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"Development of a Real-Time PCR Assay for the Direct Quantification of Circulating Cell-Free Mitochondrial DNA in Human Blood Plasma: Preanalytical Considerations, Challenges, and Clinical Implications"

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Development of a Real-Time PCR Assay for the Direct Quantification of Circulating Cell-Free Mitochondrial DNA in Human Blood Plasma: Preanalytical Considerations, Challenges, and Clinical Implications

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„Development of a Real-Time PCR Assay for the Direct Quantification of Circulating Cell-Free Mitochondrial DNA in Human Plasma: Preanalytical Considerations, Challenges, and Clinical Implications"

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Table of Contents

Eidesstaatliche Erklärung **III**

Danksagung **IV**

Table of Contents **V**

Abbreviations **VIII**

List of Figures **IX**

List of Tables **X**

Zusammenfassung **1**

Abstract **3**

1. Introduction **5**

1.1 Circulating cell-free nuclear DNA **7**

1.2 Mitochondria and circulating cell-free mitochondrial DNA **9**

1.3 Cf-DNA release mechanisms and molecular characteristics **10**

1.3.1 Release mechanisms 10

1.3.2 Cf-DNA profiling and characteristics 12

1.3.3 Clearance of cf-DNA 13

1.4 Quantification of cf-DNA **14**

1.5 Challenges regarding ccf-mtDNA quantification and preanalytical considerations **16**

1.5.1 The nature of mitochondrial sequences- Nuclear Embedded Mitochondrial Insertions 16

1.5.2 Mitochondrial fragment size 16

1.5.3 Preanalytical aspects and platelets 17

1.6 Stress and stress-related disorders and the role of mitochondria **18**

1.7 Aims **19**

2. Chapter declarations **21**

2.1 Chapter I **21**

2.2 Chapter II **22**

3. Chapter I **23**

4. Chapter II **36**

Effects of acute stress on plasma circulating cell-free mitochondrial DNA in Panic Disorder **36**

4.2 Introduction **38**

4.3 Material and Methods **40**

4.3.1 Ethical approvement 40

4.3.2 Study participants 41

4.3.3 Trier Social Stress Test 41

4.3.4 Evaluation of Chronic Psychological Stress 41

4.3.5 Blood sampling 42

4.3.6 Blood sample collection and preparation 42

4.3.7 Quantification of plasma circulating cell-free mitochondrial DNA 42

4.3.8 Statistical analysis 43

4.4 Results **44**

4.4.1 Chronic stress negatively correlates with ccf-mtDNA, while baseline levels remain unchanged between groups 44

4.4.2 Ccf-mtDNA decreases after acute stress induction in PD patients but not in healthy subjects 45

4.5 Discussion **45**

4.6 Conclusion **48**

4.7 Declarations **48**

4.7.1 Conflict of interest 48

4.7.2 Data availability 48

4.7.3 Acknowledgements 49

5. Summary of the present thesis and integrated discussion **50**

5.1 Chapter I - Direct quantification of ccf-mtDNA in human blood plasma samples **50**

5.2 Chapter I and II - Cf-DNA in chronic and acute stress in health and disease **52**

6. Concluding remarks and future perspectives **55**

References **XII**

Appendix **XXIV**

Supplementary Information **XXIV**

Curriculum vitae **XXXIV**

Abbreviations

8-OHdG ..... 8-hydroxy-2'-deoxyguanosine
BD ..... bipolar disorder
bp ..... base pairs
CAD ..... caspase-activated DNase
ccf-mtDNA ..... circulating cell-free mitochondrial DNA
cf-DNA ..... circulating cell-free DNA
cf-nDNA ..... circulating cell-free nuclear DNA
cGAS-STING ..... cyclic GMP-AMP synthase- simulator of interferon genes
COVID-19 ..... Coronavirus disease 2019
CpG-islands ..... cytosine-guanine sites
CTCs ..... circulating tumor cells
DAMP ..... damage-associated molecular pattern
DNA ..... deoxyrobonucleic acid
ELISA ..... enzyme-linked immunosorbent assays
EVs ..... extracellular vesicles
GCs ..... glucocorticoids
HPA ..... hypothalamic-pituitary-adrenal
LINEs ..... long interspersed nuclear elements
MAL ..... mitochondrial allostatic load
MD ..... major depression
mL ..... milliliters
MPO ..... myeloperoxidase
mtDNA ..... mitochondrial DNA
NE ..... neutrophil elastase
NETs ..... neutrophil extracellular traps
ng ..... nanogramm
NGS ..... next-generation sequencing
NUMTs ..... Nuclear-mitochondrial DNA segments
PD ..... panic disorder
PRRs ..... pattern recognition receptors
qPCR ..... quantitive polymerase chain reaction, quantitative PCR
RNA ..... ribonucleic acid
ROS ..... reactive oxygen species
rRNA ..... ribosomal RNA
SAD ..... social anxiety disorder
SINEs ..... short interspersed nuclear elements
SIRS ..... severe nflammatory response syndrome
SLE ..... systemic lupus erythematosus
TFAM ..... mitochondrial transcription factor A
TLR-9 ..... Toll-like receptor-9
tRNA ..... transfer RNA

List of Figures

Introduction

Figure 1 Schematic representation of liquid biopsy approaches. 6

Figure 2 Potential origins of cf-DNA that are detectable in human bodily fluids. 7

Figure 3 Selection of cf-DNA release mechanisms and forms of cf-DNA. 12

Results

Figure 4 Effect of perceived chronic stress on ccf-mtdna in healthy subjects compared to PD patients. 44

Figure 5 Kinetics of plasma ccf-mtDNA concentrations following acute stress induction and under resting conditions in healthy individuals and patients with panic disorder. 45

List of Tables

Supplementary Information

Table S1 qPCR measurements acquired from all HC and PD patient samples. XXIV

Table S2 TICS-9 score of HC and PD patients XXXII

Zusammenfassung

Mitochondrien sind multifunktionale Organellen, die aufgrund ihrer Fähigkeit, externe und interne Stressoren zu erkennen und darauf zu reagieren, zunehmend in den Fokus in der Stressforschung rücken. Als solche sind sie von zentraler Bedeutung für unser Verständnis der zellulären Resilienz und der Signalwege, die als Reaktion auf akuten oder chronischen Stress aktiviert werden. Eine Folge von anhaltendem mitochondrialem Stress und daraus resultierender Dysfunktion ist die Freisetzung mitochondrialer Komponenten, wie z. B. mitochondrialer DNA (mtDNA), in den Blutkreislauf. In diesem Zusammenhang haben sich zirkulierende, zellfreie Nukleinsäuren (cf-DNA) sowohl unter pathologischen als auch bei physiologischen Bedingungen als informative Biomarker erwiesen. Bis heute ist die Bedeutung der cf-DNA, insbesondere bei der Flüssigbiopsie, gut etabliert und wird in der klinischen Routine und in der medizinischen Praxis weitgehend angewendet. Die Quantifizierung von zirkulierend zellfreier mtDNA (ccf-mtDNA) bietet daher ein wertvolles Instrument, um Einblicke in die mitochondriale Funktionalität und die breiteren zellulären Mechanismen zu gewinnen, die unter Stressbedingungen aktiviert werden. Die Quantifizierung von ccf-mtDNA stellt jedoch aufgrund verschiedener technischer und präanalytischer Faktoren eine Herausforderung dar. In dieser Arbeit wird eine Methode zur Messung und Quantifizierung von ccf-mtDNA in menschlichen Blutplasmaproben mittels direkter quantitativer Polymerasekettenreaktion (qPCR) vorgestellt, ohne dass eine vorherige Isolierung erforderlich ist. Dieser Ansatz weist im Vergleich zu herkömmlichen Methoden erhebliche Vorteile auf, darunter eine kürzere Probenverarbeitungszeit, ein geringeres Risiko von Probenverlusten, Kosteneffizienz und eine verbesserte Gesamtleistung des Assays. Bei der Etablierung des qPCR-Assays wurden mehrere Empfehlungsrichtlinien umgesetzt, um Genauigkeit und Reproduzierbarkeit zu gewährleisten. Um die Anwendbarkeit des neu entwickelten Assays in klinischen Proben zu bewerten, wurden die ccf-mtDNA-Konzentrationen bei gesunden Personen in Abhängigkeit von ihrer chronischen Stressbelastung gemessen. Die Ergebnisse zeigten eine negative Korrelation zwischen ccf-mtDNA und der wahrgenommenen chronischen Stressbelastung bei gesunden Personen, während für cf-nDNA keine derartige Korrelation festgestellt wurde. Um diese stressassoziierte Beziehung weiter zu untersuchen, wurde die Kinetik der ccf-mtDNA nach einer akuten Stressinduktion mit dem *Trier Social Stress Test* (TSST) untersucht,

einem etablierten und kontrollierten Verfahren zur Auslösung einer physiologischen Stressreaktion sowohl bei gesunden Personen als auch bei Patienten mit Panikstörung (PD). Die Ergebnisse der vorliegenden Arbeit zeigen, dass die ccf-mtDNA-Konzentrationen in der gesunden Kontrollgruppe unverändert blieben, während sie bei den PD-Patienten 10 Minuten nach der akuten Stressinduktion signifikant abnehmen. Insgesamt bilden die Ergebnisse eine solide Grundlage für die direkte Quantifizierung von ccf-mtDNA-Konzentrationen in klinischen Proben und Eröffnen neue Möglichkeiten zur Untersuchung ihrer Rolle im Bereich der Stressforschung bei einer Vielzahl von Erkrankungen wie Entzündungskrankheiten, Krebs, neurodegenerativen Störungen und anderen.

Abstract

Mitochondria are multifunctional organelles increasingly recognized as key players in stress research due to their ability to sense and respond to external and internal stressors. As such, they are central to our understanding of cellular resilience and signaling pathways in response to acute or chronic stress. One consequence of prolonged mitochondrial stress and subsequent dysfunction is the release of mitochondrial components, such as mitochondrial DNA (mtDNA) into the circulation. In this context, circulating cell free nucleic acids (cf-DNA) have emerged as biomarker in both pathological and physiological conditions. To date the relevance of cf-DNA, particularly in liquid biopsy, is well established and widely applied in clinical routine and medical practice. Quantifying circulating cell free mtDNA (ccf-mtDNA) therefore offers a valuable tool for gaining insights into mitochondrial responses and the broader cellular mechanisms activated under stressful conditions. However, quantifying ccf-mtDNA remains challenging due to several technical and pre-analytical considerations. A method for measuring and quantifying ccf-mtDNA in human blood plasma samples using direct quantitative polymerase chain reaction (qPCR) without the need for prior isolation is presented in this thesis. This approach shows significant advantages compared to traditional methods including reduced sample processing time, lower risk of sample loss, cost efficiency and improved overall assay performance. The establishment of the qPCR-assay implemented serval recommendation guidelines to ensure accuracy and reproducibility. To evaluate the applicability of the newly developed assay in clinical samples, ccf-mtDNA concentrations were measured in healthy individuals in respect to their chronic stress load. The results revealed a negative correlation between ccf-mtDNA and the perceived chronic stress in healthy individuals, whereas no such correlation was observed for cf-nDNA. To further investigate this stress-associated relationship, ccf-mtDNA kinetics was assessed after acute stress induction using the Trier Social Stress Test (TSST), a well-established and controlled procedure for eliciting physiological stress response in both healthy individuals and panic disorder (PD) patients. The findings of the present thesis demonstrated that while ccf-mtDNA concentrations remained unchanged in the healthy control group, they showed a significant decrease 10- minutes after acute stress induction in the PD patients. Taken together the results provide a solid foundation for the direct quantification of ccf-mtDNA

concentrations in clinical samples and open new opportunities to further investigate its role in stress-research and a wide range of conditions such as inflammatory diseases, cancer, neurodegenerative disorders, and more.

1. Introduction

Over the past decades, circulating cell-free nucleic acids (cf-DNA) has gained significant attention as a biomarker in clinical research, particularly for diagnosing, monitoring and assessing disease progression. In 1948, Mandel and Metais, first described extracellular DNA fragments [1] that are detectable in all bodily fluids, including blood, saliva, urine, and the cerebrospinal fluid. While the majority of DNA is contained within the nucleus or mitochondria in eukaryotic cells (chloroplast in plants), certain physiological or pathological conditions can trigger the release of DNA into the cytoplasm and subsequently into the extracellular space. Given the growing recognition of cf-DNA as a valuable biomarker, liquid biopsy has emerged as a powerful tool for non-invasive disease monitoring and diagnosis. Traditionally, diagnosing and tracking the progression of many diseases requires invasive tissue biopsies, which can be painful, risky, and costly for both patients and healthcare providers. In contrast, liquid biopsy offers a minimally invasive alternative, enabling the analysis of biological materials such as RNA, DNA, exosomes, extracellular vesicles (EVs), and circulating tumor cells (CTCs) from bodily fluids [2] (Fig. 1).

This technique provides real-time insights into cellular processes while being cost-effective, rapid, and more comfortable for patients compared to traditional biopsies. As a result, liquid biopsy has become an essential tool in medical practice, allowing for a deeper understanding of disease mechanisms, progression, and severity.

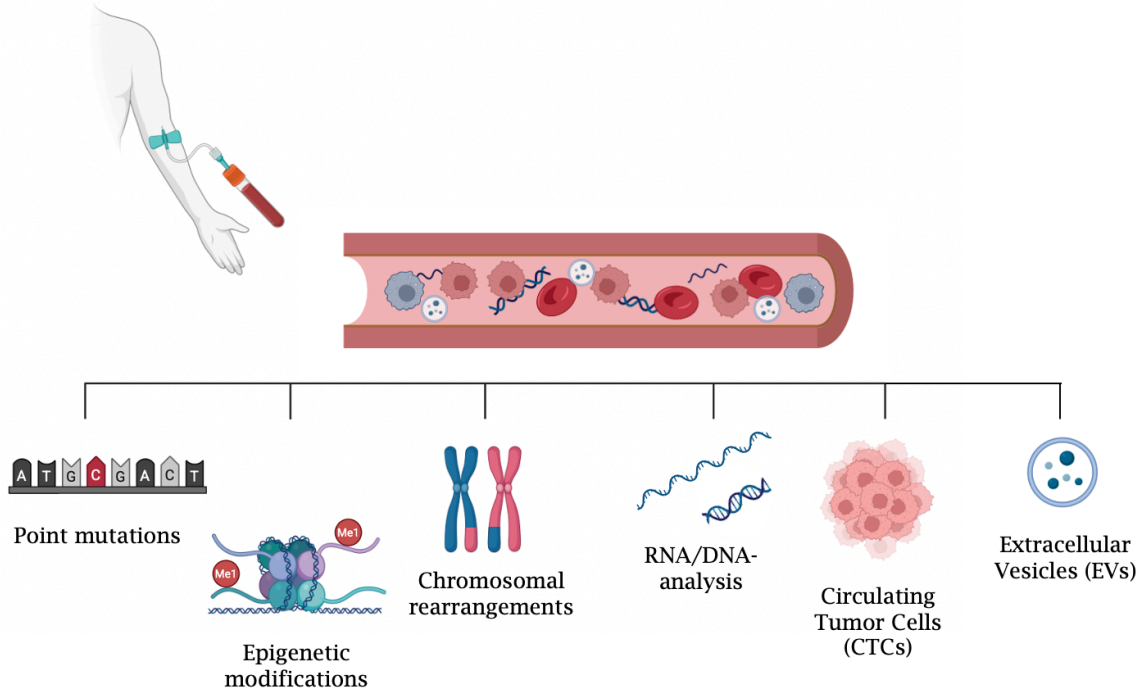

Figure 1 Schematic representation of liquid biopsy approaches. A single blood sample can be analysed for a variety of biological entities, enabling the detection and characterisation of point mutations, epigenetic modifications, chromosomal abnormalities and rearrangements, RNA/DNA profiling and analysis, as well as the identification of circulating tumour cells (CTCs) and extracellular vesicles (EVs). This figure was illustrated using BioRender.com.

To date, the biological significance of cf-DNA in liquid biopsy has been demonstrated in thousands of studies and a variety of contexts, including pathological conditions such as systemic inflammation [3], oncogenesis [4], autoimmune disorders [5], psychiatric conditions [6] as well as in response to physical activity [7] or psychological stress [8]. cf-DNA is made up of DNA fragments from various sources, including mitochondrial, nuclear, fetal, bacterial, parasitic and tumor origins[9] (Fig 2). In this study, **cf-DNA** comprises all cell-free circulating DNA fragments, including those derived from nuclear or mitochondrial sources. In contrast, **cf-nDNA** refers specifically to circulating cell-free DNA derived from the nucleus, while **ccf-mtDNA** refers to circulating cell-free DNA derived from mitochondria.

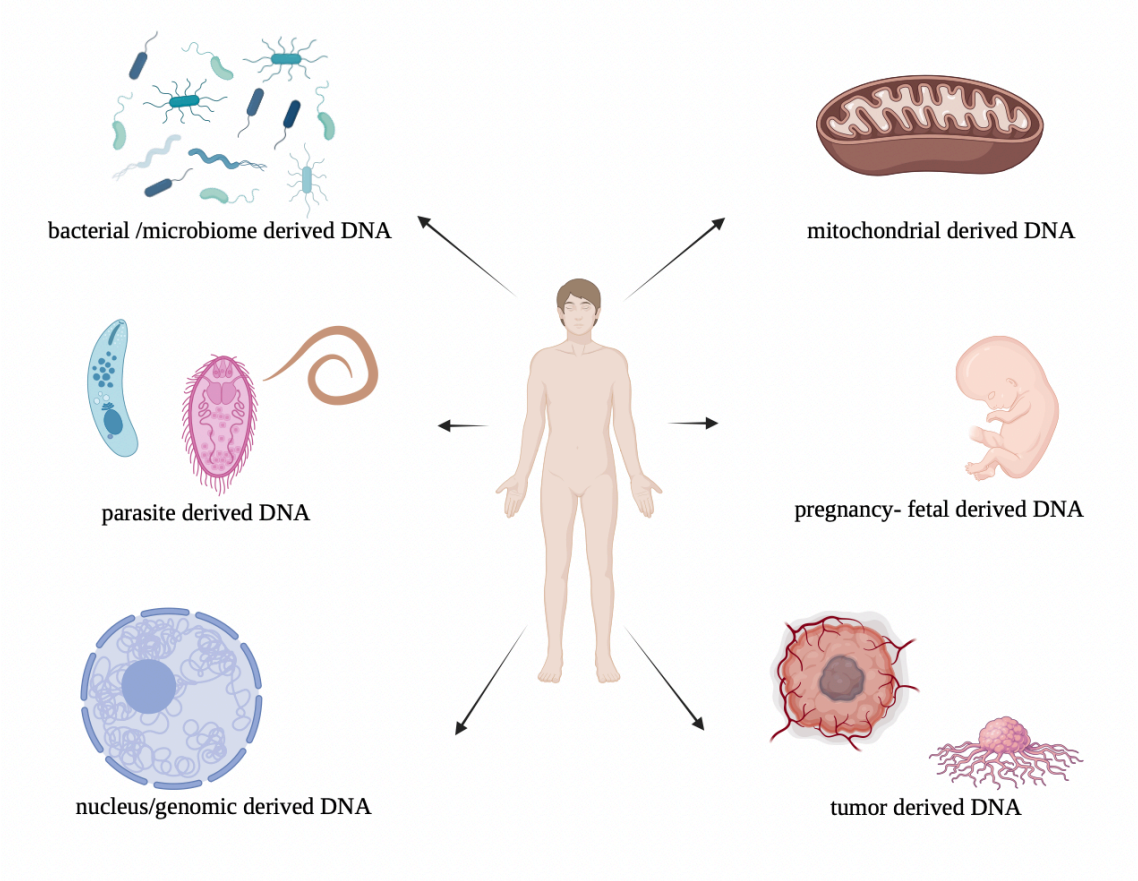

Figure 2 Potential origins of cf-DNA that are detectable in human bodily fluids. Sources of cf-DNA include "exogenous" DNA from bacteria or parasites and fetal DNA during pregnancy. "Endogenous" sources arise from nuclear DNA, mitochondrial DNA, or tumor-derived DNA. This figure was illustrated using BioRender.com.

1.1 Circulating cell-free nuclear DNA

First detected in serum of patients with systemic lupus erythematosus (SLE) [10] , cf-nDNA has since been studied in a wide range of conditions. Subsequent research revealed that cf-nDNA is not exclusive to autoimmune disorders but is also present in other pathological conditions and even in healthy individuals. Since then, cf-nDNA has been predominantly studied in human blood plasma samples, where its concentration under physiological conditions typically ranges from 1 to 40 ng/mL [11]. However, in pathological states, cf-nDNA levels can exceed 1000 ng/mL [12], reflecting increased cellular turnover or immune activation. Beyond its role in disease detection, cf-nDNA has emerged as a potential biomarker of disease progression and mortality, particularly in critical illnesses such as Coronavirus disease 2019 (COVID-19) [13]. Elevated cf-nDNA levels have been associated with poorer prognosis, increased disease severity, and reduced survival [13]. These findings highlight

the clinical relevance of cf-nDNA not only for diagnostic purposes but also for risk stratification and patient monitoring.

Therefore, several techniques have been developed to quantify cf-nDNA in biological samples. A more detailed overview of these quantification approaches is provided in section **1.5**. Among these, quantitative polymerase chain reaction (qPCR) remains the most widely used method for measuring cf-nDNA in clinical samples. Initial studies focused on the quantification of cf-nDNA using a single-copy gene target. However, subsequent research has shown that more efficient detection can be achieved by using multi-copy gene targets, such as repetitive elements, which improve sensitivity and quantification accuracy. Repetitive elements, often referred to as 'junk DNA', are present in thousands to millions of copies and make up more than 50 % of the human genome [14]. Due to their abundance and specificity, long interspersed nuclear elements (LINEs) (22 %) and short interspersed nuclear elements (SINEs) (16 %) [14] have proven to be effective targets for primer design in cf-nDNA quantification [15–17]. Some of these elements are transposable and remain active by 'jumping' around the genome, which must be considered when targeting them for quantification. However, most of these elements are consistently present in a defined amount of copy number. Notable examples include the human-specific LINE-1 element L1PA2 and various ALU subfamilies, which serve as reliable targets for cf-nDNA analysis.

While the quantification of cf-nDNA alone can give important information about the health status of an individual, composition analysis of cf-nDNA, such as epigenetic modifications, can also provide valuable insights into molecular mechanisms within cells. By analyzing cytosine-guanine sites (CpG-islands) and respective methylation patterns of cf-nDNA, their tissue of origin can be determined. For example, Neuberger *et al.*, (2022) demonstrated that the primary source of cf-DNA after physical activity are neutrophil granulocytes by comparing the methylation patterns of cf-nDNA in the circulation with CpG-island databases [18]. In summary, the quantification and analysis of cf-nDNA provides important insights into disease mechanisms. Similarly, ccf-mtDNA is emerging as an important biomarker providing unique information specifically related to mitochondria and mitochondrial functioning.

1.2 Mitochondria and circulating cell-free mitochondrial DNA

Mitochondria are organelles primarily responsible for energy production in eukaryotic cells. Beyond this essential function, they play crucial roles in calcium storage and regulation, apoptosis, steroid hormone synthesis, and reactive oxygen species (ROS) production [19–22]. As mitochondria originated from alpha-proteobacteria through an endosymbiotic process – a key event on the Tree of Life that is estimated to have taken place approximately 1200 million years ago [23]- they have a specific structure containing an inner and outer membrane, and harbor their own genome, known as mitochondrial DNA (mtDNA) [24]. The double stranded mtDNA has a circular shape and is located in the mitochondrial matrix, often associated with structures called nucleoids [25], which are primarily composed of the mitochondrial transcription factor A (TFAM) [26]. While the number of mitochondria varies between cell types, depending on the energy demands of a cell, each mitochondrion typically contains between 2 and 10 copies of mitochondrial DNA. As a result, a single cell can harbor hundreds to thousands of mtDNA copies.

The human mtDNA is 16,569 base pairs (bp) long and encodes 37 genes, including 13 protein-coding genes essential for the mitochondrial respiratory chain, 22 transfer RNA (tRNA) genes, and 2 ribosomal RNA (rRNA) genes. However, most of the genes that are encoded for mitochondrial functioning are located in the nucleus and need to be imported into the mitochondrion after their synthesis. This requires an intact signaling system to regulate the gene expression, and the adaptation to external and internal influences to optimize metabolic regulation. Therefore, mitochondrial functioning relies not only on the import of peptides and proteins but also on the export of signaling molecules, known as *mitokines*. These include proteins such as cytochrome c [27], which, when released can trigger apoptosis, as well as metabolites [28], ions, and even the mtDNA itself [29].

Among these signaling molecules, mtDNA plays a crucial role in inflammation. When released into circulation, it acts as a potent inflammatory trigger, known as ccf-mtDNA. Due to its unique molecular architecture- including unmethylated motifs and bacterial origin - mtDNA can activate the innate immune system and initiate an inflammatory response by interacting with pattern recognition receptors (PRRs) [30]. Activation of PRRs such as Toll-like receptor-9 (TLR-9) as well as cytosolic pathways like the cyclic GMP-AMP synthase- simulator of interferon genes (cGAS-STING) pathway

and NLRP3- inflammasome leading to a signaling cascade which results in the release of interferons and pro-inflammatory cytokines [31]. Therefore ccf-mtDNA is also described as a damage-associated molecular pattern (DAMP). Research has consistently demonstrated that ccf-mtDNA plasma levels are elevated in a broad range of inflammation-associated diseases, as well as in conditions where cell damage and cell death play a critical role. These include COVID-19 [32], trauma, sepsis or septic shock [33,34], severe inflammatory response syndrome (SIRS) [35], myocardial infarction [36] and cancer [37].

The release of ccf-mtDNA under these conditions further amplifies inflammatory responses and contributes to inflammasome activation. Analyzing ccf-mtDNA in pathological conditions can therefore give valuable insights into the overall well-being of a cell and might reflect mitochondrial functionality. Given this background, it is crucial to gain a deeper understanding of the mechanisms by which cf-DNA is released into the bloodstream**.**

1.3 Cf-DNA release mechanisms and molecular characteristics

1.3.1 Release mechanisms

cf-DNA can enter the circulation by two distinct mechanisms: **active** release and **passive** release. Passive release occurs through cellular damage, degradation and cell death and mainly involves necrosis and apoptosis. It has been postulated that most cf-DNA found in bodily fluids originate from apoptotic cells. Apoptosis is a highly conserved and regulated internal programmed process of cell death that plays a crucial role in maintaining tissue homeostasis, and the elimination of damaged or potentially harmful cells. During apoptosis caspase-activated DNase (CAD) cleaves the chromosomal DNA into small fragments, with additional apoptotic nucleases such as *DNAS1L3* and *DNASE1* further contributing to the degradation process. This typically results in a fragment size of cf-nDNA that peaks at around 167 base pairs in healthy individuals [38]. The length represents the double-stranded DNA wrapped around single nucleosome. However, in pathological stages, where these processes may be dysfunctional or dysregulated, the cf-nDNA size profile can be different. In contrast, necrosis, an uncontrolled form of cell death, often activated by external factors

such as trauma or infection, tends to result in less specific DNA degradation and larger fragments of cf-nDNA, up to several kilobase pairs in size [37].

Beyond passive release, cf-DNA can also actively enter the circulation through regulated biological processes. One of the most extensively studied mechanisms of active cf-DNA release is the formation of neutrophil extracellular traps (NETs). NETs are web-like structures composed of extracellular DNA associated with histones, granule proteins and other antimicrobial molecules such as neutrophil elastase (NE) and myeloperoxidase (MPO) [39]. They are formed by neutrophils, the first responders of the innate immune system, which play a crucial role in the body's defense against pathogens. The process of NET formation, also known as NETosis, enables neutrophils to capture and neutralize pathogens such as bacteria, fungi or viruses [39]. While this process is very important for the host defense, overstimulation and persistent NET formation is known to be associated in inflammatory diseases such as cystic fibrosis and therefore cf-DNA concentrations might be elevated in these conditions [40].

Another form of active release occurs when mitochondrial signaling is required. As mentioned in section **1.2** mitochondrial signaling is essential for a wide range of cellular functions, allowing mitochondria to communicate both intra- and intercellularly [41,42]. This signaling can occur through the secretion of either whole, naked mitochondria or mitochondrial components encapsulated within EVs [42]. This process is particularly relevant when mitochondrial function is compromised and requires repair to maintain cellular bioenergetics and overall cellular health [42]. The simultaneous release of cf-nDNA and ccf-mtDNA is most likely indicative of cellular death or damage, whereas the selective release of either cf-nDNA or ccf-mtDNA alone reflects a tightly regulated biological process, highlighting the complexity of cf-DNA dynamics in health and disease.

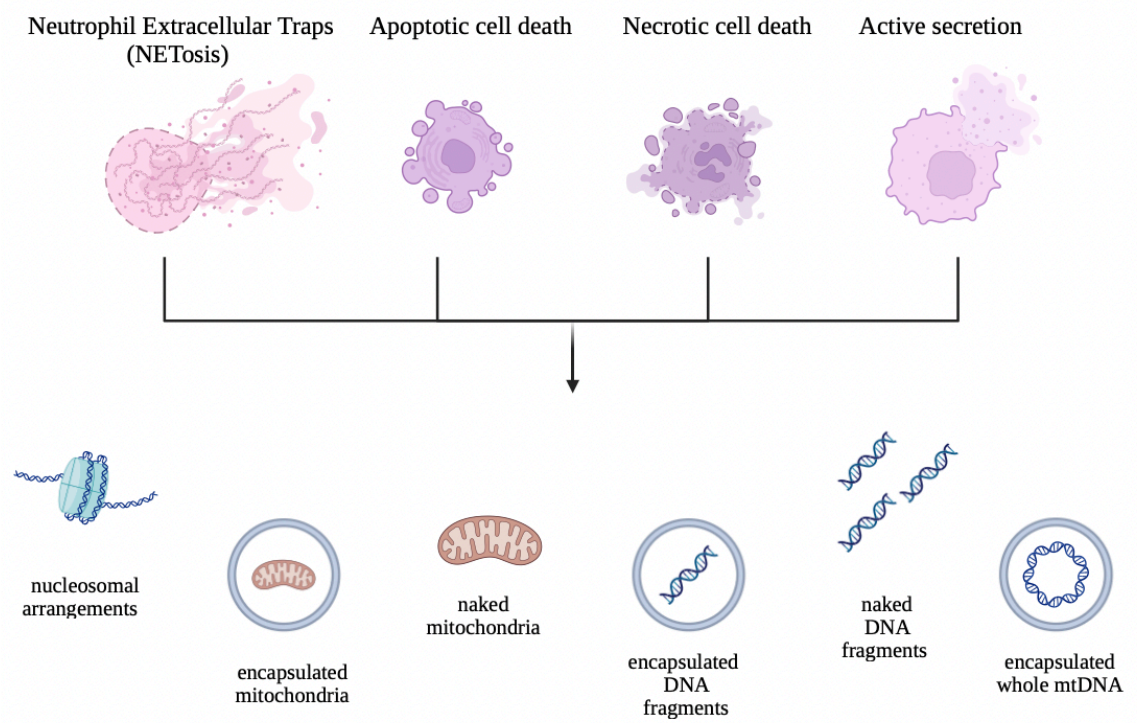

Figure 3 Selection of cf-DNA release mechanisms and forms of cf-DNA. cf-DNA enters the bloodstream through passive processes such as apoptosis and necrosis, or via active mechanisms such as NETosis and active secretion. Once in the bloodstream, cf-DNA can exist as naked DNA, bound to proteins in nucleosomal arrangements or within extracellular vesicles. This figure was illustrated with BioRender.com

1.3.2 Cf-DNA profiling and characteristics

As indicated in the previous paragraph, different release mechanisms generate distinct fragmentation patterns of cf-DNA, providing insights into the molecular processes occurring within a cell. In addition to fragmentation analysis, methylation patterns can also be used to determine the tissue of origin, as mentioned in section **1.2**. This is particularly relevant in cancer detection and treatment, where tumor-specific methylation signatures help to distinguish malignant from healthy tissue [43]. Furthermore, the characteristics of cf-nDNA fragment ends can provide additional information about the underlying cleavage processes and recent research indicates that end-motif analysis could be used as a new biomarker in liquid biopsy. For example, for those plasma DNA molecules containing more than one nucleosome, deletion of *DNAS1L3* resulted in a significant reduction in jaggedness [44]. In addition, the length distribution of cf-nDNA fragments has clinical significance as it represents the integrity. It can be determined by analyzing the ratio of long cf-nDNA to small cf-nDNA fragments. The integrity index has been shown to vary in different pathological conditions

[45], indicating its potential as a biomarker for disease progression and severity. All these features are now integrated in comprehensive cf-DNA profiling approaches. Based on these characteristics, further distinctions between cf-nDNA and ccf-mtDNA arise when considering their molecular properties and stability. For instance, apoptotic cf-nDNA exhibits a characteristic peak at around 167 bp due to nucleosomal protection, whereas necrotic cf-nDNA is often present in larger fragments and is heterogeneous in size. In contrast, ccf-mtDNA typically occurs in smaller fragments ranging from 50 bp to 400 bp, with a peak at approximately 80 bp [46]. Given that mtDNA is more susceptible to damaging agents [47] due to the lack of protective histone association as found in nuclear DNA, it is not surprising that ccf-mtDNA is less stable and more prone to degradation than cf-nDNA. This inherent instability may contribute to its generally shorter fragment length compared to cf-nDNA. Furthermore, the role of mitochondria in cellular respiration exposes mtDNA to DNA-damaging reagents like ROS, which can induce structural modifications of mtDNA such as 8-hydroxy-2'-deoxyguanosine (8-OHdG). This oxidative damage increases the instability of ccf-mtDNA and may further explain the shortening of its fragment lengths compared to cf-nDNA. In contrast to cf-nDNA, the tissue of origin cannot be determined by analyzing epigenetic modifications of ccf-mtDNA as it is not methylated. However, its oxidative state (accumulation of 8-OHdG) can serve as a valuable marker for oxidative damage within the cells, providing information on cellular stress and mitochondrial functioning [48].

1.3.3 Clearance of cf-DNA

To maintain homeostasis, cf-DNA must be efficiently cleared from the circulation. For example, Velders *et al.* (2014) demonstrated that in healthy individuals, cf-nDNA levels rise immediately after acute exercise but return to near baseline concentrations within ~30 minutes, highlighting the presence of an active and rapid clearance mechanism that prevents excessive accumulation [49]. Therefore, insufficient clearance might explain high levels of cf-DNA in pathological stages. In addition, increased cell turnover and cell death can overwhelm this system, leading to the accumulation of cf-DNA. The half-life of cf-DNA is ranging between a few minutes and up to 1-2 hours and depends on various factors, such as the presence of circulating enzymes like DNAse I and its association with protein complexes. Beyond direct enzymatic degradation, serval organs contribute to the elimination of cf-DNA from the circulation. The liver plays a dominant role in this process,

removing up to 84% of nucleosomes from the bloodstream within 10 minutes [50], primarily through the action of Kupffer cell-specialized hepatic macrophages that efficiently degrade apoptotic debris and nucleic acids [51]. Additionally, the kidneys take part in cf-DNA clearance, which may explain why cf-DNA concentrations in urine are relatively low.

1.4 Quantification of cf-DNA

In recent years, the growing impact of liquid biopsy in clinical practice has driven the development of various methods for quantifying cf-DNA in laboratories. These include for instance spectrophotometric and fluorometric assays, enzyme-linked immunosorbent assays (ELISA), next-generation sequencing (NGS) and PCR-based approaches, with the latter being widely used and routinely integrated into clinical practice. Spectrophotometric methods rely on DNA's absorbance at 260 nm, capturing all cf-DNA fragments in a sample [52]. Fluorometric assays use intercalating fluorescent dyes, where the signal intensity directly correlates with cf-DNA concentration [53]. ELISA-based methods detect protein-bound DNA fractions, such as histone-associated nucleosomes, also known as mono- and oligonucleosomes [54]. However, this increases the risk of discriminating against the non-protein-bound fraction of cf-DNA in the samples, leading to inaccurate or biased results. Furthermore, mitochondrial or bacterial DNA does not show nucleosomal arrangements and therefore will not be detected by this technique. While spectrophotometric and fluorospectrometric measurements provide a comprehensive quantification of cf-DNA regardless of its origin, ELISA is mostly limited to cf-nDNA. Therefore, specialized techniques have been developed to distinguish between different origins such as nuclear and mitochondrial cf-DNA. NGS-based methods can give an overall picture of cf-DNA composition by amplifying all DNA in the sample [55]. These approaches allow more precise analysis of cf-DNA dynamics in various biological contexts, including cancer diagnostics, prenatal screening and organ transplant monitoring. However, NGS-based techniques require advanced bioinformatics pipelines, extensive sequencing depth and higher costs, making them less accessible for routine clinical applications. Therefore, most laboratories use quantitative PCR (qPCR) to detect and quantify cf-DNA as a cost-effective and reliable alternative. The technique is based on traditional PCR amplification but incorporates a fluorescent dye or a probe that allows real-time monitoring of DNA

amplification, which is why it is known as real-time qPCR. As the reaction progresses, the fluorescence intensity increases in proportion to the amount of DNA amplified, allowing precise quantification. The detected fluorescence intensity can later be converted into a usable unit, such as ng/mL or copies/mL, by comparing it to a standard curve generated from known DNA concentrations. This allows a highly sensitive and targeted amplification of cf-DNA from any source, while being simple, inexpensive and time-efficient. However, all the above-mentioned techniques share the common requirement that cf-DNA must be isolated before the actual detection and quantification process can take place. Traditional methods for isolating cf-DNA from biological materials primarily involve silica column-based extraction, while more advanced approaches utilize high throughput and automated magnetic bead-based isolation [56]. Silica column extraction is based on a positively charged silica membrane to which DNA binds via a salt bridge formed in the presence of chaotropic salts [57]. This method is widely used because of its cost and time efficiency, making it a standard approach for cf-DNA isolation. However, it carries the risk of losing smaller DNA fragments during the washing and elution steps, as they may not bind effectively or pass through the silica membrane. This size bias can affect downstream analyses, particularly in applications where the integrity and complete representation of cf-DNA fragments is critical. To address this challenge, Neuberger *et al.* (2021) developed an assay for the quantification of human cf-nDNA directly in blood plasma samples, eliminating the need for prior isolation steps. Their study showed that the loss of cf-nDNA during isolation can range from 12% to 85%, depending on the specific kit used [15]. These findings were confirmed by other research groups and highlight the significant impact that isolation methods can have on the quality and quantity of cf-DNA, which in turn affects the reliability and accuracy of downstream analyses [58,59]. Based on this work, the main objective of this thesis was to develop and establish a qPCR-based assay for the quantification of ccf-mtDNA directly in human blood plasma samples. The aim was to develop a method that would efficiently amplify mtDNA fragments without the need for prior isolation steps, thereby minimizing losses and ensuring more accurate quantification of mtDNA in clinical samples.

1.5 Challenges regarding ccf-mtDNA quantification and preanalytical considerations

Quantifying ccf-mtDNA is significantly more challenging than measuring cf-nDNA due to a variety of biological and technical factors that can affect its detection and analysis. This section will examine these factors, providing a clearer understanding of the complexities and limitations of ccf-mtDNA quantification compared to cf-nDNA.

1.5.1 The nature of mitochondrial sequences- Nuclear Embedded Mitochondrial Insertions

One major challenge is the nature of the mtDNA itself. MtDNA is frequently transferred into the nuclear genomes of eukaryotic cells - a well-established and ongoing process that has been occurring for over 2 billion years [60]. These nuclear-embedded mitochondrial sequences, known as NUMTs , are incorporated in every eukaryotic genome [61]. Their presence complicates the detection of ccf-mtDNA using PCR-based approaches, as NUMTs can lead to amplification of nuclear sequences rather than authentic mtDNA. Although previous studies have addressed this issue, most of the literature reveals that primer design often contains at least one mismatch, which can result in the co-amplification of nuclear integrations and ultimately to conflicting results.

1.5.2 Mitochondrial fragment size

The relatively small fragment size of ccf-mtDNA [46] presents an additional challenge, as it limits the efficiency of detection by most PCR-based techniques. This is because many standard PCR protocols are optimized for DNA fragments between 150 bp and 200 bp [62], potentially leading to the loss of smaller fragments during amplification. Furthermore, primer design can be a limiting factor in PCR-based approaches, as primers typically range from 18 to 25 bp, making it challenging to design primers that effectively amplify small fragments while minimizing the risk of primer dimer formation. Consequently, shorter ccf-mtDNA fragments may be underrepresented or entirely missed, further complicating the accurate quantification of mitochondrial DNA in the circulation.

1.5.3 Preanalytical aspects and platelets

Another critical aspect to consider when quantifying ccf-mtDNA is the role of preanalytical factors, such as sample handling, sample source, storage conditions, and centrifugation procedures. While there are well-established guidelines for preanalytical factors like centrifugation protocols for cf-nDNA quantification [63], no universally accepted standards exist for ccf-mtDNA measurements.

One of the major challenges in ccf-mtDNA quantification is the presence of platelets, the smallest blood cells. Although platelets lack nuclei and therefore nuclear DNA, they contain intact mitochondria and mitochondrial DNA. It has been proposed that the majority of ccf-mtDNA in blood plasma or serum samples originates from activated platelets. As serum is obtained after clotting, this process inherently activates platelets, resulting in the release of mitochondrial DNA [17,64]. As a result, serum samples contain artificially elevated levels of ccf-mtDNA and should be avoided if the aim is to measure 'true' ccf-mtDNA levels. Instead, plasma is the preferred sample type for more accurate quantification.

However, even in plasma, the removal of platelets depends heavily on the centrifugation protocol used. Platelets can be incompletely removed during centrifugation, influencing the final ccf-mtDNA content in the sample. Numerous studies have shown that the results of ccf-mtDNA measurements are strongly influenced by platelet content [65]. For example, platelets can be activated when stored at lower temperatures or left at room temperature without anticoagulants, significantly affecting the results [66]. Roch *et al.* (2021) have shown that mtDNA levels can increase up to 67-fold when platelets are activated, highlighting the importance of careful handling during processing. Since centrifugation time and force significantly impact measurement outcomes [65], a two-step centrifugation protocol is recommended. The first step, performed at a lower speed, removes large cells, particles, and cellular debris. The second step should involve high-speed centrifugation to eliminate membrane-bound mtDNA and whole mitochondria, thereby capturing the relatively small fraction of ‘true’ ccf-mtDNA. To minimize preanalytical variability, it is strongly recommended that blood samples should be processed immediately after being drawn, using anticoagulants and keeping them at room temperature before further processing. For long-term storage, plasma should be kept at a minimum of -20°C, with -80°C preferred to maintain sample integrity [67]. However, excessive freeze-thaw cycles should be avoided to reduce the risk of ccf-mtDNA degradation. Taking all these

aspects into consideration, a standardized and careful pre-analytical handling is essential to ensure reliable and reproducible ccf-mtDNA measurements.

1.6 Stress and stress-related disorders and the role of mitochondria

The human body is constantly exposed to external and internal stressors—whether psychosocial, psychological, or mechanical—and must continuously adapt to these factors to maintain homeostasis. Homeostasis is the body's ability to preserve a stable internal environment despite external changes. It regulates factors such as body temperature, fluid balance, pH, sodium and potassium levels, and blood glucose levels [68]. The main control center for homeostasis is the nervous system, particularly the hypothalamus, which is closely related to the endocrine system to sustain equilibrium. To cope with acute stressful events the bodys 'fight-or-flight' system is activated, which is a well-conserved evolutionary process. By activating this system, the hypothalamic-pituitary-adrenal (HPA) axis is stimulated, subsequently leading to the release of stress hormones like adrenaline and cortisol [69]. The secretion of stress hormones triggers physiological changes such as an elevated heart rate, better blood flow to muscles, and heightened alertness, as well as molecular changes [69]. Increased levels of circulating glucocorticoids (GCs) have been shown to suppress various responses of immune cells, suggesting that stress may have anti-inflammatory effects by inhibiting immune activity [70]. From an evolutionary perspective, these adaptations are essential to prepare the body for a quick reaction to the stressor and enable the organism's survival. In the modern world, however, we are less likely to face life-threatening physical dangers, and instead stressors such as societal pressures, climate change, financial instability and the fast pace of daily life contribute to chronic stress. Since 2020, the American Psychological Association has reported an increase in the diagnosis of stress-related mental illnesses such as social anxiety disorder (SAD) and panic disorder (PD), leading to higher hospital admissions, increased risk of cardiovascular diseases and mortality worldwide (APA, 2020). With the rise in these diagnoses, research into the underlying molecular mechanisms that shed light on the physiological impact of acute and chronic stress has become increasingly valuable. A key focus in stress research is the role of mitochondria. As the energy suppliers of the cells, mitochondria are

capable of detecting stress signals and adapting their function in response, which is essential to meet the increased energy demands in stressful situations. Moreover, mitochondria play a crucial role in the synthesis and metabolism of all steroid hormones, including GCs such as cortisol. Therefore, their adaptation to external changes is integral for maintaining homeostasis under stressful situations, a phenomenon referred to as mitochondrial allostatic load (MAL). Prolonged stress exposure, as seen in many psychiatric disorders, can lead to a disrupted balance of this internal system, impaired cellular resistance, and recent research indicates a disrupted mitochondrial functionality [71] – also known as MAL overload. Notably, chronic stress induces mitochondrial-derived oxidative stress, disrupting the balance between reactive ROS production and antioxidant defenses, thereby compromising the body's ability to mitigate oxidative damage [72]. This can further contribute to the damage of lipids, proteins, and even mtDNA itself, ultimately leading to cell death [72] and the release of mtDNA into the circulation. As mitochondrial dynamics play a key role in the body's response to stress, there is growing interest in identifying reliable biomarkers that reflect these changes in real time. Therefore, ccf-mtDNA has emerged as a promising indicator of stress-related physiological and metabolic changes with potential applications in clinical diagnostics and research. Its concentrations have been found to vary in stress-related psychiatric conditions, including bipolar disorder (BD) major depression (MD), and SAD [73,74], thereby reflecting maladaptive or adaptive changes of mitochondrial dynamics in these disorders. In the present study, ccf-mtDNA levels were measured in individuals with PD, a condition characterized by high levels of stress and acute anxiety responses, to investigate its potential as a biomarker in this specific stress-related psychiatric disorder. By assessing ccf-mtDNA in PD, we aim to better understand the relationship between mitochondrial dysfunction and the physiological impact of chronic stress, with the goal of improving disease management and outcomes in patients with stress-related disorders.

1.7 Aims

In recent years cf-DNA has emerged as a promising biomarker in various pathological conditions including inflammatory diseases, cancer or stress-related disorders such as MD or BD. The development of stress-related disorders is influenced by a combination of psychosocial, psychological, and cellular processes that are not yet fully understood. Identifying reliable biomarkers

for stress-related disorders is crucial for monitoring disease prognosis and progression, particularly as cf-DNA offers the potential to gain a better understanding of cellular and molecular mechanisms triggered by acute and chronic stress. Within this framework, the ability of mitochondria to adapt to stress has garnered particular attention, as it is essential for maintaining cellular homeostasis. Ccf-mtDNA has therefore emerged as a potential indicator of the effects of stress on cellular function. However, quantifying ccf-mtDNA has been shown to be associated with major challenges regarding preanalytical and technical considerations. **To address these issues, the primary objective of this study was to develop a real-time qPCR-based method for quantifying ccf-mtDNA directly in human blood plasma samples, with a focus on preanalytical variables influencing ccf-mtDNA quantification.** The key advantage of this approach is the elimination of DNA isolation steps, which are inherently associated with risks such as sample loss and potential bias in DNA recovery. By enabling direct quantification, this method aims to improve both cost and time efficiency, providing a more streamlined and reliable alternative for ccf-mtDNA analysis in clinical and research settings. The secondary objectives of this thesis were aimed at further investigating the utility of ccf-mtDNA and cf-nDNA as biomarkers in the context of chronic stress, with a particular focus on individuals diagnosed with panic disorder. As PD patients are exposed to recurring episodes of intense psychological stress, this population offers a clinically relevant model to investigate whether altered ccf-mtDNA levels reflect chronic stress-induced mitochondrial dysregulation. Moreover, to explore the effects of acute stress, the Trier Social Stress Test (TSST) is used to induce a standardized psychosocial stress response in both patients with PD and healthy control participants to evaluate differences in their response. These objectives were formulated to deepen the understanding of cfDNA dynamics under different stress conditions and to explore their relevance in stress-associated pathophysiology.

2. Chapter declarations

Chapter I *of this dissertation has already been submitted and published during the preparation this thesis. The publication has been submitted to a peer-reviewed journal with the permission of all corresponding authors. The results presented in* ***Chapter II*** *are currently being prepared for publication and will be submitted to a peer-reviewed journal. The first draft of the manuscript has been included in this thesis. The citations of this section are mentioned in the reference list. Supplementary materials intended as electronic supplements are provided in the appendix of this thesis.*

2.1 Chapter I

Daubermann, C., Herhaus, B., Neuberger, E.W.I. *et al.* Methodological influences on circulating cell-free-mitochondrial and nuclear DNA concentrations in response to chronic stress.

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Contribution: First authorship

Conceptualization and Methodology, Experimental design and validation, Experimental procedures, Data acquisition and interpretation, Manuscript writing and editing

Supplementary Material: The following supporting information can be downloaded at https://static-content.springer.com/esm/art%3A10.1007%2Fs11033-025-10369-7/MediaObjects/11033_2025_10369_MOESM1_ESM.xlsx

Table S1. ccf-mtDNA concentrations of the isolated blood plasma samples in this study.

Table S2. ccf-mtDNA concentrations of the direct measurements in this study, including the direct measurements of samples at different centrifugation speeds.

Table S3. Ct values of the three independent qPCR runs including calibrator and NTC samples for the generation of the LOQ curve for the quantification of human mitochondrial DNA directly in blood plasma in this study.

Table S4. Mean Ct values and calculated copies/PCR used for the overall LOQ curve.

Table S5. Calculated values from all individual Ct values of independent measurements, derived from the LOQ curve including mean values, SD and CV.

Table S.6 Calculation for the LOB and LOD of the ND1 assay derived from the three independent qPCR runs.

Table S7. ccf-mtDNA measurements and cf-nDNA measurements with corresponding stress score of the healthy subjects in this study.

Note: Figure 5A contains an error in the log-scale transformation, resulting in all values being plotted one log unit too low. A corrected figure has been prepared and will be submitted to the journal for replacement in the online publication.

2.2 Chapter II

"Effects of acute stress on plasma circulating cell-free mitochondrial DNA in Panic Disorder"

Contribution:

The manuscript was written and edited by Carina Daubermann. The conceptualization of the study was designed by Katja Petrowski and Benedict Herhaus. Experimental design of the methodology employed was designed by Perikles Simon and Carina Daubermann. The experiments were carried out by Carina Daubermann. Data acquisition and interpretation was performed by Elmo Neuberger and Carina Daubermann.

3. Chapter I

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ORIGINAL ARTICLE

Methodological influences on circulating cell-free-mitochondrial and nuclear DNA concentrations in response to chronic stress

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Methodological influences on circulating cell-free-mitochondrial and nuclear DNA concentrations in response to chronic stress

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Abstract

Background Mitochondria are versatile eukaryotic organelles that play a crucial role in the body's stress response. Prolonged stress exposure can cause structural and functional alterations, leading to mitochondrial DNA (mtDNA) damage and subsequent release of mtDNA into the circulation. Cell-free circulating mtDNA (ccf-mtDNA) is a potential biomarker indicating cellular damage and stress. In this study we investigated the applicability of ccf-mtDNA and cf-nDNA as biomarkers of chronic stress in healthy subjects.

Methods and results We developed a quantitative polymerase chain reaction (qPCR) assay to directly measure ccf-mtDNA in human blood plasma samples, addressing numerous challenges specifically related to ccf-mtDNA quantification. We validated our 68 bp target assay based on the FDA, International Organization for Standardization (ISO) and Clinical & Laboratory Standards Institute (CLSI) guidelines for assay development, including parameters such as limit of blank (LOB), limit of detection (LOD) and limit of quantification (LOQ). Furthermore, we implemented incurred samples analysis and inter-plate samples to ensure reliability and reproducibility of the assay. In addition, we evaluated the effects of centrifugation forces on ccf-mtDNA and cf-nDNA concentrations in native plasma samples and showed that mainly ccf-mtDNA is strongly affected by centrifugation forces. We found a significant negative correlation between ccf-mtDNA levels and chronic stress. In contrast, cf-nDNA levels were not affected in response to chronic stress.

Conclusion ccf-mtDNA can directly and reliably quantified in unpurified plasma samples. However, the ccf-mtDNA levels in plasma samples of healthy subjects are close the LOQ, showing that the assay is not yet suitable for all conditions.

Keywords Circulating Cell Free mitochondrial DNA · Circulating cell free nuclear DNA · Chronic Stress · Biomarker · Assay development

Abbreviations

BMI	Body mass index
ccf-mtDNA	Circulating cell-free mitochondrial DNA
cf-nDNA	Circulating cell-free nuclear DNA
CLSI	Clinical & Laboratory Standards Institute
Cq	Quantification cycle
CV	Coefficient of variation
dPCR	Digital polymerase chain reaction
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders Fourth Edition
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
HPA	Hypothalamic–pituitary–adrenal
ISO	International Organization for Standardization
K3	Tripotassium
LINE 1	Long interspersed nuclear element 1
LOB	Limit of blank
LOD	Limit of detection
LOQ	Limit of quantification
ND1	NADH-ubiquinone oxidoreductase chain 1
NTC	Non template control
NUMTs	Nuclear-embedded mitochondrial DNA sequences

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qPCR	Quantitative polymerase chain reaction
SCID	Structured Clinical Interview
SD	Standard deviation
TICS-9	Trier Inventory for Chronic Stress

Introduction

In 2023 the American Psychological Association [1] found an increase in mental and physical health issues due to long-term stressors. Chronic diseases increased from 48% in 2019 to 58% in 2023, while mental health diagnoses increased from 31 to 45%, reaching 50% among young adults (18–34) [1]. Therefore, quantifiable biological indicators that offer critical insights into the effect of acute and chronic stress on the body would be of high value.

The stress response is an evolutionary adaptive mechanism designed to enable individuals to cope with acute threats, often referred to as the "fight-or-flight" response. Stress activates the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system, releasing hormones such as cortisol and adrenaline to increase heart rate, focus attention and mobilize energy for rapid action. However, when stress becomes chronic—whether through ongoing or repeated exposure to stressors or inadequate/insufficient coping strategies, or a combination of the two—the body remains in a state of heightened arousal, which can have a range of negative physical and mental health consequences [2]. Prolonged activation of the HPA axis results in allostatic load, resulting in immune dysregulation, inflammation, oxidative stress, and impacts on cardiovascular and metabolic health [3]. It can therefore be assumed that stress plays an important role in the dysregulation of immune and metabolic functions, contributing to a range of chronic health conditions. One key aspect of this process is mitochondrial function, which is tightly linked to cellular stress and immune responses [4, 5].

As the energy supply centers of the cell, mitochondria are essential for maintaining bioenergetics, but they are also highly responsive to stress signals to adapt to fluctuating energy demands during stressful situations. Regulating their functions to cope with ongoing cellular stress is a phenomenon known as mitochondrial allostatic load [6]. One consequence of mitochondrial stress is the release of mitochondrial DNA (mtDNA) into the circulation, referred to as circulating cell-free mtDNA (ccf-mtDNA). This release can occur by two different mechanisms: passive release resulting from dysfunctional mitochondria, cell death or cell damage and active secretion as part of regulated signaling processes. The main sources for ccf-mtDNA by passive mechanisms are necrosis and apoptosis [7] and research has shown that elevated ccf-mtDNA levels can be found in conditions such as cancer [7], sepsis [8] and trauma, where tissue damage can contribute to the passive release. In contrast, active secretion can enable intercellular and mitochondrial communication [9] through the secretion of either whole naked mitochondria or mitochondrial components encapsulated within extracellular vesicles [10]. This process typically occurs when mitochondrial function is compromised and needs to be repaired to maintain cellular bioenergetics and overall cellular health [10]. In some cases, mtDNA can also escape into the circulation as a result of immune responses. For example, immune cells such as neutrophils, release mtDNA when they form neutrophil extracellular traps [11] in response to pathogens such as bacteria or fungi.

Since mitochondria originated from alpha-proteobacteria approximately two billion years ago [12], their mtDNA is closely related to bacterial DNA and can act as an activator of the innate immune system and a potent inflammatory trigger, by binding receptors such as Toll-like receptor 9, thereby increasing pro-inflammatory cytokine production [13]. However this process must be tightly regulated to mitigate the effect of excessive immune activation. It can therefore be assumed that different pathologies may result in different mtDNA release mechanisms, influencing disease progression and immune responses.

Unlike circulating cell- free nuclear DNA (cf-nDNA), which is associated with a variety of pathological conditions, including physical and psychosocial stress [14, 15], ccf-mtDNA specifically reflects mitochondrial-related impairments [16]. As a result to cumulative stress, recent research indicates ccf-mtDNA as a potential biomarker in assessing acute as well as chronic psychological or psychosocial stress [17, 18].

However, challenges exist in detection methods due to the presence of nuclear-embedded mtDNA sequences (NUMTs) [19], variations in fragment sizes [20], and no defined guidelines regarding preanalytical considerations. Based on our previous work [21], we developed an assay for the direct quantification of ccf-mtDNA concentrations in human blood plasma samples without DNA extraction. For our quantitative polymerase chain reaction (qPCR)-based approach, we have implemented the recommendations for the development of qPCR and digital polymerase chain reaction (dPCR) assays in accordance with the bioanalytical method validation guidelines of the Food and Drug Administration (FDA) [22], Clinical & Laboratory Standards Institute (CLSI) [23] and International Organization for Standardisation (ISO) guidelines [24] concerning specificity, reproducibility, reliability, limit of quantification (LOQ), limit of detection (LOD), limit of blank (LOB) and coefficient of variation (CV). Furthermore, we addressed the previously described challenges associated with ccf-mtDNA quantification, including targeting only mtDNA without amplification of NUMTs and detection of small fragments < 70 bp. This offers the potential to monitor mitochondrial functionality in

pathological conditions as mentioned above and help manage disease severity and outcome.

In this study, we aimed to measure ccf-mtDNA and cf-nDNA levels in blood plasma samples from 22 healthy volunteers in respect to their chronic stress load. We evaluated the efficiency of a silica column-based isolation kit and compared our direct quantification approach with traditional methods using extracted samples. Furthermore, we compared three different centrifugation protocols to address preanalytical considerations related to ccf-mtDNA, which is crucial for accuracy and reliability of the measurements. Our findings provide insights into optimized methodologies for ccf-mtDNA quantification and highlight the importance of standardized protocols in biomarker research and assay development.

Material and methods

Study participants

Twenty-two healthy female (n = 19) and male (n = 3) participants were recruited through electronic announcements at the Johannes Gutenberg University Mainz. Eligibility criteria were evaluated via telephone interviews using the Structured Clinical Interview (SCID) [25] based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) [26]. Exclusion criteria included the presence of acute or chronic medical conditions, mental health disorders, medication or substance use, significant stressful life events within the past six months and smoking more than ten cigarettes daily. The average age of participants was 37.55 ± 13.80 years, with a mean body mass index (BMI) of 24.56 ± 3.14 kg/m^2. The study protocol was approved by the local Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Germany (No#2019–14188).

Study protocol

Blood samples were collected following a 30-min stationary period between 2:00 p.m. and 5:00 p.m. During this period, participants had the option to read magazines. Prior to blood collection, participants completed the Trier Inventory for Chronic Stress questionnaire [27]. The participants were asked to refrain from eating, drinking, and smoking for at least two hours before blood collection.

Evaluation of chronic psychological stress

The German short version of the Trier Inventory for Chronic Stress (TICS-9) [27] was used to measure the subjective perception of chronic stress in the previous three months. Nine items have to be answered on a five-point rating scale ranging from 'never' (0) to 'very often' (4) [27]. A validation study with 2.473 women and men showed good reliability with an internal consistency value (Cronbach's Alpha-coefficient) of α = 0.88 [27].

Blood sample collection and preparation

Venous blood samples were collected in 9 mL tripotassium ethylenediaminetetraacetic acid (K3 EDTA)-monovettes (Sarstedt, Nümbrecht, Germany). Immediately after collection, the samples were centrifuged at room temperature. For comparison studies of centrifugation protocols, the blood samples underwent a three-step centrifugation process: first at 600 × *g* for 15 min, aliquoted and centrifuged again at 2500 × *g* for 15 min, aliquoted once more, and finally centrifuged at 16,000 × *g* for 15 min. All plasma aliquots were stored at – 20 °C.

Assay validation material for ND1 assay

Linearity and accuracy of the assay was tested on a custom-made 102 bp fragment of the human mitochondrial *ND1*-gene (Table 1) (NCBI Reference Sequence: NC_012920.1). The fragment was synthesized by Eurofins MWG Operon (Eurofins MWG Operon, Ebersberg, Germany). The concentration was determined with a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) using Quant-iT PicoGreen dye (Thermo Fisher Scientific). Two calibration samples were prepared by spiking mouse plasma with sonicated DNA at known concentrations, resulting in a final dilution of 1:10. The DNA was isolated from 30 mL of pooled whole blood from four healthy donors using the Qiagen Puregene Blood Core Kit B (Qiagen, Hilden, Germany). DNA was sonicated with the Covaris S220 system (Covaris) using a microTUBE AFA Fiber Pre-Slit

Table 1 Sequence of the custom made human *ND1* gene containing an *Eco*RI restriction site. Reverse and forward primer binding sites are highlighted in grey

***ND1* gene sequence (5'→ 3')**
GTCACGAATTCAGTCACCCTAGCCATCATTCTACTATCAACATTACTAATAAGTGGCTCCTTT AACCTCTCCACCCTTATCACAACACAAGGAATTCGTCAC

Snap-Cap 6 × 16mm tube according to the manufactures instructions for DNA shearing with microTUBES for 400 bp base pair peak. The reference samples were aliquoted in 20 µL and stored at − 20 °C.

Primer design

Due to the highly hypervariable nature of mtDNA in certain regions [28], primers were designed according to the following criteria: (1) targeting conserved rather than hypervariable regions to ensure specificity (2) minimizing the risk of NUMTs co-amplification (3) keeping the target size below 70 bp to optimize efficient amplification of small fragments.

We performed a local alignment analysis with the NCBI primer BLAST [29] to show the specificity of the primer set. Furthermore, we checked for secondary structures including heterodimers and hairpins using the OligoAnalyzer™ Tool [30].

Comparison of direct quantification and isolation

Ccf-mtDNA was isolated from 200 µL of the three-times centrifuged plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions for body fluids. DNA was then eluted in a final volume of 50 µL H_2O. The extracts were stored at – 20 °C immediately after isolation.

Sample preparation and quantification of ccf-mtDNA and cf-nDNA in plasma

Quantification of cf-nDNA was based on the amplification of the repetitive human long interspersed nuclear element 1 (LINE1) (GRCh38/hg38_ chr4:68,085,016–68,085,410 / size = 395 bp / strand = +) targeting a 90 bp fragment of the gene (5′-TGCCGCAATAAACATACGTG-3′ and 5′-GACCCAGCCATCCCATTAC-3′). Detailed protocol, assay precision and specifications can be found in Neuberger et al. [21]. In brief, plasma samples were diluted 1:15 in UltraPure DNase/RNase-free H_2O (Invitrogen, Waltham, MA), each sample was measured in a final volume of 5 µL in technical replicates of three. qPCR mix consisted of 1 µL 1:15 diluted plasma sample, 0.1 µL primer mix (140 nM final concentration of each primer) and 3.9 µL of master mix. Final master mix concentrations were 1.2 × MegaFi Pro Reaction Buffer (BioCat GmbH, Heidelberg, Germany) 0.3 mM of each dNTP (BioCat GmbH), 0.15 × SYBR Green (Sigma-Aldrich, Taufenkirchen, Germany) and 0.05 U MegaFi Pro Fidelity DNA Polymerase (BioCat GmbH). The pipetting was performed by a pipetting robot (Assist Plus, Integra).

For the absolute quantification of ccf-mtDNA, plasma samples were diluted 1:10 in UltraPure DNase/RNase-free H_2O (Invitrogen) and measured using direct qPCR. Extracts remained undiluted. 3 µL of diluted plasma sample or extract was mixed with 11.7 µL of Master Mix and 0.3 µL of Primer Mix and measured in triplicates in a final volume of 5 µL. Final PCR Mix concentrations were 1.2 × MegaFi Pro Reaction Buffer (BioCat GmbH) 0.3 mM of each dNTP (BioCat GmbH), 0.2 × SYBR Green (Sigma-Aldrich), 0.05 U MegaFi Pro Fidelity DNA Polymerase (BioCat GmbH) and 300 nM of forward (for: 5′-CCTAGCCATCATTCTACTATCA-3′) and reverse (rev: 5′-TTGTGATAAGGGTGGAGAG-3-′) primers.

Amplification was performed on a Bio-Rad CFX384 system thermocycler (Bio-Rad, Hercules, CA, USA) with the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 10 s and 60 °C for 15 s including a plate reading step. A melt curve analysis from 60–90 °C with an increase of 0.5 °C every 10 s was performed in each run. If the quantification cycle (Cq) of the triplicates showed higher standard deviation (SD) than > 0.4, the plasma samples were re-diluted and re-analyzed.

Determination of assay performance and reproducibility of ND1 assay

For the determination of LOQ and LOD three standard curves were generated in water and mouse plasma. Mouse plasma is a suitable background matrix mimicking the inhibition of real native human plasma without containing the human specific mitochondrial *ND1* gene sequence. The mouse plasma was spiked with a custom-made fragment of the *ND1* gene covering a dynamic range of 1×10^6–25 copies/PCR. Final dilution of the spike in samples were 1:10 in water. The standard curve measurements were carried out in seven replicates on three different days. As non-template control (NTC) H_2O and 1:10 diluted mouse plasma was included in each run. Additionally, the two calibrator samples were included in each run on each plate. According to the CLSI- guideline EP17-A [23], LOB and LOD were defined as followed:

$LOB = mean_{blank} + 1.645(SD_{blank})$.

$LOD = LOB + 1.645$ x ($SD_{low\ copy\ number\ sample}$) [23].

Reproducibility of the assay was evaluated based on the mean Cq values of the LOQ curves, with the CV calculated using the following equation:

$$CV = \left(\frac{SD}{Mean}\right) \times 100$$

Incurred sample reanalysis

The reliability of the assay was determined by re-analyzing a subset of diluted plasma samples in two different runs. The percentage difference between the first and repeated measurement was calculated using the following equation:

$$\frac{(repeated\ measurement - first\ measurement)}{mean} \times 100$$

According to the FDA Bioanalytical Method Validation Guidance for Industry [22].

Normalization strategy—Inter-run calibration

The two calibrator samples were used to normalize the data and correct for inter-run differences, minimize the impact of technical variations and reduce background fluorescence noise. The reference samples were measured in seven replicates in three independent runs including the LOQ curve. The mean Cq values of all measurements were used for threshold adaption. Reference samples were aliquoted in 20 µL and stored at – 20 °C. Freeze–thaw cycles were limited to three.

Calculation of ccf-mtDNA and cfnDNA

To calculate the ccf-mtDNA concentrations in copies/mL from the measured Cq values the following equation was used:

$$\frac{copies}{mL} = 10^{\frac{(Cq - yIntercept)}{slope}} / 5\mu L / 0.02\ or\ 0.2 \times 1000$$

For plasma samples and extracts, the equation includes a total dilution factor of 0.02 and 0.2, respectively. This factor accounts for the initial 1:10 dilution of the plasma samples and the additional dilution in the qPCR reaction (3 µL sample in a 15 µL reaction volume). Division by 5 equals the copies/µL. Multiplication by 1000 gives the number of copies/mL. An elution factor of four was estimated for the final concentrations of the extracted samples (200 µL native plasma sample eluted in 50 µL H_2O). cf-nDNA concentrations were calculated according to the equation described in Neuberger et al. [21].

Data analysis

The qPCR analysis was performed with the Bio-Rad CFX Maestro software version 2.3 (Bio-Rad, Hercules, CA, USA) and Microsoft® Excel, 2016. For statistical analyses and graphical illustration RStudio (v4.3.3) with ggplot package [31] was used. Imprecision profile was generated using the R VFP package (v1.4.1). The data sets were transformed using log10 and tested for normal distribution using Shapiro–Wilk test. Pearson's and Spearman's correlation test were used for normal distributed and not normally distributed data, respectively. Wilcoxon rang-sum test was used as nonparametric statistical test for non-normal distributed data. *P*-values < 0.05 were considered significant.

Results

mtDNA assay performance

To evaluate the performance of the newly designed mtDNA assay three independent standard curves were generated using the custom-made fragment of the *ND1* gene. The values obtained from these measurements are provided in the Supplementary Information Table S3. The data was positively tested for normal distribution using Shapiro–Wilk test. The three independent standard curves have similar y-intercept and slope as illustrated in Fig. 1a. Efficiencies are ranging between 98.15% and 100.38%, while linearity shows $R^2 > 0.99$ (Supplementary Information Fig. S1, Tab. S4). LOB and LOD were calculated according to the CLSI guideline, yielding a LOB of 4.4 copies and a LOD of 15.41 copies (Supplementary Information Tab. S6). There are no defined guidelines for LOQ determination, but following the recommendations for qualitative real-time PCR methods [32], a CV of ≤ 25% was set as the threshold for LOQ. Values are included in the Supplementary Information Tab. S5. The imprecision profile of the assay showed a CV below 25%, meeting

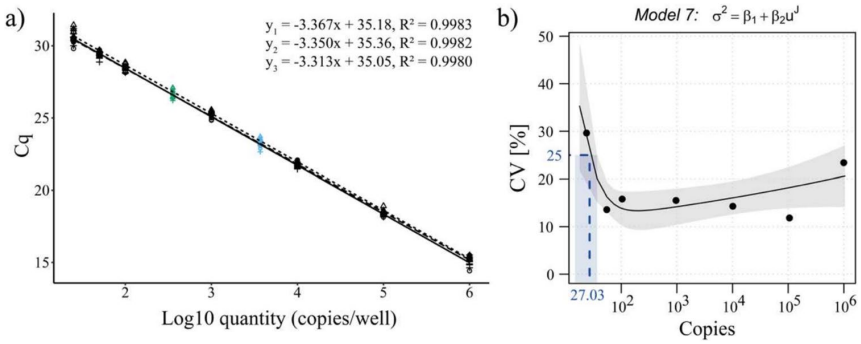


Fig. 1 LOQ curves and imprecision profile of *ND1* assay. Three standard curves were measured in septettes for each concentration, with green and blue dots representing the two calibrator samples and black dots representing the standards **a** Imprecision profile was generated using R VFP package, based on the formula $\sigma^2 = 35.68 + 0.0095 \times U^{2.108}$ **b** Figures were produced using the R ggplot2 package

the predefined threshold for precision. The LOQ of 27.03 copies/PCR was then determined based on the imprecision profile (Fig. 1b). All replicates of the low copy number sample were successfully detected within the assay range (10^6 – 27.03 copies/PCR), remaining above the LOD. Specificity of the assay was confirmed by melt curve analysis and local alignment analysis with BLAST (Fig. 2).

Incurred sample realanysis

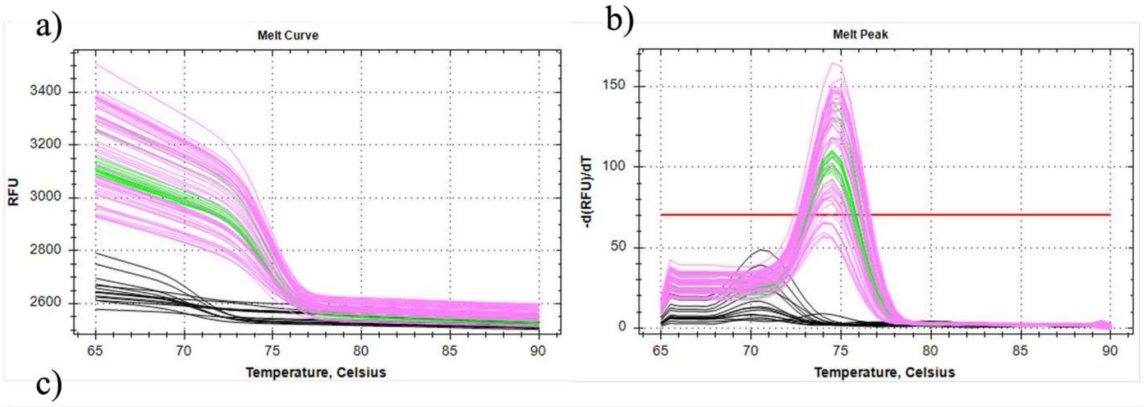
To evaluate the accuracy and reliability of the mtDNA assay, approximately 29% of the diluted plasma samples were re-analyzed in two different runs (Supplementary Information Tab. S2).The ccf-mtDNA concentrations showed a strong correlation between the initial and repeated measurements ($r = 0.93$, $p < 0.001$) (Fig. 3). According to the FDA Guidelines for Bioanalytical Method Validation [22], two-thirds of the reanalyzed samples should exhibit a percentage difference of less than 30%. In this subset, 5 samples (31.25%) exceeded the 30% threshold, while the remaining samples were within the acceptable range (Fig. 3).

Kit isolation reduces ccf-mtDNA concentrations

To compare our direct quantification approach with a kit isolation method, ccf-mtDNA was directly quantified in a subset of diluted plasma samples and compared with the corresponding isolated samples (Supplementary Information Table S1, S2). The comparison of direct quantification with isolated samples revealed a notable loss of approximately 43.3% (± 38.0) during isolation using the QIAamp DNA Blood Mini Kit (Fig. 4a). Direct and isolated measurements correlated positively, with a Spearman's correlation coefficient of 0.59 (Fig. 4b).

Centrifugation forces affect ccf-mtDNA levels

We analyzed the effect of different centrifugation forces on ccf-mtDNA concentrations in diluted native plasma samples (Supplementary Information Table S2). Ccf-mtDNA concentrations in native plasma samples significantly decreased



c)

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCTAGCCATCATTCTACTATCA	22	54.31	40.91	4.00	2.00
Reverse primer	TTGTGATAAGGGTGGAGAG	19	53.41	47.37	3.00	0.00

Products on target templates

>NC_012920.1 Homo sapiens mitochondrion, complete genome

```
product length = 68
Forward primer  1     CCTAGCCATCATTCTACTATCA  22
Template        3741  ......................  3762

Reverse primer  1     TTGTGATAAGGGTGGAGAG  19
Template        3808  ...................  3790
```

Fig. 2 Specification of the *ND1* assay. Melt curve and melt curve peak of the *ND1* target fragment (**a**, **b**). Pink and green lines indicate samples used to generate the standard curve and calibrators, respectively (**a**, **b**). Black lines indicating primer dimers in the non-template controls (**a**, **b**). Extract of the BLAST results of the primers used for the *ND1* assay, showing specificity and accuracy of the primers with no off-target amplification (**c**). (Color figure online)

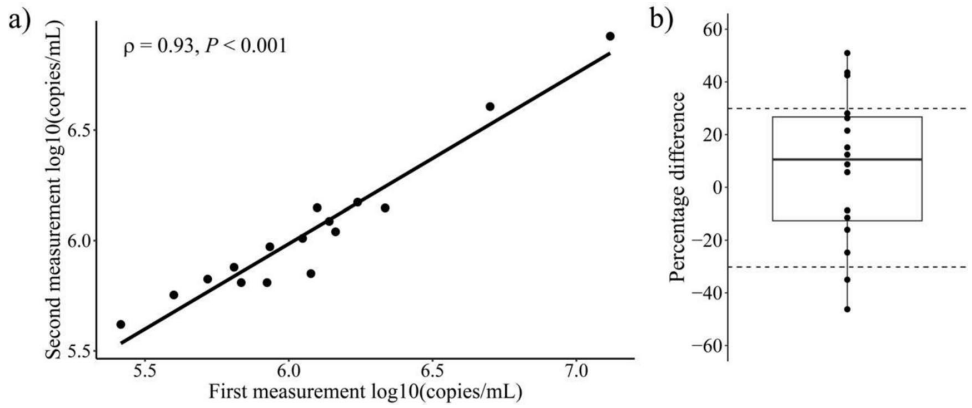


Fig. 3 Precision of *ND1* assay. Correlation between initial and repeated measurements of diluted plasma samples **a** Percentage difference between initial and repeated measurements **b** Figures were produced using the R ggplot2 package

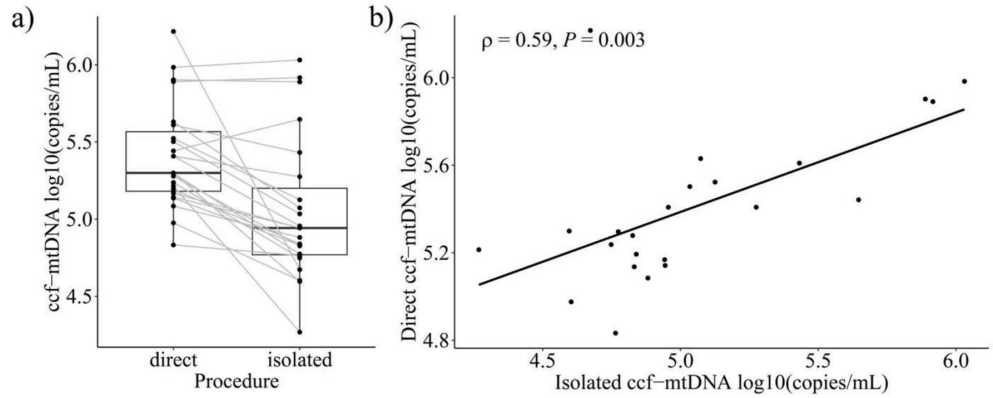


Fig. 4 Effects of Isolation. Comparison of direct quantification and silica column based isolated samples **a** Correlation between direct and isolated samples **b** Figures were produced using the R ggplot2 package

with increasing centrifugation force (Fig. 5a). All samples centrifuged at 600 × *g* and 2500 × *g* remained within the quantification range, while only 45% of those centrifuged at 16,000 × *g* were within the assay LOQ (Fig. 5a). In contrast, a significant difference in cf-nDNA levels were found between 600 × *g* and 2500 × *g* and 600 × *g* and 16,000 × *g*, respectively (Fig. 5b).

Ccf-mtDNA decreases with chronic stress but not cf-nDNA

As only 45% of the native plasma samples centrifuged at 16,000 × *g* remained above the LOQ threshold of the ccf-mtDNA assay, all samples were purified using the QIAamp DNA Blood Mini Kit (Supplementary Information Table S1). To further evaluate a potential relationship between stress and cell-free nucleic acids, we conducted the TICS-9 as a standardized questionnaire designed to assess the chronic stress load in our healthy cohort. Spearman's rank correlation showed a significant negative association between the TICS level (Supplementary Information Table S7) and ccf-mtDNA levels in purified 16,000 × *g* centrifuged plasma samples, with a correlation coefficient of − 0.51 (Fig. 6a). However, no significant correlation was found between TICS levels and cf-nDNA in native unpurified 16,0000 × *g* plasma (Fig. 6b).

Discussion

Over the past decade, cell-free nucleic acids have been recognized as a promising biomarker in liquid biopsy. Serval studies highlight its prognostic and diagnostic potential

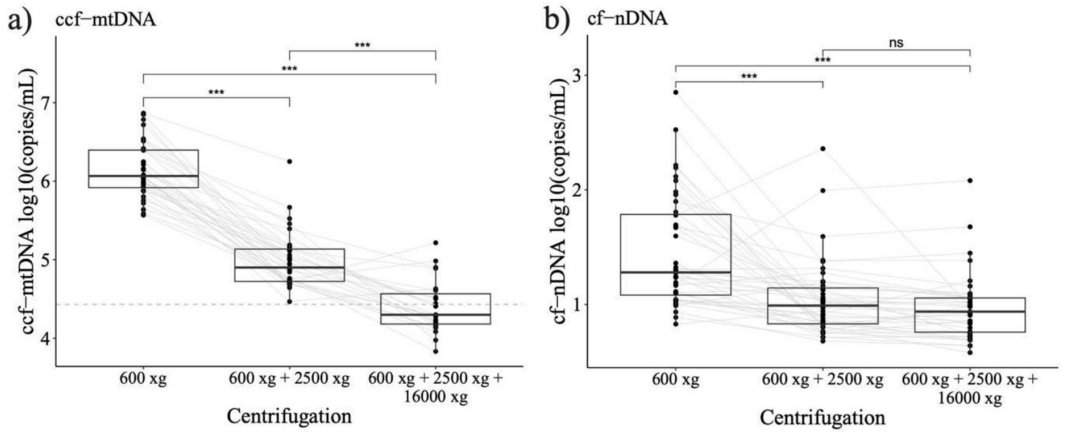


Fig. 5 Effects of centrifugation forces. Effects of different centrifugation protocols on ccf-mtDNA concentrations in unpurified plasma, with the dotted line indicating the LOQ of the assay **a** Effects of different centrifugation protocols on cf-nDNA concentrations in unpurified plasma **b** Statistical significance levels are described as $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. Figures were produced using the R ggplot2 package

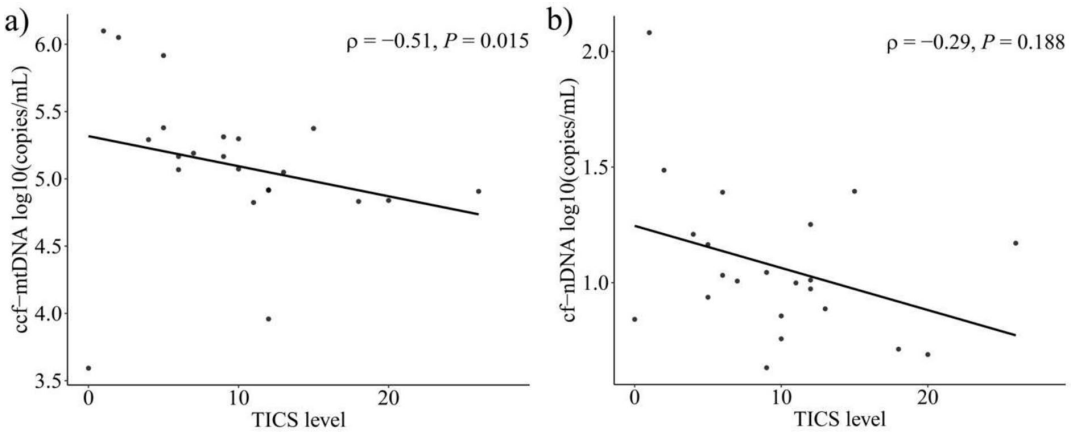


Fig. 6 Effects of chronic stress on ccf-mtDNA (**a**) and cf-nDNA (**b**) in healthy subjects. Figures were produced using R ggplot2 package

in pathological conditions including cancer [33], neurodegenerative diseases [34], mood disorders [35], and infectious conditions. Here we provide an assay for the absolute quantification of ccf-mtDNA directly in human blood plasma samples, with an LOQ of 27.03 copies (Fig. 1). Our study demonstrates that preanalytical aspects such as centrifugal forces and isolation strongly affects ccf-mtDNA measurement outcomes, but not cf-nDNA (Fig. 4, 5). Moreover, we used the new assay to quantify ccf-mtDNA in healthy subjects and found a significant reduction ($p = 0.015$) in ccf-mtDNA levels in response to chronic stress, while cf-nDNA concentrations remained unchanged (Fig. 6).

For the assay development we implemented the recommendations for developing qPCR and dPCR assays according to the ISO 20395:2019 [24], FDA [22] and CLSI EP17 -A guidelines [23]. The use of two inter-run calibration samples ensures the reliability of the assay and allows ccf-mtDNA concentrations to be compared across multiple runs on different plates. Reproducibility was verified with incurred sample reanalysis, demonstrating a strong correlation between the initial and repeated measurements, with concentrations remaining within the predefined range (Fig. 3). Our findings show that ccf-mtDNA can be directly quantified in human blood plasma samples without DNA extraction, offering a faster and more cost-effective

alternative to bead- or column-based extraction methods for ccf-mtDNA quantification. Direct quantification not only reduces processing time, but also minimizes DNA loss, a common problem of isolation procedures, as we have demonstrated in this and previous studies [36] (Fig. 4). In particular, small DNA fragments, exhibit lower binding efficiency and may pass through columns during extraction or remain attached to the silica membrane, which can lead to biased results [37]. This is important because ccf-mtDNA predominantly consists of smaller fragments, ranging in size from 50 to 400 bp and peaking at approximately 80 bp [38]. In contrast, cf-nDNA exhibits larger fragments, with a peak around 166 bp [39], making ccf-mtDNA more sensitive to loss during conventional isolation procedures. To address this issue in our direct quantification approach, we targeted a 68-bp fragment of the human mitochondrial *ND1* gene, ensuring more accurate detection of smaller fragments.

When quantifying ccf-mtDNA the presence of NUMTs in the samples should be considered in primer and or probe design. NUMTs, which are incorporated into the nuclear genome, can be released as cf-nDNA into the circulation. Due to their high similarity to mtDNA, these sequences may inevitably be co-amplified during PCR if the primer or probe design lacks precision [19]. The specificity of our primers was confirmed by melt curve analysis and BLAST results (Fig. 2). The melt curve analysis demonstrated a distinct peak corresponding to the target, and the BLAST results showed a single, specific match in the mitochondrial genome, confirming the accuracy of our primer design (Fig. 2).

We have demonstrated that different centrifugation forces influence the abundance of ccf-mtDNA in human blood plasma samples (Fig. 5a), which aligns with previous studies [40, 41]. These differences might primarily occur due to particle-bound mtDNA, such as platelet-associated DNA. Platelets, the smallest human blood cells, play a central role in blood clotting and immune response. Although they lack nuclear DNA, they contain intact mitochondria and mtDNA. Numerous studies have consistently demonstrated that platelet counts in plasma samples are highly influenced by both centrifugation force and duration, with prolonged or high-speed centrifugation significantly reducing platelet levels and mtDNA in plasma samples [42–44]. In this study, plasma samples centrifuged at high speeds resulted in ccf-mtDNA concentrations close to or below the LOQ of the direct assay, making quantification at these speeds less reliable (Fig. 5a). However, it is important to note that higher centrifugation forces reduce platelet counts and therefore yield a "purer" form of free circulating mtDNA. In contrast, lower centrifugation forces (e.g., $2500 \times g$) allow for more consistent detection, as all samples remained within the assay's LOQ for ccf-mtDNA. Despite these differences, a strong correlation was still observed between purified and unpurified ccf-mtDNA measurements at $16{,}000 \times g$ (Fig. 4b), suggesting that while absolute values may differ, trends remain comparable. Additionally, using our direct quantification approach platelet and mitochondrial membranes will be disrupted during PCR denaturation in unpurified plasma samples, leading to the release of mtDNA and subsequently increased ccf-mtDNA measurements in the lower centrifuged plasma samples.

Therefore, we recommend a two-step centrifugation protocol prior to ccf-mtDNA analysis in plasma. The first step involves low-speed centrifugation ($1000–1600 \times g$, 15 min) to remove blood cells and large particles, followed by high-speed centrifugation (e.g. $16{,}000 \times g$, 15 min). The first centrifugation separates the plasma, which should be transferred into a new tube, maintaining at least a one-centimeter gap from the buffy coat layer. The second centrifugation should be performed similarly, with both steps conducted at room temperature to avoid potential platelet activation and mtDNA release [45]. The blood should be centrifuged immediately after collection to avoid delays that could lead to cell degradation or inadvertent platelet activation. Roch et al. have shown that mtDNA levels can increase 67-fold when platelets are activated, highlighting the importance of careful handling during processing and the impact of platelets on ccf-mtDNA levels in plasma [46]. Although this procedure effectively removes cells, platelets, cellular debris and mitochondria, extracellular vesicles and exosomes, which usually pellet at ultra-high centrifugation speeds ($\geq 100{,}000 \times g$) [47] will remain in the plasma, which should be considered for downstream analysis. However, this procedure enables to the capture the rather small proportion of ccf-mtDNA human plasma, ensuring that only the freely circulating DNA is isolated, rather than membrane-bound.

Despite the fact that we observed higher cf-nDNA levels in samples centrifuged at lower speeds (Fig. 5b), previous studies have shown that lower centrifugation forces do not significantly affect cf-nDNA levels [21, 48]. This discrepancy may be due to residual cell contamination in the low-speed centrifuged plasma samples, which may result in release of DNA during PCR denaturation. In general, the presence of such contamination can artificially inflate cf-nDNA measurements and, thus, potentially bias liquid biopsy results. Therefore, it is highly recommended to adhere to preanalytical instructions in research as well as clinical routine.

Here we assess chronic stress levels in healthy subjects using the standardized and well-established TICS-9 questionnaire to evaluate perceived stress and its effects on cell-free nucleic acids. We have shown that ccf-mtDNA levels are negatively correlated with higher levels of chronic stress (Fig. 6a). In contrast, we did not observe such an effect on cf-nDNA concentrations in conjunction with the perceived stress levels (Fig. 6b). To our knowledge, this is the third

study to assess ccf-mtDNA levels in response to stress in vivo, with results conflicting with previous studies that showed elevated ccf-mtDNA levels following stress induction [18]. Trumpff et. al [17] demonstrated an increase in serum ccf-mtDNA concentrations 30 min after induced psychological stress in healthy volunteers, with consistent results in a follow-up examination one month later. However, serum samples may not be suitable to accurately reflect ccf-mtDNA levels, as most of the released mtDNA could be related to the initial clotting process of the platelets. Furthermore, the primer set used by Hummel et al. targets NUMTs as a byproduct, which could potentially contribute to the elevated ccf-mtDNA measurements observed after stress induction.

It is important to note that our study evaluated chronic stress, rather than acute stress, which could lead to different results. Interestingly, a study using a cell culture model of chronic stress simulated by continuous glucocorticoid exposure showed increased ccf-mtDNA levels [49]. Nonetheless, these findings are not necessarily transferable to in vivo conditions, as additional factors such as physiological regulatory mechanisms and inter- or intra variability in stress and immune responses may influence the results.

Although we hypothesize that ccf-mtDNA concentrations might be elevated as a result of mitochondrial dysfunction and damage, the observed negative correlation might suggest complex biological regulatory mechanisms in response to chronic stress. This could include protective adaptions of mitochondria to maintain their functionality and reduce damage to mtDNA. Acute stress typically triggers the "fight or flight" response, which involves the activation of the HPA axis and sympathetic nervous system. This activation can lead to an immune-enhancing effect, characterized by increased proinflammatory cytokine production and increased immune surveillance, whereas chronic stress and prolonged activation of HPA axis can have the opposite effect, leading to immune suppression and dysregulation [50]. Our results indicate that the observed effects are specifically related to mitochondria and mtDNA as we did not find any correlation between the chronic stress levels and cf-nDNA. Further research is needed to investigate the interplay between mitochondrial adaptions, immune regulation and activation and the body's response to prolonged stress exposure.

Limitations

Due to the small sample size, it is possible that potential changes in ccf-mtDNA or cf-nDNA were not detected. In this study, ccf-mtDNA levels are close to the LOQ of the direct qPCR assay, indicating that the assay is not yet suitable for determining ccf-mtDNA levels in healthy conditions. Improving the sensitivity of the assay could help quantifying ccf-mtDNA in conditions where concentrations are lower. However, the method could be used to reliably quantify ccf-mtDNA in human blood plasma in other pathological settings.

Conclusions

In conclusion, we have established a direct and reliable assay for ccf-mtDNA quantification in human blood plasma samples that can be used in liquid biopsy, reducing time and cost of purification. Further research should focus on optimizing this assay for broader clinical use and investigating its applicability in other diseases where liquid biopsy is a potential tool for diagnosis and prognosis. This could be an opening for studying intra- and inter-individual variability of ccf-mtDNA under different conditions and in different pathologies. The reduction in ccf-mtDNA concentrations in relation to perceived chronic stress levels in our healthy cohort suggests regulatory mechanisms specifically related to mtDNA and mitochondrial function, as we did not observe such a correlation in cf-nDNA. Future studies should investigate other biomarkers for stress related manners or in stress related diseases.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-025-10369-7.

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Data availability All data generated and analyzed during this study is included in the electronic supplementary file.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval The study involving human participants were reviewed and approved by the local Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Germany (No#2019–14188).

Consent to participate All participants gave their written informed consent to participate.

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4. Chapter II

Effects of acute stress on plasma circulating cell-free mitochondrial DNA in Panic Disorder

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Keywords: Panic Disorder (PD), Circulating cell-free mitochondrial DNA (ccf-mtDNA), Biomarker, Mitochondria, Trier Social Stress Test (TSST)

Abbreviations

BD: Bipolar Disorder, BMI: body mass index, ccf-mtDNA: circulating cell-free mitochondrial DNA, cf-nDNA: circulating cell-free nuclear DNA, CRH: Corticotrophin-releasing hormone, Cq: Quantification cycle, DSM-IV: Diagnostic and Statistical Manual of Mental Disorders Fourth Edition, EDTA: ethylenediaminetetraacetic acid, HPA: hypothalamic-pituitary-adrenal, K3: tripotassium, MD: Major Depression, mtDNA: mitochondrial DNA, ND1: NADH-ubiquinone oxidoreductase chain 1, NTC: Non template control, NUMTs: Nuclear-embedded mitochondrial DNA sequences, PD: Panic Disorder, qPCR: Quantitative polymerase chain reaction, SAD: Social Anxiety Disorder, SCID: Structured Clinical Interview, SD: Standard deviation, TICS-9: Trier Inventory for Chronic Stress, TSST: Trier Social Stress Test

4.1 Abstract

Objective Panic disorder (PD) is a stress related disorder that significantly impacts the patient's quality of life. Chronic stress is a core feature of PD, and its effects on cellular and molecular processes remain poorly understood. Mitochondria have recently gained attention in stress research due to their role in regulating multiple cellular functions and their need to adapt to both external and internal changes. This study aims to investigate the relationship between acute stress and circulating cell-free mitochondrial DNA (ccf-mtDNA) levels in PD patients and healthy subjects and in response to acute stress. **Material and Methods** In this study, we used quantitative polymerase chain reaction (qPCR) to measure ccf-mtDNA concentrations in 32 PD patients and 22 healthy subjects, targeting the human mitochondrial ND1 gene. The Trier Social Stress Test (TSST), a well-established and controlled method for inducing psychological stress, was applied to both PD patients and healthy controls. **Results** Our findings revealed that ccf-mtDNA concentrations significantly decreased 10 minutes after acute stress induction in PD patients, whereas no such change was observed in healthy controls. We found no significant reduction in baseline ccf-mtDNA concentrations in the PD cohort compared to the healthy controls. However, chronic stress was negatively correlated with ccf-mtDNA levels in both groups. **Conclusion** We have found evidence that the stress response in PD patients differ from those in healthy individuals.

4.2 Introduction

Panic Disorder (PD) is a common psychiatric disorder that profoundly impacts the patient's quality of life. It is characterized by recurrent unexpected panic attacks accompanied by symptoms of anxiety, fear of losing control, dizziness, tremors, hyperventilation, chest pain, nausea, abdominal discomfort and an elevated baseline heart rate [75]. The persistent fear of future panic attacks contributes to significant and prolonged psychological and physical distress for individuals with PD [76]. PD affects a significant portion of the population and imposes substantial costs on healthcare systems. The lifetime prevalence of PD is estimated to be approximately 4.2 - 4.7%, with a 12-month prevalence of about 2.7% [77–79]. Patients with PD often experience comorbid somatic conditions that can complicate diagnosis and treatment. Research indicates that individuals with PD are at an increased risk for various chronic physical illnesses [80]. This is explicable since individuals with PD may experience somatosensory amplification, a tendency to perceive bodily sensations as more intense or threatening, which can lead to increased reporting of somatic symptoms. This phenomenon is discussed in the context of functional somatic syndromes, irritable bowel syndrome as well as hypertensions [81]. Due to the comorbid somatic conditions and the tendency to perceive bodily sensations as threatening, individuals with PD often show increased healthcare utilization and considerable economic burdens [82,83]. Empirical data indicate that depressive and anxiety disorders in primary care settings are associated with significant healthcare costs, underscoring the economic strain these conditions place on health systems [84,85].

Stressful life events, such as trauma, loss, and a history of stressful life events, have been identified as increased risk factor as well as potential triggers for the onset and development of PD [86]. This might be explicable since the physiological response to stress involves the activation of the autonomic nervous system, leading to symptoms such as increased heart rate and hyperventilation, which are symptoms shown under a panic attack by PD patients. In individuals predisposed to PD, these stress-induced physiological changes may be misinterpreted as signs of impending danger, thereby triggering panic attacks.

In response to a stressful situation, the autonomic nervous system triggers the hypothalamus to release corticotrophin-releasing hormone (CRH), which stimulates the pituitary gland and later the

adrenal cortex. This Hypothalamic-Pituitary-Adrenal (HPA) axis regulates the body's response to stress through the release of hormones like cortisol. The model of allostasis and allostatic load of McEwen explains how the body adapts physiological and hormonal to stress and how chronic stress can negatively impact health [87]. Allostasis refers to the process by which the body achieves stability through change in response to stressors. Unlike homeostasis, which maintains fixed internal states, allostasis involves dynamic adjustments by various physiological systems (like the nervous, endocrine, and immune systems) to maintain balance during changing conditions. However, when the stress response is prolonged or frequent it can lead to an allostatic load- the cumulative "wear and tear" on the body that occurs from chronic stress exposure. When the body is unable to return to baseline after repeated stress or fails to recover adequately, these ongoing demands accumulate, leading to potential organ and system damage. Over time, this prolonged strain can contribute to various health problems, such as cardiovascular disease, depression, anxiety and immune dysfunction. Dysregulation of the stress- system has been implicated in various psychiatric disorders, including PD.

Importantly, the perturbations are not limited to systemic effects and physiological changes. Chronic stress can also disrupt cellular mechanisms such as mitochondrial biogenesis and function. Mitochondrial functioning is essential for maintaining cellular health, as mitochondria play a crucial role in many cellular processes including apoptosis [20], energy production [88], steroid hormone synthesis [21] and calcium homeostasis [19]. As the main contributors to energy production in eukaryotic cells, mitochondria are uniquely equipped to sense stress signals and adapt to the body's fight-or-flight mode, meeting the increased energy demands during such states. This process is known as mitochondrial allostatic load [89]. Prolonged stress exposure, as seen in PD, can result in maladaptive mitochondrial changes leading to an allostatic overload and ultimately to an impaired energy production, oxidative stress, and mutations in mitochondrial DNA (mtDNA), which can further compromise cellular function [89]. Due to their ability to detect stress signals and adjust their function accordingly, mitochondria play a crucial role in cell-to-cell communication. In this context, mtDNA can be released into circulation both actively—as part of cellular signaling and stress adaptation —and passively due to mitochondrial damage, cell damage or cell death [90]. This fragments of mtDNA are commonly known as circulating cell-free mitochondrial DNA (ccf-mtDNA). It has been suggested that mitochondrial dysfunction [91] plays a key role in psychiatric

disorders and is a significant factor in psychiatric illnesses. Numerous studies have shown altered levels of ccf-mtDNA in psychiatric disorders, including bipolar disorder (BD) [73], major depression (MD) [73,92] and social anxiety disorders (SAD) [74], the latter two including stress-related disorders. Additionally, psychiatric conditions are frequently associated with neuroinflammation and immune dysregulation [93]. Given its bacterial origin and function as a potent inflammatory trigger [94], ccf-mtDNA has been proposed not only as a marker of mitochondrial dysfunction but also as a contributor to neuroinflammation and oxidative stress—both key features in the pathophysiology of psychiatric disorders.

Given the role of mitochondrial dysfunction in stress-related pathophysiology [87] ccf-mtDNA may serve as valuable biomarker for investigating the molecular effects of long term and acute stress in psychiatric conditions. In this study, we utilized liquid biopsy as a minimally invasive approach to investigate the impact of acute stress exposure on circulating ccf-mtDNA levels in PD patients compared to their healthy controls. Acute stress was induced using the Trier Social Stress Test (TSST), a well-established protocol for triggering psychosocial stress under controlled conditions. By studying the effects of acute stress, we hope to gain a better understanding of how stress contributes to disease progression and the dysregulation seen in PD. This understanding could ultimately lead to the development of more effective treatments for PD in the future. In addition, by identifying biomarkers such as ccf-mtDNA, we may be able to improve the early detection and monitoring of PD, potentially leading to more timely and targeted interventions. Such advances could reduce the frequency and duration of hospital admissions, thereby reducing both healthcare costs and the overall burden on patients.

4.3 Material and Methods

4.3.1 Ethical approvement

The study protocol was approved by the local Ethics Committee of the Landesärztekammer Rheinland-Pfalz, Germany (No#2019–14188). All participants gave their written informed consent to participate.

4.3.2 Study participants

Twenty-two healthy subjects (female (n=19) and male (n=3)) and thirty-one patients diagnosed with PD (female (n=19 and male (n=12)) were recruited via electronic announcements at the Johannes Gutenberg-University Mainz. Eligibility was assessed through telephone interviews using the Structured Clinical Interview (SCID) [96] based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) [97]. Exclusion criteria included the presence of acute or chronic medical conditions, mental health disorders, medication or substance use, significant stressful life events within the past six months and smoking more than ten cigarettes daily. The average age of participants was 37.55 ± 13.80 years, with a mean body mass index (BMI) of 24.56 ± 3.14 kg/m². Rest and stress conditions were completed on different days.

4.3.3 Trier Social Stress Test

The Trier Social Stress Test (TSST) [98] is used to assess the physiological and psychological effects of acute stress. The TSST is effective in inducing a stress response due to its combination of social evaluative threat and cognitive challenge, making it a valuable tool for understanding the impact of stress on mental and physical health. The test typically consists of a series of standardized, socially challenging tasks that mimic real-life stressors. The procedure begins with a preparation phase and a simulated job interview and mental arithmetic. Throughout the test, physiological measurements are taken, including cortisol levels and heart rate, which provide indicators of the body's acute stress response. The test was performed as described in Kirschbaum *et al.* (1993) [98].

4.3.4 Evaluation of Chronic Psychological Stress

The German short version of the Trier Inventory for Chronic Stress (TICS-9) [99] was used to measure the subjective perception of chronic stress in the previous three months. Nine items have to be answered on a five-point rating scale ranging from 'never' (0) to 'very often' (4). A validation study with 2.473 women and men showed good reliability with an internal consistency value (Cronbach's Alpha-coefficient) of $\alpha = 0.88$ [99].

4.3.5 Blood sampling

Blood samples were collected between 2:00 p.m. and 5:00 p.m, following a 30-minute stationary rest period, during which participants were allowed to read magazines. Before sample collection, participants had to complete the Trier Inventory for Chronic Stress (TICS-9) questionnaire. The participants were asked to refrain from eating, drinking, and smoking for at least two hours before blood collection. Blood samples were obtained at three specific time points under rest and stress conditions: one minute before (-1), one minute after (+1), and ten minutes after (+10).

4.3.6 Blood sample collection and preparation

To determine the concentrations of ccf-mtDNA venous blood samples were collected in 9 mL tripotassium ethylenediaminetetraacetic acid (K3 EDTA)-monovettes (Sarstedt, Nümbrecht, Germany). Immediately after collection, the samples underwent a three-step centrifugation process: first at 600 *x g* for 15 min, then at 2500 *x g* for 15 min, and a final centrifugation step carried out at 16000 *x g* for 15 min. All plasma aliquots were stored at -20 °C. Ccf-mtDNA was then isolated from 200 µL of the three-times centrifuged plasma samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for body fluids. DNA was eluted in a final volume of 50 µL H_2O. The extracts were stored at - 20°C immediately after isolation.

4.3.7 Quantification of plasma circulating cell-free mitochondrial DNA

qPCR was used to quantify ccf-mtDNA by targeting a 68 bp fragment of the human mitochondrial NADH-ubiquinone oxidoreductase chain 1 (*ND1*) gene (for: 5'-CCTAGCCATCATTCTACTATCA-3' and rev: 5'-TTGTGATAAGGGTGGAGAGAG-3-'). The detailed protocol is described elsewhere [100]. Briefly, 2 µL of extract was mixed with 7.8 µL master mix and 0.2 µL primer mix and measured in duplicates in a final volume of 5 µL. Final PCR mix concentrations were 1.2 × MegaFi Pro Reaction Buffer (BioCat GmbH, Heidelberg, Germany), 0.3 mM of each dNTP (BioCat GmbH), 0.2 × SYBR Green (Sigma-Aldrich, Taufenkirchen, Germany), 0.05 U MegaFi Pro Fidelity DNA Polymerase (BioCat GmbH). The final concentration of the primer

mix was 300 mM for each primer. Reagents and samples were dispensed by a pipetting robot (Assist Plus, Integra). Each plate and each run included calibrator samples to normalize the data. Amplification was performed on a Bio-Rad CFX384 system thermocycler (Bio-Rad, Hercules, CA, USA) with the following conditions 95 °C for 5 min followed by 35 cycles of 95 °C for 10 s and 60 °C for 15 s including a plate reading step. A melting curve analysis from 60 °C to 90 °C with an increase of 0.5 °C every 10 s was performed in each run. If the measurements showed SD > 0.4, the samples were re-analyzed.

4.3.8 Statistical analysis

qPCR data were analyzed using Bio-Rad CFX Maestro software version 2.3 (Bio-Rad) and Microsoft® Excel, 2016. RStudio (v4.3.3) with the ggplot package [101] was used for statistical analysis and graphical presentation. Data were tested for normal distribution using the Shapiro-Wilk test, visualized using QQ-plots and transformed using log2 and log10 transformations, respectively. Group differences in non-normally distributed data were assessed using the Kruskal Wallis sum and Wilcoxon rank sum test. Correlations were evaluated using Kendall's tau. Bonferroni correction was applied to adjust the p-values for multiple comparisons. A p-value < 0.05 was considered significant.

4.4 Results

4.4.1 Chronic stress negatively correlates with ccf-mtDNA, while baseline levels remain unchanged between groups

The TICS scale was used to assess the chronic stress levels in healthy subjects and PD patients. A significant negative correlation was observed between perceived chronic stress and ccf-mtDNA concentrations, as indicated by Kendall's tau correlation ($\tau = -0.20$, $P = 0.0344$) (Fig.4a). However, baseline ccf-mtDNA concentrations measured at rest showed no significant difference between the healthy and PD groups (Fig. 4b).

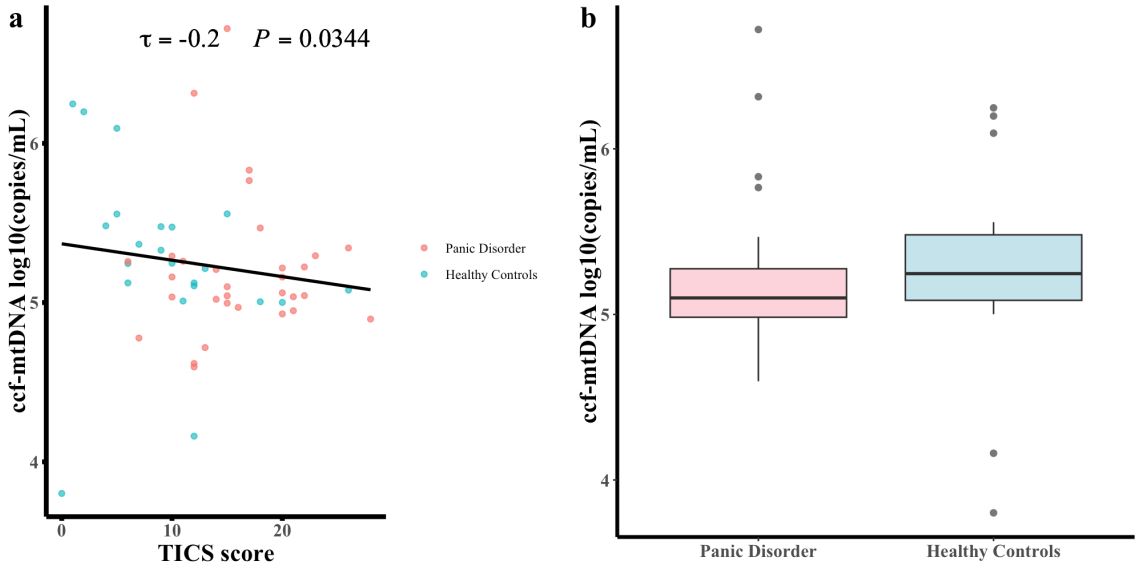

Figure 4 Effect of perceived chronic stress on ccf-mtDNA in healthy subjects compared to PD patients. Scatterplot illustrating the negative correlation between TICS and baseline ccf-mtDNA in both HC and PD patients (a). Comparison of baseline ccf-mtDNA concentrations between PD patients and HC showed no significant differenc (b). Figures were produced using the Rggplot2 package.

4.4.2 Ccf-mtDNA decreases after acute stress induction in PD patients but not in healthy subjects

The TSST was used to investigate the effects on ccf-mtDNA dynamics after acute stress induction in PD patients compared to healthy controls. Blood samples were taken at three different time points and ccf-mtDNA concentrations were measured by qPCR. We found no significant changes in ccf-mtDNA concentrations at rest in PD and healthy subjects (Fig 5). Ccf-mtDNA concentrations in our healthy cohort were not affected by TSST (Fig. 5). In contrast, ccf-mtDNA levels were significantly decreased in PD patients ten minutes after acute stress induction (p-value < 0.05).

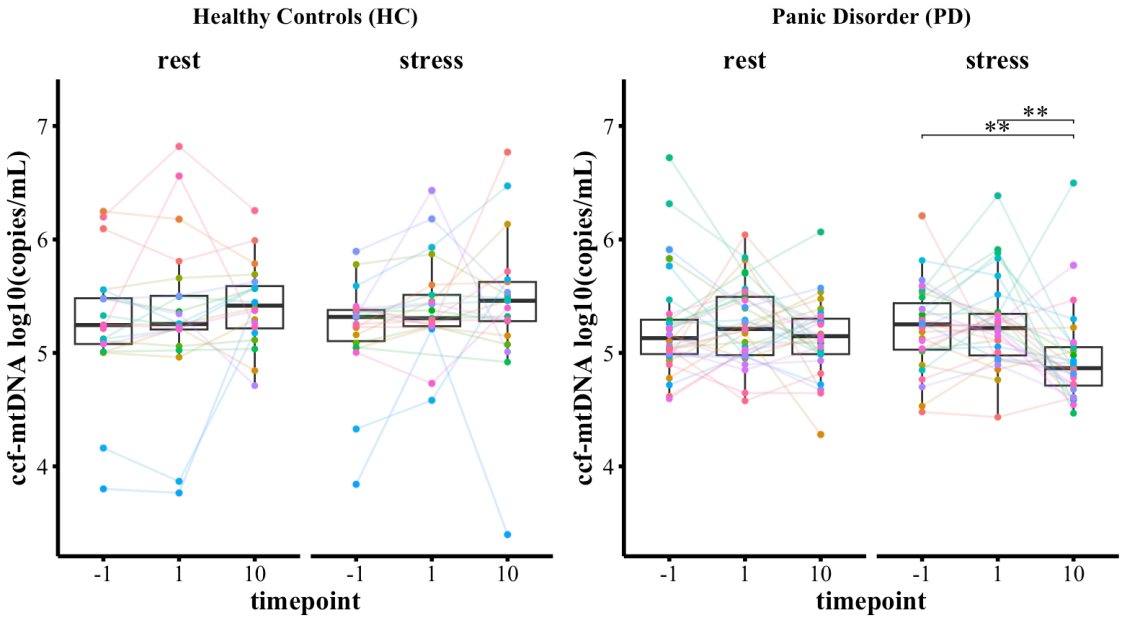

Figure 5 Kinetics of plasma ccf-mtDNA concentrations following acute stress induction and under resting conditions in healthy individuals and patients with panic disorder. Statistical significance levels are labelled as *p ≤ *0.05*, **p ≤ *0.01* and ***p ≤ *0.001*. Figures were produced using the R ggplot2 package.

4.5 Discussion

The development of mental disorders, such as PD, involves complex psychological, neurological, cellular and molecular mechanisms that are not yet fully understood. Furthermore, managing and monitoring disease progression remains challenging. In recent years, ccf-mtDNA has gained attention as a potential biomarker for psychiatric diseases and mood disorders. Building on this research, we aimed to investigate ccf-mtDNA concentrations differences in PD patients compared

to healthy controls. Additionally, we assessed ccf-mtDNA levels following acute stress induction in both cohorts to evaluate potential variations in their responses. Our results indicate that there are no significant differences in baseline ccf-mtDNA concentrations in PD patients at rest compared to healthy subjects. However, ccf-mtDNA concentrations are negatively correlated with perceived chronic stress levels. In addition, we showed that ccf-mtDNA concentrations decreased significantly ten minutes after stress induction in PD patients, whereas no such changes were observed in the healthy cohort.

To our knowledge this is the first study to assess ccf-mtDNA as biomarker in patients with PD. Previous research has demonstrated altered ccf-mtDNA and cf-nDNA levels in various psychiatric disorders, including SAD [74], MD [92], and Schizophrenia [102], with conflicting results showing lower, elevated and unchanged ccf-mtDNA levels. Additionally, altered cytokine levels, particularly the elevation of pro-inflammatory cytokines, have been reported in these psychiatric conditions [103]. In the context of PD, it has been shown that cytokines such as IL-6, IL-1β, and IL-5 are elevated [104]. These cytokines are also known to have pro-inflammatory properties, suggesting that PD may be linked to increased inflammatory reactivity and low-grade inflammation. Given this, we hypothesized that ccf-mtDNA would be altered maybe even elevated due to the inflammatory environment. However, our results indicate a tendency towards lower ccf-mtDNA concentrations, although we did not find a significant difference in ccf-mtDNA baseline concentrations between PD patients and healthy controls (Fig. 4). This lack of significance may also be due to the relatively small sample size (32 patients), which could limit statistical power. Further research should focus on replicating our findings and increasing the sample size to enhance statistical power.

The effects of acute psychological stress on cf-nDNA kinetics in healthy male subjects have been demonstrated in prior studies [8,105]. Both publications showed an immediate response after the stressor with an approximately 2-fold increase in cf-nDNA levels. However, much less is known about the effects of acute stress on ccf-mtDNA kinetics in health and disease. While Hummel *et al.* (2018) observed an increase in ccf-mtDNA concentrations following both acute psychological and physical stress [105], Limberg *et al.* (2025) recently reported no changes in plasma ccf-mtDNA kinetics after acute psychosocial and physical stress in healthy subjects [106]. Our results align with Limberg *et al.*, showing no significant alterations in ccf-mtDNA levels after acute stress, which

contradicts the findings of Hummel *et al.* To our knowledge, there are no other studies investigating the effects of acute stress on ccf-mtDNA in healthy subjects, making these results particularly noteworthy. Given the discrepancies between our results and those of Hummel *et al.*, we hypothesize that the changes observed in their study may be due to pre-analytical factors and differences in assay design. Previous studies have shown that ccf-mtDNA measurements are highly influenced by pre-analytical conditions such as centrifugation force and time [65], sample handling, storage conditions, platelet count and activation [17], all of which can affect ccf-mtDNA levels. In our study, we took steps to mitigate potential preanalytical influences by centrifuging the samples three times at room temperature, which may have minimized any impact from platelet activation. Furthermore, our assay was designed to avoid co-amplification of nuclear-embedded mitochondrial DNA sequences, so called NUMTs, a potential source of contamination noted in the methodology used by Hummel *et al.*, who employed primers that might have led to such amplification. As they showed an increase in cf-nDNA concentrations following acute stress exposure, it is possible that these mtDNA integrations were also released into the circulation, potentially contributing to the observed increase in ccf-mtDNA levels. One of the strengths of this study is that we included a cohort of healthy individuals as a control group, allowing us to reliably assess and compare ccf-mtDNA concentrations under physiological conditions. This provides a solid baseline and improves the interpretability of changes observed in patient samples. As previously shown in one of our studies, ccf-mtDNA concentrations correlate negatively with perceived chronic stress levels in healthy subjects [100]. These observations align with the findings in this study. However, when combining data from healthy subjects and PD patients, the correlation is less negative. This suggests that the mechanisms leading to ccf-mtDNA release might differ in healthy individuals and PD patients. We hypothesize that in PD, a chronic inflammatory environment might lead to altered mitochondrial function, which influences the relationship between stress and ccf-mtDNA release. Especially, in PD, factors such as neuroinflammation, autonomic dysregulation, and baseline mitochondrial dysfunction may modulate the response to stress in ways that differ from healthy individuals.

Specifically in the context of PD, we found no studies investigating the effects of acute stress on ccf-mtDNA kinetics. The primary symptom of PD is the fear of recurrent panic attacks, which themselves represent acute stress exposure. To simulate this stress response, we used the TSST to

induce acute stress in our study participants. We observed a significant decrease in ccf-mtDNA concentrations ten minutes after acute stress (Fig. 5). These findings highlight that PD may be associated with an altered immunological response compared to healthy subjects, as no such changes were observed in the control group. Furthermore, we hypothesize that PD patients may have developed compensatory mechanisms to cope with acute stress, potentially explaining the observed difference in their response to stress compared to healthy controls. Future studies should focus on replicating our findings in larger cohorts and include assessments of ccf-mtDNA to evaluate its long-term stability and potential as a reliable biomarker.

4.6 Conclusion

In this study, we found evidence of distinct patterns in ccf-mtDNA kinetics following acute stress in PD patients compared to healthy controls. Specifically, ccf-mtDNA concentrations were negatively correlated with perceived chronic stress. However, we did not observe significant differences in ccf-mtDNA baseline levels between the two study cohorts. Our results suggest that PD patients may develop compensatory mechanisms in response to prolonged psychological stress. This can be essential, as multiple cellular functions can be disrupted by chronic stress, and cellular adaptation and resilience can mitigate the negative effects of stress. Furthermore, the lack of significant differences between PD patients and HC may suggest that other biomarkers or physiological changes may be more indicative of stress or disease progression in PD. Future research should consider investigating different biomarkers, long-term stress effects, and the underlying mechanisms of stress adaptation in psychiatric disorders to better understand these complex interactions.

4.7 Declarations

4.7.1 Conflict of interest

The authors have declared that they have no conflicts of interest.

4.7.2 Data availability

All data generated and analyzed during this study is included in the electronic supplementary file.

4.7.3 Acknowledgements

The authors would like to thank M.Sc. Jakob Godde for his contribution to the experiments conducted during the preparation of his thesis.

5. Summary of the present thesis and integrated discussion

Liquid biopsy has emerged as a valuable tool in clinical practice, providing a minimally invasive approach to monitoring disease progression and response to treatment. Among its various applications, the quantification of cf-DNA has gained increasing attention due to its potential as a diagnostic and prognostic biomarker in various pathological conditions. This thesis consists of two chapters: one focused on assay development for ccf-mtDNA quantification and the other on its application in the context of stress research and stress-related diseases. The following sections summarize the main findings of this thesis, assessing the benefits and limitations of the methodologies employed. In addition, future research directions and potential improvements are discussed, addressing existing challenges and opportunities for further advances in the field.

5.1 Chapter I - Direct quantification of ccf-mtDNA in human blood plasma samples

The first step of this thesis was to develop, validate, and establish a direct qPCR-based assay for the detection and quantification of human mtDNA blood plasma samples, as presented in **Chapter I**. For the assay development, several recommended guidelines, including those for qPCR and dPCR, as well as FDA, CLSI and ISO standards, were implemented. To ensure methodological correctness, preanalytical aspects were incorporated by 1. using a multi-step centrifugation protocol, 2. comparing direct and isolated measurements, and 3. applying stringent primer design criteria. Particularly, primer design was critical to ensure the amplification of authentic mtDNA, avoiding targeting NUMTs and hypervariable regions.

As outlined in the introduction of the present thesis, NUMTs are paralogous fragments of mitochondrial origin which are integrated into the nuclear DNA of eukaryotic cells. Although NUMTs diverge and evolve differently from the genuine mtDNA sequences, with the first one evolving faster and under different functional constrains, new insertions can resemble mitochondrial

sequences — and, in case of recent integration, can be identical to the corresponding mitochondrial region. Importantly, the primers used in this thesis were carefully designed to avoid targeting 'conserved' NUMTs, that can be traced in human genomes, as confirmed by *in silico* analysis. However, the number of NUMT fragments is not fixed. Recent *in silico* studies of more than 66,000 human genomes (Genomics England Rare Disease Project) have shown – albeit not experimentally verified- that *de novo* insertions occur in the germline approximately once in every 10,000 births, and in somatic cells approximately once in every 10,000 cancers [107]. Consequently, the number and sequence of NUMTs can vary between individuals and populations with different ancestries [107]. For example, 14.2% of people carry ultra-rare NUMTs found in fewer than one in a thousand individuals [107]. Crucially, if such ultra- rare NUMTs were co-amplified during PCR they could be indistinguishable from mtDNA due to the recent integration event and absence of sequence differences. However, their overall contribution would still be negligible given the vastly higher copy number of mtDNA compared to nuclear pseudogenes. Overall, the impact of NUMTs on this study is expected to be minimal, as no fixed NUMTs were targeted. Taken together, these findings highlight the importance of constantly re-evaluating primer design criteria considering the emerging NUMT variability.

In line with these considerations, an amplicon size of 68 bp was chosen to minimize the risk of losing small DNA fragments. Furthermore, the implementation of a multi-step centrifugation protocol ensured targeting the small proportion of 'true authentic' ccf-mtDNA rather than membrane-bound particles such as platelet-associated DNA. Importantly, the results in **Chapter I** show that ccf-mtDNA levels decrease with increasing centrifugation force, supporting the notion that a substantial portion of mtDNA in plasma is associated with platelets or other membrane-bound particles. Taken together, the methodological considerations were carefully implemented to maximize the accuracy and efficiency of ccf-mtDNA quantification. Using the newly designed assay, we have shown that ccf-mtDNA can be detected and quantified directly and reliably in human blood plasma samples without the need for prior isolation. This approach offers significant advantages. Notably, it reduces sample processing time, making the method more time- and cost-efficient. Furthermore, using direct quantification the loss of DNA fragments during isolation steps can be minimized. In fact, the results of **Chapter I** demonstrate a significant loss of ccf-mtDNA during conventional isolation procedures,

further emphasizing the benefit of bypassing these steps**.** While the newly developed assay for ccf-mtDNA quantification offers significant advantages, certain limitations remain. Due to the relatively low physiological concentration of 'true authentic' ccf-mtDNA in blood plasma from healthy individuals, many samples fell outside the quantification range. Therefore, ccf-mtDNA's isolation and 'concentration' may still be required for more accurate measurements. Improving the sensitivity of the assay by expanding its dynamic range would enable lower copy numbers to be captured, reducing the need for pre-analytical enrichment and enhancing the overall performance of the assay. However, this would likely require the usage of PCR enhancers, which could further contribute to unknown variables. In addition, although targeting a 68 bp amplicon effectively captures small fragments, studies suggest that some circulating mtDNA fragments are smaller than 70 bp [46]. Consequently, fragments below this threshold may be underrepresented or lost during amplification. This should be considered for downstream analysis. However, with an amplicon size of just 68 bp, qPCR-based quantification techniques are approaching their technical lower limit. Further reducing the amplicon size would compromise primer specificity and amplification efficiency, increasing the risk of non-specific binding, reduced sensitivity and higher background noise. Despite these remaining challenges, the assay's specifications show that ccf-mtDNA can be studied directly in human blood plasma, providing opportunities to explore ccf-mtDNA concentrations in various pathological conditions in future studies. This might be particularly of interest in inflammatory diseases or mitochondrial associated disorders.

5.2 Chapter I and II - Cf-DNA in chronic and acute stress in health and disease

Stress is a psycho-physiological alarm response to external changes that occurs when an individual encounters a challenging situation which exceeds their capacity to cope with perceived threats or demands. Both acute and chronic stress can induce physiological changes, including heightened arousal, an elevated heart rate and alterations in GC synthesis and secretion.

The second section of **Chapter I** investigated how chronic stress affects ccf-mtDNA and cf-nDNA concentrations in healthy individuals. While cf-nDNA concentrations remained stable, ccf-mtDNA levels showed a negative correlation with perceived stress in the healthy study cohort. Previous research has predominantly focused on the effects of cf-nDNA kinetics in response to acute stress, consistently showing an increase in cf-nDNA concentrations after stress induction in healthy individuals [8,105]. However, to date, no studies have examined the effects *in vivo* of chronic stress on both ccf-mtDNA and cf-nDNA levels. These changes in cf-nDNA kinetics after acute stress induction are thought to be associated with the activation of immune cells and the subsequent release of nuclear DNA into the bloodstream [8]. The findings in **Chapter I** indicate that chronic or prolonged stress may engage different biological mechanisms to acute stress. This is reflected by the unchanged cf-nDNA levels observed in the study cohort. Contrary to expectation, which was based on the idea that long-term stress would increase ccf-mtDNA and cf-nDNA levels due to cumulative cellular stress or damage, the observed reduction in ccf-mtDNA concentrations suggests that mitochondrial DNA release is being suppressed. This may reflect stress-related mitochondrial dysfunction or reduced mitochondrial content.

In **Chapter II**, the applicability of ccf-mtDNA as a biomarker in stress research was further examined by comparing the ccf-mtDNA baseline concentrations of healthy individuals and PD patients. Although there has been a tendency towards lower concentrations in the PD cohort, no statistically significant difference has been observed. To investigate potential group differences in stress responsiveness further, the TSST was used to induce a standardized psychosocial stress response in both PD patients and healthy controls. This approach enabled the assessment of stress-induced changes in ccf-mtDNA kinetics and the evaluation of whether PD patients exhibit an altered mitochondrial response to acute stress. The results of **Chapter II** have revealed a significant reduction of ccf-mtDNA concentrations 10- minutes after stress induction in the PD group, whereas no such changes have been observed in the control cohort. However, the mechanisms leading to such a decrease remain unknown. While the simultaneous release of both ccf-mtDNA and cf-nDNA suggests cellular damage and death, the distinct release of ccf-mtDNA indicates that this process may be regulated rather than solely resulting from cellular injury. Therefore, the specific decrease of ccf-mtDNA in PD patients most likely points to regulated mechanism. Supporting this idea, Chang *et al.*

(2015) found a significant decrease in mtDNA copy number in the leukocytes of MD patients and significantly higher oxidative damage to mtDNA [108], which shows that psychiatric diseases can compromise mitochondrial content. Since MD can be a comorbidity in patients with PD [109], it is crucial to consider that the interplay between these psychiatric conditions may involve overlapping molecular and cellular mechanisms. Additionally, medications such as mood stabilizers or antipsychotic drugs are also known to alter mitochondrial functions, which could potentially influence mitochondrial content and, consequently, ccf-mtDNA concentrations [108]. However, the impact of these substances in the study was minimized by stringent exclusion criteria. Overall, the results indicate that mitochondrial alterations play crucial role in stress-research and highlighting the need to further investigations in future studies.

6. Concluding remarks and future perspectives

This thesis outlines two significant advances in the field of ccf-mtDNA research. **First**, it demonstrates that ccf-mtDNA can be reliably and directly measured in human blood plasma samples, providing a more robust and streamlined method for its detection and quantification. The newly developed assay addresses and overcomes serval limitations regarding contamination through cellular components, fragment loss through isolation processes and co-amplification of NUMTs.

Secondly, this thesis presents compelling evidence that ccf-mtDNA shows promise as a biomarker in stress research. It reveals distinct ccf-mtDNA patterns in response to chronic stress in healthy subjects, as well as after acute stress induction in PD patients. These results suggest that mitochondria and especially ccf-mtDNA may respond dynamically to psychological stressors and to prolonged stress exposure, possibly reflecting underlying mitochondrial stress signaling pathways. The newly designed assay provides a solid tool to further investigate the relationship between psychological stress and mitochondrial functionality. While these findings highlight the potential association between ccf-mtDNA and stress-research, certain limitations must be acknowledged. The sample sizes in both studies were relatively small to moderate, which limits the generalizability of the findings. Therefore, future research should prioritize replicating these results in larger, more diverse cohorts. Emphasis should be placed on ensuring adequate representation of diverse gender identities, age groups and other relevant demographic variables, to strengthen the validity and applicability of the observed patterns. Beyond stress-research, the direct assay opens new avenues for investigating its role in a broader range of conditions such as neurodegenerative diseases, metabolic syndromes, cardiovascular diseases and inflammatory states – all of which are known to be linked to altered mitochondrial functionality or compromised mitochondrial content.

Overall, this thesis establishes a basis for future research into the diagnostic, prognostic and disease monitoring potential of ccf-mtDNA, as well as its wider significance in different physiological and pathological conditions.

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Appendix

Supplementary Information

Table S1 qPCR measurements acquired from all HC and PD patient samples.

Study_ID	Health_status	Condition	Time_2	Sample_ID	Elution_volume	Cq	SD	copies/mL	copies/PCR	copies/PCR_ElutionfaktorFactor	copies_ml_ElutionFactor
1033	healthy	rest	-1	2	50	23,315	0,217	4972206,938	4972,206938	1243,051735	1243051,735
1033	healthy	rest	1	3	50	24,31	0,154	2571120,407	2571,120407	642,7801018	642780,1018
1033	healthy	rest	10	5	50	23,675	0,013	3916673,734	3916,673734	979,1684334	979168,4334
1033	healthy	stress	-1	13	50	26,32	0,173	678440,1376	678,4401376	169,6100344	169610,0344
1033	healthy	stress	1	14	50	26,335	0,083	671728,1397	671,7281397	167,9320349	167932,0349
1033	healthy	stress	10	16	50	20,97	0,209	23528553,27	23528,55327	5882,138318	5882138,318
1034	healthy	rest	-1	24	200	24,875	0,245	1767982,912	1767,982912	1767,982912	1767982,912
1034	healthy	rest	1	25	200	25,115	0,121	1507961,302	1507,961302	1507,961302	1507961,302
1034	healthy	rest	10	27	50	24,385	0,104	2446428,104	2446,428104	611,6070259	611607,0259
1034	healthy	stress	-1	35	200	28,435	0,189	166983,9423	166,9839423	166,9839423	166983,9423
1034	healthy	stress	1	36	200	27,125	0,064	397904,9251	397,9049251	397,9049251	397904,9251
1034	healthy	stress	10	38	50	24,945	0,159	1687825,078	1687,825078	421,9562694	421956,2694
9001	panic	rest	-1	46	50	27,295	0,135	355502,0226	355,5020226	88,87550565	88875,50565
9001	panic	rest	1	47	50	23,5	0,068	4398392,326	4398,392326	1099,598082	1099598,082
9001	panic	rest	10	49	200	28,265	0,32	186901,1394	186,9011394	186,9011394	186901,1394
9001	panic	stress	-1	57	50	28,925	0,252	120675,836	120,675836	30,168959	30168,959
9001	panic	stress	1	58	50	29,085	0,349	108532,9827	108,5329827	27,13324567	27133,24567
9001	panic	stress	10	60	50	28,485	0,046	161540,4933	161,5404933	40,38512331	40385,12331
9005	panic	stress	-1	68	200	28,565	0,355	153197,6544	153,1976544	153,1976544	153197,6544
9005	panic	stress	1	69	200	27,69323706	0,787028078	273025,4437	273,0254437	273,0254437	273025,4437
9005	panic	stress	10	71	50	26,34	0,07	669505,596	669,505596	167,376399	167376,399
9005	panic	rest	-1	79	200	28,65	0,165	144804,9795	144,8049795	144,8049795	144804,9795
9005	panic	rest	1	80	200	29,38	0,255	89256,78422	89,25678422	89,25678422	89256,78422
9005	panic	rest	10	82	50	26,535	0,309	588329,0862	588,3290862	147,0822716	147082,2716
9002	panic	stress	-1	90	50	22,92	0,039	6460353,891	6460,353891	1615,088473	1615088,473
9002	panic	stress	1	91	50	27,36	0,031	340510,712	340,510712	85,12767801	85127,67801
9002	panic	stress	10	93	50	27,60429176	0,332500708	289605,9368	289,6059368	72,40148419	72401,48419
9002	panic	rest	-1	101	50	28,445	0,119	165880,7725	165,8807725	41,47019311	41470,19311
9002	panic	rest	1	102	50	27,07	0,083	412678,6022	412,6786022	103,1696505	103169,6505
9002	panic	rest	10	104	50	26,0593283	0,053584846	806399,1899	806,3991899	201,5997975	201599,7975
9003	panic	stress	-1	112	50	26,675	0,136	536190,4375	536,1904375	134,0476094	134047,6094
9003	panic	stress	1	113	50	27,625	0,287	285657,9134	285,6579134	71,41447835	71414,47835
9003	panic	stress	10	115	50	27,06	0,082	415423,071	415,423071	103,8557677	103855,7677

9003	panic	rest	-1	123	50	26,770 98849	0,1497 52285	503138 ,1309	503,13 81309	125,7845327	125784,5327
9003	panic	rest	1	124	50	24,27	0,256	264020 1,417	2640,2 01417	660,0503543	660050,3543
9003	panic	rest	10	126	50	28,245	0,2	189395 ,3321	189,39 53321	47,34883304	47348,83304
9004	panic	rest	-1	134	50	27,89	0,392	239641 ,3405	239,64 13405	59,91033513	59910,33513
9004	panic	rest	1	135	50	26,515	0,187	596180 ,3286	596,18 03286	149,0450821	149045,0821
9004	panic	rest	10	137	50	29,615	0,005	76382, 29444	76,382 29444	19,09557361	19095,57361
9004	panic	stress	-1	145	50	28,745	0,319	135967 ,8455	135,96 78455	33,99196138	33991,96138
9004	panic	stress	1	146	50	27,295	0,343	355502 ,0226	355,50 20226	88,87550565	88875,50565
9004	panic	stress	10	148	50	28,43	1,002	167538 ,2754	167,53 82754	41,88456886	41884,56886
9006	panic	rest	-1	156	50	27,364 5204	0,0201 63338	339491 ,9727	339,49 19727	84,87299317	84872,99317
9006	panic	rest	1	157	50	26,211 33871	0,1442 39505	729107 ,2849	729,10 72849	182,2768212	182276,8212
9006	panic	rest	10	159	50	25,46	0,19	119971 0,303	1199,7 10303	299,9275757	299927,5757
9006	panic	stress	-1	167	50	27,49	0,185	312397 ,9566	312,39 79566	78,09948914	78099,48914
9006	panic	stress	1	168	50	27,94	0,254	231829 ,3592	231,82 93592	57,9573398	57957,3398
9006	panic	stress	10	170	50	29,7	0,583	72197, 81937	72,197 81937	18,04945484	18049,45484
9008	panic	rest	-1	178	200	29,06	0,04	110346 ,4564	110,34 64564	110,3464564	110346,4564
9008	panic	rest	1	179	200	28,53	0,255	156793 ,2744	156,79 32744	156,7932744	156793,2744
9008	panic	rest	10	181	50	25,77	0,154	976872 ,5233	976,87 25233	244,2181308	244218,1308
9008	panic	stress	-1	189	200	28,335	0,12	178427 ,3072	178,42 73072	178,4273072	178427,3072
9008	panic	stress	1	190	200	28,37	0,211	174335 ,5705	174,33 55705	174,3355705	174335,5705
9008	panic	stress	10	192	50	27,185	0,406	382390 ,7235	382,39 07235	95,59768088	95597,68088
1035	healthy	stress	-1	200	200	29,075	0,019	109254 ,7681	109,25 47681	109,2547681	109254,7681
1035	healthy	stress	1	201	200	28,424 16688	0,2017 79271	168187 ,2999	168,18 72999	168,1872999	168187,2999
1035	healthy	stress	10	203	50	26,59	0,018	567267 ,2142	567,26 72142	141,8168035	141816,8035
1035	healthy	rest	-1	211	200	29,19	0,122	101236 ,1911	101,23 61911	101,2361911	101236,1911
1035	healthy	rest	1	212	200	28,437 55463	0,0637 04985	166701 ,4274	166,70 14274	166,7014274	166701,4274
1035	healthy	rest	10	214	50	27,66	0,048	279107 ,1394	279,10 71394	69,77678486	69776,78486
9007	panic	stress	-1	222	200	27,374 46465	0,1094 40634	337261 ,6002	337,26 16002	337,2616002	337261,6002
9007	panic	stress	1	223	200	28,03	0,224	218403 ,9481	218,40 39481	218,4039481	218403,9481
9007	panic	rest	10	225	50	25,25	0,082	137888 5,713	1378,8 85713	344,7214282	344721,4282
9007	panic	rest	-1	233	200	28,195	0,264	195777 ,409	195,77 7409	195,777409	195777,409
9007	panic	rest	1	234	200	28,465 209	0,0501 13551	163673 ,5726	163,67 35726	163,6735726	163673,5726
9007	panic	stress	10	236	50	27,51	0,354	308283 ,9126	308,28 39126	77,07097816	77070,97816
9010	panic	rest	-1	244	200	26,32	0,174	678440 ,1376	678,44 01376	678,4401376	678440,1376
9010	panic	rest	1	245	200	27,84	0,028	247716 ,5631	247,71 65631	247,7165631	247716,5631
9010	panic	rest	10	247	50	27,04	0,043	420966 ,8853	420,96 68853	105,2417213	105241,7213
9010	panic	stress	-1	255	200	29,111 85243	0,0521 21619	106618 ,3198	106,61 83198	106,6183198	106618,3198
9010	panic	stress	1	256	200	28,825 10882	0,1512 13631	128936 ,4186	128,93 64186	128,9364186	128936,4186
9010	panic	stress	10	258	50	27,45	0,513	320791 ,4827	320,79 14827	80,19787068	80197,87068
9009	panic	rest	-1	266	200	29,055 98299	0,2050 00881	110640 ,6587	110,64 06587	110,6406587	110640,6587

9009	panic	rest	1	267	200	28,88	0,171	124329,5298	124,3295298	124,3295298	124329,5298
9009	panic	rest	10	269	50	26,815	0,149	488672,3977	488,6723977	122,1680994	122168,0994
9009	panic	stress	-1	277	200	29,16	0	103269,4301	103,2694301	103,2694301	103269,4301
9009	panic	stress	1	278	200	28	0,18	222790,3974	222,7903974	222,7903974	222790,3974
9009	panic	stress	10	280	50	28,545	0,077	155242,0747	155,2420747	38,81051866	38810,51866
1036	healthy	stress	-1	288	200	28,655	0,249	144325,8639	144,3258639	144,3258639	144325,8639
1036	healthy	stress	1	289	200	27,625	0,02	285657,9134	285,6579134	285,6579134	285657,9134
1036	healthy	stress	10	291	50	23,175	0,047	5455699,617	5455,699617	1363,924904	1363924,904
1036	healthy	rest	-1	299	200	29,205	0,101	100234,6331	100,2346331	100,2346331	100234,6331
1036	healthy	rest	1	300	200	29,34	0,042	91654,9406	91,654 9406	91,6549406	91654,9406
1036	healthy	rest	10	302	50	26,095	0,093	787555,9345	787,5559345	196,8889836	196888,9836
1037	healthy	stress	-1	310	200	28,09	0,4	209888,4393	209,8884393	209,8884393	209888,4393
1037	healthy	stress	1	311	200	28,315	0,04	180808,4168	180,8084168	180,8084168	180808,4168
1037	healthy	stress	10	313	50	25,515	0,033	1156761,305	1156,761305	289,1903262	289190,3262
1037	healthy	rest	-1	321	200	28,35	0,315	176662,076	176,662076	176,662076	176662,076
1037	healthy	rest	1	322	200	28,325	0,22	179613,9163	179,6139163	179,6139163	179613,9163
1037	healthy	rest	10	324	50	25,755	0,109	986633,565	986,63 3565	246,6583912	246658,3912
9013	panic	stress	-1	332	50	25,415	0,095	1236033,847	1236,033847	309,0084618	309008,4618
9013	panic	stress	1	333	50	23,955	0,122	3253230,869	3253,230869	813,3077173	813307,7173
9013	panic	stress	10	335	50	28,965	0,103	117518,3463	117,5183463	29,37958659	29379,58659
9013	panic	rest	-1	343	50	26,395	0,047	645537,6476	645,5376476	161,3844119	161384,4119
9013	panic	rest	1	344	50	25,33	0,057	1307672,476	1307,672476	326,9181191	326918,1191
9013	panic	rest	10	346	50	29,06	1,225	110346,4564	110,3464564	27,58661411	27586,61411
9014	panic	stress	-1	354	50	26,06	0,267	806040,2374	806,0402374	201,5100594	201510,0594
9014	panic	stress	1	355	50	24,065	0,04	3024472,308	3024,472308	756,118077	756118,077
9014	panic	stress	10	357	50	27,565	0,023	297247,5106	297,2475106	74,31187765	74311,87765
9014	panic	rest	-1	365	50	21,14	0,073	21021223,29	21021,22329	5255,305821	5255305,821
9014	panic	rest	1	366	50	24,685	0,188	2005268,926	2005,268926	501,3172316	501317,2316
9014	panic	rest	10	368	50	23,415	0,023	4653316,411	4653,316411	1163,329103	1163329,103
9012	panic	rest	-1	376	50	26,095	0,087	787555,9345	787,5559345	196,8889836	196888,9836
9012	panic	rest	1	377	50	24,65	0,01	2052333,518	2052,333518	513,0833794	513083,3794
9012	panic	rest	10	379	50	26,82	0,129	487055,5297	487,055297	121,7638824	121763,8824
9012	panic	stress	-1	387	50	25,065	0,18	1558775,252	1558,775252	389,693813	389693,813
9012	panic	stress	1	388	50	26,35792262	0,079298261	661599,084	661,599084	165,399771	165399,771
9012	panic	stress	10	390	50	27,775	0,036	258622,5223	258,6225223	64,65563058	64655,63058
9011	panic	stress	-1	398	50	25,945	0,062	869883,9642	869,8839642	217,4709911	217470,9911
9011	panic	stress	1	399	50	25,875	0,187	911196,3107	911,1963107	227,7990777	227799,0777
9011	panic	stress	10	401	50	27,19	0,014	381125,5092	381,1255092	95,28137729	95281,37729
9011	panic	rest	-1	409	50	26,215	0,138	727340,0049	727,3400049	181,8350012	181835,0012
9011	panic	rest	1	410	50	25,425	0,154	1227868,06	1227,86806	306,967015	306967,015

9011	panic	rest	10	412	50	27,16	0,125	388780,0765	388,7800765	97,19501912	97195,01912
9017	panic	rest	-1	420	50	25,49	0,127	1176089,588	1176,089588	294,022397	294022,397
9017	panic	rest	1	421	50	26,93	0,341	452807,0774	452,8070774	113,2017693	113201,7693
9017	panic	rest	10	423	50	26,08	0,299	795425,2994	795,4252994	198,8563248	198856,3248
9017	panic	stress	-1	431	50	27,645	0,252	281896,0155	281,8960155	70,47400388	70474,00388
9017	panic	stress	1	432	50	25,935	0,14	875669,0217	875,6690217	218,9172554	218917,2554
9017	panic	stress	10	434	50	21,915	0,011	12576556,6	12576,5566	3144,13915	3144139,15
9018	panic	stress	-1	464	50	25,775	0,134	973640,3498	973,6403498	243,4100875	243410,0875
9018	panic	stress	1	465	50	24,225	0,107	2720138,609	2720,138609	680,0346523	680034,6523
9018	panic	stress	10	467	50	26,84	0,145	480641,3781	480,6413781	120,1603445	120160,3445
9018	panic	rest	-1	475	50	26,34	0,055	669505,596	669,505596	167,376399	167376,399
9018	panic	rest	1	476	50	25,16	0,099	1463646,578	1463,646578	365,9116444	365911,6444
9018	panic	rest	10	478	50	26,175	0,022	746882,2177	746,8822177	186,7205544	186720,5544
9015	panic	stress	-1	486	50	25,2	0,081	1425350,187	1425,350187	356,3375469	356337,5469
9015	panic	stress	1	487	50	22,305	0,243	9711671,116	9711,671116	2427,917779	2427917,779
9015	panic	stress	10	489	50	26,975	0,344	439500,3561	439,5003561	109,875089	109875,089
9015	panic	rest	-1	497	50	22,55	0,1	8255944,593	8255,944593	2063,986148	2063986,148
9015	panic	rest	1	498	50	24,195	0,009	2774770,177	2774,770177	693,6925443	693692,5443
9015	panic	rest	10	500	50	27,15	0,398	391365,6111	391,3656111	97,84140277	97841,40277
9019	panic	stress	-1	508	50	24,285	0,107	2614081,166	2614,081166	653,5202916	653520,2916
9019	panic	stress	1	509	50	24,755	0,347	1914352,881	1914,352881	478,5882203	478588,2203
9019	panic	stress	10	511	50	28,56	0,18	153706,2214	153,7062214	38,42655535	38426,55535
9019	panic	rest	-1	519	50	26,22	0,016	724933,4585	724,9334585	181,2333646	181233,3646
9019	panic	rest	1	520	50	26,11	0,188	779764,4235	779,7644235	194,9411059	194941,1059
9019	panic	rest	10	522	50	27,135	0,024	395276,1889	395,2761889	98,81904723	98819,04723
1038	healthy	stress	-1	530	50	24,41002184	0,248849623	2406187,785	2406,187785	601,5469461	601546,9461
1038	healthy	stress	1	531	50	24,09203344	0,010707322	2970760,225	2970,760225	742,6900562	742690,0562
1038	healthy	stress	10	533	50	25,345	0,106	1294735,307	1294,735307	323,6838267	323683,8267
1038	healthy	rest	-1	541	50	25,18181194	0,180064131	1442637,784	1442,637784	360,6594459	360659,4459
1038	healthy	rest	1	542	50	24,82604471	0,29020808	1826293,748	1826,293748	456,573437	456573,437
1038	healthy	rest	10	544	50	24,715	0,078	1965787,824	1965,787824	491,446956	491446,956
1039	healthy	rest	-1	552	50	26,75135452	0,031673855	509728,8174	509,7288174	127,4322044	127432,2044
1039	healthy	rest	1	553	50	26,91106647	0,161489877	458525,536	458,525536	114,631384	114631,384
1039	healthy	rest	10	555	50	26,725	0,22	518711,3595	518,7113595	129,6778399	129677,8399
1039	healthy	stress	-1	563	50	26,82077048	0,262681435	486806,8531	486,8068531	121,7017133	121701,7133
1039	healthy	stress	1	564	50	26,30032323	0,253149304	687346,6321	687,3466321	171,836658	171836,658
1039	healthy	stress	10	566	50	26,86	0,116	474311,696	474,311696	118,577924	118577,924
9020	panic	rest	-1	574	50	24,4557895	0,090579401	2334288,647	2334,288647	583,5721618	583572,1618
9020	panic	rest	1	575	50	27,23196723	0,173700988	370669,6904	370,6696904	92,66742259	92667,42259
9020	panic	rest	10	577	50	25,89	0,281	902181,5909	902,1815909	225,5453977	225545,3977

9020	panic	stress	-1	585	50	26,341 90142	0,0168 09726	668662 ,3292	668,66 23292	167,1655823	167165,5823
9020	panic	stress	1	586	50	25,332 6423	0,0232 33733	130538 4,207	1305,3 84207	326,3460516	326346,0516
9020	panic	stress	10	588	50	26,08	0,213	795425 ,2994	795,42 52994	198,8563248	198856,3248
1040	healthy	stress	-1	596	50	25,855 0806	0,2258 78949	923306 ,8851	923,30 68851	230,8267213	230826,7213
1040	healthy	stress	1	597	50	25,816 74779	0,2022 0657	947067 ,19	947,06 719	236,7667975	236766,7975
1040	healthy	stress	10	599	50	26,145	0,019	761882 ,6837	761,88 26837	190,4706709	190470,6709
1040	healthy	rest	-1	607	50	25,442 57627	0,1847 14273	121364 6,172	1213,6 46172	303,4115429	303411,5429
1040	healthy	rest	1	608	50	25,850 4287	0,2127 54346	926158 ,2456	926,15 82456	231,5395614	231539,5614
1040	healthy	rest	10	610	50	25,15	0,013	147338 0,381	1473,3 80381	368,3450953	368345,0953
1041	healthy	stress	-1	618	50	26,948 77289	0,1615 51803	447207 ,5497	447,20 75497	111,8018874	111801,8874
1041	healthy	stress	1	619	50	27,441 09559	0,5512 43296	322690 ,4458	322,69 04458	80,67261144	80672,61144
1041	healthy	stress	10	621	50	27,396 79034	0,2611 92973	332307 ,457	332,30 7457	83,07686425	83076,86425
1041	healthy	rest	-1	629	50	27,082 83113	0,0442 84646	409183 ,6782	409,18 36782	102,2959195	102295,9195
1041	healthy	rest	1	630	50	27,036 50499	0,2892 20539	421943 ,2363	421,94 32363	105,4858091	105485,8091
1041	healthy	rest	10	632	50	26,995	0,191	433712 ,47	433,71 247	108,4281175	108428,1175
9021	panic	stress	-1	640	50	28,681 36533	0,4008 56227	141825 ,5512	141,82 55512	35,45638779	35456,38779
9021	panic	stress	1	641	50	27,310 776	0,0564 15522	351803 ,9404	351,80 39404	87,95098509	87950,98509
9021	panic	stress	10	643	50	27,445	0,209	321856 ,4075	321,85 64075	80,46410187	80464,10187
9021	panic	rest	-1	651	50	27,221 95873	0,2905 2719	373136 ,8855	373,13 68855	93,28422137	93284,22137
9021	panic	rest	1	652	50	27,235 61829	0,2070 27665	369773 ,7325	369,77 37325	92,44343312	92443,43312
9021	panic	rest	10	654	50	28,085	0,004	210585 ,2016	210,58 52016	52,64630039	52646,30039
9022	panic	rest	-1	662	50	28,098 47865	0,0654 9885	208712 ,1839	208,71 21839	52,17804597	52178,04597
9022	panic	rest	1	663	50	27,179 1878	0,0670 26913	383866 ,7393	383,86 67393	95,96668483	95966,68483
9022	panic	rest	10	665	50	26,19	0,181	739493 ,1032	739,49 31032	184,8732758	184873,2758
9022	panic	stress	-1	673	50	27,015 02735	0,2399 22269	427993 ,0467	427,99 30467	106,9982617	106998,2617
9022	panic	stress	1	674	50	26,927 43962	0,1337 84974	453576 ,1944	453,57 61944	113,3940486	113394,0486
9022	panic	stress	10	676	50	27,36	0,398	340510 ,712	340,51 0712	85,12767801	85127,67801
1042	healthy	stress	-1	684	50	26,001 2956	0,0205 05858	838022 ,5982	838,02 25982	209,5056495	209505,6495
1042	healthy	stress	1	685	50	26,054 73246	0,1575 56849	808859 ,4569	808,85 94569	202,2148642	202214,8642
1042	healthy	stress	10	687	50	26	0,367	838742 ,5756	838,74 25756	209,6856439	209685,6439
1042	healthy	rest	-1	695	50	26,688 62457	0,0134 41371	531369 ,9764	531,36 99764	132,8424941	132842,4941
1042	healthy	rest	1	696	50	25,397 44345	0,2368 88828	125050 1,734	1250,5 01734	312,6254335	312625,4335
1042	healthy	rest	10	698	50	25,565	0,078	111905 2,462	1119,0 52462	279,7631154	279763,1154
9023	panic	stress	-1	706	50	24,89	0,001	175049 1,763	1750,4 91763	437,6229406	437622,9406
9023	panic	stress	1	707	50	26,375	0,006	654152 ,3374	654,15 23374	163,5380843	163538,0843
9023	panic	stress	10	709	50	28,23	0,055	191287 ,7958	191,28 77958	47,82194894	47821,94894
9023	panic	rest	-1	717	50	26,905	0,002	460373 ,0147	460,37 30147	115,0932537	115093,2537
9023	panic	rest	1	718	50	26,17	0,085	749361 ,627	749,36 1627	187,3404067	187340,4067
9023	panic	rest	10	720	50	26,605	0,037	561655 ,0811	561,65 50811	140,4137703	140413,7703
9024	panic	rest	-1	728	50	27,13	0,336	396588 ,379	396,58 8379	99,14709475	99147,09475

9024	panic	rest	1	729	50	27,205	0,236	377354,928	377,354928	94,33873199	94338,73199
9024	panic	rest	10	731	50	28,26	0,185	187521,5912	187,5215912	46,88039779	46880,39779
9024	panic	stress	-1	739	50	28,16	0,158	200372,3956	200,3723956	50,0930989	50093,0989
9024	panic	stress	1	740	50	27,48	0,183	314475,5212	314,4755212	78,6188803	78618,8803
9024	panic	stress	10	742	50	28,475	0,074	162614,7987	162,6147987	40,65369966	40653,69966
9025	panic	stress	-1	750	50	25,785	0,312	967208,0504	967,2080504	241,8020126	241802,0126
9025	panic	stress	1	751	50	26,605	0,092	561655,0811	561,6550811	140,4137703	140413,7703
9025	panic	stress	10	753	50	28,08	0,128	211284,2768	211,2842768	52,82106921	52821,06921
9025	panic	rest	-1	761	50	26,56	0,265	578660,2723	578,6602723	144,6650681	144665,0681
9025	panic	rest	1	762	50	27,475	0,232	315519,479	315,519479	78,87986975	78879,86975
9025	panic	rest	10	764	50	27,355	0,174	341641,0983	341,6410983	85,41027457	85410,27457
9026	panic	rest	-1	772	50	26,9956258	0,100756802	433532,6034	433,5326034	108,3831508	108383,1508
9026	panic	rest	1	773	50	27,64038606	0,007534953	282759,4546	282,7594546	70,68986364	70689,86364
9026	panic	rest	10	775	50	26,925	0,074	454310,2515	454,3102515	113,5775629	113577,5629
9026	panic	stress	-1	783	50	26,74081707	0,375792816	513301,5315	513,3015315	128,3253829	128325,3829
9026	panic	stress	1	784	50	26,18848651	0,040020922	740235,3297	740,2353297	185,0588324	185058,8324
9026	panic	stress	10	786	50	24,435	0,18	2366677,88	2366,67788	591,6694699	591669,4699
9029	panic	stress	-1	794	50	26,98619398	0,185635905	436251,4305	436,2514305	109,0628576	109062,8576
9029	panic	stress	1	795	50	25,95254118	0,065614247	865546,6417	865,5466417	216,3866604	216386,6604
9029	panic	stress	10	797	50	27,87424797	0,278713615	242156,5468	242,1565468	60,5391367	60539,1367
9029	panic	rest	-1	805	50	27,04499466	0,186468013	419575,5182	419,5755182	104,8938796	104893,8796
9029	panic	rest	1	806	50	26,39548338	0,135062959	645330,8482	645,3308482	161,332712	161332,712
9029	panic	rest	10	808	50	25,99614762	0,007122692	840887,0376	840,8870376	210,2217594	210221,7594
1043	healthy	rest	-1	816	50	25,975	0,191	852757,0954	852,7570954	213,1892738	213189,2738
1043	healthy	rest	1	817	50	26,285	0,109	694363,4422	694,3634422	173,5908606	173590,8606
1043	healthy	stress	-1	827	50	26,03	0,17	822228,8397	822,2288397	205,5572099	205557,2099
1043	healthy	stress	1	828	50	25,34	0,197	1299033,413	1299,033413	324,7583531	324758,3531
1043	healthy	stress	10	830	50	25,435	0,222	1219756,22	1219,75622	304,9390549	304939,0549
1044	healthy	rest	-1	838	50	26,68866627	0,036141543	531355,2903	531,3552903	132,8388226	132838,8226
1044	healthy	rest	1	839	50	26,4	0,004	643401,7603	643,4017603	160,8504401	160850,4401
1044	healthy	rest	10	841	50	25,135	0,091	1488102,596	1488,102596	372,025649	372025,649
1044	healthy	stress	-1	849	50	25,875	0,12	911196,3107	911,1963107	227,7990777	227799,0777
1044	healthy	stress	1	850	50	25,625	0,059	1075420,919	1075,420919	268,8552299	268855,2299
1044	healthy	stress	10	852	50	25,52	0,275	1152933,934	1152,933934	288,2334835	288233,4835
9028	panic	rest	-1	860	50	26,36369922	0,070661267	659070,7064	659,0707064	164,7676766	164767,6766
9028	panic	rest	1	861	50	25,50169373	0,044860037	116700,8,925	1167,008925	291,7522312	291752,2312
9028	panic	rest	10	863	50	26,59764557	0,280128188	564399,7112	564,3997112	141,0999278	141099,9278
9028	panic	stress	-1	871	50	26,11882653	0,099759702	775215,6966	775,2156966	193,8039242	193803,9242
9028	panic	stress	1	872	50	26,46560874	0,101506985	616021,275	616,021275	154,0053188	154005,3188
9028	panic	stress	10	874	50	28,7	0,37	140084,5344	140,0845344	35,02113359	35021,13359

9027	panic	stress	-1	882	50	25,071 02116	0,0323 78626	155256 6,512	1552,5 66512	388,141628	388141,628
9027	panic	stress	1	883	50	26,104 86744	0,3523 10004	782421 ,7385	782,42 17385	195,6054346	195605,4346
9027	panic	stress	10	885	50	26,797 03244	0,2064 19417	494527 ,0592	494,52 70592	123,6317648	123631,7648
9027	panic	rest	-1	893	50	28,520 16335	0,1159 12898	157818 ,9202	157,81 89202	39,45473005	39454,73005
9027	panic	rest	1	894	50	27,025 87527	0,2213 3752	424926 ,6464	424,92 66464	106,2316616	106231,6616
9027	panic	rest	10	896	50	26,720 88033	0,0841 83989	520129 ,7244	520,12 97244	130,0324311	130032,4311
1045	healthy	rest	-1	904	50	25,185	0,044	143959 2,478	1439,5 92478	359,8981196	359898,1196
1045	healthy	rest	1	905	50	26,22	0,297	724933 ,4585	724,93 34585	181,2333646	181233,3646
1045	healthy	rest	10	907	50	25,115	0,123	150796 1,302	1507,9 61302	376,9903255	376990,3255
1045	healthy	stress	-1	915	50	25,065	0,134	155877 5,252	1558,7 75252	389,693813	389693,813
1045	healthy	stress	1	916	50	23,885	0,23	340773 2,626	3407,7 32626	851,9331566	851933,1566
1045	healthy	stress	10	918	50	22,005	0,065	118482 38,83	11848, 23883	2962,059707	2962059,707
1049	healthy	stress	-1	926	50	25,985	0,035	847911 ,0644	847,12 34042	211,7808511	211780,8511
1049	healthy	stress	1	927	50	22,145	0,275	108140 56,8	10798, 22927	2699,557318	2699557,318
1049	healthy	stress	10	929	50	27,085	0,102	408913 ,0635	408,59 58574	102,1489643	102148,9643
1049	healthy	rest	-1	937	50	25,46	0,187	120091 3,687	1199,7 10303	299,9275757	299927,5757
1049	healthy	rest	1	938	50	25,92	0,056	885249 ,1833	884,41 88244	221,1047061	221104,7061
1049	healthy	rest	10	940	50	28,12	0,097	205886 ,0399	205,75 60126	51,43900314	51439,00314
1046	healthy	stress	-1	948	50	29,445 81196	0,3311 87053	85446, 87882	85,446 87882	21,3617197	21361,7197
1046	healthy	stress	1	949	50	28,567 6717	0,2399 57784	152926 ,5968	152,92 65968	38,23164921	38231,64921
1046	healthy	stress	10	951	50	24,87	0,084	177385 2,048	1773,8 52048	443,4630119	443463,0119
1046	healthy	rest	-1	959	50	30,03	0,257	58013, 39267	58,013 39267	14,50334817	14503,34817
1046	healthy	rest	1	960	50	31,05	0,282	29505, 62589	29,505 62589	7,376406472	7376,406472
1046	healthy	rest	10	962	50	26,505	0,104	600145 ,1533	600,14 51533	150,0362883	150036,2883
9030	panic	rest	-1	970	50	25,925 81972	0,2153 64925	881013 ,7342	881,01 37342	220,2534335	220253,4335
9030	panic	rest	1	971	50	25,240 61059	0,1652 77279	138749 4,166	1387,4 94166	346,8735416	346873,5416
9030	panic	rest	10	973	50	26,244 85124	0,2518 45269	713089 ,9462	713,08 99462	178,2724866	178272,4866
9030	panic	stress	-1	981	50	26,34	0,129	669505 ,596	669,50 5596	167,376399	167376,399
9030	panic	stress	1	982	50	26,214 35039	0,2516 21358	727653 ,2521	727,65 32521	181,913313	181913,313
9030	panic	stress	10	984	50	25,497 11858	0,0543 43789	117055 3,34	1170,5 5334	292,6383351	292638,3351
1047	healthy	rest	-1	992	50	31,28	0,108	25333, 52517	25,333 52517	6,333381292	6333,381292
1047	healthy	rest	1	993	50	31,405	0,007	23319, 13101	23,319 13101	5,829782753	5829,782753
1047	healthy	rest	10	995	50	25,585	0,039	110431 5,39	1104,3 1539	276,0788475	276078,8475
1047	healthy	stress	-1	1003	50	31,14	0,239	27796, 93309	27,796 93309	6,949233272	6949,233272
1047	healthy	stress	1	1004	50	26,39	0,058	647680 ,6254	647,68 06254	161,9201564	161920,1564
1047	healthy	stress	10	1006	50	32,68	0,043	10015, 74068	10,015 74068	2,50393517	2503,93517
1048	healthy	rest	-1	1014	50	25,47	0,256	119297 8,257	1191,7 84485	297,9461213	297946,1213
1048	healthy	rest	1	1015	50	25,37	0,087	127475 0,507	1273,4 57157	318,3642892	318364,2892
1048	healthy	rest	10	1017	50	24,94	0,201	169524 9,612	1693,4 28115	423,3570288	423357,0288
1048	healthy	stress	-1	1025	50	24,01	0,153	314054 8,033	3136,7 66914	784,1917286	784191,7286

1048	healthy	stress	1	1026	50	23,015	0,341	607424 7,154	6066,0 92498	1516,523125	1516523,125
1048	healthy	stress	10	1028	50	25,26	0,025	137118 8,383	1369,7 76183	342,4440457	342444,0457
1050	healthy	rest	-1	1036	50	25,845	0,407	930379 ,2882	929,49 68793	232,3742198	232374,2198
1050	healthy	rest	1	1037	50	25,99	0,412	845104 ,996	844,32 05311	211,0801328	211080,1328
1050	healthy	rest	10	1039	50	26,2	0,184	735268 ,6891	734,60 76841	183,651921	183651,921
1050	healthy	stress	-1	1047	50	25,76	0,283	984314 ,3104	983,36 90952	245,8422738	245842,2738
1050	healthy	stress	1	1048	50	25,595	0,234	109809 9,507	1097,0 19793	274,2549484	274254,9484
1050	healthy	stress	10	1050	50	26,13	0,098	770195 ,4308	769,49 55179	192,3738795	192373,8795
9032	panic	rest	-1	1058	50	26,990 71642	0,3188 95875	434945 ,6659	434,94 56659	108,7364165	108736,4165
9032	panic	rest	1	1059	50	28,585 42705	0,0764 11683	151137 ,3694	151,13 73694	37,78434236	37784,34236
9032	panic	rest	10	1061	50	27,745 98306	0,0990 39063	263644 ,8775	263,64 48775	65,91121937	65911,21937
9032	panic	stress	-1	1069	50	26,175 55361	0,1255 97506	746608 ,1966	746,60 81966	186,6520492	186652,0492
9032	panic	stress	1	1070	50	26,735 31548	0,1454 62266	515176 ,7832	515,17 67832	128,7941958	128794,1958
9032	panic	stress	10	1072	50	27,63	0,066	284712 ,7584	284,71 27584	71,17818961	71178,18961
9031	panic	stress	-1	1080	50	27,930 01614	0,0470 12236	233368 ,6161	233,36 86161	58,34215401	58342,15401
9031	panic	stress	1	1081	50	27,092 97556	0,0568 78125	406441 ,5178	406,44 15178	101,6103795	101610,3795
9031	panic	stress	10	1083	50	28,07	0,23	212689 ,3972	212,68 93972	53,17234931	53172,34931
9031	panic	rest	-1	1091	50	27,476 57417	0,2483 87052	315190 ,4326	315,19 04326	78,79760814	78797,60814
9031	panic	rest	1	1092	50	28,343 48176	0,2353 49722	177427	177,42 7	44,35675	44356,75
9031	panic	rest	10	1094	50	28,35	0,113	176662 ,076	176,66 2076	44,16551899	44165,51899
9022. 2	panic	stress	-1	1102	50	26,216 40713	0,0586 34043	726661 ,9317	726,66 19317	181,6654829	181665,4829
9022. 2	panic	stress	1	1103	50	27,297 23701	0,0273 6143	354975 ,2848	354,97 52848	88,74382119	88743,82119
9022. 2	panic	stress	10	1105	50	27,75	0,227	262943 ,8368	262,94 38368	65,7359592	65735,9592
9022. 2	panic	rest	-1	1113	50	23,954 27721	0,0441 41043	325478 9,834	3254,7 89834	813,6974584	813697,4584
9022. 2	panic	rest	1	1114	50	25,717 79821	0,0225 52634	101126 5,101	1011,2 65101	252,8162752	252816,2752
9022. 2	panic	rest	10	1116	50	25,13	0,118	149304 2,619	1493,0 42619	373,2606548	373260,6548
1051	healthy	stress	-1	1124	50	25,69	0,34	103107 1,22	1030,0 71054	257,5177634	257517,7634
1051	healthy	stress	1	1125	50	26,29	0,395	692680 ,0403	692,06 60052	173,0165013	173016,5013
1051	healthy	rest	-1	1135	50	26,845	0,285	479439 ,0252	479,05 10823	119,7627706	119762,7706
1051	healthy	rest	1	1136	50	26,395	0,148	646100 ,9433	645,53 76476	161,3844119	161384,4119
1052	healthy	rest	-1	1146	50	26,265	0,238	704256 ,4608	703,62 97114	175,9074279	175907,4279
1052	healthy	rest	1	1147	50	26,33	0,239	674552 ,269	673,95 80615	168,4895154	168489,5154
1052	healthy	rest	10	1149	50	25,825	0,009	942797 ,8137	941,90 10005	235,4752501	235475,2501
1052	healthy	stress	-1	1157	50	27,105	0,078	403526 ,8638	403,21 49601	100,80374	100803,74
1052	healthy	stress	1	1158	50	28,05	0,1	215666 ,041	215,52 77339	53,88193347	53881,93347
1052	healthy	stress	10	1160	50	25,74	0,287	997452 ,7503	996,49 21403	249,1230351	249123,0351
1053	healthy	stress	-1	1168	50	26,235	0,017	718403 ,8307	717,76 14892	179,4403723	179440,3723
1053	healthy	stress	1	1169	50	26,125	0,163	772752 ,7711	772,04 99961	193,012499	193012,499
1053	healthy	stress	10	1171	50	26,11	0,118	780475 ,8527	779,76 44235	194,9411059	194941,1059
1053	healthy	rest	-1	1179	50	26,375	0,219	654724 ,9756	654,15 23374	163,5380843	163538,0843

1053	healthy	rest	1	1180	50	21,7	0,112	14524995,49	14502,83692	3625,70923	3625709,23
1053	healthy	rest	10	1182	50	26,32	0,085	679039,2434	678,4401376	169,6100344	169610,0344
1054	healthy	rest	-1	1190	50	22,955	0,081	6320741,918	6312,203547	1578,050887	1578050,887
1054	healthy	rest	1	1191	50	20,795	0,219	26466080,33	26422,37143	6605,592858	6605592,858
1054	healthy	rest	10	1193	50	22,76	0,042	7193061,196	7183,149188	1795,787297	1795787,297
1054	healthy	stress	-1	1201	50	25,785	0,275	968134,3575	967,2080504	241,8020126	241802,0126
1054	healthy	stress	1	1202	50	26,11	0,18	780475,8527	779,7644235	194,9411059	194941,1059
1054	healthy	stress	10	1204	50	24,625	0,216	2088962,045	2086,625885	521,6564713	521656,4713

Table S2 TICS-9 score of HC and PD patients

Study_ID	TICS-9 Score	Health_Status	sex
1033	5	healthy	2
1034	1	healthy	1
1035	18	healthy	2
1036	20	healthy	2
1037	10	healthy	2
1038	15	healthy	2
1039	12	healthy	2
1040	4	healthy	2
1041	11	healthy	1
1042	6	healthy	2
1043	9	healthy	2
1044	12	healthy	2
1045	5	healthy	1
1046	12	healthy	2
1047	0	healthy	2
1048	10	healthy	2
1049	9	healthy	2
1050	7	healthy	2
1051	26	healthy	2
1052	6	healthy	2
1053	13	healthy	2
1054	2	healthy	2
9001	21	panic	2
9002	12	panic	2
9003	15	panic	2
9004	7	panic	2
9005	10	panic	1
9006	20	panic	2
9007	10	panic	2
9008	15	panic	2
9009	22	panic	1
9010	17	panic	2

9011	11	panic	2
9012	23	panic	1
9013	14	panic	1
9014	15	panic	1
9015	12	panic	1
9017	18	panic	1
9018	22	panic	2
9019	6	panic	2
9020	17	panic	1
9021	16	panic	2
9022	13	panic	2
9023	20	panic	2
9024	15	panic	1
9025	20	panic	1
9026	10	panic	2
9027	12	panic	2
9028	20	panic	2
9029	14	panic	2
9030	26	panic	1
9031	28	panic	2
9032	21	panic	1

Curriculum vitae

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Education

Master of Science Anthropology | 2019-2022
Institute of Organismic and Molecular Evolution, Anthropology
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Bachelor of Science Biology | 2016-2019
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High School Diploma Gauß Gymnasium Worms | 2006-2015

Professional Experience

PhD Student Biology (Dr. rer. nat) | October 2022 – May 2025
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Publications

Daubermann C, Herhaus B, Neuberger EWI, Simon P, Petrowski K. (2025) Methodological influences on circulating cell-free-mitochondrial and nuclear DNA concentrations in response to chronic stress. Mol Biol Rep. 2025 Mar 13;52(1):303. doi: 10.1007/s11033-025-10369-7

Conference submissions

Poster Presentation „Direct Quantification of Circulating Cell Free Mitochondrial DNA in Human Blood Plasma without DNA Extraction" (CNAPS, 2024)