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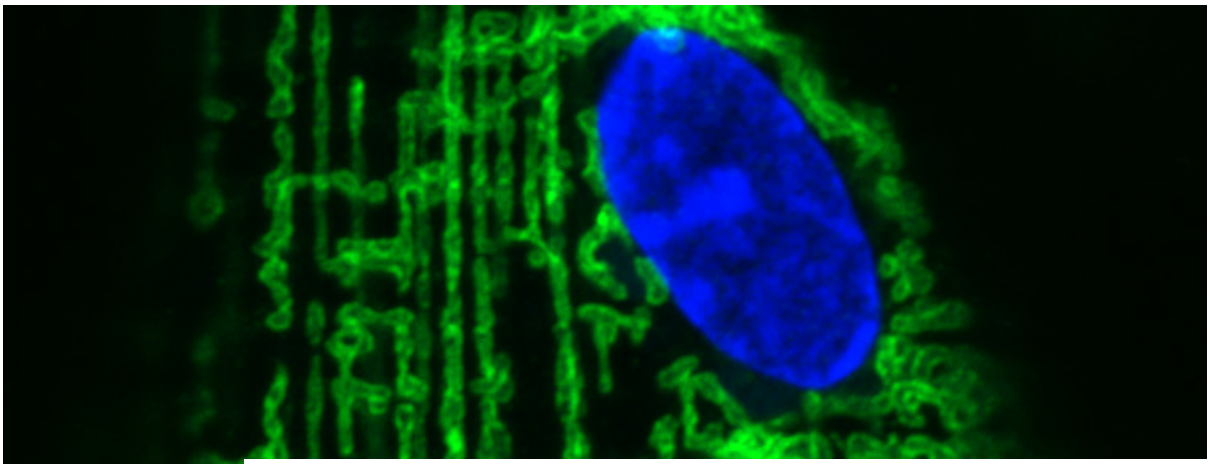

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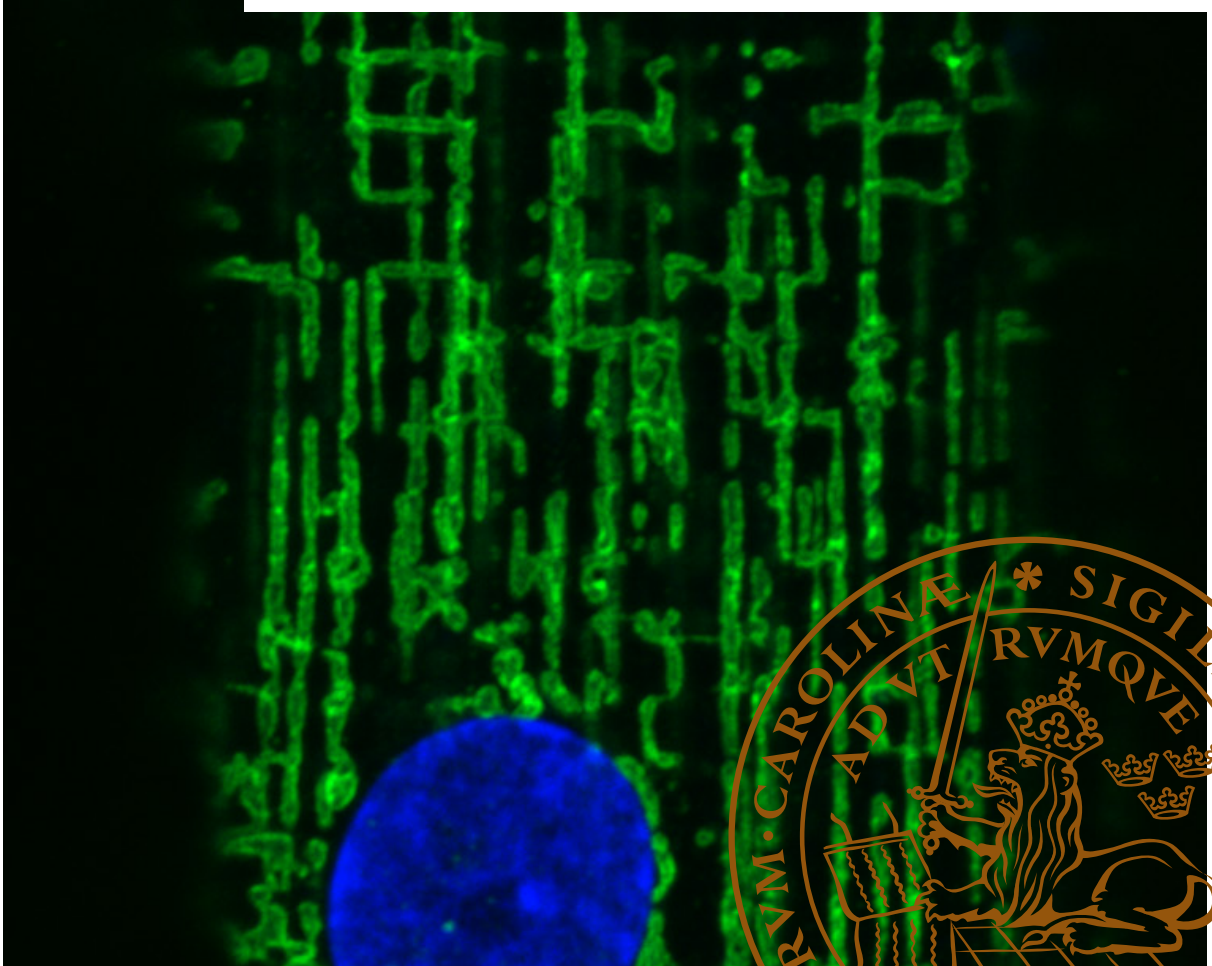

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Depression and suicidal behavior are the unfortunate result of a myriad of interactions between the affected individual and its environment, often over the course of many decades. Several lines of evidence link depression and suicidal behavior to cortisol stress signaling, inflammation, monoamine signaling, neuronal genesis and senescence. These are all processes that demand energy, created in the inner membrane of mitochondria. In this thesis, we investigate the role of cellular stress and autoimmunity, with a specific focus on mitochondria, in depression and suicidal behavior. Autoimmunity, and certain biomarkers of cellular stress and mitochondrial function are examined in several well defined, clinical cohorts of depressed and suicidal individuals and compared to healthy controls.

In a pilot study including recent suicide attempters, we found no evidence for a link between autoimmune encephalopathies and suicidal behavior. In subsquent studies we found several interesting associations between mitochondrial biomarkers, depression and suicidal behavior: i) Levels of cell-free mitochondrial DNA (cf-mtDNA, a marker of cellular stress) were significantly higher in a group of recent suicide attempters compared to controls and, ii) clinically depressed, non-suicidal, individuals also had elevated cf-mtDNA compared to controls, and cf-mtDNA levels were associated with SSRI treatment response. However, the number of mitochondrial DNA copies present within leukocytes (mtDNA-cn, an indirect marker of cellular energetics) were not significantly different between patients and controls. iii) We did not find a signficant difference in a composite index of mitochondrial respiratory chain enzymatic activity (MHI) between depressed individuals and controls, although some of the enzymes included in the index were associated with antidepressant treatment response among the depressed individuals. Finally, iv) while there was an overall decrease in cf-mtDNA in a large cohort of depressed individuals compared to controls, increased cf-mtDNA was associated with specific depressive symptoms.

In conclusion, our studies of biomarkers of mitochondrial function and cellular stress provide novel clues into the pathophysiological mechanisms of depression and suicidal behavior. Future studies are needed to validate these findings and to determine their clinical relevance.

Key words: Depression, suicidal behaviour, mitochondria, mitochondrial DNA, HPA-axis, inflammation

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To Jessica and the kids,
and to my parents.

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Abbreviations

BBB	Blood-Brain Barrier
CPRS	Comprehensive Psychopathological Rating Scale
CSF	Cerebrospinal fluid
Cf-mtDNA	Cell-free mitochondrial DNA
Ccf-mtDNA	Circulating cell-free mitochondrial DNA
DAMPs	Damage Associated Molecular Patterns
DSM	Diagnostic and Statistical Manual for mental disorders
DST	Dexamethasone Suppression Test
HDRS	Hamilton Depression Rating Scale
HPA-axis	Hypothalamus-Pituitary-Adrenal-axis
MADRS	Montgomery-Åsberg Depression Rating Scale
MDD	Major Depressive Disorder
MRI	Magnetic Resonance Imaging
mtDNA	Mitochondrial DNA
mtDNAcn	Mitochondrial DNA copy number
OXPHOR	Oxidative Phosphorylation
PRRs	Pattern Recognition Receptors
PSS	Perceived Stress Scale
RC	Respiratory Chain
SUAS	Suicide Assessment Scale
qPCR	Quantitative Polymerase Chain Reaction

Definitions

Ccf/cf-mtDNA
Circulating cell-free/cell-free mitochondrial DNA are sometimes used interchangeably in this thesis. Ccf-mtDNA is the preferred abbreviation for cell-free mtDNA *circulating* in plasma/serum, whereas cf-mtDNA refers to cell-free mtDNA in any biofluid(1).

Haplogroup
A population sharing certain mtDNA single nucleotide polymorphism variations, mutations that occurred for more than 150 000 years ago, and which correlate to the geographic origin of the population.

Insufficient treatment response
Not having achieved remission with previous and ongoing treatments during the current depressive episode.

mtDNA-cn
Mitochondrial DNA-copy number, refers to the number of mtDNA copies *per tissue cell*(2).

OXPHOS/Oxidative phosphorylation
A series of biochemical reactions occurring within the mitochondrion, whereby breathed oxygen, together with ingested calories, are utilized to synthesize ATP.

Plasma
The liquid fraction of whole blood, obtained from anti-coagulated blood.

Serum
The liquid fraction of blood obtained after clotting.

Suicidality
Contemplations, wishes, and preoccupations with death and suicide. Also referred to as suicidal thoughts or ideas.

Suicide
The act of intentionally taking one's own life.

Suicide attempt
A self-inflicted, potentially injurious behavior with a nonfatal outcome for which there is evidence (either explicit or implicit) of intent to die(3).

Introduction

Heart disease used to be the biggest killer, particularly for men in their 40s. Today, we've seen a 63-percent reduction in mortality from heart disease -- remarkably, 1.1 million deaths averted every year…

If we waited until the heart attack, we would be sacrificing 1.1 million lives every year in this country to heart disease. That is precisely what we do today when we decide that everybody with one of these brain disorders, brain circuit disorders, has a behavioral disorder. We wait until the behavior becomes manifest. That's not early detection. That's not early intervention.

-Tom Insel, former president of NIMH, at TEDxCaltech, January 2013

Scope of the problem: Major Depressive Disorder and Suicidality

Major depressive disorder (MDD) is one of the leading causes of disability in the world, affecting approximately 300 million people, according to the World Health Organization (WHO)(4). Even though this figure is too large to fully comprehend, it is most likely an underestimation of the real number. Reports of MDD incidence rates are deemed reliable only in about one third of the WHO countries(4). In general, psychiatric disorders, of which MDD is only one (albeit the most common), are underreported. The possible reasons for this underestimation include social stigma, lack of appropriate healthcare facilities, recall bias, diagnostic inconsistencies and disagreement on how to measure psychiatric disorders cross-culturally(5). In fact, the total number of individuals suffering from any psychiatric disorder, if substance use disorders are included, is likely close to 1 out of 7 (6). Or if presented as an actual number, more than 1 000 000 000 people. Considering this staggering figure, the incidence of suicides globally seems surprisingly low at approximately 700 000 individuals each year(7). However, this figure is largely based on data where the cause of death has been determined to be suicide, not adequately taking into consideration the many accidents, overdoses, or disappearances (or underreporting due to the same reasons as concerning MDD mentioned above) where suicide is the actual cause. Thus, 700 000 people is almost certainly a “best case” estimate. In Sweden, the number of people who died from suicide in 2020 was 1588 (although this number do take into account incidents

where suicidal intent was deemed probable but not certain)(8). Among people between 15-29 years, it is the second most common cause of death (following traffic accidents), a fact made even gloomier by the knowledge that many of these deaths might have been prevented. Between 30-50% of the individuals who commit suicide in Sweden have an ongoing contact with the psychiatric care, and an even larger proportion with a primary care facility(9, 10). This should provide an opportunity for intervention. However, compared to suicide attempts, completed suicide is a rare outcome and thus hard to predict. The current "gold standard" of predicting suicide rely almost solely on the assessment of a trained clinician, sometimes augmented by a risk assessment tool, i.e., a rating scale.

The use of biomarkers in predicting outcome of depression and suicide

There are currently no biomarkers of any kind that can aid the clinician in making more reliable suicide risk assessments, although some have suggested a biological predictive model for suicidal behaviour(11, 12). In fact, the very question whether biomarkers have a role in such predictions is somewhat controversial(13). The same is true for how psychiatric disorders are diagnosed. In the most recent version of the Diagnostic and Statistical manual of mental disorders (DSM-5), no biomarkers of any kind are suggested as an aid to distinguish between disorders, or even to delineate between behaviour that is considered normal, and psychopathology. This does not mean, however, that there are no biological mechanisms relevant to the understanding and treatment of psychiatric disorders, or even that they are completely unknown. On the contrary, an overwhelming number of studies quite consistently show that psychiatric disorders, MDD in particular, in many cases can be related to alterations in neurochemical systems, such as: Regulation of monoamine synthesis, transmission and metabolism, the Hypothalamus-Pituitary-Adrenal- (HPA)axis, the immune system, intracellular signalling and adaptation of a wide range of specialized cellular functions(12, 14-18). These alterations can be captured and described by measuring certain biomarkers representative of the system of interest, throughout the body. Not only are psychiatric disorders brain disorders, but rather *systemic disorders*, affecting the entire body.

What importance can mitochondria have for psychiatric disorders?

Stress is in many ways central to most, if not all, psychiatric disorders. In the popular meaning of the word stress, it is the subjective experience of inadequacy to meet demands posed on the individual by the environment, most often in a social context.

In a biological sense, stress refers to the activation of numerous systems involved in neuronal, endocrinological and immunological signalling cascades, both intra- and extracellularly. The many functions of the mitochondrion places it at a central junction, through which many of these biological systems interact. It serves both as a production site of many molecules of importance for biological signalling, and as a target for such molecules(19). Indeed, many of the processes related to psychiatric disorders also directly involve mitochondria. The following sections aim to give the reader a brief overview of the biological relationship between mitochondria and psychiatric disorders, focusing on major depression and suicidality.

An introduction to mitochondria

One could argue that mitochondria are the reason we breathe. They are present in all tissue and cell types, with the sole exception of red blood cells (as the main function of red blood cells is to carry oxygen to organs, it would be contra-productive if the oxygen were consumed during the transport). Throughout our entire body, mitochondria utilize oxygen to produce energy in a tightly controlled and highly coordinated cooperation between enzymes of the Electron Transport Chain (ETC). Without mitochondria, no higher life form than bacteria could exist. They stand out among other subcellular organelles by the fact that they carry their own genome, separate from that contained in the cell nucleus. The details of how mitochondria came to be a part of the eukaryotic cell have been long debated, although all major theories are in favour of an "endosymbiotic origin". The endosymbiotic theory poses that mitochondria, residing within either an aerobic prokaryotic, or archaic ancestor of the eukaryotic cell, were incorporated by a prokaryote to their mutual benefit. The larger anaerobic prokaryote could provide nutrients to the smaller aerobic cell, which in turn provided useful energy compounds for the host cell to utilize. Over the course of evolution, the smaller cell lost some of its genes required to survive outside the host cell, and instead came to rely on the hosts genome for encoding proteins involved in, for example, self-repair(20).

Mitochondria are in textbooks often depicted as separate, oval, and inert two-dimensional structures, randomly floating around the cell cytoplasm (Figure 1). *In vivo*, however, they are highly plastic and versatile. They are almost constantly changing form, fusing and diffusing from other mitochondria in intricate networks (Figure 2). They can be both dynamic and static, moving long distances along polarized cells such as neurons when needed, or staying put in one place where the demand for energy is high.

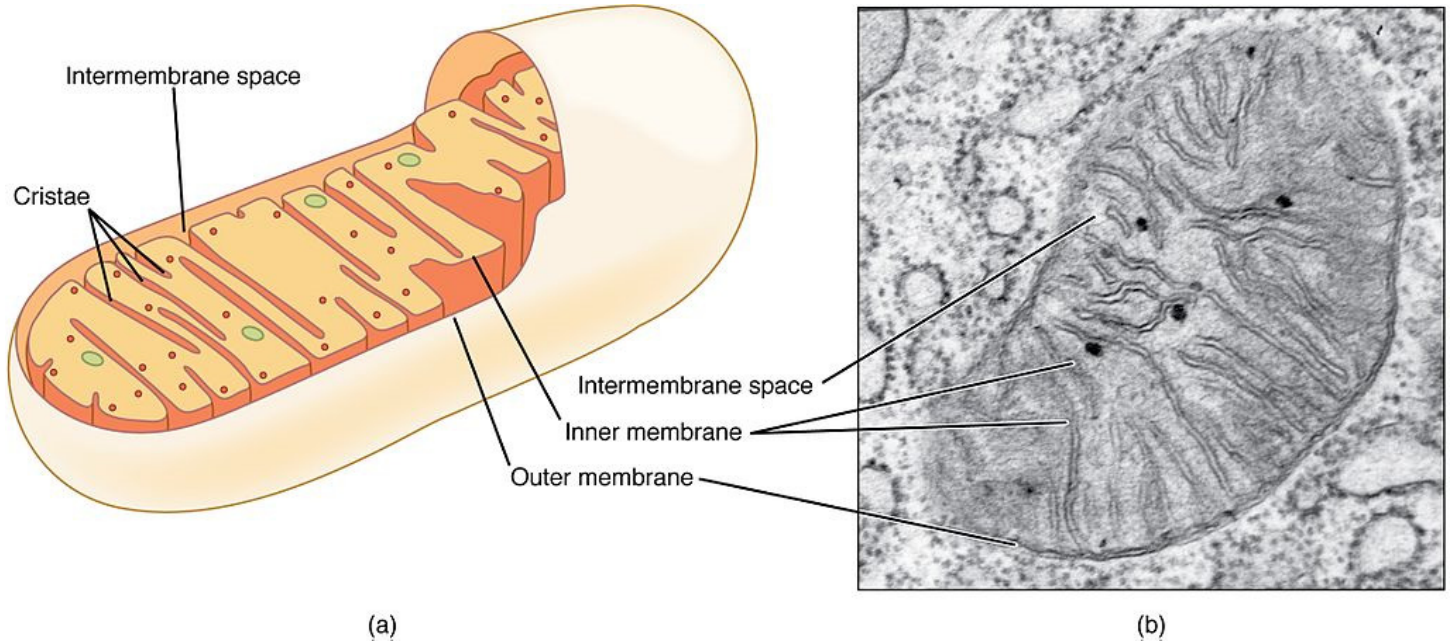

Figure 1. Structure of the mitochondrion. A) Simplified depiction of membranes B) Electron-microscopy image of a mitochondrion. 0315 Mitochondrion new.jpg by CFCF is licensed by Creative Commons Attribution 4.0 International license.

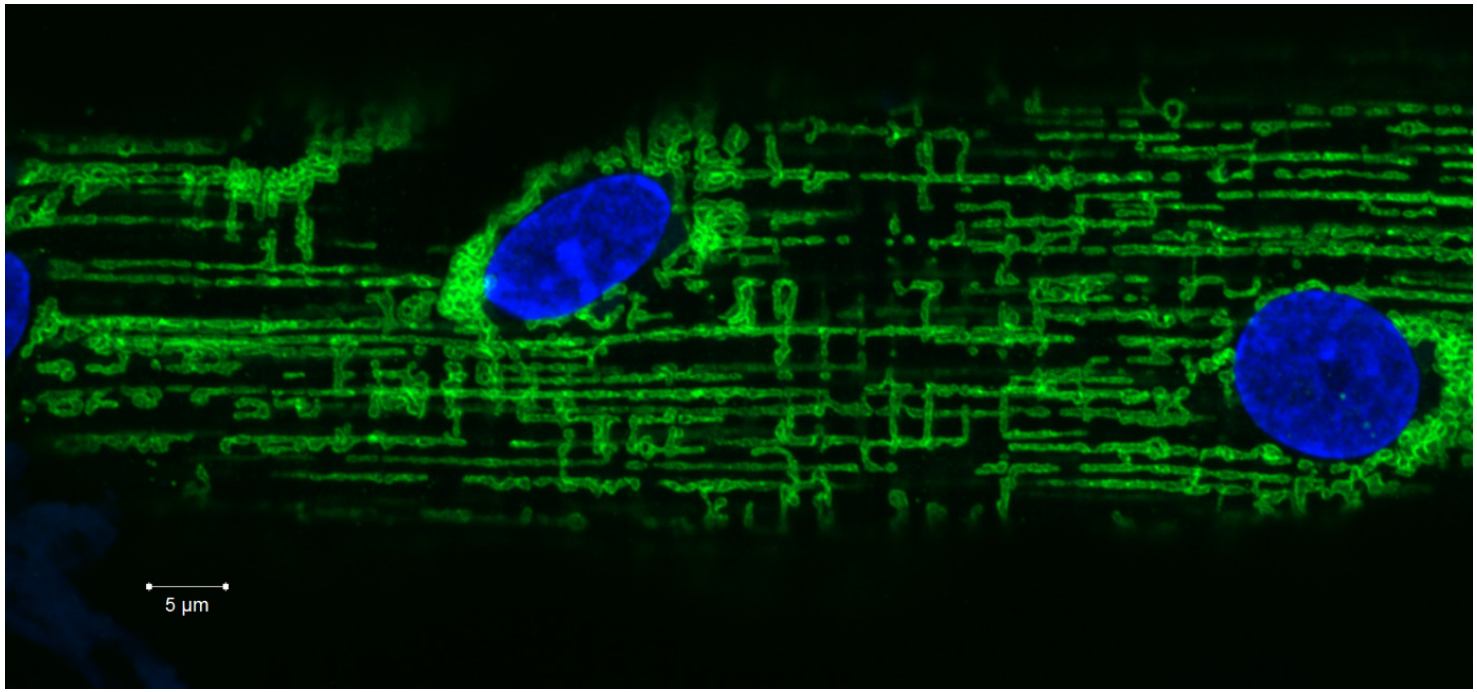

Figure 2. The figure depicts immunostaining of mitochondrial networks in human single muscle fibers (vastus lateralis). Mitochondria are stained green, cell nucleus in blue(21). Reproduced with permission from Dr. Clara Prats Gavalda.

Function of the Mitochondrion

The main purpose of the mitochondrion is to synthesize adenosine triphosphate (ATP), the molecule used throughout the body for all types of energy-demanding work. But mitochondria are also involved in a range of different processes of importance for cell homeostasis and intra- and intercellular signalling. ATP production takes place in the mitochondrion's inner membrane where electrons are passed along protein complexes. This process is called the electron transport chain (ETC). Consequently, an energy potential is maintained over the inner membrane and ultimately harnessed to yield ATP. As a by-product, highly reactive oxygen species (ROS) are produced, capable of damaging surrounding protein structures, including both nuclear and mitochondrial DNA. This eventually leads to an accumulation of DNA mutations, cellular damage and ultimately cell senescence(20). Aside from ATP production, another function of mitochondria is the storage of calcium within the mitochondrial matrix, allowing for the rapid release of calcium ions necessary for coordinated neurotransmitter and hormonal release. Other important roles of the mitochondrion, to name a few, include apoptosis, synthesis of steroid hormones, immune signalling, regulation of gene expression, cell differentiation (20).

As energy demand is constantly fluctuating in many tissue and cell types, mitochondria most be ready to increase energy production quickly (and conversely decrease production when energy demand is low). This adaptation occurs by two principal mechanisms: i) Through a change in energy production capacity, i.e., by a change in the *activity* of the enzymatic complexes in the mitochondrial inner membrane involved in energy production, and ii) through increasing the mitochondrial *content*, i.e., the number of mitochondria within a cell. These different mechanisms give a separate perspective on mitochondrial energy output(22). Much like a battery in an electrical car: A car with a bigger and fully charged battery can go a longer distance than a car with a small, uncharged battery.

The Mitochondrial Genome

Mitochondria carries its own genome in the shape of a circular structure containing approximately 16 600 base pairs. Mitochondrial DNA (mtDNA) does not by itself code for all proteins necessary for cellular energy production, but rely on a close correspondence with the cell nucleus and the proteins encoded by the more complex nuclear DNA (nDNA) molecule, in order to maintain proper function. The entire mitochondrial genome encodes 13 functional proteins in total, all components of the ETC. In comparison, nDNA encodes more than 1000 proteins involved in mitochondrial processes. Compared to nDNA, mtDNA is a relatively simple molecule (the number of mtDNA base pairs comprises about 0.001% of nDNA base

pairs). As a consequence, mitochondria lacks its own self-reparatory mechanisms, making mtDNA prone to mutation (20).

Maternal inheritance of mtDNA

In animals and plants, mtDNA are maternally inherited. Since mitochondria are present in the cell cytosol, the number of mtDNA copies transferred to the offspring are dependent on the volume of cytosol donated to the zygote from the oocyte and the sperm cell, respectively. In animal cells, the cytosol from the oocyte is many times larger, thus contributing to more than 99.999% of the mtDNA present in the zygote. Additionally, sperm cell mtDNA are actively recognized and degraded in the egg cell (20). The uniparental inheritance of mtDNA makes it possible to determine haplogroup inherency, which correlates to an individual's geographical origin.

Intra- and extracellular mtDNA

Numerous approaches to measure mitochondrial function have been proposed, and today several well defined and validated methods are in use. Good examples are respirometry, measuring the rate of oxygen consumption, or enzymatic capacity (23) (described in section "Mitochondrial respiratory chain assessment" on p. 38). However, most methods require live tissue samples, which comes with logistical obstacles that are hard to overcome in a clinical setting. MtDNA quantification have become a popular alternative to such methods, as it is a stable, easily quantified molecule (24). As briefly described on p. 21, intracellular mtDNA conveys information of the energy-producing capacity of the cells in the sample, and is typically quantified as the number of copies of the mitochondrial genome within each nucleated cell, the mtDNA *copy number* (mtDNA-cn)(25). Most often, the cell is a Peripheral Blood Mononuclear cell (PBMC), as this cell type is easily isolated and carries one copy of the nuclear genome (whereas platelets contain no nucleus and red blood cells no mitochondria). By simultaneously measuring the number of mtDNA molecules and nDNA molecules, and determine the nDNA/mtDNA ratio, the number of mtDNA molecules per cell is obtained(26). Quantified extracellularly however, mtDNA, or rather *cell-free mtDNA (cf-mtDNA)*, does not seem to reflect the energy-producing capacity of mitochondria. Instead, several studies have proposed that cf-mtDNA serves as an inflammatory marker, triggering the innate immune systems through recognition of certain molecular patterns (*Damage Associated Molecular Patterns, DAMPs)* by the Toll-like receptor 9 (TLR-9) and similar receptors of the innate immune defense, initiating an inflammatory response(27-30). This was demonstrated in a landmark, proof-of-concept study by Zhang et al., in which mtDNA levels among patients who had suffered severe but aseptic trauma were found to be several orders of magnitude higher than among controls (30).

Mitochondrial interactions with stress signaling and inflammation

The cortisol stress response system in depression and suicidality

The Hypothalamic-Pituitary-Adrenal (HPA) axis is a mechanistic term for the body's stress signalling system. Activation of the HPA-axis induces cortisol release, resulting in adaptation in all organ and tissue types in the body, and induce behavioural change. It is activated both under normal physiologic stress conditions, such as physical exercise; and under normal psychological stress conditions, such as public speaking. Similar conditions can easily be simulated in experimental paradigms, like running on a treadmill, or by submitting to the Trier Stress Test (TSST, a psychosocial stress paradigm)(31).

Cortisol can be measured in both blood and urine, but the levels vary with circadian rhythm and between individuals(32). Also, as the main function of the HPA-axis is to prepare the individual for environmental stress, it is a dynamic system. Under normal physiological conditions, HPA-axis activity is self-regulatory through inhibitory feed-back mechanisms. This limits the utility of a single cortisol measurement when seeking to assess how well the stress signalling system functions in an individual. To better make such assessments, the Dexamethasone Suppression Test (DST) was designed to test responsiveness of the HPA-axis (33, 34). In the DST, a synthetic cortisol agonist, dexamethasone, is administered intravenously. Blood samples for plasma cortisol level estimation are collected at one time-point before dexamethasone administration, and at two subsequent time-points after administration. This method enables correction for circadian rhythm variation and can thus be used for inter-individual comparison of HPA-axis.

Over the course of several decades, a substantial body of literature have established a robust correlation between a hyperactive and non-responsive HPA-axis in individuals with depression (35-37). However, not *all* individuals with depression seem to have a dysregulated HPA-axis. Results from some studies suggest that HPA-axis hyperactivity is associated with melancholic (or "endogenous") depression, whereas hypo-reactivity might be associated with "atypical" depression(38). Furthermore, it has not been clearly demonstrated that HPA-axis dysregulation causes depression (39), and the clinical utility of the DST for diagnosing depression or to make prognostic assumptions seems to be limited(40). Several reasons for these inconsistent results have been suggested, such as: i) methodological discrepancies between studies; ii) variation in demographic variables, occurrence of somatic disorders, and medication affecting the HPA-axis in different study cohorts; iii) the heterogenous nature of depression (i.e. some forms of depression might be associated with HPA-axis hyperreactivity, whereas others may not) (37).

Many studies have also suggested that HPA-axis dysregulation infers an increased risk for suicide(41, 42). But, as in the case of depression, the results have been conflicting. One meta-analysis has reported higher cortisol levels among individuals with suicidal behaviour, compared to healthy controls. But when compared to non-suicidal individuals with psychiatric disorders, the levels were lower(43). Also, studies have reported inconsistent results regarding HPA-axis reactivity in individuals who have attempted suicide, compared to individuals who have completed suicide. In several studies, the risk for suicide was higher among individuals with a hyperactive HPA-axis(44-46). Other studies have however not been able to confirm this (47). One possibility for these discrepant findings is that HPA-axis hyperactivity infers an increased risk for future suicide mainly among those individuals who have made a prior suicide attempt, *and* have a mood disorder (48). Conversely, however, *low* cortisol levels have been associated with other aspects of suicidal behaviour, such as repeated suicide attempts(49), suicidal intent(50), and ideation(51). Finally, one meta-analysis found that associations between cortisol levels and suicide attempts are age-dependent: Among individuals less than 40 years old, high cortisol levels were associated with an increased risk for suicide attempts, whereas the opposite was true for individuals older than 40 (i.e. low cortisol levels was associated with increased suicide attempts in this age group)(52). In conclusion, HPA-axis dysregulation likely infers an increased risk of suicide and suicide attempt, but the complexity of how the system is regulated, together with confounding interactions that are related to an individual's age, sex, and other demographic and environmental variables, obscures the importance of this risk.

Mitochondria and stress

Mitochondria readily respond to cortisol signals, partly through interactions mediated by the glucocorticoid receptor (GR), which is present in mitochondria(53). Activated mitochondrial GRs triggers increased mtDNA expression, ultimately increasing mitochondrial respiratory chain (RC) complex activity (54, 55). Increased cortisol signaling also leads to expression of nDNA-encoded proteins required for mitochondrial biogenesis. Thus, an acute increase in stress, mediated by an activated HPA-axis and consequent cortisol release, results in both an increased *energy producing capacity* (in the individual mitochondrion), and increased *mitochondrial content* (more mitochondria in each cell) (56). However, this effect is not linear, but rather curvilinear, like an inverted U curve. Under short-term/low-dose exposure to cortisol, mitochondrial functioning is enhanced, but during long-term/high-dose exposure, GRs are down-regulated, likely with negative effects on mitochondrial function (57).

Mitochondrial dysfunction can also lead to increased oxidative stress and production of ROS (55). The release of ROS can in turn cause damage to cellular proteins, lipid membranes and DNA. DNA damage can be quantified by measuring

of oxidative stress markers such as 8-OH 2-deoxyguanosine (8-OHgG). According to one meta-analyses, individuals suffering from depression have elevated levels of 8-OHdG (58), suggesting that DNA damage may arise in some MDD cases when ROS production exceeds the compensatory capabilities of antioxidative enzymes, such as reducing hydrogen peroxidase to water and oxygen, a reaction catalyzed by the enzyme glutathione peroxidase (GpX), protecting the cell from ROS (59).

Mitochondria in psychiatric disorders

Primary Mitochondrial disorders

Mitochondrial disorders are such illnesses that arise from genetic alterations in either mtDNA directly, or in nDNA-encoded genes that are important for normal mitochondrial function. Most often they are polygenetic, making genetic testing evasive, and the pattern of inheritance can be complex(60). Additionally, each cell can contain multiple mitochondrial genomes, some carrying a mutated gene and some being unaffected, a phenomenon referred to as *heteroplasmy(61).* If the ratio of mutated/wild-type genomes is high, the cells are generally more dysfunctional, the symptoms more pronounced and the prognosis worse(62). Unfortunately, most known mitochondrial disorders are progressive and uncurable(63).

The pathophysiology of mitochondrial disorders is in most cases related to disturbances of oxidative phosphorylation (OXPHOS), although other important biochemical processes might also be affected) (60). Symptoms are heterogenous and engage multiple organ systems, both neurological and non-neurological. Diagnosis can be difficult, especially when the onset occurs in adult life with relatively discrete symptoms.

Mitochondrial diseases typically occur early in life, or in the young adult (18-40 years old), although there are many exceptions to this general rule. Common presentations of symptoms are grouped in syndromes. Among younger children, Leighs syndrome is best described and most often diagnosed among the mitochondrial diseases. Seventy-five known monogenetic mutations engaging both mtDNA and nDNA have been associated with the syndrom (64). Affected children develop ataxia, dystonia, and ophthalmological abnormalities, often in relation to an infection. Normal neurologic and intellectual development is delayed. The course of the disease is episodic and most often fatal before 3 years if age.

Among adults, other frequently diagnosed mitochondrial disorders include Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS), and Kearns-Sayre syndrome (KSS). Apart from stroke-like episodes, deafness, diabetes mellitus, progressive loss of sight, cardiomyopathy, seizures are known symptoms of MELAS(65).

Mitochondria in depression and suicide

Many of the primary mitochondrial disorders outlined in the previous section also presents with symptoms related to several psychiatric disorders(66), such as autism(67, 68), bipolar disorder, schizophrenia(69), and depression(70). Because mitochondria are central to energy metabolism, the "mitochondrial hypothesis" of depression(71) (and other psychiatric disorders) have proposed that dysfunctional mitochondria causes alterations in energy metabolism with resulting increases in oxidative stress, inflammation and HPA-axis activity, resulting in downstream detrimental consequences for the affected individual, ranging from dysregulated blood glucose levels, hypertonia and blood lipid metabolism; to behavioral changes commonly associated with depression(55). Although such alterations in energy metabolism affect the entire body, they are likely especially important for the brain, which relies heavily on the continuous supply of ATP (72) (as glucose is not stored in neurons). Because of this, it has also been hypothesized that mitochondrial dysfunction might be particularly important for neurodegenerative and psychiatric disorders, such as depression, but also Parkinson's disease, Huntington's disease, and Alzheimer's disease (73, 74). Although the exact mechanisms are still unclear, reduced energy metabolism in the brain might contribute to diminished neurogenesis and increased neuronal cell senescence, linking findings of altered hippocampus(75-77), hypothalamus(78), and prefrontal cortex volume (79) in depression to mitochondrial dysfunction. This link have also been substantiated by results from animal studies, showing that treatment interventions commonly used in depression treatment, such as Electroconvulsive Therapy (ECT) and pharmacotherapy with antidepressants both seemed to increase the number of mitochondria in rats strains susceptible to depressive-like behavior(80, 81).

In studies of human cohorts, using same type of biomarkers as in this thesis, there have been some support of mitochondrial involvement in depression, although the results have sometimes been conflicting. In one often-cited study by Cai et al., leukocyte mtDNA-cn in blood and saliva was increased in a large-scale cohort of men and women suffering from recurrent depression compared to healthy controls(82). In the same study, results from an experimental mouse stress model were also reported, showing that both stress and glucocorticoid administration increased mtDNA-cn in the mouse model. In a commentary to their initial findings, the authors also reported that increased mtDNA-cn reflected the course of a depressive episode, when analyzed in weekly blood draws and compared to symptom rating scores(83). However, leukocyte mtDNA-cn have not been consistently increased among depressed individuals since other studies have reported unchanged(84), or even lower(85) mtDNA-cn in depressed individuals.

Ccf-mtDNA has not been extensively studied as a biomarker for depression, and the results reported from these studies have been inconsistent. Apart from the results presented in this thesis, there have only been two other studies investigating ccf-mtDNA in a human cohort of depressed individuals. One of these studies reported

lower ccf-mtDNA levels among the patients compared to controls(86), whereas the other reported higher ccf-mtDNA among individuals suffering from late-life depression(87). Among individuals with bipolar depression, the only study investing a correlation to ccf-mtDNA found no significant difference between patients and controls(88).

As for studies examining mtDNA(intra- or extracellularly) among suicidal individuals, or even suicide completers, the body of scientific literature is expectedly scarce, although one post-mortem study have reported higher mtDNA-cn in peripheral blood collected from suicide completers, compared to controls(89). The same study also reported age and sex differences in mtDNA-cn among suicide completers, and lower mtDNA-cn in prefrontal cortex tissue.

Overall aim of the thesis

This thesis aims to analyze biomarkers of cellular stress in patients with depression and suicidality, focusing on biomarkers relating to mitochondrial function and mitochondrial stress signaling. Specifically, we aim to investigate possible interactions between such biomarkers and certain psychiatric symptoms, symptom severity and suicidal behavior; correlations to other biomarkers related to cellular stress signaling, cellular aging, inflammation, and oxidative stress; and to treatment response. We also address the possibility that autoimmunity is associated with suicidal behavior. By testing the specific hypotheses of the included studies, we hope to better understand the pathophysiological mechanisms of depression and suicidality, and we also hope that the tested biomarkers can be used in future studies to inform about clinical meaningful outcomes such as prediction of treatment response and disease course.

Specific aims of the thesis

The specific aims of the thesis, and in which of the separate studies they are addressed, are provided below. In table 1, on page 31, the specific aim, methods used, and results of each study is also provided.

1. To explore the possibility that autoimmune processes may be involved in suicidal behavior (study 1).
2. To investigate whether a biomarker of cellular stress with pro-inflammatrory properties (ccf-mtDNA) is increased among individuals with depression and suicidality (study 2-5), and if this biomarker relates to certain symptom characteristics (study 5).
3. To investigate whether ccf-mtDNA relates to other established biological markers of cellular stress (study 2-4).
4. To investigate whether mtDNA measured in different cellular compartments might reflect different cellular mechanisms in depressed individuals. (study 3).
5. To investigate whether a composite index (MHI), composed of RC complex activity data, toghether with estimates of mitochondrial content, might be used to distinguish between depressed individuals and healthy controls (study 4)
6. To investigate whether ccf-mtDN,A or the Mitochondrial Health Index (MHI) predicts treatment response in depressed individuals (study 3-5).

Methods and Materials

Participants

Results from all studies included in this thesis are based on blood samples and behavioural measures obtained from five different cohorts. Common for all cohorts is that they have been recruited for participating in studies where the primary hypothesis and aim concerned stress-related biomarkers with relation to depression and suicidality. In table 1, a summary description for each cohort is provided. All study subjects have given their informed consent of participation. All studies have been approved by local ethical committees.

Summary of study aims and cohorts

In addition to the summary description of each study cohort, table 1 also provides information on the primary aim of each study included in the thesis, together with biological assays, diagnostic interviews and symptom rating scales used, and main results.

Table 1. Summary of study aims, cohorts, assays, psychometrics and results.

Number of study in thesis	Title	Main purpose of study	Patient cohort charachteristics	Control cohort charachteristics	Main assays used	Diagnostic interviews/ Symptom ratings scales	Main Results
1	Six autoantibodies associated with autoimmune encephalitis are not detectable in the cerebrospinal fluid of suicide attempters	To explore the possibility that autoimmune processes may be involved in suicidal behavior	Suicide attempters admitted to Lund University between 1987 and 2001 (n = 29)	n/a	Autoimmune Encephalitis 6 Biochip mosaics (EUROIMMUN, Lübeck, Germany)	Clinical/Diagnostic interviewing	No autoantibodies detected
						MADRS[1]	Indices of increased BBB[2] permeability in 5 subjects
2	Increased plasma levels of circulating cell-free mitochondrial DNA in suicide attempters: associations with HPA-axis hyperactivity	To determine whether cf-mtDNA[3] in cell-free plasma is increased in suicide attempters	Suicide attempters admitted to Lund University Hospital between 1992 and 2001 (n = 37)	Emplyoees or students at Lund University Hospital and seniors in Lund with no prior or current psychiatric disorders or any somatic disorders (n = 37)	DNA Isolation: QIAmp 96 DNA Blood Kit (Qiagen, Valencia, CA, USA)	Clinical/Diagnostic interviewing	Suicide attempters had significantly higher plasma levels of cf- mtDNA, compared with healthy controls
		To test the relationship between cf-mtDNA and (HPA)-axis activity			Quantitative analysis of cf-mtDNA: qPCR[4]	MADRS	Suicide attempters pre-DST
					HPA-axis[5] reactivity: DST[6]		plasma levels of mtDNA were positively correlated with post-DST cortisol levels
3	Circulating cell-free mitochondrial DNA, but not leukocyte mitochondrial DNA copy number, is elevated in major depressive disorder	To investigate levels of PBMC mtDNA-cn[7] and cf-mtDNA in MDD[8] subjects	MDD outpatients recruited through clinical referrals to (n = 50).	Recruitement of HC (n =55) between 2011-2015 by flyers, bulletin board notices, Craigslist postings and newspaper ads.	DNA Isolation: QIAmp 96 DNA Blood Kit (Qiagen, Valencia, CA, USA)	Diagnostic interviewing: SCID-I[12], Clinical Interview (by certified psychiatrist)	PBMC mtDNA did not differ between groups
		To test the relationship between mtDNA and peripheral indices of oxidative stress and telomere length	All subjects were unmedicated.	Exclusion criteria: Any history of DSM axis-1 diagnosis, acute somatic illness or infection, confounding inflammatory disorder, ongoing psychotropic medication	Quantitative PBMC mtDNA measurement: Multiplex qPCR	MDD symptom severity: HDRS-17[13]	Cf-mtDNA levels was significantly higher among MDD subjects compared to controls
		To determine if successful antidepressant treatment is associated with changes in PBMC mtDNA-cn or ccf-mtDNA.	Exclusion criteria: bipolar disorder, alcohol or substance abuse, PTSD[9], eating disorder, history of psychosis outside of a major depressive episode, or the presence of any psychotic symptoms during the current MDE[10]		Quantitative analysis of cf-mtDNA: qPCR GpX[11] activity measurent: Glutathione peroxidase activity assay (BioVision, Inc., Milpitas, California, USA)		Changes in cf-mtDNA differed between SSRI responders vs non-responders
							Baseline cf-mtDNA was positively correlated to GpX

4	Blood-based Mitochondrial Respiratory Chain Function in Major Depression	To investigate whether mitochondrial RC[14] enzymatic acitivity, mtDNA, and a composite measure of mitochondrial function (MHI) could distinguish MDD patients from healthy controls	MDD outpatients recruited through clinical referrals to (n = 47).	Recruitement of HC (n = 11) by flyers, bulletin board notices, Craigslist postings and newspaper ads.	Enzymatic activity of RC complexes: Kinetic spectrophotometric Assays	Depression symptom rating scales: HDRS-17 and HDRS-25	Mitochondrial markers did not distinguish MDDs from HC
		To investigate whether these markers could predict SSRI response (distinguish SSRI responders from non-responders)	All subjects were unmedicated. Exclusion criteria: bipolar disorder, alcohol or substance abuse within the preceding 6 months, PTSD or an eating disorder within 1 month of entering the study, history of psychosis outside of a major depressive episode, or the presence of any psychotic symptoms during the current MDE	Exclusion criteria: Any history of DSM axis-1 diagnosis, acute somatic illness or infection, confounding inflammatory disorder, ongoing psychotropic medication	mtDNA and nuclear DNA: Taqman multiplex assays for specific mtDNA and nuclear DNA genes	Percieved Stress: PSS[15] Adverse Childhood Experiences: ACE-10[16]	Mitochondrial markers (RC complex activity and mtDNAcn) could distinguish SSRI responders from SSRI non-responders
5	Plasma circulating cell-free mitochondrial DNA in depressive disorders	To investigate plasma ccf-mtDNA in a large sample of difficult-to-treat depression and healthy controls	Patients diagnosed with an affective disorder and an insufficient treatment response (n = 281), referred to study between 2012 – 2020.	HC (n = 49) were recruited through advertisements in social media and newspaper ads.	DNA Isolation: QIAmp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA)	Diagnostic interviewing: MINI 6.0[17] Clinical Interviews by senior resident or specialized psychiatrist	Mean levels in cf-mtDNA were significantly lower among depressed patients compared to healthy controls
		To investigate the relationship between cf-mtDNA and specific symptoms of depression, a history of a suicide attempt and medications that may influence cf-mtDNA	Patients were medicated Exclusion criteria: BMI < 15, pregnancy or current liver disease	Exclusion criteria: Previous or present psychiatric illness; treatment with psychotropic drugs or psychotherapy; severe or chronic somatic illness; ongoing infection, pregnancy, breast-feeding; treatment with drugs affecting the immune system.	Quantitative analysis of cf-mtDNA: Quantitative real time polymerase chain reaction (qPCR), using SYBR Green Technology (Thermo Fisher Scientific, Waltham, MA, USA).	Psychiatric Symptoms Assessment: CPRS[18]	A positive correlation between cf-mtDNA and “inflammatory depression symptoms” No correlation between cf-mtDNA and prior suicide attempt A negative correlation between treatment with mood stabilizers and cf-mtDNA

[1]. Montgomery-Åsberg Depression Rating Scale. [2]. Blood-Brain-Barrier. [3]. Cell-free mitochondrial DNA. [4]. Quantitative Real Time Polymerase Chain Reaction. [5]. Hypothalamic-Pituitary-Adrenal-Axis. [6]. Dexamethasone Supression Test. [7]. Peripheral Blood Mononuclear Cell mtDNA-copy number. [8]. Major Depressive Disorder. [9]. Post-Traumatic Stress Disorder. [10]. Major Depressive Episode. [11]. Gluthatione Peroxidase. [12]. Structured Clinical Interview for DSM-IV axis-1 disorders. [13]. Hamilton Depression Rating Scale (17-item). [14]. Respiratory Chain. [15]. Percieved Stress Scale. [16]. Adverse Childhood Experience scale (10-item). [17]. Mini International Neuropsychiatric Interview ver 6.0. [18]. Comprehensive Psychopathological Rating Scale

Biological sampling procedures, tests, and assays

Lumbar puncture

CSF samples were used in study 1. Lumbar punctures were drawn between 8 am and 9 am, following a night of fasting and bed rest. CSF was collected from the L4-L5 interspace using a standardized protocol. The samples were immediately stored at -80°C until time of analysis. Twenty of the 29 CSF samples had been previously thawed/frozen at least once before the analyses.

Autoimmune encephalitis antibody detection

The assay used for antibody detection in study 1 was the Autoimmune Encephalitis 6 Biochip mosaics (EUROIMMUN, Lübeck, Germany). The assay is designed to detect six distinct autoantibodies known to cause autoimmune encephalitis in humans. The autoantibodies detected bind to different neuronal cell structures listed below:

Table 2. Overview of antigens for the autoantibodies tested for with the Autoimmune Encephalitis 6 Biochip mosaics assay

Antigen	Properties and function of antigen
NMDAR[1]	Ionotropic glutaminergic neuronal receptor associated with learning and memory functions(90)
AMPAR[2]	Ionotropic glutaminergic neuronal receptor associated with fast synaptic transmission, plasticity, long-term potentiation, etc.(91)
GABAbR[3]	Metabotropic receptor linked to potassium channels via G-proteins. Inhibit action potential and neurotransmitter release(92)
LGI1[4]	Neuronal protein structure involved in normal brain development(93)
Caspr2[5]	Cellular adhesion molecule of the neurexin family involved in axonal structural formation(94)
DPPX[6]	Regulatory protein of potassium channels involved in neuronal signal integration and attenuation (95)

1. N-methyl-D-aspartate receptor
2.α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
3. γ-amino butyric acid B-receptor
4. leucine-rich, glioma-inactivated 1
5. contactin-associated protein-like 2
6. dipeptidyl-peptidase-like protein-6

The assay is based on the Indirect ImmunoFluorescence Test (IIFT)(96), using the specificity of the autoantibodies for their specific neuronal surface antigen (presented in table 2). If the autoantibody is present and bind to one of the antigens of interest, a secondary antibody, marked with a fluorescent dye, bind to the primary (auto)antibody-antigen complex. By excitation with light of a certain wavelength, the complex becomes visible in a fluorescence microscope. In a series of steps, the cells in the sample tested (in this case undiluted CSF), are washed, treated with the fluorescent secondary antibody, washed again, before mounted on a microscopy

cover glass treated with transfected cells expressing the antigen. The cover glass is mechanically cut into millimetre-sized fragments ("Biochips")(97) and placed in test fields of specially developed plastic slides, allowing for multiplex analysis. For each test, a negative and a positive control is included on the slide. Experienced lab personnel read and evaluate the fluorescence of the fixated cells on the slide and report the test result as either positive or negative.

Blood sampling for cortisol analysis and the DST

Cortisol level analysis was performed in study 2.

For a detailed outline of the HPA-axis and DST, please see section "The cortisol stress response system in depression and suicidality on page 22.

The DST was performed in accordance with the procedure described by Carrol et al.(98). Blood samples for cortisol was drawn in serum tubes at three consecutive time points following admission. At time point 1/baseline (before dexamethasone administration), blood was drawn at 3 pm. At 10 pm, 1 mg of dexamethasone was given intravenously. Two additional blood samples were drawn at 8 am the following day (time point 2, post DST) and 3 pm (time point 3, post DST). Serum tubes were left at room temperature before centrifugation to allow for coagulation. The tubes were centrifuged at 4 ºC and 2000 x *g* for 10 minutes within one hour of collection to separate blood components from the liquid fraction. Serum cortisol was measured using a commercial radioimmunoassay (RIA) (Orion diagnostica RIA kit). The detection limit was below 7 nmol/l, and the intra- and inter-assay coefficients of variation were below 5 and 7% respectively.

A cortisol non-suppressor was defined as an individual who did not suppress cortisol below 140 nmol/L at any time point after dexamethasone administration.

Preparation of plasma for ccf-mtDNA analysis

Ccf-mtDNA analysis was performed in study 2, 3 and 5. Similar, but not identical, methodology was used in the three studies (see table 1 for details). Blood was drawn in (lavender) EDTA tubes at specific time points, following a night of fasting. The tubes were spun at 1500-2000 *g* x 10 minutes to separate blood cells from the liquid fraction. Plasma samples were then stored at – 80 to – 70°C until ccf-mtDNA analysis was performed. Following thawing, plasma samples were spun at 10000 *g* x 10 minutes to pellet larger membrane-bound structures (such as platelets), leaving mainly mtDNA contained in smaller membrane-bound vesicles (such as extra-cellular mitochondria or exosomes), and non-membrane bound mitochondria.

Table 3. Summary of blood sampling in study 2, 3 and 5

Study number	Placed on ice before initial centrifugation?	Approx. time to initial centrifugation	Spin force at initial centrifugation	Storage temperature	Spin force second centrifugation
2	Yes	< 1 hour	20 00 g x 10 min	– 70°C	10 000 g x 10 min
3	Yes	< 1 hour	15 00 g x 10 min	– 80°C	10 000 g x 10 min
5	Yes	< 1 hour	25 00 g x 10 min	– 80°C	10 000 g x 10 min

Preparation of Peripheral Blood Mononuclear Cells (PBMC)

In study 3 and 4, PBMC's were extracted from whole blood through Ficoll centrifugation(99). In this process, whole blood is carefully layered on Ficoll (a polysaccharide solution developed for separation of whole blood cell components) in special conical test tubes. The tubes are then spun at 400 g x 30 min, which separate the whole blood sample in four layers: A liquid fraction on top; followed by the thinner "buffy coat" containing PBMC's; a Ficoll layer; and finally at the bottom of the tube, red blood cells and polymorphonuclear cells. Following separations, the buffy coat is collected and washed from remaining Ficoll, homogenized in Phosphate Buffered Saline (PBS) and spun again at 100 *g* x 10 minutes to create a PBMC pellet leaving the platelets out. PBMCs was then prepared and stored at – 80°C until further analysis.

DNA isolation from plasma

DNA (both nuclear and mitochondrial) was isolated from thawed plasma, and PBMC samples, in study 2-5. DNA isolation was performed either from a PBMC lysate using a commercial available agent (Puregene, Gentra Systems, Qiagen, Valencia, CA)(100), whereas DNA for ccf-mtDNA analysis was extracted from plasma samples using the QIAmp 96 DNA Blood Kit (Qiagen, Valencia, CA, USA)(101, 102). Both techniques use silica micro-column purification methods. These methods are based on the fact that nucleic acids of the DNA molecule may bind to a silica solid phase if the pH and salt concentration of an added buffer is just right. When isolating DNA using micro-column isolation methods, such a buffer is added to the plasma or cell lysate together with an alcohol, forming a binding solution. The binding solution are then added to the micro-column (or spin column), which is placed in a centrifuge. When the sample is spun at high velocity, the binding solution is forced through the column, allowing DNA-binding to the silica column on the way. To remove non-DNA cellular components, a second "wash" buffer is added to the column, which is then centrifuged again, this time washing out cellular debris and impurities and leaving only DNA components behind. Finally, a third "elution" buffer is added, allowing the DNA to separate from the column. When spun a third time, the DNA is collected in a trough at the bottom of

the column. The amount and purity of isolated DNA can be calculated using spectrophotometric analysis.

DNA quantification by qPCR

The quantitative analysis of DNA in studies 2-5 was performed using quantitative real time polymerase chain reaction (qPCR or real-time qPCR)(103). The method is designed for determining the presence and amount of specific DNA fragments (often specific for a particular gene) in a sample. Briefly explained, an isolated DNA fragment is amplified through repeated cycles of DNA denaturation and synthesis. For the amplification process to occur, certain reagents need to be added:

1) A heat-resistant DNA polymerase (called Taq polymerase from the bacterial species it is developed). Taq polymerase can withstand temperatures of up to 95°C without losing function, which is needed as the sample is heated and re-heated in each cycle.

2) Specific forward and reverse DNA-primers (i.e. specific DNA sequences) which serves as the starting point for the Taq polymerase to synthesize new DNA strands. The nucleotide sequence code of the primers makes them highly selective and ensure that only the desired DNA fragment is replicated. Commercially available primers are selected for their intended use. To detect and amplify mtDNA in a sample, for instance, it is possible to select a primer sequence that corresponds to DNA fragment that only exist in the mitochondrial genome. In study 2, a primer corresponding to a fragment of the Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 2 (ND2) gene was used to amplify ccf-mtDNA (see table 2).

Table 4. Primer for the mitochondrial ND2 gene, used for mtDNA quantification in study 2.

Gene	Primer forward*	Primer reverse
ND2	CACACTCATCACAGCGCTAA	GGATTATGGATGCGGTTGCT

*Note that forward and reverse primers are not complementary, i.e they cannot bind to each other as this would create “primer-dimers”, resulting in an unspecific PCR-reaction.

3) The original DNA fragment, acting as a template for the polymerase to bind and initiate DNA replication. 4) Finally, a buffer appropriate for the reaction, together with deoxynucleoside triphosphate (dNTP) building blocks are needed for DNA strand synthesis.

In the qPCR equipment (or machine), the amplification process is initiated when the sample (placed in a plate with several wells) is heated to 94°C. At this temperature, the DNA strand in denatured into two complementary strands. Following the initial heating, the sample is cooled to a temperature ideal for the specific primer to anneal to the DNA template (often somewhere between 50 - 65°C). The temperature is then

increased to 72°C, which is the optimal temperature for the *taq* polymerase to synthesize a new DNA strand. This step is then repeated in cycles, each cycle exponentially increasing the number of DNA strands present in the beginning of the process.

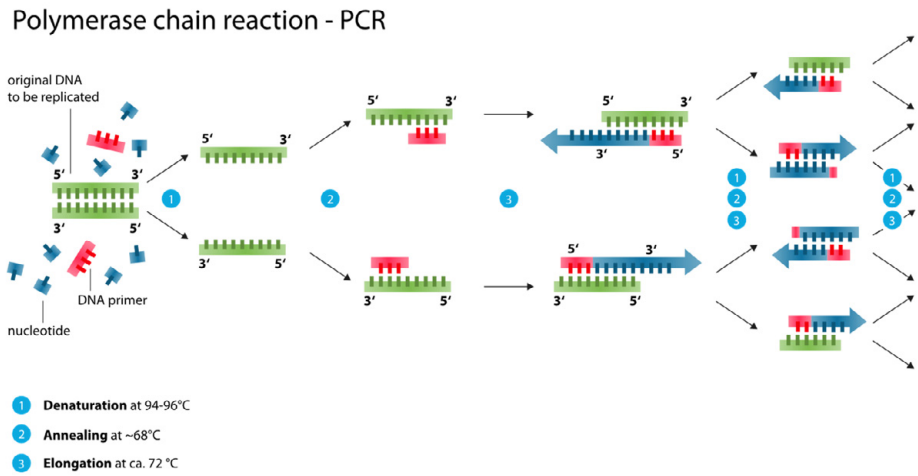

Figure 3. The different steps of the PCR by which a double-stranded DNA molecule is first denatured, then annealed to a specific primer, before Taq polymerase starts synthesizing a new DNA strand, using the old one as a template. By Enzoklop - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=32003643

To detect the number of newly formed DNA molecules, light emitted from a fluorescent dye is registered by the PCR machine. In the studies presented in this thesis, two different dyes/assays were used: Taqman (study 4) and SYBRGreen (studies 2, 3 and 5). The dyes differ somewhat in their binding properties and when in the PCR cycle light is emitted, but the principle of the technique is the same. Using SYBRGreen as an example: The dye emits light when bound to double-stranded DNA (dsDNA). As the number of dsDNA-molecules increase, so does the emitted light. In the early cycles of the amplification process, the emitted light is too low even to be detected, but as more dsDNA molecules are synthesized, the light eventually reaches the point of detection. The number of cycles required for detection of fluorescent light is known as the Ct-value. The Ct-value is then used for calculating the number of DNA molecules (matching the specific primer used) present in the samples from the beginning.

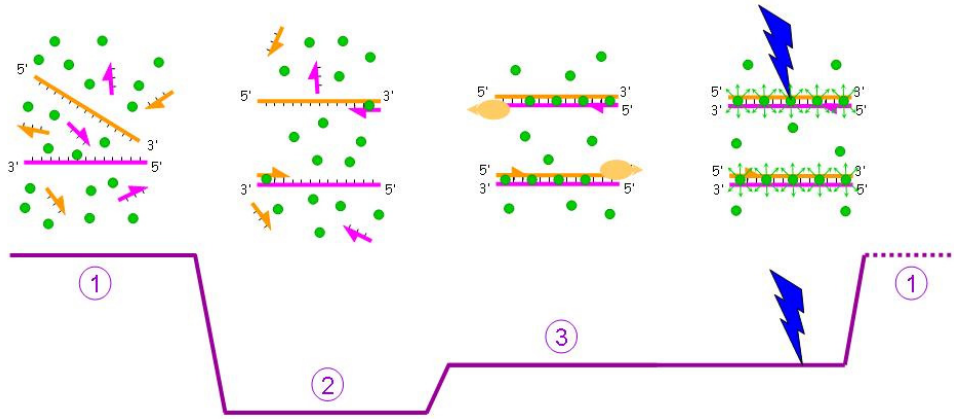

Figure 4. Fluorescent SYBRGreen dye only emits light when bound to dsDNA. The figure depicts SYBRGreen dye (green dots) in the three different steps of the PCR: 1) Denaturation 2) Annealing 3) DNA elongation. Only at the end of step 3, when the newly formed dsDNA molecule is complete, light from the SYBRGreen dye is emitted. The cycles are repeated, exponentially increasing the number of DNA molecules in each cycle. After a certain number of cycles (known as the Ct value), the light emission is large enough to be detected by photosensors in the PCR equipment.

Mitochondrial respiratory chain activity assessment

Measures of mitochondrial respiratory chain enzymatic activity were assessed in study 4. PBMC pellets were homogenized by mechanically disrupting the cell membrane by placing them a buffer solution and spinning them in a centrifuge with tungsten beads. From the homogenate, four mitochondrial RC enzymes (CS; NADH dehydrogenase/complex I; SDH/Complex II; and COX/Complex IV) were quantified using kinetic spectrophotometry. To measure the activity of a specific enzyme in a sample, the enzymatic reaction must cause a colour change in the sample. Such a colour change can be produced, for example, by adding a reactant that oxidizes the product of the enzymatic reaction, causing the sample solution to change colour as the rection proceeds. By measuring the colour change over time with a spectrophotometer, the rate of the enzymatic activity can be plotted.

Diagnostic interviews and symptom rating scales

The CPRS and the MADRS

The Comprehensive Psychopathology Rating Scale (CPRS) consists of 65 items, 45 of which are based on patient report and 20 based on observations by the interviewer(104). As the name suggest, it is comprehensive compared to many other, shorter symptom rating scales. It was designed to measure variance (specially to measure treatment response) over time and thus focus on *state* rather than *trait* psychopathology variables as these are more likely to change. The scale has demonstrated good validity and reliability in several studies(105-107). Every item

on the CPRS is clearly defined and measured on a scale from 0-3, with the possibility to rate half-points. From the CPRS, several subscales have been derived, such as The Montgomery Åsberg Depression Rating scale (MADRS)(108), focusing only on depressive symptoms. The MADRS consists of 10 items rated from 0-6 (as the half-points of CPRS have been transformed into discrete numbers), where some of the items are based on the patients' answers and others on observations. Also very common in psychiatric clinics is the 9-item, self-administered version of the scale, the MADRS-S. The MADRS was constructed with the purpose of detecting change in depressive symptom over time to evaluate the efficacy of antidepressant drugs. From the CPRS, the 17 items most often rated in depressed patients were identified. Of these, the 10 items demonstrating the greatest change over the course of antidepressant treatment were selected. Compared to the HDRS-17 (see section below), the MADRS is comparatively more focused on the "psychological" experiences related to depression, rather than on "somatic" symptoms of depression(109).

The MINI 6.0

The Mini International Neuropsychiatric Interview, version 6, is a brief diagnostic interview used for screening of the most common psychiatric disorders in DSM-IV(110). It is divided into several sections, each addressing a certain DSM diagnostic category (such as affective disorders, anxiety disorders, eating disorders, psychotic disorders, etc). The interview is "structured", i.e., the asked questions should not deviate from how they are formulated in the interview protocol. The interview should be performed by a trained interviewer. Each section of the interview starts with 2-3 questions designed to determine whether that patient have experienced the main diagnostic criteria for the specific disorder. If so, the interviewer then asks additional questions to determine i) the severity of disorder or ii) if there is any information present that contradicts or excludes for the DSM-IV diagnoses. If performed by an experienced interviewer, the interview takes about 20-30 minutes. The sensitivity and specificity of the MINI are sufficient for diagnosing affective disorders(111).

HDRS-17

The Hamilton Depression Rating Scale (17-item) is the most extensively used observer rating scale for depression(112). As in the case with MADRS, the HDRS-17 is meant for rating the severity of depressive symptoms after the diagnosis have been established. Of the 17 items included in the scale, some are based on the answers from the patient in questions, whereas others are based on the interviewer's observations. Compared to MADRS, the HDRS-17 generally require a more experienced interviewer and takes longer time to complete (generally between 20-

30 minutes). Also, the HDRS-17 is more focused on sleep disturbances, somatic symptoms of depression and anxiety, compared to MADRS(109). The discrepancies between the rating scales (which are the two most used for evaluating antidepressant efficacy in drug trials), have led to concerns that some forms of depressions are better evaluated with one of the scales rather than the other(109). However, in one quite recent meta-analysis, there was no clear evidence for this concern(113).

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) v.22, 23 or 27 (IBM Corp., Armonk, NY) was used for statistical calculations in all studies. All statistical tests were two-tailed with an alpha = 0.05.

In all studies, appropriate statistical tests were chosen based on the type of data (i.e., nominal/ordinal or continuous data); data distribution (i.e normally distributed or non-normally distributed); whether the test was used for comparison of paired (e.g. when comparing the same group of individuals at different time points) or independent groups (two different groups of individuals); if the purpose was to find correlations between variables (rather than group comparisons); and if there was a need for adjusting for covariates. In study 1, the primary outcome variable was dichotomous and negative in all cases. Thus, no statistical method was used. Demographic data were presented as groups means ± SD.

Most biological samples (Ccf-mtDNA, RC complexes, GpX, etc.) were expressed as continuous, non-normally distributed data. Consequently, most analyses comparing data based on biological samples in two groups, were performed either with non-parametric statistical methods, or by log-transforming raw data prior to analyzing with tests suitable for normally distributed data. Log transformation was successful in achieving normal distribution in almost all cases, except in study 3, were log transformation was not sufficient to achieve normal distribution in HDRS-17 baseline scores). In this case, we used Blom transformation, which replaces raw values with a rank value and adjusts scale distances between the rank values, to achieve a normal distribution.

In study 2, Mann-Whitney U was accompanied by Cohen's *d*, used for expressing the difference between two group means in standard deviation units. A Cohen's $d < 0.3$ is considered a small effect (less 0.3 SD difference between the two means), whereas Cohen's $d > 0.5$ is considered a medium affect size and > 0.8 a large effect size.

For correlations, we used either Pearson's r (for parametric correlations) or Spearman's *rho* (for non-parametric correlations). Pearson's X^2 was used for comparing proportions between groups (e.g., sex distribution). Comparisons of normally distributed data between more than two groups (e.g., when comparing demographics in controls, currently depressed patients and remitted patients in study 5), One-Way ANOVA was used, correcting for covariates (ANCOVA), where appropriate. When multiple comparisons were made, and where it was appropriate, p-values were adjusted using Bonferroni correction.

Commented Review of Study Results

Study 1: Six autoantibodies associated with autoimmune encephalitis are not detectable in the cerebrospinal fluid of suicide attempters.

Since several studies have suggested a biological connection between suicidal behaviour and neuroinflammation (114-118) and other studies have found that autoimmune reactions can trigger psychiatric symptoms (119, 120), the aim of this pilot study was to investigate whether autoimmune processes might be involved in suicidal behaviour. This had not been well studied before. For this purpose, we sought to detect the autoantibodies for six different types of autoimmune encephalopathies. CSF of 29 suicide attempters admitted to inpatient psychiatric treatment at the Psychiatric clinic of Lund University hospital, between 1987 and 2001 was used. The patients were selected from a larger study cohort. In this cohort Blood-Brain-Barrier (BBB) integrity had previously been determined by calculating the CSF/Serum albumin quota. These results have been reported previously(121) We hypothesised that even if autoimmune encephalopathy might be rare in the general population, it might be more common among individuals with suicidal behaviour than previously expected. If so, autoantibodies might be present in some of the 29 samples. The patients had all been admitted following a suicide attempt. Following physical examination, they were found to be somatically healthy. All psychotropic medication except for occasional doses of benzodiazepines had been withheld for a mean period of two weeks before lumbar puncture. Diagnoses and symptom severity ratings had been established by a certified psychiatrist. The assay used for analysis had been developed for clinical diagnoses of the six most common types of autoimmune encephalopathies (see figure 5 for details).

Of the patients included in the study, 13 were men and 16 women. Mean age were 41 years. The most common DSM-IV axis-I diagnosis was MDD (N = 8), followed by Depression, NOS (N = 5). Other axis-I diagnoses in the group were dysthymia, schizoaffective disorder, and substance use disorder. In 19 of the patients included, an axis-II diagnosis was also established.

None of the autoantibodies included in the assay were found in any of the samples.

Table 1. Autoantibodies tested in the present study, associated clinical syndromes and frequent symptoms (adapted from Wandinger et al 2011).

Autoantibody	Clinical syndrome	Frequent symptoms
Anti-glutamate receptor (type NMDA)	Anti-glutamate receptor (type NMDA) encephalitis	Psychosis, memory-/language impairment, seizures, impaired consciousness, dyskinesia, movement disorders, dysautonomia, hypoventilation
Anti-glutamate receptor (type AMPA)	Limbic encephalitis, atypical psychosis	Memory deficits, confusion, disorientation, seizures, agitation, aggressive behaviour
Anti-GABAB receptor	Limbic encephalitis	Seizures, confusion, memory deficits, behavioural disorders, paranoia, hallucinations
Anti-LGI1	Limbic encephalitis	Epilectic seizures, memory deficits, confusion, dis- orientation, hyponatriaemia, myoclonus, dysautonomia
Anti-CASPR2	Neuromyotonia, Morvan's syndrome, Limbic encephalitis	Peripheral neuronal hyperexcitability, muscle spasms/ fasciculations/myokymia, seizures, memory deficits, confusion, disorientation, neuropathic pains, sleeping disorders, dysautonomia, weight loss
Anti-DPPX	Autoimmune encephalitis	Anxiety, forgetfulness, confusion, hallucinations, muscle spasms, tremor and pleocytosis (in CSF)

Abbreviations: NMDA: N-methyl-D-aspartate, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, GABA: γ-amino butyric acid, LGI1: leucine-rich glioma-inactivated protein 1, CASPR2: contactin-associated protein 2, DPPX: dipeptidyl aminopeptidase-like protein 6

Figure 5. Review of autoantibodies tested (adapted from Wandinger et al. 2011).

Comments on Study 1

As this was a pilot study, and one of the very first to try to detect autoimmune encephalopathy among suicidal subjects, it is of scientific value to note that this does not seem to be a widely overlooked mechanism of suicidal behaviour. However, the sample size was small and a clinical investigation focusing on neurological signs and symptoms indicative of autoimmune encephalitis was not specified in the study protocol. Such a clinical investigation has been proposed to include a description of i) previously undescribed neurological focal signs in the patient, ii) presence of seizures without the presence of a known seizure disorder, iii) CSF pleocytosis, or iv) MRI features suggestive of encephalitis (122). Thus, it is impossible to rule out that autoimmune encephalopathy is a contributing factor to suicidal behaviour in *some* patients, especially in those individuals where signs and biomarkers such as those listed above are described. To properly investigate this possibility, a larger and better-defined study sample is warranted. Furthermore, it would be wise to use more than one assay with varying degrees of sensitivity in future analyses to establish a threshold for lowest detectable titre of antibodies in the sample.

Study 2: Increased plasma levels of circulating cell-free mitochondrial DNA in suicide attempters: associations with HPA-axis hyperactivity

Among suicide attempters and individuals who have committed suicide, aberrations of the cortisol signalling system have been reported in several studies(41, 42). HPA-axis hyperactivity, particularly when it is assessed by the Dexamethasone Suppression Test (DST), has been suggested as a possible biomarker of increased risk for suicide(35, 48, 123) and suicidal behaviour among women(124).

In studies of both animals and humans, mitochondrial dysfunction is involved in several pathogenic cellular pathways, including cortisol signalling, inflammation, apoptosis, calcium signalling, etc (125, 126). It has been well established that several genetic disorders affecting mitochondrial function often present with psychiatric symptoms (such as fatigue, cognitive impairment, loss of interest, etc.), and had in some studies also been directly associated with affective disorders, psychotic disorders, and autism(66-69). It thus seemed both interesting and important to explore whether indices of mitochondrial dysfunction differed between suicide attempters and healthy controls, and if it correlated to a better-established biological hallmark of increased suicide risk.

There are several methods to assess mitochondrial function, but we were limited to one that we thought would reflect some aspect of mitochondrial function and at the same time could be assessed in a plasma sample that had been frozen and stored for several years. Considering these conditions, ccf-mtDNA seemed like the best

candidate, as it is considered a very stable molecule that can be quantified through commercially available and validated assay methods.

Most previous studies, including one we consider of very high quality(82), suggested that high mtDNA levels were associated with psychopathology in humans. Thus, our hypothesis was that mtDNA levels would be higher in the patient cohort compared to healthy controls, and that these levels would also be higher among individuals in which the DST challenge indicated a less responsive, hyperactive, HPA-axis.

To test this hypothesis, 37 patients admitted to the Psychiatric clinic at Lund University Hospital between 1992-2001, following a recent suicide attempt were enrolled in the study, together with an equal number of healthy controls. The patients were selected from a cohort enrolled in a larger research project seeking to investigate the role of the HPA-axis together with inflammatory and monoamine metabolites in depression and suicidality (116, 127). Suicide attempters were diagnosed according to DSM-III by a certified specialist in psychiatry. Details about the suicide attempt (for example if the suicide attempt was violent or non-violent) were obtained. The study participants did not receive any antidepressants or antipsychotics during a washout period of 12 ± 13 days (mean ± SD), although occasional doses of benzodiazepines were allowed during the washout. Drug screening after the washout period was negative for common antidepressant and antipsychotic medication, although a few of the samples contained low concentrations of benzodiazepines. None of the patients had any known somatic disorders, or took any medications known to interfere with the results of the DST. Depressive symptom severity was assessed with the MADRS before blood sampling. As the DST requires blood samples to be drawn once before and two times after the dexamethasone challenge, blood samples were obtained at three time points (see section "The cortisol stress response system in depression and suicidality", p 23). Healthy controls consisted of students or employees at Lund university Hospital, or retired senior citizens from a senior residency complex in Lund. None of the controls had any prior somatic or psychiatric disorders, or were taking any medications known to interfere with the DST.

Patients and controls were similar in age, sex and BMI. Within the suicide attempter group, the number of days since medication discontinuation (i.e., "washout days"), had no effect on ccf-mtDNA levels. MDD was the most common DSM-IV axis-I diagnosis among the patients, followed by Adjustment disorder and Depression, NOS. Twenty of the cases met criteria for an axis-2 diagnosis, most of which were specified as within cluster B.

At all three timepoints, ccf-mtDNA was higher among patients than controls, who had their blood drawn only once since they did not partake in the DST (all $p < 2.98E-12$, Cohen's D ranging from 2.55 to 4.01, i.e., a very large effect size). Regarding ccf-mtDNA levels, there was no correlation to MADRS score; between those who

had made a suicide attempt with a violent method and those who used a non-violent method; or between those who had made only one suicide attempt and those who had made several attempts.

Ccf-mtDNA levels at timepoint 1, i.e., before the DST, correlated positively to cortisol levels at time point 2, following the DST ($p < 0.003$, rho = 0,49, i.e., a moderate effect size).

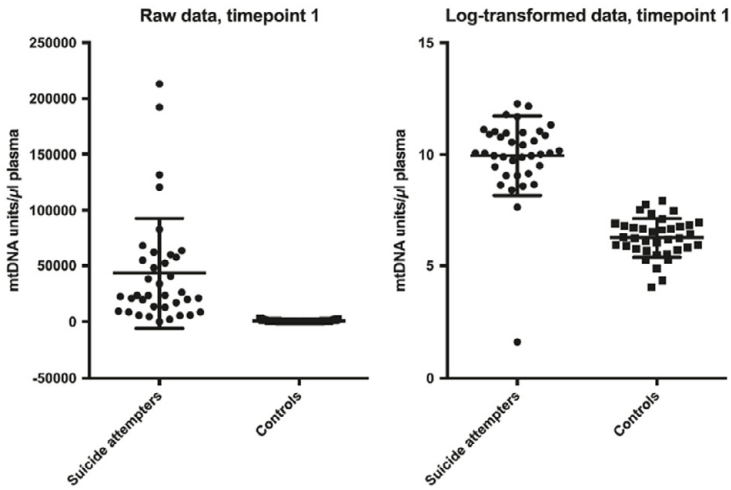

Figure 6. Ccf-mtDNA copy number. Skewed raw values on the right side, log-transformed values on the left.

Table 2. Correlations (Spearman's Rho correlation coefficients) between free-circulating plasma mtDNA units and post-DST cortisol in suicide attempters

	mtDNA units, time point 1	*mtDNA units, time point 2*	*mtDNA units, time point 3*
Post-DST cortisol 0800 hours	0.49*	0.17	0.11
Post-DST cortisol 1500 hours	0.11	0.07	−0.09

Abbreviations: DST, dexamethasone suppression test; mtDNA, mitochondrial DNA. *$P < 0.003$.

Figure 7. Correlations between plasma cortisol levels and ccf-mtDNAcn, following the DST.Comments on study 2

The results of the study showed i) a very large difference in ccf-mtDNA levels between suicide attempters and controls, and ii) that within the suicide attempter group, there was a significant correlation between higher ccf-mtDNA levels and post-DST cortisol levels. We speculated that the difference in ccf-mtDNA is likely not an effect of the suicide attempt *per se*, but rather the psychological (and to some extent physical) stress it entails on the individual. The suicide attempt occurred on average 12 days before blood sampling. There was no correlation between the number of days from when the study participant was included in the study (i.e., the number of "washout days", and ccf-mtDNA. This observation argues against the possibility that the suicide attempt is so stressful that it can be detected several days later by measurement of mtDNA. It also argues against the possibility that the physical trauma inflicted by the suicide attempt can be detected several days later, as such trauma in most patients would have healed. On this note, we recognize that partitioning of the suicide attempt into "violent", i.e. hanging, jumping from heights, etc; and "non-violent", in most cases meaning intoxication, might not be very meaningful as an intoxication might cause liver damage, which has been shown to produce an increase in ccf-mtDNA (128). It seems more likely that ongoing stress, perhaps related to both psychological (the stress of being admitted to psychiatric inpatient facility, meeting the concerns of loved ones after attempting to take your life, etc) and physiological (being more sedentary while admitted, eating differently, smoking more, sleeping less) produces the increase in ccf-mtDNA. This interpretation is also in line with the correlation between ccf-mtDNA and a less responsive, hyperactivated HPA-axis, although we cannot make any assumptions regarding causality. Thus, we conclude, it is likely that ccf-mtDNA is a generic biomarker of cellular stress, susceptible to release following both psychological and physiological/mechanical stress. This conclusion has later been reenforced by Trumpff et al., who in an experimental study demonstrated that ccf-mtDNA increase as consequence of both physical exercise and psychological stress(129).

Since the group differences in ccf-mtDNA were striking with almost non-overlapping groups, it was important to consider if any potential confounders could have contributed. Although the patients and controls had been included in the study at roughly the same time (patients were included between 1992 and 2001, controls in 1994), there were at least one known difference between patient and control samples: The control samples had at one time been thawed and re-frozen (except for one sample). Thus, it was possible that the group difference was a result of how the frozen plasma samples had been handled. However, there are several reasons to believe that the thawing of a plasma sample has no meaningful impact on the number of ccf-mtDNA copies within the sample. Firstly: DNA (both nucleic and mitochondrial) are known to be very stable molecules and are resistant to many naturally occurring denaturing processes. Secondly, there have been at least one earlier study investigating how ccf-mtDNA qPCR measurements might be affected of repeated cycles of freezing and thawing. The study reported that some bias might potentially arise from repeated freeze-thaw cycles, but that this bias were mostly

due to a few outliers, and that the agreement between measurements were generally good(130). Lastly, one of the control samples had not been thawed, and the ccf-mtDNA levels in this sample were on par with the control group median.

In addition to this methodological concern were the possibility of demographic differences between groups. Data was available on age, sex, BMI, current somatic disorder and use of medications, but lacking on smoking status, which is a potential confounder.

In conclusion, our main findings from this study demonstrate that i) ccf-mtDNA levels in suicide attempters are substantially higher than ccf-mtDNA levels in control subjects, and ii) among suicide attempters, there is a significant positive correlation between ccf-mtDNA and post-DST cortisol levels, indicating an interaction between mitochondrial and/or cellular processes and cortisol hyperactivity. We do not know however, what mechanisms that cause mtDNA release from the mitochondrion to the cytosol and from the cytosol to the extracellular space, nor do we know if ccf-mtDNA is a biomarker of mitochondrial dysfunction. In fact, later studies have suggested that ccf-mtDNA is more accurately described as a molecule that signals cellular stress(1) (please see the "General Discussion" section for an extended review of this topic).

Study 3: Circulating cell-free mitochondrial DNA, but not leukocyte mitochondrial DNA copy number, is elevated in major depressive disorder

As the results from Study 2 demonstrated very large differences in ccf-mtDNA levels between suicide attempters and controls, we wanted to investigate if these differences could be detected in a different cohort. We also wanted to learn more about the role of ccf-mtDNA and its potential benefits as biomarker of stress, and to study how ccf-mtDNA corresponded to mtDNA measured intracellularly, within PBMCs. The study cohort was composed of 50 unmedicated depressed individuals and 55 healthy controls. A subsample of the study subjects was started on antidepressant treatment (with an SSRI) during the trial and symptom ratings had been done at the beginning and end of the study period). This allowed us to study how mtDNA might relate to treatment efficacy. Levels of the antioxidative enzyme glutathione peroxidase (GpX), together with telomere length (TL) of the nuclear DNA in the PBMC had also been measured, making it possible to investigate how mtDNA relates to indices of cellular protection from oxidative stress, and cellular aging. Our study hypothesis was that i) both ccf-mtDNA and PBMC mtDNA levels would be higher in depressed individuals compared to healthy controls, as they are likely to experience more stress. We also hypothesized that ii) intracellular PBMC mtDNA and extracellular ccf-mtDNA correlated to each other positively, as increased stress on the intracellular environment will lead to activation of mechanisms of mtDNA extrusion (and consequently, increases in ccf-mtDNA).

Such stress would iii) likely cause downstream consequences of oxidative stress and activation of an antioxidative defence mechanisms, such as GpX, but nonetheless resulting in fastened cellular ageing (observable through shortened TL). Finally, iv) these changes would likely be reversed to some extent in individuals responding to SSRI treatment.

The MDD patients were all diagnostically well-defined through examination by a board-certified psychiatrist following inclusion. Symptom severity was evaluated with the 17-item Hamilton Depression Rating Scale (HDRS). All study participants were unmedicated at the time of inclusion, and also free of acute illness or infections thought to affect the results. They were also subjected to a urine toxicology test to screen for the use of narcotics. In the SSRI-treated subgroup, blood samples were drawn at baseline, and at end of week 4 and 8. HDRS scores at baseline and week 8 were compared and individuals with a rated improvement of at least 50% were defined as "responders".

There were no statistically significant demographic differences between depressed individuals and controls, except for tobacco use, which was significantly more common in the depressed group. Also, BMI among depressed individuals were trending towards being significantly higher when compared to controls. Among depressed individuals treated with SSRI, the average BMI of non-responders were bordering to be significantly higher than the average BMI among responders ($p = 0.05$). There were no differences in HDRS score between responders and non-responders.

Ccf-mtDNA were significantly higher among depressed individuals compared to controls ($t = 4.8$, $p < 0.00001$, Cohen's $D = 0.93$). This difference remained significant after adjusting for demographic covariates ($F = 20.6$, $p < 0.00002$).

However, PBMC mtDNA did not differ among the two groups, either when demographic covariates were adjusted for ($p = 0.65$) or when it was not ($p = 0.48$).

In the subsample treated with SSRIs, ccf-mtDNA levels in the non-responder group increased significantly over the 8-week period (Bonferroni adjusted $p = 0.02$), whereas it did not change significantly in the responder group ($p = 0.32$). The corresponding variations in the responder and non-responder group from baseline to week 8 could be mathematically described as a significant time x group interaction ($p = 0.02$).

Furthermore, when comparing ccf-mtDNA levels among non-responders at week 8 to those of controls at baseline, ccf-mtDNA levels were still significantly higher, whereas the difference between responders and controls, which was significantly higher among responders at baseline, had trajected to non-significance.

Again, no significant change in PBMC mtDNA levels could be detected when comparing the responder vs. non-responder groups over time (all $p > 0.4$).

There was no significant correlation between HDRS score and ccf-mtDNA or PBMC mtDNA, either cross-sectionally or over time in the groups treated with SSRIs (all $p < 0.1$).

In all subjects ccf-mtDNA and PBMC mtDNA did not inter-correlate (adjusted $p = 0.79$, unadjusted $p = 0.87$). Ccf-mtDNA (but not PBMC mtDNA), did correlate positively with GpX in all subjects ($r = 0.32$, unadjusted and adjusted $p = 0.001$). However, PBMC mtDNA (but not ccf-mtDNA) did correlate positively with PBMC TL (unadjusted: $r = 0.28$, $p = 0.005$, adjusted: $r = 0.38$, $p = 0.42$).

Comments on study 3

As in paper 2, the results from study 3 demonstrated an increase in ccf-mtDNA levels between MDD subjects and controls, with a large effect size (Cohen's $D = 0.93$). However, this was not the case for PBMC mtDNA, for which no difference between MDD subjects and controls could be established. Also, there was no inter-correlation between ccf-mtDNA and PBMC mtDNA, suggesting that mtDNA released within the cell cytosol are reflective of other processes than mtDNA released outside the cell. We speculate that PBMC mtDNA might reflect an increase in bioenergetic demand, whereas ccf-mtDNA is released to signal cellular stress and perhaps also as a pro-inflammatory molecule. This suggested function of ccf-mtDNA has long been popular in the scientific literature(27, 28, 30), although a recent paper have lately called this theory into question(1). Indeed, it is possible that there are several mechanisms by which mtDNA are released into the bloodstream. Apoptosis, or even cell necrosis following mechanical tissue trauma could be represent one aspect of such processes, whereas a more regulated release that does not concurrently imply cell senescence, might be a common factor for other types of cellular processes(1, 131).

This study also report higher ccf-mtDNA in the patient population, as was the case in study 2. However, the differences and effect-sizes are smaller. This could be due to several reasons, one of them being that this study population consisted of MDD patients at an outpatient clinic, who did not report any suicidal ideation or previous suicide attempt at the time of inclusion. This is highly speculative of course, and would need rigorous testing to be confirmed, as would other possible differences, such those relating to variance in methodology when obtaining an analysing the sample; ethnic and geographic variance; smoking status (which we did not have data on in study 2); time of day when blood samples were collected, BMI, etc.

Study 4: Blood-based Mitochondrial Respiratory Chain Function in Major Depression

In study 3, a correlation between ccf-mtDNA and major depression had been established. But as we speculated in that study, ccf-mtDNA probably does not

reflect mitochondrial energy production. Thus, we wanted to investigate how other markers, more reflective of mitochondrial energy producing capacity were affected in depressed individuals. In a previous study, a composite score relying on several enzymes involved in the mitochondrial respiratory chain (RC), together with mtDNA levels, had been shown to correlate to the subjective experience of stress among mothers to autistic children (22). In fact, the composite score, referred to as the Mitochondrial Health Index (MHI), showed a closer correlation to perceived stress than each of the individual markers. These markers included the enzymatic complexes I and IV of the RC; Citrate Synthase (CS); and mtDNA measured intra-cellularly and quantified as the number of copies per cell (mtDNA-cn). RC complexes components had been selected as they reflected functional aspects of mitochondria, whereas MtDNA-cn is a measure of mitochondrial content within the cell. These separate indices were used to create a composite estimate of the average energy output per mitochondrion, per cell, in the sample. In the paper by Picard et al., a low MHI correlated to high stress in otherwise healthy women. While these findings were novel and potentially interesting, our study was the first to investigate the MHI in MDD. As a subset of the individuals in our study started on SSRI treatment during the study, we were also interested in a possible correlation between the reported effect of antidepressant treatment, individual enzymatic activity measured, and the MHI. Our primary hypothesis was that MHI would be significantly lower among MDD subjects compared to controls. Also, we expected the MHI to increase and mitochondrial components to be significantly different among those individuals who responded to SSRI treatment, compared to non-responders.

All study participants gave written informed consent to participate in this study and were compensated for participating. The study subjects were MDD outpatients (n=47) recruited to the study through clinical referrals. Healthy controls (n=11) were recruited through ads in various media. Referred patients were assessed by a board-certified psychiatrist and diagnosis established through the Structured Clinical Interview (SCID-I). Individuals with bipolar disorder, Post-Traumatic Stress Disorder (PTSD), eating disorders, earlier psychotic episodes not related to depression, or substance use within 6 months of the time for the interview, were excluded. Healthy controls were screened for earlier psychiatric diagnosis and excluded if such had been present at any time in their life. All included study subjects had been assessed for (and found free of) potentially confounding somatic disorders, psychotropic agents, and drugs of abuse. Women of child-bearing age were asked to submit a urine sample for pregnancy test on the day of blood draw and clinical ratings. Venipuncture was performed in the morning, following rest and fasting. Depression symptom severity was assessed with the HDRS-25, subjective experience of psychological stress with the Perceived Stress Scale (PSS).

A subgroup (n = 33) of the included MDD subject underwent open label SSRI treatment for a total of 8 weeks. Primary outcome measure was change in severity

of depressive symptoms, assessed at baseline and after 8 weeks with the HDRS. Follow-up visits were scheduled at week 4 and 8 at which time additional blood samples were drawn. SSRI serum concentration was analysed in these samples to establish compliance. In all subjects undergoing SSRI treatment, the concentrations were within the expected range.

There were no significant demographic differences between MDDs and controls, and there were no significant differences regarding age, BMI or smoking between SSRI responders and non-responders. However, responders were more likely to be females.

Sex or smoking did not correlate to MHI or any individual mitochondrial marker, but BMI did ($r=0.28$, $p=0.03$). Also, MHI did not correlate to perceived stress or MDD symptom severity.

There was no significant difference in MHI between MDDs and controls. This was also true for the individual components of the MHI.

Among the patients treated with an SSRI, pre-treatment responders had significantly higher levels of mtDNA-cn ($p = 0.02$), higher CS ($p = 0.02$) and Complex I activity ($p = 0.01$).

Furthermore, complex I decreased among SSRI responders, but increased among non-responders ($p = 0.02$). A greater decrease in complex I over the 8-week period was correlated to greater improvement on the HDRS-25

Among all patients treated with SSRI, complex II (but none of the other individual markers, or MHI) increased significantly over the 8-week treatment period ($p = 0.02$).

Comments on study 4

In contrast to our main hypothesis, there was no statistically significant difference in MHI between MDDs and controls, nor did MHI increase significantly among SSRI-responders during the 8-week treatment period. This was a cross-sectional study, and mitochondrial adaptations to mood states are dynamic and may occur within hours to days(22). Thus, compensatory mechanisms could act to obscure the difference we had expected to find. For example, it is possible that a decrease in RC complex activity is compensated for by an increase in mitochondrial content over the course of a depressive episode. If so, the MHI would remain relatively unchanged, even if the individual components of the index would vary.

In post-hoc analysis, we did find some interesting correlations suggesting an effect of SSRI treatment on mitochondrial enzyme activity. Mitochondrial complexes I and IV have both been implicated in neuropsychiatric disorders(132, 133). Specifically, our findings suggest that higher mtDNA-cn (i.e., higher mitochondrial content) and CS activity predicts a better antidepressant response. This is true also

for complex I, where a decrease in activity during treatment also correlates to a greater treatment response.

That a decrease in Complex I activity correlates to an improvement in symptom ratings is seemingly counterintuitive. However, the RC is a highly complex and firmly regulated enzymatic machinery. It is possible that psychotropic medications can affect the RC, and this has in fact been reported previously(134). One study reported that administration of fluoxetine decreased complex I activity in some, but not all, brain regions (135, 136). Such complex and region-specific mechanisms are hard to disentangle and our findings should be regarded as preliminary. SSRIs are named after their function in the serotonergic neuronal synapse, but are structurally quite heterogenous molecules, and the effect of different SSRIs on mitochondria and the RC have not been extensively studied. It is also important to point out that this study come with certain other limitations: Already mentioned is the cross-sectional design which might be unsuitable to detect highly dynamic mitochondrial enzymatic processes. Also, the study sample was quite small, especially those study subjects who were started on SSRI treatment.

The study also has several strengths: Study subjects were clearly defined and screened for several potentially confounding factors. Furthermore, we used several measures of mitochondrial function and compounded them in the MHI, which provides a multi-faceted representation of mitochondrial function, available from a blood sample.

We conclude that while overall decreased mitochondrial respiratory capacity does not seem to characterize MDD as a group, antidepressant treatment might affect the RC and to some extent predict antidepressant response.

Study 5: Plasma circulating cell-free mitochondrial DNA in depressive disorders

In the "Genes, Depression and Suicidality" (GEN-DS study, patients diagnosed with an affective disorders or anxiety disorder are remitted for genotyping of the Cytochrome P450 (CYP)2D6 and CYP2C19 genes. The overall aim of the project is to investigate how knowledge of stress vulnerability and pharmacogenetics can be used to guide treatment for depressed individuals, especially those with suicidal ideation. This larger aim can be divided into smaller objectives, one being to investigate correlations between MDD, suicidality and biological stress factors (such as mitochondrial dysfunction).

In study 2-3, we had found that ccf-mtDNA were significantly higher among suicide attempters and MDD patients compared to controls. However, the study samples had been quite small, and patients were unmedicated. To better understand the role of ccf-mtDNA in depressive disorders, we wanted to measure this biomarker in a large and clinically representative cohort of patients with difficult-to-treat

depression(137), many of whom were in psychopharmacological treatment. As studies have shown that mitochondrial function can be affected by psychotropic medications(138-140), which the results from study 4 also indicated, we were interested in studying possible interactions between ccf-mtDNA and antidepressant and mood-stabilizing agents. Also, the cohorts we had studied earlier consisted of individuals who either had made a suicide attempt, but were diagnostically heterogenous (as in study 2), or individuals who were clinically depressed, but were not actively suicidal (as in study 3 and 4). Based on the results from our own previous studies, and others(86, 88, 141), we hypothesized that ccf-mtDNA would be significantly different in patients compared to controls. We also wanted to explore the potential effects of psychotropic medication, certain symptom profiles) on ccf-mtDNA. As ccf-mtDNA have been conceptualised as a pro-inflammatory marker (27-30, 131, 142-144), we were especially interested in correlations with certain depressive symptoms that previously have been associated to indices of systemic inflammation: CPRS-items "lassitude", "fatiguability", "reduced appetite" and "reduced sleep" (145). By summarizing the graded scores from these separate items, we created an "inflammation composite score".

Study 5 included 281 patients and 49 healthy controls referred to the GEN-DS project from specialists or residents in psychiatry between 2012 and 2020. Patients with a diagnosed affective disorder were referred to the project from several psychiatric clinics in southern Sweden. Most of these patients are medicated with one or several psychotropics and have had at least one insufficient treatment response. Patients with a body mass index less than 15, ongoing pregnancy or current liver disease were excluded. The patients underwent a structured and comprehensive diagnostic procedure based on the MINI 6.0, the Structured Interview for DSM Axis II Personality Disorder (SCID-II), together with semi-structured questions on psychosocial circumstances, life events, psychiatric symptoms and treatments, substance use, suicidal and self-harming behaviour. Psychiatric symptoms were rated with the CPRS. Interviews were performed by specialists or psychiatric residents with at least three years of training. The healthy controls (n = 49) were recruited through ads in social media and newspapers. If eligible for inclusion, HCs were screened with MINI to ensure they did not have any current or previous psychiatric disorders. If HCs had any severe ongoing disease or infection, psychotropic or immunomodulating treatment, pregnancy, or addiction they were excluded. Venipuncture was performed in the morning, following fasting. Ccf-mtDNA measurements were performed with qPCR (see pp. 37-39).

Depressed individuals were more likely to be smokers and have a higher BMI compared to healthy controls. Treatment with mood stabilisers were more common among patients with remitted depression, compared to patients with current depression. Among patients with a current mood disorder, recurrent unipolar depression was most the common diagnosis, followed by chronic depression. Individuals with bipolar depression constituted only about 10% of the patients with

a current mood disorder. Among the patients with a remitted mood disorder, unipolar depression was also the most common diagnosis, but bipolar depression constituted about 25% of the patients in this group.

Ccf-mtDNA was not associated with any demographic variable; nor was it associated with any particular type of somatic disorder, or the burden of several simultaneously ongoing somatic illnesses; or storage time in the freezer.

Mean levels of ccf-mtDNA were lower among patients with ongoing and remitted depression compared to controls. However, ccf-mtDNA was not significantly different when patients with an ongoing depression was compared to patients with remitted depression. Ccf-mtDNA in patients with bipolar disorder did not differ from patients without bipolar disorder.

Regarding correlations between ccf-mtDNA and the use of psychotropic medication, there was no difference between individuals taking antidepressants and individuals not taking antidepressants. This was also true for antipsychotic medications, and no psychotropic medications at all. However, ccf-mtDNA was significantly lower among patients treated with mood stabilising medications (lithium, lamotrigine and valproic acid), compared to those not taking mood stabilisers.

There were no correlations between overall depressive or suicidal symptoms and ccf-mtDNA, but we did find a positive correlation between the “information composite score”, and ccf-mtDNA, although the effect size was small. Finally, we did not find any difference in ccf-mtDNA between patients who had made an earlier suicide attempt, and those who had not.

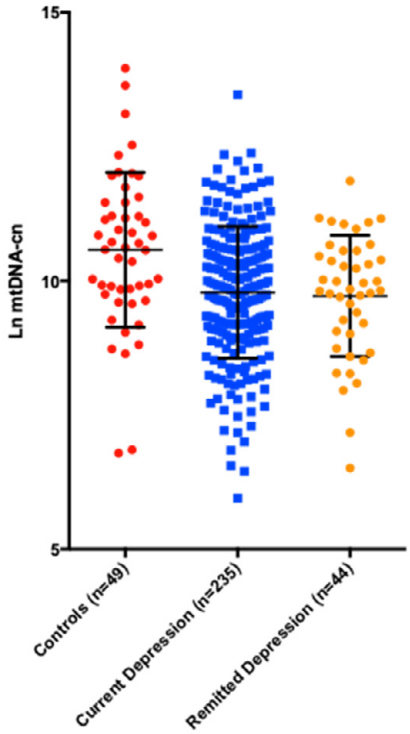

Figure 8. Log transformed ccf-mtDNA in controls, current depression, and remitted depression. Error bars represent mean, ±SD. The group effect was significant (F=8.3, p<0.001, adjusted for age and sex).

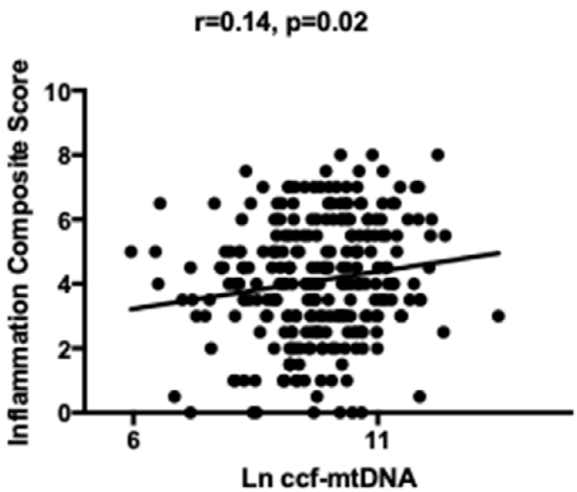

Figure 9. Correlation between ccf-mtDNA and "Inflammation Composite Score" calculated by summarizing CPRS-items "lassitude", "fatiguability", "reduced appetite" and "reduced sleep". Both patients with current and remitted depression were included.

Comments on study 5

In study 2 and 3, we found that ccf-mtDNA was increased in patients with depression and suicidality. Although the main results in study 5 were in the opposite direction, studies from other groups have reported higher, lower and no change in levels of ccf-mtDNA in depressed patients compared to controls (86, 88, 141). It should be pointed out however that in these studies, somewhat different methods were used, and thus they are not entirely comparable. In a study by Stertz et al., (141), demonstrating no ccf-mtDNA difference in twenty patients with bipolar disorder compared to controls, serum samples were used for ccf-mtDNA analysis. In the studies from our group (study 2, 3 and 5 in this thesis), plasma samples were consistently used. Serum samples likely yields higher ccf-mtDNA levels compared to plasma, since in serum samples blood platelets have had time to become activated. In this process, platelets release both whole, respiratory-competent mitochondria, and mtDNA, resulting in higher levels(1). In the study by Jeong et al. (88), also using serum samples, there was no significant difference in ccf-mtDNA levels when bipolar patients were compared to controls. The authors did report a negative correlation between depressive symptom scores and ccf-mtDNA, but this correlation lost significance when the analysis was corrected for multiple comparisons. Finally, in the study by Kageyama et al. (86), lower ccf-mtDNA levels were demonstrated in patients with depression and bipolar disorder, compared to healthy controls. This study used plasma samples but might still have overestimated ccf-mtDNA levels as the samples were spun only once at 1500 *g* x 10 minutes, possibly leaving larger membrane bound vesicles containing ccf-mtDNA in the plasma. In the studies presented in this thesis, plasma samples were spun a second time at much higher forces (10 000 g x 10 min), reducing the possibility of "vesicle contamination". However, both the study by Kageyama et al., and the study by Jeong et al. share one similarity with study 5 in this thesis: The patients were treated with psychotropic medications. This is also the main discrepancy between study 5 and studies 2 and 3 in this thesis: In the earlier studies patients were unmedicated, but in study 5 the vast majority of the patients received one, or a combination of several, psychotropics.

One very interesting finding in study 5 was that ccf-mtDNA levels were lower in patients treated with mood stabilisers. Earlier studies on the effect of mood stabilisers on mitochondria suggest that lithium increases mitochondrial RC activity(146, 147), and in an animal model of mania, inhibition of RC complexes has been demonstrated(148). It has also been suggested that lamotrigine have neuroprotective effects, mediated by mitochondrial mechanisms(149).

In addition to medication effects, we also found preliminary evidence that ccf-mtDNA might be of importance for developing "inflammatory symptoms of depression", a depression subtype that might respond better to adjunctive treatment with anti-inflammatory medications(150, 151). If future studies give additional

support to this possibility, ccf-mtDNA might be suited as a biomarker for evaluating treatment response in this subgroup.

The study comes with strengths, such as a large and well-defined patient cohort. But there are also some limitations that should be brought to reader's attention: The study was primarily designed for testing another hypothesis, and some variables that potentially could have affected ccf-mtDNA levels and confounded or results were not available to us. Examples of such variables are perceived stress, sleep pattern and quality, physical exercise, etc. Ideally, this would have been recorded. Also, the study was not powered *a priori* for detecting ccf-mtDNA differences between patients and controls (although, based on the results from study 2 and 3, the sample size in study 5 was well beyond what would have been required). However, the post-hoc analyses of the influence of psychotropics on ccf-mtDNA, some of the subgroups might have been too small. For instance, we were not able to detect a difference in ccf-mtDNA between those few patients (7%) *not* taking any medications, and all other patients (who did take medications). Future studies investigating the influence of medications on ccf-mtDNA should include larger sample populations.

General discussion

Clinical usefulness of mitochondrial markers

As stated in the introduction, there have been an increasing interest in mitochondria as both a possible contributor to the pathophysiology of depression, and as a treatment target. Since most studies on this topic, including our own, use cross-sectional designs, experimental and longitudinal studies are needed to better understand *how* mitochondria are involved in depression. In order to readily assess mitochondrial involvement in human depression and suicidality in ways that are safe, accessible and reliable, appropriate biomarkers should be identified (152). One of the main contributions of this thesis is that we assessed the utility of several novel biomarkers of cellular stress and mitochondrial function. These biomarkers reflect various aspects of mitochondrial biology and cellular stress in depressed patients and suicide attempters. Furthermore, correlations between these markers and HPA-axis activity, telomere length, and markers of oxidative stress were investigated in order to better understand how mitochondrial function and cellular stress interact with other biological systems potentially involved in depression and suicide. While some of our results are novel and may provide important clues into the pathophysiology of depression and suicidality, several outstanding questions remain before they can be translated to the clinic. There are several general conditions that should be fulfilled for a biomarker to be clinically useful: First and foremost, it should be *specific* for the condition and *sensitive* enough to enable early detection of the condition. Furthermore, it needs to be *accessible,* i.e., convenient to sample (ideally from blood, urine, or saliva), store and prepare for analysis. It should be *responsive* to treatment, i.e., the biomarker levels should vary according to treatment outcome and/or natural course of the disorder. Ideally it should also give some *insight* to the biological pathological mechanisms causing the disorder and be inexpensive to analyse(153).

So how well do the mitochondrial markers meet the conditions listed above? Based on the existing literature(30, 128, 154-161), none of the biomarkers assessed in this thesis are likely to be *specific* for depression or suicidality. Both suicidal behaviour and depression are heterogenous conditions, especially from a biological perspective(162). The current categorical approach (163) for diagnosing psychiatric disorders has been criticized(164) for not being able to capture this heterogeneity, thereby impairing the progress towards more valid and precise diagnoses. Several remedies for how to fix these issues have been proposed, but most of the suggestions rely on improvement of our current knowledge of the biological mechanisms underlying psychiatric disorders (165-167), for which biomarkers are crucial. In this regard, the markers described in this thesis might not be specific for depression as it

is currently defined, but possibly helping to define subtypes of depression in the future.

Concerning *sensitivity* of the most studied biomarker in this thesis, ccf-mtDNA, there is no real consensus on cut-offs to define values within the normal range. Both intra- and interindividual ccf-mtDNA levels can vary substantially over the course of a few days in response to both psychological stress and physical exercise, making comparisons of studies difficult (129, 154). Also, the methods used to isolate mtDNA from whole blood can have a large impact on the results (as discussed in the "Comments on study 5" section on p. 61). In order to better define a normal range of ccf-mtDNA in humans, larger studies using established methods for sampling and analyzing ccf-mtDNA are necessary.

Concerning *accessibility,* the choice of mitochondrial assessment method is essentially a trade-off between validity and feasibility. Direct measurement of cellular respiration with a high-resolution respirometer such as an Oxygraph-2k (Oroboros instruments, Innsbruck, Austria) will likely yield a highly valid approximation of mitochondrial "function", as it measures mitochondrial respiration rate with high accuracy (168). But this and similar methods require highly specialized equipment, trained lab technicians, and living cell samples. Understandably, such limitations pose severe logistical and financial problems when investigating mitochondrial function in humans. There have been successful exemptions to this general rule: Karabatsiakis et al. demonstrated that mitochondrial function was decreased in a small cohort of clinically depressed individuals (169), using high resolution respirometry. However, most human studies investigating mitochondrial function in psychiatric disorders to date have relied on biochemical assays designed to yield valid results from frozen and thawed samples derived from whole blood. MtDNA is relatively easy to isolate from whole blood, but as demonstrated in study 3, intracellular PBMC mtDNA and extracellular ccf-mtDNA reflects entirely different mitochondrial processes(1). Whereas intracellular mtDNA indirectly reflects mitochondrial respiratory capacity, ccf-mtDNA does not. Furthermore, the two markers are not correlated to each other, as we and others have demonstrated(170).

What *insights* to the mechanisms of depression does then ccf-mtDNA reflect? What causes ccf-mtDNA release from cells, and what consequences follow? In many studies, ccf-mtDNA have been suggested as a pro-inflammatory marker (27-30, 131, 143, 144) (see section on "Intra- and extracellular mtDNA", p. 21), but as recently pointed out by Trumpff et al., most circulating mtDNA molecules are in fact not freely circulating, but contained in vesicles where it is not available for receptors of the innate immune response to recognize(1). One possibility is in fact that at least some fraction of ccf-mtDNA isolated from a blood sample is contained in whole mitochondria, in the process of translocating from one to cell to the another(171). This transfer of whole mitochondria (together with its genome) have recently been given an official term: "Momioma" (mobile functions of mitochondria

and mitochondrial genome), and likely occurs in response to stress signals from neighbouring cells(172). Whole mitochondria are then transferred either through tunnelling nanotubes, gap junctions, or extruded from the healthy cell, enveloped in an extracellular vesicle(171). The transfer of ccf-mtDNA in membrane-bound structures helps explain the fact that ccf-mtDNA is abundant in blood of healthy individuals who show no signs of inflammation. This suggest that some ccf-mtDNA is either shielded from receptors of the innate immune response system, or that only a certain type of ccf-mtDNA have pro-inflammatory properties(173, 174). In study 2 and 3, we demonstrated that ccf-mtDNA is significantly higher among unmedicated suicide attempters and depressed individuals. The natural question that follows is of course why? One possibility discussed in study 2 was that mechanical trauma inflicted by the suicide attempt might trigger mtDNA release. Mechanical trauma has been associated with increased ccf-mtDNA levels(28, 29, 175), but violent suicide attempt methods was not associated with higher ccf-mtDNA in study 2. However, damage to the cell might just as well be induced by intoxication with harmful substances. In cells undergoing apoptosis, the mitochondrial inner membrane seems to undergo structural reorganisation, whereby mtDNA is released(176), so the possibility that mtDNA is released from the cell as direct consequence of the suicide attempt cannot entirely be ruled out. Another possible explanation for the differences in ccf-mtDNA levels between patients and controls in study 2-3 is that the psychological stress the patients were subjected to, triggered mtDNA release. That psychological stress can trigger mtDNA release was shown in an article by Hummel et al., where ccf-mtDNA levels in healthy young men subjected to the TSST had doubled only two minutes after the test(154), suggesting that ccf-mtDNA could be used as a biomarker of psychosocial stress. Similar results were demonstrated in one study were healthy middle-aged men and women underwent a brief stress paradigm on two separate occasions, inducing a 2-3 fold increase in ccf-mtDNA on both occasions(177). This possible role of ccf-mtDNA as a biomarker of psychological stress might constitute its major quality and should be studied further in the future.

How *responsive* are mitochondrial biomarkers? In papers 2 and 3, we found significantly higher ccf-mtDNA in unmedicated patients with depression and suicidal behavior, while this was not replicated in paper 5 including predominantly medicated patients with difficult-to-treat depression. In paper 4, MHI did not differ between patients and controls, or between SSRI responders and non-responders, but some of the individual components did: Responders had significantly higher levels of mtDNA-cn, higher CS and Complex I activity before treatment. This raises the possibility that psychotropic medication is an important confounder of the relationship between ccf-mtDNA and depression, but also that some of these markers might predict treatment response. In further support for this, we found, in paper 5, that those patients who were treated with mood stabilizers, including lithium had lower ccf-mtDNA than other patients, and among those patients who were treated with a combination of lithium and SSRI, ccf-mtDNA levels was lowest.

Most literature concerning the effects of mood stabilizers on mitochondria concerns lithium, which may have protective effects on mitochondria, as several lines of preclinical evidence suggest: For instance, lithium regulates several mitochondrial functions in neurons, such as biogenesis, bioenergetics, permeability, motility, and apoptosis(178). Furthermore, a post-mortem examination of patients with bipolar disorder found that lithium increased frontal cortex neuronal activity in RC I, II and III (146), and lithium has also been shown to increase electron transport chain complex I in leukocytes from bipolar patients (179). In order to establish the interactions between psychotropic medications and mitochondria in a clinical human cohort, however, large study populations are probably needed. Although study 5 included 281 patients, the subgroups using a specific type of psychotropic medication were often too small for making such analyses. Also, many patients were using more than one psychotropic medication, obscuring the specifics of potential interactions. However, such interactions could to some degree be explored in cell studies, relying on respirometry measures. Such studies could inform on the degree of potential interaction of specific psychotropic medications.

To conclude, the clinical usefulness of these biomarkers is still limited, but they do inform on biological processes that seem central for understanding the nature of depression and suicidality. Thus, they might have an important role in future studies of stress signaling, and possibly, when better established, in clinical diagnostic procedures and evaluation of treatment response.

Limitations

We recognize that the studies presented in this thesis come with certain limitations, as outlined in the review of the studies on page 43-62. Most important of these limitations is that the study samples are relatively small (except for study 5), which should caution the reader that interpretation of results from some of the *post hoc* analyses should be considered preliminary and in need of replication. The purpose of presenting these results is mainly to generate hypotheses that could be confirmed in future studies. Furthermore, all studies included in the thesis are cross-sectional (apart from the sub-group analyses of antidepressant treatment response in study 3 and 4). Mitochondria are highly dynamic organelles, inspiring to longitudinal studies. Finally (and more of a general limitation to this field of research), there are no guidelines on how to collect, store and analyze several mitochondrial markers, obstructing comparison of studies and facilitating misunderstanding.

Conclusions and future directions

Our studies have shown that depressed and suicidal patients exhibit alterations in mitochondrial markers, compared to individuals without any current or previous psychiatric disorder. Furthermore, our results also show that mitochondrial stress signaling are correlated to hyperactivity in neuroendocrine cortisol release, cellular aging, and indices of increased oxidative stress. However, the interactions between psychological states and biological processes are highly complex. We propose that mitochondrial function and mitochondrial stress signaling may be more closely related to certain aspects of depression, rather than a depression diagnosis *per se*. Such aspects include, but are not limited to, antidepressant treatment response, specific symptom profiles and the use of specific psychotropic medications. Future studies should pursue these hypotheses with the ultimate goal to confirm (or refute) the clinical usefulness of these biomarkers. Such studies would likely demand larger and clearly defined patient cohorts (including data on confounders likely to interact with mitochondria); a study design that allowed for repeated blood sampling; and sample collection procedures and assays that were chosen to limit the possibility of measurement error.

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Paper 1

PLOS ONE

RESEARCH ARTICLE

Six autoantibodies associated with autoimmune encephalitis are not detectable in the cerebrospinal fluid of suicide attempters

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Abstract

Previous findings suggest a link between neuroinflammatory processes and suicidality. Despite several lines of evidence supporting this link, including increased pro-inflammatory markers in blood-, cerebrospinal fluid (CSF)- and in post-mortem brain samples from suicidal individuals, the underlying mechanisms remain poorly understood. In this pilot study, we explored the possibility that autoimmune encephalopathies might be found among suicide attempters. We analysed the presence of six different autoantibodies (*N*-methyl-D-aspartate receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid receptor, the γ-amino-butyric acid B-receptor, the leucine-rich, glioma-inactivated 1, the contactin-associated protein-like 2, and the dipeptidyl-peptidase-like protein-6), all previously associated with psychopathology, in CSF samples from 29 unmedicated suicide attempters. Five of these subjects had high CSF/serum albumin ratio, indicative of increased blood-brain-barrier permeability. We were not able to detect any of these autoantibodies in the CSF samples. These pilot data do not support a role for autoimmune encephalopathies in suicidal behaviour, although the presence of lower levels of these autoantibodies cannot be ruled out in these patients.

Introduction

Several lines of evidence suggest that neuroinflammatory processes are involved in the pathophysiology of suicidal behavior [1]. Immunomodulating therapies such as interferons, used to treat certain malignancies and infections, may trigger symptoms of depression and suicidality [2–4]. Suicide attempters display increased levels of pro-inflammatory markers in blood [5, 6] and cerebrospinal fluid (CSF) [7]. Moreover, impulsivity, a personality trait associated with

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high risk for suicidal behavior, has been associated with inflammation [8]. In further support of a link between neuroinflammation and suicide, post-mortem studies have reported microgliosis [9] and elevations of pro-inflammatory cytokine-messenger-Ribonucleic acids in the brains of suicide victims [10]. These initial findings were recently synthesized in a meta-analysis providing support for a link between suicidality and aberrant cytokine levels in blood, CSF, and postmortem brain samples [11].

The underlying pathobiology behind neuroinflammation in suicidality is not fully understood. Suggested causal mechanisms include latent infections such as *Toxoplasma gondii* [12–14], as well as allergies and asthma [15, 16]. Importantly, autoimmune mechanisms have not been well studied in relation to suicidality. Increased suicide rates have been reported in individuals with autoimmune disorders such as systemic lupus erythematosus [17], multiple sclerosis [18], and celiac disease [19]. Several lines of research over the last decade have shown that autoimmune encephalopathies may generate various types of psychiatric symptoms including psychosis, depression, confusion, agitation, and emotional instability [20, 21]. A few studies have reported detection of autoantibodies in blood samples from subjects with schizophrenia [22, 23], although not all are in agreement [24, 25]. Furthermore, increased CSF levels of immunoglobin G (IgG), with affinity for dopaminergic receptors, have been reported in suicide attempters compared to healthy controls [26]. Moreover, Kruse et al. reported that suicidal behavior was one of the symptoms presented by psychiatric inpatients seropositive for autoantibodies, and in those with *N*-methyl-D-aspartate receptor (NMDAR) IgG in CSF [27].

The etiologic mechanisms behind the generation of autoantibodies in individuals with psychiatric manifestations are not fully understood, but may involve a compromised blood-brain-barrier (BBB) [28].

In the present study we set out to detect autoantibodies in CSF samples from suicide attempters, a group often characterized by depressive symptoms, emotional instability, and even psychotic symptoms. The main purpose was to explore and develop preliminary data on the possibility that autoimmune processes may be involved in suicidal behavior. To this end, we analyzed six different autoantibodies in CSF samples from 29 suicide attempters, randomly selected from a larger cohort, employing an assay used for clinical diagnosis of autoimmune encephalitis. Within this larger cohort of suicide attempters, CSF/serum albumin ratio, an indicator of BBB integrity, has been quantified and this data have been previously published [29]. In the present study, we used CSF since it is considered to be a more sensitive medium for autoantibody detection compared to serum [27, 30].

Methods

Ethical approval

All parts of this study were approved by the Lund University Medical Ethics Commitee. All subjects gave verbal informed consent to participate in the study. Written informed consent was not required per the ethical approval. Consent was registered in the patients' chart. Subjects who, in the investigator's judgment, lacked the ability to make an informed decision regarding study participation were not included in the study.

Subject recruitment

Between 1987 and 2001, 29 subjects were enrolled in the study on admission to Lund University after a suicide attempt, as defined by Beck et al. [31]. Suicide attempters were evaluated by a certified psychiatrist for DSM axis I and II diagnoses [32]. Depressive symptoms were rated using the Montgomery-Åsberg Depression Rating Scale [33]. The 29 subjects

included in this pilot study were randomly selected from a larger cohort of suicide attempters, that has been described in more detail elsewhere [7]. All subjects underwent a general physical examination before the lumbar puncture, and they were all somatically healthy except for two subjects with migraines, and two subjects with gastritis. Although some of the subjects had previously been treated with psychoactive medications, they did not receive any antidepressants or antipsychotics during a washout-period before the lumbar puncture. Occasional doses of benzodiazepines were allowed during the washout. Lumbar punctures were performed in the morning between 8 am and 9 am, after a night of fasting and bed rest. CSF was collected from the L4-L5 interspace using a standardized protocol, and immediately stored at -80˚C. Twenty of the 29 CSF samples had been previously thawed/frozen at least once before the analyses.

Assays

Six different autoantibodies previously implicated in autoimmune encephalitis [20] were analyzed; NMDAR, the α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid receptor (AMPAR), the γ-amino-butyric acid B-receptor (GABAbR), the leucine-rich, glioma-inactivated 1 (LGI1), the contactin-associated protein-like 2 (Caspr2), and the dipeptidyl-peptidase-like protein-6 (DPPX). These autoantibodies, associated clinical syndromes and frequent symptoms are summarized in Table 1 (adapted from Wandinger et al 2011 [34]).

Antibody detection was done using the Autoimmune Encephalitis 6 Biochip mosaics (EUROIMMUN, Lübeck, Germany) by trained laboratory personnel at a specialized diagnostic laboratory affiliated with Lund University Hospital (Wieslab, Malmö, Sweden). Each biochip contains transfected cells expressing a specific antigen (NMDAR, AMPAR, GABAbR, LGI1, Caspr2, and DPPX). Biochip mosaics were incubated each with 30 μl of un-diluted CSF for 30 minutes at room temperature, washed with PBS-Tween, and immersed in PBS-Tween for 5 minutes. Bound antibodies were stained with fluorescein isothiocyanate–labeled goat anti-human IgG, (Euroimmun) for 30 minutes at room temperature. Slides were washed again with a flush of PBS-Tween and then immersed in PBS-Tween for 5 minutes. Drops of PBS-buffered glycerol were placed onto a cover glass, and the biochip slides were embedded in this mounting medium simultaneously and examined by fluorescence microscopy. Positive and negative controls were included with every test procedure. Autoantibodies

Table 1. Autoantibodies tested in the present study, associated clinical syndromes and frequent symptoms (adapted from Wandinger et al 2011).

Autoantibody	Clinical syndrome	Frequent symptoms
Anti-glutamate receptor (type NMDA)	Anti-glutamate receptor (type NMDA) encephalitis	Psychosis, memory-/language impairment, seizures, impaired consciousness, dyskinesia, movement disorders, dysautonomia, hypoventilation
Anti-glutamate receptor (type AMPA)	Limbic encephalitis, atypical psychosis	Memory deficits, confusion, disorientation, seizures, agitation, aggressive behaviour
Anti-GABAB receptor	Limbic encephalitis	Seizures, confusion, memory deficits, behavioural disorders, paranoia, hallucinations
Anti-LGI1	Limbic encephalitis	Epilectic seizures, memory deficits, confusion, dis- orientation, hyponatriaemia, myoclonus, dysautonomia
Anti-CASPR2	Neuromyotonia, Morvan's syndrome, Limbic encephalitis	Peripheral neuronal hyperexcitability, muscle spasms/ fasciculations/myokymia, seizures, memory deficits, confusion, disorientation, neuropathic pains, sleeping disorders, dysautonomia, weight loss
Anti-DPPX	Autoimmune encephalitis	Anxiety, forgetfulness, confusion, hallucinations, muscle spasms, tremor and pleocytosis (in CSF)

Abbreviations: NMDA: N-methyl-D-aspartate, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, GABA: γ-amino butyric acid, LGI1: leucine-rich glioma-inactivated protein 1, CASPR2: contactin-associated protein 2, DPPX: dipeptidyl aminopeptidase-like protein 6

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against the corresponding antigen react specifically with the corresponding transfected cells and results are given as positive or negative. NMDA receptor autoantibodies react specifically with the corresponding transfected cells and induce a fine granular cytoplasmic fluorescence, while the cell nuclei are only slightly stained. Antibodies against AMPA receptors, CASPR2, LGI1, and GABAB1/B2 receptors react specifically with the cytoplasm of the corresponding transfected cells inducing a cytoplasmic fluorescence, with some fluorescence of the cell membrane, while the cell nuclei are only slightly stained. Antibodies against DPPX react with the transfected cells of the test substrate. They produce a spread, smooth to fine-speckled cytoplasmic fluorescence, partly with fluorescence of the cell membrane. The cell nuclei are only slightly stained.

Samples with no autoantibodies or negative control cells results in no fluorescent staining. The test results were interpreted manually by extensively trained personnel by reading the fluorescence of fixated cells in a microscopy and results are reported as positive or negative. There are no predetermined cut-off values, since a negative result will show no fluorescence.

Albumin was determined in serum and CSF as previously described [29]. CSF/serum albumin ratio was calculated as CSF albumin (g/l)/serum albumin (mg/l).

Results and discussion

Demographics

Demographic and clinical characteristics of the study participants are summarized in Table 2. Approximately two-thirds of the subjects had Axis II co-morbidity, cluster B being the most frequent specifier (n = 10).

Most of the subjects (n = 21) had made a non-violent suicide attempt, e.g. intoxication, while the remainder (n = 8) had made a violent suicide attempt, e.g. carbon monoxide poisoning or jumping in front of a train [35]. Ten of the subjects had attempted suicide more than once and for 19 subjects this was the first attempt.

Table 2. Demographic and clinical characteristics of study participants (n = 29).

Age (mean ± SD)	41 ± 14
Sex	13 men, 16 women
BMI (mean ± SD)	24 ± 4
Number of wash-out days (mean ± SD)	12 ± 7
MADRS score	17 ± 11
Principal Axis 1 diagnosis (n)	• MDD = 8 • Dysthymic disorder = 1 • Adjustment disorder = 5 • Depression NOS = 5 • Substance use disorder = 2 • Schizoaffective disorder = 1 • Psychosis NOS = 1 • No axis 1 disorder = 6
Axis II co-morbidity (n)	19

Abbreviations: MADS = Montgomery-Åsberg Depression Rating Scale; BMI = Body Mass Index; DSM = Diagnostic and Statistical Manual of Mental Disorders; NOS = Not Otherwise Specified

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Autoantibodies in CSF

None of the following autoantibodies were detected in any of the CSF samples: NMDAR, AMPAR, GABAbR, LGI1, Caspr2, DPPX.

CSF/serum albumin ratio

Five subjects had increased CSF/serum albumin ratio, in relation to age-adjusted reference values previously described [36]. For individuals <45 years values above 6.8 were considered high, while the cut-off was 10.2 for those aged 45 and over.

Interpretation and discussion of results

In this pilot study we investigated, the presence of autoimmune antibodies in CSF samples from a group of recent suicide attempters. Given that neuroinflammation has been implicated in the pathophysiology of suicidality [1], this is an important research question that needs to be pursued. Although classical autoimmune encephalopathies are typically associated with aberrant neurological signs and symptoms, such as seizures, affected motor functions and speech, there is also a growing interest in autoimmune encephalopathies that may present with "pure" psychiatric symptoms. The present sample was randomly selected from a larger cohort of somatically healthy and medication-free recent suicide attempters. We tested six different autoantibodies previously implicated in clinical encephalopathies characterized by a psychiatric symptoms [20]. The findings of the present study were negative; we did not detect any of these autoantibodies in any of the CSF samples. These results, however, should be interpreted with some caution as outlined below.

Various psychiatric manifestations have been documented in subjects with anti-NMDAR encephalitis and other autoimmune encephalitis. These include psychotic symptoms, cognitive impairment, depression, irritability, and personality disturbances [37, 38]. Fewer studies have investigated the presence of autoantibodies in primary psychiatric cohorts. The available studies have detected NMDAR antibodies in blood samples from subjects diagnosed with schizophrenia [22, 23], although there have also been negative studies [24, 25]. Bergquist et al showed that suicide attempters have increased CSF levels of immunoglobin G with affinity for dopaminergic receptors (DA-IgG) compared to healthy controls [26]. Comparability between that study and the present one is, however, limited since different assays were used and Bergquist et al. measured one specific antibody (DA-IgG) while we explored six different autoantibodies implicated in autoimmune encephalitis.

In those cases autoantibodies are detected in individuals with psychiatric and neurologic manifestations, their origin is not fully understood. Several potential mechanisms have been proposed including paraneoplastic processes, past influenza infections and genetic susceptibility [28, 39]. Moreover, it has been hypothesized that a compromised BBB may facilitate transportation of autoantibodies from the periphery to the brain [28]. In our sample, approximately 17% of the subjects had high CSF/serum albumin ratio, indicative of increased BBB permeability, yet CSF autoimmune antibodies were not detectable in any of these subjects. Our findings do not support the involvement of impaired BBB integrity in autoimmune processes in psychiatric patients, although larger case series are clearly needed in order to definitely confirm or refute this hypothesis.

The present study is among the first to investigate autoantibodies in CSF samples from psychiatric subjects. An important strength of the study is the inclusion of a well-characterized sample of unmedicated recent suicide attempters. The present study, however, also comes with several caveats. Firstly, the sample size was relatively small, thus any conclusions must be considered preliminary and the results should serve primarily as pilot data for testing in future

larger scale studies. Even though the test panel that was used included six different autoantibodies, thus increasing the possibility of detection, the number of subjects tested might have been insufficient. Secondly, the assays used in our study were originally designed as a diagnostic tool for targeted populations of neurology patients where autoimmune processes in the brain are suspected. Thus, we cannot rule out that a more sensitive experimental assay would have been able to detect more subtle levels of autoantibodies. Future studies might consider using experimental assays where lower concentrations of autoantibodies might be detected. Finally, this is a cross-sectional study and we cannot completely rule the possibility that autoantibodies, if present, might have been detectable immediately after the suicide attempt but not after the wash-out period at the time of the lumbar puncture. Longitudinal studies of anti-NMDA receptor encephalitis, however, have shown that although titres may decrease over time, antibodies are often detected in consecutive samples [30].

In conclusion, we were not able to detect any autoantibodies in CSF samples from recent suicide attempters. This is one of the first studies of its kind, and future larger scale studies using sensitive assays are warranted before any firm conclusions may be drawn.

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Paper 2

OPEN

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ORIGINAL ARTICLE

Increased plasma levels of circulating cell-free mitochondrial DNA in suicide attempters: associations with HPA-axis hyperactivity

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Preclinical data suggest that chronic stress may cause cellular damage and mitochondrial dysfunction, potentially leading to the release of mitochondrial DNA (mtDNA) into the bloodstream. Major depressive disorder has been associated with an increased amount of mtDNA in leukocytes from saliva samples and blood; however, no previous studies have measured plasma levels of free-circulating mtDNA in a clinical psychiatric sample. In this study, free circulating mtDNA was quantified in plasma samples from 37 suicide attempters, who had undergone a dexamethasone suppression test (DST), and 37 healthy controls. We hypothesized that free circulating mtDNA would be elevated in the suicide attempters and would be associated with hypothalamic–pituitary–adrenal (HPA)-axis hyperactivity. Suicide attempters had significantly higher plasma levels of free-circulating mtDNA compared with healthy controls at different time points (pre- and post-DST; all *P*-values $< 2.98E-12$, Cohen's *d* ranging from 2.55 to 4.01). Pre-DST plasma levels of mtDNA were positively correlated with post-DST cortisol levels (rho = 0.49, $P < 0.003$). Suicide attempters may have elevated plasma levels of free-circulating mtDNA, which are related to impaired HPA-axis negative feedback. This peripheral index is consistent with an increased cellular or mitochondrial damage. The specific cells and tissues contributing to plasma levels of free-circulating mtDNA are not known, as is the specificity of this finding for suicide attempters. Future studies are needed in order to better understand the relevance of increased free-circulating mtDNA in relation to the pathophysiology underlying suicidal behavior and depression.

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INTRODUCTION

Mitochondria are cytoplasmic organelles of the eukaryotic cells that have many important roles in cellular function. Mitochondria can be conceptualized as the 'power generators' of the cell, utilizing oxygen, energy substrates (carbohydrates and lipids) and other compounds to form energy-rich phosphates.[1,2] Furthermore mitochondria are also involved in other important cellular processes, such as apoptotic and necrotic cell death,[1,3] the generation and regulation of free radicals,[4–6] gene expression regulation[7,8] as well as signal transduction for cell proliferation and differentiation.[1,3] Each cell contains multiple mitochondria, and each mitochondrion possesses its own genome; the maternally inherited mitochondrial DNA (mtDNA) encoding for 37 different genes involved in energy production.[9–11] MtDNA is thought to be highly susceptible to oxidative damage due to limited DNA repair mechanisms.[12] Moreover, chronic stress may alter mitochondrial structure and function via increased levels of glucocorticoids, the primary mediators of the stress response.[13] Glucocorticoid levels seem to regulate mitochondrial function in neurons in an inverted U-shaped manner. Du *et al.*[14] showed, in a series of *in vivo* and *in vitro* experiments, that physiological doses of glucocorticoids increased the mitochondrial membrane potential and resistance to apoptosis, whereas long-lasting and higher doses of glucocorticoids were associated with decreased mitochondrial membrane potential and increased sensitivity for apoptosis. Furthermore, animal studies have shown that chronic stress exposure leads to mitochondrial damage and dysfunction in various brain regions such as the hippocampus, cortex and thalamus.[15–17] Yet another experimental study demonstrated that dexamethasone (a cortisol analog) may cause mitochondrial fragmentation in hepatocytes via the induction of proteins that promote mitochondrial fission.[18] Mitochondrial fragmentation, in turn, activates pro-apoptotic mechanisms that sensitize the cell toward programmed cell death.[19]

Although the exact mechanisms are still unclear, dysfunctional mitochondria, perhaps due to chronic stress, are thought to promote the release of mtDNA into the circulation. Free circulating mtDNA may activate several deleterious downstream mechanisms including systemic inflammation,[13] potentially causing depressive symptoms; however, no previous studies have measured plasma concentrations of free-circulating mtDNA in a psychiatric sample. In their seminal study, Cai *et al.* found that major depressive disorder (MDD) was associated with greater amount of mtDNA in leukocytes from saliva samples and blood.[20] Moreover, higher levels of mtDNA were (i) significantly associated with stressful life events in humans and (ii) were associated with a stress paradigm and corticosteroid administration in animals.[20] Some of the same authors subsequently confirmed, in a longitudinal study, that high leukocyte copy numbers of mtDNA (mtDNA-cn) was associated with mood disorders.[21] The difference in leukocyte mtDNA-cn

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between MDD cases and controls was most significant at week 6 of the depressive episode; however, mtDNA-cn did not correlate with severity of depressive symptoms.[21]

The present study sought to determine whether free-circulating mtDNA in cell-free plasma is increased in suicide attempters, a group often characterized by depressive symptoms, increased psychological stress and adverse childhood events.[22] On the basis of previous animal experiments, we also wanted to test the relationship between free-circulating plasma levels of mtDNA and hypothalamic–pituitary–adrenal (HPA)-axis activity in this sample. For this purpose, we measured free-circulating mtDNA in plasma samples from 37 recent suicide attempters, who had undergone a dexamethasone suppression test (DST), and 37 healthy controls. We hypothesized that plasma levels of free-circulating mtDNA would be significantly higher in the suicide attempters and that higher levels would be associated with a dysregulated stress response.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Lund University Medical Ethics Commitee, and all patients gave informed consent to participate in the study.

Subject recruitment, suicide attempters

Between 1992 and 2001, patients with a recent suicide attempt, as defined by Beck *et al.*,[23] were proposed to participate in a research project that has been described in more detail elsewhere.[24,25] Suicide attempters were evaluated by a certified psychiatrist for DSM axis I and II diagnoses.[26]

The Montgomery-Åsberg Depression Rating Scale (MADRS)[27] was used to assess depressive symptoms. Suicide attempters did not receive any antidepressants or antipsychotics during a washout period (mean ± s.d.: 12 ± 13 days) before blood sampling and the DST. Occasional doses of benzodiazepines were allowed during the washout.

In total, 37 plasma samples from medication-free subjects who had undergone the DST were selected from the above-mentioned larger study. The rationale for selecting cases that had undergone the DST was that the aim of the study, in addition to investigate the level of free-circulating mtDNA in cases and controls, was to test the association between concentration of mtDNA in cell-free plasma and post-DST cortisol levels in suicide attempters. The plasma samples from the patients had not been thawed before the analysis of mtDNA. For the control group, all plasma samples (with the exception of one unthawed sample) had been thawed and frozen once before analysis.

Twenty-nine of the included suicide attempters in this study had taken a drug overdose, four subjects had attempted to hang themselves, two subjects had used wrist-cutting and two subjects had taken a drug overdose in combination with wrist-cutting. None of the suicide attempters had a medical condition or took any medications (such as corticosteroids or insulin) known to interfere with the results of the DST. After the washout period, drug screening showed no traces of antidepressants or antipsychotics in the included subjects, but five subjects showed traces of benzodiazepines.

Subject recruitment, controls

Thirty-seven healthy controls were recruited in 1994. Healthy controls were employees or students at the Lund University Hospital or retirees. They were all medically examined by one of the co-authors (ÅW). As determined by semi-structured interviews, physical examination and blood analysis, none of the controls had any prior or current psychiatric disorders or any somatic disorders (including endocrine, nervous, hepatic, renal, cardiac, asthmatic, hypertensive or infective disorders). None of the controls took any corticosteroids, insulin or other medications that could interfere with the DST.

DST and sample preparation

One mg dexamethasone was given at 2200 hours, and blood samples were drawn, in either serum- or EDTA tubes, at 1500 hours on the day of dexamethasone administration, as well as at 0800 and 1500 hours the following day.[28] Serum tubes were left at room temperature for 30 min before centrifugation, and plasma tubes were immediately placed on ice until centrifugation. Blood samples were centrifuged at 4 °C and 2000 *g* for 10 min within 1 h of blood collection. Plasma was stored in − 70 °C until analysis of mtDNA. At all three time points, cortisol was analyzed in serum (suicide attempters only) and free-circulating mtDNA was analyzed in plasma (in all subjects). Serum analysis of cortisol was performed either on the same day or stored at − 20 °C until analysis. Serum cortisol was measured using a commercial radioimmunoassay (RIA) (Orion diagnostica RIA kit). The detection limit was 7 nmol l^{-1}, and the intra- and interassay coefficients of variation were 5% and 7%, respectively.

Measurement of free-circulating mtDNA

DNA was isolated from thawed plasma samples using the QIAmp 96 DNA Blood Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction for Blood and body-fluid protocol. Before the isolation of DNA, the plasma samples were centrifugated at 10 000 *g* for 10 min. The purity of the eluted DNA was measured using spectrophotometric analysis at 260/280 nm in a Nanodrop (ND-1000 Spectrophotometer v 3.7.1, Waltham, MA, USA).

The quantitative analysis of cell-free mtDNA was performed using quantitative real time polymerase chain reaction (PCR). The experiment was run once in triplicate reactions. A dilution series consisting of the PCR product was constructed and used to create a standard curve. The different crossing-point values from the unknown samples were compared with the standard curve, and the corresponding number of mitochondrial units was calculated using the following formula:

$$\text{mtDNA units} = \left(\frac{\frac{\times \frac{\text{gram}}{\mu l}\text{DNA}}{\text{PCR} - \text{frag}\ (\text{bp}) \times 660} \right) \times 6.022 \times 10^{23}$$

The amount of DNA (g μl^{-1}) was divided with the size of the PCR fragment (bp) and the molar mass per base pair (g mol^{-1}). The product was finally multiplied with Avogadro's constant.

The primers (Life Technologies, Paisley, UK) used for PCR amplification of mtDNA were as stated in the table below:

Gen	*Primer forward*	*Primer reverse*	*Accession nr*
ND2	CACACTCATCACAGCGCTAA	GGATTATGGATGCGGTTGCT	KJ676545

The PCR reactions were carried out using SYBR Green Technology (Thermo Fisher Scientific, Waltham, MA, USA). Each 20 µl reaction contained 5 µl of template, 1 µl of each primer (10 µM), 10 µl SYBR MIX (2×, Sensifast, Bioline, London, UK) and 3 µl of nuclease-free water. Each reaction was run in triplicate on a LC480 LightCycler from Roche, Mannheim, Germany) using the following program:

Initial denaturation at 95 °C for 10 min, followed by 45 cycles consisting of 95 °C in 10 s. for melting, 65 °C for 10 s annealing and 72 °C for 11 s extension. The program ended with a melting curve analysis measuring fluorescence continuously from 60 to 97 °C.

Statistics

The Statistical Package for the Social Sciences for Mac (version 23, IBM, Armonk, NY, USA) was used for statistical calculations. The concentration of mtDNA was skewed at all three time points. Therefore, and because some of the group-wise comparisons involved smaller subgroups, Mann–Whitney *U*-test was used to test differences in mtDNA concentration across groups. Spearman's Rho was used for correlations involving mtDNA level. Student's *t*-test was used to compare age and body mass index (BMI) between groups and Pearson's X^2 was used to compare sex distribution across groups. Effect size was calculated using Cohen's *d* on log-transformed data. Cohen's $d > 0.2$ is generally considered a small effect size, >0.5 medium and >0.8 large. All tests were two-tailed. For correlations between mtDNA units and post-DST cortisol levels, we carried out a total number of six tests (each of the three mtDNA measurements versus each of the two post-DST cortisol measurements); thus, the Bonferroni-adjusted *P*-value for the correlation analyses was set to 0.008 (0.05/6). Within the suicide attempters, we compared the mtDNA levels between repeaters versus non-repeaters, violent versus non-violent suicide attempters and suicide attempters with MDD diagnosis versus suicide

Table 1. Demographic characteristics of suicide attempters and controls

	Suicide attempters (n = *37*)	*Controls* (n = *37*)	*Cohen's* d	P-*value*
Age (years, mean ± s.d.)	39 ± 14	38 ± 17	0.06	0.65[a]
Female (%)	70	65	N/A	0.62[b]
BMI (mean ± s.d.)	23 ± 5	24 ± 3	0.24	0.80[a]
MADRS score (mean ± s.d.; *n* = 31)	18 ± 10	N/A	N/A	N/A
mtDNA units per µl plasma pre-DST, median, IQR	23 556, 11 152–58 494	503, 303–930	2.64	2.98E − 12[c]
mtDNA units per µl plasma 0800 hours post DST, median, IQR	26 902, 15 371–50 288	683, 356–1111	4.01	1.37E − 13[c]
mtDNA units per µl plasma 1500 hours post DST, median, IQR	34 388, 12 591–64 785 (*n* = 36)	755, 287–948	2.55	4.58E − 12[c]

Abbreviations: BMI, body mass index; DST, dexamethasone suppression test; IQR, interquartile range; MADRS, Montgomery-Åsberg Depression Rating Scale; mtDNA, mitochondrial DNA; N/A, not applicable. The concentration of mtDNA was skewed and, therefore, log-transformed before calculation of Cohen's *d*. Raw data are given in the table as this is more interpretable than transformed values. [a]Student's *t*-test. [b]Pearson's X^2. [c]Mann–Whitney *U*-test.

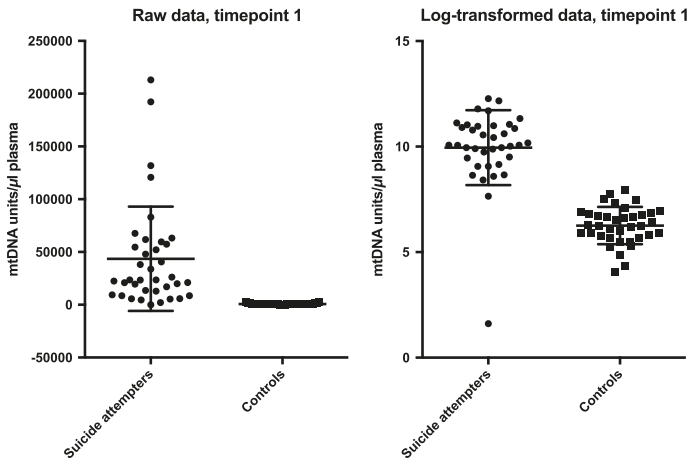

Figure 1. Free-circulating plasma mtDNA units in suicide attempters and controls at time point 1 (raw and log-transformed data, respectively). Error bars indicate 1 ± s.d. from the mean. mtDNA, mitochondrial DNA.

attempters without MDD diagnosis. For these three subgroup-analyses, the Bonferroni-adjusted *P*-value was set to 0.017 (0.05/3).

RESULTS

Demographics

Demographic and clinical characteristics of all subjects are presented in Table 1. Groups were balanced in terms of age, sex and BMI. Within the suicide attempter group, free-circulating mtDNA (measured at any of the three time points) was not significantly correlated with number of washout days or number of years stored in the freezer (all $P > 0.57$).

Eighteen of the cases had attempted suicide more than once ('repeaters') and seven had used a violent suicide method. Drug overdoses, single wrist-cutting or a combination of both are considered non-violent suicide attempts, whereas all other methods (for example, hanging, use of firearms or several deep knife cuts) are classified as violent.[29] For 14 cases, MDD was the principal axis 1 diagnosis, five cases had dysthymic disorder, six cases had Adjustment Disorder, five cases Depression Not Otherwise Specified, one case Anorexia Nervosa and six cases did not meet the criteria for an axis 1 disorder. Twenty of the cases had an axis II personality disorder, cluster B being the most frequent specifier ($n = 9$). MADRS scores were available from 31 of the suicide attempters and did not correlate significantly with mtDNA units at any of the three time points ($P > 0.29$).

mtDNA units versus clinical variables

Suicide attempters had significantly higher plasma levels of free-circulating mtDNA compared with controls at all three time points (all *P*-values $< 2.98E-12$, Cohen's *d* ranging from 2.55 to 4.01; Table 1). Raw and log-transformed data of free-circulating mtDNA units at all three time points in cases and controls are given in Figures 1-3. There was no significant difference in mtDNA levels between either suicide attempters with and without MDD, violent and non-violent suicide attempters or between repeaters and non-repeaters (NS, data not shown).

Correlations between mtDNA units and post-DST cortisol levels in suicide attempters

Correlations between mtDNA units and post-DST cortisol levels are summarized in Table 2. MtDNA units at time point 1 correlated significantly with post-dexamethasone cortisol levels at 0800 hours (rho = 0.49, $P < 0.003$). None of the other correlations were significant.

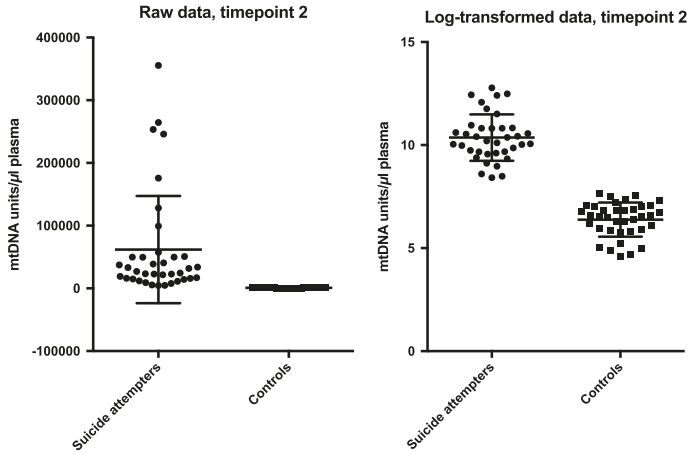

Figure 2. Free-circulating plasma mtDNA units in suicide attempters and controls at time point 2 (raw and log-transformed data, respectively). Error bars indicate 1 ± s.d. from the mean. mtDNA, mitochondrial DNA.

DISCUSSION

We here report that a group of medication-free suicide attempters display significantly higher plasma levels of free-circulating mtDNA compared with a group of healthy controls. The effect sizes were very large, with almost nonoverlapping groups. Within the group of suicide attempters, higher plasma levels of free-circulating mtDNA were significantly associated with high cortisol levels after a dexamethasone challenge, a marker of HPA-axis hyperactivity. In a series of experiments, Cai *et al.*[20] recently reported that (i) MDD was associated with high amount of mtDNA-cn in leukocytes from saliva samples and blood and (ii) increased stress and corticosteroid administration was associated with increased mtDNA-cn in an animal model. Our results are in line with this study, and extend these findings by (i) reporting a robust group difference also on cell-free plasma levels of mtDNA and (ii) showing a significant relationship between cell-free mtDNA and HPA-axis hyperactivity in a clinical sample. Moreover, we did not find a significant relationship between mtDNA levels and severity of depressive symptoms, which is also in line with a previous study on leukocyte mtDNA-cn.[21] Moreover, we did not find any significant associations between mtDNA-cn and an MDD diagnosis, suicide repetition or a violent suicide method. This suggests that high mtDNA-cn might be associated with a suicide attempt *per se* rather than certain aspects of symptom severity within this group. Another possibility is that high mtDNA-cn might reflect more long-lasting psychopathology, a hypothesis that we could not test with our cross-sectional study design. Therefore, this needs to be investigated further in longitudinal studies, taking into account the number of lifetime depressive episodes and lifetime stressors.

Increased blood levels of free-circulating mtDNA have been shown in a number of different somatic conditions such as diabetes,[30] cancers,[31] trauma,[32] myocardial infarction[33] and sepsis;[34] thus, it is unlikely that enhanced mtDNA levels are specific for suicide attempters. Rather, cellular aging, caused by metabolic stress, inflammatory triggers or (as in the case of suicidality) severe and long-lasting psychological stress,[35] may be a common denominator across these conditions potentially leading to increased apoptosis and a subsequent leakage of mtDNA from the damaged cells into the bloodstream. Accelerated aging at the cellular level is a biological characteristic of several psychiatric and somatic conditions associated with a heightened stress response.[36–38] Mental illness has been associated with an elevated risk for somatic complications and early mortality.[39] Stress-induced accelerated cellular aging and increased mtDNA may thus be common biological mechanisms causing somatic as well as psychiatric pathologies, although this hypothesis remains to be tested. Accelerated cellular aging has been associated with HPA-axis hyperactivity in stressed individuals as well as in healthy volunteers.[40,41] As reviewed by Picard *et al.*,[13] chronic neuroendocrine and metabolic stress may lead to mitochondrial dysfunction and accumulation of mtDNA damage. Activation of the HPA axis increases the demand for energy production,[42] the primary function of the mitochondria. Owing to the mitochondria's relatively poor ability to repair its own DNA and the production of reactive oxygen species, the increased metabolic rate leads to cell damage and fragmentation of mtDNA.[13] Downstream effects in this stress-disease cascade involve cellular dysfunction and senescence, and the release of free-circulating mtDNA into the bloodstream, which in turn may have detrimental effects on multiple organ systems via inflammatory mechanisms.[13,43] In the present study, we found that free circulating mtDNA levels, besides being significantly different between suicide attempters and controls, were positively correlated with cortisol levels after a dexamethasone challenge. Initially proposed as a biomarker for melancholic depression,[44] higher cortisol levels after a DST has been reported in a variety of other psychiatric disorders (ranging from psychosis to anxiety, and even in dementia),[45] as well as somatic disorders characterized by metabolic stress such as diabetes.[46] This suggests that high post-DST cortisol levels might be a general marker of stress, be it of metabolic or psychological origin. Our findings may suggest that psychological stress associated with the suicidal process may leave a biological imprint in the form of increased free-circulating mtDNA.

In addition to the hypothesis that stress-related cellular aging and apoptosis leads to leakage of cell-free mtDNA into the bloodstream, there is also the possibility that intracellular mtDNA content is increased, and that this is reflected in cell-free plasma. A few psychiatric studies have examined intracellular mtDNA-cn

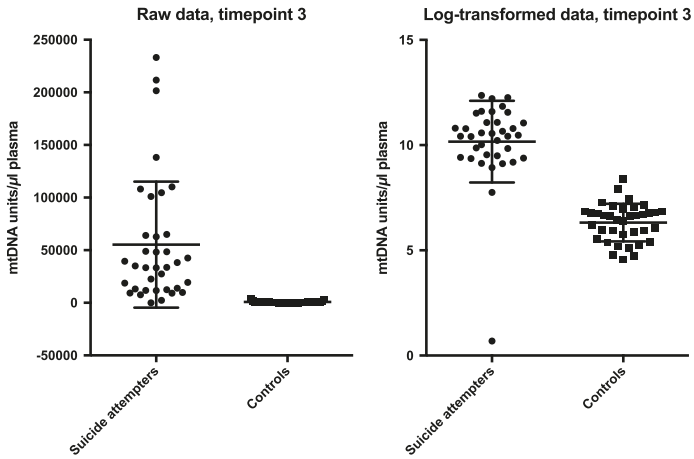

Figure 3. Free-circulating plasma mtDNA units in suicide attempters and controls at time point 3 (raw and log-transformed data, respectively). Error bars indicate 1 ± s.d. from the mean. mtDNA, mitochondrial DNA.

Table 2. Correlations (Spearman's Rho correlation coefficients) between free-circulating plasma mtDNA units and post-DST cortisol in suicide attempters

	mtDNA units, time point 1	*mtDNA units, time point 2*	*mtDNA units, time point 3*
Post-DST cortisol 0800 hours	0.49*	0.17	0.11
Post-DST cortisol 1500 hours	0.11	0.07	−0.09

Abbreviations: DST, dexamethasone suppression test; mtDNA, mitochondrial DNA. *$P < 0.003$.

(primarily in leukocytes) with conflicting results. Reduced mtDNA-cn has been reported in post-traumatic stress disorder[47] and in relation to depressive symptoms among elderly community-dwelling women without MDD.[48,49] Higher leukocyte mtDNA-cn has been reported in autism[50] and lifetime psychopathology in healthy people.[51] MtDNA-cn has been found to be lower[52] and unchanged[53] in bipolar disorder. Although the largest study to date on MDD showed significantly higher leukocyte mtDNA-cn in MDD subjects compared with controls,[20] other studies have produced mixed results with lower,[54] higher[21] or unchanged[55] mtDNA-cn in MDD. Taken together, it is unclear whether the increase in free circulating mtDNA observed in the current study is because of cell damage and subsequent mtDNA leakage into the bloodstream, increased content of intracellular mtDNA or both. Future studies on psychiatric samples should investigate these hypotheses by testing associations between free-circulating mtDNA and leukocyte mtDNA-cn as well as markers of cellular aging such as leukocyte telomere length and telomerase activity.

A major strength of this study is that the sample comprised a clinically well-characterized, somatically healthy group of medication-free suicide attempters. Moreover, the subjects underwent an inpatient washout procedure and subsequent screening for psychotropics. The present study, however, also comes with some limitations. The cross-sectional nature of our study design precludes any conclusion regarding causality, and future longitudinal studies are warranted in order to test this hypothesis. The unexpectedly large effect sizes raise the issue of whether the observed group differences were due to unaccounted factors relating to sampling, storage or health behaviors such as physical activity. Although this cannot be completely ruled out, the groups were balanced with regards to demographic characteristics such as age, sex and BMI. Moreover, all subjects were free from medications that could potentially interfere with the biological measurements. Storage time in the freezer and number of washout days were not associated with mtDNA levels, making these two factors unlikely confounders. All samples from the control group, except for one, had previously been thawed and frozen before the present analysis. Although we cannot completely rule out that this might have an impact on our results, it is unlikely as DNA is generally reported to be a very stable molecule, and a previous study found that free-circulating mtDNA levels were significantly correlated within subjects over different thaw/freeze cycles.[56] Moreover, the control sample that had not previously been thawed showed a value of 881 mtDNA units per µl plasma, which is in line with the control group median. Another possibility is that the group differences in free-circulating mtDNA were a consequence of a physical trauma potentially associated with the suicide attempt. However, this is unlikely as no significant difference in mtDNA between violent and non-violent suicide attempters was observed. Furthermore, in most cases, 2 weeks had passed between the attempt and blood sampling. Most physical injuries, unless very severe, will have substantially improved within this time. Finally, in the present study we used high post-DST cortisol levels as a surrogate marker for increased stress and a heightened stress response. We note, however, that some other stress-related conditions such as post-traumatic stress disorder[57] and depression related to long-term sick leave[58] have instead been associated with HPA-axis hyporeactivity. In some subjects this might represent a pre-existing vulnerability marker for illness rather than the consequence of prolonged stress.[58] Despite these inconsistencies across studies, we believe that our approach is justified as several lines of evidence support the notion that high post-DST cortisol is associated with increased stress in a variety of different contexts.[45]

To the best of our knowledge, this is the first study to measure free-circulating plasma mtDNA in suicide attempters and controls. Suicide attempters showed significantly higher levels of mtDNA, compared with controls, which was associated with HPA-axis hyperactivity. This peripheral index is consistent with increased cellular or mitochondrial damage. The specific cells and tissues contributing to plasma levels of free-circulating mtDNA are not known, as is the specificity of this finding for suicide attempters. Future studies are needed in order to better understand the relevance of increased free-circulating mtDNA in relation to the pathophysiology underlying suicidal behavior and depression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Paper 3

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ARTICLE OPEN

Circulating cell-free mitochondrial DNA, but not leukocyte mitochondrial DNA copy number, is elevated in major depressive disorder

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Major depressive disorder (MDD) has been linked to mitochondrial defects, which could manifest in mitochondrial DNA (mtDNA) polymorphisms or mutations. Additionally, copy number of mtDNA (mtDNA-cn) can be quantified in peripheral blood mononuclear cells (PBMC)s, indirectly reflecting cellular energetics, or in the circulating cell-free mtDNA (ccf-mtDNA) levels, which may reflect a fraction of the mitochondrial genome released during cellular stress. Few studies have examined ccf-mtDNA in MDD, and no studies have tested its relationship with intracellular mtDNA-cn or with antidepressant treatment response. Here, mtDNA levels were quantified in parallel from: (i) PBMCs and (ii) cell-free plasma of 50 unmedicated MDD subjects and 55 controls, in parallel with PBMC telomere length (TL) and antioxidant enzyme glutathione peroxidase (GpX) activity. MtDNA measures were repeated in 19 MDD subjects after 8 weeks of open-label SSRI treatment. In analyses adjusted for age, sex, BMI, and smoking, MDD subjects had significantly elevated levels of ccf-mtDNA ($F = 20.6$, $p = 0.00002$). PBMC mtDNA-cn did not differ between groups ($p > 0.4$). In preliminary analyses, we found that changes in ccf-mtDNA with SSRI treatment differed between SSRI responders and non-responders ($F = 6.47$, $p = 0.02$), with the non-responders showing an increase in ccf-mtDNA and responders not changing. Baseline ccf-mtDNA was positively correlated with GpX ($r = 0.32$, $p = 0.001$), and PBMC mtDNA correlated positively with PBMC TL ($r = 0.38$, $p = 0.0001$). These data suggest that plasma ccf-mtDNA and PBMC mtDNA-cn reflect different cellular processes and that the former may be more reflective of certain aspects of MDD pathophysiology and of the response to SSRI antidepressants.

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INTRODUCTION

Mitochondrial dysfunction may be involved in the pathophysiology of MDD [1–5]. Cells contain multiple mitochondria and, in turn, each mitochondrion contains multiple copies of its own genome, the mitochondrial DNA (mtDNA), which encodes 37 genes essential to energy production [6]. Mitochondria may be conceptualized as the "power generators" of the cell, converting oxygen, energy substrates (proteins, carbohydrates and lipids) and other substrates into adenosine triphosphate [7]. Beyond energy production, mitochondria generate other signaling molecules that influence cellular and physiological functions, [8, 9] including cellular senescence [10] and production of free radicals [11]. As a result of these mechanisms, mitochondrial impairments may be associated with, and/or lead to, psychiatric manifestations such as mood and anxiety disorders, conditions that have been associated with cellular senescence and oxidative stress. [12, 13]

mtDNA copy number (mtDNA-cn), which represents the number of mitochondrial genomes per cell [6], can be quantified in peripheral blood mononuclear cells (PBMC) and is thought to reflect variations in mitochondrial energetic function and biogenesis [6]. Additionally, mtDNA may be released at low levels into the circulation from mitochondria under cellular stress, resulting in circulating cell-free mtDNA (ccf-mtDNA) detectable in plasma [14, 15]. Ccf-mtDNA has only very recently been assessed in psychiatric patients after suicide attempt [16], and in MDD subjects [17].

Although the exact source or significance of ccf-mtDNA in psychiatric illness is yet to be determined, it is suggested to reflect cellular extrusion of mitochondria, during apoptotic or necrotic cell death or other processes, resulting in mtDNA appearing in the plasma [18, 19]. In addition, oxidative stress may promote the release of mtDNA into the cell cytoplasm, and possibly into the extracellular space [20]. Chronic neuroendocrine, inflammatory, oxidative and metabolic stress may result in mitochondrial dysfunction and mtDNA damage [21]. Downstream effects of this stress–disease cascade involve cellular dysfunction and senescence, and the release of ccf-mtDNA into

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the bloodstream, which in turn may have detrimental effects on multiple organ systems via inflammatory mechanisms [21, 22]. Although this has not been well studied in psychiatric settings, increased blood levels of free-circulating mtDNA have been reported in a number of different somatic conditions such as diabetes [23], cancers [15], and myocardial infarction [24] and have been linked to more severe illness and worse outcome [25].

Levels of ccf-mtDNA and cellular mtDNA-cn may have further significance, as they also relate to cellular oxidative stress and cellular senescence. MtDNA is highly vulnerable to oxidative stress due to the lack of protective histones and limited DNA repair mechanisms [26]. Antioxidant enzymes such as glutathione peroxidase (GpX) are therefore needed in order to protect the mitochondria and prevent cellular dysfunction [27]. Mitochondrial integrity and bioenergetics are also related to nuclear DNA senescence, due to a direct link between telomere shortening, repression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and diminished mitochondrial biogenesis in certain tissues [28]. Thus, assessment of mtDNA, in the cell-free circulation or in the intracellular compartment may also inform on cellular stress and cellular senescence [21], although the potential relevance of these interactions for MDD is currently unclear.

Despite emerging evidence for a link between high mtDNA and MDD [5, 29], there are inconsistent results across studies, [30–32] and several unresolved issues remain. These include the extent to which different measurements of mtDNA (e.g., leukocyte mtDNA-cn vs. ccf- mtDNA) inter-correlate in subjects with and without psychopathology, and how mtDNA levels in different compartments relate to other biological markers implicated in MDD, such as oxidative stress and indices of accelerated cellular aging. Furthermore, most previous studies in MDD have included subjects taking antidepressants or mood stabilizers, [5,29–31,33] highlighting the importance of studying this in well-characterized unmedicated MDD samples. Moreover, no previous studies have investigated the relationship between leukocyte mtDNA-cn or ccf-mtDNA and antidepressant treatment response. The purposes of the present study were, therefore, to (i) investigate cellular and circulating cell-free levels of mtDNA in a sample of well-characterized, unmedicated, and somatically healthy MDD subjects and healthy controls, (ii) test the relationship between mtDNA and peripheral indices of oxidative stress and accelerated cellular aging (telomere length), and (iii) determine if successful antidepressant treatment is associated with changes in cellular mtDNA-cn or ccf- mtDNA.

METHODS AND MATERIALS

Ethics statement

The Committee on Human Research of the University of California, San Francisco (UCSF) approved the study protocol. All study participants gave written informed consent to participate in this study and were compensated for participating.

Baseline recruitment procedures and study participants

MDD ($n = 50$) outpatients and healthy controls ($n = 55$) were recruited between 2011–2015 by flyers, bulletin board notices, Craigslist postings, newspaper ads and, in the case of MDD subjects, clinical referrals. Ccf-mtDNA measurements were missing for two subjects, and PMBC mtDNA-cn was missing from two subjects. All diagnoses, including MDD, were established using the structured clinical interview [34] and were verified in a separate diagnostic evaluation by a Board-certified psychiatrist. Depression symptom severity was assessed in MDD subjects using the Hamilton Depression Rating Scale (HDRS) [35]. All MDD subjects had a minimum 17-item HDRS [35] score of 17. MDD subjects were excluded if they met DSM-IV criteria (which were the extant criteria in use at the time this study was conducted) for any of the following: (i) bipolar disorder, (ii) alcohol or substance abuse within the preceding 6 months, (iii) PTSD or an eating disorder within 1 month of entering the study, and (iv) for any history of psychosis outside of a major depressive episode (MDE), or the presence of any psychotic symptoms during the current MDE. Potential healthy controls were excluded for any history of DSM-IV Axis-I diagnoses. All study participants were free of acute illnesses or infections, inflammatory disorders, neurological disorders, or any other medical conditions considered to be potentially confounding (e.g., cancer, HIV, diabetes, history of cardiovascular disease or stroke, etc.), as assessed by history, physical examinations, and routine blood screening. All subjects were free of psychotropic medications (including antidepressants), hormone supplements, steroid-containing birth control or other potentially interfering medications, including vitamin supplements above the U.S. recommended daily allowances (e.g. >90 mg/day for Vitamin C) and had not had any vaccinations for at least 6 weeks prior to enrollment in the study. For the MDD subjects, short-acting sedative-hypnotics were allowed as needed up to a maximum of three times per week, but none within 1 week prior to blood draws in the study. All subjects had to pass a urine toxicology screen for drugs of abuse (marijuana, cocaine, amphetamines, PCP, opiates, methamphetamine, tricyclic antidepressants, and barbiturates) and a urine test for pregnancy in women of child-bearing age on the same day as the blood draw.

Selective serotonin reuptake inhibitor (SSRI)-treatment

Nineteen MDD subjects underwent 8 weeks of protocol-based open-label outpatient treatment with an SSRI antidepressant (NCT00285935, https://www.clinicaltrials.gov/). In order to limit the range of potential mechanism of action of antidepressants, the choice of medication was limited to an SSRI. The decision for the specific SSRI prescribed was based on clinical information such as medical history, family history, prior medication history, subject preference and potential side effects. Compliance and clinical evaluations and assessments of drug tolerability were assessed by a telephone check-in at the end of week 1 and an in-person check-in at the end of week 4 and week 8, at which times pill counts were performed. Plasma SSRI concentrations were assessed at the week 4 and week 8 visits to evaluate medication compliance. Primary outcome measure was severity of depressive symptoms, assessed by means of the HDRS. HDRS ratings were repeated at the end of treatment (week 8). Twelve subjects were treated with sertraline (mean ± SD dose in mg = 142 ± 36), two with fluoxetine (mean ± SD dose in mg = 35 ± 7), two with citalopram (mean ± SD dose in mg = 35 ± 7), and three with escitalopram (mean ± SD dose in mg = 13 ± 6). Medication dosages were increased over the course of treatment per pre-specified protocol as tolerated and as warranted by clinical response. Sertraline dosing began with 25 mg per day and increased to a maximum of 200 mg per day; fluoxetine and citalopram dosing began with 10 mg per day and increased to a maximum of 40 mg per day; escitalopram dosing began with 10 mg per day and increased to a maximum of 20 mg per day. "Responders" were defined as subjects with greater than or equal to 50% improvement on HDRS ratings at week 8 compared to baseline. There was no significant difference in final SSRI dose (sertraline equivalents) between responders and non-responders ($p = 0.59$), and all subjects (responders and non-responders) had plasma SSRI levels within expected reference ranges.

Blood sampling and DNA extraction

Venipuncture was performed at approximately 10:00 am at the UCSF Clinical and Translational Science Institute, after 12 h of fasting (except water). Plasma was collected with a lavender EDTA vacutainer tube. Tubes were spun at 1500×*g* for 10 min and 4° C, and plasma was removed, aliquoted into tubes and frozen at −80°

C until used. PBMCs were prepared from whole blood by ficoll centrifugation [36] and were frozen at −80 °C. DNA was extracted from frozen PBMCs using commercially available reagents (Puregene, Gentra Systems, Qiagen, Valencia, CA). DNA quality and quantity were assessed using Nanodrop spectrophotometer and random samples were also assessed by agarose gel electrophoresis to assess DNA integrity.

Measurement of mtDNA-cn in PBMCs

The mtDNA-cn in PBMCs was determined by multiplex real-time polymerase chain reaction (PCR) assay simultaneously quantifying genomic mitochondrial (ND1) and nuclear (Ribonuclease P) amplicons, as described elsewhere [37]. Lab personnel who performed the assay were blind to demographic and clinical data.

Measurement of ccf-mtDNA in plasma

The isolation and quantification of mtDNA in plasma samples has been previously described [16]. Briefly, the thawed plasma samples were centrifuged for 10 min at 10,000×*g* to remove cells and cellular debris.

DNA were isolated from 200 µl of the supernatant using the QIAmp 96 DNA Blood Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction for blood and body-fluid protocol. The isolated DNA was then eluted in 200 µl and quantified using spectrophotometric analysis at 260/280 nm in a Nanodrop (ND-1000 Spectrophotometer v 3.7.1, Waltham, MA, USA). The quantitative analysis of ccf-mtDNA was performed using quantitative real time polymerase chain reaction. The experiment was run once in triplicate reactions. A dilution series consisting of the purified PCR product from a healthy control subject, not taking part in the present study, was constructed and used to create a standard curve. The DNA sequence of the PCR-fragment was determined before the dilution series was carried out. The different crossing-point values from the unknown samples were compared with the standard curve, and the corresponding number of mitochondrial units was calculated. The amount of DNA (g μl^{-1}) was divided with the size of the PCR-fragment (161 bp) and the molar mass per base pair (g mol^{-1}). The product was finally multiplied with Avogadro's constant.

Mitochondrially encoded NADH: Ubiquinone Oxidoreductase Core Subunit 2 (MT-ND2) gene was amplified using the following primers (Life Technologies, Pailsey, UK): Forward primer: [CACACTCATCACAGCGCTAA]; reverse primer: [GGATTATGGATGCGGTTGCT]. In order to verify the data, the assay was also completed with another target, the mtDNA-encoded NADH: Ubiquinone Oxidoreductase Core Subunit 1 (MT-ND1) gene. This gene was amplified in the same way as above, but using the following primers (Life Technologies, Paisley, UK): Forward primer: [CCCTAAAACCCGCCACATCT]; reverse primer: [CCGATCAGGGCGTAGTTTGA]. Both amplicons were strongly correlated ($r = 0.73$, $p < 0.001$), indicating that inter-individual mtDNA sequence variation did not affect our ability to detect ccf-mtDNA levels.

The PCR reactions were carried out using SYBR Green Technology (Thermo Fisher Scientific, Waltham, MA, USA). Each 20 µl reaction contained 5 µl of template, 1 µl of each primer (10 µM), 10 µl SYBR MIX (2 × Sensifast, Bioline, London, UK) and 3 µl of nuclease-free water. Each reaction was run in triplicate on a LC480 (LightCycler from Roche, Mannheim, Germany) using the following program:

Initial denaturation at 95 °C for 10 min, followed by 45 cycles consisting of 95 °C in 10 s. For melting, 65 °C for 10 s annealing and 72 °C for 11 s extension. The program ended with a melting curve analysis measuring fluorescence continuously from 60 to 97 °C.

Measurement of GpX

Glutathione peroxidase activity (BioVision, Inc., Milpitas, California, USA) was measured in duplicate from plasma, using a colorimetric assay according to the instructions from the manufacturer. The coefficient of variation was < 10% and LLOQ was 0.5 nmol NADPH/ml/min.

Measurement of PBMC telomere length

The telomere length measurement assay is adapted from the published original method by Cawthon [38, 39].

The telomere thermal cycling profile consists of:

Cycling for T(telomic) PCR: 96 °C for 1 min; denature at 96 °C for 1 s, anneal/extend at 54 °C for 60 s, with fluorescence data collection, 30 cycles.

Cycling for S (single copy gene) PCR: PCR: 96 °C for 1 min; denature at 95 °C for 15 s, anneal at 58 °C for 1 s, extend at 72 °C for 20 s, 8 cycles; followed by denature at 96 °C for 1 s, anneal at 58 °C for 1 s, extend at 72 °C for 20 s, hold at 83 °C for 5 s with data collection, 35 cycles.

The primers for the telomere PCR are *tel1b* [5′-CGGTTT $(GTTTGG)_5$GTT-3′], used at a final concentration of 100 nM, and *tel2b* [5′-GGCTTG$(CCTTAC)_5$CCT-3′], used at a final concentration of 900 nM. The primers for the single-copy gene (human beta-globin) PCR are *hbg1* [5′-GCTTCTGACACAACTGTGTTCACTAGC- 3′], used at a final concentration of 300 nM, and *hbg2* [5′-CACCAACTTCATCCACGTTCACC-3′], used at a final concentration of 700 nM. The final reaction mix contains 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 200 mM each dNTP; 1% DMSO; 0.4× Syber Green I; 22 ng E. coli DNA per reaction; 0.4 Units of Platinum Taq DNA polymerase (Invitrogen Inc.) per 11 µl reaction; ~6 ng of genomic DNA. Tubes containing 26, 8.75, 2.9, 0.97, 0.324 and 0.108 ng of a reference DNA (pooled human genomic DNA from leukocytest) are included in each PCR run so that the quantity of targeted templates in each research sample can be determined relative to the reference DNA sample by the standard curve method. The same reference DNA will be used for all PCR runs.

To control for inter-assay variability, eight control DNA samples are included in each run. In each batch, the T/S ratio of each control DNA is divided by the average T/S for the same DNA from 10 runs to get a normalizing factor. This is done for all eight samples and the average normalizing factor for all eight samples is used to correct the participant DNA samples to get the final T/S ratio. The T/S ratio for each sample will be measured twice. When the duplicate T/S value and the initial value vary by more than 7%, the sample will be run the third time and the two closest values were reported.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) v.22 (IBM Corp., Armonk, NY) was used for statistical calculations. All tests were two-tailed with an alpha = 0.05. Non-normally distributed variables were log-transformed to achieve normality, as was the case for both ccf-mtDNA and PBMC mtDNA-cn. In cases when log transformation was insufficient (viz., baseline HDRS scores), we used Blom transformation, a statistical procedure replacing each raw score with its rank value and adjusting the scale distances between the ranks to achieve a normal distribution. As GpX was assayed in two separate batches, we combined *z*-scores from both batches into one variable for analyses. Correlations were tested using Pearson's *r*, adjusting for covariates using partial correlations. Group comparisons are presented as unadjusted (Student's *t*-test) and adjusted (ANCOVA) analyses. Repeated measures ANOVAs were used to test the time x group interaction on the changes in mtDNA between baseline and week 8 in SSRI responders and non-responders. Covariates were age, sex, body mass index (BMI) and smoking status, analyses are presented as unadjusted and adjusted.

RESULTS

Demographic characteristics

Demographic characteristics of MDD subjects and controls and of SSRI responders and non-responders are presented in Table 1. There were no significant between-group differences with regard to age, and sex. Compared to controls subjects, MDD

Table 1. Demographic and clinical characteristics of subjects with MDD and controls and in SSRI responders and non-responders

	Controls $N = 55$	MDD $N = 50$	*P*-value	Responders $N = 11$	Non-responders $N = 8$	*P*-value
Sex (f/m)	33/22	27/23	0.54	8/3	5/3	0.64
Age (Years; mean ± SD)	37.6 ± 13.9	39.6 ± 14.7	0.47	39.2 ± 12.6	39.0 ± 14.9	0.98
BMI (kg/m^2; mean ± SD)	24.4 ± 4.9	26.1 ± 4.5	0.07	26.2 ± 5.4	30.1 ± 2.7	0.05
Current tobacco users (*n*)	3	13	< 0.01	3	2	0.91
HDRS score at baseline (mean ± SD)	N/A	20.2 ± 3.3	N/A	19.8 ± 3.0	19.6 ± 4.0	0.91

HDRS Hamilton Depression Ratting Scale, *BMI* body mass index

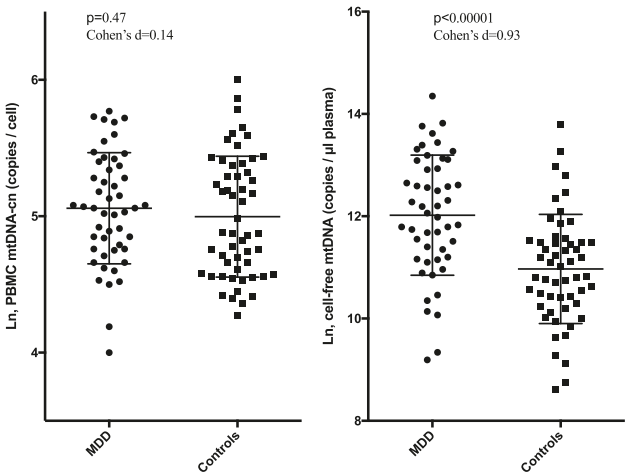

Fig. 1 Circulating cell-free mitochondrial DNA (Ccf-mtDNA) and peripheral blood mononuclear cell (PBMC) mtDNA copy number in MDD subjects and controls. Data were log-transformed. Error bars indicate ±1 SD

subjects had higher BMI, and were more likely to be current tobacco users. SSRI non-responders had higher BMI than SSRI responders ($p = 0.054$). After 8 weeks of SSRI treatment, HDRS score was 6.8 ± 2.4 (mean ± SD) for responders and 14.6 ± 4.8 (mean ± SD) for non-responders. For five subjects, this was the first depressive episode, and all other subjects had suffered from previous depressive episodes (median total number of depressive episodes = 4).

MtDNA at baseline in MDD subjects vs. controls, and correlations with depression symptom severity ratings

MDD subjects had significantly higher levels of ccf-mtDNA compared to healthy controls (mtDNA copies per µl plasma, log-transformed, mean ± SD: 12.0 ± 1.2 vs. 11.0 ± 1.1, $t = 4.8$, $p < 0.00001$, Cohen's $d = 0.93$). MDD subjects did not differ significantly from controls in mtDNA-cn measured in PBMCs (mtDNA copies per cell, log-transformed, mean ± SD: 5.1 ± 0.4 vs. 5.0 ± 0.4, $t = 0.7$, $p = 0.48$, Cohen's $d = 0.14$). After adjusting for age, sex, BMI and smoking, group difference in cell-free plasma mtDNA remained significant ($F = 20.6$, $p = 0.00002$), while PMBC mtDNA-cn remained non significant ($F = 0.2$, p = 0.65). Baseline mtDNA measurements (ccf-mtDNA and PBMCs) in MDD subjects and controls are shown in Fig. 1. In order to verify these data, we also an analyzed ccf-MtDNA using another target (MT-ND1). This yielded similar results and confirmed the difference in ccf-mtDNA levels between MDD subjects and controls ($p < 0.001$, Cohen's $d = 0.78$).

Within the MDD subjects there were no significant correlations between HDRS scores at baseline and mtDNA levels in cell-free plasma ($r = 0.03$, p = 0.86), or PBMC ($r = -0.27$, $p = 0.064$). Number of depressive episodes did not correlate significantly with either PBMC mtDNA-cn ($r = 0.21$, $p = 0.14$) or plasma ccf-mtDNA ($r = -0.12$, $p = 0.42$).

MtDNA levels after 8 weeks of SSRI treatment in a subsample of MDD subjects, preliminary analyses

Ccf ($n = 19$) and PBMC ($n = 18$) mtDNA were available from a subset of MDD subjects before and after 8 weeks of SSRI treatment, as described above. MtDNA data from healthy controls were only available at baseline. A significant time (baseline vs. week 8) × group (SSRI responders vs. non-responders) interaction was observed for ccf-mtDNA (unadjusted $F = (1,17)$ 4.43, $p = 0.05$; adjusted $F = (1,13)$ 6.47, $p = 0.02$), but not for PBMC mtDNA-cn (all $p > 0.4$). After Bonferroni correction, non-responders showed a significant increase in ccf-mtDNA between baseline and week 8 (unadjusted $p = 0.04$; adjusted $p = 0.02$), while this was not seen in responders (all $p > 0.32$). In cross-sectional analyses, ccf-mtDNA was higher in non-responders compared to responders at week 8 at trend level (unadjusted $p = 0.06$; adjusted $p = 0.08$), but did not significantly differ at baseline (all $p > 0.13$). Further, SSRI non-

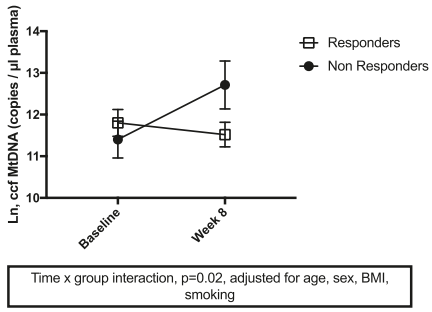

Fig. 2 Change in circulating cell-free mitochondrial DNA (ccf-mtDNA) (mean + SEM) between baseline and week 8 in SSRI responders and non-responders. Data were log-transformed

responders had significantly higher ccf-mtDNA levels at week 8 compared to healthy control levels at baseline ($p < 0.001$), whereas no significant difference was seen between SSRI responders at week 8 and healthy controls at baseline ($p = 0.37$). Adjusting for age, sex, BMI and smoking did not substantially alter these findings. Change in ccf-mtDNA between baseline and week 8 in responders and non-responders is shown in Fig. 2.

At week 8, there were no significant correlations between HDRS scores and plasma cell-free mtDNA levels, or in PBMC mtDNA-cn (all $p > 0.2$). Absolute change in HDRS scores were not correlated with absolute change in either ccf-mtDNA or PBMC mtDNA-cn (all $p > 0.1$)

Correlations between mtDNA and other biomarkers

Across all subjects, ccf-mtDNA and PBMC mtDNA-cn measurements were not inter-correlated (unadjusted: $r = -0.02$, $p = 0.87$; adjusted: $r = -0.03$, $p = 0.79$).

Across all subjects, Ccf-mtDNA, but not PBMC mtDNA-cn, was positively correlated with antioxidant enzyme glutathione peroxidase (ccf-mtDNA unadjusted and adjusted: $r = 0.32$, $p = 0.001$; PBMC mtDNA-cn unadjusted and adjusted: $r = -0.12$, $p = 0.26$). Conversely, PBMC mtDNA-cn, but not ccf-mtDNA, was positively correlated with PBMC telomere length (PBMC mtDNA-cn unadjusted: $r = 0.28$, $p = 0.005$, adjusted: $r = 0.38$, $p = 0.0001$; ccf-mtDNA: unadjusted: $r = 0.08$, $p = 0.42$, adjusted: $r = 0.11$, $p = 0.27$).

Overall, these correlations indicate that about 10% in the variance of ccf-mtDNA and PBMC mtDNA-cn (bivariate correlations) is accounted for by differences in antioxidant activity and telomere length, respectively.

Figs. 3 and 4 show these correlations in MDD and control subjects separately.

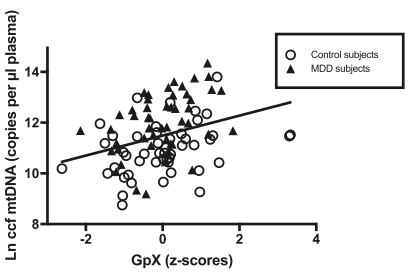

Fig. 3 Circulating cell-free mitochondrial DNA (ccf mtDNA) (log-transformed) plotted against glutathione peroxidase activity (GpX) (*z*-scores) in MDD subjects and healthy controls. Pearson's $r = 0.32$, $p = 0.001$

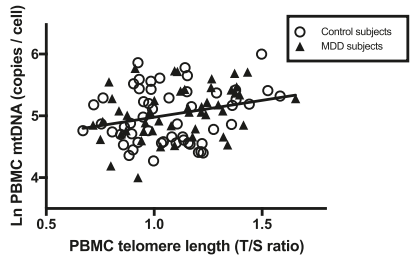

Fig. 4 Peripheral blood mononuclear cell (PBMC) telomere length plotted against PBMC mitochondrial DNA-copy number (mtDN-cn) (log-transformed) in MDD subjects and healthy controls. Pearson's $r = 0.28$, $p = 0.005$

DISCUSSION

Our results show that ccf-mtDNA is significantly higher in unmedicated MDD subjects compared to healthy controls, but that cellular (PBMC) mtDNA-cn does not significantly differ between groups. PBMC mtDNA-cn likely reflects intracellular mtDNA content and bioenergetics [6], independent of apoptosis; however, ccf-mtDNA is likely released from cells during cellular stress [19, 40] and does not contribute to cell energetics. Consistent with the idea that they may reflect different processes, ccf-mtDNA and cellular PBMC measures of mtDNA-cn were not significantly correlated. Additionally, the present study found that ccf-mtDNA levels further increased in non-responders to SSRI treatment over the 8-week course of treatment, whereas ccf-mtDNA levels in responders did not significantly change. After 8 weeks of SSRI treatment, ccf-mtDNA remained significantly elevated in non-responders compared to baseline levels of controls, whereas, at the same time point, there was no significant difference between responders and baseline levels of controls. In contrast, cellular (PMBC) mtDNA-cn did not significantly change over the course of SSRI treatment in either responders or non-responders. These data suggest that ccf-mtDNA and cellular PBMC mtDNA-cn reflect different cellular processes and that the former may be more reflective of certain aspects of MDD pathophysiology and of the response to SSRI antidepressants.

Studies of leukocyte mtDNA-cn in MDD have yielded inconsistent results, and only two previous studies have examined ccf-mtDNA in psychiatric samples. In the largest study in MDD to date, Cai et al. showed that greater mtDNA-cn in saliva and in blood leukocytes was associated with MDD [5]. The same group later confirmed, in a longitudinal study, that MDD subjects have higher cellular mtDNA-cn compared to controls over 8 weeks of antidepressant treatment. Across all time points, but not cross-sectionally, mtDNA-cn was positively correlated with depression severity [29]. Other studies have, however, reported lower [30] or unchanged [31] leukocyte mtDNA-cn in MDD compared to controls. Moreover, two population-based studies reported negative correlations between leukocyte mtDNA-cn and depressive symptoms [41, 42], while a recent longitudinal study did not find any between-person or within-person associations between depressive symptoms and leukocyte mtDNA-cn [32]. In regards to other affective disorders, one study on euthymic bipolar disorders showed lower leukocyte mtDNA-cn compared to controls [33], while another study on subjects with bipolar disorder in a depressive episode did not find a

significant difference in mtDNA-cn compared to controls, either before or after lithium treatment [43]. Reasons for the discrepancy in findings in these studies of cellular mtDNA-cn are not known, but could involve medication status of the subjects, different sample sizes across studies, somatic co-morbidities, differences in age, ethnicity or gender composition, differences in phenotypes (e.g., MDD diagnosis vs. depressive symptoms) or methodological differences (e.g., whole blood vs. isolated PBMCs). We note that our study might have been underpowered to detect a significant relationship between cellular, PBMC, mtDNA-cn and an MDD diagnosis, since some previous studies demonstrating such an effect had larger samples sizes [5, 29].

Only two studies have previously examined ccf-mtDNA in a psychiatric population. Our research group previously reported that ccf-mtDNA is strongly increased (Cohen's $d = 2.55–4.01$) in suicidal individuals (whether or not with MDD diagnoses) compared to healthy controls [16]. The present study only included subjects with MDD, and none were actively suicidal. In contrast to our study, Kageyama et al. recently reported lower ccf-mtDNA levels in MDD subjects compared to controls [17]. Differences in subject recruitment, blood sampling and statistical modeling may have contributed to the divergent findings. For example, Kageyama et al. did not actively exclude subjects with potentially confounding medical conditions, and they did not adjust for the effects of smoking or BMI on mtDNA.

Whereas cellular mtDNA-cn is a marker of mitochondrial biogenesis and energetics [6], ccf-mtDNA is believed to be released during cellular injury [19, 40]. Ccf-mtDNA molecules are subsets of so called damage-associated molecular patterns, which may have detrimental downstream effects involving immune activation [22]. Consistent with their reflecting different aspects of mitochondrial/cellular physiology, ccf-mtDNA was not significantly correlated with cellular (PBMC) mtDNA-cn in our study. The differential significant correlations we observed between ccf-mtDNA and PBMC mtDNA-cn versus antioxidant enzyme activity and telomere length, respectively, are also consistent with these functional differences.

During states of increased cellular and oxidative stress, genes encoding antioxidant enzymes, such as GpX, may increase their expression in an attempt to restore cellular homeostasis [44]. Oxidative stress can damage cellular integrity and, if unchecked, can lead to cellular endangerment or apoptosis, both of which can lead to mtDNA extrusion from the cell or release into the blood, increasing ccf-mtDNA. In fact, infusions of the anti-oxidant Vitamin C were observed to diminish ccf-mtDNA in Intensive Care Unit patients [45]. We had predicted an inverse relationship between GPx levels and ccf-mtDNA, although the opposite was found. Although speculative and in need of replication, our finding of a direct correlation between ccf-mtDNA levels and GpX activity may reflect a compensatory attempt to, ineffectively, upregulate the body's antioxidant defense mechanisms due to cellular stress. This hypothesis is in line with several preclinical studies reporting that GpX may protect against apoptotic cell death [44] and oxidative stress induced mtDNA damage [27].

Telomere shortening can reciprocally lead to cellular mitochondrial endangerment and diminished mitochondrial biogenesis via diminution of PGC-1α, the master regulator of mitochondrial biogenesis [28]. Telomeres become shorter due to each cell division, DNA damage, and exposure to oxidative stress [13]. In our study, PBMC mtDNA-cn was correlated with PBMC telomere length, a marker of cellular aging. This is in line with several previous studies reporting a positive association between mtDNA (measured in leukocytes) and telomere length [32, 41, 46, 47].

Oxidative stress and telomere shortening can both be induced by mitochondrial dysfunction, or mitochondrial allostatic load [21]. Although mitochondrial function was not measured directly here, the present data suggest that ccf-mtDNA may represent a more sensitive marker of cellular damage or cellular stress, not reflective of cellular mtDNA content or cellular energetics. The underlying mechanisms behind cellular stress and mitochondrial dysfunction in mood disorders are not yet fully understood, but might involve a wide array of pathophysiological processes. For instance, elevated stress and increased levels of pro-inflammatory cytokines may lead to accumulation of tryptophan catabolites (TRYCATS) via activation of the kynurenine pathway. TRYCATS may alter mitochondrial function both directly and via the metabolism of the endogenous antioxidant melatonin [48], with potential relevance for the pathophysiology of MDD.

In several of the previous studies investigating the relationship between mtDNA and depressive symptoms, a high proportion of depressed subjects was using antidepressant medications [5, 30, 31, 41, 42]. The participants in our study were free of any psychiatric medication for a period of at least 6 weeks prior to enrollment. This is important since experimental studies have shown that antidepressants may cause alterations in mitochondrial function [49], e.g., via inhibition of mitochondrial enzyme complexes [50], and uncoupling of oxidative phosphorylation [51]. Data from experimental studies suggest that antidepressants may have both pro-apoptotic [52], and anti-apoptotic properties [53], and that some of these effects may be mediated via mitochondria-associated pathways [54]. Interestingly, Djordjevic et al. found that fluoxetine induced apoptosis in rat liver cells, but these alterations were more pronounced in stressed animals compared to non-stressed animals [52]. Based on these data, we speculate that antidepressants may have pro-apoptotic or anti-apoptotic effects depending on symptom trajectory during the treatment course. This is in line with our observation that ccf-mtDNA continued to be higher in those subjects who did not respond to SSRI treatment, whereas there was no difference in ccf-mtDNA between SSRI responders and healthy controls. We note, however, that there was no significant difference in ccf-mtDNA between SSRI responders and non-responders at baseline. Thus, our data do not support the usefulness of ccf-mtDNA as a predictor of antidepressant treatment response, although larger samples are needed to draw any firm conclusions about this.

Strengths of the present study include (i) the simultaneous assessment of ccf-mtDNA as well as PBMC mtDNA-cn, (ii) the use of physically healthy and well-characterized subjects, (iii) the minimum 6-week medication-free period, and (iv) the exclusion of concomitant medications that could influence our results. Further, this is among the first studies to measure mtDNA, either cellular or cell-free plasma, before and after antidepressant treatment. However, the relatively small number of subjects receiving antidepressants yielded low power to test the relationship between ccf-mtDNA, cellular mtDNA-cn and SSRI response. Therefore, our results pertaining to the association between mtDNA levels and SSRI treatment response should be considered preliminary and in need of replication in larger samples. There are also several methodological factors that could have influenced our results. For example, the apparent increase of mtDNA in MDD subjects could be secondary to platelet or leukocyte lysis during the blood sampling, leading to subsequent release of mtDNA into the plasma. However, the blood sampling procedures for MDD subjects and controls was identical, so we believe that it is very unlikely that such methodological factors may have confounded our findings. Finally, another important limitation of our study is that mtDNA was measured in the peripheral blood, thus we were not able to determine if these biological markers are related to brain pathology in MDD. This would be an important area of future studies since we are not aware of any previous reports testing the relationship between peripheral measurements of mtDNA and brain imaging indices. Interestingly though, one recent post-mortem study [55] found alterations in mtDNA in the dorso-lateral prefrontal cortex of suicide completers, suggesting that changes in mtDNA might be relevant also for brain pathology.

In conclusion, our results indicate that ccf-mtDNA is a physiologically distinct marker from cellular (PBMC) mtDNA-cn. Even after adjustment for potential confounders, ccf-mtDNA was elevated in un-medicated MDD and continued to increase in subjects refractory to an 8-week SSRI treatment, whereas patients whose depressive symptoms substantively decreased post-treatment did not demonstrate changes in ccf-mtDNA levels. Together, these data suggest that elevated ccf-mtDNA may be a novel biological correlate of MDD and responsive to treatment, although the possible mechanistic relationship of this marker to depressive pathophysiology, and whether this is a state- or trait-related marker, remain unknown. Given previous inconsistencies in reported associations between intracellular leukocyte mtDNA-cn and depression, our findings point to mtDNA in cell-free plasma as a promising biological correlate of depression. The specificity of these findings for MDD is also unknown and will require further testing.

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ADDITIONAL INFORMATION

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Conflict of interest J.L. is a cofounder and consultant to Telomere Diagnostic Inc. (formerly Telome Health), a diagnostics company related to telomere biology. The remaining authors declare no competing financial interests.

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Paper 4

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Blood-based mitochondrial respiratory chain function in major depression

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Mitochondrial dysfunction has been implicated in major depressive disorder (MDD). A measure of mitochondrial respiratory chain (RC) enzymatic activity—the Mitochondrial Health Index (MHI)—has previously been found to correlate with stress and emotional states in caregivers. We here report mitochondrial RC activities, mitochondrial DNA copy number (mtDNAcn), and the composite MHI in unmedicated and somatically healthy subjects with MDD ($n = 47$) and healthy controls (HC) ($n = 11$). We also explore, in a subset of the MDD sample ($n = 33$), whether these markers are associated with response to 8 weeks of SSRI treatment. Mitochondrial RC complexes I, II, IV, citrate synthase (CS), mtDNAcn, and the MHI were assayed in peripheral blood mononuclear cells. Treatment response was defined as >50% decrease on the 25-item Hamilton Depression Rating Scale (HRDS-25). There were no significant differences in MHI or any of the mitochondrial markers between MDD subjects and HCs. Compared to SSRI nonresponders, SSRI responders had significantly higher baseline mitochondrial content markers CS ($p = 0.02$) and mtDNAcn ($p = 0.02$), and higher complex I activity ($p = 0.01$). Complex II activity increased significantly over treatment, irrespective of clinical response ($p = 0.03$). Complex I activity decreased in responders ($n = 9$), but increased in nonresponders ($n = 18$) (group x time interaction, $p = 0.02$). Absolute treatment-associated change in HDRS-25 scores correlated significantly with change in complex I activity between baseline and week 8 ($r = 0.47$, $p = 0.01$). Although mitochondrial markers did not distinguish MDD from controls, they did distinguish SSRI responders from nonresponders. If larger studies validate these mitochondrial differences, they may become useful biomarkers and identify new drug targets.

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INTRODUCTION

Major Depressive Disorder (MDD) is a prevalent debilitating condition, but its overall pathophysiology remains unknown. Further, the extent to which it is purely a "mental illness" or a "brain disease," as opposed to a disorder with peripheral somatic pathologies is uncertain [1]. Apart from its psychiatric and behavioral manifestations, MDD is associated with early mortality and increased risk for several somatic conditions [2], raising the possibility of systemic pathologies, perhaps even cellular disturbances, such as telomere length shortening, inflammation, oxidative stress, and mitochondrial dysregulation [3–8].

Mitochondria generate ATP by oxidative phosphorylation, but also have a number of other vital functions including regulating apoptosis, reactive oxygen species, steroidogenesis, inflammatory and anti-inflammatory effects, and others [9–11]. The brain is highly dependent on mitochondrial functioning, as it is the largest consumer of oxygen in the body [12, 13]. Individuals with primary mitochondrial cytopathies have a very high risk of developing psychiatric disorders, particularly MDD [14, 15]. This and other evidence have suggested a link between mitochondrial function and psychiatric symptoms, including MDD and other conditions [14, 16, 17].

While mitochondrial function may indeed be related to the pathophysiology of MDD [18], the extent to which this involves core mitochondrial enzymatic dysregulation, and whether it has clinical implications for MDD, are incompletely understood. Only a few studies have investigated mitochondrial function by quantifying electron transport chain enzymatic activity in MDD subjects. One of these studies found that activity of cytochrome c oxidase (COX)—also known as Complex IV—a large transmembrane multi-protein enzyme in the electron transport chain, measured noninvasively using near-infrared spectroscopy, was lower in the prefrontal cortex in MDD compared to controls and inversely correlated with depression severity [19]. In this small-scale pilot study, some of the patients were receiving antidepressant medication, calling for replication in larger and

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medication-free cohorts. Another study assessed mitochondrial adenosine triphosphate (ATP) production rate (MAPR) in biopsied muscle tissue from patients with MDD (who also had at least three comorbid medical conditions common in mitochondrial diseases) and found lower MAPR in MDD subjects compared to controls [20], suggestive of impaired mitochondrial efficiency in MDD. A recent meta-analysis, including only a small number of MDD studies with generally small sample sizes, found some evidence of complex I (NADH dehydrogenase) deficiency in certain brain regions in MDD, but no evidence of changes in complex IV (COX) [21]. The inclusion of medicated patients in such studies may have introduced bias [21], again highlighting the need to investigate mitochondrial markers also in unmedicated MDD patients.

Among the different methods used for assessing mitochondrial content in leukocytes, mtDNA copy number (mtDNAcn) is among the most widely used [8, 22–27]. The number of mtDNA copies per cell in leukocytes reflects mtDNA replication and degradation and has been used to indirectly reflect mitochondrial biogenesis [28–30], but it does not reflect mitochondrial function per se [31]. Total cellular mitochondrial energy production capacity is determined by both mitochondrial content (the number of mitochondria in a cell) and the mitochondrial functional capacity (the energy production capacity of each mitochondrion). To disentangle these contributors and identify the molecular nature of a potential mitochondrial perturbation, a mitochondrial health index (MHI) was developed to assess mitochondrial functional capacity in human leukocytes [32]. This metric integrates nuclear and mitochondrial DNA-encoded respiratory chain (RC) enzymatic activities and mtDNAcn into an index that reflects mitochondrial RC capacity on a per-mitochondrion basis. The MHI was previously found to be low among high-stressed caregivers compared to controls, and the MHI was also associated with mood states in this group [32]. While these findings are novel and interesting, the MHI has not, to date, been applied to psychiatric disorders and its clinical relevance remains unknown.

In this study, we report activities of mitochondrial complexes I, II, IV, mtDNAcn, citrate synthase (CS), and the composite MHI in peripheral blood mononuclear cells (PBMCs) of unmedicated MDD subjects and healthy controls. We also explored, in a subset of the present MDD sample, whether these markers (prior to antidepressant treatment as well as changes with treatment) predicted response to SSRI antidepressant treatment.

METHODS AND MATERIALS

Ethics statement

The Investigational Review Board (IRB) of the University of California, San Francisco (UCSF), approved the study protocol (UCSF IRB # 10-00825). All study participants gave written informed consent to participate in this study and were compensated for participating. The study was pre-registered at ClinicalTrials.gov (identifier NCT00285935).

Recruitment procedures

Unmedicated MDD outpatients ($n = 47$) and healthy controls ($n = 11$) were recruited by flyers, bulletin board notices, Craigslist postings, newspaper ads, and, in the case of MDD subjects, clinical referrals.

Inclusion criteria

Individuals aged 21–50 years, who met DSM-V criteria for MDD, and who had a 17-item Hamilton Depression Rating Scale (HDRS) [33] score of ≥17, or a score of ≥20 on the 25-item HDRS (to account for subjects with greater atypical symptom severity) were eligible for the study. All diagnoses, including MDD, were established using the Structured Clinical Interview (SCID) and were verified in a separate diagnostic evaluation by a Board-certified psychiatrist. The Perceived Stress Scale (PSS) [34] was used to measure the experience of psychological stress in the past month and the 10-item Adverse Childhood Experiences (ACE) questionnaire was used to assess adverse childhood experiences [35].

Exclusion criteria

MDD subjects were excluded if they met the DSM-IV criteria for any of the following: (i) bipolar disorder, (ii) alcohol or substance abuse within the preceding six months, (iii) PTSD or an eating disorder within 6 months of entering the study, and (iv) any history of psychosis outside of a major depressive episode, or the presence of any psychotic symptoms during the current major depressive episode. Potential healthy controls were excluded for any lifetime history of DSM-IV Axis-I diagnoses. All study participants (MDD and controls) were free of acute illnesses or infections, inflammatory disorders, neurological disorders, or any other medical conditions considered to be potentially confounding (e.g., cancer, HIV, diabetes, history of cardiovascular disease or stroke, etc.), as assessed by medical history, physical examinations, and blood screening, including electrolytes, lipids profile, liver, kidney, thyroid function tests, etc. All subjects were free of psychotropic medications (including antidepressants), hormone supplements, steroid-containing birth control, or other potentially interfering medications for at least 6 weeks prior to enrollment in the study. For the MDD subjects, short-acting sedative-hypnotics were allowed as needed up to a maximum of three times per week, but none within five drug half-lives prior to ratings or blood draws in the study. All subjects had to pass a urine toxicology screen for drugs of abuse (marijuana, cocaine, amphetamines, PCP, opiates, methamphetamine, tricyclic antidepressants, and barbiturates) and a urine test for pregnancy in women of child-bearing age on the same day as the blood draw and clinical ratings.

Blood sampling

Venipuncture was performed at approximately 10:00 a.m. at the UCSF Clinical and Translational Science Institute, after 12-h of fasting (except water) and seated relaxation for at least 30 min. Plasma was collected into a lavender EDTA Vacutainer tube. Tubes were spun at 1500×*g* for 10 min at 4 °C, and plasma was removed, aliquoted into tubes, and frozen at −80 °C until assayed. PBMCs were prepared from whole blood by ficoll centrifugation [36] and were frozen at −80 °C.

SSRI treatment in the MDD subgroup

Thirty-three of the MDD subjects underwent 8 weeks of protocol-based open-label outpatient treatment with an SSRI antidepressant. In order to limit the range of potential mechanisms of action of antidepressants, the choice of medication was limited to an SSRI. The decision for the specific SSRI prescribed was based on clinical information such as medical history, family history, prior medication history, subject preference, and potential side effects. The primary outcome measure was the severity of depressive symptoms, assessed by means of the 25-item HDRS [37], which was repeated at the end of treatment (week 8). Antidepressant responders were defined as those with >50% improvement on the HDRS-25. The HDRS-25, rather than the 17-item HDRS, was used to define response since the former scale includes several items (e.g., weight gain, loss of energy/fatigue) that might be linked to mitochondrial function [38, 39].

Twelve subjects were treated with sertraline (mean ± SD dose in mg = 131 ± 55), six with fluoxetine (dose in mg = 30 ± 11), four with citalopram (mean + SD dose in mg = 34 + 13), and 11 with escitalopram (mean + SD dose in mg = 18 + 5). Medication dosages were increased over the course of treatment per prespecified protocol as tolerated and as warranted by clinical response. MDD subjects had blood drawn at weeks 4 and 8 for serum antidepressant concentrations to assess medication compliance. In each of these subjects, plasma antidepressant concentrations were in the reported clinical range for that antidepressant, suggesting excellent compliance.

Rationale for calculating the MHI

The MHI was computed by integrating three enzymatic measures of respiratory chain capacity and two mitochondrial content features, as described previously [40]. Briefly, PBMC pellets were mechanically homogenized with tungsten beads in a homogenization buffer to release individual enzymes. The homogenate was then used to quantify citrate synthase (CS), NADH dehydrogenase (complex I), succinate dehydrogenase (SDH, complex II), and cytochrome c oxidase (COX, complex IV) using kinetic spectrophotometric assays. The specific activity for each enzyme was obtained by calculating the slope (first derivative) of the optical

Table 1. Demographic characteristics of subjects with MDD and controls.

	MDD $N = 47$	Controls $N = 11$	*P* value	Responders ($n = 11$)	Nonresponders ($n = 22$)	*p* value
Sex (f/m)	26/21	6/5	0.96	9/2	9/13	0.03
Age (Years; mean + SD)	35.0 ± 10.7	35.1 ± 11.1	0.99	39.0 ± 9.4	36.4 ± 11.8	0.52
BMI (kg/m^2; mean + SD)	25.1 ± 4.0	23.9 ± 2.9	0.33	25.4 ± 5.1	24.8 ± 4.0	0.72
Cigarettes smoked per day (mean ± SD)	0.19 ± 0.74	0 ± 0	0.42	0.28 ± 0.90	0.14 ± 0.64	0.61

density change, and subtracting nonspecific activity detected in the presence of specific inhibitors for each complex, or in the absence of the rate-limiting reaction substrate. All assays were performed in triplicates, at 30 °C, and final values were normalized on a per-cell basis using the qPCR-based estimates of cell numbers for each biological sample as described in ref. [32].

Mitochondrial and nuclear genome abundances were determined from 20 ul of the same homogenate used for enzymatic measurements lysed to extract genomic material (nDNA and mtDNA). The resulting lysate was used as the DNA template for two different Taqman multiplex assays for ND1 (mtDNA) and B2M (nDNA), and for COX1 (mtDNA) and RnaseP (nDNA), as previously described in details [32]. mtDNAcn was estimated using the ΔCt method, subtracting the mtDNA Ct from nDNA Ct for each amplicon pair (ND1:B2M and COX1:RNaseP), and then multiplying by 2 to account for the diploid nuclear genome. The mtDNAcn values from both assays were averaged to obtain the final mtDNAcn value for each participant.

To compute the MHI, the five mitochondrial features were mean-centered so that each parameter contributes an equal weight to the equation. Combining three features (complexes I, II, and IV) as a numerator, divided by two content features (CS and mtDNAcn) as a denominator produces a quantitative index of mitochondrial energy production capacity, or "quality" on a per cell mitochondrion basis, where a value of 100 represents the average of the cohort, and values >100 and <100, respectively, indicate higher and lower respiratory chain capacity on a per-mitochondrion basis. Previously, the composite MHI exhibited a higher predictive ability of caregiver (chronic stress) status than any of the individual MHI components alone [32].

Statistics

The Statistical Package for the Social Sciences (SPSS) v.22 (IBM Corp., Armonk, NY) was used for statistical calculations. All tests were two-tailed with an alpha = 0.05. Non-normally distributed variables were log10 transformed. Group differences were tested by independent sample *t*-tests or Chi-square. Correlations were tested using Pearson's *r*. Paired samples t-tests were used to test change in biomarkers pre- to posttreatment with SSRI. We also performed repeated-measures ANOVAs (within and between-subjects design) to test time (biomarker) × group (responder/nonresponder status) interactions on the changes in biomarkers between baseline and week 8 in SSRI responders and nonresponders. Binary logistic regression (with MDD/control and SSRI-responder/SSRI-nonresponder as dependent variable) were performed for multivariate analyses, adjusting for age and gender. Twelve MDD patients and three controls had missing baseline data on one or more mitochondrial enzymes due to technical reasons. For the purpose of calculating the MHI for these individuals, missing data were imputed using the group average. Sensitivity analyses were carried out for all calculations involving the MHI, including (i) only those subjects with full data and (ii) all subjects (including those for whom imputed data was used to replace missing values). All samples were collected over a 7-year period, and we observed a substantial effect of storage time on all enzyme activities, ranging from 5–12% activity loss per year. Therefore, all mitochondrial markers were adjusted for storage time by removing the variance (regression slope) attributable to time since collection. This is the first clinical study to test the association between these mitochondrial markers and antidepressant treatment response, thus these analyses should be considered exploratory and were not adjusted for multiple comparisons [41].

RESULTS

Demographic characteristics

Demographic characteristics of MDD subjects and controls are presented in Table 1. There were no significant group differences between MDDs and HCs with regard to age, sex, smoking, or BMI. There were no significant differences in age, BMI, or smoking between responders and nonresponders, but responders were more likely to be females.

Age was significantly correlated with mitochondrial RC complex IV ($r = -0.29$, $p = 0.04$). MHI was positively correlated with BMI ($r = 0.28$, $p = 0.03$). None of the mitochondrial biomarkers were significantly associated with sex or smoking (data not shown). MHI was not significantly correlated with ACE, PSS score, or the HDRS-25 suicidality item (all $p > 0.2$).

Mitochondrial markers in MDD subjects and controls

There were no significant differences in MHI or any of the individual mitochondrial markers between MDD subjects and healthy controls (Table 2). These results did not change when only including those with full MHI data or after adjusting for age and gender. There were no significant correlations between any of the biomarkers and depression severity ratings based on the 25-item HDRS (all $p > 0.05$).

Mitochondrial markers in SSRI responders and nonresponders

Pretreatment, SSRI responders had significantly higher mtDNAcn ($p = 0.02$), higher CS activity ($p = 0.02$), and higher RC complex I activity ($p = 0.01$) compared to SSRI nonresponders. These findings were also significant after adjusting for age and gender (all $p < 0.05$).

In all treated MDD subjects, Complex II increased significantly between baseline and after 8 weeks of SSRI treatment ($p = 0.03$). None of the other mitochondrial markers or MHI changed significantly with treatment in all treated subjects (Table 3). The results did not change for MHI when only those subjects with full data were included.

Complex I decreased in SSRI responders ($n = 9$), but increased in nonresponders ($n = 18$) (group × time interaction, $p = 0.02$, Fig. 1). There were no other significant time × group effects for any of the other biomarkers including MHI (all $p > 0.05$). Absolute change in HDRS-25 correlated significantly with an absolute change in Complex I activity between baseline and week 8 ($r = 0.47$, $p = 0.01$) (Fig. 2), such that a decrease in Complex I activity over treatment was associated with greater improvement on the HDRS-25.

DISCUSSION

We assessed the activity of RC enzymes, mtDNA, and the composite MHI in blood samples from unmedicated depressed patients and healthy controls. Although there were no significant between-group differences in any markers of mitochondrial content or RC activity, we did note significant associations between mitochondrial features and antidepressant response. Specifically, we found evidence that higher baseline (pre-SSRI treatment) mtDNAcn, as well as greater activity of CS and complex I, were associated with a better antidepressant response to an SSRI. Moreover, a greater treatment-associated decrease of Complex I was also associated with better SSRI response. Finally, complex II activity increased with SSRI treatment, irrespective of clinical response.

We found associations between certain mitochondrial features and antidepressant treatment response, whereas other features were not associated. Dysfunctional mitochondrial enzymes may

Table 2. Mitochondrial markers in MDD and controls.

	Unmedicated MDD $N = 47$	Controls $N = 11$	*P*- value	Effect size (Cohen's *d*)	Responders, Baseline ($n = 11$)	Nonresponders, Baseline ($n = 22$)	*p* value	Effect size (Cohen's *d*)
MHI (mean ± SD)	99.4 ± 14.4	95.6 ± 10.5	0.41	0.30	99.2 ± 10.9	95.4 ± 13.7	0.42	0.31
CS (mean ± SD)	45.5 ± 13.8	51.0 ± 31.4	0.59	0.23	50.1 ± 8.3	38.2 ± 13.7	0.02	1.05
Complex I (mean ± SD)	5.8 ± 1.9	5.8 ± 2.9	0.84	0.00	6.8 ± 1.8	5.0 ± 1.8	0.01	1.00
Complex II (mean ± SD)	76.0 ± 21.0	79.6 ± 33.6	0.85	0.13	79.0 ± 23.0	69.3 ± 19.2	0.27	0.46
Complex IV (mean ± SD)	10.9 ± 3.7	10.8 ± 5.6	0.79	0.02	10.1 ± 3.6	9.9 ± 2.0	0.76	0.07
mtDNAcn (mean ± SD)	437.3 ± 62.7	435.1 ± 106.7	0.93	0.03	464.6 ± 55.0	416.1 ± 54.4	0.02	0.89

Raw values are presented in the table, although *t*-tests using log-transformed data were used. Missing values for CS, $n = 4$ (four MDD, all nonresponders); complex I, $n = 3$ (three MDD, one responder, one nonresponder, one non-treated); complex II, $n = 8$ (five MDD, three nonresponders, one responder, one non-treated; three controls); complex IV, $n = 6$ (six MDD, three nonresponders, two responders, one non-treated). The group differences in MHI remained nonsignificant in sensitivity analyses including only those with full data ($p > 0.53$). Enzymatic activities are in mmol/min/106 cells, and mtDNAcn are copies per cell.

MHI mitochondrial health index, *CS* citrate synthase, *mtDNAcn* mitochondrial DNA copy number.

promote oxidative stress in various disorders [42], and increased levels of oxidative stress, in turn, have been associated with poorer SSRI treatment response [7]. Additional mechanisms for decreased mitochondrial function hindering antidepressant efficacy have also been proposed, including inflammation, apoptosis decreased activation of downstream neurotransmitter signaling, decreased vesicle release, and, possibly most importantly, decreased neuronal plasticity [43]. We found that higher baseline complex I enzyme activity, and a greater decrease over treatment, was associated with superior antidepressant treatment response. Mitochondrial RC complex I is the first enzyme of the mitochondrial RC and coordinates the transfer of electrons from Krebs cycle-derived NADH to coenzyme Q10, thus generating the proton gradient across the mitochondrial inner membrane, resulting in ATP production [44]. It is also a major site of reactive oxygen species (ROS) production within the mitochondria, which is modulated by a number of factors including membrane potential production [45]. Therefore, activity is not directly related to ROS production. RC complex I is critical for cellular functions, and deficiencies or dysfunction in complex I activity has been implicated in neurodegenerative disorders such as Alzheimer's and Parkinson's disorder [46]. Post-mortem studies have also reported lower complex I levels in brain tissue from MDD patients compared to controls [21]. RC complex 1 may suppress inflammation [47], highlighting another important mechanism linking mitochondrial function to an antidepressant response, given that systemic low-grade inflammation has been associated with lower SSRI efficacy [48–50]. Future mechanistic studies are needed to better understand how inflammation and mitochondrial dysfunction may interact to contribute to depression treatment resistance. Although this is the first clinical study, to the best of our knowledge, to investigate the association between antidepressant treatment response and specific mitochondrial enzymes, several preclinical studies have demonstrated an effect of antidepressants on mitochondrial function [43]. In a series of rodent experiments, Agostinho et al. [51, 52] reported several brain region-specific effects on mitochondrial enzyme activity of chronic and acute fluoxetine administration. They found that complex I activity decreased in the prefrontal cortex, but not in the hippocampus or the striatum, after chronic fluoxetine treatment. Complex IV activity decreased in the hippocampus, but not in the other brain regions, following chronic fluoxetine administration, and no significant effects were seen for complex II or CS [51]. In our sample, complex II increased with SSRI treatment, irrespective of clinical response. Partly in line with Agostinho et al., complex I activity decreased with SSRI treatment, but only in those patients who responded to treatment; in treatment nonresponders, on the other hand, complex I activity increased with SSRI treatment. These findings suggest that the relationship between antidepressant effect and mitochondrial function is complex, and more studies with larger samples are needed to elucidate this. Moreover, the basis underlying the specificity of our findings to complex I relative to complex IV (which similar to complex I is partially mtDNA encoded) and complex II (nuclear-encoded), is unclear and will require independent validation.

Our primary hypothesis was that unmedicated MDD subjects would have a lower MHI than controls, reflecting lower mitochondrial bioenergetic capacity. The rationale behind this was based on (i) the finding that individuals suffering from mitochondrial cytopathies, which decreases RC capacity on a per-mitochondrion basis, or MHI, often display depressive symptoms [14], (ii) several animal and human studies linking decreased mitochondrial function to depressive symptoms [14, 18, 20, 43, 53], and (iii) greater self-reported positive mood in women predicted higher MHI the following days [32]. We did not find a significant difference in MHI, or in any of the individual mitochondrial enzymes, between MDD subjects and controls. We speculate that it is possible that MDD is associated with various compensatory mechanisms that

Table 3. Pre- and posttreatment mitochondrial markers in all MDD subjects undergoing 8 weeks of SSRI treatment.

	Pretreatment, baseline	Posttreatment, after 8 weeks of SSRI treatment	*P* value	Effect size (Cohen's *d*)
MHI (mean ± SD)	70.3 ± 12.9	65.4 ± 15.3	0.56	0.10
CS (mean ± SD)	42.3 ± 13.2	48.2 ± 15.3	0.07	0.35
Complex I (mean ± SD)	5.8 ± 2.0	5.9 ± 2.5	0.77	0.05
Complex II (mean ± SD)	73.2 ± 20.8	86.6 ± 28.9	0.03	0.46
Complex IV (mean ± SD)	9.8 ± 2.7	11.5 ± 5.1	0.20	0.29
mtDNAcn (mean ± SD)	433.5 ± 59.1	460.5 ± 94.6	0.13	0.26

Raw values are presented in the table, although paired *t*-tests using log-transformed data were used (except for MHI which was normally distributed). Enzymatic activities are in mmol/min/106 cells, and mtDNAcn are copies per cell. Full data from both time points (baseline and week 8) were available for 32 subjects (MHI, including imputed values), 28 subjects (CS), 27 subjects (Complex I), 25 subjects (Complex II), 22 subjects (Complex IV), and 32 subjects (mtDNAcn).

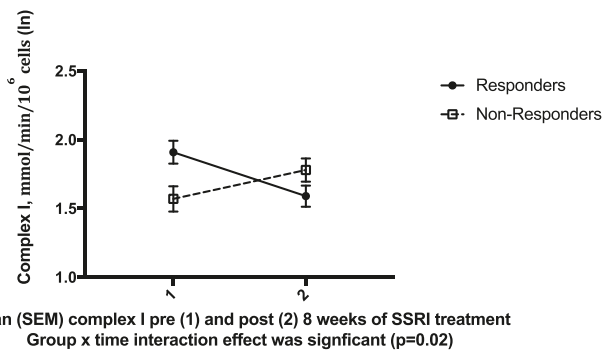

Fig. 1 Change in PBMCs RC Complex I activity between baseline and 8 weeks of SSRI treatment SSRI responders and nonresponders. Complex 1 activity is plotted on the y-axis and time point (baseline and week 8) on the x-axis.

counteract the hypothesized decrease in mitochondrial respiratory function. Also, it is possible that the cross-sectional design of the study may not have been appropriate to capture potential dynamics of mitochondrial function over time, where mitochondria may respond to mood states within hours to days [32] and may change from week to week [40]. It is also possible that mitochondrial function is not associated with the heterogeneous disorders of MDD per se, but rather certain aspects of MDD, including treatment response to antidepressants. Finally, our sample size may have been too small to detect statistically significant differences between groups, although the effect sizes of the differences between the groups somewhat argues against this.

A strength of the present study is that we have used a multivariate approach to profile potential mitochondrial alterations or recalibrations, including both mitochondrial content (CS and mtDNAcn) and RC capacity, in leukocytes. Advantages of using a composite score such as the MHI include its ability to provide an integrated representation of mitochondrial function from an easily accessible blood sample, including samples that have been previously frozen. While this is an important strength of our paper, we also note that the effects of stress and allostatic load on mitochondrial function and structure (i.e., mitochondrial allostatic load) may manifest in multiple aspects of mitochondrial behavior including DNA integrity, gene expression, protein composition, respiration, and signaling functions [54]. Future studies would gain by assessing a more comprehensive panel of mitochondrial behaviors to further our understanding of the relationship between mitochondrial health and depression. Additional strengths of our study are that: (i) depressed subjects were carefully evaluated and were unmedicated for at least six weeks prior to blood draws and were free from somatic illnesses and substance abuse; (ii) control subjects were rigorously screened for the absence of somatic and psychiatric pathologies; (iii) clinical response and adherence to the SSRI treatment protocol were closely monitored and verified. The study also has limitations, notably (i) the relatively small number of subjects, especially healthy controls and MDDs receiving antidepressant treatment (in particular, those who were "responders" to treatment), (ii) the open-label nature of SSRI treatment, although this best approximates real-world treatment conditions, and (iii) the use of PBMC cell mixtures, rather than specific purified cell types, which could have reduced our ability to detect mitochondrial alterations with greater sensitivity and specificity among particular cell populations [40]. Another limitation is that we did not have specific a priori hypotheses regarding mitochondrial component associations with SSRI treatment response, so these findings should be considered preliminary and in need of replication. While most animal studies testing the relationship between SSRI treatment and brain mitochondrial function used fluoxetine [51, 52], depressed subjects in our study were treated with a variety of SSRI agents. Although it is possible that the effect of SSRI treatment on mitochondrial function depends on the type of SSRI, we were not able to test this hypothesis given the relatively small sample size. Another important consideration when interpreting our findings is that mitochondrial health may be dynamically influenced by several factors that were not accounted for in

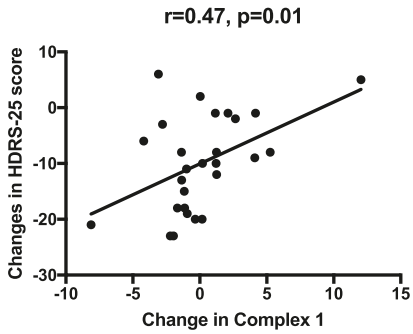

Fig. 2 Change in HDRS-25 scores correlated significantly with change in Complex I activity between baseline and 8 weeks of SSRI treatment. A negative score indicates a greater decrease over time, delta scores were computed by subtracting the week 8 value from the baseline value for HDRS-25 and Complex I respectively. This correlation was also significant using nonparametric statistics (rho = 0.4, $p = 0.04$).

the present study. Some of these include acute mood states [32] as well as exercise [55] and dietary habits [56]. Finally, we did not adjust the *p* value threshold for multiple comparisons which could be considered an additional limitation of the study. This is the first clinical study to test the relationship between these mitochondrial markers and antidepressant response. Thus these analyses should be considered exploratory and future, confirmatory, studies are needed to validate these findings.

In summary, our results suggest that, while the overall diminished mitochondrial respiratory capacity of PBMCs may not characterize MDD as a group, it may occur in relation to antidepressant treatment (with relatively higher complex I activity at baseline and relatively larger decreases with treatment associated with better antidepressant response). Future preclinical and clinical studies, especially longitudinal ones, will be required to ascertain the causes, consequences, and specific pathways involved in triggering mitochondrial recalibrations. A better understanding of the dynamic and complex mechanisms by which mitochondrial health is maintained, and how this may be altered in certain conditions may yield new strategies for treating groups of depressed patients or else current treatments might be used in a more targeted way. Lastly, further elucidation of these effects may clarify the nature and causes of certain comorbid systemic illnesses occurring with increased frequency in MDD and may broaden the perspective of depression beyond strictly monoaminergic theories [57].

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AUTHOR CONTRIBUTIONS

J.F., O.M.W., S.H.M., M.P., and D.L. formulated the specific research hypotheses. J.F. and D.L. analyzed and interpreted the data; conducted literature searches; prepared tables/figures, and cowrote the first draft of the manuscript. O.M.W., S.H.M., E.S.E., and V.I.R. designed the overall study; coordinated subject recruitment and blood sampling, and obtained funding. D.L. obtained funding for the assay. M.P. and M.A.M. assayed mitochondrial markers. C.M.H. was involved in subject recruitment and data collection. J.L. contributed to data interpretation. All authors reviewed the manuscript for intellectual content and approved the final version of the manuscript.

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The authors declare no competing interest.

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Paper 5

PLOS ONE

RESEARCH ARTICLE

Plasma circulating cell-free mitochondrial DNA in depressive disorders

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Abstract

Background

Plasma circulating cell-free mitochondrial DNA (ccf-mtDNA) is an immunogenic molecule and a novel biomarker of psychiatric disorders. Some previous studies reported increased levels of ccf-mtDNA in unmedicated depression and recent suicide attempters, while other studies found unchanged or decreased ccf-mtDNA levels in depression. Inconsistent findings across studies may be explained by small sample sizes and between-study variations in somatic and psychiatric co-morbidity or medication status.

Methods

We measured plasma ccf-mtDNA in a cohort of 281 patients with depressive disorders and 49 healthy controls. Ninety-three percent of all patients were treated with one or several psychotropic medications. Thirty-six percent had a personality disorder, 13% bipolar disorder. All analyses involving ccf-mtDNA were *a priori* adjusted for age and sex.

Results

Mean levels in ccf-mtDNA were significantly different between patients with a current depressive episode (n = 236), remitted depressive episode (n = 45) and healthy controls (n = 49) ($f = 8.3$, $p<0.001$). Post-hoc tests revealed that both patients with current ($p<0.001$) and remitted ($p = 0.002$) depression had lower ccf-mtDNA compared to controls. Within the depressed group there was a positive correlation between ccf-mtDNA and "inflammatory depression symptoms" ($r = 0.15$, $p = 0.02$). We also found that treatment with mood stabilizers lamotrigine, valproic acid or lithium was associated with lower ccf-mtDNA ($f = 8.1$, $p = 0.005$).

Discussion

Decreased plasma ccf-mtDNA in difficult-to-treat depression may be partly explained by concurrent psychotropic medications and co-morbidity. Our findings suggest that ccf-

Information and Secrecy Act. Data requests can be sent to registrator@lu.se and will be subject to a review of secrecy.

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mtDNA may be differentially regulated in different subtypes of depression, and this hypothesis should be pursued in future studies.

Introduction

Oxidative stress and apoptosis trigger the release of mitochondrial DNA (mtDNA) from the cell into the systemic circulation [1]. Circulating cell-free mtDNA (ccf-mtDNA), as measured in blood plasma, triggers inflammatory cascades but may also have beneficial antibacterial effects and contribute to cell-to-cell communications [1]. Increased ccf-mtDNA has been reported in various somatic disorders including sepsis, diabetes, and traumatic injury [2–5]. Recent studies show that also psychological stress may trigger ccf-mtDNA release [1, 6, 7], suggesting that this biomarker might be useful in certain psychiatric disorders.

We have previously shown that unmedicated patients with suicidal [8] and non-suicidal [9] major depressive disorder (MDD) have increased levels of plasma ccf-mtDNA, and that increased ccf-mtDNA levels are associated with hypothalamic-pituitary-adrenal axis hyperactivity [8]. While these findings suggest that depression and suicidality may be accompanied by increased amounts of cellular stress, other studies have reported unchanged [10, 11], or decreased [12] ccf-mtDNA in mood disorders compared to healthy controls. As recently reviewed elsewhere [1], there are several factors relating to assay methodology and study design that might explain divergent findings across studies. Moreover, the use of psychotropic medications may influence mitochondrial function and cellular health [13–15]. For instance, preclinical studies have shown that SSRIs, antipsychotics, and mood stabilizers may improve cellular health and have neuroprotective effects [16–21], but the relationship between these medications and cellular stress marker ccf-mtDNA has not yet been investigated in a real-life clinical sample of depression. Moreover, no previous studies that have investigated the relationship between ccf-mtDNA and specific symptom profiles of depression.

The main aim of the current study was to investigate plasma ccf-mtDNA in a large and diagnostically well-characterized clinical sample difficult-to-treat depression and healthy controls. Moreover, we aimed to test the relationship between ccf-mtDNA and specific symptoms of depression, a history of a suicide attempt and medications that may influence ccf-mtDNA.

Methods and materials

Ethical approval

All patients included in the study have given written informed consent to participate. The GEN-DS project was approved by the Regional Ethical Board in Lund, Sweden (2011/673).

Subject recruitment, patient cohort

This study is a part of a more comprehensive cohort named "Genes, Depression and Suicidality" (GEN-DS), seeking to investigate pharmacogenetic aspects among patients who have made suicide attempts and those who have not. Patients who were previously diagnosed with an affective disorder and had an insufficient treatment response were referred to the GEN-DS study. In this study insufficient treatment response was defined as not having achieved remission with previous and ongoing treatments during the current depressive episode. Recruitment procedures have been described in a previous study [22]. Briefly, 281 patients were referred to the project from four psychiatric clinics in southern Sweden between the years of

2012 and 2020. All referrals of patients with clinical depression according to referring specialist or resident in psychiatry were included in the project. Exclusion criteria were body mass index less than 15, pregnancy or current liver disease.

After inclusion, all patients were diagnosed according to DSM-IV by either a specialist in psychiatry or a senior resident in psychiatry under supervision by a specialist in psychiatry.

The diagnostic procedure included a standardized research protocol including Mini International Neuropsychiatric Interview (MINI) 6.0 [23] and the Structured Clinical Interview for DSM-IV Personality Disorders (SCID-II) [24]. Psychiatric symptoms were assessed using the Comprehensive Psychopathological Rating Scale (CPRS) [25]. We extracted the Montgomery-Åsberg Rating Scale (MADRS) from the CPRS [26]. The structured research protocol also included questions on psychiatric symptoms, suicidal and self-harm behaviour, alcohol and substance use, psychiatric and somatic diagnoses and treatments. Remitted depression was defined as referring only to the nine DSM-IV-TR criterion symptom domains for MDD [27].

Subject recruitment, controls

Forty-nine healthy controls were recruited through advertisements in social media and through newspaper ads. If deemed eligible for inclusion, controls subjects underwent a MINI. Any previous or present psychiatric illness; addiction disorder; treatment with psychotropic drugs or psychotherapy; somatic illness deemed severe or chronic; ongoing infection; present pregnancy, breast-feeding or treatment with drugs influencing the immune system were considered to be an exclusion criterium. Healthy controls received 500 SEK in compensation after the blood draw.

Measurement of cell-free mtDNA

Plasma was sampled in the morning after a night of fasting and instructions to avoid taking medications and smoking in the morning. Samples were stored at -80C until analyses.

DNA was isolated from thawed plasma samples using the QIAmp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction for Blood and bodyfluid protocol. Before the isolation of DNA, the plasma samples were centrifugated at 10 000 g for 10 min.

The quantitative analysis of cell-free mtDNA was performed using quantitative real time polymerase chain reaction (PCR). The experiment was run once in triplicate reactions. A dilution series consisting of the PCR product was constructed and used to create a standard curve. The different crossing-point values from the unknown samples were compared with the standard curve, and the corresponding number of mitochondrial units was calculated using the following formula:

The amount of DNA (g μl – 1) was divided with the size of the PCR fragment (bp) and the molar mass per base pair (g mol– 1). The product was finally multiplied with Avogadro's constant. The primers (Life Technologies, Paisley, UK) used for PCR amplification of mtDNA were as stated in the table below:

Gen Primer forward	Primer reverse	Accession nr
ND2 CACACTCATCACAGCGCTAA	GGATTATGGATGCGGTTGCT	KJ676545

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The PCR reactions were carried out using SYBR Green Technology (Thermo Fisher Scientific, Waltham, MA, USA). Each 20 μl reaction contained 5 μl of template, 1 μl of each primer (10 μM), 10 μl SYBR MIX (2 Å~, Sensifast, Bioline, London, UK) and 3 μl of nuclease-free water. Each reaction was run in triplicate on a LC480 LightCycler from Roche,

Mannheim, Germany) using the following program: Initial denaturation at 95˚C for 10 min, followed by 45 cycles consisting of 95˚C in 10 s. for melting, 65˚C for 10 s annealing and 72˚C for 11 s extension. The program ended with a melting curve analysis measuring fluorescence continuously from 60 to 97˚C.

Statistics

The Statistical Package for the Social Sciences for Mac (SPSS version 27, IBM, Armonk, NY, USA) was used for statistical calculations. One-way ANOVA or Student's t-test were used to compare demographic data between groups. Since the ccf-mtDNA levels were skewed this variable was log-transformed. Bivariate correlations were calculated using Pearson's r or Spearman's Rho, as appropriate. All analyses involving ccf-mtDNA were *a priori* adjusted for age and sex using either ANCOVA or partial correlations. Pearson's chi-2 was used to compare proportions between-groups. All tests were two-tailed and the significance level was set to $p < 0.05$.

Ccf-mtDNA, as a danger-associated molecular pattern–DAMP—may trigger of chronic low-grade inflammation. Systemic low-grade inflammation has been implicated in the pathophysiology of depression [28, 29], particularly in those subjects with symptoms of low energy, fatigue and sleep and appetite disturbances [30]. We therefore calculated a composite symptom score of "inflammatory depression" using CPRS items lassitude, fatiguability, reduced appetite and reduced sleep–and investigated the association between this composite score and ccf-mtDNA.

Results

Demographic characteristics

Demographic characteristics and ccf-mtDNA levels in current depression, remitted depression and healthy controls are summarized in **Table 1.**

As expected, MADRS scores were significantly higher among currently depressed individuals compared to those with remitted depression. Treatment with mood stabilizers (either lithium, lamotrigine or valproic acid) was more common in remitted depression than current depression.

Diagnostic characteristics for all patients are summarized in Table 2.

Patients with remitted depression did not fullfill the DSM criteria for a depressive episode at the time of the diagnostic evaluation, although comorbidity with other psychiatric disorders was common as shown in **Table 3** below.

Table 1. Demographic characteristics and ccf-mtDNA in patients and controls.

	Controls (n = 49)	Current depression (n = 236)	Remitted depression (n = 45)	P-value
Age Mean ± SD	36.7 ± 13.1	38.2 ± 13.5	34.0 ± 10.2	0.13
Sex N (%) females	36 (73.5)	154 (65.3)	30 (66.7)	0.54
BMI Mean ± SD	23.8 ± 4.0	26.3 ± 5.3	25.3 ± 4.7	0.006
Current smokers, N (%)	3 (6.1)	48 (20.5)	12 (26.7)	0.03
MADRS score (mean, SD)	na	22.9 ± 7.9	14.6 ± 9.1	<0.001
Current treatment with mood stabilizer N (%)	na	49 (20.8)	16 (35.6)	0.03
Current treatment with antipsychotic N (%)	na	45 (19.1)	9 (20.0)	0.90
Current treatment with antidepressant N (%)	na	192 (81.7)	32 (71.1)	0.20
Ccf-mtDNA (C/μl plasma) (mean, SD)	105 578 ± 207 848	35 683 ± 59 906	26 762 ± 26 688	p<0.001

Data was missing for BMI (n = 14), smoking (n = 2) and MADRS (n = 17).

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Table 2. Detailed diagnostic characteristics including type of affective disorder.

Diagnostic group	*Number of patients*
Current mood disorder	*236*
Depression, single episode	*8*
Recurrent depression	*120*
Chronic depression	*79*
Depression NOS	*3*
Dysthymia	*59*
Bipolar disorder, depressive episode	*22*
Remitted mood disorder	*45*
Recurrent depression, in remission	*31*
Bipolar depresson, in remissiom	*11*
No affective disorder	*3*

Patients could be assigned to more than one diagnosis.

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Ccf-mtDNA was not significantly associated with age (p = 0.70), sex (p = 0.34), BMI (p = 0.66) or smoking (p = 0.74). The most common somatic comorbidities among the patients were musculoskeletal disorders (24.9%), gastrointestinal disorders (16.7%), respiratory disorders (8.5%), cardiovascular disorders (7.1%), neurological disorders (12.5%), endocrinological disorders (13.5%). We calculated a "composite somatic co-morbidity score" in which one point was added for each organ system affected. There was no significant correlation between this score and ccf-mtDNA (p = 0.63). Ccf-mtDNA was not significantly correlated with freezer time (p = 0.68).

Ccf-mtDNA in patients and controls

Mean levels in ccf-mtDNA were significantly different between patients with a current depressive episode, remitted depressive episode and healthy controls (F = 8.3, $p<0.001$, adjusting for age and sex,). Post-hoc tests revealed that both patients with current ($p<0.001$) and remitted (p = 0.002) depression had lower ccf-mtDNA compared to controls. There was no significant difference in ccf-mtDNA between current and remitted depression (p = 0.86). Patient samples had been stored longer in the freezer compared to control samples (median 4 vs 2 years, $p<0.001$, Mann-Whitney U-test). Storage time was, however, not significantly correlated with ccf-mtDNA as shown above and the group differences in ccf-mtDNA (controls vs ongoing depression and controls vs remitted depression) were still significant even after adding storage time as a covariate (F = 9.3, $p<0.001$).

Ccf-mtDNA was plotted in current depression, remitted depression and controls in Fig 1.

Table 3. Psychiatric comorbidity in patients with current and remitted depression.

	Controls (n = 49)	Current depression (n = 236)	Remitted depression (n = 45)	P-value
Bipolar disorder N (%)	na	24 (10.2)	12 (26.7)	0.002
Personality disorder N (%)	na	81 (34.3)	20 (44.4)	0.53
Anxiety disorder N (%)	na	132 (55.9)	24 (53.3)	0.49
Substance or alcohol use disorder N (%)	na	12 (5.1)	2 (4.4)	0.82

Chi-square tests were carried out to compare current depression vs remitted depression

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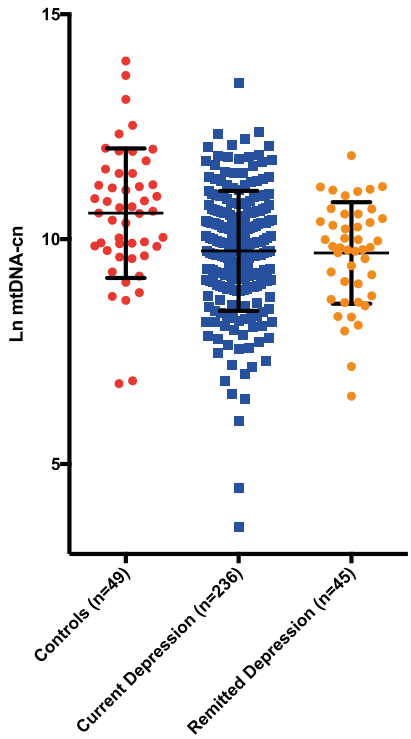

Fig 1. Log transformed ccf-mtDNA in controls, current depression and remitted depression. Error bars represent mean, SD. The group effect was significant (F = 8.3, p<0.001, adjusted for age and sex). Post-hoc tests revealed that patients with current (p<0.001) and remitted (p = 0.002) depression had lower ccf-mtDNA compared to controls.

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Associations between ccf-mtDNA and psychotropic medications

Patients taking mood stabilizers (n = 65) had significantly lower ccf-mtDNA compared to those not taking mood stabilizers (n = 216) (F = 8.1, p = 0.005, adjusted for age and sex). There were no differences in ccf-mtDNA between those taking antidepressants or not, or between those taking antipsychotics or not (all p>0.27). Patients with bipolar disorder did not differ significantly in ccf-mtDNA compared to patients without bipolar disorder (p = 0.42).

Nineteen patients (6.8%) took no psychotropic medications at the time of the diagnostic evaluation. There was no significant difference in ccf-mtDNA between those patients and all other patients (p = 0.28).

Associations between ccf-mtDNA and psychiatric symptoms and suicidality

Ccf-mtDNA was not significantly correlated with MADRS or the SUAS subscale comprising items 16–20 (all p>0.25). As shown in Fig 2, the inflammatory depression composite score

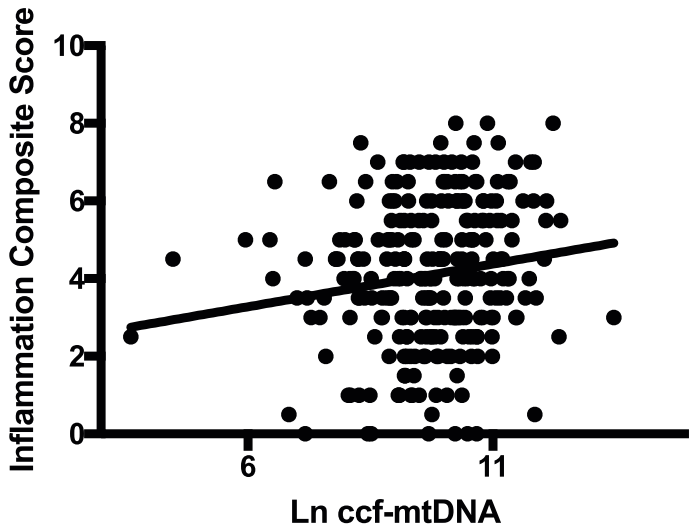

Fig 2. Correlation between ccf-mtDNA and "inflammation composite score" calculated by summarizing CPRS-items "lassitude", "fatiguability", "reduced appetite" and "reduced sleep". Both patients with current and remitted depression were included. The correlation was significant (r = 0.15, p = 0.02, df = 264, adjusting for age and sex).

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correlated positively and significantly in all patients (r = 0.15, p = 0.02, df = 264, adjusted for age and sex). Patients with a history of a suicide attempt (n = 83) did not differ in ccf-mtDNA levels compared to those who had not made a suicide attempt (n = 191) (p = 0.18, adjusting for age and sex). Information regarding previous suicide attempts was missing for seven patients.

Discussion

Our most salient finding was that patients with depression in secondary psychiatric care had lower ccf-mtDNA levels compared to healthy controls. These findings contrast our previous reports on unmedicated and suicidal depressed patients [8, 9], but are more in line with other studies on mood disorders [10–12]. Although low ccf-mtDNA was found in the depressed group overall, an "inflammatory depression symptom profile" [30] was conversely associated with higher ccf-mtDNA, suggesting that ccf-mtDNA may be differentially regulated across depression symptom profiles. Finally, treatment with mood stabilizers was associated with the lowest ccf-mtDNA within the depressed group, consistent with animal studies showing that these medications may promote cellular and neuronal health [31].

Previous studies have reported inconsistent findings regarding between-group differences in ccf-mtDNA in patients with mood disorders and healthy controls. The divergent results across studies may be explained by cohort-specific factors such as medication status, illness chronicity, symptom profiles and somatic and psychiatric co-morbidity. In the present study, 93% medicated with one, or a combination of several, psychotropics. Moreover, somatic as well as psychiatric comorbidities were common in the current sample. These sample

characteristics are largely in contrast to one of our previous studies, where we reported *increased* ccf-mtDNA in depression, in which all patients were unmedicated, somatically healthy and had no or minimal psychiatric co-morbidity [9]. In the other study from our group in which increased ccf-mtDNA was reported patients versus controls, all patients were also unmedicated and had recently attempted suicide at the time of the blood sampling [8]. In line with the findings of the present study, Kageyama et al. also reported decreased ccf-mtDNA in unipolar and bipolar depression compared to controls [12]. In another recent study, Jeong et al. found no difference in ccf-mtDNA between adolescents with bipolar disorder and healthy controls [10]. Moreover, Jeong et al. also reported that severity of depressive symptoms was negatively correlated with ccf-mtDNA, although specific depression symptom profiles were not investigated in this partciluar study. In both of these studies [10, 12], a substantial part of the patients medicated with one or more psychotropic. In the present study, we found, in exploratory analyses, that patients within the depressed group treated with mood stabilizers had the lowest levels of ccf-mtDNA, suggesting that at least part of the between-group differences in ccf-mtDNA may be accounted for by medication status. Psychotropic medications, and lithium in particular, are known to influence mitochondrial biology and cellular health [20]. In one study based on pluripotent stem cell technology, mitochondrial abnormalities were found in neurons from patients with bipolar disorders. Furthermore, lithium could normalize some of these alterations [32]. Similar effects of lithium were shown in a post-mortem study, in which activity of electron transport chain (ETC) enzyme complex I-III was increased in human frontal cortex after exposure to lithium [33]. Moreover, an animal model of mania, in which mitochondrial dysfunction was induced using d-amphetamine, both lithium and valproate were found to reverse some of these alterations [34]. Also lamotrigine may have neuroprotective effects mediated via its actions on mitochondrial function, according to some preclinical studies [35, 36]. We show, for the first time, that medication with mood stabilizers is associated with lower plasma levels of ccf-mtDNA in a large clinical sample of depression. These findings may have future implications for treatment response and the understanding of mechanistic actions of mood stabilizers. Such issues should be pursued in future studies.

Both clinical and preclinical studies suggest that ccf-mtDNA is an immunogenic molecule [37–41], although this assumption has also recently been challenged [1]. Mitochondrial DNA can act as damage associated molecular patterns (DAMPs) triggering the innate immune response primarily through binding to the toll-like receptor 9 (TLR-9). Interestingly, we found a significant positive relationship between ccf-mtDNA and depressive symptoms that have been more closely linked to low-grade inflammation [30]. While highly preliminary and in need of replication, these findings suggest that ccf-mtDNA may be differentially regulated in different subtypes of depression. Specifically, our findings point to a role of ccf-mtDNA in "inflammatory depression"; a depression subtype that has been associated with worse treatment response to conventional SSRIs and a better treatment anti-inflammatory compounds [29, 42]. Future clinical trials testing the antidepressant efficacy of such interventions should consider measuring ccf-mtDNA as a potential biomarker of treatment response.

While the present study has several notable strengths including the large sample size and the thorough diagnostic assessments, it also comes with several limitations. There are many factors that potentially may influence ccf-mtDNA levels that were not accounted for in the present study. As described above, both exercise and psychological stress can induce changes in ccf-mtDNA within minutes [6, 7]. We attempted to mitigate these effects by standardizing blood sampling procedures to be done fasting in the morning. We did not, however, record levels of subjective perceived stress preceding the blood draw, sleep patterns or other health behaviors, thus we can not rule out that this may have influenced our results. Sample storage

time in freezer differed significantly between patients and controls, but was not significantly correlated with ccf-mtDNA. Moreover, the main group effects remained significant even after taking this factor into account, making it unlikely that storage time confounded our results. The current study was originally designed to test another primary hypothesis, namely the relationship between genetic variants and suicidal behavior. Thus, we did not, *a priori*, power the study with the main intent to investigate differences in ccf-mtDNA between patients and controls. Although this might be considered a weakness of the study, our previous experiences using the same biomarker assay in other MDD/control cohorts (with substantially smaller sample size than the present study) showed effect sizes ranging between Cohen's d of 0.9 [9] to Cohen's d >2 [8]. Therefore, we believed that the current sample size would be large enough to detect a significant group difference between patients and controls. Finally, while we argue above that psychotropic medication might be a factor leading to lower ccf-mtDNA in the patient group, we did not find a significant difference in ccf-mtDNA between a small subset of the patients not taking any medications (7%) and all other patients. Potential reasons for this include that the unmedicated group might have been too small to detect a significant effect, and future larger studies should further investigate this hypothesis.

In conclusion, we found that plasma ccf-mtDNA is decreased in individuals with depressive disorders compared to controls. These findings may be partly explained by concurrent psychotropic medications and psychiatric co-morbidity. Specifically, our results suggest that treatment with mood stabilizers may affect ccf-mtDNA levels, possibly as a down-stream consequence of their effect on mitochondrial enzymatic processes. Our findings suggest that ccf-mtDNA may be differentially regulated in different subtypes of depression. This hypothesis might be valuable to pursue in future studies.

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