Faculteit Geneeskunde en Levenswetenschappen
School voor Levenswetenschappen

master in de biomedische wetenschappen

Masterthesis

Mitochondrial DNA mutations in association with birth weight and BMI in the early life of twins

Laurien Geebelen

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen, afstudeerrichting milieu en gezondheid

PROMOTOR :
Prof. dr. Michelle PLUSQUIN

COPROMOTOR :
dr. Esmee BIJNENS

BEGELEIDER :
Mevrouw Charlotte COSEMANS
Mevrouw Hanne SLEURS

2018
2019

Faculteit Geneeskunde en Levenswetenschappen
School voor Levenswetenschappen

master in de biomedische wetenschappen

Masterthesis

Mitochondrial DNA mutations in association with birth weight and BMI in the early life of twins

Laurien Geebelen
Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen, afstudeerrichting milieu en gezondheid

PROMOTOR :
Prof. dr. Michelle PLUSQUIN

COPROMOTOR :
dr. Esmee BIJNENS

BEGELEIDER :
Mevrouw Charlotte COSEMANS
Mevrouw Hanne SLEURS

Content

List of abbreviations

ATP	Adenosine Triphosphate
BMI	Body Mass Index
CI	Confidence Intervals
CPEO	Chronic Progressive External Ophthalmoplegia
ddPCR™	Droplet Digital™ Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
DOHaD	Developmental Origins of Health and Diseases
ENVIR*ON*AGE	ENVIRonmental influence on early AGEing
LHON	Leber Hereditary Optic Neuropathy
LOD	Limit of Detection
LOQ	Limit of Quantification
LS	Leigh Syndrome
MAF	Minor Allele Fraction
MDM	Maternally Inherited Diabetes Mellitus
MELAS	Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes
MERRF	Myoclonic Epilepsy and Ragged Red Fibres
MIDD	Maternally Inherited Diabetes and Deafness
MILS	Maternally Inherited Leigh Syndrome
mtDNA	Mitochondrial DNA
mtSNP	Mitochondrial Single Nucleotide Polymorphism
NARP	Neurogenic Weakness, Ataxia and Retinitis Pigmentosa
ND	NADH Dehydrogenase
PCR	Polymerase Chain Reaction
PS	Pearson Syndrome
qPCR	Quantitative Polymerase Chain Reaction
REML	Restricted Maximum Likelihood
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
SNP	Single Nucleotide Polymorphism
TCA	Tricarboxylic Acid Cycle
tRNA	Transfer RNA
WHO	World Health Organization

Acknowledgments

Abstract

INTRODUCTION – Recently published papers dispute the importance of mitochondrial DNA mutations in birth weight, BMI and common metabolic disorders like diabetes. Heteroplasmy (i.e. differences in mutations between individuals or between diverse tissues and organs in one individual) of the mitochondrial DNA might be a missing part explaining the heritable susceptibility to obesity. However, not only the association of heteroplasmy with BMI or birth weight is unclear, but also the nature of the mutations is still under discussion. Twin studies provide a very useful way to study the effects of nature versus nurture on various processes. Therefore, we hypothesize that these specific mtDNA mutations, namely MT-ND4L$_{10550A \to G}$, MT-ND4$_{11719A \to G}$ and MT-ND5$_{13780A \to G}$, are mainly determined by the genetic inheritance in twins and that they are indeed associated with birth weight and BMI, using the Limburg Twin Study.

MATERIALS & METHODS - The Limburg Twin Study, as part of the ENVIR*ON*AGE birth cohort, includes 43 twin pairs born at the East-Limburg Hospital in Genk between April 1, 2014, and January 24, 2017, with 25 twin pairs around the age of four in May 2019, making them eligible for a follow-up visit. DNA extractions were carried out using cord blood samples of a subset of twins (n = 35) and peripheral blood of the mother (n = 35). Next, the DNA samples were used for mutational analysis using Droplet Digital™ PCR in 63 twin children and 28 mothers. This technique was first optimized for the three assays used: MT-ND4L$_{10550A \to G}$, MT-ND4$_{11719A \to G}$, and MT-ND5$_{13780A \to G}$. Also, BMI z-scores for 19 twin children were computed using WHO standards. For the statistical analysis, mixed modelling was used to investigate the associations of heteroplasmy of these mutations with birth weight and BMI z-scores in the twins. Linear regression was used to associate heteroplasmy of both mutations with BMI in the mothers.

RESULTS - For the mutational analysis, it was apparent that only MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$ were usable for the analysis. In brief, this research within the framework of the Limburg Twin Study was set up to investigate four different points. Firstly, this research is indicative of a possible genetic background. For the transmission of the mutations from the mother to her offspring, we observed transmissions of the mutations from the mother to her offspring, but *de novo* mutations were also present. Secondly, we associated heteroplasmy of both mutations with BMI in the mothers. For MT-ND4L$_{10550A \to G}$, a significantly higher BMI (9.99 kg/m²; 95% CI 3.7 to 16.2 kg/m²; p = 0.006) was observed in the group of mothers with the highest levels of heteroplasmy in comparison to the mothers that presented no heteroplasmy levels. For MT-ND5$_{13780A \to G}$, a significantly higher BMI (5.33 kg/m²; 95% CI 0.3 to 10.3 kg/m²; p = 0.01) was only found between the group of mothers with a low level of heteroplasmy compared to the group of mothers that had no heteroplasmy detected. Thirdly, for MT-ND4L$_{10550A \to G}$ heteroplasmy in mothers, a significant lower birth weight was found in the twin groups that showed moderate (2158.6 g; 95% CI 1969.8 to 2347.3 g; p = 0.04) and high (1991.8 g; 95% CI 1779.5 to 2204.1 g; p = 0.02) levels of heteroplasmy in comparison to the twin group where no mutation was detected in the blood sample of the mother. The same trend was seen in the twin groups that showed moderate (2187.1 g; 95% CI 2047.9 to 2327.0 g; p = 0.03) and high (2111.1 g; 95% CI 1960.6 to 2261.6 g; p = 0.02) levels of heteroplasmy when the child presented heteroplasmy levels. For MT-ND5$_{13780A \to G}$, for the mutation of the children, there was a significant lower birth weight in the moderate heteroplasmy level group (2284.5 g; 95% CI 2214.9 to 2354.0 g; p = 0.007). Finally, the association between either the heteroplasmy levels of the mother or the twin and the BMI z-scores was not significant for both mutations.

DISCUSSION & CONCLUSION – These specific mutations are mainly determined by the genetic inheritance, with the genetic bottleneck as a possible mediator. Our results show that a higher frequency of MT-ND4L$_{10550A \to G}$ mutation poses a potential risk factor for a higher BMI and thus obesity. These results indicate that having a higher frequency of mutation of MT-ND4L$_{10550A \to G}$ of mother or child is disadvantageous for the birth weight. For MT-ND5$_{13780A \to G}$, our results are contrary to what we expected, namely that a low frequency of heteroplasmy is potentially hazardous for a higher BMI. Also, we observe that having a low frequency of MT-ND5$_{13780A \to G}$ is negative for the birth weight. In conclusion, twin studies provide the opportunity to explain the background of mutations. In addition to this, Droplet Digital™ PCR proves its advantages for mutational analysis. For the future, more mutations can be confirmed and analysed using this twin population, however, this research might be seen as an ideal pilot study to confirm the findings in a larger study. Lastly, the identification of these mutations around the age of four might have important implications for health in later life.

Samenvatting

INLEIDING – Recent gepubliceerde papers betwisten het belang van mitochondriële DNA-mutaties voor het geboortegewicht, BMI en veelvoorkomende metabole aandoeningen zoals diabetes. Heteroplasmie (verschillen in de mutaties tussen individuen of tussen verschillende weefsels en organen in één individu) van het mitochondriale DNA kan een ontbrekend stuk zijn in de verklaring van de erfelijke vatbaarheid aan obesitas. Niet enkel de associatie tussen heteroplasmie en BMI of het geboortegewicht is onduidelijk, maar ook de natuur van de mutaties staat nog onder discussie. Tweelingstudies vormen een handige manier om de effecten van nature versus nurture op verschillende processen te onderzoeken. De hypothese van dit onderzoek is dus dat deze specifieke mtDNA mutaties, namelijk $MT\text{-}ND4L_{10550A \to G}$, $MT\text{-}ND4_{11719A \to G}$ and $MT\text{-}ND5_{13780A \to G}$, voornamelijk bepaald worden door de genetische overerving en dat ze inderdaad geassocieerd zijn met geboortegewicht en BMI in tweelingen, in het kader van de Limburgse Tweelingstudie.

MATERIAAL & METHODEN – De Limburgse Tweelingstudie, als onderdeel van het Limburgs Geboortecohort, bevat 43 tweelingparen geboren in het Ziekenhuis Oost-Limburg in Genk tussen 1 april 2014 en 24 januari 2017. In mei 2019 waren er 25 tweelingparen ongeveer vier jaar oud, waardoor zij in aanmerking kwamen voor een follow-up bezoek. DNA-isolaties werden uitgevoerd op navelstrengbloed van een subpopulatie tweelingparen (n = 35) en perifeer bloed van een subset moeders (n = 35). Vervolgens werden deze DNA-stalen gebruikt voor mutatie-analyse middels Droplet Digital™ PCR in 63 tweelingindividuen en 28 moeders. Deze techniek werd eerst geoptimaliseerd voor de drie gebruikte assays: $MT\text{-}ND4L_{10550A \to G}$, $MT\text{-}ND4_{11719A \to G}$ en $MT\text{-}ND5_{13780A \to G}$. Ook werden BMI z-scores berekend met behulp van de WHO-groeistandaarden. Voor de statistische analyse werd mixed modeling gebruikt voor het bestuderen van de associaties van heteroplasmie van deze mutaties met het geboortegewicht en de BMI z-scores in de tweelingparen. Lineaire regressie werd gebruikt voor het associëren van heteroplasmie van beide mutaties met BMI in de moeders.

RESULTATEN – Voor de mutatie-analyse is het duidelijk dat alleen $MT\text{-}ND4L_{10550A \to G}$ and $MT\text{-}ND5_{13780A \to G}$ bruikbaar waren voor analyse. Deze research in het kader van de Limburgse Tweelingstudie trachtte vier punten te bestuderen. Ten eerste is dit onderzoek een indicatie van een mogelijke genetische achtergrond. Voor de overdracht van de mutaties van de moeder naar haar nakomelingen observeerden wij inderdaad overdracht van de mutaties naar haar nakomelingen, maar nieuwe mutaties in de nakomelingen konden ook optreden. Ten tweede associeerden we heteroplasmie van beide mutaties met het BMI van de moeder. Voor $MT\text{-}ND4L_{10550A \to G}$ werd er een significant hoger BMI (9.99 kg/m²; 95% BI 3.7 tot 16.2 kg/m²; p = 0.006) geobserveerd in de grote moeders met de hoogste niveaus heteroplasmie in vergelijking met de groep moeders die geen heteroplasmie vertoonden. Voor $MT\text{-}ND5_{13780A \to G}$ observeerden we een significant hoger BMI (5.33 kg/m²; 95% BI 0.3 tot 10.3 kg/m²; p = 0.01) in de groep met een lage hoeveelheid heteroplasmie in vergelijking met de moeders die geen heteroplasmy vertoonden. Ten derde vonden we voor heteroplasmy van $MT\text{-}ND4L_{10550A \to G}$ in de moeders een significant lager geboortegewicht in de groepen die gemiddelde (2158.6 g; 95% BI 1969.8 tot 2347.3 g; p = 0.04) of hoge (1991.8 g; 95% BI 1779.5 tot 2204.1 g; p = 0.02) heteroplasmie vertoonden. Dezelfde trend konden we zien in de tweelinggroepen die gemiddelde ((2187.1 g; 95% BI 2047.9 tot 2327.0 g; p = 0.03) en hoge (2111.1 g; 95% BI 1960.6 tot 2261.6 g; p = 0.02) heteroplasmie-niveaus vertoonden. Wanneer de tweelingen gemiddelde heteroplasmie van $MT\text{-}ND5_{13780A \to G}$ vertoonden, vertoonden zij een significant lager geboortegewicht (2284.5 g; 95% BI 2214.9 tot 2354.0 g; p = 0.007). Ten laatste was de associatie tussen zowel de heteroplasmie-niveaus van de moeder of van de tweelingen en de BMI z-scores voor beide mutaties niet significant.

DISCUSSIE & CONCLUSIE – De achtergrond van deze specifieke mutaties werd voornamelijk bepaald door de genetische overerving, met de genetische bottleneck als mogelijk mechanisme. Onze resultaten laten zien dat een hogere frequentie van gemuteerd $MT\text{-}ND4L_{10550A \to G}$ een mogelijke risicofactor is voor het ontwikkelen van een hoog BMI en dus obesitas. Deze resultaten zijn ook een indicatie dat het hebben van een hogere frequentie van mutaties in $MT\text{-}ND4L_{10550A \to G}$, zowel in moeders als in haar nakomelingen, nadelig is voor het geboortegewicht. Voor $MT\text{-}ND5_{13780A \to G}$ zijn de resultaten tegengesteld aan wat we verwachtten, namelijk dat een lage frequentie van heteroplasmy mogelijk risicovol is voor een hoger BMI en de ontwikkeling van obesitas. Daarenboven observeren we dat het hebben van een lage frequentie van $MT\text{-}ND5_{13780A \to G}$ negatief is voor het geboortegewicht. In conclusie is het zo dat tweelingstudies de mogelijkheid bieden om de achtergrond van mutaties te bestuderen. Hiernaast bewijst Droplet Digital™ PCR zijn voordelen voor het uitvoeren van mutatie-analyse. In de toekomst kunnen meer mutaties bevestigd en geanalyseerd worden met gebruik van deze tweelingstudie, maar dit onderzoek kan ook gezien worden als een pilootstudie om deze bevindingen te bevestigen in een grotere studie. Ten laatste kan men stellen dat de identificatie van deze mutaties rond de leeftijd van vier jaar belangrijke gevolgen kan hebben voor gezondheid in het volwassen leven.

1 Introduction

The ENVIRonmental influence ON early AGEing (ENVIR*ON*AGE) birth cohort is a prospective, population-based cohort study that studies the interactions of humans with their way of ageing and the involved environmental processes. This cohort recruits mother-newborn-pairs at the East-Limburg Hospital in Genk (Belgium) and follows procedures approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital (3). The ENVIR*ON*AGE birth cohort is designed to follow the mother-newborn pairs, to gather evidence about the processes that might elevate the risk of health issues in later life through *in utero* exposures (3). Even though there are not many studies that research the *in utero* period and its exceptional vulnerability, the interest in this field is growing rapidly. The Developmental Origins of Health and Diseases (DOHaD) or Barker hypothesis, has given attention to this vulnerability to influences from the outside. This hypothesis namely states that a lower birth weight can give rise to physiological adaptations that might elevate the risk for several health problems in later life. Most of the information known to date about the Barker hypothesis associates nutritional deprivation early in life with the risk for chronic diseases. These risks are related to nutrition or cognitive development, however, more studies on birth cohorts like the ENVIR*ON*AGE cohort are necessary to elucidate the exact effects (4).

A special part of this cohort is dedicated to twins, namely the Limburg Twin Study. Between April 2014 and January 2017, 43 twins were recruited at the East-Limburg Hospital. These twins will also be followed throughout their life, with a first follow-up around the age of four, starting in January 2019. During this follow-up visit, different biological samples, as well as data about the lifestyle and medical history, are collected. Also, a cognitive test of the children is conducted (**Figure 1**)(3).
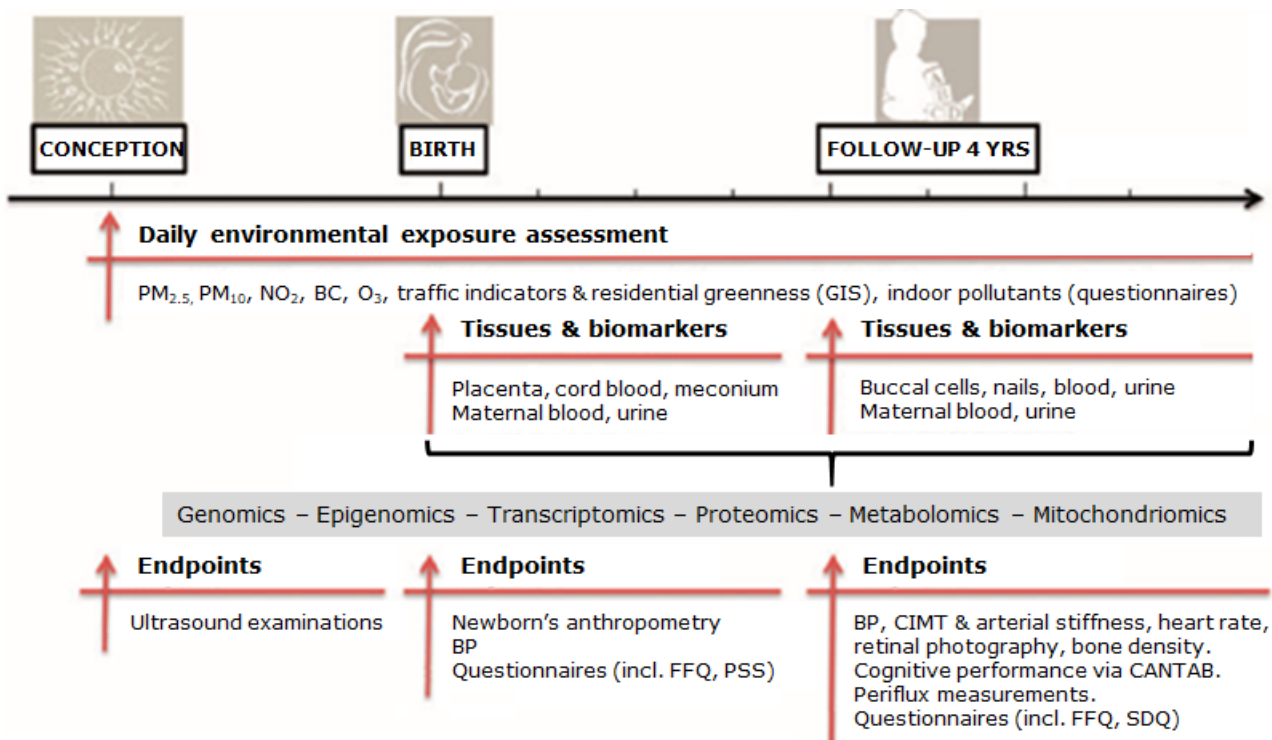
Figure 1. Study scheme of the Limburg Twin Study as part of the ENVIR*ON*AGE birth cohort. PM, particulate matter; NO_2, nitrogen dioxide; BC, black carbon; O_3, ozone; GIS, geographical information system; BP, blood pressure; FFQ, Food Frequency Questionnaire; PSS, Perceived Stress Scale; CIMT, carotid intima-media thickness; CANTAB, Cambridge Neuropsychological Test Automated Battery; SDQ, Strengths and Difficulties Questionnaire. Adapted from Janssen *et al.* (3).

One of the most important objectives of the Limburg Twin Study, and also the ENVIR*ON*AGE birth cohort, is to explore the possible associations between molecular targets of ageing, as for example mitochondria, with clinical outcomes in childhood. Next to this, both cohorts tend to assess the association between environmental or lifestyle factors with these childhood clinical outcomes (3). For these objectives, twin research has proven to be effective (5, 6). Twin research provides the opportunity to gain insight into the relative importance of genes and environment. Monozygotic twins are twins that are genetically identical. Therefore, a difference between two siblings of the same pair is ultimately caused by these environmental effects. This is an ideal study set up to control for genetic influences. On the other hand, dizygotic twins only share half of their genes. If dizygotic twins show a greater deal of variance than monozygotic twins, this is caused by genetic factors, assuming that monozygotic and dizygotic twins share their common environment to the same extent (5-7).

Research has proven that this classical twin model is ideal to use in low-level variation analysis, such as the variation that might be present in the mitochondrial sequence (6). The mitochondrion is a critical cellular organelle for the metabolism, thermogenesis, cell signalling, apoptosis and the control of multiple biochemical pathways as for example the urea cycle and the tricarboxylic acid (TCA) cycle (8-10). However, the most important role of mitochondria is controlling the oxidative phosphorylation, since their primary function is to generate energy in the form of adenosine triphosphate (ATP) (8). Although this energy is indispensable to all forms of life, by-products of this energy production can damage almost every cell in the body. During the generation of ATP, the mitochondria consume oxygen and substrates, which in turn produces reactive oxygen species (ROS) or free radicals. Too much ROS is harmful to the DNA, proteins, and lipids present in the body, and causes so-called oxidative stress (8, 11).

The human mitochondrial DNA (mtDNA) is a double-stranded, closed and circular molecule consisting of 16,569 nucleotides (10, 12). Together, these nucleotides encode for the 13 subunits of the components for the oxidative phosphorylation, the two ribosomal RNAs (rRNAs) and the 22 transfer RNAs (tRNAs). In any of these structures, mutations might be apparent (10). The mtDNA is exceptionally vulnerable to mutations since it lacks the protection mechanisms of the genomic DNA, like histones (13, 14). It is clear that mtDNA has some kind of a repair system, although not sufficient to counteract all the mutational damage (15).

The presence of mutations in the mitochondrial sequence can lead to a heterogenic population of sequences between cells or in different tissues (16). This heterogeneity in mitochondria is called heteroplasmy, which is defined as the mixed population of sequences of mtDNA in tissues or cells, and it is mostly expressed as the percentage of minor-allele genomes that are present in the tissue studied (13, 16, 17). This is also called the minor allele fraction (MAF), and it is known that a higher MAF or level of heteroplasmy can be related to disease (6, 13, 17). This phenomenon results from two different causes: (A) the fact that the cell carries a lot of mitochondria and (B) the fact that the mitochondrial genome has a much higher mutation rate than the nuclear genome, about 10- to 17-fold higher (6, 8, 15).

Over 250 pathogenic mtDNA mutations, both point mutations and rearrangements, have been characterized to date, with many more to be yet described. Many of these mutations are said to be involved in a lot of mitochondrial diseases, as for example Leigh's disease (10, 18); mitochondrial diabetes mellitus (MDM) (10, 19); mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (10, 19); and maternally inherited diabetes deafness (MIDD) (16, 20) (**Figure 2**).

A very important implication of the heteroplasmy effect for these diseases is the so-called threshold effect: a minimum amount of mutated mtDNA is necessary before any defects or dysfunction can become apparent. Although different for each mutation and amongst diverse tissues, this threshold is situated somewhere between 60 and 90% mutant to wild type mtDNA (15).
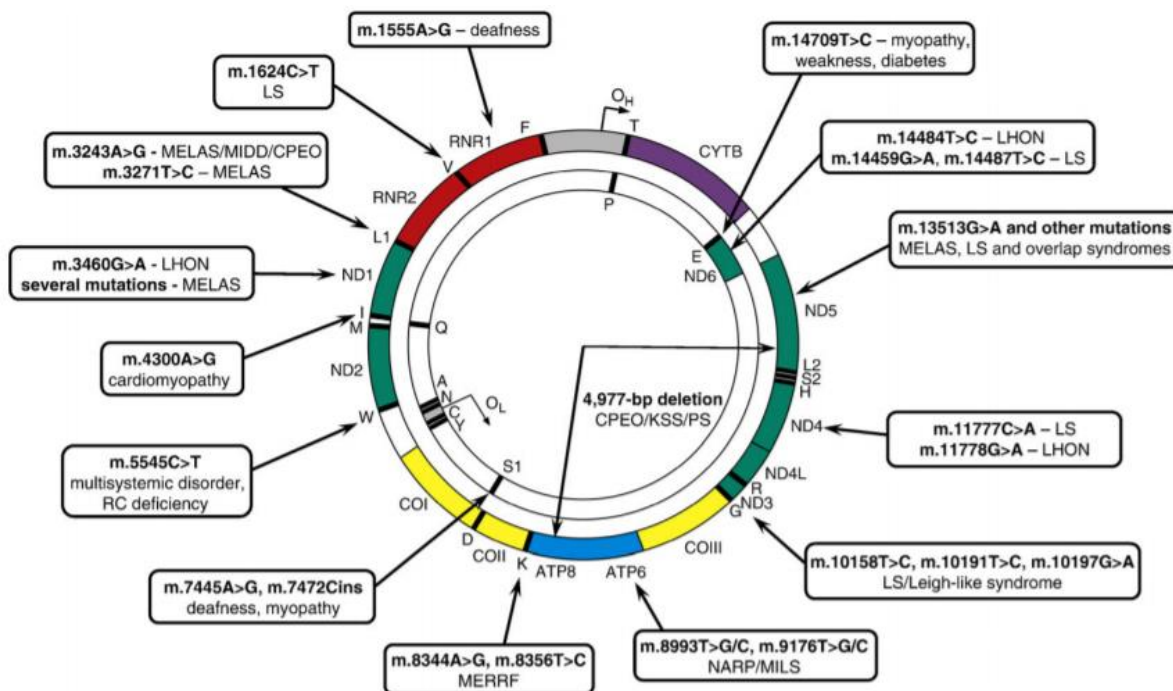
Figure 2. Most important mtDNA mutations and their associations with mitochondrial disease. The circular, double-stranded human mitochondrial genome is depicted with all genes, some of the most common mutations and their associated pathologies. CPEO, chronic progressive external ophthalmoplegia; LHON, Leber Hereditary Optic Neuropathy; LS, Leigh Syndrome; MELAS, Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis , and Stroke-like episodes; MERRF; Myoclonic Epilepsy and Ragged Red Fibres; MILS, Maternally Inherited Leigh Syndrome; NARP, Neurogenic weakness, Ataxia, and Retinitis Pigmentosa; PS, Pearson syndrome (15).

The involvement of mtDNA mutations in mitochondrial diseases is clear, but the role of these mutations in more common, complex disorders has not been unquestionably proven (12, 13, 17, 21). Given the essential function of mitochondria for the generation of energy and the fact that they are more prone to mutations, there is a possibility that the malfunctioning of mitochondria might contribute to cellular energy imbalance (22), leading to different kinds of metabolic disorders (11, 22, 23). These mutations might influence the metabolic system in different ways, for example by coupling the mitochondrial electron transport too tight or by interfering with mitochondrial function (13, 17, 21). It is also proven that mitochondrial dysfunction results in a dysregulation of lipid and glucose metabolism, which are characteristics of metabolic diseases (21).

Therefore, this research is focused on obesity and its association with mitochondrial mutations and heteroplasmy. By the World Health Organization (WHO), obesity is defined as an abnormal or excessive accumulation of fat with possible impairment of health. In 2016, more than 1.9 billion adults worldwide were overweight and in this group, 650 million adults were obese and for children and adolescents, the amount of overweight and obese persons has risen dramatically (24). For adults, obesity is defined as a body mass index (BMI) greater than or equal to 30, while overweight is classified as having a BMI greater than or equal to 25 (24). Not only does obesity cause major discomfort for the patient, but it might also elevate the risks for comorbidities such as cardiovascular diseases (8, 24, 25), diabetes (8, 24, 26), respiratory problems (24, 27) and even some types of cancer (8, 24).

The most important cause of obesity is an imbalance between the number of calories consumed and expended (24). This energy imbalance might be provoked by many factors, as for example environmental, behavioural and genetic factors, but the molecular mechanisms have not been fully elucidated yet (26).

Although the genetic background of obesity has been greatly appreciated, this heritability might be overestimated (28). Other mechanisms might be the missing link in order to explain the full genetic inheritability (29). As mentioned above, given the crucial function of mitochondria in the energy metabolism, it might be plausible that the deregulation of the mitochondrial function could play an important role in obesity and also in its comorbidities. Therefore, Yang *et al.* suggest that genetic variants of mtDNA might be very important in the susceptibility to obesity, a topic that has been studied by various research groups (**Table 1**) (8, 11, 13, 28, 30, 31).

Flaquer *et al.* also researched the association of mtDNA variants and BMI (8). They found that several significant SNPs were located on the NADH subunit dehydrogenase (ND) gene of complex I. The ND gene is central to energy production and its malfunctioning might be very important in obesity pathogenesis (8, 9, 32). Its malfunctioning may result in a decreased activity of complex I or an increased production of ROS, both detrimental for energy production (8, 9). One of the mutations studied by Flaquer *et al.*, MT-ND4L$_{10550A \to G}$, is a mutation in association with BMI ($p = 2.8 \times 10^{-4}$) (8). Another mutation, studied by Saxena *et al.*, MT-ND5$_{13780A \to G}$, was also significantly associated with BMI ($p = 0.025$), but other associations with type 2 diabetes, the insulinogenic index or cholesterol levels were not significant (22).

The last mutation that will be used to test the association with BMI, MT-ND4$_{11719A \to G}$, was not significantly associated with either BMI or parameters that predict metabolic diseases. However, this mutation is, to our knowledge, the mutation with the highest grade of heteroplasmy (MAF = 49.7%), which might also be very interesting to study in this setting (22). Unfortunately, other research groups failed to replicate significant associations between mtDNA variants and BMI or found associations with other metabolic parameters (11, 13, 22, 28, 31).

Table 1. Research studying the association between mtDNA variants and obesity/BMI in different ethnicities during different stages of life. Details of the population, number of samples, analysed mtDNA variants and most interesting results have been listed. SNP, single nucleotide polymorphism; mtDNA, mitochondrial DNA. Adapted from Ebner *et al.* (30).

Population studied	Number of samples	Analysed mtDNA variants	Results	Reference
Germans	6582 adults	984 mtDNA SNPs	Association with obesity found for mt3336, mt4851, mt9698, mt10550 and mt6663.	(8)
Germans and Frenchmen	192 extremely obese children, 192 lean adults	252 D-loop polymorphisms	Association with obesity found for mt16292 and mt16189.	(11)
	1158 obese children and adolescents, 453 adult controls	35 mtDNA SNPs	Association with obesity found for mt8994. Confirmation of these results failed.	(11)
European-Americans	1080 obese children, 2500 lean children	138 mtDNA SNPs	No association with obesity for any SNP and no differences in heteroplasmy.	(13)
Caucasians of Northern European origin	2286 adults	72 mtDNA SNPs	Mt4823 and mt8873 associated with lower BMI and reduced body fat mass.	(28)
Americans	7219 adults	40 mtDNA SNPs	No association with obesity.	(31)

Nonetheless, not all recent publications are approving a possible relationship between mtDNA variants, heteroplasmy, and obesity (11, 13, 31). Grant *et al*. even state a lack of relationship between mitochondrial heteroplasmy and obesity. Even though they believe that the mitochondrial genome could play a role in obesity, they failed to identify differences in genotype between obese and lean individuals (11, 13). It is clear that these publications do not see eye to eye in their conclusions and that more research is needed. Different research groups have all together analysed over 1000 mtDNA single nucleotide polymorphisms (SNPs) (**Table 1**). Of these, only a few mutations have gained significant results, of which most of them failed to stay significant after correction for multiple testing.

On the other hand, there has been no research studying the association of mtDNA variants and birth weight. Lately, the so-called germ-line bottleneck has received a lot of attention (15, 33). This bottleneck is responsible for the transmission of mtDNA, and thus also mtDNA mutations, from the mother to her offspring. The fact that mtDNA is inherited from mother to offspring has been an established fact for many years, however, new research abolishes this fact (34). For deleterious heteroplasmic mutations, the possibility of a bottleneck might have serious consequences: a low frequency of a particular variant in the mother might be transformed into a high frequency of mutations in her offspring. This might cause life-threatening diseases or might be implied in different kinds of complex diseases (33). Since mtDNA mutations have been observed to accumulate with age, it might be of great importance to detect these variants at an early age (12).

Apart from the fact that the association between mtDNA variants, heteroplasmy, and BMI or obesity has not been unambiguously proven, the nature of this mutation is even more of a mystery. As mentioned before, the twin study design is very useful to determine the environmental or genetic nature of a mutation (5, 35). An extra advantage of twin research is the fact that very low levels of variation between twins can be detected in the mitochondrial sequence (6). By comparing monozygotic and dizygotic twins and their concordance for a particular trait, it is possible to conclude whether the mutation was inherited or acquired (35). This will contribute to more understanding of the heritability of these mitochondrial mutations and the background of complex, metabolic diseases like obesity (28). As mtDNA variation has an impact on many biological processes and might be implied in different pathologies, more knowledge about the variation and heteroplasmy could give more insights in mutation and selection (6).

As mentioned before, an advantage of the use of twin studies is the fact that low levels of variation can be detected (6). A technique that allows for the detection of these low variation levels is Droplet Digital™ PCR (ddPCR™). This technique enables the detection and analysis of nucleic acids at a level of precision and sensitivity not matched by other techniques before (36, 37).

In regard to all the evidence shown before, it is questioned whether these specific mtDNA mutations are associated with birth weight and/or BMI in children. Together, all of these studies deliver conflicting evidence that there are mutations of the mitochondrial genome that are associated with metabolic processes in the body. We thus hypothesize that these specific mtDNA mutations, namely MT-ND4L$_{10550A \rightarrow G}$, MT-ND4$_{11719A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$, are mainly determined by the genetic inheritance in twins and that they are indeed associated with birth weight and BMI.

The following objectives will be investigated during this project:

- Mutational analysis using Droplet Digital™ PCR for MT-ND4L$_{10550A\rightarrow G}$, MT-ND5$_{11719A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$.

- Investigation of the association of a mutation of MT-ND4L$_{10550A\rightarrow G}$ and the birth weight and/or BMI in twins aged four.

- Investigation of the association of a mutation of MT-ND4$_{11719A\rightarrow G}$ and the birth weight and/or BMI in twins aged four.

- Investigation of the association of a mutation of MT-ND5$_{13780A\rightarrow G}$ and the birth weight and/or BMI in twins aged four.

- Determination of the genetic or environmental nature of the observed mtDNA mutations in twins.

2 Materials & methods

2.1 Study population, data collection and sample collection at birth

The Limburg Twin Study is a prospective cohort study that recruited 43 newborn twins at the East-Limburg Hospital in Genk between April 1, 2014, and January 24, 2017. Mothers that were interested in participating in the Limburg Twin Study provided written informed consent and whether they were able to fill out questionnaires in Dutch, was the only inclusion criterium. These questionnaires provided detailed information about age, socioeconomic status, ethnicity, smoking behaviour, residential details, BMI before the pregnancy and parity (Table 2). Reasons not to participate in the study were the following: failure to ask for participation, failure to fill in questionnaires, complications and/or emergency caesarean section. This study was approved by the ethical committee of East-Limburg Hospital (Genk, Belgium) and Hasselt University (Hasselt, Belgium).

Maternal education was categorized as either 'low' (no diploma, primary school or high school) or 'high' (college or university degree). Based on the information on the ethnicity of the twins, the twin was either European (when two or more grandparents were European) or non-European (when at least three grandparents were of non-European ancestry).

Samples of placental tissue (n = 80) and umbilical cord blood (n = 78) were collected right after delivery. Also, perinatal parameters such as the sex of the newborn, information about the zygosity and chorionicity, date of birth, birth weight and length, head circumference, and gestational age were collected. Cord blood was collected immediately after delivery in Vacutainer® Plus Plastic K2EDTA Tubes (Bd, Franklin Lakes, NJ, USA). Samples were centrifuged at 3,200 rpm for 15 minutes to divide the sample into red blood cells (RBC), buffy coat and plasma. These substances were immediately frozen, first at -20°C and afterwards at -80°C for storage until use. Placentas were obtained in the delivery ward. Biopsies were taken immediately after birth or the placenta was frozen first before taking the biopsies. Biopsies of the villous tissue were taken from the foetal side of the placenta and were stored at -80°C.

2.2 Study population, data collection and sample collection at the age of four

In January 2019, a major part of the studied twins had already turned four years of age and they were re-invited for a follow-up visit in a child-friendly room, either at the University of Hasselt (Diepenbeek, Limburg, Belgium) or the Field Research Center of the University of Hasselt (Maasmechelen, Limburg, Belgium).

Biological samples, as for example nails, buccal cells, urine and blood, were collected and questionnaires were provided to obtain updated information of the twins. Also, anthropometric and cardiovascular parameters were tested, alongside cognitive testing. The parameters particularly valuable for this research were the weight, measured with the Omron Body Composition Monitor BF511; the height, measured with a stadiometer; and the abdominal circumference, measured with measuring tape. These parameters are used to calculate the BMI of the child. The body mass index or Quetelet index is a measure for the human body shape and it is defined as the body weight of the individual divided by the square of the height (kg/m²) (8). However, since the definitions of childhood overweight and childhood obesity are complicated, an adapted version of BMI, the BMI z-score was used in this research (38).

2.3 Literature search for eligible mutations

All relevant literature in the PubMed database was studied until 02/22/2019. The search term used was "mitochondrial DNA obesity [Title]". This search of all English-language literature was extended with a manual search of the cited references of the selected articles. All studies performed on human subjects, until February 2019 and describing possible mtDNA variants in association with either BMI or obesity were included. Exclusion of studies was based on any of the following reasons: no mtDNA variants discussed, only mtDNA haplogroups discussed or use of animal models and/or cell cultures.

2.4 DNA isolation from cord blood samples

DNA was extracted from cord blood samples (n = 63) and whole blood samples from the mother (n = 28) using the Qiagen QIAamp DNA Mini and Blood Mini Kit (Qiagen, Venlo, the Netherlands). The following adaptations were made: the incubation of the samples at 56°C was carried out for an hour instead of ten minutes and the second incubation step with the AE buffer was carried out for five minutes.

Purified DNA was quantified on a NanoDrop ND-1000 UV-Vis spectrometer (Thermo Scientific, Wilmington, DE, USA). After measurements, the samples were further diluted to a concentration of 30 ng/µl and both undiluted and diluted DNA were stored at -20°C until further use.

2.5 Mutational analysis with Droplet Digital™ PCR

The used ddPCR™ system included the QX200™ Droplet Generator, the QX200 Droplet Reader, C100 Touch™ Thermal Cycler with 96-Deep Well Reaction Module and PX1™ PCR Plate Sealer (Bio-Rad, Temse, Brussels, Belgium). ddPCR™ was performed with purified DNA previously obtained using newly designed ddPCR™ assays (**Figure 3**). These ddPCR™ assays for the detection of mutations were run across a thermal gradient (50-60°C) to determine the optimal annealing temperature. Before the actual handling of the samples, the optimal DNA concentration for both the samples and the positive controls, and limit of detection (LOD) were also determined.

For mtDNA mutational analysis, the custom assays were ordered from Bio-Rad as well. Sequences and other information can be obtained from www.bio-rad.com with the following ID numbers: MT-ND4$_{11719A \to G}$ (assay ID: dMDS913515317), MT-ND4L$_{10550A \to G}$ (assay ID: dHsaMDS169630047) and MT-ND5$_{13780A \to G}$ (assay ID: dHsaMDS257109922) (**Appendix 1**). For each assay, a suitable positive control, namely gBlocks Gene Fragments, was ordered from Integrated DNA Technologies (IDT, Leuven, Flemish Brabant, Belgium) (**Appendix 1**).
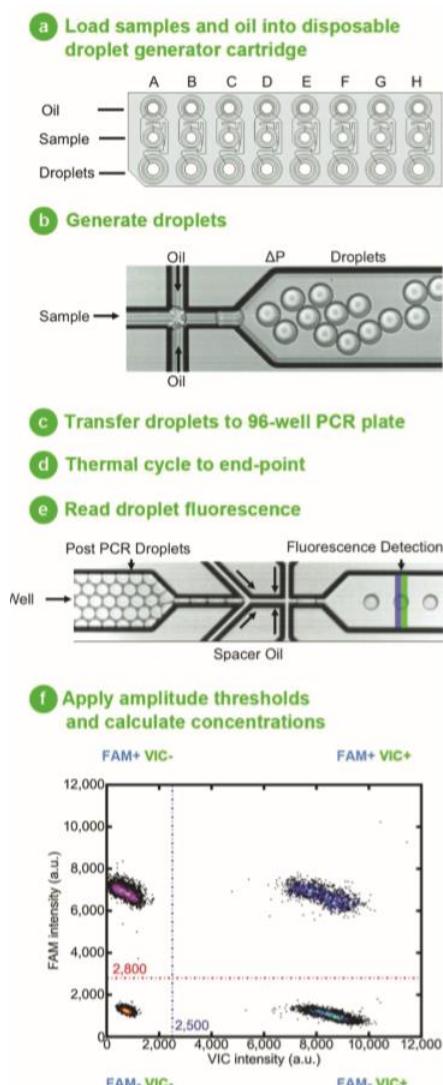
Figure 3. Droplet digital PCR workflow (1).

The mutant target and wild type probes were mixed together and provided in a single tube, with FAM probes targeting the mutant allele and HEX probes targeting the wild type allele. The ddPCR™ method was performed according to the instructions provided by the manufacturer, except no restriction enzyme was used (**Figure 3**). Data were analysed using QuantaSoft™ Software (Bio-Rad, Temse, Brussels, Belgium).

2.6 Statistical analysis

The statistical analysis will be carried out using SAS software (Version 9.4; SAS Institute, Cary, NC, USA). All reported p-values are two-sided and are significant when $p \leq 0.05$. For the statistical analysis, the heteroplasmy values, in copies/µl, were categorized (**Table 2**). For the investigation of the association between the prevalence of the mutations in both mothers and offspring and the birth weight or BMI z-score, mixed modelling was used. The twins were analysed as individuals in a multilevel regression analysis to account for relatedness between twin members by the addition of a random intercept to the model. Restricted maximum likelihood estimation (REML) was used to estimate the coefficients and standard errors and using the LSMEANS option, we studied mean values of either birth weight, BMI or BMI z-scores, together with 95% confidence intervals (CI).

Table 2. Categories of heteroplasmy. The different levels of heteroplasmy in MT-ND4L$_{10550A \rightarrow G}$ were grouped as following: none (no heteroplasmy levels detected), low (less than 0.8 copies/µl), moderate (between 0.8 and 1.0 copies/µl) and high (more than 1.0 copies/µl). The different levels of heteroplasmy were grouped as following for MT-ND5$_{13780A \rightarrow G}$: none (no heteroplasmy levels detected), low (less than 0.8 copies/µl and high (more than 0.8 copies/µl). Dependent on the analysis performed, fewer observations than the total number will be used, based on other missing values.

	Mother	Twin
MT-ND4L$_{10550A \rightarrow G}$	n = 28	n = 63
None (< 0 copies/µl)	n = 18 (64.3)	n = 41 (65.1)
Low (< 0.8 copies/µl)	n = 5 (17.9)	n = 13 (20.6)
Moderate (≥ 0.8 – 1.0 copies/µl)	n = 3 (10.7)	n = 4 (6.4)
High (≥ 1.0 copies/µl)	n = 2 (7.1)	n = 5 (7.9)
MT-ND5$_{13780A \rightarrow G}$	n = 28	n = 63
None (< 0 copies/µl)	n = 6 (21.4)	n = 15 (23.8)
Low (< 0.8 copies/µl)	n = 13 (46.4)	n = 32 (50.8)
High (≥ 0.8 copies/µl)	n = 9 (32.2)	n = 16 (25.4)

Covariates were selected *a priori*, but differed for the analyses at the different levels, namely at birth and at follow-up. For the association between MT-ND4L$_{10550A \rightarrow G}$, MT-ND5$_{13780A \rightarrow G}$ and the BMI of the mother, the generalized linear model was adjusted for parity, maternal educational level, and maternal age. For the association between MT-ND4L$_{10550A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$ in the twins themselves and the birth weight of the twins, the mixed model was adjusted for zygosity, chorionicity, maternal educational level, maternal age, gestational age. For the association between MT-ND4L$_{10550A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$ in the mother and the birth weight of the twins, the previous mixed model was further adjusted for parity and weight before pregnancy. At follow-up, BMI z-scores were calculated, since these scores are more trustworthy and precise than the general calculation of BMI as in adults (38). This analysis was performed with the statistical software R (Version 3.4.1; R Project for Statistical Computing, Vienna, Austria) using the WHO Child Growth Standards R igrowup package. In short, this package provides the z-score for BMI-for-age based on the WHO Child Growth Standards and corrected for age and gender of the child. This z-score for BMI-for-age was subsequently used in a mixed model, which was only corrected for maternal education since the BMI z-score was already corrected for gender and age of the child.

3 Results

3.1 Study population, data collection, and sample collection

The entire Limburg Twin Study comprises 43 twin pairs (**Appendix 2**). For this research, we used cord blood samples of a subpopulation of the Limburg Twin Study, including 17 male and 40 female newborns (n = 57) (**Table 3; Figure 4**). The mothers (n = 28) had an average age of 31 ± 4.1 years, ranging from 23 to 39 years. The mothers had a mean pre-gestational BMI of 25.1 ± 4.7 kg/m². In this study population, 82.5% was classified as Europeans. Birth weight of the twins averaged 2386 ± 441.7 g.

Of the 43 twins that were recruited at birth, 25 twins were eligible for a follow-up visit around the age of four. Of these 25 twins, 14 twin pairs have visited the University of Hasselt (**Table 3; Figure 4**). The overall participation rate was 56 %. Reasons not to participate in the follow-up were: failure to ask for participation and failure to contact (n = 4), failure to find a suited appointment (n = 3), not interested (n = 2) or failure to contact the mothers after cancellation of an appointment (n = 2).

The study population used in the follow-up of the Limburg Twin Study included 7 boys and 12 girls (n =19). The mothers (n = 10) had an average age at follow-up of 37 ± 4.0 years, ranging from 31 to 43 years. The mothers had a mean BMI of 24.9 ± 3.4 kg/m². The children of the twin pairs had an average BMI z-score of 0.6 ± 0.7 kg/m². Weight at follow-up averaged 17.5 ± 1.9 kg and length at follow-up averaged 103.9 ± 4.3 cm.

Table 3. Characteristics of the study population of the Limburg Twin Study. On behalf of missing values, some statistical models use less than the maximum study population.

	Birth	Follow-up
	n (%) or mean [10th – 90th]	n (%) or mean [10th – 90th]
Mothers	**n = 28**	**n = 10**
Parity		
1	11 (39.3)	4 (40.0)
> 1	17 (60.7)	6 (60.0)
Age, years	31 [25 – 37]	37 [31.5 – 42]
BMI, kg/m²	25.1 [20.6 – 32.2]	24.9 [20.7 – 29.8]
Maternal education [a]		
Low	15 (53.6)	5 (50.0)
High	13 (46.4)	5 (50.0)
Newborn/child	**n = 57**	**n = 19**
Complete pairs	54 (94.7)	18 (94.7)
Gestational age, weeks	36 [32 -38]	
Age		4.3 [3.83 – 4.75]
Sex		
Female	40 (70.2)	12 (63.2)
Ethnicity [c]		
European-Caucasian	47 (82.5)	19 (100.0)
Zygosity		
Monozygotic	24 (42.1)	8 (42.1)
Dizygotic	33 (57.9)	11 (57.9)
Chorionicity		
Dizygotic-dichorial	36 (63.2)	11 (57.9)
Monozygotic-monochorial	2 (3.5)	2 (10.5)
Monozygotic-dichorial	12 (21.1)	4 (21.1)
Undetermined	7 (12.2)	2 (10.5)
Birth weight, g	2386 [1759 – 3045]	
BMI z-score, kg/m²		0.6 [-0.21 – 1.67]
Length, cm		103.9 [98.5 – 110]
Weight, kg		17.5 [14.5 – 21.1]
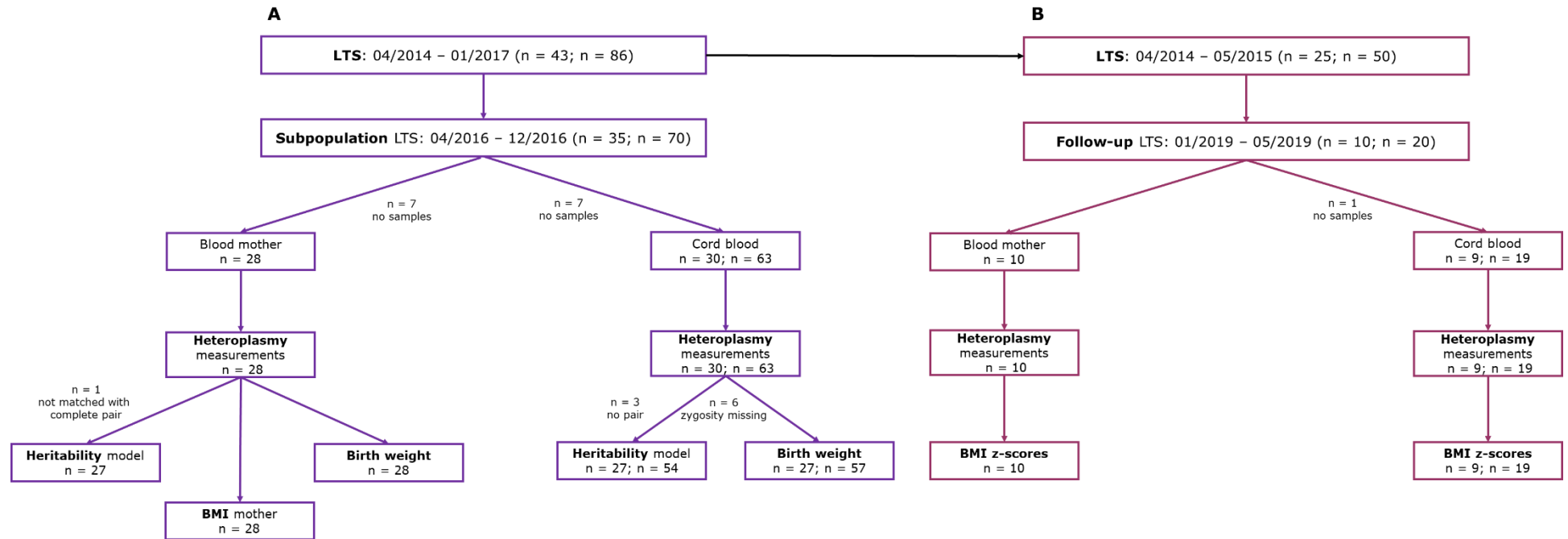
Figure 4. Flowchart for the selection of subpopulations of the Limburg Twin Study. Numbers in the flowchart indicate either the amount of mothers (n = X) or the amount of complete twin pairs and the amount of individuals (n = X; n = X) **A:** The Limburg Twin Study recruited 43 twins (n = 86) and 43 mothers at birth from April 1, 2014, to January 24, 2017. Of this group, a subpopulation (n = 35; n = 70) was chosen for DNA extractions, however, not all children (n = 7) and mothers (n = 7) has samples available. For the heteroplasmy measurements, the samples of 30 complete pairs and 3 siblings (n = 57) and 28 mothers were measured. The models that corrected for heteroplasmy levels of the mother always used all samples analysed for heteroplasmy (n = 28), except for the heritability model (n = 27). However, for the twins, heritability was only estimated for the 27 complete pairs (n = 54) that had zygosity information present. For the model that used the birth weights of the twins, 27 complete pairs and three siblings (n = 57) were used. **B:** From the whole Limburg Twin Study (n = 43), 25 twin pairs (n = 50) and 25 mothers were eligible for a follow-up visit around the age of four. A subpopulation (n = 10; n = 20) from the 14 twin pairs (n = 28) that had a follow-up visit, was used for statistical analysis. However, for one sibling, samples were missing, so the heteroplasmy measurements were only carried out for nine complete twin pairs and one sibling (n = 19). BMI z-scores were calculated for nine complete pairs and one sibling (n = 19) and adjusted for the mother (n = 10).

3.2 Literature search for eligible mutations

The search term used was "mitochondrial DNA obesity [Title]", resulting in a total of five papers. In addition, the references for these papers were searched for other relevant papers, which gained an additional amount of four papers (**Figure 5**). These nine papers together yield a total amount of 308 mtDNA variants, of which 109 mtDNA variants were present in more than one paper. Of these 199 unique mtDNA variants, the MAF is known for only 61 variants (**Appendix 3**).
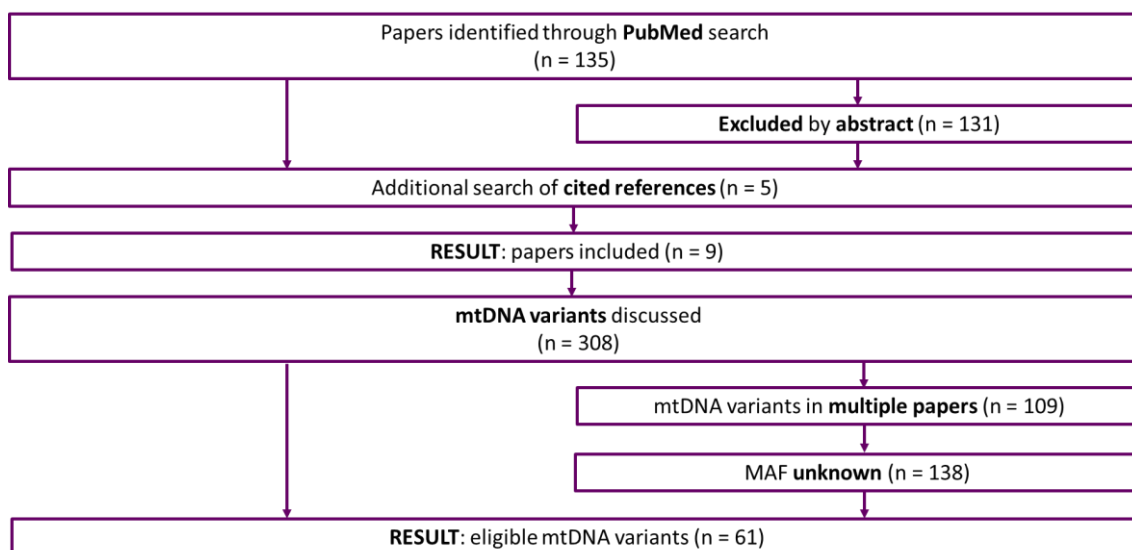
Figure 5. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart of included papers. The search started with 135 papers and after extensive reduction, only five papers were included. An additional search of references of these five papers delivered an additional four papers, bringing the total to nine papers. These nine papers yielded a total of 308 mtDNA variants, of which 199 mutations were unique. Of only 61 mtDNA variants, the MAF was known (Appendix 3). mtDNA; mitochondrial DNA, MAF; minor allele frequency.

3.3 Optimization of the mutational analysis with Droplet Digital™ PCR

3.3.1   Determination of the optimal annealing temperature

In order to determine the optimal annealing temperature for all three assays, a thermal gradient was performed from 50°C to 60°C. The optimal annealing temperature for all three assays was around the temperature of 54°C (**Appendix 4.1**).

3.3.2   Determination of the optimal DNA concentration

For the quantification of mtDNA mutations, it is important to determine the optimal DNA load. Starting from a concentration of 10 ng/µl DNA, the DNA was diluted in different steps: 10 ng/µl, 5 ng/µl, 2 ng/µl, 0.2 ng/µl, 0.1 ng/µl, 0.02 ng/µl, 0.005 ng/µl, 0.0025 ng/µl, 0.002 ng/µl, 0.00125 ng/µl, 0.000625 ng/µl, 0.0003125 ng/µl, 0.00015625 ng/µl, 0.000125 ng/µl, 0.0000125 ng/µl and 0.00000125 ng/µl. The optimal DNA concentration was set at 0.00125 ng/µl (Appendix 4.2). For the concentration of the positive control (gBlocks), a dilution of 1:40 000 was recommended. However, extra dilutions of 1:400 000 and 1:4 000 000 were needed. The dilution of 1:4 000 000 was optimal (**Appendix 4.2**).

3.3.3   Spiking experiment

To determine the limit of detection (LOD) and limit of quantification (LOQ), a spiking experiment was performed. The obtained thresholds were used to obtain the amount of copies per microliter in the actual sample plates (**Appendix 4.3**). This spiking experiment was used to determine the mutant thresholds as well as the wild type thresholds.

3.4 Mutational analysis with Droplet Digital™ PCR

For MT-ND4L$_{10550A \rightarrow G}$, heteroplasmy levels of the mutant allele were found in 22 of the 63 twin samples (34.9%) and 10 of the 28 mother samples (35.7%) (**Figure 6; Appendix 5**). For a mutant threshold of 6000, copies per microliter ranged from 0.1 to 454 for the twin samples and from 0.24 to 87 for mother samples. In the statistical analysis, the samples for each individual of the twin and for the mother were grouped based on the amount of copies per microliter, which resulted in four different groups: no heteroplasmy detected (0 copies/µl), heteroplasmy levels lower than 0.8 copies/µl, heteroplasmy levels equal to or higher than 0.8 and lower than 1.0 copies/µl and heteroplasmy levels equal to or higher than 1.0 copies/µl.
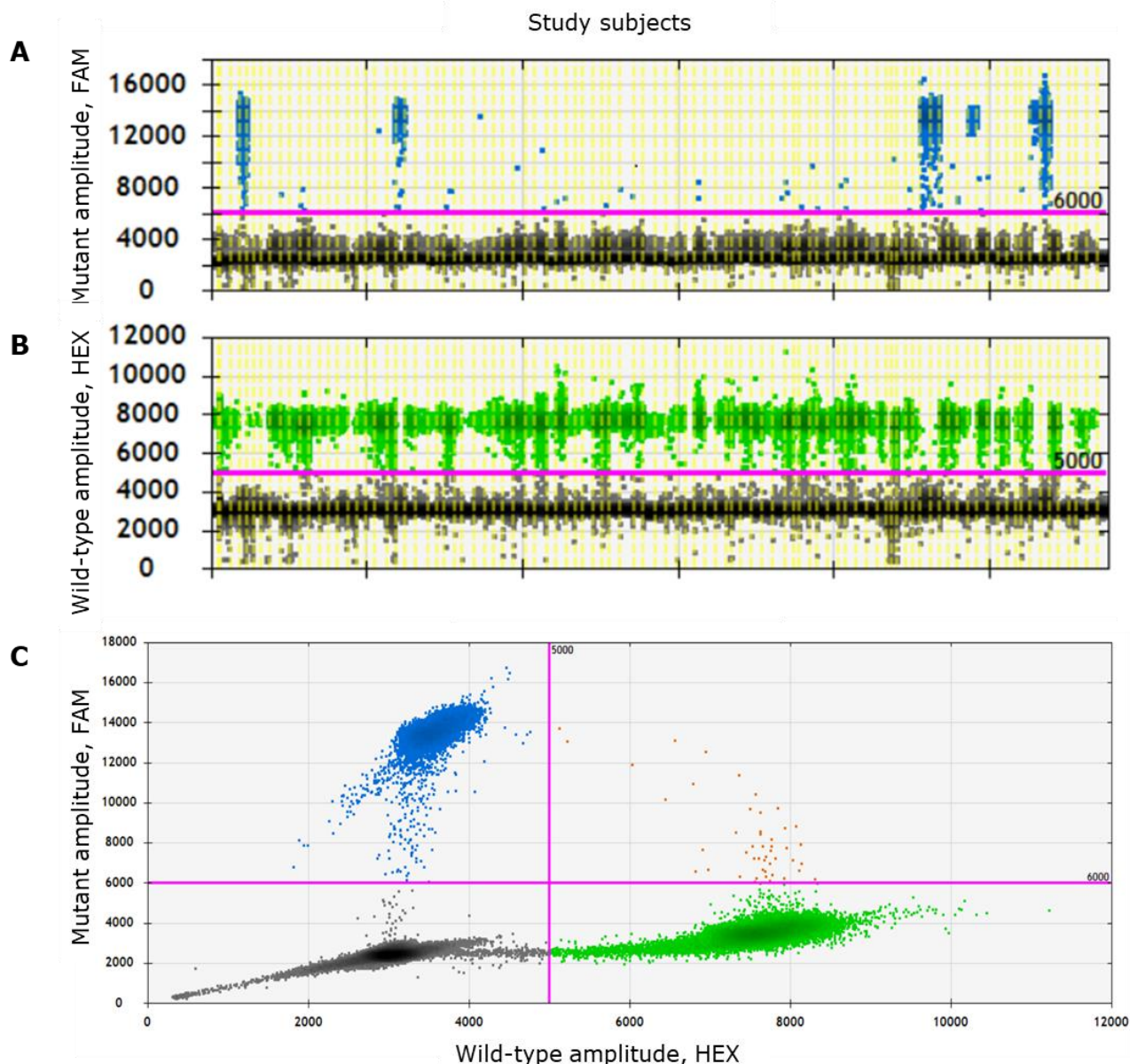
Figure 6. Mutational analysis for MT-ND4L$_{10550A \rightarrow G}$ **A:** 1D scatter plot of ddPCR™ results of twin samples for MT-ND4L$_{10550A \rightarrow G}$. The y-axis shows the event number, while the x-axis shows the fluorescent amplitude for the mutant allele. The threshold for the mutant allele was set at 6000 as determined with the spiking experiment. **B:** 1D scatter plot of ddPCR™ results of twin samples for MT-ND4L$_{10550A \rightarrow G}$. The y-axis shows the event number, while the x-axis shows the fluorescent amplitude for the wild type allele. The threshold for the mutant allele was set at 6000 as determined with the spiking experiment, where the threshold for the wild type allele was set at 5000. **C:** 2D scatter plot of ddPCR™ results of twin samples for MT-ND4L$_{10550A \rightarrow G}$. The y-axis shows the fluorescence amplitude of the FAM probe, which only hybridizes to the mutant allele (blue). The x-axis shows the HEX probe (green), which only hybridizes to the wild-type reference allele. The orange droplets show double-positive droplets, which both carry the mutant and the wild-type reference allele. The gray droplets show double-negative droplets, which do not carry any reference allele.

14

For MT-ND4$_{11719A \to G}$, the plate has failed (**Figure 7**). In both twin and mother samples, no heteroplasmy levels have been detected using the threshold levels obtained in the spiking experiment. Therefore, the heteroplasmy of this mutation was not included in the statistical analysis.
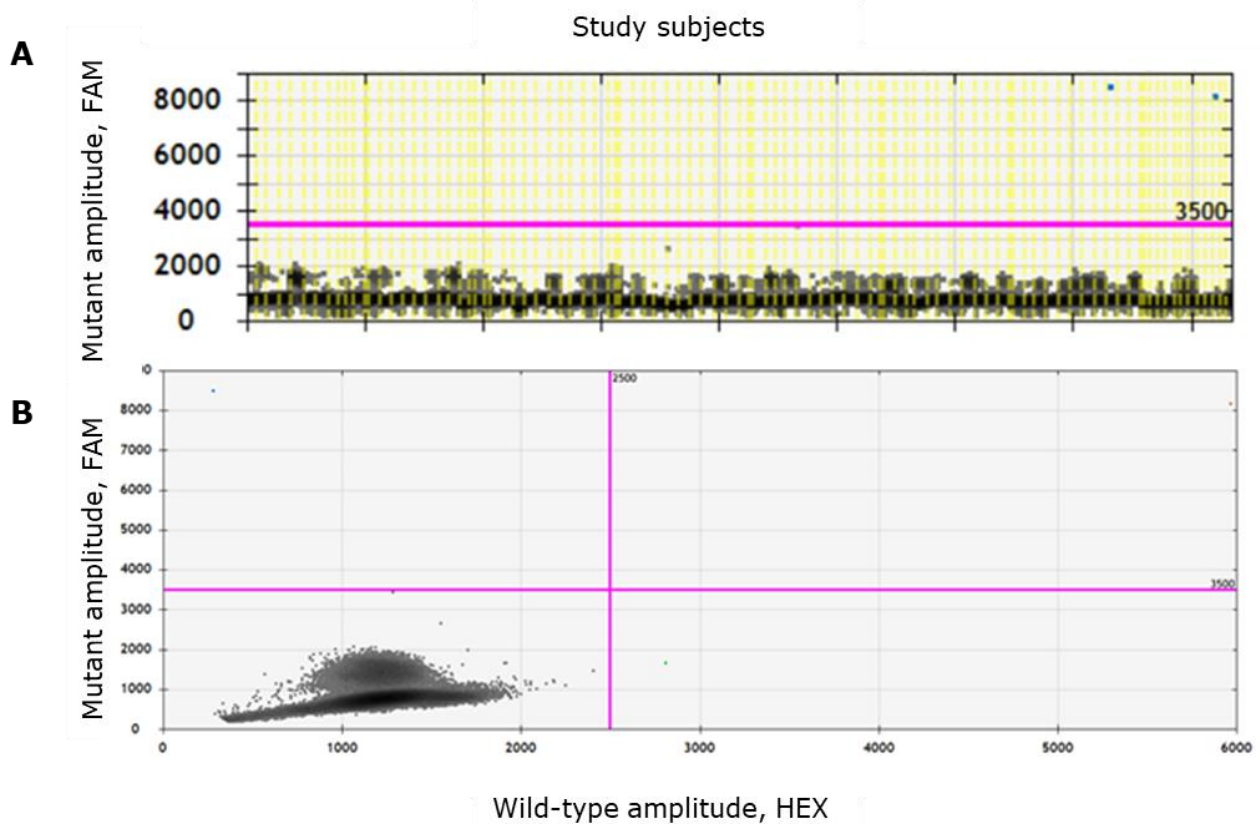
Figure 7. Mutational analysis for MT-ND4$_{11719A \to G}$ A: 1D scatter plot of ddPCR™ results of twin samples for MT-ND4$_{11719A \to G}$. The y-axis shows the event number, while the x-axis shows the fluorescent amplitude for the mutant allele. The threshold for the mutant allele was set at 3500 as determined with the spiking experiment, where the threshold for the wild type allele was set at 2500. **B:** 2D scatter plot of ddPCR™ results of twin samples for MT-ND4$_{11719A \to G}$. The y-axis shows the fluorescence amplitude of the FAM probe, which only hybridizes to the mutant allele (blue). On the x-axis, the fluorescence amplitude of the HEX probe (green) is shown, which only hybridizes to the wild type reference allele. The grey droplets show double-negative droplets, which do not carry any reference allele. This is an indication of failure of the mutational analysis.

For MT-ND5$_{13780A \to G}$, heteroplasmy levels of the mutant allele were found in 48 of the 63 twin samples (76.2%) and 22 of the 28 mother samples (78.5%) (**Figure 8; Appendix 5**). For a mutant threshold of 7000, copies per microliter ranged from 0.1 to 1.4 for the twin samples and from 0.1 to 6.8 for mother samples. In the statistical analysis, the samples for each individual of the twin and for the mother were grouped based on the amount of copies per microliter, which resulted in three different groups: no heteroplasmy detected (0 copies/µl), heteroplasmy levels lower than 0.8 copies/µl and heteroplasmy levels equal to or higher than 0.8 copies/µl.
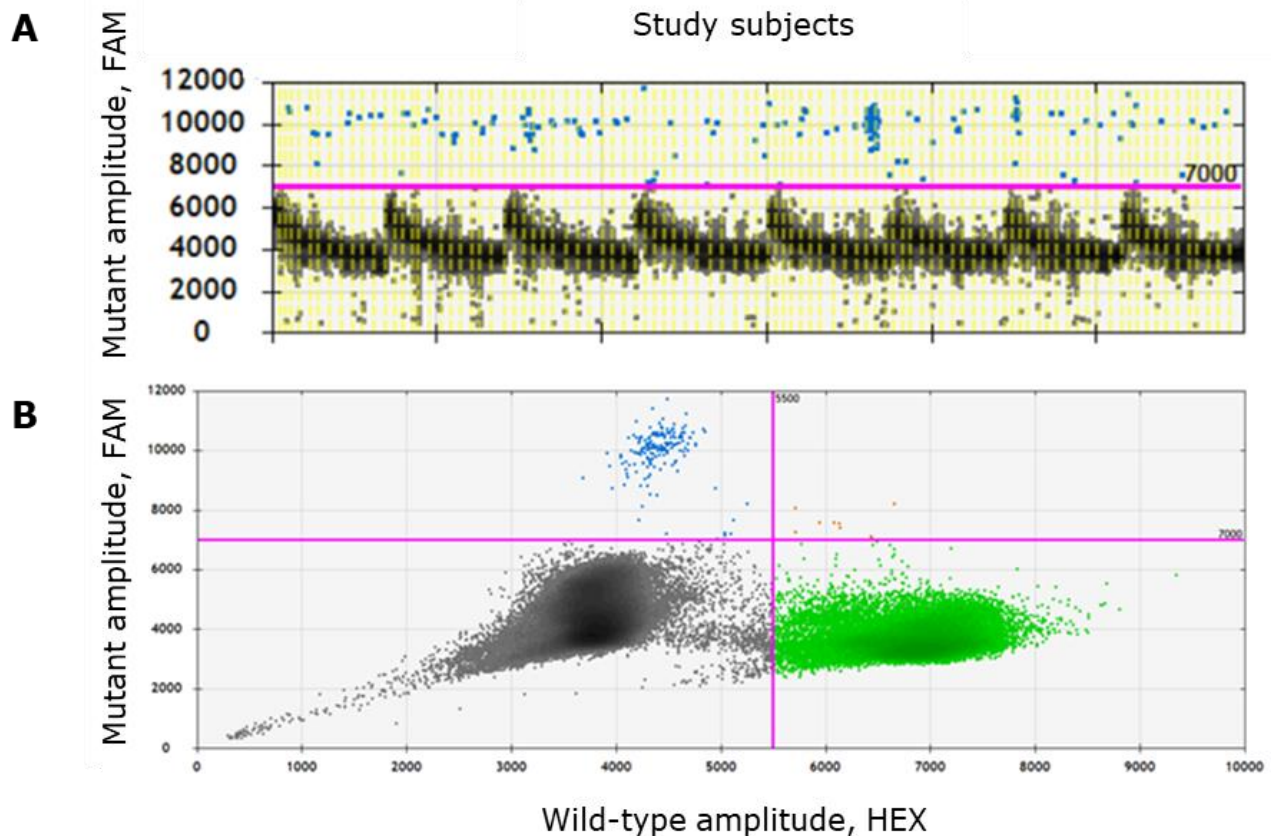
Figure 8. Mutational analysis for MT-ND5$_{13780A \to G}$. A: 1D scatter plot of ddPCR™ results of twin samples for MT-ND5$_{13780A \to G}$. The y-axis shows the event number, while the x-axis shows the fluorescent amplitude for the mutant allele. The threshold for the mutant allele was set at 7000 as determined with the spiking experiment, where the threshold for the wild type allele was set at 2500. **B:** 2D scatter plot of ddPCR™ results of twin samples for MT-ND5$_{13780A \to G}$. The y-axis shows the fluorescence amplitude of the FAM probe, which only hybridizes to the mutant allele (blue). On the x-axis, the fluorescence amplitude of the HEX probe (green) is shown, which only hybridizes to the wild type reference allele. The y-axis shows the fluorescence amplitude of the FAM probe, which only hybridizes to the mutant allele (blue). The HEX probe (green) only hybridizes to the wild type reference allele. The orange droplets show double-positive droplets, which both carry the mutant and the wild type reference allele. The grey droplets show double-negative droplets, which do not carry any reference allele.

3.4.1 Heritability of MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$

To make an assumption about the genetic or environmental background of the different mutations, the amount of mutations in each group were studied for complete twin pairs in both monozygotic and dizygotic twins. For MT-ND4L$_{10550A \to G}$, the groups were classified as follows: none (no heteroplasmy detected), low (less than 0.8 copies/µl), moderate (between 0.8 and 1.0 copies/µl) and high (more than 1.0 copies/µl) (**Table 4**). For the heritability, only complete pairs where both samples of both children underwent mutational analysis were used (n = 27; n = 54), namely 12 monozygotic twins (n = 24) and 15 dizygotic twins (n = 30). A significant effect was seen for the mutation in monozygotic twins ($p < 0.0001$; r = 0.72), but this was not seen in dizygotic twins ($p = 0.41$; r = 0.15).

For MT-ND5$_{13780A \to G}$, the groups were classified as follows: none (no heteroplasmy detected), low (less than 0.8 copies/µl) and high (more than 0.8 copies/µl) (**Table 5**). For the heritability, only complete pairs where both samples of both children underwent mutational analysis were used (n = 27; n = 54), namely 12 monozygotic twins (n = 24) and 15 dizygotic twins (n = 30). A significant effect was seen for the mutation in monozygotic twins ($p = 0.008$; r = 0.52), but this was not seen in dizygotic twins ($p = 0.46$; r = 0.13).

16

Table 4. Heritability table for MT-ND4L$_{10550A\rightarrow G}$ in monozygotic and dizygotic complete twin pairs (n = 27). The different levels of heteroplasmy were grouped as following: none (no heteroplasmy levels detected), low (less than 0.8 copies/µl), moderate (between 0.8 and 1.0 copies/µl) and high (more than 1.0 copies/µl). For every group, twin 1 was compared to twin 2.

Heteroplasmy of MT-ND4L$_{10550A\rightarrow G}$	Monozygotic twins p < 0.0001 r = 0.72				Dizygotic twins p = 0.41 r = 0.15			
Twin 1	None	Low	Moderate	High	None	Low	Moderate	High
Twin 2								
None	**12**	6	0	0	**10**	4	4	2
Low	4	**0**	0	0	4	**0**	0	0
Moderate	0	0	**0**	0	4	0	**0**	0
High	0	0	0	**2**	0	0	0	**2**

Table 5. Heritability table for MT-ND5$_{13780A\rightarrow G}$ in monozygotic and dizygotic complete twin pairs (n = 27). The different levels of heteroplasmy were grouped as follows: none (no heteroplasmy levels detected), low (less than 0.8 copies/µl and high (more than 0.8 copies/µl). For every group, twin 1 was compared to twin 2.

Heteroplasmy of MT-ND5$_{13780A\rightarrow G}$	Monozygotic twins p = 0.008 r = 0.52			Dizygotic twins p = 0.46 r = 0.13		
Twin 1	None	Low	High	None	Low	High
Twin 2						
None	**4**	2	0	**2**	0	2
Low	2	**6**	6	8	**6**	6
High	0	2	**2**	0	4	**2**

Besides the possible genetic or environmental background, the possible transmission of the mutation from the mother to her offspring was studied (**Table 6**). The categories were classified in the exact same manner as previously explained for both MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$, with inclusion of all mothers (n = 27) that had heteroplasmy levels measured in combination with the heteroplasmy levels of the complete twin pair (n = 54). A significant effect for the transmission of the mutation from the mother to her offspring was seen in both MT-ND4L$_{10550A\rightarrow G}$ (p = 0.0006; r = 0.43) and in MT-ND5$_{13780A\rightarrow G}$ (p = 0.008; r = 0.23).

Table 6. Transmission from mother to offspring in MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$. The different levels of heteroplasmy in MT-ND4L$_{10550A\rightarrow G}$ were grouped as follows: none (no heteroplasmy levels detected), low (less than 0.8 copies/µl), moderate (between 0.8 and 1.0 copies/µl) and high (more than 1.0 copies/µl). The different levels of heteroplasmy were grouped as following for MT-ND5$_{13780A\rightarrow G}$: none (no heteroplasmy levels detected), low (less than 0.8 copies/µl and high (more than 0.8 copies/µl).

	MT-ND4L$_{10550A\rightarrow G}$ p = 0.0006 r = 0.43				MT-ND5$_{13780A\rightarrow G}$ p = 0.008 r = 0.23		
Heteroplasmy mother	None	Low	Moderate	High	None	Low	High
Heteroplasmy twin							
None	25	9	3	1	3	9	2
Low	8	0	2	0	6	13	9
Moderate	2	1	0	0			
High	0	0	0	3	2	3	7

3.4.2 Association of MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$ with BMI in mothers

In order to present an indication whether these mutations might impact BMI in later life, both mutations were associated with the BMI of the mothers before pregnancy (**Figure 9**). The groups were classified as mentioned before and all groups were compared to the group without heteroplasmy. For MT-ND4L$_{10550A \to G}$, compared to mothers without a mutation, mothers with the highest level of heteroplasmy had a 9.99 kg/m² higher BMI (95% CI 3.7 to 16.2 kg/m²; p = 0.006). For MT-ND5$_{13780A \to G}$, compared to mothers without a mutation, mothers with a low level of heteroplasmy had a 5.33 kg/m² higher BMI (95% CI 0.3 to 10.3 kg/m²; p = 0.01).
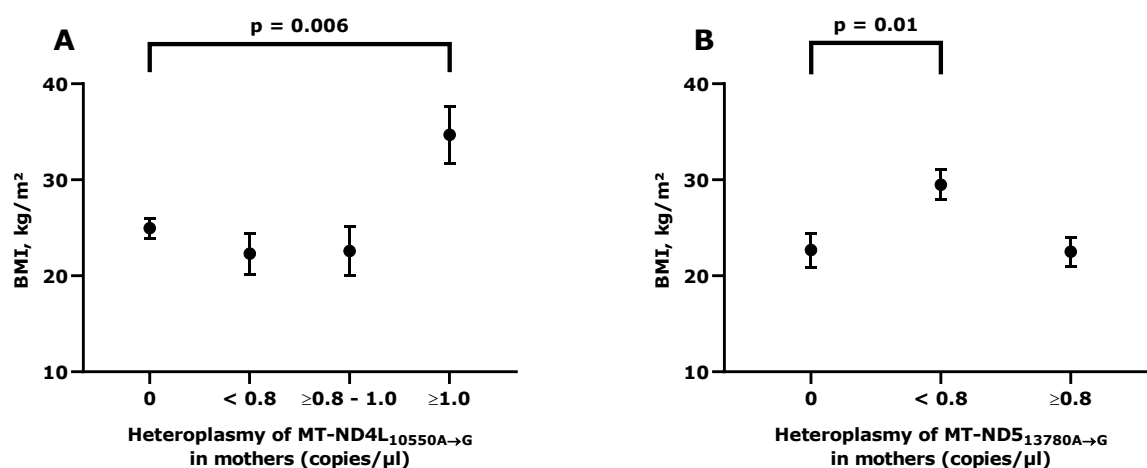
Figure 9. Mean (95% CI) maternal BMI in function of heteroplasmy levels of MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$ measured in blood of the mother (n = 28). A. Heteroplasmy levels of mutations in MT-ND4L$_{10550A \to G}$, presented as the mean BMI (kg/m²) with a 95% confidence interval. The used model was adjusted for the following variables: parity, educational level of the mother and age of the mother. **B.** Heteroplasmy levels of mutations in MT-ND5$_{13780A \to G}$. The used model was adjusted for the variables mentioned above.

3.4.3 Association of MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$ with birth weight in twins

The first main research question remains whether both mutations have a profound effect on birth weight in twins. Therefore, both mutations in mother and in children have been associated with birth weight (**Figure 10**). The groups were classified as mentioned before and all groups were compared to the group without heteroplasmy.

For MT-ND4L$_{10550A \to G}$, a significantly lower birth weight was found in the twins of mothers showing moderate (2158.6 g; 95% CI 1969.8 to 2347.3 g; p = 0.04) and high (1991.8 g; 95% CI 1779.5 to 2204.1 g; p = 0.02) levels of heteroplasmy compared to the mothers without heteroplasmy. On the other hand, when the child presented heteroplasmy levels, the birth weight was also significantly different in the moderate (2187.1 g; 95% CI 2047.9 to 2327.0 g; p = 0.03) and high level (2111.1 g; 95% CI 1960.6 to 2261.6 g; p = 0.02) groups. For MT-ND5$_{13780A \to G}$, no significant differences in birth weight were found in the twin groups when the mother presented heteroplasmy levels. However, for the mutation of the children, there was a significant difference in birth weight in the moderate heteroplasmy level group (2284.5 g; 95% CI 2214.9 to 2354.0 g; p = 0.007). To confirm an effect was not only seen because of heteroplasmy of the mother, a sensitivity analysis was performed for models that were corrected for heteroplasmy of the twins (**Appendix 6**). Conversely, to ensure an effect in the children was not only seen because of possible heteroplasmy of the mother, a sensitivity analysis was performed for models that were corrected for heteroplasmy of the mother (**Appendix 7**). Lastly, a sensitivity analysis was performed to see whether the mutations had a different effect in boys and girls (**Appendix 8**).
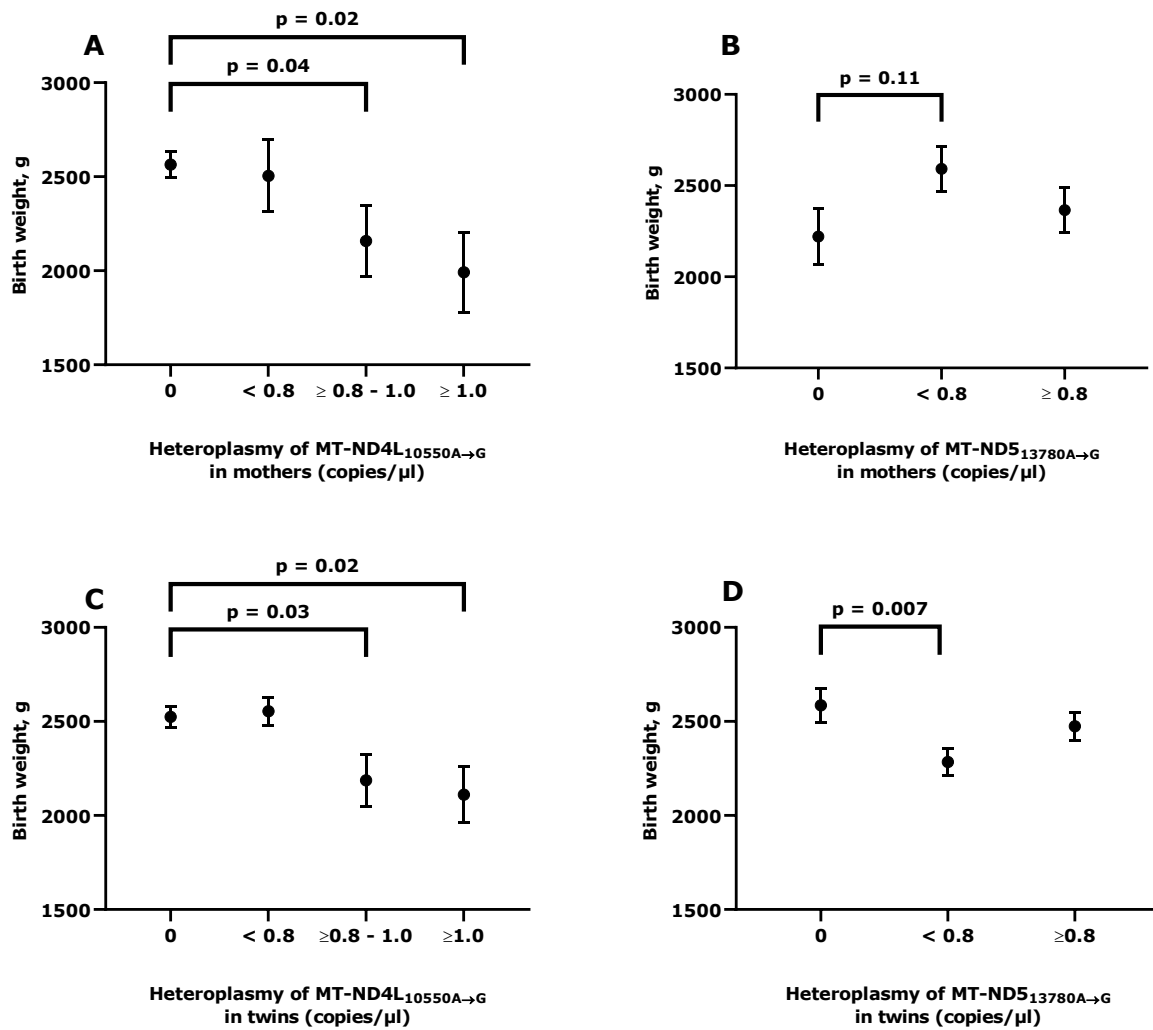
Figure 10. Mean (95% CI) birth weight of twins in function of heteroplasmy levels of MT-ND4L$_{10550A\to G}$ and MT-ND5$_{13780A\to G}$. Windows **A** and **B** show the associations for birth weight in twins (n = 27; n = 57) with mutations in the mother (n = 28), measured in blood of the mother, respectively for MT-ND4L$_{10550A\to G}$ and for MT-ND5$_{13780A\to G}$. The models that study the mutation of the mother were corrected for zygosity, chorionicity, parity, educational level of the mother, age of the mother, gestational age and weight before pregnancy. Windows **C** and **D** show the associations for birth weight in twins (n = 27; n = 57) with mutations in the twins (n = 57), measured in cord blood, respectively for MT-ND4L$_{10550A\to G}$ and for MT-ND5$_{13780A\to G}$. The models that study the mutation of the twin were corrected for zygosity, chorionicity, gender of the child, educational level of the mother and gestational age.

3.4.4   Association of MT-ND4L$_{10550A\to G}$ and MT-ND5$_{13780A\to G}$ with BMI z-scores in twins

The second main research aim questioned whether both mutations have a profound effect on BMI in twins around the age of four. Both associations of heteroplasmy in mothers and children for MT-ND4L$_{10550A\to G}$ with the BMI z-scores were not significant (**Figure 11**). This was also the case for the heteroplasmy in mothers and children for MT-ND5$_{13780A\to G}$ on the BMI z-scores.
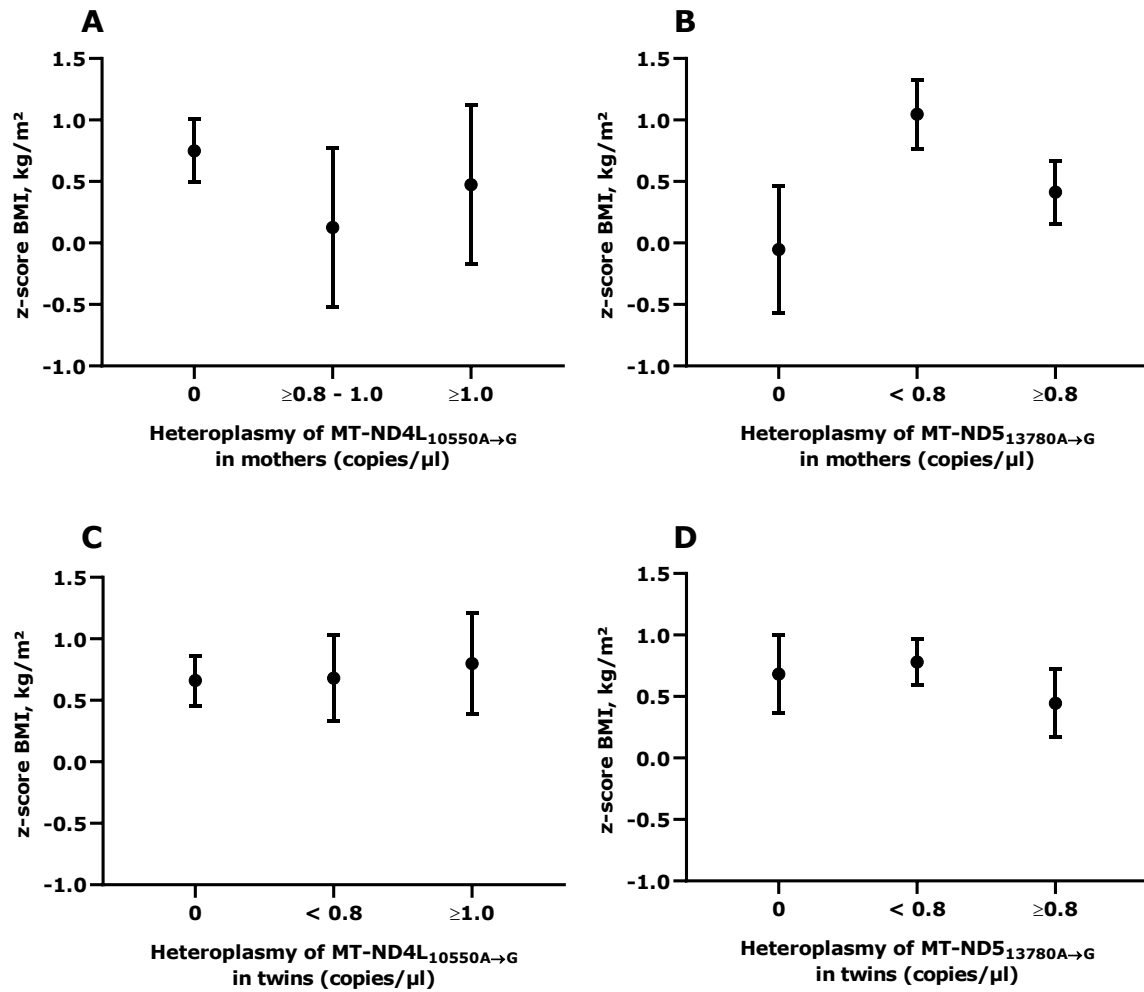
Figure 11. Mean (95% CI) BMI z-scores of twins in function of heteroplasmy of MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$. Windows **A** and **B** show the mutations in the mother (n = 10), measured in blood of the mother, in association with the BMI z-scored for the twins (n = 9; n = 19), respectively for MT-ND4L$_{10550A\rightarrow G}$ and for MT-ND5$_{13780A\rightarrow G}$. The model was only adjusted for the educational level of the mother since the BMI z-scores were already corrected for age and gender of the child. Windows **B** and **D** show the mutations in the twins (n = 9; n = 19), measured in cord blood, respectively for MT-ND4L$_{10550A\rightarrow G}$ and for MT-ND5$_{13780A\rightarrow G}$. The model was adjusted for the abovementioned variable.

4 Discussion

4.1 Most important findings

In brief, this research within the framework of the Limburg Twin Study was set up to prove four different points. Firstly, the genetic or environmental background of both mutations was tested. On the one hand, it is clear that the concordance between monozygotic twins is greater than the concordance between dizygotic twins, which is indicative of a possible genetic background of both mutations. On the other hand, the possible transmission from mother to her offspring was tested. In this population, it is apparent that the transmission from the mother to her offspring is present in the major portion of the group. However, *de novo* mutations in offspring that are not present in the mother or either a lower or a higher level of heteroplasmy in the offspring is also possible.

Secondly, the association between the heteroplasmy levels of the mother and the pre-gestational BMI was evaluated. For MT-ND4L$_{10550A \rightarrow G}$, there was a significant higher BMI for the mothers in the high heteroplasmy group compared with the mothers in the group that showed no heteroplasmy. For ND5$_{13780A \rightarrow G}$, there was a significant higher BMI for mothers in the low heteroplasmy group in comparison to the mothers in the group that showed no heteroplasmy.

Thirdly, the association between either the heteroplasmy levels of the mother or the twin and the birth weight in the twins was studied. For MT-ND4L$_{10550A \rightarrow G}$, both for the heteroplasmy levels of the mother and her offspring, there was a significant lower birth weight in both the high and moderate heteroplasmy groups in comparison to the group that had no heteroplasmy levels presented. For ND5$_{13780A \rightarrow G}$, the association between the heteroplasmy levels of the mother and birth weight in the twins was not significant, but the association of birth weight with heteroplasmy levels in the twins themselves were significant for the group that presented low levels of heteroplasmy.

Lastly, the association between either the heteroplasmy levels of the mother or the twin and the BMI z-scores was evaluated. Unfortunately, no significant associations were found for the BMI z-scores.

4.2 Mutational analysis with Droplet Digital™ PCR

To detect the smallest levels of mutations in mtDNA, the techniques used need to be extremely sensitive (39). Because these heteroplasmic mutations occur in a random matter, all mutations that were acquired are present in low levels and need highly sensitive and specific detection in order to detect the mutation (40). Several other methods were used for decades in research including sequencing, real-time quantitative PCR (qPCR) and techniques that were derived from qPCR (39). The sequencing technique was regarded as the gold standard for the detection of mutations for a long time, but its application is limited since this technique is expensive and relatively low in sensitivity (39). Also, the techniques that were derived from qPCR have several similar limitations, as for example the lower sensitivity and specificity (41). Nowadays, ddPCR™ is seen as the gold standard for mutational analysis, this is only a recent development in this field of research (37). ddPCR™ is a technique that finds several applications in molecular research, as for example the identification of cancer-associated gene mutations, the identification of genetic markers in diagnosis and the monitoring of treatment response. However, a challenging application for ddPCR™ is the detection of rare mtDNA mutations, as in this research. The detection of this mutant mtDNA is demanding owing to the heteroplasmy effect (39).

In terms of specificity, sensitivity and day-to-day reproducibility, ddPCR™ is superior to all the techniques mentioned above (39, 41). The use of ddPCR™ also reduces the background fluorescence, which makes this approach less prone to inhibitors. All these advantages together suggest the promising application of ddPCR™ for the detection of rare mutations with unseen precision. However, this method still has multiple technical limitations. This procedure requires highly specific probes, in order to minimize cross-reactivity and the occurrence of false positives. Studies with a large sample size are also required, in order to ensure that the targeted mutations are found (39).

Another issue that should be addressed in regard to the use of ddPCR™ is tissue inhomogeneity. Patterns of heteroplasmic mutations have been known to vary among the different tissues in an individual. Non-dividing tissues as for example the muscles and the brain are said to be more prone to heteroplasmic mutations, in comparison to peripheral blood or cord blood (17, 22). Previous research indicates that heteroplasmic mtDNA mutations that were present in cancer cells, were absent in blood from that same individual (42). Therefore, using DNA samples that are derived from blood rather than other tissues that might be more directly implicated in obesity might pose a problem. By using blood DNA instead of muscle tissue or adipocytes, the ability to identify heteroplasmy levels might be confounded (13). Fortunately, the smaller sample size and the use of cord blood for the detection of MT-ND4L$_{10550A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$ have not impacted our research.

Even though ddPCR™ is seen as the new gold standard for mutational analysis and other kinds of very sensitive analyses, the failure of the mutational analysis for MT-ND4$_{11719A \rightarrow G}$ could not be prevented. Since the analyses for MT-ND4L$_{10550A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$ have succeeded, it is not the case that there was any damage done to the samples or the obligatory components of the ddPCR™ reaction. However, this particular plate for MT-ND4$_{11719A \rightarrow G}$ was left overnight on the C100 Touch™ Thermal Cycler. According to Memon *et al.*, this additional step of overnight incubation should increase the droplet count (41). In this case, it is possible that this has had negative effects on the mutational analysis in a way that it was no longer possible to determine whether there were any mutant alleles present. This observation is strengthened by the fact that the plates that were not incubated overnight, were eligible for analysis. This is one possible explanation, but it is for example also possible that there was a problem concerning the compatibility of the assay with the samples.

4.2.1 Heritability of MT-ND4L$_{10550A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$

To study whether a mutation has a possible genetic or environmental background, a twin model is ideal. In the so-called classical twin model, the degree of variance between monozygotic and dizygotic twins is compared, as mentioned before. A genetic background can be concluded upon when the concordance is higher in monozygotic twins than in dizygotic twins, whereas an environmental background is hinted when the variation is greater in a monozygotic pair than in a dizygotic pair (23, 40). In our research, the concordance in monozygotic twins is significantly higher than in dizygotic twins, therefore, a genetic inference plays an important role here. In the case of a heteroplasmic mutation, this genetic background has more consequences for the genetic material. Monozygotic twins do not only share all of their genetic material but because of the heteroplasmy, they also share the same mitochondrial cell composition. This is caused by the fact that monozygotic twins are derived from the same zygote, while conversely, dizygotic twins are derived from two separate zygotes (23).

Therefore, the mitochondrial cell composition in dizygotic twins will not be exactly the same (23). Whether a twin is classified as monozygotic or dizygotic thus has a strong effect on the amount and state of mtDNA that is inherited, which is further complicated by the presence of a genetic bottleneck (**Figure 12**) (23).

During development, this genetic bottleneck is present and it affects the amount of mutated mtDNA that is transmitted from the mother to her offspring (2, 40, 43). This implies that the frequency of intraindividual mtDNA polymorphisms can change dramatically over generations, which can affect the disease phenotype in the offspring. For example, an allele that was present at a low, harmless frequency in the mother, can be transformed into an allele that is present at a high, disease-causing frequency in her offspring (33). The exact mechanism for the occurrence of this genetic bottleneck is not known to date (15). The transformation from a low to a high frequency is seen in only a minor fraction of our population, namely in 1.85% for MT-ND4L$_{10550A\rightarrow G}$ and in 5.56% for MT-ND5$_{13780A\rightarrow G}$, however, we cannot conclude on the disease-causing properties of this amount of heteroplasmic mutation.
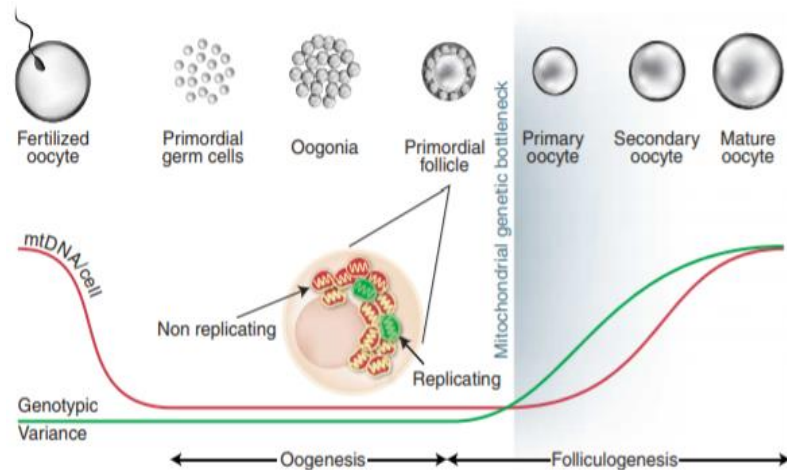
Figure 12. Model for maternal transmission of mtDNA. After fertilization, the amount of mtDNA per cell rapidly declines. Early in folliculogenesis, the so-called genetic bottleneck rapidly segregates sequence variants. Adapted from Wai et al. (2)

Previous research indicates that common mtDNA heteroplasmy is only transmitted from mother to offspring in 30% of the cases (44). In this research, however, it is clear that the mtDNA heteroplasmy of both MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$ is often transmitted from the mother to her offspring. In this population, maternal transmission of heteroplasmy was more likely, but this might also be biased by the rather small sample size. Sondheimer *et al.* state that mtDNA heteroplasmy occurs more often than not *de novo* in offspring, or that the mutation is present in mothers, but not in her offspring (44). Indeed, in MT-ND4L$_{10550A\rightarrow G}$, 18.5% of the twin population was presented with heteroplasmy that was absent in the mothers. For MT-ND5$_{13780A\rightarrow G}$, this was 14.8%.

It is possible that the exposure to environmental toxicants, of which biologic agents, therapeutic drugs, toxic gases, and chemical substances are a few examples, give rise to mutations that were not present in the mother but are present in their offspring and even exert an effect (43). Indeed, mitochondrial toxicity is seen as one of the most dangerous consequences of exposure to environmental toxicants. During pregnancy, this exposure to a range of different chemical substances or other toxicants, can exert deleterious effects on the unborn child (43, 45). Although there is relatively little information within the context of human pregnancy and the *in utero* exposures, it was established early on that this is the most sensitive period in life for environmental damage (4). Whether the mitochondrial disturbances were inherited from the mother or acquired during pregnancy, had no effect on the consequences for the newborn (43).

A rather new finding in this population, is the fact that for ND4L$_{10550A \to G}$ 24.1% of the children have an absence of heteroplasmy where the mothers do present various levels of heteroplasmy. For MT-ND5$_{13780A \to G,}$ this is 20.4% This gives rise to a possible inverse mechanism of the genetic bottleneck. Where normally a non-disease-causing allele would be turned into a disease-causing allele in a normal bottleneck, this population implies that a higher amount of heteroplasmy was not readily transmitted to her offspring (33). Indeed, Yanicostas *et al.* state that the genetic bottleneck might operate in a way that it enables selection against deleterious mutations (45). In addition to this, 3.7% and 16.7% of the children with respective mutations of ND4L$_{10550A \to G}$ and ND5$_{mt13780A \to G}$ present a lower mutational frequency than their mothers. This might also imply an inverse genetic bottleneck. Unfortunately, the mechanisms by which this genetic bottleneck operates are unknown, nor is it known when it functions in favour of negative mutations or not.

4.2.2 Association of MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$ with BMI in mothers

In order to present an indication whether these mutations might be related to BMI in adulthood, both mutations were associated with the BMI of the mothers before pregnancy. For MT-ND4L$_{10550A \to G,}$ a significantly higher BMI (9.99 kg/m²; 95% CI 3.7 to 16.2 kg/m²; p = 0.006) was found in the group of mothers with the highest levels of heteroplasmy. This implies that mothers, or in general, women that carry high levels of the MT-ND4L$_{10550A \to G}$ mutation, have a significantly higher risk of having a high BMI in comparison to women with no MT-ND4L$_{10550A \to G}$ mutation. Indeed, Flaquer *et al.* already found that this particular mutation might pose a risk factor for different metabolic disorders, including BMI and phenotypes related to BMI (p = 2.8 x 10^{-4}) (8). And even though the population of the Limburg Twin Study only uses women, the findings are similar in the mixed adult cohort (8). However, other researches have failed to identify MT-ND4L$_{10550A \to G}$ as a possible obesity-causing mutation in adults (13, 22, 28).

There are different reasons why this mutation might have an influence on the metabolism in a disadvantageous way. The first reason is that MT-ND4L$_{10550A \to G}$ is a synonymous mutation mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4L. This kind of mutation is a change of a single nucleotide and does not lead to a change in the protein. It is still under investigation how an excess of this kind of mutations could have an impact on BMI. An explanation might be that different codons might lead to a difference in protein expression levels, without changing the protein itself (8). Secondly, it is also possible that nucleotide changes that are not pathogenic themselves may affect other, more damaging mtDNA mutations, and exert their effects indirectly (15). Lastly, it might be the case that a nucleotide change, regardless of whether this causes a protein change, in high levels can alter the mitochondrial genome expression or even directly trigger mitochondrial dysfunction (8).

For MT-ND5$_{13780A \to G,}$ a significantly higher BMI (5.33 kg/m²; 95% CI 0.3 to 10.3 kg/m²; p = 0.01) was only found between the group of mothers with a low level of heteroplasmy compared to the group of mothers that had no heteroplasmy detected. In contrast to what we would expect, we did not find a higher BMI in the group that shows a high amount of the mutation in comparison to the group with none or a low amount of mutation. MT-ND5$_{13780A \to G}$ was also established as a mutation that was implied in the development of a BMI phenotype (22). Yet again, other papers failed to identify MT-ND5$_{13780A \to G}$ as a mutation involved in obesity (8, 13, 28). A possible explanation for this finding is that there is some kind of confounding factor in the group of mothers that have a low level of heteroplasmy of MT-ND5$_{13780A \to G}$. Another explanation might be the inverse mechanism of disease-causing mutations, namely that a high level of heteroplasmy of a certain mutation might have advantageous consequences for the carrier.

MT-ND5$_{13780A \rightarrow G}$ is a mutation of the mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 5 and was significantly associated with BMI in a cohort of Scandinavian, Swedish, Canadian, American and Polish adults (p = 0.025) (22). MT-ND5$_{13780A \rightarrow G}$ is a missense mutation that leads to a change in amino acid, which might pose a possible working mechanism. An elevated BMI might be contributable to an excess of this missense mutation since individuals with this excess might carry a significant fraction of a protein that was altered by MT-ND5$_{13780A \rightarrow G}$ (8).

4.2.3   Association of MT-ND4L$_{10550A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$ with birth weight in twins

Both the mutations in mother and in children have been associated with birth weight. For MT-ND4L$_{10550A \rightarrow G}$ heteroplasmy in mothers, a significant different birth weight was found in the twin groups that showed moderate (2158.6 g; 95% CI 1969.8 to 2347.3 g; p = 0.04) and high (1991.8 g; 95% CI 1779.5 to 2204.1 g; p = 0.02) levels of heteroplasmy in comparison to the twin group were no mutation was detected in the blood sample of the mother. This would imply that the heteroplasmy of the mother leads to a lower birth weight in her offspring, regardless of whether the child presents any heteroplasmy of this mutation. This finding was confirmed by the sensitivity analysis (**Appendix 6**) since all associations remained significant.

The same trend is seen in the twin groups that showed moderate (2187.1 g; 95% CI 2047.9 to 2327.0 g; p = 0.03) and high (2111.1 g; 95% CI 1960.6 to 2261.6 g; p = 0.02) levels of heteroplasmy when the child presented heteroplasmy levels. To exclude the possibility of the heteroplasmy of the mother causing this relationship, a sensitivity analysis was carried out for the mutation of the mother (**Appendix 7**). All associations remained significant, except for the high heteroplasmy group where birth weight was not significantly lower than birth weight in the group without a mutation.

In the case of an association between the heteroplasmy of MT-ND4L$_{10550A \rightarrow G}$ and birth weight in twins, possible mechanisms could be linked to the *in utero* environment. It is plausible that the genetic bottleneck has operated in such a way that the heteroplasmy was transferred from mother to her offspring (33). Nonetheless, there were cases noted where the mother presented heteroplasmy that was not transferred to her offspring. Therefore, it might be that the heteroplasmy in the mother has an effect on the energy metabolism in the placenta, and subsequently might affect the birth weight of the child (43). On the other hand, a physiological state that requires a high energy demand, as for example pregnancy, may require greater activity of the mitochondria. If the mitochondria are not able to meet these demands, this might lead to impaired energy provision to the unborn child (43).

For MT-ND5$_{13780A \rightarrow G}$, no significant differences in birth weight were found in the twin groups when the mother presented heteroplasmy levels. However, if the mother presented a low level of heteroplasmy for MT-ND5$_{13780A \rightarrow G}$, this would lead to a higher birth weight, although not significant (2592.0 g; 95% CI 2466.9 to 2717.1 g; p = 0.11). Contrary to this, for the mutation of the children, there was a significantly higher birth weight in the low heteroplasmy level group (2284.5 g; 95% CI 2214.9 to 2354.0 g; p = 0.007). Similar to our observations of BMI in the mothers, the low level heteroplasmy group has a stronger effect than the high level heteroplasmy group. Again, the explanation for this might be the inverse mechanism of disease-causing mutations, as mentioned before (45). It is clear that this effect is recurrent in multiple stages of life, as well in the mothers as in the newborn twins. Therefore, it is possible that this is an inherent characteristic of the MT-ND5$_{13780A \rightarrow G}$ heteroplasmy.

This is in contrast with the threshold effect that has been widely debated in mitochondrial genetics. When heteroplasmy is present, there is also a certain threshold level observed. Different studies have shown that mitochondrial mutations are mostly recessive, but above this threshold, the high levels of mutational variance definitely lead to the presence of a disease phenotype (10, 40). Typically, the threshold levels are situated between 60% and 90% mutant mtDNA, but the threshold levels vary for different mutations and for different tissues (15). A threshold level is not observed in $MT\text{-}ND5_{13780A \rightarrow G}$, since a higher mutational frequency of $MT\text{-}ND5_{13780A \rightarrow G}$ does not automatically lead to a lower birth weight in this population.

Intuitively, it is clear that mitochondria are very important for foetal development. Especially during the early stages, foetal development has a high energy demand. It makes active use of the respiratory chain of the mitochondria, in order to produce enough energy to adequately complete all stages of fertilization and implantation. Even after these stadia, there is a lot of energy needed to provide for the development of the embryo, for constant cell division, cell migration and cell differentiation (43, 45). It might be a surprising fact that in most cases, a deficiency in the respiratory chain does not have a significant impact on foetal or infant life. Nonetheless, this research observed an association of both mutations with birth weight, even though it is apparent that most consequences only become visible during adulthood (45). If there are indeed mutations present in genes that are particularly important for the energy provision, as for example the mutations studied here, this might impair the energy provision during the foetal development, although major health issues are not visible. Impaired energy provision might, in turn, imply a lower birth weight and imply physiological adaptations in adulthood.

The identification of both mutations in association with a decrease in birth weight in twins has important consequences in later life. As first described by Barker and Osmond in 1986, the 'Developmental Origins of Health and Diseases' (DOHaD) poses that poor *in utero* growth, and thus a lower birth weight, are potential risk factors for many chronic diseases in later life. Obesity is only one of these diseases, next to diabetes, hypertension, mental health problems, and an impaired cognitive performance. In addition to chronical illnesses, a lower birth weight poses a major threat for higher mortality rates (4). All this evidence leads to the conclusion that a lower birth weight is a possible predictor for pathologies and physiological adaptations in adult life. Even if these changes are genetically embedded in the genome, the question remains whether there are any measures to be made in order to decrease the severity of these health problems in later life.

4.2.4   Association of $MT\text{-}ND4L_{10550A \rightarrow G}$ and $MT\text{-}ND5_{13780A \rightarrow G}$ with BMI in twins

Our results show an association of $MT\text{-}ND4L_{10550A \rightarrow G}$ and $MT\text{-}ND5_{13780A \rightarrow G}$ with birth weight, this association was suggested in this research. To investigate possible consequences later in childhood, we studied the association of $MT\text{-}ND4L_{10550A \rightarrow G}$ and $MT\text{-}ND5_{13780A \rightarrow G}$ with BMI around the age of four is studied. In brief, no significant associations of both mutations with BMI z-scores in the Limburg Twin Study were found.

There are several potential reasons why we did not identify an association with both mutations around the age of four, although an association of both mutations with BMI was present in the mother population. Even more than the population at birth, the population around the age of four was smaller with 19 twin children and ten mothers. Secondly, the follow-up population did not contain any overweight children.

For BMI z-scores, the WHO states that overweight is defined as a z-score two standard deviations higher than the growth standards and obesity is defined as a z-score three standard deviations higher than the growth standards, all before the age of five. Conversely, thinness and severe thinness are defined as respectively two and three standard deviations lower than the growth standards for children under the age of five published by the WHO (38). In our population at four years of age, no children were categorized in the groups of thinness or overweight. The ability to identify an association with BMI z-scores is strongly influenced by having a healthy population. At last, it might not be possible to confirm this association in this substantially young population. Grant *et al.* state that the development of heteroplasmy is time-dependent, thus the selection of a paediatric group might not show differences that will be present in later life (13). As mentioned above, most consequences of perturbations in early life, as for example mitochondrial mutations, only become visible during adulthood (45).

Even though there is no direct observation of differences in z-scores in association with both mutations around the age of four, this does not imply that there are no changes in progress at this life stage. There are different underlying mechanisms that contribute to the accumulation of mitochondrial damage. As mentioned before, the mitochondrial genome is particularly sensitive to damage. Different reasons for this sensitivity lie in the absence of an extensive repair system, the uniparental inheritance and the polyploid nature of the mitochondrial genome (15, 46). There is a possibility that the mitochondrial genome is able to tolerate a certain amount of mutations before the clinical presentation of these mutations is visible. This is illustrated by the threshold effect, as explained before (10, 40). In childhood, it might be the case that the accumulation of these mutations has not reached the threshold yet, but in adulthood, the threshold might be exceeded, so the effects start showing. In obesity, this mitochondrial dysfunction caused by the accumulation of mutations is expressed as a failure to oxidize fatty acids and a disturbed glucose homeostasis (46).

Again, also at the age of four, this twin model proves to be very useful. It is widely known that genes and environment interact and work together in the promotion of obesity. Speiser and colleagues indicate that monozygotic twins with the same genotype are more likely to respond evenly to an overflow in energy than subjects with different genotypes (47). In this research, we also tried to study the intrapair difference in BMI at the age of four in association with both mutations, however, no significant results were found here (data not shown). This might also be caused by either a too small study population or the presence of a clinically healthy study population.

4.3 Strengths and weaknesses

The main weakness of this study at the follow-up level is the sample size. It should be noted that this is a very small group, which of course lowers the power of the study (30). At birth, a low sample size does not seem to pose a threat, since multiple significant results were obtained here. Another challenge that this research is facing, is the fact that it is difficult to associate mtDNA mutations with the severity and the form of dysfunction in mitochondria (10). In addition, the distinction between neutral mtDNA variants and mutations that cause diseases is often difficult. This is caused by the high mutational rate of the mtDNA and presence of a lot of polymorphisms (15).

On the other hand, one of the main strengths of this research is the use of twins, even in a smaller group. As mentioned before, twin studies provide the opportunity to gain insight into the relative importance of genes and environment. The classical twin model contrasts the degree of concordance within monozygotic and dizygotic twins. This model is based upon the assumption that monozygotic twins share all of their nuclear and mitochondrial DNA, while dizygotic twins only share half of their genetic material. Genetic interference is hinted when the concordance in monozygotic twins is higher than the concordance in dizygotic twins (23). Now, this study alludes upon a possible association between metabolic parameters and mtDNA variants, this study might be seen as a pilot study to conclude about the genetic or environmental background of the variants.

This is, however, not the only advantage of this study. As explained before, the Limburg Twin Study was started in 2014 and the recruited twins are now around the age of four. To our knowledge, none of the publications mentioned above were able to use a population that was this young of age. It is also a major advantage that there are samples available from birth onwards, which makes it possible to also associate the mtDNA mutations with possible variations in birth weight. On the other hand, this might pose a major threat to the study design, since the selection of a paediatric group might conceal possible differences that play an important role in later life (13).

At last, another important strength of the Limburg Twin Study is the fact that we collect information and samples at different time points in life. As with the ENVIR*ON*AGE birth cohort, these repeated measurements allow the researchers to associate different exposures and mechanisms, as the mtDNA variants, in early life and possible onset of diseases in later life (3).

5 Conclusion

This research was set up in order to establish the association between three mitochondrial mutations with birth weight of twin children and their BMI at the age of four, in the framework of the Limburg Twin Study. Based on extensive literature search, three mutations were investigated, i.e. MT-ND4$_{11719A\rightarrow G}$, MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$. In addition, the association of these three mitochondrial mutations with the BMI of the mothers enrolled in the Limburg Twin Study was studied.

The mutational analysis was carried out using ddPCR™ since this type of analysis requires an extremely sensitive and specific technique in order to detect the low amounts of mutation present. From this research, it can be concluded that ddPCR™ indeed possesses the needed sensitivity and specificity the conduct mutational analysis, even when mutations have a low prevalence in the population.

Based on the literature search, it was not clear whether these mutations have an environmental or genetic background. Since twin studies prove to be ideal for the establishment of the background, this was the first analysis to be carried out. For both MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$ heteroplasmy, a genetic background was most apparent since there was a stronger concordance in monozygotic twin pairs than in dizygotic twin pairs. Next to this genetic background, maternal transmission of heteroplasmy of both mutations was present in this study population. However, there was also evidence suggesting the operation of a genetic bottleneck, causing the frequency of the mutations to change over generations.

For MT-ND4L$_{10550A\rightarrow G}$, a significant higher BMI was found in the group of mothers with the highest levels of heteroplasmy in comparison to the group of mothers that showed no heteroplasmy levels for MT-ND4L$_{10550A\rightarrow G}$. This implies a significantly higher risk of having a high BMI in comparison to women with no MT-ND4L$_{10550A\rightarrow G}$ mutation. For MT-ND5$_{13780A\rightarrow G}$, a significant higher BMI was only found between the group of mothers with a low level of heteroplasmy compared to the group of mothers that had no heteroplasmy detected. In contrast to our expectations, women that show a high amount of this mutation do not have a higher BMI in comparison to the group with none or a low amount of mutation.

The second association tested was between MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$ with birth weight in the twins of the Limburg Twin Study. For MT-ND4L$_{10550A\rightarrow G}$, both for the heteroplasmy levels of the mother and the twins, there was a significantly lower birth weight between the group that showed no heteroplasmy and both the moderate and high heteroplasmy level groups, which indicates that having a higher frequency of mutation is disadvantageous for the birth weight. For ND5$_{13780A\rightarrow G}$, the association between the heteroplasmy levels of the mother and birth weight in the twins was not significant, for the mutation of the children, there was a significantly higher birth weight in the low heteroplasmy level group.

Lastly, the association between either the heteroplasmy levels of the mother or the twin and the BMI z-scores was evaluated. Unfortunately, no significant associations were found between the level of heteroplasmy and the BMI z-scores. This is in contradiction to the expectations of this research. However, since there are associations found of both mutations with BMI in adulthood as mentioned before, it might just be the case that the selection of this paediatric and clinically healthy population might obscure effects that might become apparent in adulthood.

To conclude, our hypothesis, that these specific mtDNA mutations, namely MT-ND4L$_{10550A\rightarrow G}$, MT-ND4$_{11719A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$, are mainly determined by the genetic inheritance in twins and that they are indeed associated with birth weight and BMI, is only partly proven correct. It is clear that there is a genetic influence, both because of the concordance in heteroplasmy of MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$ seen in monozygotic twins that is not observed in dizygotic twins and the transmission of these two mutations from the mother to her offspring. However, we found an association with birth weight but not with BMI around the age of four. In addition to this, we have identified an association of both mutations with BMI of the mother in the maternal population of this study. To confirm the results found in this study, we recommend testing these mutations in a larger study population.

5.1 Future perspectives

Starting from these results, a lot of future research steps can be taken. Because of the small study population, and particularly in the follow-up, this study might be seen as a pilot study for further research. The classical twin model is very useful to establish the genetic or environmental background of certain mutations, as mentioned before. On the other hand, there is a more complicated model to identify these influences, in addition to the identification of a possible interplay between genetic or environmental influences. Using Open MX software, structural equation modelling can be performed in complete pairs to estimate the genetic and environmental components of variance for the traits. This variance can be separated into additive genetic (A), common environmental (C) and unique environmental (E) effects, using the so-called ACE-model (35). Fortunately, this study can already conclude upon a genetic influence for both MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$ heteroplasmy so in regard to further steps, it is not necessary to investigate twins.

In order to strongly confirm the associations that were established in this research, it is for example possible to use a larger cohort of singletons. As mentioned in the very, the Limburg Twin Study is part of the ENVIR*ON*AGE birth cohort, which is a very large birth cohort of singletons (3). Studying the association of ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$ and birth weight and/or BMI in this birth cohort would be an ideal next step. On the other hand, if we would like to investigate other mutations in association with birth weight and/or BMI, it would be advantageous for the Limburg Twin Study to start recruiting at birth again to increase the study population, which would be beneficial for further, new research.

Finally, mitochondrial mutations and the phenomenon of heteroplasmy provide more options to explore. Research on the most prevalent mitochondrial mutations, and particularly on those that are associated with monogenic mitochondrial diseases, has been carried out a lot (Figure 2), however, the involvement of heteroplasmy of mitochondrial mutations in complex diseases has been debated. As much as 250 mutations for the mtDNA have been described up to date, and without a doubt, there are more mutations to discover (10). These new mutations, together with the mutations that have been discovered in the past, pave the way for new research since they might be associated with different, complex diseases. Given the essential function of mitochondria for the energy provision, it is clear that mitochondrial mutations might be implied in a lot of metabolic diseases, as for example diabetes (17, 28), but also neurodevelopmental diseases like Parkinson's disease (29, 40) and Alzheimer's disease (40). Even in different forms of cancer, mtDNA variants might play an important role through mitochondrial ROS production (12). To conclude upon the future, there is no debate that mtDNA variants are a great study subject, and in combination with a twin set-up, this could lead to the discovery of missing links in future research.

6 References

1. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical chemistry. 2011;83(22):8604-10.
2. Wai T, Teoli D, Shoubridge EA. The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. Nature genetics. 2008;40(12):1484-8.
3. Janssen BG, Madhloum N, Gyselaers W, Bijnens E, Clemente DB, Cox B, et al. Cohort Profile: The ENVIRonmental influence ON early AGEing (ENVIRONAGE): a birth cohort study. International journal of epidemiology. 2017;46(5):1386-7m.
4. Hoffman DJ, Reynolds RM, Hardy DB. Developmental origins of health and disease: current knowledge and potential mechanisms. Nutrition reviews. 2017;75(12):951-70.
5. Guo SW. Does higher concordance in monozygotic twins than in dizygotic twins suggest a genetic component? Human heredity. 2001;51(3):121-32.
6. Bouhlal Y, Martinez S, Gong H, Dumas K, Shieh JT. Twin Mitochondrial Sequence Analysis. Molecular genetics & genomic medicine. 2013;1(3):174-86.
7. Mustelin L, Pietilainen KH, Rissanen A, Sovijarvi AR, Piirila P, Naukkarinen J, et al. Acquired obesity and poor physical fitness impair expression of genes of mitochondrial oxidative phosphorylation in monozygotic twins discordant for obesity. American journal of physiology Endocrinology and metabolism. 2008;295(1):E148-54.
8. Flaquer A, Baumbach C, Kriebel J, Meitinger T, Peters A, Waldenberger M, et al. Mitochondrial genetic variants identified to be associated with BMI in adults. PloS one. 2014;9(8):e105116.
9. Flaquer A, Rospleszcz S, Reischl E, Zeilinger S, Prokisch H, Meitinger T, et al. Mitochondrial GWA Analysis of Lipid Profile Identifies Genetic Variants to Be Associated with HDL Cholesterol and Triglyceride Levels. PloS one. 2015;10(5):e0126294.
10. Zhu Z, Wang X. Significance of Mitochondria DNA Mutations in Diseases. Advances in experimental medicine and biology. 2017;1038:219-30.
11. Knoll N, Jarick I, Volckmar AL, Klingenspor M, Illig T, Grallert H, et al. Mitochondrial DNA variants in obesity. PloS one. 2014;9(5):e94882.
12. Wallace DC. Mitochondrial DNA mutations in disease and aging. Environmental and molecular mutagenesis. 2010;51(5):440-50.
13. Grant SF, Glessner JT, Bradfield JP, Zhao J, Tirone JE, Berkowitz RI, et al. Lack of relationship between mitochondrial heteroplasmy or variation and childhood obesity. International journal of obesity (2005). 2012;36(1):80-3.
14. Janssen BG, Munters E, Pieters N, Smeets K, Cox B, Cuypers A, et al. Placental mitochondrial DNA content and particulate air pollution during in utero life. Environmental health perspectives. 2012;120(9):1346-52.
15. Tuppen HA, Blakely EL, Turnbull DM, Taylor RW. Mitochondrial DNA mutations and human disease. Biochimica et biophysica acta. 2010;1797(2):113-28.
16. Aryaman J, Johnston IG, Jones NS. Mitochondrial Heterogeneity. Frontiers in genetics. 2018;9:718.
17. Avital G, Buchshtav M, Zhidkov I, Tuval Feder J, Dadon S, Rubin E, et al. Mitochondrial DNA heteroplasmy in diabetes and normal adults: role of acquired and inherited mutational patterns in twins. Human molecular genetics. 2012;21(19):4214-24.
18. Virgilio R, Ronchi D, Bordoni A, Fassone E, Bonato S, Donadoni C, et al. Mitochondrial DNA G8363A mutation in the tRNA Lys gene: clinical, biochemical and pathological study. Journal of the neurological sciences. 2009;281(1-2):85-92.
19. Harihara S, Nakamura K, Takubo K, Takeuchi F. Spontaneous event of mitochondrial DNA mutation, A3243G, found in a family of identical twins. Mitochondrial DNA. 2013;24(2):158-62.
20. de Laat P, Zweers HE, Knuijt S, Smeitink JA, Wanten GJ, Janssen MC. Dysphagia, malnutrition and gastrointestinal problems in patients with mitochondrial disease caused by the m3243A>G mutation. The Netherlands journal of medicine. 2015;73(1):30-6.
21. Bournat JC, Brown CW. Mitochondrial dysfunction in obesity. Current opinion in endocrinology, diabetes, and obesity. 2010;17(5):446-52.
22. Saxena R, de Bakker PI, Singer K, Mootha V, Burtt N, Hirschhorn JN, et al. Comprehensive association testing of common mitochondrial DNA variation in metabolic disease. American journal of human genetics. 2006;79(1):54-61.
23. Andrew T, Calloway CD, Stuart S, Lee SH, Gill R, Clement G, et al. A twin study of mitochondrial DNA polymorphisms shows that heteroplasmy at multiple sites is associated with mtDNA variant 16093 but not with zygosity. PloS one. 2011;6(8):e22332.
24. World Health Organisation (WHO). Fact sheet about obesity and overweight 2018 [updated 16 February 2018. Available from: https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight.
25. Naukkarinen J, Heinonen S, Hakkarainen A, Lundbom J, Vuolteenaho K, Saarinen L, et al. Characterising metabolically healthy obesity in weight-discordant monozygotic twins. Diabetologia. 2014;57(1):167-76.

26.   Nardelli C, Labruna G, Liguori R, Mazzaccara C, Ferrigno M, Capobianco V, et al. Haplogroup T is an obesity risk factor: mitochondrial DNA haplotyping in a morbid obese population from southern Italy. BioMed research international. 2013;2013:631082.

27.   Murugan AT, Sharma G. Obesity and respiratory diseases. Chronic respiratory disease. 2008;5(4):233-42.

28.   Yang TL, Guo Y, Shen H, Lei SF, Liu YJ, Li J, et al. Genetic association study of common mitochondrial variants on body fat mass. PloS one. 2011;6(6):e21595.

29.   Hudson G, Gomez-Duran A, Wilson IJ, Chinnery PF. Recent mitochondrial DNA mutations increase the risk of developing common late-onset human diseases. PLoS genetics. 2014;10(5):e1004369.

30.   Ebner S, Mangge H, Langhof H, Halle M, Siegrist M, Aigner E, et al. Mitochondrial Haplogroup T Is Associated with Obesity in Austrian Juveniles and Adults. PloS one. 2015;10(8):e0135622.

31.   Liu C, Yang Q, Hwang SJ, Sun F, Johnson AD, Shirihai OS, et al. Association of genetic variation in the mitochondrial genome with blood pressure and metabolic traits. Hypertension (Dallas, Tex : 1979). 2012;60(4):949-56.

32.   Guo LJ, Oshida Y, Fuku N, Takeyasu T, Fujita Y, Kurata M, et al. Mitochondrial genome polymorphisms associated with type-2 diabetes or obesity. Mitochondrion. 2005;5(1):15-33.

33.   Rebolledo-Jaramillo B, Su MS, Stoler N, McElhoe JA, Dickins B, Blankenberg D, et al. Maternal age effect and severe germ-line bottleneck in the inheritance of human mitochondrial DNA. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(43):15474-9.

34.   Luo S, Valencia CA, Zhang J, Lee N-C, Slone J, Gui B, et al. Biparental Inheritance of Mitochondrial DNA in Humans. Proceedings of the National Academy of Sciences. 2018;115(51):13039-44.

35.   Neale MC, Cardon LR. Methodology for genetic studies of twins and families. STATISTICS IN MEDICINE. 1994;13:199-.

36.   Wang F, Zhu L, Liu B, Zhu X, Wang N, Deng T, et al. Noninvasive and Accurate Detection of Hereditary Hearing Loss Mutations with Buccal Swab Based on Droplet Digital PCR. Analytical chemistry. 2018;90(15):8919-26.

37.   Sofronova JK, Ilinsky YY, Orishchenko KE, Chupakhin EG, Lunev EA, Mazunin IO. Detection of Mutations in Mitochondrial DNA by Droplet Digital PCR. Biochemistry Biokhimiia. 2016;81(10):1031-7.

38.   Cole TJ, Lobstein T. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. Paediatric obesity. 2012;7(4):284-94.

39.   Tong Y, Shen S, Jiang H, Chen Z. Application of Digital PCR in Detecting Human Diseases Associated Gene Mutation. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2017;43(4):1718-30.

40.   Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nature reviews Genetics. 2005;6(5):389-402.

41.   Memon AA, Zoller B, Hedelius A, Wang X, Stenman E, Sundquist J, et al. Quantification of mitochondrial DNA copy number in suspected cancer patients by a well optimized ddPCR method. Biomolecular detection and quantification. 2017;13:32-9.

42.   He Y, Wu J, Dressman DC, Iacobuzio-Donahue C, Markowitz SD, Velculescu VE, et al. Heteroplasmic mitochondrial DNA mutations in normal and tumour cells. Nature. 2010;464(7288):610-4.

43.   Moren C, Hernandez S, Guitart-Mampel M, Garrabou G. Mitochondrial toxicity in human pregnancy: an update on clinical and experimental approaches in the last 10 years. International journal of environmental research and public health. 2014;11(9):9897-918.

44.   Sondheimer N, Glatz CE, Tirone JE, Deardorff MA, Krieger AM, Hakonarson H. Neutral mitochondrial heteroplasmy and the influence of aging. Human molecular genetics. 2011;20(8):1653-9.

45.   Yanicostas C, Soussi-Yanicostas N, El-Khoury R, Benit P, Rustin P. Developmental aspects of respiratory chain from fetus to infancy. Seminars in foetal & neonatal medicine. 2011;16(4):175-80.

46.   Wlodarczyk M, Nowicka G. Obesity, DNA Damage, and Development of Obesity-Related Diseases. International journal of molecular sciences. 2019;20(5).

47.   Speiser PW, Rudolf MC, Anhalt H, Camacho-Hubner C, Chiarelli F, Eliakim A, et al. Childhood obesity. The Journal of clinical endocrinology and metabolism. 2005;90(3):1871-87.

7   Appendices

7.1 Appendix 1: Assay information

Table 7. Assay information. For every assay, the context sequence and the positive control sequence are shown. The position of the mutation is shown between brackets: [major allele/minor allele]. MT-ND; mitochondrially encoded NADH dehydrogenase subunit 1.

MT-ND4L$_{10550A\rightarrow G}$	
Assay ID dHsaMDS169630047	ATATTATACTAGCATTTACCATCTCACTTCTAGGAATACTAGTATATCGCTCACACCTCAT[A/G]TCCTCCCTACTAT GCCTAGAAGGAATAATACTATCGCTGTTCATTATAGCTACTCTCATAA
Positive control	CCCATGAGACATACAAAAAGGTAATGCCGCCTCGCTAGGTGAGCTACAGCTCGATTGTCACGTTAAGCTGGCCGTG ACTACAAAAAGGATTAGACTGAGCCGAATTGGTATATAGTTTAAACAAAACGAATGATTTCGACTCATTAAATTATG ATAATCATATTTACCAAATGCCCCTCATTTACATAAATATTATACTAGCATTTACCATCTCACTTCTAGGAATACTAGT ATATCGCTCACACCTCATGTCCTCCCTACTATGCCTAGAAGGAATAATACTATCGCTGTTCATTATAGCTACTCTCAT AACCCTCAACACCCACTCCCTCTTAGCCAATATTGTGCCTATTGCCATACTAGTCTTTGCCGCCTGCGAAGCAGCGG TGGGCCTAGCCCTACTAGTCTCAATCTCCAACACATATGGCCTAGACTACGTACATAACCTAAACCTACTCCAATGC TAAAACTGGTCTCGACTATACGCCCGTTTTCGGATC
MT-ND4$_{11719A\rightarrow G}$	
Assay ID dMDS913515317	CCATTCTCATCCAAACCCCCTGAAGCTTCACCGGCGCAGTCATTCTCATAATCGCCCACGG[G/A]CTTACATCCTCA TTACTATTCTGCCTAGCAAACTCAAACTACGAACGCACTCACAGTCGCA
Positive control	CCCATGAGACATACAAAAAGGTAATGCCGCCTCGCTAGGTGAGCTACAGCTCGATTGTCACGTTAAGCTGGCCCAT ACTCTTCAATCAGCCACATAGCCCTCGTAGTAACAGCCATTCTCATCCAAACCCCCTGAAGCTTCACCGGCGCAGTC ATTCTCATAATCGCCCACGGACTTACATCCTCATTACTATTCTGCCTAGCAAACTCAAACTACGAACGCACTCACAG TCGCATCATAATCCTCTCTCAAGGACTTCAAACTCTACTCCCACGGTCTCGACTATACGCCCGTTTTCGGATC
MT-ND5$_{13780A\rightarrow G}$	
Assay ID dHsaMDS257109922	CCTATTCGCAGGATTTCTCATTACTAACAACATTTCCCCCGCATCCCCCTTCCAAACAACA[A/G]TCCCCCTCTACCT AAAACTCACAGCCCTCGCTGTCACTTTCCTAGGACTTCTAACAGCCCT
Positive control	CCCATGAGACATACAAAAAGGTAATGCCGCCTCGCTAGGTGAGCTACAGCTCGATTGTCACGTTAAGCTGGCCAGC ACTCGAATAATTCTTCTCACCCTAACAGGTCAACCTCGCTTCCCCACCCTTACTAACATTAACGAAAATAACCCCACC CTACTAAACCCCATTAAACGCCTGGCAGCCGGAAGCCTATTCGCAGGATTTCTCATTACTAACAACATTTCCCCCGC ATCCCCCTTCCAAACAACAGTCCCCCTCTACCTAAAACTCACAGCCCTCGCTGTCACTTTCCTAGGACTTCTAACAG CCCTAGACCTCAACTACCTAACCAACAAACTTAAAATAAAATCCCCACTATGCACATTTTATTTCTCCAACATACTCG GATTCTACCCTAGCATCACACACCGCACAATCCCCTATCTAGGCCTTCTTACGAGCCAAAACCTGCCCCTACTCCTC CTAGACCTGGTCTCGACTATACGCCCGTTTTCGGATC

Table 8. Characteristics of the overall study population of the Limburg Twin Study.

	Birth	Follow-up
	n (%) or mean [10th – 90th]	n (%) or mean [10th – 90th]
Mothers	n = 43	n = 14
Parity		
1	20 (50.0)	20 (71.4)
> 1	20 (50.0)	8 (28.6)
Age, years	31 [25 – 37]	36 [31 – 43]
BMI, kg/m²	24.5 [20.4 – 31.4]	25 [21.4 – 30.9]
Maternal education [a]		
Low	25 (58.1)	6 (42.9)
High	18 (41.9)	8 (57.1)
Newborn/child	n = 86	n = 28
Gestational age, weeks	36 [32 – 38]	
Age		
Sex		
Female	56 (65.1)	16 (57.1)
Ethnicity [c]		
European-Caucasian	76 (88.4)	26 (92.9)
Zygosity		
Monozygotic	30 (34.9)	14 (50.0)
Dizygotic	44 (51.1)	14 (50.0)
Undetermined	12 (14.0)	
Chorionicity		
Dizygotic-dichorial	16 (18.6)	20 (71.4)
Monozygotic-monochorial	2 (2.3)	2 (7.1)
Monozygotic-dichorial	58 (67.4)	4 (14.4)
Undetermined	10 (11.6)	2 (7.1)
Birth weight, g	2348.5 [1650 – 3025]	
BMI z-score, kg/m²		0.49 [-0.21 – 1.53]
Length, cm		104.3 [98.5 – 110]
Weight, kg		17.4 [15.5 – 19.7]

Table 9. List of mutations resulting from the literature search. For each mutation, the name, position, minor allele, major allele, MAF and gene were listed, together with an indication whether the listed references found a significant association. From this list, the mutations elaborately studied in this research were chosen.

mtSNP	Position	Minor allele	Major allele	MAF	Gene	Significant association?	Reference(s)
mt348	348	G	A	0.024	D-loop	No	(28)
mt705	705	G	T	0.119	12S rRNA	No	(28)
mt752	752	A	G	0.018	12S rRNA	No	(28)
mt759	759	G	T	0.024	12S rRNA	No	(28)
mt1438	1438	A	G	0.030	12S rRNA	No	(11, 22, 28)
mt1700	1700	C	T	0.011	16S rRNA	No	(11, 28)
mt1811	1811	G	A	0.130	16S rRNA	No	(11, 22, 28)
mt1888	1888	A	G	0.110	16S rRNA	No	(11, 22)
mt2706	2706	A	G	0.463	16S rRNA	No	(11, 22, 28)
mt3010	3010	A	G	0.244	16S rRNA	No	(11, 22, 28)
mt3197	3197	C	T	0.082	16S rRNA	No	(11, 13, 22, 28)
mt3336	3336	T	G	0.033	MT-ND1	Yes	(8)
mt3506	3506	C	T	0.020	MT-ND1	No	(28)
mt3608	3608	A	G	0.011	MT-ND1	No	(28)
mt4217	4217	C	T	0.200	MT-ND1	No	(28)
mt4580	4580	A	G	0.035	MT-ND2	No	(11, 22)
mt4716	4716	A	G	0.032	MT-ND2	No	(28)
mt4769	4769	C	G	0.031	MT-ND2	No	(11, 22)
mt4823	4823	C	A	0.042	MT-ND2	Yes	(28)
mt5461	5461	A	G	0.029	MT-ND2	No	(28)
mt5496	5496	G	A	0.020	MT-ND2	No	(28)
mt6168	6168	T	A	0.013	COI	No	(28)
mt6186	6186	G	A	0.013	COI	No	(28)
mt7028	7028	C	T	0.458	COI	No	(11, 22, 28)
mt8252	8252	A	G	0.055	COII	No	(28)
mt8861	8861	A	G	0.013	ATPase6	No	(28)
mt8873	8873	A	G	0.029	ATPase6	Yes	(28)
mt8994	8994	A	G	0.013	ATPase6	Yes	(11, 22)
mt9055	9055	A	G	0.066	ATPase6	No	(11)
mt9123	9123	A	G	0.016	ATPase6	No	(11, 22, 28)
mt9698	9698	C	T	0.067	COIII	Yes	(8, 11, 22, 28)
mt10238	10238	C	T	0.031	MT-ND3	Yes	(11, 13, 22, 28)
mt10399	10399	G	A	0.193	MT-ND3	No	(28)
mt10463	10463	C	T	0.105	tRNA Arg	No	(11, 22, 28)
mt10550	10550	G	A	0.066	MT-ND4L	Yes	(8, 11, 22, 28)
mt10590	10590	C	T	0.010	MT-ND4L	No	(28)
mt10916	10916	G	A	0.012	MT-ND4L	No	(28)
mt11147	11147	A	G	0.026	MT-ND4L	No	(28)
mt11251	11251	G	A	0.194	MT-ND4L	No	(11, 13, 22, 28)

mt11299	11299	C	T	0.061	MT-ND4	Yes	(22, 28)
mt11467	11467	G	A	0.216	MT-ND4	No	(11, 13, 22, 28)
mt11674	11674	T	C	0.014	MT-ND4	No	(11, 28)
mt11719	11719	A	G	0.497	MT-ND4	No	(11, 22, 28)
mt11812	11812	G	A	0.067	MT-ND4	No	(11, 22, 28)
mt11914	11914	A	G	0.023	MT-ND4	No	(11, 22, 28)
mt11948	11948	G	A	0.021	MT-ND4	No	(11, 28)
mt12007	12007	A	G	0.016	MT-ND4	No	(11, 22, 28)
mt12247	12247	C	G	0.035	tRNA Ser	No	(28)
mt12308	12308	G	A	0.218	tRNA Leu	No	(11, 13, 22, 28)
mt12502	12502	T	C	0.035	MT-ND5	No	(28)
mt12612	12612	G	A	0.089	MT-ND5	No	(22, 28)
mt12634	12634	A	C	0.024	MT-ND5	No	(11, 28)
mt12705	12705	A	C	0.078	MT-ND5	No	(11, 13, 22, 28)
mt13106	13106	C	T	0.024	MT-ND5	No	(28)
mt13368	13368	A	G	0.101	MT-ND5	No	(11, 22, 28)
mt13617	13617	C	T	0.081	MT-ND5	No	(11, 22, 28)
mt13708	13708	A	G	0.101	MT-ND5	No	(11, 22, 28, 33)
mt13780	13780	A	G	0.040	MT-ND5	Yes	(22)
mt14470	14470	C	T	0.026	MT-ND5	No	(11, 22, 28)
mt14794	14794	G	A	0.067	Cyt B	No	(28)
mt14905	14905	A	G	0.102	Cyt B	No	(11, 22, 28)
mt15043	15043	A	G	0.042	Cyt B	Yes	(11, 22, 28)
mt15218	15218	G	A	0.044	Cyt B	No	(11, 22, 28)
mt15302	15302	T	C	0.012	Cyt B	No	(28)
mt15326	15326	A	G	0.015	Cyt B	No	(11, 28)
mt15452	15452	A	C	0.187	Cyt B	No	(11, 22, 28)
mt15607	15607	G	A	0.102	Cyt B	No	(11, 22, 28)
mt15785	15785	G	A	0.011	Cyt B	No	(28)
mt15885	15885	T	C	0.020	Cyt B	No	(28)
mt15924	15924	G	A	0.042	tRNA Thr	Yes	(11, 22, 28)
mt16093	16094	G	A	0.066	D-loop	No	(28, 30)
mt16140	16140	C	T	0.063	D-loop	Yes	(11, 28)
mt16173	16173	C	T	0.040	D-loop	No	(28)
mt16225	16225	A	G	0.075	D-loop	No	(28)
mt16258	16258	T	C	0.085	D-loop	No	(28)
mt16272	16272	T	C	0.090	D-loop	No	(28)
mt16313	16313	C	T	0.201	D-loop	No	(28)
mt16321	16321	A	G	0.014	D-loop	Yes	(28)
mt16521	16521	T	C	0.337	D-loop	No	(28)

7.4 Appendix 4: ddPCR™ optimization results

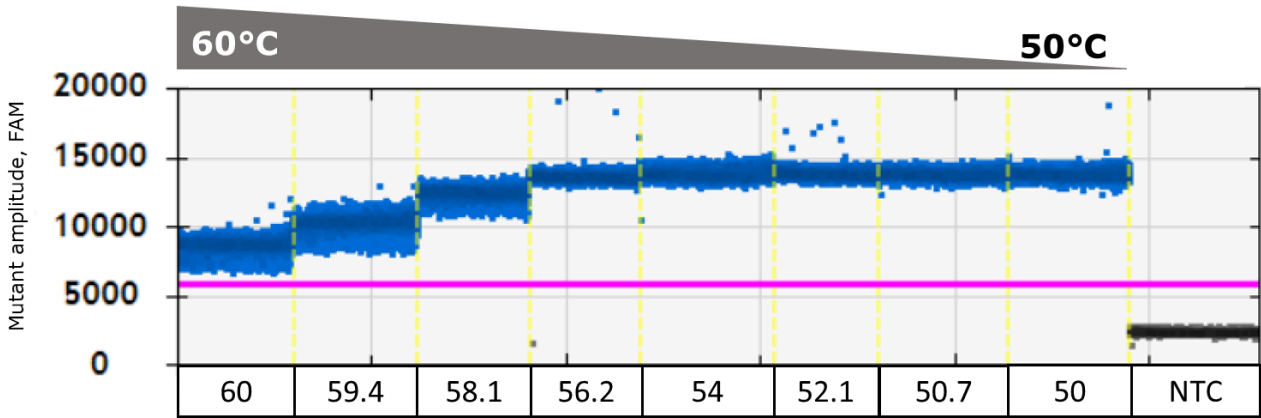### 7.4.1 Appendix 4.1: Determination of the optimal annealing temperature

Figure 13. Determination of the optimal annealing temperature. The gradient PCR was performed according to the standard ddPCR™ protocol. The graph shown is the gradient PCR for MT-ND4L$_{10550A \rightarrow G}$, however, the results were similar for MT-ND4$_{11719A \rightarrow G}$ and MT-ND5$_{13780A \rightarrow G}$ (data not shown) The optimal temperature for all three assays was set at 54°C.

7.4.2 Appendix 4.2: Determination of the optimal DNA concentration
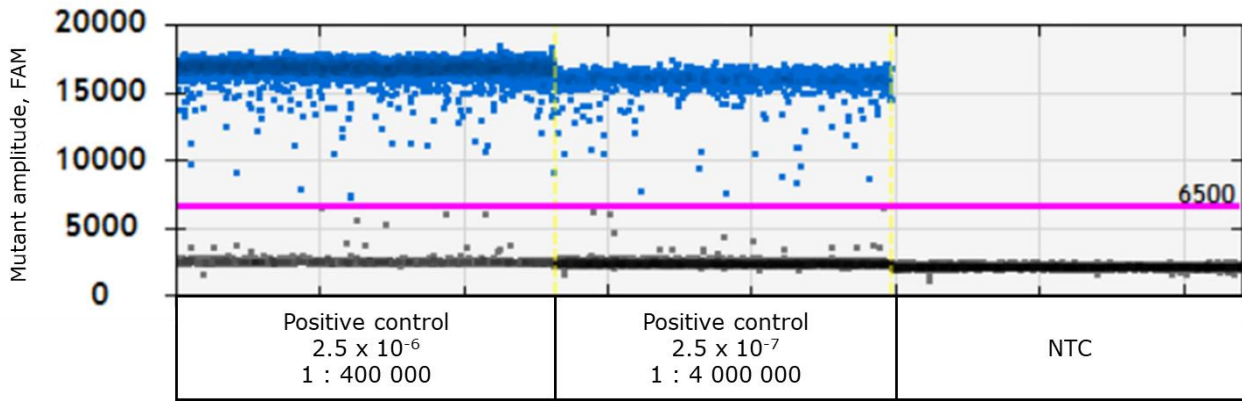
Figure 14. Determination of the optimal concentration for the positive controls (gBlocks). Previous experiments (data not shown) have determined that dilutions of 1:200 and 1:40 000 were insufficient. In addition, 1:400 000 and 1:4 000 000 dilutions were tested. In conclusion, 1:4 000 000 was chosen as the optimal concentration for the positive controls since this is the concentration that shows the best band of positive droplets with less false positive droplets.
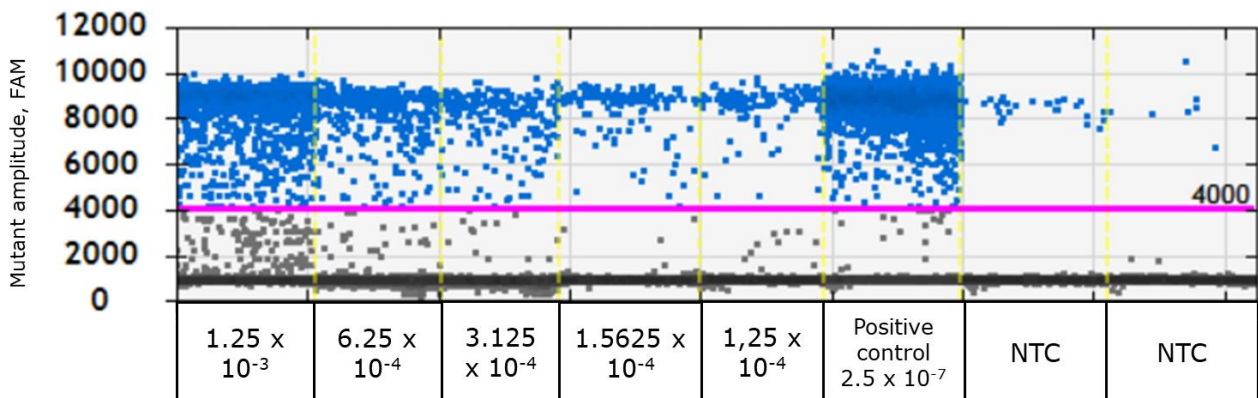

Figure 15. Determination of the optimal DNA concentration. Previous experiments (data not shown) have determined the optimal range of the dilution between 1.25 x 10-3 and 1.25 x 10-4. In conclusion, 1.25 x 10-3 was chosen as the optimal DNA concentration, since this is the concentration that shows the best band of positive droplets.
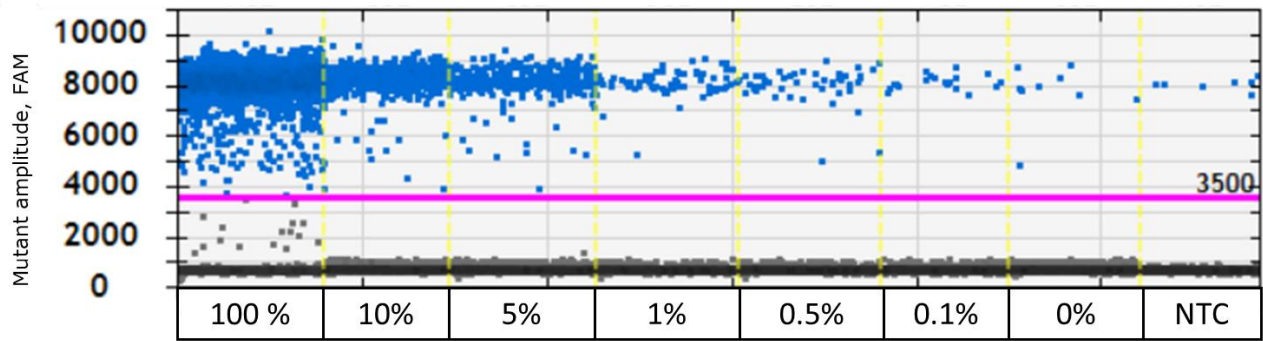
37

Figure 16. Spiking experiment to determine the LOD. In order to determine the LOD, a wild type sample was spiked with different concentrations of a mutant sample (here: gBlocks MT-ND4$_{11719A\rightarrow G}$), in concentrations ranging from 100% to 0%. In conclusion, this experiment led to a threshold of 3500 for the mutant threshold of MT-ND4$_{11719A\rightarrow G}$. Thresholds for other assays were determined in a similar manner (data not shown).

Table 10. Table of results for mutational analysis of MT-ND4L$_{10550A\to G}$ and MT-ND5$_{13780A\to G}$. Family numbers that underwent analysis for at least one family member were listed, together with values for mother (n = 28), twin 1 (n = 32) and twin 2 (n = 31) in copies/µl.

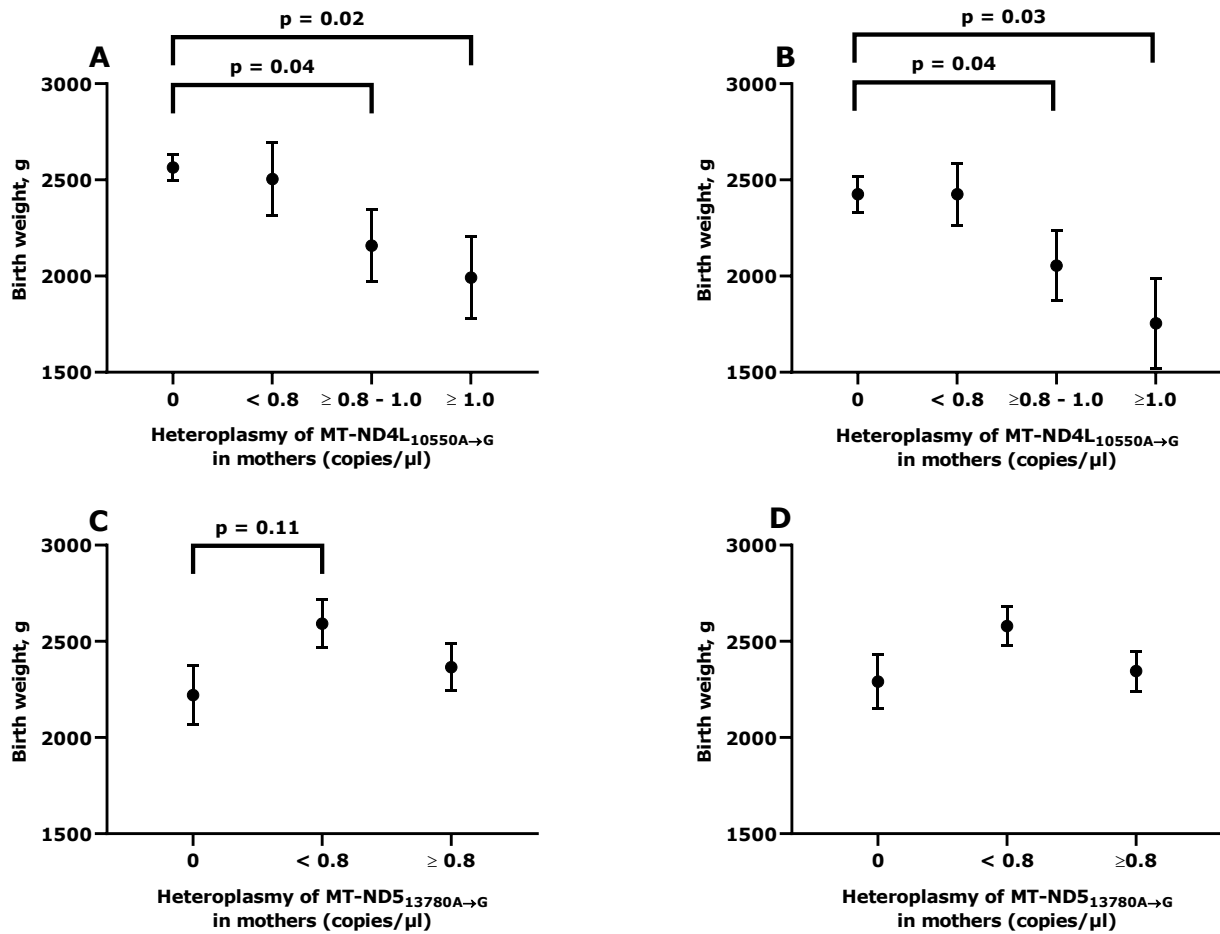| Family number	Heteroplasmy levels of MT-ND4L$_{10550A\to G}$ (copies/µl)			Heteroplasmy levels of MT-ND5$_{13780A\to G}$ (copies/µl)		
	Mother	Twin 1	Twin 2	Mother	Twin 1	Twin 2
1	0.07	0	0	0.24	0	0
2	0.8	0	0	0.17	0	0.14
3	0.27	0	0	1.6	0.9	0.12
4	0	0	0	0	0.1	0.9
5	87	22	0	0.13	0.9	0.17
7	0	0		0.15	0.12	
9	0.8		0.21	0		0.12
10		0.3	0.11		0.1	0.9
11	0	0	0	0.9	0.9	0.9
12	0.7	0	0	0	0	0.11
13		0	0		0.9	0.1
14	0.9	0.15	0	0	0	0.12
15	0	0	0.11	0.17	0	0
16	0	0	0.2	0.17	0.11	0.2
17	0	0	0.16	0.98	0.17	0.15
18	0	0.17	0.33	0.8	0.8	0
20	0.24	0	0	0.8	0.34	0.9
21	0	0	0	0.1	0.24	0.21
23		311	454		0.1	0
25	0	0	0.9	0.1	0.3	0.1
26	0	0	0	6.8	1.4	0.5
27	0	0	0	0.9	0.26	0.23
28	7.5	398	3	0	0	0.19
29	0	0.11	0	0	0.18	0.9
32		0.9	0		0.8	0.8
34	0.28	0	0.8	0.8	0	0.29
35	0	0.8	0	0.8	0.9	0.1
37	0	0	0.1	0.1	0	0.23
38		0.29			0.22	
39	0	0	0	0.1	0.24	0.15
40	0	0	0	0.15	0.8	0.42
41	0	0.24	0	0.17	0	0
42	0	0	0	0.17	0	0.9

Figure 17. Mean (95% CI) birth weight in twins (n = 57) in function of heteroplasmy levels of MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$: sensitivity analysis of heteroplasmy levels in the twins (n = 57). Windows **A** and **C** show the uncorrected models, respectively for MT-ND4L$_{10550A\rightarrow G}$ and for MT-ND5$_{13780A\rightarrow G}$, while windows **B** and **D** show the models that were corrected for the heteroplasmy levels of the mother (n = 28), measured in blood of the mother, respectively for MT-ND4L$_{10550A\rightarrow G}$ and for MT-ND5$_{13780A\rightarrow G}$. In addition, both models were also adjusted for zygosity, chorionicity, educational level of the mother and gestational age. The data are presented as mean birth weight (g) with 95% confidence intervals.
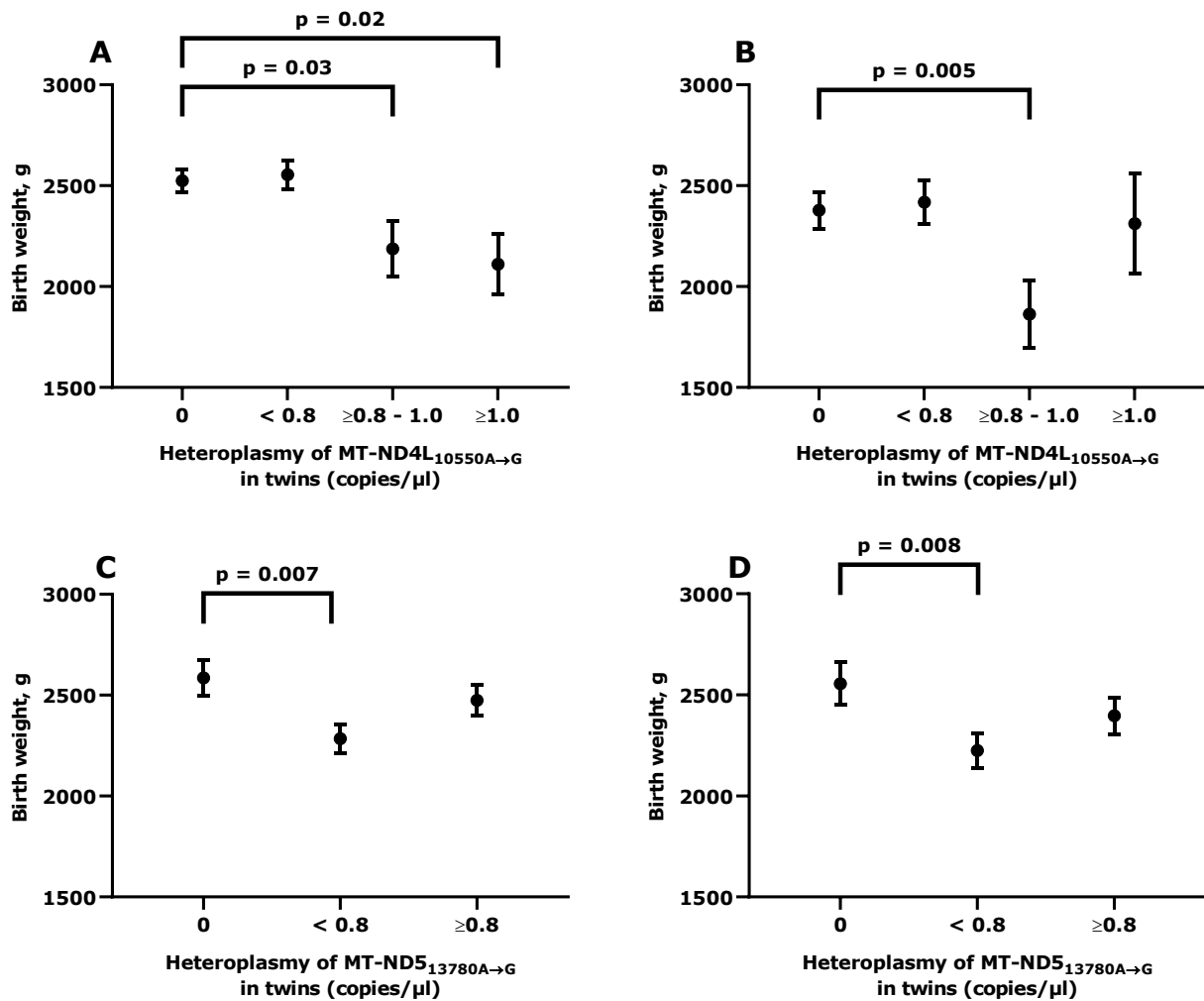
Figure 18. Mean (95% CI) birth weight in twins (n = 57) in function of heteroplasmy levels of MT-ND4L$_{10550A\rightarrow G}$ and MT-ND5$_{13780A\rightarrow G}$: sensitivity analysis of heteroplasmy levels in the mother (n = 28). Windows **A** and **B** show the uncorrected models, respectively for MT-ND4L$_{10550A\rightarrow G}$ and for MT-ND5$_{13780A\rightarrow G}$, while windows **C** and **D** show the models that were corrected for the heteroplasmy levels of the mother, measured in blood of the mother, respectively for MT-ND4L$_{10550A\rightarrow G}$ and for MT-ND5$_{13780A\rightarrow G}$. In addition, both models were also adjusted for zygosity, chorionicity, educational level of the mother and gestational age. The data are presented as mean birth weight (g) with 95% confidence intervals.
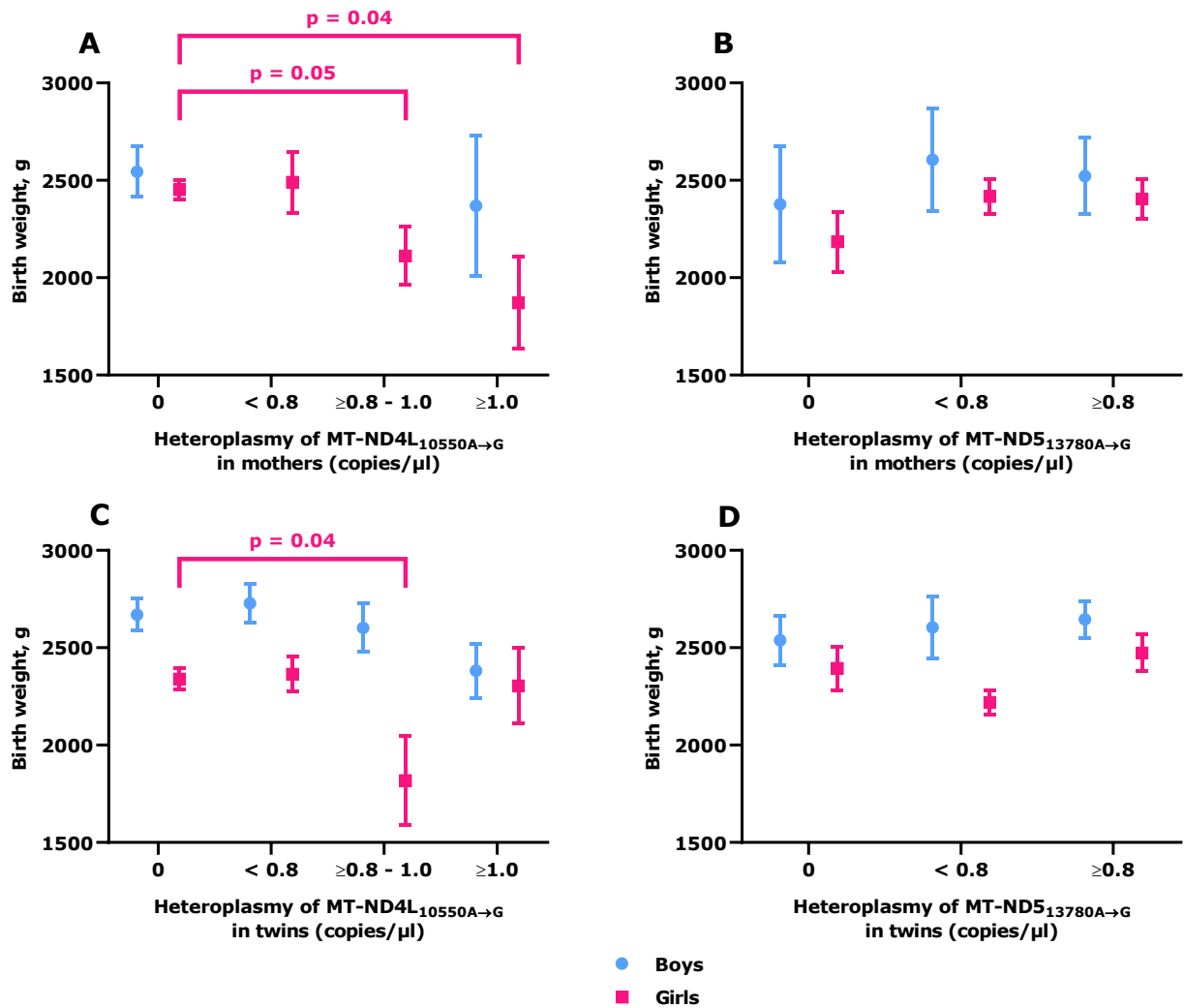
Figure 19. Mean (95% CI) birth weight in twins (n = 57) in function of heteroplasmy levels of MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$: sensitivity analysis for gender of the child. Windows **A** and **B** show the association of birth weight with the mutations in the mothers (n = 28) of respectively MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$, separately for boys (blue) and girls (pink). The models that study the mutation of the mother were adjusted for zygosity, chorionicity, parity, educational level of the mother, age of the mother, gestational age and weight before pregnancy. Windows **C** and **D** show the association of birth weight with the mutations of the boys (blue) and girls (pink) separately of respectively MT-ND4L$_{10550A \to G}$ and MT-ND5$_{13780A \to G}$. The models that study the mutation of the twin were adjusted for zygosity, chorionicity, gender of the child, educational level of the mother and gestational age.