

CELL BIOLOGY-BASED STRATEGIES FOR THE DIAGNOSIS AND TREATMENT OF MITOCHONDRIAL DISEASES.

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EPILOGUE.

INTRODUCTION.

Mitochondrial disease is a term used usually to describe a diverse set of conditions caused by genetic defects in the assembly and/or

functioning of OXPHOS proteins, altered transport of substrates or ions into and out of the organelle or changed mitochondrial biogenesis. They cause reduced ATP synthesis and present with a diverse set of symptoms, which makes them difficult to diagnose without molecular approaches that are now performed in a small set of clinics experienced in these diseases.

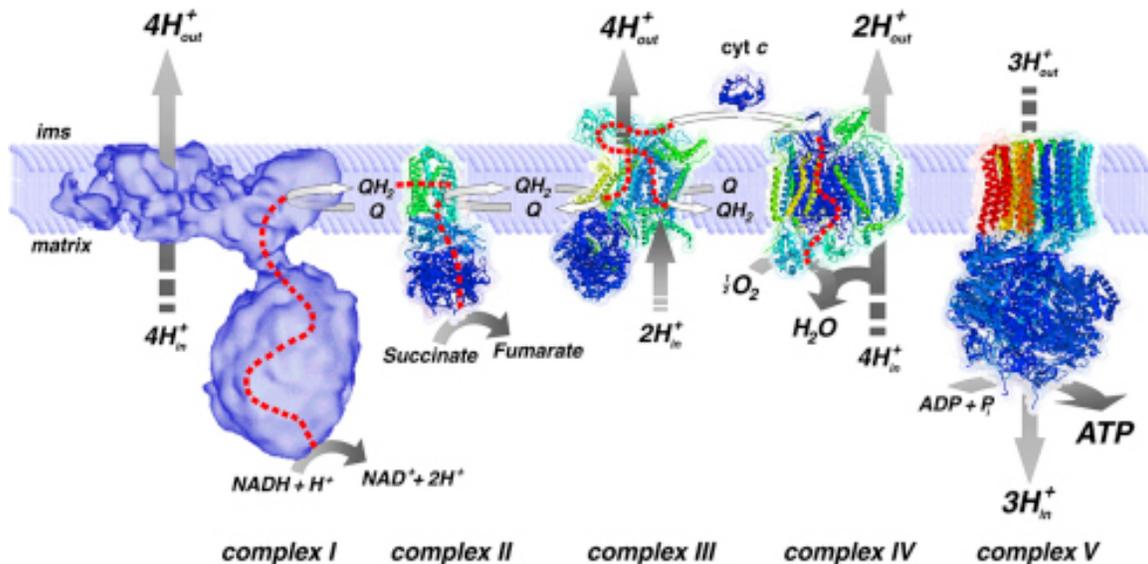


Fig 1. The mitochondrial oxidative phosphorylation complexes. Reproduced from Yu-Wai-Man P, Griffiths PG, Chinnery PF. Prog Retin Eye Res (2010)

There are other inherited genetic diseases of mitochondria that are not covered directly here, although the methodologies can equally be applied to these conditions. In terms of the location in the cell, inherited diseases in fatty acid metabolism, in the Krebs cycle, and in the urea cycle, along with Friedreichs ataxia are all mitochondrial diseases, as are other conditions described in an earlier review in this series.

The goals of ongoing research are to better diagnose mitochondrial diseases and to better treat these conditions. Treatment strategies for OXPHOS deficiency are evolving rapidly and now include behavioral changes such as increased exercise, and modifications to diet. Also various nutraceuticals are being recommended to lessen symptoms, such as lipoic acid and coenzyme Q. New therapies are being developed in several pharmaceutical companies, with at least one

compound in phase 2 trials. To date, these therapies are mostly aimed at minimizing the symptoms rather than correcting the defect.

Further progress in both diagnosis and in development of effective therapies depends heavily on being able to identify the cause, in this case the mutations in the genome. This, in turn, requires that any observed mutation(s), particularly novel mutations, is (are) shown to be responsible for mitochondrial dysfunction and resulting cell stress. Development of effective therapies also requires knowledge of the interplay between mitochondrial functioning and overall viability of the cells of different tissues. New methodologies are now making such evaluations easier and more accurate, particularly in providing a rapid and accurate diagnosis of mitochondrial disease.

Previously in this set of reviews I have covered the structure-function relationships of mitochondria, and have provided a summary description of the many age-related conditions associated with mitochondrial dysfunction e.g. neurodegenerative diseases and cancer. I have also reviewed overall cell metabolism with emphasis on energy production by mitochondria. This review follows on from the previous two in the series and shows how recent advances in understanding the structure and functioning of mitochondria, and the integration of this organelle in overall cell metabolism described previously, are improving diagnosis, and identifying new therapeutic interventions for patients with mitochondrial diseases, with particular emphasis on defects in oxidative phosphorylation.

PART 1. THE NEW PARADIGM: USING GENETIC AND BIOCHEMISTRY APPROACHES IN CONCERT TO DIAGNOSE MITOCHONDRIAL DISEASES.

Two novel broad screening approaches have been introduced recently that greatly aid in identifying mutations in the genetic material (DNA and RNA) of cells. These two methods, called exome sequencing and RNAseq respectively, greatly increase the chance of detecting potentially pathogenic mutations when used in parallel.

Both are now being exploited for the diagnosis of mitochondrial diseases.

However, the genomics alone does not establish that any mutation is the cause of an observed pathology. This requires establishing that the mutation causes a change in the amount or activity of the gene product i.e. the encoded protein, that is sufficient to alter mitochondrial functioning (see later). Hence there is an important place for cell biology in establishing a diagnosis of a mitochondrial disorder. Here I explain both the new genomic and the cell biology methods that are changing how mitochondrial diseases are diagnosed, and in elucidating the complex biology that must be understood in order to develop rational therapies for these conditions.

GENETIC ANALYSIS.

a). EXOME SEQUENCING

Exome sequencing, also known as whole exome sequencing (WES or WXS), is a technique for analyzing all of the expressed genes in a genome (known as the exome). It involves two steps: 1) separation of the subset of regions that encode proteins using microarrays constructed with bound oligonucleotides that hybridize to each of the 180,000 different exons in humans, (constituting about 1% of the human genome). 2). Sequencing of the exonic DNA using high-throughput sequencing technology.

A good example of applying whole exon sequencing to diagnose mitochondrial disease has been reported recently by Nafisinia et al (2017). The patient presented with late onset Leighs disease, the symptoms of which included seizures, ptosis, scoliosis, dystonia and symmetrical putaminal abnormalities. There was a lactate peak on brain MRS, but normal mitochondrial respiratory chain enzymology in muscle and liver. Using whole exome sequencing allowed detection of a heterozygous mutation in NADH dehydrogenase flavoprotein NDUFV1.

b). RNAseq

Exome sequencing does not identify disease-causing mutations if they are in a non-coding region, which is sometimes the case. However a second genetic approach, RNA seq, provides a method to

detect mutations in these non-coding regions and identifies alternative gene spliced transcripts, post-transcriptional modifications, gene fusion mutations/SNPs, as well as changes in gene expression over time.

RNA-Seq uses so called deep-sequencing technologies. A population of RNA molecules is first converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). The data are typically 30–400 bp, long. The resulting reads are then either aligned to a reference genome or reference transcripts, or assembled de novo without the genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and level of expression for each gene. Then by simply counting the number of molecules with a given sequence the expression level of any given gene or gene product can be determined.

As an example, a recent study Kremer et al. 2017 showed the power of such a transcriptosome analysis by identifying pathogenic mutations in fibroblasts of patients with mitochondrial disease that had not been detected by exome sequencing. They identified a novel alteration in the spicing of mRNA of the complex I assembly factor TIMMDC1 not identifiable by exome sequencing.

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PART 2. CELL BIOLOGICAL APPROACHES IN ANALYZING OXPHOS DISEASES.

Once a mutation has been identified by the genetic approaches above, the next step is to establish that it causes a change in level or activity of a protein(s) that is sufficient to alter mitochondrial functioning. This requires cell biological studies. In both the studies by Nafasinia et al. and Kremer et al. the genetic analysis was followed up by cell biological analyses to show that the identified mutations were pathogenic and gave rise to mitochondrial disfunction.

Cell biological as well as biochemical analysis is directed at answering two key questions:

- i) Is the gene product containing the mutation present in mitochondria in normal amount?*
- ii) If the gene product is present, is it functionally active?*

For mutations in components of the OXPHOS complexes, question 1 can be expanded to include:

- a) is the complex containing a mutated subunit or with a mutated assembly factor fully assembled i.e. are all of the subunits present?*
- b) is the complex present in normal amounts relative to the other complexes of the respiratory chain or ATP synthase.*

The method of choice for evaluating protein composition and levels depends on several factors. First it is important to know whether the putative pathogenic mutation is in a mitochondrial or a nuclear encoded gene. Approximately 15% of cases of mitochondrial disease are due to mutations in the mt DNA, the remainder are chromosomal and can be dominant or recessive. If the mutation is in a nuclear encoded gene, analysis is simplified by the fact that it will be ubiquitous to all cells and tissues with only a few exceptions such as if the gene involved encodes a tissue specific protein. This means that the proteomic analysis can be conducted on easily accessed samples such as skin fibroblasts, urine sediment or buccal cheek swabs, with every expectation that of the mutation, if they occur, will be detected.

Evaluating cases where the mutation is in mtDNA is more difficult. Cells contain a relatively large volume of mitochondria with anywhere up to several hundred copies of the mtDNA present in total. In general, the mtDNA copies are heterogeneous, having inherited mutations in some but not all of the organellar genome, along with multiple mutations to various copies accumulated from environmental factors.

Further, mitochondria are inherited only from the mother, and with fertilization are subject to the so-called bottleneck effect in which the number of copies of the mtDNA is greatly reduced. The consequence is that mutant mitochondria are distributed unevenly between different tissues. As a result, pathogenic mutations in mtDNA often do not show up in fibroblasts, urine etc. and the biochemistry must be done on tissue samples selected based on the patient symptoms.

Cell biological approaches that can be done on intact cells in culture include gel electrophoretic separation of proteins to evaluate levels and modifications, antibody detection of proteins in situ by ICC, and assay of various mitochondrial functions using dyes.

Antibody detection of proteins can also be done in tissue slices, but for gel electrophoresis and for activity measurements, mitochondria must be prepared first from the tissue samples.

DETECTING ALTERED ASSEMBLY/STRUCTURAL DEFECTS BY GEL ELECTROPHORETIC METHODS.

Gel electrophoresis can be used on samples that have been dissolved in non-denaturing buffers e.g. containing Triton X100, lauryl maltoside or digitonin as the solubilizing detergent, by using agarose as the gel material. In this method proteins remain as complexes through the electrophoresis step so that protein associations are detected. Alternatively, proteins can be separated for detection after dissolving samples in denaturing detergent e.g. sodium dodecyl sulfate (SDS). So-called SDS-PAGE uses polyacrylamide as the gel material and proteins are separated as individual polypeptides. Non-denaturing gel electrophoresis, also called blue native gel electrophoresis (BNPAGE), has been employed extensively to

identify defects when mutation of a component of the mitochondrial OXPHOS complexes is suspected. In this method the 5 complexes are separated based on size in a low percentage non-denaturing agarose gel. These are all MW greater than 120K and therefore well separated from the bulk >100K proteins when the gels are run for a prolonged time. (Complex I is 900K, ATP synthase is 550K, Complex III is 300K, Complex IV is 240K and Complex II is 120K)

An indication of dysfunction in patients is that one or more of the complexes migrate to a different position of the gel, either because it is not fully assembled, or because the mutation makes it unstable to the mitochondrial membrane dissociation condition.

While BNPAGE is useful, there are limitations and concerns. Before the analysis, mitochondria must be isolated from the tissue or cell line being investigated. The purer the preparation and the more intact the mitochondria after the procedure, the better the data obtained.

Isolating mitochondria is not always possible from the samples available. This is particularly true for pediatric cases.

Another issue is that the OXPHOS complexes exist in super-complexes, which are sometimes retained, and thereby complicate analysis when optimal cell dissociation and membrane protein solubilization conditions are not used.

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ANTIBODY BASED METHODS TO DETECT ALTERED ASSEMBLY/STRUCTURAL DEFECTS.

Antibody-based approaches provide an alternative to purely gel separation techniques, and have the advantage that analysis is greatly simplified, and importantly, can be done on cell culture

material or tissue material without the need to isolate mitochondria. When combined with the genetic approaches e.g. exon seq or RNA seq, analysis can be focused on that protein encoded by the mutated gene and those proteins associated with the target protein, whether directly through binding to it i.e. subunits of a complex, or indirectly as components in the pathway(s) to which the target protein contributes. A good example of the power of antibody detection of altered OXPHOS complexes in patients is presented in Murray et al.(2004) and key data shown in FIGURE 2.

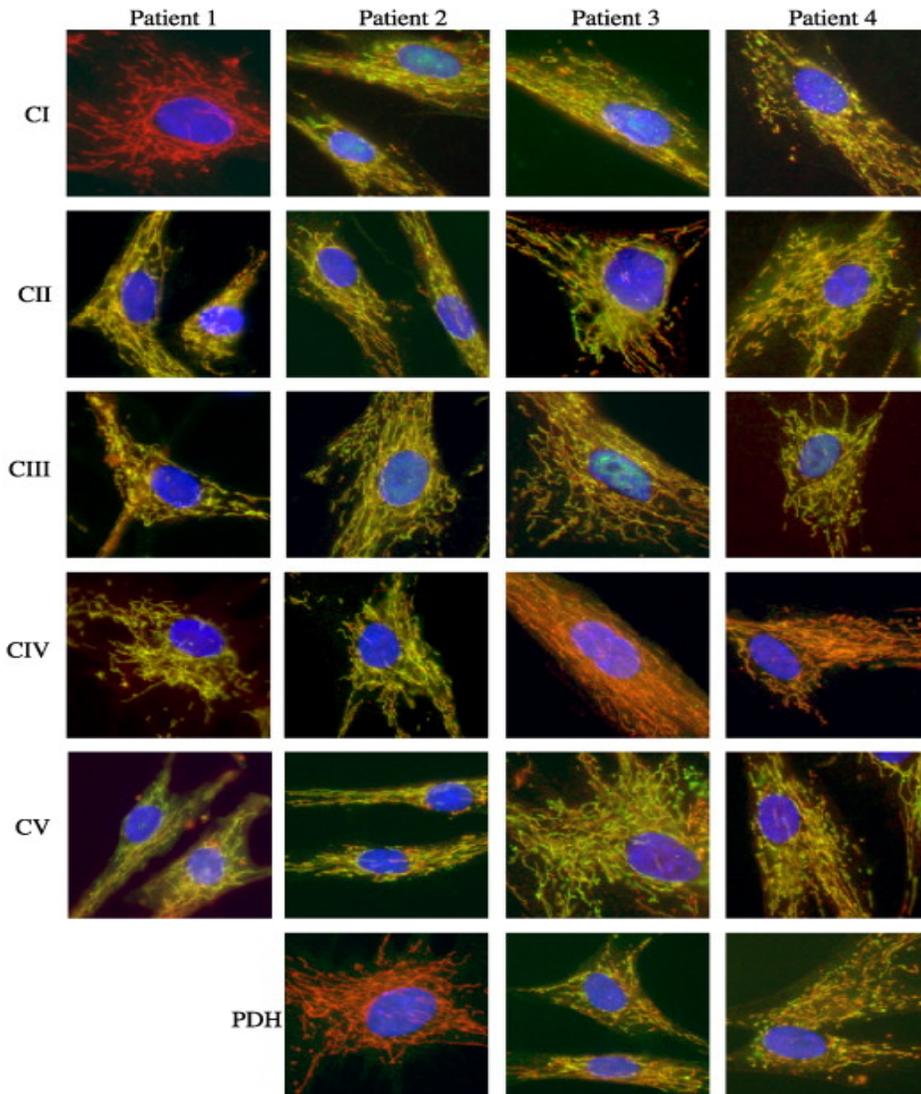


FIGURE 2. Images of fibroblasts from 4 patients each stained with a anti-porin antibody (red fluorescence) to highlight mitochondria and antibodies to each of the OXPHOS complexes and PDH (green fluorescence).

In this study fibroblasts from 4 patients, one with a mutation in complex I, two with mutations in complex IV and one a PDH mutant were evaluated for the presence of assembled complexes and PDH by immunocytochemistry (ICC). A green fluorescent probe attached to a secondary antibody was used to detect binding of antibodies to the different complexes in each case and a secondary antibody with a red dye was used to detect binding of anti-porin antibody to identify mitochondria in these fibroblast cell lines. The presence of altered respiratory chain complexes I and IV and the alteration of PDH are clearly evident from this screening approach (as red)

Note that there are now antibodies to many of the subunit proteins involved in OXPHOS, allowing for careful and systematic analysis of the state of assembly of any of the complexes when this level of analysis is desired. Further, although not reviewed in any detail here, there are good antibodies to most of the enzymes of fatty acid oxidation, several urea cycle enzymes, frataxin (the protein involved in Friedreichs ataxia), the ADP/ATP translocase and other mitochondrial proteins that can be used to screen for genetic defects.

Antibodies are particularly useful in evaluating protein composition in mitochondrial disease patients where the phenotype indicates tissue specificity of the defect, as the approach can be used with tissue samples. FIGURE 3 shows the use of antibodies against complex I, Complex IV, SDH and porin respectively to examine the heterogeneity of expression of a large scale deletion in mtDNA that affects both complex I and cytochrome c oxidase. The heteroplasmy of the defect can be clearly seen in these images of muscle fibers.

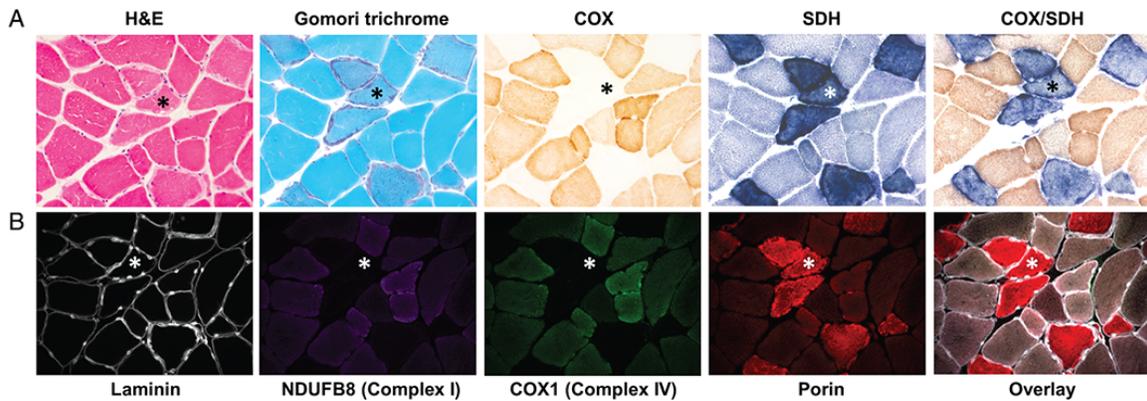


Figure 3. Serial skeletal muscle sections from a patient with a single, large-scale sequential COX/SDH. (B) Quadruple immunofluorescence assay of the levels of complex I (NDUFB8 subunit), complex IV (COX1 subunit), laminin, and porin respectively, within a single 10- μ m section. * COX-deficient fibre (figure kindly provided by Rob Taylor) is from Alston et al. J.Pathol. 241. 238-250.

Mutations in mitochondrially-encoded subunits of complexes I, III, IV and the ATP synthase are not the only source of tissue specific mitochondrial disease. Mutations in proteins responsible for DNA replication are a significant disease burden. Studies of Copeland and colleagues have identified large numbers of mutations in DNA polymerase gamma, some of which cause Leighs disease, progressive external ophthalmoplegia (PEO), and several ataxias. POLG mutations lead to altered levels of mtDNA, which can be quantified in cells by the newer sequencing methods, and visualized in cells in culture using fluorescence in-situ hybridization

Not all antibodies available for purchase are equally good. It is critical that the antibody is specific for the target protein, and recent reviews have emphasized that specificity has not been adequately confirmed for many commercial antibodies. The monoclonal antibodies most often used in the study of mitochondria were made in my laboratory and at Mitosciences, a company I formed to expand the availability of high quality antibodies for medical research. All of the antibodies were tested for specificity in several ways before they were used in experiments. First they had to react with a single band in SDS PAGE, except in cases where there were processed forms in the cell, which is sometimes the case, or mixture of isoforms of the protein. Second, the antibody had to immunocapture the target protein from cell or

tissue samples, and this immunoprecipitated material was subjected to mass spectrometry protein sequencing to confirm it was against the target protein. Thirdly the antibody had to react with native protein, as in immunocytochemistry, and when possible in thin-sectioned tissue samples to ensure the utility of the antibody in mitochondrial studies. (MitoSciences was bought several years ago by AbCam and I receive no continuing remuneration from them, so there is no profit in my lauding these antibodies, other than the pride of contributing them to the field!)

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MONITORING OXPHOS FUNCTIONING WITH ACTIVITY ASSAYS.

Altered assembly of the OXPHOS complexes is readily detected by the approaches above. The consequences for turnover of each of the complexes, and overall efficiency of the energy-producing apparatus to make ATP, provide supporting proof of the pathogenicity of the identified mutation. In the case of OXPHOS defects caused by mutations in mtDNA, the assay of activity, both of the individual complexes and the overall levels of ATP synthesis, are particularly

import in that they provide an indication of the extent of normal versus defective cells in the tissue.

One relatively set of simple assays of the effect of any mitochondrial mutation on mitochondrial functioning is the measurement of ATP generation, membrane potential or both simultaneously using a combination of luminescence to measure ATP levels and a dye to measure membrane potential. These assays can be done with cell material e.g. fibroblasts, cheek swap samples, and with care can be performed on thin sliced biopsy material. A pathogenic mutation in the OXPHOS proteins is likely to reduce ATP levels and decrease the membrane potential. The possibility that the effect on these parameters is due to cellular effects other than OXPHOS functioning can be evaluated by doing the assay in the presence of glucose and comparing with that in galactose. In glucose, cells in culture prefer to use glycolysis for ATP production, but in galactose, cells are “forced” to use OXPHOS. Therefore defective mitochondrial functioning is much more evident in galactose-grown cells than those grown in glucose.

Another more global measure of OXPHOS functioning is to measure the rate of oxygen consumption in respiring cells (see references). Oxymetry, the use of the Seahorse technology and fluorescent dyes all measuring oxygen consumption rates.

To evaluate the cause and consequences of a mutation more specifically activity assays for each of the 5 complexes must be done. Relatively simple assays are available to do this, but these require that mitochondria are isolated first and as discussed above, this is not always possible because of the amount of sample available. More crude solubilized tissue fractions are also used sometimes. The isolation of mitochondria is not straightforward, and damage to the organelle can release some membrane material and/or alter the protein content. The consequence is that activity based on a per mg protein basis are unreliable when compared to the controls. A solution is to correlate the activity with a spectrophotometric heme measurement in the cases of Complex III and IV, but such analyses require significant amounts of material.

Antibodies offer another strategy for assaying the activity of OXPHOS proteins. Antibodies are available that can immunocapture intact and

functionally active complexes I-IV, the ATP synthase and PDH. (FIGURE 4). In each case an activity assay is possible for the immunocaptured protein attached in the wells of a microplate. Activity measurements can be followed up in the same well by using a second antibody to the same protein (complex) to quantify the amount of protein in each well. Thus a relative specific activity is derived for comparison with controls analyzed at the same time in the microplate.

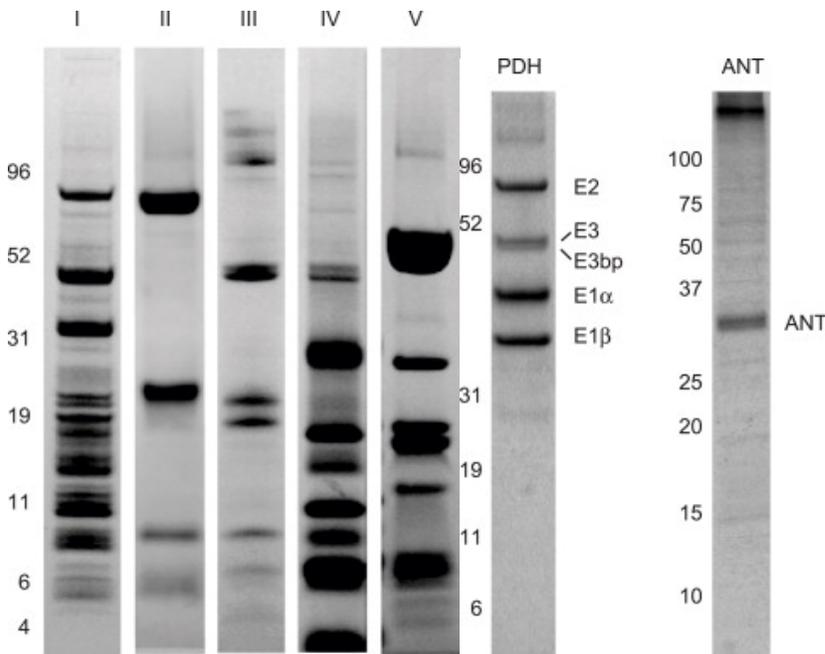


Figure 4. SDS PAGE of immunocaptured complexes I,II,III,IV and V (ATP synthase) respectively as well as PDH and ANT. In each case the antibody brings down the complex with a full compliment of subunits and in an active form

Antibody immunocapture, and then activity measurements combined with immunodetection of the amount of enzyme complex present, can also be done using lateral flow devices i.e. dipsticks, as first done in my laboratory. These have been used but sparingly for diagnosis of mitochondrial diseases. (see readings) They are cheap, very fast and accurate.

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PART 3. USE OF MODEL ORGANISMS TO DETERMINE THE PATHOGENICITY OF MUTATIONS.

Many of the mutations in genes for the OXPHOS system are pathogenic because the protein fails to fold properly, does not interact properly with other subunits of the complex, or less often, is in a catalytic site, i.e. the mutation alters key regions of the protein, which more often than not are highly conserved between species. Thus for

some mutations pathogenicity can be established by experiments using other species including E.coli, as in the case of several mutations in the ATP synthase, and yeast for complexes II-IV and the ATP synthase. Drosophila and C. elegans have also been used to model mitochondrial mutations.

One interesting approach is to use the vertebrate, zebrafish, as a model system. These fish are more representative of the human condition than unicellular organisms. Zebrafish studies are particularly useful because they yield information about the effect of a mutation on development as well as physiology of the defect.

In one study COX deficiency similar to that expected from pathogenic mutations in structural subunits or assembly factors, was induced using morpholinos. In this way expression of CoxVa, a structural subunit, and Surf1, an assembly factor, were each reduced to 50%, in each case leading to reduced assembly of the enzyme.

Developmental defects in endodermal tissue, cardiac function, and swimming behavior, were observed. There was a significant reduction in cardiac size and major neuronal defects. Cellular investigations revealed different underlying mechanisms in tissues. Apoptosis was dramatically increased in the hindbrain and neural tube, and secondary motor neurons were absent or abnormal, explaining the motility defect. In contrast, the heart lacked apoptotic cells but showed increasingly poor performance over time, consistent with energy deficiency.

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PART 4. THE EXPANDING CATALOGUE OF MUTATIONS KNOWN TO CAUSE MITOCHONDRIAL DISEASES.

The number of proteins involved in mitochondrial functioning is in the several 1000s. The number required for one function of the organelle alone i.e. OXPHOS, is large, with the 4 electron transfer complexes and ATP synthase together composed of nearly 100 proteins, and with each complex assembled via a number of assembly factors to generate the final functional complex. Mutations have been found in around half of these component proteins that reduce the efficiency of energy production by OXPHOS (listed in Figure 5). The rate of identification of pathogenic mutations in the OXPHOS system is likely to increase significantly as gene sequencing methods advance and biochemical analysis of these mutations are refined.

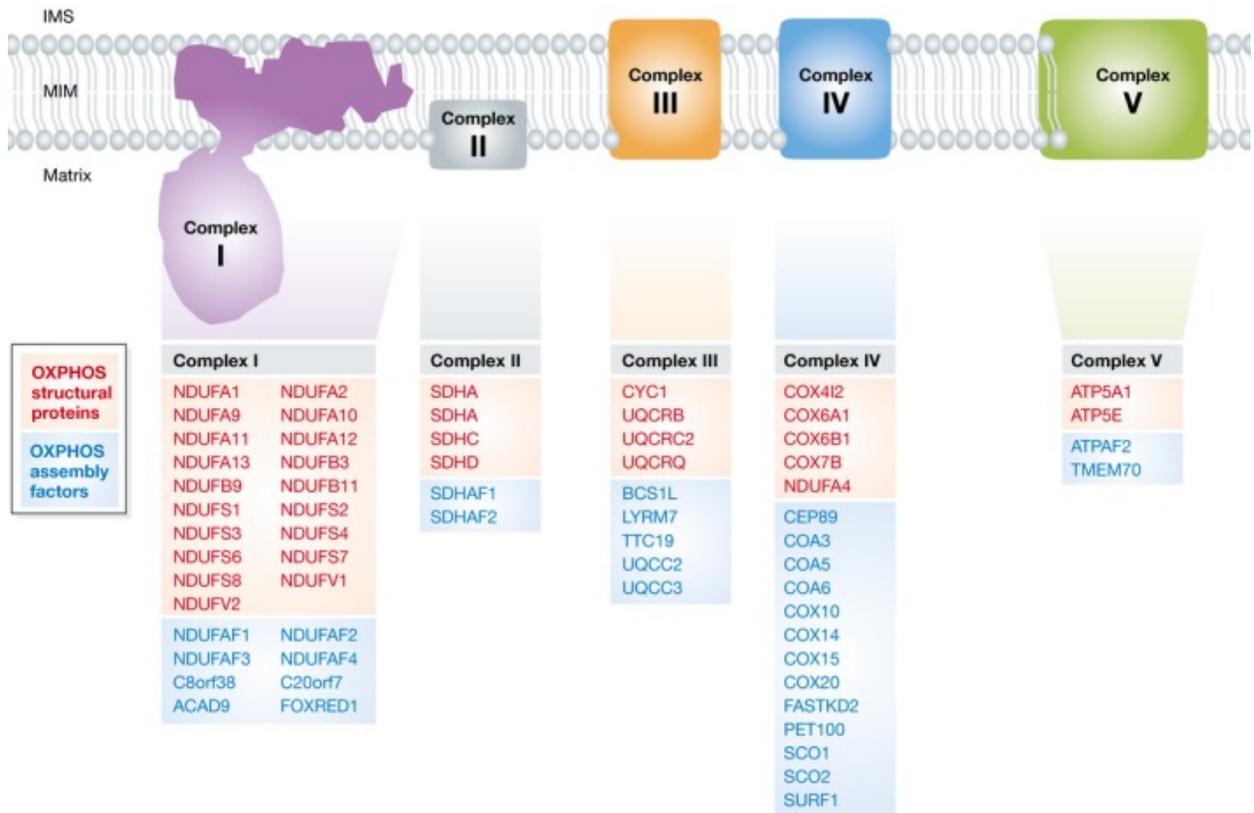


Figure 5. Subunits of each of the OXPHOS complexes in which mutations have been identified. reproduced from Koopman et al. *EMBO Mol Med.* (2016) 4: 311–327.

PART 5. CONSEQUENCES OF PATHOGENIC MITOCHONDRIAL MUTATIONS ON THE FUNCTIONING OF CELLS.

Mutations that affect OXPHOS have a wide range of effects on the functioning of cells. They reduce ATP levels, increase the ratio of NADH to NAD⁺ which is problematic as NAD⁺ is a substrate for many cell reactions, dramatically increase ROS production, alter mitochondrial morphology, and affect movement of ions into and out of mitochondria, including Ca⁺⁺ ions which flood the cytosol. All of these effects are signaled throughout the cell by a set of signaling pathways, and can end up in altered substrate use e.g. glycolysis versus oxidative phosphorylation, up-regulation of anti-oxidants, altered interplay between mitochondria and other organelles, and ultimately, to fates such as autophagy/mitophagy, or apoptosis and other mechanisms of cell death. Obtaining a full understanding of

how altered OXPHOS affects cellular functioning is now a key goal in the search for treatments that can minimize the symptoms of these diseases. In particular, the effects of excess ROS production are a major focus for researchers interested in maintaining the cell viability of high energy-requiring tissues such as skeletal muscle, heart and brain. As yet the information on what is called retrograde signaling of mitochondrial dysfunction is limited.

Proteomics is the gold standard for analyzing the broad effect of mutations on cellular proteins. However, the method is expensive when quantification is needed, and only available in selected centers. One relatively cheap way to screen for altered cell functioning in response to a mitochondrial defect when the mutation is known is In-Cell Western analysis. In this approach patient and control cells are grown in 96 (or 384) well plates, antibodies to the specific proteins of interest are reacted in different wells and a pattern of global changes is developed. Cells are made permeable before addition of antibodies. Secondary antibodies to which a fluorescent dye is attached provide the read out of the change in levels of protein (or modification of proteins e.g. phosphorylation, acetylation), between the patient and control.

In the study in Figure 6, cells were grown in the presence and absence of rotenone a powerful inhibitor of complex I) for 24hr to mimic a genetic defect in complex and then analyzed. In this experiment a total of 64 different antibodies were used covering OXPHOS, Krebs cycle, fatty acid synthesis, fatty acid oxidation, urea cycle, glycolysis, protein synthesis and anti-oxidant proteins.

PART 6. EMERGING TREATMENTS FOR MITOCHONDRIAL DISEASES: A BRIEF SUMMARY.

From the foregoing, mitochondrial diseases induce more alterations in cell function than just reducing ATP levels. Generation of excessive amounts of free radicals by damaged mitochondria is a particular cause of cell disruption. Reduced repair and/or replacement of damaged proteins, along with destruction of mitochondria by autophagy/mitophagy, and ultimately cell death, all provide significant symptomology.

Most attempts to treat mitochondrial diseases at this stage revolve around trying to minimize the symptoms, with particular emphasis on reducing ROS generation, or in trying to provide alternative substrates for energy production such as ketone bodies and fatty acids. An excellent review of the compounds now being tested has been present recently by Koopman et al.2016. Therefore the following is only brief summary of ongoing work.

Studies using idebenone, ubiquinone (coenzyme Q), curcumin, elamipretide and lipoic acid, all antioxidants, are aimed at reducing oxidative stress.

Idebenone and ubiquinone both pass electrons directly to Complex III thereby potentially bypassing any mutation in Complex I. However neither compound is likely to increase the levels of NAD⁺, which is required for multiple cellular functions. In addition to its antioxidant properties, curcumin also alters several cell signaling pathways.

KH 171 is another free radical scavenger, being developed by Khondrion, that shows promise in the trials to date.

Other strategies include using compounds (e.g. EPI-743 and RP103) to enhance glutathione levels and thereby reduce oxidative damage. Treatment with 2'3'5'-tri-O-acetyl uridine is being tested as a way to increase uridine synthesis, which is reduced by defective OXPHOS.

The synthesis of multiple OXPHOS genes is controlled by peroxisomal proliferator-activated receptors (PPARs). Studies are ongoing to test the ability of bezafibrate, an activator of PPARs to increase mitochondrial levels.

A recent report describes a novel approach to increasing the NAD⁺ to NADH ratio. Titov et al. used a bacterial flavin adenine dinucleotide dependent H₂O-forming NADH oxidase expressed in mitochondria of HeLa cells to increase NAD⁺. The effect was to increase electron transfer activity over 2 fold and the activity of enzymes that require NAD⁺ as substrate was significantly increased.

Progress in bringing treatments to the clinic is being made. Stealth BioTherapeutics recently presented the results of a phase 2 trial of elamipretide (SS131) and is seeking permission to conduct a phase 3 trial. Elamipretide is claimed to restore electron transport as well as reducing oxidative stress through an interaction at the cytochrome/cytochrome oxidase interface.

SELECTED READING.

The addition of ketone bodies alleviates mitochondrial dysfunction by restoring complex I assembly in a MELAS cellular model.

Frey S, Geffroy G, Desquirit-Dumas V, Gueguen N, Bris C, Belal S, Amati-Bonneau P, Chevrollier A, Barth M, Henrion D, Lenaers G, Bonneau D, Reynier P, Procaccio V. (2017) Biochim Biophys Acta. 1863:284-291

Mitochondrial disorders in children: toward development of small-molecule treatment strategies. *Koopman WJ, Beyrath J, Fung CW, Koene S, Rodenburg RJ, Willems PH, Smeitink JA. (2016) EMBO Mol Med. 1, 311-27*

Complementation of mitochondrial electron transport by manipulation of the NAD⁺/NADH ratio. Titov D.V, Cracan V & Mootha VK. (2016) Science 35. 231-235.

The mitochondrial cocktail: rationale for combined nutraceutical therapy in mitochondrial cytopathies Tarnopolsky MA (2008)Advanced drug delivery reviews 60. 1561-67

First-in-class cardiolipin-protective compound as a therapeutic agent to restore mitochondrial bioenergetics. *Szeto HH (2014)Br J Pharmacol. 171:2029-50.*

EPILOGUE.

These are hopeful times for patients with mitochondrial diseases and for their families. As described here, the improved understanding of the biology of mitochondria is leading to better diagnostic methods and to novel treatments of these conditions. With respect to diagnosis, it remains a long, tedious and expensive experience for patients and their families. However the recent development of high coverage and good resolving gene sequencing methods is likely to greatly aid in diagnosis. One advantage is that the “needle in the haystack” i.e. the mutations likely to underlie the disease can in most cases be identified relatively quickly by these DNA or RNA sequencing methods. This review highlights methods, particularly antibody based methods, that can easily and relatively cheaply determine whether the mutations identified by sequencing are pathogenic and cause disease. If so, then a positive diagnosis has been made, and done relatively quickly and without the plethora of tests that take months, are costly, generally require biopsy, generate prolonged angst to families, as well as delaying implementation of treatment regimens. Progress toward rational treatments is moving fast, and organizations like to UMDF are at the forefront of promoting the increased understanding of these diseases.