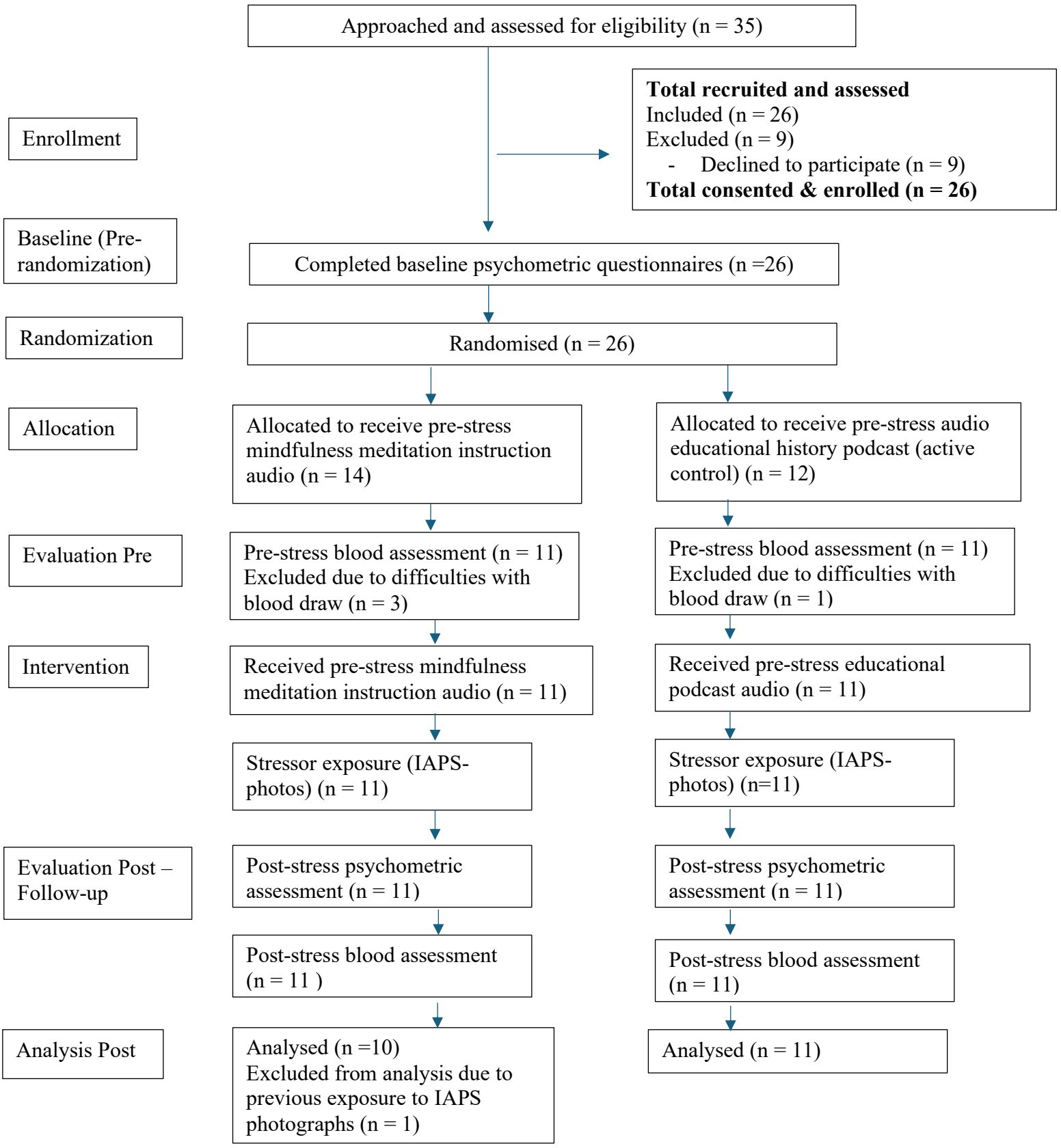

Figure 5. CONSORT Flow Diagram

Table 2. Baseline Characteristics of Participants in the MM and Control Groups.

Variable		MM (n = 10)	Control (n = 11)	*P*
Age, mean (SD)		24.70 (5.38)	22.18 (3.60)	0.22
BMI, mean (SD)		24.80 (5.98)	22.74 (3.24)	0.33
Ethnicity, n (%)				
	White	1 (10.0)	2 (18.2)	0.73
	Black	2 (20.0)	1 (9.1)	
	South Asian	5 (50.0)	6 (54.5)	
	Chinese	0 (0.0)	1 (9.1)	
	Other	2 (20.0)	1 (9.1)	
Prior Blood Draw, n (%)		10 (100.0)	11 (100.0)	NA
Blood draw distress, mean (SD)		2.40 (1.78)	2.00 (1.00)	0.53
IPAQ Activity Levels, n (%)				
	Low	1 (10.0)	2 (18.2)	0.87
	Moderate	4 (40.0)	4 (36.4)	
	High	5 (50.0)	5 (45.5)	
Last Menstrual Period, n (%)				
	Within the last week	3 (30.0)	3 (27.3)	0.65
	Within the last 2 weeks	2 (20.0)	4 (36.4)	
	Within the last 3 weeks	4 (40.0)	2 (18.2)	
	Difficult to estimate due to irregular cycles	1 (10.0)	2 (18.2)	
MEDI-Q, mean (SD)[a]				
	Menstrual Symptoms	6.30 (3.34)	7.27 (5.95)	0.65
	Symptoms Distress	2.22 (1.00)	2.23 (1.00)	0.98
	Specificity Index	0.63 (0.35)	0.62 (0.35)	0.99
POMS-2, mean (SD)				
	Anger - Hostility	1.00 (1.41)	2.36 (3.61)	0.28
	Confusion - Bewilderment	7.50 (5.68)	12.18 (6.03)	0.08
	Depression - Dejection	2.30 (2.83)	4.00 (5.39)	0.38
	Tension - Anxiety	2.30 (1.64)	5.45 (4.50)	0.05
	Fatigue - Inertia	4.20 (3.33)	7.09 (7.87)	0.30

Note. Values are presented as mean (SD) or n (%)

[a]. *Menstrual Symptoms* refers to the number of symptoms that were more distressing during the menstrual phase compared to the intermenstrual phase (i.e. the time after menstruation ends and before the next premenstrual phase); *Symptoms distress* refers to the average distress of menstrual symptoms that were more distressing during the menstrual phase compared to the intermenstrual phase. This score ranges from 0 to 5; *Specificity index* refers to the proportion of symptoms that were more distressing during the menstrual phase compared to the premenstrual and intermenstrual phases. This score ranges from 0 to 1, where 0 indicates that all menstrual symptoms were equally distressing across all phases, and 1 indicates that all symptoms were more distressing in the menstrual phase compared to the other phases.

4.2. POMS-2

For the Tension-Anxiety subscale, as reflected in Table 3 (see page 38) there was a significant main effect of group ($p = 0.03$) and time ($p < 0.01$), but the group x time interaction was not significant ($p = 0.12$). The between-group Hedges' effect size was large ($g_s = 0.90$), a finding that supports the role of MM in reducing psychological stress responses subsumed under the labels of tension and anxiety. Additionally, the within-group effect sizes were large for both the MM group ($g_{rm} = 0.96$) and the control group ($g_{rm} = 0.93$) participants, suggesting that the IAPS stressor was effective at inducing stress in both groups.

For the Anger-Hostility subscale, a significant main effect of time ($p = 0.02$) was found, but there was no significant group ($p = 0.13$) or group x time interaction ($p = 0.17$). The between-group Hedges' effect size was moderate-to-large ($g_s = 0.67$). However, the within-group effect size was moderate for both groups ($g_{rm} = 0.49$), confirming, again, no significant within group differences.

For the Confusion-Bewilderment subscale, a significant main effect of time ($p = 0.01$) was found, but there was no significant group ($p = 0.29$) or group x time interaction ($p = 0.41$). The between-group Hedges' effect size was small ($g_s = 0.24$), confirming minimal differences between groups. Also, the within-group effect size was moderate for the MM group ($g_{rm} = 0.47$) and small for the control group ($g_{rm} = 0.25$).

For the Depression-Dejection subscale, a significant main effect of time ($p = 0.02$) was found, but there was no significant group ($p = 0.13$) or group x time interaction ($p = 0.20$). The between-group Hedges' effect size was large ($g_s = 0.69$), suggesting that the MM group participants demonstrated less increases in depressive mood compared to the control group.

For the Fatigue-Inertia subscale, there were no significant main effects of group ($p = 0.22$), time ($p = 0.53$) or group x time interaction ($p = 0.71$). The between-group Hedges' effect size was moderate

(g_s = 0.57), and the within-group effect size was very small for MM group (g_{rm} = 0.05) and the control group (g_{rm} = 0.10) participants, confirming minimal changes in fatigue for both groups.

Lastly, as shown in Figure 8 (see page 44), both the MM and control groups showed increases in negative mood subscales from pre to post stress, with participants in the MM group generally showing lesser increases than the participants in the control group. For both groups, baseline (pre-stress) negative mood score means were relatively low, in the context of POMS-2 normative data, suggesting that participants who joined the study were at normative stress levels. Following the IAPS stressor, mean negative mood scores generally increased for both groups which suggests that the stressor did have some degree of stress induction effect.

Table 3. Changes in POMS-2 negative mood subscales from pre- to post- IAPS stressor exposure in the MM and CTRL groups

POMS-2	Group	Pre	Post	*P* (Group)	*P* (Time)	*P* (Group x Time)	g_s (between-groups)	g_{rm} (within-groups)
Anger - Hostility	MM	1.00 (1.41)	2.30 (3.37)	0.13	0.02	0.17	0.67	0.49
	CTRL	2.36 (3.61)	7.00 (8.73)					0.49
Confusion - Bewilderment	MM	7.50 (5.68)	12.30 (9.48)	0.29	0.01	0.41	0.24	0.47
	CTRL	12.18 (6.03)	14.73 (10.00)					0.25
Depression - Dejection	MM	2.30 (2.83)	4.40 (4.58)	0.13	0.02	0.20	0.69	0.52
	CTRL	4.00 (5.39)	10.55 (10.95)					0.66
Tension - Anxiety	MM	2.30 (1.64)	7.10 (6.33)	0.03	0.00	0.12	0.90	0.96
	CTRL	5.45 (4.50)	15.82 (11.29)					0.93
Fatigue - Inertia	MM	4.20 (3.33)	4.40 (3.69)	0.22	0.53	0.71	0.57	0.05
	CTRL	7.09 (7.87)	7.91 (7.42)					0.10

Note. Results presented as mean (SD); POMS-2: Profile of Mood States, Second Edition.

Table 4. Changes in ln ND1 from pre- to post- IAPS stressor exposure in the MM and CTRL group.

ND1	Group	Pre	Post	*P* (Group)	*P* (Time)	*P* (Group x Time)	g_s (between-groups)	g_{rm} (within-groups)
Ln ND1	MM	7.11 (0.45)	7.21 (0.39)	0.38	0.88	0.12	0.62	0.22
	CTRL	7.08 (0.23)	7.00 (0.25)					0.32

4.3. ln ND1 (representation of ccf-mtDNA levels)

Table 4 demonstrated that the natural log-transformed ND1 values from pre to post stress showed no significant main effects of group ($p = 0.38$), time ($p = 0.88$) or group x time interaction ($p = 0.12$). The between-group Hedges' effect size was moderate-to-large ($g_s = 0.62$), and the within-group effect sizes were small for both the MM group ($g_{rm} = 0.22$) and the control group ($g_{rm} = 0.32$). These results suggest that ccf-mtDNA levels remained relatively stable from pre to post stress in both groups as minimal changes were observed.

4.4. ccf-mtDNA Data- Description of Results that were < Limit of Detection

Due to lab analysis difficulties, the data related to ccf-mtDNA responses received from collaborators (Psychobiology Lab, Columbia University, Martin Picard, Director) were incomplete due to various findings being below the limit of detection (LOD). When findings are below detection limits, the validity of the finding is compromised and reduced although causes can remain unclear. As collaborators (at Columbia and York), we are unable to explain these findings currently, although we believe further investigations may provide better answers. The effectiveness and reliability of these findings are critical for Columbia's research program and for projects with multiple collaborative groups. However, as this thesis is focused on the reliable findings received (those valid and beyond the LOD), at this time we can only speculate without direct empirical support.

Once our blood results reached Columbia (via FedEx-same day delivery services), the serum samples were stored in their lab freezer (at -80°C). When Columbia began analyses, these serum samples were thawed at room-temp and aliquoted into a 96-well plate (80 µL / well). This plate was stored at -80° C until being implemented in this analysis. 10 µL of each of our (York University) samples were then transferred from storage plate to two replicate lysis plates loaded with 190 µL lysis buffer per well. Lysis plates were shaken on an orbital shaker (1,200 RPM x 1 minute), centrifuged (1,000 x G pulse), then

incubated at 55 °C for 16 hours for lysis, followed by 10 minutes at 95 °C to deactivate proteinase. Lysates were then held at 4 °C until preparation for qPCR the following day.

The qPCR plate setup involved the lysates being shaken (950 RPM x 1 minute) and pulse centrifuged. Then 8 µL of lysate from each replicate lysis plate was transferred in triplicate to a 384-well plate loaded with 12 µL of qPCR buffer per well. DNA extracted from primary human dermal fibroblasts (hFB1), quantified by digital droplet PCR, was serially diluted and added to qPCR plates as a standard curve. Concentrations of marker genes for mtDNA (**ND1**) and nDNA (**B2M**) in dilutions ranged from **1.6 million to 100 copies per reaction** and **6,000 to <1 copy per reaction**, respectively. The qPCR plates were mixed (600 RPM x 1 minute) and pulse centrifuged before analysis. qPCR was run on a Quantstudio 7 Flex real-time PCR system with the program as followed: 1) 50 °C for 2 minutes, 2) 95 °C for 20 seconds; 3) followed by 40 cycles of 95 °C for 1 second, and 60 °C for 20 seconds. Fluorescence threshold was set at 0.08 for both VIC and FAM probes. ROX (included in TaqMan MasterMix) was used as a passive reference.

Coefficients of variation (CVs) for ND1 and B2M cycle threshold measurements (CtND1 and CtB2M) were calculated for each sample on each replicate qPCR plate. For samples with CVs that were > 0.5% and calculated from three Cts, the Ct that most greatly deviated from the mean of the three was rejected, and **the mean of the two remaining Cts** was used to calculate copies of target gene per reaction. If the CV for a sample's three measurements was ≤ 0.5%, the mean of all three measurements was used.

As can be seen, the Columbia Lab engages in a careful process of analysis and the validation of analyses undertaken. Beyond these cautions, we have in our analyses deleted all the samples that were associated with an LOD determination. While this has limited the sample size, it assists in the interpretation of thesis data and enables communication to assist subsequent data analyses.

4.4.1. Induced Acute Psychological Stress Did Not Increase ccf-mtDNA Levels

Disconfirming hypothesis 1, participants in the mindfulness meditation (MM) instruction group did not demonstrate lower levels of serum ccf-mtDNA after exposure to the IAPS lab stressor when compared to the control group participants who listened to an educational podcast.

Indeed, we found no significant increase in ccf-mtDNA content post-stress induction in either experimental or control subjects. When evaluating the ln (ND1) / ln (B2M) ratio, the majority of included experimental group participants (63.6%) showed a slight increase in ln ND1 (0.02%), whereas the majority of control group participants (54.5%) showed a slight decrease (0.06%) (see Figure 6, page 43). A paired two tailed t-test was used to compare the ratio of ln (ND1) / ln (B2M) between the pre and post blood samples for both groups. In the MM group, the mean ccf-mtDNA levels, reported as ln (ND1) / ln (B2M), increased slightly from the baseline value of 1.92 to 1.96 at 30 min post-stress, exhibiting no statistically significant increase ($t(6) = -0.45$, $p = 0.67$). In the CTRL group, mean ccf-mtDNA levels decreased slightly from baseline values of 1.97 to 1.93 at 30 min post-stress, with no significant change ($t(5) = 0.69$, $p = 0.52$).

Serum samples were also analyzed for nuclear DNA (nDNA) content in order to examine whether the increased ccf-mtDNA was specific to mitochondria or due to a general increase in circulating cellular genomic material. The cellular genomic material nDNA (B2M) did **not** increase after the stressor test and was nearly equal to the pre-stress levels.

When evaluating only the natural log-transformed ND1, the majority of included experimental group participants (90.9%) showed a slight increase in ND1 (0.097%) after stress exposure, while the control group participants demonstrated a slight decrease (0.08%) (see Figure 7, page 43). A paired two tailed t-test was used to compare the ln ND1 between the pre and post blood samples for both groups. In the MM group, the mean ccf-mtDNA levels, reported as ln ND1, increased slightly from the baseline value of 7.11 to 7.21 at 30 min post-stress, exhibiting no statistically significant increase ($t(9) = -1.24$, $p = 0.25$).

In the CTRL group, mean ccf-mtDNA levels decreased slightly from baseline values of 7.08 to 7.00 at 30 min post-stress, also with no significant change ($t(10) = 1.08$, $p = 0.30$).

4.4.2. Relationship of POMS-2 and ccf-mtDNA Results

The POMS-2 results suggested that both groups showed increases in negative mood subscales after the IAPS stressor, with the MM group generally showing lesser increases than the CTRL group. For instance, despite the absence of significant differences, there were large between-group effect sizes for the Anger-Hostility, Depression-Dejection, and Tension-Anxiety subscales, suggesting that negative mood responses were attenuated, to some degree, for the MM group. However, there were no significant group × time interactions observed for any of the negative mood subscales. Additionally, the higher stress levels evident in psychometric findings were not evident in the ccf-mtDNA blood results. This enables the observation that such discrepancies may not be unusual and that experiences reflected in self-report may not be represented in blood analyses of ccf-mtDNA levels.

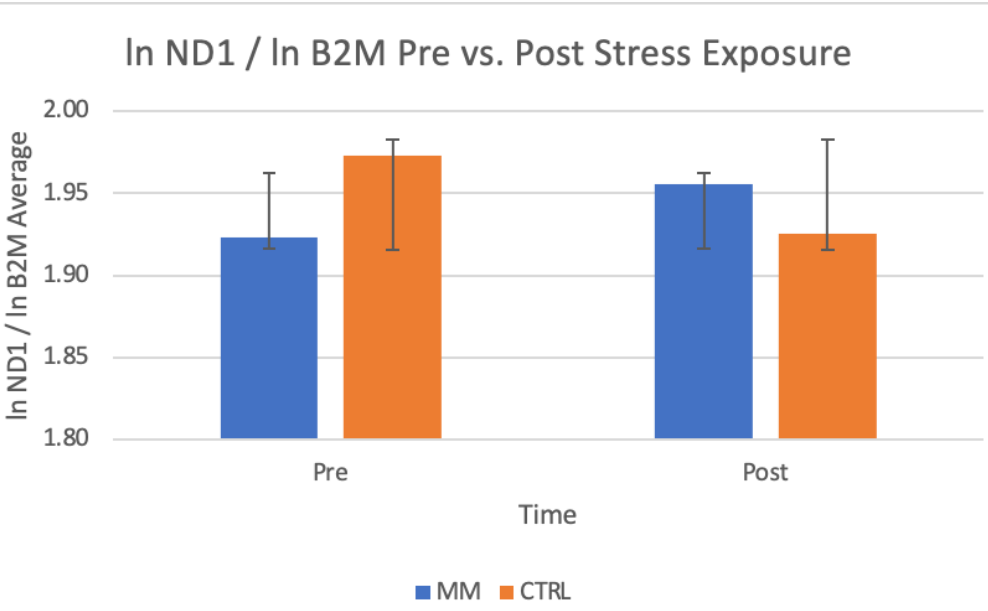

Figure 6. Average ln ND1 / ln B2M, represents ccf-mtDNA content, before and after stress exposure in MM and CTRL groups.

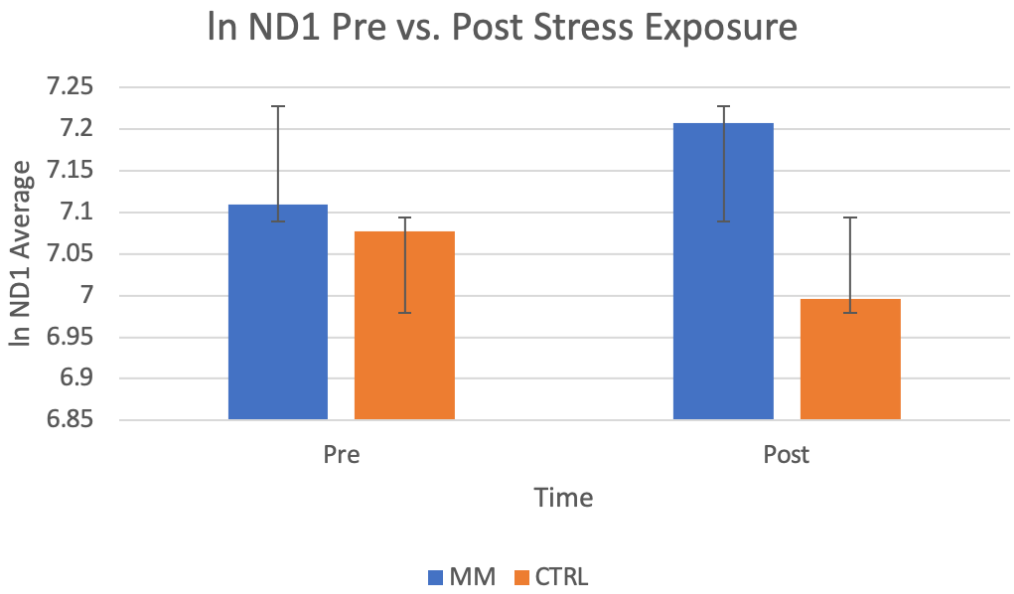

Figure 7. Average ln ND1, also represents ccf-mtDNA content, before and after stress exposure in MM and CTRL groups.

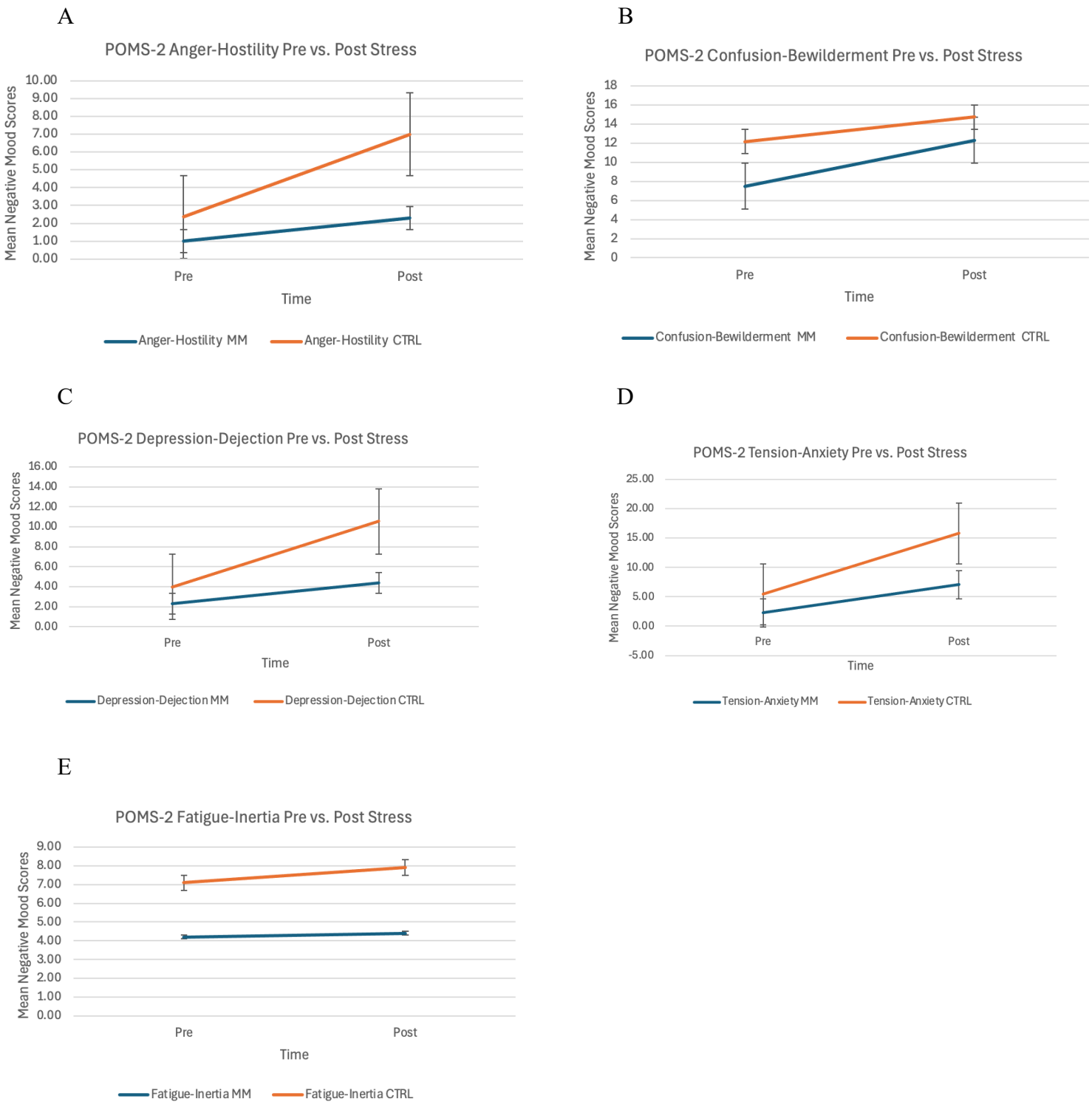

Figure 8. Changes in POMS-2 negative mood scores before and after stress exposure in MM and CTRL groups. A) Anger-Hostility, B) Confusion-Bewilderment, C) Depression-Dejection, D) Tension-Anxiety, and E) Fatigue-Inertia subscales are presented as means and standard errors.

5. Discussion

5.1. Effects of Mindfulness Meditation on Mood and Physiological Markers

The study's main findings indicated no significant differences (within a RCT design) in the blood results compared between the experimental group (who received mindfulness meditation) and the control group (who received an educational podcast). This study specifically examined whether individuals exposed to the mindfulness meditation (MM) instruction showed attenuated psychophysiological responses to stress when compared to the control group listening to the podcast. According to Bergin & Pakenham (2016), increased levels of mindfulness have been associated with reductions in depression and anxiety and increases in life satisfaction. In this study, while there were no significant group x time interactions, large between-group effect sizes were found for Anger-Hostility, Depression-Dejection, and Tension-Anxiety subscales, with the MM group showing lesser negative mood increases compared to the controls (post-stress). Therefore, mindfulness may act as a relatively mild stress buffer, under certain conditions (Lucas-Thompson et al., 2021; Lucas-Thompson et al., 2024). However, our findings did not show a significant difference in ccf-mtDNA levels between the experimental and control group. If there was stress buffering in the experimental (mindfulness) group, it was not observed in the blood results.

According to Pollock et al. (1979), higher scores on the POMS-2 Depression-Dejection and Tension-Anxiety subscales are significantly associated with elevations in physiological markers such as heart rate (HR) and diastolic blood pressure (DBP). Similarly, higher scores on the Anger-Hostility subscale were also linked to increased HR (Pollock et al., 1979). Furthermore, Trumpff et al. (2019) showed how acute psychological stress (induced by the TSST) was associated with increases in negative mood as indicated in the Anger-Hostility and Tension-Anxiety subscales (POMS-2) and in elevated systolic blood pressure (SBP), DBP, HR, and ccf-mtDNA. However, our study revealed no significant differences in the ccf-mtDNA levels after exposure to the IAPS lab stressor between participants in the MM group and the

control group. We did not measure SBP, DBP and HR. As stated, results from other studies suggest negative mood states, as assessed by the POMS-2 subscales, correlate with physiological markers.

Additionally, a study done by Nuissier et al. (2007) found that individuals who reported higher scores on the Depression-Dejection subscale also showed the greatest decreases in parasympathetic nervous system activity, as measured by heart rate variability (HRV). This study showed that the POMS subscales were correlated with the autonomic nervous system function measures like HRV (Nuissier et al., 2007). Although, our current study did not measure HRV, it would be appropriate to incorporate this physiological measure in future studies of mindfulness and physiologically manifested stress.

5.2. Effects of Physical Activity on Mood

Additional results suggested that the responses of subjects differed in accord with their self-report on the IPAQ reflecting low, moderate, and high levels of physical activity. According to Poole et al. (2011), physical activity levels are inversely associated with depressive mood symptoms, as measured by the Centre of Epidemiological Studies Depression scale (CES-D) and POMS, such that higher physical activity levels are associated with lower negative mood states. Their study also showed that individuals who engaged in light to moderate physical activity had better mood scores (Poole et al., 2011). In our study, there were no significant associations between physical activity levels and POMS-2 mood measures.

5.3. ccf-mtDNA and its Relation to Psychological Distress and Mindfulness Protection

In the statistical modeling of predictors of outcome stress levels, the level of anxiety attributed to the blood draw procedure did not appear to significantly affect outcomes. This result provides more support for subjects responding to lab stress induction rather than the particular stress associated with blood draws. This finding was also reflected in no associated elevations of ccf-mtDNA levels.

In the past relevant literature, several studies were performed with psychiatrically diagnosed and healthy populations. For instance, two cross-sectional studies performed in violent and non-violent suicide

attempters (Lindqvist et al., 2016), and patients with major depressive disorder (Lindqvist et al., 2018) showed significantly elevated ccf-mtDNA levels when compared to matched controls. In non-diagnosed populations, another two studies assessed whether induced lab stress was associated with increases in ccf-mtDNA levels using the TSST for inductions. While Hummel et al. (2018) found a 1.7-fold statistically significant *plasma* ccf-mtDNA increase in 20 young, healthy males, Trumpff et al. (2019) assessed 50 healthy middle-aged female and male subjects and found significant differences (Cohen's d values ranging from 0.85-1.23) in *serum* ccf-mtDNA elevations.

Our study was a partial replication of the above studies in the use of lab stress induction. However, our study differed in that a RCT design was employed to assess stress reduction methods, along with lab stress induction methods. In this RCT, a mindfulness protocol was purposely inserted, *following* baseline assessments of mood and a blood draw, to assess whether mindfulness had a protective effect when the stress induction was implemented. In accord with POMS-2 results, there appeared to be a limited stress inoculation effect, i.e. implementation of the stress reduction protocol was associated with a diminished response to the stress induction protocol. Stress inoculation effects are valued because they can promote homeostasis if subsequent stress exposures are not overwhelming but challenging enough to induce adaptive emotional-cognitive processing (Ashokan et al., 2016; Meichenbuam & Cameron, 1989). Adaptive processing in specific situations can then contribute to adaptive coping with subsequent stressors. The novelty of the current study involved testing whether stress inoculation effects influenced mitochondrial activity. In the simplest interpretation of the observed mitochondrial evidence, the stress inoculation effect was disconfirmed. As stress inoculation is both a mental and physical phenomena (Meichenbuam & Cameron, 1989), our approach to assessing it was appropriate (based on both psychometric and mitochondria assessments). Nonetheless, our results were not definitive, likely because the lab stress induction might have been too mild to thoroughly evaluate the stress inoculation phenomena. Alternately, the stress reduction protocol was perhaps too conservative. For example, longer mindfulness

meditation sessions might have been more effective in reducing the stress levels reflected in mtDNA dynamics. Further investigation is required re: how mitochondria might be affected by stress inoculation procedures.

5.4. Differences in Study Design and Potential Explanation for Findings

Trumpff et al. (2019) investigated 50 middle-aged female and male subjects, while this study focused only on 21 female university students aged 18-30 years with the aim of controlling for sex and age differences. It is typical for university students to experience higher levels of anxiety during public speaking tasks, which is why this study utilized the photograph-viewing approach (IAPS) as the stress protocol rather than the public-speaking stressor (TSST). There are advantages to using the IAPS images as more intensely stress evoking images can be selected for future studies.

Once again, despite findings from Trumpff et al. (2019) and Mirzadeh et al. (2023), the latter being a study conducted at the same site with similar subjects, our findings did not reflect significant differences between intervention and control conditions. Further explanation for the lack of significant differences includes the possibility that the necessity of switching sites for where the experimental stressor and stress reduction conditions were undertaken and where the lab where the blood samples were drawn was a factor. The environmental changes and the need to walk between measurement sites might have had a reduction effect on the responses that were observed. While the experimental stressor (IAPS) has previously been shown effective, the current study did not show the expected effect possibly because this set of participants had prior imagery exposure that attenuated their stress response. Finally, the current study had different blood analysis protocols when compared to the Trumpff et al. study (specific centrifugation differences) and the Mirzadeh et al. study (2023).

The lack of significant differences between the experimental and control conditions could have occurred due to the possibility that the educational podcast may had some relaxing or calming effect. Although Azam et al. (2015) showed that mindfulness meditation has a reductive effect on chronic stress,

previous studies have not explored whether listening to an educational podcast could elicit similar stress reduction effects. There is not enough research exploring psychological stress responses associated with listening to educational podcasts to explain the lack of significant difference observed in this study.

5.5. Strengths and Limitations

5.5.1. Strengths

One study strength was a partly successful manipulation of the independent variable (i.e. lab-induced stress), as indicated by significant main effects of time across several POMS-2 negative mood subscales using a well-known standardized stressor (selected IAPS photos) with 60 selected images. We ascertained that the images evoked were negative and emotionally stress inducing as indicated by large between-group effect sizes on the Anger-Hostility, Depression-Dejection, and Tension-Anxiety subscales. These comparisons were psychometrically measured after the stressor task and before the 2nd blood draw.

Another strength is that the scheduling time and the protocol instructions were standardized to reduce potential confounds with daily stress factors related to circadian rhythms and to daily stress exposures associated with university attendance. Another strength of this RCT study design is the inclusion of a control group which allowed for comparisons of various outcomes (ex. POMS, IPAQ, MEDI-Q, ccf-mtDNA) between the two different groups. This design improved the internal validity of the study. Additionally, the administration of the MEDI-Q was another strength because it examined how different menstrual cycle phases could affect stress levels.

5.5.2. Limitations and Future Directions

One of the limitations of this study is that the 30-minute mindfulness meditation audio may not have been long or sufficiently impactful to buffer the IAPS-based lab stress exposure. Future studies could incorporate a) a longer, single mindfulness session or b) shorter mindfulness sessions done across multiple visits to examine whether the more extended training in or timing of meditative activity affects ccf-mtDNA

levels. Lastly, the findings of the present study only apply to female participants between the ages of 18 to 30 years. As a result, the generalizability is limited since changes in the ccf-mtDNA levels were only examined in a specific sex and age group. Therefore, future studies should include male participants to examine potential sex-related differences in ccf-mtDNA levels.

Another potential limitation that resulted in the absence of significant differences in serum ccf-mtDNA levels between groups from pre- to post- IAPS stress exposure could be that the IAPS images were not fear-or-stress provoking enough. This lesser stress effect may have been more predominant in the large South Asian female population who due to cultural norms and lived experiences may have been less sensitized to the IAPS images. Research has shown that a large number of stress-inducing factors in the female South Asian population includes: loss of social support, economic uncertainty, downward social mobility, mechanistic lifestyle, difficulties in accessing healthcare services, lack of social health insurance, and changes in climate and food (Ahmad et al., 2005). Due to real-life stressors that have an impact on mental health, exposure to IAPS images may not have provoked elevated psychophysiological stress responses in the South Asian female population.

5.6. Conclusions

This study investigated the potential protective effects of mindfulness meditation (MM) on ccf-mtDNA levels in female participants after an artificial lab stressor (IAPS). This study showed no significant protective effects when compared to a control condition (listening to a podcast). Psychometrically, large between-group effect sizes were found for Anger-Hostility, Depression-Dejection, and Tension-Anxiety subscales, suggesting that MM may act as a potential stress buffer but in a limited way.

This RCT appears to be the first study to explore the possibly protective effects of MM on both ccf-mtDNA levels and mood after an acute artificial lab stressor. Future studies should explore repeated mindfulness sessions (rather than a single MM session) and use multiple mitochondrial markers (based on

saliva as well as blood samples) to assess the psychophysiological effects of mindfulness on stress responses.

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Appendix A:

Self-Administered Comorbidity Questionnaire (SCQ)

Date: _____

Time: _____

Please select your response (✓ Yes or ✓ No) for each item in the box provided.

Problem	Do you have the problem?	Do you receive treatment for it?	Does it limit your activities?
Heart disease	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
High blood pressure	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Lung disease	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Diabetes	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Ulcer or stomach disease	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Kidney disease	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Liver disease	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Anemia or other blood disease	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Cancer	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Depression	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Osteoarthritis, degenerative arthritis	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Back pain	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Rheumatoid arthritis	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No
Other medical problems (please write in)	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No

Appendix B:

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

Date: _____

Time: _____

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?
_____ days per week
No vigorous physical activities → Skip to question 3

2. How much time did you usually spend doing vigorous physical activities on one of those days?
_____ hours per day
_____ minutes per day
Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.
_____ days per week
No moderate physical activities → Skip to question 5

4. How much time did you usually spend doing moderate physical activities on one of those days?
_____ hours per day
_____ minutes per day
Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?
_____ days per week
No walking → Skip to question 7

6. How much time did you usually spend walking on one of those days?
_____ hours per day
_____ minutes per day
Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the last 7 days, how much time did you spend sitting on a week day?
_____ hours per day
_____ minutes per day
Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Appendix C:

Profile of Mood States (POMS)

Date: _____

Time: _____

Directions: Describe HOW YOU FEEL RIGHT NOW by highlighting the most appropriate number after each of the words listed below.

Item	Not at all	A little	Moderate	Quite a bit	Extremely
1. Friendly	1	2	3	4	5
2. Tense	1	2	3	4	5
3. Angry	1	2	3	4	5
4. Worn out	1	2	3	4	5
5. Unhappy	1	2	3	4	5
6. Clear-headed	1	2	3	4	5
7. Lively	1	2	3	4	5
8. Confused	1	2	3	4	5
9. Sorry for things done	1	2	3	4	5
10. Shaky	1	2	3	4	5
11. Listless	1	2	3	4	5
12. Peeved	1	2	3	4	5
13. Considerate	1	2	3	4	5
14. Sad	1	2	3	4	5
15. Active	1	2	3	4	5
16. On edge	1	2	3	4	5
17. Grouchy	1	2	3	4	5
18. Blue	1	2	3	4	5
19. Energetic	1	2	3	4	5
20. Panicky	1	2	3	4	5
21. Hopeless	1	2	3	4	5
22. Relaxed	1	2	3	4	5
23. Unworthy	1	2	3	4	5
24. Spiteful	1	2	3	4	5
25. Sympathetic	1	2	3	4	5
26. Uneasy	1	2	3	4	5
27. Restless	1	2	3	4	5
28. Unable to	1	2	3	4	5
29. Fatigued	1	2	3	4	5
30. Helpful	1	2	3	4	5
31. Annoyed	1	2	3	4	5
32. Discouraged	1	2	3	4	5
33. Resentful	1	2	3	4	5
34. Nervous	1	2	3	4	5

Please continue on the next page....

Item	Not at all	A little	Moderate	Quite a bit	Extremely
35. Lonely	1	2	3	4	5
36. Miserable	1	2	3	4	5
37. Muddled	1	2	3	4	5
38. Cheerful	1	2	3	4	5
39. Bitter	1	2	3	4	5
40. Exhausted	1	2	3	4	5
41. Anxious	1	2	3	4	5
42. Ready to fight	1	2	3	4	5
43. Good-natured	1	2	3	4	5
44. Gloomy	1	2	3	4	5
45. Desperate	1	2	3	4	5
46. Sluggish	1	2	3	4	5
47. Rebellious	1	2	3	4	5
48. Helpless	1	2	3	4	5
49. Weary	1	2	3	4	5
50. Bewildered	1	2	3	4	5
51. Alert	1	2	3	4	5
52. Deceived	1	2	3	4	5
53. Furious	1	2	3	4	5
54. Efficacious	1	2	3	4	5
55. Trusting	1	2	3	4	5
56. Full of pep	1	2	3	4	5
57. Bad-tempered	1	2	3	4	5
58. Worthless	1	2	3	4	5
59. Forgetful	1	2	3	4	5
60. Carefree	1	2	3	4	5
61. Terrified	1	2	3	4	5
62. Guilty	1	2	3	4	5
63. Vigorous	1	2	3	4	5
64. Uncertain about things	1	2	3	4	5
65. Bushed	1	2	3	4	5

Please check to see that you have responded to all the items....thank you

Appendix D:

Menstrual Distress Questionnaire (MEDI-Q)

Date: _____

Time: _____

Instructions - Please carefully review the list of provided symptoms. Please answer question A, for each symptom that you have experienced during your periods in the last 12 months. If you did not experience a particular symptom, please answer "No" and skip to the next symptom on the list. However, if you did experience a symptom, please also answer questions B, C, and D regarding the impact of that symptom on your functioning and quality of life.

Are you currently menstruating? _____ Yes _____ No

When were you last menstruating? ☐ Within the last week ☐ Within the last 2 weeks ☐ Within the last 3 weeks ☐ Difficult to estimate due to irregular cycles

A. On average, in the past year on the days you had your period, did you…				**If you had this symptom, to what degree it interfered with your quality of life, your recreational or work activities, or your social relationships…**												
				B. …on days when you were menstruating?				**C. …during the premenstrual phase (in the 7 days before the start of menstruation)?**					**D. …during the other days (outside the menstrual/premenstrual phase)?**				
	Yes, more than half of the times I've had my period	Yes, less than half the times I've had my period	No *(Skip to next item)*	*Not at*	*A little*	*Moderatel*	*Very*	*Not at*	*A little*	*Moderatel*	*Very*	Never had this symptom during the premenstrual phase	*Not at*	*A little*	*Moderatel*	*Very*	Never had this symptom during the other days
1. …have pain in your lower abdomen?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
2. …have pain when urinating?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
3. …have pain during bowel movement?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
4. …have muscle/bone/joint pain?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
5. …feel bloated or did you experience breast tenderness?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
6. …experience nausea?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
7. …have headaches?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

8. …have digestive problems (heartburn, uncomfortable sense of fullness after meals ...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
9. …have diarrhea?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
10. …have constipation?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
11. …have discomfort due to vaginal bleeding (fear of stains or odors, discomfort from the tampon, difficulty or embarrassment during sexual activities...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
12. …have the feeling of being dirty?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
13. …feel excessively sad (easily crying, little drive to do things, loss of interest in usual activities ...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

A. On average, in the past year on the days you had your period, did you…				**If you had this symptom, to what degree it interfered with your quality of life, your recreational or work activities, or your social relationships…**												
				B. …on days when you were menstruating?				**C. …during the premenstrual phase (in the 7 days before the start of menstruation)?**					**D. …during the other days (outside the menstrual/premenstrual phase)?**			
	Yes, more than half of the times I've had my period	Yes, less than half the times I've had my period	No *(Skip to next item)*	*Not at*	*A little*	*Moderatel*	*Very*	*Not at*	*A little*	*Moderatel*	*Very*	Never had this symptom during the premenstrual phase	*Not at*	*A little*	*Moderatel*	*Very*	Never had this symptom during the other days
14. …feel emotionally unstable (fluctuating mood, rapid transition from one mood to	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

another even in response to minimal stimuli...)?																	
15. …feel irritable or short-tempered (feeling nervous, not being able to bear unexpected events, people or situations, feeling angry easily...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
16. …feel impulsive (driven to act without thinking or planning...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
17. …feel anxious (agitated, tense, excessively insecure or indecisive, fearful that something bad could happen at any moment ...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
18. …excessively hungry (desire to overeat, loss of control over food…)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
19. …feel a lack of hunger?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
20. …have insomnia (inability to fall or stay asleep)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
21. …experience excessive sleepiness (sleeping during the day, not being able to get up	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

in the morning ...)?																	
22. …feel excessively tired (sluggish, with little energy...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
23. …have low sexual desire (reduced drive to have sexual activities, lack of sexual fantasies ...)?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
24. …have difficulty concentrating?	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

25. Did you have sexual interactions that included vaginal penetration in the last year?	☐ Yes		☐ No *(end the questionnaire) (0)*		
25A. On average, in the last year on the days you had your period, did you have pain during sexual interactions that included vaginal penetration?	☐ Yes, more than half of the times I've had my period I had pain during vaginal penetration (2) ☐ Yes, less than half of the times I've had my period I had pain during vaginal penetration (1) ☐ No, I never had pain during vaginal penetration *(end the questionnaire) (0)* ☐ I never had vaginal penetration on the days I had menstrual flow because I would have had too much pain (2) ☐ I never had vaginal penetration on days when I had menstrual flow for reasons other than pain *(end the questionnaire) (0)*				
25B. On days when you were menstruating, to what degree this pain (or avoiding vaginal penetration) interfered with your quality of life, your recreational or work activities and your social relationships?	☐ Not at all	☐ A little	☐ Moderately	☐ Very much	
25C. During the premenstrual phase (in the 7 days before the start of menstruation), if you had this symptom, to what degree it interfered with your quality of life, your recreational or work activities, or your social relationships?	☐ Not at all	☐ A little	☐ Moderately	☐ Very much	☐ Never had this symptom during the premenstrual phase
25D. During the other days (outside the menstrual/premenstrual phase), if you had this symptom, to what degree it interfered with your quality of life, your recreational or work activities and your social relationships?	☐ Not at all	☐ A little	☐ Moderately	☐ Very much	☐ Never had this symptom during the other days

Appendix E:

COVID-19 Screening Form

Time: _____

Participant Name: _____

Have you tested positive for COVID-19 during the past 14 days? Yes__ No___

Do you have any of the following new or worsening symptoms? (*symptoms should not be chronic or related to other known causes or conditions*)

Fever or Chills	Yes___ No___
Difficulty breathing or shortness of breath	Yes___ No___
Cough	Yes___ No___
Sore throat	Yes___ No___
Runny nose/stuffy nose or nasal congestion	Yes___ No___
Decrease or loss of smell or taste	Yes___ No___
Nausea, vomiting, diarrhea, abdominal pain	Yes___ No___
Not feeling well, extreme tiredness, sore muscles	Yes___ No___
Have you travelled outside of Canada in the past 14 days?	Yes___ No___

Have you had close contact with a confirmed or probable case of COVID-19 during the past 14 days?

Yes___ No___

Participant signature: _____ Date: _____

Appendix F:

BMI and Demographic Self-Report Questionnaire

Date: _____ **Time:** _____

Please answer the following questions. Your responses will be kept confidential and used for research purposes only.

Section A: Body Mass Index (BMI)

1. **What is your current weight?** _____ kilograms (kg) _____ pounds (lbs)
2. **What is your current height?** _____ meters (m) _____ feet and inches (ft. in.)

Note: BMI will be calculated by the research team using the formula: BMI = weight (kg) / [height (m)]^2

BMI (to be completed by the research investigator): _____

Section B: Demographic Self-Report

1. **Please state your country of birth**: _____
2. **How many years have you lived in Canada?**
   ☐ Less than 1 year
   ☐ 1-5 years
   ☐ 6-10 years
   ☐ More than 10 years
   ☐ Born and raised in Canada
3. **Please identify your race or ethnicity according to the following:**
   ☐ White
   ☐ Black
   ☐ South Asian
   ☐ Chinese
   ☐ Other (please specify): _____
4. **Have you experienced what you consider a highly stressful event in the last 12 months?**
   ☐ Yes ☐ No

Appendices G:

Pre - Blood Draw Questionnaire

Date: _____

Time: _____

Please answer the following questions. Your responses will be kept confidential and used for research purposes only.

1. Have you ever had your blood drawn before?
   ☐ Yes ☐ No

2. How are you feeling about the upcoming blood draw?
   Please indicate your feelings using the 7-point scale below:

   1 2 3 4 5 6 7
   Calm…….Distress

3. Are you concerned about any of the following? (Check all that apply)
   ☐ Pain or discomfort
   ☐ Bruising
   ☐ Becoming dizzy or faint
   ☐ Fear of needles
   ☐ Other (please specify): _____
   ☐ None of the above

4. Have you ever felt faint during a previous blood draw?
   ☐ Yes ☐ No

Post - Blood Draw Questionnaire

Date: _____

Time: _____

Please answer the following questions. Your responses will be kept confidential and used for research purposes only.

1. How did you feel about having your blood drawn?
   Please indicate your feelings using the 7-point scale below:

   1 2 3 4 5 6 7
   Calm…….Distress

2. Did you experience any of the following? (Check all that apply)
   ☐ Pain or discomfort
   ☐ Bruising
   ☐ Becoming dizzy or faint
   ☐ Fear about needles
   ☐ Other (please specify): _____
   ☐ None of the above