Master Thesis

Correlation between mitochondrial DNA copy number and the Waist/Hip-Ratio in females

Establishing mitochondrial DNA copy number measured with qPCR as a potential biomarker for female health

submitted by

Tabea Ettinger, BSc

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Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

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Zusammenfassung

Korrelation zwischen der Kopienzahl mitochondrialer DNA und Waist/Hip-Ratio in weiblichen Probanden

Als das Kraftwerk der Zelle sind Mitochondrien weitläufig bekannt. Doch diese Zellorganellen haben noch viel mehr zu bieten. Sie besitzen ihre eigene DNA, deren kodierende Proteine eine wichtige Rolle in der oxidativen Phosphorylierung spielen. Vor allem die Kopienzahl dieser DNA rückt immer mehr in den Fokus biomedizinscher Forschung. Hier geht es allem voran um die Frage, welchen Einfluss die DNA-Menge auf das Altern und den Erhalt der Gesundheit im menschlichen Körper hat.

Die vorliegende Studie hatte zum Ziel, einen Zusammenhang zwischen der Kopienzahl mitochondrialer DNA in Leukozyten und körperlicher Fitness des Menschen zu finden.

Für dieses Projekt wurde ein Set an Blutproben verwendet, das bereits aus einer früheren Studie von Hassler et al. stammt. Ein mitochondriales Gen sowie ein Gen der Kern-DNA wurden ausgewählt und mithilfe der qPCR quantifiziert. Das mitochondriale Gen MT-ND1 und das chromosomale Gen RPPH1 wurden mit der delta-Ct-Methode in Relation gebracht. Von der vorangegangenen Studie waren Messungen verschiedener Parameter vorhanden, die zur weiteren Analyse zur Verfügung standen. Der Datensatz inkludierte Angaben zu Fitnessverhalten, Ernährungsverhalten sowie Messungen verschiedener Muskel– und Fettverteilungswerte und einige Blutwerte. Nach Zusammenfügen der beiden Datensätze wurde eine statistische Auswertung durchgeführt.

Die bivariate Korrelationsanalyse nach Aufteilung der Proben nach Geschlecht zeigte einen statistisch signifikanten Zusammenhang zwischen der mitochondrialen DNA-Ratio und der Waist/Hip-Ratio in Frauen. Der Fund deutet darauf hin, dass eine schmale Taille in Relation zum Hüftumfang mit einer höheren Anzahl an DNA-Kopien in den Mitochondrien zusammenhängt. Um die Beziehung dieser beiden Parameter zu bestätigen, sind weitere Studien notwendig.

Abstract

Correlation between mitochondrial DNA copy number and the Waist/Hip-Ratio in females

Mitochondria are widely known as the powerhouse of the cell. But these cell organelles have much more to offer. They possess their own genome, which encoded proteins play an important role in oxidative phosphorylation. Above all, the copy number of this DNA is increasingly becoming the focus of biomedical research. The main question concentrates on the influence DNA quantity has on aging and the maintenance of health in the human body.

The present study aimed to find a correlation between the copy number of mitochondrial DNA in leukocytes and physical fitness in humans.

For this project, we used a set of blood samples from a previous study by Hassler et al. One mitochondrial gene and one nuclear gene were selected and quantified by qPCR. The mitochondrial gene MT-ND1 and the chromosomal gene RPPH1 were put into relation using the delta Ct method. Measurements of several variables were available for further analysis due to the previous study. The data set included information on fitness and dietary behavior, measurements of various muscle and fat distribution values as well as a variety of blood biomarkers. After merging the two data sets, a statistical analysis was performed.

After stratification of the sample set into two groups by sex, the bivariate correlation analysis showed a statistically significant association between mitochondrial DNA Ratio and Waist/Hip-Ratio in all females. The finding suggests that a narrow waist in relation to hip circumference can be associated with a higher number of DNA copies in mitochondria. Further studies are needed to confirm the relationship between these two variables.

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List of abbreviations

ADP	adenine diphosphate
ATP	adenine triphosphate
BMI	Body Mass Index
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine-triphosphate
dGTP	deoxyguanosine-triphosphate
dTTP	deoxythymidine-triphosphate
FISH	Fluorescence in situ hybridization
H-strand	heavy strand
LCPUFA	long-chain polyunsaturated fatty acid
LDL	low-density-lipoprotein
LHON	Leber’s hereditary optic neuropathy
L-strand	light strand
MDS	mitochondrial DNA depletion syndromes
MRI	magnetic resonance imaging
mtDNA-CN	mitochondrial DNA copy number
NGS	next generation sequencing
NUMT	nuclear-mitochondrial DNA
PCOS	polycystic ovary syndrome
POLRMT	mitochondrial RNA polymerase
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
SSB protein	single-stranded binding proteins

TAS	termination associated sequence
UMM	universal master mix
VDAC	voltage-dependent anion-selective channels
WES	whole exome sequencing
WHR	Waist/Hip-Ratio
WGS	whole genome sequencing

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1 Introduction

Research on mitochondria and their function is an active area of biomedical science as it offers insights into energy production, aging and various disease mechanisms.

1.1 Mitochondria

As the main energy producer of the cell through respiration, mitochondria are also known as the powerhouse of the cell and therefore almost indispensable in eukaryotic organisms. However, these organelles are involved in the regulation of many cellular processes like apoptosis as well as the metabolism of carbohydrates, lipids and amino acids. Even the calcium levels in the cell are managed by absorbing and releasing calcium ions as needed. The tubular cell organelles are approximately the same dimension as bacteria with a size of two to five µm. Mitochondria are surrounded by a double membrane. While the outer membrane surrounds the organelle, the inner membrane is strongly folded and forms cristae. Authorizing small molecules to diffuse through the outer membrane, mitochondrial porins serve as transportation canals. Additionally, these voltage-dependent anion-selective channels (VDAC) play an important role in complex regulatory processes for metabolism of the organelle as well as the whole cell. The components of the electron transport chain are located within the inner membrane of the mitochondrion. These create a proton gradient through oxidation and reduction reactions that is further processes by ATP synthase to convert adenosine diphosphate (ADP) into adenosine triphosphate (ATP) through phosphorylation. The space inside the inner membrane is called the matrix, containing 2/3 of all mitochondrial proteins and playing an important role in ATP production. Mitochondria even contain their own ribosomes attached to the inner membrane and responsible for protein synthesis. These mitoribosomes differ from cellular ribosomes regarding structure and function as well as possession of mitochondrial-specific proteins. Mitochondria can vary in shape and size and are dynamic and mobile. They possess their own circular genome, which undergoes replication independently from the nuclear genome. (1–3) A schematic representation of mitochondrial structure is shown in Figure 1.

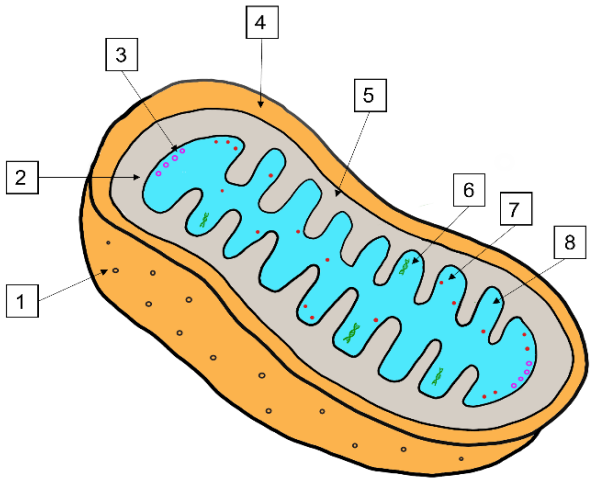

Figure 1: Schematic representation of a mitochondrion: 1: porins serve as transport channels for small molecules; 2: inner membrane; 3: ATP – synthesis sites are located at the inner membrane; 4: outer membrane serves as a protective layer; 5: intermembrane space; 6: mitochondrial DNA (as described in chapter 0 mitochondrial DNA is circular; the simplified illustration as a double helix strand serves the clear recognition of this structure and does not match its actual shape); 7: mitoribosomes are responsible for mitochondrial – specific protein synthesis; 8: matrix contains 2/3 of all mitochondrial proteins. This image was created with a Gaomon Graphics Tablet.

1.2 Mitochondrial DNA

The genome of mitochondria is a circular, intron free, haploid, double stranded DNA that is inherited maternally. The structure is composed of a strand rich in guanine, known as the heavy (H) strand and another strand rich in cytosine, called the light (L) strand. The 16,5kb contain 37 genes which encode for 13 proteins, mainly essential for the oxidative phosphorylation complex, as well as two rRNAs and 22 tRNAs involved in mitochondrial translation of these 13 polypeptides. This means that the majority of mitochondrial genes are non-protein coding. Proteins encoded from the mitochondrial genome also influence the expression of many nuclear genes for metabolism, growth, differentiation and apoptosis. (4) The mitochondrial gene products contain seven subunits of complex 1, one subunit of complex 3, three subunits of complex 4 and two subunits of complex 5, all relevant for generating ATP. However, the nuclear genome encodes most mitochondrial proteins and imports them into mitochondria via translocation systems in the inner and outer membrane. (5)

Mitochondrial DNA is up to 20 times more prone to mutations than nuclear DNA. This higher mutation rate can be attributed to various factors such as the absence of introns or histones in the mitochondrial genome, the asymmetrical division pattern during replication and its close proximity to reactive oxygen species (ROS) sites. ROS is generated as a by-product during oxidative phosphorylation which – if present in excessive amounts – can damage proteins, lipids and nucleic acids possibly causing neurological diseases like Huntington's disease or Parkinson's disease to worsen. (1,4) Another factor is that unlike the available repair pathways in the nucleus, repair of mitochondrial DNA is limited. While mitochondria in mammals provide a resilient base excision repair pathway for repairing oxidative damage and base deamination, they lack nucleotide excision repair, ribonucleotide excision repair, mismatch repair and end-joining pathways – all critical for repairing double-strand breaks. The consequences of these absent repair mechanisms can be managed due to the mitochondria's ability to replicate independently from the cell cycle. (6)

When additional copies of mitochondrial DNA are synthesized, the damage is reduced because the majority of mutations in the mitochondrial genome are recessive. This means that they only lead to defects in the oxidative phosphorylation if the number of effected copies reaches a certain threshold. Depending on the type of mutation this threshold can be as high as 90%. With this mechanism mutations mostly occur only concerning a small part of these copies, allowing mitochondria to maintain normal respiratory-chain function. This presence of different genomes in one cell is called heteroplasmy. This proportion can shift through mitotic segregation and random distribution to daughter cells. Also, in high proliferating tissues, deliberate elimination of specific forms of mutations was detected. This happens through negative selection. There is another mechanism influencing the heteroplasmy levels of mitochondrial DNA. During germline development in highly heteroplasmic cells the absolute copy number of mitochondrial DNA is reduced to a small fraction leading to daughter cells with highly varying heteroplasmy levels. Primordial germ cells contain the lowest amount of DNA before it is increased by replicative segregation. This is called genetic bottleneck and gives a possible explanation for the drastic differences in allele frequency seen between two generations. However, the bottleneck mechanism could occur in other cell lines as

well, leading to the high variety of the number of mutated alleles seen in some individuals. (7)

Having an effect on the respiratory chain, mutations of mitochondrial DNA are associated with age-related human diseases and the aging process in general. (8)

1.3 Inheritance

As early as 1980, human mitochondrial DNA was found to be inherited purely maternally, even though evidence for this non – Mendelian form of inheritance of mitochondrial DNA in other species had existed even years before that. In this study Giles et al. isolated platelets from peripheral blood collected from different families consisting of several members. Then DNA was extracted and restriction endonuclease digestions were performed. A horizontal agarose slab gel was used to make the restriction endonuclease fragments visible. Whenever a notable disparity between the parents' cleavage patterns was detected, the offspring inherited the maternal cleavage. The biggest family with informative results provided 33 family members extending over three generations. These findings prove that human mitochondrial DNA is inherited uniparentally by the mother. (9)

During the process of fertilization, the paternal mitochondria including their DNA get passed into the cytoplasm of the oocyte alongside the nuclear genome of the sperm. However, the paternal mitochondrial DNA is undetectable after a certain point in the further development of the blastocyst. There have been many studies trying to reveal the exact mechanism of this loss of the paternal proportion in different species. Currently, two hypotheses are competing to describe the mechanism responsible for this. The simple dilution model suggests that the mitochondrial DNA of the father is not actively eliminated but rather diluted with the mother's until its presence is no longer detectable and seen as clinically irrelevant. Conversely, the active degradation model proposes a deliberate breakdown of the DNA in question. Depending on the species, this degradation happens at different stages of embryogenesis, some even before fertilization. In mammals, these studies suggest a labeling of the paternal mitochondria with ubiquitin. There was evidence proposing a ubiquitination of the sperm mitochondria long before fertilization is happening, when the sperm is still present in the male reproductive tract. Regardless of the time

of marking ubiquitin serves as a signal for autophagy resulting in degradation of mitochondrial DNA by proteasomes and lysosomes. Although no obvious ubiquitination was detected in any other species, degradation in *C. elegans* was found to be mediated by autophagy as well. During this process, the involved membranes isolate different cell components presenting them to lysosomes for degradation. (10)

Since the establishment of this dogma there has only been one publication claiming to have found evidence for the existence of biparental inheritance of mitochondrial DNA in humans. When Luo et al. found an uncommonly high amount of heteroplasmy in mitochondrial DNA variants – detected by long-range PCR and next-generation-sequencing – this discovery led them to consider this the result of biparental inheritance. (11) As this proposition challenged the common believe of purely maternal inheritance of mitochondrial DNA, many research groups tried to replicate these findings soon after. However, the hypothesis that paternal DNA participates in the inheritance of mitochondrial DNA was quickly refuted. Rius et al. studied genome sequencing data of DNA extracted from patients with potential mitochondrial diseases a year after the controversial study had been published. They only found variants to be either of de novo occurrence or passed on by the mother. This concluded the transmission of paternal mitochondrial DNA as unlikely. (12) Another research group proposed a possible explanation for this false believe. They suggested that the detected segments of mitochondrial DNA were actually NUMTs, short for nuclear-mitochondrial DNA. This term stands for segments of mitochondrial DNA embedded in the nuclear genome. Usually, these NUMTs consist of only one copy and are therefore present at very low and undetectable levels. In this case the sequence probably involved a series of interlinked repeats of mitochondrial DNA resulting in higher levels. (13)

Luo et al. never disputed the non – Mendelian maternal heredity of mitochondrial DNA as they clearly stated paternal transmission as a rare occurrence in individual cases in need of further assessment. A proposition of this proportion can lead to re-evaluation of the current examinations and treatments concerning mitochondrial diseases. (11)

1.4 Transcription and Replication

There are three transcription sites in mitochondrial DNA, two located on the H-strand (HSP1 and HSP2) and one located on the L-strand (LSP). At each of these locations, one of the three primary transcripts is generated. For further process of these transcripts into RNAs they undergo cleavage, polyadenylation and tRNA – as well as – rRNA modifications. Transcription is managed by the mitochondrial RNA polymerase (POLRMT), the transcription factors TFAM and TF2BM, an elongation factor (TEFM) and a transcription termination factor (MTERF1) as well as many proteins relevant for transcription of nuclear genes. As described in the previous chapter, these originate from the nuclear genome. The frequency of transcript generation is – among other factors – dependent on ATP levels in each cell. (5)

Another difference between the nuclear and mitochondrial genome is that mitochondrial replication is happening regardless of the cell cycle. Even post mitotic cells can replicate independently through relaxed replication. Replication of mitochondrial DNA in mammals can be divided into two categories of mediator. One form of replication is in many features similar to conventional synthesis, in which leading- and lagging-strands are synthesized in parallel. The other form differs in the absence of synchronous DNA synthesis of the lagging-strand. There are two competing theories as to how this can occur with a time delay after the synthesis of the leading strand. The template for the lagging-strand is provided either by encapsulation with proteins or by systematic hybridization with processed mitochondrial transcripts until DNA synthesis of the lagging-strand occurs. This asynchronous type of synthesis underlies the so-called strand-displacement-mechanism. (14)

The major differences to the replication of the nuclear genome are that no special proteins are required for the recognition of the origins and that no Okazaki fragments are formed. Three important enzymes involved in mitochondrial replication are DNA polymerase gamma, helicase TWINKLE and POLRMT.

One important step in the beginning of mitochondrial DNA replication is RNA processing of the L-strand transcripts. From these transcripts primers for the initiation of DNA synthesis are generated. They originate from the region between

the promoter LSP up to nucleotide 191 in the non-coding region of human mitochondrial DNA identified as the origin of replication OriH. (15)

Explained more precisely, the promoter LSP recruits POLRMT to transcribe toward OriH. The formed RNA binds to the template, resulting in the formation of an RNA-DNA hybrid. The G-rich sequence CSB2 on the H-strand can form four-stranded structures with the also G-rich RNA strand. This G-quadruplex is also called R-loop and can cause polymerase to stall leading to termination of transcription. RNase H1 is present to solve this problem by degrading the RNA structure. This degradation happens with the exception of the RNA portion involved in the R-loop, since this part is four-stranded, but RNase can only destroy single strands. The single stranded H-strand is protected from degradation by mitochondrial single strand binding (mtSSB) proteins. These proteins then attract TWINKLE and DNA-polymerase to the ssDNA, although it is unclear how this recruitment happens. The DNA-polymerase binds to the 3'-end of the RNA strand and continues elongation.

95% of the time, after approximately 650 nucleotides, polymerase falls off and replication stops due to the termination associated sequence (TAS). The generated DNA strand is referred to as 7S DNA. The "S" stands for Svedberg, a unit used in ultracentrifugation to measure the rate of sedimentation of biomolecules. This sequence is tightly bound to the L-strand leaving the complementary sequence on the H-strand left out. This part of the H-strand is called the displacement-loop (D-loop). There is a sequence shortly upstream of CSB1 that matches a sequence of TAS. How these facts play together to form the D-loop and terminate replication is still unclear, just like the general function of the loop. The whole process could serve the regulation of copy numbers generated per mitochondria.

If this regulatory termination does not occur and OriL is reached, the replication from OriL is initiated by the formation of a stem loop structure. When the origin on the L-strand is unwound by TWINKLE, the ssDNA forms a loop structure with a stem of 11 nucleotides and the actual loop of 12 nucleotides. MtSSB proteins occupy all ssDNA except for the loop structure as the enzymes need at least 15-20 nucleotides to bind. POLRMT – again recruited by the binding proteins – links to the single-stranded loop structure. During transcription the stem of the loop structure is unwound by the enzyme. The generated RNA sequence stays tightly bound to the

DNA strand. Since the complementary strand is involved in replication, the loop structure serves as a single-stranded template for transcription, making the process unstable. Usually, the polymerase stays in contact with the complementary strand even after unwinding the DNA, stabilizing the enzyme's activity. This instability results in an aborted transcription after around 25 nucleotides. Here again, DNA polymerase is attracted by mtSSB proteins, causing elongation from the 3'-end of the RNA sequence away from the OriL. Helicase and polymerase are now moving in opposite directions, making movement of polymerase independent of helicase movement.

When the enzyme reaches the region of origin, termination of replication is initiated. This happens through 1-3 nucleotides left out by RNase H1 earlier. Polymerase is moving forward resulting the formation of an RNA/DNA flap which then causes the enzyme to stop. A nuclease like FEN1 or EXOG – although to date unclear which one exactly – then removes the flap which allows DNA-ligase 3 to connect the pieces.

The OriH termination differs slightly. Reaching the G-quadruplex TWINKLE unwinds this structure, allowing RNase H1 to degrade the RNA. This degradation leaves small parts of RNA behind. A flap is also formed on this strand, although this happens even before DNA polymerase arrives at the 5'-end of the RNA strand. The flap is approximately 191 nucleotides long, which corresponds exactly to the length to the presumed position of OriH. Endonuclease MGME1 digests the flap to ensure that polymerase can continue elongation up to the 5'-end of the RNA strand and then terminate replication. Here, too, DNA-ligase 3 links the two separate sequences. The completed molecules are still entangled in a chain-like structure. For division by double-strand break the enzyme topoisomerase alpha is on site, resulting in two individual mitochondrial DNA molecules. (5,14,15)

1.5 Mitochondrial DNA copy number

In contrast to the nuclear genome, which in healthy individuals only consists of two copies of each gene per cell, the mitochondrial genome possesses 100 up to 10 000 copies per cell, causing a relatively high gene density. The mitochondrial DNA copy number depends on the amount of ATP required by each cell type as generating

ATP through oxidative phosphorylation is one of the main tasks of mitochondria. Therefore, the more ATP is needed the more copy numbers are present. There is variation in the number of mitochondria per cell as well as DNA copies per mitochondrion.

The previous mentioned transcription factor TFAM plays a crucial role in controlling mitochondrial DNA copy number. This has been determined by several studies, showing the significant influence of a TFAM-containing region on chromosome 10 on mitochondrial DNA levels. When TFAM is disrupted in a heterozygous state, this leads to a reduction of about 50% of mitochondrial DNA. On the other hand, mild TFAM overexpression results in an increase of 50% or more in the amount of mitochondrial DNA. (8)

A close relation of energy reserves, oxidative stress and changes in mitochondrial membrane potential to the level of mitochondrial DNA copy number can be seen when the copy number is reduced. This decline results in decreased expression of vital protein complexes, altered cell appearance and decreased activity of respiratory enzymes. (4)

In addition to the mutation rate, absolute copy number of mitochondrial DNA is also thought to have an impact on age-related diseases. There have been several studies attempting to find a correlation between the mitochondrial DNA copy number and the aging process or several neurodegenerative diseases. A study by Mengel-From et al. conducted in the year 2014 measured relative mitochondrial DNA copy number in comparison to nuclear DNA by quantitative PCR. The results showed a significant yearly decline in mitochondrial DNA copy number in participants older than 50 years. Additionally, in terms of parameters like cognitive skills and physical strength participants with higher mitochondrial DNA copy number performed better than those with low mitochondrial DNA copy number. (16)

Autosomal recessive diseases, in which mutations in specific genes result in a profound tissue-specific decrease in the amount of mitochondrial DNA, are called mitochondrial DNA depletion syndromes (MDS). The affected genes all play an important role in preservation processes of mitochondrial DNA like nucleotide metabolism, replication of mitochondrial DNA and assurance of quality of the DNA molecules directly linking decreased amount of DNA to pathological maladies.

Studies found that mitochondrial DNA copy number can also have an influence on the penetrance of a disease. In patients with Leber's hereditary optic neuropathy (LHON), those with limited eyesight showed lower amount of absolute mitochondrial DNA than the mutation-carriers without any signs of symptoms. (8)

1.6 Waist/Hip-Ratio

This quotient is used to indicate the ratio between waist and hip circumference. The value is calculated by dividing the measurement of the waist with that of the hip. Therefore, the greater the difference between the waist circumference and the hip circumference the smaller the WHR. Due to the natural difference of fat distribution in men and women, this parameter ranges vary greatly depending on sex. The explanation for this sexually dimorphic body fat distribution is that fat storage is coordinated by sex hormones. Estrogen – the female sex hormone – suppresses the accumulation of fat in the abdominal area but promotes its deposition in buttocks, thighs and hips while the male sex hormone testosterone does exactly the opposite by inhibiting fat deposition in the lower part of the body while stimulating its storage in the abdominal area. Low estrogen levels in women as seen in patients suffering from polycystic ovary syndrome (PCOS) lead to a shift in fat distribution towards the male range resulting in higher WHR compared to age-matched control subjects. The same effect appears in menopausal women due to a decrease in estrogen production. (17)

Especially in women, this parameter is highly associated with attractiveness and reproductive potential. There have been studies as early as 1993 suggesting that women with lower WHR are viewed as significantly more attractive and healthier than those with higher WHR. The exclusively male participants also ranked women with normal Body Mass Index (BMI) but lower WHR as potential mate partners. (18) One year later, the same scientist published another study presenting the results of a survey in which male and female physicians were questioned. Here too, women with lower WHR were ranked higher in terms of health, fertility and overall attractiveness by both sexes with only minor differences. (19) Even with different preferences in BMI by different cultures all over the world the preference in WHR only varied slightly. Overall, a lower WHR is desired. (20)

However, there is more to this than just optical advantages in mate selection. Gluteofemoral fat stores higher amounts of long-chain polyunsaturated fatty acids (LCPUFAs) essential for the brain development of fetus and infant. Abdominal fat on the other hand increases the synthesis of these essential fatty acids from dietary intake by reducing the quantity of a synthesis-inhibiting enzyme. To produce human breast milk and for pregnancy in general, the body relies significantly more on LCPUFAs stored in gluteofemoral fat rather than on newly synthesized acids resulting in a notable decrease of these fat storages during pregnancy. This could be a good explanation for the natural difference in fat distribution in male and female individuals. Additionally, it may rationalize why female individuals with normal weight according to BMI, higher gluteofemoral fat storages and therefore lower WHR are viewed as more fertile and attractive. A sufficient presence of LCPUFAs is even more relevant in teenage pregnancies as the mother herself still requires larger amounts of these acids for her own brain development making mother and child compete for these resources. Thus, early pregnancy carries a higher risk for a compromised cognitive development of the child. Young mothers with lower WHR seem to birth children with less cognitive impairment than does with higher WHR. (20)

Endless studies show associations between WHR and several health parameters and disease risk markers. According to a report published in 2008 by the World Health Organization, measures of abdominal obesity such as WHR are better suited for predicting cardiovascular disease risk than general obesity measures like BMI. In women, a WHR of 0,8 or lower is associated with a low risk of cardiovascular diseases whereas in men the risk limit sets at 0,95. However, in both men and women, a WHR of 1,0 or higher drastically increases health risks. (21) This is also reflected in a publication from 2006 which correlates higher WHR in older persons to a higher mortality rate. The authors also discovered that health risks caused by excess weight based on BMI are exaggerated. (22)

A further study from the year 2018 investigated the Waist/Hip-Ratio in context to mortality in heart failure. The results showed a positive correlation between the two factors in female subjects only which indicated WHR to be a sex-specific measurement. (23)

Lockie et al. conducted a study in the year 2018 to examine whether body fat distribution may influence the physical performance in fitness tests. The participants were all law enforcement agency recruits before they started their training. For body composition the measurements of waist circumference and WHR were used. Natural differences between the sexes were considered, in terms of fat distribution and general fitness performance. The results showed only one significant correlation regarding WHR. They saw that recruits with a greater WHR were able to perform a lower amount of push-ups. (24)

1.7 Scope and Aim

It is known that physical activity increases the mitochondrial density and thus also the copy number of mitochondrial DNA in the affected muscle cells. Whether the same effects can also be found in other cell types has not yet been investigated. Therefore, we hypothesized that mitochondrial DNA copy number in leukocytes can provide information about the patient's physical fitness regarding muscle and fat distribution. The minimal-invasive sample collection qualifies mitochondrial copy number from leukocytes as a suitable biomarker for the general population.

To investigate this hypothesis main aims were defined:

1. Choosing the appropriate sample set.
2. Preliminary tests to ensure the accuracy of the used method and reproducibility of the results.
3. Quantification of a specific mitochondrial gene in relation to a specific nuclear gene by quantitative PCR.
4. Relating the two measured genes using the delta Ct method.
5. Statistical evaluation of potential associations between mitochondrial DNA copy number and biomarkers of physical fitness using SPSS.

2 Material and methods

This chapter describes which methods were used to test the hypothesis and which materials were necessary for this. The project was conducted at the Clinical Institute of Medical and Chemical Laboratory Diagnostics at the Medical University of Graz under the supervision of Assoz. Prof. Univ.-Doz. Mag. Dr.rer.nat. Wilfried Renner.

2.1 Sample set

A sample set generated for a previous study conducted by Hassler et al. was used to test the hypothesis. In that study the adipose tissue distribution – both intermuscular and subcutaneous - of the mid-thigh was measured by MRI to investigate its relationship with serum adiponectin levels and different risk factors for cardio-metabolic diseases. (25)

The sample set included 190 samples. DNA isolation was performed as part of another study by Hassler et al. that was published under the title “Sex-Specific Association of Serum Anti-Oxidative Capacity and Leukocyte Telomere Length” in 2021. According to the publication, the isolation was achieved using a MagNA Pure instrument by Roche Diagnostics. (26)

2.2 Target Genes

The selected mitochondrial specific target gene was ND1, the nuclear-specific gene was RPPH1. The mitochondrial target was marked with fluorescent dye VIC (540-580 nm), the nuclear target with fluorescent dye FAM (465-510 nm). The selection of target genes and fluorescent dyes was adopted from a previous publication. (27)

ND1 – also called mitochondrially encoded NADH dehydrogenase 1 – is located in the mitochondrial membrane and activates dehydrogenase of NADH to ubiquinone as well as the assembly of mitochondrial respiratory chain complex 1. It is associated with and used as a biomarker for multiple neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease. (28)

RPPH, 1 or ribonuclease P RNA component H1, is a gene located on the q arm of chromosome 14. The encoded protein plays an important role in the formation of

the mature 5-prime termini of their tRNA sequences by cleaving the tRNA precursor molecules. (29) There are minor disease associations for the RPPH1 gene like visual epilepsy, hereditary spastic paraplegia and anauxetic dysplasia. (30)

2.3 Preliminary tests

For this project, a universal master mix (UMM) was used, containing all the necessary components except primers, probes and DNA template. The PerfeCTa Multiplex qPCR Supermix was supplied at 2X concentration by Quantabio consisting of reaction buffer with multiplex qPCR-optimized MgCl2, dATP, dCTP, dGTP and dTTP, ultra-pure AccuStart hot start Taq DNA Polymerase, reference dye and proprietary performance enhancing additives and stabilizers. For detection with quantitative PCR, target-specific ready to use Assay Mixes supplied at 20X concentrations from applied biosystems by Thermo Fisher Scientific were used. Those provided the required primers and probes. For the chromosomal gene the TaqMan® Gene Expression Assay with the Assay ID Hs03297761_s1 was used, for the mitochondrial gene the TaqMan® Non-coding RNA Assay with the ID Hs02596873_s1 was used. In the preparation of the Master mix the UMM and the Assay Mixes were diluted with distilled water to 1X concentrations in the desired final volume. Both Assay Mixes were added to one final Master mix. The calculated amounts were pipetted as listed in Table 1.

Table 1: Master mixes used in the preliminary tests with 96-well-plates.

Reagent:	**1 sample (20 µl):**
20x AssayMix (mt)	1 µl
20x AssayMix (nc)	1 µl
2x UMM	10 µl
H_2O	4 µl
Sample DNA	4 µl

In the run-up, samples 1 to 15 as well as laboratory-internal control samples were used to test the accuracy of the method and reproducibility of the results. All preliminary tests were performed on 96-well-plates and pipetted manually. The qPCR runs were performed on the cobas® z 480 Analyzer by Roche Diagnostics. Several conditions were changed from run to run to see if or how they would affect

the qPCR results. The samples were each measured multiple times in triplet assays. The position of the sample triplets on the well plate was changed to exclude possible deviations due to the position. Since both the triplets per assay and the assays among each other provided comparable values, the preliminary tests confirmed the reproducibility of the results. Furthermore, one sample was diluted in the ratio 1:4 with distilled water. This dilution was measured in one run in addition to the undiluted sample and then compared with it. Here, too, the ratios – calculated with the 2^-delta Ct method – were similar. Since 384-well-plates require less total volume per well, the ratio of master mix to DNA sample changed. The used quantities were also tested on the 96-well-plate. Volumes were used as shown in Table 2. In each run, regardless of the total volume, 4 µl of sample DNA were added.

Ct values are a relative parameter, depending on the respective threshold, and can therefore be different on each plate. To make the values of different plates comparable, a reference sample was measured on all plates.

2.4 Multiplex quantitative PCR

There are multiple methods suitable for quantifying mitochondrial DNA copy number. Establishing Fluorescence in situ hybridization (FISH) has been attempted with limited success. Southern blot hybridization was considered the gold standard technique for a long period of time. However, due to several disadvantages different methods took over. Currently, PCR-based methods are commonly used for determination of the amount of mitochondrial DNA, often as a relative measure compared to a nuclear gene. There are already promising approaches using sequencing data from next generation sequencing (NGS) technologies like whole exome sequencing (WES) and whole genome sequencing (WGS) suggesting that these methods will be increasingly applied in mitochondrial DNA related aims. (8) Taking these into consideration the method of choice for this project was quantitative PCR. More specifically, multiplex quantitative PCR since two target genes were amplified in the same reaction using the same reagent Master mix.

For the main experiment Hard-Shell 384-well skirted PCR plates by BioRad were used. Before the experiment, the master mix was prepared as listed in Table 2.

Table 2: Master mix used for the runs on the QIAgility. These volumes were also used once in 96-well-plates as part of the preliminary tests.

Reagent:	**1 sample (10 µl):**
20x AssayMix (mt)	0,5 µl
20x AssayMix (nc)	0,5 µl
2x UMM	5 µl
H_2O	-
Sample DNA	4 µl

Originally, the pipetting of master mix and samples into the 384-well-plates was supposed to be performed on the automated workstation Biomek i7 by Beckman Coulter. During the first try, problems with the workstation occurred due to malfunctioning pipette tips in one specific batch, which resulted in a discard of the whole 384-well-plate and changing to another automated PCR Setup System by QIAGEN – QIAgility, that is shown in Figure 2.

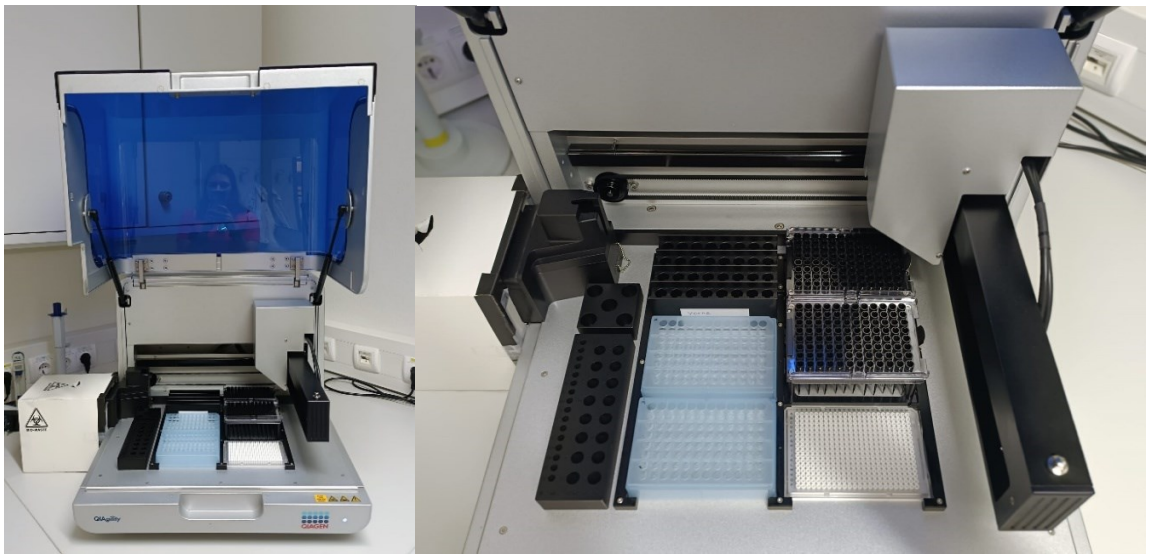

Figure 2: QIAgility, an automated PCR Setup System by QIAGEN, was used to pipette the master mix and samples into the 384-well-plates. On the right picture the compact arrangement of the pipette tips and the 384-well-plate as well as two racks for the required reagents like the Master mix can be seen.

The workstation was programmed to make a triplicate from each sample with both genes of interest. The 190 samples required two 384-well-plates – 126 samples on the first plate and 64 samples on the second plate.

The PCR runs were performed on the CFX384 Touch Real-Time PCR Detection System by Bio-Rad. This PCR-Cycler is located at the Center for Medical Research at the Medical University of Graz (ZMF) and is shown in Figure 3.

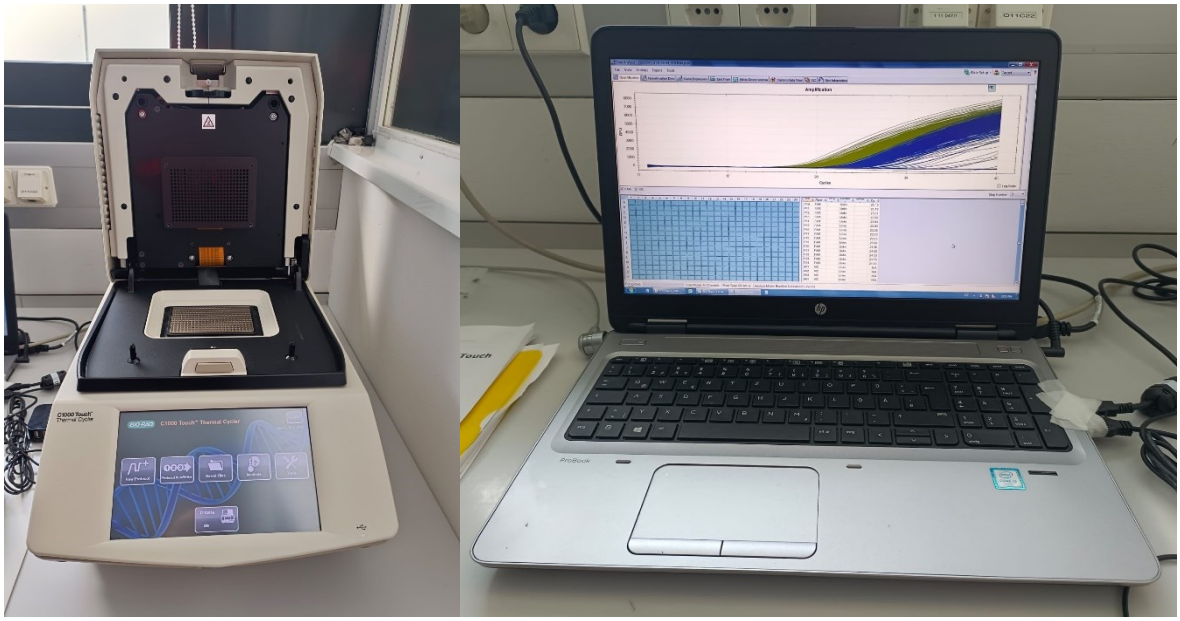

Figure 3: For the qPCR runs the CFX384 Touch Real-Time PCR Detection System by Bio-Rad was used. On the left picture you get an insight into the cycler through the opened lid. This is where the 384-well-plate was placed. On the right picture you can see a completed qPCR run with its qPCR graph and a graphical representation of the plate arrangement.

The plates were sealed with optically clear Plate Sealing Film by BioRad, compatible with the PCR-Cycler, to prevent the reaction sets from evaporating during qPCR. The temperatures used in the different phases of the PCR and how many cycles the reaction consisted of, can be seen in Table 3.

Table 3: The exact temperatures and number of cycles used on the CFX384 Touch Real-Time PCR Detection System.

Phase	Temperature	Duration	Repetitions
Activation phase	95°C	1:00min	1x
Denaturation	95°C	0:10min	40 cycles
Hybridization and polymerization	60°C	0:30min	
Plate Read	--	--	

2.5 ΔCt-method

The ΔCt-method was used to calculate the ratio of mitochondrial DNA to nuclear DNA. For this calculation, the Ct value of the reference gene is first subtracted from the value of the gene of interest and the formula is used to calculate the relative expression difference between the target and reference group as shown below. This formula indicates the multiplicative factor by which the presence of the target gene varies compared to the reference gene.

ΔCt = Ct (gene of interest) – Ct (reference gene)

2^ (- ΔCt) = relative expression difference

Then the mean value and standard deviation of the triplicates as well as the ratios were calculated using Microsoft Excel. Samples with Ct values of 30,0 or higher were excluded from further analysis as we suspected too little sample material as the reason for this. Seven samples did not meet this requirement.

2.6 Statistical evaluation

The statistical analysis was performed with the platform IBM® SPSS Statistics. The previous study by Hassler et al. provided a large data set of information about the measured patients. In addition to information such as sex, age, weight, height and diet (vegetarian diet, low carb diet, supplementing, alcohol and tobacco consumption), the available data of the patients included body measurements (BMI, waist circumference, hip circumference, Waist/Hip-Ratio, Waist/Height-Ratio), measurements of muscle and fat deposition obtained by MRI (intermuscular and subcutaneous adipose tissue), frequency of exercise (endurance training sessions per week, strength training sessions with body weight and additional weight per week, total amount of training per week), pre-existing conditions and risk factors as well as several blood values (adiponectin, osteocalcin, leptin, high density lipoprotein, low density lipoprotein, total cholesterol, triglycerides, interleukin 6, arylesterase). The two datasets were merged to facilitate comparison of the data. A sample was only included in the common data set if previous data was available and Ct values were meeting inclusion criteria.

First, the coefficient of variation for the ratio was calculated. A coefficient of variation of more than 20% was set as an exclusion criterion for further assessment. This filter excluded another 29,3% leaving 133 samples that met all inclusion criteria. The data set was divided into 2 batches according to the PCR-plate on which they were measured. The calculated mean values for the batches differed widely. Therefore, the batches had to be aligned to make the values between the two PCR-runs comparable. For this purpose, the mean values were zeroed and the new variable was called mtDNA_ratio_normalized.

Different restrictions to the included samples were used. Statistical significance was set at $p<0,05$. For the first part of the analysis all samples were tested regardless of sex. For the second part the sample set was divided into two groups (male and female), since there are natural differences in body composition and fat distribution when it comes to sex. As the hypothesis claimed that mitochondrial DNA copy number is increased by factors regarding physical fitness and exercise, we searched for a correlation with relevant parameters. The following parameters were tested with the bivariate correlation analysis: Age, amount of strength training sessions per

week, amount of endurance training sessions per week, BMI, Waist/Hip-Ratio, fat and muscle distribution parameters.

3 Results

For this master thesis we investigated whether the copy number of mitochondrial DNA in leukocytes is dependent on the patient's fitness, the more physically fit a patient, the higher the copy number. To test this hypothesis the mitochondrial gene MT-ND1 as well as the nuclear gene RPPH1 were selected and quantified with qPCR. The Ct values were put into relation and then analyzed along with a dataset containing information about the subject's fitness behavior and various parameters concerning muscle and fat distribution.

3.1 Multiplex qPCR and ΔCt-method

The reactions were set in triplets. The mean values and standard deviations of the ΔCt values and Ratios were calculated. The evaluated data from the qPCR runs of some samples are shown as examples in Table 4. The seven samples that did not meet the set inclusion criteria of Ct value<30,0 and were therefore excluded from statistical analysis are marked in red: 1, 17, 23, 77, 84, 99, 101.

Table 4: Exemplary results of the multiplex qPCR after using the ΔCt-method to compute the ΔCt values and Ratios and Microsoft Office to calculate the mean values and standard deviations of ΔCt values and Ratios in all measured samples. The marked sample number (1) was not used for further analysis as the Ct values did not meet the set inclusion criteria of Ct value<30,0. Other excluded sample numbers that are not shown in this table: 1, 17, 23, 77, 84, 99, 101.

Sample Nr.	ΔCt (mean)	σ ΔCt	Ratio (mean)	σ Ratio
1				
2	1,387237045	0,460686386	0,38229625	0,126637788
3	4,042522146	0,780698108	0,060684751	0,038690697
4	3,184782819	0,466279733	0,109972688	0,034027182
5	2,921852027	0,398449295	0,131957749	0,038345618
6	3,229723236	0,548850997	0,106599809	0,047033358
7	3,71414946	0,233492692	0,076195549	0,013020595
8	2,592252341	0,399016807	0,165826635	0,051248569
9	2,916623695	0,628555886	0,132436831	0,066369276
10	3,149387349	0,260888015	0,112704158	0,021711927
11	3,535083454	0,394870305	0,086264844	0,025952785
12	4,231776855	0,120654675	0,053224088	0,004527222
13	4,004560736	0,244298731	0,062302733	0,010852753
14	3,3010102	0,341659323	0,10146048	0,023804034
15	2,862875451	0,53252743	0,137463885	0,058929052

3.2 Analysis of all study participants (men and women)

First a descriptive statistic of the study participants including variables like age and anthropometric measurements was conducted to create an overview over the previously existing data. For all included variables mean value as well as standard deviation was calculated for the whole sample set. Additionally, the values were calculated after dividing the sample set into female and male samples. In general, 133 samples met all inclusion criteria and were therefore included in analysis. These divide into 72 female and 61 male samples. For the fat distribution variables SAT, IMAT and SAT/(SAT+IMAT) data for only 105 samples (53 female and 52 male) were available. For the low-density-lipoprotein (LDL) three female samples were without available measurements. These descriptive statistics can be seen in Table 5.

Table 5: To form a good overview over the preexisting data of the study participants descriptive statistics were performed. This table shows mean values and standard deviation of anthropometric measurements for all samples as well as divided into female and male samples.

ANTHROPOMETRIC MEASUREMENTS	TOTAL (N=133)	FEMALE (N=72)	MALE (N=61)
AGE (YEARS)	42.10± 11.07	43.33± 10.63	40.66± 11.49
BMI (KG/M^2)	23.56± 3.07	22.67± 3.30	24.61± 2.40
WAIST CIRCUMFERENCE (CM)	81.49± 10.50	76.44± 8.91	87.44± 9.05
HIP CIRCUMFERENCE (CM)	99.36± 7.68	97.68± 8.22	101.34± 6.50
WHR	0.82± 0.07	0.78± 0.06	0.86± 0.07
SAT (AREA IN CM^2)	5423.11± 2767.47	7216.66± 2682.05	3595.08± 1238.32
IMAT (AREA IN CM^2)	761.83± 488.76	940.34± 558.64	579.88± 319.49
SAT/(SAT+IMAT)	0.88± 0.05	0.89± 0.05	0.87± 0.05
TOTAL BLOOD CHOLESTEROL (MG/DL)	192.76± 34.25	196.82± 32.14	187.97± 36.26
HDL (MG/DL)	67.27± 18.96	74.68± 19.18	58.52± 14.55
LDL (MG/DL)	102.12± 32.28	99.97± 29.64	104.54± 35.11
TRIGLYCERIDES (MG/DL)	117.83± 92.95	111.86± 102.12	124.87± 81.08

It is well known that age has a significant impact on the body and various health measurements, as aging is a natural process causing numerous changes in the organism over time. With an advanced age, muscle mass decreases, while the amount of body fat tends to increase, which can affect variables such as BMI or WHR. In addition, metabolic processes slow down with age, leading to changes in cholesterol levels. The organ function can also decline with age, which can impact various biochemical measurements in blood samples.

These changes should be considered in the evaluation. Therefore, the first analysis was performed to verify whether there was a significant relationship between mtDNA copy number and the age of the study participants. All 133 samples that met the inclusion criteria were included in the analysis. Contrary to expectations that age would also have an influence on the mtDNA quantity present, no correlation was discernible. The Pearson's test resulted in a p-value of 0.417, which can be seen in Figure 4. This missing relationship between the two measurements is visualized in Figure 5.

Korrelationen

		Alter	mtDNA_ratio_normalized
Alter	Pearson-Korrelation	1	-,071
	Sig. (2-seitig)		,417
	N	188	133
mtDNA_ratio_normalized	Pearson-Korrelation	-,071	1
	Sig. (2-seitig)	,417	
	N	133	133

Figure 4: This figure shows the result of the bivariate correlation analysis between the variables age and mitochondrial DNA copy number. The p-value (0.417) indicates no relationship between the two variables.

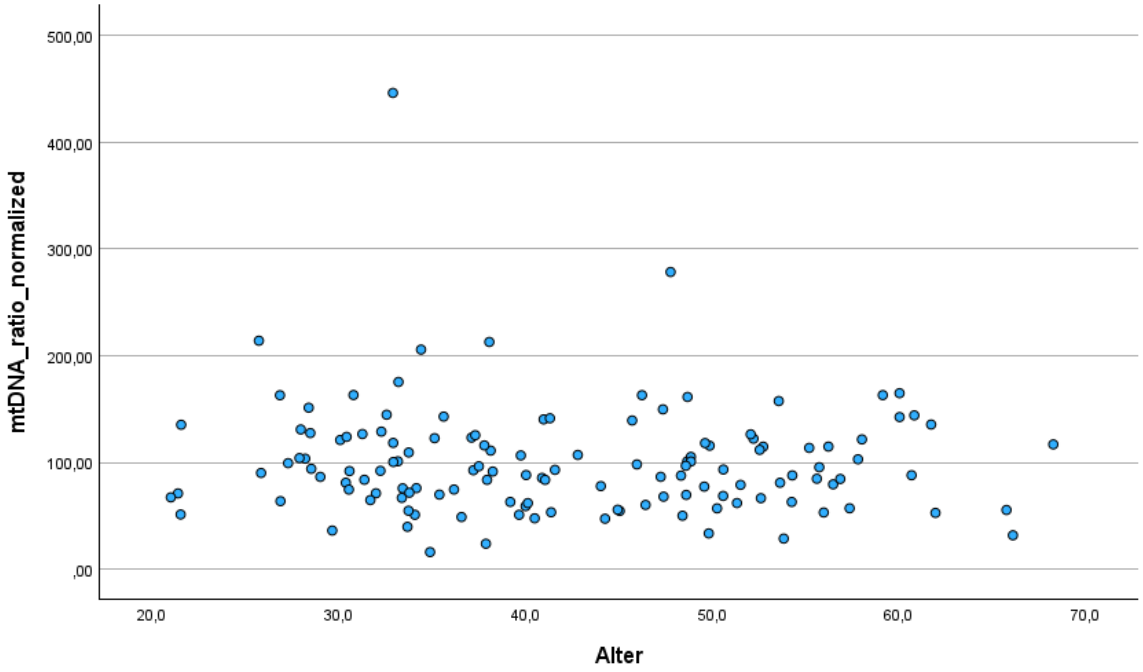

Figure 5: The graph is presented as a scatterplot with the variable normalized Ratio of mitochondrial DNA to nuclear DNA plotted on the vertical axis and the variable age plotted on the horizontal axis. Each dot represents one individual in the studied sample set. The filter was set to include both male and female samples. It shows no significant impact of age on the amount of mitochondrial DNA in humans.

Since the decline of mtDNA-CN in leukocytes is described in various publications only after the age of 50 years, the data set was restricted according to age and the analysis was repeated. (16) However, in participants 50 years of age or older no decline in mtDNA-CN was detected either. The test resulted in a p-value of 0.585. Because the data set consists of younger subjects, only 35 samples were included in this analysis, which may reduce the accuracy of the result.

Then the bivariate correlation analysis was performed including all study participants (male and female). This analysis showed one 2-sided significance between mitochondrial DNA Ratio and the amount of strength training sessions per week. The correlation is weakly significant with a p-value of 0.032. The found correlation can be seen in Figure 6.

Korrelationen

		mtDNA_ratio_normalized	Anzahl Kraft/Woche
mtDNA_ratio_normalized	Pearson-Korrelation	1	,188*
	Sig. (2-seitig)		,032
	N	133	130
Anzahl Kraft/Woche	Pearson-Korrelation	,188*	1
	Sig. (2-seitig)	,032	
	N	130	185

*. Die Korrelation ist auf dem Niveau von 0,05 (2-seitig) signifikant.

Figure 6: This figure shows the result of the bivariate correlation analysis between the mitochondrial DNA Ratio and the number of strength training sessions per week. 2 – sided significance can be seen with a p-value of 0.032. The significance level in this analysis was set at 5%. The found significance of 3,2% is weakly significant.

A weak p-value alone does not necessarily indicate a statistically relevant correlation. It simply indicates the likelihood of the observed correlation occurring by chance. To further investigate whether this finding has significant meaning, we created a scatterplot as visual representation. Although the p-value indicated a correlation the scatterplot showed no statistically significant relationship between mtDNA copy number and the amount of strength training sessions per week. The created scatterplot is shown in Figure 7.

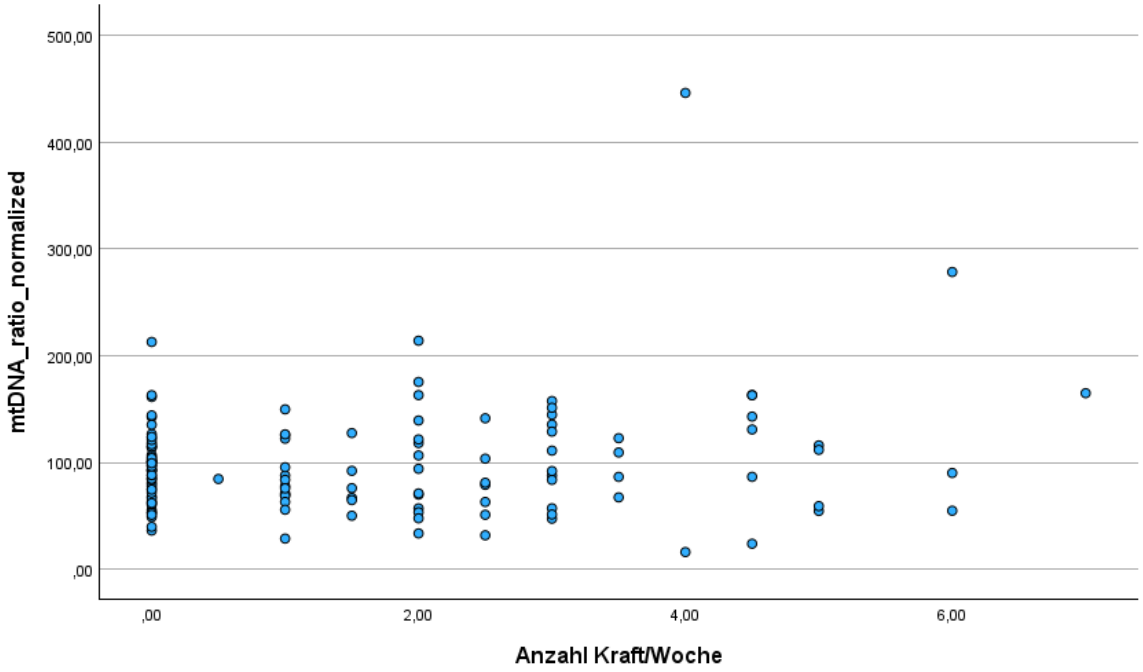

Figure 7: The graph is presented as a scatterplot with the variable normalized Ratio of mitochondrial DNA to nuclear DNA plotted on the vertical axis and the variable strength training sessions per week plotted on the horizontal axis. Each dot represents one individual in the studied sample set. The filter was set to include all samples that met the previous set inclusion criteria. It shows no significant impact of the amount of strength trainings performed per week on the amount of mitochondrial DNA in humans.

However, the scatterplot made an uneven distribution of the number of patients per category visible. It can be seen clearly that the category with participants performing no strength training at all has the largest number of contestants.

Therefore, we took a closer look at the breakdown of the available data. A detailed listing of the data regarding the subjects' strength training intensity showed an uneven distribution of the patients in the categories. Almost half of the included subjects (55 out of 130) stated that they did not participate in any strength training at all. More than two third of the study participants (96 out of 130) placed themselves in four (0, 1, 2 and 3 training sessions per week) of the available 13 categories leaving two categories (0,5 and 7 training sessions per week) with only one subject and eight categories with each less than 5% of all participants. This listing of categories and included subjects can be seen in Figure 8.

Bericht

mtDNA_ratio_normalized

Anzahl Kraft/Woche	Mittelwert	N	Std.-Abweichung
,00	94,9887	55	33,15651
,50	84,5262	1	.
1,00	86,7450	15	32,00798
1,50	79,5401	6	27,26914
2,00	104,5966	14	53,84476
2,50	78,6597	7	35,97587
3,00	103,9704	12	39,80685
3,50	96,4535	4	24,53310
4,00	231,0374	2	304,02539
4,50	118,3739	6	54,20641
5,00	85,3495	4	33,02187
6,00	141,0714	3	120,16562
7,00	164,8398	1	.
Insgesamt	98,7502	130	51,20037

Figure 8: This figure shows a precise listing of the subjects' data on their strength training behavior. The categories 0, 1, 2, and 3 strength training sessions per week include more than two third of all subjects. 55 out of 130 probands listed that they never take part in strength training sessions at all. This unequal apportionment casts doubt on the richness of the found significance.

This imbalanced number of subjects in each category, in addition to the strongly scattering mean values and standard deviations, indicated that the group of subjects is too small and the provided information for some categories too little to make significant statements. Therefore, this finding was declared to be not conclusive and was not investigated further.

3.3 Sex-stratified analysis

Since the analysis including all samples, regardless of sex, did not lead to any significant results, the next step was to divide the sample set into male and female subjects by sex. The male group included 61 samples, while the remaining 72 samples formed the female cohort. Then the bivariate correlation analysis was performed for both sexes separately. For men the analysis - including a variety of variables - revealed no significant findings.

When we performed the bivariate correlation analysis including only female samples, a correlation was demonstrated. The analysis showed a 2-sided significance between the aligned mitochondrial DNA Ratio and the Waist/Hip-Ratio. The correlation is strongly significant with a p-value of 0.002 as shown in Figure 9.

Korrelationen

		mtDNA_ratio_normalized	W/Hip Ratio
mtDNA_ratio_normalized	Pearson-Korrelation	1	-,357**
	Sig. (2-seitig)		,002
	N	72	72
W/Hip Ratio	Pearson-Korrelation	-,357**	1
	Sig. (2-seitig)	,002	
	N	72	98

**. Die Korrelation ist auf dem Niveau von 0,01 (2-seitig) signifikant.

Figure 9: This figure shows the result of the bivariate correlation analysis between mitochondrial DNA Ratio and Waist/Hip-Ratio in all female samples. 2-sided significance can be seen with a p-value of 0.002. The correlation is significant at the 0,01 level, indicating a strong significance.

The scatterplot in Figure 10 was created to better display this correlation. With an increased WHR, a decrease in mitochondrial DNA Ratio is evident. This indicates a negative relationship between the two variables. It is important to note that the diagram only shows a correlation and not a causal relationship.

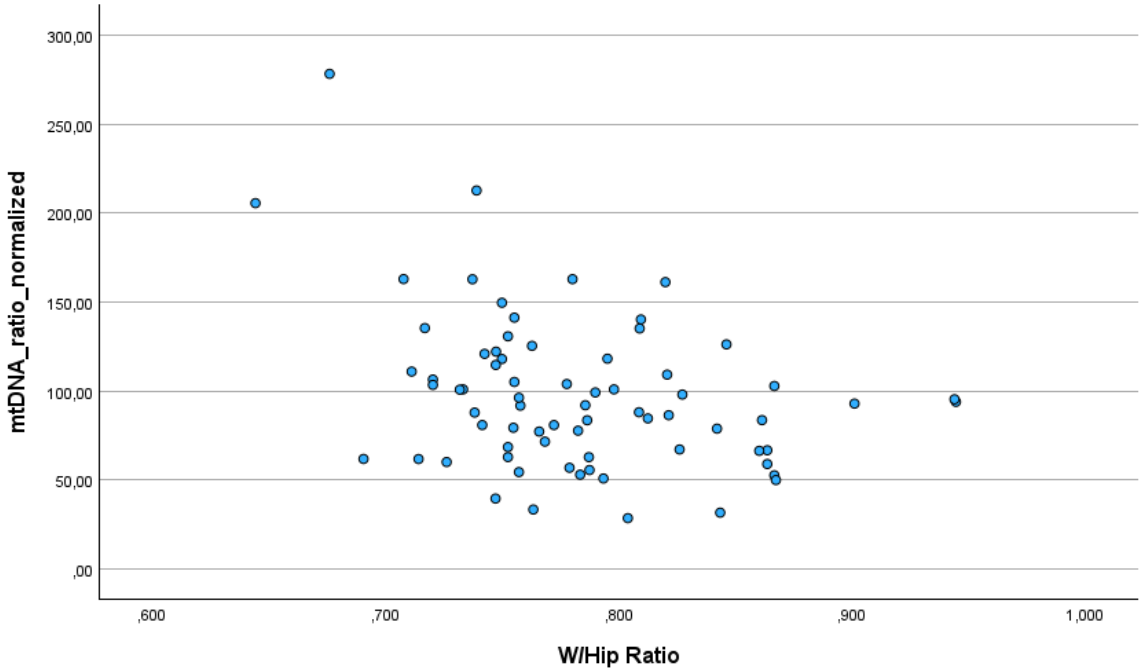

Figure 10: The graph is presented as a scatterplot with the variable normalized Ratio of mitochondrial DNA to nuclear DNA plotted on the vertical axis and the variable WHR plotted on the horizontal axis. Each dot represents one individual in the studied sample set. The filter was set to include only female samples. This scatterplot visualizes the 2-sided significance between the two variables. With an increased WHR, a decrease in mitochondrial DNA Ratio is evident, indicating a negative relationship between the two variables.

To test the assumption that the correlation occurs in female participants only, the entire data set including both sexes was subsequently testes for this correlation, as well as the separated male cohort. Both analyses are presented with scatterplots as well. The analysis of the entire data set including both sexes is listed in Figure 11 while a scatterplot is shown in Figure 12. The analysis for the male cohort can be seen in Figure 13 with the associated scatterplot in Figure 14.

Korrelationen

		mtDNA_ratio_normalized	W/Hip Ratio
mtDNA_ratio_normalized	Pearson-Korrelation	1	-,084
	Sig. (2-seitig)		,338
	N	133	133
W/Hip Ratio	Pearson-Korrelation	-,084	1
	Sig. (2-seitig)	,338	
	N	133	188

Figure 11: This figure shows the result of the bivariate correlation analysis between the variables mtDNA Ratio and WHR in all samples (male and female). The p-value clearly shows no correlation between these two variables in the present data set.

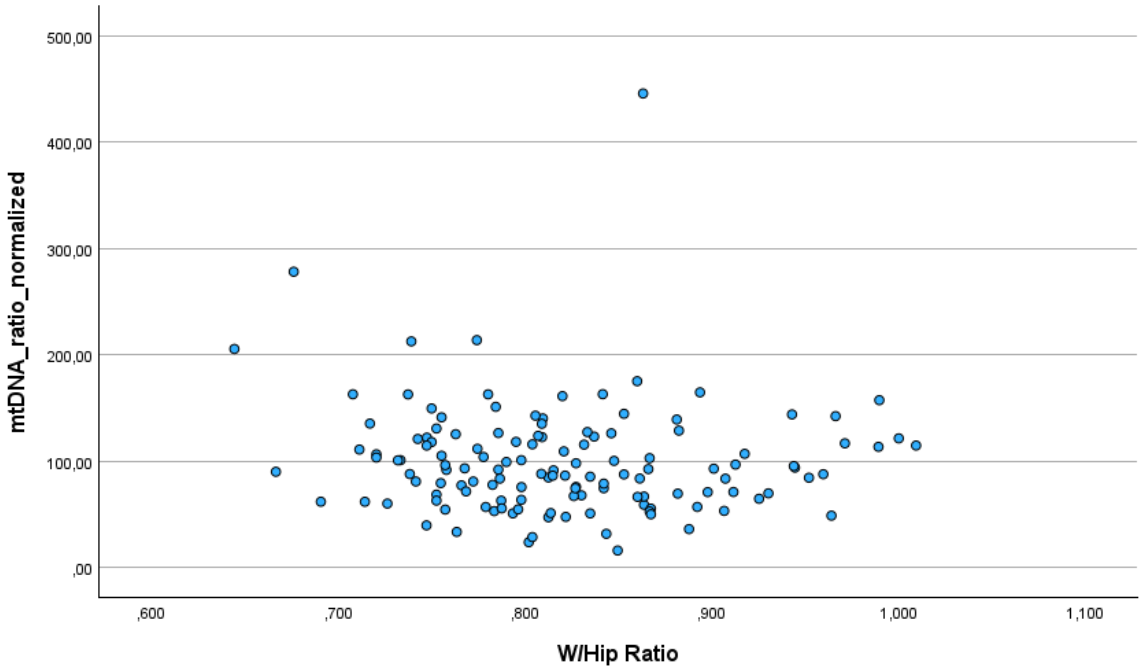

Figure 12: In this scatterplot all samples, male and female, were included in the analysis. Here again, the variable normalized Ratio of mitochondrial DNA to nuclear DNA is plotted on the vertical axis while the variable WHR is plotted on the horizontal axis. It indicates no relationship between the two tested variables.

Korrelationen

		mtDNA_ratio_normalized	W/Hip Ratio
mtDNA_ratio_normalized	Pearson-Korrelation	1	,034
	Sig. (2-seitig)		,793
	N	61	61
W/Hip Ratio	Pearson-Korrelation	,034	1
	Sig. (2-seitig)	,793	
	N	61	90

Figure 13: This figure shows the result of the bivariate correlation analysis between the variables mtDNA Ratio and WHR in the male cohort. The p-value indicates no relationship between these two variables in the present data set.

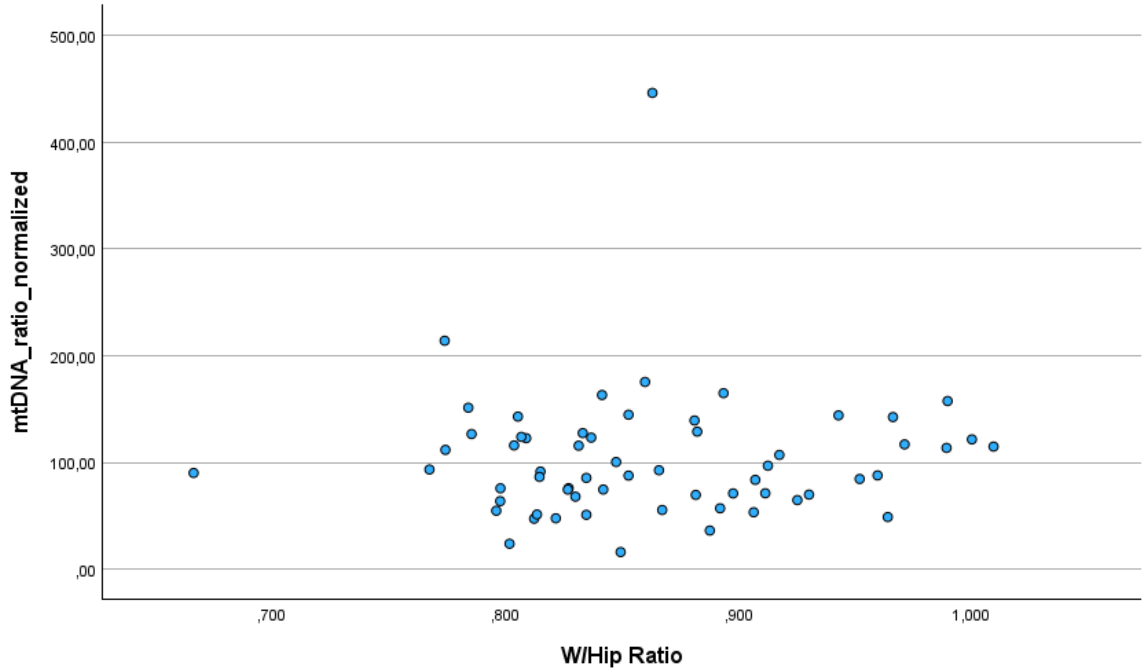

Figure 14: This scatterplot visualizes the analysis of all male samples. The variable normalized Ratio of mitochondrial DNA to nuclear DNA is plotted on the vertical axis and the variable WHR is plotted on the horizontal axis. Here too, no relationship between the two tested variables can be seen.

The p-values for the analysis of all samples as well as the analysis of the male cohort indicated no relationship between the analyzed variables in those sample groups. In addition, the scatterplots illustrate no discernible correlation between the two measurements in neither all samples nor male samples only. The points are distributed seemingly randomly throughout the graphs, with no apparent systematic relationship between the two variables. This lack of clear correlation indicates that changes in one of the variables cannot be predicted or explained based on the values of the other variable. Thus, the scatterplots serve as a visual tool to illustrate the independence and lack of a linear relationship between the variables in those groups. This serves to illustrate the assumption that the found correlation in female samples is indeed gender specific.

4 Discussion and conclusion

Although mitochondrial DNA and their copy number have been subject of intensive biomedical research for some time, their influence on human disease and aging has not been completely clarified. This is because the copy number of mitochondrial DNA is a complex parameter that can be influenced by a variety of factors including both genetic and environmental aspects as well as internal regulatory mechanisms, which complicates research in this field. Despite this complexity, or perhaps because of it, we attempted to make a contribution with our project. By intensively studying the topic we gained insight into the complex interrelationships.

In the present project, 190 DNA samples, obtained from peripheral blood were examined for the interaction of physical fitness and changes in DNA copy number. To evaluate the physical fitness of the participants, data from a previous study conducted by Hassler et al. was used, in which several parameters regarding exercise and fat and muscle distribution as well as some blood values were measured in the same sample set.

Since aging leads to major physical changes in the human body and thus to changes in various laboratory measurements, the relationship between mtDNA copy number and the age of the study participants was investigated first. With a p-value of 0.417, a correlation between these variables could be clearly excluded in the data set used. Additionally, a filter was set to include only samples of participants at the age of 50

years or older as some publications indicate a decline in mtDNA-CN starting at that age. This analysis showed no relationship between these two variables as well (p-value of 0.585). One reason for this result could be that the cohort was too small. As the participants were mainly younger than 50 years this age filter excluded almost 75% of all samples leaving only 35 samples for the analysis.

At first, the correlation analysis regardless of sex showed a significance with a p-value of 0.032 between mitochondrial DNA Ratio and the amount of strength training sessions performed per week. This suggested a relationship between those variables. After creating a scatterplot to visualize the finding, an uneven distribution of the participants into the categories was visible. Especially the category including participants that do not perform any strength training sessions contained a large number of study contestants. This was further investigated by looking closer into the available data for the variable strength training sessions per week. We realized that the available data was too incomplete to draw any further conclusions. To investigate the possible finding, more studies with a larger number of participants that distribute more evenly across the categories are required.

The results of the bivariate correlation analysis in all female samples showed a significant negative correlation between the Waist/Hip-Ratio and mitochondrial DNA copy number with a p-value of 0.002, suggesting that a lower waist circumference in relation to the hip circumference can lead to an increased number in mitochondrial DNA. The relationship between a low WHR and increased health has been described many times. The result of previous research indicates an association of lower WHR with lower risks for many diseases like cardiovascular diseases. (23) Taking these relationships into consideration, the finding that a lower WHR is linked with higher amount of mitochondrial DNA supports the assumption that a greater abundance of DNA in the mitochondria is related to improved physical health in the human body. As mitochondria are the primary source of cellular energy, a higher DNA content might signify more efficient energy production and better cellular maintenance, contributing to enhanced physical resilience and vitality. To illustrate that this is a gender-specific finding, the correlation analysis was repeated with the entire data set as well as the male cohort only. Neither analysis revealed a significant relationship. While the scatterplot of the data set of both sexes

demonstrated a slight tendency due to the included female samples, the male cohort did not show any correlation at all.

There were several limitations in our project. Since blood samples were only taken at one time, we can only assess the differences between patients per se and only at one point, but not evaluate the subjects' individual development due to different fitness or body changes. Multiplex qPCR allowed us to analyze relative values but was not suitable for measuring absolute amounts of DNA. Still, this finding is further indication for the assumption that the copy number of mitochondrial DNA is well suited to be used as a biomarker for overall fitness in the general population.

Despite the limitations, the present project provides valuable insights into the role of mitochondrial DNA in fitness related matters.

5 Summary and Outlook

In conclusion, the aim of this study was to investigate the informative value of mitochondrial DNA copy number measured in leukocytes on the general fitness and health status of the human body. Therefore, an appropriate data set of 190 blood samples, previously cultivated and used by Hassler et al. for another study, was chosen. We selected a mitochondrial gene (MT-ND1) and a nuclear gene (RPPH1) and were able to successfully quantify these genes using multiplex qPCR. The Ct values were put in relation with the delta Ct method and the data set including the calculated Ratios, means and standard deviations was merged with an existing data set containing various variables about the subjects. A statistical analysis of the results was performed, after defining the following exclusion criteria: Ct values of 30,0 or higher, coefficient of variation of Ratios>20%, data existing in only one of the two merged data sets.

Contrary to expected assumptions, analysis of the data revealed that age has no statistically significant influence on mitochondrial DNA copy number in this data set. This result goes against previous studies suggesting a relationship between age and mtDNA-CN due to aging processes and potential accumulation of mitochondrial damage leading to this decrease in copy number. These studies showed a yearly decline in mtDNA-CN starting at the age of 50 years. Analysis of participants of this

age group showed no influence of age on the amount of mitochondrial DNA either. As the participants were mainly younger than 50 years this filter excluded almost 75% of all samples. To clarify whether there is a relationship between these variables or not, further studies with a larger data set including older subjects should be conducted.

One correlation found between the mitochondrial DNA Ratio and the amount of strength training sessions per week in all participants was discarded after closer examination, with a p-value of 0.032 and a scatterplot indicating no correlation between the two variables. A closer look at the number of subjects in the existing categories revealed that the distribution of participants was insufficient. Further studies are needed to clarify whether this correlation is justified or a coincidental occurrence. This testing would require a larger number of participants, making sure that all training intensities are covered by a sufficient number of participants. Additionally, it would be useful to divide the participants into groups and have them follow specific training programs over a set period of time including repeated blood sample collections over time. Differences and changes can be detected between the variation of training programs but also between the blood samples of one individual.

After separating the data set into groups according to sex, a correlation was found in the female group with high significance (p-value 0.002). As described in chapter 1.6, there are already several studies that have linked health benefits in women to a lower WHR. These observations could rarely be found in men. Taking our new findings as well as previous studies into consideration, WHR could be established as a sex-specific biomarker for women’s physical health.

The present project provides evidence that female body fat distribution (measured with WHR) correlates with mitochondrial DNA copy number present in leukocytes. It has been observed that a higher amount of mitochondrial DNA copy number may be associated with a lower WHR. This suggests that body weight distribution in women may play a role in generating mitochondrial DNA copies. Additionally, as a lower WHR has been associated with decreased risk for many diseases and can therefore be seen as a parameter for beneficial health, the observed relationship

indicates a connection between higher mitochondrial DNA copy number in leukocytes and increased health in humans.

However, it is important to point out that further research is needed to better understand the underlying mechanisms and causal relationship between the two tested variables. Additional studies and consideration of other influencing factors are necessary to confirm a causal relationship between WHR and mitochondrial DNA copy number in women. Only through more comprehensive research can we gain a more detailed understanding of this correlation and its influence on the human body.

In this project we were able to obtain single-point measurements of the patients as only one blood collection was performed. This can limit the validity of the results. In addition, many parameters, such as the number of trainings sessions per week, were based on the patients' own statements. Here, a certain degree of dishonesty or different perception of the patients must be taken into account. Future research could benefit from employing longitudinal study designs including repeated blood collections after certain time durations to establish causality and investigate how the relationship between WHR and the amount of mitochondrial DNA copies changes over time. Thus, not only can a comparison be made between subjects, but also the patients' individual development through different lifestyle changes can be assessed. This approach may also help to clarify which variable is dependent on which. The correlation found does not allow to determine whether a low WHR promotes mitochondrial DNA replication or a higher copy number has a positive effect on fat distribution in women.

The method used also comes with limitations, since only relative quantities could be determined. For further assessment it could be beneficial to rely on other methods that allow measurements of absolute DNA copy number as well. As already mentioned in chapter 2.4 attempts to study mitochondrial DNA using sequencing methods showed promising results. More focus should be placed on these methods in the future.

These limitations may have had an influence on the reliability of our results. Still, the conducted research raises important questions and opens new possibilities for future investigations.

Although additional and more detailed studies are necessary to confirm these results, this project could contribute to the establishment of mitochondrial DNA copy number and WHR as biomarkers for general physical health in humans in the long run. Especially in women, there is already great evidence for this.

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