Damascus University

Faculty of Agriculture

Patho-physiological Mechanisms of Dominant Optic Atrophy

Thesis Submitted to Fulfillment the Partial Requirements for Master Degree in Biotechnology

Prepared By

Alia Mohamad Shams

Supervised by

Dr. Chadi Soukkarieh

Faculty of Science -Damascus University

Dr. Salam Lawand

Faculty of Agriculture-Damascus University

In Collaboration With

Dr. Guy Lenaers

Institute for Neurosciences of Montpellier-France

The Board of Examiners

This thesis was discussed and approved on 30.10.2014 at the Faculty of Agriculture- Damascus University

Prof. Dr. **Essam Qassem** - Damascus University - Faculty of Science

Dr. **Chadi Soukkarieh -** Damascus University - Faculty of Science

Dr. **Aida Jaluol** - Damascus University - Faculty of Agriculture

قرار لجنة الحكم

نوقشت الرسالة بتاريخ 2014/10/30 في كلية الهندسة الزراعية – جامعة دمشق وأقرت من قبل السادة المحترمين أعضاء لجنة الحكم:

أ.د **عصام قاسم** – جامعة دمشق– كلية العلوم

د. **شادي سكرية** – جامعة دمشق– كلية العلوم

د. **عايدة جلول** – جامعة دمشق– كلية الزراعة

Certificate

We certify that the work described in this thesis, is the result of a scientific research, carried out by the candidate **Alia Mohamad Shams**, under the supervision of Dr. **Chadi Soukkarieh** a professor in Animal Biology Department, Faculty of Science, Damascuc University, and Dr. **Salam Lawand**, a professor in the Agronomy Department, Faculty of Agriculture, Damascus University. Any other references discussed in this thesis are documented in the text.

Candidate	Supervisor	Supervisor
Alia Mohamad Shams	Dr.Chadi Soukkarieh	Dr. Salam Lawand

شهادة

نشهد بأن العمل الموصوف في هذه الأطروحة، نتيجة بحث علمي قامت به المرشحة **عالية محمد شمس**، تحت إشراف الدكتور **شادي سكرية** مدرس في قسم علم الحياة الحيوانية، في كلية العلوم بجامعة دمشق، والدكتورة **سلام لاوند**، الأستاذ المساعد في قسم المحاصيل الحقلية، في كلية الزراعة بجامعة دمشق. وإن أي مراجع أخرى بحثت في هذه الأطروحة موثقة في النص.

المرشحة	الدكتور	الدكتورة
عالية محمد شمس	شادي سكرية	سلام لاوند

Declaration

I declare that the research described in this thesis titled: **Patho-Physiological Mechanism Of Dominant Optic Atrophy**, is never previously presented to obtain any other university degree, currently is not submitted so by any other researcher, and that all the work and the results listed are my personal effort and under the guidance of the scientific supervisor. Any information or other results in the thesis have been reported to its sources and authors in the list of references.

Candidate

Alia Mohamad Shams

تصريح

أصرح بأن البحث الموصوف في هذه الأطروحة تحت عنوان: **دراسة الآليات الفيزيولوجية لمرض ضمور العصب البصري الراجح**، لم يسبق أن قُدم للحصول على أي درجة جامعية أخرى، وغير مقدم حالياً لذلك من قبل باحث آخر، وأن كافة الأعمال والنتائج المذكورة هي جهودي الشخصية وبتوجيه من المشرف العلمي. وقد نسبت أية معلومات أو نتائج أخرى ذكرت في الأطروحة إلى مصادرها ومؤلفيها في قائمة المراجع.

المرشحة

عالية محمد شمس

Acknowledgements

It is a humbling experience to acknowledge those people who have helped along the journey of my master; I am indebted to so many for encouragement and support.

Special note of thanks to my family, you should know that your support and encouragement was worth more than I can express on paper.

My sincerest thanks are extended to my supervisors, for their encouragement and guidance, to the jury for their valuable comments on the research.

To all members of technical and support staff from neurogenetics platform, imaging platform and others in INM (Institut des Neurosciences de Montpellier), to the patients and their families for their appreciated collaboration.

To many friends I am grateful they are always around when needed, to those who are away; you are missed.

INDEX

ABBREVIATION TABLE

OA	Optic Atrophy
OPA	Optic Atrophy
ADOA	Autosomal Dominant Optic Atrophy
AROA	Autosomal Recessive Optic Atrophy
DOA	Dominant Optic Atrophy
LHON	Leber's Hereditary Optic Neuropathy
IM	(Mitochondrial) Inner Membrane
OM	(Mitochondrial) Outer Membrane
OXPHOS	Oxidative Phosphorylation
mtDNA	Mitochondrial DNA
OH	Origin of H strand DNA replication
OL	Origin of L strand DNA replication
PCR	Polymerase Chain Reaction
RMC	Random Mutation Capture
Gen	Genistein
SIF	Soy Isoflavone
ROS	Reactive Oxygen Species
RGC	Retinal Ganglion Cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MRI	Magnetic resonance imaging

LIST OF FIGURES

LIST OF DIAGRAMS

LIST OF TABLES

ABSTRACT

Dominant Optic Atrophy (DOA) is a neuro-ophthalmic condition characterized by a bilateral degeneration of the optic nerves, causing insidious visual loss, typically starting during the first decade of life. The disease affects primary the retinal ganglion cells (RGC) and their axons forming the optic nerve, which transfer the visual information from the photoreceptors to the lateral geniculus in the brain. Two genes (OPA1, OPA3) encoding inner mitochondrial membrane proteins and three loci (OPA4, OPA5, OPA8) are currently known for DOA. Additional loci and genes (OPA2, OPA6 and OPA7) are responsible for X-linked or recessive optic atrophy. All OPA genes yet identified encode mitochondrial proteins are embedded in the inner membrane and ubiquitously expressed. Optic nerve degeneration is therefore due to disturbed mitochondrial function. However, the trigger for RGC loss is much more complex than a simple bioenergetic crisis. More than 200 mutations in the OPA1 gene have been found to cause optic atrophy type 1 (Dominant form). The most conmen mutation in this gene is R445H. While, a nonsense mutation in the TMEM126A gene which is also known as OPA7 has been shown to be related to optic atrophy type 7 (receive form).

A set of different aspects of optic nerve atrophy had been studied in this work, where a search for mutations in the gene TMEM 126A took place with a group of patients and all samples illustrated negative results. Then a search for quality and quantity mutations in mitochondrial DNA concerning a group of mice with genotype OPA1 +/- where carried out and results showed no relationship between the mutation on OPA1 and mitochondrial DNA. Finally in this thesis we studied the impact of Genistein as an anti-oxidant on the fibroblast cells obtained from patients carrying the mutation R445H OPA1, where it was found that this product have a positive impact in increasing cell viability, a slight improvement in the structure of the network and an effect on the mitochondrial membrane potential.

LITERATURE REVIEW

Mitochondria

Mitochondria are eukaryotic subcellular organelles which are commonly referred as the powerhouse of the cell, since they produce most of the cell's energy supply. These organelles, however, are the main players in many other cellular processes, not only the energy production, but also thermogenesis, apoptosis, reactive species of oxygen production and calcium homeostasis.

Mitochondria present a complex double membrane system, where the outer membrane (OM) bounds the organelle, and the inner membrane (IM) separates the matrix space from the intermembrane space. The IM is organized in distinguished compartments, the peripheral inner membrane and the cristae, defined by several lines of evidence as the active site of oxidative phosphorylation (Perotti *et al.,* 1983; D'Herde *et al.,* 2001; Vogel *et al.,* 2006), separated from the peripheral inner membrane by narrow tubular structure, called cristae junction (Perkins *et al.,* 2001; Frey *et al.,* 2000).

In the cytosol, mitochondria form a highly dynamic reticulum, which morphology varies according to cell types and contexts, also depends on continuous fission and fusion of both mitochondrial OM and IM (Chen *et al.,* 2004, 2005; Liu *et al.,* 2009). The ultrastructure and the reticular organization of the organelle are determined by mitochondria-shaping proteins that regulate the equilibrium between fusion and fission processes (Chen, X. *et al.,* 2005).

Having multiple copies of own DNA, mitochondria exhibit unique structures among the cytoplasmic organelles, and their DNA encodes a small number of proteins, essential for their energetic function

(Anderson *et al.,* 1981). However, nuclear genes encode the majority of the proteins that are fundamental for mitochondrial physiology, including those responsible for expression and maintenance of the mtDNA. Proper mitochondrial function thus relies on the coordinate expression of two separate genetic systems, the mitochondrial and the nuclear genomes. (Bar-Yaacov *et al.,* 2012).

The critical importance of this organelle is highlighted by the escalating number of human disorders that in recent years have been shown to be related to or in connection with mitochondrial dysfunctions. Many rare genetic disorders are caused by mutation either in mitochondrial DNA or in nuclear genes encoding for mitochondrial proteins. Mitochondrial dysfunction has also been implicated in more common human diseases, such as several degenerative diseases, cancers, diabetes, and even in the natural process of aging (Zeviani and Antozzi, 1997; Wallace, 2005; Mao and Holt, 2009; Trifunovic, 2006).

Mitochondrial Ultra-structure

As dual-membrane organelles, the OM in mitochondria is highly permeable, containing many pores for small molecules to pass through. In contrast, the mitochondrial IM is impermeable to small molecules and ions, a property required for maintenance of an electrochemical gradient between the matrix and the IMS (Figure 1) (Nicholls *et al.,* 2002). The IM folds into lamellar structures double-membrane interdigitations called cristae, (Figure 1) (Frey and Mannella, 2000) which increase its surface area and mass. These cristae were initially assumed to be loose unfoldings of the IM, but subsequent electron tomography studies have revealed that they form a structurally distinct compartment

(Perkins *et al.,* 1997). Thus, the IM system is now best described as two contiguous yet distinct membranes: the inner boundary membrane, which faces the OM, and the cristae membranes, which extend into the mitochondrial interior as tubules or lamellae. The cristae and inner boundary membranes are joined by a limited number of discrete sites called cristae junctions, which typically have a consistent diameter of 15-20 nm, but may be altered by matrix volume and respiratory activity (Frey *et al.,*2002; Frey and Mannella 2000; Mannella *et al.,* 1997; Mannella *et al.,* 1994).

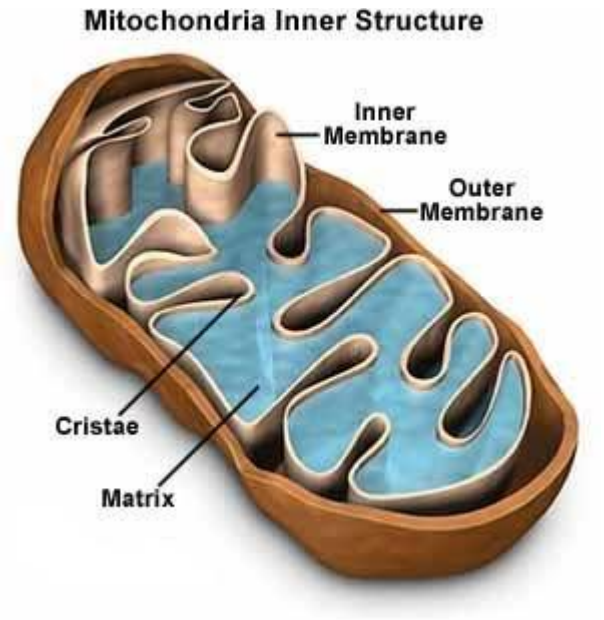

Figure 1: Mitochondrial Structure
From: http://micro.magnet.fsu.edu/cells/mitochondria/mitochondria.html

It has been estimated that three quarters of the mass of the IM are protein, and many of these proteins are components of the oxidative phosphorylation (OXPHOS) system. The interior compartment of mitochondria is composed of a gel-like substance, the matrix, which contains enzymes of the tricarboxylic acid cycle, and β-oxidation. These

oxidation-reduction reactions and energy transfer processes make mitochondria the powerhouses of eukaryotic cells (Mao and Holt, 2009).

The structural organization of the inner membrane strongly suggests that diffusion between internal compartments is restricted. This can have profound functional implications for the mitochondrial function. Because oxidative phosphorylation relies on rapid diffusion of ions and substrates to sites of transport or reaction on the mitochondrial IM, the number and shape (diameter and length) of cristae junctions could regulate rates of ATP phosphorylation under certain conditions (Perotti *et al.,* 1983; D'Herde *et al.,* 2001). Furthermore the shape and volume of cristae can be expected to affect the diffusion of cytochrome c between intracristal and intermembrane compartments (Bernardi and Azzone, 1981; Scorrano, 2002). The morphologies of the cristae and the crista junctions are highly dynamic, and might be sensitive to or controlled by matrix volume and the energetics of protein–lipid membrane folding (Renken *et al.,* 2000).

Mitochondria and Energy Production

The mitochondrion is the site of the electron transport chain and OXPHOS oxidative phosphorylation System, which provides the bulk of cellular energy in the form of ATP (Balaban, 1990; Hatefi *et al.,* 1985).

The OXPHOS system is composed of five enzymatic complexes (complex I-V) (Figure 2) and consists of approximately 90 subunits, of which 13 are encoded by the mtDNA. All the complexes are embedded in the lipid bilayer of the mitochondrial IM. Complexes I-IV together with two mobile electron carriers, ubiquinone (CoQ) and cytochrome c, make up

the electron transport chain. In the electron transport chain is coupled to the generation of a proton gradient across the inner mitochondrial membrane, which is further used by the fifth enzymatic complex, complex V (F0F1-ATP synthase), to synthesize ATP from ADP and inorganic phosphate (Saraste, 1999).

Complex I (NADH dehydrogenase) is the largest of the respiratory complexes, comprising forty-five subunits, of which seven are mitochondrially encoded (Carroll et al.,2002).

Complex II is the only respiratory enzyme completely encoded by the nucleus (Rustin et al., 2002). Complex III, the middle segment of the respiratory chain, and this complex has only one mtDNA-encoded subunit, cytochrome b; the other 10 subunits are nucleous-encoded, and at least one of these has been reported to be essential for the complex assembly (Berry et al., 2000; Yu et al., 1998; Zeviani et al., 2003). Complex IV is the terminal component of the respiratory chain, composed of 13 subunits, of which three are encoded by the mtDNA. The energy released by the flow of electrons through the respiratory chain is used to pump protons through the mitochondrial inner membrane by complexes I, III, and IV. This electrochemical gradient is finally utilised by the ATP synthase (F1F0-ATPase), to generate ATP (Saraste, 1999). Complex V has two subunits encoded by mtDNA (ATPase6 and ATPase8), that take part to the membrane-bound portion (F0) of the complex, and about 13 other subunits encoded by nDNA (Abrahams et al., 1994).

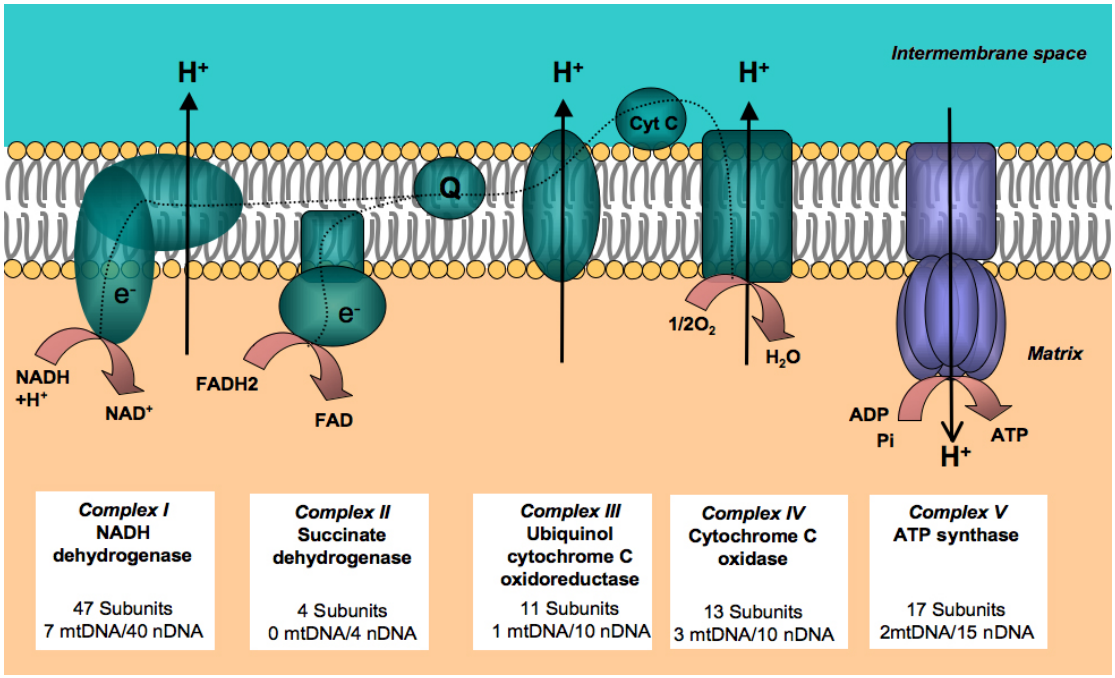

Figure 2: Mitochondrial Respiratory Chain
From: Frontiers in Bioscience 14, 4015-4034, January 1, 2009

Even though the structure of each individual OXPHOS complex is fairly well known, it is unclear how the complexes are organized in relation to one another. It has been suggested that the enzymatic complexes of OXPHOS exists as supercomplexes, so called "respirasomes", where several complexes are organized and function together. These supercomplexes have been found in mitochondria of yeast, plants and mammals (Krause et al., 2004, Schafer et al., 2006; Schagger and Pfeiffer 2000). The supramolecular organization of the OXPHOS system is considered to be of great functional importance. Formation of supercomplexes plays a role in the assembly and stability of the complexes, suggesting that the supercomplexes are the functional state of the respiratory chain (Vonck et al., 2009).

Mitochondrial Network

It remains an intriguing mystery how a cell generates and regulates the morphology of its organelles. Consider the mitochondria. They are the powerhouse of the cell, centers of respiratory, ATP production, and key organelles in essential lipid metabolism pathways. Within a cell, mitochondria appear in a range of forms from numerous small individual organelles, as most often depicted in textbook illustrations, to a single large interconnected membrane-bound tubular network, depending on environmental conditions, cell type, and organism.(Rafelski SM *et al.,* 2013).(Figure 3)

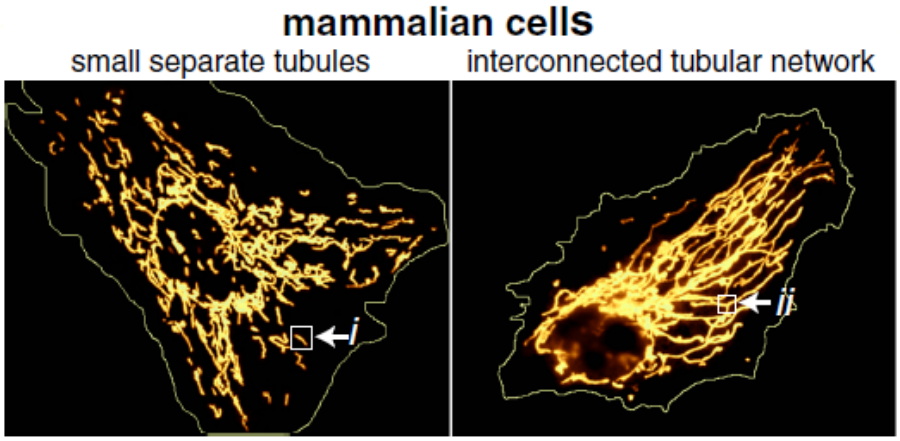

Figure3: Mitochondrial Network morphology
Mitochondrial networks can vary from separated structures to interconnected networks.
From: Rafelski SM: Mitochondrial network morphology: building an integrative, geometrical view. BMC Biology 2013 11:71.

Formally, the term 'mitochondrial dynamics' includes all of the different ways that the geometrical features of mitochondrial networks change over time. More commonly, 'mitochondrial dynamics' refers to the fission and fusion dynamics that constantly remodel the mitochondrial network. Fission cuts a tubule into two while fusion can link two tubules together to form a longer tubule or a branch. The balance of fission and

fusion is important for shaping mitochondrial networks; more fission than fusion leads to over-fragmented networks while more fusion than fission leads to over-connected networks compared to normal, wild-type networks. (Shaw JM *et al.*, 2002).

Computational modeling of mitochondrial networks

Understanding the interdependence between the geometrical features of mitochondrial networks will require a combination of quantitative experimentation and computational modeling. From the computational side, models of mitochondrial network organization can be generated based on any given set of assumptions. The key requirement for these sorts of computational models to be useful is the ability to compare them to 'real' networks. Models need to be based on experimentally perturbable parameters with output properties that can be experimentally measured. Several studies have begun carefully documenting the types of fission and fusion events and the biophysical requirements for them to occur. (liu X. *et al.,* 2009. Twig G. *et al.,* 2010).

Several computational models of mitochondrial networks have been generated. On one hand there exist models that attempt to tie together fission and fusion dynamics with their effect on maintaining mitochondrial health, testing the 'mitochondrial quality control mechanism'. (Mouli PK *et al.,* 2009. Figge MT *et al.,* 2012).

These models only take into account the fission and fusion dynamics themselves, not the physical size, shape and distribution of the mitochondria within the cell. Recently, a new model has been built that for the first time incorporates the fact that mitochondria move in space, can form networks, and undergo continuous biogenesis that is not

necessarily coupled to mitochondrial turnover. (Patel PK *et al.,* 2010). This model makes testable predictions about where in parameter space real mitochondrial networks should fall to maintain mitochondrial function most successfully. On the other hand, models can be built to determine the underlying requirements for generating the organization of mitochondrial networks without considerations of their functional impact. An example is a recent graph-based model that was built based on a set of simple rules of network connectivity. (Sukhorukov VM *et al.,* 2012). This model predicts several topological properties that mitochondrial networks should display, and thus can now be tested with directed experiments measuring network topology. A key limitation of this model is that it is purely topological and does not take any spatial information into account. The next generation of computational models will depend on more extensive quantitative experimental measurements of the interdependent geometric features of mitochondrial network morphology and, eventually, simultaneous mitochondrial functional state.

Mitochondrial DNA

Human mitochondrial (mtDNA) is a double-stranded, circular molecule of 16569bp. (Figure 4). The two strands of mtDNA, based on their nucleotide composition, can be separated in a cesium chloride gradient and are therefore called the 'heavy strand' (H-strand), rich in guanine, and 'light strand' (L- strand) strand, rich in cytosine (Fernandez-Silva et al., 2003). The mitochondrial genome sequence and gene organization have been fully elucidated (Anderson *et al.*, 1981). mtDNA in mammals and other vertebrates exhibits extreme economy of sequence organization, thus there are essentially no repetitive sequence families,

introns or intergenic regions. Some respiratory chain protein genes overlap and certain stop codons necessary for proper mRNA translation are only generated after polyadenylation (Anderson *et al.,* 1981; Ojala *et al.,* 1981).

There are only two non-coding regions in the mtDNA and they contain most of the known regulatory functions; it contains sequences that are vital for the initiation of both mtDNA replication and transcription. The major one is the 1 kb displacement D-loop, characterized by the presence of a triple strand structure, due to association of the new H-strand in this region (Fernandez-Silva *et al.,* 2003). The D-loop contains the origin of H-strand DNA replication (OH) and is also the site of transcription from opposing heavy and light strand promoters (Clayton, 2000; Scarpulla, 2008). The second non-coding region is about 30 nucleotides long and contains the origin of L- strand replication (OL). This region is located in a cluster of five tRNA genes around two thirds of the mtDNA length from the OH (Anderson *et al.,* 1981; Fernandez-Silva *et al.,* 2003).

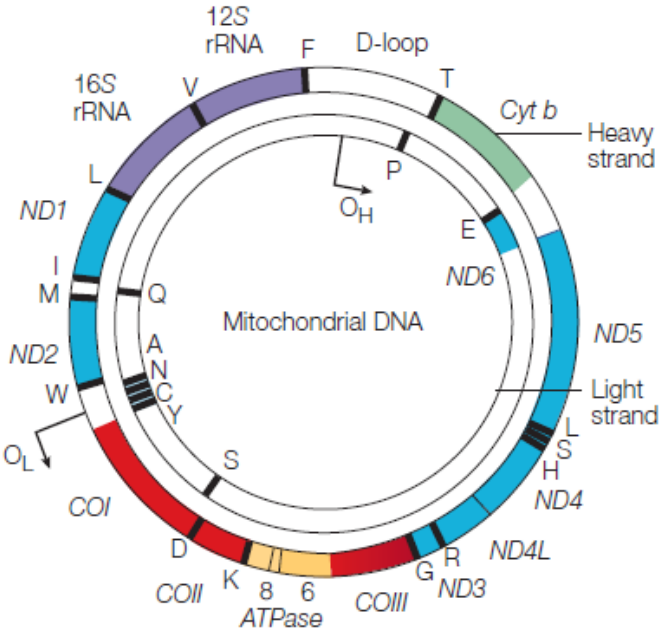

Figure 4: Mitochondrial DNA

Map of the human mitochondrial genome. The displacement loop (D-loop), or non-coding control region, contains sequences that are vital for the initiation of both mtDNA replication and transcription, including the proposed origin of heavy-strand replication (shown as OH). The origin of light-strand replication is shown as OL.

From: MITOCHONDRIAL DNA MUTATIONS IN HUMAN DISEASE Robert W. Taylor and Doug M. Turnbull 2005 Nature Publishing Group.

Most mitochondria contain between one and ten copies of mtDNA. (Robin *et al.,* 1988; Satoh *et al.,* 1991). In a single cell the copy number of mtDNA is typically over 1,000. This huge number of mtDNA molecules is organized into discrete loci called mitochondrial nucleoids, which are believed to be tethered to the IM. Mt-nucleoids comprise multiple copies of mtDNA and an unknown number of proteins. Nuclear DNA encodes all the proteins involved in mt-nucleoid metabolism. Most of the little that is known about mammalian mt-nucleoids has come from studies of human diseases caused by mtDNA perturbation. (Chih-Chieh *et al.,* 2009).

Despite its small size, mtDNA encodes 22 transfer RNAs, 2 ribosomal RNAs and 13 polypeptides which are required for OXPHOS. Although the number 1000 is widely quoted as the mtDNA complement of a typical cell, there is in fact considerable variation in the mtDNA copy number among different cell types and tissues. The key determinant of the copy number is energy demand; thus, cardiac muscle has a considerably higher mtDNA copy number than skin cells. A high mtDNA copy number appears to protect against defects in mtDNA because OXPHOS can still be maintained without a significant cellular phenotype, until or unless a large proportion of mtDNA molecules is defective. (Chih-Chieh *et al.,* 2009).

Mutation in Mitochondrial DNA

The human mitochondrial genome is extremely small compared with the nuclear genome, and mitochondrial genetics presents unique clinical and experimental challenges. (Robert et al., 2005). Despite the diminutive size of the mitochondrial genome, mitochondrial DNA (mtDNA)

mutations are an important cause of inherited disease. Considerable progress in understanding basic mitochondrial genetics and the relationship between inherited mutations and disease phenotypes have been reported recently, and in identifying acquired mtDNA mutations in both ageing and cancer. However, many challenges remain, including the prevention and treatment of these diseases. (Robert et al., 2005).

The increasing ease with which the mitochondrial genome can be analyzed, and the availability of a consensus human sequence, have both helped to recognize mtDNA disorders as a frequent cause of genetic disease. It is difficult to estimate the true prevalence of mtDNA disease owing to its many clinical guises, presentations and the involvement of numerous causative mutations. (Andrews et al., 1999).

In addition, there is increasing evidence from animal models (Trifunovic, et al., 2004) and human studies (Muller-Hocker, et al., 1990) that acquired mtDNA mutations and mitochondrial dysfunction are involved in ageing and age-related diseases such as diabetes. (Mootha et al., 2003; Petersen et al., 2003). Although our present knowledge already indicates that mtDNA mutations are an important cause of disease, but the true impact of these mutations on human health remains to be determined.

Quantification of Mitochondrial DNA Mutation Load

Mitochondrial DNA mutations have been shown to accumulate with age in a number of postmitotic [e.g. brain (Cottrell *et al.,* 2001a), heart (Cortopassi and Arnheim, 1990), skeletal muscle (Cooper *et al.*, 1992) human tissues. These mutations have been shown to clonally expand to a level, which causes respiratory chain deficiency. These respiratory

chain deficient cells can be detected using histochemical techniques (Old and Johnson, 1989) and fractions of respiratory chain deficient cells calculated with ease. However, quantifying the total mtDNA mutation load in ageing tissues is challenging but of fundamental importance if we are to understand the importance of mtDNA defects in human ageing.

A number of previous studies in human brain (Simon *et al.*, 2001) skeletal muscle (Del Bo *et al.*, 2002) and blood (Wilding *et al.*, 2006), and numerous tissues in the mitochondrial mutator mouse (Trifunovic*et al.*, 2004; Kujoth *et al.*, 2005) have employed a post-PCR cloning strategy. This technique involves DNA extraction followed by PCR amplification of a target sequence in the mitochondrial genome with a high-fidelity DNA polymerase. These PCR products are then cloned in a bacterial vector, and the plasmid DNA re-amplified and sequenced. Each individual PCR product is a copy of one mtDNA molecule and by sequencing a large number of clones; the mutation load per base pair can be quantified.

An alternative method is single-molecule PCR (Kraytsberg and Khrapko, 2005). This involves extraction of mtDNA from tissue sections followed by serial dilution of the DNA down to a dilution in which only one in every five PCR reactions contains an amplifiable mtDNA molecule. This ensures that only one molecule is being studied. This method allows any polymerase errors to be identified.

A third method has recently been developed, the random mutation capture (RMC) assay (Vermulst *et al.*, 2007). This assay avoids the possibility of both polymerase errors and fixation of base adducts into mutations. The template DNA undergoes restriction digestion prior to PCR, so that all molecules which are wild-type at that restriction site will be cut and only molecules with a mutation in that site remain intact. PCR

primers which flank the restriction site are then used to amplify the mutant molecules only. The starting template copy number is quantified using real-time PCR, and the mutation frequency calculated as the number of mutant bases per total number of base pairs screened. (Greaves *et al.,* 2009).

Human Mitochondrial Diseases

Mitochondrial diseases are a diverse group of human diseases characterized by defects in any aspect of mitochondrial function. Often for simplicity, defects relating to energy production, especially OXPHOS, are considered separately. Since the components of OXPHOS are encoded by mtDNA and nuclear DNA, and mtDNA is itself dependent on nuclear genes for its maintenance and expression, mitochondrial diseases of OXPHOS fall into three categories, mtDNA mutations as the primary cause, nuclear DNA defects affecting OXPHOS but unrelated to mtDNA, and nuclear DNA defects as a cause of mtDNA perturbation. (Chih-Chieh *et al.,* 2009).

1- Mitochondrial DNA as the primary cause

Mitochondrial DNA mutations can themselves be divided into two broad categories, those that affect translation, and those affecting a specific protein- encoding gene. The 22 tRNA and 2 rRNA genes are all required for mitochondrial translation; hence, deleterious mutations in any one will affect the production of all 13 mitochondrially-encoded components of OXPHOS.

2- Nuclear DNA defects affecting OXPHOS rather than mtDNA

Most of the 1000 or more mitochondrial proteins encoded in nuclear DNA do not directly affect mtDNA stability, but relate to mitochondrial

metabolism and OXPHOS. (Poyton *et al.,* 1996; Sue *et al.,* 2000). However, since OXPHOS is formed through the complementation of nuclear and mitochondrial DNA, mutations in nuclear genes encoding either structural component of OXPHOS, (Smeitink *et al.,* 1999; Budde *et al.,* 2003). Or factors involved in the assembly of OXPHOS, (Pecina *et al.,* 2004) can all lead to clinical phenotypes similar to mtDNA mutations.

3- Nuclear DNA defects as a cause of mtDNA perturbations

Nuclear genes encode all the mtDNA binding proteins. (Bar-Yaacov *et al.,* 2012). Some are essential for replication and others are presumed to play roles in maintenance and segregation. As mitochondria are the major site of free radical production, protection from oxidative stress is another key function of the mtDNA binding proteins. Mutations in these nuclear genes can therefore produce mutations in mtDNA, but unlike primary mtDNA mutations, these are heterogeneous (multiple deletions and/or point mutations). Predictably, the clinical phenotypes associated with nuclear gene defects of proteins involved in mtDNA metabolism are often similar to those linked to specific mtDNA mutations, but the transmission follows Mendelian rules rather than a maternal pattern of inheritance. (Chih-Chieh *et al.,* 2009).

Optic Atrophy

- Optic atrophies (OA) refer to a specific group of hereditary optic neuropathy in which the cause of the optic nerve dysfunction is inherited either in an autosomal dominant or autosomal recessive pattern. Autosomal dominant optic atrophy (ADOA), type Kjer, is the most common OA, whereas autosomal recessive optic atrophy (AROA) is a rare form. Prevalence of ADOA ranges from 1:50,000 to

1:10,000. The frequency of AROA is unknown but the disease seems rare. (Christophe *et al.,* 2003).

- On other hand the Leber's hereditary optic neuropathy (LHON) is a distinct type of inherited optic atrophies or hereditary optic neuropathies. LHON refers to an optic nerve dysfunction due to mutations in the mitochondrial DNA (mtDNA) and is transmitted in a non-mendelian or maternal pattern. However, sporadic forms and singleton cases of LHON are numerous. (Christophe *et al.,* 2003).

Autosomal Dominant Optic Atrophy

Mutations in the OPA1 gene are responsible for autosomal dominant optic atrophy. This disease is believed to be the most common of the hereditary optic neuropathies, which is the most common form of inherited optic neuropathy, and the second leading cause of blindness in the world. (Delettre *et al.,* 2002). With an estimated disease prevalence of 1:50,000. DOA, also known as Kjer's optic atrophy, affects retinal ganglion cells and the axons forming the optic nerve, leading to progressive visual loss (Kjer *et al.,* 1996).

Clinical Features and Histopathology

Autosomal dominant optic atrophy, first described clinically by Batten (Batten, 1896) and Kjer (Kjer, 1959), is characterized by a slowly progressive bilateral loss of central vision starting in childhood and variably progressing in adult life (Carelli *et al.,* 2004; Yu-Wai-Man *et al.,* 2009; Delettre *et al.,* 2002; Cohn *et al.,* 2008). DOA has an insidious onset, presenting usually between ages four and six years, although in mild cases it may remain subclinical until early adult life (Hoyt 1980).

Rapid decline in visual acuity in adult life has been reported but it is rare (Kjer *et al.,* 1996).

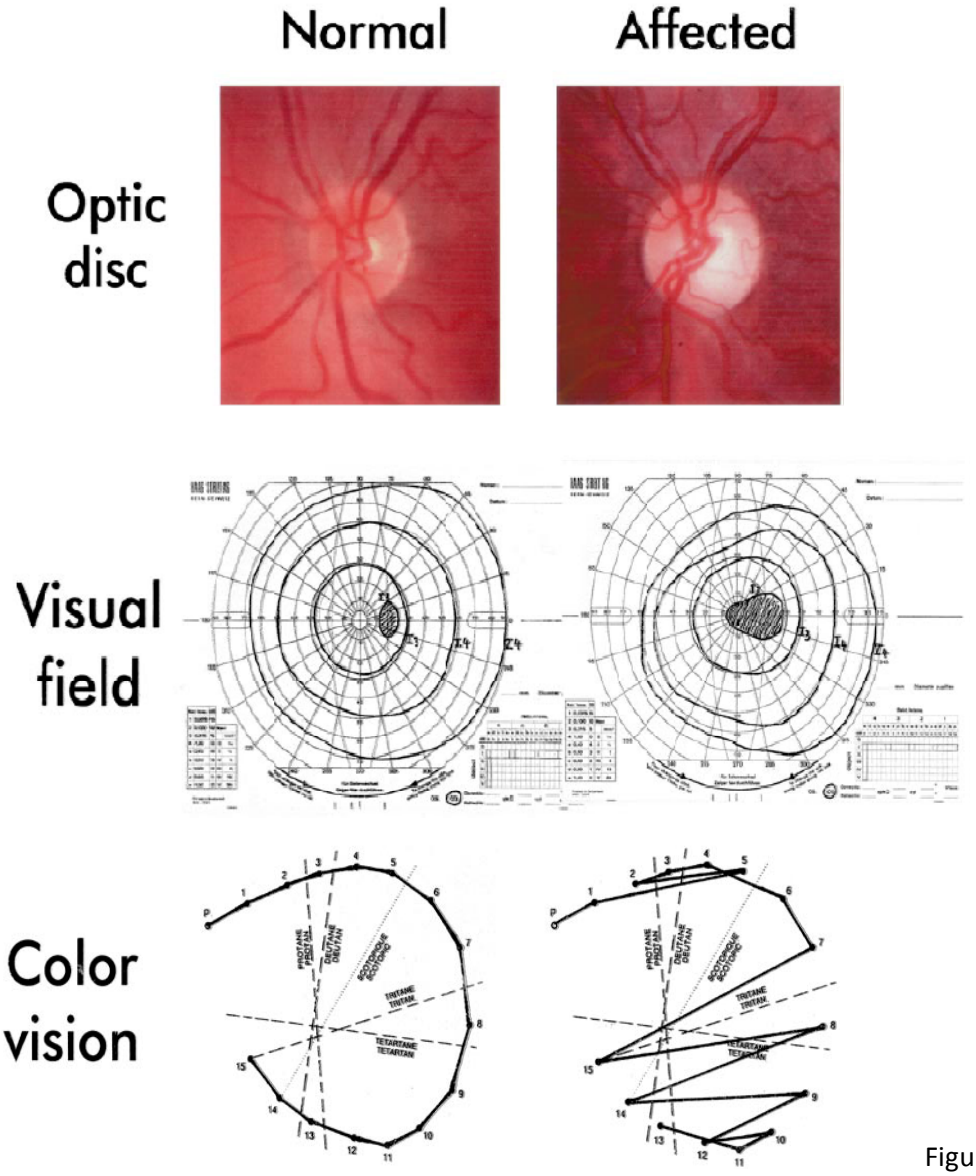

Figure 5: Appearance of diagnosis & testing at the level of Optic disc, visual field and color vision in normal and affected individuals
From: www.inmfrance.com/us/1_team1_lenaers.php

The disease is highly variable in clinical expression (Figure 5) and shows incomplete penetrance in some families (Hoyt *et al.,* 1980; Johnston *et al.,* 1999; Votruba *et al.,* 1998.). Visual impairment is usually moderate (6/10 to 2/10), but ranges from mild or even insignificant to severe (legal blindness). The visual loss is usually symmetrical, visual fields in patients with DOA characteristically show central, paracentral, and often appears

as a large defect in individuals with severe disease (Manchester and Calhoun 1958; Hoyt *et al.,* 1980; Votruba *et al.,* 1998). The colour vision defect is often reported as an acquired blue-yellow loss, or tritanopia (Eliott *et al.,* 1993), but most individuals have a generalized nonspecific dyschromatopsia and some families have been described in which affected individuals have red-green defects (Jeager *et al.,* 1972; Kok-van-Alphen, 1970; Mantyjarvi *et al.,* 1992).

Post mortem studies of two patients with DOA identified similar histopathological changes, with disperse atrophy of the retinal ganglion cells layer, loss of myelin and fibrillary gliosis along the anterior visual pathways extending to the lateral geniculate body (Johnston *et al.,* 1979; Kjer *et al.,* 1983). The optic nerve showed axonal loss and swelling, demyelination, and an increased content of collagen especially in the temporal aspect, suggesting specific vulnerability of the papillomacular bundle fibres, without signs of inflammation. MRI data from patients with DOA have also confirmed significant tissue loss and thinning of the optic nerve along its entire length (Votruba *et al.,* 2000).

Although there are dominantly inherited syndromes of optic atrophy associated with neurologic dysfunction, most of the patients with the syndrome of autosomal-dominant optic atrophy have no additional neurologic deficits. However, sensorineural hearing loss is not uncommon, and tends to cluster within families. The hearing loss ranges from severe and congenital to subclinical (i.e., identified by specific audiologic testing only). In most cases, it is unclear whether these pedigrees represent a phenotypic variant of dominant optic atrophy, a

genetically distinct disorder, or a genetically heterogeneous group of disorders with a similar phenotype (Amati-Bonneau *et al.,* 2005).

Genetics of the Disease

Two genes (OPA1 on chromosome 28) and (OPA3 on chromosome 19) encoding inner mitochondrial membrane proteins and three loci (OPA4 on chromosome22), (OPA5 on chromosome 12) and (OPA8 on chromosome 16) are currently known for DOA. Additional loci and genes (OPA2 for X-linked), (OPA6 on chromosome 8) and (OPA7 on chromosome 11) are responsible or recessive optic atrophy. All OPA genes yet identified encode mitochondrial proteins embedded in the inner membrane and ubiquitously expressed, as are the proteins mutated in the Leber Hereditary Optic Neuropathy. (Lenaers *et al.,* 2012). OPA1 mutations affect mitochondrial fusion, energy metabolism, control of apoptosis, calcium clearance and maintenance of mitochondrial genome integrity. OPA3 mutations only affect the energy metabolism and the control of apoptosis. (Lenaers *et al.,* 2012).

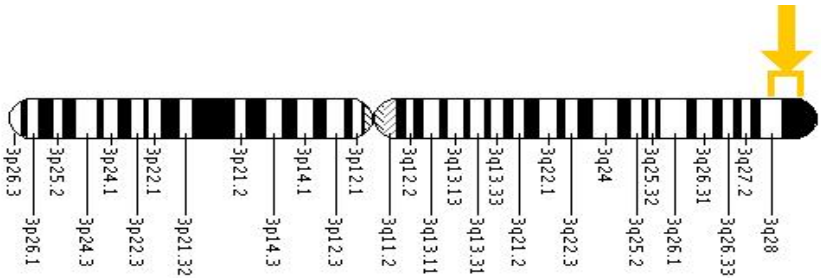

Figure 6: The major locus has been mapped to chromosome 3q28 and the responsible gene, OPA1, is identified.
From: http://ghr.nlm.nih.gov/gene=opa1

In 2000, two simultaneous studies reported OPA1 mutations in patients affected by autosomal dominant optic atrophy (Alexander *et al.,* 2000, Delettre *et al.,* 2000). DOA is a genetically heterogeneous disease, and

five loci have been linked to the disease (named OPA1 to OPA5) (Olichon *et al.,* 2007b). Among these, the most common form of DOA is caused by mutations in the OPA1 gene. (Figure 6).

The OPA1 gene (GenBank accession no. AB011139) is 6031 nucleotides long and is composed of 31 exons spanning >114 kb of genomic DNA. According to the eOPA1 database, 205 OPA1 gene mutations have been identified, which are basically family specific. (Ferre *et al.,* 2005). These mutations are spread throughout the protein, but the GTPase domain (32,8%), dynamin domain (18,1%), the C-terminus (18,1%) and the N-terminus region (6,9%) are more frequently affected.

R445H OPA1 Mutation

The R445H mutation is the only OPA1 mutation that has been associated with this unique syndrome of optic atrophy, sensorineural hearing loss, ptosis, and ophthalmoplegia in two unrelated families (Li et al., 2005).

Dominant optic atrophy, ptosis, ophthalmoplegia and sensorineural deafness have previously been described in a Belgian and North American family with the R445H OPA1 GTPase-domain mutation (Payne et al., 2004; Li et al., 2005). Intriguingly, R445H is one of the most common OPA1 mutations, but the phenotype usually only involves the visual system (Hudson et al., 2008). It remains to be established why the same mutation can cause an organ specific phenotype in some families, but a multi-system disorder in another (Hudson et al., 2008).

The heterozygous R445H mutation in OPA1 was found in patients with optic atrophy and deafness. Skin fibroblasts showed hyper

fragmentation of the mitochondrial network, decreased mitochondrial membrane potential, and adenosine triphosphate synthesis defect. Thus, optic atrophy and deafness may be related to energy defects due to a fragmented mitochondrial network (Amati-Bonneau et al., 2005).

The R445H mutation in OPA1 might be the cause of the association between dominant optic atrophy and moderate deafness, a phenotype that may be currently under diagnosed (Amati-Bonneau et al., 2003).

Note: As mentioned before retinal ganglion cells are specifically affected by mutations in OPA1. Since all derived cells from patients were skin fibroblasts, therefore it was necessary to apply a stress effort on the cells by using staurosporine, where staurosporine is a natural alkaloid. It is widely employed as an inducer of apoptosis in many mammalian cell types. (Kruman et al., 1998; Posmantur et al., 1997).

TMEM 126A : Transmembrane Protein 126A

Defects in this gene are a cause of optic atrophy type 7 (OPA7). Hereditary optic atrophy is a group of neurodegenerative disorders characterized by a sudden or gradual loss of retinal ganglion cells function. The disease primarily involves the macular beam of the optic nerve and may progress to peripheral fibers. Leber hereditary optic neuropathy (LHON) and autosomal-dominant optic atrophies (adOAs) are by farmore frequent than recessive forms.

Typically, patients with LHON and ADOA have no relevant health problems apart from optic atrophy. However, the disease may be associated with a wide range of extraocular features of mitochondrial dysfunction, including brain, cardiac, muscle, or auditive signs. (Hanein

et al., 2009). In contrast with LHON and ADOA, autosomal-recessive optic atrophies (AROAs) usually involve the central nervous system and other organs. Nonsyndromic arOA are less common. (Barbet et al., 2003).

Considering that all known optic atrophy genes encode mitochondrial proteins, (Carelli et al., 2007) the selected in the interval three genes predicted to encode mitochondrial products. (Calvo et al., 2006). Systematic sequence analysis identified a homozygous nonsense mutation in the gene encoding TMEM126A; a transmembrane protein. Where, a nonsense mutation is a point mutation in a sequence of DNA that results in a premature stop codon, or a nonsense codon in the transcribed mRNA, and in a truncated, incomplete, and usually nonfunctional protein product.

48 additional unrelated patients affected with nonsyndromic OA (AROA n = 14; ADOA n = 10; sporadic n = 24) have been screened for TMEM126A mutations. All affected patients and their relatives, including all members of family. In all cases, OPA1 mutations and the most frequent LHON mutations (mtDNA G11778A, G3460A, T14484C, and G15257) were previously excluded by direct sequencing. None of the sporadic and adOA cases carried TMEM126A mutations. (Hanein et al., 2009).

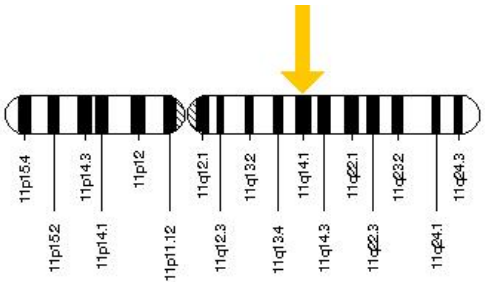

Figure 7: The TMEM126A gene is located on the long (q) arm of chromosome 11 at position 14.1. http://ghr.nlm.nih.gov/gene/TMEM126A

The TMEM126A gene spans 8.5 kb on chromosome 11 (Figure 7) and encodes a single ubiquitous transcript (770 bp) made of one noncoding and four coding exons. (Cizkova et al., 2008).

TMEM126A have been identified as a gene that encodes a mitochondrial protein of higher eukaryotes and whose mutations are to our knowledge the first known genetic cause of nonsyndromic autosomal-recessive optic atrophy. This finding gives further support to the view that mitochondrial dysfunctions are a hallmark of optic neuropathies, suggesting that common therapeutic approaches may be developed to improve the long-term prognosis of patients whatever the gene involved. (Delettre et al., 2000 ; Chevrollier et al., 2008).

Pathophysiology of the Disease

OPA1 gene sequencing in patients with hereditary optic neuropathies indicates that the clinical spectrum of DOA is larger than previously thought. The systematic molecular screening of OPA1 in patients with DOA has revealed a wide range of phenotypic variations of the disease. Specific OPA1 mutations are responsible for several distinct clinical presentations in DOA patients, in DOA with deafness (DOAD), and in severe multi-systemic syndromes, the so-called “DOA plus” disorders (Amati-Bonneau *et al.,* 2008; Hudson *et al.,* 2008). The study of the various clinical presentations of DOA in conjunction with the investigation of OPA1 mutations in fibroblasts from patients with optic atrophy provides new insights into the pathophysiological mechanisms of the disease, while underscoring the multiple physiological roles played by OPA1 in energetic metabolism, mitochondrial structure and

maintenance, and cell death (Amati-Bonneau *et al.,* 2009; Lenaers *et al.,* 2009).

What are the effects of the mutations on mitochondria?

From the observations, two hypotheses arise that can link mutations in OPA1 and mitochondrial dysfunction. One is that truncated or partially inactivated proteins might somehow destabilize the mitochondrial inner membrane and therefore interfere with the function of the respiratory complexes, reducing the amount of energy produced and increasing the amount of derived reactive-oxygen species (ROS). The second hypothesis is that mutated proteins might partially disorganize the mitochondrial network to the extent that the energetic pools of ATP would be unevenly spread in the cell. (Delettre *et al.,* 2002).

Why retinal ganglion cells?

Despite the ubiquitous expression of OPA1 gene in all organs; retinal ganglion cells are specifically affected by mutations in OPA1. One possibility reasons is that OPA1 is predominantly expressed in the retinal ganglion cell layer. (Pesch *et al.,* 2001). Moreover retinal ganglion cells are fundamentally highly susceptible to the reactive-oxygen species. (Kortuem *et al.,* 2000). Vulnerability of retinal ganglion cells could also be related to specific requirements in the distribution of the energy along their axons and lead to the failure of the transport in the optic nerve. Another hypothesis is that the optic nerve mitochondrial network in necessary to maintain transmission of action potential along the axon and particularly in the non-myelinated laminar proteins which are rich in mitochondria. (Andrews *et al.,* 1999).

Mitochondrial network dynamics in DOA

Fibroblasts from DOA patients possess either normal or fragmented mitochondrial networks in comparison with controls (Olichon *et al.,* 2007b; Zanna *et al.,* 2008; Chevrollier *et al.,* 2008). Mitochondrial structure alterations have been frequently reported in fibroblasts (Amati-Bonneau *et al.,* 2005, 2008; Olichon *et al.,* 2007b; Zanna *et al.,* 2008), in myotubes (Spinazzi *et al.,* 2008), in skeletal muscle from DOA patients (Amati-Bonneau *et al.,* 2008) as well as in OPA1 mouse models (Alavi *et al.,* 2007). The identification of a single OPA1 GTPase mutation that specifically disrupts the fusion-fission equilibrium, without affecting respiration, supports the concept that in some cases OPA1 mutation has a direct implication in the fusion machinery (Spinazzi *et al.,* 2008). Since this alteration of mitochondrial shape is not associated to altered bioenergetics or increased sensitivity to apoptosis, it could be assumed that pro-fusion activity of OPA1 per se is relevant in DOA pathogenesis, at least in this case.

DOA Plus

Mutations in OPA1 have been linked also to syndromic form of DOA associated with sensorineural deafness, ataxia, axonal sensory-motor polyneuropathy, chronic progressive external ophthalmoplegia and mitochondrial myopathy with cytochrome c oxidase negative and Ragged Red Fibres. (Amati-Bonneau *et al.,* 2008) This clinical phenotype has been defined as “DOA plus” syndrome. Most remarkably, these patients all harboured multiple deletions of mtDNA in their skeletal muscle, thus revealing an unrecognized role of the OPA1 protein in mtDNA stability (Hudson *et al.,* 2008).

Gain or loss of function of the protein activity is responsible for mtDNA instability. The current hypothesis states that OPA1 is a mechano-enzyme that uses GTP hydrolysis to switch between distinct conformations that either facilitate membrane fusion directly or recruit machinery for it (Praefcke and McMahon, 2004; Hoppins *et al.,* 2007). GTPase activity is critical for OPA1 function and DOA miss-sense mutations found in the GTPase domain adjacent to its active site (A357T, G439V, R445H, S545R) may impair GTP hydrolysis locking the protein in an 'on' or 'off ' state (Amati-Bonneau *et al.,* 2008; Hudson *et al.,* 2008). Where, a miss-sense mutation is a point mutation in which a single nucleotide change results in a codon that codes for a different amino acid.

However, it must be noted that a number of other miss-sense mutations in the OPA1 gene have been described and listed in the eOPA1 website (http://lbbma.univ-angers.fr) (Ferre *et al.,* 2005), including the exons building up the GTPase domain. Concerning these latter mutations there is no report of 'plus' features in these patients, which seem to be affected by classic DOA. Thus, it is reasonable to assume that mis-sense mutations in the GTPase domain not necessarily lead to 'OPA1 plus' phenotypes, which may be strictly dependent on amino acid location and/or change.

Genistein

Genistein is a natural compound belonging to the class of isoflavones. While isoflavones are widely distributed in plant Kingdome, the concentration of these compounds is relatively high in particular in soybeans. (Fukutake *et al.,* 1996; Barnes *et al.,* 1995).

However it was also isolated from fermentation broth of various kinds of microorganisms (Streptomyces sp., Pseudomonas sp.). (Asahi *et al.,* 1981).

Despite the relatively simple chemical structure of genistein its mode of action remains still fairly unexplored. Numerous experiments have been undertaken show that genistein interferes with many biochemical pathways and its mode of action in the live cell is complex and multidirectional. Several biochemical targets of genistein in the cell were defined. However these proteins targets may partly serve to account for varied genistein effects that can be observed in cell or tissue cultures. One of the biological activities of genistein explored in cell is antioxidant effects among many other effects. Thus the recent studies are focused on application of this natural isoflavone in the treatment of cancer as well as many other chronic diseases. (Polkowski *et al.,* 2000).

Genistein can inhibit both the priming events necessary for high level of reactive Oxygen species (ROS) production or can directly inhibit agonist-stimulated ROS production. (Peterson *et al.,* 1995).

Genistein as an inhibitor of mitochondrial oxidative damage

It is well recognized that mitochondrial dysfunction is associated with neurodegenerative disease (Moreira *et al.,* 2010). Functional food, especially the dietary consumption of antioxidant compounds has been the subject of much interest in protection against neurodegenration. Soy isoflavone (SIF) is the major flavonoid found in the human diet (Song *et al.,* 2007). A number of beneficial effects of SIF especially genistein (Gen), the main active component in SIF, has been shown on several

disease such as osteoporosis (Stechell and Lydeking-Olsen 2003; Bitto *et al.,* 2009), cancer (Li *et al.,* 2010) and cardiovascular disease (Park *et al.,* 2005; Si and Lui 2007). Recently Genistien had gained attention due to its potential health benefits in preventing neurodegeneration (Valles e *et al.,* 2010; Lee 2008; Wang *et al.,* 2005). Previous study has indicated that Gen could clear reactive oxygen species (ROS) partially and play an antioxidant role. (Ma *et al.,* 2010).

Although past studies have extensively explored its antioxidative (Ma *et al.,* 2010), anti-inflammatory (Valles *et al.,* 2010), and estrogenic effects (Zeng *et al.,* 2004), there is little evidence regarding the protective effect of Gen on mitochondrial dysfunction induced by oxidant production. Thus, we hypothesize that oxidation pressure may damage the mitochondrial membrane and resulting in the reduction of antioxidant capacity, subsequently affecting the cell function and ultimately lead to neuronal death. (Xi *et al.,* 2011).

JUSTIFICATIONS & AIMS

Justifications of the Project:

Since of the great importance of the visual hereditary neuropathy, especially dominant optic nerve atrophy in the conception of sight loss in humans, as well in the light of continuous and systematic research for a new genes and new mutations related to the disease, additionally of the detection whether there is a relationship between nuclear mutations causing the disease, and the appearance of mutations in mitochondrial genes, and to investigate for the role of Genistein in protecting cells taken from DOA patients against the effect of oxidation factors research aims to:

Aims of the Project

1. Systematic sequence analysis to identify a mutation in the gene encoding TMEM126A.
2. Detection of mutations in mitochondrial DNA; extracted from OPA1+/- mouse (model animal).
3. Studying the effect of Genistein on the Fibroblast R445H OPA1, and characterize the effect of this product on the mitochondrial network and mitochondrial membrane potential.

MATERIALS & METHODS

All the following experiments took place in laboratories of Institut des Neurosciences de Montpellier - St Eloi Hospital, Montpellier – France.

1:0 Genetic diagnoses TMEM126A Gene

1:1 AmpliTaq Gold PCR (Applied Biosystem)

Same reagent are going to be used in each PCR reaction concerning the five different exones only forward and backward oligos are changed regarding to the suitable exone were amplified.

1. Thaw and gently mix each of the reagents. Avoid generating bubbles when mixing the enzyme. Spin the enzyme solution down in micro centrifuge before pipetting. Pipet the enzyme and buffer slowly and carefully:

Table 1: The components of AmpliTaq Gold PCR reaction

AmpliTaq Gold (1.25 units/ reaction)	0. 4 µl
10X PCR Gold Buffer	5 µl
MgCl2(25mM)	5 µl
Oligo F (0.5 µM)	2. 5 µl
Oligo R (0.5 µM)	2. 5 µl
dNTPs 10mM each)	0.8 µl
DNA Tamplate 50ng	4 µl
Water	30 µl
Total Volume	50µl

2. Optimal PCR amplification achieved using the thermal profiles outlined bellow, please note that for each exone a specific and most appropriate program were set where annealing temperature is substantial:

Table 2: Thermal cycling steps of the 1st and 5th Exons of TMEM126A

Step	Temperature,° C	Time	Numbers of Cycles
Initial denaturation	95	5min	1
Denaturation	95	30s	35
Annealing	**58**	30s	
Extension	72	30sec	
Final extension	72	10min	1
Hold	22	∞	Hold

Same program were set for each of the other exons, but annealing temperature were adjust in convenient with each exon as following:

Exon #1: Annealing Tem. 58° C

Exon #2: Annealing Tem. 60° C

Exon #3: Annealing Tem. 55° C

Exon #4: Annealing Tem. 48° C

Exon #5: Annealing Tem. 58° C

►Oligos to amplify TMEM126A (designed and prepared by the laboratory):

Table 3: List of primers of the TMEM126A exons

	Sens	Anti-sens
Exon1:	CTT CTC AGC CCA AAG CCG CT	CGT CGT GCT TCT CCT GAC AC
Exon2:	CAG ATT AGC TCT ACA GTA TTA T	CCT TCT CAC TTC CAT GTT G
Exon3:	GGG ATA GAT GTC GTA TCC AG	ATT ACA GCA TAC AGT ACT TGG
Exon4:	CAA TTA CAT TTG ATA TAT TAC CTG	GGG TAA CAT TGC CTT TGG TC
Exon5:	TTT ATC TTA AGA CTT CTA GGA C	CCT TTT GTT GTA GGT CCC AG

1:2 DNA sequencing

1:2:1Purification

ExoSAP-IT prepares PCR products for numerous applications, such as sequencing. When PCR amplification is complete, unconsumed dNTPs and primers remaning in the PCR product mixture will interfere with further methods. ExoSAP-IT is used to remove these unwanted nucleotides and primers and provide PCR products suitably prepared for DNA sequencing.

ExoSAP-IT (usb) is added directly to the PCR products. The enzymes are active in the buffer used for PCR. After treatment ExoSAP-IT is inactivated simply by heating to 80˚C for 15˚C.

Table 4: The components of ExoSAP-IT reaction

PCR Products	10 µl
ExoSAP-IT	1.5 µl
Water	1.5 µl

1:2:2 Sequence Reaction

1. Set the Sequence Reaction:

Table 5: The components of the sequence reaction

Purification Products (200-300ng)	2µl
Big Dye (Applied Biosystems)	2µ
Buffer	2µl
Oligo (f or b)	1µl
Deionized water	3µl
Total Volume	10µl

2. Pipet up and down to mix well, seal the plate of 96 wells with adhesive film or caps, then spin the plate briefly.

3. Run the reactions in a thermal cycler:

Table 6: Thermal cycling steps for sequencing

Temperature,° C	**Time**	**Numbers of Cycles**
96	1min	1
96	10s	25
50	5s	
60	4min	
20	Hold	1

1:2:3 The Precipitation:

1. For each sequence reaction add a mixture composed of:

Table 7: The components of the precipitation reaction

Sodium Acetate 3M	1µl
Ethanole 95%	25 µl
1 EDTA (125mM)	1µl
Sequence reaction products	10µl

2. Centrifuged at 4000 rpm for 45 min.

3. Eliminate the supernatant by two successive centrifuges.

1st centrifugation 1200 rpm 30 sec to remove the supernatant

2nd centrifugation 3000 rpm 1 min

Check that there is no residue on the walls of the wells. If this is the case, perform the 3rd centrifugation at 3000 rpm for 1 min to remove all the residues.

4. Add 35µl of 70% ethanol to each well. Reseal the strips with caps or set a sticky film on the 96-well plate.

5. Centrifugal 3600 rpm for 15 min

6. Eliminate the supernatant by two successive centrifuges:

As described in step (3).

Check that there is no residue on the walls of the wells. If this is the case, perform the 3rd centrifugation at 3000 rpm for 1 min to remove all the residues.

7. Resuspend the pellet in 15µl formamide and homogenize.

8. The products are sequenced by Four capillary sequencer (upgradable to 16 capillaries) – 5 fluorochromes. And data analyzed on Specific software: Sequencing Analysis. Sequences obtained were compared with the references on NCBI Reference Sequence Database (BLAST).

2:0 OPA1 +/- mouse mt-DNA analyzing

2:1 Post PCR Cloning

The flow chart below outlines the experimental steps necessary to clone the PCR product:

Produce blunt PCR products

⇩

TOPO cloning reaction

⇩

Transform into bacterial cells

⇩

Select and analyze colonies

⇩

Isolate plasmid DNA and sequence

To look at mutation load by PCR cloning, DNA was extracted from cells delivered from both wild type mice and OPA1 +/- mice.

2:1:1 Produce Blunt PCR Products

The PCR reaction was completed by the using of specific primers of non coding region in the mtDNA called the D-Loop region which is the major control site for mtDNA expression because it contains the leading for origin of replication and major promoters for transcription.

Set up the following 50 µl PCR reaction (Promega):

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Place a thin-walled PCR tube on ice and add the following components for each 50 µl reaction:

Table 8: PCR reaction Components to produce blunt products

Sterile water	Variable (add to a final volume)
10X Pfu Buffer with MgSO4	5 µl
dNTP Mix, 2 mM each (Promega)	5 µl (0.2 mM of each)
Forward primer	0.1-1.0 µM
Reverse primer	0.1-1.0 µM
Template DNA	50 pg - 1 µg
Pfu DNA Polymerase (Promega)	1.25-2.5 u
Total volume	50 µl

3. Gently vortex the samples and spin down.
4. Perform PCR using the following thermal cycling conditions:

Table 9: Thermal cycling steps to produce blunt products

Step	**Temperature,° C**	**Time**	**Numbers of Cycles**
Initial denaturation	95	5min	1
Denaturation	95	30s	35
Annealing	62	30s	
Extension	72	2min	
Final extension	72	10min	1

►Two sets of primers were (designed and prepared by the laboratory) of non coding region in the mice mtDNA called the D-Loop region:

Table 10: List of primers of the D-loop region

	Sens	Anti-sens
Set 1:	AGA AGA AGG AGC TAC TCC CCA C	GGA TAC AAT TAT CCA TCT AAG C
Set 2:	CAC CAG CAC CCA AAG CTG GT	TAA GGC CAG GAC CAA ACC TTT G

5. 10 µL blunt-end PCR product electrophoresis by agarose gel (1.5%) to verify the quality and quantity of the PCR product. And make sure that we have a single, distinct band of the correct size. Then the band on gel were visualized and photographed over UV light.

Direct cloning of DNA amplified by proofreading polymerases including Pfu into TOPO Cloning vectors is often difficult because of very low cloning efficiencies. This is because proofreading polymerases remove the 3´ A-overhangs necessary for Cloning. Invitrogen has developed a simple method to clone these blunt-ended fragments.

This is just one method for adding 3´ adenines:

After amplification with the proofreading polymerase, place tube on ice and add 0.7-1 unit of Taq polymerase (Invetrogen) per tube. Mix well. A sufficient number of PCR products will retain the 3´ A-overhangs.

2. Incubate at 72°C for 8-10 minutes (do not cycle).

3. Place on ice and use immediately in the TOPO Cloning reaction.

TOPO Cloning Kits for Sequencing (Invetrogen) provide a highly efficient one-step cloning strategy "TOPO Cloning" for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector for sequencing. No ligase, or PCR primers containing specific sequences are required.

Taq polymerase has a nontemplate-dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

2:1:2 TOPO Cloning Reaction

Use the following procedure to perform the TOPO Cloning reaction. Set up the TOPO Cloning reaction using the reagents in the order shown:

Table 11: The components of the TOPO cloning reaction

Reagent	Volume
Fresh PCR product	1 µL
Salt Solution	1 µL
Sterile water	Variable add to a total volume of 6 µL
TOPO vector*	1 µL
Final Volume	6 µL

*The plasmid vector (pCR®4-TOPO®)

1. Mix the reaction gently and incubate for 15 minutes at room temperature (22–23°C).

2. Place the reaction on ice to stop it.

2:1:3 One Shot Chemical Transformation

1. Add 2 µL of the final product of TOPO Cloning reaction from the last step into a vial of One Shot Chemically Competent E.coli and mix gently. Do not mix by pipetting up and down.

2. Incubate on ice for 5–30 minutes.

3. Heat-shock the cells for 30 seconds at 42°C in water bath without shaking.

4. Immediately transfer the tubes to ice.

5. Add 250 µL of room temperature S.O.C. medium.

6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for

1 hour.

7. Spread 10–50 µL from each transformation on a prewarmed selective plate. (LB plates containing 50–100 µg/mL kanamycin) To ensure even spreading of small volumes, add 20 µL of S.O.C. medium.

8. Incubate plates at 37°C, visible colonies should appear within 8 hours.

9. An efficient TOPO Cloning reaction should produce several hundred colonies. pCR®4-TOPO® allows direct selection of recombinants via disruption of the lethal E. coli gene, ccdB. The vector contains the ccdB gene

fused to the C-terminus of the LacZα fragment. Ligation of a PCR product disrupts expression of the lacZα-ccdB gene fusion permitting growth of only positive recombinants upon transformation. Cells that contain non-recombinant vector are killed upon plating. Therefore, blue/white screening is not required.

2:1:4 Analyze Transformants:

1. Inoculate as much of the white single colony as possible in LB containing 50–100 µg/mL ampicillin.

2. Incubate in a horizontally shaker (200 rpm) at 37°C for overnight.

2:1:5 Isolate Plasmid DNA

To purify the plasmids follow the manufacturer's protocol concerning (QIAprep Spin Miniprep) Kit.

This protocol is designedfor the purification of plasmid DNA from 1-5ml overnight E.coli culture in LB medium centrifuge at 10000rpm for 5 minutes or 5000rpm for 10 minutes.

Things to do before starting:

- Add RNase a solution to buffer P1.
- Add ethanol (96-100%) to buffer PE
- Check buffers P2 and N3 for salt precipitation and redissolve at 37°C if necessary.

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Centrifuge at 10000 rpm for 5 min.
2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.
3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
4. Centrifuge for 10 min at 13,000 rpm in a table-top microcentrifuge.
5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
6. Centrifuge for 30–60 s. Discard the flow-through.
7. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
9. Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

2:1:6 Plasmids DNA Sequence

Same protocol was applied as mentiend in page 45 in this review for sequence reaction and precipitation.

2:2 SYBR Green-based real-time RT-PCR:

1. Prepare a PCR master mix as follows:

Table 12: The components of the SYBR Green RT-PCR reaction

2× iQ SYBR Green Supermix	2 µl
10 µM Forward primer	0.5 µl
10 µM Reverse primer	0.5 µl
Water	5 µl
DNA sample	2 µl
Total Volume	10µl

2. Mix the PCR master mix with DNA by gently pipetting up and down several times (Note: Try your best to minimize bubble formation).

3. Seal the 96-well plate with a BioRad film. Spin down all droplets by centrifugation at 4,000 rpm for 2 min.

4. Warm up the iCycler system for 15−20 min before use. Use the following protocol for a general SYBR Green PCR assay:

Table 13: Thermal cycling steps for SYBR Green RT-PCR reaction

Step	Temperature,° C	Time	Numbers of Cycles	
Cycle 1	95	30sec	1	End of cycle2: Data collection and real-time analysis enabled.
Cycle 2	95	20sec	45	
	60	30sec		
	72	30sec		
Cycle 3	95	1min	1	End of cycle5: Melt curve data collection and analysis enabled.
Cycle 4	55	1min	1	
Cycle 5	55	10sec	80	

2:3 Long-Range PCR:

Long Range PCR refers to the amplification of DNA lengths that cannot typically be amplified using routine PCR methods or reagents. For DNA templates, polymerases optimized for Long Range PCR can amplify up to 20 kb and beyond. This method needs three steps to be accomplished as three PCR reactions take place as the following:

First PCR TaKaRa LA Taq (TAKARA BIO INC)

1. Keep all reagents on ice until use.
2. Set the reaction mixture on ice:

Table 14: The components of the first TaKaRa La PCR reaction

TaKaRa LA Taq (5units/ µl)	0. 5 µl
10X LA PCR Buffer	5 µl
MgCl2(25mM)	5 µl
Oligo F (0.5 µM)	0.5 µl
Oligo R (0.5 µM)	0. 5 µl
dNTPs 2.5mM each)	8 µl
DNA Tamplate	5 µl
Water	25.5 µl
Total Volume	50µl

3. set a thermal cycler ready to start with the designated program and set the tubes in to start the thermal cycling:

Table 14: Thermal cycling steps of the first TaKaRa La PCR reaction

Temperature,° C	**Time**	**Numbers of Cycles**
94	1min	1
98	10s	30
68	15min	
72	10min	1

<u>Second and Third PCR TaKaRa Ex Taq</u> (TAKARA BIO INC)

1. Keep all reagents on ice until use.
2. Set the reaction mixture on ice:

Table 15: The components of the 2nd and 3rd TaKaRa La PCR reaction

TaKaRa Ex Taq (5units/ µl)	0. 25 µl
10X Ex Taq Buffer	5 µl
MgCl2(25mM)	5 µl
Oligo F (0.5 µM)	0.5 µl
Oligo R (0.5 µM)	0. 5 µl
dNTPs 2.5mM each)	4 µl
DNA Tamplate	5 µl
Water	34.75 µl
Total Volume	50µl

2. set a thermal cycler ready to start with the designated program and set the tubes in to start the thermal cycling:

Table 16: Thermal cycling steps of the 2nd and 3rd TaKaRa La PCR reaction

Temperature,° C	Time	Numbers of Cycles
94	30sec	1
98	10sec	30
68	1min	
72	1min	1

4. Analyze 5–10 µL from the products by agarose gel (0.8%) electrophoresis.

3:0 Cell Biological Tests

3:1 Cell Culture

Skin fibroblasts were derived, from DOA patients with mutation on OPA1 gene. R445H OPA1.

Fibroblast cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Euroclone) supplemented with 10% fetal bovine serum (FBS) (Euroclone), 2 mmol/L L-glutamine (Euroclone), 100 units/mL penicillin (Euroclone), and 100µg/mL streptomycin (Euroclone), at 37°C in a 5% CO2 humidified incubator. For incubations in galactose medium, DMEM glucose-free medium supplemented with 5mM galactose (Euroclone), 2 mM L-glutamine, 5 mM Na-pyruvate and 5% FBS was used.

The following are the steps used to transfer the cells from the growing flask to wells preparing them to the treatment:

1. Remove spent medium from the flask.
2. Wash the cell monolayer one time with PBS 5 ml.

3. Remove PBS, Pipette trypsin onto the washed cell monolayer 0.25X EDTA incubate for 5 min in 37°C
4. Rotate flask to cover the monolayer with trypsin.
5. Once the cells start to sheet and the media becomes cloudy, move on to the next step.
6. Re-suspend the cells in a 5 times volume to the volume of Trypsin of fresh serum-containing medium to inactivate the Trypsin.
7. Transfer the solution to 15ml tube and centrifuge at 1500rpm for 5 min.
8. Remove the supernatant and then add 3ml of PBS or fresh medium and centrifuge once again then remove the supernatant again. Add 3ml of fresh medium.
9. Determine cell density and calculate the split ratio required to seed the new wells depending on the desired final volume using the Haemocytometer (counting chamber) (Bright-Line) and a microscope.

Cells per ml = the average count per square x the dilution factor x 10^4 (count 10 squares)

- Example: If the average counts per square is 45 cells x 5 x10^4 = 2,250,000 or 2.25 x 10^6 cells/ml.

Total cell number = cells per ml x the original volume of fluid from which cell sample was removed.

- Example: 2.25 x 10^6 (cell per ml) x 10 ml (original volume) = 2.25 x 10^7 total cells.

3:2 Cell Viability Test: MTT after treating with Genistein

1. Transfer the required number of cells (2500 cell/well) to the new plates with appropriate final volume of new medium. (Test performed in 96 wells plate).

2. Prepare the different dilution of Genistein diluted by DMSO: 0.1µM, 0.3µM, 1µM, 3µM, 10µM and 30µM.

3. Remove the medium, add 100µl from each dilution to 3 different wells in the plate. For the negative and positive control add the same volume of medium and DMSO. Incubate for 24hours in 37°C.

4. Remove the medium, wash with PBS. Add 100µl staurosporine (STS) (SIGMA-ALDRICH) 1.75µM for 3 hours in 37°C. For positive control add STS diluted with the medium to the same concentration. For the negative control add appropriate volume of DMSO and medium.

5. After incubation remove the solution and wash one time with PBS, add 100µl medium to each well and incubate in 37°C for 24 hours.

6. Remove the medium, add 100µl of 1:10 solution; MTT (SIGMA) 5mg/ml: DMEM. Incubate for 3 hours in 37°C

7. Spectrophotometrically measure absorbance at a wavelength of 570nm-655nm is applied using Bio Rad machine and microplate manager as a soft ware analysis.

3:3 Mitochondrial Network Structure (Mitotracker) after treating with Genistein

MitoTracker (according to Invitrogen manual and protocol sheet) is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential. To label mitochondria, cells are simply incubated with MitoTracker probes, which passively diffuse across the plasma membrane and accumulate in active mitochondria.

Then we have to recognize the proportion filamentous phenotype, fragmented or interspersed on 100 cells.

1. Culture the cells in a 6-well plate (35mm) and 2 ml of RPMI medium supplemented.

2. Seed 100,000 cells / well and incubated 24 hours at 37°C.

3. Rinse wells with PBS and put fresh medium.

4. Remove the medium. Add the tested product with the propitiate concentration incubate for 24hours in 37°C.

5. Remove the medium. Add the STS 2µM incubate for 3 hours in 37°C.

6. Remove the medium in each well, rinse with PBS and add 2 ml of a 100 nM Mitotracker (Invitrogen).

7. 30 min incubation in 37°C.

8. Remove the solution of Mitotracker then rinse with PBS.

9. mtDNA and nuclear DNA were labelled by adding to the cells 0.5 µl DAPI (SIGMA) 3µM incubate for 5 min in room temperature.

10. Observed by a microscope and images were captured by a confocal microscope (Zeiss, LSM 510 Meta) with a 40X oil objective (Diaphot, Nikon, Japan) using Metamorph acquisition/analysis software (Universal Imaging Corp., Downingtown, PA, USA).

3:4 Quantitative Measurement of Mitochondrial Membrane Potential (JC1) after treating with Genistein

This assay is applied to visualize mitochondrial membrane potential (ΔΨ) by the utilization of a derivative of Tetramethyl Rhodamine (TMRE). The TMRE is a cell permeable cationic dye that has a strong fluorescent signal. In most healthy cell lines, accumulation of TMRE in mitochondria results in a quenched fluorescence intensity. In apoptotic cells, where the mitochondrial membrane potential is compromised, TMRE is not accumulating in the mitochondria and therefore its fluorescence is not quenched.

1. Culture the cells in a 6-well plate (35mm) and 2 ml of RPMI medium supplemented.

2. Seed 100,000 cells / well and incubated 24 hours at 37°C.

3. Rinse wells with PBS and put fresh medium.

4. Remove the medium. Add the tested product with the propitiate concentration incubate for 24hours in 37°C.

5. Remove the medium. Add the STS 2µM incubate for 3 hours in 37°C.

6. Remove the medium in each well, rinse with PBS and add 2 ml of a 5µg/ml JC1 (SIGMA-ALDRICH).

7. 30 min incubation in 37°C.

8. Remove the solution of JC1 then rinse with PBS

9. mtDNA and nuclear DNA were labelled by adding to the cells 0.5 µl DAPI 3µM incubate for 5 min in room temperature.

The cells were observed with two different wavelengths of emission:

538 nm (green): mitochondrial mass.

590nm (red): mitochondrial membrane potential.

The ratio 590/538 provides the mitochondrial membrane potential in relation to mitochondrial mass.

Red: strong

Yellow: Medium

Green: low

RESULTS

1:0 Genetic Diagnoses of TMEM126A Gene

After the Medical Diagnosis, if patients show any Inherited Optic Atrophy (ION) symptoms, Genetic Diagnosis will take place in the diagnostic process. Blood samples from the patients are used to extract DNA. DNA analysis methods can indicate any occurrence of Dominant Optic Atrophy (DOA) such as mutation in OPA1, OPA3 and OPA8, or Recessive Optic Atrophy (ROA) such as mutation in TMEM126A gene.

• Considering that all known optic atrophy genes encode mitochondrial proteins. Systematic sequence analysis identified a mutation in the gene encoding TMEM126A, a transmembrane protein.

12 DNA samples from 12 different patients have no molecular diagnosis as they show no mutation in neither OPA1 nor OPA3 and OPA8 genes. Amplifying the DNA samples; using specific oligos for each of the five exons then sequencing TMEM126A exons to identify any mutation in this gene. (Figure 8).

The results of the sequencing were negative indicating any mutation on the TMEM126A five exons. To continue the diagnosis for further information Mitochondrial DNA Analysis and Cell Biological tests will take place in the process but not in this project.

TMEM126A Sequence

```
  1 aacctccgtc ccgcagtcca attagcagcc gcgacccggc gcccgcccac gccgcgtcac
 61 gagtcagcca aagatggctg cgcccaggta atttgagcaa aggccacagt gaactccggc
121 gtggctgagg aaggaggagg cacccacagg ctgctgggag gagagcataa ggctcaaaat
181 ggaaaatcat aaatccaata ataaggaaaa cataacaatt gttgatatat ccagaaaaat
241 taaccagctt ccagaagcag aaaggaatct acttgaaaat ggatcggttt atgttggatt
301 aaatgctgct ctttgtggcc tcatagcaaa cagtcttttt cgacgcatct tgaatgtgac
361 aaaggctcgc atagctgctg gcttaccaat ggcagggata ccttttctta caacagactt
421 aacttacaga tgttttgtaa gttttccttt gaatacaggt gatttggatt gtgaaacctg
481 taccataaca cggagtggac tgactggtct tgttattggt ggtctatacc ctgttttctt
541 ggctatacct gtaaatggtg gtctagcagc caggtatcaa tcagctctgt taccacacaa
601 agggaacatc ttaagttact ggattagaac ttctaagcct gtctttagaa agatgttatt
661 tcctattttg ctccagacta tgttttcagc ataccttggg tctgaacaat ataaactact
721 tataaaggcc cttcagttat ctgaacctgg caaagaaatt cactgatttt aaacaaatat
781 gtaaacaaaa ataaaatggt aaaaacagtt tatgtcta
```

Figure 8: the sequence of TMEM126A gene

Table18: Length feature of the different five exons of TMEM126A gene

Position in the open reading frame	**Exon Length**	**Fragment Amplified Length**
Exon1: 1 to 89	90nt	259nt
Exon2: 90 to 182	92 nt	298 nt
Exon3: 183 to 376	193 nt	409 nt
Exon4: 377 to 491	114 nt	345 nt
Exon5: 492 to 726	234 nt	420 nt

The following is the result of the electrophoresis regarding the amplifying products of all 12 patients, after that product were treat to be appropriate to the sequencer as well results were BLAST but no serious mutation had been found. (Figure 9).

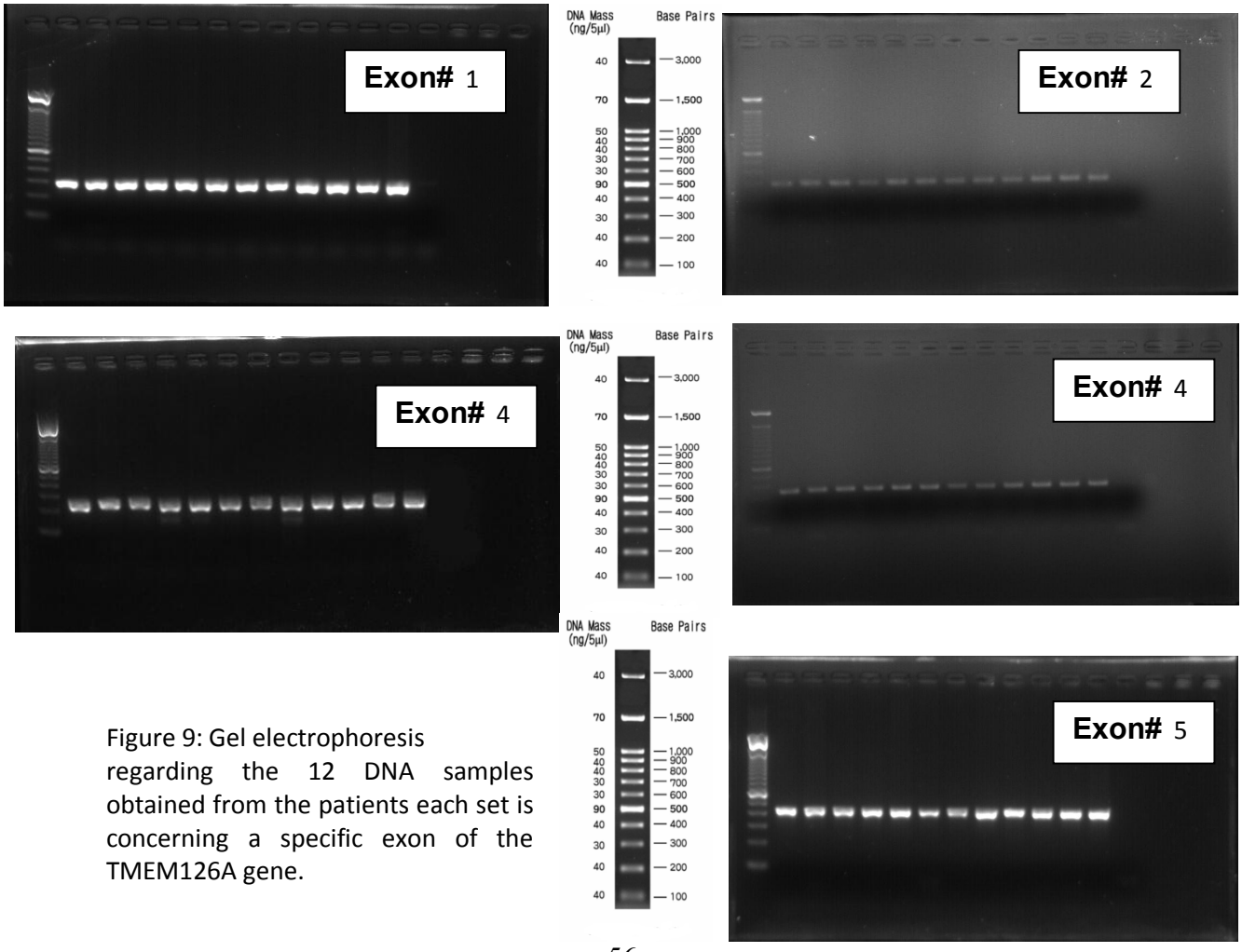

Figure 9: Gel electrophoresis regarding the 12 DNA samples obtained from the patients each set is concerning a specific exon of the TMEM126A gene.

→ According to the size of the bands on the gel all bands signify the exact size regarding the representative exon in addition that the sequencing results on BLAST showed no serious mutation had been found in any of the 12 samples.

2:0 OPA1 +/- mouse mt-DNA analyzing

• As the OPA1 +/- model animal is created; indications showed that deletions in mitochondrial DNA and susceptibility to apoptosis are activated, but yet mutation in mitochondrial DNA is not obviously obtained whether if it's activated or not. Mitochondrial DNA mutations are important cause of genetic diseases and have been proposed to play role in the aging process.

Since there are multiple copies of the mitochondrial genome in a single cell the quantification of total mitochondrial mutations is difficult, moreover the detecting of individual mtDNA mutated molecules is beyond the sensitivity of most sequencing based techniques.

The method currently used to document the incidence of mtDNA mutations is Post-PCR cloning. The strategy of this method depends on the amplification of a target sequence in the mitochondrial genome with a high fidelity DNA polymerase. These PCR products are then cloned in a bacterial vector, and the plasmid DNA purified then sequenced. Each individual PCR products is a copy of one mtDNA molecule and by sequencing a large number of clones; the mutations can be quantified.

The PCR reaction is completed by the using of specific primers of non coding region in the mtDNA called the D-Loop region which is the major control site for mtDNA expression because it contains the leading for origin of replication and major promoters for transcription. The D-Loop region is a hyper variable area therefore it is a hot spot for somatic mutations.

In next step mtDNA extraction is going to be tested for both depletion and deletions.

Mitochondrial Depletion is a disorder involving a quantitative defect of mtDNA. To establish a range of mtDNA levels; real-time quantitative polymerase chain reaction (PCR) analyzing method will be carried out. Deletions in mtDNA are going to be identified by long range PCR.

2:1 Post PCR Cloning

Over 100 samples of mitochondrial DNA had been sequenced and the sequences obtained were compared with the references on NCBI Reference Sequence Database over BLAST. With the consideration to choose the clean sequence which lack of baseline noise so real peaks are still easy to call for analysis.

OPA1+/- sample no. 22

```
Score =  887 bits (480),  Expect = 0.0
 Identities = 482/483 (99%), Gaps = 0/483 (0%)
 Strand=Plus/Minus

Query  81     TTGTTAATGTTTATTGCGTAATAGAGTATGATTAGAGTTTTGGTTCACGGAACATGATTT  140
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16299  TTGTTAATGTTTATTGCGTAATAGAGTATGATTAGAGTTTTGGTTCACGGAACATGATTT  16240

Query  141    TGTAAAATTTTTACAAGTACTAAAATATAAGTCATATTTTGGGAACTACTAGAATTGATC  200
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16239  TGTAAAATTTTTACAAGTACTAAAATATAAGTCATATTTTGGGAACTACTAGAATTGATC  16180

Query  201    AGGACATAGGGTTTGATAGTTAATATTATATGTCTTTCAAGTTCTTAGTGTTTTTGGGGT  260
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16179  AGGACATAGGGTTTGATAGTTAATATTATATGTCTTTCAAGTTCTTAGTGTTTTTGGGGT  16120

Query  261    TTGGCATTAAGAGGAGGGGGTGGGGGGTTTGGAGAGTTAAAATTTTGTATTGAGTAGCAT  320
              |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  16119  TTGGCATTAAGAGGAGGGGGTGGGGGGTTTGGAGAGTTAAAATTTGGTATTGAGTAGCAT  16060

Query  321    TTATGTCTAACAAGCATGAATAATTAGCCTTAGGTGATTGGGTTTTGCGGACTAATGATT  380
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16059  TTATGTCTAACAAGCATGAATAATTAGCCTTAGGTGATTGGGTTTTGCGGACTAATGATT  16000

Query  381    CTTCACCGTAGGTGCGTCTAGACTGTGTGCTGTCCTTTCATGCCTTGACGGCTATGTTGA  440
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15999  CTTCACCGTAGGTGCGTCTAGACTGTGTGCTGTCCTTTCATGCCTTGACGGCTATGTTGA  15940

Query  441    TGAAAGTAGGCCAAAATAAAAAGATACCAAATGCATGACACCACAGTTATGTTGGTCATG  500
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15939  TGAAAGTAGGCCAAAATAAAAAGATACCAAATGCATGACACCACAGTTATGTTGGTCATG  15880

Query  501    GGCTGATTAGACCCGATACCATCGAGATGTCTTATTTAAGGGGAACGTATGGGCGATAAC  560
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15879  GGCTGATTAGACCCGATACCATCGAGATGTCTTATTTAAGGGGAACGTATGGGCGATAAC  15820

Query  561    GCA  563
              |||
Sbjct  15819  GCA  15817
```

Figure 10: A result of one of the mitochondrial DNA molecule which had been sequenced and compared with the references on NCBI Reference Sequence Database over BLAST.

The result came screening that the bars show what places along our sequence are similar to other published sequences concerning Mice mitochondrial DNA database.

Several sequence gaps and misalignments were detected over all the samples analyzed but no serious mutation had been detected.

→ The data showed in (figure 10) represent one example of the 100 plasmids that had sequenced. After applying the technique (Post PCR Cloning); sequencing of the inserted fragment in the plasmid did not appear with any serious mutation.

2:2 Long-Range PCR

→ According to the size of bands the outputs of Long-Range PCR did not show any deletion in in any samples of the mitochondrial DNA compared with the reference and DNA ladder on agarose gel. (Figure 11).

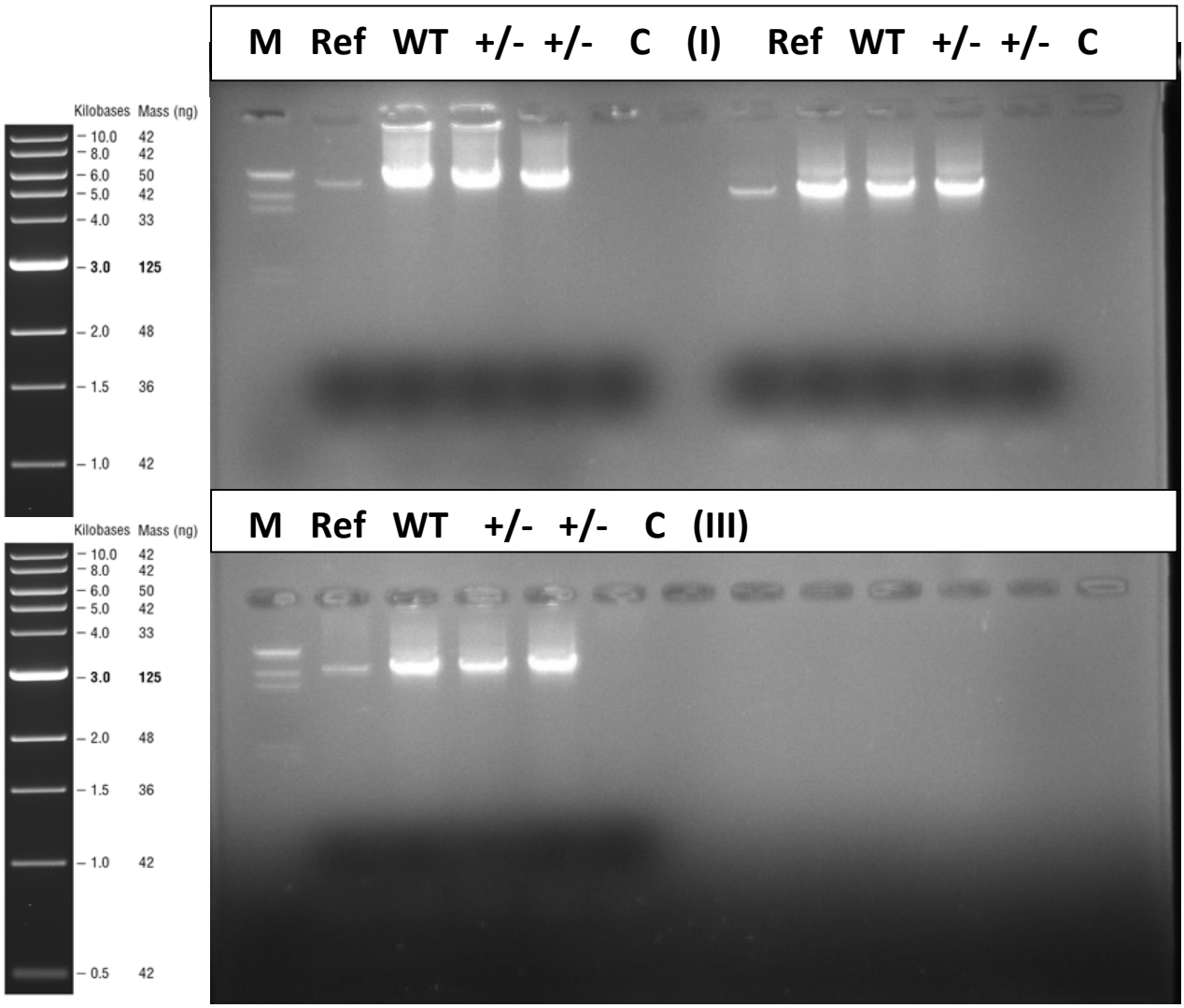

Figure 11: **M:** Marker, **Ref:** reference, **wt:** wilde Type, **+/-:** OPA1+/- DNA sample from model animal muscles tissue, **C:** Control, **(I),(II)**and**(III):** 1st 2nd and 3rd PCR respectively.

2:3 SYBR Green-based real-time RT-PCR

The mtDNA copy number ratio was essentially unaltered when comparing the mutated samples with those from the wild type measured up to the nuclear genome. (Diagram 1).

The threshold line is placed within the exponential increase phase of the amplification curve.

- PCR amplification plot (PCR Base Line Subtracted):

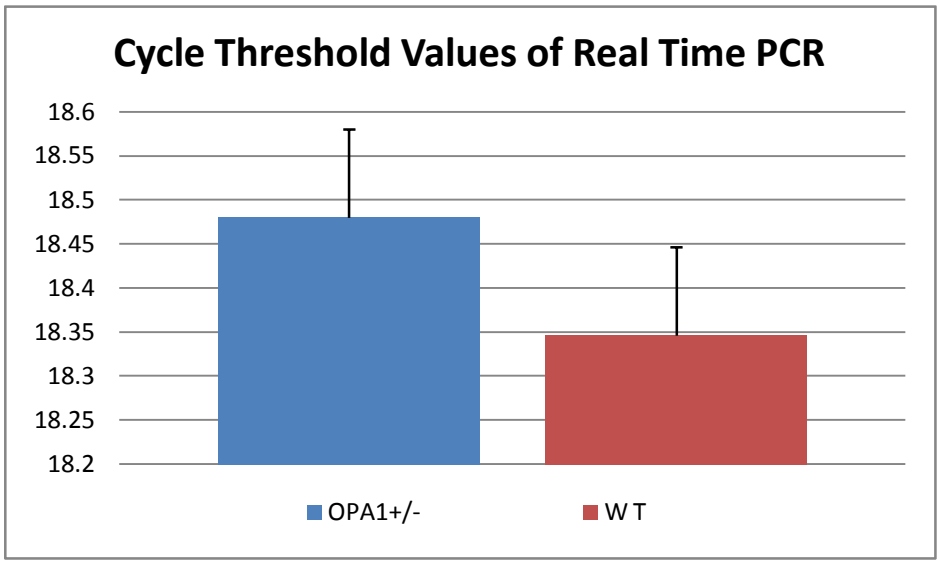

Diagram 1: Values of real time PCR of mutated and wild type samples of mtDNA

- Melt-curve analysis:

The amplification of our target sample is represented by a single peak emerged at about 80-90°C. As the result came out there were no minor peaks at lower and higher temperatures implies the presence of non-specific PCR products or any contamination.

→ The outputs of quantitative Rt-PCR did not show any difference in the number of copies of mtDNA between samples taken from mutated mice and samples taken from the wild type mice.

3:0 Cell Biological Tests

• Since mutation in all genes encode mitochondrial proteins can be clearly expressed in every single type of cells; Fibroblasts had been selected for Cell Biological tests because of their ability to be obtained easily by -*in vitro* culture- methods.

Studying the effect of Genistein on the Fibroblast R445H OPA1:

• MTT based test to measure the growth of the cells by counting the viable cell after treating with Genistein in different concentration. The MTT assay is a colorimetric assay for assessing cell viability. dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color.

• And characterize the effect of this product on the:

- Mitochondrial network by using Mitochondrion-selective probes MitoTracker.
- Mitochondrial membrane potential by JC1.

3:1 Cell Viability Test: MTT after treating with Genistein

The following are the result (Diagram 2) is the average of two times experiment to figure the optimal concentration of Genistein that affect on cell viability:

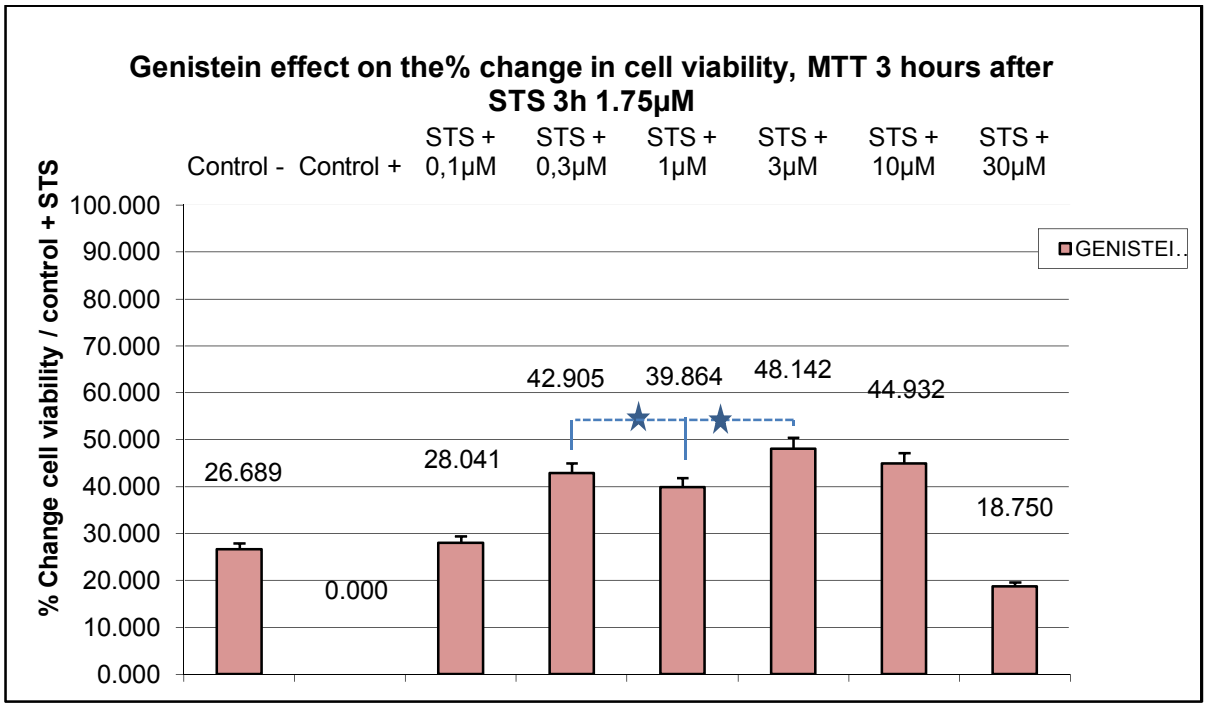

Diagram 2: Average of two times experiment to measure the effect of Genistein on cell viability percentage after applying apoptotic stress caused by STS. ★ $P < 0.05$ which indicate to significant difference

The above chart is the sum results of the two times experiment of the optimal concentration of Genistein that affect on cell viability.

To compare statistical differences between means tow sided t-test had been applied where significant value indicate the optimum concentration of the drug.

Genistein 3µM had best promotion of the cell viability compared with the other concentration in the experiment, consequently of the previous result which induced an increase in the percentage of cells viability in this concentration; all further experiments to measure or test the membrane potential and network structure of the mitochondria in

cultured cells are applied to study the affect of the product in this concentration (3µM).

→ Cell viability MTT test after treating with six different concentrations of Genistein ranged between 0.1µM and 30µM showed a better ratio of viability in 3µM, where viability ratio increased by approximately 50%. Thus this concentration used (3µM) of Genistein was used in the further experiments.

3:2 Mitochondrial Network Structure (Mitotracker) after treating with Genistein

Recognize the filamentous phenotype and fragmented phenotype on 100 cells repeated twice. (Table 23 and Figure 12).

Table 19: The percentage of different phenotypes of the mitochondrial network among different treatments

Control			
	1st Experiment	2nd Experiment	Percentage %
Fragmented	75	83	79
Filamentous	25	17	21
Genistein			
	1st Experiment	2nd Experiment	Percentage %
Fragmented	78	89	83.5
Filamentous	22	11	16.5
Genistein + Staurosporine			
	1st Experiment	2nd Experiment	Percentage %
Fragmented	62	55	58.5
Filamentous	38	44	41.5

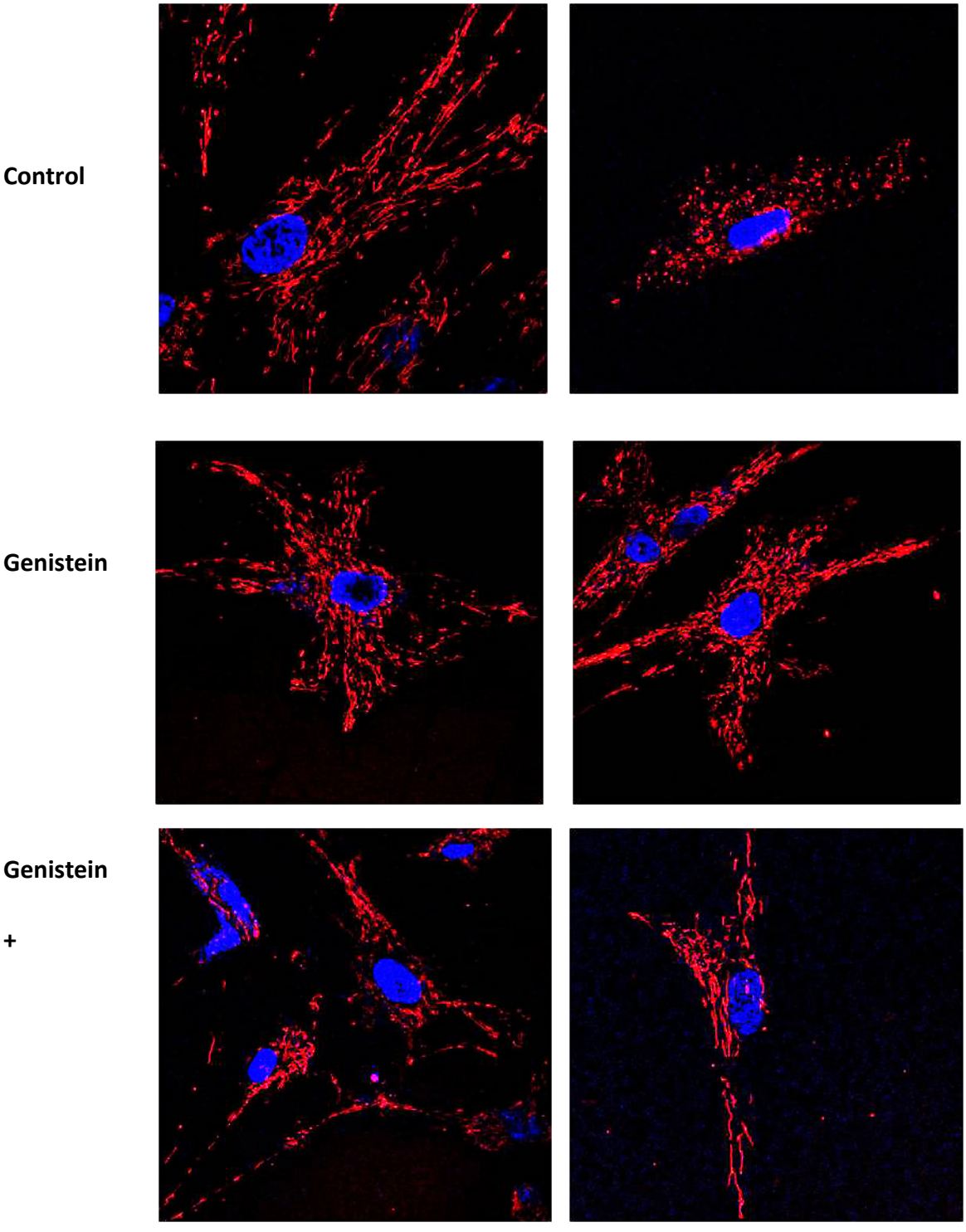

Figure 12: Representative images of mitochondrial morphologies of DOA fibroblasts incubated and captured with different treatment to indicate any recognizable changes in mitochondrial network.

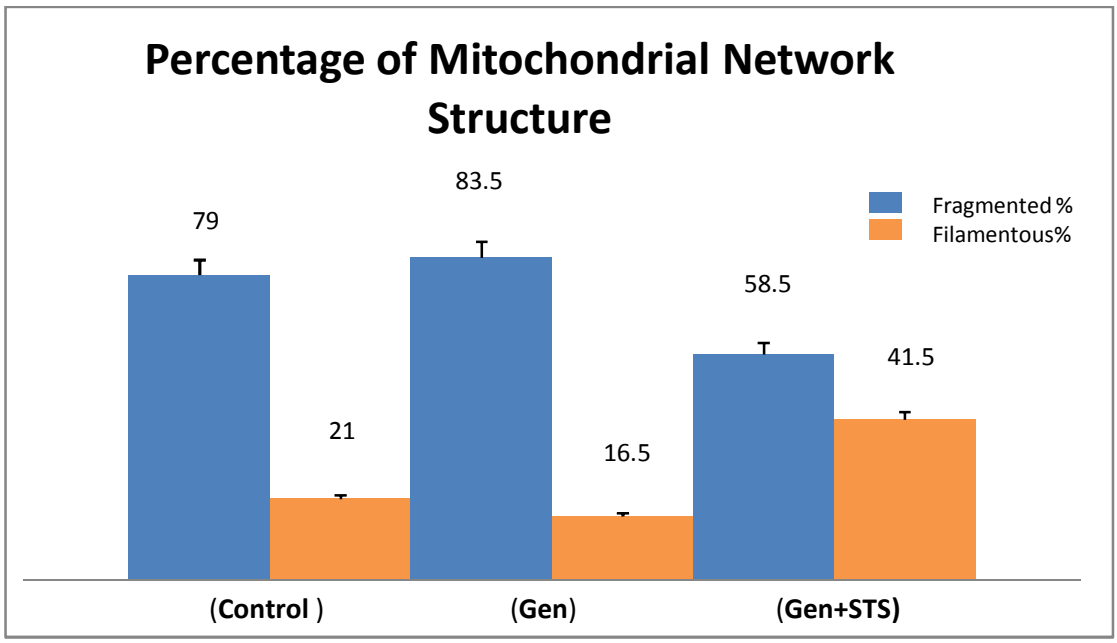

Diagram3: Percentage of different phenotypes of mitochondrial network among 100 cells with each treatment.

Results are reported (Diagram 3) as the mean standard deviation of two different experiments. Our results indicate that mitotracker is a sensitive indicator of relative changes in mitochondrial network morphology, whereas Genistien is relatively effective on network changes under apoptotic stress caused by staurosporine, as the results above we can notice the notable changes in the percentage of cells with filamentous network after treating with both Genistien and staurosporine. Yet there is no notable changes can be mentioned caused by the lone effect of Genistien its self compared with the control.

→ The results of mitotracker which is the pigment that used to study mitochondrial network structure shows that the mitochondrial network in fibroblast cells R445H OPA1 is fragmented in about 80% of the cells, and this percentage is significantly improved after treating with Genistein where network appears more filamentous in about 60% of the cells . This result indicates the role of this drug in maintaining the structure of the mitochondrial network.

3:3 Quantitative Measurement of Mitochondrial Membrane Potential (JC1) after treating with Genistein

Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio (Diagram 4). The potential-sensitive colour shift is due to concentration-dependent formation of red fluorescent aggregates; where a higher red means a high membrane potential while a higher green means a low membrane potential. The results in experiment show a slight changes on the comparative measurements of membrane potential when treated by both Genistein and staurosporine, in other hand those results do not reasonably determine the percentage of mitochondria within a population that respond to an applied stimulus by Genistein.

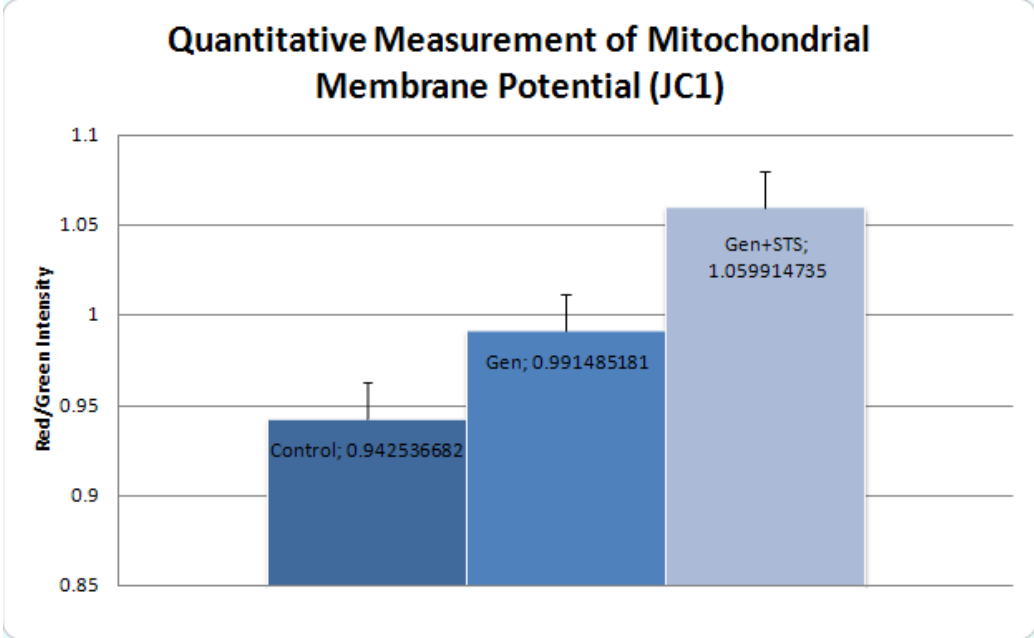

Diagram 4: Quantitative results of mitochondrial membrane potential for 100 cells after treating with Genistein and staurosporine.

→ The results of JC1, which is the pigment that use to study the mitochondrial membrane potential where parts for high-potential appears in red while parts of the low-potential appears in green, a slight increase in the relative measurements of potential when treating with Genistein.

DISSCUSION & CONCLUSIONS

► Researches have identified *TMEM126A* as a gene that encodes a mitochondrial protein of higher eukaryotes and whose mutations are to our knowledge the first known genetic cause of nonsyndromic autosomal-recessive optic atrophy (optic atrophy type 7). This finding gives further support to the view that mitochondrial dysfunctions are a hallmark of optic neuropathies, suggesting that common therapeutic approaches may be developed to improve the long-term prognosis of patients whatever the gene involved (Delettre *et al.,* 2000, Chevrollier *et al.,* 2008).

TMEM126A may be important to the assembly of a protein complex required for the function of retinal ganglion cells in higher eukaryotes. In other tissues, it is possible that another protein of the TMEM family could adequately substitute for the defective function of TMEM126A. It is also possible that TMEM126A may be required to accelerate the assembly of a protein complex. In the absence of TMEM126A, the complex would constitute slowly, and only high-energy-demanding retinal ganglion cells would be affected (Chevrollier *et al.,* 2008). Investigation must carry out to define the exact cause of the symptoms within TMEM family or OPA family and more.

To date, there is no preventative or curative treatment in DOA; severely visually impaired patients may benefit from low vision aids. Genetic counseling is commonly offered and patients are advised to avoid alcohol and tobacco consumption, as well as the use of medications that may interfere with mitochondrial metabolism. The ultimate goal is to translate these research advances into clinical practice and new treatment strategies are currently being investigated to improve the

visual prognosis for patients with mitochondrial optic neuropathies. Gene and pharmacological therapies for DOA are currently under investigation.

► Somatic mutations provide exquisitely specific biomarkers for cancer diagnostics (Sidransky 2002). Somatic mutations in mitochondrial DNA are particularly well-suited for such diagnostic applications because; they are 500 to 1000 times more numerous in the cell than nuclear DNA mutations (Jones *et al.,* 2001, Wong *et al.,* 2003, Fliss *et al.,* 2000).

Plasma of two colorectal cancer patients prior to and following surgery to remove their cancers was studied. In both patients, nuclear DNA mutations were evident in the plasma before but not after surgery (Diehl *et al.,* 2008). Mutant mtDNA template molecules were also identified in the plasma of these two patients at much higher concentrations before surgery than after surgery, confirming their tumor-specificity. The concentration of mutant mtDNA molecules in the plasma greatly exceeded the concentration of the mutant nuclear genes. (He *et al.,* 2010).

Since we know that studies indicate that the mutation in OPA1 may affects the stability of the mtDNA by the existence of ROS. Thus several techniques involved measuring and specify the stability of mtDNA were applied in model animal OPA1 +/- and compared to the wild type mice.

Overstatement the results do not indicates the high quality of the used methods yet the conditions and qualifications of the experiments must be modified to achieve intelligible results. For instance the age of the mice have to be taken in account, five months old mice may be less effective to give voluble indicators rather than the older mice. Moreover

in the published study the samples were driven from human cancer patients, whereas our samples are driven from mice model animal with reference to OPA1. In addition for any further studies number of samples must be multiply by 100 to obtain statistical and more realistic results.

► Mitochondria are the major organelle of essential energy production for cell activity, and have been suggested to be involved in many important physiological activities (Moreira *et al.,* 2010; Robertson and Orrenius 2002). The mitochondrial respiratory chain is a major site of ROS production in the cell, and thus mitochondria are suggested as a prime target for oxidative damage (Trifunovic and Larsson 2008). In a physiological state, most mammalian cells keep a balance between the generation of ROS and its removal. However, when the mitochondria suffer from injuriesthis balance is likely broken resulting in oxidative stress that leads to cell death (Moreira *et al.,* 2010; Hirai *et al.,* 2001; Robertson and Orrenius 2002; Trifunovic and Larsson 2008; Sas *et al.,* 2007). Intracellular oxidative stress is also known to alter a cell's mitochondrial potential (Lee *et al.,* 2011).

DOA is a mitochondriopathy, as the genes responsible for DOA encodes proteins ubiquitously expressed, imported into the mitochondria and associated to the inner membrane. Mutations in these genes generally affect mitochondrial fusion, energy metabolism, maintenance of mitochondrial genome integrity and balance between the generation of ROS and its removal. Our findings are in agreement with data of previous studies which have indicated that Genistein as a dietary antioxidant plays a protective role in neurodegeneration (Valles *et al.,* 2010; Lee and Lee 2008; Wang *et al.,* 2005; Ma *et al.,* 2010; Yu *et al.,*

2009; Ma *et al.,* 2009). Researches focused on mitochondria of PC12 cells and found Genistein maintained the mitochondrial redox system disordered by beta-amyloid peptide 25–35 (Aβ25-35) and then maintained mitochondrial membrane potential.

Based on the present results that Genistein maintains mitochondrial membrane potential and reduction-oxidation system in mitochondria of PC12 cells (Ma *et al.,* 2009, 2010), we speculate the data obtained in our study show that Genistein pretreatment considerably increases the viability percentage of fibroblast cells obtained from DOA patients with a mutation on OPA1 gene, and affect positively on other vital factors in mitochondria. Further research is needed to clarify the relationship between mitochondrial dysfunction and OPA1 mutations, and the mechanism of Genistein to protect mitochondrial systems and functions. However, pre-treatment with Genistein might reverse the damage of the mitochondrial membrane and increase mitochondrial antioxidant function. Further studies are needed to explore the exact signalling pathway(s) by which Genistein protects mitochondria. Although using fibroblast is recommended in researches because of its ease of cultivation feature and mutation in all genes encode mitochondrial proteins can be clearly expressed in these cells, but research might be useful on other types of cell expressly on ganglion cells if it's possible. On other hand experiments should be applied in a larger number of cells so statistical results can be more valuable.

Conclusions:

1- TMEM126A have been identified as a gene encodes a mitochondrial protein. However, No serious mutations in this gene had identified in any of the 12 samples. Hence analytical approaches must carried out to improve the long term prognosis of patients whatever the gene involved.

2- Mutation in OPA1 obtained from mice model animal (OPA1+/-) had no effect on the quantity or the quality of mtDNA. Defective in mitochondrial gene can be reason to increases the possibility to affect the stability of the mtDNA by the existence of ROS.

3- Pre-treatment with Genistein affect positively on vital factors in mitochondria and considerably increases the viability percentage of fibroblast cells obtained from DOA patients with a mutation on OPA1 gene. In addition of an effect on mitochondrial network and membrane potential.

Perspective:

1- To date, there is no preventative or curative treatment in DOA. The ultimate goal is to translate these research advances into clinical practice and new treatment strategies are currently being investigated to improve the visual prognosis for patients with mitochondrial optic neuropathies.

2- In experiments obtained to identify the mtDNA mutation load; conditions and qualifications of the experiments must be modified to achieve intelligible results. For example the age of the mice, the type of cells are used to obtain the materials of study and the technique itself.

3- Further studies are needed to explore the exact signalling pathway(s) by which Genistein protects mitochondria.

4- Beside fibroblast; research might be useful on other types of cell expressly on ganglion cells if it's possible.

References:

1- Abrahams, J.P., Leslie, A.G., and Lutter, R. (1994). Structure at 2.8 A resolution of F1-ATPase from bovine heart mitochondria. Nature. 370:621-628.

2- Alavi, M.V., Bette, S., and Schimpf, S. (2007). A splice site mutation in the murine Opa1 gene features pathology of autosomal dominant optic atrophy. Brain. 130:1029-42.

3- Alexander, C., Votruba, M., and Pesch, U.E. (2000). OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat Genet. 26:211-5.

4- Amati-Bonneau, P., Derrien, C., Pasquier, L., Malthiéry, Y., Reynier, P., and Bonneau, D. (2003). The association of autosomal dominant optic atrophy and moderate deafness may be due to the R445H mutation in the OPA1 gene. American Journal Of Ophthalmology Issue 6, p973-1252.

5- Amati-Bonneau, P., Guichet, A., and Olichon, A. (2005). OPA1 R445H mutation in optic atrophy associated with sensorineural deafness. Ann Neurol. 58:958-63.

6- Amati-Bonneau, P., Milea, D., and Bonneau, D. (2009). OPA1-associated disorders: phenotypes and pathophysiology. Int J Biochem Cell Biol. 41:1855-65.

7- Amati-Bonneau, P., Valentino, M.L., and Reynier, P. (2008). OPA1 mutations induce mitochondrial DNA instability and optic atrophy plus phenotypes. Brain 131:338-51.

8- Anderson, S., Bankier, A.T., and Barrell, B.G. (1981). Sequence and organization of the human mitochondrial genome. Nature. 290:457-465.

9- Andrews, R. M. (1999). Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature Genet. 23, 147.

10- Asahi K. I., Ono I., Kusakabe H., Nakamura G., Isono K. (1981). Studies on differentiation inducing substances of animal cells. I: Differenol A, a differentiation inducing substance against mouse leukemia cells.Antibiot. 34, 919.

11- Balaban, R.S. (1990). Regulation of oxidative phosphorylation in the mammalian cell.Am J Physiol. 258:C377-89.

12- Barnes S., Peterson T.G., Coward L. (1995). Rationale for the use of genistein-containing soy matrices in chemoprevention trials for breast and prostate cancer. Cell Biochem 22 (suppl.), 181.

13- Bar-Yaacov, D., Blumberg, A., Mishmar, D. (2012). Mitochondrial-nuclear co-evolution and its effects on OXPHOS activity and regulation. Biochim Biophys Acta 1819: 1107–1111. doi: 10.1016/j.bbagrm.2011.10.008.

14- Barbet, F., Gerber, S., Hakiki, S., Perrault, I., Hanein, S., Ducroq, D., Tanguy, G., Dufier, J.L., Munnich, A., Rozet, J.M. (2003). A first locus for isolated autosomal recessive optic atrophy (ROA1) maps to chromosome 8q. Eur. J. Hum. Genet. 11, 966–971.

15- Bernardi, P. and Azzone, G.F. (1981). Cytochrome c as an electron shuttle between the outer and inner mitochondrial membranes. J. Biol. Chem. 256, 7187-7192.

16- Berry, E.A., Guergova-Kuras, M., and Huang, L.S. (2000). Structure and function of cytochrome bc complexes. Annu Rev Biochem. 69:1005-1075.

17- Bitto, A., Burnett, B.P., Polito, F., Levy, R.M., Marini, H., Di-Stefano, V., Irrera, N., Armbruster, M.A., Minutoli, L., Altavilla, D., and Squadrito, F. (2009). Genistein aglycone reverses glucocorticoid-induced osteoporosis and increases bone breaking strength in rats: a comparative study with alendronate. Br J Pharmacol 156:1287–1295.

18- Budde, S.M., van-den-Heuvel, L.P., Smeets, R.J., Skladal, D., Mayr, J.A., Boelen, C., Petruzzella, V., Papa, S., and Smeitink, J.A. (2003). Clinical heterogeneity in patients with mutations in the NDUFS4 gene of mitochondrial complex I. J Inherit Metab Dis 26:813-5.

19- Calvo, S., Jain, M., Xie, X., Sheth, S.A., Chang, B., Goldberger, O.A., Spinazzola, A., Zeviani, M., Carr, S.A., and Mootha, V.K. (2006). Systematic identification of human mitochondrial disease genes through integrative genomics. Nat. Genet. 38, 576–582.

20- Carelli, V., LaMorgia, C., Iommarini, L., Carroccia, R.,Mattiazzi, M., Sangiorgi, S., Farne, S., Maresca, A., Foscarini, B., and Lanzi, L. (2007). Mitochondrial optic neuropathies: How two genomes may kill the same cell type? Biosci. Rep. 27, 173–184.

21- Carelli, V., Ross-Cisneros, F.N., and Sadun, A.A. (2004). Mitochondrial dysfunction as a cause of optic. Prog Retin Eye Res. (1):53-89.

22- Carroll, J., Shannon, R.J., and Fearnley, I.M. (2002). Definition of the nuclear encoded protein composition of bovine heart mitochondrial complex I. Identification of two new subunits. J Biol Chem. 277:50311-50317.

23- Chen, H., and Chan, D.C. (2004). Mitochondrial dynamics in mammals. Curr Top Dev Biol. 59:119-44.

24- Chen, H., Chomyn, A., and Chan, D.C. (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem. 280:26185-92.

25- Chen, X.J., and Butow, R.A. (2005). The organization and inheritance of the mitochondrial genome. Nat Rev Genet. 6: 815-25.

26- Chevrollier, A., Guillet, V., Loiseau, D., Gueguen, N., de Crescenzo, M.A., Verny, C., Ferre, M., Dollfus, H., Odent, S., Milea, D. (2008). Hereditary optic neuropathies share a common mitochondrial coupling defect. Ann. Neurol. 63, 794–798.

27- Chih-Chieh, M., and Ian, J.H. (2009). Mitochondrial DNA instability Chang Gung Med J Vol 32 No. 4.

28- Christophe, O. (2003). Leber's Hereditary Optic Neuropathy. Orphanet Encyclopedia.

29- Cizkova, A., Stra -necky, V., Mayr, J.A., Tesarova, M., Havlı-ckova, V., Paul, J., Iva-nek, R., Kuss, A.W., Hansı-kova, H., Kaplanova, V., et al. (2008). TMEM70 mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalocardiomyopathy. Nat. Genet. 40, 1288–1290.

30- Clayton, D.A. (2000). Vertebrate mitochondrial DNA: a circle of surprises. Exp Cell Res. 255:4–9.

31- Cohn, A.C., Toomes, C., and Hewitt, A.W. (2008). The natural history of OPA1-related autosomal dominant optic atrophy, Br. J. Ophthalmol. 92:1333–1336.

32- Cortopassi, G.A., and Arnheim, N. (1990). Detection of a specific mitochondrial DNA deletion in tissues of older humans.Nucleic Acids Res. 18(23):6927-33.

33- Cooper, J.M., Mann, V.M., and Schapira, A.H. (1992). Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. J. Neurol Sci. 113(1):91-8.

34- Cottrell, D.A., Blakely, E.L., Johnson, M.A., Ince, P.G., Borthwick, G.M., and Turnbull, D.M. (2001). Cytochrome c oxidase deficient cells accumulate in the hippocampus and choroid plexus with age. Neurobiol Aging. 22(2):265-72.

35- D'Herde, K., De-Prest, B., Mussche, S. (2001). Evidence and age-related distribution of mtDNA D-loop point mutations in skeletal muscle from healthy subjects and mitochondrial patients. Ultrastructural localization of cytochrome c in apoptosis demonstrates mitochondrial heterogeneity. Cell Death. Differ. 7:331-337.

36- Del-Bo, R., Bordoni, A., Martinelli- Boneschi, F., Crimi, M., Sciacco, M., Bresolin, N., Scarlato, G., and Comi, G.P. (2002). J. Neurol Sci. 202(1-2):85-91.

37- Delettre, C., Lenaers, G., Griffoin, J.M., Gigarel, N., Lorenzo, C., Belenguer, P., Pelloquin, L., Grosgeorge, J., Turc-Carel, and C., Perret, E. (2000). Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat. Genet. 26, 207–210.

38- Delettre, C., Lenaers, G., and Pelloquin, L. (2002). OPA1 (Kjer type) dominant optic atrophy: a novel mitochondrial disease, Mol. Genet. Metab. 75:97–107.

39- Diehl, F., Schmidt, K., Choti, M.A., Romans, K., Goodman, S., Li, M., Thornton, K., Agrawal, N., Sokoll, L., Szabo, S.A., Kinzler, K.W., Vogelstein, B., and Diaz, L.A. (2008). Circulating mutant DNA to assess tumor dynamics. Nat Med. 14(9):985-90.

40- Eliott, D., Traboulsi, E.I., and Maumenee, I.H. (1993). Visual prognosis in autosomal dominant optic atrophy (Kjer type). Am J Ophthalmol. 1993;115:360-7.

41- Fernandez-Silva, P., Enriquez, J.A. and Montoya, J. (2003). Replication and transcription of mammalian mitochondrial DNA.Exp Physiol. 88, 41-56.

42- Ferre, M., and Amati-Bonneau, P. (2005). eOPA1: an online database for OPA1 mutations. Hum Mutat. 25:423–8.

43- Figge, M.T., Reichert, A.S., Meyer-Hermann, M., and Osiewacz, H.D. (2012). Deceleration of fusion-fission cycles improves mitochondrial quality control during aging. PLoS Comput Biol 8:e1002576.

44- Fliss, M.S., Usadel, H., Caballero, O.L., Wu, L., Buta, M.R., Eleff, S.M., Jen, J., and Sidransky, D. (2000). Facile detection of mitochondrial DNA mutations in tumors and bodily fluids.Science. 287 (5460):2017–2019.

45- Frey, T.G. and Mannella, C.A. (2000). The internal structure of mitochondria. Trends Biochem Sci. 25:319-324.

46- Frey, T.G., Renken, C.W., and Perkins, G.A. (2002). Insight into mitochondrial structure and function from electron tomography.Biochim Biophys Acta. 1555:196-203.

47- Fukutake M., Takahashi M., Ishida K., Kawamura H., Sugimura T., Wakabayashi K. (1996). Quantification of genistein and genistin in soybeans and soybean products. Food. Chem. Toxicol. 43, 457.

48- Greaves L.C., Beadle N.E., Taylor G.A., Commane D., Mathers J.C., Khrapko K. and Trunbull D.M. (2009). Quantification of mitochondrial DNA mutation load. Aging Cell 8, pp566–572.

49- Hanein, S., Perrault, I., Roche, O., Gerber, S., Khadom, N., Rio, M., Boddaert, N., Jean-Pierre, M., Brahimi, N., Serre, V., Chretien, D., Delphin, N., Fares-Taie, L.,

Lachheb, S., Rotig, A., Meire, F., Munnich, A., Dufier, J.L., Kaplan, J., and Rozet, J.M. (2009). TMEM126A, Encoding a Mitochondrial Protein, The American Journal of Human Genetics 84, 493–498.

50- Hatefi, Y. (1985). The mitochondrial electron transport and oxidative phosphorylation system. Annu Rev Biochem. 54:1015-69.

51- He, Y., Wu, J., Dressman, D.C., Lacobuzio-Donahue, C., Markowitz, S.D., Velculescu, V.E., Diaz-Jr, L.A., Kinzler, K.W., Vogelstein, B., and Papadppoulos, N. (2010). Heteroplasmic mitochondrial DNA mutations in normal and tumour cells Nature. 464(7288): 610–614.

52- Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R.L., Atwood, C.S., Johnson, A.B., Kress, Y., Vinters, H.V., Tabaton, M., Shimohama, S., Cash, A.D., Siedlak, S.L., Harris, P.L., Jones, P.K., Petersen, R.B., Perry ,G., and Smith, M.A. (2001). Mitochondrial abnormalities in Alzheimer's disease. J. Neurosci 21:3017–3023.

53- Hoppins, S., Lackner, L., and Nunnari, J. (2007). The machines that divide and fuse mitochondria. Annu Rev Biochem. 76:751-80.

54- Hoyt, C.S. (1980). Autosomal dominant optic atrophy. A spectrum of dis- ability. Ophthalmology. 87: 245-251.

55- Hudson, G., Amati-Bonneau, P., and Blakely, E.L. (2008). Mutation of OPA1 causes dominant optic atrophy with external ophthalmoplegia, ataxia, deafness and multiple mitochondrial DNA deletions: a novel disorder of mtDNA maintenance. Brain. 131:329-37.

56- Jaeger, W., Früh, D., and Lauer, H.J. (1972). Types of acquired colour deficiencies caused by autosomal-dominant infantile optic atrophy. Mod Probl Ophthalmol. 11:145-7.

57- Johnston, P.B., Gaster, R.N., Smith, V.C., and Tripathi, R.C. (1979). A clinicopathologic study of autosomal dominant optic atrophy. Am J Ophthalmol. 88:868-875.

58- Johnston, R.L., Seller, M.J., Behnam, J.T., Burdon, M.A., and Spalton, D.J. (1999). Dominant optic atrophy. Refining the clinical diagnostic criteria in light of genetic linkage studies. Ophthalmology. 106:123-128.

59- Jones, J.B., Song, J.J., Hempen, P.M., Parmigiani, G., Hruban, R.H., and Kern, S.E. (2001). Detection of mitochondrial DNA mutations in pancreatic cancer offers a “mass”-ive advantage over detection of nuclear DNA mutations. Cancer Res. 61 (4):1299–1304.

60- Kjer, B., Eiberg, H., Kjer, P., and Rosenberg, T. (1996). Dominant optic atrophy mapped to chromosome 3q region. II. Clinical and epidemiological aspects. Acta Ophthalmol Scand. 74:3-7.

61- Kjer, P., Jensen, O.A., and Klinken, L. (1983). Histopathology of eye, optic nerve and brain in a case of dominant optic atrophy. Acta Ophthalmol. 61:300-312.

62- Kjier, P. (1959). Infantile optic atrophy with dominant mode of inheritance: a clinical and genetic study of 19 Danish families. Acta Ophthalmol Suppl. 164:1-147.

63- Kok-van-Alphen, C.C. (1970). Four families with the dominant infantile form of optic nerve atrophy. Acta Ophthalmol (Copenh). 48:905-16.

64- Kortuem, K., Gieger, L.K., and Levin, L.A. (2000). Differential susceptibility of retinal ganglion cells to reactive oxygen species. Invest Ophthalmol Vis Sic 41:3176-3182.

65- Krause, F., Reifschneider, N.H., Vocke, D., Seelert, H., Rexroth, S., and Dencher, N.A. (2004). "Respirasome"-like supercomplexes in green leaf mitochondria of spinach. J Biol Chem. 279:48369-75.

66- Kraytsberg, Y., Khrapko, K. (2005). Single-molecule PCR: an artifact-free PCR approach for the analysis of somatic mutations. Expert Rev Mol Diagn. 5(5):809-15.

67- Kruman, I., et al ., (1998). Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. J. Neurosci. Res., 51,293-308.

68- Kujoth, G.C., Hiona, A., Pugh, T.D., Someya, S., Panzer, K., Wohlgemuth, S.E., Hofer, T., Seo, A.Y., Sullivan, R., Jobling, W.A., Morrow, J.D., Van-Remmen, H., Sedivy, J.M., Yamasoba, T., Tanokura, M., Weindruch, R., Leeuwenburgh, C., and Prolla, T.A. (2005). Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science. 309:481–484.

69- Lee, Y.W., and Lee, W.H. (2008). Protective effects of genistein on proinflammatory pathways in human brain microvascular endothelial cells. J. Nutr Biochem 19:819–825.

70- Lee C.M., Huang, S.T., Huang, S.H., Lin, H.W., Tsai, H.P., Wu, J.Y., Lin, C.M., and Chen, C.T. (2011).C60 fullerenepentoxifylline dyad nanoparticles enhance autophagy to avoidcytot oxic effects caused by the beta-amyloid peptide. Nanomedicine 7:107‑114.

71- Lenaers, G., Reynier, P., Elachouri, G., and Soukkarieh, C. (2009). OPA1 functions in mitochondria and dysfunctions in optic nerve.Int J Biochem Cell Biol. 41:1866-74.

72- Lenaers G., Hamel C., Delettre C., Amati-Bonneau P., Procaccio V., Bonneau D., Reynier P., Milea D. (2012). Dominant Optic Atrophy. Orphanet J Rare Dis. 7:46. doi: 10.1186/1750-1172-7-46.

73- Li, C., Kosmorsky, G., Zhang, K., Katz, B.J., Ge, J., and Traboulsi, E.I. (2005). Optic Atrophy and sensorineural hearing loss in a family caused by an R445H OPA1 mutation. Am J Med Genet A. 138A:208–11.

74- Li, W., Frame, L.T., Hirsch, S., and Cobos, E. (2010). Genistein and hematological malignancies. Cancer Lett 296:1–8.

75- Liu, X., Weaver, D., Shirihai, O., and Hajnóczky, G. (2009). Mitochondrial 'kiss-and-run': interplay between mitochondrial motility and fusion-fission dynamics. EMBO J. 28:3074-89.

76- Ma, W.W., Xiang, L., Yu, H.L., Yuan, L.H., Guo, A.M., Xiao, Y.X., Li, L., and Xiao, R. (2010). Genistein as a neuroprotective antioxidant attenuates redox imbalance induced by β-amyloid peptides 25-35 in PC12 cells. Int J Devl Neurosci 28:289–295.

77- Ma, W.W., Xiang, L., Yu, H.L., Yuan, L.H., Guo, A.M., Xiao, Y.X., Li, L., and Xiao, R. (2009). Neuroprotection of soyabean isoflavone co-administration with folic acid against b-amyloid 1-40-induced neurotoxicity in rats. Br J Nutr 102:502–505.

78- Manchester, P.T. and Calhoun, F.P. (1985). Dominant hereditary optic atrophy with bitemporal field defects. AMA Arch Ophthalmol. 60:479-84.

79- Mannella, C.A., Marko, M., and Buttle, K. (1997). Reconsidering mitochondrial structure: new views of an old organelle. Trends Biochem Sci. 22:37-8.

80- Mannella, C.A., Marko, M., Penczek, P., Barnard, D., and Frank, J. (1994). The internal compartmentation of rat-liver mitochondria: tomographic study using the high-voltage transmission electron microscope. Microsc Res Tech. 27:278-83.

81- Mäntyjärvi, M.I., Nerdrum, K., and Tuppurainen, K. (1992). Color vision in dominant optic atrophy. J Clin Neuroophthalmol. 12:98-103.

82- Mao, C.C. and Holt, I.J. (2009). Clinical and molecular aspects of diseases of mitochondrial DNA instability. Chang Gung Med J. 32:354-69.

83- Mootha, V. K. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nature Genet. 34, 267–273.

84- Moreira, P.L., Zhu, X., Wang, X., Lee, H.G., Nunomura, A., Petersen, R.B., Perry, G., and Smith, M.A. (2010). Mitochondria: a therapeutic target in neurodegeneration. Biochim Biophys Acta 1802:212–220.

85- Mouli, P.K., Twig, G., and Shirihai, O.S. (2009). Frequency and selectivity of mitochondrial fusion are key to its quality maintenance function. Biophys J. 96:3509–3518.

86- Muller-Hocker, J. (1990). Cytochrome *c* oxidase deficient fibres in the limb muscle and diaphragm of man without muscular disease: an age-related alteration. *J. Neurol. Sci.* 100, 14–21.

87- Nicholls, D.G. (2002). Mitochondrial function and dysfunction in the cell: its relevance to aging and aging-related disease. Int J Biochem Cell Biol. 34:1372-81.

88- Ojala, D., and Montoya, J. (1981). tRNA punctuation model of RNA processing in human mitochondria. Nature. 290: 470-4.

89- Old, S.L., Johnson, M.A. and Histochem, J. (1989). Methods of microphotometric assay of succinate dehydrogenase and cytochrome c oxidase activities for use on human skeletal muscle. 21(9-10):545-55.

90- Olichon, A., Landes, T., Arnauné-Pelloquin, L., Emorine, L.J., and Mils, V. (2007). Effects of OPA1 mutations on mitochondrial morphology and apoptosis: relevance to ADOA pathogenesis. J Cell Physiol. b; 211:423-30.

91- Park, D., Huang, T., and Frishman, W.H. (2005). Phytoestrogens as cardioprotective agents. Cardiol Rev 13:13–17.

92- Patel, P.K., Shirihai, O., and Huang, K.C. (2010). Optimal dynamics for quality control in spatially distributed mitochondrial networks. PLoS Comput Biol. in press.

93- Payne, M., Yang, Z., Katz, B.J., Warner, J.E., Weight, C.J., and Zhao, Y. (2004). Dominant optic atrophy, sensorineural hearing loss, ptosis, and ophthalmoplegia: a syndrome caused by a missense mutation in OPA1. Am J Ophthalmol 138: 749–55.

94- Pecina, P., Houstkova, H., Hansikova, H., Zeman, J., and Houstek, J. (2004). Genetic defects of cytochrome c oxidase assembly. Physiol Res 53 Suppl 1:S213-23.

95- Perkins, G., Renken, C., and Martone, M.E. (1997). Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts. J Struct Biol. 119:260-72.

96- Perkins, G., Renken, C.W., Frey, T.G., and Ellisman, M.H. (2001). Membrane architecture of mitochondria in neurons of the central nervous system. J Neurosci. Res 66.

97- Perotti, M.E., Anderson, W.A., and Swift, H. (1983). Quantitative cytochemistry of the diaminobenzidine cytochrome oxidase reaction product in mitochondria of cardiac muscle and pancreas. J. Histochem. Cytochem. 31:351-365.

98- Pesch, U.E., Fries, J.E., Alexandre, C., Wheeler-Schilling, T.H., Kohler, K., and Wissinger, B. (2001). Spatial and temporal retinal expression of OPA1 involved in autosomal optic atrophy. Invest Ophthalmol Vis Sic 42:A3521.

99- Peterson, G. (1995). Evaluation of the biochemical targets of genistein in tumor cells. Nutr. 125 (3 Suppl):784S-789S.

100- Petersen, K. F. (2003). Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300, 1140–1142.

101- Plkowski, K., and Mazurek A. (2000). Biological Properties of Genistein. Polish Pharmaceutical Society 135-155.

102- Posmantur, R., McGinnis, K., Nadimpalli, R., Gilbertsen, R.B., Wang, K.K. (1997). Characterization of CPP32-like protease activity following apoptotic challenge in SH-SY5Y neuroblastoma cells. J Neurochem; 68:2328–2337.

103- Poyton, R.O., and McEwen, J.E. (1996). Crosstalk between nuclear and mitochondrial genomes. Annu Rev Biochem 65:563-607.

104- Praefcke, G.J., and McMahon, H.T. (2004). The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol. 5:133-47.

105- Rafelski, S.M. (2013). Mitochondrial network morphology: building an integrative, geometrical view. BMC Biology 11:71.

106- Renken, C.W. (2000). Crista junctions reflect the osmotic pressure on the inner membrane of mitochondria. Biophys. J. 78:140.

107- Robert, W.T. and Doug, M.T. (2005). Mtiochondrial DNA Mutations in Human Disease. Nature Publishing Group.

108- Robertson, J.D., Orrenius, S. (2002). Role of mitochondria in toxic cell death. Toxicology 181‑182:491‑496.

109- Robin, E. D. and Wong, R. (1988). Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. J. Cell. Physiol. 136, 507–513 .

110- Rustin, P., and Rotig, A. (2002). Inborn errors of complex II - unusual human mitochondrial diseases. Biochim Biophys Acta. 1553:117-122.

111- Saraste, M. (1999). Oxidative phosphorylation at the fin de siècle.Science. 283:1488-93.

112- Sas, K., Robotka, H., Toldi, J., and Vécsei, L. (2007). Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders J Neurol Sci 257:221–239.

113- Satoh, M. and Kuroiwa, T. (1991). Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. Exp. Cell Res. 196, 137–140.

114- Scarpulla, R.C. (2008). Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev. 88:611-638.

115- Schäfer, E., Seelert, H., and Reifschneider, N.H. (2006). Architecture of active mammalian respiratory chain supercomplexes. J Biol Chem. 281:15370-5.

116- Schägger, H. and Pfeiffer, K. (2000). Supercomplexes in the respiratory chains of yeast and mammalian mitochondria.EMBO J. 19:1777-83.

117- Scorrano, L., Ashiya, M., and Buttle, K. (2002). A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell. 2:55-67.

118- Setchell, K.D., and Lydeking-Olsen, E. (2003). Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies. Am J Clin Nutr 78:593S–609S.

119- Shaw, J.M., and Nunnari, J. (2002). Mitochondrial dynamics and division in budding yeast. Trends Cell Biol 12:178–184.

120- Simon, D.K., Lin, M.T., Ahn, C.H., Liu, G.J., Gibson, G.E., Beal, M.F., and Johns, D.R. (2001). Low mutational burden of individual acquired mitochondrial DNA mutations in brain. Genomics. 73(1):113-6.

121- Si, H., and Liu, D. (2007). Phytochemical genistein in the regulation of vascular function: new insights. Curr Med Chem 14:2581–2589.

122- Sidransky, D. (2002). Emerging molecular markers of cancer. Nat Rev Cancer. 2 (3):210–219.

123- Smeitink, J., and van-den-Heuvel, L. (1999). Human mitochondrial complex I in health and disease. Am J Hum Genet. 64:1505-10.

124- Song, W.O., Chun, O.K., Hwang, I., Shin, H.S., Kim, B.G., Kim, K.S., Lee, S.Y., Shin, D., and Lee, S.G. (2007). Soy isoflavones as safe functional ingredients. J. Med Food 10:571–580.

125- Spinazzi, M., Cazzola, S., Bortolozzi, M., Baracca, A., and Loro, E. (2008). A novel deletion in the GTPase domain of OPA1 causes defects in mitochondrial morphology and distribution, but not in function. Hum Mol Genet. 17:3291-302.

126- Sue, C.M., and Schon, E.A. (2000). Mitochondrial respiratory chain diseases and mutations in nuclear DNA: a promising start? Brain Pathol 10:442-50.

127- Sukhorukov, V.M., Dikov, D., Reichert, A.S., and Meyer-Hermann, M. (2012). Emergence of the mitochondrial reticulum from fission and fusion dynamics. PLoS Comput Biol. 8:e1002745.

128- Trifunovic, A. (2006).Mitochondrial DNA and ageing.Biochim Biophys Acta. 1757:611-7.

129- Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly-Y, M., Gidlöf, S., Oldfors, A., Wibom, R., Törnell, J., Jacobs, H.T., and Larsson, N.G. (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature. 429(6990):417-23.

130- Trifunovic, A., and Larsson, N.G. (2008). Mitochondrial dysfunction as a cause of ageing. J. Intern Med 263:167‑178.

131- Twig, G., Liu, X., Liesa, M., Wikstrom, J.D., Molina, A.J.A., Las, G., Yaniv, G., Hajnoczky, G., and Shirihai, O.S. (2010). Biophysical properties of mitochondrial fusion events in pancreatic -cells and cardiac cells unravel potential control mechanisms of its selectivity. Am J Physiol Cell Physiol, 299:C477–C487.

132- Valles, S.L., Dolz-Gaiton, P., Gambini, J., Borras, C., Lloret, A., Pallardo, F.V., and Viña, J. (2010). Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression in cultured astrocytes. Brain Res 1312:138–144.

133- Vermulst, M., Bielas, J.H., Kujoth, G.C., Ladiges, W.C., Rabinovitch, P.S., Prolla, T.A., and Loeb, L.A (2007). Mitochondrial point mutations do not limit the natural lifespan of mice. Nat Genet. 39(4):540-3.

134- Vogel, F., Bornhövd, C., Neupert, W., and Reichert, A.S. (2006). Dynamic subcompartmentalization of the mitochondrial inner membrane. J Cell Biol. 175:237-47.

135- Vonck, J., and Schäfer, E. (2009). Supramolecular organization of protein complexes in the mitochondrial inner membrane. Biochim Biophys Acta. 1793:117-24.

136- Votruba, M., Fitzke, F.W., and Holder, G.E. (1998). Clinical features in affected individuals from 21 pedigrees with dominant optic atrophy. Arch Ophthalmol. 116: 351-358.

137- Votruba, M., Leary, S., and Losseff, N. (2000). MRI of the intraorbital optic nerve in patients with autosomal dominant optic atrophy. Neuroradiology 42:180-3.

138- Wallace, D.C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine.Annu Rev Genet. 39:359-407.

139- Wang, X., Chen, S., Ma, G., Ye, M., and Lu, G. (2005). Genistein protects dopaminergic neurons by inhibiting microglial activation. Neuroreport 16:267–270.

140- Wilding, C.S., Cadwell, K., Tawn, E.J., Relton, C.L., Taylor, G.A., Chinnery, P.F., and Turnbull, D.M. (2006). Mitochondrial DNA mutations in individuals occupationally exposed to ionizing radiation. Radiat Res. 165(2):202-7.

141- Wong, L.J., Lueth, M., Li, X.N., Lau, C.C., and Vogel, H. (2003). Detection of mitochondrial DNA mutations in the tumor and cerebrospinal fluid of medulloblastoma patients. Cancer Res. 63 (14):3866–3871.

142- Xi, Y.D., Yu, H.L., Ma, W.W., Ding, B.J., Ding, J., Yuan, L.H., Feng, J.F., and Xiao, R. (2011). Genistein inhibits mitochondrial-targeted oxidative damage Journal of Bioenergetics and Biomembranes. Volume 43, Issue 4, pp 399-407.

143- Yu, C.A., Xia, D., Kim, H., Deisenhofer, J., and Zhang, L. (1998). Structural basis of functions of the mitochondrial cytochrome bc1 complex.Biochim Biophys Acta. 1365:151-8.

144- Yu-Wai-Man, P., Griffiths, P.G., Hudson, G., and Chinnery, P.F. (2009). Inherited mitochondrial optic neuropathies. J Med Genet. 46:145-58.

145- Zanna, C., Ghelli, A., and Porcelli, A.M. (2008). OPA1 mutations associated with dominant optic atrophy impair oxidative phosphorylation and mitochondrial fusion. Brain. 131:352–67.

146- Zeng, H., Chen, Q., and Zhao, B. (2004). Genistein ameliorates beta-amyloid peptide (25-35)-induced hippocampal neuronal apoptosis. Free Radic Biol Med 36:180–188.

147- Zeviani, M., and Antozzi, C. (1997). Mitochondrial disorders. Mol Hum Repr. 3:133-148.

148- Zeviani, M., Spinazzola, A., and Carelli, V. (2003). Nuclear genes in mitochondrial disorders. Curr Opin Genet Dev. 13:1-9.

جامعـــة دمشق
كلـــية الزراعة

دراسة الآليات الفيزيولوجية لمرض ضمور العصب البصري الراجح

أطروحة أعدت لنيل درجة الماجستير في التقانات الحيوية

إعداد الطالبة
عالية محمد شمس

إشـــــراف

د. شادي سكرية
كلية العلوم – جامعة دمشق

د. سلام لاوند
كلية الزراعة – جامعة دمشق

وبالتعاون مع

د. غي ليناغس
معهد دراسات العلوم العصبية مونبيليه - فرنسا

2014

المقدمة

ضمور العصب البصري:

يشير ضمور العصب البصري إلى مجموعة من الاعتلالات العصبية وراثية المنشأ والتي تؤدي إلى تموت الخلايا العقدية في الشبكية وهي الخلايا التي تشكل محاورها الأسطوانية العصب البصري. والمورثات المسؤولة عن هذه الاعتلالات الرئيسية هي مورثات جسمية إما راجحة أو متنحية. يعد النمط الوراثي الراجح من ضمور العصب البصري (ADOA) الأكثر شيوعاً (تواتر المرض يتراوح من 1:50000 إلى 1:10000)، في حين يعد النمط الوراثي المتنحي (AROA) من الأشكال نادرة. (Christophe O. et al., 2003). بينما يوجد اعتلال بصري وراثي ثالث يعرف بالاعتلال العصبي ليبر (Leber's hereditary optic neuropathy) أو (LHON) هو نوع من ضمور العصب البصري الموروث والمرتبط بمجموعة من الطفرات في DNA الميتوكوندري (mtDNA) وينقل بطريقة غير مندلية عن طريق الأمهات (Christophe O. et al., 2003).

ضمور العصب البصري الوراثي الجسمي الراجح (ADOA):

وصف لأول مرة سريرياً من قبل كل من باتن (Batten, 1896) وكجر(Kjer, 1959)، وهو خسارة تصاعدية في الرؤية المركزية ثنائية الجانب (أي تحدث في الطرفين معاً) تبدأ في مرحلة الطفولة، عادة عند الأطفال الذين تتراوح أعمارهم بين أربع وست سنوات، وتتطور بنسب مختلفة مع التقدم في العمر (Carelli et al., 2004; Yu-Wai-Man et al., 2009; Delettre et al., 2002; Cohn et al., 2008). يعد هذا المرض أكثر الاعتلالات العصبية البصرية الوراثية شيوعاً، والسبب الرئيسي الثاني للعمى في العالم (Delettre C., et al., 2002). بالرغم من وجود متلازمات من ضمور العصب البصري الوراثي المرتبطة مع خلل وظيفي عصبي، فإن معظم المرضى الذين يعانون من متلازمة الضمور العصبي الجسمي الراجح ليس لديهم خلل عصبي إضافي. أولى الدراسات التي ذكرت السبب الوراثي للمرض تعود للعام 2000 حيث نشرت دراستان في وقت واحدة عدد من الطفرات في المورثة OPA1 لدى المرضى المصابين بالضمور الوراثي للعصب

البصري ذو النمط الراجح (Alexander et al., 2000, Delettre et al., 2000). بعدها تتالت الدراسات التي كشفت اشتراك طفرات على مورثات أخرى في إحداث المرض (OPA3 وOPA8)، ولكن تبقى طفرات المورثة OPA1 الأكثر شيوعاً لدى المرضى (Olichon et al., 2007b).

تتوضع المورثة OPA1 على الصبغي 28 وتتكون من 31 إكسوناً وتضم 6031 نيوكليوتيداً (GenBank accession no: AB011139). وقد حددت حوالي 205 طفرة على هذه المورثة (Ferre et al., 2005). ترمز المورثة OPA1 بروتيناً متوضعاً في الغشاء الداخلي للميتوكونري لجميع الخلايا وهو عبارة عن دينامين dynamin يحوي مجال من GTPase ويؤدي هذا البروتين عدداً من الأدوار الفزيولوجية ضمن الميتوكوندري كتنشيط عملية الاستقلاب، وصيانة الغشاء بالإضافة إلى دوره في حادثة الموت الخلوي (Amati-Bonneau et al., 2009; Lenaers et al., 2009). والسؤال المطروح: كيف يمكن للطفرات التي تصيب المورثة OPA1 أن تؤثر في عمل الميتوكوندري وكيف تقود إلى تموت الخلايا العقدية في الشبكية دون سواها من خلايا الجسم وتؤدي بالتالي إلى ضمور العصب البصري؟ توصلت الأبحاث في هذا المجال إلى وضع فرضيتين الأولى تقول بأن البروتينات الطافرة تزعزع استقرار الغشاء الداخلي للميتوكوندريا، وتتداخل مع وظيفة المعقد التنفسي، وبالتالي تقليل كمية الطاقة المنتجة، مما يسبب في زيادة كمية الجذور الحرة (ROS)، أما الثانية فتقول أن البروتينات الطافرة قد تشوش جزئيا" شبكة الميتوكوندريا مما يؤدي إلى توزع غير متجانس للأدينوزين ثلاثي الفوسفات ATP في الخلية (Delettre et al., 2002). لقد بينت الدراسات أن ضعف الخلايا العقدية في الشبكية يمكن أن يعود إلى كونها واحدة من أكثر الخلايا حساسية للجذور الحرة للأوكسجين التفاعلية (Kortuem et al., 2000)، كما يمكن لهذا الضعف في الخلايا الشبكية العقدية يمكن أيضا أن يكون ذا صلة بمتطلبات محددة في توزع الطاقة "ATP" على طول المحاور العصبية وأي خلل في هذا التوزع سيؤدي إلى فشل في نقل الإشارة في العصب البصري. بالإضافة إلى أن شبكة الميتوكوندري في العصب البصري ضرورية للحفاظ على انتقال كمونات العمل على طول المحاور العصبية غير المغمدة المكونة للعصب البصري (Andrews et al., 1999).

درست مختلف الآليات الفيزيولوجية للمرض في خلايا الأرومة الليفية مأخوذة من المرضى الذين يعانون من ضمور العصب وذلك بالتزامن مع التحقق من نمط الطفرات التي تصيب المورثات المسؤولة عن المرض وخاصة المورثة OPA1. بينت هذه الدراسات أنه يطرأ تغيرات على هيكل الميتوكوندري لخلايا الأرومة الليفية للمرضى بينما تكون الشبكة الميتوكوندرية إما طبيعية أو مجزأة مقارنة مع الخلايا الشاهدة المأخوذة من أشخاص أصحاء (Olichon et al., 2007b; Zanna et al., 2008; Chevrollier et al., 2008) (Amati-Bonneau et al., 2005, 2008; Olichon et al., 2007b; Zanna et al., 2008). كما أن الطفرة في المجال GTPase للمورثة OPA1 والمحدثة في النماذج الحيوانية "الفئران" (Alavi et al., 2007) تؤثر في التوازن القائم بين عمليات الانصهار والانفصال التي تصيب الشبكة الميتوكوندرية دون التأثير على التنفس (Spinazzi et al., 2008)، وبالتالي يمكن افتراض أن دور المورثة OPA1 في تغير شكل الشبكة الميتوكوندرية على علاقة بالمرض.

الطفرة R445H OPA1:

تعد الطفرة R445H التي تصيب مجال GTPase في المورثة OPA1 (Payne et al., 2004; Li et al., 2005) من أكثر الطفرات شيوعاً وهي الطفرة الوحيدة التي ارتبطت مع هذه المتلازمة الفريدة من ضمور العصب البصري وفقدان السمع الحسي العصبي وقد حددت أولاً في عائلتين غير مرتبطتين (Li et al., 2005). أظهرت الخلايا الليفية لهؤلاء المرضى فرطاً في تجزئة الشبكة الميتوكوندرية، وانخفاضاً في كمون الغشاء، وخللا" في تركيب ATP. وبالتالي، ضمور العصب البصري والصمم على صلة مع عيوب الطاقة الناتجة من وجود شبكة ميتوكوندرية مجزأة (Amati-Bonneau et al., 2005).

المورثة TMEM 126A:

إن البحث المستمر والممنهج عن المورثات المسؤولة عن ضمور العصب البصري والتي ترمز بروتينات ميتوكوندرية، توصل حديثاً إلى تحدد طفرة من النوع nonsense متماثلة اللواقح في المورثة TMEM 126A والتي ترمز بروتيناً عابراً للغشاء الميتوكوندري يعبر عنه في جميع الخلايا. تتوضع هذه المورثة على الصبغي رقم 11 وتتألف من 5 إكسونات إحداها غيرمرمز (Cizkova, A. et al., 2008) (Carelli, V., et al., 2007).

بينت الدراسات الحديثة أن الطفرات التي تحدث في هذا المورثة هي السبب الأول المعروف لضمور العصب البصري الجسمي المتنحي غير المتلازم. هذا الاكتشاف يعطي مزيدا من الدعم للنظرية التي تقول أن الخلل الميتوكوندري هو السمة المميزة لاعتلالات العصب البصري، مما يوحي بأن النهج العلاجية قد تكون مشتركة ويمكن تطويرها لتحسين التشخيص على المدى الطويل أيا كان المورثة المشارك والمسبب للمرض (Delettre, C. et al., 2000 ; Cherollier, A. et al 2008).

الجنيستين Genistein:

Genistein هو مركب طبيعي ينتمي إلى فئة من الايزوفلافون، والتي تنتجها مجموعة كبيرة من النباتات، ولعل من أهمها فول الصويا (Fukutake et al., 1996; Barnes et al., 1995). أثبتت العديد من التجارب أن جينيستين يتداخل مع العديد من المسارات البيوكيميائية في الخلية الحية وهذا التداخل معقد ومتعدد. واحدة من الفعاليات البيولوجية للجينيستين التي تم اكتشافها في الخلية هو التأثير المضاد للأكسدة حيث يمنع الجينيستين كل من الأحداث الأولية اللازمة لإنتاج مستوى عال من الأشكال الفعالة من الأوكسجين (ROS) أو يمكن أن يحول دون إنتاج هذه الجذور (Peterson et al., 1995). ونظراً لهذا الأثر للجينيستين تركزت الدراسات الحديثة على أثره في علاج السرطان، بالإضافة إلى العديد من الأمراض الأخرى، كالأمراض التنكسية العصبية (Valles et al,. 2010; Lee 2008; Wang et al,. 2005) (Polkowski et al., 2000).

بالرغم من أن دوره في الوقاية من الجذور الحرة بات مثبتاً إلا أن الأدلة على تأثيره الوقائي على الميتوكوندريا الناجمة عن إنتاج المؤكسدات ما تزال قليلة جداً.

أهمية البحث:

نظراً للأهمية الكبيرة للاعتلال العصبي البصري وراثي المنشأ وخاصة ضمور العصب البصري السائد في إحداث فقدان البصر لدى البشر، وفي ضوء البحث المستمر والممنهج عن مورثات جديدة أو طفرات جديدة على علاقة بالمرض، ولكشف فيما إذا كان هناك علاقة بين الطفرات المسببة للمرض والتي تصيب المورثات النووية وظهور الطفرات في المورثات الميتوكوندرية، وللبحث في دور الجينيستين في حماية الخلايا المأخوذة من المرضى من أثر عوامل الأكسدة هدف البحث إلى:

أهداف البحث:

1. تحديد التتابع النكليوتيدي للـــ DNA المستخلص من عينات دم مأخوذة من عدد من المرضى لتحديد وجود أي تغير في المورثة المرمزة للـــ TMEM 126A.
2. الكشف عن الطفرات في DNA الميتوكوندري المستخلص من فئران متخالفة اللواقح للطفرة OPA1 +/− (نموذج حيواني للمرض).
3. دراسة تأثير جينيستين على شبكة الميتوكوندري وكمون الغشاء لخلايا الأرومة الليفية R445H OPA1 المأخوذة من المرضى.

المواد وطرائق البحث:

تم إجراء كل التجارب العملية الخاصة بهذا البحث في مخابر معهد دراسات العلوم العصبية مونبيليه. مونبيليه− فرنسا.

1− كشف الطفرات في المورثة TMEM 126A:

تم الحصول على عينات من DNA من 12 مريضاً أظهر التشخيص الطبي إصابتهم بضمور العصب البصري، ولم يظهر التشخيص الجزيئي أي طفرات في الجينات OPA1 أو OPA3 أو OPA8. ومن ثم ضخمت الإكسونات الخمسة العائدة للجين TMEM126A بوساطة PCR وتم تحديد التتابع النكليوتيدي لكل الشدف لتحديد وجود أي طفرة في هذه الإكسونات.

2- تحليل DNA الميتوكوندري mtDNA المستخلص من النموذج الفأري الحامل للطفرة:

تحوي الخلية الواحدة نسخاً متعددة من الجينوم الميتوكوندري وبالتالي فإن التقصي الكمي عن إجمالي الطفرات في DNA الميتوكوندري ليس بالأمر السهل، فضلا عن ذلك فإن الكشف عن الجزيئات الفردية الطافرة من mtDNA هو أبعد من حساسية معظم التقنيات المعتمدة للسلسلة. تعرف الطريقة المستخدمة حاليا لتحديد الطفرات في mtDNA بـــPost-PCR cloning. تعتمد إستراتيجية هذه الطريقة على تضخيم تسلسل مستهدف في الجينوم الميتوكوندري باستخدام أنزيم بوليميراز عالي الوثوقية. ثم يتم تنسيل منتجات PCR في بلاسميدات خاصة، ومن ثم يتم تنقية البلاسميدات و تحديد تتابعها النكليوتيدي. كل منتج PCR فردي هو نسخة من جزيء mtDNA واحد ومن خلال سلسلة عدد كبير من الجزيئات المستنسخة يمكن تحديد الطفرات كمياً. ينجز تفاعل PCR باستعمال بادئات محددة من المنطقة غير المرمزة في جزيئات mtDNA تسمى D-Loop region وهي منطقة عالية التباين والاختلاف لذلك فهي تعد بقعة ساخنة للطفرات الجسدية.

في الخطوة التالية سوف يتم اختبار جزيئات mtDNA للكشف عن طفرات من نمط الحذف (Deletion) وتغير في الكمية (انخفاض عدد نسخ mtDNA في الخلية الواحدة) (Depletion). لتحديد التغير الأول تم اللجوء إلى تقانة long range PCR بينما حدد التغير الثاني بتطبيق Real-Time PCR أو PCR الكمي.

3- دراسة تأثير جينيستين على خلايا الأرومة الليفية R445H OPA1 المأخوذة من المرضى:

استنبتت خلايا الأرومة الليفية الحاملة للطفرة OPA1 R445H المأخوذة من المرضى، ودرس تأثير الجينيستين عليها، حيث عرضت الخلايا إلى ضغط أكسدة Oxidative stress بمعالجتها بتركيز ثابت (1.75µM) من أحد محفزات الموت الخلوي المبرمج وهو الستوروسبورين، ومن ثم معالجة هذه الخلايا المستنبتة بستة تراكيز متزايدة من الجنيستين ودراسة أثر هذه المعالجة على حيوية الخلايا من خلال استعمال اختبار MTT وعلى الشبكة المتيكوندرية باستعمال الصباغ MitoTracker وعلى كمون الغشاء الميتوكوندري باستعمال الصباغ JC1.

النتائج:

1- كشف الطفرات في الجين TMEM 126A:

نتائج الرحلان الكهربائي على هلام الاغاروز لنواتج الـــ PCR تدل على عدم وجود أي طفرة من نمط حذف أو إضافة في عينات الـــ DNA الاثنتي عشرة، بإضافة إلى أن مقارنة تسلسل العينات بقواعد البيانات المرجعية NCBI باستعمال برنامج BLAST لم يكشف عن أي تغير نكليوتيدي وبالتالي لا يوجد أي طفرة نقطية في إكسونات الجين TMEM 126A للمرضى الذين تم اختبارهم.

2- تحليل الحمض النووي الميتوكوندري المستخلص من النموذج الفأري الحامل للطفرة:

لم تظهر نواتج تحديد التتابع النكليوتيدي للقطع المنسلة ضمن البلاسميدات بعد تطبيق تقانةPost PCR Cloning وجود أي طفرة، كما أن نواتج Long-Range PCR لم تظهر أي حذف في DNA الميتوكوندري مقارنة مع الشاهد. كما أن نواتج PCR الكمي لم تظهر أي اختلاف في عدد نسخ mtDNA بين العينات المأخوذة من الفئران الحاملة للطفرة والعينات المأخوذة من الفئران الشاهدة.

3- أثر جينيستين على خلايا الأرومة الليفية R445H OPA1 المأخوذة من المرضى:

أظهر اختبار الحيوية الخلوية MTT بعد المعالجة بست تراكيز من الجينيستين تراوحت بين 0.1µM و30µM أن أقل تركيز يعطي أفضل نسبة حيوية هو 3µM، حيث زادت نسبة الحيوية بمقدار يقارب 50%. من هذه النتيجة استعمل هذا التركيز (3µM) للجنيستين في التجارب اللاحقة.

- الأثر على بنية الشبكة الميتوكوندرية:

تشير نتائج استعمال mitotracker هو الصباغ الذي يظهر بنية الشبكة الميتوكوندرية، أن الشبكة الميتوكوندرية في خلايا الأرومة الليفية R445H OPA1 تكون مجزأة في حوالي 80% من الخلايا، هذه النسبة تتحسن بشكل ملحوظ بوجود الجنيستين حيث تأخذ الشبكة الميتوكوندرية الشكل الخطي المتصل في حوالي 60% من الخلايا. هذه النتيجة تدل على دور هذا العقار في الحفاظ على بنية الشبكة الميتوكوندرية.

- الأثر على كمون الغشاء الميتوكوندري:

تشير نتائج استعمال JC1، هو الصباغ الذي يظهر كمون الغشاء الميتوكوندري بتلوينه باللون الأحمر للأجزاء ذات الكمون المرتفع و باللون الأخضر للأجزاء ذات الكمون المنخفض ، إلى زيادة طفيفة في القياسات النسبية لكمون الغشاء عند المعالجة بالجنستين.

وبالنتيجة، درس في هذا العمل مجموعة من الأوجه المختلفة لضمور العصب البصري حيث تم البحث عن طفرات في الجين TMEM 126A لدى مجموعة من المرضى أعطت كلها نتائج سلبية، كما بحث عن طفرات نوعية وكمية في DNA الميتوكوندري لمجموعة من الفئران ذات النمط الجيني OPA1 +/− تبين بنتيجتها عدم وجود علاقة بين طفرة OPA1 وحدوث طفرات على DNA الميتوكوندري. وأخيراً درس أثر الجنيستين كمادة مضادة للأكسدة على خلايا الأرومة الليفية لأشخاص يحملون الطفرة R445H OPA1 حيث تبين أن لهذا العقار أثر ايجابي في زيادة عشوية الخلايا وفي تحسن في بنية الشبكة الميتوكوندرية وفي كمون الغشاء.