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BIOLOGICAL AGEING: A STUDY OF TELOMERE LENGTH AND MITOCHONDRIAL DNA COPY NUMBER IN ANOREXIA NERVOSA

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DECLARATION

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LIST OF ABBREVIATIONS

AN	Anorexia Nervosa
APA	American Psychiatric Association
ARFID	Avoidant Restrictive Feeding Disorder
ATP	Adenosine Triphosphate
ADP	Adenosine diphosphate
BDNF	Brain-Derived Neurotrophic Factor
BED	Binge Eating Disorder
BMI	Body Mass Index
BN	Bulimia Nervosa
CBT	Cognitive Behavioural Therapy
COX	Cytochrome c oxidase
COI	Cytochrome c oxidase subunit 1
COII	Cytochrome c oxidase subunit 2
COIII	Cytochrome c oxidase subunit 3
Cyt B	Cytochrome B
Cyt C	Cytochrome C
CV	Coefficient of variation
DKC1	Dyskerin 1
DNA	Deoxyribonucleic Acid
DTT	Dithiothretitol
dsDNA	Double stranded Deoxyribonucleic Acid
DSM-5	Diagnostic and Statistical Manual, Version 5

ED	Eating Disorder
EDE	Eating Disorders Examination questionnaire
ETC	Electron Transport Chain
FADH	Flavin adenine dinucleotide + hydrogen
FAD	Flavin adenine dinucleotide
GAR1	Glycine and arginine rich protein 1
GDPR	General Data Protection Regulations
GR	Glucocorticoid Receptor
HSE	Health Service Executive
HSP	Heavy Strand Promoter
hTERC	Human Telomerase RNA Component
hTERT	Human Telomerase Reverse Transcriptase
ICD-10	International Classification of Diseases, Version 10
LSP	Light Strand Promotor
MARSIPAN	Management of Really Sick People with Anorexia Nervosa
MANTRA	Maudsley Model of Anorexia Nervosa Treatment for Adults
MDT	Multidisciplinary Team
MINI	Mini International Neuropsychiatric Interview
mtDNAcn	Mitochondrial DNA Copy Number
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + hydrogen
NCP-ED	National Clinical Guideline for Eating Disorders
ND	Nicotinamide adenine dinucleotide

NGF	Nasogastric Feeding
NICE	The National Institute for Health and Care Excellence
NOLA2	Nucleolar protein family A, member 2
NOLA3	Nucleolar protein family A, member 3
O_L	Origin of the light strand
O_H	Origin of the heavy strand
PGC-1α	Peroxisome Proliferator-activated Receptor-ɣ Coactivator 1α
POT1	Protection of telomeres 1
Q10	Co-enzyme Q10
qRT-PCR	quantitative Real Time - Polymerase Chain Reaction
RAP1	Repressor/activator protein 1
RC	Respiratory chain
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEAN	Severe Enduring Anorexia Nervosa
IBM SPSS	International Business Machines Corporation Statistical Package for Social Sciences
SJH	St James's Hospital
SPMHS	St Patrick's Mental Health Services
TCAB1	Telomerase cajal body protein 1
TCD	Trinity College Dublin

TCIN	Trinity College Institute of Neuroscience
tHcys	Total Homocysteine
TEN	Telomerase Essential N-terminal domain
TL	Telomere Length
TRF1	Telomere repeat-binding factor
TRF2	Telomere repeat-binding factor 2
tRNA	Transfer Ribonucleic Acid
TIN2	TRF1 interacting nuclear factor 2
TPP1	Tripeptidyl peptidase 1
WHO	World Health Organisation

SUMMARY

INTRODUCTION

Patients with anorexia nervosa (AN) experience significant psychological and physical stress, which are risk factors for accelerated biological ageing. Telomeres are protective caps at the ends of chromosomes that prevent damage to the chromosome and preserve chromosomal integrity. Telomere length (TL) provides a measure of normal biological ageing and reduces with age. TL can also be reduced by stressors. Mitochondria, known as the “powerhouses” of the cell, are responsible for the production of energy. They are unique in that they possess their own DNA, distinct from nuclear DNA, of which there are many copies within the cell. Mitochondria may reflect stress via changes such as alterations in mitochondrial DNA copy number (mtDNAcn).

I sought to test the following hypotheses: (1) TL is reduced in patients with AN compared to healthy controls; (2) TL is reduced with increasing duration and severity of illness; (3) mtDNAcn is increased in patients with AN compared to healthy controls; (4) mtDNAcn is increased with duration and severity of AN; and (5) mtDNAcn is correlated with TL.

METHODS

For this cross-sectional case control study, TL and mtDNAcn were assessed in peripheral whole blood DNA collected from patients with AN and age- and sex-matched healthy controls, using quantitative real-time polymerase chain reaction. Clinical and

demographic characteristics were also obtained. Severity of illness was measured using the Eating Disorders Examination, a clinician-evaluated questionnaire.

RESULTS

Patients with AN (*n*=23; 21 females; mean age 30.3 years, SD 13.1) and age- and sex-matched controls (*n*=33; 32 females, mean age 30.6 years, SD 12.2) were included in the study. TL was significantly longer in patients with AN compared to healthy controls, both before and after adjusting for age, sex, level of educational attainment, smoking status, body mass index and antipsychotic use. TL was not associated with duration of illness after adjusting for age. TL was not associated with severity of illness. There was no difference between mtDNAcn in patients with AN compared to controls. mtDNAcn was positively associated with duration of illness, but not with illness severity. There was no correlation between TL and mtDNAcn.

CONCLUSION

Contrary to my hypotheses, the results indicate that TL does not reflect accelerated biological ageing in patients with AN. Further investigation may identify the reason for increased TL in those with AN. Interestingly, mtDNAcn was elevated in those with a longer duration of illness. Studies with larger sample sizes are required to clarify whether compensatory mechanisms increase mtDNAcn with increased illness duration.

LAY ABSTRACT

Eating disorders are a group of psychiatric illnesses where one develops abnormal eating behaviours and problems with normal weight management, which interfere with normal functioning. Anorexia nervosa (AN) is an eating disorder characterised by distorted cognitions about eating, shape and / or weight. Those with AN usually restrict food intake relative to energy output by extreme dieting while engaging in other behaviours to lose weight. Body mass index (BMI) is a measure of weight relative to height and is calculated by dividing weight (kilograms) by height (metre) squared (kg/m^2). A normal BMI is between 18.5 and 24.9 kg/m^2. Patients with AN are often underweight with a very reduced BMI.

Those with mental illness, and specifically AN, experience increased physical and psychological stress and have a shorter life expectancy compared to those without mental illnesses. Age is commonly measured in chronological terms; however, a more accurate approach may be to measure biological age. Biological age is a way of measuring one's age based on results of objective physical tests. There are many ways to measure biological parameters such as the investigation of specific components of blood results, body imaging results or measuring physical performance during exercise.

Telomeres are protective caps at the ends of our chromosomes. Chromosomes are collections of DNA and genes that are found in every cell in our body. Telomeres become shorter as we age. Telomere length (TL) is an accurate and well-established method of measuring biological age. Mitochondria are also present in every cell in the human body and are responsible for producing energy. Mitochondria have their own DNA, of which

there are multiple copies (quantified as copy number) in each cell. Changes in mitochondrial DNA copy number (mtDNAcn) may reflect increased stress.

I sought to investigate TL and mtDNAcn in patients with anorexia nervosa. Contrary to my hypothesis, I found increased TL in patients with anorexia nervosa compared to healthy controls. There was no difference in mtDNAcn between patients with anorexia nervosa and controls. TL was not related to duration or severity of AN. mtDNAcn was related to duration of illness but not illness severity. There was no association between TL and mtDNAcn.

VALUE OF RESEARCH

This research explores whether those with anorexia nervosa (AN), who undergo substantial physical and psychological stress, experience changes on a molecular level because of their illness. Stress is associated with an accelerated rate of ageing. There are no molecular studies to date exploring whether those with AN experience accelerated ageing as measured by telomere length (TL) and mitochondrial DNA copy number (mtDNAcn).

Research would (i) provide a better understanding of the effects of AN on the ageing process, (ii) enable clinicians to better inform patients of effects of AN on physical health, and (iii) potentially identify those at increased risk of age-related illness.

OUTPUTS

Published papers:

Doody E, Ryan MK, O'Toole C, McLoughlin DM (2022). Telomere length in patients with anorexia nervosa. *Psychiatry Research Communications*, p100022. Doi.org/10.1016/j.psycom.2022.100022 (appendix 1)

Doody E, Ryan MK, O'Toole C, McLoughlin DM (2024). Duration of anorexia nervosa is positively associated with whole blood mitochondrial DNA copy number: a cross-sectional case-control study. *The European Journal of Psychiatry, 100265.*

Doi.org/10.1016/j.ejpsy.2024.100265 (appendix 2)

Related published work:

Ryan, K.M., **Doody, E.** and McLoughlin, D.M., 2023. Whole blood mitochondrial DNA copy number in depression and response to electroconvulsive therapy. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *121*, p.110656 (appendix 3)

Poster Presentations

1. "Mitochondrial DNA copy number is associated with illness duration in anorexia nervosa", **Doody E,** Ryan MK, O'Toole C, McLoughlin DM. Presented at the British Association of Psychopharmacology (BAP) Summer Meeting, 23rd – 26th July 2023, Manchester, United Kingdom.

2. "Telomere length in patients with anorexia nervosa", **Doody E,** Ryan MK, O'Toole C, McLoughlin DM. Presented at the European College of Neuropsychopharmacology (ECNP) Annual Congress 7th – 10th October 2023, Barcelona, Spain.

Oral Presentation

"Impact of anorexia nervosa on biological ageing", **Doody E,** Ryan MK, O'Toole C, McLoughlin DM. Runner up prize awarded for my oral presentation at the Royal Academy of Medicine, Ireland (RAMI), 5th October 2023, Dublin, Republic of Ireland.

CHAPTER 1. INTRODUCTION

1.1 Anorexia nervosa

1.1.1 Classification

Eating disorders are a broad but well-conceptualised group of psychiatric disorders comprising anorexia nervosa (AN), bulimia nervosa (BN), binge eating disorder (BED), and avoidant restrictive food intake disorder (ARFID). These disorders have been re-classified as Feeding and Eating Disorders (FEDs) in the most recent revision of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) (American Psychiatric Association, 2013) and in the latest revision of the *International Statistical Classification of Diseases and Related Health Problems* published in 2019 (ICD-11) (World Health Organization, 2019).

Eating disorders are characterised by significant abnormalities in eating behaviour and weight management. Anorexia nervosa, the focus of this thesis, is a severe and complex psychiatric disorder that is often accompanied by significant physical consequences secondary to starvation (Phillipou et al., 2018b). This psychiatric disorder is characterised by distorted cognitions about eating, shape and weight in addition to obsessional and anxious traits, leading to a restriction of energy intake relative to energy needs (American Psychiatric Association, 2013). Psychiatric co-morbidities are common and include anxiety disorders, substance misuse disorders and mood disorders (Kaye et al., 2004, Herpertz-Dahlmann et al., 2001).

1.1.2 Epidemiology

The lifetime prevalence rates of AN are up to 4% and 0.3% respectively among females and males, (van Eeden et al., 2021b). Based on an Irish census population in 2022 of 5,123,536, this equates to an estimated 220,312 individuals having experienced AN at some point in their lives in Ireland. The incidence rate of AN ranges from 0.5 to 318 per 100,000 women-years (Martínez-González et al., 2020). However, the true community incidence of AN is unknown as many patients delay or avoid seeking treatment (Hay, 2020). The incidence of AN is reportedly increasing in younger females aged <15 years (van Eeden et al., 2021a). Thus, AN appears to be occurring at a younger age. Although AN predominantly affects young females in the Western world, AN does in fact affect both sexes, in all age groups and in non-Western countries.

1.1.3 Aetiology

The aetiology of AN comprises a range of factors including biological and psychological vulnerabilities that in combination with environmental circumstances increase the risk of developing and maintaining AN (Klump et al., 2009).

Genetic factors influence the risk of developing AN. Relatives of persons with AN have an increased risk of developing AN in comparison to healthy controls who do not have an affected relative (Strober et al., 2000). Genetic studies suggest that serotonin genes may be implicated in the genetic aetiology of AN (Baker et al., 2017). Twin-based

studies report heritability estimates of 50-60% (Paolacci et al., 2020). Reduced serotonin activity is implicated in the aetiology of AN (Hartman, 1995), however replacing serotonin has not been shown to successfully treat AN. Furthermore, an imbalance between dopamine and serotonin, neurotransmitters that regulate reward and inhibitory control in the cortico-striatal circuits, is suggested in animal models of AN based on increased cognitive control and reduced reward processing (Huang and Foldi, 2022). Indeed, the difficulty in understanding the biological aetiology of AN has posed challenges in the development of novel treatments (Collier and Treasure, 2004).

Psychosocial and interpersonal factors may trigger disease onset. Those with tendencies towards perfectionism, inhibition and risk avoidance have an increased risk of AN (Herpertz-Dahlmann et al., 2011). Perceptual and cognitive inflexibility and, thus, the inability to change learnt behaviours, are commonly seen in AN (Strober, 2004).

Inflammation may also be involved in the pathophysiology of AN. An exploratory study by Dalton et al. (2018) found that concentrations of interleukin (IL) – 6, IL – 15, brain-derived neurotrophic factor (BDNF) and vascular cell adhesion molecule (VCAM) – 1 were significantly increased in those with AN (n = 27) compared with healthy controls (n = 13). The authors also reported that vascular endothelial growth factor (VEGF) – A and tumor necrosis factor (TNF) – β were significantly reduced in those with AN compared to the control group. Several studies have reported increased levels of oxidative stress in AN (Solmi et al., 2015, Vignini et al., 2010, Vannacci et al., 2006). Interestingly, Gibson and Mehler (2019) suggest a link between inflammation and oxidative stress in AN. Oxidative stress is caused by free radicals, or atoms with unpaired electrons, which are extremely unstable and have the potential to damage tissues by disrupting the normal functioning

of cells (Chapple, 1997). The authors report that elevated levels of pro-inflammatory cytokines in AN may be caused by the increase in reactive oxygen species (ROS). This suggests a complex interplay between the immune system and molecular stress in AN. ..

Furthermore, changes in neural networks may contribute to disease maintenance (Zipfel et al., 2015). Malnutrition often leads to significant changes of metabolism, both centrally and peripherally, in particular neuroendocrine and neurotransmitter alterations, which have the potential to cause neural restructuring during period of brain development, i.e. during adolescent years, when the incidence of AN is increased (Herpertz-Dahlmann et al., 2011). Chronic starvation and alterations in neuroendocrine and neurotransmitter functioning may lead to "biological scars" that sustain the disorder (Herpertz-Dahlmann et al., 2011), making successful treatment more challenging.

1.1.4 Diagnosis

Diagnosis is confirmed using criteria provided in the DSM-5 (American Psychiatric Association, 2013) or ICD-11 (World Health Organization, 2019) as mentioned in paragraph 1.1.1. The use of either classification tool depends on clinician preference. For this thesis the DSM-5 was used for diagnosis and classification of AN. AN is defined by the Diagnostic and Statistical Manual version 5 (DSM-5) (American Psychiatric Association, 2013) as:

a. Persistent restriction of energy intake, leading to significant low body weight (in the context of what is minimally expected for age, sex, developmental trajectory, and physical health)
b. Intense fear of gaining weight or of becoming fat, or persistent behaviour that interferes with weight gain (even though at significantly low weight)
c. Disturbance in the way one's body weight or shape is experienced, undue influence of body shape and weight on self-evaluation or persistent lack of recognition of the seriousness of the current body weight.

Anorexia nervosa can be further sub-grouped into two types:

1. Restricting type where individuals restrict their intake leading to severe weight loss.
2. Binge/purging type (based on last 3 months) where individuals engage in occasional episodes of binge-eating and purging.

1.1.5 Treatment

While most patients can be managed on an outpatient or day patient basis, more severe cases require admission to an inpatient unit with a dedicated specialist eating disorder programme. To provide a holistic approach, a multi-disciplinary team (MDT) is required to address nutritional, physical, and psychological co-morbidities. The aims of treatment are to restore a normal body weight, manage medical complications, develop a healthy

relationship with weight, shape and eating behaviours and to treat co-morbid psychological symptoms.

Psychotherapy is one of the most effective forms of treatment, including Cognitive Behavioural Therapy (CBT) for eating disorders and Maudsley Anorexia Nervosa Therapy for Adults (MANTRA) (Hay, 2020) for both inpatients and outpatients.Schmidt et al. (2015) reported average increases in body mass index (BMI) of 1.8 after 12 months of psychotherapy. CBT for eating disorders (CBT-E), is usually delivered over 40 sessions. Research shows that those with AN who complete a course of CBT-E have an average weight gain of 7.5kg and this weight gain was maintained at follow up (60 weeks) (Fairburn et al., 2013). Furthermore, 90% of participants reported minimal symptoms of their eating disorder. Family-based therapy is often recommended due to the high incidence of familial expressed emotion (Fisher et al., 2019). A randomised controlled trial (RCT) by Lock et al. (2010) reported that 89% of participants (n = 121) reached full or partial remission by end of treatment. At the 12-month follow up, 78% of participants were either in full or partial remission. Pharmacotherapy is considered to help weight gain, to reduce AN cognitions and psychological symptoms and prevent relapse (Attia and Schroeder, 2005). Both antipsychotics and antidepressants are prescribed (Kishi et al., 2012, Marvanova and Gramith, 2018). Fassino et al. (2002) reported an increase of 1.3 in BMI after 12 weeks of treatment with Citalopram. An RCT by Attia et al. (2019) reported that participants who were prescribed olanzapine had a higher BMI at end of treatment compared to controls. However, there were no differences in psychological symptoms between the two groups. In severe cases of starvation, where a patient requires medical interventions, the Management of Really Sick Patients with Anorexia Nervosa

(MARSIPAN) guidelines provide a treatment guide to prevent re-feeding syndrome (Royal College of Psychiatrists, 2014).

Treatment is complicated by challenges in maintaining patient motivation and engagement during the process of recovery (DeJong et al., 2012). A significant proportion of those with AN delay seeking treatment for at least a decade due to multiple factors including stigma, shame or a lack of insight into their illness (Brelet et al., 2021).

1.1.6 Co-morbidity

Psychiatric co-morbidity is high in AN (Berkman et al., 2007). Substance misuse, personality disorders, and mood and anxiety disorders are common in AN (Papadopoulos et al., 2009). In fact, two thirds of those with AN and one third of those who have recovered from AN have at least one other psychiatric illness (Halvorsen et al., 2004).

Physical co-morbidity is common and almost every organ system is affected by complications of AN (Mitchell and Crow, 2006). Medical complications are usually related to the chronicity of illness and severity of starvation (Miller et al., 2005) and account for more than 50% of all deaths in patients with AN (Mehler and Brown, 2015). Physical complications are summarised in Table 1 (Mehler and Brown, 2015).

Dermatologic	dry skin, alopecia, lanugo hair, starvation-associated pruritis
Cardiac	bradycardia, hypotension, mitral valve prolapse, sudden death – arrhythmia, refeeding syndrome, changes on echocardiogram including reduced left ventricular volume and increased risk of pericardial effusion
Gastroenterologic	constipation, refeeding pancreatitis, acute gastric dilation, delayed gastric emptying, hepatitis, dysphagia
Endocrine	amenorrhoea, infertility, osteoporosis, thyroid abnormalities, hypercortisolaemia, hypoglycaemia, neurogenic diabetes insipidus, arrested growth
Haematologic	pancytopaenia due to starvation, decreased sedimentation rate
Neurologic	cerebral atrophy
Ophthalmic	lagophthalmos
Pulmonary	aspiration pneumonia, respiratory failure, spontaneous pneumothorax, emphysema

Table 1.1 Physical complications of anorexia nervosa

1.1.7 Prognosis and mortality

Prognosis for those with AN is poor in terms of treatment and risk of death, with less than 63% of individuals achieving recovery from their illness (Eddy et al., 2017). Longer follow up periods tend to be associated with better recovery rates and reduced mortality rates (Jagielska and Kacperska, 2017). However, lifetime mortality rates in AN are the highest of all psychiatric disorders (Papadopoulos et al., 2009). Arcelus et al. (2011) reported a

mortality rate of 5.1 per 1,000 individuals per year and standardised mortality rates (SMRs) are reported to be 10 to 12 times higher than that of the general population (Löwe et al., 2001, Herzog et al., 1997). Both prognosis and mortality risk depends on severity of AN, including requirement for hospitalisation and nasogastric tube feeds, age of onset, duration of illness and co-morbidity (Jagielska and Kacperska, 2017).

1.2 Ageing

1.2.1 Introduction

The World Health Organisation (WHO) describes ageing as a process that occurs because of time-dependent molecular and cellular damage, leading to a steady reduction in physical and cognitive capacity and an increased risk of disease and death (World Health Organization, 2022). The impacts of ageing are numerous. There is an increase in risk of chronic health conditions such as cardiovascular disease, stroke, diabetes, respiratory diseases, and cancer (Halpin et al., 2010). The effects of ageing place extra pressure on our economy, including public finances and healthcare resources. In Ireland, an ageing population will result in a reduced rate of economic expansion and an increase in age-related public expenditure (Department of Finance, 2021). Thus, it is imperative to focus on the process of ageing, develop our understanding of this process, and identify the risk

factors that make certain people more vulnerable to an accelerated rate of ageing and therefore more susceptible to age-related decline.

1.2.2 Biological and chronological age

Although advancing age results in reductions in functionality (Jazwinski and Kim, 2017), not all individuals will experience the same rate of loss of function and health outcomes vary considerably. Indeed, there is much heterogeneity in the health outcomes of individuals (Lowsky et al., 2013). Those who age successfully present as physically active and engaged in life activities with low levels of disease and disability and higher levels of cognitive functioning (Rowe and Kahn, 1997). Others experience an increased rate of reduced functionality, and this may be only loosely related with that person's age.

Ageing is commonly measured in chronological terms. However, ageing may be more accurately quantified in biological terms, accounting for differences between successful and accelerated ageing, and reflecting heterogeneity amongst individuals in the ageing process. Chronological age refers to the amount of time a person has lived. Biological age, first considered by Alex Comfort in 1969 (Comfort, 1969), may be examined by the identification of biological changes that occur with increasing age. Almost every aspect of the organism's phenotype is affected by the process of ageing, which results in an accumulation of cellular and molecular damage to cause impaired functioning (Jylhävä et al., 2017). It is well established that chronological age is not consistent with biological age (Kaeberlein, 2013). While chronological age may be viewed as a proxy for the rate of

ageing, biological age may provide a more accurate account of the individual's functionality, the presence or absence of accelerated ageing processes, and risk for morbidity and mortality.

1.2.3 Quantifying biological ageing

Many methods exist that attempt to quantify biological age using data from multiple organ systems. However, the examination of DNA and changes that occur on a cellular and molecular level have proved to be of major interest in quantifying biological age due to their accuracy. Over time, cells experience distinctive phenotypic alterations that affect the whole organism and result in ageing as we know it (Zole and Ranka, 2018). This process causes several changes on a biological level, which have been described as the hallmarks of ageing (López-Otín et al., 2013). Such hallmarks include telomere length attrition and mitochondrial DNA copy number, which have both been investigated in relation to the ageing process (see Sections 1.3 and 1.4 below).

Briefly, telomeres play an important role in the protection of chromosomes (O'Sullivan and Karlseder, 2010). Telomeres are protective caps on the ends of chromosomes that prevent chromosomal damage (Saretzki, 2018). Telomere length (TL), which reduces with age, is one of the most reliable markers of ageing and is well established and extensively researched (Vaiserman and Krasnienkov, 2021). There is a large body of evidence describing reduced telomere length in psychiatric and physical illnesses (Price et al., 2013). Additionally, telomeres are shortened when the organism is

exposed to stress (Shalev et al., 2014). Mitochondria, organelles primarily responsible for energy production, are essential in ensuring cellular integrity (Tatsuta and Langer, 2008). Mitochondria possess their own DNA. Mitochondrial DNA copy number (mtDNAcn), a quantification of mitochondrial DNA, also plays an important role in biological ageing, stress and age-related illnesses (Billard and Poncet, 2019, Lagouge and Larsson, 2013). mtDNAcn has been reported to both increase and decrease with age and, thus, no conclusive trend has been identified (Picard and McEwen, 2018c). mtDNAcn has been reported to be associated with stress (Ridout et al., 2018a). Similarly, both increases and decreases have been noted, suggesting that is it not possible to identify a conclusive trend between the stress response and mtDNAcn (Cai et al., 2015b, Liu and Zhou, 2012b). In acute stress, such as would be experienced during a relapse of AN, there is an increase in mtDNAcn per cell.

Finally, telomeres and mitochondria have been shown to potentially regulate one another (Tyrka et al., 2015b), with suggestions that shortening of TL may trigger mitochondrial alterations in mtDNAcn (Kelly, 2011).

1.2.4 Factors affecting the rate of biological ageing

The process of ageing is dependent on the interplay of multiple factors such as genetic differences, lifestyle variations, and the body's resilience or innate ability to manage stress (Levine, 2013). Those who are exposed to psychosocial stressors are more likely to have an increased risk of accelerated ageing (Palma-Gudiel et al., 2020). Certain lifestyles such

as excessive alcohol consumption, smoking, and reduced physical activity are known to accelerate the ageing process (López-Otín et al., 2016). Diet and calorie intake has a significant impact on the rate of ageing (Moore et al., 2018). It has been noted that individuals with chronic illnesses, both physical and mental, have an increased risk of accelerated ageing and dependency (Fransquet et al., 2019).

Anorexia nervosa is a disease where there is significant physical and psychological stress imposed on the body. AN has the highest mortality rate of all mental illnesses (Papadopoulos et al., 2009) with many deaths in AN attributed to medical complications due to the effect of starvation (Holmes et al., 2012). Such a degree of physical and psychological stress is likely to trigger changes on a cellular and molecular level. Furthermore, there are often age-related illnesses identified in patients with AN such as brain atrophy (Ehrlich et al., 2008) and osteoporosis (Fazeli, 2016). However, there are no known studies to date investigating mtDNAcn or telomere length in AN.

1.3 Telomeres

1.3.1 Introduction

Chromosomes are structures that contain proteins, known as histones, that bind to deoxyribonucleic acid (DNA) (Figure 1.1). DNA is a molecule containing genetic information that enables the organism to develop and function (Crick et al., 1979). Each end of a DNA molecule is numbered referred to as either 5´ (prime) or 3´. These numbers

are references to the number of carbon atoms in a deoxyribose sugar molecule that a phosphate group bonds. The genetic information in DNA is made up of four chemical bases: adenosine (A), guanine (G), cytosine (C) and thymine (T). These bases forms pairs with one another, adenosine forms a base pair with thymine, and guanine forms a base pair with cytosine. These base pairs are attached to a sugar and a phosphate molecule, forming a nucleotide. Nucleotides bind together to form two long strands that are arranged in a double helix structure.

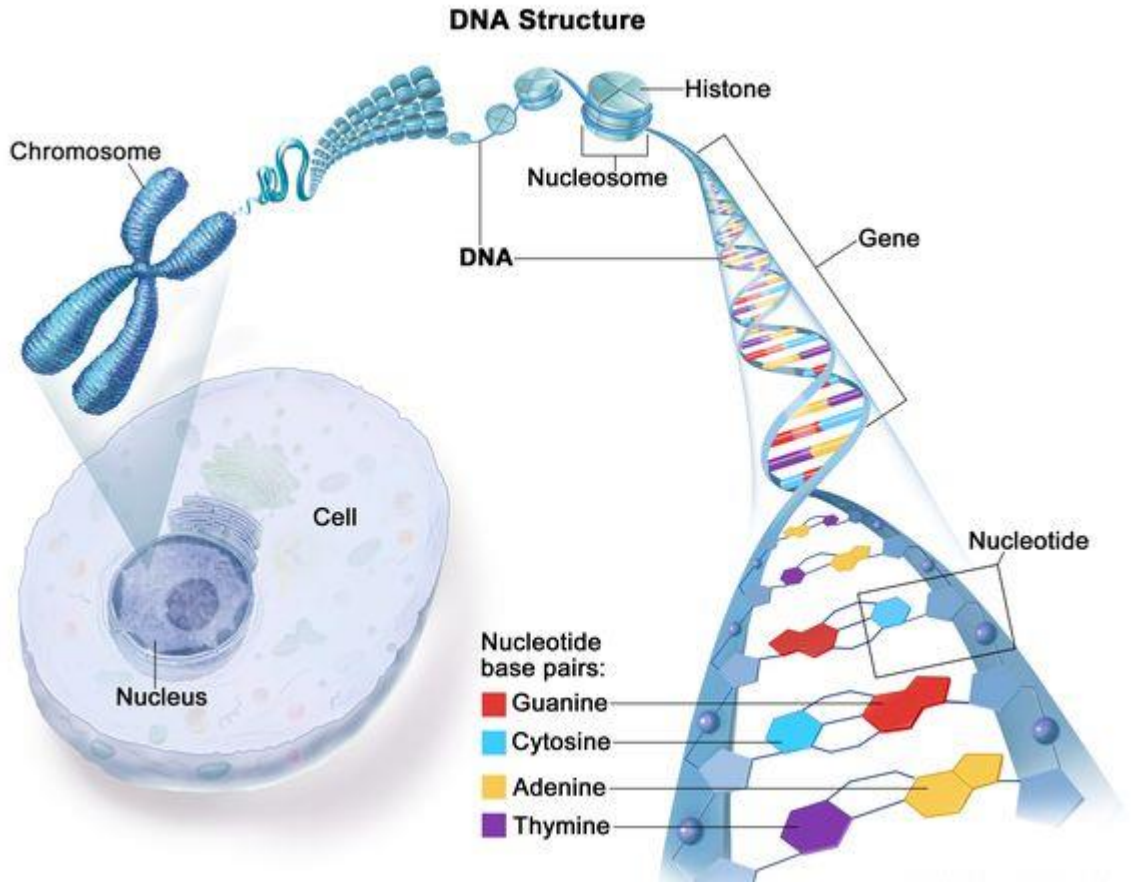

Figure 1.1 Deoxyribonucleic acid. Source: National Human Genome Research Institute; Glossary of Genomic and Genetic Terms. https://www.genome.gov/genetics-glossary/Deoxyribonucleic-Acid).

Telomeres are DNA-protein complexes that are located at the end of chromosomes and protect the chromosome. These telomere complexes are composed of a variable number of tandem TTAGGG repeats of DNA sequence (Meyne et al., 1989). TTAGGG repeats range from a few to 15 kilobases in length and are required for the maintenance of chromosomal and genetic stability (Blackburn, 2005). The number of telomeric repeats, known as “telomere length”, varies significantly between species, populations, individuals, and cell types (Dlouha et al., 2014, Blasco, 2005). Telomere length can be measured from specific tissues or from peripheral blood, the latter being the most frequent biological specimen evaluated in telomere research studies. Peripheral whole blood measurements can be used, or specific blood groups, usually leukocytes, can be used to examine telomere length (TL) (Montpetit et al., 2014).

Hexamers are molecules made up of six structural subunits. The hexameric TTAGGG DNA sequence is organised into a looped structure known as a T-loop. The looped structure consists of two strands, one strand being longer than the other, thereby creating an overhang known as a single stranded G-rich overhang (Blackburn, 2001). The overhang is made up of 50-300 nucleotides. This overhang loops back into the double stranded structure creating a D-loop within the T-loop structure. The D-loop protects loose DNA ends within the nucleoprotein structure (Figure 1.2).

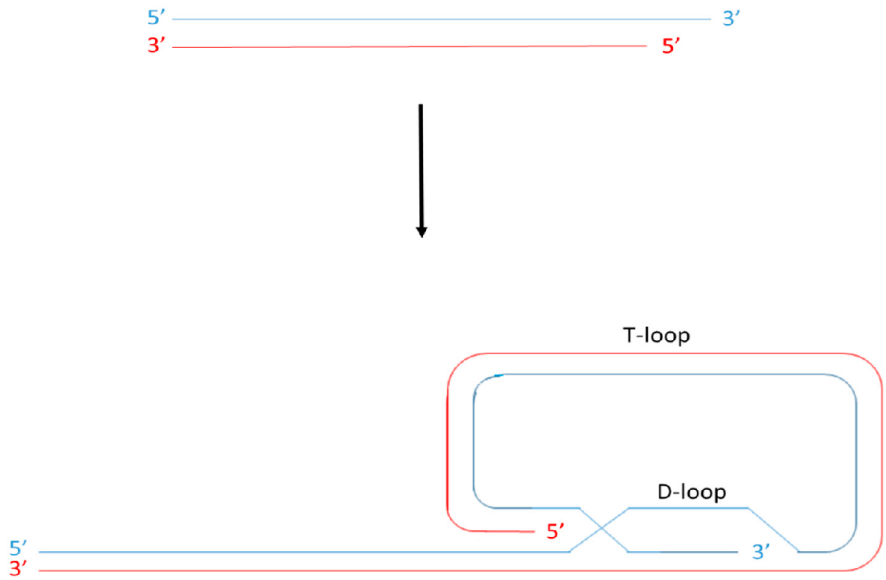

Figure 1.2 T-loop and D-loop structure. Source: Telomere Biology and Human Phenotype (Turner et al., 2019).

The shelterin complex (Figure 1.3) is an important protein structure that is associated with the telomere. It comprises six different proteins that are connected to the T-loop to create a capping structure at the ends of the telomere. This complex serves to safeguard telomeres (de Lange, 2005). The six proteins within the shelterin complex are essential in the regulation of telomere length. Interactions between proteins in the shelterin complex and the telomere hexameric DNA sequence provide stability to the telomere structure and regulate access of proteins involved in DNA maintenance, including DNA repair and lengthening (Kong et al., 2013). This specialised nucleoprotein structure protects the ends of chromosomal arms from inappropriate DNA repair mechanisms, thereby inhibiting chromosomal fusions (Amir et al., 2020). It also prevents the degradation of genes at the ends of chromosomes from incomplete DNA replication.

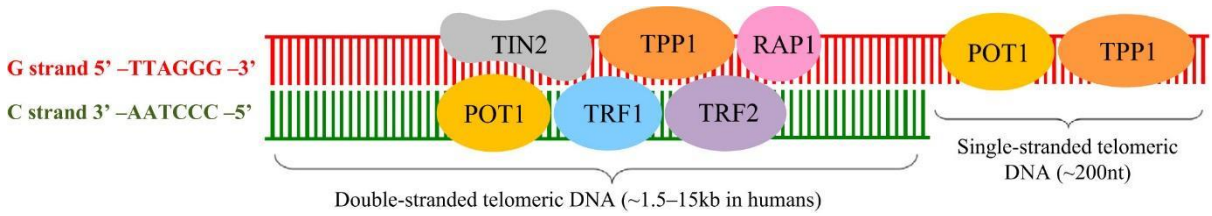

Figure 1.3 Telomere sequence and key protective proteins. Source: Cardiac telomere length in heart development, function, and disease (Booth and Charchar, 2017).

Abbreviations: *TRF1, telomere repeat-binding factor 1; TRF2, telomere repeat-binding factor 2; POT1, protection of telomeres 1; TIN2, TRF1 interacting nuclear factor 2; TPP1, tripeptidyl peptidase 1; RAP1, repressor/activator protein 1.*

Telomerase is an enzyme that is responsible for the maintenance of telomere length by the addition of guanine (G)-rich repetitive sequences (Cong et al., 2002). Telomerase is an intracellular ribonucleoprotein complex that consists of hTERT (human telomerase reverse transcriptase), hTERC (human telomerase RNA component), a non-coding human telomerase RNA, and associated cofactors (Nguyen et al., 2018) (Figure 1.4). Telomerase is recruited to the telomere through the telomerase essential N-terminal domain (TEN) of hTERT. Telomerase has a significantly reduced function in most mammalian somatic cells as cell proliferation is strictly limited and senescence follows approximately 50-70 cell divisions (Zvereva et al., 2010). While telomerase is responsible for the synthesis of telomere repeats by elongating the 3´ ends of chromosomes, DNA polymerase fills the complementary strand (Hug and Lingner, 2006). Thus, both telomerase and DNA polymerase are involved in creating new DNA during the DNA replication process (Greider, 1996).

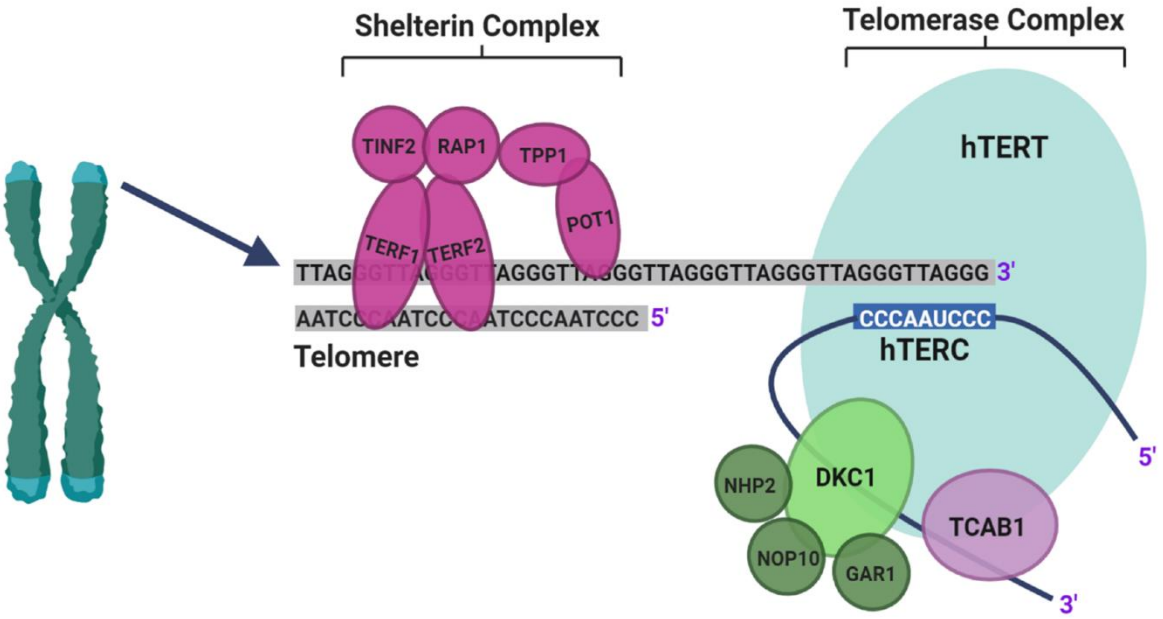

Figure 1.4 Telomere and telomerase components. Source: Investigating the role of telomere and telomerase associated genes and proteins in endometrial cancer (Bradfield et al., 2020).

Abbreviations: *GAR1, glycine and arginine rich protein 1; hTERT, human telomerase reverse transcriptase; hTERC, human telomeric RNA component; DKC1, dyskerin 1; RAP1, repressor/activator protein 1; TCAB1, telomerase cajal body protein 1; TINF2, TRF1 interacting nuclear factor 2; TPP1, tripeptidyl peptidase 1; NOP10, NOLA3 nucleolar protein family A, member 3; NHP2, NOLA2 nucleolar protein family A, member 2; POT1, protection of telomeres 1; TRF1, telomere repeat-binding factor 1; TRF2, telomere repeat-binding factor 2.*

1.3.2 The end replication problem

Cells undergo a process of mitosis whereby they divide and replicate. However, cells normally have a limited ability to replicate, known as the “Hayflick limit” (Hayflick and Moorhead, 1961), or the “end replication problem” (Figure 1.5). During the DNA replication process fragments of DNA are lost secondary to priming failure (Ohki et al., 2001). The loss of bases at template strand means that DNA polymerase is then unable to

synthesize new DNA leading to blunt ends, translating to the loss of DNA and contributing to telomere attrition (Chakhparonian and Wellinger, 2003). As a result, the cell is unable to replicate any further. When telomeres have shortened to a critical level, the shelterin complexes are no longer able to carry out their function in capping the end of the chromosome (de Lange, 2005). In addition, oxidative stress from reactive oxygen species (ROS) damages telomeres (Von Zglinicki, 2002). This results in the progressive shortening of telomeres with every mitotic event and the loss of their protective capacity (Bonnell et al., 2021). Thus, a major limiting factor in the function of the telomere is its length.

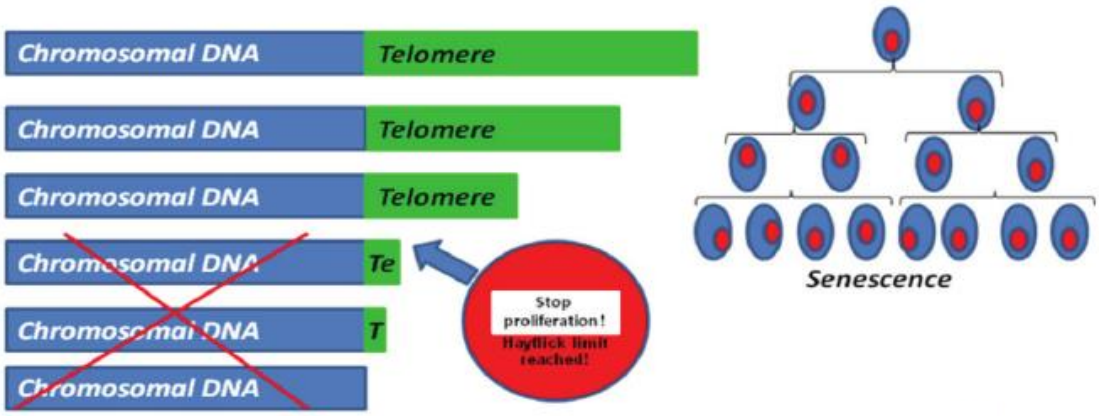

Figure 1.5 The hayflick limit. Source: Cancer Stem Cells – The Cutting Edge (Shostak, 2011)

With each DNA replication cycle telomeres shorten; TL therefore serves as a biomarker of biological ageing (Bernadotte et al., 2016). When telomeres become critically short, cells may enter senescence or undergo apoptosis (Blackburn et al., 2015). While cellular senescence leads to cell arrest, metabolic functions are still performed. In

the case of cellular apoptosis cells die and no longer participate in metabolic functioning. Cellular senescence and apoptosis are significant because cells are no longer able to carry out essential functions that maintain cell integrity (Bernadotte et al., 2016). This leads to the process of ageing as we know it (Zole and Ranka, 2018). TL is thus an accurate reflection of the cell's biological age, as opposed to the cell's chronological age (Epel et al., 2004).

1.3.3 Telomere length and anorexia nervosa

Due to the significant physical and psychological stress experienced by those with AN, I hypothesised that those with AN would experience accelerated biological ageing, which would be measured using TL. As the literature on TL and AN is currently non-existent, I reviewed the literature on TL and states that mimicked AN, including those with a low body mass index, regular intense exercise, and calorie restriction. Furthermore, because those with AN experience psychological stress, I reviewed the literature on TL and psychological stress.

1.3.3.1 Telomere length and body mass index

A key diagnostic criterion of anorexia nervosa is reduced body mass index (BMI) in the context of age, sex, developmental trajectory, and physical health (American Psychiatric

Association, 2013). Although the World Health Organisation's definition of a reduced BMI is <18.5kg/m^2, a diagnosis of anorexia nervosa can still be presumed if a person has a normal BMI in the context of significant and rapid weight loss that is not due to another illness.

Several meta-analyses have examined the relationship between BMI and TL and discovered that TL shortens with an increasing BMI (Gielen et al., 2018, Mundstock et al., 2015, Müezzinler et al., 2014). The meta-analysis by Gielen et al. (2018) included 87 studies and consisted of 146,114 adults, of which 40% were male and 83% were white. Mundstock et al. (2015) examined 63 studies which comprised 119,439 participants. Müezzinler et al. (2014) report on the results of 16 studies. However, these aforementioned meta-analyses have examined only those with a normal or increased BMI (Gielen et al., 2018), or those with a mean BMI in the overweight and obese categories (24.5 – 30.4) (Müezzinler et al., 2014, Mundstock et al., 2015). In addition, many studies included in these meta-analyses were cross-sectional studies, with a paucity of longitudinal studies. Potential associations between TL and BMI require further investigation with prospective studies, controlling for various confounders. Furthermore, many studies include mostly white participants, which limits interpretation of ethnic variations in TL. The meta-analysis by Gielen et al. (2018) reported that each unit increase in BMI equated to a -3.99 base pair difference in TL and that each unit increase in BMI equated to a -1.58 x 10 (-3) unit T/S ratio difference in TL (*n* = 146,114). T/S ratio correlates with the average TL and is calculated by comparing the amount of telomere repeat amplification product (T) to a single copy gene (S) product. A systematic review by Qiao et al. (2020) examined weight loss interventions and reported an increased TL among

overweight or obese adolescents or adults post-weight loss intervention. Of note, those with an increased BMI are more likely to experience co-morbid illnesses, which may potentially confound these results and many studies involved small sample sizes. Saha et al. (2022) reported no difference in TL between healthy children and those suffering from severe acute malnutrition (*n* = 80).

Although there is some evidence that TL is reduced with increasing BMI, there are no studies investigating TL and severely reduced BMI, as occurs in a condition such as AN. TL may be reduced in cases of increased BMI due to the increased stress on the body secondary to low grade inflammation and oxidative stress that occurs in obesity (De Heredia et al., 2012). Cachexia results in systemic inflammation (Evans et al., 2008) and thus, one could speculate that a severely reduced BMI secondary to starvation in AN may have a similar impact on reducing TL.

1.3.3.2 Telomere length and physical activity

Abrahin et al. (2019) compared TL between elite athletes (*n* = 306) and sedentary controls (n = 322) and reported longer telomeres in the elite athletes' group. All elite athletes had competed at national or international level and the authors concluded that high levels of chronic physical activity may preserve telomere length. Denham et al. (2013) compared TL in ultra-marathon runners with healthy non-marathon runners (*n* = 56) and discovered that ultra-marathon runners (*n* = 67) have 1% longer telomeres than healthy controls after adjusting for age and cardiovascular risk factors. Aguiar et al. (2021) reported that master

athletes (*n* = 240) had longer telomeres than age-matched non-athletes (*n* = 209). A systematic review by Arsenis et al. (2017) found that increased levels of physical activity in both healthy and chronically ill persons were associated with longer telomeres. This finding was particularly pronounced in middle-aged and older individuals. Du et al. (2012) reported that calisthenics or an aerobics-associated exercise is associated with longer leukocyte telomere length in active women compared to sedentary women (*n* = 7813). Loprinzi et al. (2015) reported a dose-relationship between movement-based behaviours and leukocyte telomere length (*n* = 6503). Movement–based behaviours included moderate intensity and vigorous intensity physical activity, walking and cycling for transportation and engaging in muscle-strengthening activities. Cherkas et al. (2008) reported longer telomeres in the leukocytes of those who are physically active, with those who are most active having the longest telomeres (*n* = 2401). Long term moderate exercise was associated with longer TL compared to short term moderate exercise in a study of 782 males (Savela et al., 2013). A meta-analysis by Lin et al. (2019) reported that physical exercise was associated with longer TL and that moderate exercise was particularly associated with longer TL compared to lower levels of exercise (*n* = 19,292). Moderate levels of exercise were associated with significantly longer TL compared with both the lowest and highest levels of exercise in a group of 69 participants (Ludlow et al., 2008). However, some studies have reported no difference in TL between participants assigned to an exercise regime versus controls (Shin et al., 2008, Mason et al., 2013, Sjögren et al., 2014). Mathur et al. (2013) compared healthy sedentary subjects with marathon runners and found no difference in TL (*n* = 32). A systematic review by Mundstock et al. (2015) reported mixed results. Furthermore, frequent exercise is

required to elicit the beneficial effects of telomere protection (Saßenroth et al., 2015), with evidence that over ten years of regular exercise is required.

Possible underlying mechanisms responsible for the preservation of TL with moderate exercise include lower oxidative stress and chronic inflammation (Borghini et al., 2015) as well as enhanced shelterin protein expression and telomerase activity (Nickels et al., 2020). Regular exercise can stimulate the expression of anti-inflammatory cytokines and reduce the concentration of pro-inflammatory cytokines such as C-Reactive Protein, Interleukin 1 and Tumour Necrosis Factor (Pedersen and Febbraio, 2012). Furthermore, regular exercise increases the activity of telomerase and telomerase reverse transcriptase (TERT), proteins necessary for the synthesis of telomere DNA through the addition of base pairs on the ends of chromosomes via reverse transcription (Denham et al., 2016, Chilton et al., 2014).

Although there is much evidence for the benefits of exercise on TL, there is no evidence for the effects of intensive exercise in situations where the body is under stress due to starvation and a severely low BMI, such as in AN. The stress of intensive exercise in cases where the body is physically and physiologically stretched to the limit is therefore unknown. There is evidence for a U shaped association between levels of exercise and TL (Ludlow et al., 2008), suggesting that while low levels of exercise are associated with reduced TL, intensive exercise in AN may also negatively affect TL.

1.3.3.3 Telomere length and nutrition

Calorie restriction is the reduction of calorie intake without causing malnutrition. Vera et al. (2013) found that calorie restriction attenuates telomere erosion associated with ageing and that synergizes with TERT over-expression in increasing "health span" and extending mouse longevity. Kark et al. (2012) conducted a longitudinal observational study of 609 participants and reported that there was an inverse relationship between calorie intake and leukocyte telomere length in men, but not in women. Leukocyte TL was determined by Southern blots. Energy intake was determined at baseline using a self-reported, semi quantitative food-frequency questionnaire. Four hundred and five men and 204 women were included in the study. Inverse associations were identified for all macronutrients; however, these associations were strongest for unsaturated fatty acids. Interestingly, Kiefer et al. (2008) examined dietary restraint, defined as chronic preoccupation with weight and attempts at restricting food intake, and found that higher levels of dietary restraint were associated with shorter leukocyte telomere length, independent of BMI, smoking status, and age (n = 56). This cross-sectional study included 36 pre-menopausal women, aged 20-50 years, and 20 post-menopausal women, aged 53-69 years. TL was examined from extracted DNA from peripheral blood mononuclear cells (PBMCs) using polymerase chain reaction (PCR). Dietary restraint was determined by a self-reported questionnaire. The authors concluded that dietary restraint serves as a source of chronic psychological and possibly biochemical stress, which may lead to telomere erosion. This may indicate that those with AN may have a reduced TL secondary to chronic dietary restraint.

A number of studies have also examined TL and association with vitamin B12, due to its involvement in the metabolism of nucleic acid precursors. A Vitamin B12 deficiency may lead to increased levels of homocysteine, which in turn may decrease methylation potential and increase oxidative stress, which negatively impacts on TL (Praveen et al., 2022). Shin and Baik (2016) found no association between TL and serum vitamin b12 levels in adults aged > 55 years without systemic inflammation, and these results was replicated in a studies by which report no association between TL and plasma concentration of vitamin B12, or ingestion of vitamin B12 (Paul et al., 2009, Xu et al., 2009). However, the study by Xu et al. (2009) did report that women who ingest vitamin B12 as a dietary supplement have increased TL in comparison to those who do not take vitamin B12 supplements.

1.3.3.4 Telomere length and psychological stress

Patients with AN are likely to experience stress due to extreme physical stress due to reduced BMI and psychological stress because of their mental illness. Stress is the body's response to physical, mental or emotional pressure and can be categorised as acute, episodic or chronic stress (Fink, 2010). Stress is comprised of an individual's level of exposure to stressors, the individual's perception of stress and the physiological stress response (Chida and Hamer, 2008).

An expanding body of literature has suggested that there is a relationship between psychosocial stress and reduced telomere length (Mathur et al., 2016). The literature suggests that physiological changes in response to stressors and perceived stress may be

responsible for the activation of mechanisms through which stress causes telomeres to be shortened (Von Zglinicki, 2002). Tyrka et al. (2016) reported that TL was shortened in individuals with major depression, depressive and anxiety disorders, in addition to those who has experienced maltreatment in childhood and parental loss (*n* = 290). One hundred and seventy-seven women and 113 men were included in this study and the majority of participants were white (*n* = 241). Childhood maltreatment was determined using the 28-item version of the Childhood Trauma Questionnaire. This questionnaire assessed moderate-severe levels of emotional and physical neglect, in addition to emotional, sexual and physical abuse. Parental loss included the loss of a parent before the age of 18 years and / or at least six months of separation from a parent. Whole blood DNA was analysed for TL using PCR. In fact, many studies now report on the association between childhood trauma, often termed early life sress (ELS), and shortened telomeres (O'Donovan et al., 2011, Boeck et al., 2018). Ridout et al. (2018a) reported that adults with a history of early life stress have shorter telomeres. A meta-analysis and systematic review by Mathur et al. (2016) described how increased short-term psychological stress was associated with a very small decrease in TL after adjusting for age (*n* = 8948). Schutte and Malouff (2016) found that higher levels of perceived stress were associated with shorter telomere length (*n* = 1143). A meta-analysis of non-human vertebrates reported that exposure to stressors was correlated with shorter telomeres or increased rates of telomere shortening (Chatelain et al., 2020). A large body of literature indicates that TL is shortened in those with mental illnesses and in those exposed to other forms of psychological stress, suggesting that those with AN may have a reduced TL. It is worth keeping in mind, however, that many reported studies on TL and mental illness include participants who

are prescribed psychotropic medications, the impact of which on TL is unknown. The literature on psychotropic medication and TL is difficult to interpret due to a range of conflicting results. Although one study reports that mood stabilizers are associated with longer TL (Squassina et al., 2020), a study by (Needham et al., 2015) found that participants with major depressive disorder who were prescribed antidepressants had a significantly shorter TL compared to healthy controls, while those with a major depressive disorder who were not prescribed antidepressant medication, did not have a shorter TL in comparison to controls. It may be that the latter group present with a less severe form of depression, however, results on psychotropic medication and mental illness need to be interpreted with caution.

1.4 Mitochondria

1.4.1 Introduction

Mitochondria, are double-membrane organelles located in the cell cytoplasm, consisting of an inner and outer mitochondrial membrane. These membranes form an inter-membrane surface with folds known as cristae, in the inner matrix, which increase the surface area (Figure 1.6).

Mitochondria are responsible for the production of ATP, or adenosine triphosphate, the energy currency of the cell. This is accomplished via the respiratory chain (RC), otherwise known as the electron transport chain (ETC). The RC is positioned

in the cristae and consists of five enzyme complexes (I, II, III, IV and V), each of which consists of multiple proteins (Figure 1.7). Sugars are catabolised via the Kreb's cycle while fats are catabolised via the beta-oxidation pathway, creating protons (H^+) through complexes I and II, respectively (Figure 1.7). Complex IV, otherwise known as cytochrome *c* oxidase, or COX, combines oxygen with the protons creating energy in the form of ATP (Nicholls, 2013).

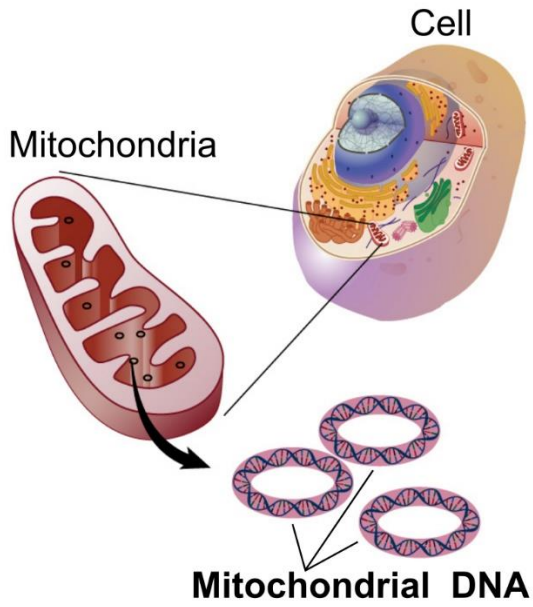

Figure 1.6 The mitochondrion. Source: The National Human Genome Research Institute, Mitochondrial DNA (https://www.genome.gov/genetics-glossary/Mitochondrial-DNA?id=129)

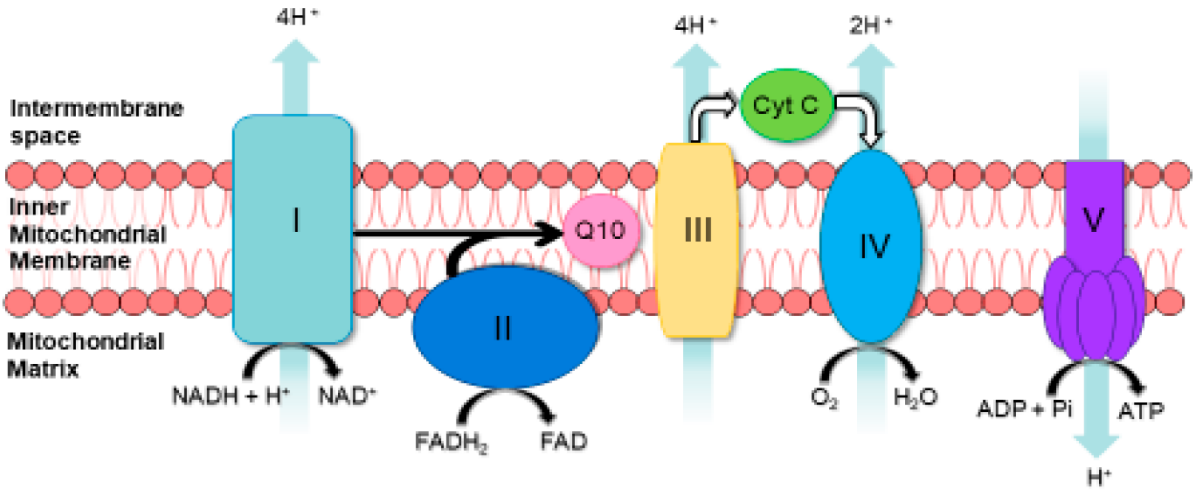

Figure 17. The Mitochondrial Respiratory Chain. Source: The Biochemical Assessment of Mitochondrial Respiratory Chain Disorders (Turton et al., 2022).

Abbreviations: H^+, protons; Cyt C: Cytochrome C; Q10: Co-enzyme Q10; 0_2: oxygen; H_20: water; NADH: Nicotinamide adenine dinucleotide + hydrogen; NAD: Nicotinamide adenine dinucleotide ; FADH: Flavin adenine dinucleotide + hydrogen; FAD: Flavin adenine dinucleotide; ADP: adenosine diphosphate; P: phosphate; ATP adenosine triphosphate.

The mitochondrion is responsible for many other functions apart from ATP production, including: intracellular signalling and apoptosis; the biosynthesis of lipid, nucleic acids, heme and iron-sulphur clusters; the production of reactive oxygen species; and calcium homeostasis (Wallace, 1992, Vakifahmetoglu-Norberg et al., 2017).

1.4.2 Mitochondrial DNA

Mitochondria are distinct in that they possess their own genome, known as mitochondrial DNA (mtDNA), which is separate from the nuclear DNA of the cell and is maternally inherited (Giles et al., 1980). The mitochondrial genome consists of a circular, double

stranded DNA (dsDNA) molecule, comprising a heavy strand and a light strand (Figure 1.8). The displacement loop (D-loop), a noncoding control region, contains the promotors required for the transcription of the light strand (otherwise known as light strand promoter, LSP) and the heavy strand (heavy strand promotors, HSP1 and HSP2). The D-loop is also the origin of replication of the H strand (red arrow shown in Figure 1.8 as O_H). The origin of the light strand replication is shown as O_L (Figure 1.8, red arrow). The dsDNA molecule is made up of 16,569 DNA base pairs and 37 genes. These genes are responsible for encoding 13 protein subunits of the oxidative phosphorylation system, 22 tRNAs for mitochondrial translation, and 2 rRNAs – 12S rRNA and 16S rRNA (Anderson et al., 1981, Bibb et al., 1981). The mitochondrial genome contains all the machinery necessary for its expression, i.e., to replicate, transcribe and translate the genetic information contained within the genome (Gustafsson et al., 2016).

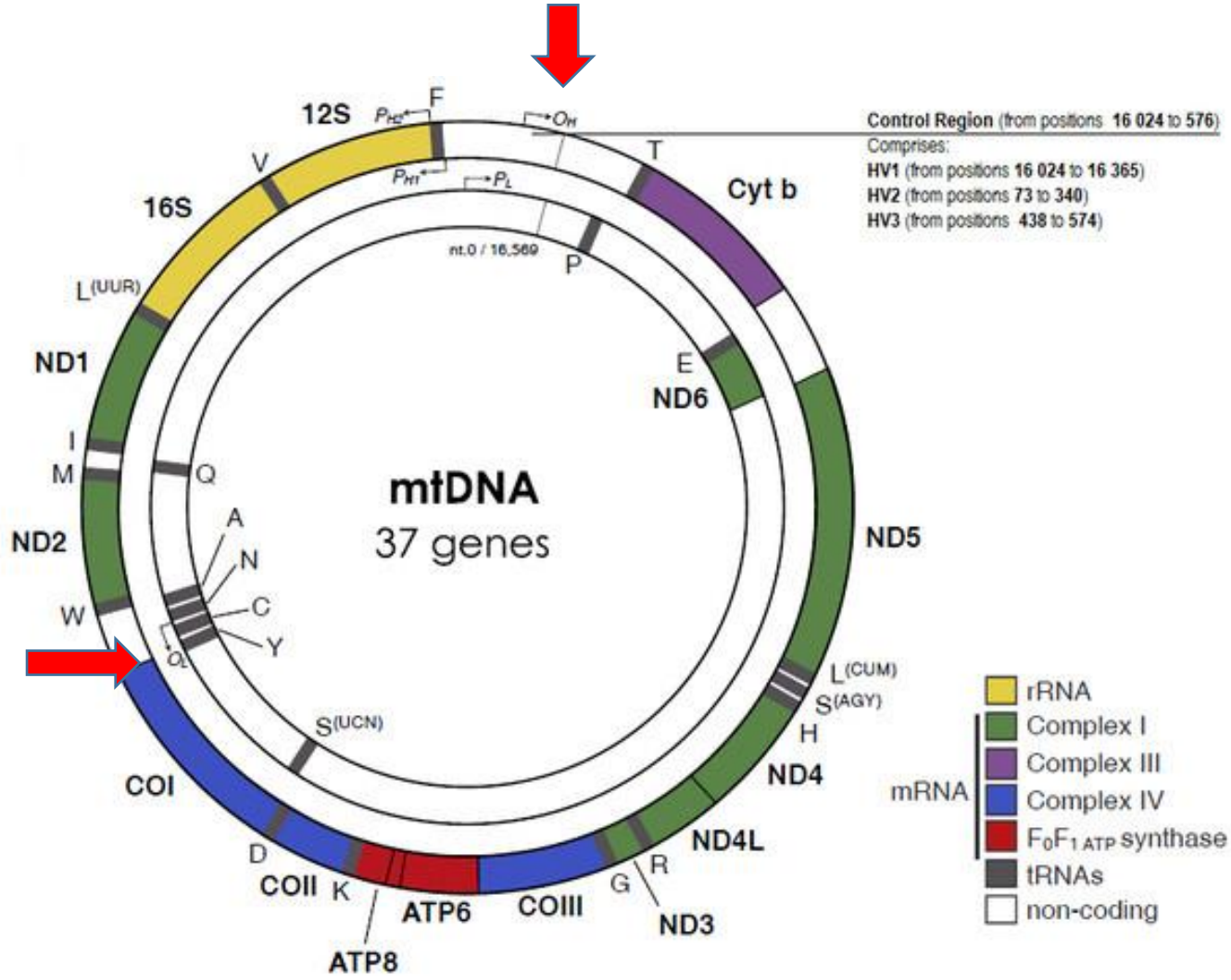

Figure 1.8 Mitochondrial DNA. Source: The rise of mitochondria in medicine (Picard et al., 2016).

Abbreviations: ATP: Adenosine Triphosphate Synthase; ND: Nicotinamide adenine dinucleotide; COI: Cytochrome c oxidase subunit 1; COII; Cytochrome c oxidase subunit 2; COIII: Cytochrome c oxidase subunit 3; Cyt b: Cytochrome b; O_L: origin of the light strand; O_H: origin of the heavy strand.

Unlike cellular nuclear DNA, mtDNA is not protected by histone proteins and has an inefficient DNA repair mechanism in comparison to that of nuclear DNA, making it more susceptible to damage compared to nuclear DNA (Yakes and Van Houten, 1997). In addition, the mtDNA is near the respiratory chain and is therefore subjected to damage by reactive oxygen species (ROS) production secondary to oxidative phosphorylation (Lee

and Wei, 2005). As a result, mitochondrial DNA has a higher rate of mutation in comparison to nuclear DNA (Alexeyev et al., 2013, Ishikawa et al., 2008). In fact, Harman (1955) first proposed the mitochondrial free radical theory of ageing whereby damage to mitochondrial DNA secondary to ROS production during cellular respiration contributes to the ageing process.

1.4.3 Mitochondrial DNA copy number

While the nuclear genome contains two copies per cell, the mitochondrial DNA differs as there are multiple copies of mtDNA per cell. mtDNA copy number varies greatly between cell types and depends on the energy requirements of the cell. Copy numbers in the heart average around 4-6 x 10^3 in comparison to copy numbers of approximately 0.5-2.0 x 10^3 in the liver, lungs, and kidney (D'Erchia et al., 2015b). Furthermore, mtDNAcn differs across different cell types (Robin and Wong, 1988).

Regulation of copy number is poorly understood to date, making it difficult to fully interpret copy number changes. Copy number can both increase and decrease with age (Picard and McEwen, 2018c). Studies in those aged >50 years report more consistent results where there is a reduction in mtDNAcn with increasing age (Mengel-From et al., 2014). Mechanisms may also exist where copy number is increased via a process of upregulation when required by the cell as an adaptive response to stress (Picard and McEwen, 2018c). However, over time, a decline in the mitochondrion's ability to replicate

and a reduction in mitochondrial bioenergetics can occur due to an accumulation of defective mitochondria and increases in reactive oxygen species (Malik and Czajka, 2013). This results in a reduction in mtDNAcn. It is thought that chronic stress may downregulate brain-derived neurotrophic factor (BDNF) in the cerebral cortex and hippocampus, resulting in impaired glucocorticoid receptor (GR) signalling and subsequent glucocorticoid resistance (Jeanneteau and Arango-Lievano, 2016). This may result in a reduction of mtDNAcn secondary to chronically elevated levels of glucose that damage mitochondria and mitochondrial DNA through the generation of toxic products and systemic inflammation (Picard et al., 2014).

Mitochondrial DNA copy number indirectly reflects the functional capacity of mitochondria and is a proxy measure of mitochondrial dysfunction (Picard and McEwen, 2018d). Mitochondrial dysfunction has significant implications as it leads to reduced capacity of the cell to produce ATP due to inefficiencies in oxidative phosphorylation, which in turn results in an increased production of reactive oxygen species (Pello et al., 2008). Damage subsequently occurs to the mitochondrion with a negative impact on the metabolism, growth, differentiation, and apoptotic ability of the cell, leading to ageing and increasing the risk of chronic illness (Nicolson, 2014).

1.4.4 Mitochondrial DNA copy number and anorexia nervosa

Due to the significant physical and psychological stress experienced by those with AN, I hypothesised that those with AN would experience accelerated biological ageing, which would be reflected via altered mtDNAcn. Although mtDNAcn is reported to both increase

and decrease with age, there is evidence that mtDNAcn increases with acute stress and in mental illness due to compensatory mechanisms to combat stress exposure (Cai et al., 2015b). As there are no available studies on mtDNAcn and AN, I researched mtDNAcn and states that mimicked AN including low BMI, regular intense exercise, calorie restriction and stress.

1.4.4.1 mitochondrial DNA copy number and body mass index

Studies examining mtDNAcn and body mass index (BMI) report mixed results overall and sample sizes are small, limiting interpretation of the results. Bordoni et al. (2019) reported a negative relationship between mtDNAcn and BMI in females (*n* = 69) from samples taken from buccal swabs. Meng et al. (2016a) found an inverse relationship between leukocyte mtDNAcn and weight, waist size, BMI, and waist-hip ratio in women (*n* = 1700). All participants reported in those results were within a normal BMI range or overweight/obese. The authors excluded the underweight group due to "potential differences in their aetiology". This study also examined bi-directional associations between mtDNCcn and changes in weight over time. The results show that women who gained weight over time (5 and 10 years) had significantly lower mtDNcn compared with those who maintained a stable weight over time (± 2 kg). Furthermore, a lower mtDNAcn at baseline was found to be negatively associated with future weight gain, meaning that those with a lower mtDNAcn tended to gain more weight over time (5 and 10 years). Similarly, Kaaman et al. (2007) demonstrated that mtDNA copy number in adipocytes decreased with an increasing BMI (*n* = 148). Notably, 64% of the participants were obese.

Obese participants were placed on a 10–week energy-restricted diet and achieved a reduction in BMI, however, this reduction in BMI was not associated with mtDNAcn. In contrast, Jokinen et al. (2018) conducted a weight loss programme (*n* = 38) and reported an increase in mtDNAcn in adipose tissue in those who regained weight at five months; however, this returned to baseline levels by 12 months. Those in the weight loss group experienced a reduction in mtDNAcn between 5 and 12 months. Of note, the participants in this case control study who were assigned to the weight loss programme (*n* = 19) were all obese, with an average BMI of 34.6 kg/m^2.

1.4.4.2 mitochondrial DNA copy number and physical activity

Most studies report an increase in mtDNAcn with physical activity, however, sample sizes remain small, again limiting interpretation of the results. Chen et al. (2017) reported a correlation between higher mtDNAcn and exercise efficiency (*n* = 15) using saliva samples. Constantin-Teodosiu et al. (2020) found that mtDNAcn was highest in a non-sedentary control group and lowest in a sedentary group with type 2 diabetes mellitus (*n* = 30). Similarly, Baykara et al. (2016) examined mtDNAcn in peripheral blood lymphocytes in three groups: highly trained swimmers, normal trained swimmers, and non-athlete participants (*n* = 24). The authors found that mtDNAcn was increased in highly trained swimmers compared to the other two groups. Balakrishnan et al. (2010) reported a statistically significant increase in muscle mtDNAcn in participants with moderate to severe chronic kidney disease who exercised in comparison to those who did not exercise (*n* = 23). An experimental-control, cross-sectional study by Kim et al. (2012) found that

mtDNAcn was significantly higher in those who exercised. Additionally, a cross-sectional study involving 144 post-menopausal women found that those who engaged in regular exercise, of a moderate and/or vigorous intensity, had higher leukocyte mtDNAcn compared with those who did not engage in regular exercise (Chang et al., 2016). In contrast, a larger study by Vyas et al. (2020) reported that there were no significant associations between physical activity measures in terms of frequency, amount, or intensity, and mtDNAcn (*n* = 391). The authors found no association between mtDNAcn and BMI. Similarly, a case-control study by Thyagarajan et al. (2012) reported no difference in mtDNAcn between participants who participated in regular physical activity compared to those who did not (*n* = 874).

1.4.4.3 mitochondrial DNA copy number and nutrition

Much evidence exists to suggest that a diet rich in fruit and vegetables and low in salt and sugar increases mtDNAcn (Wu et al., 2019b, Hernández-Ríos et al., 2013). Fruit and vegetables serve as sources of exogenous antioxidants, which protect mitochondrial function by reducing oxidative stress (Sun et al., 2002). Those with AN are more likely to follow a similar diet to achieve or maintain weight loss due to the lower calorie count in fruit and vegetables. However, most studies on mtDNAcn and nutrition are conducted with small sample sizes and these studies do not include participants with a significantly reduced BMI, as is the case in AN. A relatively large study with 2769 participants by Wu et al. (2019b) reported a positive association between leukocyte mtDNAcn and intake of fruit and flavonoids. Flavonoids are a group of phytochemicals that have antioxidant properties

(Barreca et al., 2017). In terms of calories intake, Ma et al. (2020) found that calorie intake was negatively correlated with mtDNAcn after adjusting for confounders (*n* = 403). A pilot study by Cancello et al. (2022) reported an increase in mtDNAcn in participants assigned to a 12 month weight loss intervention (*n* = 20). In contrast, Rabøl et al. (2009) found no difference in mtDNAcn after a weight loss intervention (*n* = 9). Similarly, Kaaman et al. (2007) reported no association between adipocyte mtDNAcn and energy-restricted diets (*n* = 148), however, most participants' weight was in the obese category, with an average BMI of 33. Although there are many studies reporting beneficial properties of fruit and vegetables, including increasing mtDNAcn, none of these studies investigate those with a greatly reduced BMI, in fact most studies investigate participants with a significantly increased BMI, usually within the obese category. Therefore, the results of these studies need to be interpreted with caution considering the significant body composition differences, and thus physiology, between those with AN, and those with obesity.

1.4.4.4 mitochondrial DNA copy number and psychological stress

Cai et al. (2015b) reported an increase in mtDNAcn in samples of blood and saliva in mice exposed to stressors (*n* = 16). The authors also conducted a study on 11670 women and discovered a significant positive correlation between mtDNAcn and major depression and stress-related illness. This study assessed mtDNAcn from whole-genome sequencing of salivary DNA samples. In contrast, a study by Liu and Zhou (2012b) reported a significant decrease in hippocampal and striatal mtDNAcn in rats exposed to stress compared to controls (*n* = 60). A study conducted by the research department in St Patrick's Mental

Health Services reported elevated mtDNAcn in patients with depression (*n* = 100), whom are likely to experience significant stress, compared to healthy controls (*n* = 89) (Ryan et al., 2023). Tyrka et al. (2016) reported on adults (n = 290) who experienced early life stress in the form of childhood maltreatment and parental loss before the age of 18 years; they had statistically significant increased levels of mtDNAcn in comparison to controls. The results of these studies suggest a complex dynamic between stress and mtDNAcn balance. It may be that while stress may increase mtDNAcn secondary to a compensatory response such as activation of the hypothalamic-pituitary-adrenal axis (McEwen, 1998) and changes in the functioning of the neuroimmune system (Graham et al., 2006), in other instances stress may reduce mtDNAcn due to other factors. Furthermore, changes may be tissues specific, Cai et al. (2015b) reported increases in mtDNAcn in liver and a decrease in muscle tissues, with no changes observed in mtDNAcn in ovarian nor hippocampal tissues.

1.5 Relationship between telomere length and mitochondrial DNA copy number

There is evidence that a positive relationship exists between TL and mtDNAcn (Lindqvist et al., 2018b). Tyrka et al. (2015b) examined the relationship between TL and mtDNAcn in a sample of 392 healthy adults. The authors reported a positive association between mtDNAcn and TL in the sample after adjusting for age, smoking and BMI. Participants were sub-grouped - depending on history of adversity and presence of psychiatric disorder - and the association between TL and mtDNAcn remained significant within all subgroups.

These associations also remained significant after controlling for confounders including age, smoking and BMI. Kim et al. (2013b) examined 129 female participants, living in the community. The authors found that mtDNAcn was positively associated with TL. Likewise, Meng et al. (2016a) reported a strong positive association between mtDNAcn and TL in a cohort of 1700 healthy participants. However, a study by Tyrka et al. (2016) reported an inverse relationship between TL and mtDNAcn; a decrease in TL was associated with an increase in mtDNAcn. Our own group has recently reported no association between mtDNAcn and TL (Ryan et al., 2023).

We do not yet fully understand the relationship between TL and mtDNAcn. There may be a co-regulation of telomeres and mitochondrial function; however, mechanisms underlying this relationship are poorly understood. Such a relationship may be important in understanding the pathophysiology of biological ageing. It is thought that damage to mitochondria and mitochondrial DNA, secondary to oxidative stress, may lead to a reduction in TL and trigger cellular senescence (Picard and McEwen, 2018c). In turn, peroxisome proliferator-activated receptor-ɣ coactivator 1α (PGC-1 α) is a key element in mitochondrial biogenesis and is downregulated by tumour suppressor protein p53 in the presence of telomere dysfunction, leading to apoptosis and reduced mitochondrial biogenesis (Gonzales-Ebsen et al., 2017). Furthermore, telomerase reverse transcriptase may also be involved in in the functioning of mitochondria. Telomerase reverse transcriptase appears to translocate to the mitochondrion from the nucleus in the presence of oxidative stress, where it influences respiratory chain function, production of ROS, damage to the mitochondrion and apoptosis (Ale-Agha et al., 2014, Jaiswal et al., 2013).

1.6 Conclusion

Patients with AN experience acute stress during a relapse of AN. Due to their lifelong perfectionistic and obsessional tendencies, a core characteristic of patients with AN, they are also more likely to experience chronic stress. Stress accelerates the ageing process (de Magalhães and Passos, 2018). Deciphering the cellular processes involved in ageing, and the complex interactions of multiple factors involved in this process, both genetic and environmental, may help to reduce the rate of ageing and improve quality of life. Understanding these processes may help us to identify effective strategies to alleviate the burden of disease and disability and to encourage healthy ageing. Ideally, interventions to reduce the rate of ageing and limit the ageing process should be implemented while individuals are still young (Fontana et al., 2014). Furthermore, research in younger cohorts on ageing may prove more effective due to the lower risk of morbidity and polypharmacy in a younger age group. That said, many illnesses start in early life, unknown to individuals, and are only diagnosed later in life. However, by developing an in-depth understanding on the process of ageing, the identification, assessment, and management of this process can potentially help us to delay or reverse some of the elements of ageing to improve quality of life. The knock-on effect of this is enormous, with the potential to result in reduced carer burden and reduced dependence on our health care services, and to alleviate the impact on the economy.

Despite undeniable reductions in an individual's capacity with age, the process can be managed to optimise ageing. As the global population ages, the prevalence of disease

and disability is increasing (Vos et al., 2012), the treatment of which place significant costs on our healthcare systems and economies (Goldman et al., 2013). Research has shown that the most effective method of reducing these costs is to improve the health of the population to increase the number of years of life free from disease or disability (Burch et al., 2014). Identifying those at an increased risk of ageing, particularly those with substantial physical and psychological stress, such as in AN, may reduce the burden on our healthcare system and our economy in the future. Thus, focussing on issues associated with ageing, including increased dependence on services and on others, and attempting to intervene exogenously to delay the ageing process, may be one of the most effective ways to reduce disease and disability (Gavrilov and Gavrilova, 2001).

1.7 Objectives and hypothesis

1.7.1 Objective

To conduct a case-control, observational study comparing mitochondrial DNA copy number (mtDNAcn) and telomere length (TL) between patients with anorexia nervosa (AN) and age- and sex-matched healthy controls, and to explore the relationship between TL and mtDNAcn, and duration and severity of illness. The aim of my study is to provide a better understanding of the effects of AN on the ageing process and to characterise the effects of AN at a molecular level through the measurement of mtDNAcn and TL.

1.7.2 Hypothesis

(1) TL is reduced in patients with AN compared to healthy controls; (2) TL is reduced with increasing duration and severity of illness (3) mtDNAcn is increased in patients with AN compared to healthy controls; (4) mtDNAcn is increased with duration and severity of AN; and (5) mtDNAcn is associated with TL.

CHAPTER 2. MATERIALS AND METHODS

2.1 Laboratory materials

2.1.1 General laboratory ware

• Becton Dickenson (BD) Vacuette® winged blood collection device	Becton Dickenson, UK
• BD 10ml potassium ethylenediaminetetraacetic acid (K_2EDTA) tubes	Becton Dickenson, UK
• BD Sharps container 3 Litre (L)	Becton Dickenson, UK
• Micropipettes	Thermo Scientific, UK
• Micropipette tips	Thermo Scientific, UK
• DNaseErase® 500ml Decontamination solution	Fisher Scientific, UK
• Polymerase Chain Reaction (PCR) tubes 0.2ml, 1.5ml, 2ml	Sarstedt, Germany
• 96 well PCR plates and covers	Fisher Scientific, UK
• Vortex IKA MS3 basic	IKA Works Inc, NC, USA
• Mini Centrifuge	Fisher Scientific, UK
• Centrifuge 5417C	Eppendorf, US
• StepOnePlus™ Real-Time Polymerase Chain	Thermo Fisher Scientific, USA

Reaction system

- Sterile purified water (H_20) — Sigma Aldrich, Ireland

2.1.2 Telomere Length Assay

- Normalisation controls — Sigma Aldrich, Ireland
- Henrietta Lacks (HeLA) Deoxyribonucleic Acid (DNA) — Sigma Aldrich, Ireland
- Dithiothreitol (DTT) — Thermofisher Scientific, USA
- Primers — Eurofins Genomics, Germany
- Quantitect SYBR™ Green PCR Kit — Qiagen, USA

2.1.3 Mitochondrial DNA copy number assay

Human Mitochondrial DNA monitoring primer set — Takara Bio Inc., Sweden

- Primers (ND1, SLCO2B1, ND5, SERPINA1)
- TB Green® *Premix Ex Taq (thermus aquaticus)* II (Tli RNaseH Plus)
- ROX™ reference dye

2.2 Phenotyping materials

2.2.1 Demographic variables

Demographic variables were obtained at baseline from patient and control interviews and patient clinical records. Only the minimum required data were collected. Identifiable data were not included. Demographic data included: age, sex, ethnicity, occupational status, occupation, educational attainment level, marital status, alcohol and substance use, and smoking status. Educational attainment was measured as number of years in education.

2.2.2 Clinical variables

Clinical variables including weight, height, body mass index (BMI) were collected on all participants. BMI was calculated based on the weight in kilograms divided by height in metres squared (kg/m^2). Clinical data collected on cases included: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) diagnosis (anorexia nervosa (AN) restrictive type or AN binge-purge type), age at first onset of illness, duration of AN, previous psychiatric admissions for the treatment of AN, and history of nasogastric feeding. A list of prescribed medication was obtained. Full blood count (FBC) cellular components (neutrophil, lymphocyte, and platelet count) for cases only were collected. Patients with AN were also asked about other therapies (including engagement with allied

health professionals) and programmes that they might have been attending while in hospital.

2.2.3 Study assessments

2.2.3.1 Mini-International Neuropsychiatric Interview for DSM 5

The Mini-International Neuropsychiatric Interview (M.I.N.I.) for DSM-5 version 7 (Sheehan, 2014) was used for both cases and controls. This is a brief structured interview for the major psychiatric disorders in DSM-5. The M.I.N.I has similar reliability and validity properties as other instruments such as the Structured Clinical Interview for DSM-5 (S.C.I.D); however, the M.I.N.I can be delivered in a shorter period of time. The M.I.N.I is divided into modules that correspond to major psychiatric disorders such as major depressive episode, manic and hypomanic episode. Screening questions and further detailed questions are asked in each module. All questions are rated as “yes” or “no”. Diagnoses of AN and AN sub-types, restrictive type and binge-purge type, were confirmed with this assessment in the cases cohort. This assessment was also used for controls to screen for psychiatric illness.

2.2.3.2 Eating Disorders Examination

The Eating Disorders Examination (EDE) edition 17.0D (https://www.credo-oxford.com/pdfs/EDE_17.0D.pdf) was used for both case and controls. This instrument is considered the "gold standard" for the identification of eating disorders. The EDE edition 17.0D was updated from edition 16.0D (Fairburn CG, 2008) due to the need to generate DSM-5 eating disorder diagnoses. This instrument has good interrater reliability, with correlation coefficients ranging from 0.96 – 0.99, and good internal consistency, with a range of correlation coefficients from 0.75 for the restraint sub-scale to 0.90 for the eating concern scale (Guest, 2000b). In addition, high levels of treatment validity, construct validity, and discriminant validity were also found in the assessment of eating disorders (Guest, 2000a).

The purpose of using this assessment for cases was to rate the severity of illness and to categorise sub-type of AN (restrictive type or binge-purge type). Individuals without eating disorders are still likely to score, albeit on a low level, on this assessment and thus this was also used for controls. All questions in this interview are based on eating behaviours, and weight and shape cognitions over the previous 28 days. Items are rated on a seven-point severity scale ranging from 0 to 6, with a high score reflecting a higher burden of eating disorder severity. There are 23 items in total. Scores from all 23 items are added together to provide an overall global score. Subscales scores may also be obtained. Subscale scores provide information on the severity of four different aspects of eating disorder psychopathology: restraint, eating concern, weight concern and shape concern. To obtain a particular subscale score the ratings from the relevant items are

added together and the sum is divided by the total number of items forming the subscale (Table 2.1).

Eating Disorder Examination Subscale items	
Restraint	**Shape concern**
Restraint over eating	Flat stomach
Avoidance of eating	Preoccupation with shape or weight
Food avoidance	Importance of shape
Dietary rules	Fear of weight gain
Empty stomach	Dissatisfaction with shape
	Discomfort seeing body
	Avoidance of exposure
	Feelings of fatness
Eating concern	**Weight concern**
Preoccupation with food, eating or calories	Importance of weight
	Reaction to prescribed weighing
Fear of losing control over eating	Preoccupation with shape or weight
Eating in secret	Dissatisfaction with weight
Social eating	Desire to lose weight
Guilt about eating	

Table 2.1 Eating Disorders Examination Sub-scales (Fairburn CG, 2008)

2.3 Laboratory methods

2.3.1 Blood sample collection

All participants were fasting overnight. Peripheral whole blood samples were taken between 07:30 and 09:30am at St Patrick's Mental Health Services (SPMHS). Samples were obtained using a BD Vacuette® winged blood collection device and collected in 10ml potassium ethylenediaminetetraacetic acid (K_2EDTA) tubes from the ante-cubital fossa of the non-dominant arm. Samples were inverted 8 – 10 times immediately after blood collection. These samples were stored within 20 minutes of collection at -20°C for 24 hours at SPMHS. Blood samples were then transported to St James's Hospital within a 30-minute time frame by the researcher in a 10 litre Styrofoam box containing ice. Blood samples were stored at -80°C in St James's Hospital until DNA extraction and further analysis.

2.3.2 DNA extraction

Whole blood DNA was extracted using an Autopure LS ® (Qiagen, Germany) system by Trinity College Dublin's Biobank facility (https://www.tcd.ie/ttmi/facilities/trinity-biobank/). The Biobank facility is located at St James's Hospital and the DNA extractions were completed by the core facility. DNA is made up of nucleic acids as discussed in Chapter 1. The quantification of nucleic acids per sample is performed to determine the

concentration and purity of DNA in that sample. This is achieved by calculating the optical density (OD). DNA is exposed to ultraviolet (UV) light at a wavelength of 260 nanometres (nm). The amount of light that passes through the sample is measured. The remaining light that is absorbed by the nucleic acids contributes to the optical density. A higher nucleic acid concentration in a sample will lead to a higher optical density as less light passes through the sample. Nucleic acids have an absorbance maximum at 260 nm (A260). One A260 unit equates to the amount of nucleic acid contained in 1ml that produces an optical density of 1. The ratio of this absorbance maximum (A260nm) to the absorbance at 280 nm (A280) has been used to quantify purity in DNA extractions. An A260/280 ratio of ~1.8 is generally considered "pure" for DNA (Wilfinger et al., 1997). For this study purified DNA had an A260/A280 ratio of 1.6–1.9.

Extracted DNA samples were transported to and stored at -20°C at the McLoughlin Laboratory, Trinity College Institute of Neuroscience (TCIN) until analysis. The Laboratory was locked with limited staff access. I transported the from St James's Hospital to TCIN within a 30-minute time frame in a 10 litre Styrofoam box containing ice.

2.3.3 General laboratory preparation

Unless otherwise stated, all solutions were prepared with sterile purified water (H_20). General laboratory techniques were adapted from "Practical Skills in Biomolecular Sciences (Reed and Reed, 2007). All bench surfaces and equipment were decontaminated using DNaseErase® before use. A separate lab coat was used specifically for work involving

PCR to avoid cross-contamination with other laboratory work. Windows in the laboratory remained closed to avoid potential DNA contamination of sample. Laboratory analysis was conducted at the McLoughlin Lab, Trinity College Institute of Neuroscience (TCIN), The Lloyd Building, Trinity College Dublin, Dublin 2. All mandatory health and safety procedures were followed.

2.3.4 Telomere length assay

2.3.4.1 Introduction

Peripheral whole blood telomere length (TL) was measured using quantitative real-time polymerase chain reaction (qRT-PCR) (Cawthon, 2002). Discovered by Kary Mullis in 1983, PCR is a method of amplifying samples of DNA (Mullis, 1990). The samples are initially heated causing the double stranded DNA to “denature” or separate into two pieces of single-stranded DNA (Figure 2.1). Thermus aquaticus (Taq) polymerase, an enzyme responsible for the synthesis of new strands of DNA, uses the single stranded DNA as a template for the new strands, and duplicates the DNA. The process is repeated several times creating over one billion copies of the original DNA.

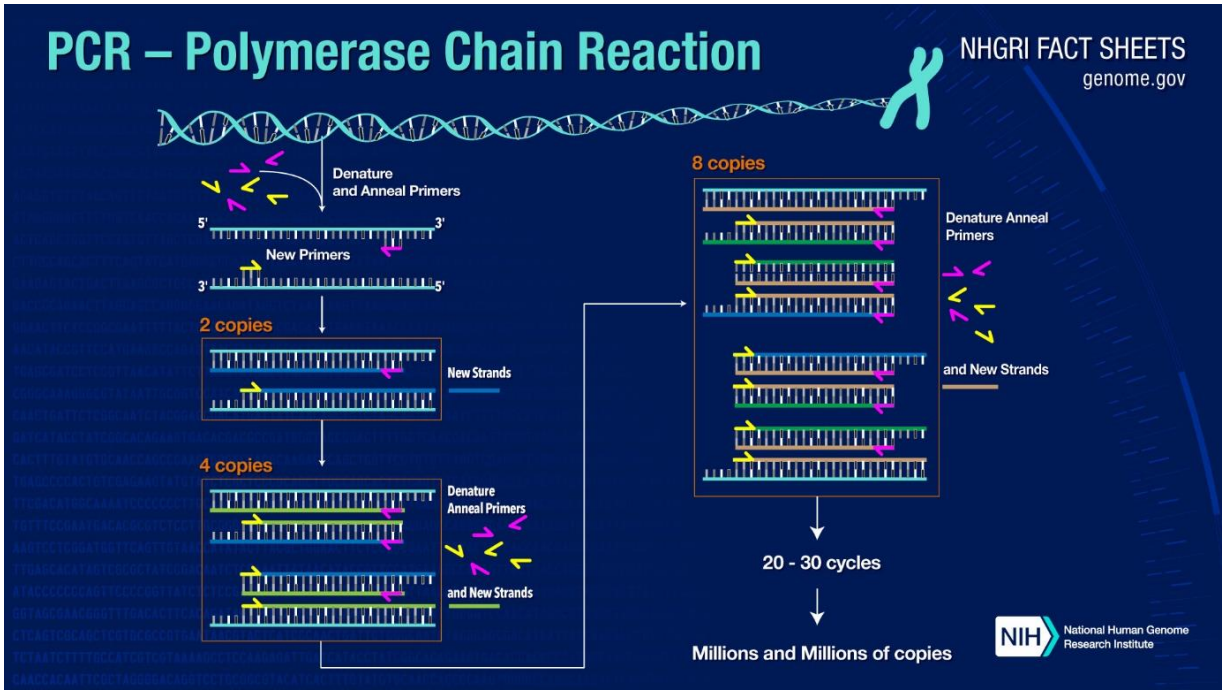

Figure 2.1 Polymerase Chain Reaction. Source: https://www.genome.gov/about-genomics/fact-sheets/Polymerase-Chain-Reaction-Fact-Sheet.

DNA samples are prepared and then pipetted into a 96-well plate. qRT-PCR quantifies, for each well containing samples of DNA, the $C_{t,}$ or fractional cycle number. This is the point at which the DNA's accumulating fluorescence crosses a set threshold, which is several standard deviations (SDs) above baseline fluorescence (Higuchi et al., 1993). A linear graph is created from these amplifications (Figure 2.2) which reflects the C_t (amount of input target DNA). This allows us to determine the simple relative quantification of unknown DNA (in the samples) in comparison to the standard curve (serial dilutions) of known DNA quantities as a reference. This is known as the absolute quantification method.

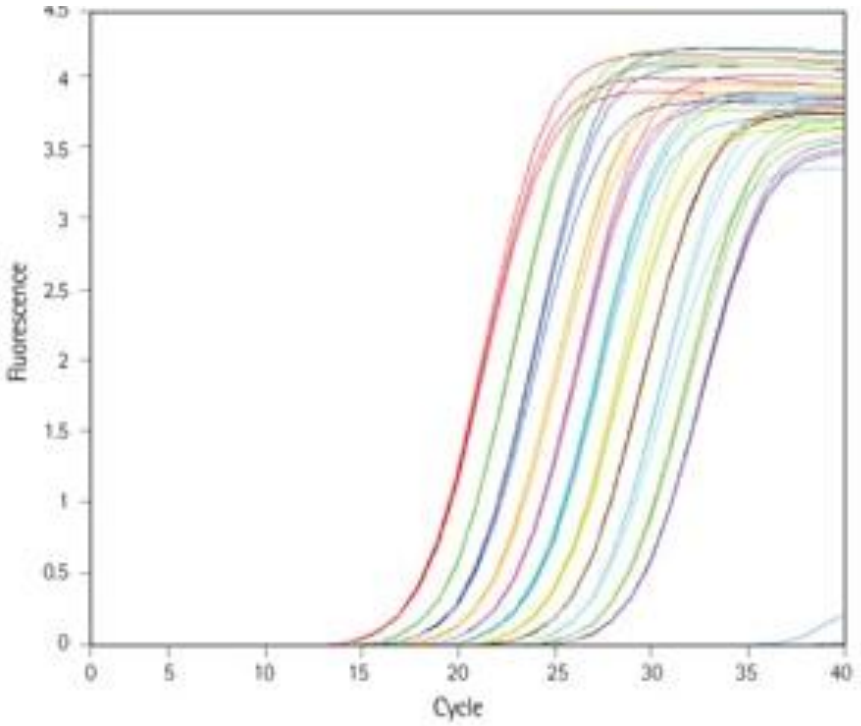

Figure 2.2 Linear graph created from DNA amplifications during PCR (X-axis: cycle; y-axis: fluorescence). Source: https://www.merckmillipore.com/IE/en/life-science-research/genomic-analysis/dna-preparation-cloning/PCR/qPCR/7Rub.qB.GXwAAAFEdVlXHZkB,nav.

TL is estimated by comparing the amount of telomere repeat amplification product (T) from the genomic DNA sample (the study participant), to a reference DNA sample referred to as a single copy gene (S) product. The single copy gene product used was the β-globin gene (hgb). The T/S ratio correlates with the average TL. Telomere (T) PCRs and single copy (S) PCRs are performed in separate 96-well PCR plates. TL is therefore expressed as a telomere to single-copy gene (T/S) ratio.

Each sample was assayed in duplicate wells on three separate plates, with identical well positions used across all plates. Repeated measures of the T/S ratio on the same DNA sample provide the lowest variability when the DNA sample well position remains

consistent across plates, i.e. row and column coordinates for the first T PCR plate matches the row and column coordinates for the S PCR on the second plate (Cawthon, 2002).

Each well contained either study participant's sample or a control sample in addition to the standard curve and the "master mix". The "master mix" consisted of quantitect SYBR™ Green, Dithiothreitol (DTT) and primers. All chemicals and reagents used will be further discussed in the following sections. Three interplate calibrators from healthy controls, not included in the study sample, were included on each plate to account for inter-run and intra-run variability.

2.3.4.2 DNA samples and normalisation controls

To reduce pipetting error when performing PCR, a working concentration DNA plate was generated which included DNA samples that were all at consistent and known concentrations. These are known as normalisation controls. Normalisation controls contained exact known quantities of DNA to ensure accurate lab technique and measurement. A perfect normalisation control is a gene whose expression does not vary at all, however, no such gene exists. The expression of all genes is variable for several reasons including the origin of the cell or tissue, the developmental stage of the cell or tissue, and the conditions of the laboratory experiment. Thus, to reduce bias, at least two reference genes should be selected that are shown to have minimal variability. Five reference genes were used as PCR normalisation controls in this study:

1. MCF7: Michigan Cancer Foundation-7, a breast cancer cell line.

2. UM-UC-3: an epithelial-like cell urinary bladder cell line.
3. HEK293: Human Embryonic Kidney cell line.
4. 1301: a T-cell leukaemia cell line.
5. K562: a myelogenous leukaemia cell line.

The samples and normalisation controls were prepared as follows:

- Samples and normalisation controls (MCF7, UM-UC-3, HEK293, 1301, K562) (100 ng/µl) were taken from the freezer and placed on ice.
- Samples/controls were allowed to defrost, then the tubes were vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tubes.
- Two sets of 0.2 ml PCR tubes were labelled to match the sample/control names. Permanent marker was used to mark an "a" on one tube and a "b" on the other.
- "a" tubes (2 ng/µl; 1:50 dilution): 98 µl H_2O was added to all tubes. Then, 2 µl of stock DNA was added to the respective tubes. The tubes were Vortexed gently to mix contents and centrifuged briefly to ensure all contents were at the bottom of the tubes.
- "b" tubes (0.2 ng/µl; 1:10 dilution): 180 µl H_2O was added to all tubes. Then, 20 µl of sample/control from the "a" tubes was added to their counterpart "b" tubes. The tubes were vortexed gently to mix contents and centrifuged briefly to ensure all contents were at the bottom of the tubes.
- The sample/control from the "b" tube will be used for plating on the PCR plate later (0.2 ng/µl x 10 µl per well = 2 ng DNA per well of the PCR plate).

2.3.4.3 Standard Curve

A standard curve is a tool that allows the quantification of DNA of unknown samples by comparing them with samples of known DNA concentrations known as standards. A five-point standard curve of Henrietta Lacks (HeLa) cell DNA ranging from 0.31–25.0 ng was included on each plate so that the quantity of targeted templates in each sample could be determined relative to the standard curve. All samples and standards were run in duplicate to ensure a high-quality standard curve. During each PCR cycle, the amount of fluorescent signal for each standard in the dilution series is estimated. When the fluorescent signal crosses the detection threshold the cycle number is recorded as a C_t value, or threshold cycle value, and this is used to create the standard curve. The C_t values are inversely proportional to the concentration of the DNA in the standards. Each C_t value for each dilution of the standard curve is plotted on a graph. The standard curve was prepared as follows:

- Eight x 0.2 ml tubes were labelled as follows: 75 ng, 25 ng, 8.33 ng, 2.78 ng, 0.93 ng, 0.31 ng, 0.10 ng. Tubes were placed on ice from a -20°C freezer.
- HeLa DNA stock tube was taken from the -80°C freezer and allowed to thaw on ice.
- HeLa stock tube was gently vortexed and centrifuged briefly to ensure all contents are at the bottom of the tube.
- *25 ng standard:* 15 µl HeLa DNA stock was added to 185 µl H_2O in a 75 ng tube. 65 µl was taken from the 75 ng tube and added to 135 µl H_20. The tubes were vortexed

gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.

- *8.33ng standard:* 65 µl was taken from the 25 ng tube and added to 135 µl H_20. The tubes were vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- *2.78 ng standard:* 65 µl was taken from the 8.33 ng tube and added to 135 µl H_20. The tubes were vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- *0.93 ng standard:* 65 µl was taken from the 2.78 ng tube and added to 135 µl H_20. The tubes were vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- *0.31 ng standard:* 65 µl was taken from the 0.93 ng tube and added to 135 µl H_20. The tubes were vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.

2.3.4.4 Dithiothreitol (DTT)

DTT, alternatively known as Cleland's agent (Cleland, 1964), is a reagent used to stabilise enzymes and other proteins that possess free sulfydryl groups. DTT reduces disulfide bonds and maintains monothiols in a reduced state. DTT is bought at a concentration of 1 M stock but I required 2.5 mM in 0.5 µl for the master mix; therefore, to make 125 mM stock (1 in 8 dilution):

- 62.5 µl DTT was added to 437.5 µl H_2O in a 1.5 ml tube.
- The tube was vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- The tube was placed on ice.

2.3.4.5 Primers

Primers are short pieces of single-stranded DNA, consisting of approximately 20 nucleotides in length. In every PCR reaction, two primers are used, to enable DNA synthesis or amplification and later quantification. The region of DNA that is copied in PCR reactions depends on the primers used.

The telomere primers used in this reaction were tel1b (CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT) at a concentration of 100 nM, and tel2b (GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) at a concentration of 900 nM. The primers for the single-copy gene (*Hgb*) were Hgb1 (GCTTCTGACACAACTGTGTTCACTAGC) at a concentration of 300 nM, and Hgb2 (CACCAACTTCATCCACGTTCACC) at a concentration of 700nM. Primers were salt-free purified (Eurofins Genomics, Germany).

2.3.4.5.1 Tel1b (100nM)

Tel1b primer is bought as 100 µM stock, however for this experiment a concentration of 2.5 µM is required. This requires a 1 in 4 dilution, followed by a 1 in 10 dilution. 2.5 µM of

the Tel1b primer is then later diluted in 25 µl Master Mix giving a final concentration of 0.1 µM.

- Two aliquots of Tel1b primer were taken from freezer and placed in ice. These were allowed to defrost then combined contents into one tube, vortexed gently and centrifuged briefly to ensure all contents are at the bottom of the tube.
- Two x 1.5 ml tubes were labelled as follows: Tel1b(1), Tel1b(2).
- Tube Tel1b(1) [1 in 4 dilution]: 15 µl of Tel1b stock primer was taken and added to 45 µl sterile purified H_2O. The tube was vortexed gently to mix contents and centrifuged briefly to ensure all contents were at the bottom of the tube.
- Tube Tel1b(2) [1 in 10 dilution]: 35 µl from Tel1b(1) tube was taken and added to 315 µl double distilled H_2O. The tube was vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- The tube was placed on ice.

2.3.4.5.2 Tel2b (900nM)

Tel2b primer is bought as 100 µM stock, however for this experiment a concentration of 22.5 µM is required. This requires a 1 in 4.44 dilution. 22.5 µM of the Tel2b primer is then later diluted in 25 µl Master Mix giving a final concentration of 0.9 µM.

- Eight aliquots of Tel2b primer were taken from the freezer and placed in ice. These were allowed to defrost then the contents were combined into one tube, vortexed gently and centrifuged briefly to ensure all contents were at the bottom of the tube.
- One x 1.5 ml tube was labelled as follows: Tel2b.
- 72.07 µl Tel2b stock primer was add to 247.93 µl H_2O [1 in 4.44 dilution].
- The tube was vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- The tube was placed on ice.

2.3.4.5.3 Hgb1 (300nM)

Hgb1 primer is bought as 100 µM stock, however for this experiment a concentration of 7.5 µM is required. This requires a 1 in 13.33 dilution. 7.5 µM of the Hgb1 primer is then later diluted in 25 µl Master Mix giving a final concentration of 0.3 µM.

- Three aliquots of Hgb1 primer were taken from freezer and placed in an ice block. These were allowed to defrost then the contents were combined into 1 tube, vortexed gently and centrifuged briefly to ensure all contents were at the bottom of the tube.
- One x 1.5 ml tube was labelled as follows: Hgb1.
- 24.76 µl Hgb1 stock primer was taken (from freezer) and added to 305.24 µl H_2O [1 in 13.33 dilution].
- The tube was vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- The tube was placed on ice.

2.3.4.5.4 Hgb2 (700nM)

Tel2b primer is bought as 100 µM stock, however for this experiment a concentration of 17.5 µM is required. This requires a 1 in 5.71 dilution. 17.5 µM of the Hbg2 primer is then later diluted in 25 µl Master Mix giving a final concentration of 0.7 µM.

- Three aliquots of Hgb1 primer were taken from freezer and placed in an ice block. These were allowed to defrost then the contents were combined into 1 tube, vortexed gently and centrifuged briefly to ensure all contents were at the bottom of the tube.
- One x 1.5 ml tube was labelled as follows: Hgb1.
- 56.04 µl Hgb2 stock primer was taken (from freezer) and added to 263.96 µl H_2O [1 in 5.71 dilution].
- The tube was vortexed gently to mix contents and centrifuged briefly to ensure all contents are at the bottom of the tube.
- The tube was placed on ice.

2.3.4.6 Master Mix

The master mix contained DTT, the primers and quantitect SYBR™ Green. Quantitect SYBR™ Green is a fluorescent nucleic acid dye used for DNA detection. When bound to DNA molecules these dye molecules fluoresce brightly allowing DNA to be quantified. Three 2 ml tubes were labelled “Master Mix”. The volumes of diluted reagents were added to each tube (total volume in tube was 1500 µl) as described in Table 2.2.

Reagent	Volume
Quantitect SYBR™ Green	1250 µl
Tel1b (2) or Hgb1	100 µl
Tel2b or Hgb2	100 µl
DTT	50 µl

Table 2.2 Master mix recipe

2.3.4.7 Plating

Plating involved adding the master mix and all standards, samples or controls to each well on a 96-well PCR plate. A plate plan (Figure 2.3) was devised prior to experimentation to plan the layout of all samples, controls and standards (where applicable) to ensure that all DNA samples were accounted for. To account for potential errors while plating, blank wells were included on the plating layout. The plate was then sealed with a PCR plate sealing film and placed in a centrifuge at 800 revolutions per minute (rpm) for one minute to remove bubbles which would potentially interfere with the PCR analysis. The protocol for plating was as follows:

- Take three x 96-well PCR plate from the box, holding only the edges, and place in plate holders.
- Label the plates Run “X”, plate A,B,C.
- Prepare a “plate plan” which identifies the exact positions of all standards, controls and samples on the 96 well PCR plate.

- According to the plate plan, plate 10 µl of standards, controls, or samples in duplicate.
- Turn the plates around.
- Plate 15 µl of Master Mix in each well of the 96-well PCR plate. Use a separate Master Mix tube for each plate.
- Place plate sealing film on plates, centrifuge at 800 x rpm for 1 min. Check for bubbles and re-centrifuge if necessary.
- Put two plates in the fridge.
- Run one plate on the StepOnePlus™ Real-Time PCR system using the appropriate cycling conditions.

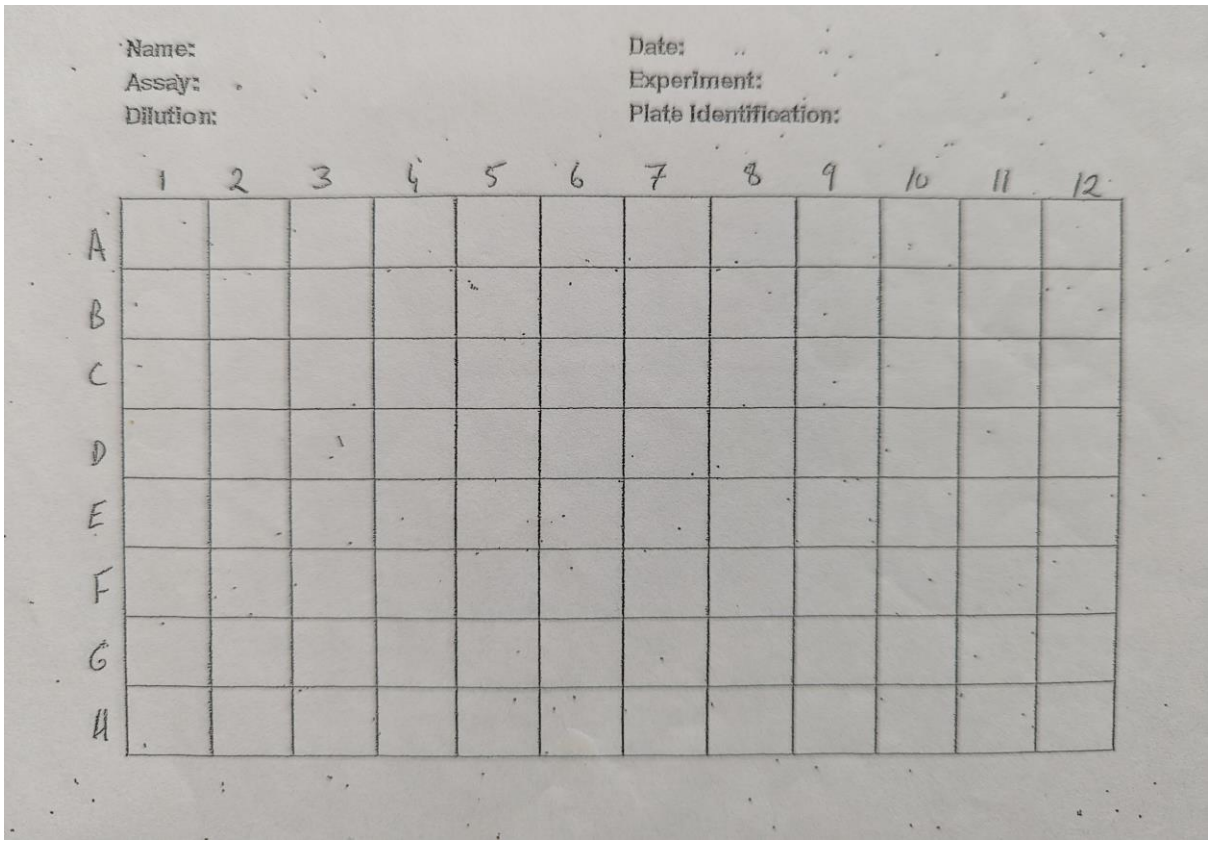

Figure 2.3 Copy of a blank plate plan for a 96-well PCR plate.

2.3.4.8 PCR cycling conditions

The qRT-PCR cycling profiles for the telomeres were as described in Table 2.3 below. The qRT-PCR cycling profile for the single-copy gene was as described in Table 2.4.

Step	Temperature	Time
Enzyme activation	95°C	5 minutes
PCR (30 cycles) Denature Anneal / extend	 95°C 54°C	 15 seconds 2 minutes

Table 2.3 qRT-PCR Cycling conditions for telomeres.

Step	Temperature	Time
Enzyme activation	95°C	5 minutes
PCR (40 cycles) Denature Anneal / extend	 95°C 58°C 72°C	 15 seconds 20 seconds 28 seconds

Table 2.4 qRT-PCR Cycling conditions for the single copy gene (Hgb).

All data from the StepOnePlus™ Real-Time PCR system were transferred via password protected USB from to the Thermofisher Connect® website, accessed via a password protected laptop registered to Trinity College Dublin, and stored on the Thermofisher cloud (private account) until analysis.

2.3.4.9 T/S ratio calculation

As described earlier, TL is expressed as a T/S ratio. Briefly, TL is estimated by comparing the amount of telomere repeat amplification product (T) to a single copy gene (S) product (*HGB*), the T/S ratio correlates with the average TL. Telomere (T) PCRs and single copy (S) PCRs are performed in separate 96-well plates. TL is expressed as a telomere to single-copy gene (T/S) ratio.

2.3.4.10 PCR parameters

2.3.4.10.1 Normalisation factor

Four control genomic DNA samples were included in each PCR run as inter-plate calibrators to calculate a normalizing factor. The T/S ratio for each of the control DNA samples was divided by the average T/S ratio for the same DNA for the telomere and Hgb runs to obtain a normalizing factor (Nudelman et al., 2019). The normalization factor was then used to correct for inter-run variability to generate the final T/S ratio. The control genomic DNA samples were obtained from healthy controls, aged 29 to 55 years, not

included in the study sample. These samples were included on each plate to account for inter-run variability. Both inter- and intra-assay co-efficient of variation (CV) were calculated.

2.3.4.10.2 Efficiencies

The efficiency is calculated from the slope. A slope of 3.3 indicates that the PCR reaction is 100% efficient; the target DNA is doubled with every cycle. The PCR efficiency is dependent on the assay, the master mix performance, and sample quality. An efficiency between 90% and 110% is considered acceptable.

2.3.4.10.3 r^2 (Coefficient of determination)

This is a statistical term that indicates how good one value is at predicting another value. An r^2 value >0.99 provides good confidence in correlating two values. This value measures how well the regression line fits the data points. A line that fits the data points perfectly has an r^2 of 1.

2.3.5 Mitochondrial DNA copy number assay

2.3.5.1 Introduction

A mitochondrial DNA monitoring primer set was used to determine relative mtDNAcn by qRT-PCR. As per the manufacturer's recommendations, this set was stored at 4°C as

it was being used within a short period of time (1 month). This set is designed to quantify the relative number of copies of human mitochondrial DNA (mtDNA) using nuclear DNA (nDNA) content as a standard. This set contains primer pairs for the amplification of four regions: two primer pairs for the detection of mtDNA and two primer pairs for the detection of nDNA. Relative quantification of mtDNAcn was determined from the difference in C_t (threshold cycle) values for four primers for mtDNA (*ND1* and *ND5*) and nuclear DNA (*SLCO2B1* and *SERPINA1*). The general principles of PCR are discussed earlier in this chapter. However, the mitochondrial DNA copy number uses a different method of assessing gene expression in comparison to the method used to assess TL. The comparative CT method is used for mitochondrial DNA copy number. For this method no standard curve is required, less well space is required on the 96-well plates and dilution errors are eliminated. This method assumes that the amplification efficiency of the target gene and the control gene is 100%. The reaction mixture was prepared on ice.

2.3.5.2 Method

24 µl of the master mix (containing TB Green Premix Ex Taq II (thermus aquaticus), genomic DNA sample, sterile purified H_20 and ROX reference dye) was dispensed into each tube as per the quantities specified in Table 2.5.

Master Mix Components	Volume
TB Green *Premix Ex Taq* II	12.5 µl
Genomic DNA sample	2 µl
Sterile purified H_20	9 µl
ROX reference dye	0.5 µl

Table 2.5 Mitochondrial DNA copy number master mix recipe.

1 µl of the primer was then added to the master mix. Four reactions are performed for each DNA sample (one reaction each of the four primer pairs). Preparation of the reaction mixture using this procedure minimises the amount of variation due to pipetting errors in the amount of template dispensed and provides more reliable results. In summary, each sample was run in duplicate in a 25 µl reaction. To account for potential errors while plating, blank wells were included on the plating layout.

2.3.5.3 Polymerase Chain Reaction (PCR) cycling conditions

The PCR cycling profile for mitochondrial DNA copy number was as described in Table 2.6.

Step	Temperature	Time
Enzyme activation	95°C	30 seconds
PCR (40 cycles) Denature Anneal / extend	 95°C 60°C	 5 seconds 30 seconds

Table 2.6 PCR cycling profile for mitochondrial DNA copy number.

2.3.5.4 Mitochondrial DNA copy number (mtDNAcn) calculation

Relative mtDNAcn per diploid genome was calculated using the formula below, which calculates differences in threshold cycles (C_t):

$2^{-\Delta Ct}$ for the values ΔC_t 1 and ΔC_t 2

ΔC_t 1 = (C_t for *SLCO2B1* – C_t for *ND1*)

ΔC_t 2 = (C_t for *SERPINA1* – C_t for *ND5*)

In summary:

- Determine the difference in the C_t values for the *ND1/SLCO2B1* pair (ΔC_t 1 = C_t for *SLCO2B1* - C_t for *ND1*)
- Determine the difference in the C_t values for the *ND5/SERPINA1* pair in the same manner (ΔC_t 2 = Ct for *SERPINA1* - C_t for *ND5*)

- Find 2Δ C_t for the values for Δ C_t 1 and Δ C_t 2
- Use the average of the 2 values found in the above step to calculate mtDNA copy number

For analysis of mtDNAcn for the study participants, an excel file was used to calculate mtDNA copy number using Microsoft Office Excel. The file was downloaded from the product page on the Takara ™ website (https://www.takarabio.com/products/stem-cell-research/accessories/human-mitochondrial-dna-monitoring).

2.3.5.5 PCR parameters

As previously described in section 2.3.4.10.

2.4 Phenotyping methods

2.2.2 Study approval and governance

The study entitled "*Impact of Anorexia Nervosa on Biological Ageing*" (SPMHS Protocol 09/18) was approved on the 17th of April, 2018, by the Research Ethics Committee at St Patrick's Mental Health Services (appendix 4) and adhered to the Declaration of Helsinki (World Medical Association, 2013). I was the main applicant for the study and responsible

for the design, ethical approval, recruitment, analysis and anonymisation of the data and dissemination of results for the study.

2.2.3 Personal data and General Data Protection Regulation (GDPR)

All participants were given a study identification (ID) number. This study ID was used on all documents used to record clinical and demographic data for each participant. Only the minimum information required was collected and identifiable information such as the participant's name, date of birth or address were never documented on collected data. The key did contain the participant's name and study ID. The key was stored in a separate location to the participant's study data.

All data were stored both electronically and on paper format. Electronic documents were stored on a password protected computer registered to St Patrick's Mental Health Services that was used only by me. The computer was kept in a locked filing cabinet in a locked office that was only used by me. The office was located at St Patrick's Mental Health Services in the Research Building that had limited access for staff. Electronic documents were stored on a secure hospital drive and were password protected. The principal investigator (PI) and I were the only individuals who had approved access to the folder on the hospital drive. Paper documents were stored in a locked filing cabinet in a separate locked room in the same research building. All data were irreversibly anonymised following analysis of the data.

2.2.4 Study location

This study took place at St Patrick's Mental Health Services (SPMHS), Dublin 8, a psychiatric hospital which consists of 241 beds. Regarding the recruitment of cases, eligibility for this study was restricted to inpatients of SPMHS. SPMHS is a not-for profit hospital and Ireland's largest independent-sector mental health service provider. Most inpatients in SPMHS have private health insurance. In 2021, 47.1% of the Irish population had private health insurance (Health Insurance Authority, 2023).

2.2.5 Participants and consent

2.2.5.1 Recruitment of cases

Patients admitted to St Patrick's Mental Health Services for the treatment of anorexia nervosa (AN) under the care of the Eating Disorders Multidisciplinary Team (MDT) were considered for inclusion in the research study. Recruitment for the study started on 01.08.2018 was completed on 30.04.2019, a recruitment period of 9 months in total. The Consultant Psychiatrist specialising in eating disorders identified new admissions to the eating disorders unit who were under her care and informed me daily. I then approached the identified patients and provided both verbal and written information on the research study. I designed a participant information leaflet (appendix 5) for this study with all relevant study information and contact details. Verbal consent was obtained to return to the patient after a minimum 24-hour period. This was to provide the patient with enough

time to make an informed decision regarding participation in the study. More time was given if requested. The patients had an opportunity to ask questions and I was happy to meet with their families if they had any further questions or concerns. I returned to the patient after he or she had made an informed decision regarding the study. All patients who were interested in participating in the study signed a participant consent form (appendix 6). Participants were then screened for suitability for inclusion in the study.

Inclusion criteria for cases

I. Primary diagnosis of anorexia nervosa as diagnosed using the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5) (American Psychiatric Association, 2013)
II. Minimum age of 18 years
III. Ability to speak English with adequate fluency
IV. Capacity to consent to the study

Exclusion criteria for cases

I. Involuntary admission status
II. Active alcohol / substance misuse disorder in the previous six months
III. Current physical illness unrelated to anorexia nervosa

The Consultant Psychiatrist specialising in eating disorders and the participant's own local General Practitioner (GP) were informed by writing regarding all cases who were recruited to the study (appendix 7) and information regarding the study was provided with my contact details.

2.2.5.2 Recruitment of controls

Healthy controls were recruited though online advertisement on i-VOL (appendix 8), word of mouth and social media using a standard recruitment advertisement (appendix 9). I attended a volunteer recruitment fair where further advertisement was completed. Recruitment leaflets were distributed containing information on the study. Potential controls contacted me via email or telephone to express an interest in the study. Further information on the study was provided both verbally and via email. If interested, an appointment was scheduled to meet me during normal working hours at St Patrick's Mental Health Services. All potential controls who were interested in participating in the study signed a consent form. Participants were then screened for suitability for inclusion in the study using purpose made screening form. Healthy controls were considered if there was no history of psychiatric illness, alcohol or substance misuse in the previous six months, current physical illness and a normal body mass index (BMI). Controls were age- and sex- matched with cases. Recruitment for the study started on 01.08.2018 was completed 0n 30.04.2019, a recruitment period of 9 months in total.

Inclusion criteria for controls

I. Normal BMI
II. Minimum age of 18 years
III. Ability to speak English with adequate fluency
IV. Capacity to consent to the study

Exclusion criteria for controls

I. Psychiatric illness
II. Alcohol / Substance misuse disorder in the previous six months
III. Physical illness
IV. Prescribed medications

2.2.5.3 Documentation of clinical and demographic variables

Clinical and demographic variables for all participants were documented in a purpose-made participants demographics sheet (appendix 10).

2.5 Statistical methods

SPSS (statistical Package for the Social Sciences), version 27 (IBM Corporation, NY, USA), was used to analyse all data. Data were assessed for normality using the Shapiro-Wilk test. Normally distributed data require parametric tests, and non-normally distributed data will require non-parametric tests for analysis. Non-normally distributed data can be log-transformed to help increase the chances of normalising the distribution. All the data collected in my study were assessed for normality.

Differences in demographic and clinical characteristics between groups were determined using Fisher's Exact test and Chi Square for categorical data (Camilli, 1995). Independent *t* tests and Mann Whitney U tests were used for continuous data (Neely et al., 2003). Spearman's rho was used to assess associations between continuous variables (Fredricks and Nelsen, 2007), while point-biserial correlations were used to determine associations between continuous and dichotomous variables (Demirtas and Hedeker, 2016).

General linear models (Dobson and Barnett, 2018) were used to determine differences between groups while adjusting for covariates, where appropriate. In addition to age, variables such as sex, level of educational attainment, smoking status, vitamin B12, antipsychotic medication and body mass index (BMI) are known to affect telomere length (TL) and mitochondrial DNA copy number (mtDNAcn) (Lulkiewicz et al., 2020, Astuti et al., 2017, Praveen et al., 2020, Polho et al., 2015, Hu et al., 2023, Wang et al., 2020, Wu et al., 2019c, Skuratovskaia et al., 2019, Kumar et al., 2018). Therefore, these variables were included as covariates. Analyses were conducted as follows: (1) unadjusted and (2) adjusted for age, sex, level of educational attainment, smoking status, vitamin B12, antipsychotic medication and BMI.

TL data are presented as mean T/S ratio ± SD and mtDNAcn data are presented as mean mtDNAcn ± SD. mtDNAcn data are presented as mean mtDNAcn ± SD (standard deviation). Differences with a p value ≤ 0.05 were deemed statistically significant. Analyses were two-tailed. Power analysis was performed using G*Power (version 3.1.9.7) (https://www.psychologie.hhu.de/en/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower). With a sample size of 56 there is 91%

power at a 0.05 level of significance to be able to detect an effect size (Cohen's *d*) of at least 0.4 for the difference in TL and mtDNAcn between the AN and control groups. In general, an effect size 0.2 and 0.5 is considered to be a medium effect size (Cohen, 1977).

CHAPTER 3. RESULTS: TELOMERE LENGTH

3.1 Introduction

The structure and function of the telomere are described in detail in Chapter 1, sections 1.3.1 and 1.3.2. Telomere length (TL) is a well-established biomarker of ageing and reduces with increasing age (Zole and Ranka, 2018). There is much evidence for the shortening of TL in psychiatric illnesses, including affective disorders, anxiety disorders and psychotic disorders (Mathur et al., 2016, Tyrka et al., 2016, Ridout et al., 2018b). TL is also reduced secondary to stress (Schutte and Malouff, 2016, Chatelain et al., 2020).

Anorexia nervosa (AN) is a psychiatric illness that involves significant physical and psychological stress. Those with AN experience physical stress secondary to excessive exercise and extreme calorie restriction, with a resulting low body mass index (BMI). Common physical consequences of AN are described in Chapter 1, section 1.1.6.Those with AN experience significant psychological stress and often suffer from co-morbid anxiety and depressive disorders (Papadopoulos et al., 2009). Patients with AN tend to display perfectionistic traits, placing very high standards upon themselves and increasing psychological stress due to internal sources (Herpertz-Dahlmann et al., 2011). Furthermore, those with AN often have poor flexibility and problem-solving skills, again increasing the burden of psychological stress (Strober, 2004). AN tends to be a chronic illness; thus, those with AN tend to experience AN symptomatology throughout their lives (Eddy et al., 2017). Relapses will inevitability result in increased periods of acute-on-chronic stress.

TL is reduced secondary to stress (Mathur et al., 2016). Physical stress, such as that experience in chronic illnesses such as diabetes mellitus and cardiovascular disease is associated with reduced TL (D'Mello et al., 2015, Haycock et al., 2014). Several studies investigating psychiatric disorders including depression, post-traumatic stress disorder (PTSD) and psychotic disorders report reduced TL compared to healthy controls (Darrow et al., 2016). Both acute and chronic stress result in reduced TL (Mathur et al., 2016, Monroe and Simons, 1991). Those who experience early-life childhood trauma have reduced TL compared to controls (Ridout et al., 2018b, Tyrka et al., 2016, Tyrka et al., 2010). External sources of stress, or "major stressors", such as unemployment, work-related issues and poverty can reduce TL (Oliveira et al., 2016). In addition, internal sources of stress including perfectionistic traits, obsessive tendencies and poor problem-solving skill have an increased risk of depressive and anxiety disorders, thus increasing the risk of reduced TL (Darrow et al., 2016).

There is a paucity of research examining TL and eating disorders. In fact, to date, there is only one study investigating TL in AN (Uziel et al., 2023). This study on female adolescents with AN (n = 44) and healthy controls (n = 22) and found that there was no difference in TL between the two groups. The authors did, however, report shorter TL in the AN binge/purge-type (n= 18) compared to the AN restricting-type (n=26).

Given the extensive evidence that TL is associated with stress, and that those with AN suffer from significant stress, my aim was to explore the relationship between TL and AN. I sought to test the hypothesis that TL would be reduced in patients with AN, compared to healthy sex- and age-matched controls. I also hypothesised that TL would be reduced with increasing duration of illness, and with increasing severity of illness.

3.2 Materials and methods

Materials and methods are discussed in detail in Chapter 2. Briefly, this is a cross-sectional, case-control study involving inpatients with AN and healthy age- and sex-matched controls. Cases were recruited from the Eating Disorder Unit at St Patrick's Mental Health Services (SPMHS). Healthy controls were recruited through word of mouth, through advertisements on a volunteering website, i-Vol (https://www.i-vol.ie/), and at a volunteering recruitment fair. Peripheral whole blood samples are used to analyse TL using quantitative Real Time Polymerase Chain Reaction (qRT-PCR; for methodological details see Chapter 2, section 2.3.4). Samples were analysed using PCR at Trinity College Institute of Neuroscience (TCIN).

Demographic and clinical cvariables were recorded as described in Chapter 2. Blood results and prescribed medications were also documented. Severity of illness was assessed using the Eating Disorder Examination Questionnaire (EDE) (Fairburn and Beglin, 2008). The EDE was also conducted in healthy controls to exclude any eating disorders. While it is normal for healthy controls to score on the EDE, scores are significantly lower than that compared to those with eating disorders. The Mini International Neuropsychiatric Interview (M.I.N.I.) for Diagnostic and Statistical Manuel for Mental Disorders, version 5 (DSM-5) (Sheehan, 2014) was used to confirm a diagnosis of AN and exclude any other major psychiatric disorders. AN type, restrictive or binge/purge type, was recorded.

3.3 Statistical analysis

SPSS (statistical Package for the Social Sciences), version 27 (IBM Corporation, NY, USA), was used to analyse all data. Further information on the statistical analyses can be found in Chapter 2, section 2.5 “Statistical methods”.

3.4 Results

3.4.1 Participant recruitment

The flow diagram in Figure 3.1 summarises reasons for ineligibility and non-recruitment for the study. Recruitment took place over a nine-month period between August 2018 and April 2019, inclusive. During this time there were 44 admissions to the Eating Disorder Unit. Twenty-six (60%) of these admitted patients met the inclusion criteria and were eligible to participate in the study. Of the 18 (40%) that were not eligible, one patient was admitted involuntarily, seven patients had a diagnosis of bulimia nervosa, six patients had a diagnosis of eating Disorder Not Otherwise Specified (EDNOS) and four were repeat admissions. Twenty-three (53%) potentially eligible patients consented to participate in the study.

Controls were recruited through word of mouth, volunteer fairs and a national online volunteering website, i-VOL (https://www.i-vol.ie/). Thirty-eight potential participants expressed an interest in volunteering. Five applicants were excluded: one potential participant was <18 years, one was taking prescribed medications and had a chronic physical illness, and three participants had a body mass index (BMI) outside (above) the normal range (18.5 – 24.9 kg/m^2). Thirty-three control participants met the inclusion criteria and were consented to take part in the study (Figure 3.2).

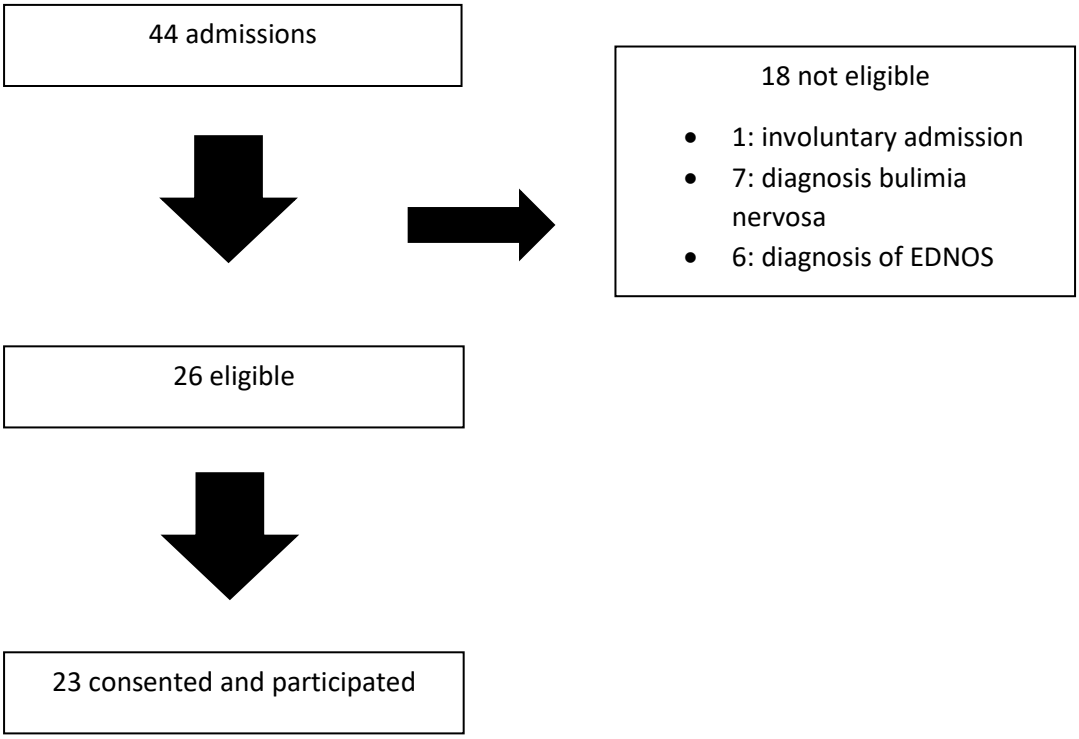

Figure 3.1 Flow diagram: recruitment of cases with anorexia nervosa

Abbreviations: EDNOS, Eating Disorder Not Otherwise Specified

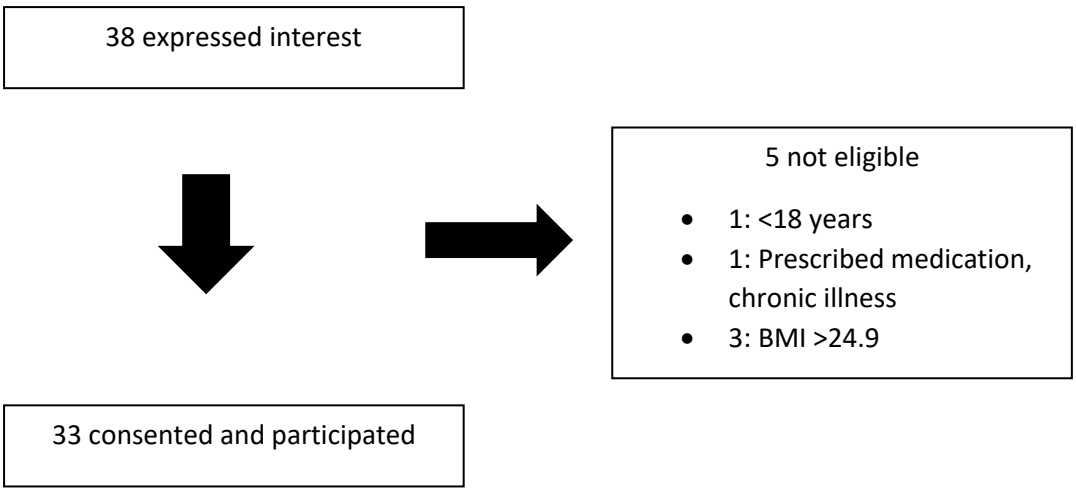

Figure 3.2 Flow diagram: recruitment of healthy controls

Abbreviations: BMI, Body Mass Index

3.4.2 Polymerase Chain Reaction (PCR) quality control

3.4.2.1 Deoxyribonucleic acid (DNA) extraction

Purified DNA from this study had an Absorbance 260/280 ratio (A260/A280) of 1.6–1.9. The absorbance ratio is explained in further detail in chapter 2, section 2.3.2. Briefly, an A260/280 ratio of ~1.8 is generally considered "pure" for DNA (Wilfinger et al., 1997). Therefore, an absorbance ratio of 1.6-1.9 is within acceptable parameters.

3.4.2.2 Inter- and intra-assay Co-efficient of Variation (CV)

The intra-assay CV for Telomere length (TL) runs was <3.5% and the inter-assay CV was <4.5%. For the single copy gene (Haemoglobin gene (*HGB*)) runs, the intra-assay CV was <2.5% and the inter-assay CV was <2.5%. CVs are explained in further detail in chapter 2, section 2.3.4.10.1. These results are within acceptable parameters.

3.4.2.3 Efficiencies

The average efficiencies for the telomere length and *HGB* runs were 96.24% and 90.75%, respectively. PCR efficiencies are explained in further detail in chapter 2, section 2.3.4.10.2. These results are within acceptable parameters.

3.4.2.4 Average r^2 (Coefficient of determination)

The r^2 is a statistical term that indicates how good one value is at predicting another value. The average r^2 of the PCR runs were 0.991 and 0.989 for the telomere and single copy (*HGB*) runs, respectively. r^2 is explained in further detail in Chapter 2, section 2.3.4.10.3. These results are within acceptable parameters.

3.4.3 Characteristics of the participants

3.4.3.1 Demographic characteristics

Demographic characteristics of the sample are summarised in Table 3.1. Both groups were reasonably balanced in terms of age and sex. There were three smokers in the AN group and one smoker in the control group. The control group had a statistically significant higher educational attainment. Fifty-five participants (98%) of the participants were white, and one participant from the AN group was Asian. As expected, the anorexia nervosa group had a statistically significant lower BMI compared to the control group. The mean BMI was 15.1 (kg/m^2) in the AN group and 22.2 (kg/m^2) in the control group.

Characteristic	**AN patients (n=23)**	**Controls (n=33)**	**Test Statistic**	***p* value**
Age (years), mean ± SD	30.3 ± 13.1	30.6 ± 12.2	*U = 346.5, r* = -0.8	0.582
Sex, No. Male Female	 2 21	 1 32	*Fisher's exact test* *OR* = 0.328	0.562
Smokers, No.	3	1	*Fisher's exact test* *OR* = 0.208	0.295
Educational Attainment, No. Primary Secondary Tertiary/Quaternary	 1 15 7	 1 9 23	*Chi Square goodness-of-fit test* $\chi^2 = 23.3$	≤0.001
Ethnicity, No. White Other	 22 1	 33		
BMI (kg/m^2), mean ± SD	15.1 ± 2.4	22.2 ± 1.9	*U = 25.0, r* = -0.7	≤0.001

Table 3.1 Demographic characteristics of the two groups of participants

Abbreviations: AN, Anorexia nervosa; BMI, body mass index; SD, Standard Deviation.

3.4.3.2 Clinical characteristics: Eating Disorders Examination (EDE)

As expected, the AN group had statistically significantly higher EDE scores compared to the Control group (Table 3.2). The results of all the sub-scales in the EDE were similar with the Restraint sub-scale and Shape Concern sub-scale, scoring slightly higher than the other sub-scales (Eating Concern, Weight Concern) in the AN group. The control group had low scores in the EDE, which would be considered normal. The following questions were noted to be more likely to generate scores (in the mild range, 0-2) in the control group:

1. *Over the past four weeks have you been consciously trying to restrict (cut back) the overall amount that you eat, whether or not you have succeeded?"*
2. *Over the past four weeks have you tried to avoid eating any foods which you like, whether or not you have succeeded?*
3. *Over the past four weeks have you wanted to weigh less?*
4. *Over the past four weeks have you had a definite desire to have a completely flat stomach?*

However, there was no impairment of functioning and controls were not pre-occupied by the above cognitions.

Characteristic	AN patients (n=23)	Controls (n=33)	Test Statistic	*p* value
EDE score, mean (range)				
Global score	3.9 (2.1–5.7)	0.3 (0.0–1.1)	*U = 759.0, r* = 0.80	≤0.001
Restraint	4.1 (1.2–6.0)	0.3 (0.0–2.4)	*U = 754.5, r* = 0.86	≤0.001
Eating Concern	3.8 (0.0–6.0)	0.1 (0.0–0.4)	*U = 736.5, r* = 0.85	≤0.001
Shape Concern	4.1 (2.1–6.0)	0.5 (0.0–1.4)	*U = 736.0, r* = 0.84	≤0.001
Weight Concern	3.9 (0.8–6.0)	0.4 (0.0–1.8)	*U = 749.5, r* = 0.83	≤0.001

Table 3.2 Eating Disorder Examination Scores for both groups

Abbreviations: AN, Anorexia nervosa; EDE, Eating Disorders Examination.

3.4.3.3 Characteristics of the anorexia nervosa group

The mean age of onset of illness was 17.1 years (standard deviation ± 4.6), and the mean duration of illness was 13.2 years (± standard deviation 13.7). The mean length of index admission was 68 days (range seven to 178 days). Nineteen patients (83%) had previous admissions for the treatment of anorexia nervosa. Six patients (26%) had previously received nasogastric tube feeding due to medical complications arising from severe weight loss. Nineteen patients (83%) were diagnosed with anorexia nervosa restrictive sub-type and four were diagnosed with binge/purge sub-type (17%) (Figure 3.3).

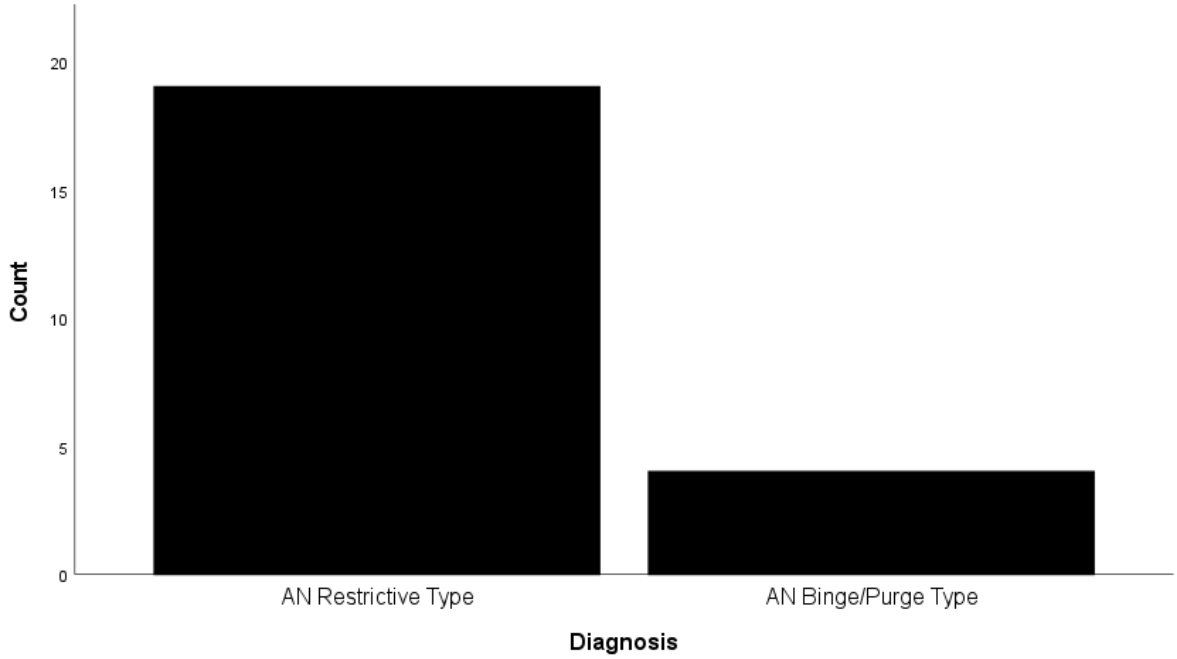

Figure 3.3 Anorexia nervosa (AN) group (n=23) divided by AN sub-type (AN restrictive type and AN binge/purge type).

Abbreviations: AN, Anorexia nervosa.

3.4.3.4 Prescribed medications – Anorexia nervosa group

The most commonly prescribed medications were multivitamin supplements, for which 18 out of 23 participants with AN were prescribed (Table 3.5). Other frequently prescribed supplements included calcium carbonate and colecalciferol (Calcium, Vitamin D3), thiamine and vitamin B12. Eleven patients were prescribed selective serotonin re-uptake inhibitors (SSRIs) and 10 patients were prescribed anti-psychotic medication. All prescribed medications and supplements are summarised in Table 3.3.

Prescribed medications	
SSRI	11 *(Fluoxetine n = 8, Escitalopram n = 2, Sertraline n = 1)*
SNRI	3 *(Venlafaxine n = 2, Duloxetine n = 1)*
Mirtazapine	4
Antipsychotics	10 *(Risperidone n = 4, Olanzapine n = 5, Quetiapine n = 1)*
Benzodiazepines	3 *(Alprazolam n = 2, Clonazepam n = 1)*
Non-benzodiazepine hypnotics	6 (*Melatonin n = 5, Z-drugs n = 1)*
Pregabalin	2
Lactulose	4
Movicol®	7
Thiamine	11
Vitamin B12	11
Multivitamins	18
Calcium carbonate / colecalciferol (vitamin D3)	15
Fortisip®	6

Table 3.3 Prescribed medications for the anorexia nervosa group

Abbreviations: SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin-norepinephrine reuptake inhibitor

3.4.4 Telomere length and correlations with clinical and demographic characteristics

3.4.4.1 Telomere length and age

As displayed in Figure 3.4, Telomere length (TL) was negatively associated with age for the group as a whole (r = -0.496, p ≤0.001) and individually for both the control (Figure 3.5; r = -0.524, p = 0.002) and case groups (Figure 3.6; r = -0.505, p = 0.014).

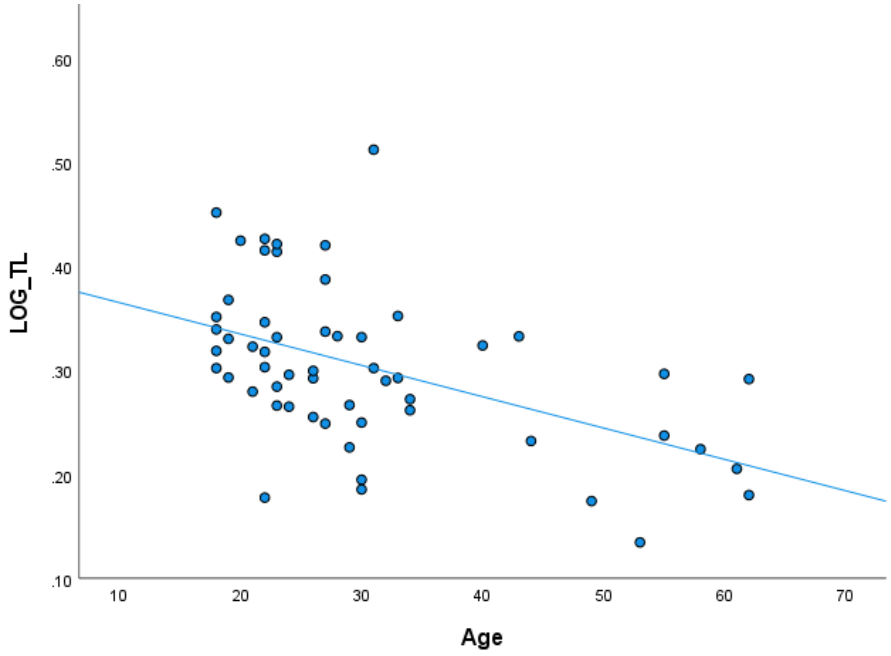

Figure 3.4 Scatterplot of age (years) and telomere length (log-transformed) for full group (n=56)

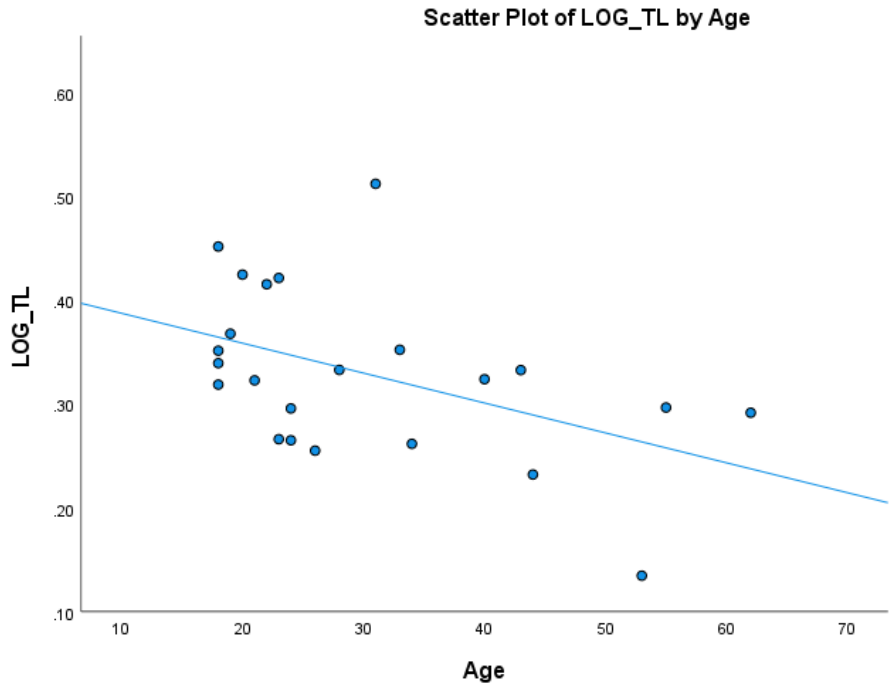

Figure 3.5 Scatterplot of age (years) and telomere length (log-transformed) for cases (n=23)

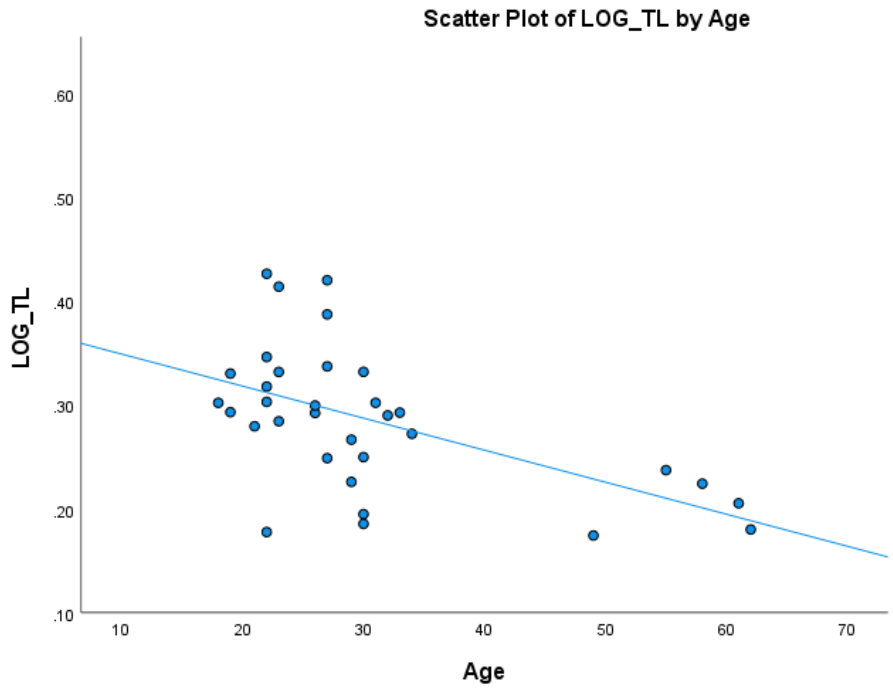

Figure 3.6 Scatterplot of age (years) and telomere length (log-transformed) for controls (n=33)

3.4.4.2 Telomere length and correlation with BMI

TL was not significantly associated with BMI in the group as a whole (all p >0.05), nor when both groups were examined individually.

3.4.5 Telomere length in anorexia nervosa group versus control groups

Data for telomere length (TL) was not normally distributed, however, following log-transformation TL data was normally distributed. There was a significantly longer TL in the AN group compared to the control group (t = -2.15, df = 54, p = 0.04, r = 0.28) with a mean TL of 0.33 (SD ± 0.08) in the AN group and 0.29 (SD ± 0.07) in the control group (Figure 3.7). These results remained statistically significant after adjusting for age, sex, level of educational attainment, smoking status, antipsychotic medication and BMI ($F_{(1, 48)} = 5.45$, p = 0.02).

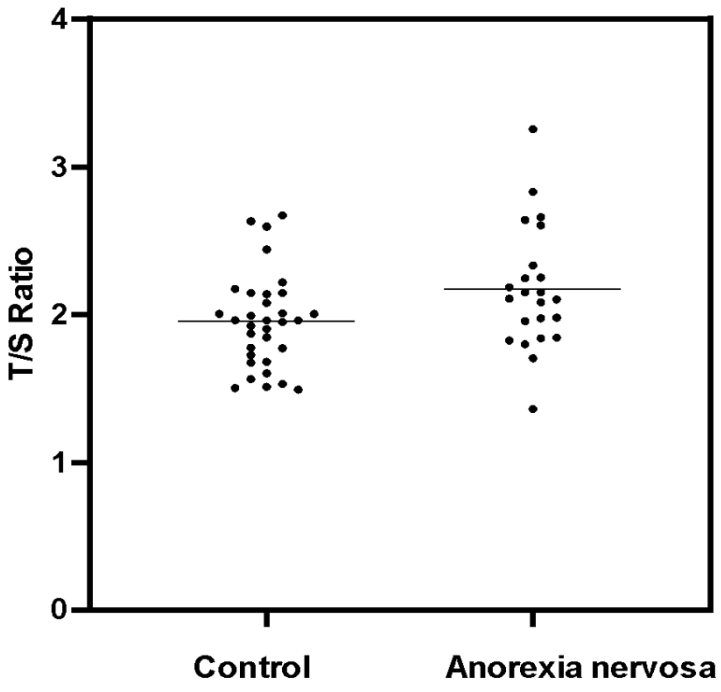

Figure 3.7 T/S (telomere/single copy gene) ratio in controls versus cases with AN

3.4.6 Telomere length and duration of illness

Telomere length (TL) was negatively associated with illness duration in the AN group (r = -0.477, p = 0.021) (Figure 3.8). However, this test did not reach statistical significance after adjusting for age (r = -0.262, p = 0.239).

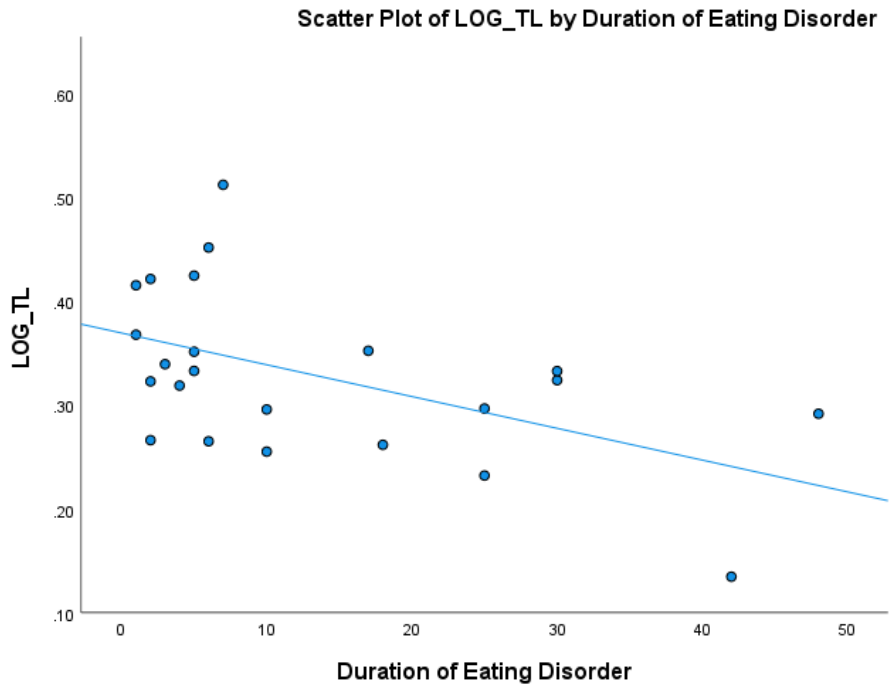

Figure 3.8 Correlation between duration of AN (years) and telomere length (log-transformed)

3.4.7 Telomere length and illness severity

Additionally, I analysed telomere length (TL) and severity of illness as measured by the Eating Disorders Examination (EDE). There was no association found between TL and global or sub-scale scores of the EDE (all p >0.05), both before and after (Table 3.4) adjusting for age.

	r	***p***
Global EDE score	0.17	0.46
Restraint	-0.10	0.68
Eating Concern	-0.16	0.48
Shape Concern	-0.01	0.95
Weight Concern	0.17	0.46

Table 3.4 Correlation between telomere length and EDE scores, after adjusting for age.

Abbreviations: EDE, Eating Disorders Examination

3.5 Discussion

This was the first study to report on the relationship between TL and anorexia nervosa (Doody et al., 2022). Contrary to my initial hypothesis, I found TL to be longer in the underweight AN group compared to healthy controls. There may be several reasons for this. First, many studies report a positive relationship between exercise and TL (Lin et al., 2019). In particular, regular exercise for more six months is associated with greater TL (Song et al., 2022). Aerobic, strength and endurance exercise have all been associated with increased TL (Song et al., 2022, Buttet et al., 2022). Regular exercise may preserve TL by reducing inflammation and increasing antioxidant gene expression (Ludlow et al., 2013). Physical activity has the potential to stimulate progenitor cells which increases proliferation, helping to maintain the length of telomeres. Patients with AN are likely to exercise significantly more than healthy controls, which may contribute to increased TL. Secondly, calorie restriction may also affect TL. García-Calzón et al. (2014) found that TL was increased in adolescents after two months of an energy-restricted diet. Buttet et al. (2022) also reported that dietary restriction contributed to increased TL. Energy-restricted diets may promote the maintenance of TL by increasing insulin sensitivity, which is associated with longer telomeres (Demissie et al., 2006). Thus, the combination of increased exercise and reduced calorie intake may have had an impact on the preservation of TL in the AN group compared to the healthy control group. However, to date the literature remains unclear regarding the overall impact of calorie restriction and dietary restraint on TL. Kiefer et al. (2008) reported premature leukocyte telomere shortening in premenopausal and post-menopausal women who practised chronic dietary restraint. Tomiyama et al. (2017) reported shorter peripheral blood mononuclear cell

telomer length in those practising calorie restriction compared to controls. Thus, there is no clear evidence that calorie restriction delays ageing related to telomere length.

As regards my second hypothesis, I found no significant relationship between TL and duration of illness after controlling for age. Again, this may be due to the lifestyle of those with AN where regular exercise and a restricted calorie intake take priority and have a protective effect on TL. Those with a longer duration of AN are likely to have a significant history of exercising daily and restricting their calorie intake over long periods of time which may contribute to the maintenance of TL. Additionally, those with a longer duration of AN may experience chronic physical and psychological stress and compensatory mechanisms may be activated secondary to stress, which may in fact contribute to the lengthening of telomeres. Telomerase, an enzyme that elongates telomere length, is stress-responsive (Beery et al., 2012) and may drive the increase in TL in those with AN who are exposed to physical and psychological stress in comparison to the controls. In relation to my hypothesis on TL and severity of AN, as measured by the EDE (Eating Disorders Examination), I found no significant relationship between the severity of AN and TL. The EDE provides both a global score, and a sub-scale score and there was no relationship between either the global or the sub-scale scores and TL.

This study is the first published study to examine telomere length (TL) in anorexia nervosa (AN) and currently only one of two published studies on this topic that I am aware of. While my study reports on adult inpatients with AN, Uziel et al. (2023) reports on female adolescents aged 13 to 19 years who were admitted to an inpatient Eating Disorder Unit. A younger population are more likely to have a shorter duration of illness. The authors of this study found no difference in TL between patients with AN (n = 44) and

controls (*n* = 18) and no change in TL from admission to discharge in a subset of 18 patients with AN, despite an improvement in body mass index (BMI). However, due to the small number, this does not enable any conclusion about the impact of inpatient treatment and weight gain on TL. Similar to the results of my study, age was inversely correlated with TL. The authors did, however, report shorter TL in patients with AN-binge/purge type compared with those with AN-restricting type. It was not possible for me to analyse these data in my study due to the small number of patients with AN-binge/purge type (*n* = 4) compared to AN-restrictive type (*n* = 19). There are a number of limitations in the study by Uziel et al. (2023). Firstly, the authors did not assess for non-eating disorder comorbid psychopathology in the control group, and therefore did not rule out psychiatric illness in this group. I screened for major mental illness in the control using the Mini International Neuropsychiatric Interview (M.I.N.I.) for Diagnostic and Statistical Manuel for Mental Disorders, version 5. The group of patients with AN were significantly younger than their control group (15.4 years versus 16.3 years), although the authors did control for age when comparing TL between the two groups.

There are a number of limitations to my study. The TL measurements were cross-sectional, and so I was unable to examine changes over time. I measured TL from peripheral blood samples and a change in TL in the peripheral blood may not indicate the dynamics of telomere length in the brain. Furthermore, we measured telomere length in whole blood as opposed to specific blood cell types. A study by Lin et al. (2010) reported that B cells have the longest telomere length compared to other lymphocyte subtypes; alterations in cell subtypes may thus account for increased telomere length in the AN group. There is evidence that exercise and dietary restriction may affect TL (García-Calzón

et al., 2014; Lin et al., 2019); however, I do not have a measure of physical exercise nor exact calorie intake in this group. Similarly, data on childhood trauma, a significant psychological stressor, was not collected and may affect TL. The sample size examined in this study is small, thus limiting the generalisability of the results. Furthermore, this study included inpatients only, limiting the generalisability of results to populations with less severe AN. Finally, most patients with AN were prescribed psychotropic medications and dietary supplements. The effect of prescribed medication and supplements on TL is unknown. The duration of use of medications was not incorporated into the study and this could be investigated in future studies. Studies have shown that human TL is positively associated with nutritional status (Paul, 2011), and most of the patients with AN were prescribed supplements that may have affected TL.

Ideally, future studies investigating TL should have a prospective design to provide insights into longitudinal trajectories of the symptoms of AN with TL. Investigating TL in those with AN in the acute phase of their illness and comparing these results to TL when recovered, may shed some light on the longer-terms effects of AN, and whether compensatory mechanisms increase TL during the acute phase of illness. The study of purified cellular populations would differentiate between the impact of differing TL in different cell types. Whole blood contains red blood cells, different sub-types of white blood cells, and platelets. TL results may depend on the distribution of these various cells, having an impact on the overall result.

In conclusion, I discovered that patients with AN had a longer mean TL than controls but there was no correlation between TL and duration of illness, or illness severity, after adjusting for covariates. It was not possible for me to determine whether

TL is preserved due to characteristics commonly present in this illness, such as increased exercise and reduced calorie intake, or whether perhaps there is a compensatory mechanism at play during the acute phase of AN that preserves TL in the presence of increased physical and psychological stress. Further, larger scale, prospective studies are required to clarify whether TL is increased in those with AN and in AN-like states.

CHAPTER 4. RESULTS: MITOCHONDRIAL DNA COPY NUMBER

4.1 Introduction

The structure and function of the mitochondrion is discussed in detail in Chapter 1. Briefly, mitochondrial DNA is present in various quantities, or copy numbers, within the mitochondrion, depending on several factors including the specific energy requirements of that cell (Robin and Wong, 1988). Mitochondrial DNA copy number (mtDNAcn) indirectly reflects that functioning of the mitochondrion (Picard et al., 2016).

Anorexia nervosa (AN) is a psychiatric illness that involves significant physical and psychological stress, as described in detail in Chapter 1, section 1.1.6 and Chapter 3, section 3.1. Those with AN experience physical stress secondary to excessive exercise, extreme calorie restriction, resulting in abnormally and dangerously low body mass index (BMI). Furthermore, those with AN are likely to experience psychological stress secondary to the symptoms of their mental illness and personality traits. mtDNAcn can both increase and decrease in response to stress depending on the type of stress, whether acute or chronic (Picard and McEwen, 2018a, Malik and Czajka, 2013). In the presence of acute stress, mtDNAcn has been shown to increase (Cai et al., 2015b). Furthermore, those who experience psychological stress secondary to depression and early life stress have increased mtDNAcn compared to healthy controls (Ryan et al., 2023, Tyrka et al., 2016).

In addition, studies have reported co-regulatory pathways between mtDNAcn and telomere length (TL) and suggested that a positive association exists (Tyrka et al., 2015b, Kim et al., 2013b, Meng et al., 2016a). Reactive oxygen species (ROS) may lead to reduced

TL and result in telomere dysfunction and cellular senescence (Picard and McEwen, 2018b). Telomere dysfunction can then adversely affect mitochondrial biogenesis by downregulating peroxisome proliferator-activated receptor-ɣ coactivator 1α (PGC-1 α), impacting on mitochondrial DNA copy number (Gonzalez-Ebsen et al., 2017). Several studies show that there is a relationship between TL and mtDNAcn (Lindqvist et al., 2018a, Tyrka et al., 2015a, Meng et al., 2016b, Kim et al., 2013a). Interestingly, in Study 1 (Chapter 3), I reported that TL was increased in patients with AN compared to age- and sex-matched healthy controls. The same group of patients are investigated in Study 2 in relation to mtDNAcn, which will be examined for an association with TL.

Despite the significant stress experienced by those with AN, and the impact of stress on mtDNAcn, there are no studies to date examining mtDNAcn in patients with AN. I sought to test the hypothesis that mtDNAcn would be increased in inpatients experiencing an acute relapse of AN, compared to healthy sex- and age-matched controls. I also hypothesised that mtDNAcn would be increased with increasing duration of illness, and with increasing severity of illness. As described in chapter 1 (section 1.3.3.4), TL is reported to be reduced secondary to stress, and both mtDNAcn and TL may be co-regulated. Therefore, I also hypothesised that there would be an association between mtDNAcn and TL.

4.2 Materials and methods

The materials required, and methods used, for the collection of samples and analysis of mtDNAcn is described in detail in Chapter 2. In summary, for this cross-sectional, case-control study, patients with AN were recruited from St Patrick's Mental Health Services

(SPMHS). All patients were admitted to an Eating Disorder Unit. Healthy age- and sex-matched controls were recruited through word of mouth, via an online volunteering website, i-Vol (https://www.i-vol.ie/), and through a volunteering recruitment fair.

The patients and controls recruited for the study of mtDNAcn were the same cohort of participants that are described in Chapter 3 for Study 1.

Peripheral whole blood was used to measure mtDNAcn using quantitative real-time polymerase chain reaction (qRT-PCR) (Bai and Wong, 2005). The comparative C_t (threshold cycle) method is used for mitochondrial DNA copy number. For this method primer pairs for the amplification of four regions are required. There were two primer pairs for the detection of mtDNA (mitochondrial DNA) and two primer pairs for the detection of nDNA (nuclear DNA). Relative quantification of mtDNAcn was determined from the difference in C_t values for four primers for mtDNA (*ND1* and *ND5*) and nuclear DNA (*SLCO2B1* and *SERPINA1*). All participants were fasting overnight, and samples were taken at SPMHS between 07:30 and 09:30am. Samples were analysed at Trinity College Institute of Neuroscience (TCIN).

4.3 Statistical analysis

SPSS (statistical Package for the Social Sciences), version 27 (IBM Corporation, NY, USA), was used to analyse all data. Further information on the statistical analyses can be found in Chapter 2, section 2.5 “Statistical methods”.

4.4 Results

4.4.1 Participant recruitment

Participant recruitment is reported in Chapter 3, section 3.4.1. Briefly, 33 healthy age- and sex-matched healthy controls and 23 patients with AN were recruited to the study. The same study ccohort was used for both Study 1 on telomere length and study 2. The flow diagrams in Figures 3.1 and 3.2, Chapter 3, summarise the recruitment of cases and controls.

4.4.2 Polymerase Chain Reaction (PCR) quality control

4.4.2.1 Deoxyribonucleic acid (DNA) extraction

Purified DNA used in this study had an absorbance 260/280 ratio (A260/A280) of 1.6–1.9. The absorbance ratio is explained in further detail in Chapter 2, section 2.3.2. An A260/280 ratio of ~1.8 is considered acceptable (Wilfinger et al., 1997).

4.4.2.2 Inter- and intra-assay Co-efficient of Variation (CV)

The intra-assay coefficient of variation (CV) was <3.7% and the inter-assay CV was <2.3%. CVs are explained in further detail in Chapter 2, section 2.3.4.10.1. These results are within acceptable parameters.

4.4.3 Characteristics of the participants

The clinical and demographic characteristics of all participants are described in Chapter 3, sections 3.4.3. Characteristics described include age, sex, level of educational attainment,

smoking status, body mass index (BMI), prescribed medications, diagnoses, duration of illness and severity of anorexia nervosa (AN) in addition to sub-type of AN (binge-purge type or restrictive type-AN).

In addition, haematological investigations were recorded, as these results can affect mtDNAcn (Picard, 2021). Haematological investigations were completed as per the standard protocol for new admissions to the Eating Disorders Unit. Full blood count (FBC) results for the AN group were recorded. FBC results were recorded as close as possible to date of admission blood samples for TL analysis. Cell components from the FBC included the neutrophil count, lymphocyte count and platelet count (Table 4.1). Twenty-two out of 23 patients had FBC results recorded. Eight of 22 patients had a reduced neutrophil count, and six of 22 patients had a reduced lymphocyte count. One patient had a reduced platelet count, and one had an elevated platelet count.

Cell Type	**Patient Range (No. outside normal range)**
Neutrophil Count *	Range: 0.66 – 5.69 × 10^9/L (8/22 reduced)
Lymphocyte Count *	Range: 0.46 – 2.14 × 10^9/L (6/22 reduced)
Platelet Count *	Range: 125 – 428 × 10^9/L (1/22 reduced; 1/22 elevated)

* Neutrophil normal range: 2.0 – 7.0 × 10^9/L; Lymphocyte normal range: 1.0–3.0 × 10^9/L; platelet normal range: 150–410 × 10^9/L

Table 4.1 Full blood count (grouped by cell type) results for the anorexia nervosa group

4.4.4 Mitochondrial DNA copy number and correlations with clinical and demographic characteristics

4.4.4.1 Mitochondrial DNA copy number and age

As displayed in Figure 4.1 and 4.2 respectively, mtDNAcn was weakly positively correlated with age for the group as a whole (r=0.39, p=0.003) and moderately positively correlated for the AN group (r=0.49, p=0.02). However, there was no significant correlation between age and mtDNAcn in the control group (r=0.31, p=0.08) (Figure 4.3).

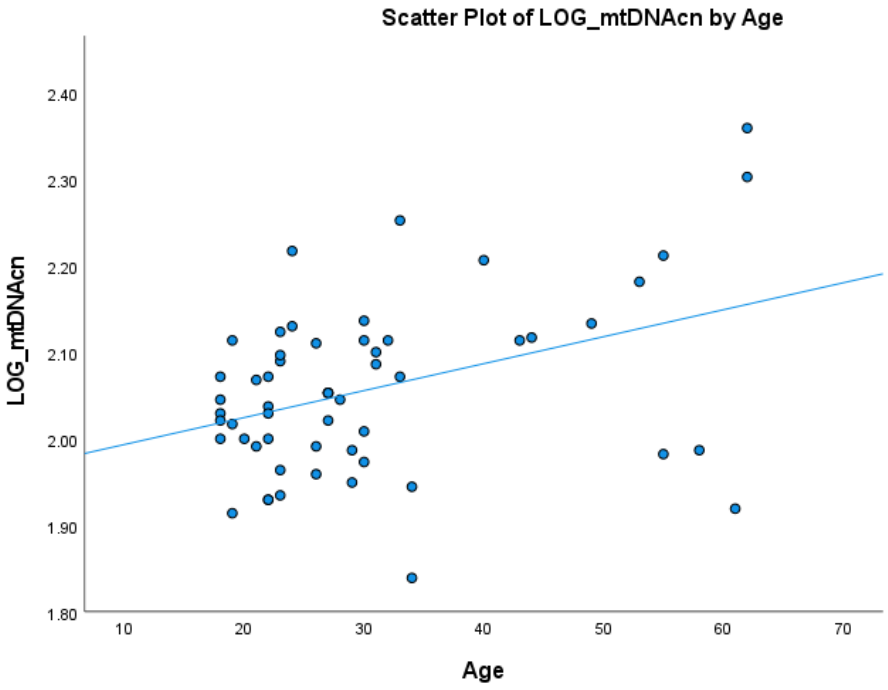

Figure 4.1 Scatterplot of age (years) and mitochondrial DNA copy number (log-transformed) for full group (n=56)

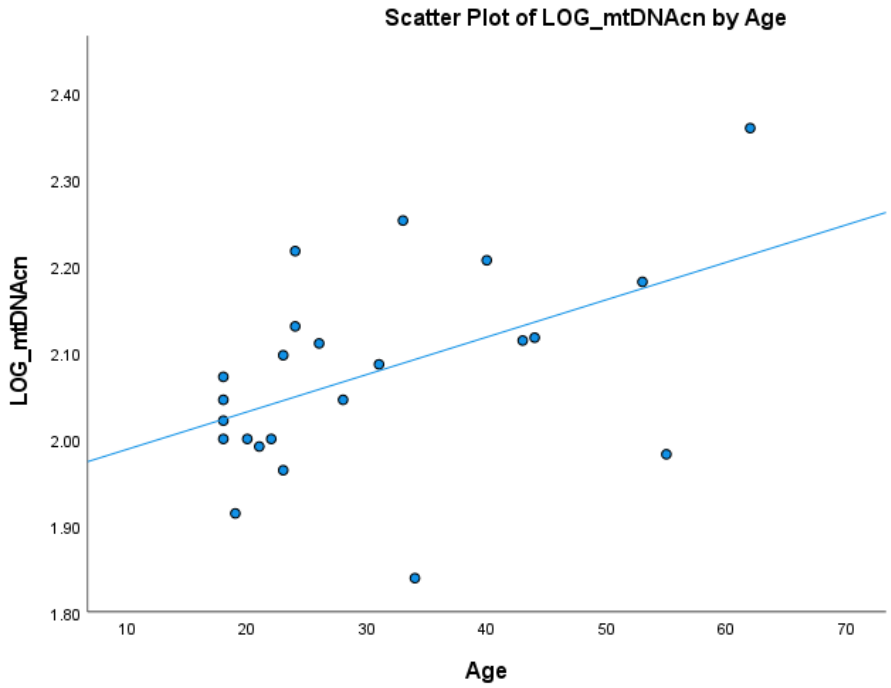

Figure 4.2 Scatterplot of age (years) and mitochondrial DNA copy number (log-transformed) for cases (n=23)

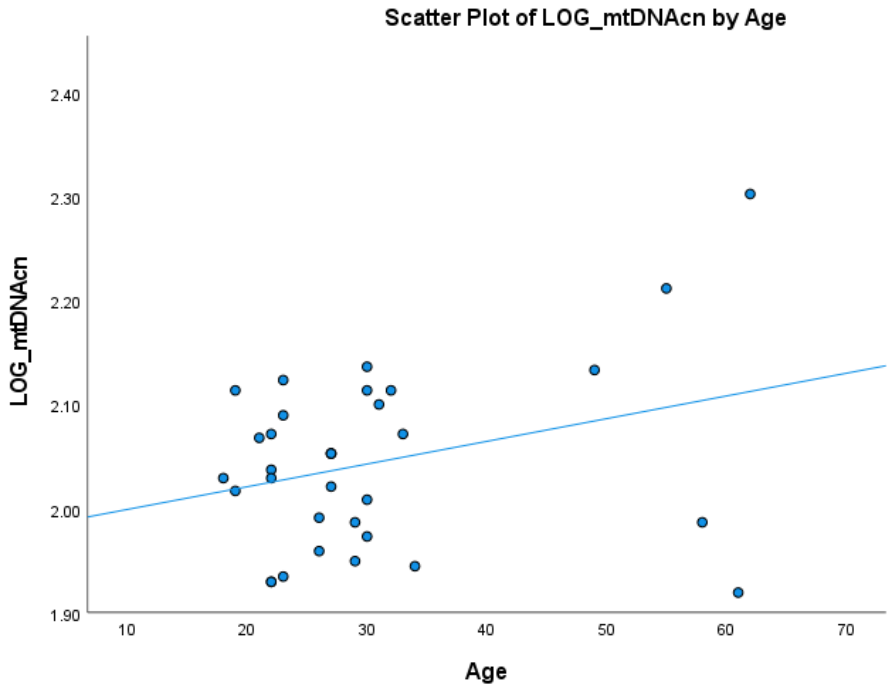

Figure 4.3 Scatterplot of age (years) and mitochondrial DNA copy number (log-transformed) for healthy controls (n=33)

4.4.4.2 Mitochondrial DNA copy number and haematological investigations

There was no association between mitochondrial DNA copy number and lymphocyte and platelet count, both before and after adjusting for age (Table 4.2). There was a significant correlation between mitochondrial DNA copy number and neutrophil count, both before (r = -0.50, p=0.02) and after adjusting for age (Table 4.2).

	r	*p*
Neutrophils	-0.45	0.04
Lymphocytes	-0.02	0.93
Platelets	-0.3	0.19

Table 4.2 Correlation between mitochondrial DNA copy number and full blood count sub-types, after adjusting for age (AN group only).

4.4.4.3 mitochondrial DNA copy number and body mass index (BMI)

There was no statistically significant association between mtDNAcn and BMI either in the whole group or when both groups were individually examined (p>0.05).

4.4.5 Mitochondrial DNA copy number in AN versus control groups

Raw mtDNAcn data were not normally distributed, but following natural log transformation data were normally distributed. Mean mtDNAcn was 2.08 ± 0.12 in the AN group and 2.04 ± 0.09 in the control group (effect size: *d*=0.18), and no significant difference was noted between the groups (Fig. 4.4: *t*=-1.17, *df*=54, *p*=0.25). Statistical adjustment for covariates did not change the results ($F_{(1, 48)}$=1.25, *p*=0.27).

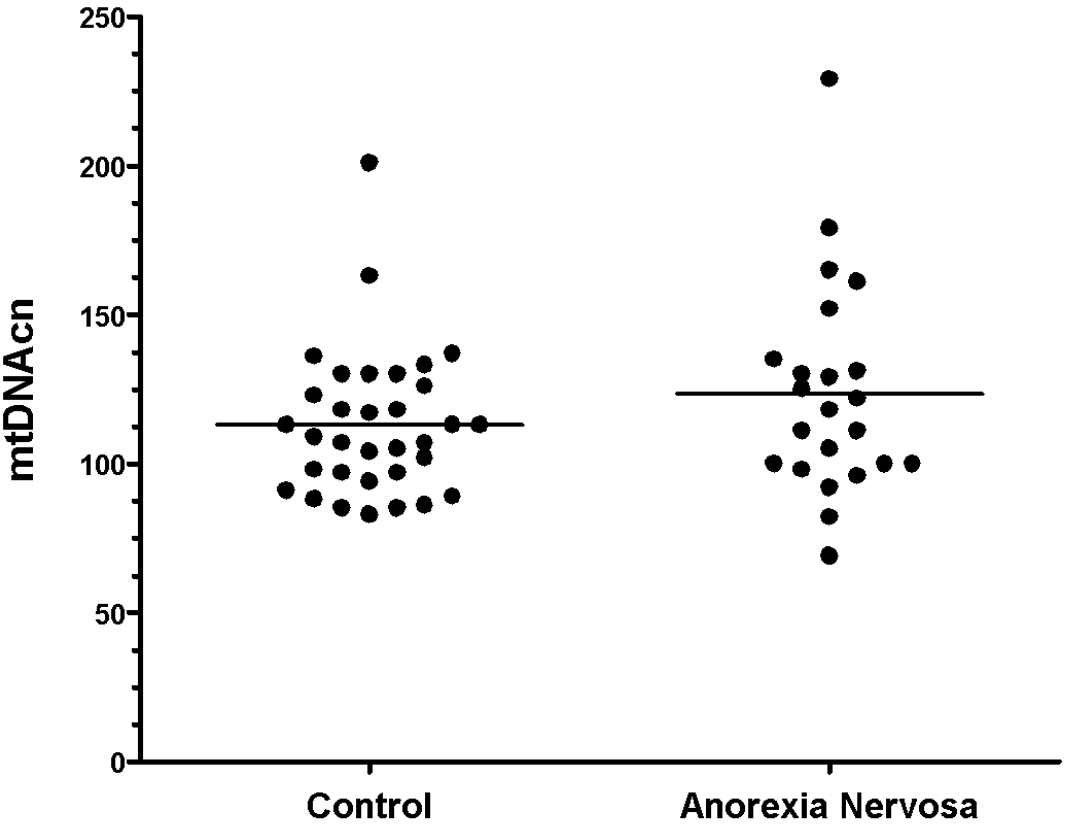

Figure 4.4 mtDNAcn in healthy controls versus cases with anorexia nervosa

Abbreviations: mtDNAcn, mitochondrial DNA copy number.

4.4.6 Mitochondrial DNA copy number and duration of illness

mtDNAcn was significantly positively correlated with illness duration (Fig. 4.5; r=0.59, p=0.003), which remained significant following adjustment for age (r=0.44, p=0.04).

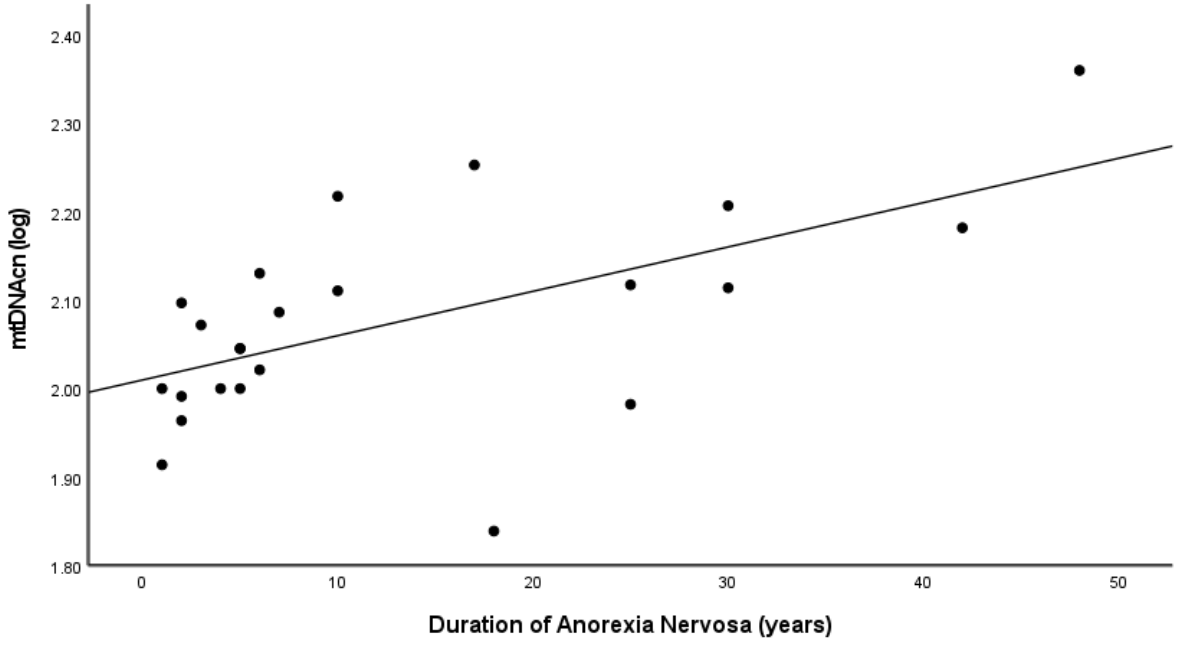

Figure 4.5 Correlation between duration of AN (years) and mitochondrial DNA copy number (log-transformed)

4.4.7 Mitochondrial DNA copy number and illness severity

Additionally, I analysed mitochondrial DNA copy number (mtDNAcn) and severity of illness as measured by the Eating Disorders Examination (EDE). There was a significant association between mtDNAcn and shape concern before adjusting for age (r = -0.54, p = 0.01), however this did not reach statistical significance after adjusting for age (Table 4.3)

There was no association found between mtDNAcn and the global EDE or other sub-scale scores of the EDE (all p >0.05), both before and after adjusting for age (Table 4.3).

	r	***p***
Global EDE score	-0.08	0.73
Restraint	0.22	0.32
Eating Concern	0.03	0.89
Shape Concern	-0.40	0.06
Weight Concern	0.18	0.43

Table 4.3 Correlation between mitochondrial DNA copy number and EDE scores, after adjusting for age.

Abbreviations: EDE, Eating Disorders Examination

4.4.8 Correlations between mitochondrial DNA copy number and telomere length (TL)

There was no statistically significant correlation between mtDNAcn and TL in the group as a whole (*r*=-0.24, *p*=0.78), even following adjustment for age as seen in Figure 4.6 (*r*= 0.59, *p*=0.67), or in the AN group, both before and after controlling for age (*r*=-0.19, *p*=0.34 and *r*=-0.05, *p*=0.82, respectively). In the control group, there was a statistically significant inverse correlation between mtDNAcn and TL (*r*=-0.42, *p*=0.01); however, this was no longer statistically significant after controlling for age (*r*=-0.32, *p*=0.08).

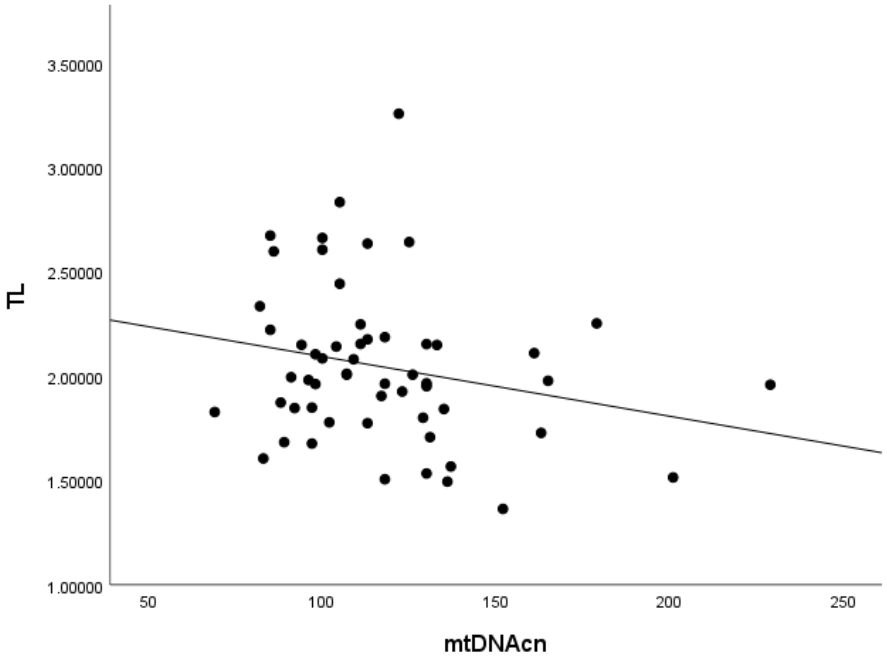

Figure 4.6 Correlation tests between mtDNAcn and TL in the whole group, n = 56

Abbreviations: mtDNAcn, mitochondrial DNA copy number; TL, telomere length.

4.5 Discussion

To my knowledge, this study is the first to examine mitochondrial DNA copy number (mtDNAcn) in blood from patients with AN. Contrary to my primary hypothesis, these results showed no significant difference in mtDNAcn between patients with AN compared to healthy controls. Regarding my second hypothesis, interestingly, there was a positive correlation between mtDNAcn and illness duration. There was no significant correlation identified between mtDNAcn and severity of illness. Finally, I found no correlation between mtDNAcn and telomere length (TL).

I did identify a positive association between mtDNAcn and duration of illness. There may be several reasons for the association between mtDNAcn and the duration of illness noted here. First, an increase in mtDNAcn might occur as a mechanism to compensate for reduced mitochondrial energetics induced by physical or psychological stressors (Picard, 2021). Picard and McEwen (2018d) describe an increase in mtDNAcn secondary to compensatory upregulation of mitochondrial biogenesis as an adaptive response to stress by the cell. Animal studies of mtDNAcn and chronic stress report an increase in mtDNAcn as measured in peripheral tissues following chronic stress (Cai et al., 2015a, Cai et al., 2015c). Furthermore, studies examining rodents trained with treadmill running reported that mtDNA was either partially or completely protected against stress-induced reductions in copy numbers (Liu and Zhou, 2012a). Similarly, in humans, chronic physical activity and exercise may have also have a protective effect on mtDNA, therefore preserving copy number (Puterman et al., 2011). Those with AN experience numerous physical stressors in the form of severely reduced BMI, intense exercise, and reduced calorie intake. Psychological stress is common and those with AN are more likely to have

co-morbid psychiatric illnesses such as post-traumatic stress disorder, obsessive compulsive disorder, generalised anxiety disorder and mood disorders (Furtado and Katzman, 2015). Second, studies show that patients with AN can have altered cell distributions and develop haematological abnormalities. For example, up to 17% of hospitalised patients with severe AN develop thrombocytosis (Sabel et al., 2013). Different cell types show vast differences in mtDNAcn (Picard, 2021). For instance, lymphocytes have a mtDNAcn in the range of 150–600 copies per cell, neutrophils have an mtDNAcn in the region of ~150 copies per cell, and platelets, which have no nucleus, contain an "infinite" mtDNAcn (Picard, 2021). Thus, as mtDNAcn calculations are based on the relative number of copies of mitchondrial DNA using the nuclear genome as the denominator, in the case of platelets this denominator is zero; therefore, an increased number of platelets in the circulation, as is the case with thrombocytosis, may artificially increase the mtDNAcn and subsequently affect the overall mtDNAcn count (Picard, 2021). However, most of the patients in this study had normal platelets counts; thus, it is very unlikely that the platelets count affected my results. Patients with AN are also found to have increased lymphocyte numbers (Gibson and Mehler, 2019). Since there is lower mtDNAcn in neutrophils compared to lymphocytes, a higher neutrophil to lymphocyte ratio would result in lower mtDNAcn (Picard, 2021). Full blood counts were not acquired for patients included in this study on the same day as sample collection; thus, I was unable to adjust our analyses to take into account variations in blood cell numbers. However, I did have full blood count data available for patients taken an average of five days prior to recruitment. Using these data, ~27% of our patient cohort had a lymphocyte count below the normal range and ~36% had a reduced neutrophil count (these were not the same

people). The majority of this patient cohort had a platelet count that was within the normal range of 150–410 × 10^9/L, with the data showing that only one patient had a platelet count just slightly above the normal range (i.e., 428 ×10^9/L) and one had a count slightly below the normal range (i.e., 125 ×10^9/L). Thus, it is possible that blood cell counts may have impacted on my results as interpretation is limited by the lack of same day blood cell counts. Therefore, future studies should ensure that full blood count data are acquired at the same time as study sample collection to enable statistical adjustment for blood cell numbers to gain a better picture of mtDNAcn in this patient population.

I found no significant correlation between mtDNAcn and severity of illness. It is worth noting that the Eating Disorders Examination (EDE) may not accurately reflect the biological severity of the disease as this clinician-rated questionnaire is based on the patient's concerns regarding their weight, shape, eating habits, and restraint tendencies and does not quantify the level of exercise or calorie expenditure or analyse body mass index (BMI).

It is worth noting that the measurement of mtDNAcn is only one of many ways to indirectly quantify mitochondrial content, and the measurement of mtDNAcn alone does not inform us of the efficiency of the whole mitochondrion (Picard, 2021). Mitochondria are considerably complex organelles that are involved in a range of cellular functions. Assessment of a range of different mitochondrial function measurements, such as mitochondrial content, integrity and respiratory capacity, would be a more in-depth and accurate way to report on mitochondrial content and function. Overall, the comparison of studies on mtDNAcn remains challenging because multiple factors can affect mtDNAcn, including the cell population studied, the tissue type, and changes in energy

requirements. Factors such as age, stress and diurnal variation can affect cell distribution. Furthermore, not all mtDNAcn quantification methods are equivalent and kits used for DNA extraction can also affect mtDNAcn quantification (Picard, 2021). Therefore, it is vital that there is a consistency of approach in future studies of mtDNAcn in psychiatry, in particular for AN.

A strength of this study is the fact that this is the first report of mtDNAcn in patients with AN compared to a healthy control group. In addition, though the sample size was small, a positive relationship was identified between mtDNAcn and duration of illness. This study has several limitations. First, mtDNAcn was determined using whole blood instead of using isolated cell-subtypes. Furthermore, I did not have a full blood count for these patients taken at the timepoint of blood collection for this study; so, I was unable to conduct analyses to control for platelet count. Second, I measured mtDNAcn in peripheral blood samples. A change in mtDNAcn in the peripheral blood may not be indicative of mtDNA content or function in the brain. Third, the mtDNAcn measurements were cross-sectional, and so it was not possible for me to assess changes in mtDNAcn over time, limiting conclusions about causality. Fourth, this sample size (n = 56) was small, which limits the generalisability of the results. Additionally, as this was an exploratory study, I did not correct for multiple comparison and larger scale studies would be required to validate our findings. Fifth, physical exercise and dietary restraint may affect mtDNAcn; however, I did not have a measure of physical exercise or details about the exact caloric intake for this cohort. Finally, most of these patients were receiving treatment with prescribed psychotropic medications, the effects of which on mtDNAcn are unknown.

Furthermore, many of the patients with AN are prescribed dietary supplements, including folate and vitamin B12, the effect of which is unknown on mtDNAcn. A study by Praveen et al. (2020) reported a significant positive correlation between plasma folate and plasma vitamin B12 levels with mtDNAcn in those aged ≥60 years. However, in those aged <60 years, while there was a significant positive correlation between mtDNAcn and plasma folate levels, there was no correlation between mtDNAcn and plasma vitamin B12 levels. Furthermore, when grouped by gender, there was no significant correlation between mtDNAcn and plasma vitamin B12 levels in men. The authors of this study acknowledge that although the plasma vitamin B12 levels were higher in the >60 years of age group, this cohort also had increased levels of plasma creatinine and lower eGFR (estimated Glomerular Filtration Rate) levels, most likely secondary to kidney dysfunction, which can artificially increase the plasma vitamin B12 levels. In addition, those aged > 60 years had higher levels of total homocysteine (tHcys) which translates to a functional deficiency of plasma vitamin B12 despite having increased plasma vitamin B12 levels (McMahon et al., 2015). Vitamin B12, B6 and folate break down homocysteine in order for this amino acid to be used by the body. Raised levels of tHcys indicate that these micronutrients are deficient. I did not have plasma folate and vitamin B12 levels for the participants in my study, and thus I was unable to conduct an exploratory analysis for an association between plasma folate and vitamin B12 levels and mtDNAcn.

In summary, these results reflect the complexity of stressors and their effect on mtDNAcn and highlight the need for studies examining mtDNAcn and various types of stressors, such as those that vary in nature (physical stress, psychological stress) and with duration (acute and chronic). Although I reported no correlation between mtDNAcn and

illness severity, it would be important to examine changes in copy number with varying severity of stress (mild, moderate, severe), and investigate lifetime exposure to stress to examine the effect of stress in early life versus later life. Larger scale studies are required to increase the sample size and overcome some of the limitations of the present study. Longitudinal and prospective study designs would help to establish the association between stressors to fully examine mtDNAcn and mitochondrial function in AN and AN-like states and advance our understanding of the impact of AN on the mitochondrion.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Introduction

The study of patients with anorexia nervosa (AN), particularly inpatients with an established diagnosis and severely low body mass index (BMI), provides a distinct population of study participants with no other physical illness, who are otherwise healthy, except for physical issues that are secondary to their eating disorder. In addition, these patients are subject to a substantial amount of stress, both physically and psychologically, because of their illness. This condition provides a model for the study of the body's response to significant stress, from both the physical and psychological sources, which can be readily quantified. There is an established association between psychological stress and accelerated biological ageing (Palma-Gudiel et al., 2020), and between physical stress and biological ageing (Fransquet et al., 2019). Therefore, the study of this cohort of patients provides an opportunity to quantify stress and explore its relationship with biological age through the investigation of telomere length (TL) and mitochondrial DNA copy number (mtDNAcn). Understanding the effect of stress on a molecular level, may indicate if certain conditions, or certain stressors, accelerate our risk of ageing. The study of conditions that may accelerate ageing is important as it helps with the identification of persons at increased risk of reduced functionality and increased risk of morbidity and mortality.

5.2 Study 1: Telomere Length

5.2.1 Conclusions

Patients with anorexia nervosa (AN) experience significant psychological and physical stress secondary to the effects of their illness (Phillipou et al., 2018a, Herpertz-Dahlmann et al., 2001). This places this cohort of patients at an increased risk of accelerated biological ageing. Telomeres are protective caps at the ends of chromosomes that protect the chromosome from damage (Blackburn, 2005). The length of telomeres provides a measure of biological age and reduces with increasing age (Bonnell et al., 2021). Telomere length (TL) can also be reduced by stressors (Mathur et al., 2016), such as those experienced by patients with AN. I hypothesised that TL would be reduced in patients with AN compared to healthy age- and sex-matched controls. I also hypothesised that TL would be reduced with duration and severity of illness. Of note, this is the first published study investigating TL in AN.

I found that telomere length (TL) was longer in patients with AN compared to healthy age- and sex-matched controls, contrary to my hypothesis. There was no significant association between TL and duration of illness, or severity of illness as measured by the Eating Disorders Examination (EDE). To date, there is only one other published study that investigates TL in a cohort of patients with AN (Uziel et al., 2023). However, my study is the only study to examine TL in adults with AN, while the study by Uziel et al. (2023) investigates adolescent patients (n = 44), aged between 13 and 19 years, who have a much shorter duration of illness.

The literature is well established as regards the effect of age on telomeres and the benefits of regular and moderate exercise and healthy diet on preserving telomere length (Sun et al., 2012). However, we have little understanding of the effect of intensive exercise and / or excessive dietary restraint, that results in a significantly reduced body mass index (BMI) as found in AN, on our telomeres. The main limitation of this study was the sample size, which limits generalisability of the results, however, there are no other studies investigating TL in adults with AN, and the results of my study suggest that further investigation would be worthwhile in expanding our understanding of the impact of AN on TL.

5.2.2 Future Research

Larger case-control studies, with a longitudinal design, would help develop our understanding of the effects of AN on TL. This design, with repeated measurements of TL, may help to identify variables that may either reduce or increase TL, such as weight loss, weight gain, acute stressors, history of significant trauma such as childhood trauma, and changes in intensity of exercise, to name but a few. Duration of illness could be more accurately defined and investigated with a prospective-style study. Reporting the length of each acute illness, and the number of relapses over time would help to clarify duration of illness. Measurements of exercise frequency and intensity, extent of dietary and calorie restriction, extent and rate of weight loss, presence of amenorrhoea and requirement for naso-gastric feeding should be included in future research studies in this area as these measures would provide more accurate measures of severity of AN. Furthermore, a larger sample size would increase the generalisability of the results, and it would also be possible

to investigate differences between sub-types of AN (binge-purge and restricting sub-types) and TL. Although the EDE is considered the *gold standard* in the measurement of severity of AN, additional measures of severity of psychological stress could be employed, as those with AN display perfectionistic and obsessive-compulsive tendencies, which increase psychological stress, and may impact on TL. The use of several different measures of severity of illness, including the EDE, measures of stress and the quantification of exercise and calorie restraint may provide a more holistic result on TL and severity of illness.

Future research should also investigate the effects of medications and supplements on TL. Most inpatients with AN are prescribed psychotropic medications including anti-depressants and anti-psychotics, the effect of which on TL largely is unknown. Measuring the effects of these medications using detailed measures, for example quantifying anti-psychotic strength using a uniform system such as conversion of anti-psychotic doses to chlorpromazine equivalents, or the use of therapeutic drug monitoring (TDM) where plasma levels of prescribed medications are measured, may help identify the impact that medications have on TL in AN. Plasma levels of micronutrients such as vitamin B12 and folate could be investigated, as many patients who are initially admitted to an inpatient unit are deficient in these nutrients, and replacement of same with supplements may affect TL (Praveen et al., 2020). Studies should also account for haematological variability to avoid confounding results. The study of purified cell populations would differentiate between naturally differing TL in different cell types. Whole blood contains red blood cells, different sub-types of white blood cells and platelets, and TL results may depend on the distribution of these various cell types,

influencing the overall result and future studies should account for this variability. Future studies on AN and TL have the potential to provide valuable insights into the effect of physical and psychological stress on telomeres. Furthermore, larger scale, prospective-style studies may also have the potential to investigate whether preserved TL may reflect better outcomes in those with AN.

5.3 Study 2: Mitochondrial DNA copy number

5.3.1 Conclusions

Mitochondria, known as the "powerhouses" of the cell, are responsible for the production of energy, amongst several other functions (Nicholls, 2013). The are unique in that they possess their own DNA, distinct form nuclear DNA, of which there are many copies within the cell (D'Erchia et al., 2015a). Alterations in mitochondrial DNA copy number (mtDNAcn) may reflect stress. Several studies report an increase in mtDNAcn secondary to stress (Tyrka et al., 2016, Ryan et al., 2023). As previously mentioned, those with AN experience significant physical and psychological stress. I hypothesised that mtDNAcn would be increased in those with AN compared to healthy age- and sex-matched controls, and that mtDNAcn would be increased with duration and severity of illness. I also hypothesised that mtDNAcn would be associated with TL.

This study found no difference in mitochondrial DNA copy number (mtDNAcn) in those with AN compared to healthy age- and sex-matched controls. The results of this study reported that mtDNAcn is not associated with severity of illness, as measured by

the EDE. However, there was a positive association between mtDNAcn and duration of illness. There was no association between mtDNAcn and TL.

To my knowledge, this is the first study to investigate mtDNAcn in AN. To date, we have a limited understanding of the regulation mtDNAcn and the results of studies are varied. While mtDNAcn is reported to both increase and decrease in response to stress, it has also been reported to increase and decrease with increasing age (Picard and McEwen, 2018c), although results in older adults (aged >50 years) are more consistent with a reduction in copy number with advancing age (Mengel-From et al., 2014). We have little knowledge on the sequelae of increasing or decreasing mtDNAcn, making the study of mtDNAcn challenging but fascinating. While some studies report a relationship between TL and mtDNAcn (Lindqvist et al., 2018b, Tyrka et al., 2015b), with evidence that they may be co-regulated, many studies find no association between mtDNAcn and TL. The study of mtDNAcn in those with AN is particularly interesting as this population possess a very distinct phenotype with a reduced BMI secondary to excessive exercise and chronic calorie restriction generally in the absence of any other illness, e.g., infection, cancer. One would expect that the physical and psychological stress of this illness would activate the stress response and impact on mtDNAcn, which is very responsive to the endogenous stress hormones and the immune response, and further investigation is required to understand this relationship.

5.3.2 Future Research

There are many gaps in our knowledge of mitochondria, and mtDNAcn, that need be resolved to maximise the results of future scientific studies in this area, and to help

understand the significance of variations in mtDNAcn in relation to the mitochondrion as a whole, and health outcomes. As mentioned in Study 1, future studies should be larger in size, with a prospective design, to help identify variables that affect copy number over time, including age and the development of disease. Increasing the accuracy of the definitions of severity and duration of illness would be beneficial. The interpretation of mtDNAcn is not uni-directional, so both an increase and a decrease in copy number can be indicative of dysfunction. The investigation of mtDNAcn in AN should incorporate detailed information on exercise intensity and frequency, the quantification of calorie intake, measures of severity of illness and the measurement of the effects of prescribed medications and supplements, to help identify variables that impact on copy number in AN. Because mtDNAcn is very responsive to the stress response the measurement of specific stressors, including acute and chronic stress, internal and external sources of stress, early life stress and later life stress, to name but a few, should all be considered. Furthermore, mtDNAcn exhibits diurnal variation. Therefore, future studies should incorporate multiple daily measures, where feasible. mtDNAcn also varies between cell type, and thus future researchers need to be cognisant of the heterogenous cell type composition of leukocytes and the abundance of platelets. For accurate results, purified cell populations should be used in the measurement of mtDNAcn.

Mitochondria are complex organelles that have several independent functions that can affect copy number. To truly understand mtDNAcn in AN, or in any illness, examining the whole mitochondrion is necessary. The investigation of mtDNAcn in parallel with other measures of mitochondrial health would help develop our understanding of the reasons for increase and decreases in copy number, and the significance of these

changes. Therefore, different tests are required to measure different aspects of mitochondrial health. mtDNAcn is only one way to (indirectly) measure mitochondrial function and tells us very little about the actual health of the mitochondrion and, therefore, stress and biological ageing. Ideally, to understand the significance of alterations in copy number, the study of mtDNAcn should be in parallel with other measures of mitochondrial functions in addition to the study of mitochondrial content. Tissues vary as regards metabolic requirements, and thus, mitochondria can differ in terms of protein composition or function. The study of mitochondria should include the investigations that directly assess mitochondrial function (for example mitochondrial respiration, respiratory chain enzymatic activity, and production of reactive oxygen species), or those that measure alterations in the structure or morphology of mitochondria via electron microscopy. Combining measures of mitochondrial function and content into mitochondrial health indices would result in studies that are more accurate and sensitive than individual markers alone.

Future studies of mtDNAcn should consider both the method of measurement of mtDNAcn and the DNA extraction methodology or kit used. Not all methods of measuring copy number are equivalent. Thus far, the most robust mtDNAcn estimates are from whole genome-based sequencing studies. The absolute copy number can also be influenced by DNA extraction methods, emphasising the importance of the use of a validated method of extraction and importance of the reporting of the method of extraction used. The study of mtDNAcn is both fascinating and challenging and future research is required to contribute to our understanding of the role of the mitochondrion in human health and ageing.

In conclusion, both TL and mtDNAcn can reflect stress, a risk factor for accelerated biological ageing. Those with AN are placed under significant physical and psychological stress, despite being otherwise healthy. This cohort of patients have an abnormally reduced body mass index secondary to extreme calorie control and/or intensive exercise in addition to suffering from internal sources of stress due to perfectionistic and obsessive-compulsive traits. There is a high rate of psychiatric co-morbidity as a result of these traits. Due to the increased levels of physical and psychological stress, which can be readily measured and quantified, AN is a potential model for future studies of stress and stress-related biological changes, which can increase the risk of accelerated biological ageing.

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APPENDICES

Appendix 1 Publication: Telomere length in patients with anorexia nervosa

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Telomere length in patients with anorexia nervosa

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ABSTRACT

Patients with anorexia nervosa (AN) experience substantial physical and psychological stress exerted on the body as part of the condition. Telomere length (TL) provides a measure of normal biological ageing that can be further reduced by additional stressors. We sought to test the following hypotheses: (1) TL is reduced in patients with AN compared to healthy controls, and (2) TL is reduced with increasing duration of illness. For this cross-sectional study, we assessed TL in whole blood DNA collected from patients with AN (n = 23; 21 females; mean age 30.3 years, SD 13.1) and age- and sex-matched healthy controls (n = 33; 32 females, mean age 30.6 years, SD 12.2) using quantitative real-time polymerase chain reaction. Clinical characteristics were obtained. Our results indicate that TL is significantly longer in patients with AN compared to healthy controls, both before and after adjusting for age, sex, level of educational attainment, smoking status, body mass index and antipsychotic use. TL was not associated with duration of illness after adjusting for age. Contrary to our hypotheses, our results indicate that TL does not reflect accelerated biological ageing in patients with AN. Further investigation may identify the reason for increased TL in those with AN.

1. Introduction

Although ageing is commonly measured in chronological terms, it may also be quantified in biological terms. Key components of ageing include molecular changes within our cells (López-Otín et al., 2013). Telomeres, caps at the end of chromosomes that protect them from degradation, are composed of a variable number of tandem TTAGGG repeats of DNA (Meyne et al., 1989). Cells normally have a limited ability to replicate, known as the "Hayflick limit", resulting in progressive shortening of telomeres with every mitotic event (Hayflick et al., 1961). This eventually leads to cellular senescence and the ageing process (Campisi et al., 1996; Harley, 1997). Therefore, telomere length (TL) is suggested to reflect the cell's biological age, as opposed to its chronological age (Epel et al., 2004).

Shortened telomeres are associated with stress (Shalev et al., 2013). Indeed, those with anorexia nervosa (AN) experience substantial both psychological and physical stress due to the effects of this mental illness. Mental illness is a form of psychological stress and TL has been found to be significantly reduced in those with post-traumatic stress, anxiety, mood and psychotic disorders, in comparison to healthy controls (Darrow et al., 2016). Severely reduced body mass index (BMI) due to starvation, as experienced by those with AN, places a substantial physical stress on the body affecting all systems with an accumulation of damage over time (Treasure et al., 2020). Furthermore, chronic dietary restraint has been reported to be a risk factor for premature telomere shortening (Kiefer et al., 2008). However, despite the well-reported physical and psychological stress experienced by those with AN, there are no studies to date that have investigated TL in AN.

We therefore sought to test the following hypotheses using a cross-sectional, case-control design: (1) TL is shorter in patients with AN compared to healthy controls, and (2) TL is reduced with increasing duration of illness with AN.

2. Methods

2.1. Participants

This study was approved by St Patrick's University Hospital Research Ethics Committee and adhered to the Declaration of Helsinki (World Medical Association, 2013). All participants provided written informed consent. Patients with AN were recruited between August 2018 and April 2019 in St Patrick's University Hospital, Ireland. Inclusion criteria were:

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Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5) diagnosis of AN and ≥18 years old. Diagnoses of AN and AN subtypes, restrictive and binge-purge, were confirmed with the Mini-International Neuropsychiatric Interview (M.I.N.I) for DSM-5, version 7 (Sheehan, 2014). Exclusion criteria were: involuntary status, active substance dependence, and chronic physical illness unrelated to anorexia nervosa. Healthy controls, with no history of psychiatric or current physical illness and a normal body mass index (BMI), were recruited though advertisement in local volunteer groups, word of mouth and social media.

2.2. Measures

Demographic and clinical data were documented for all participants. Data on medication, including generic name and class of medication, was collected at the time of sampling. Data on treatment length was not collected. Severity of AN was assessed using the Eating Disorders Examination edition 17.0D (Fairburn et al., 2008), which provides an overall global score and is divided into four subscale scores based on eating disorder psychopathology: restraint, eating concern, weight concern and shape concern.

2.3. DNA extraction

Fasting, peripheral whole blood samples were collected in K_2EDTA tubes (BD, UK). Samples were initially stored at −20 °C for 24 h and then at −80 °C until DNA extraction and further analysis. Whole blood DNA was extracted using an Autopure LS ® (Qiagen, Germany) system by Trinity College Dublin's Biobank facility (https://www.tcd.ie/ttmi/facilities/trinity-biobank/). Purified DNA had an A260/A280 ratio of 1.6–1.9. Extracted DNA samples were stored at −20 °C until analysis.

2.4. Telomere assay

Blood TL was measured using qRT-PCR as previously reported (Ryan and McLoughlin, 2020). Primer and PCR run details are provided in Supporting *Information*. TL is expressed as a telomere to single-copy gene (T/S) ratio.

Each sample was assayed in duplicate wells on three separate plates, with identical sample/well positions used across plates. Samples and inter-plate calibrators were run using 2.0 ng DNA per well. The four values from two plates that had an SD < 0.5 Cts were used to calculate the mean quantity for the telomere and Hgb PCR runs, from which the T/S ratio was generated. Four control genomic DNA samples were included in each PCR run as inter-plate calibrators in order to calculate a normalizing factor. The T/S ratio for each of the control DNA samples was divided by the average T/S ratio for the same DNA for the telomere and *Hgb* runs to obtain a normalizing factor (Nudelman et al., 2019). The normalization factor was then used to correct for inter-run variability to generate the final T/S ratio.

2.5. Statistical analyses

SPSS, version 27 (IBM Corporation, NY, USA), was used to analyse all data. Data were assessed for normality using the Shapiro-Wilk test. Differences in demographic and clinical characteristics between groups were determined using Fisher's Exact test and Chi Square for categorical data. Independent *t* tests and Mann Whitney U tests were used for continuous data. General linear models were used to determine differences between groups while adjusting for covariates. Spearman's rho was used to assess associations between continuous variables, while point-biserial correlations were used to determine associations between continuous and dichotomous variables. Variables such as age, sex, level of educational attainment, smoking status, BMI and antipsychotic medication are known to affect TL (Astuti et al., 2017; Lulkiewicz et al., 2020; Polho et al., 2015). Therefore, these variables were included as covariates. Analyses were conducted as follows: (1) unadjusted and (2) adjusted for covariates. TL data are presented as mean T/S ratio ± SD. Differences with a p value ≤ 0.05 were deemed statistically significant. Analyses were two-tailed.

3. Results

3.1. Demographic and clinical characteristics

Forty-four patients were admitted to the Eating Disorders Unit at St Patrick's Mental Health Services during the study period. Of these, 18 were ineligible: one patient was admitted involuntarily, 13 did not have a primary diagnosis of anorexia nervosa and four were repeated admissions. Twenty-six patients were deemed eligible, 23 of whom consented to participate. We recruited 33 control participants.

Participants' demographic and clinical details are summarised in Table 1. Patient and control groups did not differ significantly regarding age, sex and smoking status (all $p > 0.05$). The groups differed in educational attainment, with the control group having a higher level of education overall ($p \leq 0.001$). As expected, the control group had a higher BMI ($p \leq 0.001$) and lower EDE scores ($p \leq 0.001$). Prescribed psychotropic medications included anti-depressants, antipsychotics, pregabalin, benzodiazepine and non-benzodiazepine hypnotics. Information on duration of medication use was not collected. Patients' medications are summarised in Supporting *Information* (Table S1).

Table 1
Demographic and clinical characteristics of the anorexia nervosa and healthy control participants.

Characteristic	AN patients (n = 23)	Controls (n = 33)	Test Statistic	*p* value
Age (years), mean ± SD	30.3 ± 13.1	30.6 ± 12.2	$U = 346.5$, $r = -0.8$	$p = 0.582$
BMI (kg/m^2), mean ± SD	15.1 ± 2.4	22.2 ± 1.9	$U = 25.0$, $r = -0.7$	$p \leq 0.001$
Sex, No.	2	1	*Fisher's exact test*	$p = 0.562$
Male	21	32	$OR = 0.328$	
Female				
Smokers, No.	3	1	*Fisher's exact test*	$p = 0.295$
			$OR = 0.208$	
Educational Attainment, No.	1	1	*Chi Square goodness-of-fit test*	$p \leq 0.001$
	15	9		
Primary	7	23	$\chi^2 = 23.3$	
Secondary				
Tertiary/Quaternary				
Age of onset of illness (years), mean ± SD	17.1 ± 4.6			
Duration of illness (years), mean ± SD	13.2 ± 13.7			
Ethnicity, No.	22	33		
White	1			
Other				
AN subtype	19			
Restrictive	4			
Binge/purge				
EDE score, mean (range)	3.9 (2.1–5.7)	0.3 (0.0–1.1)	$U = 759.0$, $r = 0.80$	$p \leq 0.001$
Global score	4.1 (1.2–6.0)	0.3 (0.0–2.4)	$U = 754.5$, $r = 0.86$	$p \leq 0.001$
Restraint	3.8 (0.0–6.0)	0.1 (0.0–0.4)	$U = 736.5$, $r = 0.85$	$p \leq 0.001$
Eating Concern	4.1 (2.1–6.0)	0.5 (0.0–1.4)	$U = 736.0$, $r = 0.84$	$p \leq 0.001$
Shape Concern	3.9 (0.8–6.0)	0.4 (0.0–1.8)	$U = 749.5$, $r = 0.83$	$p \leq 0.001$
Weight Concern				

AN, Anorexia nervosa; BMI, body mass index; EDE, Eating Disorders Examination.

3.2. Comparison of TL between anorexia nervosa and control groups

Data for TL were non-normally distributed but were normally distributed following natural log transformation. We found a significantly longer TL in the AN group compared to the control group ($t = -2.15$, $df = 54$, $p = 0.04$, $r = 0.28$) with a mean TL of 0.33 (SD ± 0.08) in the AN group and 0.29 (SD ± 0.07) in the control group (Fig. 1). These results remained statistically significant after adjusting for age, sex, level of educational attainment, smoking status, antipsychotic medication and BMI ($F_{(1, 48)} = 5.45$, $p = 0.02$).

3.3. Correlations between TL and illness duration

TL was negatively associated with illness duration in the AN group ($r = -0.477$, $p = 0.021$). However, this test did not reach statistical significance after adjusting for age ($r = -0.262$, $p = 0.239$). Additionally, we analysed TL and severity of illness as measured by the EDE. There was no association found between TL and global or sub-scale scores of the EDE (all $p > 0.05$).

TL was negatively associated with age for the group as a whole ($r = -0.496$, $p \leq 0.001$) and individually for both the control ($r = -0.524$, $p = 0.002$) and case groups ($r = -0.505$, $p = 0.014$). TL was not significantly associated with sex, BMI, level of educational attainment or smoking status in the group as a whole, nor when both groups were examined individually (all $p > 0.05$).

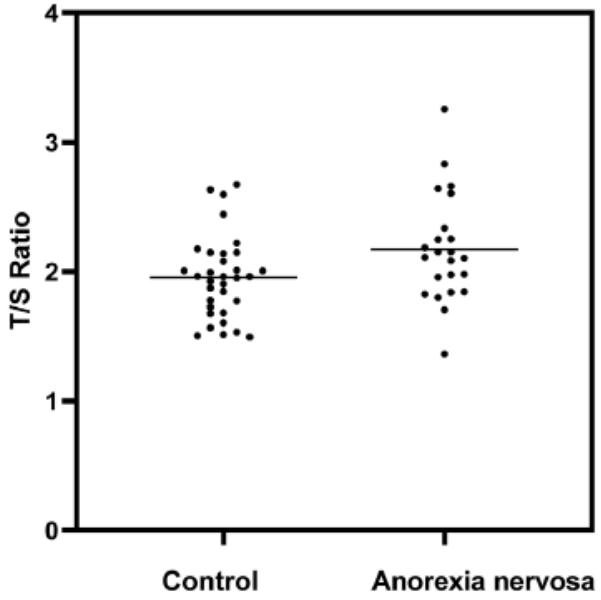

Fig. 1. Unadjusted raw T/S ratio values in healthy controls compared to patients with anorexia nervosa.

4. Discussion

This study is the first to examine the relationship between TL and anorexia nervosa. Contrary to our hypothesis, we found TL to be longer in the underweight AN group compared to healthy controls. There may be a number of reasons for this. First, many studies show a positive relationship between exercise and TL (Lin et al., 2019). Those with AN are likely to exercise significantly more than the controls, which may contribute to longer TL. Secondly, calorie restriction may also affect TL. García-Calzón et al. (2014) found that TL lengthened in adolescents after two months of an energy-restricted diet. Thus, the combination of increased exercise and reduced calorie intake may have contributed to the preservation of TL in the AN group compared to the control group. Interestingly, Kiefer et al. (2008) described premature leukocyte telomere shortening in premenopausal and post-menopausal women who reported chronic dietary restraint. However, Tomiyama et al. (2017) reported shorter peripheral blood mononuclear cell telomeres in those practising calorie restriction compared to controls, concluding that there was no clear evidence that calorie restriction delays ageing related to telomere length.

As regards our second hypothesis, we found no significant relationship between TL and duration of illness after controlling for age. Again, this may be due to the aforementioned frequent exercise and restricted calorie intake in this cohort. Additionally, compensatory mechanisms may be activated secondary to stress, which may increase TL. Telomerase, an enzyme that elongates telomere length, is stress-responsive (Beery et al., 2012) and may drive the increase in TL in those with AN who experience physical and psychological stress compared to healthy controls.

There are some limitations to our study. Our TL measurements were cross-sectional, and so we were unable to examine changes over time. We measured TL from peripheral blood samples and a change in TL in the peripheral blood may not indicate the dynamics of telomere length in the brain. Furthermore, we measured telomere length in whole blood as opposed to specific blood cell types. A study by Lin et al. (2010) reported that B cells have the longest telomere length compared to other lymphocyte subtypes; alterations in cell subtypes may thus account for increased telomere length in the AN group. There is evidence that exercise and dietary restriction may affect TL (García-Calzón et al., 2014; Lin et al., 2019), however we do not have a measure of physical exercise nor exact calorie intake in this group. The sample size examined in this study is small, thus limiting the generalisability of the results. Finally, most patients with AN were prescribed psychotropic medications and dietary supplements. The effect of prescribed medication and supplements on TL is unknown. The duration of use of medications was not incorporated into the study and this could be investigated in future studies. Studies have shown that human TL is positively associated with nutritional status (Paul, 2011), and most of the AN participants were prescribed supplements which may have affected TL.

In conclusion, we discovered that patients with AN had a longer mean TL than controls but there was no correlation between TL and duration of illness after adjusting for age. It was not possible for us to determine whether TL is preserved due to characteristics commonly present in this illness, such as increased exercise and reduced calorie intake, or whether perhaps there is a compensatory mechanism at play during the acute phase of AN that preserves TL in the presence of increased physical and psychological stress. Further, larger scale studies are required to identify the reason for increased TL in those with AN.

Author statement

All authors have seen and approved the final version of the manuscript. The manuscript is the authors' original work, it has not received prior publication and is not under consideration for publication elsewhere.

Data availability statement

The data used in this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

Declan McLoughlin has received speaker's honoraria from Mecta and Otsuka and an honorarium from Janssen for participating in an esketamine advisory board meeting. The other authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.psycom.2022.100022.

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Supporting Information

Telomere Assay

A five-point standard curve of HeLa cell DNA ranging from 0.31–25.0 ng was included on each plate so that the quantity of targeted templates in each sample could be determined relative to the standard curve.

The telomere primers used were tel1b (CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT), at a final concentration of 100 nM, and tel2b (GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT), at a final concentration of 900 nM. The primers for the single-copy gene (*Hgb*) were Hgb1 (GCTTCTGACACAACTGTGTTCACTAGC), at a final concentration of 300 nM, and Hgb2 (CACCAACTTCATCCACGTTCACC), at a final concentration of 700nM. Primers were salt-free purified (Eurofins Genomics, Germany).

Cycling profile for the telomere and single-copy gene qRT-PCR reaction

Telomere qRT-PCR reaction: 95°C for 5 min followed by 30 cycles of 95°C for 15 s and 54°C for 2 min.

Single-copy gene: 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 58°C for 20 s, and 72°C for 28 s.

Average R^2, efficiencies, inter- and intra-assay CVs for telomere and Hgb runs

The average r^2 of the PCR runs were 0.991 and 0.989 for the telomere and *Hgb* runs, respectively, and the average efficiencies for the telomere and *Hgb* runs were 96.24% and 90.75%, respectively.
The intra-assay CV for TL runs was <3.5% and the inter-assay CV was <4.5%. For the *Hgb* runs, the intra-assay CV was <2.5% and the inter-assay CV was <2.5%.

TABLE S1. Prescribed medications for the anorexia nervosa group

Medications	
SSRI	11 *(Fluoxetine n = 8, Escitalopram n = 2, Sertraline n = 1)*
SNRI	3 *(Venlafaxine n = 2, Duloxetine n = 1)*
Mirtazapine	4
Antipsychotics	10 *(Risperidone n = 4, Olanzapine n = 5, Quetiapine n = 1)*
Benzodiazepines	3 *(Alprazolam n = 2, Clonazepam n = 1)*
Non-benzodiazepine hypnotics	6 (*Melatonin n = 5, Z-drugs n = 1)*
Pregabalin	2
Lactulose®	4
Movicol®	7
Thiamine	11
Vitamin B12	11
Multivitamins	18
Calcium carbonate / colecalciferol (vitamin D3)	15
Fortisip®	6

SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin-norepinephrine reuptake inhibitor.

Appendix 2 Publication: Duration of anorexia nervosa if positively associated with whole blood mitochondrial DNA copy number: A cross-sectional case-control study.

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ORIGINAL ARTICLE

Duration of anorexia nervosa is positively associated with whole blood mitochondrial DNA copy number: A cross-sectional case-control study

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KEYWORDS
Anorexia nervosa;
Feeding and eating disorders;
Mitochondria;
Mitochondrial DNA;
Telomere;
Aging

Abstract
Background and Objectives: Anorexia nervosa (AN) is associated with significant physical and psychological stress. Stress causes changes at a cellular level and mitochondria may reflect this stress via changes such as alterations in DNA quantity or in their function. Mitochondria are unique in that they possess their own DNA, distinct from nuclear DNA, of which there are many copies within the cell. An indirect measure of mitochondrial function is the assessment of mitochondrial DNA copy number (mtDNAcn). Telomere length (TL) represents a marker of normal biological ageing, and TL can be shortened by stressors. We sought to test the following hypotheses: (1) that increased mtDNAcn would be found in patients with AN in comparison to healthy controls, (2) that mtDNAcn is related to duration and severity of AN, and (3) mtDNAcn is correlated with TL.
Methods: Using quantitative real-time polymerase chain reaction, we calculated mtDNAcn and TL using peripheral whole blood DNA collected from patients with AN (n = 23) compared to samples from age- and sex-matched healthy controls (n = 33). Clinical characteristics were also obtained.
Results: We found no difference between mtDNAcn in patients with AN in comparison to controls. mtDNAcn was positively associated with duration of illness, but not with illness severity. There was no association between mtDNAcn and TL.
Conclusions: Further investigations may clarify whether compensatory mechanisms increase mtDNAcn with increased illness duration.

Abbreviations: AN, anorexia nervosa; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; BMI, body mass index; GR, glucocorticoid receptor; mtDNAcn, mitochondrial DNA copy number; qRT-PCR, quantitative real time polymerase chain reaction; ROS, reactive oxygen species; TL, telomere length.

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Introduction

The mitochondrion is a double-membraned cell organelle that uses the mitochondrial respiratory chain to produce energy in the form of adenosine triphosphate (ATP), and regulates intracellular signalling, cellular homeostasis, and apoptosis.[1] Mitochondria are unique in that they possess their own DNA, which is independent of the nuclear DNA genome. Mitochondrial DNA contains: 37 genes, encoding 13 proteins that participate in oxidative phosphorylation; 22 tRNAs; and two rRNAs.[1] Hundreds of mitochondria are contained within each cell, with each mitochondrion containing 2–10 copies of the mitochondrial DNA genome.

Compared to nuclear DNA, mitochondrial DNA has an increased risk of being exposed to oxidative damage from reactive oxygen species and this, twinned with its limited DNA repair system, can have a negative impact on functioning of the mitochondrion.[1] Stress or damage to the mitochondrion is thought to result in changes in the number of copies of mitochondrial DNA. Indeed, mitochondrial DNA copy number (mtDNAcn) can increase or decrease in response to stressors. Mechanisms may exist where mtDNAcn is increased as an adaptive response to stress through a process known as upregulation.[2] However, when a stressful event persists, the continued exposure to reactive oxygen species (ROS) leads to a reduction in the mitochondrion's ability to replicate and results in an accumulation of defective mitochondria.[3] This then contributes to a reduction in mtDNAcn. Furthermore, chronic stress may downregulate brain-derived neurotrophic factor (BDNF) in the cerebral cortex and hippocampus, leading to impaired glucocorticoid receptor (GR) signalling and resulting in glucocorticoid resistance.[4] Chronically increased levels of glucose can damage mitochondria and mitochondrial DNA due to the production of toxic products and systemic inflammation and thereby contribute to reduced mtDNAcn.[5] Furthermore, alterations in mtDNAcn are associated with cardiovascular disease, cancer and renal disease and with overall mortality.[6] This suggests that the measurement of mtDNAcn in peripheral blood may be an accessible and readily available method of identifying those with increased risk of comorbid disease in states where the body experiences significant physical stress, such as AN.

Patients with anorexia nervosa (AN) experience major stressors in the form of extreme physical stress, with severely reduced body mass index (BMI) secondary to calorie restriction and/or excessive exercise. Studies suggest that mtDNAcn increases with a reducing BMI in females.[7] There may be an inverse relationship between mtDNAcn and weight, waist-hip ration, waist size, and BMI.[8] mtDNAcn has also been shown to increase with exercise intensity.[9] However, while many studies of mtDNAcn have been conducted in participants with a normal or raised BMI,[10] there are no studies investigating mtDNAcn and severely reduced BMI and, to our knowledge, there have been no studies conducted to date examining mtDNAcn in AN.

Those with AN also experience significant psychological stress secondary to the effects of their psychiatric illness. In fact, mitochondrial dysfunction has been linked to various psychiatric conditions associated with stress such as mood disorders.[11] Tyrka, et al.[12] reported significantly increased mtDNAcn in those who had experienced psychological stress in the form of early life stress, compared to controls without a history of childhood maltreatment and parental loss. We have recently reported elevated blood mtDNAcn in severely depressed patients undergoing treatment with electroconvulsive therapy.[13] Similarly, Cai, et al.[14] reported increased mtDNAcn in major depression in females.

Telomeres, which contain repetitive DNA sequences, are located at the terminal ends of chromosomes and protect chromosomes from degradation.[10] We have previously reported increased TL in patients with AN compared to healthy controls.[15] The preservation of TL in patients with AN may occur secondary to the protective effect of diet and exercise on TL. However, there have been no studies examining the relationship between TL and mtDNAcn in patients with AN to date. Alterations in both TL and mtDNAcn may be indicative of the biological aging process, and studies show that mtDNAcn and TL appear to be co-regulated and positively associated.[10] Reactive oxygen species (ROS) may result in shortened TL and lead to telomere dysfunction and cellular senescence.[2] Telomere dysfunction can then negatively impact on mitochondrial biogenesis by downregulating peroxisome proliferator-activated receptor- γ coactivator 1α (PGC-1 α), affecting mitochondrial DNA copy number.[16] Several studies show that there is a positive relationship between TL and mtDNAcn.[8,17–19]

We therefore sought to test the following hypotheses using a cross-sectional, case-control design: (1) mtDNAcn is increased in patients with AN compared to healthy controls, (2) mtDNAcn is increased with increasing duration and severity of illness, and (3) mtDNAcn is positively correlated with TL.

Material and methods

Participants and sample collection

Participant recruitment and outcome measures for this study are described in a previously published study using the same participant cohort.[15] Briefly, we recruited patients with AN from August 2018 to April 2019 in St Patrick's University Hospital, Ireland. The inclusion criteria were as follows: ≥18 years old; diagnosis of AN per Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5). We confirmed a diagnosis of AN, or the restrictive and binge-purge AN subtypes, using the Mini-International Neuropsychiatric Interview (M.I.N.I.) for DSM-5, version 7.[20] The exclusion criteria for this study were as follows: chronic physical illness unrelated to AN, active substance dependence, involuntary status. We recruited healthy controls who had no history of psychiatric or current physical illness and a normal BMI using word of mouth, social media, and advertisements in local volunteer groups.

We collected fasting, peripheral whole blood samples into K_2EDTA tubes (BD, UK). Tubes were stored at −20 °C for 24 h and then at −80 °C prior to analysis.

Demographic and clinical data

We documented demographic and clinical data for all participants. The Eating Disorders Examination (EDE) edition 17.0D[21] was used to assess the severity of AN. This scale provides a score based on eating disorder psychopathology including restraint, and concern regarding eating, weight, and shape. All prescribed medications for the AN group were

documented. Full blood count (FBC) samples were taken from patients with AN following admission to the eating disorders ward.

DNA extraction

Blood samples were sent to the Trinity College Dublin Biobank facility (https://www-tcd-ie.elib.tcd.ie/ttmi/facilities/trinity-biobank/) for DNA extraction. DNA was extracted using the Autopure LS ® (Qiagen, Germany) and subsequently stored at −20 °C until analysis. Purified DNA had an A260/A280 ratio of 1.6–1.9.

Mitochondrial DNA copy number assay

As previously described,[13] we used the Takara® Human Mitochondrial DNA monitoring primer set (Takara Bio Inc., Sweden) to assess relative mtDNAcn using quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, each sample was run in duplicate in a 25 μl reaction, as outlined in Table 1. Each sample was run using two primers for mtDNA (ND1 and ND5) and two primers for nuclear DNA (SLCO2B1 and SERPINA1), included in the Takara set. qRT-PCR was performed using a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, USA) with the following cycling conditions: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

Relative mtDNAcn per diploid genome was calculated using the mtDNA Copy Number Tool from Takara using the following formula: $2^{-\Delta Ct}$ for the values ΔCt1 and ΔCt2, where ΔCt1 = (Ct for SLCO2B1−Ct for ND1) and ΔCt2 = (Ct for SERPINA1−Ct for ND5). An example calculation is provided in Supplemental File 1.

To control for inter-assay variability, three interplate calibrator DNA samples, obtained from healthy volunteers aged 29 to 55 years, were included on each PCR plate. The intra-assay coefficient of variation (CV) was <3.7 % and the inter-assay CV was <2.3 %.

Telomere length assay

The method for the telomere length (TL) assay is reported in a previously published study on the same cohort of participants.[15] TL data obtained in that study[15] were included in analyses reported here. Therefore, the same participant sample is used for both the TL and mtDNAcn assays. Briefly, each sample for the TL analyses was run in duplicate plates. A five-point standard curve of HeLa cell DNA was prepared so that the quantity of targeted templates in each sample could be quantified relative to the standard curve. The cycling profile for the telomere rection was 95 °C for 5 min followed by 30 cycles of 95 °C for 15 s and 54 °C for 2 min. The cycling profile for the Single-copy gene was 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 58 °C for 20 s, and 72 °C for 28 s.

Statistical analyses

All statistical analyses were performed using SPSS, version 27 (IBM Corporation, USA). A Shapiro-Wilk test was used to assess data for normality. The Fisher's Exact test and Chi Square Goodness-of-fit test were used to determine differences in categorical demographic and clinical data. Independent *t* tests and Mann Whitney U tests were used to analyse continuous demographic and clinical data. Differences between groups were assessed using general linear models, adjusting for covariates where appropriate. Associations between continuous variables were assessed using Pearson's and Spearman's correlation tests, while associations between continuous and dichotomous variables were determined using point-biserial correlations. Age, sex, educational attainment, smoking status, BMI,[13] antipsychotic medication,[22] and vitamin B12 supplementation[23] were included as covariates as these can affect mtDNAcn. We conducted both (1) unadjusted analyses and (2) analyses adjusted for age, sex, level of educational attainment, smoking status, antipsychotic medication, BMI, and vitamin B12. Because this was an exploratory study no correction was made for multiple comparison. Data are presented as mean mtDNAcn ± standard deviation (S.D). Differences with a p value ≤ 0.05 were deemed statistically significant. Analyses were two-tailed. Because there are so few previous studies reporting mtDNAcn in AN, it was not possible to definitively estimate a sample size for this exploratory study. However, we performed a post hoc power analysis, using G*Power (version 3.1.9.7) (https://www.psychologie.hhu.de/en/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower). With a sample size of 23 patients and 33 controls and setting alpha at 0.05 (two-sided), there is 80 % power to be able to detect an effect size of 0.8 for the difference in mtDNAcn between the AN and control groups; this represents a large and clinically relevant effect size.

Table 1 qRT-PCR Reaction set-up.

Reagent	Volume per reaction	Final Conc.
TB Green Premix Ex Taq II (Tli RNaseH Plus) (2X)	12.5 μl	1 x
ND1 Primer Mix (mtDNA primer)	1 μl	0.4 μM
SLCO2B1 Primer Mix (nuclear DNA primer)	1 μl	0.4 μM
ND5 Primer Mix (mtDNA primer)	1 μl	0.4 μM
SERPINA1 Primer Mix (nuclear DNA primer)	1 μl	0.4 μM
Genomic DNA sample (5 ng/μl)	2 μl	
RNase/DNase-free water	9.5 μl	

Ethics

This study adhered to the Declaration of Helsinki[24] and was approved by the St Patrick's University Hospital Research Ethics Committee. All ethical procedures were performed, and the privacy of participants was always observed. Participants provided written informed consent for experimentation before being included in the study.

Results

Demographic and clinical characteristics

Fifty-six participants were recruited to this study, as previously described.[15] Briefly, 23 patients with AN (21 females; mean age 30.3 years, SD 13.1) and 33 healthy controls (32 females, mean age 30.6 years, SD 12.) were included. There was no significant difference between the groups with regard to age, sex, or smoking status (all $p > 0.05$). However, the control group had a higher level of education ($p \leq 0.001$), and as expected, a higher BMI ($p \leq 0.001$), and lower EDE scores ($p \leq 0.001$). The mean age of onset of AN was 17.1 years and the mean duration of AN was 13.2 years. Nineteen patients had a diagnosis of restrictive-type AN, and four patients had a diagnosis of binge-purge-type AN. A summary of clinical and demographic details for the whole group, and prescribed medications for the AN group, are described in the online supplemental file 2. This file supplemented a previously published study that used the same participant cohort.[15] The control group was not being treated with any prescribed medications. Full blood count samples were obtained from the AN cohort following admission and are described in Table 2.

MtDNAcn was positively correlated with age for the group as a whole ($r = 0.39$, $p = 0.003$) and for the AN group ($r = 0.49$, $p = 0.02$). However, there was no correlation between age and mtDNAcn in the control group ($r = 0.31$, $p = 0.08$). There was no significant correlation between mtDNAcn and sex, BMI, level of educational attainment, or smoking status either in the whole group or when both groups were individually examined (all $p > 0.05$).

Comparison of mtDNAcn between AN and control groups

Raw mtDNAcn data were not normally distributed, but following natural log transformation data were normally distributed. Mean mtDNAcn was 2.08 ± 0.12 in the AN group and 2.04 ± 0.09 in the control group (effect size: $r = 0.18$), and no significant difference was noted between the groups (Fig. 1; $t = -1.17$, $df = 54$, $p = 0.25$). Moreover, statistical adjustment for potential covariates did not change the results ($F_{(1, 47)} = 0.53$, $p = 0.47$).

Correlations between mtDNAcn and severity of illness and illness duration

In the AN group, mtDNAcn was significantly positively correlated with illness duration (Fig. 2; $r = 0.59$, $p = 0.003$), which remained significant following adjustment for age ($r = 0.44$, $p = 0.04$). There was no statistically significant correlation between mtDNAcn and illness severity measured by the EDE global score ($r = 0.08$, $p = 0.57$), and this did not change following adjustment for age ($r = 0.13$, $p = 0.35$).

Correlations between mtDNAcn and TL

There was no statistically significant correlation between mtDNAcn and TL in the group as a whole (Fig. 3, $r = -0.24$, $p = 0.08$), even following adjustment for age ($r = 0.06$, $p = 0.67$), or in the AN group, both before and after controlling for age ($r = -0.19$, $p = 0.34$ and $r = -0.05$, $p = 0.82$, respectively). In the control group, there was a statistically significant inverse correlation between mtDNAcn and TL ($r = -0.42$, $p = 0.01$); however, this was no longer statistically significant after controlling for age ($r = -0.32$, $p = 0.08$).

Discussion

To our knowledge, this study is the first to examine mtDNAcn in blood from patients with AN. Contrary to our primary hypothesis, our results show no significant difference in mtDNAcn between patients with AN compared to healthy controls. Regarding our second hypothesis, there was a positive correlation between mtDNAcn and illness duration, though there was no significant correlation identified between mtDNAcn and severity of illness. It is worth noting that the EDE may not accurately reflect the biological severity of the disease as this clinician-rated questionnaire is based on the patient's concerns regarding their weight, shape, eating habits, and restraint tendencies and does not quantify the level of exercise or calorie expenditure or analyse BMI. Finally, we found no correlation between mtDNAcn and TL.

There may be several reasons for the link between mtDNAcn and the duration of illness noted here. First, an increase in mtDNAcn might occur as a mechanism to compensate for reduced mitochondrial energetics induced by

Table 2 Full blood count results.

Cell Type	Range
Neutrophil Count (No. outside normal range)	Range: 0.66–5.69 × 10^9/L (8/22 reduced)
Lymphocyte Count (No. outside normal range)	Range: 0.46–2.14 × 10^9/L (6/22 reduced)
Platelet Count (No. outside normal range)	Range: 125–428 × 10^9/L (1/22 reduced; 1/22 elevated)

Neutrophil normal range: 2.0–7.0 × 10^9/L; Lymphocyte normal range: 1.0–3.0 × 10^9/L; Platelet normal range: 150–410 × 10^9/L.

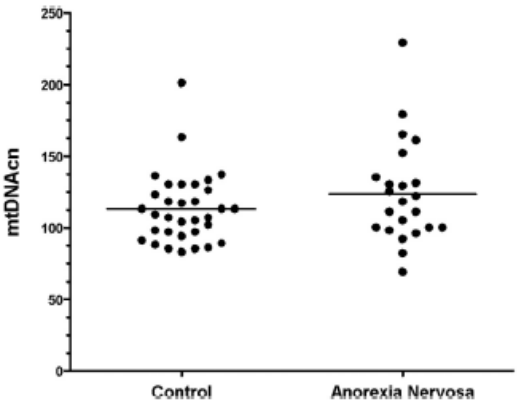

Fig. 1 Raw unadjusted mitochondrial DNA copy number (mtDNAcn) values in Healthy Controls (mean 2.04 ± 0.09) compared to patients with Anorexia Nervosa (mean 2.08 ± 0.12). The mean mtDNAcn values for each group are indicated by each horizontal line. There was no statistically significant difference between the groups, either before or after adjusting for covariates ($p > 0.05$).

physical or psychological stressors.[25] Second, studies show that patients with AN can have altered cell distributions and develop haematological abnormalities. For example, up to 17 % of hospitalised patients with severe AN develop thrombocytosis.[26] Different cell types show vast differences in mtDNAcn.[25] For instance, lymphocytes have an mtDNAcn in the range of 150–600 copies per cell, neutrophils have an mtDNAcn in the region of ~150 copies per cell, and platelets, which have no nucleus, contain an "infinite" mtDNAcn.[25] Thus, as mtDNAcn calculations are based on the relative number of copies of mitochondrial DNA using the nuclear genome as the denominator, in the case of platelets this denominator is zero; therefore, an increased number of platelets in the circulation, as is the case with thrombocytosis, may artificially increase the mtDNAcn and subsequently affect the overall mtDNAcn count.[25] Patients with AN are also found to have increased lymphocyte numbers.[27] Since there is lower mtDNAcn in neutrophils compared to lymphocytes, a higher neutrophil to lymphocyte ratio would result in lower mtDNAcn.[25] Full blood counts were not acquired for patients included in this study on the same day as sample collection; thus, we were unable to adjust our analyses to take into account variations in blood cell numbers. However, we did have full blood count data available for patients taken an average of five days prior to recruitment. Using these data, ~27 % of our patient cohort had a lymphocyte count below the normal range and ~36 % had a reduced neutrophil count (these were not the same people). The majority of our patient cohort had a platelet count that was within the normal range of 150–410 $\times$ 10^9/L, with the data showing that only one patient had a platelet count slightly above the normal range (i.e., 428 $\times$ 10^9/L) and one had a count slightly below the normal range (i.e., 125 $\times$ 10^9/L). Thus, it is possible that blood cell counts may have impacted on our results as interpretation is limited by the lack of same day blood cell counts. Therefore, future studies should ensure that full blood count data are acquired at the same time as study sample collection to enable statistical adjustment for blood cell numbers to gain a better picture of mtDNAcn in this patient population.

It is worth noting that the measurement of mtDNAcn is only one of many ways to indirectly quantify mitochondrial content, and the measurement of mtDNAcn alone does not inform us of the efficiency of the whole mitochondrion.[25] Mitochondria are involved in a range of functions. Assessment of a range of different mitochondrial function measurements, such as mitochondrial content, integrity, and respiratory capacity, may be a more holistic and accurate way to report on mitochondrial content and function. On the whole, the comparison of studies on mtDNAcn remains challenging because multiple factors can affect mtDNAcn, including the cell population studied, the tissue type, and changes in energy requirements. Factors such as age, stress and diurnal variation can affect cell distribution. Furthermore, not all mtDNAcn quantification methods are equivalent and kits used for DNA extraction also affect mtDNAcn

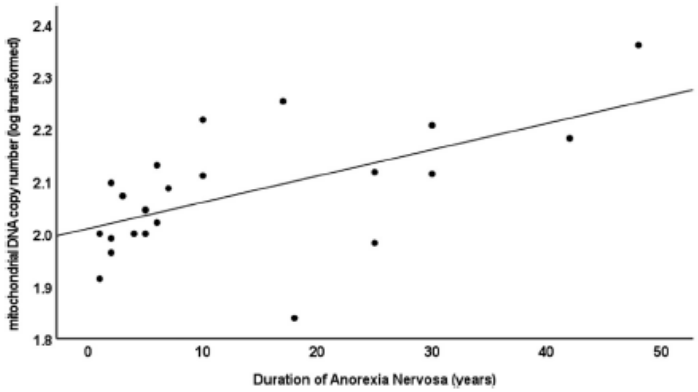

Fig. 2 Scatterplot representation of a statistically significant positive correlation between mitochondrial DNA copy number (mtDNAcn) values (log transformed) and duration of Anorexia Nervosa ($p = 0.003$).

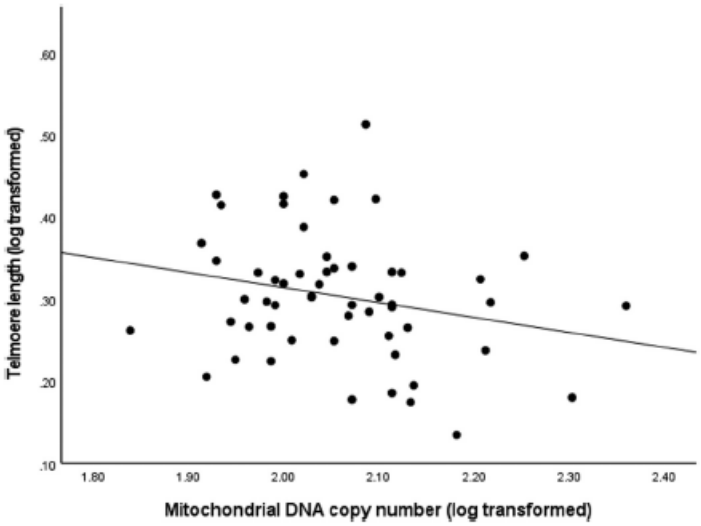

Fig. 3 No statistically significant correlation was found between telomere length (TL) and mitochondrial DNA copy number (mtDNAcn) values either before or after adjusting for age ($p > 0.05$) in the group as a whole ($n = 56$).

quantification.[25] Therefore, it is vital that there is a consistency of approach in future studies of mtDNAcn in psychiatry, in particular for AN.

A strength of this study is the fact that this is the first report of mtDNAcn in patients with AN compared to a healthy control group. This is interesting from a translational perspective as further larger scale studies may shed light on the impact of AN on the mitochondrion. In addition, though the sample size was small, a positive relationship was identified between mtDNAcn and duration of illness. This study has several limitations. First, mtDNAcn was determined using whole blood instead of using isolated cell-subtypes. Furthermore, we do not have a full blood count for these patients taken at the timepoint of blood collection for this study; so, we were unable to conduct analyses to control for platelet count. Second, we measured mtDNAcn in peripheral blood samples. A change in mtDNAcn in the peripheral blood may not be indicative of mtDNA content or function in the brain. Third, our mtDNAcn measurements were cross-sectional, and so it was not possible for us to assess changes in mtDNAcn over time, limiting conclusions about causality. Fourth, our sample size was small, which limits the generalisability of our results. Additionally, as this was an exploratory study, we did not correct for multiple comparison and larger scale studies would be required to validate our findings. Fifth, physical exercise and dietary restraint may affect mtDNAcn; however, we did not have a measure of physical exercise or details about the exact caloric intake for this cohort. Finally, most of our patients were receiving treatment with prescribed psychotropic medications, the effects of which on mtDNAcn are unknown. Furthermore, many of the AN patients are prescribed dietary supplements, including vitamin B12 that has a positive correlation with mtDNAcn.[23]

Conclusions

We found no overall difference in mtDNAcn between patients with AN and healthy controls. However, we did identify a positive association between mtDNAcn and duration of illness, suggestive, perhaps, of a mechanism of mitochondrial adaptation with progression of AN. Picard and McEwen[28] describe an increase in mtDNAcn secondary to compensatory upregulation of mitochondrial biogenesis as an adaptive response to stress by the cell. Animal studies of mtDNAcn and chronic stress report an increase in mtDNAcn as measured in peripheral tissues following chronic stress.[14,29] Furthermore, studies examining rodents trained with treadmill running reported that mtDNA was either partially or completely protected against stress-induced reductions in copy numbers.[30] Similarly, in humans, chronic physical activity and exercise may have also have a protective effect on mtDNA, therefore preserving copy number.[31] Our results reflect the complexity of stressors on mtDNAcn and highlight the need for studies examining mtDNAcn and various types of stressors, such as those that vary with nature (physical stress, psychological stress) and with duration (acute and chronic) of illness with AN. Although we reported no correlation between mtDNAcn and illness severity, it would be important to examine changes in copy number with various levels of stress (mild, moderate, severe), and investigate lifetime exposure to stress to examine the effect of stress in early life versus later life. Larger scale studies are required to increase the sample size and overcome some of the limitations of the present study. Longitudinal and prospective study designs would help to establish the association between stressors to fully examine mtDNAcn and mitochondrial function in AN and AN-like states and advance our understanding of the impact of AN on the mitochondrion.

Declaration of competing interest

Declan McLoughlin has received speaker's honoraria from Mecta, Otsuka and Janssen and an honorarium from Janssen for participating in an esketamine advisory board meeting. The other authors have no conflicts to declare.

Ethical considerations

This study adhered to the Declaration of Helsinki[24] and was approved by the St Patrick's University Hospital Research Ethics Committee. All ethical procedures were performed, and the privacy of participants was always observed. Participants provided written informed consent for experimentation before being included in the study.

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Declaration of Generative AI and Assisted technologies in the writing process

No generative AI or assisted technologies were used in the writing process.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ejpsy.2024.100265.

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Appendix 3 Publication: Whole blood mitochondrial DNA copy number in depression and response to electroconvulsive therapy.

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Whole blood mitochondrial DNA copy number in depression and response to electroconvulsive therapy

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ABSTRACT

Mitochondrial dysfunction may play a role in various psychiatric conditions. Mitochondrial DNA copy number (mtDNAcn), the ratio of mitochondrial DNA to nuclear DNA, represents an attractive marker of mitochondrial health that is easily measured from stored DNA samples, and has been shown to be altered in depression. In this study, we measured mtDNAcn in whole blood samples from medicated patients with depression ($n = 100$) compared to healthy controls ($n = 89$) and determined the relationship between mtDNAcn and depression severity and the therapeutic response to electroconvulsive therapy (ECT). We also explored the relationship between mtDNAcn and telomere length and inflammatory markers. Our results show that mtDNAcn was significantly elevated in blood from patients with depression when compared to control samples, and this result survived statistical adjustment for potential confounders ($p = 0.002$). mtDNAcn was significantly elevated in blood from subgroups of patients with non-psychotic or unipolar depression. There was no difference in mtDNAcn noted in subgroups of ECT remitters/non-remitters or responders/non-responders. Moreover, mtDNAcn was not associated with depression severity, telomere length, or circulating inflammatory marker concentrations. Overall, our results show that mtDNAcn is elevated in blood from patients with depression, though whether this translates to mitochondrial function is unknown. Further work is required to clarify the contribution of mitochondria and mtDNA to the pathophysiology of depression and the therapeutic response to antidepressant treatments.

1. Introduction

Mitochondria are double-membraned cell organelles involved in energy production, cell proliferation, apoptosis, and intracellular calcium homeostasis that are present in almost all eukaryotic cells (Filograna et al., 2021; Malik and Czajka, 2013). Every cell in the body depends on the production of energy by mitochondria. Some tissues with varying metabolic demands contain mitochondria that differ with regard to their function or protein composition, even if they are contained within the same cell (Picard and McEwen, 2018). Mitochondria are the only cell organelle to have their own separate DNA, termed mitochondrial DNA (mtDNA). This is comprised of 37 genes (Filograna et al., 2021; Gimenez-Palomo et al., 2021) and is a double-stranded circular molecule that is present in multiple copies per cell (Filograna et al., 2021) and is maternally inherited (Giles et al., 1980).

Different cell types have different mtDNA copy numbers (mtDNAcn). Those with higher energy demands have a higher mtDNAcn (e.g., heart, skeletal muscle) and differences have also been noted within tissues with, for example, variance in mtDNAcn observed across brain regions (Picard, 2021). MtDNA replication is independent of the cell cycle (Sasaki et al., 2017) and the number of mtDNA copies per nucleated cell is said to reflect the health of the mitochondria (Picard, 2021). MtDNA is susceptible to damage from reactive oxygen species (ROS) (Gimenez-Palomo et al., 2021), though mitochondria have many mechanisms to maintain their function, including antioxidant defenses to neutralize ROS, biogenesis, and mitophagy (Gimenez-Palomo et al., 2021). The

Abbreviations: BMI, body mass index; DAMP, damage-associated molecular pattern; ECT, electroconvulsive therapy; HAM-D24, Hamilton Depression Rating Scale 24-item version; MQC, mitochondrial quality control; mtDNA, mitochondrial DNA; mtDNAcn, mitochondrial DNA copy number; PBMC, peripheral blood mononuclear cell; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SNRI, selective noradrenergic reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TFAM, transcription factor A; TLR, toll-like receptor..

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endosymbiotic theory of the origin of mitochondria suggests that these organelles originated from bacteria; thus, the immune system recognises mitochondria as "foreign" molecules and can mount an immune response against mtDNA since it has similarities to the bacterial chromosome (De Gaetano et al., 2021).

Mitochondrial dysfunction appears to play a role in various psychiatric conditions such as schizophrenia, anxiety, and depression (Bergman and Ben-Shachar, 2016; Filiou and Sandi, 2019; Gimenez-Palomo et al., 2021; Klinedinst and Regenold, 2015). The mitochondrial hypothesis states that mood disorders are caused by mitochondrial dysfunction such as changes in oxidative phosphorylation and membrane polarity (Allen et al., 2018). In addition, mitochondrial diseases often present with psychiatric symptoms such as altered mood and cognition (60–93% of cases) (Klein et al., 2021). Stress, a known risk factor for depression (de Kloet et al., 2005), can lead to dysregulated energy homeostasis or even energy depletion (Ostergaard et al., 2018). In line with this, a recent study reported that peripheral blood mononuclear cells (PBMCs) from depressed patients exposed to oxidative stress (H_2O_2) show impairment in the repair and degradation of mtDNA compared to controls, suggesting that mitochondria in depressed patients cannot cope with increased oxidative stress or the combination of stress and elevated mtDNA damage (Czarny et al., 2020). In vivo studies in rodents have shown that psychological stress can have significant adverse effects on mitochondria, occurring in an inverted U-shaped manner, with acute stress found to damage the morphology and ultrastructure of mitochondria within hours while chronic stress increases mtDNAcn and the size of the mitochondria; this is thought to reflect compensatory biogenesis and replication of mitochondria in response to deficient energy supply (see Picard and McEwen, 2018). As the brain has the highest energy demand of all organs in the body, even small disruptions in energy production could lead to large effects therein (Pei and Wallace, 2017).

Measuring the number of mtDNA copies compared to the diploid nuclear genome, which has two copies per cell, using quantitative RT-PCR (qRT-PCR) yields a mtDNA:nuclear DNA (nDNA) ratio, which represents the most common means of assessing mtDNAcn (Picard, 2021). mtDNAcn is an attractive marker of mitochondrial health as it is easily measured from stored DNA samples or determined from genotyping data (Picard, 2021). mtDNAcn has the potential to be used as a measure of mitochondrial function clinically since, unlike other methods, it is readily scalable (Castellani et al., 2020). mtDNAcn was first denoted as a measure of mitochondrial dysfunction in rare genetic disorders but variations in mtDNAcn have now been observed in neurodegenerative disease, cardiovascular disease, diabetes, cancers, and aging (Castellani et al., 2020; Picard, 2021). Alterations in this ratio have also been reported in patients with psychiatric conditions, such as depression, anxiety, stress, adjustment disorders, and schizophrenia in comparison to healthy controls (Li et al., 2015; Wang et al., 2017).

Few studies have examined mtDNAcn in relation to antidepressant treatment outcomes (de Souza et al., 2014; Fernstrom et al., 2021a) and none has assessed mtDNAcn in relation to clinical response to electroconvulsive therapy (ECT), the most acutely effective antidepressant treatment available (UK ECT Review Group, 2003; Kirov et al., 2021). Notably, shifts in mtDNAcn might contribute to the increased inflammation and oxidative stress often observed in patients with psychiatric conditions (Klinedinst and Regenold, 2015; Malik and Czajka, 2013) since mtDNA can act as a damage-associated molecular pattern (DAMP) and initiate an immune response once it enters the cytoplasm or extracellular space (Grazioli and Pugin, 2018). Changes in mtDNA and telomeres, the protective caps on the end of each chromosome, are said to reflect processes that are central to the aging process, and the two have been shown to be associated and may be co-regulated (Tyrka et al., 2015). There are, however, conflicting reports regarding the direction of this association, i.e., whether mtDNAcn and telomere length are positively (Lindqvist et al., 2018; Tyrka et al., 2015; Tyrka et al., 2016) or negatively (Cai et al., 2015a; Edwards et al., 2016) correlated.

In this study we measured mtDNAcn in whole blood samples from medicated deeply-phenotyped severely-depressed inpatients referred for treatment with ECT compared to healthy controls. As described above, clinical reports on mtDNAcn in depression are limited and the results are conflicting. Therefore, based on in vivo studies reporting elevated mtDNAcn in chronically stressed animals, we hypothesized that mtDNAcn would be elevated in our severely depressed patient population compared to healthy controls. Moreover, we hypothesized that elevated mtDNAcn would positively correlate with depression severity, i.e., higher mtDNAcn would be associated with more severe depressive symptoms. Since previous reports have suggested differences in mtDNAcn between patients with unipolar compared to bipolar depression (Tsujii et al., 2019), we conducted a subgroup analysis of patients with unipolar vs. bipolar disorder in an attempt to replicate these findings, and also explored mtDNAcn in patients with psychotic vs. non-psychotic depression as, to our knowledge, this had not been previously reported on. In addition, as Lindqvist et al. (2018) previously reported a difference in circulating cell-free (ccf)-mtDNAcn in SSRI responders and non-responders, we also explored if there were differences in mtDNAcn between ECT responders/non-responders and ECT remitters/non-remitters, and subsequently explored whether mtDNAcn could be used to predict clinical outcomes following ECT. We also explored the relationship between mtDNAcn and telomere length and inflammatory markers (IL-6, IL-10, TNF-α, CRP) previously reported on in this cohort (Ryan and McLoughlin, 2020, 2022).

2. Methods

2.1. Participants

Depressed patients were recruited into the EFFECT-Dep Trial (Enhancing the Effectiveness of Electroconvulsive Therapy in Severe Depression; NCT01907217) from 2008 to 2012 in St. Patrick's Mental Health Services, Ireland (Semkovska et al., 2016). Healthy controls, with no history of psychiatric illness, were recruited in three batches through advertisement in local newspapers and social media from 2008 to 2016. This study received approval from St Patrick's University Hospital Research Ethics Committee and adhered to the Declaration of Helsinki (World Medical Association, 2013). All participants provided written informed consent. All participants were of White Irish ethnicity.

2.2. Electroconvulsive therapy

The EFFECT-Dep trial was a pragmatic, patient- and rater- blinded, non-inferiority trial comparing the effects of twice-weekly moderate-dose bitemporal (1.5× seizure threshold) and high-dose unilateral (6× seizure threshold) ECT in real-world practice (Semkovska et al., 2016). ECT was administered to patients as previously described with hand-held electrodes using methohexitone (0.75–1.0 mg/kg) for anesthesia and succinylcholine (0.5–1.0 mg/kg) as muscle relaxant (Semkovska et al., 2016). Patients were randomly allocated to moderate dose bitemporal (1.5× seizure threshold) or high-dose unilateral (6× seizure threshold) ECT and maintained on pharmacotherapy as usual throughout the treatment course. To be included in the trial, patients had to be over 18 years old, referred for ECT for treatment of a major depressive episode as diagnosed by the Structured Clinical Interview for DSM-IV Axis I Disorders (First et al., 1996), and have a pre-treatment Hamilton Depression Rating Scale 24-item version (HAM—D24) score ≥ 21 (Beckham and Leber, 1985). Patients were excluded from the trial for the following reasons: substance misuse in the previous 6 months, medically unfit for general anesthesia, ECT in the previous 6 months, dementia or other axis I diagnosis, involuntary status or inability/refusal to consent.

2.3. Blood sampling and DNA extraction

Fasting peripheral whole blood samples were collected in K$_2$EDTA tubes (BD, UK) between 07:30–09:30 and stored at −80 °C until analysis. Blood was collected from the patients on the morning of the first ECT treatment and from controls on the assessment day. Whole blood samples for DNA analysis were available at the baseline/pre-ECT timepoint only. DNA was extracted by Trinity College Dublin's Biobank facility using an Autopure LS® (Qiagen), as previously described (Ryan et al., 2018). Purified DNA had an A260/A280 ratio of 1.7–1.9.

2.4. mtDNA assay

The mtDNA assay was performed using the Takara® Human Mitochondrial DNA monitoring primer set (Takara Bio Inc., Sweden) to determine relative mtDNAcn using qRT-PCR per manufacturer's instructions (Soltesz et al., 2019) (see Supplementary Methods for full detail). qRT-PCR was performed using a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, USA). The mtDNAcn was calculated as the difference in Ct values between mtDNA (ND1 and ND5) and nuclear DNA (*SLCO2B1* and *SERPINA1*) using the formula: $2^{-\Delta Ct}$ for the values ΔCt1 and ΔCt2, where ΔCt1 = (Ct for *SLCO2B1*–Ct for ND1) and ΔCt2 = (Ct for *SERPINA1*–Ct for ND5). Four interplate calibrators from healthy controls not included in the study sample were included on each plate to account for inter-run variability. The intra-assay CV was <3% and the inter-assay CV was <1.5%. Samples from participants with co-morbid chronic inflammatory or neurological conditions were excluded from the mtDNA assay.

2.5. Telomere length assay

The telomere length assay was published on previously (Ryan and McLoughlin, 2020). Data from that study were included in analyses performed here.

2.6. Inflammatory marker analysis

IL-6, IL-10, TNF-α, and CRP concentrations were assessed using V-PLEX kits from Meso Scale Diagnostics, LLC, USA as previously described (Ryan and McLoughlin, 2022). Data from that study were included in analyses performed here.

2.7. Statistical analysis

Data were analyzed using SPSS, version 26 (IBM Corporation, NY, USA). Data were tested for normality using the Shapiro-Wilk test and $\log_{10}$ transformed where required. Differences in demographic and clinical characteristics between groups were determined using chi-squared tests or independent *t*-tests, where appropriate. General linear models were used to determine differences between groups. Pearson's or Spearman's correlation tests were used to assess associations between continuous variables. Logistic regression models were used to analyze the association between mtDNAcn and therapeutic outcomes, conducted as follows: (1) unadjusted or (2) adjusted for age, sex, body mass index (BMI), smoking, electrode placement. Data are presented as mean mtDNAcn ± standard deviation (SD). Differences with a *p*-value <0.05 were deemed statistically significant.

3. Results

3.1. Demographics and clinical characteristics

The demographics and clinical characteristics of the participants included in this study can be found in Table 1. Blood DNA samples were available from 110/138 patients from the EFFECT-Dep trial. Ten participant samples were excluded from the final analysis owing to the presence of co-morbid chronic inflammatory or neurological conditions or for the purposes of age and sex matching with healthy controls. Thus, we compared a total of 100 patients with depression to 89 age- and sex-matched controls. The groups differed significantly with respect to BMI, education, and smoking, with the patient group having a higher BMI and more smokers than the control group and achieving a lower level of education overall.

Table 1
Demographic and clinical characteristics of participants.

Characteristic	Depressed ($n = 100$)	Controls ($n = 89$)	Statistical test
Age, years	56.36 (14.28)	53.42 (10.39)	$t = 1.63$, $p = 0.10$
Sex, No. (%)			$\chi2 = 0.38$, $p = 0.54$
Male	38 (38)	30 (33.71)	
Female	62 (62)	59 (66.29)	
BMI, mean (SD)	26.72 (4.98)	25.35 (4.10)	$U = 3588.50$, $p = 0.03$
Smokers, No. (%)	41 (41)	18 (20.22)	$\chi2 = 9.47$, $p = 0.002$
Education, No. (%)			$\chi2 = 50.12$, $p = 1.31 \times 10^{-11}$
Primary	18 (18)	4 (4.49)	
Secondary	56 (56)	16 (17.98)	
Tertiary/Quaternary	26 (26)	69 (77.53)	
Bipolar depression, No. (%)	21 (21)		
Psychotic depression, No. (%)	24 (24)		
Medications, No. (%) taking			
SSRI	22 (22)		
SNRI	50 (50)		
TCA	25 (25)		
MAOI	0 (0)		
Mirtazapine	38 (38)		
Bupropion	2 (2)		
Lithium	38 (38)		
Agomelatine	4 (4)		
Trazodone	4 (4)		
Buspirone	1 (1)		
Anticonvulsant	32 (32)		
Antipsychotics	78 (78)		
Benzodiazepines	60 (60)		
Non-benzodiazepine hypnotics	50 (50)		
Baseline HAM—D24, mean (SD)	30.35 (6.49)	2.85 (2.32)	$U = 0.000$, $p < 0.001$
Post-ECT HAM—D24, mean (SD)	11.28 (8.43)		
Episode duration (weeks), mean (SD)	28.42 (32.76)		
Electrode placement, No. (%)			
Unilateral	52 (52)		
Bitemporal	48 (48)		
Number of ECT sessions, mean (SD)	8.03 (2.51)		
Previous ECT, No. (%)	37 (37)		
Responders, No. (%)	60 (60)		
Remitters, No. (%)	48 (48)		

Data are presented as means with standard deviations (SD) or number (%) per group where appropriate.
Abbreviations: BMI, body mass index; ECT, electroconvulsive therapy; EEG, electroencephalogram; HAM—D24, Hamilton depression rating scale, 24-item version; MAOI, monoamine oxidase inhibitor; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant.

3.2. mtDNAcn in patients with depression and healthy controls

Fig. 1 shows mtDNAcn in patients with depression at baseline/pre-ECT compared to healthy controls. The unadjusted GLM analysis showed a significantly higher mean mtDNAcn in patients with depression (mean ± SD: 86.26 ± 15.65) versus controls (76.42 ± 21.90) ($F(1,187) = 17.50$, $p = 0.000044$). The result remained statistically

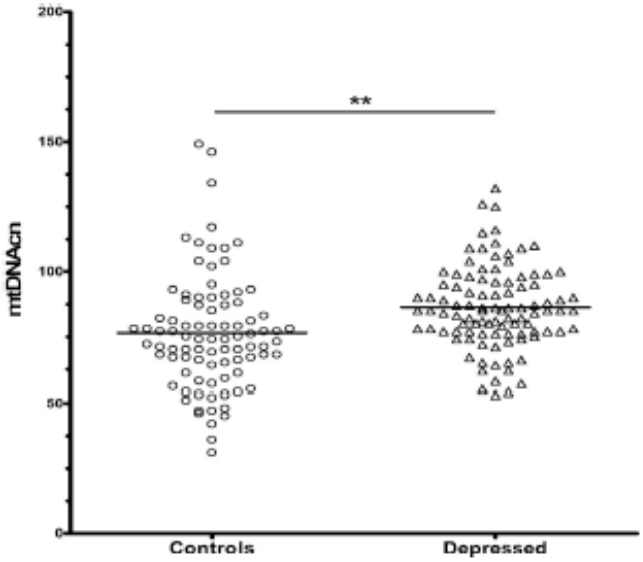

Fig. 1. mtDNAcn is increased in whole blood from patients with depression compared to healthy controls. ** $p < 0.01$.

significant after adjusting the GLM for age, sex, BMI, education, and smoking status ($F(1,180) = 9.92$, $p = 0.002$).

With regard to comparisons between healthy controls and depression subgroups, one-way ANOVA identified a significant difference in mtDNAcn across groups of controls, patients with psychotic depression, and patients with non-psychotic depression, which remained significant after adjusting for covariates (Table 2). Post-hoc analysis revealed a significant difference between patients with non-psychotic depression and controls ($p < 0.001$), but not between patients with psychotic depression compared to non-psychotic depression or controls. One-way ANOVA revealed a significant difference in mtDNAcn across groups of controls, patients with bipolar depression, and patients with unipolar depression, which remained significant after adjusting for covariates (Table 2). Post-hoc analysis identified a significant difference between controls and patients with unipolar depression ($p < 0.001$) but not between patients with bipolar disorder compared to those with unipolar depression or controls.

Table 2
mtDNAcn in controls and depression subgroups.

Group	mtDNAcn (mean ± SD)	ANOVA unadjusted	ANOVA adjusted	Post-hoc
Control	76.42 ± 21.90	$F(2,188) = 10.41$, $p = 0.00005$	$F(2,179) = 6.49$, $p = 0.002$	$p < 0.001$ controls vs. non-psychotic depression
Psychotic depression	80.04 ± 14.22			
Non-psychotic depression	88.22 ± 15.65			
Control	76.42 ± 21.90	$F(2,188) = 8.82$, $p = 0.0002$	$F(2,179) = 4.96$, $p = 0.008$	$p < 0.001$ controls vs. unipolar depression
Unipolar depression	86.78 ± 16.09			
Bipolar depression	84.29 ± 14.04			

3.3. mtDNAcn and ECT

We first assessed mtDNAcn in ECT responders vs. non-responders (see Supplementary Table 1 for demographic and clinical characteristics). No significant difference was noted between the groups (85.82 ± 16.48 vs. 86.93 ± 14.49, respectively; unadjusted GLM: $F(1,98) = 0.21$, $p = 0.65$; adjusted GLM: $F(1,89) = 0.27$, $p = 0.61$). The presence of higher levels of mtDNAcn did not confer increased odds for being an ECT non-responder, as assessed using an unadjusted ($\chi 2 = 0.22$, $p = 0.64$) or fully adjusted ($\chi 2 = 2.35$, $p = 0.13$) logistic regression model.

We next assessed mtDNAcn in ECT remitters vs. non-remitters (see Supplementary Table 2 for demographic and clinical characteristics). No significant difference was noted between the groups (86.52 ± 17.42 vs. 86.02 ± 13.98, respectively; unadjusted GLM: $F(1,98) = 0.001$, $p = 0.98$; adjusted GLM: $F(1,89) = 0.52$, $p = 0.48$). The presence of higher levels of mtDNAcn did not confer increased odds for being an ECT non-remitter, as assessed using an unadjusted ($\chi 2 = 0.001$, $p = 0.98$) or fully adjusted ($\chi 2 = 0.58$, $p = 0.45$) logistic regression model.

mtDNAcn did not significantly differ between patients who had previously been treated with ECT compared to those who had not (84.76 ± 14.75 vs. 87.14 ± 16.20, respectively; unadjusted GLM: $F(1,98) = 0.42$, $p = 0.52$; adjusted GLM: $F(1,88) = 0.00004$, $p = 0.10$).

3.4. Correlations between mtDNAcn and mood score

In the total group of depressed patients mtDNAcn was not associated with HAM-D24 score at baseline ($\rho = 0.06$, $p = 0.54$; Fig. 2a) and there was no correlation between baseline mtDNAcn and the absolute change in HAM-D24 score post-ECT ($r = 0.03$, $p = 0.76$; Fig. 2b). There was a trend towards an association between mtDNAcn and the duration of the current depressive episode in weeks ($\rho = 0.20$, $p = 0.05$).

Exploratory correlation analyses were performed to assess the relationship between mtDNAcn and baseline HAM-D24 scores and the absolute change in HAM-D24 score post-ECT in ECT responders vs. non-responders. No significant correlation was identified (responders: $\rho = 0.12$, $p = 0.36$; non-responders: $r = -0.06$, $p = 0.73$, respectively). There was no association between mtDNAcn and the absolute change in HAM-D24 post-ECT (responders: $\rho = -0.14$, $p = 0.28$; non-responders: $r = 0.14$, $p = 0.39$, respectively).

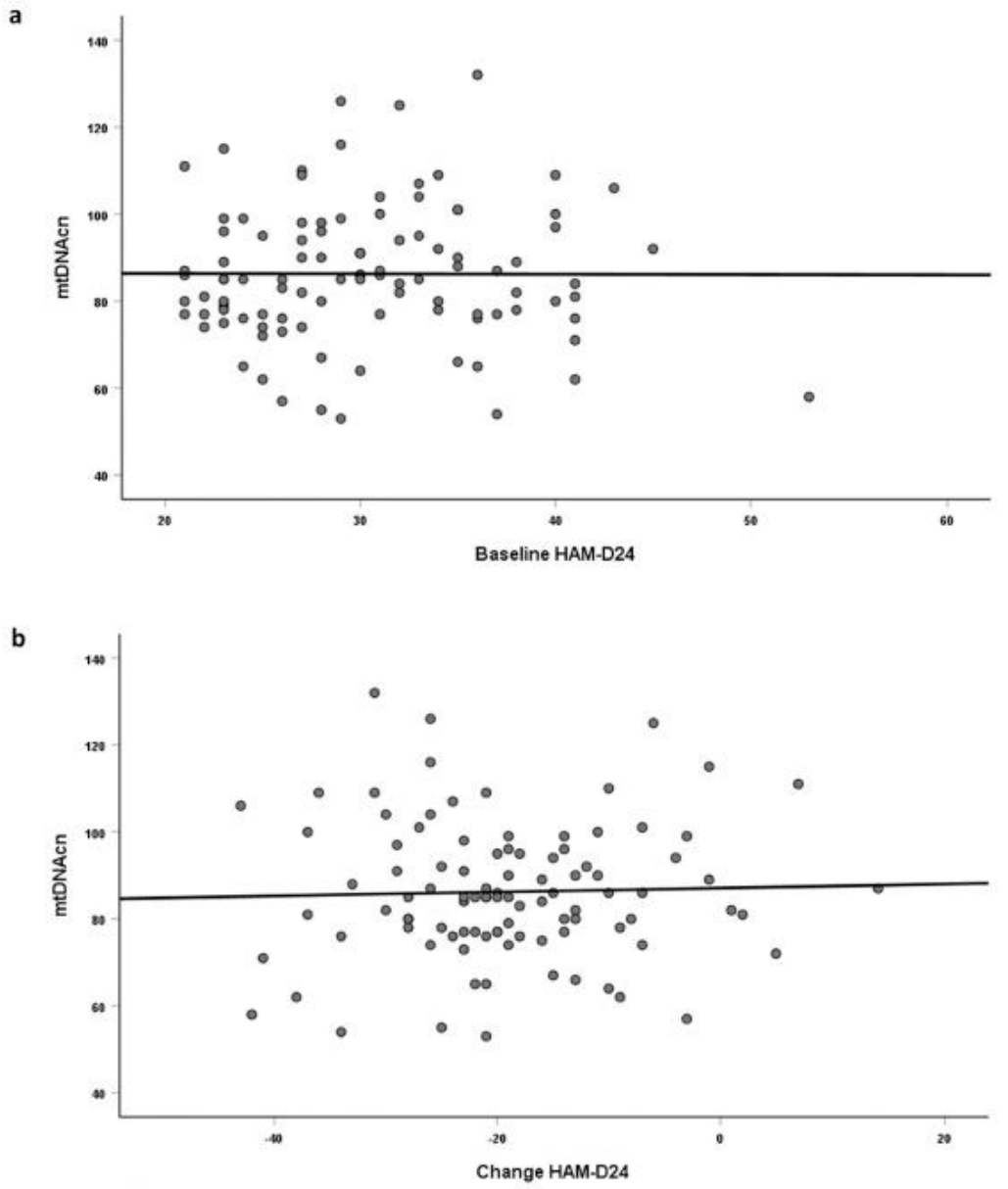

Fig. 2. Relationship between mtDNAcn and mood score in patients with depression.
A. Correlation between baseline mtDNAcn and baseline HAM-D24 score. B. Correlation between baseline mtDNAcn and the absolute change in HAM-D24 score post-ECT.

Exploratory correlation analyses were also performed to assess the relationship between mtDNAcn and baseline HAM-D24 scores and the absolute change in HAM-D24 score post-ECT in remitters vs. non-remitters. No association was identified between mtDNAcn and mood at baseline (remitters: $\rho = 0.20$, $p = 0.17$; non-remitters: $\rho = -0.11$, $p = 0.46$, respectively). There was no association between mtDNAcn and the absolute change in HAM-D24 (remitters: $r = -0.15$, $p = 0.30$; non-remitters: $r = 0.23$, $p = 0.11$, respectively).

3.5. Correlations between mtDNAcn and telomere length

There was no association between mtDNAcn and telomere length in the total group of participants ($\rho = 0.01$, $p = 0.86$) or in the control or depressed groups individually ($\rho = -0.05$, $p = 0.69$; $\rho = 0.13$, $p = 0.24$; respectively).

3.6. Correlations between mtDNAcn and inflammatory markers

We assessed the relationship between mtDNAcn and plasma concentrations of the inflammatory markers IL-6, IL-10, TNF-α, and CRP, which we have previously reported on in this cohort (Ryan and McLoughlin, 2022). No significant correlations were identified (data not shown).

4. Discussion

To our knowledge, this is the first study to report on mtDNAcn in a cohort of deeply-phenotyped severely-depressed inpatients referred for treatment with ECT. The results of this study show that, in line with our hypothesis, mtDNAcn was significantly elevated in whole blood samples from medicated patients with depression compared to controls, even after adjusting for covariates. mtDNAcn did not differ between subgroups of patients with unipolar vs. bipolar depression, psychotic vs. non-psychotic depression, ECT remitters vs. non-remitters, or ECT responders vs. non-responders. Contrary to our second hypothesis, there was no association between mtDNAcn and mood score. Moreover, there was no association between mtDNAcn and the duration of the current depressive episode. There was no correlation between mtDNAcn and telomere length or inflammatory markers either in the entire sample or in groups of controls and patients with depression.

Results from studies analyzing mtDNAcn in depression are contrasting. Our own results are in line with studies showing elevated mtDNAcn in peripheral blood cells in medicated patients with depression compared to controls (Chung et al., 2019), which occurred irrespective of whether the patients had experienced a single episode or recurrent episodes of illness (Chung et al., 2019). Studies assessing the relationship between exposure to adversity and mtDNAcn have shown higher mtDNAcn in those exposed to adversity (Cai et al., 2015a; Tyrka et al., 2016); a limitation of our study is that we did not have adversity scores for this cohort. In contrast, others have reported low mtDNAcn in patients with bipolar disorder compared to controls (Angrand et al., 2021; Wang et al., 2020; Wang et al., 2018), in particular during manic episodes (Angrand et al., 2021), and in medicated patients with unipolar depression compared to controls (Chang et al., 2015; Kim et al., 2011), with antipsychotic use found to negatively associate with mtDNAcn (Chang et al., 2015). On the other hand, some reports indicate no difference in mtDNAcn between unmedicated or medicated depressed subjects compared to controls (Czarny et al., 2020; de Sousa et al., 2014; Fernstrom et al., 2021a; He et al., 2014; Lindqvist et al., 2018), though it has been reported that ccf-mtDNAcn is raised in patients with depression compared to controls despite no change in PBMC mtDNAcn being noted (Lindqvist et al., 2018). However, the ccf-mtDNAcn literature is also conflicting with other reports of lower ccf-mtDNAcn in patients with either a current depressive episode or those in remission compared to controls (Fernstrom et al., 2021b). In contrast to the results from our subgroup analyses, Tsujii et al. (2019) showed a difference in whole blood mtDNAcn in medicated patients with bipolar depression compared to those with unipolar depression, with lower mtDNAcn reported in those with bipolar disorder and higher mtDNAcn in those with unipolar depression compared to controls.

We found no relationship between mtDNAcn and the severity of depressive symptoms. This is in line with some studies that have shown no association between mtDNAcn and mood in patients clinically diagnosed with bipolar disorder (de Sousa et al., 2014) or major depressive disorder (Fernstrom et al., 2021a; He et al., 2014), or in a community sample (Verhoeven et al., 2018); though this contrasts with other studies that have shown a negative relationship between mtDNAcn and mood and psychotic symptoms (Angrand et al., 2021; Chung et al., 2019; Czarny et al., 2020). However, we found a trend towards a positive association between mtDNAcn and the duration of the depressive episode, similar to the result of a previous report showing a significant positive relationship between the duration of the depressive episode and mtDNAcn (Edwards et al., 2016). These results suggest that this molecular change may be a consequence of the disease course in depressed patients.

Differences between our results and those of others may have occurred for a number of reasons (see Picard, 2021). First, there are differences in the primers used and mtDNA and nDNA genes targeted across studies. Second, there are differences in the ethnic origin of participants across studies. Ethnic diversity can influence nDNA and in turn the overall mtDNA:nDNA ratio since some genes can have evolutionary duplications and the number of nDNA repeat elements varies between people. Malik et al. (2011) recommended that validated assays targeted towards single copy nDNA genes be used to assess mtDNAcn. Third, differences in the extraction methodology can influence the mtDNA:nDNA ratio owing to differences in the retention of smaller or larger DNA molecules and the exact chemistries used across techniques. Fourth, differences in the sample types (whole blood, saliva, PBMCs) used across studies may also have contributed to differences in results. Thus, there is a strong need for consistency in methodologies to be implemented across studies of mtDNAcn in order to gain a better understanding of how this might vary in depression. Fifth, previous studies have indicated ethnic differences in risk genes and related pathophysiologies for depression across cohorts of patients of European compared to East-Asian descent (Giannakopoulou et al., 2021). Moreover, a recent meta-analysis of mtDNAcn in patients with bipolar disorder also showed diversity in mtDNAcn when ethnicity was taken into account, with no difference in mtDNAcn noted across all patients compared to controls but lower mtDNAcn identified in patients of Asian descent (Yamaki et al., 2018). Thus, caution is warranted with regard to generalizing findings about mtDNAcn across populations and it is therefore essential that ethnicity be clearly reported and considered in any future studies.

Little is known about the mechanisms by which mtDNA is released into the cytosol or from cells (De Gaetano et al., 2021), or how mtDNA increases in blood. Upregulation of mtDNAcn is thought to reflect compensation for poor mitochondrial function and energetics (Picard, 2021), and might represent a mechanism to overcome mtDNA mutation-induced bioenergetic defects (Filograna et al., 2021). However, while mtDNA serves as the template for the production of proteins that participate in energy production, mtDNAcn does not necessarily directly reflect the energy production capacity of the cell, and cells can lose a large proportion of mtDNA but still be able to maintain normal energy production capacity (Picard, 2021). The interpretation of changes in mtDNAcn is complicated since it can also be influenced by fluctuations in cell types and changes in energy requirements (Picard, 2021). Variations in loci located near the mitochondrial genes *TFAM* (transcription factor A), which regulates mtDNA transcription, and *CDK6* are related to variations in mtDNA amount (Cai et al., 2015b) and might also contribute to the variations in mtDNAcn observed in this study, although we do not have mtDNA genotyping data available for this cohort.

mtDNA contains glucocorticoid response elements; thus, glucocorticoids, which are reported to be elevated in depression (Nandam et al.,

2019), have a role in regulating mtDNA transcription (Demonacos et al., 1995; Ioannou et al., 1988). An in vivo study in mice chronically administered the glucocorticoid corticosterone showed that corticosterone induced an increase in mtDNAcn in saliva and blood, though this study also showed that changes in mtDNAcn may reflect altered metabolic strategies in times of perceived or expected stress and seem to be transitory as they appear to rectify once the stressor is removed (Cai et al., 2015a). Notably, a small study of medication-free suicide attempters with a clinical diagnosis of psychiatric illness ($n = 37$) reported significantly elevated plasma mtDNAcn compared to controls, and, in line with the results from the mouse study, elevated mtDNAcn was moderately associated with impaired negative feedback of the hypothalamic-pituitary-adrenal (HPA)-axis (Lindqvist et al., 2016). Suicide is one of the most serious outcomes of mental illness. Another study of a large cohort of suicide completers ($n = 508$) also found elevated mtDNAcn in peripheral blood, though postmortem analysis in a small subgroup ($n = 20$) showed lower mtDNAcn in the prefrontal cortex compared to controls (Otsuka et al., 2017). These results suggest that the psychological stress associated with suicidal behaviour may leave a biological imprint in the form of leakage of mtDNA into the circulation (Lindqvist et al., 2016). Thus, it is plausible that the increased mtDNAcn apparent in our study may be a transient occurrence owing to leakage of mtDNA into the circulation following stress that transpires in advance of the patients receiving ECT treatment. Therefore, it is important that future studies measure the extent and persistence of changes in mtDNAcn over time.

As mentioned, accumulation of mtDNA in the circulation may be a consequence of cell damage induced by stressors (Picca et al., 2021). For example, neutrophils with mitochondrial damage can extrude mtDNA, which represents a possible alternative mechanism to the removal of mtDNA by mitophagy, though neutrophils can also spontaneously release mtDNA into the extracellular milieu (Caielli et al., 2016), mainly through a cell death pathway known as NETosis (De Gaetano et al., 2021; Delgado-Rizo et al., 2017). Another way in which mtDNA could be released from a cell is following the production of ROS, which can trigger the mitochondrial permeability transition pore to open, altering mitochondrial function and inducing the release of mtDNA into the extracellular space (Ma et al., 2020; Oka et al., 2012; Riley et al., 2018). Lymphocytes (B, T and NK cells), monocytes, and eosinophils have also been found to secrete webs containing mtDNA that prime type I interferon responses (Ingelsson et al., 2018; Yousefi et al., 2008).

With regard to blood studies, mtDNAcn is known to vary across circulating immune cells, with leukocytes having ~150–600 copies per cell depending on the subtype analyzed, e.g., monocytes and neutrophils have about 100–200 copies, T and B lymphocytes have about 45–600 copies (Picard, 2021). As noted earlier, mtDNAcn measured in one tissue does not necessarily reflect mtDNAcn in the entire individual since variance has been observed across different organs, with one study showing no correlation between blood mtDNAcn and that of other tissues (Picard, 2021). Platelets contain on average five mitochondria (i.e., 5 mtDNA copies) but have no nucleus, with each millilitre of blood containing approximately 40 platelets for each leukocyte (Picard, 2021). Thus, platelet count can have a significant impact on mtDNAcn as it uses the nuclear genome as the denominator, and since platelets have no nucleus the mtDNAcn is infinitely large and inflates the overall mtDNAcn in blood (Picard, 2021). A limitation of our study is that we did not conduct full blood count measures at the time of blood sampling and so we are unable to adjust for platelet count.

Few studies have examined the relationship between mtDNAcn and antidepressant treatment outcomes. In one study depressed patients who went on to become selective serotonin reuptake inhibitor (SSRI) responders following eight weeks of treatment were shown to have higher baseline PBMC mtDNAcn and markers of mitochondrial respiratory chain activity (Fernstrom et al., 2021a). However, this contrasts the results of Lindqvist et al. (2018) who showed that ccf-mtDNAcn differed between SSRI responders and non-responders, but there was no difference in PBMC mtDNAcn. Another study showed that six weeks of lithium treatment had no impact on mtDNAcn in patients with bipolar disorder (de Sousa et al., 2014). Our results indicate that there is no relationship between mtDNAcn and clinical outcomes following ECT. It must be reiterated that all of the patients included in our study were receiving psychotropic medication as usual at the time of blood collection and throughout the study. Psychotropic medications are known to impact on mitochondria, and may act to enhance mitochondrial function by reducing oxidative stress, increasing mitochondrial biogenesis, preventing excessive mitochondrial calcium influx, and through antioxidant effects; thus, novel interventions that target mitochondria may be useful as adjunctive therapies for mood disorders (Gimenes-Palomo et al., 2021). However, the data are conflicting since antidepressants, such as fluoxetine, amitriptyline, and citalopram, antipsychotics, such as chlorpromazine, clozapine, and olanzapine, benzodiazepines, and the mood stabilizer lithium have all been documented to induce mitochondrial damage (Neustadt and Pieczenik, 2008). For instance, the SSRI fluoxetine can cross the mitochondrial membrane with ease and affect membrane-bound proteins and induce pro-apoptotic events (de Oliveira, 2016), while the selective noradrenergic reuptake inhibitor (SNRI) venlafaxine increases the expression of mitochondria-related anti-apoptotic and antioxidant genes (Tamasi et al., 2014). Thus, the raised mtDNAcn observed in this study may be owing to the effects of chronic pharmacotherapy.

Mitochondrial quality control (MQC) mechanisms (biogenesis, autophagy, etc.) are in place to safeguard the integrity and function of mitochondria (Picca et al., 2021). Defective MQC has been documented in several age-related conditions that have chronic low-grade inflammation, which might be caused/initiated by the displacement of mtDNA into the extracellular space (Picca et al., 2021). Aberrant methylation of mtDNA on the CpG islands marks this as "non-self" and triggers an inflammatory response (Bae et al., 2019; Caielli et al., 2016). Thus, mtDNA can act as a DAMP and, through binding to pattern recognition receptors such as toll-like receptors (TLRs), can initiate an immune response, primarily an innate immune response, once it enters the cytoplasm or extracellular space (Grazioli and Pugin, 2018). The release of DAMPs, or alarmins, acts as a stress signal for the body (Grazioli and Pugin, 2018). This ties to the idea of sterile inflammation in depression, whereby the low-grade inflammation observed in patients appears to occur in the absence of pathogens or tissue damage (Fleshner et al., 2017). We have previously reported on inflammatory markers in this patient group (Ryan and McLoughlin, 2022). However, there were no strong correlations identified between mtDNAcn and any inflammatory markers measured to date in this cohort.

There are several limitations to this study. First, the study was cross-sectional as bloods were only collected in the EFFECT-Dep trial at a single time-point for DNA analyses. Thus, we were unable to determine the effect of ECT on mtDNAcn. Second, we did not include a full blood count measurement at the exact time-point of blood sample collection; therefore, we were unable to make any correction for platelet abundance or variations in leukocyte subtypes in the samples. Third, as already discussed, we did not have measures of recent or childhood adversity or mitochondrial genotype data. Fourth, we only assessed mtDNAcn and had no measure of mitochondrial function in this study. While mtDNAcn may be indirectly reflective of mitochondrial biogenesis, it does not reflect mitochondrial function (Fernstrom et al., 2021a); thus, further studies are required. Fifth, the controls were recruited over a longer time period than patients. Thus, the duration of time for which some samples were stored may have impacted on the results owing to degradation; however, all samples underwent processing as a single batch to reduce variability and DNA is generally considered a very stable sample type.

5. Conclusion

Our results indicate that mtDNAcn is elevated in patients with depression compared to controls, even after adjusting for potential

covariates. Whether this finding translates to dysfunctional mitochondria as well as the exact mechanism underlying this phenomenon are as yet unknown. Thus, further work is required to clarify the contribution of mitochondria and mtDNA to the pathophysiology of depression and the therapeutic response to antidepressant treatments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2022.110656.

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Data statement

Data are available from the corresponding author upon reasonable request.

Ethical statement

This study received approval from St Patrick's University Hospital Research Ethics Committee and adhered to the Declaration of Helsinki (World Medical Association, 2013). All participants provided written informed consent.

CRediT authorship contribution statement

Karen M. Ryan: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Eimear Doody:** Writing – review & editing. **Declan M. McLoughlin:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

DM has received speaker's honoraria from MECTA, Otsuka, and Janssen and an honorarium from Janssen for participating in an esketamine advisory board meeting. KR and ED have no competing interests to declare.

Data availability

Data will be made available on request.

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Appendix 4 Research Ethics Committee, Study approval

St Patrick's Mental Health Services

PRIVATE & CONFIDENTIAL

April 17th, 2018

Dr Eimear Doody

Research Registrar

St Patrick's Mental Health Services

(eidoody@tcd.ie)

Re: Impact of Eating Disorders on Biological Ageing *(Protocol 09/18)*

Dear Dr Doody,

Your application was considered at the Research Ethics Committee (REC) meeting held on **April 10th, 2018**, in the Conference Room at St Patrick's University Hospital.

Your application was granted full ethical approval by the following committee members who were in attendance at this meeting:

Very Revd Dermot Dunne (CHAIR)

Mr Terence Coghlan	Prof. Jim Lucey
Dr Frank Doyle	Mr Ercus Stewart
Dr Adam Kavanagh	Dr Bré Sullivan
Dr Emer Keeling	Ms Marie Tuohy

Approval was granted subject to the following standard conditions:

1. You must adhere fully to the terms and conditions set out in your research protocol.

2. All persons involved in this research who are not employees of St Patrick's Mental Health Services are required to obtain an honorary contract from the hospital. This process should be initiated by your study supervisor. It will be your responsibility to ensure that these contracts are renewed and kept up-to-date as necessary throughout the duration of your study.

3. If there are any material changes to be made to Protocol 09/18 in the next 12 months, you must contact the Research Ethics Committee for approval.

4. You must report back to the Research Ethics Committee no later than 12 months subsequent to this approval letter (April 17th, 2019), with a summary report on the progress of this research. This report can be downloaded from the hospital website. Failure to complete this report may result in ethical approval being withdrawn for your research.

5. The committee encourages all researchers to publish the results of their study once it is completed, whether these appear to be positive or negative. If your study generates a publication or a poster that is presented, we would ask that a copy be forwarded to us for our records.

6. Please complete the attached form and send it via email to regionallibrary@hse.ie. The Directorate of Clinical and Quality Care in the HSE have requested that details of all research projects in Ireland be pooled together in one location, namely, www.lenus.ie, for the purpose of promoting a culture of research and in order to deliver evidence based clinical care. The HSE hope to gain a complete picture of the quality and quantity of healthcare and healthcare-related research in Ireland, and St. Patrick's Mental Health Services have agreed to cooperate with this process. This repository of research activity is managed by

Health Librarians based in Dr Steevens' Hospital, and we thank you in advance for emailing this relatively simple form to them at your convenience.

In addition, the committee would appreciate a brief response to the following minor issues:

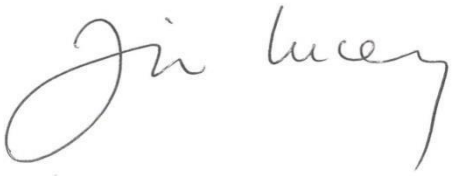
- Is it appropriate to seek fasting blood samples from patients with eating disorders?
- The power of the study is low (70%). Would recruiting more controls enhance the power?
- The application form indicates consultants/GPs will not be informed but a letter to consultants was supplied with the documentation.

Please could we invite you to reply to these queries **on or before May 1, 2018**?

Thank you for your cooperation and we wish you well in your research.

With very best wishes.

Yours sincerely,



JAMES V. LUCEY MD., Ph.D., FRCPI., FRCPsych.

Secretary to the Research Ethics Committee | Medical Director

Medical Council 00646

Encl.

cc. Prof. Declan McLoughlin, Principal Investigator

St Patrick's
Mental Health Services

St Patrick's Mental Health Services – Research Ethics Committee

www.stpatricks.ie

PRIVATE & CONFIDENTIAL

May 16th, 2018

Dr Eimear Doody
Research Registrar
St Patrick's Mental Health Services
(eidoody@tcd.ie)

Re: Impact of Eating Disorders on Biological Ageing *(Protocol 09/18)*

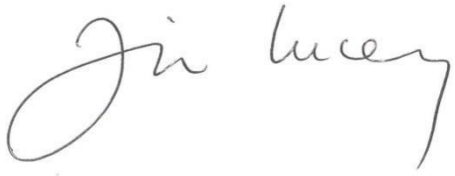
Dear Dr Doody,

Thank you for your email dated May 1st, 2018, in response to the conditions of approval outlined in our letter dated April 17th, 2018.

Your response has been reviewed and we are grateful for your clarifications.

We hope your research is going well and we look forward to receiving reports on its progress in due course.

With very best wishes.
Yours sincerely,



JAMES V. LUCEY MD., Ph.D., FRCPI., FRCPsych.

Secretary to the Research Ethics Committee | Medical Director

Medical Council 00646

cc. Prof. Declan McLoughlin, Principal Investigator

St Patrick's Mental Health Services – Research Ethics Committee

Please reply to: James Braddock, Research Ethics Committee Administrator, St Patrick's Mental Health Services
P.O. Box 136, James's Street, Dublin 8 (Tel: (01) 2493614; email: jbraddock@stpatsmail.com)

St Patrick's
Mental Health Services
Empowering recovery

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Participant Information Leaflet

Impact of Eating Disorders on Biological Ageing

Principal investigator's name:	**Prof. Declan McLoughlin**
Principal investigator's title:	**Research Professor in Psychiatry**
Telephone number of principal investigator:	**01 249 3385**
Data Controller's/joint Controller's Identity:	**1. Prof Declan McLoughlin**
	2. Dr Clare O' Toole
	3. St Patrick's Mental Health Services
	4. Trinity College Dublin
Data Controller's Contact Details:	**01 249 3385**
Data Protection Officer's Identity:	**Mr John Woods**
Data Protection Officer's Contact Details:	**jwoods@stpatsmail.com**

OBJECTIVES OF THE STUDY:

We are currently undertaking a study in St. Patrick's University Hospital looking at the effects of Eating Disorders on telomere length and mitochondrial DNA. Eating Disorders are associated with premature ageing and earlier onset of age-related diseases, including osteoporosis. These adverse outcomes may be mediated by the effects of prolonged dietary restriction on telomere length and mitochondrial DNA.

What are telomeres? These are microscopic molecular caps that protect the ends of your chromosomes – the microscopic structures that contain your DNA and genes. However, as cells in your body divide, the telomere caps get shorter and shorter with each division cycle, ultimately leading to instability of the genome, i.e. the genetic information encoded in your DNA. This shortening process occurs with normal ageing but can be exacerbated by biological and psychological stress.

What is mitochondrial DNA? Mitochondria are structures within cells that convert the energy from food into a form that cells can use. They are involved in energy production in the body. Mitochondria also have a small amount of their own DNA. This genetic material is known as mitochondrial DNA. Mitochondrial DNA is involved in protein synthesis. Mitochondrial dysfunction is proposed to play a role in various psychiatric conditions and may be altered in psychiatric illnesses such as Eating Disorders.

Shortened telomere length and altered mitochondrial DNA has thus been proposed to be a marker of "biological", as opposed to "chronological", age. We do not yet know the effect of Eating Disorders on telomere length nor mitochondrial DNA but expect this to be pronounced.

The main **purpose** of this study is to compare telomere length and mitochondrial DNA between patients with Eating Disorders and a matched group of healthy controls and explore the effect of duration and severity of illness on telomere length and mitochondrial DNA. Using a specialist molecular technique, called Polymerase Chain Reaction (PCR), we can measure the length of telomeres and mitochondrial DNA obtained from the white blood cells that are circulating around your body. These white blood cells can be readily obtained from a small (e.g. 10-20 mls) sample of your blood in the same way you may have previously given a blood sample to your doctor.

WHY HAVE I BEEN CHOSEN?

You have been chosen because you are being treated for an Eating Disorder or you have volunteered to be a control participant in this study.

DO I HAVE TO TAKE PART?

It is your decision whether you take part of not. If you do decide to get involved with the study, you will be given this Information Sheet to keep and asked to sign a Consent Form, a copy of which will also be given to you. You may withdraw from the study at any time you wish and without giving a reason and also at your request, any identifiable data or blood samples will be destroyed.

If you are a patient, this will not affect the care you receive from your doctor or any other person with whom you are involved.

WHAT WILL HAPPEN IF I AGREE TO TAKE PART IN THIS STUDY?

If you agree to help us, a member of our research team will interview you. We will ask you for your permission to review your medical health records (if you are a patient), participate in a brief interview and complete some standardised questionnaires about your dietary restrictions and eating behaviours. Together, this will take about 45-60 minutes and it will be possible to take some breaks. A medical doctor researcher will obtain a small blood sample. After that you are finished, although the study itself will take about 2 years.

WHAT WILL BE THE POSSIBLE BENEFITS OF TAKING PART?

This study will be of no direct benefit to you at this stage, but you may learn some more about Eating Disorders and a bit about Molecular Biology. The results will help us understand better the effects of Eating Disorders on telomere length, mitochondrial DNA and biological ageing. Providing future patients with information at a molecular level about the adverse effects of eating disorders on their bodies will provide them with more information to facilitate making important healthcare decisions.

WHAT ARE THE POSSIBLE DISADVANTAGES OF TAKING PART?

A possible disadvantage to you is the time it takes to be interviewed, completing questionnaires and assessments over about 60 minutes. To minimise any discomfort, multiple breaks can be taken.

Usually giving a blood sample is straightforward but some people find the procedure too uncomfortable and others may develop a bruise at the needle insertion site. To minimise these possibilities, blood samples will be collected by a trained medical doctor. Some people have a strong dislike of needles and giving blood and we will enquire about this early on as this study may not be suitable for you to join.

You can withdraw from the study at any time point you wish without any effect upon your treatment.

WILL MY TAKING PART BE CONFIDENTIAL?

Yes. All information collected about you, during the course of the research study, will be kept strictly confidential and will only be accessed by members of the research team. Your name will not be attached to any information about you that leaves the hospital. Any personal information you give us will be confidential and stored in a safe research facility.

Your data will initially be coded to protect your anonymity and when the study is completed all the data will be irreversibly anonymised. At a later stage, these irreversibly anonymised data may form part of a collaborative study with other researchers in Ireland and abroad but at that stage it will not be possible to link the data to any individual.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results will be published in scientific journals and presented at conferences, again without any breach of confidentiality.

LEGAL ISSUES

The researchers involved in this study are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

PERMISSION

This study has been approved by the St. Patrick's University Hospital Research Ethics Committee (protocol number 09/18).

Appendix 6 Participant consent form

Consent Form

Impact of Eating Disorders on Biological Ageing

Study Site: St Patrick's University Hospital, James's St, Dublin 8 and Trinity College Dublin

St Patrick's Research Ethics Committee Protocol Number: 09-18

Principal Investigator: Prof Declan McLoughlin

Telephone: 01 2493385 Email: d.mcloughlin@tcd.ie

PLEASE READ THROUGH EACH ITEM CAREFULLY AND INDICATE YOUR RESPONSE BY TICKING THE APPROPRIATE BOX

I have read and understood the **Information Leaflet** about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that I don't have to take part in this study and that I can opt out at any time. I understand that I don't have to give a reason for opting out and I understand that opting out won't affect my future medical care.	**Yes** ☐	**No** ☐
I am aware of the potential risks, benefits and alternatives of this research study.	**Yes** ☐	**No** ☐
I give permission for researchers to look at my medical records to get information. I have been assured that information about me will be kept private and confidential.	**Yes** ☐	**No** ☐
I have been given a copy of the Information Leaflet and this completed consent form for my records.	**Yes** ☐	**No** ☐
I consent to take part in this research study having been fully informed of the risks, benefits and alternatives.	**Yes** ☐	**No** ☐
I give informed explicit consent to have my data processed as part of this research study.	**Yes** ☐	**No** ☐

I consent to be contacted by researchers as part of this research study.	**Yes** ☐	**No** ☐

	FUTURE CONTACT [please choose one or more as you see fit]		
	OPTION 1: I consent to be re-contacted by researchers about possible future research **related** to the current study for which I may be eligible.	**Yes** ☐	**No** ☐
	OPTION 2: I consent to be re-contacted by researchers about possible future research **unrelated** to the current study for which I may be eligible.	**Yes** ☐	**No** ☐

STORAGE AND FUTURE USE OF INFORMATION		
RETENTION OF RESEARCH MATERIAL IN THE FUTURE [choose one or more as appropriate]		
OPTION 1: I give permission for material/data to be stored for *possible future research **related*** to the current study ***only if consent is obtained*** at the time of the future research but only if the research is approved by a Research Ethics Committee.	Yes ☐	No ☐
OPTION 2: I give permission for material/data to be stored for *possible future research **related*** to the current study ***without further consent being required*** but only if the research is approved by a Research Ethics Committee.	Yes ☐	No ☐
OPTION 3: I give permission for material/data to be stored for *possible future research **unrelated*** to the current study ***only if consent is obtained*** at the time of the future research but only if the research is approved by a Research Ethics Committee.	Yes ☐	No ☐
OPTION 4: I give permission for material/data to be stored for *possible future research **unrelated*** to the current study ***without further consent*** being required but only if the research is approved by a Research Ethics Committee.	Yes ☐	No ☐
OPTION 5: I agree that some future research projects may be carried out by researchers working for **commercial/pharmaceutical companies.**	Yes ☐	No ☐
OPTION 6: I understand **I will not be entitled to a share of any profits** that may arise from the future use of my material/data or products derived from it.	Yes ☐	No ☐

Patient/Participant Name (Block Capitals) | Patient/Participant Signature | Date

---------- ---------- --------

Translator Name (Block Capitals) Translator Signature Date

---------- ---------- ---

Legal Representative/Guardian Name Legal Representative/Guardian Signature Date

To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

Name (Block Capitals)	Qualifications	Signature	Date

Appendix 7 Information letter for participants' GP and Consultant

XX/XX/2018

Dear Dr XXXXX,

Re: *Impact of Eating Disorders on Biological Ageing*

This is to inform you that your patient XXXX YYYYY has consented to participate in the above study in St. Patrick's University Hospital on the effects of Eating Disorders on telomere length and mitochondrial DNA. Shorter telomere length and mitochondrial DNA alterations may be a mechanism for premature ageing and earlier onset of age-related diseases seen in Eating Disorders. The attached Participant Information Sheet will provide you with more information about this case control study that has been approved by the SPMHS Research Ethics Committee (protocol number 09/18).

The study involves a review of clinical notes and the use of standardised assessments about dietary restrictions and eating behaviours. The interview will be led by a trained researcher (Dr Eimear Doody) and should take about 45-60 minutes. Patients will also be asked to provide a blood sample, after which their active participation will be finished. All data collected with be anonymised and confidential.

If you have any questions or need further clarification on details of the study please do not hesitate to contact us. Thank you for supporting this research study.

Yours sincerely,

_____

Dr Eimear Doody
MCRN 408902
Research Registrar

Healthy Volunteer Needed for Study on Eating Disorders

St. Patrick's Mental Health Services

About St. Patrick's Mental Health Services
St. Patrick's Mental Health Services is Ireland's largest, independent, not-for-profit mental health service and aspires to be the recognised leader in:
1. the provision of quality mental health care
2. the promotion of mental health awareness
3. the protection of the rights and integrity of those experiencing mental health difficulties

What's Involved
We are a multidisciplinary team of researchers based at St Patrick's University Hospital and Trinity College Institute of Neuroscience. Our research aims to better our understanding of eating disorders and the impact that these disorders have on the ageing process.
We are currently looking for healthy volunteers to help us with our study: **Impact of Eating Disorders on Biological Ageing**. In this study, healthy volunteers will act as controls; a comparison group to the participants with a diagnosis of an eating disorder.

We are looking for volunteers who are:
(1) Aged 18+
(2) BMI between 18.5 - 25.0
(3) Willing to provide a blood sample
(4) Have no history of psychiatric illness including substance dependence.

Healthy volunteers will be asked to attend St Patrick's Mental Health Services to meet with a doctor on the research team and provide a fasting blood sample. Participants will then be asked to complete a clinical assessment with the doctor. The entire process is expected to last no more than 90 minutes.

Every attempt will be made to ensure that meetings are scheduled to suit the needs of the participants and reimbursements for travel costs will be made available.
We hope that this study will help clarify the biological effects of eating disorders upon the body with regards to the ageing process.

What's required for this position
We are looking for volunteers who are:
(1) Aged 18+
(2) BMI between 18.5 - 25.0

(3) Willing to provide a blood sample
(4) Have no history of psychiatric illness including substance dependence.

- Fitness/ Health/ Beauty
- Research/ Journalism/ Media

Level of English required for this role:
Fluent English

Times and commitment required for this role

	Mon	Tue	Wed	Thu	Fri	Sat	Sun
Morning	✓	✓	✓	✓			
Afternoon							
Evening							

Minimum time commitment: Once Off
Hours required: 1hrs Once off

Other Details
Location of volunteering opportunity:
St Patrick's Mental Health Services, James' Street, Dublin 8 K7YW

Also available in these counties
Dublin - City, Dublin - Dun Laoghaire/ Rathdown, Dublin - Fingal, Dublin - South Dublin County, Kildare, Louth, Meath, Wicklow

Screening details for this role:

- Formal Interview

Volunteers will be provided with the following supports:

- Reimbursement of expenses

Age restrictions:
Over 18

Is this opportunity suitable for groups/teams of volunteers?
No

Benefits you will receive as a volunteer

The information gathered during this study will contribute to scientific knowledge on the ageing process and inform further research. Knowledge gained may be useful in clarifying the biological effects of Eating Disorders upon the body.

Getting there
The following transport options are within a 5-minute walk from the hospital:
1. Rail: Heuston Station
2. Luas: Red Line Heuston Station stop
3. Bus: 13 and 123 to James' Street or 25A, 25B, 66X, 67X, 79, 79A to Heuston station

Apply Now Click the apply now button to register your interest in this opportunity.

- Tweet
- **This opportunity is posted by:**
- **Volunteer Centre:** Dublin City

← **New Search**
Bottom of Form

Impact of Eating Disorders on Biological Ageing

We are currently undertaking a study in St. Patrick's University Hospital and Trinity College Dublin looking at the effects of Eating Disorders on the ageing process.

Eating Disorders are associated with premature ageing and earlier onset of age-related diseases, including osteoporosis.

Molecules in the bloodstream may be used as markers of premature ageing. We do not yet know the effect of Eating Disorders on these molecules but expect this to be pronounced.

To express an interest in this study, please contact:

Dr Eimear Doody

Tel: 01 249 3376

Email: edoody@stpatsmail.com

Approved by St Patrick's Research Ethics Committee Protocol Number 09/18

St Patrick's Mental Health Services
Empowering recovery

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Impact of Eating Disorders on Biological Ageing

Participant Demographics Sheet

Participant Protocol Number: _____
Date Completed: _____
Completed By: _____

Participant Details

- Age at time of assessment: _____
- Gender:
  - Male / Female
- Education Years: _____
- Education Level:
  - Primary / Secondary / Tertiary / Quaternary
- Marital Status:
  - Single
  - Married / Co-habiting
  - Divorced / Separated
  - Widowed
- Ethnicity:
  - Irish
  - Irish Traveller
  - African
  - Chinese
  - Any other white background
  - Any other black background
  - Any other Asian background
- Occupational Status:
  - Employed
  - Unemployed
  - Student
  - Retired
  - Other
- Occupation: _____

- SES:
    1. Professional
    2. Managerial
    3. Skilled Non-Manual
    4. Partly Skilled
    5. Unskilled

Current Treatment Details

Status:

- Inpatient
- Outpatient
- Day Patient

Medications:

Name	Dose	Frequency

Other therapies: _____
_____
_____

BMI on admission: _____

- Weight: _____ kg
- Height: _____ cm
- Date: _____

BMI on discharge: _____

- Weight: _____ kg
- Height: _____ cm
- Date: _____

Date of admission: _____
Date of discharge: _____
Total Length of stay: _____

Patient History

- Age of Eating Disorder (ED) Onset: _____
- Age of first attendance to ED specialist care service: _____
- Number of hospitalisations for treatment of Eating Disorder: _____
- Number of hospitalisations for medical treatment secondary to Eating Disorder: _____

- Other psychiatric history: _____
  _____
- Other medical history: _____
  _____
- Family history of mental illness: _____
  _____
  _____
- Units of Alcohol / week: _____
- History of alcohol abuse?
  - No
  - Yes
    If yes, duration of abstinence: _____
- History of substance abuse?
  - No
  - Yes
    If yes, duration of abstinence: _____
- Smoker:
  - No
  - Yes
    If yes, number of cigarettes per day? _____