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Review

How do changes in the mtDNA and mitochondrial dysfunction influence cancer and cancer therapy? Challenges, opportunities and models

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ABSTRACT

Several mutations in nuclear genes encoding for mitochondrial components have been associated with an increased cancer risk or are even causative, *e.g.* succinate dehydrogenase (SDHB, SDHC and SDHD genes) and iso-citrate dehydrogenase (IDH1 and IDH2 genes). Recently, studies have suggested an eminent role for mitochondrial DNA (mtDNA) mutations in the development of a wide variety of cancers. Various studies associated mtDNA abnormalities, including mutations, deletions, inversions and copy number alterations, with mitochondrial dysfunction. This might, explain the hampered cellular bioenergetics in many cancer cell types. Germline (*e.g.* m.10398A>G; m.6253T>C) and somatic mtDNA mutations as well as differences in mtDNA copy number seem to be associated with cancer risk. It seems that mtDNA can contribute as driver or as complementary gene mutation according to the multiple-hit model. This can enhance the mutagenic/clonogenic potential of the cell as observed for m.8993T>G or influences the metastatic potential in later stages of cancer progression. Alternatively, other mtDNA variations will be innocent passenger mutations in a tumor and therefore do not contribute to the tumorigenic or metastatic potential. In this review, we discuss how reported mtDNA variations interfere with cancer treatment and what implications this has on current successful pharmaceutical interventions. Mutations in MT-ND4 and mtDNA depletion have been reported to be involved in cisplatin resistance. Pharmaceutical impairment of OXPHOS by metformin can increase the efficiency of radiotherapy. To study mitochondrial dysfunction in cancer, different cellular models (like ρ^0 cells or cybrids), *in vivo* murine models (xenografts and specific mtDNA mouse models in combination with a

Abbreviations: ATP, adenosine triphosphate; OXPHOS, oxidative phosphorylation system; mtDNA, mitochondrial DNA; ECT, electron transport chain; nDNA, nuclear DNA; ROS, reactive oxygen species; SODs, superoxide dismutases; MnSOD, manganese superoxide dismutase; GSH, glutathione; GSSG, glutathione disulfide; HIF, hypoxia inducible factor stabilization; SDH, succinate dehydrogenase; RCCs, renal cell carcinoma; TFAM, mitochondrial transcription factor A; SNPs, single nucleotide polymorphisms; rCRS, revised Cambridge Reference Sequence; MT-ND4, NADH dehydrogenase subunit 4; ρ^0, rho-zero; IR, ionizing radiation; AMPK, adenosine monophosphate activated protein kinase; 2-DG, a glucose analogue, 2-deoxyglucose; Etbr, ethidium bromide; NDUFS4, NADH dehydrogenase ubiquinone Fe-S protein 4; Tp, thymidine phosphorylase; Bcl-2, B-cell lymphoma 2; MCT1, monocarboxylate transporter 1; MPCs, mitochondrial pyruvate carriers.

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spontaneous cancer mouse model) and small animal models (*e.g. Danio rerio*) could be potentially interesting to use. For future research, we foresee that unraveling mtDNA variations can contribute to personalized therapy for specific cancer types and improve the outcome of the disease.

Contents

1. Introduction: the cancer's cell energy supply

In normal physiology, mitochondria are very important in the cell as they produce most of the adenosine triphosphate (ATP) *via* the oxidative phosphorylation system (OXPHOS), which is a necessary energy supply for cellular processes.

The OXPHOS system consists of five protein complexes (complex I–V). All complexes are partly encoded by the mitochondrial DNA (mtDNA), except for complex II, which is completely nuclear encoded [1]. The OXPHOS system also consists of electron carriers that are situated in the inner mitochondrial membrane (Fig. 1B) [2]. Approximately 0.15–2% of the electrons that enter the electron transport chain (ETC) [3] can escape the OXPHOS cycle, resulting in the formation of superoxide. Increased exposure to this OXPHOS-related superoxide may not only affect the nearby located mtDNA but also the nuclear DNA (nDNA), proteins and lipids, resulting in impaired proteins and/or enhanced reactive oxygen species (ROS) production [4]. mtDNA mutations can lead to a decreased efficiency of the ETC and can cause more ROS production [5,6]. It has been shown both *in vitro* and *in vivo* that the OXPHOS system and OXPHOS-related superoxide can have a major influence on tumor progression [7]. As the electron transport chain is the most important site of ROS generation, mutations in mtDNA can cause more ROS production [8]. Evidence suggests when mitochondrial integrity is compromised by excessive ROS formation or mtDNA instability, cancer progression is enhanced. Increased ROS levels and oxidative damage are observed in fibroblasts of patients with mtDNA mutations [6]. To protect against ROS, cells have an antioxidant network from which important antioxidant enzyme groups are catalases, glutathione peroxidases, thioredoxins, peroxiredoxins, glutathione transferases and superoxide dismutases (SODs). SODs are capable of catalyzing the dismutation of superoxide into hydrogen peroxide and oxygen [9]. One of these SOD family members, manganese superoxide dismutase (MnSOD) (Fig. 1B), is located in the matrix of mitochondria [10]. Many types of human cancer cells have reduced MnSOD activity compared to their originated cells [11]. Increased MnSOD expression was able to suppress tumor cell growth and tumor formation in MCF7, A172R, U118, SCC25 and DU145 cells [12]. It has been suggested that MnSOD is a tumor suppressor gene [13] and that mutations found in its promoter region could explain the reduced MnSOD activity [14]. Additionally, also antioxidants are involved in this network. The hydrophilic non-protein thiol, glutathione (GSH), is an important ROS scavenger. In a cell, GSH mainly exits in its reduced form, but is able to oxidize into glutathione disulfide (GSSG), with a ratio between 30:1 and 100:1. However, this ratio can decrease upon oxidative stress [15]. Additionally, GSSG can be converted back into GSH driven by glutathione reductase, the catalyzer for this reaction [16]. An inadequate antioxidant response, where antioxidants are not able to maintain 'normal' physiological ROS levels, will lead to a vicious circle of ROS production and create cellular damage [6,17].

Warburg observed in 1927 that cancer cells rely less on the OXPHOS system, but rather on glycolysis for ATP production and therefore produce high amounts of lactate, even in the presence of oxygen. This phenomenon is known as the Warburg effect, or aerobic glycolysis [18]. Many cancer types have a tendency to be highly glycolytic and mitochondria in cancers cells also show altered cristae, membrane composition and membrane potential [4,19], resulting in an aberrant mitochondrial function influencing ROS production and apoptosis [4,20]. ROS production can be increased due to aberrant mitochondrial function causing cells to be more prone to apoptosis by activating the mitochondrial permeability transition pore [21–23]. However, increased apoptosis could also be independent of ROS as observed in for instance the mutator mouse model [24]. In cancer, inhibition of factors initiating apoptosis could

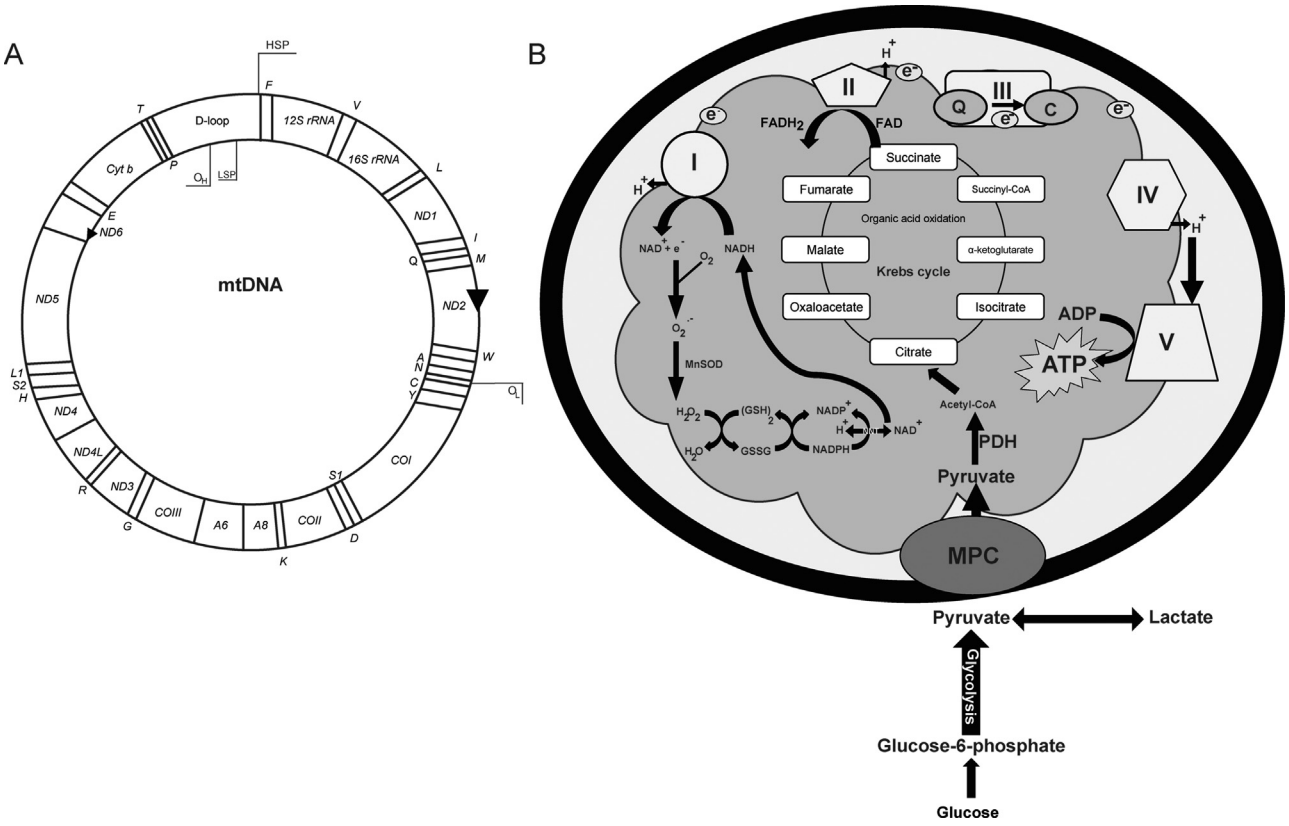

Fig. 1. Global representation of the OXPHOS system and the link to glycolysis in a cell. (A) Overview of the mitochondrial DNA. The genes coding for subunits of OXPHOS complex I are *ND1-ND6* and *ND4L*. Genes for cytochrome c oxidase (complex IV) are *COI-COIII*. The gene encoding for Cytochrome B of complex III abbreviated with *Cyt B*. Additionally the subunits of Complex V (ATP synthase) are *ATPase 6* and *8* abbreviated with *A6* and *A8* respectively. The 22 tRNAs are indicated with a single letter. The two ribosomal RNAs encoded by the mtDNA are *12S* and *16S*. The displacement loop is represented as D-loop and contains sequences for initiation for replication and transcription including the origin of heavy-strand replication (OH). The light strand replication origin is indicated by OL. The position of the Light strand promoter is shown as LSP and the position of the heavy-strand promotor as HSP. (B) Overview of OXPHOS system. When glucose enters the cell it is converted to glucose-6-phosphate, after which it undergoes glycolysis. The final step of glycolysis is the formation of pyruvate and reduced nicotinamide adenine dinucleotide (NADH). Pyruvate can be either transformed into lactate and nicotinamide adenine dinucleotide (NAD) by lactate dehydrogenase (LDH) or can be transported immediately into the mitochondria. This transport is mediated by a mitochondrial pyruvate carrier (MPC) which transports pyruvate across the outer mitochondrial membrane (OMM), the intermembrane space (IMS) and the inner mitochondrial membrane (IMM) into the mitochondrial matrix, where pyruvate dehydrogenase (PDH) catalysis it is converted into acetyl coenzyme A (acetyl CoA). At the same time, the MPC transports hydroxide out of the mitochondria. Acetyl CoA is able to enter the Krebs cycle, where organic acid oxidation takes place and results in succinate production as part of the Krebs cycle and NADH. Electrons from NADH can enter the electron transport chain (ETC), formed by complex I, complex II, complex III and complex IV (I–IV). CI is also known as NADH dehydrogenase/NADH ubiquinone oxidoreductase, the largest of the oxidative phosphorylation (OXPHOS) complexes, and is one of the starting points of enrolling the electrons into the ETC [5]. The ETC transports electrons through the IMM, toward coenzyme Q (Q), after which they are transferred to complex III (III). The electrons can also skip CI and be transported *via* flavin containing enzyme complexes directly toward Q [2]. Another route for electron donation to the ETC is *via* complex II, succinate dehydrogenase/succinate ubiquinone oxidoreductase (II), where succinate is reduced and electrons are transferred toward coenzyme Q and complex III (III). *Via* complex III and cytochrome *c* (C), electrons are moved toward CIV. During electron transport, a proton gradient is formed over the IMM by CI, CIII and CIV, which drives protons into the mitochondrial matrix *via*, CV, ATP synthase (V) with concomitant production of ATP [2,260]. All complexes combined are called OXPHOS. In total, OXPHOS can produce up to 36 mol of ATP per mol of glucose [261]. Additionally reactive oxygen species can be formed which can be transferred into H_2O_2 by manganese superoxide dismutases (MnSOD). H_2O_2 and glutathione can be transformed into H_2O and oxidized under the influence of glutathione peroxidase (GPx). *Via* glutathione reductase (GR) and nicotinamide (NNT) eventually NAD^+ can be formed again.

drive cells toward an actively proliferating state with ROS escalation and DNA damage. The paradox between ROS and apoptosis, in aging or cancer has been extensively reviewed by Wallace [22,25]. Mitochondrial dysfunction can be observed in tumors, particularly in those that are aggressive and growing rapidly [26,27]. Simonnet et al. compared mitochondrial enzymes and DNA contents in renal cell carcinoma (RCC) to normal kidney tissue. Mitochondrial impairment was increased from the less aggressive to the most aggressive RCCs and correlated with a considerably decreased content of OXPHOS complexes [27]. To maintain optimal ATP levels, some tumor cells are able to stimulate the glycolysis pathway upon pharmacological inhibition of the OXPHOS system. In contrary, after inhibition of glycolysis, the OXPHOS system cannot compensate for the loss of ATP production in these same cells [28,29]. This is, however, not universal as other tumor cell lines are still capable of maintaining high ATP contents when they are deprived of glucose [30,31]. The extent of changes in mitochondrial respiratory capacity seems to depend on the tumor type and its micro-environmental characteristics, such as hypoxia and hormones [32–34]. Adaptation of these processes might affect the tumor responsiveness to therapies, which points to a potential therapeutic target for new cancer therapies.

2. Mutations in nuclear-encoded mitochondrial genes can cause cancer

Next to the conventional cancer causing mutations (*e.g. TP53* encoding for p53 which is involved in proliferation, apoptosis and DNA repair [35–37]), some cancers (*e.g.* paraganglioma) are found to be associated with a mutation in one of the nuclear mitochondrial genes encoding for OXPHOS subunits or parts of the Krebs cycle.

Patients suffering from this disease have a dysfunctional succinate dehydrogenase (*SDH*), better known as complex II, which

is composed of four subunits. Whereas mutations in subunit A are mostly responsible for Leigh's disease (a rare neurometabolic disorder), mutations in subunits B, C or D mainly cause paraganglioma [38–41]. In a xenograft study studying a *SDHC* missense mutation it was found that mice formed benign tumors comparable with paragangliomas [41]. In 2 other studies investigating families with paragangliomas carrying mutations in *SDHD* [39] or *SDHC* [40] a causative role was found for these mutations and a tumor suppressor function for the genes was suggested [39,40].

Nevertheless, a family with mutated *SDHA* was recently reported to have paraganglioma as well [42]. A proposed mechanism in patients could be that mitochondrial ROS increases and more oxidative damage occurs. This could result in oncogenic transformation as occurs for paragangliomas [41].

Additionally, mutations in succinate dehydrogenase have been found in breast cancer, gastrointestinal stromal cancer and renal carcinomas [43–45]. For other cancers like uterine leiomyomas and RCCs [46,47], associations with mutations were found in other nuclear-encoded mitochondrial genes such as fumarate hydratase. Heterozygous mutations have been reported in the gene encoding for fumarate hydratase, which catalyzes the reversible hydration of fumarate to malate, a Krebs cycle substrate (see Fig. 1B) [48]. Similarly, for iso-citrate dehydrogenase, catalyzing the conversion of isocitrate into 2-glutarate in the Krebs cycle, mutations in the cytoplasmic (*IDH1*) and the mitochondrial (*IDH2*) isoforms are reported in a wide variety of human cancer types such as: acute myeloid leukemia [49,50], angioimmunoblastic T-cell lymphomas [51], cartilaginous tumors [52], colorectal cancer [53], glioblastomas [54], glioma [55] and prostate cancer [56]. For a more extended review see Gaude and Frezza [57].

Although, evidence for a causative role of the latter mentioned mutations in oncogenesis is mostly indirect, the consequent changes in metabolite levels such as succinate have been shown to influence hypoxia inducible factor (HIF) stabilization [58].

3. Mitochondrial DNA mutations and cancer

Over recent years, accumulating evidence suggests that mtDNA mutations may also contribute to a cell's potential to become a cancer cell [59–61], ultimately leading to tissue invasion and metastasis. mtDNA variations, such as deletions, point mutations and copy number differences, are associated with a wide variety of cancer types [62–72].

3.1. Genetic insight into mtDNA

3.1.1. mtDNA characteristics

The mtDNA is a 16.5 kb double-stranded, circular-shaped DNA molecule that is distinct from nDNA in several ways: (1) mtDNA is only maternally inherited; (2) mammalian cells can contain thousands of mtDNA molecules, but they nevertheless represent only a minor percentage of the total DNA [73]. As mtDNA is polyploid, a mitochondrion contains five to ten copies of mtDNA, which can differ in composition from each other. Homoplasmy indicates that the mtDNA copies in a cell are all identical, while a mixture of wild-type and variant copies is referred to as heteroplasmy [74]; (3) mtDNA is almost completely comprised of coding sequence. It codes for 13 OXPHOS subunits, 22 tRNAs and 2 rRNAs; less than 10 percent of the entire mtDNA is non-coding [1] (Fig. 1A). This non-coding region is mainly located in the displacement (D)-loop, a 1.1 kb region involved in mtDNA replication and transcription. In addition, mtDNA encoded genes do not contain introns and have none or only a few non-coding bases between them. The coding sequences of some of the encoded genes are even overlapping, *i.e.* they share several mtDNA bases [1]. Consequently, changes in the mtDNA are more likely to have functional consequences than nDNA variations [68,75,76]; (4) in the mitochondrial genome, transcription starts from one of the two mitochondrial promoters and can take place on both DNA strands. Subsequently, transcription produces a polycistronic precursor RNA that results in individual tRNA, rRNA and mRNA molecules after processing; (5) mtDNA replication occurs independent of the cell cycle which is therefore referred to as relaxed replication. This means that the number of mtDNA molecules replicated per cell cycle is only restricted by the levels of available replication machinery factors and nucleotides [77,78]; (6) mtDNA has a higher mutation frequency compared to the nDNA in mammals [79,80]. Possible explanations are: mtDNA is located close to the ROS producing OXPHOS and mtDNA lacks protective histones [81,82]. However, there is a debate about the protective role of histones, as there is also evidence showing that the electrons can transfer easily from histones to DNA leading to damage [83] and under some conditions (exposure to $Cu(II)/H_2O_2$) histones can even enhance DNA damage [84]. It is also suggested that DNA-binding proteins of mitochondrial nucleoids can be as equally protective as histones for mtDNA under H_2O_2 or X-ray exposure [85,86].

3.1.2. Mitochondrial DNA maintenance

The replication of mtDNA is maintained *via* DNA polymerase γ (*POLG1* and *POLG2* genes) [87], mitochondrial transcription factor A (*TFAM*) [88], Twinkle (*C10orf2*) [42], mitochondrial 12S rRNA dimethylase 1 and mitochondrial 12S rRNA dimethylase 2 (*TFB1M* and *TFB2M*, synonyms: *mtTFB* or *Mtf1*) [48], mitochondrial single stranded DNA binding protein (mtSSB) [89] and mitochondrial RNA polymerase gene (*POLRMT*) [72]. Both polymerase γ and TFAM are essential for maintaining mtDNA copy number and integrity [90–94]. More frequent point mutations and deletions were observed when introducing mutations in the exonuclease domain of POLG resulting in misincorporation of nucleotides [95,96]. Furthermore, compared to nDNA, mtDNA damage persists longer [82].

In murine models having a complete knockout for TFAM, loss of mtDNA and severe OXPHOS defects are observed leading to embryonic death [94]. Inducible knock-down leads to cell death of the targeted cell type [97,98]. In 2011, Balliet and colleagues showed that fibroblasts lacking TFAM exhibited mitochondrial dysfunction and increased oxidative stress due to the loss of certain OXPHOS components and over-production of hydrogen peroxide and lactate [99].

3.1.3. mtDNA dynamics

Mitochondria are able to change their number and shape in different cell types under varying physiological conditions [100,101]. One possible way for mitochondria to maintain their integrity and thus to ensure a healthy population is by exchanging mtDNA through constant fission and fusion processes. It can guard a cell by allowing the mitochondria to fuse or divide and thereby protect the cell from detrimental effects of (accumulating) mtDNA mutations [102].

Mitophagy, a degradation process of mitochondria through autophagy, controls the number of mitochondria in the cell and initiates the removal of dysfunctional and damaged mitochondria [103]. Previous studies had already shown that defective mitochondria displayed increased mitophagy [104,105]. So when a mutation leads to a reduced mitochondrial membrane potential (depolarization of mitochondria) the mutation causes a phenotypical change and mitochondria loose therefore their ability to function normal. Consequently, the mitochondria are not able to re-fuse with the mitochondrial network after fission and are recycled [106].

When a somatic mtDNA mutation first occurs, it will be in one mtDNA molecule. The process of random genetic drift, *i.e.* the change in the frequency of a variant in a population due to random

sampling, will at first determine the persistence and expansion or loss of this mutation since it will not have significant functional effects and thus selection advantage as a single copy. These mutations are not targeted by mitophagy and *via* fusion dominant mutations can become homoplasmic [68]. It is known from haplogroup studies that mtDNA variation can have an effect on how efficient electron transport and ATP production are coupled in the mitochondria [107]. In that sense, varying percentages of mutant mtDNA can lead to bioenergetic defects ranging from mild mitochondrial dysfunction to severe metabolic distress and cell death [108] or they can lead to more optimal functioning of the OXPHOS process. In combination with the proposed model that cancer metabolism switches from mainly glycolysis to preferentially OXPHOS and back during the cancer process [109], this suggests that mutations reaching certain heteroplasmy levels by random genetic drift resulting in a detrimental (stimulating glycolysis) or beneficial effect on OXPHOS will be selected and clonogenic expanded in these respective metabolism waves.

3.2. Germline mtDNA mutations associated with cancer

Different studies have explored the association between germline mtDNA mutations and cancer. There are indications that different germline mutations could actually contribute to the development of a certain cancer type in a specific population.

Different human populations can be distinguished by different human mtDNA haplogroups which are defined by unique sets of mtDNA polymorphisms, reflecting mutations accumulated by a discrete maternal lineage [110]. The haplogroups are associated with region-specific mtDNA sequence variation as a result of genetic drift and/or adaptive selection for an environment-favored mitochondrial function [107,111]. Tanaka et al. classified 30 different haplogroups in a retrospective study and found that the risk in the population M7b2 haplogroup was related to increased risk for hematopoietic cancer [112]. Furthermore, Booker et al. showed that haplogroup U was related to a 2.5-fold increased risk for developing renal cancer in Caucasian American men [113]. They also reported a two-fold increased risk for development of prostate cancer, a finding which was supported by Canter et al. [113,114].

Bai et al. identified two different haplogroups that influence breast cancer risk in a Caucasian population: haplogroup K indicates an increased risk whereas haplogroup U is a protective haplogroup [115]. Additionally, they identified four different single nucleotide polymorphisms (SNPs) that influence cancer risks in certain populations.

The m.10398A is especially of interest as it also defines the European haplogroup N [116]. The revised Cambridge Reference Sequence (rCRS) [117] has an A as reference nucleotide. In the paper of van Oven et al. a different sequence was published based on a phylogenetic approach. Here it was shown that the original wild-type nucleotide on this position should be a G and this site is prone for mutations as the G>A polymorphism occurs in different haplogroups [116]. Annotations in this paper are based on the rCRS nomenclature and HGVS guidelines.

The m.10398A>G and m.16519T>C variants were found to be related to an increased risk of breast cancer. Two other SNPs, m.13708G>A and m.3197T>C, were identified as protective variations [115]. Interestingly, in a population of African-American women, the m.10398A variant led to an increased risk of invasive breast cancer [118]. In an Indian population with haplogroup N, an association was found between m.10398A and breast and esophageal cancer [119]. However, in another study by Francis et al. within an Indian population no correlation was found between the m.10398A polymorphism and increased breast cancer risk [120]. In a study performed by Mosquera-Miguel et al. also no association was found for this variation in a Spanish population [121]. Recently, Salas et al. reanalyzed all studies and no correlation could be found regarding the m.10398A>G SNP [122].

For prostate cancer, Petros et al. have demonstrated an association for four different germline mutations in cytochrome oxidase subunit I (m.6253T>C, m.6340C>T, m.6261G>A, and m.6663A>G) in a cohort study. Additionally in a proof of principle study they showed for the germline mutation m.8993T>G in ATP6 a 7-fold enhanced tumor volume for mutants compared to wild-type tumors. This indicates that mtDNA germline variations could play a role in tumor growth for prostate cancer [66].

For pancreatic cancer Wang et al. investigated if pancreatic cancer risk increase was associated with mtDNA SNPs however no correlation could be observed [123]. Navaglia et al. showed by sequencing the D-loop region that germline m.16519T worsened the prognosis in pancreatic cancer patients. However, somatic D-loop mutations seemed not to be involved [124]. Also Half-danarson et al. investigated if survival outcome in pancreatic cancer could be predicted based on mtDNA variations by using a SNP approach; but they did not find a relationship between certain SNPs and pancreatic survival [125]. However, Lam et al. found using genome wide sequencing an association with pancreatic cancer and the variant m.5460G>A encoding for a non-synonymous p.A331T substitution in the ND2 gene. In the same study it was found that haplogroup K was correlated with a reduced risk compared to haplogroup H, however this could be a false positive finding, since these results were inconsistent with previous data and the amount of haplogroup K participants was very low. Additionally, in haplogroup N the amount of rare singleton (variants unique to a single participant) variations in HV2 and 12S RNA regions was increased compared to controls. The same was found for singletons ND4 and ND5 among patients with haplogroup L [65], These findings indicate that mtDNA variations might contribute to pancreatic cancer risk, however this needs to be further investigated.

The mentioned studies above took different approaches to identify mtDNA variations. Multiple studies only looked at certain SNPs [66,115,118,120,121,123–125] and others performed complete mtDNA sequencing to pick up variants [65], which give more inside in the different types of variations. It should be taken into account that also individual non-synonymous polymorphisms are able to influence mitochondrial function and that restricting analysis to haplogroup variation does not cover the load of mtDNA variation. Epistatic interactions (interactions between 2 or more variations) between the mtDNA and the nDNA are worth investigating in relation to cancer predisposition or development. There seems to be some evidence that germline polymorphisms and haplogroups are related to different cancer types. Note that studies finding germline mtDNA variations often face high frequencies of functional polymorphisms and high mutation rates can lead to the rise of the same mutation in different populations and therefore should be adjusted for population substructures as it is done in similar to Genome Wide Association studies. Otherwise, the SNPs can just mark different ancestral populations and not causal mechanisms [126].

Identifying germline mutations related to a specific cancer type in a defined population can be very difficult, as the same mutation can lead to a different risk in another population. From this perspective, somatic mtDNA mutations have an advantage as they are only carried in the tumor and not the patients' normal tissue [68].

3.3. Somatic mtDNA mutations related to cancer

In 1998, Polyak et al. demonstrated that somatic variations in the mtDNA were present in the primary tumors of colorectal cancer patients [68]. In the following years, numerous somatic

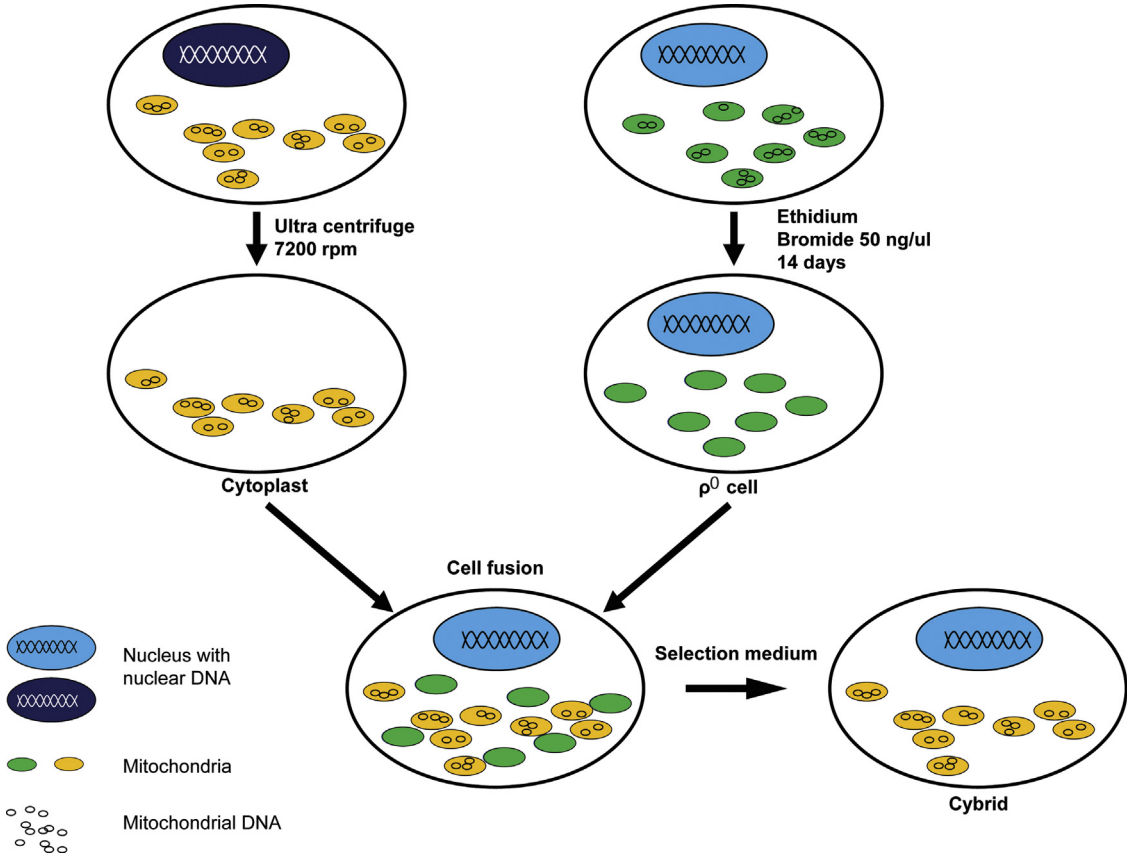

Fig. 2. Cybrid model for investigating the influence of mtDNA on cancer. Cybrids are produced by combining nucleated cells without mtDNA (ρ^0 cells) with cytoplasm from non-nucleated cells (cytoplasts). A cytoplast is made by removing the nucleus through ultracentrifugation (7200 RPM), leaving a cell that only contains mitochondria with mtDNA. Nucleated cells without mtDNA (ρ^0 cells) are formed by the depletion of the mtDNA by exposing the cells to ethidium bromide (Etbr). As these cells contain nuclear DNA, they define the nuclear background of the cybrid. Fusion of ρ^0 cells with the cytoplasts leads to the formation of cybrids.

mtDNA mutations were reported in a wide variety of tumors (*e.g.* colorectal, breast, bladder, esophageal, head and neck, ovarian, renal, leukemia, lung and thyroid cancer) [67–69,127–131]. Furthermore, somatic mutations have been shown to influence cancer progression and metastasis [66,71,132,133]. Data analysis by Larman et al. showed that, across 5 different cancer types, displayed somatic mtDNA mutations ranging, from 13% in glioblastoma to 63% frequency in rectal adenocarcinomas [133]. Some data suggests that the effect of the somatic mtDNA mutations and the degree or nature of the tumorigenesis effect depend on the functional and threshold effect of the mutation [134–136]. For instance, the m.3460G>A/*MT-ND1* mutation (decrease in complex I activity) result in different tumorigenic potential as determined by colony forming efficiency and tumor growth of osteosarcoma cybrids (cytoplasmic hybrids, Fig. 2) [135] compared to the m.3571insC/*MT-ND1* and the m.3243A>G/*MT-TL1* mutation (severe structural and functional complex I alteration). More severe alterations in complex I (m.3571insC/*MT-ND1* and the m.3243A>G/*MT-TL1* mutation) resulted in a reduced tumorigenic potential both *in vitro* and *in vivo*, compared with cells displaying milder complex I dysfunction (m.3460G>A/*MT-ND1* mutation) [135].

3.4. Changes in mtDNA copy number and its association with cancer

The number of mtDNA molecules could be another factor of consideration since this can affect proper mitochondrial function too [137]. Other reviews describe this subject in more depth or have been exclusively dedicated to this subject (see [138]). Here we give a short summary on this subject; however, this is not a complete list regarding observed changes of mtDNA copy number in cancer. The association between copy number variation and cancer is still subject of debate. Different trends have been reported in multiple tumor types. Both an increase and a decrease in mtDNA copy number have been reported to be associated with an increased risk for tumorigenesis. In genomic DNA extracted from blood an elevated copy number (compared to matched control subjects) has been observed in of patients with various cancers *e.g.* breast cancer [139], RCCs [140], non-small cell lung cancer [63]. Additionally, there are also studies investigating mtDNA copy number in tumor tissues. In tumor tissues compared to matched control subjects an elevated copy number was found for endometrial cancer [141], glioblastoma [142], head and neck cancer [143] and ovarian cancer [144]. Also in tumor tissue compared with paired non-tumor tissue from the same patient an elevated copy number have been found for colorectal carcinoma [145], esophageal squamous cell carcinoma [146], metastatic fibrolamellar carcinomas [147], prostate cancer [148] and thyroid carcinoma [149].

Increased mtDNA copy numbers in tumors could possibly be explained by an increase of oxidative stress [150]. However, the precise mechanisms have not been fully understood. Potentially mtDNA replication could be increased to compensate for metabolic effects caused by mtDNA variations or oxidative stress [138].

In contrast, decreased mtDNA copy numbers have been reported in patients compared to matched tissue subjects for astrocytoma [151] and Ewings sarcoma [152], and in tumor *versus* non-tumor tissue samples from the same patient for breast cancer [149], lung cancer [153], primary fibrolamellar carcinoma [147], gastric cancer [154], hepatocellular carcinoma [147] and RCC

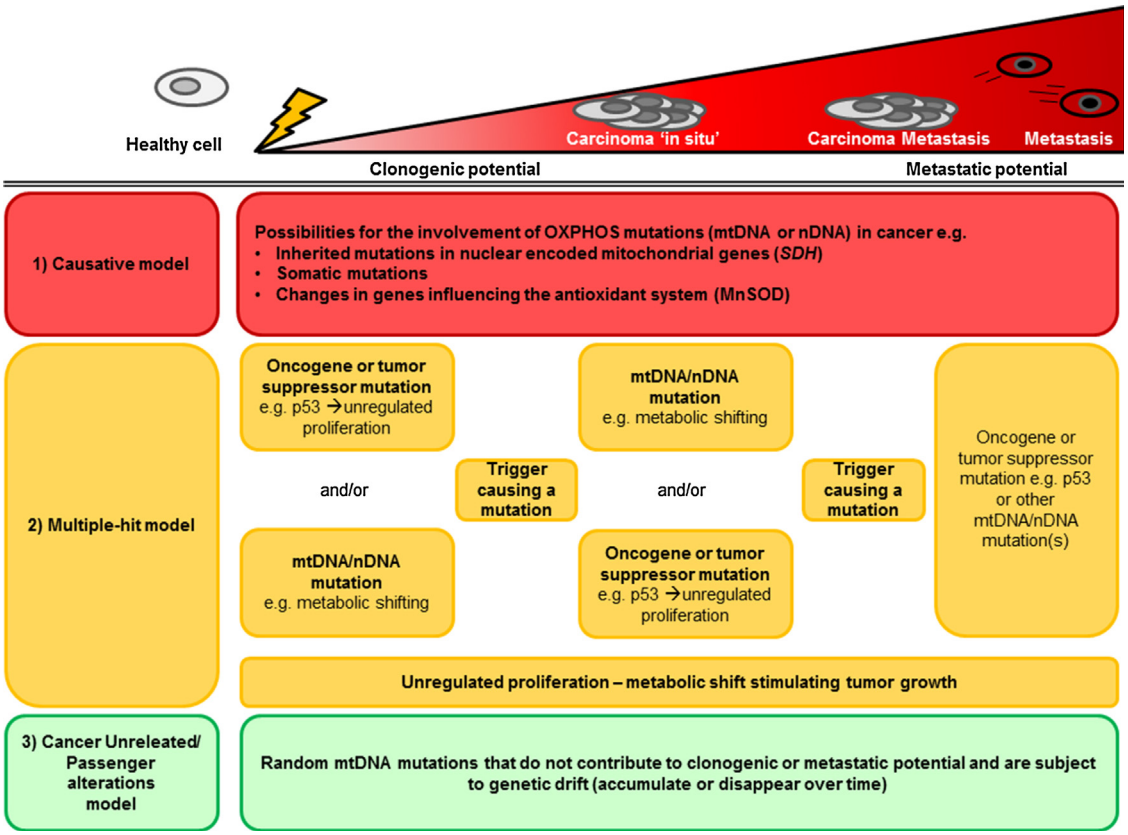

Fig. 3. Representation of different scenarios possibly explaining how mitochondrial genes encoded in nDNA or mtDNA could contribute to cancer. When a cell gains clonogenic potential and evolves into a primary tumor or metastasis, different alterations occur in nuclear encoded mitochondrial genes and mtDNA. Potentially three different scenarios could describe and explain these observations. In the causative model, mutations in mitochondrial genes encoded either in the nDNA or mtDNA cause the development of a carcinoma and/or metastasis. The multiple-hit model describes a scenario where different types of mutations (in classic oncogenes or tumor suppressor genes and in mtDNA or nDNA OXPHOS genes) together contribute to the clonogenic potential and the development of a carcinoma or metastasis. Mutations can follow sequentially or a driver mutation needs additional mutations after which it can express its mutagenic or metastatic potential resulting in a pathogenic phenotype. Additionally, also low heteroplasmic mtDNA mutation levels can be increased *via* drift. This increased mutation percentage could thereby exhibit the pathogenic effect of the mutation. The third scenario consists of passenger mutations observed in the tumor but not contributing to an increased clonogenic or metastatic potential. The mutations can accumulate or disappear over time (genetic-drift). All different models can exist in the same tumor site, tumor or tumor type.

[155]. A twin study found that a reduced mtDNA copy number was related to an increased risk for the development of RCC [156].

Furthermore, there are indications that the depletion of mtDNA in cancer cells can be responsible for disease progression [157]. Lowered mtDNA is correlated with metastasis in patients suffering from Ewing's sarcomas [152]. In addition, shorter survival time was related to low mtDNA copy numbers in patients suffering from astrocytoma [151]. Again, a reverse observation was made for head and neck tumors where increased mtDNA copy number was found to be associated with a decreased survival [158].

Low mtDNA content can be caused by p53 or *POLG1* mutations, resulting in tumor initiation or progression [152,159–161]. The *POLG1* gene was found to be mutated in 63% of breast tumors, and thought to be responsible for a depletion of mtDNA in these tumors [160]. TFAM mutations associated with a lowered TFAM protein and a decreased mitochondrial copy number were found in colorectal cancers with microsatellite instability while no TFAM mutations were observed in colorectal cancers with microsatellite stability. Additionally TFAM mutated cells (TFAM frame-shift mutation) were able to grow larger tumors *in vivo* [93] and lowered mtDNA copy number and more oxidative mtDNA damage was observed in $TFAM^{+/-}$ mice [94,162]. When this model was crossbred with the adenomatous polyposis coli multiple intestinal neoplasia ($APC^{-/+}$) mouse cancer model, mtDNA instability enhanced tumorigenesis [162]. These findings suggest that both polymerase γ and TFAM might play an important role in tumorigenesis. Although, it is not investigated if *POLG1* and *TFAM* mutations influence oncogenic transformation as such, a promotion of oncogenic potential after primary transformation has been shown especially for TFAM [93].

There have been several reports of higher cancer incidence with both increased and decreased copy numbers in the same tumor type. An example is RCC, for which both increased [140] and decreased copy numbers has been described [155,156]. Furthermore, there seems to be no specificity for tumor type or primary site. The exact molecular and metabolic differences between the cancer types that relate to these copy number differences should be examined in more detail for a plausible rationale to be found.

Previously, we discussed several types of nuclear encoded mitochondrial and mtDNA mutations exhibiting mitochondrial dysfunction and how they relate to cancer and cancer therapy (see Fig. 3). (1) Certain mutations display a causative effect for nuclear encoded mitochondrial and mtDNA genes in cancer. Some of the mutations in nuclear encoded mitochondrial genes have been reported to behave like tumor suppressor genes (like SDH or MnSOD) and loss of function or lowered expression increases tumor formation. Additionally, it has also been reported that mtDNA depletion can result in a tumorigenic phenotype *in vitro* as well as *in vivo* [51]. (2) The multiple-hit model could be an explanation for the observed alterations of mtDNA variations observed in cancer as most scientific findings presented only an association of mtDNA mutations in cancer. These mutations are not directly involved in generating the neoplastic phenotype but can

be responsible for tumor progression or play an important role in the metastatic potential of the tumors. Additionally, such mutations can also influence the outcome of disease by promoting cancer treatment insensitivity. However, there is also a second mechanism for the multiple-hit model possible. In this mechanism some driver mutations, without direct selective value, can be randomly fixed *via* random-drift. In this manner they only express their clonogenic or metastatic potential at a later stage and only in combination with other driver mutations. (3) Another option could be that neutral mtDNA mutations occur random and are not related to clonogenic potential or metastatic potential and are subject to genetic drift (= can disappear or accumulate) and are so called bystander (or passenger) mutations. However, it is likely that combination of the different models takes place in a tumor.

4. mtDNA and cancer treatment implications

In cancer patients, the choice of therapy depends on multiple factors such as the histopathology of the tumor, the stage of the disease and the patient's condition. The three most commonly used treatment options, sometimes combined with one another, are surgery, chemotherapy and radiotherapy.

4.1. Chemotherapy

mtDNA abnormalities affect the response and outcome of therapies in cancer patients. As described in a review by Singh et al. different studies have already shown that alterations in mtDNA can result in chemotherapy resistance [4]. It has been reported that mutations in the NADH dehydrogenase subunit 4 (*MT-ND4*) contributed to acquired chemoresistance during paclitaxel carboplatin treatment [163].

However, it also has been suggested that mtDNA variations can be induced by chemotherapy. In a case report a patient with ovarian carcinoma had a mtDNA mutation in the *MT-ND4* gene (m.10875T>C) which occurred after chemotherapy treatment [163]. A second study observed that in a leukemia patient 6 months after chemotherapy more mtDNA variations were found compared to the samples withdrawn prior to treatment [8]. In the same study, leukemia patients treated with a fludarabine/alkylator-based chemotherapy regimen displayed an increase in mtDNA mutations in primary leukemic cells compared to non-treated patients [8]. Additionally, accumulation of mtDNA damage even persisted after ending doxorubicin treatment and was associated with adverse effects such as cardiotoxicity [164]. Therefore it seems that *de novo* variations can be induced by chemotherapy. Another possibility is that due to selection a very low heteroplasmy mutation load could be increased in the patients due to its clonogenic advantageous property. Both possible mechanisms should be further investigated as well as the observations should be validated in larger study populations before any conclusions could be drawn.

Another chemotherapeutic drug, cisplatin, is able to accumulate in the mitochondria, causing impairment of mtDNA and mtRNA synthesis [165]. This drug has been shown *in vitro* to induce more adduct formation in the mtDNA compared to the nuclear DNA [166]. In addition, cisplatin leads to caspase-dependent apoptosis by changing the mitochondrial membrane permeability and resulting in the release of cytochrome c into the cytosol, subsequently activating caspase 8 and 9 [167]. For the intestines, a correlation was observed between the mitochondrial density and cisplatin sensitivity. Cisplatin sensitivity was limited in normal ileum tissue having a low mitochondrial density, while the opposite was observed for the normal duodenum tissue, which has a high mitochondrial density. In line with these findings, *in vitro* cultured cells depleted from their mtDNA, rho-zero cells (ρ^0, Fig. 2) generated from normal intestinal epithelial cell lines (IEC-6) showed a four- to five-fold increased resistance against cisplatin compared to their parental counterparts [168]. This can be explained by the fact that cisplatin-induced adduct formation in mtDNA measured in head and neck squamous cell carcinoma cell lines can be up to 500 times higher compared to nDNA [169]. However, it has also been suggested that mtDNA mutations lead to an increased ROS production and that combination with chemotherapeutics with the same effect (like cisplatin) can lead to exhaustion of a cell's antioxidant capacity/response and thereby eventually lead to apoptosis [170–172]. Correlations of ROS production and sensitivity to 2-methoxyestradiol, a ROS generating agent, have already been reported [173,174].

There seems to be some evidence that mtDNA variations could contribute to chemotherapy resistance and some drugs directly interact with the mtDNA. The observations also apply to radiotherapy, another conventional cancer therapy.

4.2. Radiation therapy

Ionizing radiation (IR) is a widely used cancer therapy that results in cellular damage in a direct or indirect cellular manner. The direct mechanism involves the transfer of energy from incident photons or particles to target molecules in their path. ROS are formed in the presence of oxygen, resulting in secondary damage [175]. Upon DNA damage, cells might undergo a temporary cell cycle arrest to repair the damage or die, mainly by mitotic catastrophe and only partly by apoptosis [176]; both processes require ATP [177,178], and might involve directly or indirectly mitochondria as both are at least in part dependent on mitochondrial membrane permeabilization. This suggests that mitochondria are potentially involved in downstream irradiation effects [179,180].

Studies have shown that ρ^0 cells are more radioresistant than parental cells containing wild-type mtDNA [181,182]. Human fibroblast and pancreatic cancer ρ^0 cells have shown higher survival rates following radiation, which could be related to decreased G2/M cell cycle arrest [181] or decreased apoptosis without alterations in cell cycle distribution [182]. Improved survival could not be linked to differences in antioxidant enzyme expression [181]. In contrast, other studies did not identify differences in survival after irradiation between human fibroblast and osteosarcoma in parental *versus* ρ^0 cells, although less micronuclei (fragments found in the cytoplasm which originates from nuclear DNA and correlates with dose/quality of irradiation [183]) formation was observed in ρ^0 cells after irradiation [184,185].

A differential radiation response was observed between a normal B-lymphoblastoid cell line and the mitochondrial mutant cell lines for Leigh's syndrome (m.8993T>G mutation in MTATP6 ATP synthase gene) or Leber's optic atrophy (m.11778G>A mutation in MTND4 gene) [186,187]. In both cell lines, the apoptosis-related genes showed a remarkable up-regulation compared to the control cell line. However, the repair of the irradiation-induced double-stranded breaks was different for each of the mutant cell lines. Repair was significantly decreased in cells with Leigh's mutation, leading to worse short-term radiation survival, while for the Leber's cells, double-strand breaks could be repaired resulting in better short-term radiation survival although the studies were not performed in cell lines with the same background [186,188]. In a recently published *in vivo* study it was shown that irradiation of a SiHa cell line with induced mitochondrial dysfunction (showing a decrease of oxygen consumption upon exposure to ethidium bromide) lead to an increased time to reach the endpoint compared to their parental counterpart [189].

There are also indications that mtDNA variation may not only affect radiotherapy outcome, but that radiotherapy and chemotherapy itself also can induce mutations in the mtDNA. Wardell et al. observed an increased number of point mutations and

deletions in patients treated with radiotherapy and chemotherapy [190]. Additionally, a decrease in mtDNA content has been reported as a result of radiotherapy in cancer patients [191]. However, another study found no evidence that radiotherapy for pediatric cancer, which resulted in scatter radiation to the ovaries, is associated with the mitochondrial genome mutation frequency in female cancer survivors and their children [192]. In conclusion, mtDNA mutations (induced by radiotherapy or germline) can also affect the response and therefore the outcome of radiotherapy although the precise role and mechanisms are not yet fully understood.

4.3. Mitochondrial function interventions in cancer therapy

ROS is one of the major processes implicated in cancer. It may be possible to reduce the ROS escalation caused by dysfunctional OXPHOS using metformin, a type II diabetes drug that is used to suppress gluconeogenesis and, among others, inhibits ROS production as well as mitochondrial complex I [193–195]. A previous study showed that type II diabetes patients have an increased risk of developing cancer with a poorer prognosis [196]. In 2005, Evans et al. observed a correlation between a reduced risk of developing cancer and treatment of type II diabetes patients with metformin [197], which was recently confirmed in a large cohort study [198].

Metformin activates adenosine monophosphate activated protein kinase (AMPK) and therefore stimulates muscles to take up glucose from the blood, a process also activated by exercise [197]. Furthermore, the activation of the cell metabolism regulator AMPK results in inhibition of mTORC1 and its downstream signaling pathway, thereby decreasing protein synthesis and cell proliferation crucial for tumors [199,200]. The combination of metformin and radiation therapy was shown to be successful *in vitro* since cellular survival was decreased [201]. Song et al. found that metformin was able to increase radiosensitivity of cancer cells *in vitro* and enhance radiation-induced growth delay of fibrosarcoma tumors as well as non-small cell lung cancer [202,203]. When metformin treatment was combined with 2-deoxyglucose (2-DG, a glucose analog), it resulted in an energetic stress cell death [204,205]. Instead of using a glucose analog, glucose uptake could be inhibited by other substances (*e.g.* Pholertin, WZB117 or Fasentin) to enact a similar mechanism [206–208]. Inhibition of glucose uptake of the cell could also be a potential target for tumors with a dysfunctional OXPHOS system [209].

Both chemo- and radiotherapy could be influenced by mtDNA variations. Additionally, a combination therapy including conventional therapies and drugs influencing mitochondrial function could be explored as a therapeutic option for cancer patients.

5. Opportunities and pitfalls in studies using mtDNA models

Multiple studies have identified potential associations between mtDNA variations and cancer [62–71,210]. Mutations/deletions and copy number variations in the mtDNA are able to affect proper OXPHOS function and consequently change predisposition for a disorder. mtDNA variations (*de novo*, inherited, caused by ROS production in tumors or by cancer treatments) can promote tumor development and progression. Different mtDNA models are available for diagnostic and research purposes. Table 1 presents a short representation of *in vitro*, *in vivo* and *ex vivo* models.

5.1. In vitro *mtDNA models*

Effects of mtDNA depletion are commonly investigated in the ρ^0 cell model, mammalian cells depleted of mtDNA by exposure to ethidium bromide (Etbr) [211]. Consequently, ρ^0 cells rely completely on glycolysis instead of oxidative phosphorylation for energy production. Removal of Etbr leads to mtDNA repopulation and therefore it should be used throughout the entire experiment. Etbr treatment can lead to stalled proliferation [212], is genotoxic to cells *in vitro* (http://ntp.niehs.nih.gov) and can lead to off-target effects on the nDNA. Therefore this model is not suitable for *in vivo* studies.

Most studies on pathophysiology have exploited fibroblasts derived from patients with mtDNA diseases or mutations. These models allow for high-throughput screening [75,76,213] and testing of potential therapies *in vitro*. Unfortunately, the consequences of OXPHOS-related defects are sometimes less pronounced in *in vitro* fibroblast cultures, since cells are capable of switching from oxidative phosphorylation toward glycolysis under specific culturing conditions [214]. In addition, these cells are generally not able to form tumors by themselves. Therefore fibroblasts are not suitable for investigating the link between cancer and mtDNA.

A cybrid cell line is another model used to study the effect of mtDNA variations in cancer. These are cell lines with the nuclear DNA background of a tumor cell line [132] and the mtDNA of another cell line (Fig. 2). The mtDNA can come from a patient-derived cancer cell

Table 1
Comparison of different *in vitro*, *in vivo* and *ex vivo* mitochondrial models.

	In vitro				*In vivo*			*Ex vivo*
	Tumor cell lines	ρ^0 cells	Fibroblast cell lines	Cybrids	Small organisms	Rodent models: mtDNA	Rodent models: Xenografts	Primary tissue
Availability	+++	++	+	+/−	+++	+	++	−
High throughput	+++	++	+	+	+	+/−	++	+++
Pathological similarity	+/−	−	++	+	+/−	++	+	+++
Main advantage	Easy to manipulate	Easy to manipulate	Patient-derived	For comparison of nDNA and mtDNA background effects	High throughput	Pathological similarity	Implantation of patient material in a biological environment	No translation necessary
Main disadvantage	Reduced phenotype	Not applicable for *in vivo* experiments	Availability	Reduced phenotype	Evolutionary distance	Costs	Less biologically relevant than mitochondrial mouse models	Availability
Applications: Study pathology	+/−	+/−	+	++	+/−	++	+/−	+++
Therapy testing	−	+/−	++	++	+++	++	+	+/−

+++, excellent; ++, good; +, satisfactory, but better options are available; +/−, sufficient but other models are more desirable; −, insufficient.

line, a fibroblast or a lymphocyte cell line. The main advantage of these cybrids is the ability to investigate whether phenotypes and biochemical changes are related to changes in the nDNA or mtDNA. Furthermore, cybrid models enable the investigation of *in vitro* and *in vivo* effects of different mtDNA mutations in cancer cell lines and tumors with the same nuclear background [215]. A possible disadvantage is the dependency on naturally occurring patient mtDNA variations.

5.2. Mitochondrial animal models

Patients with variations in their mtDNA are often unique, so grouping them is very difficult. Therefore, data obtained from patient studies should be carefully interpreted with respect to large inter-individual variation. The use of murine models is in this respect advantageous, as experimental settings can be better controlled than in patient studies. For instance, cell lines with the same nuclear genetic background harboring one mtDNA variation can be used as xenograft models. Additionally, to easier investigate the molecular mechanisms, specific mitochondrial murine models, such as the POLG mutator mouse, can be used either combined with mouse models which spontaneously develop cancer.

Currently, different mouse models are available to unravel the pathways involved in mtDNA mutations and cancer (Table 1). The most studied mouse model created for translational research in this particular field is the POLG knock-in mouse. These mice lack the proofreading function of pol γ and therefore show an accumulation of mutations and deletions in their mtDNA [216]. In homozygous mice, but not in heterozygous mice, a premature aging phenotype resulting in effects such as heart enlargement and hearing impairment has been observed. However, no cancer development has been reported [216,217]. Another mouse model carries a mutation in the complex I subunit NADH dehydrogenase ubiquinone Fe-S protein 4 (NDUFS4). These mice displayed a phenotype similar to that in human patients carrying a NDUFS4 mutation, and a full knockout was lethal at approximately seven weeks of age [218,219]. A heterozygote mutant of the NDUFS4 mice was viable and still showed the biochemical changes observed in patients suffering from Leigh's syndrome [220].

A third model, the thymidine phosphorylase (Tp) and uridine phosphorylase (Upp) double knockout mouse model ($Tp^{-/-}Upp\text{-}1^{-/-}$) has been developed for studying mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). Murine UPP-1, unlike human UPP-1, cleaves thymidine as well as uridine; therefore, the full capacity to cleave mouse thymidine is abolished in this mouse model [221–223]. In addition, deficiencies of complex I and IV could be observed in the brain of very old $Tp^{-/-}Upp\text{-}1^{-/-}$ mice [223]. This thymidine-induced dNTP imbalance has been recently demonstrated to be overcome by the metabolic switch into the salvage pathway in acute lymphoblastic leukemia cells, therefore avoiding the lethal replication stress and the subsequent tumor progression. Accordingly, strategies focused on inhibition of this switch have been suggested as metabolic intervention in acute lymphoblastic leukemia [224]. Tp is found to be overexpressed in different tumors (*e.g.* non-small-cell lung cancer and colorectal cancer) and seems to play an important role in angiogenesis, invasion and metastasis of tumors [225,226]. A study by Moghaddam et al. found that Tp has angiogenic properties and, in a MCF7 breast cancer xenograft model, Tp overexpression led to faster tumor growth [227]. In contrast, low Tp expression correlates to the favorable prognosis of gastric cancer treated with chemotherapy [228,229] and promoter methylation has been proposed as a mechanism of its expression down regulation in different cancer cells [230,231].

Mice can be given a trigger (*e.g.* a toxin such as nitrosaminoketone) or have a nuclear gene background such as the $K\text{-}ras^{LA}$ mouse model to develop cancer [232,233]. This in combination with a murine model with a pathological mtDNA background could lead to the creation of a model containing both the mtDNA phenotype and the cancer phenotype to investigate the contribution of both parameters to the phenotype of the tumor.

As every mouse strain has specific differences and it is labor intensive to make genetic mouse models for every human phenotype, another option is to use small animal models specially created for studying the mitochondrial DNA mutation pathology. Animal models, such as *Danio rerio* (zebra fish) [234], *Drosophila melanogaster* (fruit fly) [235] and *Caenorhabditis elegans* (round worm) [236], develop rather quickly and the model can be manipulated by directly adding compounds to the food, medium or water, or irradiating their culture dish. Because the evolutionary distance to humans is larger than for mice, some of these organisms do not contain all the organs affected in humans [237].

5.3. OXPHOS inhibitors in cellular models for therapeutic interventions

Several studies demonstrated that inhibition of glycolysis could be a possible treatment option for cancers with compromised mitochondrial function (see review Pelicano et al. [209]). As these cancers rely more on aerobic glycolysis (Warburg effect), driving the tumors to use OXPHOS instead would lead to sever energetic stress. Cells with mtDNA mutations or with a lack of oxygen (hypoxia) are not able to use alternative energy sources such as fatty acids and amino acids to produce metabolic intermediates which can be used in the Krebs cycle for ATP production through OXPHOS. In these cells inhibition of glycolysis could induce cell death [238,239]. For instance, in an *in vitro* system using human leukemia cells (HL-60) and human lymphoma cells (Raji), cells with a respiration defect were less sensitive to cytostatic drugs like 1-β-D-arabinofuranosylcytosine (ara-C), doxorubicin (Adriamycin), taxol and vincristine [29]. However, lonidamine, 3-bromopyruvate and 2-deoxyglucose are known inhibitors of glycolysis that showed promising results [29,204,205,240]. In addition, partial inhibition of OXPHOS can be achieved by inhibition of the different complexes of the OXPHOS system. The pesticide rotenone is known for its ability to inhibit complex I [219]. It can be used in an experimental set-up at low concentrations, but it is not likely to obtain U.S. Food and Drug Administration (FDA) approval for usage in humans due to toxic effects (*e.g.* irritation of the respiratory tract and apoptosis of erythrocytes) [241,242]. Metformin, an FDA approved drug prescribed to patients with type II diabetes [199], is another inhibitor of complex I [193]. However, as discussed above metformin is also used as an anti-cancer drug by inhibiting the mTOR signaling through activation of AMPK. A more potent drug for complex I inhibition is phenformin [243] a drug related to metformin from the biguanide class that is able to inhibit the development and growth of MCF7 and MDAMV231 tumors in a xenograft mouse model [244]. Additionally, it also affects the mTOR signaling pathway [245]. In xenograft melanomas and in genetically modified mice for melanomas ($BRAF^{V600E}$) combined therapy of phenformin and PLX4720 showed a significant growth reduction whereas treatment with only phenformin or PLX470 resulted in growth inhibition but no tumor regression [245]. However, FDA has redrawn phenformin from the North-American market in 1977, due to its association with fatal lactic acidosis in diabetic patients.

6. Future prospects

There is still a lot of debate about the precise relationship between mtDNA variants, OXPHOS abnormalities and cancer. Are these mtDNA variations driving disease, involved in disease

progression or implicated in treatment response and adaptation to treatment? Or are they merely passenger observations?

6.1. Detecting mtDNA mutations

Various platforms can be used to identify mtDNA mutations in tumor material or other specimens. Using mutation specific restriction digestion or restriction fragment length polymorphism (RFLP) analysis, screening for common mtDNA mutations becomes possible [246]. Denaturing high performance liquid chromatography can be used to determine heteroplasmy mtDNA mutations [247]. Pyrosequencing allows accurate quantification of the heteroplasmy levels of a variation [248]. For detection of mtDNA deletions long-range PCR [249,250] or quantitative real-time PCR based methods [251] and southern blotting [252] could be used. Though, currently there is more desire for high-throughput methods of which some of these are listed below.

Random Mutation Capture (RMC) assay is based on single molecule amplification which facilitates the user in measuring relative values of the spontaneous mutation frequency and mtDNA deletions [253]. Another method is digital deletion detection (3D assay) and is based on the RMC method; with 3D it is also possible to identify rare deletion events [254]. Currently, next generation sequencing (NGS) of mtDNA is the standard for determination of homo- and heteroplasmic variations. Heteroplasmy depends on tissue type; therefore somatic mutations in tumor tissue are mainly heteroplasmic since tumors exist out of different cell types. NGS has an enhanced sensitivity for detecting low levels of heteroplasmy and has as well an increased coverage [255]. The previously used platform, MitoChip, is commonly used for the identification of homoplasmic mutations. Although the detection range is limited, also some heteroplastic variants can be found [256,257]. mtDNA variants detected with MitoChip usually have to be confirmed using conventional Sanger sequencing.

6.2. Identification of potential drug targets

mtDNA research is not only pursuing the identification of variations of mtDNA that are involved in cancer and its treatment, but also the identification of potential drug targets. The ideal treatment would be to repair the appropriate defective mtDNA sequence, although this would be almost impossible for cancer-related somatic mtDNA mutations. Different strategies to achieve this goal have been suggested for severe germline mtDNA mutations. For example, blocking the replication of mutated mtDNA by peptide nucleic acid might result in repopulated cells with unaffected wild-type DNA [258]. However, as interventions specifically for mtDNA are difficult to implement, mitochondria and mtDNA can be targeted at multiple downstream steps of physiological processes. Drugs can interact with mitochondrial permeability, membrane potential, energy supply, antioxidants, ROS production and apoptosis. For instance, influencing the function of B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein, might be used as a target. Bcl-2 is often overexpressed in solid tumors and contributes to resistance to conventional therapies [259]. Possible targets in the cell are the mitochondrial outer membrane proteins (*e.g.* monocarboxylate transporter 1 (MCT1) and mitochondrial pyruvate carriers (MPCs)) or inner membrane proteins (complex I, complex II, complex III, complex IV and complex V), the ETC and the Krebs cycle.

Alternatively, variations in mtDNA can be exploited to tailor patients' therapy. It is unlikely that all patients with different variations causing OXPHOS deficiency will benefit from the same treatment. To illustrate, for tumors with heteroplasmic driver mutations interventions that stimulate mtDNA quantity can result in high mutation levels and should be avoided to prevent that tumors become more therapy resistant. In this case, stimulating mitophagy could be a better option to eliminate the mutation. Altogether, personalized treatments should be carefully evaluated in respect to the pathological process in the tumors of the cancer patients.

7. Conclusions

Over the last decade, the role of mitochondria and, more specifically, mtDNA in cancer has started to be explored. Different signaling processes and ROS production have been implicated in the development and progression of cancer, and these processes can be affected by changes in the mtDNA. However, the exact molecular mechanisms are not yet known. The link between mtDNA and different processes (DNA damage, ROS and apoptosis) and how this plays a role in cancer remains especially vague and is not well defined. In this review, we discussed 3 different types of models (Fig. 3) of nuclear encoded and mitochondrial encoded DNA mutations and how they relate to cancer and cancer therapy. The mutations can have a causative effect, could depended on the multiple hit model or can be just neutral the so-called passenger mutations. Although a combination of these models could occur in the tumor.

Future research should focus on establishing whether these processes are causes or consequences and under which circumstances, and exploring whether they are connected or unrelated to changes in the mtDNA. Upon progressive knowledge about the role of mtDNA, it will be possible to better understand cancer initiation and progression and to improve the prognosis and treatment of patients.

Conflict of interest-statement

There are no conflicts of interest.

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