

# Analysis of Mitochondrial Metabolic Defects

Qualifying Exam Proposal

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## Description

Mitochondria harness energy obtained by combining oxygen with fuel molecules to make ATP, and are regarded as the power plants of cells. Any disruption in the process can have disastrous consequences. Each year in the U.S., nearly 4,000 children are born with mitochondrial disease. The mortality for the most severe forms of mitochondrial disease in childhood is up to 50 percent per year. Some children born with mitochondrial disease do not show symptoms until adulthood: over 50 million adults are affected by diseases in which mitochondrial disorders are suspected. Many diseases of aging, such as Parkinson's, Alzheimer's, cancer, diabetes and heart disease are thought to involve mitochondrial defects. Currently, there are no effective treatments that address the underlying genetic problem, though intense research in this area is ongoing.

This research will bring together knowledge of mitochondrial metabolic processes under a modeling framework. This system will be able to characterize a snapshot of an individual's unique disease state based on their mitochondrial genetic component and functional metabolic capacity. Moreover, therapeutic treatments can be designed that address the shortcomings of that individual's impaired energy metabolism, and evaluated for possible success. The long-term goals of this project are: (1) computational characterization of the mitochondrial metabolic mechanism, with the appropriate resolution of disease states, (2) analysis of existing data to determine what influence a disease state has on the performance of the mitochondria and the whole tissue and (3) execution of critical experiments to fill gaps in the existing knowledge and investigate the predicted therapeutic benefit of alterations to the metabolic network.

## Performance Sites

- Department of Bioengineering, University of Washington, Seattle, WA, USA
- Mitochondria and Disease Research Group, Medical School, University of Newcastle upon Tyne, Newcastle, Scotland

## Key Personnel

| Name                 | Organization                      | Department     | Degree     | Role                   |
|----------------------|-----------------------------------|----------------|------------|------------------------|
| Michael G. Dodds     | University of Washington          | Bioengineering | M.S.       | Principal Investigator |
| Douglass M. Turnbull | University of Newcastle upon Tyne | Neurology      | M.D./Ph.D. | Collaborator           |

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## Glossary and Abbreviations

|              |  |              |  |
|--------------|--|--------------|--|
| <b>LHON</b>  | Leber Hereditary Optic Neuropathy  | <b>CIPO</b>  | Chronic Intestinal Pseudoobstruction with myopathy and Ophthalmoplegia |
| <b>AD</b>    | Alzheimer's Disease  | <b>CPEO</b>  | Chronic Progressive External Ophthalmoplegia                           |
| <b>ADPD</b>  | Alzheimer's Disease and Parkinson's's Disease  | <b>MMC</b>   | Maternal Myopathy and Cardiomyopathy                                   |
| <b>NARP</b>  | Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa; alternate phenotype at this locus is reported as Leigh Disease | <b>FICP</b>  | Fatal Infantile Cardiomyopathy Plus, a MELAS-associated cardiomyopathy |
| <b>MM</b>    | Mitochondrial Myopathy   | <b>LIMM</b>  | Lethal Infantile Mitochondrial Myopathy                                |
| <b>DMDF</b>  | Diabetes Mellitus + Deafness   | <b>MHCM</b>  | Maternally inherited Hypertrophic CardioMyopathy                       |
| <b>DM</b>    | Diabetes Mellitus  | <b>BFSN</b>  | Familial Bilateral Statal Necrosis                                     |
| <b>MELAS</b> | Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes   | <b>LDYT</b>  | Leber's hereditary optic neuropathy and DYsTonia                       |
| <b>MERRF</b> | Myoclonic Epilepsy and Ragged Red Muscle Fibers  | <b>DEAF</b>  | Maternally inherited DEAFness or aminoglycoside-induced DEAFness       |
| <b>PEM</b>   | Progressive encephalopathy   | <b>mtDNA</b> | Mitochondrial DNA  |
| <b>nDNA</b>  | Nuclear DNA  | <b>ROS</b>   | Reactive oxygen species  |

## Detailed Budget for Initial Period (4/1/02 - 3/31/03)

### Personnel

| Name                               | Role on Project        | Time Appt (Months) | % Effort | Institutional Base Salary | Salary Requested (effort*base) | Fringe Benefits (15%) | Total (salary+fringe) |
|------------------------------------|------------------------|--------------------|----------|---------------------------|--------------------------------|-----------------------|-----------------------|
| Michael G. Dodds                   | Principal Investigator | 12.0               | 0.50     | \$80,000                  | \$40,000                       | \$6,000               | \$46,000              |
| Graduate Student                   | Research Assistant     | 12.0               | 1.00     | \$20,000                  | \$20,000                       | \$3,000               | \$3,000               |
| Douglass M. Turnbull               | Collaborator           | 12.0               | 0.00     | \$120,000                 | \$0                            | \$0                   | \$0                   |
| Animal Technician                  | Research Assistant     | 12.0               | 0.10     | \$60,000                  | \$6,000                        | \$900                 | \$6,900               |
| Administrative Assistant           | Program Assistant      | 12.0               | 0.20     | \$30,000                  | \$6,000                        | \$900                 | \$6,900               |
| Consultants                        |                        |                    |          |                           |                                |                       | \$0                   |
| <b>Subtotal Personnel Expenses</b> |                        |                    |          |                           |                                |                       | <b>\$62,800</b>       |

## Direct Costs

| Item  | Totals           |
|---|------------------|
| LINDO Software License  | \$8000           |
| MATLAB Software with Optimization Toolbox   | \$500            |
| FBA3 Software   | \$1,000          |
| (2) Dell P4 Desktop Computers, Operating System, Software, Warrantee                                | \$6,000          |
| Histological, histochemical, imaging of samples   | \$8,000.00       |
| Animals (housing, operation requirements)   | \$25,000         |
| Myoblast isolation and culture  | \$12,000         |
| Miscellaneous experimental supplies   | \$6,000.00       |
| Mailings, office supplies, meeting supplies   | \$2,000          |
| Travel for PI (one per year) to UK  | \$2,500          |
| Travel for collaborator, graduate student, and PI (one per year) to present results at a conference | \$7,500          |
| Graduate Student's Tuition (out-of-state)   | \$18,000         |
| Consortium/Contractual Direct Costs   | \$64,000.00      |
| Consortium/Contractual Administration Costs   | \$18,000         |
| <b>Subtotal Direct Costs</b>  | <b>\$178,500</b> |
| <b>Total Direct Costs for Initial Budget Period</b>   | <b>\$241,300</b> |

## Budget for Entire Proposed Period of Support (4/1/02 – 3/31/05)

|   | Initial Budget Period | 2 <sup>nd</sup> | 3 <sup>rd</sup> | Total Cost       |
|---|-----------------------|-----------------|-----------------|------------------|
| Personnel   | \$62,800              | \$62,800        | \$62,800        | \$188,400        |
| Direct Costs  | \$178,500             | \$178,500       | \$178,500       | \$535,500        |
| <b>Total Direct Costs for Entire Proposed Period of Support</b> |                       |                 |                 | <b>\$723,900</b> |

## Resources

### Laboratory

- University of Washington. Work will take place in the Resource Facility for Population Kinetics. This laboratory occupies approximately 1200 square feet of office space, subdivided into cubicles and offices.
- University of Newcastle upon Tyne. Work will take place in the Medical School facilities in the Mitochondria and Disease Research Group laboratories.

### Clinical

- University of Newcastle upon Tyne, Medical Center. Newcastle is a major referral center for patients with mitochondrial disease and provides an excellent diagnostic facility.

### Animal

- University of Newcastle upon Tyne, animal housing facility. Animals will be housed in separate cages and cared for by certified veterinarian technicians. Food storage and proper sanitary facilities are also available on-site. Operation and monitoring equipment and resources necessary for work set forth herein are also available.

### **Computer**

- University of Washington, two Pentium 4 machines. Existing infrastructure (networking, backup, etc.) is in place for support of these machines. They are required to develop modeling and analysis techniques described herein.

### **Office**

- University of Washington. The PI will continue to use his existing office.
- University of Newcastle upon Tyne. The collaborator will continue to use his existing office.

### **Other**

### **Major Equipment**

- University of Newcastle upon Tyne.
  - Microcentrifuge: 10,000 g capability.
  - Bio-Rad MRC 600 laser-scanning confocal microscope: tissue immunoassay imaging.
  - CM100 transmission electron microscope: tissue immunogold imaging.
  - Reichert Frigocut 28000N cryostat microtome: tissue sample cyrosectioning.
  - Hybaid Touchdown thermal cycler: PCR
  - PhosphorImager system: automated gel electrophoresis.
  - ABI 377: automated DNA sequencer

## A. Specific Aims

Each year in the U.S., nearly 4,000 children are born with mitochondrial disease. The mortality for the most severe forms of mitochondrial disease in childhood is up to 50 percent per year. Some children born with mitochondrial disease do not show symptoms until adulthood. Over 50 million adults are affected by diseases in which mitochondrial disorders are suspected. Many diseases of aging, such as Parkinson's, Alzheimer's, cancer, diabetes and heart disease are thought to involve mitochondrial defects. Currently, there are no effective treatments that address the underlying genetic problem, though intense research in this area is ongoing. This project seeks to provide a basis by which the impact of a particular genetic mutation in mitochondrial DNA can be understood, in terms of the metabolic network within the diseased tissue. As gene therapy technology becomes available, this research can serve as a means to evaluate the prospective success of a therapeutic intervention. Thus our proposed research is timely.

The first step of realizing a tool by which therapies can be guided is to create an accurate and appropriately detailed model of mitochondrial function. Mitochondria encode only a small fraction of the genes involved in respiration, but as these products serve as the terminus of the respiratory chain, defects there can affect upstream processes in a debilitating way. It is most appropriate, then, to construct a model that allows for the greatest detail where the suspected pathogenicity lies. For this to occur, the second step towards this project is required: an analysis of the currently understood mitochondrial diseases in order to identify which metabolic processes are affected. Each disease will map onto the metabolic model, and simulations can be performed to demonstrate qualitatively the biochemical and histological predictions seen in a clinical setting. Finally, the third step of this project will involve resolving, by simulation, the best therapy (dietary and supportive) that can be applied to most efficiently use the suboptimal metabolic pathways available to a diseased tissue.

In summary, the long-term goals of this project are: (1) computational characterization of the mitochondrial metabolic mechanism, with the appropriate resolution of disease states, (2) analysis of existing data to determine what influence a disease state has on the performance of the mitochondria and the whole tissue and (3) execution of critical experiments to fill gaps in the existing knowledge and investigate the predicted therapeutic benefit of alterations to the metabolic network.

### A.1 Hypothesis

We hypothesize that the metabolism of a cell or tissue is organized in such a way as to maximize the synthesis of ATP relative to the substrates required to produce this product ( $O_2$  and fuel molecules). In the case of a defective metabolic network (defined by the reduction of efficiency), this objective will still be maintained.

This leads to the specific aims of this project:

#### A.1.1 Specific Aim #1

We will develop a mathematical model to describe the metabolic processes in mitochondria. The solution to this problem will be verified experimentally or by using existing clinical data.

#### A.1.2 Specific Aim #2

We will modify the above metabolic network to represent a damaged or missing step in the network present in a disease state. The simulation of various disease cases will be verified using existing data or experimentation.

#### A.1.3 Specific Aim #3

Supportive and dietary supplements used to treat mitochondrial disorders will be examined by this metabolic model, and the outcome of these therapies will be attributable to changes in the metabolic network.

## B. Background and Significance

### B.1 Mitochondrial Structure, Function and Genotype

Oxidative metabolism has the function of oxidizing fuel molecules into the primary energy currency of the cell (ATP). The aerobic oxidation of energy substrates takes place within the mitochondria through a variety of metabolic pathways. Highly energetic tissues (e.g. muscle and neurological) rely critically on oxidative metabolism for their energy requirements.[9] In addition to producing energy, mitochondria also perform many other functions within a cell, all of which are essential for survival. Anabolic processes such as the synthesis of heme, amino acids, lipids and nucleotides are all carried out in the mitochondria.[11] Recently, much attention has been given to the mitochondrial role in programmed cell death (apoptosis).[12] From this, it is clear that malfunctioning mitochondria have a severe impact on the survival of a cell, especially in those tissues that have high energetic requirements. Figure 1 gives an overview of the catabolic processes in the mitochondria.

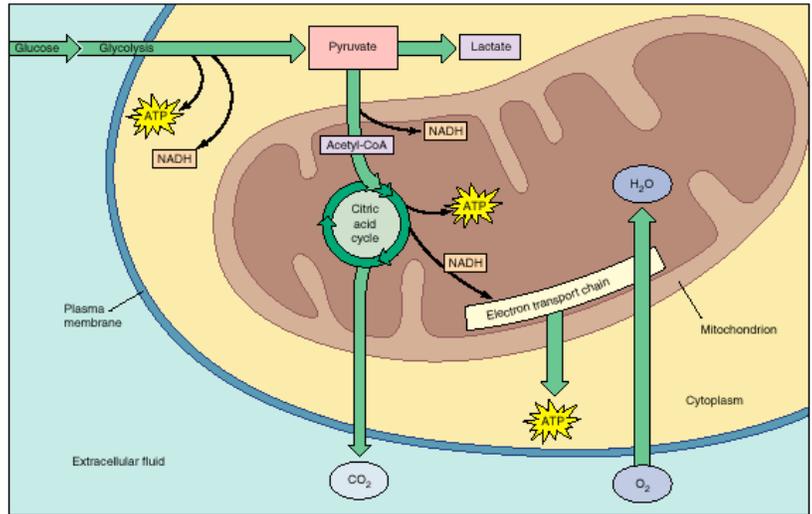


Figure 1 Overview schematic of energy metabolism in a cell. From [1].

From this, it is clear that malfunctioning mitochondria have a severe impact on the survival of a cell, especially in those tissues that have high energetic requirements. Figure 1 gives an overview of the catabolic processes in the mitochondria.

Mitochondrial genes are primarily stored in nuclear DNA (nDNA) with only 13 being encoded from mitochondrial DNA (mtDNA). MtDNA also encodes 2 rRNA and 22 tRNA for synthesis of those proteins encoded within the mitochondria.[13] All of the remaining proteins are encoded in the nucleus of the cell, synthesized in the cytoplasm and imported to the mitochondria. However, the mitochondria is responsible for its own DNA replication and repair. It has been shown that mtDNA are ten times more susceptible to DNA mutations, owing to the lack of protection systems (histones) and a low-efficiency repair system.[14] In summary, mitochondria function is a shared responsibility of the cell and the mitochondria itself. A defect in carrying out these tasks on the part of the cell or the mitochondria can disrupt the symbiotic relationship between them.

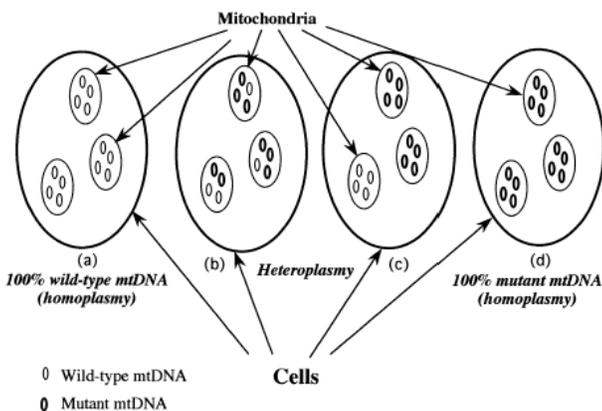


Figure 2 Heteroplasmy of mutant mtDNA. Each cell (large circles) in a tissue has a different complement of mutant mtDNA (small bold circles) in their mitochondria (medium circles). From the left we have the cases (a) where all the mtDNA is normal, (b) where the mitochondria have a mixed proportion of mutant and normal mtDNA, (c) where each mitochondria has only mutant or normal mtDNA, and (d) where every mitochondria has only mutant mtDNA. From [2].

An important concept when considering the impact mutant mtDNA has on the cell (see below) is the concept of *heteroplasmy*. Most cells contain hundreds of mitochondria and each mitochondria contains two to ten mtDNA. MtDNA is a circular, covalently closed DNA with 16,569 base pairs, which resembles a classic plasmid structure. A population of cells with mutant mitochondria can take on a variety of genotypes.[2] Figure 2 shows the four different genotypes, with respect to mtDNA complement, a cell may take on under this model. This has important impact on the consideration of what an mutation in the mtDNA has on the function of the overall respiration of the cell. When there is a mutation, not all of the mtDNA copies necessarily have this mutation, so that we find a mixture of normal (wild-type) and abnormal (mutant) mtDNA molecules in that mitochondria. This mixture can be expressed as a quasi-continuous fraction of mutant mtDNA molecules in the entire mtDNA population in a mitochondria. This concept can be expanded first to the cellular level, then to the tissue level. It becomes clear that a great deal of diversity can be expected in a

tissue with variable amounts of heteroplasmy in its mtDNA complement. Indeed, the stochastic distribution of mutant mtDNA molecules in a tissue has suggested a *bioenergetic mosaic*, wherein the cells in a tissue will have a range of bioenergetic capacities.[9]

## B.2 Mitochondrial Disorders

### B.2.1 Clinical Relevance

A recent survey in the northeast of England showed a conservative prevalence of 1:8070 for those individuals at risk for developing a mtDNA disease, with 1:15,217 to be affected by a disorder.[15] The effects of these diseases are progressive and insidious, but as the disorders all relate to the terminus of respiration, there is no effective drug therapy. Typical treatments often are dietary, supplemental, and supportive efforts. Table 1 offers a summary of suspect clinical features that might indicate a mitochondrial disorder.

### B.2.2 Major Categories of Disorders

Defects in metabolism leading to disease

| Organ System              | Possible Problems  |
|---------------------------|--|
| Brain                     | Developmental delays, mental retardation, dementia, seizures, neuropsychiatric disturbances, atypical cerebral palsy, migraines, strokes   |
| Nerves                    | Weakness (which may be intermittent), neuropathic pain, absent reflexes, gastrointestinal problem (ge reflux, constipation, pseudo-obstruction), fainting, absent or excessive sweating resulting in temperature regulation problems |
| Muscles                   | Weakness, hypotonia, cramping, muscle pain   |
| Kidneys                   | Proximal renal tubular wasting resulting in loss of protein, magnesium, phosphorous, calcium and other electrolytes  |
| Heart                     | Cardiac conduction defects (heart blocks), cardiomyopathy  |
| Liver                     | Hypoglycemia (low blood sugar), liver failure  |
| Eyes                      | Visual loss and blindness  |
| Ears                      | Hearing loss and deafness  |
| Pancreas and Other Glands | Diabetes and exocrine pancreatic failure (inability to make digestive enzymes), parathyroid failure (low calcium)  |
| Systemic                  | Failure to gain weight, short stature, fatigue, respiratory problems including intermittent air hunger, vomiting   |

Table 1 Clinical features that may indicate a mitochondrial disorder. From [3].

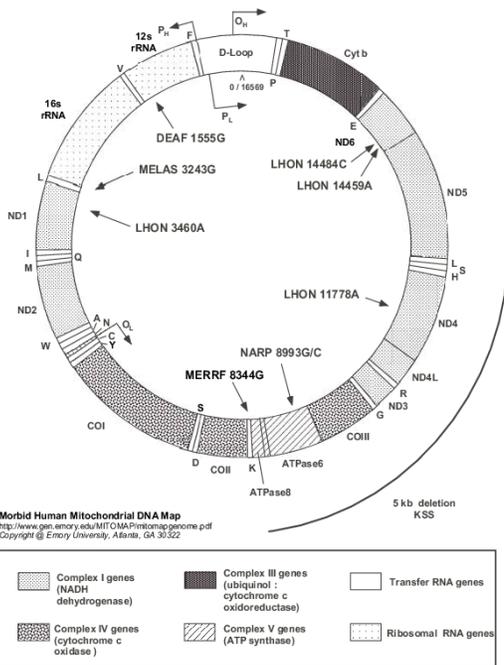


Figure 3 Map of the human mitochondrial DNA, showing common point mutations with disease association (bold text with disease name), as well as the common deletion of 5kbp. From [4].

states can occur at any point within the overall metabolic path. However, mtDNA mutations are limited to the electron transport chain and transcriptional machinery, by virtue of sole genetic encoding. However, as this is an interdependent system, upstream processes can still be affected. One clear interdependency can be seen in the ratio of NAD/NADH in the cytosol and mitochondrial matrix.

Two major categories of mtDNA disorders are characterized by either point-mutations in protein-coding genes or point-mutations in translation-related genes.[4] Mitochondrial disorders shown to be involved with protein-coding regions of the genome are LHON, NARP (also Leigh disease) and LDYT. Disease states related to defects in translation genes are further subdivided into two categories: those affecting rRNA genes and those affecting tRNA genes. Disorders that are involved with rRNA defects are DEAF, DM, APDP and Rett Syndrome. Those disorders related to tRNA-encoding genes are more numerous than any other, and are seen in MERRF, MELAS, myoglobinuria, MM, AMDF, DMDF, CPEO, KSS, MMC, PEM, Ocular myopathy, FICP, CPIO and LIMM. A third category of mtDNA-related disorders are characterized by large-scale re-arrangements of mtDNA are usually seen in single deletions, or more rarely, partial duplications. Disorders related to these defects are KSS, CPEO, Ocular myopathy or the rare Pearson's bone marrow-pancreas syndrome.[9] Figure 3 details the location of some of the identified point-mutations on the human mtDNA molecule.

### B.2.3 Threshold Effect

One of the interesting features of mitochondrial disorders is that different tissues, all of which have a particular mtDNA mutation, show different levels of pathology. This heterogeneity has been answered by attention to two salient features of mitochondria: heteroplasmy in mutant mtDNA complement and the fact that mitochondria meet different demands in different tissues.[2] This feature, the so-called *threshold effect*, has been extensively studied for relevance to a variety of disorders. Cell lines with the MELAS point-mutation required a mtDNA complement of around 90% before their respiratory rate began to drop rapidly. This phenomenon has been reported for MERRF, CPEO and cytochrome *c* oxidase deficiency both in isolated cell lines and isolated enzymatic studies.[16][17] In general, maintaining a 10% population of wild-type (normal) mtDNA in a cell is sufficient to avoid any clinical pathology: a remarkable compensatory feature. Findings indicate that the level of heteroplasmy do not dictate the level of product in mitochondria: a small minority of wild-type mtDNA maintains a wild-type phenotype.

*In vitro* studies of each of the respiratory chain complexes have shown a sigmoid inhibition shape when presented with a gradient of chemical inhibitors.[5][18] Figure 4 shows a typical set of curves for rat kidney mitochondria complex IV respiratory function when titrated against KCN, a known inhibitor of this complex. A mild response to the inhibitor is in evidence until a threshold inhibition is reached, where upon the respiratory rate drops dramatically. Again, we see a compensatory feature in the metabolic network: even as the function of a particular unit in metabolic network drops, the system as a whole is able to compensate. The ability to compensate has limits, which we will examine in subsequent sections.

This has positive implications for finding therapies for these diseases. If a therapeutic intervention can create even a modest impact on the ratio of wild-type to mutant mtDNA or gene product in a tissue, that tissue can be rescued from a disease state. This has suggested a simple gene therapy approach, whereby mtDNA replication is selectively inhibited, thereby giving a replicative advantage to wild-type DNA. This has been demonstrated to be partially successful in cell cultures.[19]

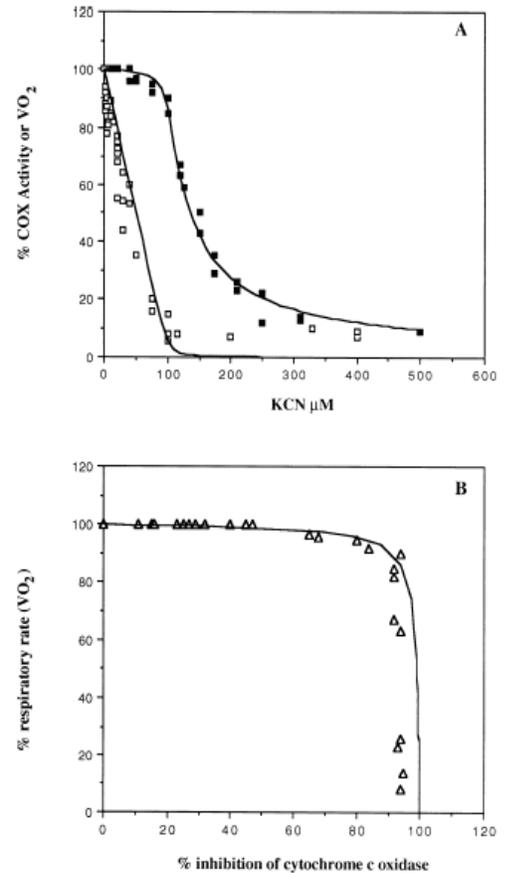


Figure 4 Titration and threshold curves of cytochrome c oxidase in rat kidney mitochondria. (A) KCN titration of respiration rate (solid squares) and cytochrome c oxidase rate (open squares) as a function of KCN (B) Threshold curve with percentage of respiratory rate as a function of complex IV inhibition by KCN. From [5].

## C. Preliminary Studies

### C.1 Mitochondrial Model - Specific Aim #1

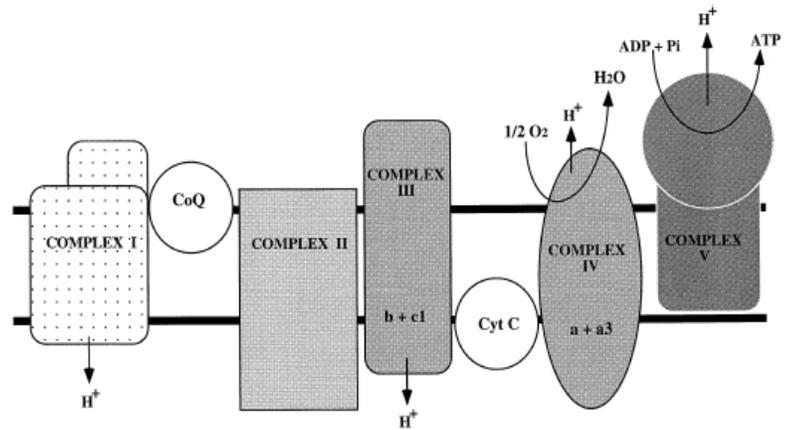
MtDNA mutations are limited to the electron transport chain and transcriptional machinery, by virtue of sole genetic encoding in the mitochondria. However, the electron transport chain serves at the terminus for oxidative metabolism, so we expect that problems here will affect upstream processes. Indeed, one of the first signs that an individual has a mitochondrial deficiency is detected in elevated blood or CSF lactate, creatine kinase and/or amino acids, though this is hardly definitive.[20]

Figure 5 shows a schematic of the respiratory chain, as well as the genes responsible for encoding the components (see Figure 3 for the mtDNA locations for each of these subunits). As previously stated, the respiratory chain should be the focus of the mitochondrial metabolic model, but sufficient detail about the upstream components (glycolysis and the TCA cycle) should be supplied.

We present here preliminary work towards detailing such a model. This analysis capitalizes on the knowledge of the stoichiometry of the biochemical reaction network, which thereby generates constraints on the mathematical representation of the network by invocation of mass-balance theory.

#### C.1.1 Flux-Balance Analysis

The fundamental principles underlying flux-balance analysis (FBA) is the conservation of mass combined with metabolic pathway knowledge.[7][21][22][23] Figure 6 shows the overall fundamental arrangement of FBA. A flux balance can be written for each metabolite ( $X_i$ ) of interest in the metabolic network to create dynamic mass balance equations that relate the metabolites to the processes in the network. These ordinary differential equations can be written as a function of the stoichiometric matrix ( $S_{ij}$ , number of moles of metabolite  $X_i$  formed by reaction  $j$ ) times the reaction fluxes ( $v_j$ , reaction  $j$  that produces or consumes metabolite  $X_i$ ) minus the losses to processes and sinks outside the defined system,  $b_i$ , as written in equation (1).



| Enzyme Complex                     | mtDNA-encoded subunits            |
|------------------------------------|-----------------------------------|
| I (NADH dehydrogenase)             | 7 (ND1,2,3,4,4L,5,6)              |
| II (Succinate dehydrogenase)       | 0                                 |
| III (CoQH2-cytochrome c reductase) | 1 (Cytochrome b)                  |
| IV (Cytochrome c oxidase)          | 3 (Cytochrome oxidase I, II, III) |
| V (ATP synthase)                   | 2 (ATPase 6,8)                    |

Figure 5 Schematic of the respiratory chain in the mitochondrial inner membrane. Each subunit is encoded by either the mtDNA or nDNA. Only complex II is entirely encoded by the nDNA. From [6].

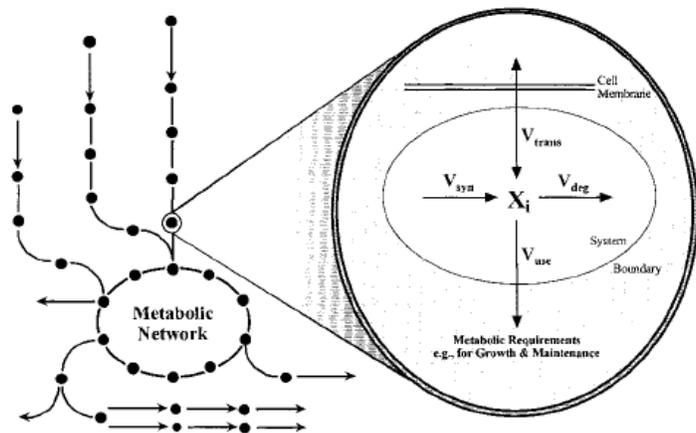


Figure 6 Fundamentals of Flux-balance analysis. From [7].

$$\frac{dX_i}{dt} = \sum_j S_{ij} \cdot v_j - b_i \quad (1)$$

The stoichiometric matrix is then an  $i \times j$  matrix, where  $i$  is the number of metabolites and  $j$  is the number of fluxes in the network. Losses from the system are enumerated as  $j$  input and output fluxes from beyond the boundary of the metabolic network. Thus, we have a clear definition of what is “internal” to the system and what is “external”.

The pathway information we are seeking should be an invariant property of the metabolic network, so a steady-state condition is applied to (1). This develops a set of linear homogenous equations whereby it becomes possible to calculate the flux values. This system is typically underdetermined, having more fluxes in the system than metabolites.

$$\sum_j S_{ij} \cdot v_j - b_i = 0$$

$$S \times v - b = 0 \quad (2)$$

The matrix  $S_{ij}$  is not square ( $j > i$ ), so there exists many solutions to equation (2). All possible solutions to (2) lie in “null space”, a basic property of an underdetermined system. The concept of a null space in terms of biochemical networks has been well studied for more than a decade.[24] This null space contains all the capabilities of the metabolic network: what it can and cannot do. It is then up to the investigator to create a well posed question to reduce the possible solutions in this null space. In other words, when an overall objective is hypothesized, this reduces the solution space to those fluxes that optimize that objective. Additionally, constraints are placed on the flux components of this problem,  $\alpha_j$ , typically ruling out negative fluxes or incorporating prior (experimental) knowledge by the explicit inclusion of the maximum allowable value of that flux. Bi-directional fluxes, typical because of the reversible nature of most metabolic processes, are decomposed into two reactions, thus allowing the non-negativity constraint to hold. Fluxes from or to outside the system under investigation are allowed to be unconstrained, resulting in a set of constraints that complete the specification of this problem:

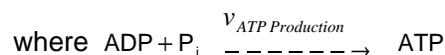
$$0 \leq v_j \leq \alpha_j, \forall j$$

$$-\infty \leq v_{external} \leq +\infty \quad (3)$$

This problem is tractable by restatement as a linear programming (LP) problem whereby the flux vector is adjusted to maximize or minimize a specified objective. Typically, convex analysis is employed to yield a set of “extreme pathways”, which are a subset of the elementary modes of a reaction network.[22] The set of extreme pathways is systematically independent, to use the analogous term “linearly independent” from classic linear algebra. This means, mathematically, that these pathways cannot be formed by positive combinations of any other pathways. As a visualization aid, consider that each of these pathways represent an extreme phenotype, such as maximized growth rate or maximized protein synthesis. It is likely that no one pathway will meet the overall objective, so a combination of these pathways is selected, each with a variable amount of utilization. It is in this way that the overall objective is met by combining the efforts of each extreme pathway. Subject the constraints in equation (3), equation (2) can be optimized with a specific objective in mind. The hypothesis put forward in our Specific Aims, written in these terms is:

$$\operatorname{argmax}_c \left[ Z = \sum c_i v_i \right]$$

$$\text{where } Z = v_{ATP \text{ Production}} \quad (4)$$



$Z$  is the objective function, representing a phenotypic property, where we have included our hypothesis that the objective is to produce the maximum amount of ATP as efficiently as possible.  $c_i$  is the cost or benefit of each flux if it is brought into the solution: some reactions will cost ATP, while others will create ATP.  $v_{ATP \text{ Production}}$  is the set of reactions in the network that produce ATP from ADP. As a detail, we note that GTP is created in the citric acid cycle, but this is converted on an equimolar basis to ATP, so this reaction can be treated equivalently.

Several software systems are available that can implement the LP problem. A commercially available software package was used (Lindo Systems, Chicago, IL) in conjunction with a graphical prototyping tool (Genetic Circuits Research Group, UCSD, California) designed for this task.

### C.1.2 Mitochondrial Metabolic Map

Table 2 summarizes the fluxes accounted for in the existing model by Ramakrishna et al.[8] This model incorporates glycolysis, the citric acid (TCA) cycle, and oxidative phosphorylation (also the respiratory chain and electron transport system, ETS). The relevance of the pentose phosphate shunt has been questioned in mitochondrial metabolism, and has been left out.[9] Glycolysis is performed in the cytosol, while the TCA and ETS are performed in the

| Enzyme/Input Reaction                    | E.C. Number | Enzyme/Input Reaction                    | E.C. Number |
|--|-------------|--|-------------|
| Glucose input                            |             | Citrate synthase                         | 4.1.3.7     |
| Oxygen input                             |             | Pyruvate dehydrogenase                   | 1.2.4.1     |
| Lactate input                            |             | Aconitase                                | 4.2.1.3     |
| Palmitate output                         |             | Isocitrate dehydrogenase                 | 1.1.1.42    |
| Lactate drain                            |             | $\alpha$ -Ketoglutarate dehydrogenase    | 1.2.4.2     |
| $\alpha$ -Ketoglutarate drain            |             | Succinyl-CoA synthetase                  | 6.2.1.5     |
| Oxaloacetate drain                       |             | Succinate dehydrogenase                  | 1.3.99.1    |
| Hexokinase                               | 2.7.1.2     | Fumarate reductase                       | 1.3.99.1    |
| Phosphoglucosomerase                     | 5.3.1.9     | Fumarase                                 | 4.2.1.2     |
| Phosphofructokinase                      | 2.7.1.11    | Malate dehydrogenase (isozyme)           | 1.1.1.37    |
| Fructosebiphosphatase                    | 3.1.3.11    | NADH dehydrogenase (complex I)           | 1.6.5.3     |
| Aldolase                                 | 4.1.2.13    | Ubiquinone cytochrome c-oxidoreductase   | 1.10.2.2    |
| Triose-P-isomerase                       | 5.3.1.11    | Cytochrome c-oxidase                     | 1.9.3.1     |
| Glyceraldehyde-3-phosphate dehydrogenase | 1.2.1.12    | ATP synthase                             | 3.6.1.34    |
| Phosphoglycerate kinase                  | 2.7.2.3     | Palmitate thiokinase                     | 6.2.1.3     |
| Phosphoglycerate mutase                  | 5.4.2.1     | Acyl-CoA dehydrogenase                   | 1.3.99.3    |
| Enolase                                  | 4.2.1.11    | Enoyl-CoA hydratase                      | 1.3.99.2    |
| Pyruvate kinase                          | 2.7.1.40    | 3-Hydroxyacyl-CoA dehydrogenase          | 4.2.1.17,   |
| PEP synthase                             | 2.7.9.2     | Acyl-CoA acetyltransferase               | 4.2.1.74    |
| PEP carboxy kinase                       | 4.1.1.49    | Aspartate aminotransferase               | 1.1.1.35    |
| PEP carboxylase                          | 4.1.1.31    | Cytosolic glycerol-3-P-dehydrogenase     | 2.3.1.16    |
| Lactate dehydrogenase                    | 1.1.2.4     | Mitochondrial glycerol-3-P-dehydrogenase | 2.3.1.9     |
| Malate- $\alpha$ -ketoglutarate exchange |             | Glutamate-aspartate exchange             | 2.6.1.1     |

Table 2 The metabolic reactions included in the existing model of the mitochondrial metabolic network. The E.C. classification number is included for reference in the standard databases. From [8].

mitochondria. This compartmentalization requires that specialized shuttle systems be described. The first is the malate-aspartate shuttle, which by a coordinated exchange of malate and  $\alpha$ -ketoglutarate with aspartate and glutamate via two antiporters, allows the transport of reducing equivalents in the form of NADH from the cytosol into the mitochondria.[25] The second is the glycerol phosphate shuttle, which delivers reducing equivalents from cytosolic NADH in the form of FADH<sub>2</sub>. [26] This is a less-desirable pathway, supposing our hypothesis true, as a molecule FADH<sub>2</sub> produces only two molecules of ATP, versus the three created by NADH. This, then, would be preferentially used if the malate-aspartate shuttle were defective or less desirable for lack of required intermediates.

Figure 7 shows the flux distribution when the metabolic network is metabolizing glucose only. The units displayed here are normalized to one unit of substrate molecule, or 1 mol\*unit time<sup>-1</sup>\*unit mass dry weight<sup>-1</sup> or "1 unit". Under the conditions specified, this network can produce 38 units of ATP per unit of glucose consumed, which is in agreement with the understanding of metabolism. Other substrates have been examined using this model (lactate and palmitate), and are shown in Figure 7 with dashed lines, indicating they do not play a role in the analysis shown here. However, the model does accurately predict the overall ATP yield for each substrate: 6.33, 5.83, 5.61 (mol ATP per mole oxygen) for unlimited glucose, lactate and palmitate, respectively. Moreover, the predicted preferential utilization of the malate-aspartate shuttle over the glycerol phosphate shuttle was seen. Thus, Specific Aim #1 is at least partially satisfied by the preliminary work done with this model.



### C.1.4 Case Study: Effects of Malate Dehydrogenase Deficiency

The malate-aspartate shuttle, as previously described, is key in moving reducing equivalents from the cytosol to the mitochondria. By altering the constraints on this system's fluxes, a profile for ATP production dependence (sensitivity) on this shuttle was developed, in a similar manner as the previous case study. When the level of activity falls below a certain threshold, the glycerol phosphate shuttle becomes necessary. However, recall that the efficiency of this shuttle is less than that of the malate-aspartate shuttle, so the overall ATP production efficiency falls. The interesting feature of this response is that it is bi-phasic, as shown in Figure 9. This is interesting because it mimics the threshold effect seen in disease pathology and in respiratory unit inhibition experiments. This feature of our model will be very important when attempting to emulate what is seen *in vivo*.

### C.1.5 Summary of Preliminary Work - Specific Aim #1

We have presented a model of mitochondrial metabolism that is capable of capturing the salient features with a resolution level down to specific enzymes and fluxes. Three different metabolic substrates were simulated, and good agreement was found with experimental data. By placing additional constraints on specific fluxes, simulation of disease states is possible. However, we have not specified how a certain disease state will modify our model, nor have we described how heteroplasmy will impact the function of the metabolic system. These points will be addressed in our methods. We have demonstrated an example, inactive succinate dehydrogenase, that qualitatively presents features seen in actual pathological reports. Moreover, this example is exactly at the metabolic location, the inner membrane of the mitochondria, that we are interested in exploring. The example of malate dehydrogenase deficiency also indicates that threshold effects can be successfully incorporated in our model. We believe that flux-balance analysis of this model is an excellent demonstration of the preliminary work already in place to answer Specific Aim #1.

## C.2 Mapping of Genetic Defects to Disease Expression - Specific Aim #2

The task of delineating the exact impact mutations in mtDNA have on mitochondrial functions is somewhat daunting. However, we can divide the effects of the mutations into two categories. The first are those mutations that take place in the protein-coding regions of the mtDNA. These defects will affect only one subunit of the ETS, or impair the replication of the mtDNA, in the case of the rRNA gene defects. The second are those mutations that affect the tRNA and rRNA genes, which would affect all proteins synthesized in the mitochondria. This second category is difficult to quantify in terms of impact. Indeed, the associated disease phenotypes seen in clinical practice do not segregate cleanly by genotypic defect.

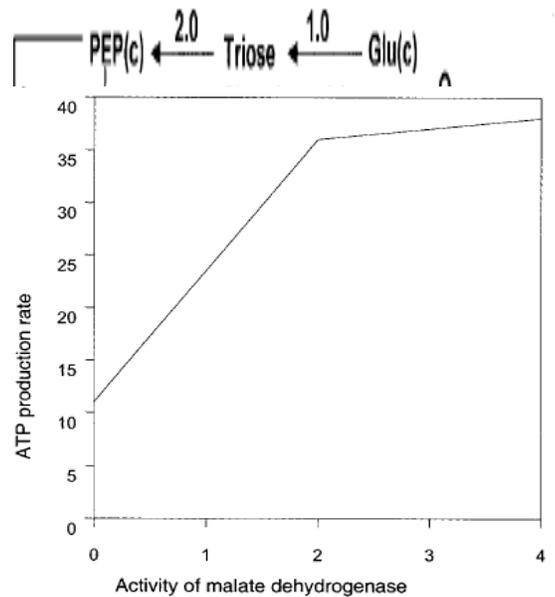


Figure 9 The predicted effect of malate dehydrogenase on ATP production. Note the qualitatively different phases of response. From [8].

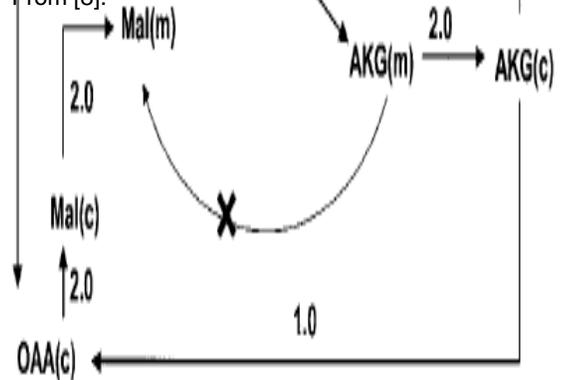


Figure 8 The predicted optimal flux distributions when the so-called "lower span" of the TCA cycle is inactive. The carbon entering the TCA cycle is partially metabolized. From [8].

### C.2.1 Gene Mutations in mtDNA and Their Consequences

Specific Aim #2 clearly defines the problem at hand to be one where the developed metabolic network can be modified to reflect a damaged or missing step in the network. Therefore, we will restrict ourselves for the moment to the defects in mtDNA that affect a definite step in the metabolic network. Table 3 shows the known (confirmed by multiple investigators) point mutations and deletions in the protein-coding region of the mitochondrial genome. Appendix B contains a more comprehensive list of these mutations, but are unconfirmed or suspected to be non-pathogenic polymorphisms. Clearly, Leigh disease (also known as NARP), FBSN, LHON and KSS are of interest. Mutations in tRNA and rRNA are expected to have impact on all the functional units of the respiratory chain. Teasing out the exact implications on each unit will be difficult, and will be addressed later in our Methods.

### C.2.2 Mouse Model

A major hurdle in taking proven in vitro strategies through further development is the lack of an animal model which expresses heteroplasmic pathogenic mtDNA.[28] To overcome this problem, an animal model was developed using severe combined immunodeficiency (SCID) mice.[29] Previous work with this animal model has shown that injection of normal human myoblasts into the tibialis anterior led to development of muscle fiber that expressed human dystrophin. Satellite cells, though more readily available via biopsy, researchers in this field have shown that these cells do not contain a relevant level of mitochondrial mutations. Two questions relevant to proposing this mouse model as appropriate for mutant mtDNA expression studies were asked. First, would the SCID mice produce muscle fiber that are stable and continue to express human proteins? Second, if myoblasts with mutant mtDNA are injected, would the regenerated tissue retain the mutant mtDNA, and to what level? The methods employed in this study will be presented in our Methods section.

The results of this study are as follows. Muscles that had been irradiated and injected with PBS showed regeneration but no human  $\beta$ -spectrin. Muscles that had been irradiated and injected with normal human myoblasts or mutant mtDNA myoblasts showed expression of  $\beta$ -spectrin. This demonstrated that normal and mutant myoblasts could contribute to muscle regeneration. A colocalization of acetylcholine esterase activity at the surface of  $\beta$ -spectrin positive fibers was established, indicating motor end plate formation and innervation. Finally, human cytochrome c oxidase immunostaining showed positive expression of human mitochondria in both the T8993C and control myoblasts.

To examine the retention and expression level of mutant mtDNA, the ratio of fragmented 430bp (into 130- and 300bp fragments) to unfragmented was compared to the original myoblasts of the patient. The original patient heteroplasmy was ~85%, and  $\beta$ -spectrin positive fibers from five animal subjects showed similar heteroplasmy of ~82%, ~73%, ~85% and ~81%. The slight decrease in mutant mtDNA load can probably be attributed to the decreased fitness of the mutant mtDNA myoblasts. A similar experiment with mutants involved in MERRF was performed (tRNA<sup>Lys</sup> gene), with similar results. As regeneration and similar mutant mtDNA can be obtained from myoblasts containing either of these mutations, there is no reason why this method could not be used for any mtDNA mutation that is expressed in myoblasts. Beyond this, it has been shown that skin fibroblasts may be converted to myoblasts in MDX (X-linked muscular-dystrophy) mouse muscle, so it may be quite possible to generate equivalent mouse models using dermal fibroblasts gathered from humans.[30]

| Gene     | Protein     | Mutation | Disease    |
|----------|-------------|----------|------------|
| ND1      | Complex I   | G3460A   | LHON       |
|          |             | T4216C   | LHON       |
| ND2      | Complex I   | A4917G   | LHON       |
| ND3      | Complex I   | 5kb CD   | KSS        |
| ND4      | Complex I   | G11778A  | LHON       |
|          |             | 5kb CD   | KSS        |
| ND5      | Complex I   | 5kb CD   | KSS        |
| ND6      | Complex I   | G14459A  | LHON       |
|          |             | T14484C  | LHON       |
| COX1     | Complex IV  |          |            |
| COX2     | Complex IV  |          |            |
| COX3     | Complex IV  | 5kb CD   | KSS        |
| CytB     | Complex III |          |            |
| ATPase 6 | ATPase      | T8993G/C | Leigh/NARP |
|          |             | 5kb CD   | KSS        |
|          |             | T9176C   | FBSN/Leigh |
| ATPase 8 | ATPase      | 5kb CD   | KSS        |

Table 3 Confirmed point mutations and deletions in protein-coding regions in mtDNA. Point mutations are given by the original nucleotide, location in the genome, and nucleotide substitution. The so-called "5kb common deletion" is also a source of disease, and is denoted "5kb CD". The associated disease(s) are also listed. Compiled from [4].

### C.2.3 Summary of Preliminary Work - Specific Aim #2

We have categorized the effects of mutations in mtDNA into those that involve the all products of mtDNA (tRNA and rRNA genes) and those that produce a specific defective product (protein-coding genes). An exhaustive presentation of the known or suspected mutations in mtDNA was presented (Table 3 and Appendix B). We located the impact on the mitochondrial metabolism of those mutations known to be associated with disease. The case-study detailed in section C.1.3 provides a clear methodology for implementation of a model of each pathological phenotype. However, beyond these two related case studies, no work has been done to implement mitochondrial diseases in this context, which will be addressed in our methods. The issue of heteroplasmy in mtDNA mutations remains to be addressed, however. A SCID mouse model was specified to emulate patient-specific mutations. We believe that the necessary tools to provide a model for various disease cases has been put forward in preliminary response to Specific Aim #2.

### C.3 Therapeutic Interventions - Specific Aim #3

At this point, there are no effective treatments for mitochondrial disorders. The purpose of the treatments are to alleviate symptoms of the disease and to slow the progression of the disease.[3] The effectiveness of the treatments vary from patient to patient. In general, individuals with milder disorders respond better to treatments. Generally, the treatments currently offered are empirical in nature, and rarely tailored to an individual's precise disease state. As this project is at a very preliminary stage, no significant effort has been made to incorporate our model with existing clinical data in a quantitative manner. However, understanding the possible therapeutic modes will be key in using information gained from this project in a clinical setting.

#### C.3.1 Dietary Supplements

Three key points are stressed in long-term care for patients with mitochondrial diseases: dietary adjustment, vitamin and cofactor supplementation, and stress avoidance.[3]

| Supplement                       | Dose Range  | Supplement                             | Dose Range        |
|----------------------------------|---|--|-------------------|
| CoQ10                            | 4.3 mg/kg/day or 60-120 mg per day or a dose to reach a target blood level          | Calcium                                | variable          |
| levo-carnitine (Carnitor®)       | variable, 25-100 mg/kg/day, some argue against carnitine unless carnitine deficient | Magnesium                              | variable          |
| Thiamine (B1)                    | 50-100 mg a day   | Phosphorous                            | variable          |
| Riboflavin (B2)                  | 50-100+ mg a day  | Vitamin K <sub>1</sub> or <sub>3</sub> | 5 - 30 mg per day |
| Nicotinamide (B3)                | 50-100 mg a day   | Succinate                              | 6 gm per day      |
| Vitamin E                        | 200-400 IU; 3 times a day   | Citrates                               | variable          |
| Vitamin C                        | 100-500 mg SR; 3 times a day  | Prednisone                             | variable          |
| Lipoic Acid ( $\alpha$ -lipoate) | 12.5 mg/kg/day in 3 divided doses   |  |                   |
| Selenium                         | 25-50 micrograms a day  |  |                   |
| $\beta$ -carotene                | 10,000 IU; every other day to daily   |  |                   |

Table 4 A list of supplements (vitamin, cofactors and minerals) suggested for mitochondrial disorders. From [3].

Dietary therapy is recommended. Avoidance of fasting is stressed, as even rest overnight may be dangerous for some patients. Illness is of special concern, especially if it involves vomiting and loss of appetite, such as flu, and should be treated with hospitalization and intravenous glucose. Small, frequent meals are recommended, as is a bed-time snack consisting of complex carbohydrates. An increase of fat in the diet in the case of complex I deficiency is advisable, as metabolism of fatty-acids partially bypasses this complex, delivering electrons directly to complex II. However, in disorders involving fatty-acid metabolism, care should be taken to deliver the correct type of fatty-acid. Fatty-acid disorders are difficult to manage, in general, as a balance must be struck between delivering fatty-acids and the ability to process them, as unprocessed fatty-acids inhibit adenosine nucleotide translocase, which is responsible for exchanging cytosolic ADP for mitochondrial ATP.

Vitamin and cofactor supplements are also recommended. These supplements serve two possible roles: enzyme function enhancement and serving as antioxidants which may slow the progression of the disease. Table 4 shows some of the supplements that may be useful in supporting a mitochondrial disorder. The use of supplements remains controversial, as no benefit has been proven under controlled conditions.

Avoidance of physiological stress conditions is also recommended. Factors such as heat, cold, starvation, and lack of sleep have all been suggested to be detrimental to individuals with mitochondrial disorders. Body temperature regulation is often abnormal, as it is related to uncoupling processes in the metabolic network, and a sudden gain or drop in body temperature can trigger a metabolic crisis. Toxins, obviously, can be an insult to metabolic demand in the body, and alcohol has been associated with onset of a crisis. For some, these stresses may result in a temporary decompensation, while in others it may lead to a progression of the disease.

This suggested regimen, while apparently haphazard, offers some insight to the possible success of potential targeted therapies. Recall, a heteroplasmy of around 90% is required for most disorders to become pathological. The pathological behavior of these disorders is based on a threshold effect. These supplements, in essence, seek to make a modest, but critical, change on the pathological threshold. So, any successes of these modest changes is very hopeful indeed.

### C.3.2 The Future: Targeted Gene Therapy

As gene therapy technology improves, the possibility grows that the underlying genetic defects inherent in mitochondrial disorders could be remedied. Before any strategy can be attempted, some way to convey genetic material directly to the mitochondria must be found. Peptide nucleic acids (PNA) oligomers are good candidates for delivery into mitochondria. They are synthetic DNA analogues with aminoethyl glycine units used to replace the deoxyribose phosphate backbone, and are connected to conventional bases by a methyl carbonyl linker.[31] As this structure is linear, and as it has the same spacing as nucleic acids, it hybridizes with complementary DNA and RNA sequences. The neutral PNA backbone increases both binding affinity relative to DNA and resistance to nucleases and proteases. These attributes make PNA oligomers very attractive anti-sense reagents.

However, they cannot transport through lipid bi-layers with any efficiency, so several methods have been suggested to facilitate transport. One is through the linkage of these PNAs to a mitochondrial signal peptide derived from ornithine transcarbamylase.[32] This vector system has been shown to be effective, capable of transporting passenger molecules of up to 3000bp into the mitochondria, via the mitochondrial protein transport machinery. However, the PNA backbone is susceptible to proteolysis, and as this transport system is relatively slow, is of concern. Another method that has been suggested is to conjugate the PNA to the triphenylphosphonium cation.[33][34] These lipophilic cations easily permeate lipid bilayers and are taken up into the cytoplasm, driven by the plasma membrane potential. Once in the cytoplasm, they are preferentially concentrated inside the mitochondria, due to the much greater potential there. This method is perhaps most attractive for transport of native DNA bases in the form of linear plasmids, due to the low residence time in the cytoplasm, where the vector will be subject to degradation. Neither of these vectors seems to be preferred at present, though refinements will certainly

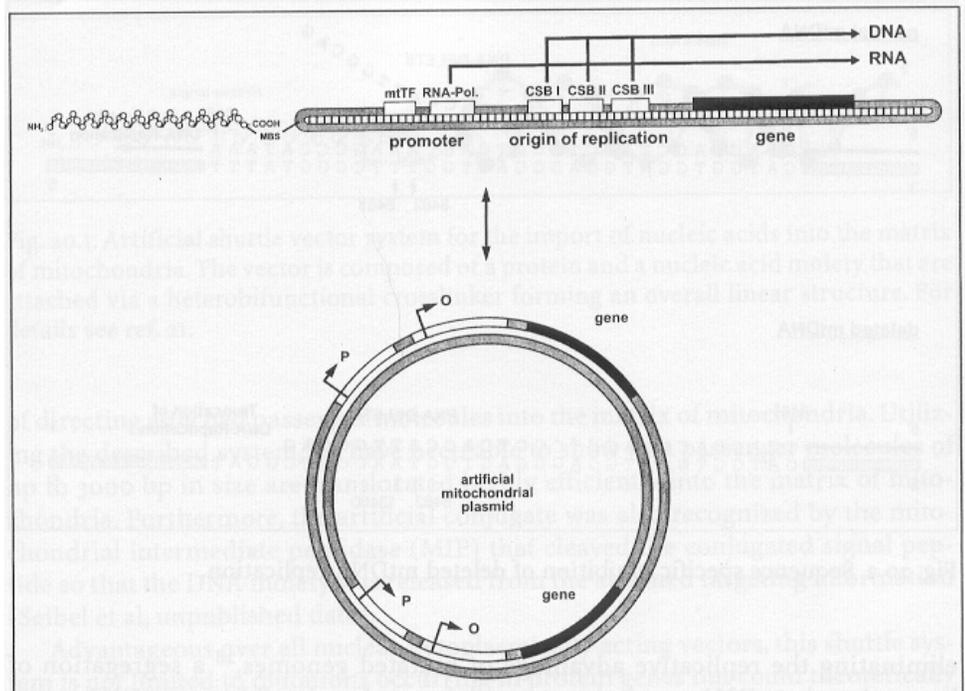


Figure 10 Model of a self-replicating mitochondrial plasmid. From [9].

take place to bring one of both forward as a viable method.

Once a reliable vector system is in place, two possible strategies have been suggested as a therapeutic intervention. The first is that of an anti-genomic approach, where single-stranded DNA or RNA is transported to the mitochondria, where it interferes with mutant DNA replication or RNA translation. The premise is that by inhibiting mutant protein expression or mutant mtDNA replication, an advantage can be given to the wild-type mtDNA. Recall, the disease pathology indicates a threshold effect, so even a modest and temporary suppression of mutations may well put the disease into a remission. This work has been pioneered by Taylor and others, and shows much promise. However, *in vitro* cell culture experiments reveal no improvement of heteroplasmy towards the normal genotype.[33] New thoughts have arisen from this, and current research is geared towards PNAs that are capable of forming a stable triple helices with non-replicating mtDNA. Another approach, more appealing for its permanency, would be to transport a autonomously replicating mitochondrial gene in the form of a linear plasmid. Figure 10 diagrams such a construct, attached to a signal peptide. The issue of construct size is a glaring one, but this treatment seems eminently suitable for single gene additions, especially those genes encoding tRNA. These genes are very small, and a construct containing the entire complement of tRNA genes would be immensely beneficial. Again, this work is ongoing, so no statement can be made as to the virtue of either.

These therapies were included in this discussion to highlight the utility of this project. Once we have established a model that is capable of incorporating heterogeneity and predicting metabolic state based on this information, these therapies will have an existing method by which the efficacy of a “dose” of genetic modification can be evaluated for potential success. For example, if an anti-genomic therapy's dose-response profile for heteroplasmy can be developed from an animal model, that information can be translated to effect on the metabolic network developed in this project. No work towards this end has been made, due to the availability of these therapies, but efforts to integrate them into our model will be discussed in our methods.

### **C.3.3 Summary of Preliminary Work – Specific Aim #3**

We have documented the kind of therapies, supportive and supplemental, that are currently in practice. As of now, dietary adjustment, vitamin and cofactor supplements, and metabolic stress avoidance are the only available treatments, though the efficacy of these are sporadic, at best. We have shown the current thinking towards a more rational therapy, namely gene therapy. An anti-genomic therapy has been suggested, whereby the normal mtDNA in an affected tissue would be given replicative or expressive advantage and therefore bring the mutant heteroplasmy level below the threshold for disease expression. A gene addition approach has been outlined, where a more permanent solution is sought by providing normal genetic information to the mitochondria so that the supplied corrected gene products can complement the defective products. Our brief statement at the interaction with our project with these therapies will be expanded in a later section. We believe that the knowledge of the possible therapeutic modes available now and in the future should guide the progression of this project, and we have reviewed them here as a preliminary response to Specific Aim #3.

## D. Research Design and Methods

### D.1 Mitochondrial Model - Specific Aim #1

The objective of this phase of the project is to develop a model that can accurately reflect the metabolic processes in healthy tissue. This will yield a set of normal operating parameters for our model. Of course, population variation is to be expected, so a range of allowable flux values will be developed. The general strategy is as follows. Biochemical assays will be performed on biopsied muscle tissue to determine the activities of each of the functional units of the respiratory chain while quantifying oxygen usage as a terminal chain acceptor during the metabolism of standard substrates. Our submodel will then be checked for quantitative agreement with these data. This will then generate a "normal" range of flux values that specify a typical muscle tissue's metabolism. Thus, this set of experiments will be used to validate our model.

#### D.1.1 Quantification of Flux Parameters

*Patients.* Biopsies used for this control study will be taken from patients that undergo a muscle biopsy for neuromuscular symptoms, but are subsequently found free of any mitochondrial disease. Muscle biopsy will be taken from the quadriceps muscle, with informed consent, under general anesthesia. A small incision is made, squamous tissue parted, and a "punch" taken, yielding a cross-section of tissue. As this process is quite invasive, and should only be performed on individuals that are suspected to have a neuromuscular disorder, no account can be given at this time as to the nature of the patient population. Also, it is vital that we stress the importance of our collaborators in Newcastle. Their proximity to a facility that regularly handles diagnoses of mitochondrial disorders means a certain supply of biopsy data. The reproducibility of mitochondrial function is quite good, so specifying a single individual will require a single biopsy be taken.[35] However between-individual differences can be expected, and it is not clear from the literature the ranges we can expect from healthy donors.

*Protocol.* Our protocol follows Rossignol et al. (2000).[18]

- *Isolation of mitochondria.* Fresh muscle is immersed in high-EDTA buffer and freed from tendons and paratendinous tissue, visible fat, and connective tissue. Mitochondria are isolated by differential centrifugation as described by Morgan-Hughes et al.[36] Muscle tissue is collected in isolation medium I (210mM mannitol, 70mM sucrose, 50mM Tris/HCl, pH 7.4 and 10mM EDTA) and digested by trypsin (0.5mg/g of tissue) for 30 minutes. The reaction is quenched with the addition of a trypsin inhibitor (soya bean inhibitor to trypsin: 3:1). The homogenate is centrifuged at 1,000g for 5 minutes. The supernatant is strained on gauze and recentrifuged at 10,000g for 10 minutes. The resulting pellet is resuspended in ice-cold isolation medium II (222mM mannitol, 75mM sucrose, 10mM Tris/HCl, pH 7.4 and 0.1mM EDTA) and a new series of centrifugations (1000g and 7000g) is performed. The final pellet is resuspended in a minimum volume of isolation medium B to obtain a mitochondrial concentration between 50 and 70 mg/mL. This is sufficient mitochondria for around 50 subsequent experiments.
- *Polarographic measurements.* Mitochondrial oxygen consumption is monitored at 37C in a 1mL thermostatically controlled chamber equipped with a Clarke oxygen electrode, in the following respiration buffer: 75mM mannitol, 25mM sucrose, 100mM KCl, 10mM Tris/phosphate, 10mM Tris/HCl, pH 7.4, 50µM EDTA plus respiratory substrate (10mM pyruvate in the presence of 10mM malate or 25mM succinate). The mitochondrial concentration used in this study is 1mg/mL and 2mM ADP is added to ensure an excess. The respiration rates are expressed in natom of oxygen/min/mg-protein. Replicates from the same biopsy offer information as to the precision of these measurements.
- *Isolated-step activity.* These assays are colorimetric, and rely on precisely controlling the available electron acceptors to specify which complex is allowed to function. These data represent the maximal respiration output by each subunit.
  - *Complex I (NADH dehydrogenase).* The oxidation of NADH by complex I is measured using the ubiquinone analogue decylubiquinone as an electron acceptor. The basic assay medium (above) is supplemented with defatted BSA (2.5mg/mL), antimycin A (5µg/mL), 65µM decylubiquinone and 0.13mM NADH in a final volume of 1mL. The enzyme activity is measured by starting the reaction with 50µg of mitochondrial protein. The

decrease in absorption at 340nm measures NADH oxidation.

- **Complex III (ubiquinol cytochrome c reductase).** The use of cytochrome c (III) as an electron acceptor for the oxidation of 20 $\mu$ M decylubiquinone determines complex III activity. Again, the basic assay medium is supplemented, this time by defatted BSA (2.5mg/mL) and cytochrome c (III) (15 $\mu$ M). The addition of 10 $\mu$ g of mitochondrial protein begins the reaction, and the oxidation of cytochrome c (III) is measured at 550nm.
- **Complex IV (cytochrome c oxidase).** To isolate complex IV from the rest of the system, the basic assay medium is supplemented with rotenone (10 $\mu$ g/mL), antimycin A (10 $\mu$ g/mL) and defatted BSA (2.5mg/mL). Ascorbate (3mM) and tetramethyl-*p*-phenylenediamine (TMPD, 0.5mM) functions as an electron donor. Cytochrome c (II) serves as an electron acceptor, and the enzyme activity is measured at 550nm.
- **Global-flux inhibition.** Oxygen consumption is measured in respiration buffer while titrating the system with a known inhibitor of each complex. Negative controls are included, with no inhibitor. Vehicle controls are included to account for media absorbance.
  - Complex I: rotenone from 0 to 60ng/mL
  - Complex III: antimycin from 0 to 80ng/L
  - Complex IV: KCN from 0 to 150 $\mu$ M
  - ATP synthase: oligomycin from 0 to 300 pM
- **Isolated-step inhibition.** Once at a steady state, the samples from the global-flux inhibition are immediately subjected to the isolated-step activity protocol, above.

| Name       | Site of Action                |
|------------|-------------------------------|
| Rotenone   | Complex I                     |
| Antimycin  | Complex III                   |
| KCN        | Complex IV                    |
| Oligomycin | OSCP fraction of ATP synthase |

Table 5 A list of inhibitors of the ETS. Compiled from [9][10].

**Analysis.** Information from the isolated-step activities represents the maximal allowable flux for the respiratory chain units throughout this analysis, effectively placing hard upper-bounds in equation (3). For complexes I, III and IV, direct fitting to inhibition curves can be performed using the isolated-step inhibition data. For ATP synthase, this step is inaccessible experimentally, so we must consider that oligomycin behaves by a non-competitive quasi-irreversible model. A non-linear fitting method described by Gellerich et al. (1990) is used to determine the respiratory flux of this subunit based on the oxygen-consumption flux.[37] This information, along with the overall respiratory rate derived from oxygen consumption allows us to calculate the effect on overall respiration by inhibition of each of the subunits. With this, we can approach our mitochondrial model, and generate simulations that mimic this behavior.

**Expected results, limitations, and remedies.** We do not expect any problems with the execution of the outlined methodology.

- The maximal respiratory data should be in accordance with published data.[35] Rasmussen et al. (2000) report that *in vitro* data extrapolate quite well to *in vivo* characterization, though care should be taken to keep in mind the 30-fold activity rate increase in exercising muscle. Rasmussen et al report that the *in vitro* rate of aerobic free energy production can account for 80-85% of the maximal work rate in skeletal muscle if anaerobic process are accounted for.[35] Hence, we can be reasonably assured that we can extrapolate our *in vitro* data to *in vivo* conditions over a variety of work ranges.
- The maximal respiration environment (no inhibitors) is simulated with the proper substrate used in the study, by adjusting the maximal bounds of each flux in equation (3). The system responds by finding a flux solution for the remaining units that maximize ATP production under these circumstances. The measured oxygen uptake flux from the global-flux inhibition data (with no inhibitor) is compared to that of the simulation. A stable solution, internal or equal to the maximum bounds imposed by the isolated-step information is found.
- The isolated-step inhibition data for each complex are expressed in terms of a percent activity relative to the isolated-step activity data. The oxygen global-flux data (in percentage units) can then be expressed as a function of the percent inhibition of the isolated-step inhibition. Simulating a zero to one-hundred percent inhibition of a complex can be performed in our model by multiplying the upper bound of the flux in equation (3) by that percentage. Oxygen utilization predicted by our model will match that seen in the data.

- Our model should be able to develop threshold curves from the data, such as seen in Figure 4. We expect a gradual decrease in global respiratory function, followed by a sharp decrease. We base this on our previously discussed case-study of section C.1.4, particularly Figure 9. There, we see a bi-phasic ATP production response to malate dehydrogenase activity. However, that was due to the availability of an alternate shuttle to compensate, so the drop-off was not very severe.
- Regulatory processes are not accounted for in this model. The question of where the regulation takes place is still a matter of discussion in the literature. Neither simple output activation (ATP usage) or input-output activation (substrate dehydrogenation and ATP usage) can adequately describe the data.[38] Korzeniewski went on to show, at least partially, that an adequate model of oxidative phosphorylation in hepatocytes demonstrates that vasopressin, adrenaline and glucagon must simultaneously activate the majority of the processes in this system. This led the authors to put forward the idea that the regulatory system controls the overall process at each step. While it is unlikely that the level of activation at each step is the same, this system appears to act in concert, rather than relying on regulation at certain steps. Therefore, our model should be valid regardless of the regulatory process. If it becomes clear that a certain flux is likely regulated, we can integrate that information by treating the regulatory processes as an effector of the flux boundary values.

### D.1.2 Summary of Research Design and Methods – Specific Aim #1

We have outlined the methodology required to assess the capability of our mathematical representation to accurately represent the function of mitochondria. We intend that this model be most accurate and realistic around the components known to be encoded by mtDNA, therefore the data extracted in these studies examine the respiratory chain. These data place upper bounds on the possible maximum function of each of these subunits, and will form the basis by which a unit can be defined as dysfunctional. Later analysis of defective systems will be compared to these values.

### D.2 Mapping of Genetic Defects to Disease Expression - Specific Aim #2

The goal of this portion of the project is to quantify the impact a disease state has on the metabolic network. Figure 11 shows the respiratory chain units, and indicates where mutations are predicted to have deleterious effects on this network. Refer back to Table 3 for the known mutation locations. First, we must create a screening method that identifies mutations in mtDNA and specifies the level of that mutation (heteroplasmy). Second, we must address the issue of heteroplasmy as it relates to our mitochondrial model.

#### D.2.1 Mutation Detection and Heteroplasmy Assessment

Taylor and Turnbull et al. (2001) have set forward a technique to simultaneously detect mutations and to determine the level of the mutation (heteroplasmy) in a cell.[39]

**Patients.** Control muscle is taken from a patient with no findings of mutations. Sample muscle is taken from patients with a suspected or known mitochondrial mutation. Since this methods is diagnostic, it can be used in the normal course of identifying mtDNA mutations.

**Protocol.** Biopsied tissue is cryosectioned and the sections biochemically assayed for complex activity, allowing for identification of fibers with reduced function. Individual muscle fibers are microdissected using fine glass capillaries and placed in a sterile centrifuge tube with lysis buffer (50mM Tris/HCl, pH 8.5, 1mM EDTA, 0.5% Tween-20, 200 µg/mL proteinase K). Cells are incubated for 2 hours at 55C, followed by thermal inactivation of proteinase K at

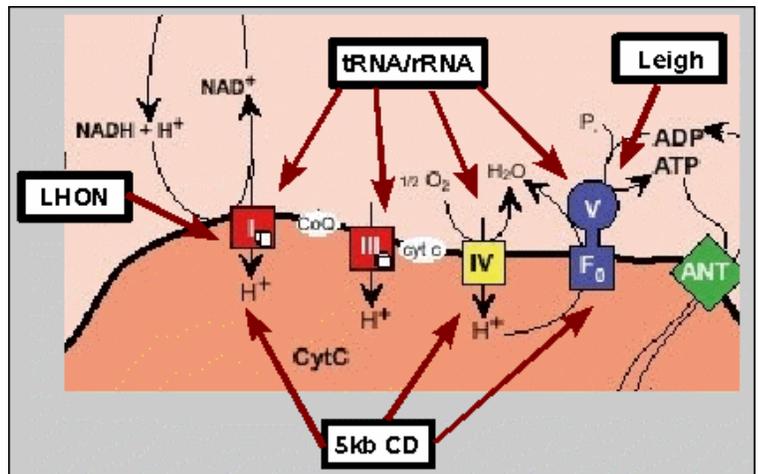


Figure 11 Predicted activity decrease locations for each type of mtDNA mutation. The two specific protein-coding region mutations have impact of limited scope, while the 5kb CD, tRNA, and rRNA mutations are likely to impact many sites of the respiratory chain. Adapted from [4].

95C for 10 minutes. Nine PCR primers are available for primary amplification of mtDNA in ~2kbp fragments. The inability of these primers to amplify nucleus-embedding mtDNA pseudogenes has been studied with human osteosarcoma cell lines, cultured as previously described by King and Attardi (1989), either containing mtDNA (143B.TK; p<sup>+</sup>) or lacking mtDNA (143B206, p<sup>0</sup>). [40] Lack of mtDNA in the p<sup>0</sup> cells are confirmed by Southern blot analysis. Twenty-eight secondary PCR primers have been created to amplify between 600 and 700 bp overlapping fragments to produce enough mtDNA to provide accurate sequence information.

*Analysis.* After the secondary amplification, the PCR-products are purified (QIAquick PCR purification kit; QIAGEN) and sequenced directly with BigDye terminator cycle sequencing chemistry (PE Biosystems) performed on an automated DNA sequencer (ABI 377). Sequences generated are compared to the standard sequence (revised Cambridge Reference Sequence, rCRS) using Sequence Navigator and Factura software (PE Biosystems). The level of the mutation was determined by PCR-RFLP analysis by the amplification of the gene fragment essentially encompassing the mutation site. The mutation site, in combination with forward PCR primer gene creates an additional restriction site, yielding a different band pattern when separated on a gel. Careful controls are in place at each amplification step to ensure reliable quantification.

*Expected results, limitations and remedies.*

- This method has been shown to be very specific for amplifying only the section of mtDNA of interest, and can detect mtDNA variants in as low as 30% of the mtDNA genotype in a cell. Replicates will be required to get an accurate accounting of variation in a cell population extracted from tissue.
- This method does not determine the level of gene product (complex) heterogeneity, but rather the potential for heterogeneity. The suggestion that detection of mutant mRNA would be definitive, and findings indicate that the level of wild-type mRNA is higher than the wild-type mtDNA complement would suggest. [41] However, the activity of the produced complex is higher than the corresponding wild-type mRNA, so mRNA complement will not tell the entire story either. For example, van den Bogert demonstrated a normal rate of ATP synthesis with only 40% wild-type mtDNA. So, we can state that the heteroplasmy level in a cell is the lower bound for heterogeneity in product. Gene product function in a heteroplasmic environment will be addressed in the next section.

## D.2.2 Heteroplasmy in mtDNA

At first blush, heteroplasmy in mtDNA mutations in a cell and tissue appears to be a stumbling block towards creating an accurate and diagnostic model of mitochondrial function.

*Hypothesis.* We will describe heteroplasmy within a mitochondria as two separate pathways within the metabolic network. As such, we can effectively create a double of each of the pathways under direct control of the mitochondria, specifically the respiratory chain complexes. As an aid in visualizing this scheme, let us consider a specific example, a mutation in the gene encoding complex I, such as is found in LHON. If that mutation exists in a mitochondria in various amounts (heteroplasmy), then the gene products will be formed from a mixture of the mutant mtDNA and the wild-type mtDNA. Thus, we can think of the products being two distinct entities. The wild-type product will have the expected activity, while the mutant product will have less than the expected activity. Effectively, this reduces the possible flux through the mutant gene product. After adding a branch in the respiratory chain model, this would result in a further underspecification of the model. Figure 12 shows a cartoon of this branch addition. However, knowledge of the level of heteroplasmy allows for an additional bound to be placed on the model. Specifically, equation (3) must be amended to include this information.

$$\begin{aligned}
 0 \leq v_j &\leq \alpha_j, \quad \forall j \\
 \gamma_{ik} &= v_i / v_k \\
 0 \leq v_i + v_k &\leq \alpha_{ik} \quad (3a) \\
 0 \leq v_k &\leq \frac{\alpha_{ik}}{1 + \gamma_{ik}}, \quad 0 \leq v_i \leq \frac{\alpha_{ik}}{1 + 1/\gamma_{ik}}
 \end{aligned}$$

In equation (3a) we have added an additional constraint such that the fluxes for the branched pathways,  $v_i$  and  $v_k$ , are tied by a proportionality constant,  $\gamma_{ik}$ . This constant is related to the heteroplasmy value in the mitochondria,

and will be referred to henceforth as the heteroplasmy constant. Additionally, the bound for the two combined fluxes is specified. If  $v_i$  is the flux for the wild-type complex I, and the mitochondria is nearly all wild-type (approaching 0% heteroplasmy), then  $\gamma_{ik}$  is approaching infinity. As this occurs, the flux  $v_k$  is constrained to approach zero, while the flux  $v_i$  is allowed to approach the bound of the sum of these two fluxes,  $\alpha_{ik}$ . It is perhaps unclear why this particular scheme is used, rather than a more straightforward allowance for the heteroplasmy to have a more natural range (zero to one). This is to avoid creating a situation where the solution to equation (2) contains nonlinear terms, which would result in serious problems applying LP techniques.

*Protocol.* Biopsies taken from those patients that are subsequently found to have a mutation in a gene encoding only a single complex or genes that cause multiple defects in the respiratory chain. Establishment of a mutation in the mtDNA and the level of heteroplasmy will be determined by the detection and assessment protocol described (section D.2.1). Functional aspects of the sampled mitochondria will be determined by the flux analysis protocol discussed above (section D.1.1). Our mitochondrial model will be refit with the new constraints, equation (3a), and the novel branches added to the pathway network.

*Analysis.* Identification of a mtDNA mutation leading to a respiratory chain defect will be made in concert with heteroplasmy information for that mutation. This places a reasonable lower bound on our heteroplasmy constant,  $\gamma_{ik}$ , as the activity of the wild-type pathway is expected to be greater than the proportion of wild-type mtDNA to mutant mtDNA. The flux bound on the sum of the fluxes through the branched pathway,  $\alpha_{ik}$ , is available from the respiration studies of mitochondria bearing mutant mtDNA.

*Expected results, limitations, and remedies.*

- We expect that this set of protocols will allow us to test our hypothesis that mutant and wild-type gene products can be expressed as discrete pathways in our metabolic network. This analysis will provide insight into the relative contributions of the wild-type and mutant gene products, as well as their relationship with the genetic heteroplasmy that gives rise to them.
- A possible limitation may be the inability of this hypothesis to explain the data. A more complex formulation of the branched pathways would be attempted, perhaps by altering state “B” in Figure 12 that arises from flux to the mutant complex to have a flux that “drains” a fraction of the flux normally routed to state “C”. This would, in effect, generate a “loss” term in the flux-balance equations, indicating the inability of this complex to 100% efficiently perform its function. It should be noted here that model construction is often an iterative process, relying on further data to refine its description of the system. Here, we have presented a set of experiments that supply the data necessary for hypothesis testing in this kind of developmental framework.
- A possible limitation to this method may be the inability to discriminate between defects in multiple locations in the metabolic network. However, we believe that the isolated-step and complex inhibition data should address this issue. The reduction in global-flux activity should be attributable to isolated complexes, but this relationship may not be easily dissected. More advanced data analysis may be required to address this issue.

### D.2.3 Summary of Research Design and Methods – Specific Aim #2

We have detailed a method by which the level of genetic heteroplasmy can be determined: the cause of the dysfunction. We have also described how we extract data pertinent to the function of a defective pathway: the effect of the dysfunction. One of the key benefits and contributions of our model will be to explore the relationship between heteroplasmy and pathway function. We have put forward a reasonable structural model: the defective gene products function as a (less efficient) parallel pathway to the normal gene products. If this relationship holds over a range of diseases and patients, it may be sufficient to start guiding therapies.

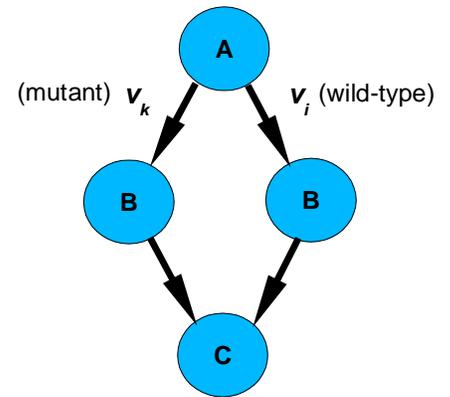


Figure 12 Cartoon of a branched pathway to describe two distinct “states” of metabolic capacity. Wild-type and mutant reactions yield the same product.

### D.3 Therapeutic Interventions - Specific Aim #3

The objective of this portion of the project is to apply the predictive power of our mitochondrial model to investigations of therapeutic applications. The concept of a “rate-limiting” step in a metabolic network has fallen by the wayside since the establishment of metabolic control theory. We contend that targeted supportive therapies (e.g. coenzyme Q) have failed to improve the metabolic function of a disease tissue due their lack of treatment of the metabolic system as a whole. However, the supportive general (shotgun) therapy discussed in section C.3.1 has seen some benefit, at least in helping mild cases. This indicates to us that a better approach would be to examine a therapy in the context of the entire network. Through the use of an animal model and our computational model, such a therapy can be designed and examined for efficacy. Beyond this, emergent gene therapy outlined in section C.3.2 dovetails well with the approach. The only limitation to this effort is the creation of a suitable animal model.

#### D.3.1 Animal Model

Our protocol follows Clark and Turnbull et al. (1998) , and exact details of the method are available in the literature.[42]

*Patients.* Myoblast cultures are obtained from biopsies performed on subjects subsequently found to have no mitochondrial disorders and subjects that are found to have a protein-coding gene mutation, under informed consent.

*Protocol.* Animals used are immunodeficient BALB/CSCID mice (Fox Chase Suppliers, Charles River, UK). X-ray irradiation is used on the right hind limb to render the normal muscle incapable of regeneration before the myoblasts are injected. All the mice injected with the mutant mtDNA are sacrificed after thirty days, while those mice injected with normal (control) myoblasts are sacrificed after eight weeks, to establish stability of the regenerated tissue. Histological, histochemical and immunocytochemical analysis are performed on the regenerated tissue.

*Analysis.* Human specific primary antibodies for  $\beta$ -spectrin and subunit II of cytochrome c oxidase were combined with a rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO A/S, Glostrup, Denmark) to detect fibers formed from human myoblasts. PCR detection for the gene mutation capitalize on additional restriction sites, leading to an additional fragmentation of the normal PCR product into two smaller fragments, which are detectable using standard techniques such as the PCR-RLFP protocol described in section D.2.1.

*Expected results, limitations, and remedies.* This techniques has been found to work quite well in two cases: a T8344C MERRF mutation and a T8993C NARP mutation (covered in section C.2.2). In each case, the mutation was expressed stably and with a heteroplasmy level consistent with the original myoblasts.

#### D.3.2 Supportive Therapeutic Design

*Patients.* As before, myoblast cultures are obtained from biopsies performed on subjects subsequently found to have no mitochondrial disorders and subjects that are found to have a protein-coding gene mutation, under informed consent.

*Protocol.* The objective to our design is to examine the effect of supportive therapies by establishing a dose-response curve for the kinds of dietary supplements given in Table 4. Myoblasts from normal and diseased biopsies will be transplanted to our animal model, outlined above. Standard clinical trial techniques will be employed to best characterize the dose-response of the diseased tissue compared to the normal tissue.

*Analysis.* In this case, the dose-response we wish to characterize is (a) possible reduction in heteroplasmy attributable to the therapy and (b) improved overall flux through the affected metabolic pathway attributable to the therapy. Each of these data are available from the protocols outlined prior.

*Expected results, limitations, and remedies.*

- We expect no issue in the execution of the protocol itself. However, it is unlikely that any of the supportive therapies will greatly improve the metabolic network. Improvements will be seen in the overall global-flux data, which can then be attributed to specific or multiple isolated-step activities. Failure the ability to match

improvements to isolated-step activities will be regarded as improvements elsewhere in the system. If a large improvement was found elsewhere in the system, such a finding would warrant studies in candidate metabolic blocks (glycolysis and the TCA cycle).

- Even in the likely event that no discrimination is possible between healthy and diseased tissue, such as when the therapy is generally supportive to the overall metabolic process, this information is still valuable when assessing how to tailor a therapy to a particular disease. Iron, for example, is cited as a suspicious therapeutic agent, as it on one hand aids in synthesis of heme subunits in the mitochondria, but on another may be responsible for creating reactive oxygen species (ROS) via the Fenton reaction, which is known to cause damage to the mitochondria.[3] There may be other examples when supplying too much supportive therapy is deleterious.
- We also expect that a synergy effect is likely, as the metabolic network depends on a balance of functioning subunits. Therefore, there is likely a regimen that optimally supports the flux of each subunit in such a way that optimizes the combined flux network. Such a finding, again, would be most useful when designing a therapy to support a particular disease.

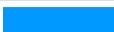
### D.3.3 Future Work: Gene Therapy

As presented in section C.3.2, we are poised on the verge of being able to deliver a gene therapy to a diseased tissue. Whether this therapy will be anti-genomic or gene-additive is speculative. However, as such technologies are explored in the Mitochondria and Disease Research Group, our animal model and computational model will be used to guide a therapeutic goal. Once an empirical, or semi-empirical, relationship between heteroplasmy and pathway function is established, then the parameters for an effective therapy can be defined by heteroplasmy reduction needed to bring a disrupted pathway to a necessary level of function. At that time, a renewal or new funding source will be sought for this work.

### D.3.4 Summary of Research Design and Methods – Specific Aim #3

We have detailed a mouse model that is capable of expression wild-type and mutant mitochondria of whatever particular disease state. We have focused on diseases that cause a deleterious effect at a specific location in the metabolic network, but this is not a requirement of our experimental methods. Rather, this allows us to attribute therapeutic benefit to an increase in a specific damaged step in our network. This promotes rational therapy design tailored to a particular disease state. Our system, however, is quite capable of discerning benefit from a melange of supportive therapies (vitamins, cofactors and minerals) for a diseased tissue with a variety of dysfunctions.

## D.4 Timeline

| Task (Specific Aim)                                | Duration  | Year 1  | Year 2  | Year 3  |
|--|-----------|---|---|---|
| Quantification of Flux Parameters (1)              | 6 months  |  |   |   |
| Mutation Detection and Heteroplasmy Assessment (2) | 6 months  |   |   |   |
| Heteroplasmy in mtDNA (2)                          | 12 months |   |  |   |
| Animal Model (3)                                   | 3 months  |  |   |   |
| Supportive Therapeutic Design (3)                  | 12 months |   |   |  |
| Future Work: Gene Therapy (3)                      | future    |   |   |  |

## D.5 Summary of Research Design and Methods

We have outlined the methodology required to assess the capability of our mathematical representation to accurately represent the function of mitochondria. These studies provide upper bounds on the possible maximum function of each of the subunits in the respiratory chain, and will form the basis by which a unit can be defined as dysfunctional. We have shown method to determine the cause of the dysfunction: genetic heteroplasmy. The same methods employed to assess our model in healthy states can be used to determine the effect of the

dysfunction: complex activity information. One of the key benefits and contributions of our model will be to explore the relationship between heteroplasmy and pathway function. We have put forward a reasonable model of how the effect of the genetic dysfunction plays out in complex performance. We have a mouse model that is capable of expression of whatever particular disease state is found in mitochondria. In the cases where a particular gene mutation resolves to a definite defect in the network, we can look for therapies that improve that function. In the future, as gene therapies become available, our system will be able to specify the therapeutic benefit of improving the heteroplasmic complement of wild-type mtDNA. We have designed a modeling and experimental system robust enough to search of benefit from a variety of supportive therapies (vitamins, cofactors and minerals) that we would not predict, *a priori*, to have any benefit, but is seen in the clinical setting to provide therapy.

## E. Human Subjects

The genotype of many individuals will be required for this project. This information is publicly available, using blinded identity tags. During this course of this project, collaboration with other researchers in this area may bring additional sequencing information, and any unique identification information associated with these transmittals will be removed and replaced with identifying tags, in accordance with 56 CFR 46 (<http://ohrp.osophs.dhhs.gov/humansubjects/guidance/45cfr46.htm>).

In addition, biopsies will be gathered from human subjects for the purposes of isolating myoblasts from muscle tissue. However, this biopsy procedure is very invasive, requiring surgery and anesthesia in most cases, and results in a scarring in the area biopsied on the order of one to two inches. In light of this, we will use biopsies gathered with informed consent in the course of diagnosing mitochondrial diseases when blood samples are inconclusive. The Mitochondria and Disease Research Group at the University of Newcastle upon Tyne is commonly involved in such diagnosis, and will present an invaluable resource in terms of access to biopsies.

As such, the Inclusion of Women and Minorities Policy (<http://www.nih.gov/grants/guide>) and Inclusion of Children Policy (<http://www.nih.gov/grants/guide/notice-files/not98-024.html>) cannot be strictly adhered to. However, it must be said that incorporating racial diversity into any mitochondrial studies is vital to the success of mitochondrial research as a whole. Mitochondrial DNA has recently come into the spotlight as a way to track the migration over time of the human species. As such, we can expect this ethnic diversity to be played out in the genetic diversity of mitochondrial DNA. Therefore, to fully understand the breadth of diversity (pathological and non-pathological polymorphisms), a cross-sectional approach should be made to fully understand what genotypes exist, as well as to establish the prevalence of mitochondrial disorders across ethnic groups. However, this is outside the scope of this project. Moreover, mitochondrial DNA is inherited strictly from the maternal line. Therefore, the typical (nuclear DNA) classification of "race" does not apply here. As an example, consider the case where the first generation of a family line is race "A". If we track the maternal line through ten generations off offspring with race "B", we will still find the mitochondrial complement normal for race "A" in female offspring, even though the phenotype will, in all likelihood, be predominantly race "B".

## F. Vertebrate Animals

A separate Animal Welfare Assurance has been filed with the Office for Protection from Research Risks for the Mitochondria and Disease Research Group laboratories at the University of Newcastle upon Tyne. Additionally, the laboratory has on-file all the necessary supporting documents with the parallel structure in Great Britain. These facilities have carried out vertebrate research for many years, and are committed to the ethical and humane treatment of laboratory animals.

## G. Recombinant DNA

Chimeric protein-DNA molecules derived for this research fall outside the provisions set forward by the NIH, as the DNA portion of this molecule is derived solely from same-species sequences, and will be re-introduced into that same species.

From "NIH Guidelines for Research Involving Recombinant DNA Molecules, January 1991"  
(<http://www4.od.nih.gov/oba/rac/guidelines/GUIDELINjan01rev.pdf>):

*Section III-F-4. Those that consist entirely of DNA from an eukaryotic host including its chloroplasts, mitochondria, or plasmids (but excluding viruses) when propagated only in that host (or a closely related strain of the same species).*

Moreover, any anti-sense PNA entities synthesized for this project have no ability to self-replicate, and therefore fall outside the guides set forth, above.

Although clear from the Methods section of this proposal, it should be re-iterated that **no plans** are made to re-introduce any modified myoblasts back into human subjects.

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## I. Consortium/Contractual Agreements

### I.1 Mitochondria and Disease Research Group, Medical School, University of Newcastle upon Tyne

This research group (Professor DM Turnbull, Professor RN Lightowlers, Dr PF Chinnery) has been established at the Medical School for over ten years. The Group has a strong international reputation for working at the interface

between basic and clinical science. Newcastle is a major referral center for patients with mitochondrial disease. As well as providing an excellent diagnostic facility, the Group is interested in the molecular etiology of these disorders and in particular those disorders that are due to defects of the mitochondrial genome. The Group has many ongoing projects which focus on mitochondrial genetics and disease.

Fiscal support from this project for this group will go towards the costs of housing, feeding, and experimentation on the animal models previously described. Synthesis of novel oligonucleotides is already ongoing at this facility, and supported by existing grants. These animals are already present at this site, and the experimentation described has already been done in a published study. Therefore, it is more economical to support this group than to face capitalization costs involved in setting up a duplicate resource here at the University of Washington. Moreover, locating the experimental animals proximally with the oligonucleotide research also represents a savings to this project.

The Mitochondria and Disease Research Group is proximal to the Medical School, which is a common referral site for patient suspected of having a mitochondrial disease. As such, this location presents a vital component to this research: gathering human subject samples.

Letters confirming the administrative and programmatic support from the University of Washington and the University of Newcastle upon Tyne in accordance with PHS consortium policy are attached.

## **J. Consultants/Collaborators**

### ***J.1 Douglass Turnbull, M.D./Ph.D., University of Newcastle upon Tyne, Newcastle, Scotland***

- Execution of *in vivo* mouse model experiments.
- Synthesis of mitochondrial gene therapy vectors.

## Appendix A. Qualifying Exam Question 2

A variety of metabolic diseases effecting skeletal muscle and/or cardiac function (e.g., myophosphorylase deficiency) result from a missing or malfunctional enzyme that would otherwise catalyze an essential metabolic pathway. How would you computationally characterize the central metabolic systems involved in one or more of these diseases? You might use a constraint-based approach (Edwards et al., *Nat. Biotechnol.* 19:125-130, 2001, and references therein), a more conventional kinetic approach (Bassingthwaighe, *Phil. Trans. R. Soc. Lond. A* 359:1055-1072, 2001, and references therein), or develop some hybrid method. You must design experiments, or use existing experimental data for evaluation/validation of the model. You must motivate the development of modeling technology with relevant applications. A first step would be to evaluate the disease phenotypes at the metabolic level. In addition, pharmaceutical therapies could be evaluated and optimal treatments, based on model predictions, determined.

## Appendix B. Probable or Unconfirmed Point Mutations in Mitochondrial Protein Coding Regions

From most current databases [4].

| Gene     | Mutation | Amino Acid Change | Disease                             |
|----------|----------|-------------------|-------------------------------------|
| 12S rRNA | A1555G   |                   | DEAF                                |
| 16S rRNA | 2835T    |                   | Rhett syndrome                      |
| 16S rRNA | 3196A    |                   | ADPD                                |
| ND1      | T3308C   | M-T               | MELAS                               |
| ND1      | G3316A   | A-T               | NIDDM; LHON; PEO                    |
| ND1      | T3394C   | Y-H               | LHON                                |
| ND1      | T3394C   | Y-H               | NIDDM                               |
| ND1      | A3397G   | M-V               | ADPD                                |
| ND1      | G3496T   | A-S               | LHON                                |
| ND1      | C3497T   | A-V               | LHON                                |
| ND1      | A4136G   | Y-C               | LHON                                |
| ND1      | T4160C   | L-P               | LHON                                |
| ND2      | G5244A   | G-S               | LHON                                |
| ND2      | G5460A   | A-T               | AD                                  |
| ND2      | G5460T   | A-S               | AD                                  |
| COX1     | G5920A   | W-Ter             | Myoglobinuria, Exercise Intolerance |
| COX1     | G6930A   | G-Ter             | Multisystem Disorder                |
| COX1     | G7444A   | Ter-K             | LHON                                |
| COX2     | T7587C   | M-T               | Mitochondrial Encephalomyopathy     |
| COX2     | T7671A   | M-K               | MM                                  |
| COX2     | G7896A   | W-Ter             | Multisystem disease                 |
| COX2     | 8042     | M-Ter             | Lactic acidosis                     |
| ATPase 6 | T9101C   | I-T               | LHON                                |
| ATPase 6 | T9176G   | L-R               | Leigh Disease                       |
| COX3     | G9438A   | G-S               | LHOQuaN                             |
| COX3     | G9738T   | A-S               | LHON                                |
| COX3     | G9804A   | A-T               | LHON                                |
| COX3     | G9952A   | W-Ter             | Mitochondrial Encephalopathy        |
| COX3     | T9957C   | F-L               | PEM; MELAS                          |
| ND3      | T10191C  | S-P               | ESOC                                |
| ND4      | T10663C  | V-A               | LHON                                |
| ND4      | A11084G  | T-A               | MELAS                               |
| ND4      | G11832A  | W-Ter             | Exercise Intolerance                |
| ND4      | A12026G  | I-V               | DM                                  |
| ND5      | G13513A  | D-N               | MELAS                               |
| ND5      | G13708A  | A-T               | LHON                                |
| ND5      | G13730A  | G-E               | LHON                                |
| ND6      | C14568T  | G-S               | LHON                                |
| CytB     | 14787    | I-frameshift      | PD / MELAS                          |
| CytB     | G15059A  | G-Ter             | MM                                  |
| CytB     | G15242A  | G-Ter             | Mitochondrial Encephalomyopathy     |
| CytB     | G15257A  | D-N               | LHON                                |
| CytB     | G15615A  | G-D               | Exercise Intolerance                |
| CytB     | G15762A  | G-E               | MM                                  |
| CytB     | G15812A  | V-M               | LHON                                |

## **Appendix C. Digital Media**

The hardcopy of this work includes a reference CD, with this manuscript, bibliography list, and full-text articles cited in this work. This information is also available online:

**<http://the-magister.com/projects/Qual/>**