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*Review Article*

# Mitochondrial Markers for Cancer: Relevance to Diagnosis, Therapy, and Prognosis and General Understanding of Malignant Disease Mechanisms

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Cancer cells display changes that aid them to escape from cell death, sustain their proliferative powers, and shift their metabolism toward glycolytic energy production. Mitochondria are key organelles in many metabolic and biosynthetic pathways, and the adaptation of mitochondrial function has been recognized as crucial to the changes that occur in cancer cells. This paper zooms in on the pathologic evaluation of mitochondrial markers for diagnosing and staging of human cancer and determining the patients' prognoses.

## 1. Introduction

Mitochondria are membrane-enclosed cell organelles that can be found in all human cells, except for the peripheral red blood cells. An eukaryote cell contains around 1000 to 2000 mitochondria, with diameters varying between 0.5 and 1.0 $\mu$m. The mitochondrion is a key venue for cellular metabolism and the powerhouse of the cell. As a consequence, mitochondrial content is influenced by cellular energy demand. Exercise training for instance, increases the amount of mitochondria per fiber and the volume of organelles in skeletal muscle tissue [1, 2].

*1.1. Mitochondrial Metabolic Pathways.* To understand the crucial role played by mitochondria in cancer, it is necessary to fully grasp the extent of their metabolic activity.

*1.1.1. Glycolysis.* Glycolysis is the sequence of cellular reactions that converts glucose into pyruvate, with the concomitant production of a relatively small amount of energy. Glycolysis occurs throughout the cell and is considered the basis of all energy processes. The 2 ATP and 2 NADH molecules it produces can enter the oxidative phosphorylation (OXPHOS) cycle, which then produces larger quantities of ATP.

*1.1.2. Oxidative Phosphorylation.* In the Krebs or OXPHOS cycle, sequential oxidation and reduction reactions take place upon a chain of four multiprotein complexes: (1) complex I is composed of 45 protein subunits and displays NADH dehydrogenase activity. In the process, four hydrogen ions are pumped out of the mitochondrial matrix. (2) Complex II: the succinate dehydrogenase complex catalyzes the oxidation of succinate to fumarate, with concomitant reduction of ubiquinone. (3) In two cycles, complex III or coenzyme Q cytochrome c oxidoreductase reduces coenzyme Q, extracting 4 protons from the mitochondrial matrix. (4) On complex IV or cytochrome c oxidase, electrons are donated one at a time to cytochrome c and passed on to $O_2$, producing 2 $H_2O$ molecules. In addition to the protons utilized in the reduction of $O_2$, there is electron transfer-linked transport of 2 protons from the matrix to the mitochondrial intermembrane space.

The translocation of protons across the inner mitochondrial membrane, established by the respiratory chain, creates an electrochemical gradient ($\Delta\psi$) that is utilized by complex V, the ATP synthase, to generate ATP from ADP and inorganic phosphate. Complex V is composed of a catalytic component on the matrix side (termed F1) and a hydrophobic membrane component (termed F0) that contains the proton

channel, linked by a stalk. The membrane-associated OXPHOS complexes are co-assembled into higher-order supercomplexes within the inner mitochondrial membrane [3].

*1.1.3. Tricarboxylic Acid Cycle.* In the tri-carboxylic acid (TCA) cycle, a series of chemical reactions generates energy through the oxidization of acetate derived from carbohydrates, fatty acids, and proteins. In addition, the cycle provides precursors (amino acids for instance) as well as the reducing agent NADH that is used in numerous biochemical reactions. The series of oxidation reactions that occurs is connected to the electron transport chain and generates ATP. Acetyl CoA is oxidized by the pyruvate dehydrogenase complex, which is located in the mitochondrial matrix.

*1.1.4. Fatty Acid Oxidation.* $\beta$-oxidation is the process by which fatty acids are broken down to generate acetyl CoA, the latter being the entry molecule for the TCA cycle. The $\beta$-oxidation pathway involves 4 enzymes that are present in the mitochondrial matrix and function in a repetitive cycle. The process continues until the original fatty acid molecule is completely degraded to acetyl coA. Acetyl coA can subsequently enter the TCA cycle, undergoing oxidation to $CO_2$. Under conditions of starvation, acetyl coA can alternatively be converted into ketones.

*1.1.5. Urea Cycle.* The urea cycle is a cycle of biochemical reactions that produces urea from ammonia at the cost of 3 ATP molecules. The urea cycle consists of five reactions of which the first two occur in the mitochondrial matrix.

*1.1.6. Gluconeogenesis.* Gluconeogenesis is a metabolic pathway that generates glucose from substrates such as lactate, glycerol, and glucogenic amino acids. It is one of the two main mechanisms to keep blood sugar levels from dropping when glucose intake is low and takes place mainly in the liver. Gluconeogenesis is a pathway consisting of a series of eleven enzyme-catalyzed reactions, starting in the mitochondria with the formation of oxaloacetate by the carboxylation of pyruvate. Oxaloacetate is reduced to malate using NADH, a step required for its transportation out of the mitochondria, and then reoxidized in the cytosol using $NAD^+$. Oxaloacetate is decarboxylated and then phosphorylated to form phosphoenolpyruvate using the enzyme phosphoenolpyruvate carboxykinase. Phosphoenolpyruvate carboxykinase activity is present in both the cytosol and the mitochondria. The enzymes that convert phosphoenolpyruvate to glucose are found in the cytosol. Transport of phosphoenolpyruvate across the mitochondrial membrane is accomplished by dedicated transport proteins.

*1.2. Dual Genetic Origin of Mitochondria.* A unique feature of mitochondria is that these organelles contain their proper genome. The human mitochondrial genome (mtDNA) is a 16 569 base-pair long circular DNA molecule that contains genes for 13 structural subunits of oxidative phosphorylation: (1) 7 subunits of complex I encoded by ND1, ND2, ND3, ND4L, ND4, ND5, and ND6 genes, (2) one complex III subunit encoded by the CYTB gene, (3) 3 complex IV subunits encoded by COXI, COXII, and COXIII genes, and (4) 2 complex V subunits encoded by the ATP6 and ATP8 genes. The mtDNA also contains sequence information for 2 rRNA's and 22 tRNA's essential for translation of mitochondrial proteins. The mtDNA is exclusively maternally inherited and a variety of polymorphisms is present in the human population [4]. Multiple copies are present in each mitochondrion. When mtDNA with different sequences coexists within a single cell or tissue, this is defined as heteroplasmy. Many disease-causing mtDNA mutations have been shown to display heteroplasmy, and the percentages of mutated mtDNA determine the severity of mitochondrial dysfunction [5].

*1.3. Mitochondrial Morphology.* Historically, mitochondria were described as free-floating vesicles in the cell [6]. They consist of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM) that contains convolutions termed cristae, forming the compartments of intermembranous space (IMS) and mitochondrial matrix. The development of cellular imaging techniques, however, has since cast new light onto mitochondrial structure, revealing it as a network of long tubules rather than a collection of individual small vesicles. Mitochondria are highly dynamic organelles that can fuse, divide, and form an interconnected network throughout the cell that stretches from the nucleus to the plasma membrane [7].

The mitochondrial network is shaped through the equilibrium obtained by regulation of the mitochondrial fusion/fission machinery. Two mitofusins, MFN1 and MFN2, are dynamin-related GTPases located to the OMM. They can form homo- and heterodimers and, fueled by GTP, will make the OMM of adjacent mitochondria fuse. MFN2 is also responsible for the tethering between mitochondria and the endoplasmic reticulum. The related dynamin GTPase optic atrophy 1 (OPA-1) is located to the IMM and controls fusion at this level. Other proteins that are involved in mitochondrial fusion are leucine zipper-EF-hand containing transmembrane protein 1 (LETM1), phospholipase D, and prohibitins. Dynamin-related protein 1 (DRP1) is a cytosolic GTPase that, when recruited to the OMM, oligomerizes forming ring-shaped structures and inducing mitochondrial constriction and subsequent fission. Mitochondrial fission factor (MFF) is an adaptor of DRP1 on the OMM, and mitochondrial elongation factor (MIEF) binds DRP1 inhibiting the latter's activity [8]. Fission 1 (FIS1) can interact and sequester MIEF function. Other proteins involved in mitochondrial fission are endophilin B1, a fatty acyl transferase that acts downstream of DRP1, mitochondrial 18 kDa protein (MTP18) [9], mitofusin binding protein (MIB), and ganglioside-induced differentiation associated protein 1 (GDAP1) [10].

Mitochondrial morphology and OXPHOS function are strongly associated. Impaired OXPHOS function leads to mitochondrial network fragmentation. Structural defects in complex V lead to severe morphological alterations to

the mitochondria [11], and the oligomeric state of F1F0 ATPsynthase determines cristae morphology [12].

## 2. Metabolic Cancer Markers

Cancer is strongly associated with perturbations in cellular metabolism, with a characteristic metabolic shift toward aerobic glycolysis in transformed cells.

*2.1. Markers of Aerobic Glycolysis.* In cancer cells, pyruvate is abundantly transformed to lactate by aerobic glycolysis [13], and the overexpression of glycolysis genes is the general rule [14]. Physical association between glycolytic enzymes and the mitochondrion has been firmly established using the yeast cell model [15].

Enolases catalyze the conversion of 2-phosphoglycerate to phosphoenolpyruvate. These enzymes are classically regarded as cytosolic, yet they tightly associate with the mitochondrial surface [16]. In human tissues, three genetic loci, termed $\alpha$, $\beta$, and $\gamma$, encode the different enolase isoforms. Enolase 1 is present in almost all adult tissues, enolase 2 is found in neuronal and neuroendocrine tissues, and enolase 3 is found mainly in muscle. They form hetero- and homodimers that dehydrate 2-phosphoglycerate in the glycolysis or the reverse reaction in the gluconeogenesis. The enzyme is upregulated under stress conditions, via activation of hypoxia-inducible factor-1 (HIF-1). Overexpression of $\alpha$-enolase is associated with tumor development and represents a potential diagnostic and prognostic marker [17]. In breast cancer, $\alpha$-enolase gene expression correlates with tumor size and shorter disease-free interval [18]. In head and neck [19] and non-small-cell lung cancer [20], higher expression of $\alpha$-enolase is associated with poorer clinical outcome in patients.

Pyruvate kinase (PK) is a rate-limiting glycolytic enzyme that converts phosphoenolpyruvate into pyruvate with the generation of one ATP molecule. Its PKM1 and PKM2 isoforms are encoded by the same gene and are generated by alternative splicing. PKM1 is found mainly in normal cells, whereas PKM2 is an embryonic isoform expressed in cancer cells [21]. Both plasma and fecal PKM2 can be used as a marker for gastrointestinal cancer, showing good diagnostic accuracy [22]. In addition, PKM2 plasma levels appear predictive for disease severity and outcome. Elevated levels have been found to associate with aggressive breast carcinomas [23]. In breast cancers lacking caveolin-1, PKM2 immunostaining is confined to the stromal cells and sometimes the extracellular matrix [24].

Pyruvate, the end product of the glycolysis, enters the mitochondria where it is transformed to acetyl CoA, producing NADH and $CO_2$. Acetyl CoA can subsequently enter the TCA cycle. This process is regulated by the pyruvate dehydrogenase complex (PDH), which is composed of the E2 core bound to the E1 and E3 components. PDH activity is controlled by pyruvate dehydrogenase kinases (PDK) 1 to 4, which phosphorylate and suppress the E1 subunit [25]. PDH levels are dramatically decreased in hepatomas [26] and skin carcinomas [27]. While normal lung tissue stains strongly for PDH and PDK1, a large proportion of cancer cells in patients with non-small-cell lung carcinomas show diminished expression. The lack of PDH expression was shown to be associated with HIF-1$\alpha$ stabilization [28].

*2.2. Oxidative Phosphorylation Markers.* Warburg hypothesized as early as the thirties of the last century that the increased rates of aerobic glycolysis he observed in tumor cells, might be due to their impaired respiratory capacities [13]. Reduced respiration is indeed associated with cancer, however, the OXPHOS is not always compromised as a whole. For instance, the OXPHOS enzyme activities and protein levels are not uniformly decreased in breast cancer cell lines. The spectrum of decrease is MCF7 (complexes II, III, and V), T47D (complexes I, III), SKBr3 (complexes III, IV, and V), and MDA-MB-231 (complexes I, III, IV, and V) [29]. Interestingly, the most aggressive breast cancer line (MDA-MB-231) displays the broadest OXPHOS defect. In support, a correlation was shown between low overall OXPHOS activity and tumor aggressiveness in renal carcinoma [30].

*2.2.1. Complex I.* Complex I loss of function has been shown many times in cancer tissues. In renal oncocytomas, complex I activity is reduced by 50% [31] to 65% [32], with blue native PAGE immunoblot revealing the lack of assembled complex I. All 17 oncocytic thyroid adenomas tested display greatly reduced or absent complex I subunit NDUFS4 immunoreactivity, compared to expression levels of complex II, III, IV, V, and porin, which are higher in the tumor cells than in the adjacent normal tissue [33].

*2.2.2. Complex II.* A reduction of complex II activity is associated with human cancer. In a series of 31 renal carcinomas, complex II activities are 6-fold lower than in controls [34]. Complex II activity was found decreased in breast cancer cell lines [29].

*2.2.3. Complex III.* In addition to reduced activities [29], complex III activation has also been reported in cancer. Complex III subunits UQCRFS1 and UQCRH are overexpressed in a variety of tumors. The complex III subunits Rieske iron-sulfur protein and Hinge protein are encoded by the UQCRFS1 and UQCRH gene, respectively. Expression studies reveal that transcripts of UQCRFS1 and UQCRH are increased in breast tumors compared to normal breast tissue. Immunohistochemical staining for UQCRFS1 shows higher expression scores in breast cancer compared to benign breast tissue [29]. Also, UQCRFS1 gene amplification has been detected in breast tumors [35].

*2.2.4. Complex IV.* Both compromised and induced complex IV function have also been observed in cancer. COXI, COXII, and COXIV, mRNA levels are increased in hepatoma compared with normal liver [36]. A proteomic study shows a 2-fold upregulation of COXII protein in breast cancer cells [37]. On the other hand, complex IV activity is 5-fold decreased in renal carcinomas compared with controls [34]. Also, COXIII mRNA levels are lower in colonic carcinoma versus normal mucosa samples [38]. Differential complex

IV activity could be relevant to therapeutic outcome. Adriamycin-resistant leukemia cells, for instance, contain alterations in complex IV subunits and decreased activity of the complex [39].

*2.2.5. Complex V.* The relationship between complex V function and cancer appears to be complicated. Complex V activity was shown to be reduced in high grade but normal in low grade renal carcinomas [30], but another report did not find a tumor grade correlation [34]. A proteomic study shows a 2-fold increase of the ATP synthase f chain in breast cancer cells [37].

*2.3. Other Metabolic Markers.* Being such a crucial venue of metabolism, many other mitochondrial metabolic factors are continuously surfacing as possible biomarkers for cancer. A selection is discussed hereunder.

*2.3.1. Hydratases.* Fumarate hydratase (FH) is a nuclear-encoded mitochondrial enzyme that takes part in the TCA cycle, catalyzing the formation of L-malate from fumarate. Mutations in the FH gene are associated with an autosomal dominant tumor syndrome causing uterine, skin, and kidney cancer [40] and hereditary leiomyomatosis and renal cell cancer [41]. Mitochondrial enoyl-coA hydratase 1 catalyzes the second step of the $\beta$-oxidation. Its expression is 10-fold increased in a human gastric cancer cell line [42]. NAD-dependent bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD) regulates the biosynthesis of tetrahydrofolate, providing precursors for nucleotides and methylation reactions. There is a strong association between MTHFD genetic variants and gastric [43] and bladder [44] cancer risk. MTHFD2 protein content is 3-fold decreased in breast cancer cell lines [37].

*2.3.2. Dehydrogenases.* Isocitrate dehydrogenases (IDH) are important players in the exchange of metabolites within the cell, and two IDH isoforms can be found within the mitochondrion. NADP-dependent IDH2 has a role in shuttling of electrons between the mitochondrion and the cytosol. The mitochondrial matrix enzyme NAD-dependent IDH3 is involved in the TCA cycle. Breast cancer cell lines display high levels of IDH2, and expression is positively associated with overall survival in breast cancer patients [45], possibly due to enhanced reactive oxygen species (ROS) protection.

*2.3.3. Oxidases.* Coproporphyrinogen III oxidase (HemN), an enzyme required for heme synthesis, is present in the IMM. It's expression is increased in adriamycin-resistant breast cancer cells [46].

## 3. Genetic Cancer Markers

Due to their genetic complexity, both the nuclear (nuDNA) and the mitochondrial genome are suitable for investigation in regard to carcinogenesis.

*3.1. mtDNA Alterations in Cancer.* The mtDNA is particularly susceptible to mutations due to its proximity to ROS generation and the relatively inefficient DNA repair system the mitochondrion possesses [47]. The frequency of mtDNA mutations in cancer cells is 10-fold higher than nuDNA mutations [48], and many alterations to the mtDNA that can be detected in tumor cells potentially alter mitochondrial function. Also, mtDNA alterations can often already be detected in the premalignant stage. mtDNA is abundant and readily detectable in blood, urine, and saliva samples, making it an attractive subject for diagnostic investigations.

*3.1.1. Alterations in ND Genes.* The mtDNA encodes for seven complex I subunits (ND1–ND6 and ND4L) and alterations in cancer cells are plenty. Somatic mutations of ND genes are associated with thyroid oncocytoma [49]. A study of 10 colorectal cancer cell lines reveals 1 ND1, 1 ND4L, and 1 ND5 sequence alterations [50]. Of 45 colorectal carcinomas tested, 3 contain ND1 and 3 ND5 gene mutations [51]. Of 18 primary oral squamous cell carcinomas tested, 3 contain ND2, 1 ND3, and 1 ND4 somatic mutations [52]. In a series of 15 patients with renal oncocytoma and compromised complex I function, 4 patients harbor ND1, 3 patients ND4, and 3 patients ND5 sequence alterations. The alterations are most often single-nucleotide insertions (6/10) or deletions (3/10) [32]. In 31 urothelial cell carcinoma patients, tumor cells contain ND1 ($n = 2$), ND2 ($n = 3$), ND4 ($n = 2$), and ND6 ($n = 1$) frequency variations [53]. In a study examining 38 bladder tumors, 73% contain at least one base-pair alteration in the ND1 gene [54].

*3.1.2. Alterations in the CYTB Gene.* The mtDNA contains one gene that encodes a structural unit of complex III, namely, cytochrome b (CYTB). In 10 colorectal cancer cell lines, 2 CYTB sequence alterations could be found [50]. Tumor cells from 3 of 31 patients with urothelial cell carcinoma contain CYTB alterations [53]. In 81% of bladder carcinomas, at least one base-pair substitution is present in the CYTB gene [54]. In a human bladder cancer model, CYTB alteration is shown to generate higher levels of ROS and lead to increased tumor growth [55].

*3.1.3. Alterations in COX Genes.* The mtDNA encodes three structural components of complex IV: COXI, COXII, and COXIII. In 10 colorectal cancer cell lines, 1 COXI, 1 COXII, and 1 COXIII sequence alterations are present [50]. Of 18 oral squamous cell carcinomas, 1 contains a COXIII somatic mutation [52]. In 31 urothelial cell carcinoma patients, tumor cells are found to contain altered sequence in COXI ($n = 2$) and COXIII ($n = 2$) genes [53]. 11% of prostate cancer patients harbor COXI mutations in conserved sequences compared to less than 2% in healthy controls [56].

*3.1.4. Alterations in ATPase Genes.* The mtDNA encodes two structural units of complex V, being ATPase6 and ATPase8. ATPase6 is central to the proton channel of the ATPase [57]. Mutations in the ATP6 gene are present in 24 of

39 patients with osteosarcoma [58]. In 1 of 31 urothelial cell carcinoma patients, an ATP6 sequence variant is found [53]. 71% of bladder tumors tested contain at least one base-pair substitution in the ATP6 gene [54]. In a series of 102 epithelial ovarian tumors, 12 contain ATP6 and 21 contain ATP8 sequence variations [59]. ATP6 mutant cybrids of prostate cancer cells display enhanced ROS generation accompanied by increased tumorigenecity [56].

*3.1.5. Alterations in tRNA Genes.* The mtDNA encodes 22 transfer RNA (tRNA) molecules necessary for mitochondrial protein translation. tRNA is the adaptor used to bridge the four-letter genetic code in messenger RNA (mRNA) to the twenty-letter code of amino acids present in proteins. In one of the 15 tested patients with renal oncocytoma, the A3243G mutation was shown in the mtDNA tRNALeu gene [32]. In urothelial cell carcinoma patients, tumor cells contain tRNA sequence variants in 4 of 31 patients tested [53].

*3.1.6. Alterations in rRNA Sequences.* The mtDNA encodes both a small (12S) and a large (16S) ribosomal RNA (rRNA) gene. In 10 colorectal cancer cell lines, 3 16S rRNA and 1 12S rRNA sequence alteration are found [50]. In 31 urothelial cell carcinoma patients, 5 tumors contain rRNA somatic gene variations: 4/31 in the 16S and 1/31 in 12S rRNA [53].

*3.1.7. mtDNA Displacement-Loop Alterations.* Replication of mtDNA starts in the displacement loop (D-loop) region located between nucleotides 16024 and 16576. mtDNA replication involves DNA polymerase $\gamma$ (POLG) and transcription factor A mitochondrial (TFAM), the latter being the key transcription factor regulating mtDNA copy numbers [60]. Mutations in the D-loop can therefore lead to decreased mtDNA copy numbers and subsequent OXPHOS dysfunction and increased ROS. Intriguingly, the most frequent mtDNA alterations in human cancers are in fact located in the D-loop region [61]. mtDNA alterations within the D-loop could be detected in 77% [52] or 64% [62] of oral squamous cell carcinomas and 44% of colorectal carcinomas ($n = 45$) [51]. Of 9 renal cell tumors tested, 3 contain D-loop sequence changes [63]. In breast cancer patients, the occurrence of D-loop mutations is associated with an older onset age [64], suggesting that age-related DNA damage contributes to the disease mechanism.

A homopolymeric C-stretch within the D-loop, between nucleotides 303 and 315 termed the 310 microsatellite sequence, is a relatively conserved region. It includes a replication origin for the mtDNA heavy-strand, and sequence variations in D310 are thought to affect mtDNA replication [65]. Many reports have shown D310 sequence alterations in human cancers. In 34% of rectal carcinomas ($n = 38$) and 38% of sigmoid carcinomas ($n = 25$), D310 sequence alterations could be detected [66]. In breast cancer patients, 68% of ductal carcinomas in situ ($n = 23$) and 71% of invasive ductal carcinomas ($n = 26$) harbor D310 sequence alterations [67]. In 11 of 18 breast cancers, mtDNA mutations could be detected, of which 42% are D310 alterations [68]. 38% of gallbladder carcinomas ($n = 123$) contain D310 alterations [69] and 62% of multicentric hepatocellular carcinomas [70].

D310 mutation detection could represent a potential early marker for premalignant human disease. Histologically normal breast epithelial cells, adjacent to invasive ductal carcinomas that carry D310 mutations, already display D310 alterations [67]. D310 alterations are also present in normal-appearing (46%) and dysplastic (57%) gallbladder epithelia accompanying gallbladder carcinomas [69]. It seems that D310 alterations are an early event in malignity, and detection could serve as an early detection strategy. In addition, D-loop alterations have been put forward as a possible prognostic marker for therapeutic outcome. Three-year survival rates of patients with stage III colorectal cancer are 70% without and 47% with D-loop mutations. In combination with fluorouracil as adjuvant chemotherapy, the difference is even more pronounced, being 78% versus 45%, respectively [71].

*3.1.8. Alterations in mtDNA Abundance.* Each mitochondrion contains multiple copies of the mtDNA, and this copy number changes in reponse to energy demands. Either increased or reduced mtDNA content has been reported in cancer cells [72, 73]. mtDNA content is elevated in head and neck [74] and esophageal [75] squamous cell carcinoma and increased in malignant compared to premalignant lesions [76]. Also, mtDNA levels are increased in papillary thyroid carcinomas [77], endometrial cancer [78], and oncocytomas [79]. Quantitative real-time PCR amplifying mtDNA-encoded COXI and nuDNA-encoded $\beta$-actin revealed a significant increase of mtDNA content in the tumors of 76% of urothelial cell carcinoma patients compared to the matched lymphocytes [53]. Nonetheless, increased mtDNA content does not appear to be firmly associated with carcinogenesis. In prostate cancer, it has been observed that cells with an amplified mtDNA copy number coexist with cells displaying a diminished or depleted copy number [80]. In a study that quantified mtDNA content using RT-PCR with ND1 primers in 31 gastric cancers, 23% of patients displayed a significant increase, but 55% displayed a significant decrease of mtDNA content [81]. Moreover, the relative ratio of mtDNA over nuclear 18S rDNA, as determined by Southern blot, is 3-fold lower in renal carcinoma tissues than in controls [34]. Also, mtDNA content was found reduced in the majority of breast tumors, with mean mtDNA content, as determined by quantitative RT-PCR using ND1 gene primers, being significantly lower than that of the adjacent nontumorous tissue [64]. In lung cancer, mtDNA depletion has been associated with progressive disease [82].

Circulating mitochondrial nucleic acids could be a prognostic marker for cancer. Patients with prostate, head and neck, kidney and colorectal cancer, and blood mitochondrial RNA concentrations above the 95th percentile found in healthy subjects demonstrate decreased survival after a two-year follow-up period [83]. Also, mtDNA content may be a potential biomarker for prediction of the response to chemotherapy. Breast cancer patients with lower copy number of mtDNA have better disease-free survival than patients

with high mtDNA content, when treated with anthracycline after surgery [84]. Possibly, low mtDNA content enhances ROS and sensitizes the cancer cells to anticancer agents.

*3.2. nuDNA Alterations Affecting Mitochondrial Function in Cancer.* The repertoire of nuclear-encoded genes involved in mitochondrial function continues to expand [85]. They include, but are not limited to, mutations in genes encoding structural OXPHOS subunits, OXPHOS assembly factors, and components of the mitochondrial protein translation machinery. nuDNA alterations have not been studied as extensively as mtDNA alterations, but are more and more recognized in cancer. SNP-analysis of the mitochondrial biogenesis pathway (25 genes), for instance, reveals a pathway-wide association with a risk of developing epithelial ovarian carcinoma [86]. Mitochondrial biogenesis is crucial because of the constant battle of the organelle against ROS and DNA damage.

*3.2.1. Complex I.* The nuDNA encodes several structural complex I subunits, as well as complex-specific assembly factors. Single-nucleotide polymorphisms in NDUFA9, NDUFS2, and NDUFB9 genes are associated with prostate [87] and NDUFB10, NDUFA11, and NDUFA12 with epithelial ovarian [86] cancer risk.

*3.2.2. Complex II.* Complex II contains four nuclear-encoded structural subunits designated SDHA to D. The subunits are imported into the mitochondria, where they are modified, folded, and assembled to functional complex II. The genes encoding complex II subunits behave as tumor suppressors. Hereditary paraganglioma has been linked to SDHA [88], SDHB [89], SDHC [90], and SDHD [91] gene mutations. SDHB mutations have also been shown in pheochromocytoma [92] and renal cell carcinomas [93]. Also, mutations in SDH assembly factor 2 (SDHAF2) cause head and neck paragangliomas [94]. SDHAF2 is essential for the incorporation of FAD cofactor in the SDHA subunit. It has been suggested that loss of complex II function induces HIF, which promotes glycolysis and angiogenesis and thus tumor growth and survival.

*3.2.3. Complex III.* Of the 11 structural subunits that make up complex III, 10 are encoded by nuclear genes. Genetic amplification of the UQCRFS1 gene has been reported in leukemia [95], ovarian [96], and breast carcinoma [35].

*3.2.4. Complex IV.* In addition to structural subunits, the nuclear genome encodes many assembly factors necessary for complex IV formation. Single-nucleotide polymorphisms in the COX7A2 gene have been linked to an increased risk of developing prostate cancer [97], while COX7A1 and COX8C alterations have been shown to associate with epithelial ovarian cancer risk [86].

*3.2.5. DNA Polymerase γ.* POLG is responsible for the replication of the mtDNA, and mutations cause multiple large scale deletions and mtDNA depletion, leading to compromised OXPHOS functioning. Mutations in the POLG gene have been detected in breast cancers and are associated with mtDNA depletion in cancer cells [98]. The POLG gene contains a polymorphic CAG repeat of a dominant length of 10. Men with homozygous deviations from this length have an increased risk of developing seminomas [99]. The percentage of the homozygous non-10 allele is significantly higher in cancer patients than in the general population, yet is only present in low frequencies.

## 4. Mitochondrial Stress Markers

*4.1. ROS Damage Control.* Mitochondrial respiration inherently produces ROS, and OXPHOS dysfunction further increases its generation. In cancer cells, a consistent increase of ROS can be observed. NADPH oxidase 1, a major source of ROS in the cells, predominantly localizes to the mitochondria and is highly expressed in breast (86%) and ovarian (71%) tumors [100]. To counter the damaging effect of ROS, cells contain a multilayered system of antioxidant defences executed by three types of enzymes: superoxide dismutases (SOD), peroxidases (POD), and catalases (CAT).

MnSOD is constitutively present in the mitochondrial matrix, but expression can be further induced by hypoxia. MnSOD levels are low or below the detection limit in healthy human pleural mesothelium, but highly increased in tumor biopsies of malignant mesothelioma [101]. MnSOD levels are increased in ovarian cancer [102]. In breast cancer patients, strong MnSOD staining can be observed in neoplastic cells, moderate-to-strong staining in adjacent hyperplastic ducts and weak-to-moderate staining in normal epithelium [103]. A histochemical study on the other hand reveals lower expression in breast cancer cells compared to the adjacent normal epithelia [104]. MnSOD expression levels have been put forward as a prognostic factor for gliobastoma [105, 106].

Glutathione POD is present in different cellular compartments, including the mitochondria. A polymorphism variant of the glutathione POD1 gene is associated with lung cancer risk [107]. In women with papillary serous ovarian cancer, serum glutathione POD3 levels are significantly lower than in controls, and the decrease is stage-dependent [108]. In endometrial adenocarcinoma on the other hand, glutathione POD3 is uniformly downregulated regardless of tumor grade or histopathological subtype, which would imply that it is an early event in tumorigenesis [109].

Mitochondrial expression of CAT has been shown, and in vitro studies have indicated that decreased levels of CAT correlate with invasive and migratory capacities of cell lines and resistance to chemotherapeutic drugs [110]. Gene therapy that results in mitochondrial CAT overexpression renders transgenic mice less susceptible to metastatic breast cancer [111]. Thus, increasing the antioxidant capacity of the mitochondrial compartment could be a rational therapeutic approach.

Prolonged hypoxia and failing ROS neutralization activates HIF proteins that in turn increase the expression of the oncogenes RAS, MYC, and p53. MYC stimulates

mitochondrial biogenesis, which will increase ROS further. Induction of p53 induces mitochondrial apoptotic pathways, and mitochondrial p53 physically and functionally interacts with the mtDNA and POLG. p53 provides exonuclease activity enabling error-repair, and thus functions as a guardian of the mitochondrial genome [112]. Loss of p53 is a marker for advanced progression in prostate cancer [113].

*4.2. Apoptosis Markers.* Mitochondria are regulators of apoptotic processes. Oxidative stress accompanied by calcium overload, ATP depletion, and elevated phosphate levels induce mitochondrial permeability transition (MPT) with formation of nonspecific MPT pores (MPTP). Three proteins are key structural components of the MPTP: adenine nucleotide translocase (ANT) in the IMM, cyclophilin D in the matrix, and the voltage-dependent anion channel (VDAC) in the OMM [114]. Opening of MPTP causes mitochondria to become permeable to all solutes up to a molecular mass of 1500 Da. Pore opening results in mitochondrial dysfunction, through uncoupling of OXPHOS from ATP hydrolysis. Osmotic forces make the mitochondria swell and the OMM rupture, releasing apoptogenic proteins into the cytosol. Released proteins include cytochrome c, apoptosis inducing factor (AIF), and endonuclease and second mitochondria-derived activator of caspases/direct IAP-associated binding protein with low pI (Smac/DIABOLO), which, in conjunction with apoptosis protease activating factor (APEF-1), activate caspase 9. The ultimate result is DNA fragmentation and cell death. Additionally, mitochondrial membrane permeability is further regulated by Bcl-2 proteins, including the proapoptotic Bax, Bak, Bid, and Bad [115]. Bax is a known tumor suppressor protein [116]. The MPTP, that contains both repressors and inducers of apoptosis, has been ascribed a target for anticancer drugs [117].

Repression of apoptosis is a hallmark of tumorigenesis [118]. Many immunohistochemical markers of apoptosis have become available to the pathologist, including cleaved caspase 3 and AIF, and have become indispensable for tissue evaluation. Cyclophilin D was found significantly increased in tumors of the breast, uterus, and ovarium but not in kidney, gastro-intestinal, and lung cancers [119]. Proapoptotic Bcl-2 interacting protein 3-like (BNIP3L) immunostaining is elevated in the stroma of human breast cancers that lack caveolin-1 [120]. Cofilin, an inducer of the release of cytochrome c, is a small cytoskeletal protein that depolymerizes actin filaments. Apoptosis is mediated through mitochondrial shuttling of actin- and cofilin-interacting protein 1 [121]. Gene associated with retinoid-interferon-induced mortality-19 (GRIM-19) is a cell-death regulator that works through inhibition of signal transducer and activator of transcription 3 (STAT3). Loss or dysregulation of GRIM-19 provides growth advantage to cancer cells. Intriguingly, it has been demonstrated that GRIM-19 localizes to OXPHOS complex I [122], and that its dysfunction results in complex I disassembly and disruption of electron transfer [123]. GRIM-19 mRNA and protein expression are severely reduced in primary renal cell [124], colorectal [125], and cervical [126] carcinoma. In normal prostatic tissues, the epithelium stains intensely, while in prostatic adenocarcinomas, GRIM-19 staining is focally lost [124]. In lung cancer, GRIM-19 expression is reduced and translocates to the nucleus. GRIM-19 downregulation in non-small-cell lung carcinoma correlates with advanced disease stage [127]. Delivery of GRIM-19 has been put forward as a plausible therapeutic approach [128].

*4.3. Molecular Chaperones.* Mitochondrial molecular chaperones play important roles in protein transport, protein complex assembly, refolding of misfolded proteins, and, when all else fails, sentencing proteins to degradation by the proteasome. They represent a heterogeneous group of proteins subdivided into families according to their molecular weight: large HSP, HSP90, HSP70, HSP60, and HSP40 and small HSP families. In cancer cells, the network of molecular chaperones appears altered compared to normal cells.

*4.3.1. HSP90 Family.* Molecular chaperones of the HSP90 gene family are considered indispensable regulators of protein folding. The HSP90 proteins HSP86 and HSP84 are mainly found in the cytoplasm of normal cells, but in contrast are found to accumulate in the mitochondrial matrix and IMS of tumor cell mitochondria [129]. Bladder specimens stain strongly for HSP90 in all cells, but in primary bladder transitional cell carcinoma, loss of expression is observed in 24% of tumors. In superficial bladder carcinoma, loss of HSP90 expression is associated with the risk of developing an infiltrating recurrence [130]. TNF receptor-associated protein 1 (TRAP1) is a member of the HSP90 family that is considered mostly mitochondrial. In vivo studies in the rat have shown that TRAP1 protects against hypoxia, by reducing generation of ROS, improving mitochondrial complex IV activity, and preserving ATP levels [131]. TRAP1 expression is induced in tumor cells. As revealed by immunohistochemistry, TRAP1 staining appears intense in pancreas, breast, colon, and lung adenocarcinomas, while normal matched epithelia stain weakly [132].

Accumulating evidence points to a protective role against apoptosis for the HSP90 family. TRAP1 and HSP90 are involved in the mitochondrial pathway that antagonizes the proapoptotic activity of cyclophilin D [132]. This interaction occurs in a multichaperone complex that is selectively assembled in tumor cells and is not present in normal mitochondria [133]. Also, TRAP1 has been shown to directly interact with members of the MPTP, inhibiting its opening and the subsequent release of cytochrome c [134].

*4.3.2. HSP70 Family.* The human HSP70 family encompasses a member present in the mitochondrial matrix and two cytosolic members: HSP73 is largely constitutive, while HSP72 can be induced by cellular stress. HSP70 levels are increased in cancer cells, including osteosarcoma [135] and renal cell carcinoma [136]. In urothelial carcinoma of the bladder, HSP70 immunostaining is significantly linked with tumor grade [137]. This upregulation increases the tumorigenic potential through the chaperone's anti-apoptotic properties.

*4.3.3. HSP60 Family.* In human cells, HSP60 is largely but not exclusively mitochondrial and localizes to the mitochondrial matrix and the OMM. HSP60 is constitutively expressed, but its expression increases in response to mitochondrial damage and mtDNA depletion [138]. The chaperone appears to have both prosurvival and proapoptotic functions, and the relationship between HSP60 and tumor prognosis and clinical outcome remains unclear. Upregulation has been described in several cancers including colorectal [139] and cervical [140] carcinomas. Western blot analysis revealed significantly higher HSP60 protein levels in cervical cancer compared to normal tissue, but semiquantitative RT-PCR showed no difference in expression of the corresponding mRNA [141]. HSP60 protein is overexpressed in prostate cancers compared with normal prostate epithelium and hyperplasia. Immunostaining is relatively homogeneous, coarsely granular and confined almost exclusively to neoplastic epithelial cells. Western blotting analyses show that high Gleason score tumors have significantly higher levels of HSP60 protein than tumors of low to moderate Gleason score [142]. In oesophageal squamous cell carcinoma [143] and ovarian cancer [144] on the other hand, positive HSP60 expression correlates with good prognosis.

*4.3.4. Small HSP Family.* HSP27 is mainly cytosolic, but a small fraction localizes to the mitochondria. HSP27 expression may be a useful prognostic marker of poor survival in human cancers. HSP27 is upregulated in the serum of breast cancer [145] and ovarian cancer [146] patients and correlates with poor clinical outcome. mRNA and protein levels are significantly higher in epithelial ovarian cancer with peritoneal metastasis than without [147]. A clinical evaluation of breast cancer and melanoma patients correlates expression of HSP27 with tumor aggressiveness and decreased survival [148]. HSP10 staining is present in the mitochondria and correlates with high grading of urothelial bladder carcinoma [137].

## 5. Mitochondrial Membrane Markers

*5.1. Mitochondrial Depletion and Proliferation Markers.* Mitochondrial membrane proteins can serve to quantify the mitochondrial load of tumors, and porin (VDAC) is often used as a marker for mitochondrial abundance. Quantified to porin content measured by Western blot, no obvious mitochondrial reduction was seen in renal carcinoma compared to control kidney. However, citrate synthase activity, another frequently used marker for mitochondrial load, was 2-fold lower in renal carcinoma tissues [34]. Oncocytomas show a 5-fold increase of citrate synthase activity and an increase of the number of mitochondria [30].

*5.2. Mitochondrial Import Channels.* Mitochondrial function is profoundly dependent on the import of cytosolic proteins. Complex protein structures form channels that allow preproteins to translocate from the cytosol to the mitochondrial matrix. The proteins that constitute these channels are the translocase of the outer mitochondrial membrane (TOMM) and translocase of the inner mitochondrial membrane (TIMM) proteins [149]. TOMM20 selectively stains metastatic breast cancer cells and is largely absent from the adjacent metastatic lymph node stroma [150]. Proteomic analysis shows a 5-fold increase of TIMM17A protein levels in breast cancer cells, and the increase could be validated by western blot and immunohistochemistry. The latter shows strong staining in ductal carcinoma in situ and invasive ductal carcinoma of the breast, while the adjacent normal epithelia and stromal cells are negative. All normal breast tissues are TIMM17A negative. Quantitative RT-PCR confirms significantly higher levels in invasive carcinoma compared to normal breast tissue [151]. A recent study confirms upregulation of TIMM17A mRNA, when normalized against the housekeeping gene cytokeratin 19 [152]. Both studies have shown TIMM17A expression to be associated with poorer disease-free and overall survival. Thus, TIMM17A offers a promising diagnostic as well as prognostic marker for breast cancer patients.

*5.3. Other Mitochondrial Membrane Proteins.* Translocator protein (TSPO), also known as peripheral-type benzodiazepine receptor, is a well-conserved protein located at the OMM-IMM contact sites and is closely associated with VDAC and ANT. TSPO has been shown to participate in apoptotic processes but has been ascribed both anti- and proapoptotic properties. Administering TSPO ligands increases the antineoplastic properties of cytostatic drugs in human hepatocellular cancer cells [153]. Overexpression of TSPO is associated with aggressive tumor subtypes in breast, colorectal, and prostate carcinomas and correlates with advancing stages of malignancy. Metastatic breast and colorectal adenocarcinomas manifest increased TSPO expression relative to their primary malignancies. In contrast, adrenocortical hepatomas display decreased TSPO levels compared to the strongly staining normal hepatocytes [154].

## 6. Mitochondrial Morphology Markers

The classical way to study mitochondrial morphology is transmission electron microscopy. Using such techniques, changes in mitochondrial morphology have been shown to accompany alterations in mitochondrial function. Increasing evidence shows the involvement of mitochondrial dynamics in cancer development, but the structural mitochondrial alterations appear heterogeneous and nonspecific to neoplasias. In addition, in all instances where mitochondrial pleomorphism could be observed, normal mitochondria were equally present.

Mitochondrial morphological changes associated with cancer are varied and include: (1) increased mitochondrial mass as seen in breast carcinomas [49] and pancreatic tumors [155] (2) reduced numbers and degradation to mitochondrial remnants in hepatocellular carcinoma [156] (3) swollen mitochondria as in clear cell carcinoma [157] and undifferentiated retinoblastoma [158] (4) elongated tubular

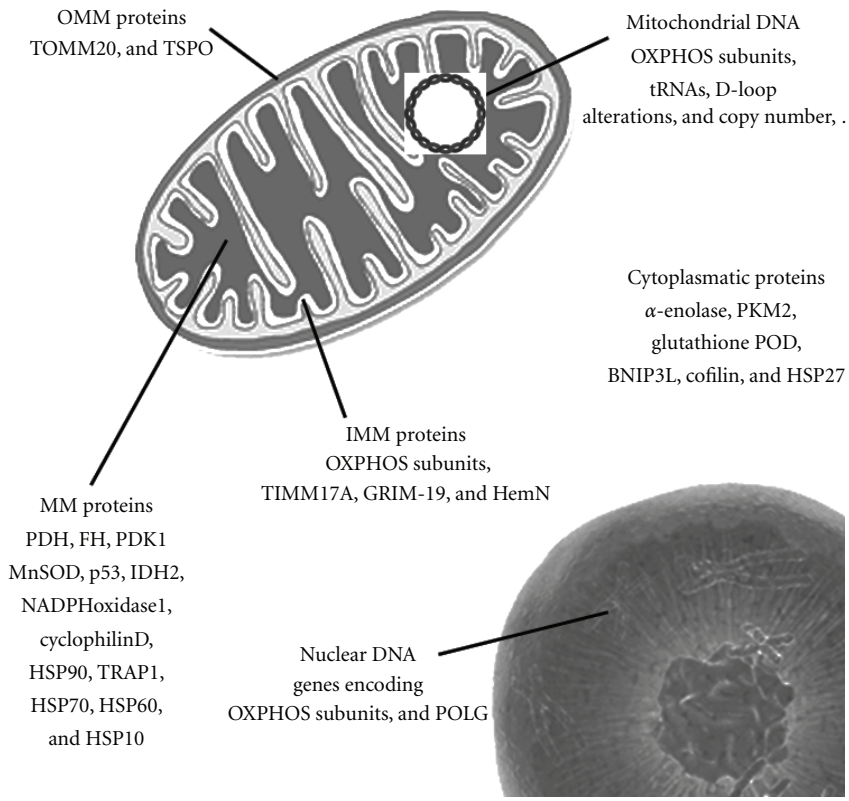


Figure 1: Mitochondrial diagnostic and prognostic biomarkers and their dominant intracellular location. Bcl-2 interacting protein-3 like (BNIP3L), fumarate hydratase (FH), gene associated with retinoid-interferon-induced mortality (GRIM), coproporphyrinogen III oxidase (HemN), heat shock protein (HSP), inner mitochondrial membrane (IMM), isocitrate dehydrogenase (IDH), mitochondrial matrix (MM), outer mitochondrial membrane (OMM), oxidative phosphorylation (OXPHOS), pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase (PDK), pyruvate kinase type M (PKM), peroxidase (POD), polymerase $\gamma$ (POLG), translocase of the inner mitochondrial membrane (TIMM), translocase of the outer mitochondrial membrane (TOMM), tumor necrosis factor-associated protein (TRAP), translocator protein (TSPO).

mitochondria in aldosterone-producing adrenal cortical adenoma [159] and (5) aberrations to the cristae varying from reduced amounts in osteosarcoma cell lines [160] to densely packed villiform and lamelliform cristae in Warthin's tumor [161].

When comparing a human gastric cancer cell line with normal rat gastric mucosal cells, electron microscopy reveals a significant decrease in both the numbers per cell and the size of mitochondria [162]. An ultrastructural study of renal cancer observes most severe mitochondrial aberrations in eosinophilic clear cell renal cell carcinomas, where most mitochondria appear swollen with loose matrix and short attenuated cristae [163]. Percentages of mitochondria with damaged cristae are 72% in a primary mammary carcinoma cell culture, compared to 20% in a control mammary epithelial cell line [164].

Several cristae remodeling markers have been described and include Mitofilin [165]. Many mitochondrial fusion/fission factors are involved in maintaining mitochondrial integrity. tBid binds to cardiolipin, a negatively charged phospholipid present in the IMM [166, 167]. Optimal mitochondrial membrane potential is necessary for mitochondrial fusion [168]. Cancers exhibit an imbalance of mitochondrial fission factors. In tumor tissue from lung cancer patients, increase of DRP1 is accompanied by decrease of MFN2 levels when comparing with adjacent healthy tissue [169]. Immunohistochemistry and immunoblotting reveals DRP1 overexpression in 89% of tested lung adenocarcinomas ($n = 202$). DRP1 is mostly found sequestered within the nucleus [170]. Nuclear DRP1 overexpression correlates with tumor staging, smoking, resistance to chemotherapy, and worse prognosis. FIS1 is upregulated in subtypes of melanoma [171].

## 7. Conclusions and Perspectives

Assaying mitochondrial factors has long been recognized as a diagnostic approach for metabolic disorders. Convenient methods have been developed for human tissues [172] and cultured skin fibroblasts [173, 174]. In addition, diagnostic and prognostic mitochondrial markers have been developed for cancer. An overview is given in this paper (Figure 1). These include classical histopathological evaluation of mitochondrion-related proteins, as well as molecular techniques. Detection of mtDNA alterations offers the advantage of allowing low invasive methods, including analysis in urine for bladder cancer or saliva for head and neck cancers [70]. Also, screening tools such as MitoChip

oligonucleotide arrays [175] make it possible to screen for many factors at the same time.

Mitochondria have not only become valuable subjects for the early detection of cancer, but, moreover, may become cellular targets for future cancer therapy. Mitocans, that is, anticancer agents that act through the mitochondria, provide a strategy for targeting of mitochondrial metabolism and apoptotic processes. The vitamin E analog alpha-tocopheryl succinate (alpha-TOS) is a mitocan that inhibits the mitochondrial electron redox chain by interfering with the ubiquinone-binding sites of complex II [176], by which processes are suppressed that are essential for tumor neovascularization [177]. The mitochondrially targeted analog of alpha-TOS, termed MitoVES, is more potent in preventing tumor neovascularization and suppressing cancer growth. This selectivity is attributed to the greater mitochondrial inner membrane potential of proliferating endothelial cells and translates in reduced tumor progression in an in vivo breast cancer model [178]. The clinic awaits results of therapeutic trials with great interest.

## Conflict of Interests

The author declares to have no conflict of interests.

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