MASTER THESIS

MITOCHONDRIAL DYSFUNCTION IN REPRODUCTIVE MEDICINE TREATMENT

Chances for new therapeutical approaches

submitted by

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in fulfillment of the requirements for the degree of

Master of Science
(MSc)

at the

Medical University of Graz

conducted in the course of

Master of Science in Medical Genetics
at the
Diagnostics and Research Institute of Human Genetics
under the supervision of

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Graz, August 2022

Declaration of Academic Integrity

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Graz, 08/19/2022 Gerit Verena Mayrhuber m.p.

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I. Abbreviations

AFC	antral follicle count
ATAD3	ATPase family AAA domain- containing protein 3
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
Ca^{2+}	Calcium
Cas9	CRISPR-associated protein 9
CB	cytochalasin B
CICR	Ca^{2+}-induced-Ca^{2+}-release
CIRCR	Ca^{2+}-induced-Ca^{2+}-release
CL	corpus luteum
CNS	central nervous system
CPEO	Chronic Progressive External Ophthalmoplegia
CPEO	Chronic Progressive External Opthalamoparesis
CRISPR	clustered regularly interspaced short palindromic repeats
D-loop	displacement loop
DNA	deoxyribonucleic acid
DOR	diminished ovarian reserve
AMH	anti- Mullerian hormone
DPV	Diabetes Patienten Verlaufsdokumentation
e.g.	for example
ER	endoplasmic reticulum
ESC	embryonic stem cells
ETC	electron transport chain
GV	germinal vesicle
HVR	hypervariable regions
ICSI	intracytoplasmic sperm injection
IMM	inner mitochondrial membrane
IMS	inter membrane space
iPSC	induced pluripotent stem cells
IVF	in vitro fertilization

IVM	in vitro maturation
kb	kilobase
LH	luteinizing hormone
LHON	Leiber's Hereditary Optic Neuropathy
MAM	mitochondrial-associated endoplasmic reticulum membrane
MELAS	Mitochondrial Encephalopathy Lactic Acidosis and Strokes like episodes
MERRF	Myoclonic Epilepsy with Ragged Red Fibers
MGE	mitochondrial genome editing
MII	metaphase II
MILS	maternally inherited Leigh syndromes
mitoTALEN	mitochondrial-targeted TALENS
MMP	mitochondrial membrane potential
MRCA	most recent common ancestor
mRNA	messenger ribonucleic acid
MRT	mitochondrial replacement therapy
MST	maternal spindle transfer
mtDNA	mitochondrial deoxyribonucleic acid
mtDNAcn	mitochondrial deoxyribonucleic acid copy number
NARP	Neuronic Ataxia and Retinitis Pigmentosa
NCR	non- coding region
ND4	NADH-ubiquinone oxidoreductase chain 4
nDNA	nuclear deoxyribonucleic acid
NGS	Next Generation Sequencing
NUMTs	nuclear mitochondrial deoxyribonucleic acid
NumtS	nuclear mitochondrial deoxyribonucleic acid sequences
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PB1T	polar body 1 transfer
PB2T	polar body 2 transfer
PBT	polar body genome transfer
PGC	primordial germ cell
PGD	preimplantation genetic diagnosis
pM	picomolar

PMD	primary mitochondrial disease
PND	prenatal diagnosis
PNT	pronuclear transfer
POI	primary ovarian insufficiency
POLG	deoxyribonucleic acid polymerase gamma
PPN	pre-pronucleus
ROS	reactive oxygen species
RP-Loop	RNA transport-derived stem loop
rRNA	ribosomal ribonucleic acid
SER	smooth surfaced endoplasmic reticulum
SQSTM1	Sequestrosome 1
TALEN	transcription activator-like effector nuclease
TFAM	mitochondrial transcription factor A
tRNA	transfer ribonucleic acid
WES	whole exome sequencing
WGS	whole genome sequencing
ZFN	zinc finger nucleases

II. List of figures

III. Zusammenfassung

Mitochondrien sind hochdynamische Zellorganellen, die eine Vielzahl von Funktionen erfüllen. Sie sind vor allem für die ATP-Produktion über die Atmungskette bekannt. Mitochondriale und nukleare DNA sind an der zellulären Energieproduktion beteiligt, weshalb die in den Mitochondrien und im Kern kodierten Gene für die Befruchtung und die Einnistung des Embryos und damit für eine erfolgreiche Embryonalentwicklung entscheidend sind.

Die Tatsache, dass es in einer Zelle zahlreiche Kopien der mtDNA gibt, führt zu einer komplexen Genetik. Die Koexistenz von Wildtyp- und mutierter mtDNA in einer Zelle wird als Heteroplasmie bezeichnet. Der Grad der Heteroplasmie kann zwischen verschiedenen Geweben variieren, sich im Laufe der Zeit verändern und zu unterschiedlichen Phänotypen mitochondrialer Krankheiten führen. Bislang gibt es keine Heilung für mitochondriale Erkrankungen, sondern nur eine palliative Behandlung zur Unterstützung der betroffenen Patient*innen.

Mitochondrien spielen eine wichtige Rolle bei der Reifung der menschlichen Eizelle, der Präimplantation und der Einnistung des Embryos, da sie für diese energieaufwendigen Prozesse unerlässlich sind. Sie wirken sich sowohl auf die weibliche als auch auf die männliche Fruchtbarkeit aus. Die Rolle der Mitochondrien im Zusammenhang mit der Reproduktionsmedizin wird immer besser verstanden. Die mitochondriale Ersatztherapie wird bereits vereinzelt eingesetzt. Diese neue Form der IVF soll dysfunktionale mtDNA ersetzen und so mitochondriale Erbkrankheiten verhindern und das Reproduktionspotenzial von Eizellen erhöhen. Da bei MRT-Techniken genetisches Material einer dritten Person verwendet wird, wirft dies ethische Fragen auf. Neue Techniken scheinen sich schneller zu entwickeln, als ethische Dilemmata gelöst werden können. Insbesondere auf dem Gebiet des mitochondrialen Genome Editings gibt es vielversprechende Methoden, um mitochondriale Krankheiten in Zukunft heilen und verhindern zu können.

IV. Abstract

Mitochondria are highly dynamic cell organelles that perform a variety of functions. Above all, they are known for ATP production via the respiratory chain. Mitochondrial and nuclear DNA are involved in cellular energy production, which is why the mitochondrial and nuclear encoded genes are crucial for fertilization and embryo implantation and consequently for successful embryonic development.

The fact that there are numerous copies of mtDNA within a cell entails complex genetics. The coexistence of wild-type and mutant mtDNA in a cell is called heteroplasmy. The degree of heteroplasmy can vary between different tissues, can change over time, and leads to different phenotypes of mitochondrial diseases. To date, there is no cure for mitochondrial diseases, only palliative treatment to support affected patients.

Mitochondria play an important role in human oocyte maturation, preimplantation and embryo implantation because they are indispensable for these energy-demanding processes. They have an impact on female, as well as on male fertility. The role of mitochondria in the context of reproductive medicine is becoming more understandable. Mitochondrial replacement therapy is already used to a limited extend. This new form of IVF is intended to replace dysfunctional mtDNA and thus prevent mitochondrial genetic diseases and increase reproductive potential of oocytes. Since MRT techniques involve genetic material from a third person, this raises man ethical questions. New techniques seem to be developing faster than ethical dilemmas can be resolved. Particularly in the field of mitochondrial genome editing, there are promising methods for curing and preventing mitochondrial diseases in the future.

1 Introduction

Mitochondria are highly dynamic cell organelles that perform a variety of functions. Their name is derived from the Greek words for thread (*mitos*) and granule (*khondors*) (1).

Above all, they are known for adenosine triphosphate (ATP) production via the respiratory chain. Therefore, they are also considered the powerhouse of the cell. In addition to energy production, they play an important role in cell metabolism (e. g. lipid metabolism and amino acid metabolism) and are involved in the regulation of cell death (apoptosis). Processes like iron-sulfur biogenesis, protein degradation, protein translocation and metabolite exchanges are located within the mitochondrial structure (2). Both mitochondrial and nuclear deoxyribonucleic acid (DNA) are involved in cellular energy production, which is why these mitochondrial and nuclear encoded genes are crucial for fertilization and embryo implantation and consequently for successful embryonic development (3).

1.1 Human mitochondria

Depending on the type of cell and the metabolic state, mitochondria can take on different shapes like spherical, filamentous, or branched tubular (4,5).

The organelles contain a double membrane consisting of a smooth, outer mitochondrial membrane (OMM) and a highly folded inner mitochondrial membrane (IMM). The outer membrane serves as a barrier for macromolecules, its pores allow passage of solutes with a molecular mass of a few thousand Daltons. The inner membrane spans around the matrix space of the mitochondrion. In contrast to the outer membrane, it is impermeable even to small solutes such as ions and metabolic substrates. These molecules can only pass via carrier proteins (6).

The inner membrane is subdivided into the inner boundary membrane and the cristae membrane. The inner limiting membrane is located close to the outer membrane and, together with the outer membrane, spans the mitochondrion. The cristae membrane forms invaginations that protrude into the matrix space. The cristae are connected to the inner limiting membrane via cristae junctions. These

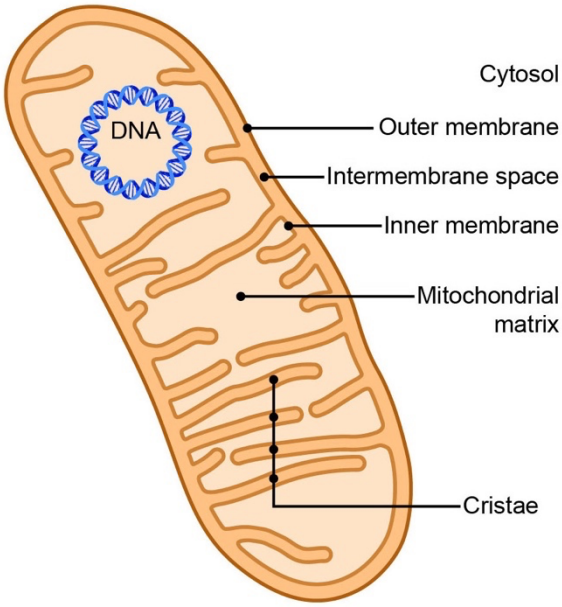

Fig.1: Schematic representation of a mitochondrion. The ring-shaped mtDNA and further copies thereof are located in the mitochondrial matrix. The surface of the IMM is enlarged by cristae invaginations into the matrix. Between OMM and IMM extends the intermembrane space. Mitochondria are surrounded by the cytoplasm (144)

structures can also highly differ depending on their morphology (4,5). The protein-rich lipid bilayers harbor various protein complexes, especially those involved in oxidative phosphorylation (OXPHOS) (2).

The intermembrane space (IMS) extends between the outer and inner membrane (7). The IMS contains a wide range of proteins. Although the cell compartment houses only about 5% of the mitochondrial proteome, it has flexible protein transport mechanisms across the OMM. Porin channels in the OMM allow passaging of intermediates of the respiratory chain. Enzymes such as creatine kinase or adenylate kinase convert or synthesize ATP before. The OMM is permeable to small molecules such as ions and sugars, so their concentration is the same as in the cytosol. The protein composition in the IMS differs from the cytosol. For transport across the membrane, large proteins require specific signal sequences. Through this mechanism, cytochrome c, an important electron carrier of the respiratory chain, is retained in the IMS (8,9).

The organelles can provide up to 90 percent (%) of cellular energy in the form of ATP through OXPHOS in mammalian cells (6). The mitochondrial matrix contains enzymes, ribosomes, and the ring-shaped DNA molecules. The number of mitochondria in a cell depends on the energy demand. A mature egg cell contains more than 100,000 mitochondria, while muscle, liver, and brain cells contain between 2,000 and 10,000. Mitochondria are present in most eukaryotic cells with few exceptions like erythrocytes.

1.2 Mitochondrial DNA

Mitochondrial DNA was discovered in 1960, when electron micrograph images of chicken liver mitochondria were published by Nass M. *et al.,* and mitochondrial DNA was isolated from yeast. These discoveries indicated the presence of DNA in mitochondria for the first time (10,11).

1.2.1 Structure

The endosymbiont theory assumes that mitochondria evolved about two billion years ago. Mitochondria were formed through endosymbiosis with an alpha proteobacteria in eucaryotic precursor cells.
Among other things the existence of ribosomes and the ring-shaped DNA in the mitochondrial matrix support this theory. Mitochondria have retained their ancestors' double membrane and ATP production but have acquired additional functions within the cell.
During evolution, most of the genetic material of the bacterium was transferred to the nuclear genome. A small circular genome has remained in the human cells, the present mitochondrial DNA (mtDNA). In contrast to the nuclear chromosomes, there are numerous copies of mtDNA within the cell (12,13).Each mitochondrion carries multiple copies of compact organized mtDNA, and each cell in turn contains numerous functional mitochondria (1). This results in a much higher gene density in mtDNA than in nuclear DNA. In addition, mitochondrial messenger ribonucleic acid (mRNA) makes up a strikingly high portion of the total cellular mRNA, e.g., up to 30% of cellular mRNA in the heart or 5 to 25% in other human tissues with lower energy demands like adrenal and ovary (14,15).
MtDNA is essential for the functionality of the cell, although it represents less than 1% of the total number of DNA. The circular, double-stranded mtDNA genome has a size of 16.569 kilobases (kb) and is located within the IMM in the mitochondrial matrix, closely linked to the cristae of the mitochondrion (16). It consists of a heavy and a light strand, encoding 37 genes for 13 proteins, 22 transfer ribonucleic acids (tRNAs) and 2 ribosomal ribonucleic acids (rRNAs) (3,17).
Although mtDNA encodes for its own tRNAs and rRNAs, the transcriptional and translational processes work semi-autonomous, depending on chromosomally

encoded genes (18). The proteins are synthesized independently by the mitochondrial translation system. They are part of the protein complexes (complex I, III, IV and V) required for OXPHOS. Nevertheless, more than 1000 proteins required for mitochondrial function are encoded by the nuclear genome. Proteins like mitochondrial ribosomal proteins, metabolic enzymes, mtDNA replication factors, as well as those proteins required for complex II are translated in the cytoplasm and imported into the organelles subsequently (14,17).

Up to 10 copies of the circular mtDNA molecule are located in the mitochondrial matrix. These mtDNA molecules form mtDNA- protein complexes, better known as nucleoids (19). The mtDNA packaging is different from that of nuclear DNA (nDNA), as there are no histones. Like with bacterial chromosomes, mtDNA is surrounded by a range of proteins, e.g., mitochondrial transcription factor A (TFAM), prohibitins, a catalytic subunit of DNA Polymerase gamma (POLG), and ATPase family AAA domain- containing protein 3 (ATAD3). As the main protein of the nucleoid, the transcription factor TFAM is crucial for maintenance of mtDNA and very important for nucleoid distribution and organization (20,21).

MtDNA fragments are generated during the life span of organisms in both somatic and germ cells by reactive oxygen species (ROS) in mitochondria. The fragments are transferred and inserted into the nucleus during the repair of double-strand breaks (non- homologous repair). These fragments are referred to as nuclear mitochondrial DNA (NUMTs). This occasional transfer of mtDNA into the nucleus represents a process that has occurred many times during genome evolution.

So called mitochondrial- associated endoplasmic reticulum membranes (MAMs) connect the mitochondria with the endoplasmic reticulum (ER). This is important for mitochondrial fission and fusion. Proteins and lipids of the MAMs are also located in the OOM and ER membranes. The connection between mitochondrion and ER affects initiation of mitochondrial transcription and mediates distribution of replicated mtDNA to daughter mitochondria. These ER- mitochondria contact sites have been illustrated in imaging experiments, highlighting their importance for nucleoid dynamics, segregation, and transportation processes (22).

Chromosomally encoded transcription and replication factors are interacting with the displacement loop (D-loop). The D-loop shapes a triple strand, consisting of a heavy and light DNA strand and an additional heavy strand. The third strand is hydrogen bonded to the light strand (23). The D-loop is part of the only non- coding region

(NCR) of the mitochondrial genome and anchored to the IMM through a multiprotein complex, which ensures distribution throughout the mitochondrion (17,24). The NCR is about 1 kb in size and is the most polymorphic site within mtDNA. It can be divided into two hypervariable regions (HVR) with several known polymorphisms (25,26).

HVRs evolve at a much faster rate than average and are usually considered mutational hotspots. HVRs can be identified by human mtDNA variation analysis of the noncoding regions (26). Sequences of HVRs can be assigned to mitochondrial haplogroups and contribute to trace genetic lineages of human populations (27). Mitochondrial haplogroups are defined by differences in human mtDNA. They help trace the origin and spread of humans all over the world. Scientists have theorized a “Mitochondrial Eve”. The Mitochondrial Eve is the hypothetical woman at the roots of all mitochondrial haplogroups. She is therefore not the first woman of our species, but the woman who passed on mitochondrial DNA without interruption. She represents the matrilineal most recent common ancestor (MRCA) for all currently living humans in biology and genetic genealogy.

The NCR serves as a regulatory unit by controlling mitochondrial transcription and translation. Besides the origin of replication for one strand, the mtDNA control region also contains the origin of transcription for both strands (1,28).

In the mtDNA genome, intron sequences between coding regions are missing, hence, coding regions might overlap, e.g., ATPase6 or ND4L. Some other genes lack termination codons because these are generated via post- transcriptional polyadenylation (17,18).

1.2.2 Heredity

MtDNA is maternally inherited. This phenomenon is also known as uniparental inheritance in a non-Mendelian manner. That is why some mitochondrial diseases due to mtDNA mutations are also maternally inherited (29,30).

Paternal mitochondria from the sperm enter the egg cell, coexist with maternal mitochondria, but can no longer be detected at around the four to eight cell stage after fertilization. Although it is generally accepted that mtDNA is inherited exclusively from the mitochondria of the oocyte, there are two different hypotheses

to explain the underlying mechanisms of degradation of paternal mtDNA during fertilization (29).
Active degradation occurs through ubiquitin labelling. Labelled mitochondria are recognized and degraded by proteasome and/or lysosomes after fertilization. Another hypothesis is based on the ratio of maternal to paternal mitochondria, is easier to explain. Paternal mitochondria are simply diluted, because in contrast to the egg cell, containing more than 100,000 mitochondria, only about one hundred of these organelles can be found in the sperm. Endonuclease G and mitophagy are thought to contribute to the removal of paternal mtDNA during fertilization, however, the degradation mechanisms are not fully understood (6).

1.3 Mitochondrial diseases

Mitochondrial defects lead to failure of cellular energy metabolism. Classical mitochondrial diseases include respiratory chain defects, the underlying mechanisms are also the best studied. They cause a cellular energy deficiency and can cause progressive neurodegenerative disease patterns. By establishing Next Generation Sequencing methods, new mutations of the mtDNA as well as defect of numerous nuclear genes are constantly being described. The etiology of mitochondrial disease (mitochondrial or nuclear gene defects) is not yet treatable. The date on pre-implantation diagnostics as prevention and risk reduction of inheritance of pathogenic mtDNA mutations is limited. Progress is being made in the field of mitochondrial replacement therapy.

1.3.1 Causes

Mitochondrial diseases are caused by genetically determined dysfunction of the mitochondria and belong to the most common hereditary diseases (with a lifetime risk of 1:150,000). Pathogenic mtDNA mutations have been declared the main cause of human hereditary disease because, according to epidemiological studies, one in 5,000 people are affected. Pathogenic alleles are found in more than one of 200 newborns. Inherited mtDNA mutations develop multi-system diseases, often accompanied with damaging effects in the nervous system. As treatment options

are quite limited, it is very important to find reliable techniques to avoid transmission of mtDNA mutations (31).

Mitochondrial diseases can underlie nuclear gene defects or mutations of the mtDNA. Nuclear gene defects affect proteins that are encoded in the nucleus and follow Mendelian rules. They can be inherited autosomal dominant or X-chromosomal but are mostly autosomal recessive. Mutations of the mtDNA are maternally inherited or occur sporadically. Pathogenic mitochondrial variants therefore follow maternal inheritance patterns, because only oocytes pass on mtDNA to embryos. Maternally inherited, but also *de novo* mutations, have the potential to cause diseases (32).

The diseases are clinically, biochemically, and genetically heterogeneous and are often not diagnosed correctly, because of the extreme phenotypic- genotypic heterogeneity. Mitochondrial diseases are predominantly multisystem diseases. Any tissue or organ can be affected, whereby mainly organs with high energy requirements, e.g., brain, eye-, heart- and skeletal muscles, are damaged. The severity ranges from the most severe diseases in childhood to relatively mild diseases in old adulthood. Childhood mitochondriopathies differ from adult mitochondriopathies in that they have a broader clinical spectrum, ranging from severe perinatal lethal forms to relatively mild myopathic diseases. The central nervous system (CNS) is very often affected. Typical manifestations in adults include epileptic seizures, stroke-like events, hearing loss, retinopathy, external ophthalmoparesis, muscular strain intolerance and diabetes mellitus. Constellations of two or more symptoms are particularly suspicious. Only a sensible combination of clinical, biochemical, morphological, and molecular genetic examinations leads to a specific diagnosis.

As mentioned before, mitochondria are involved in crucial metabolic pathways and participating in intracellular signaling networks to control distinct cellular functions. According to this, mitochondrial defects or dysregulations are observed in ageing processes and in cytopathological mechanisms based on cancer, neurodegenerative and other diseases. The most frequent mitochondrial diseases are Leigh Syndrome, Pearson Syndrome, Myoclonic Epilepsy with Ragged Red Fibers (MERRF), Leber's Hereditary Optic Neuropathy (LHON), Neuronic Ataxia and Retinitis Pigmentosa (NARP), Chronic Progressive External Ophthalmoplegia

(CPEO) and Mitochondrial Encephalopathy Lactic Acidosis and Strokes like episodes (MELAS) (33).

Disease- causing defects in energy metabolism have been described in over four hundred genes so far. MtDNA is much more vulnerable than nuclear DNA. Genes coding for subunits of the respiratory chain or for mitochondrial assembly proteins may be mutated. Genes which influence the translation of mtDNA, or genes that control the phospholipid compositions of the inner mitochondrial membrane or those involved in mitochondrial dynamics can also be affected by mutations. In addition, defects in mtDNA maintenance also contribute to mitochondrial diseases.

MtDNA shows a 10-to-20-fold higher mutation rate than the nuclear genome - they do not possess efficient repair mechanisms compared to the nuclear genome. The higher rate of mutagenesis is caused by different factors. Introns and histones are missing in mtDNA, the mitochondrial genome is located next to the sites of ROS production, and its replication is based on asymmetrical division. Structural rearrangement (e.g. deletions), quantitative changes (like depletion or reduction of mtDNA copy number) or point mutations occur.

Replication efficiency decreases with tissue- dependent mtDNA depletion. Mainly responsible for impaired mtDNA are lesions caused by oxidative damages, which can be repaired by DNA repair mechanisms only to a certain degree. Generating ATP for the cell relies on OXPHOS and entails the generation of ROS. About 90% of ROS are produced by OXPHOS, first and foremost in Complex I and III (34).

1.3.2 Heteroplasmy

The fact that there are numerous copies of mtDNA within a cell entails complex genetics. The coexistence of wild-type and mutant mtDNA in a cell is called heteroplasmy (Fig.2).

The degree of heteroplasmy can vary between different tissues and change over time. Different mtDNA mutations can coexist within a tissue and a mosaic of different mitochondria develops. If a spontaneous mutation occurs early in life, this can lead to a mosaic organism with affected and unaffected tissues and organs. As part of the ageing process, mtDNA mutations accumulate in certain tissue types.

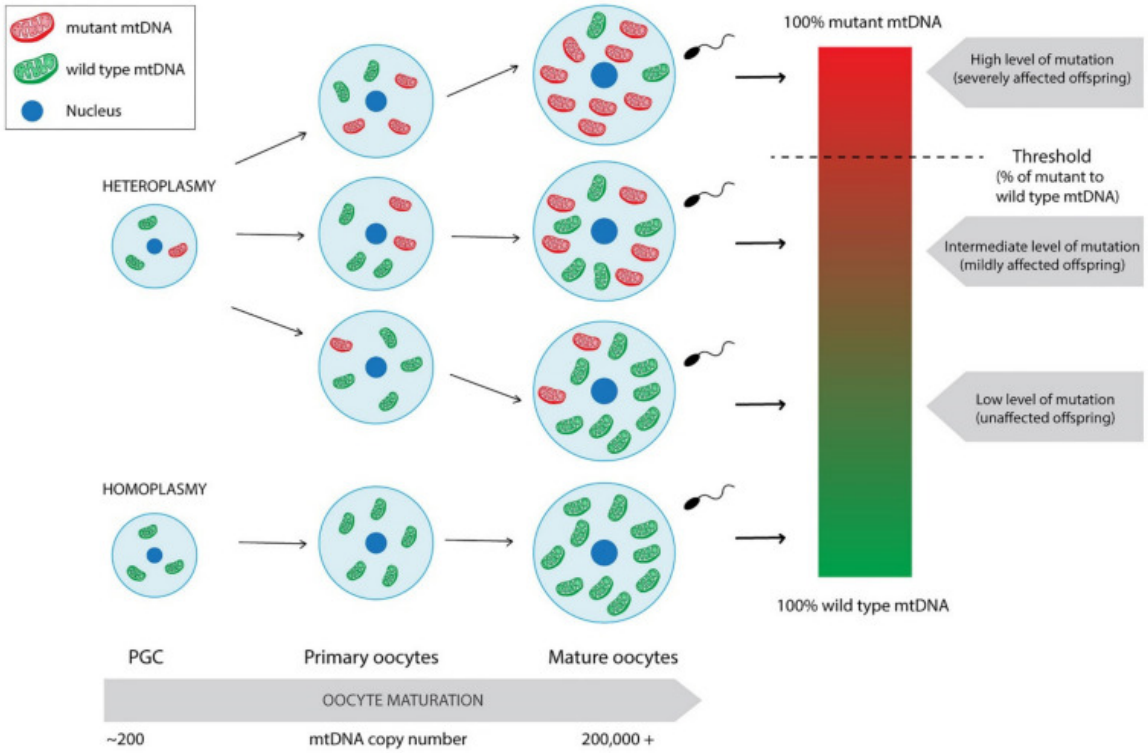

Fig.2: Differentiation of homoplasmic and heteroplasmic PGCs during oocyte maturation with mtDNA copy number expansion. Mature oocytes derived from heteroplasmic PGC may present varying levels of mutation frequency leading to unaffected or mildly or severely affected offspring. Paternal linkage was not taken into consideration. "Differentiation of homoplasmic and heteroplasmic PGCs during oocyte maturation with mtDNA" by Amira Podolak *et. al* (2022) is licensed under CC BY 4.0

De novo mutations occur in all kinds of body cells. The proportion of mutant mtDNA must reach a certain threshold, at which the wild type mtDNA can no longer compensate for the biochemical damage, resulting in phenotypic abnormalities. Depending on the tissue, this threshold lies between 50 and 90% of mutated mtDNA. The phenotypic threshold effect is also applicable at the single cell level, as demonstrated by experiments performed directly on single muscle fibers (35). The ratio of healthy to mutated mitochondria determines the severity of the phenotype. For example, 90% of mutant mtDNA must be present in the muscle for MERRF syndrome to arise (3,35).

The germline mtDNA bottleneck explains and facilitates changing heteroplasmy allele frequencies between generations. The bottleneck effect describes the reduction in mtDNA content during oogenesis (36). During the formation of immature oocytes, only a few mtDNA molecules can be transferred. Then the oocytes mature, resulting in a rapid increase in the number of mtDNA molecules already present - the principle of the bottleneck effect. A small number of initial mtDNA molecules are

responsible for the basis of all subsequent mtDNA copies in the entire organism (37). This phenomenon can be observed in mothers with a low level of mutant mtDNA molecules having offspring with a disproportionately high level of mutant mtDNA. The ration of mutant to original genes is random. This process allows proportionally more molecules of mutant mtDNA to be inherited than it would correspond to the status of the mother (the carrier of the original mutation). Mutations causing changes in a protein are subject to strong prenatal selection and subsequently cannot be passed on to the offspring, which has already been demonstrated in a paper by Nils Goran *et al*. In contrast, silent mutations must not be disregarded. Although they represent changes in a gene, they have no effect on the translated protein. The silent mutations of mtDNA are not subject to prenatal selection (38).

1.3.3 Homoplasmy

If a cell carries identical mtDNA copies with molecules of the same or no mutation, this is called homoplasmy. Women carrying homoplasmic mtDNA mutations pass them on to their offspring. The mechanisms behind mtDNA inheritance have been debated for decades, but the most important molecular and cellular questions have now been clarified by working with mouse models. It has been shown, that in mice selection occurs during the transmission of heteroplasmy mtDNA mutations. This leads either to accumulation of pathogenic mtDNA mutations or, more desirably, to their loss (31).

1.3.4 Mitochondrial damage

Damage to mitochondria has a variety of consequences. Mitochondria can respond differently to damage. Denatured proteins ca be folded by mitochondrial heat shock proteins (Hsp10 and Hsp60), which form a mitochondrial folding complex, to prevent protein aggregate formation and protect them from degradation by proteases. Hsp70 prevents proteins from forming aggregates. The mitochondrial matrix is harboring a proteasome- like complex (LON- complex) and an ATP- driven protease complex (ClpP). In addition, there are proteases in the IMM to degrade defective

membrane proteins. Fusion among mitochondria is also a way to exchange functional components and thereby prevent the degradation of the organelle. When mitochondria are exposed to low stress levels from toxic chemicals or from nutrient deficiencies, increased fusion events can be observed. When components of the cytoplasm are digested by macro-autophagy due to nutrient deficiency, the cell protects its mitochondria by converting them into elongated tubes. They appear to be resistant to autophagy and inhibit fission but promote fusion (39).

1.3.5 Examples and consequences for carriers

The most common syndromes caused by mtDNA mutations are MERRF, MELAS, NARP and Leigh syndrome. These examples illustrate, that the phenotypes of different syndromes can overlap, and that the same mutation can develop in different syndromes depending on its mutation value.

The MERRF syndrome is a mitochondrial encephalomyelopathy characterized by myoclonic cerebral seizures. The prevalence in European population has been determined to be 0.9: 100,000. However, the disease appears to be more common in the USA. Affected individuals usually suffer from myoclonus epilepsy in early adulthood or adolescence. Symptoms such as sensory-neural hearing loss, optic atrophy, short stature, or peripheral neuropathy may also appear. The disease is progressive, which means that the epilepsy worsens, and patients suffer from additional symptoms including ataxia, hearing loss, muscle weakness, and dementia. The clinical symptoms vary from patient to patient, both, within a family and between families.

MERRF syndrome is caused by mutations in mtDNA. More than 80% of the patients carry a 8344A>G mutation in the mitochondrially encoded tRNA lysine- gene (*MTTK*). The gene usually codes for lysine transfer RNA (tRNA Lys). The remaining mutations are in genes for other tRNAs or in the mitochondrially encoded NADH Dehydrogenase 5- gene (*MTND5*), coding for the ND5 subunit of respiratory chain complex I. They can cause a so-called MERRF/MELAS overlap- syndrome, in which patients (as in MELAS) also suffer from stroke-like episodes. The diagnosis of MERRF syndrome is based on the detection of abnormally elevated lactate levels in blood or in cerebrospinal fluid and following findings in muscle biopsy- cytochrome c oxidase- negative muscle fibers and ragged red fibers. A cytochrome c oxidase

deficiency or a combined respiratory chain defect can often be detected biochemically. Heteroplasmy must always be considered and makes genetic counselling more difficult. The level of mutated DNA can highly vary between different tissues. However, experience has shown, that the level of mutated mtDNA in MERRF syndrome is very high (about 90%) in all tissues, so the mutation can also be detected in blood samples. There is no specific treatment strategy for this mitochondrial syndrome. Because of its progression, MERRF generally has a poor prognosis, although the severity varies and some patients, especially those without cerebral symptoms, live longer and with little disability (40). In general, the phenotypic threshold should be around 60% for deletions and 90% for point mutations (3,35).

Under the condition that a threshold value of 60% has been reached, a m.8993T>G mutation in mitochondrially encoded ATP synthase 6- gene (*MT-ATP6*) either leads to mild symptoms like headaches or mild pigmentary retinopathy or has no consequences at all (32). If a mutation value of 70 to 90% is reached, a phenotype matching with NARP syndrome appears. Mutation levels above 90% develop into severe Leigh syndrome.

The NARP syndrome is clinically heterogeneous. It often manifests as a combination of sensory-motor neuropathy with cerebellar ataxia and night blindness. The prevalence is estimated 1:12,000. NARP usually occurs in young adults. It is caused by mutation 8993T>G in the mitochondrial *MTATP6* gene, which codes for the ATPase 6 subunit. The evolutionarily highly conserved leucine in position 156 is replaced by arginine (L156R), resulting in severe reduction of mitochondrial ATP synthesis and cellular energy reserves. Especially tissues depending heavily on oxidative phosphorylation (brain and retina) are affected, the mutation leads to cell death.

The same mutation is found in 8 to 10% of Leigh syndrome cases. These Leigh syndrome versions are called MILSs (maternally inherited Leigh syndromes). MILS is the most severe manifestation of NARP syndrome and manifests earlier and earlier from generation to generation (pseudo-anticipation). Treatment is supportive and treatment options are limited, but recently a treatment with antioxidants has been proposed, based on in vitro findings (41). Mutation m.8993T>C in the same

gene seems to be less severe, occurring in patients with levels above 90%. The phenotypic threshold is for guidance and should not be considered a specific value (32).

The how and when of the inheritance of health-threatening mitochondrial mutations have so far been controversially discussed. Mitochondrial tRNAs have been studied as potential hotspots for mitochondrial diseases. Using a mouse model, a pathogenic mutation of a mitochondrial gene coding for tRNA Methionine was further investigated by Nils Goran *et al*. It is known that mutated mitochondrial tRNA genes are responsible for a large proportion of known mitochondrial diseases, although tRNA genes make up only a fraction of mtDNA.

In three of the best- studied mitochondrial diseases - MELAS, MERRF and CPEO - mutations in tRNA genes have been identified (42). All three mainly affect the brain, the nervous system, as well as muscles. As a carrier of the mutation, the mother can be completely healthy, and the disease only appears in the offspring due to the bottleneck effect and thus the concentration of mutated mtDNA.

The mouse model by Nils Goran *et al.* was intended to clarify questions about the mixing ratio between mutated and non- mutated genes in the different phases of the inheritance process. Germ cells of mouse embryos were used, and the respective degree of mutation was analyzed. This can vary from germ cell to germ cell. When a mouse was born, the mutation levels of the immature oocytes were determined again. Finally, the mutation levels of mtDNA of the offspring of that mouse were evaluated. With this experiment, it became clear that the female germline does not screen out tRNA mutations in genes. Thus, it is already largely decided during development of the maternal germ cells, when the mother herself is still in the embryonic stage, whether and to what extent mutated genes can be passed on to the next generation. Since the corresponding mixing ratio can vary from oocyte to oocyte, the mutation level is randomly passed on to the next generation. This also explains the differences within a family. While there is no selection in germ cells, there is a strict selection of high-grade mutations after fertilization, i.e. during embryonic development (43).

1.3.6 Diagnostic breakthroughs

Mitochondrial diseases pose enormous challenges for diagnostics. Damages in mtDNA and nuclear genes ultimately affect ATP synthesis or OXPHOS, but the disease mechanisms behind them are quite complex.

At both the clinical and genetic levels, molecular diagnosis of a patient with mitochondrial disease can be challenging, since the genotype- phenotype relationship is not always clear. Sometimes, mutations of different genes cause the same phenotype (e.g. Leigh Syndrome). There are over 75 causes of this disorder, concerning both the nuclear genome and mtDNA. It can also happen that an acknowledged pathogenic mutation leads to various phenotypes (44).

Some common concomitants of mitochondrial disorders, such as migraine, diabetes mellitus and hearing loss, may also be present in the unaffected population. It sometimes proves difficult to attribute isolated clinical features of a patient or relative to a familial disorder or to prove that it is a mere coincidence. According to the German-/ Austrian- DPV ('Diabetes Patienten Verlaufsdokumentation') register, diabetes mellitus has been diagnosed as a mitochondrial disease in only 0.02% of patients younger than 30 years (44,45).

It is difficult for non-specialist clinicians to diagnose mitochondrial diseases right away. Often there are enormous diagnostic delays. Sometimes additional clinical features need to appear, or related family member must develop suggestive symptoms for the diagnosis to become clear. Grier *et al.* have visualized the complex diagnostic odyssey of patients with mitochondrial diseases. According to them, patients affected must see an average of 8.19 doctors before a mitochondrial disease is diagnosed. Finding a diagnosis is time-consuming or often does not lead to clear and conclusive results (46).

The understanding of the molecular background of some primary mitochondrial diseases (PMDs) has been greatly improved using Next-generation sequencing (NGS) techniques (47).

Due to the establishment of sequencing panels for nuclear mitochondrial candidate genes and exome sequencing and finally whole genome sequencing (WGS), the number of mitochondrial disease gene suddenly increased exponentially (48). Sequencing trios is used to describe *de novo* dominant mutations, and new genes

are included in diagnostic panels that had not previously been associated with mitochondrial diseases (44,49).

In contrast to what was initially anticipated, some predicted mitochondrial genes were classified as non- significant, due to their functional redundancy. Hence, mutations in those genes may not cause diseases. To assess pathogenic variants in patients affected by mitochondrial diseases, global access to large-scale exome and whole genome sequencing data (both from controls and people with common diseases) is of high importance and necessary for scientific breakthroughs (44).

By using ultra-deep mtDNA sequencing, it is possible to detect mtDNA heteroplasmy at a very low level (around 0.5%) (50). Pathogenic mutations with this level of heteroplasmy are, however, widespread in the population (in every 200 individuals), with the potential to proliferate throughout life alongside *de novo* mutations in cells and subsequently whole tissues (44). They do not necessarily have to be clinically relevant. Variant analysis of the directly affected tissue (e.g. skeletal muscles) is used in such unclear cases, biochemical testing may also be helpful. This clarifies whether the variant of the affected patient detected in the blood sample can be held responsible for the disease. Among other things, such a variant analysis can also help to separate genuine mtDNA variants from false positive results due to nuclear mtDNA sequences (NumtS) (51,52).

Diagnostic WGS generally covers the nuclear genome 40 to 60-times to reliably detect nuclear genetic variants (53). In contrast to nuclear DNA, there are more copies of mtDNA, resulting in around 2000-fold coverage for mtDNA (51).

This explains why mtDNA heteroplasmy is detectable with lower variant numbers in samples. WGS enables the detection of sequence variants (variant calling) of the mitochondrial and nuclear genome in a single blood DNA analysis (44).

With the use of the WGS, it is now possible to perform analyses and evaluate data from nuclear mitochondrial genes, established mitochondrial disease genes and new potentially pathogenic variants in already known mitochondrial disease genes (54). Patients with mtDNA deletions usually show a characteristic clinical phenotype. In those cases, targeted genotyping is performed first, since it is a cost- and time-efficient alternative to WGS methods (44).

As probably disease- causing mutations in new disease genes are also recorded in databases, WGS will facilitate future diagnostic interpretations. In addition, likely-pathogenic mutations in genes outside the mitochondria will also be identified. Data

show that this is a quite relevant group of mutations that have numerous phenotypic overlaps between mitochondrial diseases and other inherited diseases (44,55).

The use of WGS has great potential to diagnose mitochondrial diseases in patients with less specific disease progressions. It would be optimal if WGS was used primarily to identify heteroplasmic mtDNA variants and nuclear gene defects in patients suffering from neurological or muscular disorders. The rapid application of WGS is particularly relevant in young children, as the parents can be informed and treatment initiated as soon as possible, in addition to comprehensive genetic counselling and discussion of possible future reproductive options.

If WGS is inconclusive, histochemical, and biochemical tests are performed on a tissue biopsy. Soon, bioinformatic approaches may allow reliable detection of mtDNA deletions from WGS data (56,57).

Although NGS have greatly advanced the knowledge of the molecular background of PMDs, patients with such a suspected diagnosis still sometimes remain without a genetic diagnosis. As a result, affected patients are denied access to comprehensive genetic counselling and access to clinical trials, for example. Despite all, sequencing with targeted gene panels and whole genomes sequencing (WES) have made an enormous contribution to mitochondrial research. NGS have led to a significant increase in molecular diagnoses achieved.

In the past, genome sequencing was only used in the most difficult cases, but it is now increasingly applied for initial screening in rare genetic neurological diseases. As part of the 3rd generation of sequencing methods, long- read- sequencing is seen as having the potential to make advances in finding genetic causes of mitochondrial diseases. Long- read- sequencing is expected to solve phasing issues, to improve RNA- and mtDNA- analyses through direct sequencing and to further enhance coverage (47).

NGS methods for sequencing the mitochondrial and nuclear genome still reach their limits- the molecular diagnostic procedures are often not automated and time-consuming. However, there are good prospects for solving these challenges in the fields of genomics and transcriptomics (47).

2 Mitochondria in germ cells and their implications to fertility

Mitochondria play an important role in human oocyte maturation, preimplantation and embryo implantation because they are indispensable for these energy-demanding processes. Mitochondria have an impact on female, as well as on male fertility (3). In the following chapter, the role of these essential cell organelles and the importance of their DNA in human germ cells and embryos are summarized.

2.1 Oogenesis

Oogenesis describes the developmental process of oocyte maturation, starting from oogonia to mature oocytes. When the primordial germ cells have arrived at the developing gonads of a female organism, they differentiate into oogonia. Oogonia are the stem cells of oogenesis. In the embryonic gonads, oogonia undergo a series of mitotic divisions. While the majority of oogonia continue to divide, some are differentiating into much larger primary oocytes. The primary oocytes enter the prophase of the first maturation division during embryonic period. Each oocyte surrounds itself with a layer of flat follicular epithelial cells – as a result, primordial follicles are formed. In the primordial follicle, the first maturation division is arrested at the end of the prophase.
In the 5th month of development, the total number of germ cells in the ovary reaches its maximum, estimated at 7 million cells. From then on, cell death sets in and most oocytes perish. During the 7th month, all remaining primary oocytes are in the dictyotene stage. The dictyotene is a resting stage into which the primordial follicles enter and in which the follicles stop maturing until puberty. Surrounded by a layer of flat epithelial cells, the primordial follicles are found close to the ovarian surface epithelium (58).

The total number of primary oocytes at birth is estimated to be about 600,000 to 800,000, of which only about 40,000 cells remain at the onset of puberty. Less than 500 of these oocytes are ovulated during the female reproductive phase. In each ovarian cycle, 15 to 20 primordial follicles enter further development into primary, secondary, and tertiary follicles. Only one of these follicles matures to ovulation. The

oocyte completes the first maturation division (meiosis I) shortly before ovulation and expels a polar body. The secondary oocyte immediately enters the meiosis II. But the second meiotic division is arrested in metaphase and only terminated at fertilization with the expulsion of the second polar body (58).

2.1.1 Ovarian aging

Since the number of first pregnancies of women in an advanced reproductive age is increasing, the ageing process of the female reproductive tract has become a central topic in reproductive medicine. Here, the first signs of ageing are detected earlier compared to other organ systems. From the age of 35, women are already said to be in advanced reproductive age. The trend of belayed childbearing will continue – factors such as education and career ambitions are important for today's women and must be considered in family planning (59).

The aging of the female ovaries can be observed in the progressive decline of their functions. In reproductive medicine, this refers to the decreasing quantity and quality of the oocytes (59,60). A diminished ovarian reserve (DOR) is the quantitative consequence of ageing ovaries. An anti- Mullerian hormone (AMH) level of <1.1 ng/ml, a follicle stimulating hormone level of >10 IU/L or an antral follicle count (AFC) of <5-7 total follicles are associated with a low total number of oocytes left in the ovaries (61). Oocyte quality is measured by the competence of the cells to re-initiate meiosis and the ability to undergo fertilization and further preimplantation development accurately.

According to Broekmans *et al.*, the average age at which women enter menopause among Caucasian women in the USA is 51 years. For women affected by primary ovarian insufficiency (POI), the average age of menopause is much lower (under 40 years). There are significant variations due to genetic and environmental factors (61). Menopause is the final stage of ovarian ageing. The process begins with initial cycle irregularities due to the decrease in the number of follicles and qualitative decrease in oocytes and ends with the final cessation of menstruation (62).

From a clinical point of view, ovarian aging does not occur in parallel with the ageing process of other tissues and the biological woman's age on a regular basis (63) Moderate oocyte quality or a reduced number of oocytes may prompt in vitro

fertilization (IVF). Despite advances in reproductive medicine, the efficiency of IVF treatment can be limited by parameters such as low oocyte count, poor fertilization rates and lack of embryo development. If IVF fails, patients may have to consider the use of donor oocytes. As the desire for fertility at later ages will continue, research continuous to delay or even reverse ovarian ageing (61).
Available therapeutic interventions and promising advances in reproductive medicine are discussed in more detail in the following chapter 3.

2.1.2 Oocyte quality

The prerequisite for successful fertilization and subsequent embryonic development is nuclear and cytoplasmic oocyte maturation (64,65). Metaphase II of meiosis results in a polar body, which indicates the maturity of the oocyte nucleus. Cytoplasmic oocyte maturity is assessed by the distribution pattern and activity of the mitochondria (65).
During oocyte maturation, two meioses take place- meiosis I at the time of ovulation and the second meiosis at the time of fertilization (meiosis II). As soon as the mature oocyte has been exposed to the luteinizing hormone (LH), meiosis I is completed. Before ovulation in the middle of the cycle, the LH concentration increases significantly (preovulatory LH peak). The assembly and disassembly of microtubules within the oocyte is one of the most energy- demanding processes but is of great importance for the correct alignment and adequate segregation of chromosomes (66). If the spindle built from microtubules is no longer appropriately formed in the ageing oocyte and if chromosome segregation errors occur, aneuploidy or unbalanced chromosomes will accumulate. Non-extrusion of the first polar body is also possible due to meiosis defects. The second meiosis is more susceptible to age-related aneuploidy compared to the preceding meiosis I (67).

The most common genetic cause of spontaneous abortions and developmental disorders are aneuploidies, which belong to numerical chromosomal aberrations. Aged female oocytes lack strong cohesive bonds that hold chromosomes together. The decrease in these bonds over time could predispose aged oocytes to aneuploidy. The oocyte is not able to notice the absence of these forces during meiosis I, which is why it does not react to them and does not block cell division. It

is not clear whether the oocyte does not respond due to age or does not have the capacity to respond appropriately to the loss of cohesion and the accompanying segregation defects (68).

The DNA- binding to spindles is crucial for the segregation and migration of chromosomes or chromatids to the corresponding daughter cells (69). Mitochondrial activity is related to chromosomal chaotic mosaicism in preimplantation embryos (70). The aim of competent mitochondria is to migrate to the required sites to supply the organelles with sufficient energy (71,72).

Follicular atresia and premature ovarian failure can result from mitochondrial mutations and associated decreases in ATP levels. Oxidative stress may cause lesions in the gene encoding ATP synthase. Subsequently, this can cause ovarian insufficiency and loss of function. Increasing levels of the 4977 bp-deletion have been detected in ovarian tissue, oocytes, and the supporting cells of the follicle. Decreased expression of mitochondrial genes and the presence of the 4977 bp-deletion are found in oocytes that cannot be fertilized and in embryos that have stopped development (73).

In general, the mitochondria number is characteristic for the activity of the cell (18). In mature oocytes, but also for example in neurons and muscle cells, plenty of mtDNA copies are present compared to other somatic cells. ATP generation is highly relevant for the maturation of the cytoplasm, and the nucleus for the upcoming fertilization and completion of meiosis II (74,75). A high quality oocyte has the optimal mitochondrial number with an ATP content of at least 2 pmol per oocyte and will be able to develop into a higher quality blastocyst after fertilization (76).

2.1.3 Mitochondrial dynamics and functions in oogenesis

Using electron microscopy, the morphology of mitochondria in cells developing from primordial follicles to mature oocytes is described as naive, roundish to oval. Compared to mitochondria in somatic cells, they possess less structure, resulting in unstructured cristae with limited capacities for ATP generation. In differentiated somatic cells, mitochondria occur as mature, forming structured networks (77).

For continuous transcription and translation processes, the oocyte requires large amounts of ATP, which are provided by mitochondria. The mtDNA copy number and

the distribution of mitochondria therefore change during oocyte maturation. Mitochondrial dysfunction affects the whole oocyte. If conditions are suboptimal, as during in vitro maturation (IVM), this can negatively affect mitochondrial morphology and mitochondrial gene expression. As mentioned earlier, mitochondrial dysfunction affects oocyte quality and thus oocyte maturation. For example, mitochondria can no longer counteract ROS production. Antioxidant reagents targeting the mitochondria could be used in IVM to neutralize ROS. According to this, it is important to understand the intracellular distribution of mitochondria in the oocyte and the mitochondrial genome during in vivo/ in vitro maturation to improve the developmental potential of the oocyte (78).

At the beginning, the primordial germ cells (PGCs) enter the niche of the ovary. Then proliferation of mitochondria in the PGCs begins and the number of mtDNA increases in the subsequent stages of oogenesis.
About 200 mtDNA copies rise to more than 400,000 copies. As illustrated in Fig.2, the most mtDNA copies are present in the mature oocyte. On the time axis from the primordial follicle to the MII oocyte, two steep increases in mtDNA copy number can be observed- the first on the section from the primordial to the primary follicle and a second one from the GV (germinal vesicle) to the MII (metaphase II) oocyte at the late antral stage (79,80). Oocyte maturation requires more energy, and energy demand peaks at the time of ovulation. Matthew Cotterill and coworkers investigated the actual amount of mtDNA copy number across the stages of oogenesis for the first time, and the mitochondrial activity in GV an MII oocytes was also measured. They studied the increasing mtDNA copy number during oogenesis, reflecting the changing ATP demand of the cell. The changing energy demand is explained by the necessary reorganization of the cytoskeleton and cytoplasm, and oocyte maturation, as well as the need to resume meiosis (81).
As the fertilized oocyte develops into a blastocyst of the preimplantation embryo, an intracellular decrease in mitochondrial copies is recorded again. MtDNA replication does not take place until the blastocyst has hatched. Embryonic mitochondrial replication resumes after the implantation stage (73). As a result, MII oocytes, fertilized oocytes, and early cleavage stage embryos are dependent on the available mitochondrial reserve (73,82). Mature oocytes at fertilization need the present mtDNA molecules to maintain OXPHOS activities. The mtDNA molecules are

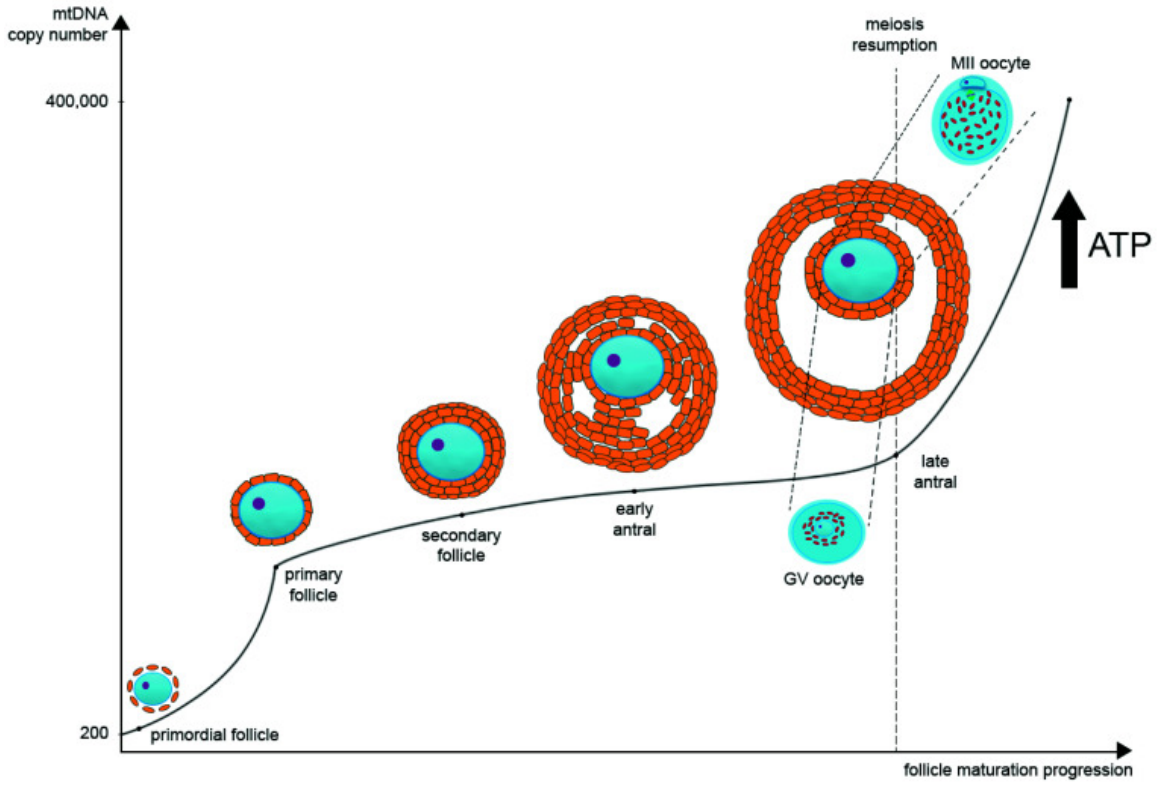

Fig.3: The change in mtDNA copy number during follicle maturation. As oocytes mature, mtDNA copies increase significantly with a maximum at the MII stage. As oogenesis progresses, a parallel increase of ATP level occurs and is illustrated as an arrow on the right. The distribution of mitochondria substantially changes at the late follicle stage. Mitochondria migrate form the center of the ooplasm (in GV oocytes) to the pericortical region (in MII oocytes), distributing evenly through the whole ooplasm (78).

indispensable for energy production and subsequently for embryonic development by providing ETC (electron transport chain) components. MtDNA is amplified in advance during oocyte maturation in the ovary to be able to adapt to this developmental stage (82).

Mitochondria also serve to sequester calcium, in addition to their function as energy suppliers for cell division and chromosome segregation in the oocyte. A successful oocyte activation and embryonic development require an increased calcium concentration in the cytoplasm. Somatic cells are directly involved in the regulation of intracellular free calcium (Ca^{2+}) in response to a variety of signals that can sequester and release these ions. Among them are molecular signals related to apoptosis activation. The signal for Ca^{2+}-induced-Ca^{2+}-release (CICR) can originate from storage sites such as the smooth surfaced endoplasmic reticulum (SER),

specialized granule cells, or from Ca^{2+} releases by other mitochondria (mCICR). Changes in cell physiology such as local changes in Ca^{2+} levels can up- and down-regulate the respiratory activity of the affected mitochondrion - an important function of somatic cells undergoing morphodynamical changes that cause mitochondrial redistributions, thus balancing ATP supply and demand at different sites in the cytoplasm (71).

Ca^{2+} oscillations in germ cells occur after sperm attachment to the oocyte membrane or after intracytoplasmic sperm injection (ICSI). If OXPHOS is inhibited in the mitochondria, Ca^{2+} is released from the ER to counteract local ATP depletion. Oocyte fertilization completing meiosis II is energy demanding and acquires elevated ATP concentrations. After the increased Ca^{2+} flux directly stimulates the cell to express enzymes of the respiratory chain, ATP production is then driven by OXPHOS (83).

2.2 Spermatogenesis

Spermatogenesis comprises the development form spermatogonia to sperm. The stem cells of spermatogenesis are called spermatogonia. While in female development the differentiation of the oocytes already begins in utero, the differentiation of the spermatogonia starts after puberty.
Spermatogonia are taken up in the embryonic testis together with Sertoli cells into the solid germinal cords, where they rest until puberty. At puberty, spermatogonia develop from the germ tubes. From then on, the spermatogonia reproduce mitotically.
Primary spermatocytes develop continuously, with 2 secondary spermatocytes and finally 4 spermatids that emerge in meiosis divisions. The differentiation of spermatids into spermatozoa is called spermiogenesis. The spermatid undergoes several changes during its development into a sperm. A lysosome is erected as an acrosome over the anterior pole of the nucleus, the nuclear chromatin condenses in a confined space, and the mitochondria arrange themselves in a ring around the tail filament extending from the centriole. The development from human spermatogonia to sperm takes 74 days, and about 300 million spermatozoa are produced daily (58).

2.2.1 Mitochondrial functionality in male fertility

The mitochondrial genome exerts an effect on sperm fertility, centered on energy production and apoptosis during spermatogenesis. The mitochondria also participate in other processes of spermatogenesis (e.g. redox, signaling pathways, steroid hormone production) and thus play a crucial role in male fertility.
These cell organelles are located at the periphery of the tail microtubules, as they provide the energy supply for motility. The volume of the mitochondria can change depending on the flagella (84,85). Using mitochondrial sperm parameters such as mitochondrial membrane potential (MMP), mtDNA copy number (mtDNAcn), mtDNA integrity, and apoptotic indices, semen quality can be determined (85).
For both fertilization and sperm fertility, sperm DNA integrity is of great importance, because the fetus can be endangered or even damaged by endogenous and/ or exogenous factors. Moderate to severe sperm DNA damage is suspected to be behind idiopathic male infertility.
The greatest influence on this DNA damage appears to be oxidative stress, which occurs predominantly in the mitochondria through free radical formation. The membrane of sperm cells has a high proportion of unsaturated fatty acids and a low cytoplasmic volume, which favors oxidative attacks. Mitochondrial DNA aberrations are causal for structural damage in the mitochondria, allowing free radical formation (86,87).
Sperm fertility is physically lost if mitochondria do not ensure that sperm are supplied with motor energy. Sperm motility, acrosomal response, interaction with the oocyte - these are factors depending on ROS regulation by mitochondria (88). Low sperm count (oligospermia) and low sperm motility (asthenospermia) define male infertility. It has been possible to detect mtDNA mutations in patients with unfulfilled desire to have children, suggesting that defects in the mitochondrial respiratory chain may probably contribute to male infertility.

Research on the mitochondrial genome has revealed that mitochondrial genes are involved in regular growth, differentiation processes, and sperm motility. Consequently, mutations in mtDNA lead to alterations in normal sperm properties and abnormal sperm flagellar movements, resulting in infertility (89).

The three major mtDNA deletions (4977 base pair (bp)-, 7345 bp-, 7599 bp-mitochondrial genome deletions) appear to be more common in patients suffering from azoospermia (complete absence of sperm in the seminal fluid) or oligospermia than in fertile individuals (85).
In 2020, M. Karimian *et al.* studied the association between frequent mtDNA deletions and male infertility. Their data showed a significant association between a frequent 4977 bp-deletion and an increased risk of male infertility, the association turned out to be ethnicity-infertility phenotype-dependent. Therefore, this mtDNA deletion has the potential to act as a genetic biomarker for male infertility. The 4977 bp-deletion has not been associated with oligospermia and normospermia (normal quality and volume of semen). This lack of association and the additional positive association between the deletion and various types of asthenospermia (reduced sperm motility) illustrate the importance of this finding for sperm motility. However, further studies with larger samples from different populations are needed to obtain a more comprehensive conclusion concerning mtDNA deletions (89).

2.2.2 Mitochondrial regulation in spermatozoa

The nematode *C. elegans* has turned out to be a good model organism to better understand mitochondria. Due to the high degree of mitochondrial protein conservation between mammal and nematodes, the observations made in nematodes seem to be largely transferable to mammals. The transparency of nematodes offers the opportunity to label mitochondria and study their motility, structure, and functions. With small adaptations, it is possible to link studies on human mitochondria with those of *C. elegans*, which contributes to an enormous progress in the elucidation of mitochondrial function (90). *C. elegans* mitochondria migrating via microtubules, fusion and division with other mitochondria are depending on age and metabolism as is human mitochondria (91). Through fusion with neighboring mitochondria, small mitochondrial protein defects or mtDNA can be supplemented by functional proteins or mtDNA. Fission occurs during cell division to distribute mitochondria (39).

The mitochondria of the spermatozoa are capable of metabolizing various energy substrates (e.g. pyruvate, lactate) and thus perform a central metabolic role. They

have highly differentiated structure and supply the spermatozoa with energy on their way to fertilization. Mitochondria influence the redox balance, calcium regulation and apoptosis. These processes play a major role in flagellar movement, capacitation, and the acrosome reaction with final gamete fusion (92).

Developing from late spermatocytes to mature spermatozoa, the mitochondrial structure change to a condensed, more active structure (93). Residual bodies of cell cytoplasm containing mitochondria are degraded and consequently few mitochondria remain in the mature germ cells. Simultaneously, the germ cells migrate across the germinal epithelium, which also adjusts the supply of energy substrate - Sertoli cells supply lactate and pyruvate from then on (94). ATP is used by spermatozoa for the following processes: motility, capacitation acrosome reaction and oocyte penetration. This illustrates once again the crucial role of mitochondria in fertility. The flagella of spermatozoa can move by an interplay of axonemal microtubules and dynein arms. ATP is obligatory for locomotion because dynein proteins possess adenosine triphosphatase (ATPase)- activity. Mitochondrial ATP generation takes place in the intermediate piece of the sperm cell. Whether the diffusion of ATP from the intermediate piece contributes to the energy supply of the principal piece remains unclear. The principal piece consists of about 90% flagella, so the energy requirement is correspondingly high (92,95). In the midpiece of spermatozoa, ATP is likely to be generated via OXPHOS, in the principal piece ATP is likely to be available via anaerobic glycolysis (96).

The respiratory chain is provided with the required reduction equivalents from the cytosol for OXPOS via three shuttles (lactate/pyruvate-shuttle, malate/aspartate-shuttle and glycerol-3-phosphate-shuttle). In addition, the ß-oxidation of fatty acids and the citric acid cycle provide $FADH_2$ and $NADH_2$ molecules for ATP production in the mitochondria (96). By using different metabolic pathways and obtaining different energy sources, spermatozoa can adapt to the available substrates in their environment. The surrounding substrates change as they move through the female genital tract (97).

If the membrane potential cannot be maintained, fusions between mitochondria are no longer possible to protect intact mitochondria from the damaged components. Dysfunctional mitochondria are degraded by mitophagy. Mitophagy is a mitochondrion-specific form of autophagy. In some cases, the membrane potential

can be restored by protein synthesis, and the mitochondrion is not degraded (98). Mitophagy, fusion and fission are working together to counteract heteroplasmy (99).

The presented mitochondrial functions in spermatozoa underline that mitochondria can be attributed a crucial role in spermatogenesis and during fertilization of the oocyte. After fertilization of the female germ cell, however, they no longer seem essential, as they appear functionless afterwards. Despite controversial publication, the scientific community agrees that human mtDNA is inherited uniparentally through the mother (30,100). This is applicable to most sexually dimorphic eukaryotes. The mechanisms of elimination of parental mitochondria have been studied in *C. elegans* and *Drosophila* (101).

In sexually reproducing organisms, the sperm usually provides DNA, centrioles with cytoplasm and organelles to the oocyte. Al Rawi *et al.* used spermatozoa in *C. elegans* to show how autophagosomes are recruited within minutes of fertilization to degrade the paternal mitochondria. The membrane organelles of nematode spermatozoa are marked in the form of ubiquitination before the formation of the autophagosome, whereas mitochondria are not. Autophagy markers have been identified in fertilized mouse embryos, suggesting that this autophagy process is evolutionarily conserved. It is thought to prevent paternal transmission of mtDNA to the offspring and to counteract heteroplasmy (102).

The endonuclease G homologue CPS-6, which is normally located in the mitochondrial interspace, was also found to be involved in mtDNA degradation after fertilization in *C. elegans*. CPS-6 was previously known in a different context- CSP-6 is involved the degradation of nDNA during apoptosis (103). Contrary, mtDNA degradation by endonuclease G was discovered before fertilization in *Drosophila*. The mitochondrial nucleoids are shed from the spermatids in endonuclease G mutants by cellular remodeling that truncates the spermatid tails. Thus, the ability to pass on the paternal mtDNA to the offspring is already lost during spermatogenesis (104).

Various mechanisms are known to prevent paternal mtDNA transmission, but there is still no consensus on the process in human reproduction (29). The massive mtDNA reduction during spermatogenesis is well known. On the one hand, cytoplasm including mitochondria is removed via residual bodies (104), on the other

hand, the downregulation of mitochondrial proteins (e.g. TFAM) is linked to downregulation of mtDNA copy number (105). As mentioned above, paternal mtDNA of invertebrates is almost completely removed in mature spermatozoa due to endonuclease G working during spermatogenesis (104).

Another possibility of uniparental mtDNA transmission can arise from passive dilution during fertilization (106). By comparing the concentration of maternal to paternal mitochondria, a significant difference can be observed. In a mature oocyte there are about 250,000 mitochondria, but in spermatozoa only between 1 to 1,000 copies (107,108).

Pyle *et al*. (2015) could not detect paternal mtDNA haplotypes by deep sequencing in human cheek swab samples of the offspring (109). This argues against the dilution model of uniparental mtDNA transmission. Due to unequal mitochondrial distribution of spermatozoa in early embryonic stage, paternal mtDNA could be distributed only to certain cells and tissues. Thus, hypothetically, paternal mtDNA would be detectable in certain tissues of the offspring (110).

Mitochondria are labelled for degradation during spermatogenesis by ubiquitinated substrates (111). These tags facilitate the degradation of defective spermatozoa in epididymis or that of paternal mitochondria after fertilization in the ooplasm. Mitochondrial membrane and matrix proteins are targets for ubiquitination, among others. Various molecular pathways are involved in the degradation process of mitochondria in the ooplasm (e.g. mitophagy, ubiquitin-proteasome-system). As a cargo protein, Sequestrosome 1 (SQSTM1), also known as ubiquitin-binding-protein-p62, can recognize ubiquitinated spermatozoa for selective autophagy (112). Specific endonucleases appear to be used for removal of damaged mitochondrion.

Nevertheless, it remains unexplained why paternal mitochondria and their mtDNA must be eliminated from the zygote and transmission of the mtDNA occurs through the mother. A plausible explanation remains that paternal mtDNA is exposed to ROS and spermatozoa swim a long distance towards the mature oocyte, resulting in damage that should not be inherited (29).

3 Therapeutical approaches

To date, there is no cure for mitochondrial diseases, only palliative treatment to support affected patients. Preimplantation genetic diagnosis (PGD) offers the possibility of avoiding nDNA mutations in embryos of carriers by selecting only embryos that are unlikely to be affected. The embryo biopsy only reflects the level of heteroplasmy in that sample material and is rarely at zero. PGD therefore only serves to reduce the risk of mutated mtDNA transmission. Egg donation would prevent the transmission of maternal mtDNA mutations but is not an option for many couples who wish to have children, as the offspring is not genetically related to the mother. Prenatal diagnostics can also provide information about the degrees of heteroplasmy in amniotic fluid or chorionic villus biopsies, but do not provide information about the severity of a disease or the degree of heteroplasmy in other tissues (113).

The role of mitochondria in the context of reproductive medicine is becoming more understandable. Mitochondrial dysfunction and mtDNA have implications to fertility and early embryonic development. Advancing mitochondrial research has great potential for clinical application. Mitochondrial replacement therapy (MRT) is already used to a limited extent. This new form of IVF is intended to replace dysfunctional mtDNA and thus prevent mitochondrial genetic diseases and increase reproductive potential of oocytes.

Since the techniques involve genetic material form a third person, this raises many ethical questions. New techniques seem to be developing faster than ethical dilemmas can be resolved.

3.1 Mitochondrial replacement therapy (MRT)

In general, MRT is based on the principle of replacing the mother`s defective mtDNA with mtDNA from a healthy donor. This new form of therapy is used in IVF and includes various techniques, e.g., pronuclear transfer (PNT), maternal spindle transfer (MST), and polar body genome transfer (PBT). PNT and MST are currently in clinical application (3,114). The focus is on developing healthy offspring without genetic disorders and eliminating lethal mitochondrial disorders. In addition, these

techniques are intended to counteract ovarian aging by replacing defective cytoplasm through IVF to increase the expectation of pregnancy rates (114).

In general, MRT can be divided into autologous and heterologous therapies. Furthermore, the classification can be made according to its treatment goal:

1.) MRT preventing vertical transmission of mitochondrial disease
2.) MRT improving oocyte quality

The former includes PNT, MST, and PBT, where the whole ooplasm is replaced. The second category includes autologous and heterologous mitochondrial transfer, using germline cells, autologous stem cells, and heterologous oocyte cytoplasm (115).

3.1.1 Pronuclear transfer (PNT)

PNT implies the separation of the pronuclei from the mother's zygote, followed by a transfer to the enucleated donor zygote. This should reduce the transmission of maternal mitochondria to the offspring (115).
Before the nDNA (in a karyoplast, consisting of cytoplasm surrounded by plasma membrane) can be transferred, both the maternal and donor oocyte must be fertilized (Fig.4) (3). The patient's oocyte with abnormal mitochondria is fertilized with the partner's sperm; the donated oocyte is also fertilized but contains normal mitochondria (116). After successful fertilization, the pronucleus containing the maternal genome and the other one with paternal genome are visible under the light microscope (117). Both zygotes are developing in vitro (114).
While the donor's zygote with normal mitochondria is enucleated, the patient's pronuclei are removed from their zygote to be transferred to the enucleated donor's zygote (116). It is difficult to distinguish the human female pronucleus from the paternal one, which is why pronuclei are always transplanted together in PNT (117). Thus, a reconstructed zygote with healthy mitochondria is obtained. The PNT is completed, and the cleaving embryo is ready to be implanted into the patient's uterus (116).

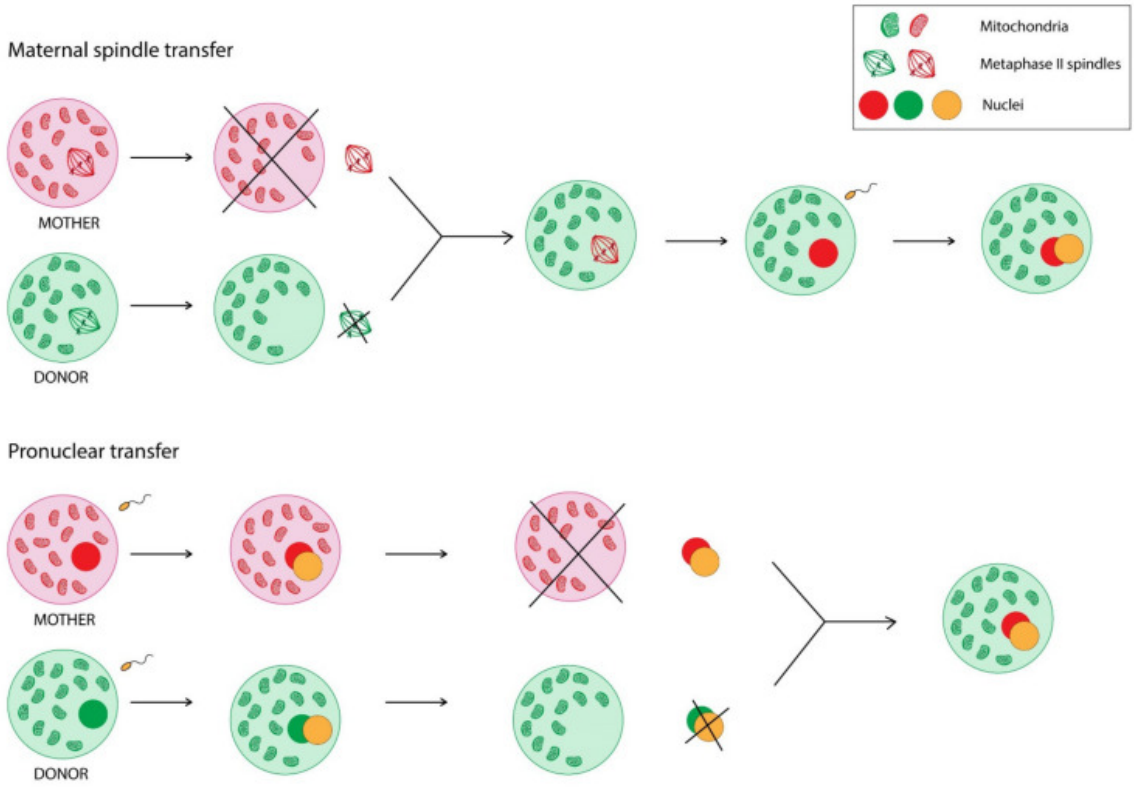

Fig.4: Comparison of pronuclear transfer and maternal spindle transfer. The donor cells are shown in green, those of the mother in red. During MST the donor's nDNA is removed, leaving the part of the oocyte containing healthy mitochondria. After inserting the mother's metaphase II spindle, the oocyte finally gets fertilized and is ready to be implanted into the uterus. On the contrary, PNT requires fertilization of both oocytes before transferring the nDNA of the mother to the donor's cell. "Comparison of two mitochondrial replacement technologies- maternal spindle transfer and pronuclear transfer." by Amira Podolak *et. al* (2022) is licensed under CC BY 4.0

J. McGrath *et al.* (1983) were the first to perform nuclear transplantation on mouse embryos. The zygotes pronuclei were removed micro surgically and fused with the donor nucleus in a virus-mediated manner. Over 90% of the manipulated embryos survived the nuclear transplantation, and their development did not differ significantly from the control group, which successfully resulted in offspring. With that, an efficient exchange between genetically different inbred mouse strains was carried out for the first time (118).

Akitsugu Sato *et al.* (2005) performed zygote nuclear transplants on trans mitochondrial mice models (mito- mice) to investigate the use of the PNT method against mitochondrial genetic diseases. The experimental animals suffered from respiratory defects and mitochondrial diseases due to a large deletion in the mtDNA. Nuclear transplantation from the mito- mice was administered into normal enucleated zygotes. The result was offspring in the F_0 generation that did not

express any respiratory defects throughout life. A. Sato *et al.* thus demonstrated a potential method to prevent offspring from developing mitochondrial disease by eliminating pathogenic mtDNA through nuclear transplantation (119).
PNT and MST have been shown to be compatible with the development of human embryos up to the blastocyst stage. The mtDNA of the mutation carrier relocated during nuclear transfer of the nDNA constitutes only a small percentage of the total mtDNA of the constructed embryo (117). A mtDNA carryover of less than 2% indicates that PNT has the potential to prevent transmission of human mtDNA diseases (113). Although the mtDNA carryover in embryos after MRT treatment seems low, embryonic stem cells (ESC) show a progressive increase in heteroplasmy. The resulting clinical significance is still unclear, but it highlights the importance of further minimizing the carryover of mtDNA. PBT may be predestined for this purpose (117).

Hyslop *et al.* (2016) established a new transplantation procedure for PNT to improve the survival rate of reconstructed embryos from normally fertilized zygotes (113). The 4% mtDNA carryover of a blastocyst by PNT could be measured in a heteroplasmy increase in ESC (120). But they “have therefor developed an alternative approach based on transplanting pronuclei shortly after completion of meiosis rather than shortly before the first mitotic division. This promotes efficient development to the blastocyst stage with no detectable effect on aneuploidy or gene expression” (120). As a result, carryover in blastocyst was below 2% in 79% of blastocysts and no carryover above 5% occurred (113). This means that PNT generally minimizes the risk of developing mtDNA disease. Nevertheless, PNT cannot guarantee prevention of mtDNA disease (120).

Keliang Wu *et al.* (2017) questioned the use of cytoskeletal disruptors during MRT. This is because in order to isolate the chromosome spindle complex or the pronuclei it requires cytoskeletal disruptors such as cytochalasin B (CB) or nocodazole. Since clinical use of these cytoskeletal disruptors in this context has not been sufficiently evaluated, Keliang Wu *et al.* presented a method that does not require these disruptors. “Female pre-pronucleus (PPN) can be isolated shortly after fertilization in a condition without cytoskeleton disruptors and then be successfully used for MRT, leading to the generation of human ESC lines with mtDNA carryover at very

low levels." The PPN is smaller in size than the chromosome spindle or pronucleus, and consequently has fewer mitochondria. As a result, fewer (defective) mitochondria are transferred during MRT. In addition, premature activation of the oocyte is avoided, as PPNT manipulation takes place only after fertilization. Kelian Wu *et al.* showed that PPNT was followed by high preimplantation rates. PPNT resulted in blastocysts and ESCs with a low mtDNA carry over and had no negative influence on embryonic development (121).

Since the donor's mtDNA is transferred to a new nuclear environment by MRT, doubts arose that this could lead to harmful interactions between nDNA and mtDNA. MRT has already been used in human, macaques, and mice, but most of these experiments state that MRT is not injurious (122). Hyslop *et al.* (2016) showed that oocytes with a given nucleus are usually less likely to turn into a blastocyst than oocytes reinjected with their own nucleus (120). Whether this effect occurred because of the deleterious mito-nuclear interactions (DMNIs) or because that oocyte was previously frozen remains unclear. Further studies do not give indication that MRT could be harmful. Animal experiments were carried out where mtDNA of one strain of a species was recombined with the nuclear genome of another strain of the same species - negative effects of MRT were not observed (122–125). Blastocysts developed from MRT show regular gene expression (120).
Population genetic theory defines mitochondrion/nucleus interactions as no more frequent in individuals conceived by MRT than in normal reproductions. It is thought that selection in humans is not strong enough to create imbalance between nuclear and mitochondrial genomes. The risk of DMNIs due to MRT appears to be lower than the risk of a pathogenic mtDNA carrier producing an affected child naturally (122).

3.1.2 Maternal spindle transfer (MST)

Another method for avoiding mitochondrial disorders besides predictive testing such as pre-implantation genetic diagnoses (PGD) and prenatal diagnosis (PND), is the maternal spindle transfer (126).
The maternal spindle complex is removed from the defective oocyte of the prospective mother. The donor's spindle complex is also removed, and the donor's

oocyte remains containing healthy mitochondria (Fig.4) (3). At this point, the spindle is in the metaphase stage, which can be exploited to its fullest. Arresting in metaphase of meiosis II and preparing for fertilization, the spindle apparatus can be made visible by polarized light. This allows the spindle and the corresponding chromosomes to be removed (117). The extracted metaphase II spindle of the mother's oocyte is inserted into the donor's cell (3) The enucleated donor cell harbors healthy mitochondria (114). The reconstructed oocyte is fertilized and both, the haploid paternal and maternal, genomes are placed in pronuclei. Pronuclei can be easily identified with a light microscope and implanted into the woman's uterus. Transplantation is always done with both pronuclei, as it is not possible to reliably distinguish the male and female pronucleus in the zygotes (117).

In 2013, the first human MRT studies were published, both Tachibana *et al.* and Paull *et al.* focused on MST of human oocytes. They concentrated on avoiding premature oocyte activation, optimizing karyoplast fusion and timing during MRT. These studies indicated for the first time that MST is associated with accurate preimplantation development and low mtDNA transmissions (113). In their 2013 paper, Paull *et al.* confirmed that nuclear genome transfer does not negatively affect the progress of development to the blastocyst stage, and that "genome integrity was maintained, provided that spontaneous oocyte activation was avoided through the transfer of incompletely assembled spindle-chromosome complexes". The mtDNA carryover during MST was initially detected at less than 1% but was subsequently at undetectable levels or not at all present in the blastocyst stage and stem cell lines. This was monitored for a year but transmitted maternal mtDNA was also not found in differentiated or reprogrammed cells – results that underline the great potential of MST against the transmission of mitochondrial mutations in humans (127). The mtDNA heteroplasmy levels remained low or undetectable (113).

In the same year, Tachibana *et al.* reported abnormal fertilization in 53% of cases after nuclear transfer, but the remaining embryos still developed to the blastocyst stage and ESCs developed similar to the controls. Those ESCs lines had no maternal mtDNA transmission and were euploid (113,124).

Finally, both PNT and MST appear to be capable of embryonic development up to the blastocyst stage. The co-transfer of mtDNA remains in a small fraction, with further optimization of the transfer (117).

3.1.3 Polar body genome transfer (PBT)

In females, after each meiotic division, one of the two nuclei produced is discarded as the polar body. Thus, after meiosis I, a secondary oocyte and polar body I are formed, and after meiosis II, a haploid oocyte and polar body II are formed.

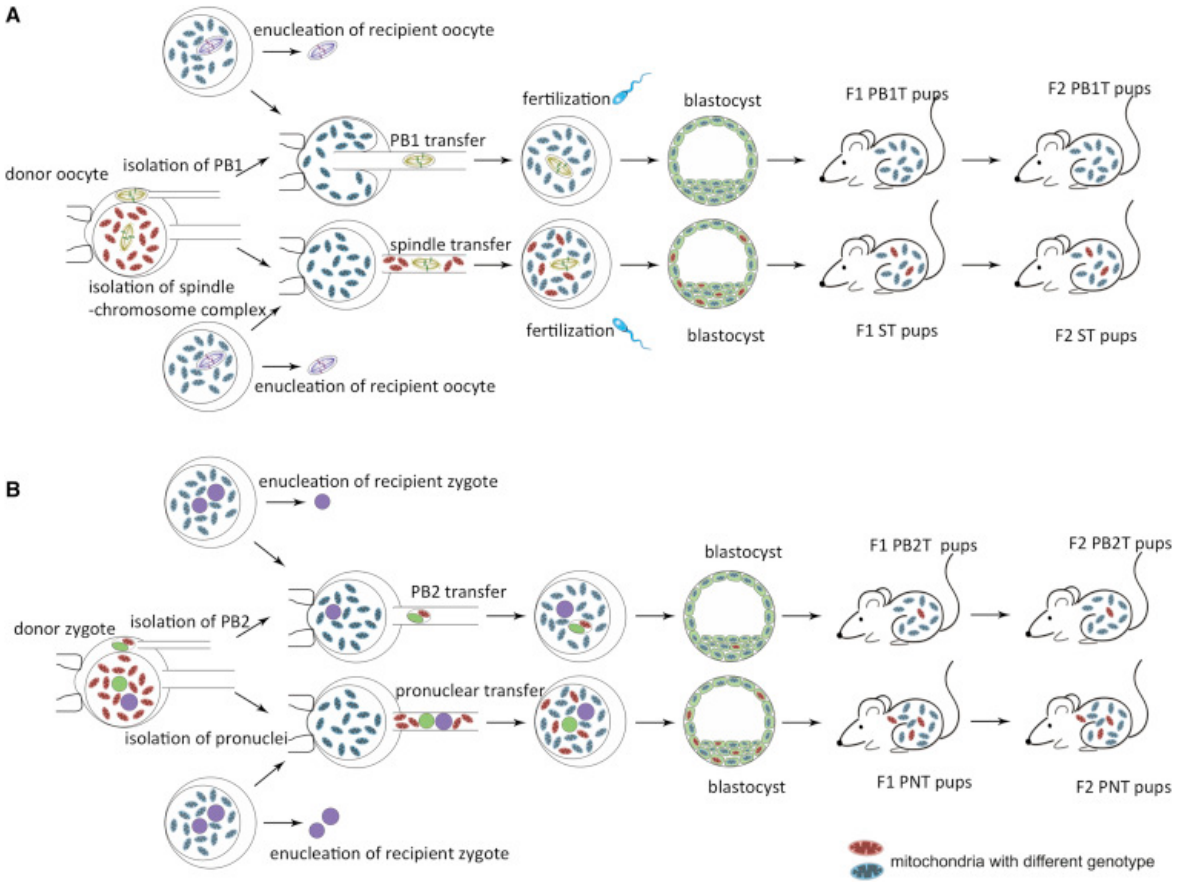

Fig.5: Different MRT methods to exchange mtDNA genotypes. A) PB1 genome transfer is compared to MST. Oocytes were obtained from mice strains with different mitochondria genotypes (red vs. blue). The resulting heteroplasmy levels in F_1 and F_2 generation are outlined. B) PB2 transfer vs. PNT. PB2 and pronuclei were transferred to half-enucleated/ enucleated recipient zygotes. The offspring were delivered from foster mice (131)

PGD makes use of the polar body, because the genome of the first polar body contains the haploid chromosome set of the mother, the second polar body carries the homologous chromatids from the second maturation division. However, this procedure can only be used to examine the maternal genome and only makes sense if the mother is either a carrier of a severe monogenic disease or a carrier of a balanced chromosome aberration (58). New attempts are being made to make use of the polar body in MRI as well.

Polar bodies are defined as two small bodies with unequal cytoplasm but the same number of chromosomes as in the nucleus of the primary oocyte, formed during oogenesis (128). A distinction can be made between the transfer of the first diploid polar body (PB1T) and that of the second (PB2T), which is haploid. The PB1T is performed on an unfertilized oocyte, the PB2T requires a fertilized zygote (113).
Since 1997, it has been known that mouse genomes from both polar bodies can contribute to normal embryonic development (129,130). Wakayama *et al.* first recognized the potential of polar body transfer in 2014 (114). But T. Wang *et al.* (2014) were the first to introduce PBT in mice. The assumption that polar body transfer is a suitable method to replace the defective mtDNA of the carrier (Fig.5) was confirmed in their mouse study, normal development of the progeny was validated (125). PBT does not require cytoskeletal disruptors and is performed with a micromanipulation procedure (128).
PB1 genome transfer is used to exchange the mtDNA genotypes in mice. Oocytes are obtained from strains with different mitochondria genotypes. PB1 is isolated from the donor's oocyte to be transferred into an enucleated recipient oocyte. Followed by fertilization, PB1T leads to regular blastocyst formation. What distinguishes PB1T from other MRT is that the mtDNA genotype remains stable until F2 generation. The transfer of PB2 requires fertilization. The PB2 of the donor zygote is transferred into an enucleated recipient zygote. The transmission of mtDNA cannot be completely avoided (131).

The fact that there is little cytoplasm and thus few mitochondria in the polar bodies appears to be very favorable when it comes to reducing mtDNA transmission or, desirably, to avoiding it (114). Another advantage is the cellular membrane surrounding the polar bodies. This makes them easy to be visualized and the

chromosomes can be transferred without loss using a micropipette (132). In order to increase efficiency in reproductive medicine, the spindle or pronuclei of the individual donor eggs could be used in addition to the polar bodies, which would drastically reduce the demand for donor cells (125).

MRTs in human embryos have shown promising results for the avoidance of mtDNA-related diseases. However, these methods should be further investigated in animal models to test the safety and efficacy more accurately before they are applied in clinical practice (133). The preclinical models show that PBT probably will be the best way to prevent inherited human mtDNA diseases in the future (131).

3.1.4 Humanin-mediated regulation of reproduction

In context of Alzheimer's research, the peptide humanin was found on the mitochondrial MT-RNR2 gene. Neuroprotective and anti-apoptotic properties are attributed to this peptide (134). Various protective functions in many tissues (e.g., cerebrum, testis, blood vessels, ovaries), especially in nervous tissue, have already been described.

Recently, reproductive medicine has also become aware of the humanin peptide, as it seems to have a regulatory function in the ovaries and testes during oxidative stress and apoptosis. Humanin influences various signaling pathways as soon as the body is in a state of emergency. The underlying mechanisms are being elucidated to understand the exact role of humanin - this could be useful for the development of new therapeutic approaches for male/female infertility, glucose metabolism in polycystic ovarian syndrome or male contraception (135).

Humanin expression in ovarian tissue changes during the luteal phase of the menstrual cycle. It increases significantly in the mid to late luteal body in monkeys increases significantly. The corpus luteum hormone plays an important role in maintaining pregnancy. It seems to play an important role in promoting the survival of corpus luteum (CL) in monkeys (135,136).

M. Rao *et al*. (2019) measured high humanin concentrations in human ovarian cells (stromal cells, granulosa cells in the follicle, CL, oocytes in the primordial and primary follicle). Increased humanin in the follicular fluid was associated with an increased likelihood of clinical pregnancy in the course of IVF-ICSI treatment. An

opposite effect between humanin concentration and pregnancy termination could not be found. In conclusion it shows that humanin is significantly involved in follicular development and ovarian regulation (135,137). In addition, a correlation between downregulated expression of humanin and oxidative stress in GCs from PCOS patients was revealed (138).
Humanin also plays a significant role in male fertility. Depending on the sperm morphology, the localization of humanin also changes. Humanin is associated with sperm quality, as it is also thought to prevent sperm apoptosis. Via molecular signaling pathways (STAT-3 signaling and p38 mitogen-activated protein kinase-(p38 MAPK) pathway), the initiation of apoptosis is prevented by not releasing the apoptosis-inducing Bax protein into the mitochondria (139).

3.2 Mitochondrial genome editing for human fertility health

In the century of the well-known CRISPR/Cas9 genome editing method, it seems obvious to apply genome editing to mitochondria as well. Mitochondrial genome editing (MGE) seems to be another opportunity to combat mitochondrial disease transmission. Using MGE, intracytoplasmic microinjection attempts to introduce mitochondria-targeted nucleases into mitochondria that modify human oocytes or embryos (3).

Mitochondrial genome editing aims to target modifications in human zygotes or oocytes at risk of mtDNA disease by preventing germline transmission of mutant mtDNA haplotypes using mitochondria-targeted nucleases (e.g. endonucleases, zinc finger nucleases (ZFN) or transcription activator-like effector nucleases (TALEN)) via intracytoplasmic microinjection. Precise and direct gene editing of the mtDNA could be made possible by targetable nucleases, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9)-CRISPR/Cas9 (140).
Induced pluripotent stem cells (iPSCs) are predestined to study the diseases caused by mtDNA. Yahata *et al.* (2017) isolated iPSCs from m.13513G>A mutation carriers and reprogrammed them to produce two clones (one with, one without mutation). The concentration of mutated mtDNA could be reduced by using platinum

transcription activator-like effector nucleases (TALENs). TALENs were injected into the mitochondria to recognize the mtDNA sequence carrying the mutation to cleave this sequence segment. Through this mechanism, the heteroplasmy level in iPSCs from MELAS patients was thereby decreased (141).
The MELAS-specific mutation m.3243A>G was also eliminated using mitochondrial-targeted TALENS (mitoTALENs). Off-target editing was not observed in the iPSC clones - compared to nuclear localized TALENs, mitoTALENs have difficulty reaching the nucleus. The treated iPSCs were capable of energy production and showed regular mitochondrial respiration. MitoTALEN-mRNAs were also successfully applied in porcine oocytes (142).

The CRISPR/Cas9 genome editing is another method that has the potential to fight pathogenic mutant mtDNA. Initially, the biggest obstruction to this method was the fact that importing RNA into the mitochondria is inherently infeasible (140). S.Hussain *et al*. (2021) applied gene editing to mitochondrial DNA by appending NADH-ubiquinone oxidoreductase chain 4 (ND4) targeting guide RNA to a RNA transport-derives stem loop element (RP-loop) and were able to express Cas9 enzyme with a preceding mitochondrial localization sequence (143). Cleavage of mtDNA by endonuclease Cas9 is possible, but it will still take time before the method is used clinically (3)

4 Conclusion

The importance of mitochondria in fertility and early embryonic development remains undisputed. Mitochondria will serve as a treatment target to cure mitochondrial disease and prevent it in advance. Particularly in the field of mitochondrial genome editing, the great scientific interest and the rapid development of the possible methods can be observed in continuously published studies.

The use of genome editing for gene therapy raises ethical questions with regard to the safety of the application for those being treated, for third parties and the environment, the weighing of benefits and risks, the recording of the benefits of the therapy beyond a medically defined purpose (for example, what quality of life is achieved or maintained during or after the application), the right to self-determination as well as social justice in access to the mostly highly expensive therapies. In view of the rapid scientific development, however, ethical questions are also posed in a new way or with new urgency. While ethical discussions have been hypothetical, speculative in nature, the discovery and ease of use and proliferation of genome editing tools such as the CRISPR-Cas9 method have not only made medical interventions possible, but also encouraged researchers to take steps towards their application.
Currently, the application of genome editing to the human germline for reproductive purposes is not considered by the majority. However, ethical hurdles already arise in purely basic research, in the genetic modification of germ cells or early, single-cell embryos, as well as in preclinical research. As soon as human embryos are to be used for research purposes, the question arises as to what is ethically justifiable in dealing with those embryos and what is not. On an international level, as well as within Europe, countries take different positions depending on how the moral status of the embryo is assessed. Thus, it happens that in some countries the use of embryos for research purposes is permitted, and in others the use of an embryo outside the body for a purpose not serving its preservation is punishable. The cultivation of an embryo in vitro is therefore already prohibited if it is not intended to induce a pregnancy and thus represents an ethically impermissible

instrumentalization of the embryo. Some research projects on genome editing on the human embryo serve to create the prerequisites for future medical applications (germ line therapy). The possible effects of germline intervention affect the treated individuals for the rest of their lives, as well as their possible offspring. The consequences of an intervention are irreversible - the genetic change in the embryo affects only one or a few cells; for a revision at a later stage of development, an incalculable number of cells would have to be treated. At present, neither the consequences of such interventions are known nor concrete test plans for clinical trials exist. The presumably small number of test persons and the necessity of long-term observation over several generations will make the implementation of clinical studies problematic. Findings from animal studies cannot be adequately transferred to humans, which is why such studies can only serve as a limited risk assessment for treatment in humans. Preimplantation and prenatal diagnostics could check whether interventions were carried out successfully or whether any undesired effects in the DNA or mosaic effects may have occurred, but it is largely unclear how possible effects can be classified over several generations. Most scientists agree that a clinical application of germline interventions would not be sensible, even irresponsible, now. However, it is emphasized that the path to clinical trials and their implications need to be discussed and reflected upon.
Mitochondrial replacement therapy and mitochondrial genome editing methods do not directly interfere with the germline but target mitochondrial genetic information. The mtDNA is in continuous exchange with the nDNA, genes are read and converted into proteins, and as already mentioned, the mtDNA is also able to incorporate itself into the nDNA. The interaction of mitochondria with the cell nucleus is thus undisputed. The figurative comparison with the simple replacement of a battery would be too simplistic for mitochondria replacement therapy.

Mitochondrial replacement therapy is already being used in a few countries. In Mexico, the first child was born in April 2015 after the application of MST. The 36-year-old mother came from Jordan and was a carrier of a mutation in the ATPase gene. This pathogenic mutation is associated with Leigh syndrome. Afterwards, in January 2017, the second child was delivered in Ukraine after MRT. PNT was applied to the oocyte of a 34-year-old Ukrainian woman. As of 2018, a total of 8 clinically normal babies were born in that Ukrainian clinic. In Europe, the first child

after MST treatment was born in Greece in April 2019. The treatment was not due to a genetic disease, but to the infertility of the 32-year-old Greek woman. The legalization of MRTs by the parliament of the UK in 2015 is still considered very controversial. A bioethics committee was consulted for the decision, but the decision was only made based on oocyte studies of 15 women, 3 macaques and 10 mice. A profound risk assessment could not be presented (3)

MRT requires the healthy DNA of the expectant mother, a donor egg cell, and the sperm of the genetic father. The method therefore involves 3 sexually mature individuals. Therefore, the term "three parent babies" has already become established in society. However, it seems to be negatively tainted with many prejudices, some of which are unfounded. Considering the social skepticism, the fact that the child biologically carries the genetic information of three parents could possibly lead to (identity) problems for the child. The very strict legal regulations prohibiting the use of MRT are partly unfounded. The laws were passed based on the erroneous assumption that this method interferes with the germ line. This possibility of reproduction should not be denied to couples who wish to have genetically identical offspring. This desire to reproduce appears neither immoral, nor illegal, nor disrespectful to cultures or religions. Provided that regulations are adhered to, comprehensive clinical studies are carried out, adequate risk management is in place and ethical concerns are critically evaluated, MRT should hopefully one day be available to every patient if all the conditions are met.

Manipulation of embryos is heavily debated by critics and considered morally reprehensible. The rarity of mitochondrial diseases will lead to a low evidence base for the frequency of expected complications after MRT application and will complicate clinical establishment.

The reproductive medicine sector is largely privatized. A negative example of the unreasonable, too rapid commercial approval of a new treatment method was provided by AUGMENT, an autologous germline mitochondrial energy transfer therapy, whereby autologous mitochondria were isolated from an oogonial stem cell and injected via ICSI. At the time of the introduction of the therapy method, numerous questions remained unanswered, and the clinical trial also appeared to be incomplete (3) Consequently, AUGMENT was withdrawn from the market, and it is to be hoped that lessons have been learned. The same scientific standards should

apply to a highly privatized sector, an evidence-based policy and, of course, the highest safety and quality standards.

The final hope is that science will shape our future responsibly, that science will not be abused, and that it will be possible to ensure that health and the healing of diseases have top priority.

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