INVESTIGATION OF THE COMMON MELAS MUTATION IN THE NORTHWESTERN PENNSYLVANIA AMISH COMMUNITY: MUTATION FREQUENCY AND EFFECTIVENESS OF AN EDUCATIONAL INTERVENTION

by

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ABSTRACT

Although mitochondrial respiratory chain deficiencies (often called "mitochondrial disease") affect an estimated 1 in 5,000 individuals world-wide, prior to the recent diagnosis of this study's index patient, mitochondrial DNA (mtDNA) mutations were not previously reported in Amish communities. The index patient from the Northwestern Pennsylvania Amish community was diagnosed with MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like Episodes), the most common maternally-inherited mitochondrial disorder, and carries the common MELAS mutation, m.3243A>G in the *MT-TL1* gene. Subsequently, two additional Amish families with different mitochondrial mutations were identified. These findings prompted a study with three aims: characterizing the incidence and clinical features of this MELAS mutation in this community, constructing a detailed pedigree of the Amish community, and assessing by pre- and post-intervention questionnaires the efficacy of an educational intervention designed to increase understanding of MELAS and mitochondrial disease.

A study visit to the community was attended by the index patient's extended family. During this visit, an educational intervention was presented, and questionnaires were administered. Interviews were conducted to gather family history information, including family structure and the presence of mitochondrial disease symptoms in family members. Samples were collected from 13 adults and two children for genetic testing. Samples were analyzed using high-resolution melt profiling for targeted assessment of the m.3243A>G mutation. While data analysis of

questionnaires demonstrated limited increased understanding of educational intervention material, anecdotal experiences support increased understanding. Genetic testing revealed the mutation of interest in 13 participants, with tissue-specific variations in the levels of heteroplasmy. At a follow-up visit, test results and their implications were disclosed and discussed through genetic counseling.

This study's findings suggest maternally-inherited mitochondrial disease may be under-recognized given the lack of previous diagnosis in 13 participants reported here. The public health significance demonstrated through this is the potential for similarly unrecognized mitochondrial disease in the larger Amish community and in the general population due to the challenges related to diagnosis. Results of efficacy analysis of an educational intervention in this community can also inform the development of educational interventions for the general population and for health care providers about mitochondrial and other rare genetic disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 INTRODUCTION

A 15 year-old female first presented to the Children's Hospital of Pittsburgh of UPMC with acute vomiting, altered mental status, status epilepticus, and lactic acidosis. A brain MRI revealed a small focal, left occipital lobe infarct, after which she developed other stroke-like episodes in the left occipital and temporal areas. It was revealed that she also had a history of developmental delay, short stature, hearing loss, fatigability, and poor appetite. Confirmation of a MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like Episodes) diagnosis was made through molecular testing of saliva, which showed 74% heteroplasmy for the most common MELAS-causing mutation, m.3243A>G in the *MT-LT1* gene. This patient was a member of the Mercer County, Pennsylvania Amish community, where mtDNA mutations had not previously been identified. This patient's presentation was coupled with an extensive family history of MELAS-related symptoms, including the following: developmental delay, migraine headaches, hearing loss, mild hypotonia, renal failure, diabetes mellitus, and recurrent miscarriages. Knowledge that a significant portion of patients in the Amish community with chronic disease remain undiagnosed[1] combined with familiarity with the significant monetary and health-care impact of the Clinic for Special Children on the Amish community in Lancaster, Pennsylvania led to development of a program to assess the Amish communities of Northwestern Pennsylvania, initially for MELAS, but ultimately for other mitochondrial conditions.[2; 3]

Based on identification of MELAS in the community, the potential benefits of early diagnosis and treatment, and the recognition that this and other conditions are under-diagnosed in this population, investigators developed a study whose aims were three-fold: to characterize the incidence and clinical features of this MELAS mutation in this Amish community, to construct a detailed extended pedigree of the index family and other key kinships in the community, and to assess by pre- and post-intervention questionnaires the efficacy of an educational intervention designed to increase understanding of MELAS and mitochondrial disease in this community.

This document will serve to outline the background information used to design, implement, and analyze this study, provide information about the potential significance of this work, detail the methods used in the study, and describe and discuss study results. Additionally, the public health competencies developed and practiced throughout the study will be considered. Finally, conclusions about the study will be presented, as well as information about future directions of continued research including further investigation of MELAS and potential identification of other mitochondrial disorders in this community and in the general population at large.

2.0 HYPOTHESES AND SPECIFIC AIMS

2.1 HYPOTHESES

Hypothesis 1: There is a higher incidence of the m.3243A>G mutation in the *MT-TL1* gene in mitochondrial DNA in the Northwestern Pennsylvania Amish community than is currently clinically recognized.

Hypothesis 2: The proposed educational intervention will increase understanding of MELAS and mitochondrial disease in the Northwestern Pennsylvania Amish community.

2.2 SPECIFIC AIMS

Aim 1: To characterize the incidence and clinical features of the m.3243A>G *MT-TL1* gene mutation in the Northwestern Pennsylvania Amish community.

Aim 2: To construct a detailed extended pedigree of the index family and other key kinships in the Northwestern Pennsylvania Amish community.

Aim 3: To assess the efficacy of an educational intervention designed to increase understanding of MELAS and mitochondrial disease in the Northwestern Pennsylvania Amish community using pre- and post-intervention surveys.

3.0 BACKGROUND

3.1 MITOCHONDRIA

Mitochondria are the powerhouse of the cell because they are the cell's energy-producing organelles. Mitochondria synthesize adenosine tri-phosphate (ATP), which is the main energy currency of life. Each mitochondrion carries multiple copies of its own DNA called mitochondrial DNA, or mtDNA.

3.1.1 Mitochondrial Energy Production

A key function of mitochondria is to synthesize ATP via oxidative phosphorylation (OXPHOS), as shown in Figure 1.[4] As much as 90% of the body's energy is produced as mitochondria convert reducing equivalents from cellular metabolism plus oxygen into ATP.[5] The carbohydrates consumed by an individual begin the energy conversion process through glycolysis, and the products of this reaction enter the tricarboxylic acid (TCA) cycle. During times of stress or fasting, fatty acids are oxidized inside the mitochondrion to produce NADH+, hydrogen ions, electrons, and $FADH_2$. The products go through OXPHOS via respiratory complexes I, II, III, and IV to produce water.[6] Flow through the electron transport chain is used to create an electrochemical gradient that allows for the storage of potential energy, which is later used to bring proteins and calcium into the mitochondrion, to generate heat, and to synthesize ATP inside the mitochondrial matrix.[7]

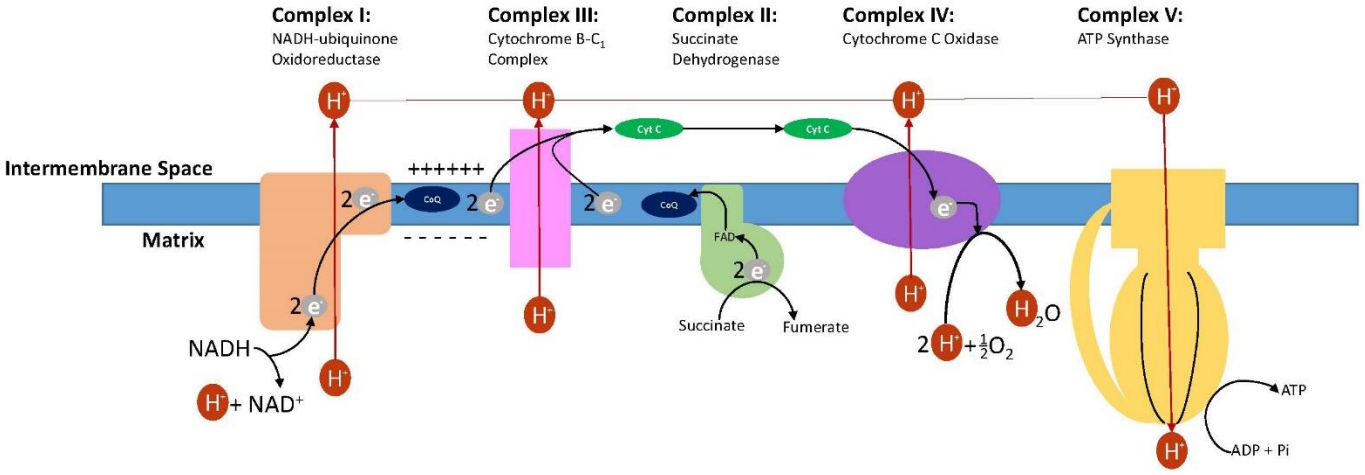

Figure 1. Oxidative Phosphorylation

3.1.2 Mitochondrial Genetics

Mitochondrial DNA is a double-stranded circular genome of 37 genes encoding 13 proteins, 22 transfer RNAs, and two ribosomal RNAs that play a role in formation of the respiratory complexes I, III, IV, or V.[7; 8]

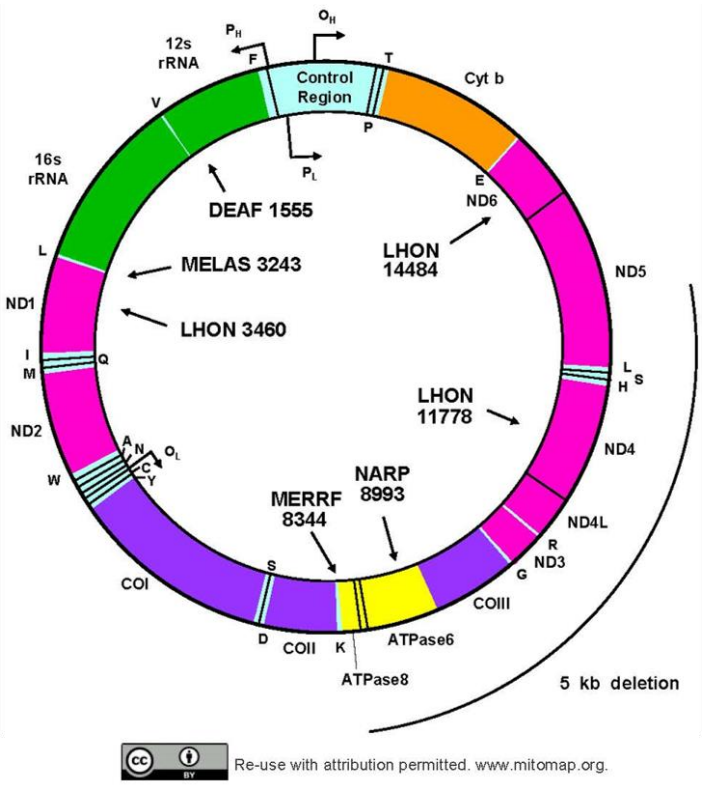

Figure 2. Mitochondrial DNA Map ©[9]

Each mitochondrion has multiple copies of mtDNA. There is typically only one type of mtDNA found in individuals without mitochondrial disease. However, in some cases, such as those of mitochondrial disease, there may be different forms of mtDNA within a cell. This is known as heteroplasmy. Some of the mitochondria within the cell may contain normal mtDNA while others may contain mutant mtDNA.

Mitochondrial DNA is transmitted from one generation to the next through the mother. This is called mitochondrial, or maternal, inheritance. Sperm cells contain little or no cytoplasm, which is where the mitochondria reside in cells. Thus they do not transfer mitochondria to the offspring. Alternatively, egg cells contain considerable cytoplasm and cytoplasmic organelles. As egg cell cytoplasm is sequestered from the progenitor germ cell, multiple and different copies of mtDNA, if present, are randomly apportioned to the egg cell. Thus, the heteroplasmy present in

the progenitor cells may be variably represented in the next generation. While a single cell can be heteroplasmic, so can an individual. Cells in an individual who carries mutant mtDNA may have variable amounts of mutant DNA, creating heteroplasmy. An individual who carries mitochondria with relatively few mutant mtDNA may not exhibit features associated with the corresponding condition. Whether or not one shows clinical features associated with a mitochondrial mutation is dependent on the mutational load, which refers to the amount of mutant mtDNA present. In order to show a disease phenotype, one's mutational load must exceed a threshold in a particular tissue. This concept is known as the threshold effect, which states that accumulation of mutations does not result in phenotypic changes until a cell's capacity to suppress mutation expression, or threshold, is crossed. This threshold is different for different tissues, meaning a particular mutational load may result in no or little phenotypic effect in one tissue, while causing disease features in another.

3.1.3 Mitochondrial Disease

While ATP production through OXPHOS is a prominent function of mitochondria, they also support other biochemical activities including the production and subsequent detoxification of reactive oxygen species (ROS), regulation of calcium signaling, and initiation of apoptosis.[10] Dysfunction of any of these myriad tasks can present as clinical disease. Mitochondrial disease can be caused by a mutation in nuclear DNA (nDNA) or mtDNA. Mitochondrial DNA is particularly susceptible to accumulation of mutations through oxidative stress, polymerase dysfunction, and exogenous toxins.[11] Since mitochondria have fewer DNA repair mechanisms than the nucleus, mtDNA is more prone to the accumulation and propagation of mutations.

Different tissues of the body have different energetic needs based on function, and these needs typically correlate with the number of mitochondria in that tissue. For example, a neuron that is constantly firing requires a greater amount of energy than a skin cell. The brain uses 20 percent of the body's energy while comprising only two percent of the total body weight.[5] Cells containing more mitochondria will be more susceptible to dysfunction with a lower mutation load. This is a part of what results in the clinical variability of mitochondrial disorders, as the mutation load in a particular tissue will dictate if and which symptoms arise in an individual's clinical disease course.

Mitochondrial respiratory chain defects (often referred to simply as "mitochondrial disease") affect approximately 12.48 in 10,000 individuals world-wide.[12] These conditions vary widely in presentation. Age of onset can vary from infancy to adulthood depending on the condition, and there are multiple clinical features variably associated with mitochondrial disease.[11] These features include myopathy, seizures, low energy, muscle weakness, and ragged red fibers as determined by a histological studies of a muscle biopsy specimen. The specific combination of these features leads to a suspected clinical diagnosis of a specific mitochondrial disease.

3.2 MITOCHONDRIAL ENCEPHALOMYOPATHY, LACTIC ACIDOSIS, AND STROKE-LIKE EPISODES – MELAS

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) is a multi-system mitochondrial disorder. Although the exact incidence of MELAS is unknown, in part because of its variability of clinical presentation, and it is a rare condition, it is the most common of the mitochondrially-inherited disorders.[13]

3.2.1 Molecular Basis

MELAS is caused by mutations in the mtDNA. The most common cause of MELAS, implicated in 80% of MELAS cases, is a m.3243A>G mutation in the *MT-TL1* gene, first described by Goto et al in 1990. This mutation prevalence is estimated to be 0.06%, or 60 in 100,000 individuals in the general population.[13] This mutation is known to cause dysfunction of $tRNA^{Leu(URR)}$.[14] There are at least 28 other mtDNA mutations and at least seven mitochondrial tRNA gene mutations that have been identified as leading to MELAS, including *MT-ND1*, *MT-CO3*, *MT-ND4*, *MT-ND6*, and *MT-CYB*. [13; 15; 16]

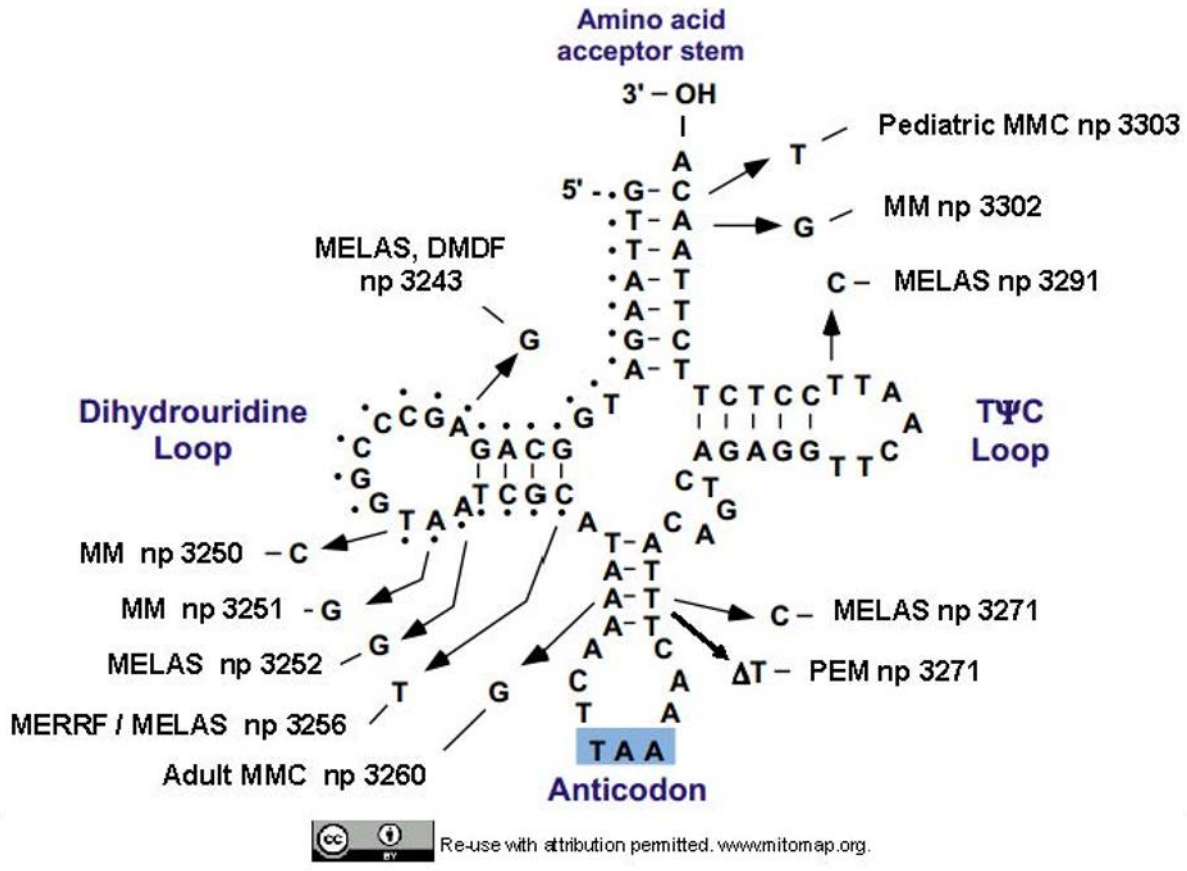

Figure 3. Pathogenic Mutations in $tRNA^{Leu(UUR)}$ © [17]

3.2.2 Clinical Manifestations

The age of onset, clinical features, and severity of MELAS differ significantly among affected individuals due to cellular heteroplasmy and differences in the threshold effect in different tissues. While all individuals with MELAS carry the mutation from birth, few affected individuals show symptoms early on. In fact, a distinguishing feature of MELAS is that affected individuals typically do not show symptoms from birth; rather, they typically have normal birth and early development. This is likely due because energy output at that time is enough to meet the body's energy requirements. Symptoms do not begin to manifest until the demands of the body exceed the mitochondrial energy output.[13] The range of age of onset of symptoms is most commonly 2-40 years old, but usually individuals with MELAS begin to present with symptoms in childhood, between the ages of five and 15 years-old.[13; 15]

There are six core features of MELAS that are present in 90% of cases: (1) age of onset of symptoms at less than 40 years old; (2) encephalopathy, frequency appearing as seizures, dementia, or both; (3) exercise intolerance; (4) lactic acidosis; (5) ragged red fibers on muscle biopsy; and (6) stroke-like episodes (before age 40 years). There are three additional features commonly identified, two of which are typically required to make a diagnosis. These features are normal early development, recurrent headaches, and recurrent vomiting.[11; 13; 16; 18] Additional features of MELAS affect several body systems. Neurological features associated with MELAS, in order of likelihood of occurrence include hemiparesis, hearing loss, hemianopsia, learning disabilities, basal ganglia calcification, myoclonus, cerebellar signs, memory impairment and episodic comas. Ophthalmologic symptoms include external opthalmoplegia, optic atrophy, and pigmentary retinopathy. Cardiovascular symptoms seen in patients with MELAS include cardiomyopathy, chronic heart failure, pulmonary hypertension, and angiopathy. Endocrine

symptoms include diabetes mellitus and growth failure, while gastrointestinal symptoms include abdominal discomfort, constipation, or pseudo-obstruction. Nephropathy and vitiligo are other symptoms of MELAS include.[11; 13]

3.2.3 Diagnosis

Mitochondrial disease is often difficult to recognize clinically because the features overlap many other conditions. However, once suspected, a diagnosis can be confirmed with biochemical and molecular testing. Biochemical testing including blood lactate and pyruvate levels, urine organic acids, blood amino acid levels, and muscle respiratory chain enzyme analysis may lead to a suspicion of MELAS, but molecular testing is ultimately necessary to confirm it. Molecular testing includes allele specific techniques and full mitochondrial chromosomal sequencing. Due to heteroplasmy, it is possible for one tissue type, such as blood, to test negative for the mutation, while another tissue, such as muscle, tests positive; thus analysis of multiple tissues may be necessary. The challenges with diagnosing MELAS often result in diagnosis being a multi-step process. This process could require multiple visits to one or more health care providers, which may pose additional challenges to diagnosis of MELAS in populations without affordable access to advanced healthcare.

3.2.3.1 Sample Specifications

Research has been conducted to determine which sample type is most suitable for providing the best possible reflection of heteroplasmy in an individual.[19] Because it is known that different tissues will show different levels of heteroplasmy, it may be best to find the most clinically affected tissues in order to have the highest likelihood of identifying abnormal mtDNA. One study

compared five tissue types including blood leukocytes, skin fibroblasts, hair roots, urinary sediment, and cheek mucosa to determine which provided the best estimate of mutational load.[19] Molecular testing of skeletal muscle has long been considered the gold standard of molecular diagnosis, but this may no longer be the case when looking for an initial diagnosis in patients.[20] Testing blood is a less invasive sample type for testing; however, while the m.3243A>G mutation is detectable in blood, the mutational load will deteriorate over time in blood leukocytes.[20] In some cases, testing for this mutation in blood in a severely clinically affected individual reveal continued reduction of mutational load with aging, and results can even be negative.[19] Thus, when considering testing for asymptomatic individuals, it is paramount to use something other than a blood sample in order to reduce the likelihood of returning a false negative result. One study showed that buccal mucosa and urinary sediment were the best non-invasive tissue types to test, as both samples provided for mutation detection in cases when blood samples did not.[19] Several studies have also supported the use of urinary epithelial tissue for primary diagnosis when trying to use a non-invasive sample collection method.[19-21]

3.2.4 Management & Treatment

Treatment of MELAS is two-fold. First, supportive care must be based on the symptoms in the affected individual.[11] Patients should be monitored for progression of their condition and for development of new symptoms through routine evaluation by ophthalmology, cardiology, endocrinology, and neurology. Second, some medications may be of some benefit in mediating the cellular damage caused by the underlying energy defect. There are no consensus guidelines for specific drug regimens for MELAS patients.[13] Biochemical treatment plans for mitochondrial diseases, including MELAS, include an array of nutritional supplements, vitamins, and cofactors

of uncertain efficacy.[16] Currently, these treatments include agents such as coenzyme Q_{10} (CoQ_{10}), L-arginine, B vitamins, idebenone, creatinine, and levocarnitine. These treatments aim to minimize the demands of mitochondria and maximize their function by augmenting the production or utilization of ATP and reducing the effects of excess production of reactive oxygen species.[13] CoQ_{10} is the most commonly used agent to treat patients with mitochondrial disorders due to the key role of Co-enzyme Q as an electron acceptor within respiratory chain complex III. Results of CoQ_{10} use in the treatment of MELAS have been mixed, and it has not resulted in sustained clinical benefit even though it appears to improve some biochemical markers of MELAS.[13] L-arginine is another commonly used agent in MELAS treatment regimens. It is proposed to play a role in reducing the frequency of stroke-like episodes in individuals with MELAS due to its role in nitric oxide metabolism, and several studies have shown its utility for this purpose.[13; 22-24] However, its true efficacy remains controversial. L-carnitine and idebenone have been used as antioxidants, but the latter has fallen out of favor more recently. Creatinine is a high energy phosphate compound that becomes depleted in muscles of patients with respiratory chain defects.[25] However, controlled studies of its use have given mixed results on efficacy. Overall, when designing a drug therapy regimen for a patient with MELAS, clinicians must balance the potential benefits of particular treatments with the risk for adverse reactions, financial burden to the patient, the patient's symptoms, and other individualized health factors.[13]

Other treatments aimed at reducing effects of MELAS include non-drug therapies, or lifestyle modifications including diet and exercise programs. In particular, the effects of exercise and training in patients with mitochondrial disorders have been examined.[26-31] These few studies suggest that there are deleterious effects of limited physical activity because they perpetuate the mitochondria's oxidative impairment. In contrast, endurance training appears to improve

mitochondrial function in vitro and overall muscle strength in vivo.[26; 29; 31] The mechanism for this improvement may include inducing "gene shifting," a process by which the molecular events leading to expression of metabolic myopathy are reversed.[32] As understanding of the pathways involved in mitochondrial functions are elucidated, perhaps the development of more regimented treatment and management plans for patients with MELAS will occur. However, it has been proposed that the most promising treatments lie not in pharmacological therapies but in developing gene therapies aimed at manipulating heteroplasmic mtDNA genotypes in favor of wild-type mtDNA.[33]

3.3 THE AMISH

The Amish are a group of people with converging beliefs regarding religion, society, and plain living. They are known as the Plain Community, believing in plain living as a part of their religious beliefs.

3.3.1 Amish Culture

The Amish community arose as the result of a split in religious views that occurred in the Protestant Reformation in Europe. Anabaptists, who believed that Baptism should occur later in life as an intentional act by an informed adult, ultimately established the Anabaptist church.[34] Later, there was a further separation of the Anabaptists into the Mennonites and Amish based on treatment of individuals excommunicated from the church, with the Amish being followers of Jakob Ammann, who believed in stricter adherence to excommunication laws.[35]

Throughout the early 16th to the mid-17th centuries, there was a migration of the Old Order Amish from Europe to America in pursuit of freedom from religious persecution. Today the Amish living in the United States are clustered in about 200 settlements across the country.[35] A 2010 report showed that the highest populations of Amish in the United States were located in Ohio, Pennsylvania, and Indiana (Figure 4).[36] While the foundation of each of these communities is rooted in the same history, there are slightly divergent beliefs among the different settlements, just as there were between the Mennonites and the Amish. The foundation of Amish culture is plain living, which is a way of life that rejects modern conveniences and technologies whenever possible. They believe in making choices that are best for the community, and they separate themselves from popular culture. Religion is at the core of the values by which the Amish live their lives.

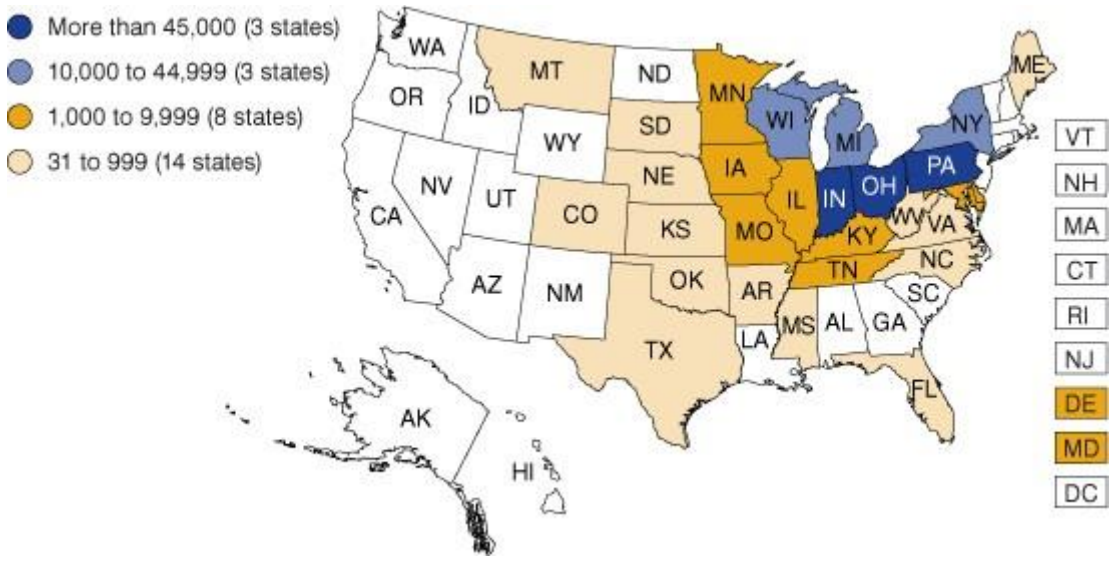

Figure 4. Number of Amish Adherents by State, 2010©[36]

The Amish believe that, "true grace can only be achieved if one lives in isolation from the non-Amish world." [35] This separates them from some laws governing other United States citizens.

In order to maintain their separation from the non-Amish, or "English" world, the Amish depend on one another and their community to provide assistance when self-reliance is not possible.

3.3.2 Amish Medical Beliefs

The combination of the core Amish beliefs and their desire to avoid use of modern conveniences and technologies once led to the belief that seeking health care outside of the Amish community was unacceptable. However, over time many settlements have come to accept the life-saving qualities modern medicine has to offer. This facet of Amish culture is exemplified in cases where the Amish must seek English medical care for their children. In many of these cases, a single family is often unable to cover the cost of all expenses related to this care, so the community contributes whatever additional funds are required to help the family afford the care their child needs. This practice has developed because the Amish beliefs in self-reliance, commitment to community aid, and belief that God's will is supreme limit their participation in traditional or government-subsidized health insurance. Due to the ever-rising costs of health care in the United States, the Amish's reliance on English healthcare has been difficult for many communities. As a result, the Amish Hospital Aid Fund has been growing since the 1960s and serves as a source of communal monies to be put toward the healthcare of those who are unable to bear the entire financial burden associated with seeking healthcare.[35]

3.3.3 The Amish and Genetics

Over time, an increased frequency of specific autosomal recessive (AR) conditions has been recognized in Amish people due to founder effects, resulting from a "genetic bottleneck," seen

when a small group of founders immigrate to a new location. In a self-isolating community such as the Amish, this results in the gene pool being much more limited than in larger or more geographically and ethnically diverse populations. In this situation, any autosomal recessive mutation in a founder individual will result in an increased prevalence of that mutation in subsequent generations in the community due to inbreeding. While the overall rate of genetic disease in the Amish is similar to the general population, its spectrum is different. There is an increased incidence of numerous autosomal recessive conditions, for example glutaric acidemia and propionic academia, in some Amish settlements. There is limited evidence of mitochondrial disorders in Amish populations; prior to recent diagnoses made in Western Pennsylvania by this principle investigator and colleagues, mitochondrial disease in the Amish had been unrecognized. This discovery of a mitochondrial condition in the Amish is significant for three reasons: the family sizes often found in Amish communities result in risk to a significant number of individuals; the potential for significant morbidity related to these conditions; and the potential for benefits from early recognition and intervention.

3.4 SELF-ADMINISTERED QUESTIONNAIRES AS A MEASURING TOOL

Questionnaires have long been used as a method for eliciting information from research subjects. When trying to elicit information about knowledge gained through an educational intervention, a pre- and post-educational intervention, or a "before and after" model, has commonly been used. Many studies have shown the efficacy of utilizing self-administered questionnaires for this purpose in different study populations. Most commonly, this model is used in a school setting to determine if students are gaining the knowledge associated with learning objectives.[37; 38] Questionnaires can

only provide useful information in research when they are properly designed and administered.[39] Many factors can affect whether questionnaire responses provide the desired information to further research goals. Good formatting is paramount in effectively utilizing a questionnaire as a measuring tool because a well-formatted questionnaire can reduce measurement error and increase response rates.[40] This includes word choices and order of questions, the placement of instructions, the layout of the questions, the length of questionnaire, the response categories, the cover page, and many other features. All of these can work to provide better measurement outcomes.[40] When determining formatting, the primary focus is the audience, or respondents. Concentrating attention on the specific needs of respondents will help guide choices related to the aforementioned questionnaire features. For example, when measuring knowledge using questionnaires, it is important to place knowledge questions before attitude questions.[39] Asking respondents if they are familiar with or aware of a concept before moving forward to gain deeper insight into their understanding or attitudes toward that subject may provide a more accurate representation of their true level of knowledge regarding that concept. Another factor that may play a role in questionnaire administration is the physical format of the questionnaire. For example, when administering questionnaires to the general population, a personal electronic device might be the easiest questionnaire method, but in the Amish community, this is not an option. Rather a booklet format was selected because this was thought to be a format with which they might be most familiar.

3.4.1 Use of Questionnaires in Amish Communities

In the Amish and other communities, there are several factors to be considered when administering questionnaires. A key consideration is the level of comprehension by the respondent based on factors such as reading level and prior exposure to questionnaire content. Formal schooling in this

community generally ends after the eighth grade. When administering questionnaires to the Amish population, modifications must be made to ensure the comprehension of the respondents in order to obtain the most accurate representation of respondent data. Modifications made include targeting the reading level to sixth to eighth grade, as is recommended for general population studies, and altering word choice to exclude words with culturally-inappropriate connotations or that make referral to technologically-advanced ideas. Some studies have suggested that personal interviews may be beneficial to ensuring this comprehension. However, these and other studies have also found that answers given in personal interviews may differ from those provided on self-administered questionnaires.[41] Overall, there is limited research conducted in this population to determine the best means of assessing knowledge gained from an intervention to conclude that self-administered questionnaires would provide a more accurate representation of knowledge than personal interviews.

3.4.2 Skip Patterns

A skip pattern is a questionnaire tool used to allow respondents to skip one or more questions based upon their selected answer to a particular question, called the directive question rather than being required to answer every question.[42] Well-designed skip patterns allow a respondent to easily identify the next question he or she should answer based on their answer to the directive question. When utilized visually or in basic language, skip patterns can aid respondents who have lower reading levels.[42] However, skip pattern development is a nuanced process that benefits from extensive pre-testing, preferably by a member of the population being surveyed, to be most effective.[42] There are several forms of skip patterns, all with the same goal: trying to reduce errors of both commission, the tendency not to skip a question when directed to do so, and of omission,

the tendency to skip questions when not directed to do so.[43; 44] Most commonly, visual skip patterns can be utilized to provide the highest level of compliance.[42-44]

3.5 HIGH-RESOLUTION MELT PROFILING

High-resolution melt (HRM) profiling is a process by which sequence variants can be identified through the exploitation of thermal denaturation of nucleic acids.[45] Denaturation of DNA containing more G-C base pairs occurs at a higher temperature than that of DNA containing more A-T base pairs due to the extra hydrogen bond in a G-C base pair. The HRM profiling process was first described in 2003 by Wittwer *et al.*[45-47] In this method, the region of interest is amplified through real-time PCR, then the double-stranded PCR product, or amplicon, is denatured in an HRM-capable real-time PCR machine. The denaturing process is monitored closely using intercalating dyes, which fluoresce brightly when bound to double-stranded DNA (dsDNA) but lose their fluorescence as the dsDNA denatures in single-stranded DNA (ssDNA). The result is the generation of a post-PCR melt profile of the sample, which can be compared to that of a sample containing a known genetic sequence to determine the presence of a sequence variation.[45-47]

3.5.1 High-Resolution Melt Profiling in mtDNA

HRM profiling using amplicon-based genotyping has proven to be sensitive enough to identify some mtDNA point mutations, including the m.3243A>G MELAS mutation, at a heteroplasmy level at or below 10%.[47] In this method, the melt profiles of test samples are compared to those of control samples. Comparison controls are comprised of samples of the mtDNA reference

sequence, samples homoplasmic for the mutation of interest, and samples made by diluting the homoplasmic mutant mtDNA, resulting in samples with different levels heteroplasmy for the mutation of interest. These are used to define standard melt profiles for comparison with the test sample. If the dilutions used for comparison are specific enough, HRM can provide resulting heteroplasmy levels ±1%.[47]

4.0 SIGNIFICANCE

4.1 PUBLIC HEALTH AND MITOCHONDRIAL DISEASE

Over the past several decades, research has shown that mitochondrial respiratory chain deficiency is one of the most prevalent classes of genetic disorders. The minimum prevalence of mitochondrial disease caused by either nDNA or mtDNA mutations has been estimated to be approximately 1 in 5,000 individuals world-wide.[48] As described previously, mitochondrial diseases are characterized by phenotypic and genetic heterogeneity and frequent multisystem involvement.[49] Primarily because of the phenotypic heterogeneity, it is often difficult to identify a mitochondrial disease. There are many different symptoms that can arise with mitochondrial disease. However, many of these symptoms can be seen in isolation in the general population in those who do not have mitochondrial disease. Many individuals who suffer from mitochondrial disease are misdiagnosed with other conditions.[12] These may include atypical cerebral palsy, various seizure disorders, and other childhood or age-related diseases. In other cases, a pattern of symptoms may not have been recognized, and an individual may carry multiple complex disease diagnoses without a caretaker having recognized their relatedness.[12] For example, an individual may have a diagnosis of insulin-dependent diabetes mellitus and suffer from hearing loss. While it is possible these two diagnoses are unrelated, they can also be seen in combination in patients with the common MELAS mutation. It is only when a caretaker recognizes the pattern of symptoms in an individual or a family as being related that diagnosis of a mitochondrial disorder is considered.

Even in cases when mitochondrial disease is suspected, it may be difficult to confirm a diagnosis due to an inability to identify a specific respiratory chain defect or an inability to detect a genetic mutation leading to disease. In many cases, mutations and defects can escape identification using blood, saliva, or urine sediment testing. While a muscle biopsy has long been considered the gold standard in mitochondrial disease diagnosis, sometimes diagnosis requires multiple biopsies. Due to the invasiveness of this procedure, the landscape is shifting in favor of genetic testing as a gold standard of diagnosis. However, this mechanism can also encounter difficulty due to variable detection of mutations in different tissue types. This long process of diagnosis can leave patients and caregivers frustrated and ultimately result in an undiagnosed patient due in part to testing fatigue or limited financial resources to pursue additional testing.

Providing education regarding MELAS and other mitochondrial diseases to members of the general population and health care providers could help promote recognition of mitochondrial disease in individuals and families. In 2004, the Surgeon General's Office launched the Family Health History Initiative, which was an effort to promote awareness and improve family health history ascertainment and utilization in order to better identify individuals at risk for diseases and disorders based on family history.[50; 51] While this effort has been geared primarily toward complex diseases such as heart disease and diabetes, this initiative could also be used to help families identify symptoms related to mitochondrial disease in order to help healthcare professionals identify patterns in families. It is especially important to promote this discussion among families because research has shown, at least historically, that many primary care physicians were not eliciting adequate family histories for identification of familial or hereditary conditions.[52] Obtaining family health history can be combined with increased efforts to promote education about

mitochondrial disease to help increase the number of affected individuals and families who are being diagnosed and treated accordingly.

Increased diagnosis might promote increased demand for additional research into treatment and management options for individuals affected with mitochondrial conditions. Diagnosis and proper treatment may help to decrease the financial burden on society and the public healthcare system that is associated with undiagnosed individuals where symptom-based treatments can improve long-term outcomes.

4.2 THE IMPACT OF MELAS

Because of the variable presentation of MELAS and the sometimes cumbersome diagnostic process, those who have this condition may not be recognized and diagnosed for many years.[53] This means that individuals affected with the condition often do not get appropriate care to maintain their best possible quality of life, and women who are mildly, or even moderately, affected may not understand that they are at risk to pass the condition to their offspring.[16]

MELAS has a broad spectrum of possible clinical features with variable severity. When MELAS is expressed in its most severe form, it is a debilitating condition, and patients required detailed attention and medical care to maintain a reasonable quality of life. Treating each feature of the condition without understanding the way the symptoms are connected to an underlying comprehensive diagnosis may leave gaps in care for individuals with MELAS.

While there is limited data on the subject, it is likely that one of the best ways to ensure the highest quality of life and best treatment or management possible is to identify MELAS as early as possible. Early diagnosis of MELAS allows for prevention or faster treatment of neurological

symptoms. For example, identification of the condition would make parents and physicians aware of the potential for seizures and stroke-like episodes before a child experiences those symptoms. Another example would be screening for hearing loss and diabetes since those are commonly related symptoms. Specifically in regards to diabetes, there is evidence that earlier detection and treatment may lead to better outcomes.[54] The benefits of prevention or better control of these and other symptoms resulting from early diagnosis may truly impact an affected individual's future.[12; 55]

4.3 AMISH HEALTH CARE

Dr. D. Holmes Morton and Caroline Morton established The Clinic for Special Children in 1989 to provide care to the children with genetic disorders in the Pennsylvania Amish and Mennonite communities. The work of this clinic dramatically demonstrates the impact of simple improvements in care and early detection of disease. In addition, parental and community education have been shown to change the course of a child's disease and life. One example of this impact is the discovery of glutaric acidemia, type 1 as the cause of what was once called "Amish cerebral palsy" in 16 Amish children who had common ancestors, or one "founding" couple. Children found to have this condition through early screening live healthier lives as a result of comprehensive follow-up care.[34]

The lifestyle of the Amish is one that does not easily allow them to spend large amounts of money on traditional health care. In 2010, a $1.5 million operating budget allowed the clinic to provide care that saved the community an estimated $20 to $25 million in caring for their children with autosomal recessive conditions.[2] For example, there has been a 94% decrease in hospital costs

associated with maple syrup urine disease (MSUD), saving at least $4.3 million in annual health care costs for the Lancaster County Amish community.[2] If this same paradigm is applicable to the lives and care of individuals with mitochondrial disease, it may result in a significant impact on the affected families in the Amish community. Since the Amish are such a communal group, positively impacting just a few families will in turn positively impact the community as a whole.

Given the recent identification of a family in the Mercer County Amish community with the common MELAS mutation, along with the large family sizes in traditional Amish communities, it is hypothesized that there will be a significant number of members of the community who have some degree of symptomatology related to undiagnosed MELAS. According to clinicians at The Clinic for Special Children who serve the Lancaster County Amish communities, as many as 50% of patients in their community with chronic disease remain without a specific diagnosis.[1] Assessment of appropriate individuals from this and related populations for MELAS and other mitochondrial disorders may lead to provision of targeted medical care for them.

5.0 MATERIALS AND METHODS

The study was designed to determine the frequency of MELAS in the Mercer County Amish community, to provide an investigator-designed educational program to community members who may be affected or know individuals who are affected by MELAS, to study the effectiveness of the educational session, and to construct an extended family pedigree of the index family. This research protocol was submitted to the University of Pittsburgh's Institutional Review Board (IRB) and was approved on November 13, 2014 (Appendix A).

5.1 RECRUITMENT PROCEDURES

Assessment of family interest in participation in a research study of MELAS in the Amish community was completed through outreach to the index family that is followed clinically at the Children's Hospital of Pittsburgh of UPMC by the primary investigator and a co-investigator. A letter was sent to the family asking them to contact the primary investigator if they were interested in learning about the study (Appendix B). A conversation was then arranged to briefly explain the goals and plans of the proposed research and to see if the family was interested in participating. Since the family was interested, per local customs, they were asked to arrange for communication between the study team and the local Deacon. The Deacon granted permission to conduct research in the community (Appendix C).

The family then confirmed that they would organize a meeting at their home to facilitate the study activities. The index family invited their extended family members to attend the first study visit. This extended family was primarily composed of maternal family members.

5.2 STUDY VISIT ONE

5.2.1 Pre-Education Questionnaires

After introductions of the study staff in attendance and a brief explanation of the research study's aims and activities, participants were informed that they had the option to either continue or decline further participation in the study. At this point, a pre-education questionnaire (Appendix D) was distributed. The purpose of this questionnaire was to gauge each study participant's understanding of and exposure to information about genetics, mitochondrial disease, and MELAS prior to the study's start. Questionnaires were distributed in a manila envelope to all individuals willing to participate and over the age of 14 years. Each manila envelope was labeled with a code, and each envelope contained both a pre-education questionnaire and a post-education questionnaire. Each questionnaire was also coded to allow for pre- and post-education questionnaires to be compared while maintaining anonymity. After taking the pre-education questionnaire, participants retained their manila envelope until later in the session. Those who did not want to participate were able to decline participation by returning the questionnaire without answers.

Investigators were able to administer the pre-education questionnaire prior to obtaining written informed consent. This was permissible because the IRB provided approval for a "waiver for written consent" for completing the pre-education questionnaire. This waiver can be seen

within the approval for the research study, which is shown in Appendix A. This "waiver for written consent" was requested for this study activity because investigators were concerned that the informed consent process would bias responses to the questionnaire because the informed consent document contained information describing the topics queried in the pre-education questionnaire.

5.2.1.1 Questionnaire Construction

The questionnaire was composed of 16 multiple-choice questions, which were divided into four sections. The first section contained questions about basic genetics, and the second contained questions about autosomal recessive genetic disease. The third section contained questions about mitochondrial disease, and the fourth contained questions about MELAS specifically.

A skip pattern was utilized to allow participants to skip any of the four sections if they did not have prior knowledge of the topic covered in that section. This was achieved by using the first question in each section to ask a fundamental question related to the topic in that section and making one of the multiple choice answers, "I have never heard of [topic]." If a participant chose this option, it directed the participant to skip the remainder of that section and move to the next one. Each question also had the multiple-choice answer: "I am unsure of the correct answer." This option was offered in an attempt to mitigate skewing of results due to random guessing.

Finally, at the end of the questionnaire, there was a section to be completed only by participants who had or knew someone who had a mitochondrial disease. This final section included several quality of life questions developed by modifying the Newcastle Mitochondrial Disease Scales.[56] While approved for collection from this group of study participants, these data will be analyzed by team members in parallel with work for this study and are not reported here.

This questionnaire was developed with the aid of Dr. Todd M. Bear, who directs the Office of Health Survey Research in the Institute for Evaluation Science, conducts numerous health

surveys yearly, and currently teaches the "Health Survey Methods" and "Measurement in Social and Behavioral Sciences" courses for the Graduate School of Public Health at the University of Pittsburgh. Additionally, this questionnaire was reviewed during a core educational conference for professional genetic counselors and genetic counseling students at the Children's Hospital of Pittsburgh of UPMC, where these professionals were given the opportunity to provide feedback on the questionnaire.

5.2.2 Informed Consent

A comprehensive informed consent process was completed following the completion of the pre-education questionnaires. The two informed consent documents to be used, adult (Appendix E) and pediatric (Appendix F), were approved on November 13, 2014. The components of the consent form were reviewed in a group with a co-investigator to answer questions and facilitate written informed consent for each subject. Adult subjects who were not mentally impaired were asked to consent to the study. Adults who were mentally impaired could assent to the study while their parents were asked to provide consent. Children over the age of 14 were also asked to assent to the study while their parents and the parents of children under the age of 14 years were asked to consent for study participation.

Subjects and parents/guardians were informed that their participation and their child's participation were completely voluntary, and their decision to participate in the study would not impact the clinical care of themselves or their child at UPMC facilities. The informed consent form included consent for the following:

- An educational intervention

- Pre- and post-educational questionnaires for comparison and assessment of efficacy of the educational intervention
- Blood, urine, and buccal samples for analysis of the m.3243A>G mutation in the *MT-TL1* gene
- One-on-one session to elicit individual and family medical history
- Release of medical information from UPMC and other facilities
- Genetic counseling for genetic test results

The subjects were given as much time as necessary to consider whether or not they would like to participate in research while the primary or co-investigators were available to address any questions or concerns they had. Those who did not want to participate retained their pre-education questionnaires. If an individual chose to consent to the study, he or she signed and returned the consent form. If an individual chose not to consent to the study, he or she was encouraged to remain for the group educational session that followed the consent process, but was not included in the remaining study activities. This allowed community members who did not want to participate in research the opportunity to learn valuable information about this condition due to its potential implications for them, their families, and their community.

5.2.3 Educational Intervention

The educational intervention was done following the informed consent process. The intervention was an educational session with verbal and visual components that was designed to provide participants with information about MELAS and mitochondrial disease. The session included information about the following:

- mitochondria

- different modes of inheritance for genetic diseases
- mitochondrial DNA
- the meaning and involvement of heteroplasmy and mutational loads
- mitochondrial inheritance and its relationship to MELAS and the participants' community
- information about the common MELAS mutation and its related phenotype
- the basic clinical features of MELAS
- the treatment and management options for patients with MELAS

5.2.3.1 The Presentation

The educational intervention was presented verbally by this co-investigator using a visual aid handout (Appendix G) developed by this co-investigator with input from the study team and others including genetic counselors at the Children's Hospital of Pittsburgh and faculty members in the Department of Human Genetics in the Graduate School of Public Health at the University of Pittsburgh. Clarification was provided per participant questions and feedback.

The presentation was designed to be interactive, similar to a genetic counseling session, in order to assess the understanding of participants throughout the presentation. This design allowed for minor adjustments to be made in the way information was being presented based on this investigator's perception of participant understanding. This would be similar to a genetic counselor making adjustments in a genetic counseling session based on his or her assessment of a patient's level of understanding.[57] For example, changing the language used when discussing a particular topic may provide an opportunity for better participant comprehension, and the need to do so may be based on an assessment of facial expressions, body language, or participant verbalizing of lack

of understanding, all of which can be assessed using an interactive educational intervention. The presentation also allowed for additional time for questions.

5.2.4 Post-Education Questionnaires

A post-education questionnaire (Appendix H) was administered following the educational session. This questionnaire was nearly identical to the pre-education questionnaire except for the quality of life questions and acknowledgment of participation at the end. All participants were given time to complete the questionnaire prior to individually moving forward with additional study activities. After completing the post-education questionnaire, participants were asked to return their manila envelope containing both their pre- and post-education questionnaire to a study staff member. The staff member confirmed the presence of both questionnaires before accepting each envelope.

5.2.5 One-on-One Interview Sessions

Following the educational intervention and post-session questionnaires, one-on-one interviews were conducted between either the primary or a co-investigator and a family historian. This typically included either an individual study participant or a nuclear family of participants. The first activity completed in each one-on-one interview was privately and confidentially to confirm informed consent and willingness to participate in the study. This was done to ensure that no participant was participating unwillingly due to a sense of social obligation or pressure that may have arisen due to the group setting or the participants' intrinsic sense of community.

The purpose of the interview was to collect a detailed family and medical history from all participants. Using a list of clinical features associated with MELAS and mitochondrial disease

(Appendix I), each historian was asked to identify any features associated with mitochondrial disease in themselves or their family members. This list was used by investigators to ensure uniformity in recording information since sessions were conducted by different investigators.

Each historian provided information that was to be used for construction of a pedigree of all individuals discussed during the session. The historian would answer questions about him- or herself, his or her immediate family, and the nuclear families of any extended family members not present at the study visit. Standard pedigree construction allows for collection of health information about family members which is regarded as hearsay; this means that the information has not been confirmed by the family member in question, and there is no clinical evidence present to support the information being recorded.[58] Regarding each family member, the historian was asked whether he or she exhibited the symptoms of mitochondrial disease listed on the investigators' checklist.

During the one-on-one interviews, participants also signed medical release forms for records from facilities where the individual was seen for an issue that may be related to mitochondrial disease.

5.2.6 Sample Collection

Following the one-on-one interview session, each participant was asked to provide urine and blood samples. This collection was coordinated by study staff and completed by a medical doctor (MD) investigator. Blood samples were collected using lancets to pierce the finger and the blood drops were blotted onto newborn screening filter paper to store the sample. Urine was collected in urine cups privately by each participant without investigator oversight. All samples, both blood and urine, were labeled with the participants' coded identification numbers. Filter papers were dried

before storing, and for traveling; urine samples were stored on ice in a cooler until they were transferred to the primary investigator's laboratory freezer pending sample analysis.

5.3 SAMPLE ANALYSIS

DNA analysis of samples was conducted in the laboratory of the primary investigator under the supervision of the co-investigator Steven Dobrowolski, PhD at the Children's Hospital of Pittsburgh by co-investigator Lina Ghaloul Gonzalez, MD. Samples were analyzed for the presence of the m.3243A>G mutation in the *MT-TL1* gene using HRM profiling as described by Dobrowolski.[47] HRM profiling identifies sequence variants by deviation in the shape of a post-PCR melting profile performed in a 96-well formatted LightScanner96 instrument. Controls of 100% A (wild type allele), 100% G (mutant allele), and defined mixtures of each were used to generate a standard curve for quantification of each allele in subject DNA.

Briefly, participants' DNA was extracted from dried blood spots and urine samples, and the mtDNA was amplified by a polymerase chain reaction (PCR) with validated primers.[47] PCR was performed on each sample and on control DNA in duplicate in a 96-well plate. Then, using a 96-well formatted LightSCanner96 instrument, a post-PCR melt profile was generated. Data were normalized, temperature-shifted, and converted to difference plots to allow for easier distinguishing of different genotypes by plotting the fluorescence differences between normalized and temperature-shifted curves as described.[46] Melt calibration was performed using a single internal oligonucleotide calibrator.[59] Analysis was performed with LightScanner software version 2.0 Call-IT (v2.0.0.1131; Idaho Technology) using the melt calibration module.

5.3.1 High-Resolution Melt Profiling Method

This method of sample analysis was chosen because of its cost efficiency, rapid turnaround time, and its previous utility in identifying the specific mutation of interest. This method was previously published by one of the co-investigators, which allowed for increased consultation and guidance regarding its use in this study. The co-investigator who performed the sample analysis practiced the method multiple times using volunteer samples for quality assurance purposes prior to performing study sample analysis.

5.4 PEDIGREE CONSTRUCTION

At the study visit, pedigree construction was initiated following the educational intervention, prior to the start of the one-on-one interview sessions. During this time, the primary and co-investigators asked the participants as a group to help identify and order family members of the index patients parents' generation in order to save time and reduce repetitive information being taken during one-on-one interviews. Progeny Clinical 6.0 software was used to construct a pedigree of this kinship. Study materials were saved in a private database behind the University of Pittsburgh firewall, accessible only with a password known to study staff. The first step of this pedigree construction was the creation of a database compatible with the requirements of data entry, after which the applicable data fields could be populated with information gathered in the one-on-one interview sessions. A legend was devised to depict the symptoms reported in individuals. This allowed for representation of the most commonly seen symptoms in the community.

5.5 QUESTIONNAIRE DATA ANALYSIS

Questionnaire data was compiled and organized utilizing both Microsoft Excel and GraphPad's QuickCalc software. Excel served as a repository for compilation and organization of data prior to inputting data into QuickCalc for statistical analysis, after which Excel was used to develop graphs of this analysis.

Because the questionnaire data was paired and could be dichotomized, or put into two distinct categories, McNemar's Test was the best tool to compare the differences in answers in the pre- and post-education questionnaires for statistical significance. Statistical significance and power of the results were determined based on the number of questionnaires completed by participants.

5.6 STUDY VISIT TWO

After completing sample analysis to determine the heteroplasmy level of all participants, a return visit was scheduled via letter to the index family on December 05, 2014 (Appendix J). After no communication was received in response to this first attempt at scheduling the visit, a second letter was sent to the family on January 9, 2015 (Appendix K). The patriarch of the index family then called but was unable to speak to the PI. During this phone call, the patriarch requested that rather than travel to Mercer County for a second visit, the study team mail individual sealed envelopes containing the results of each participant to the home of the index family for distribution to study participants at their next family gathering. Given the nature of this study, specific IRB approvals for the study, and the ethical concerns with distributing results of genetic testing without genetic

counseling, the PI sent a letter in response requesting that the index family and participants move forward with the results disclosure visit as previously planned (Appendix L). Given concerns about the burden of travel in winter weather on participants, the study team and index family opted to wait to disclose test results until March 2015.

5.6.1 Results Disclosure Session

The test results for each participant who had testing were disclosed by an MD, a licensed certified Genetic Counselor (CGC), or a supervised Masters of Genetic Counseling student. Results were disclosed to the participant who had testing along with supportive, patient-designated family members. For children who received results, the results were disclosed to the parent/guardian of the child in the child's presence.

To ensure consistency among all results disclosure sessions, before Study Visit Two an outline of information to be reviewed with participants was created to serve as a guide for the disclosure session (Appendix M). Results disclosure sessions were designed to follow the format of a traditional one-on-one genetic counseling session. Information to be provided included exploration of the subject's recollections about mitochondrial inheritance and MELAS, a basic review of information regarding MELAS, the participant's genetic test results, and a discussion of the implications of the results for the individual patients on their health and their children's risks for health problems, including management options. During the disclosure visit, the psychosocial effects of the disclosure on the participants was further explored through questions and active listening.

5.6.1.1 Information Reviewed with All Participants

Some information was reviewed briefly with all participants regardless of their test results. This information included a review of mitochondrial inheritance, with emphasis on maternal transmission of the mtDNA mutation to children and the lack of mitochondrial disease risk for children and grandchildren of men who had a positive test. The concepts of heteroplasmy and mutational loads were reviewed in the context of both likelihood of passing the mutation on to one's children and for disease prognosis. The clinical features of MELAS and mitochondrial disease were also reviewed, with emphasis on the variability that can be seen in individuals due to heteroplasmy.

5.6.1.2 Disclosing Negative Results

For participants who had "No Heteroplasmy Detected" or "Negative" test results, after disclosing the result, study team members emphasized participant understanding of his or her result and the limits of the testing. In the case of such a negative result, this includes ensuring that the individual understands their extremely low likelihood of having children with the m.3243A>G mutation due to the mitochondrial inheritance pattern of the condition. This discussion was done based on the participant's gender.

5.6.1.3 Disclosing Positive Results

For participants who had "Heteroplasmy Detected" or "Positive" test results, additional information was reviewed. The session included a review of the mitochondrial inheritance pattern, the clinical features of MELAS, the treatment and management options available to the participant, and next steps moving forward based on the participant's heteroplasmy level.

5.6.2 Consenting Additional Study Participants

At the time of Study Visit Two, investigators were prepared to consent additional participants for mutation studies and collect samples from them. These participants did not participate in the educational intervention or the associated questionnaires. These results are to be disclosed outside the scope of this project in the same manner as those disclosures included in this study.

6.0 RESULTS

The first study visit occurred on November 15, 2014 in the home of the index family in Mercer County in Northwestern Pennsylvania (Figure 5).[60] Study staff travelled to this location for all study-related activities that occurred for study visit one. This group included the primary investigator Jerry Vockley, MD, PhD; co-Investigators Amy Goldstein, MD; Lina Ghaloul Gonzalez, MD; Cate Walsh Vockley, MS, LCGC; this co-Investigator, and first year MS Genetic Counseling student Bess Wayburn, PhD.

Figure 5. Mercer County, PA©[60]

Approximately 35 individuals from four generations of the extended family of the index family attended this visit. After setting up all materials for the study activities, approximately 20 individuals remained in the community space for the study staff's presentation. Other family members moved to the main house with the children. This group of people was primarily composed of men, the majority of whom were the spouses of women who stayed for the study activities.

After a brief review of the study, 18 pre-education questionnaires were distributed. Two individuals chose not to participate prior to distribution of the questionnaires. Approximately 20 minutes were given to allow all participants time to complete the questionnaire to their satisfaction. The informed consent process was then led by Cate Walsh Vockley, MS, LCGC, with review of the informed consent forms in a group setting. She and primary investigator Dr. Jerry Vockley fielded questions about the study. Sixteen individuals signed consent forms to participate in the study; two of these 16 participants were parents signing for their children. After collecting signed consent forms, the educational intervention commenced.

6.1 EDUCATIONAL INTERVENTION

The educational intervention information was presented verbally using visual aids. Each participant was given a color-printed copy of the educational handout in Appendix G. This co-investigator presented information verbally while using the handout and participant feedback to present information. The presentation lasted approximately 20 minutes with additional time for questions, which were answered by the primary and co-investigators.

After the educational intervention, post-education questionnaires were administered. This questionnaire was completed in a slightly different time-frame than the pre-education questionnaire because some participants chose to complete their questionnaires immediately, while others took breaks, and still others moved into one-on-one interview sessions with study staff members prior to completing the questionnaire. All participants were given ample time to complete the questionnaire and could ask questions at any point during the questionnaire if needed. Ultimately, 15 manila envelopes containing both pre- and post-education questionnaires were

collected. The three remaining questionnaires were not returned. One of these was from the original index patient, and two others were from other participants.

Analysis of the data obtained from the questionnaires was completed after returning from the study visit. Two questionnaires, Q003 and Q007, were discarded from analysis. In both cases, the pre-education questionnaire was answered, but the post-education questionnaire had a note from the participant saying, “same [answers] as before.”

The results of the questionnaires are shown in Table 1, which shows the complete set of answers given by participants for each questionnaire that was returned to the study team.

Table 1. Questionnaire Answers

Question	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Q002.1	S	S	S	S	S	S	S	0	S	S	0	0	1	0	0	S
Q002.2	S	S	S	S	S	S	S	S	S	S	S	1	S	0	0	S
Q003.1	1	1	0	1	0	0	1	1	IDK	1	0	1	1	0	1	0
Q003.2	Wrote "Same [answer] as before"															
Q004.1	1	0	0	1	0	0	1	1	IDK	1	S	1	1	1	0	0
Q004.2	1	1	1	1	0	0	1	0	1	1	0	0	1	0	1	0
Q005.1	1	0	0	1	0	1	1	0	IDK	IDK	0	0	0	1	1	0
Q005.2	1	1	0	0	0	0	1	1	0	1	0	1	1	1	0	1
Q006.1	1	0	0	0	0	0	0	1	IDK	IDK	0	1	1	0	0	0
Q006.2	1	0	0	0	0	0	0	1	0	IDK	0	1	1	1	0	1
Q007.1	1	1	0	0	0	1	0	0	IDK	1	0	1	1	1	1	1
Q007.2	Wrote "Same [answer] as before"															
Q008.1	IDK	IDK	IDK	1	IDK	1	0	0	1	0	1	1	0	0	0	0
Q008.2	S	S	S	S	S	S	0	0	IDK	0	0	0	0	0	0	0
Q010.1	1	1	1	0	0	0	0	1	IDK	IDK	0	0	1	1	0	0
Q010.2	S	S	S	S	S	S	0	0	IDK	1	0	1	1	1	1	1
Q011.1	1	0	0	1	0	0	1	1	0	0	0	1	1	0	0	0
Q011.2	1	0	0	0	0	0	0	1	0	IDK	0	1	1	0	1	1
Q012.1	1	0	1	1	0	0	S	S	S	S	S	S	S	1	0	0
Q012.2	1	0	0	0	0	0	1	1	IDK	0	0	1	1	1	1	1
Q013.1	1	0	0	0	0	0	1	0	0	0	S	0	1	0	1	1
Q013.2	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	0
Q014.1	1	0	1	1	0	0	0	1	IDK	0	S	1	1	1	1	0
Q014.2	1	0	0	0	1	1	0	1	IDK	0	0	1	1	1	1	1
Q015.1	1	0	IDK	IDK	IDK	IDK	IDK	1	IDK	IDK	0	1	1	1	0	0
Q015.2	1	1	0	0	0	0	1	1	0	1	0	1	1	1	1	0

1 = Correct Answer; 0 = Incorrect Answer; IDK = “I am uncertain of the correct answer”; S = Skipped question

Table 2. Pre-Education versus Post-Education Total Counts of Types of Answers

	Pre-Education	Post-Education
Total Number of Correct answers	**59**	**75**
Total Number of Incorrect Answers	**76**	**70**
Total Number of "Uncertain" Answers	**19**	**5**
Total Number of Skipped Questions	**22**	**25**
Total Number of Questions Answered	**154**	**151**
Total Number of Questions from Section #1 Correct	**13**	**14**
Total Number of Questions from Section #2 Correct	**12**	**9**
Total Number of Questions from Section #3 Correct	**9**	**14**
Total Number of Questions from Section #4 Correct	**25**	**37**

There were a total of 176 questions that could be answered on either of the pre- or post-education questionnaires (16 questions per questionnaire, and 11 completed questionnaires included in the analysis). All analyses were completed using proportions in order to negate the biasing impact of the skipped questions on the statistical analysis. Table 1 shows all answers given to each questionnaire, and Table 2 shows a comparison of the total counts of the types of answers. As shown from this table, the number of correct answers in the post-education questionnaires, 75, exceeds the number of correct answers in the pre-education questionnaires, 59. Figure 6 graphically depicts this comparison using the proportion of correct answers in pre-education questionnaires (0.38±0.15) as compared with the post-education questionnaires (0.50±0.15),

which shows that this difference is not statistically significant. Table 2 also shows that a comparison of the number of incorrect answers between the pre- and post-education questionnaires shows a decrease from 76 to 70. Figure 6 shows that the proportion of incorrect answers in pre-education questionnaires is 0.49±0.15 and is 0.46±0.15 in the post-education questionnaires, which again shows that this difference is not statistically significant. Table 2 shows that a comparison of the number of "Uncertain" answers between the pre- and post-education questionnaires shows a decrease from 19 to 5. Figure 6 shows this with the proportion of "Uncertain" answers in pre-education questionnaires (0.12±0.10) being greater than that of the post-education questionnaires (0.03±0.05), which shows that this difference is not statistically significant. Finally, Table 2 also shows that the comparison of the number of skipped questions in the pre-education questionnaire and post-education questionnaire shows an increase from 22 to 25 questions. This is graphically depicted in Figure 6, which shows that the proportion of skipped questions in pre-education questionnaires (0.14±0.11) is less than that of the post-education questionnaires (0.17±0.11), but again, this difference is not statistically significant. While data does not show statistically significant differences in responses in pre- and post-education questionnaires, trends were identified and will be discussed.

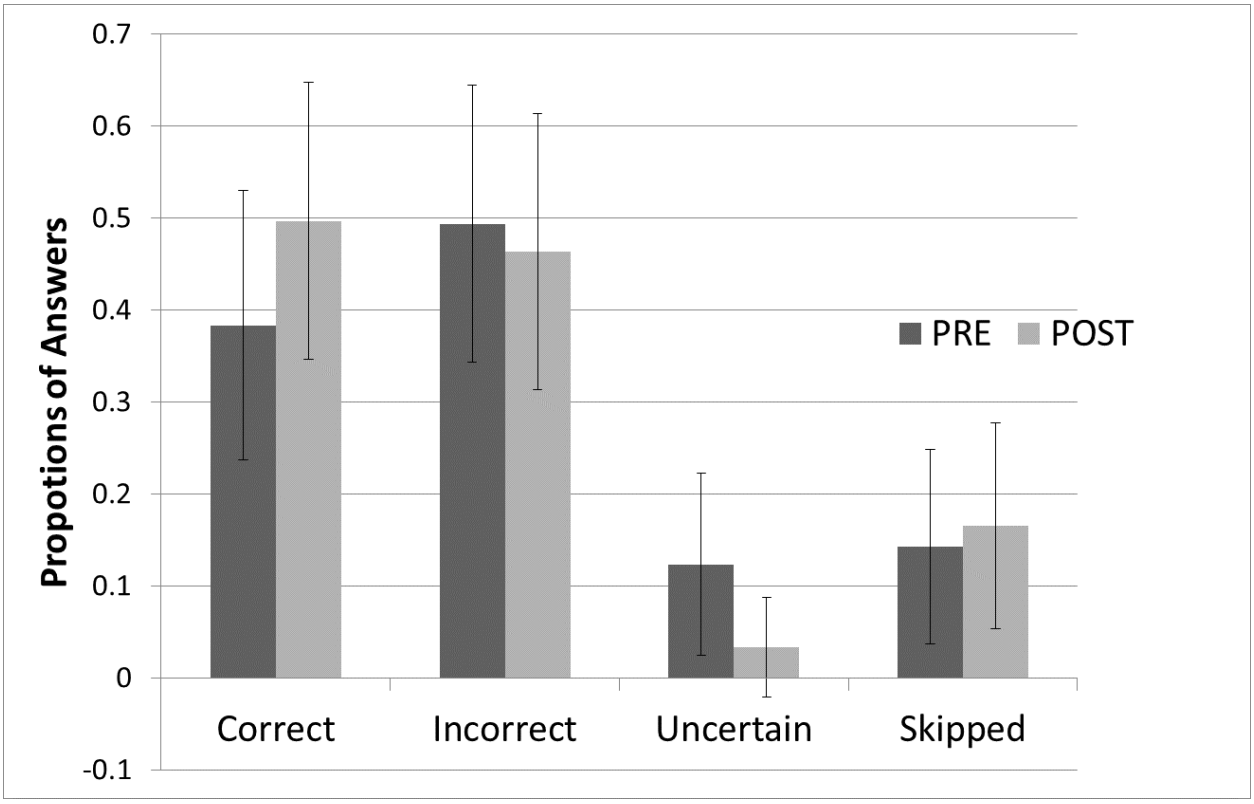

Figure 6**.** Comparison of Proportions of All Answer Types in Pre- and Post-Education Questionnaires with Error Bars to Show Standard Error (SE**)**

Additionally, Table 2 shows information about the number of correctly answered questions in each of the four sections of the questionnaire. This information is graphically depicted in Figure 7, which shows a comparison of the proportions of correct answers from each section in the pre- and post-education questionnaires. It can be seen that the proportion of correct answers in section one did not significantly change (0.43±0.09 and 0.41±0.09). The same can be said for the proportion of correct answers in section two, which decreased from 0.30±0.08 to 0.25±0.08, again not statistically significant. An increase in the proportion of correct answers was seen in both sections three and four (respectively 0.30±0.08 to 0.35±0.09 and 0.48±0.09 to 0.67±0.09), but this was not statistically significant.

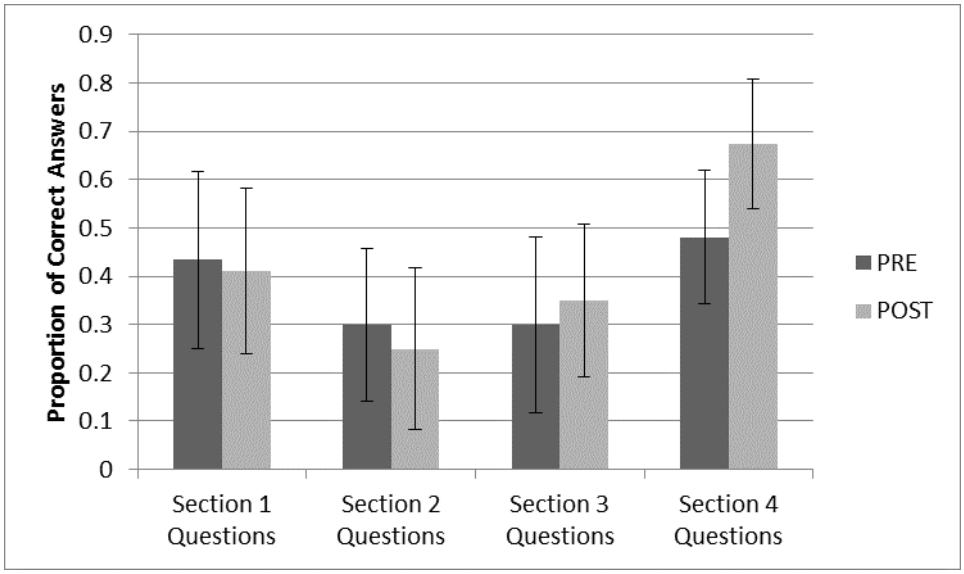

Figure 7. Comparison of Proportions of Correct Answers from Each Questionnaire Section in Pre- and Post-Education Questionnaires with Error Bars to Show Standard Error (SE)

Due to the small sample size and other factors including several skipped questions, seven key questions were identified prior to data analysis as being the most descriptive of displaying participant knowledge. One question each was identified from questionnaire sections one, two, and three, and four questions were identified from section four as being the most representative of understanding the topic discussed in each section. Because the bulk of information presented in the educational intervention was from section four, more questions were selected from this section to provide additional information regarding the participants' deeper understanding of this topic. The key questions selected from each section were question numbers 1, 4, 8, and 13-16 and are shown in Table 3. Each participant's answers are shown to these key questions in Table 4, and the number of correct answers to each question in both pre- and post-educational intervention questionnaires are shown in Table 5.

Table 3. Key Questions by Section

Section	Question Number	Question	Correct Answer
1	1	"An inherited, or genetic, disorder can be passed to a child by which of the following people?"	a. His or her parents
2	4	"A carrier of an autosomal recessive disorder is:"	b. Someone who does not show symptoms of a disorder, but whose children can have it.
3	8	"A mitochondrial disorder can be passed to a child by which of the following people?"	b. The mother
4	13	"MELAS can be passed to a child by which of the following people?"	b. The mother
	14	"Which of the following is true of MELAS?"	c. A child who has MELAS may not show all the symptoms of MELAS.
	15	"Which of these symptoms is seen in patients with MELAS?"	d. All of the above
	16	"In general, do women with MELAS-causing mitochondrial changes pass these changes on to all of their children?"	a. Yes

Table 4. Participant Answers to Key Questions

Questionnaire #	Q1	Q4	Q8	Q13	Q14	Q15	Q16
Q002.1	S	S	0	1	0	0	S
Q002.2	S	S	S	S	0	0	S
Q004.1	1	1	1	1	1	0	0
Q004.2	1	1	0	1	0	1	0
Q005.1	1	1	0	0	1	1	0
Q005.2	1	0	1	1	1	0	1
Q006.1	1	0	1	1	0	0	0
Q006.2	1	0	1	1	1	0	1
Q008.1	IDK	1	0	0	0	0	0
Q008.2	S	S	0	0	0	0	0
Q010.1	1	0	1	1	1	0	0
Q010.2	S	S	0	1	1	1	1
Q011.1	1	1	1	1	0	1	0
Q011.2	1	0	1	1	0	1	1
Q012.1	1	1	S	S	1	0	0
Q012.2	1	0	1	1	1	1	1
Q013.1	1	0	0	1	0	1	1
Q013.2	1	1	0	1	1	1	0
Q014.1	1	1	1	1	1	1	0
Q014.2	1	0	1	1	1	1	1
Q015.1	1	IDK	1	1	1	0	0
Q015.2	1	0	1	1	1	1	0

1 = Correct Answer; 0 = Incorrect Answer; IDK = "I am uncertain of the correct answer"; S = Skipped question

Table 5. Number of Correct Answers to Key Questions in Pre- and Post-Education Questionnaires

Key Question	Number of Correct answers in Pre-education Questionnaire	Number of Correct answers in Post-education Questionnaire
Question 1	9	8
Question 4	6	2
Question 8	6	6
Question 13	8	9
Question 14	6	7
Question 15	4	7
Question 16	1	6

Figure 8 shows there is variability of undefined statistical significance in the proportion of correct answers to the key questions before and after the educational intervention. Rather, McNemar's Test was used to determine the significance of the difference in the proportion of correct answers to these key questions between the pre- and post-education questionnaire responses. The results of these tests are presented in Table 6.

Table 6. McNemar's Test Results for Differences in Proportions of Correct Answers to Key Questions

	Two-tailed P-value	Statistically Significant?	Chi Squared	DF	Odds Ratio	95% CI
Question 1	Could not be calculated (more than one discordant value is zero)					
Question 4	0.3711	No	0.800	1	0.250	0.005-2.526
Question 8	1.0000	No	0.000	1	0.500	0.008-9.605
Question 13	1.0000	No	0.000	1	Cannot be calculated (a discordant value is zero)	
Question 14	1.0000	No	0.000	1	2.000	0.104-117.994
Question 15	0.3711	No	0.800	1	0.250	0.005-2.526
Question 16	0.1306	No	2.286	1	6.000	0.728-275.986

6.2 COMMUNITY MUTATION FREQUENCY

At Study Visit One, samples were collected from 14 individuals from the extended family of the index patient. Twelve of these participants were adults, and two of them were children of adult participants. One participant consented to the study but declined to participate in sample collection and the associated testing. All participants were related to the index family through the matriarch of the index family except subjects AF004 and AF017, who were married into the family from the Amish community. Urine samples were collected from all participants, and blood spots on filter cards were collected from all participants except subject AF026, who declined to provide a sample for blood heteroplasmy level testing. Prior to sample collection, all participants took part in interviews, which began with a confirmation of willingness to consent. During this time, no participant who had previously consented declined to confirm consent.

High-resolution melting (HRM) profiling was completed twice (Trial 1 and Trial 2) by co-investigator Lina Ghaloul Gonzalez, MD. Trial 1 took place on all samples on November 19, 2014, and Trial 2 took place on all samples on November 20, 2014. These results were recorded in 20% increments, except for the 5% heteroplasmy level, which was the only dilution made in a 5% increment. In order to improve accuracy of the results, the testing was repeated in Trial 3 on December 19, 2014 using further dilutions of the control samples. Results of this testing are reported in Table 7. This allowed interpretation of results in approximately 10% increments, except for the 5% heteroplasmy level, which was the only dilution made in a 5% increment. Examples of results from HRM testing are shown in Figure 8.

Table 7. Results of Heteroplasmy Testing

Subject ID	Urine Heteroplasmy Trial 1	Blood Heteroplasmy Trial 1	Urine Heteroplasmy Trial 2	Blood Heteroplasmy Trial 2	Urine Heteroplasmy Trial 3	Blood Heteroplasmy Trial 3
AF001	40%	10%	40-60%	~10%	40%	10%
AF002	90-100%	~60%	~100%	40-60%	100%	~60%
AF003	Not Done	Not Done	80-100%	~20%	90%	~30%
AF004	0%	0%	~0%	~0%	0%*	0%
AF005	40-60%	10-20%	~40%	~10%	~20%*	~5%
AF007	N/A	N/A	N/A	N/A	N/A	N/A
AF008	Not Done	Not Done	~80%	~20%	~80%	~20-30%
AF009	>~80%	40-60%	60-80%	~40%	~70%	~40%
AF010	~60%	~20%	~80%	~20%	70-80%	20%
AF012	~70%	~20%	40-60%	~20%	~70%*	~30%
AF015	60-80%	~20%	80%	~20%	70%	~20%
AF017	Not Done	Not Done	0%	0%	0%*	0%
AF019	Not Done	Not Done	~100%	~80%	100%	~70%
AF025	~10%	~0%	~20%	0-5%*	~10%*	0-5%
AF026	Not Done	Not Done	~20%*	N/A	~20%*	N/A
AF027	Not Done	Not Done	90-100%!	80%	~90%	~70%

! = greater than 90%, and slightly less than 100%

* = value is slightly shifted from the expected laboratory value

"Not Done" = Sample not run in that trial; N/A = Sample did not provide adequate results for interpretation

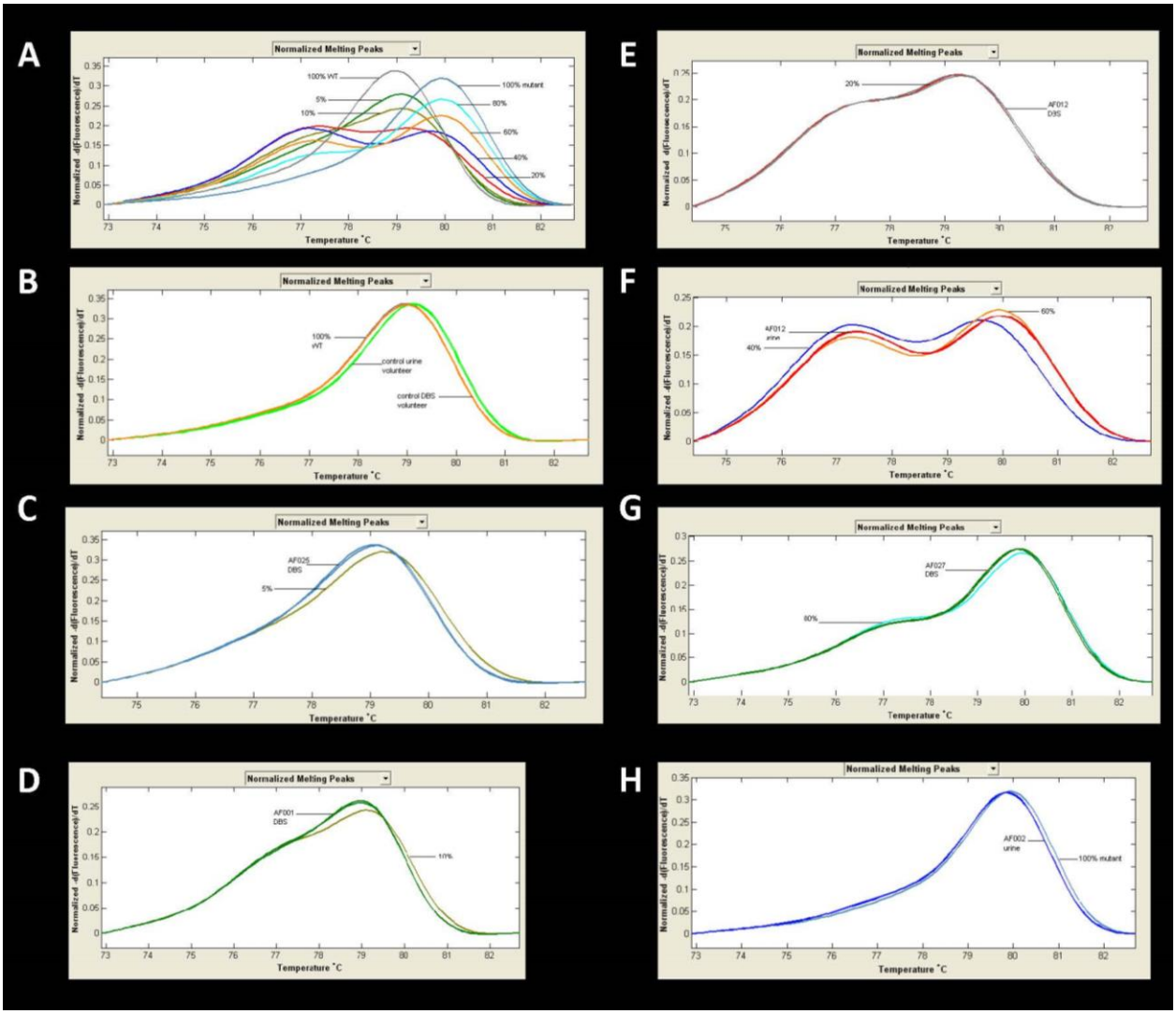

Figure 8. Selected HRM Profiles from Analysis by Lina Gonzalez, MD

A. Result of all controls from trial 2 including the wild-type sample and samples with heteroplasmy of 5%, 10%, 20%, 40%, 60%, 80%, and 100%

B. 100% wild-type control samples from volunteers

C. Results of subject AF025's trial 2 dried blood spot (DBS) HRM testing, approximately 5% heteroplasmy

D. Results of subject AF001's trial 2 DBS HRM testing, approximately 10% heteroplasmy

E. Results of subject AF012's trial 2 DBS HRM testing, approximately 20% heteroplasmy

F. Results of subject AF012's trial 2 urine HRM testing, approximately 40-60% heteroplasmy

G. Results of subject AF027's trial 2 DBS HRM testing, approximately 80% heteroplasmy

H. Results of subject AF002's trial 2 urine HRM testing, approximately 100% heteroplasmy

Table 8. Highest Detected Heteroplasmy Levels in Participants

Subject ID	Provided Sample?	Heteroplasmy Detected?	Highest Level of Heteroplasmy	Sample Tissue Type
AF001	Yes	Yes	40-60%	urine
AF002	Yes	Yes	100%	urine
AF003	Yes	Yes	90%	urine
AF004	Yes	No		
AF005	Yes	Yes	20%*	urine
AF007	No			
AF008	Yes	Yes	80%	urine
AF009	Yes	Yes	70%	urine
AF010	Yes	Yes	70-80%	urine
AF012	Yes	Yes	70%*	urine
AF015	Yes	Yes	70%	urine
AF017	Yes	No		
AF019	Yes	Yes	100%	urine
AF025	Yes	Yes	10%*	urine
AF026	Yes	Yes	20%*	urine
AF027	Yes	Yes	>90%	urine

* = value is slightly shifted from the expected laboratory value

The Study Visit two activities occurred on March 21, 2015. The study team met with participants to review the results of their MELAS heteroplasmy testing. Results were disclosed to some participants individually and to others in nuclear family groups as per their preference. Parents of both of the child participants had results disclosed to the parent to confirm understanding. The disclosure was made by investigators meeting in pairs with individuals or selected immediate family members. Before arriving, each group was assigned to disclose to specific participants to ensure that those with the highest levels of heteroplasmy would be speaking with a physician to assure that specific treatment and management guidelines for each of those individuals was appropriately communicated. All participants were provided with a copy of their individual testing results during their counseling session.

During the disclosure process, several of the women whose results identified heteroplasmy indicated interest in having their children tested. Two of the participants, AF005 and AF025, were each accompanied by a daughter for whom they provided consent for testing. These two participants also expressed great interest in pursuing testing for their other children, either during a home visit or at a planned clinic at Hermitage Hospital.

Participant AF008 was unable to attend study visit two. However, study staff will follow-up via mail and/or telephone to provide him with his test results and answer any questions he has about management and treatment guidelines.

For all study participants, a letter summarizing the genetic counseling/results disclosure session along with a copy of their test results will be mailed to them. An anonymized example of a letter sent to participant is shown in Appendix N. However, each letter was tailored to encompass all information discussed in the genetic counseling session.

6.3 EXTENDED FAMILY PEDIGREE

Information to construct pedigrees was collected during study visit one interviews. Each interview was conducted by an investigator with pedigree-taking experience including, the primary investigator, Jerry Vockley, MD, PhD and co-investigators, Catherine Walsh Vockley, MS, LCGC; Amy Goldstein, MD; and Afifa Irani, BS. Each interview was conducted with individuals or nuclear family members. All interviews began with confirmation of willingness to consent to study participation, during which no participant who had previously consented to the study declined to confirm consent. Additionally, it was noted that multiple members of the extended family who were present declined to participate in interviews and the study as a whole. Each

participant was questioned about his or her own health using the MELAS-related symptoms checklist (Appendix I), and asked to keep these symptoms in mind when providing information about other family members' health. Prior to the interviews, as a group, participants aided investigators in constructing a brief pedigree that provided information on the birth order of the index patient's maternal sibship (the children of AF010) in order to later combine all pedigrees constructed during interviews. While taking family history information, all data were recorded, but difficulties were encountered in how to report uncertainty.

Using Progeny 6.0 and Microsoft Office PowerPoint, a pedigree was constructed for the extended family related to the index patient through maternal lineage after returning from study visit one. This pedigree is not shown here. Rather, a smaller pedigree, shown in Figure 9 includes only the index patient's mother' sibship. In this pedigree, each member of the sibship is labeled with a letter. Additional pedigrees were constructed to provide information about each nuclear family and symptoms of mitochondrial disease, and these are shown in Figures 10-14. These pedigrees each have a letter in the upper left-hand corner that corresponds to the letters with which the siblings in Figure 9 are labeled.

Because the community historically limits outsiders' access to their community, any interaction with the extended community can only occur with permission of the community elders. These study activities were conducted with approval of a community Deacon. However, given time constraints of the study, the team was unable to extend the study activity permissions and interaction beyond the extended index family. This means that pedigree construction did not extend beyond the index family.

All MELAS-related clinical features reported in the family are recorded in the pedigree key in Figure 9. Health problems that are likely unrelated to MELAS are listed below each individual's pedigree symbol.

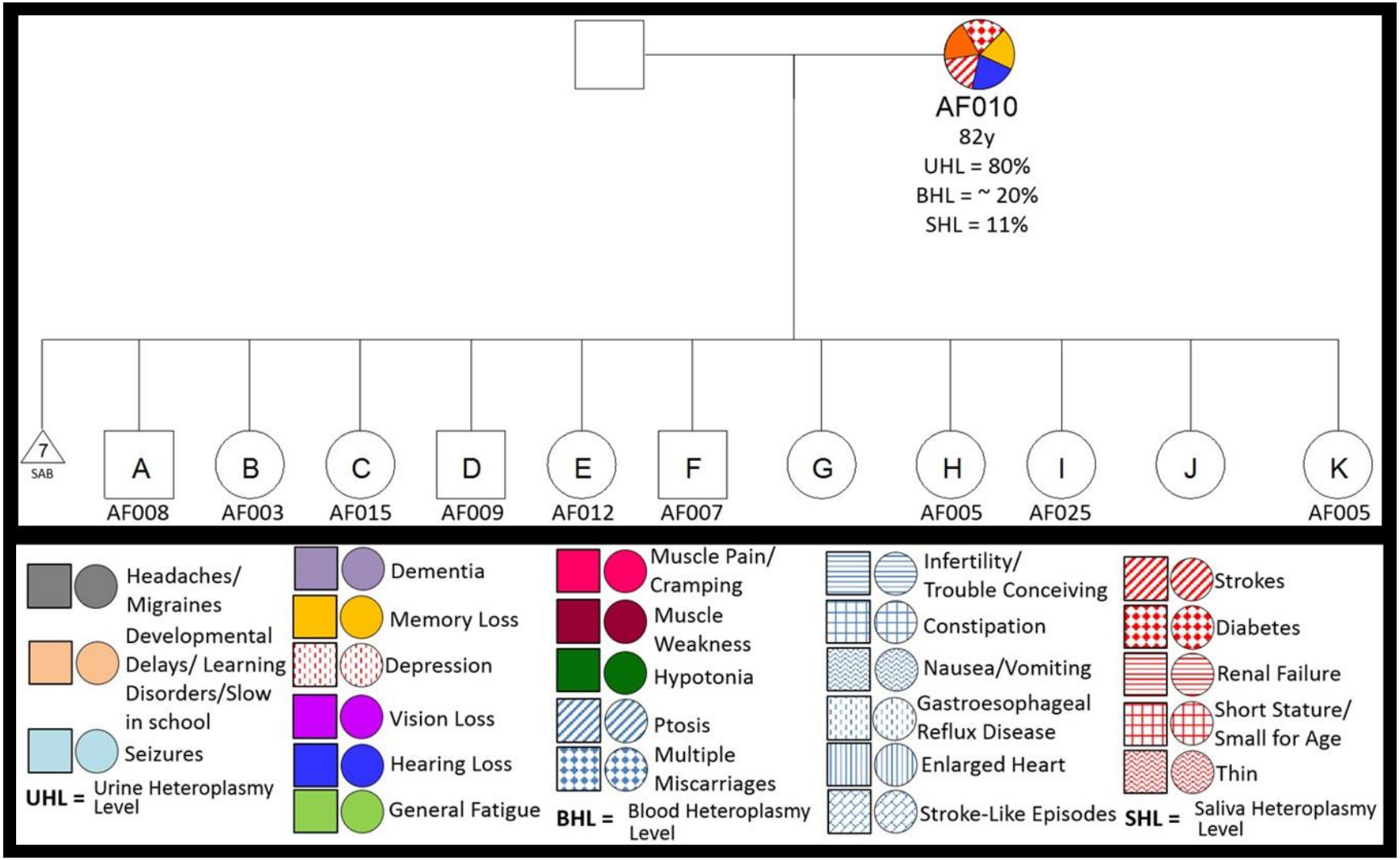

Figure 9. Extended Maternal Family of Index Patient and Pedigree Key

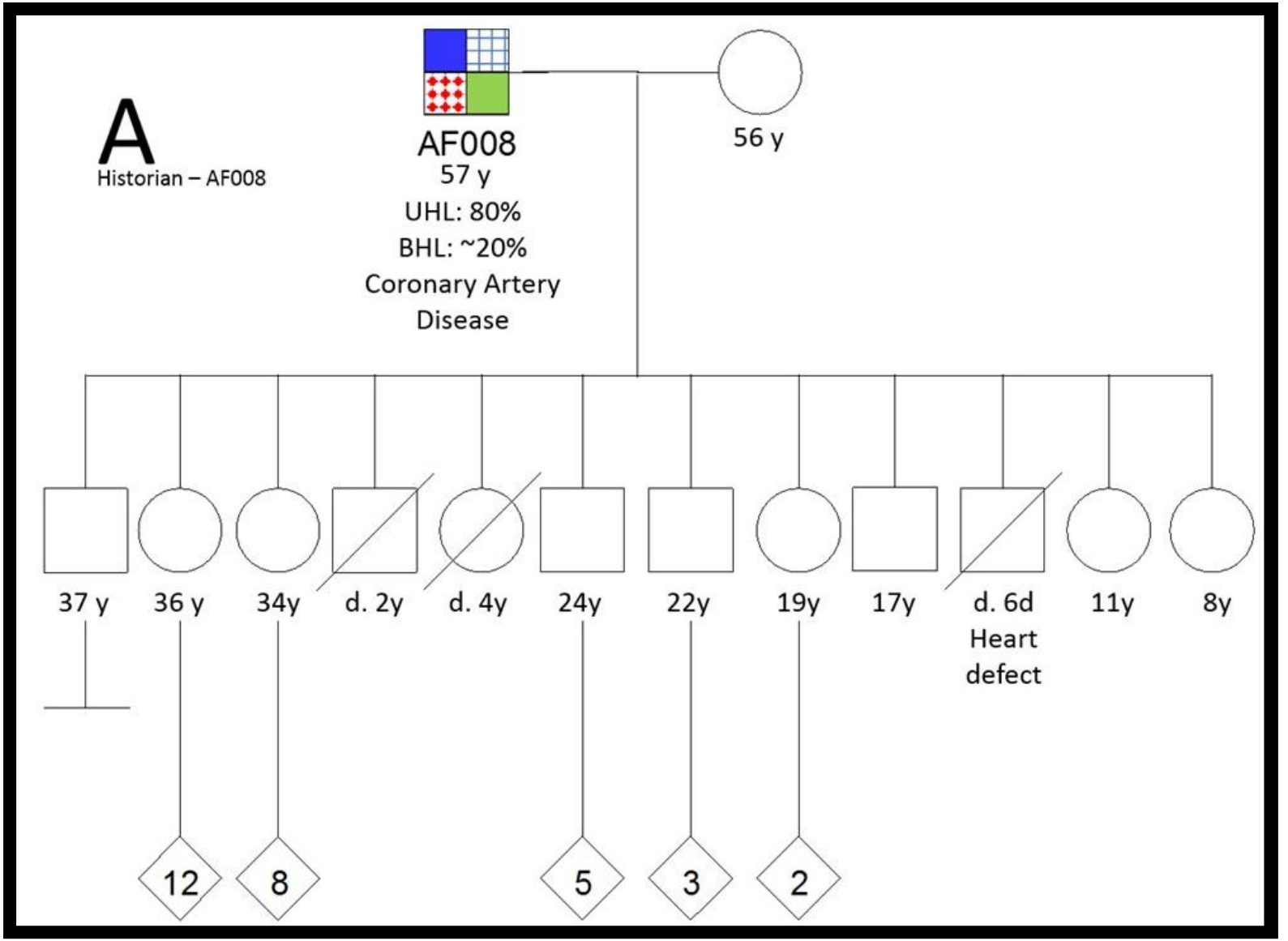

Figure 10. Family A

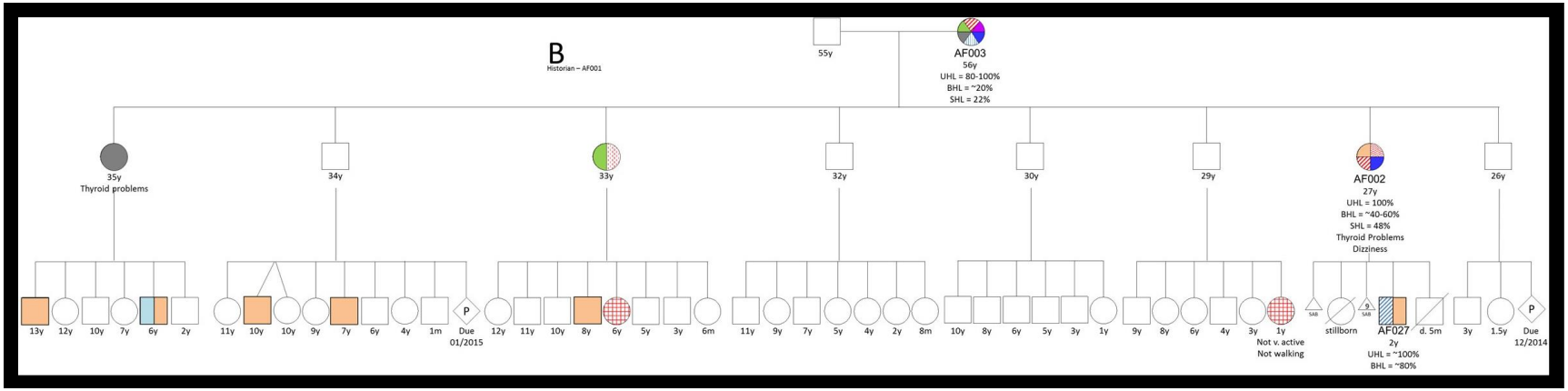

Figure 11. Family B

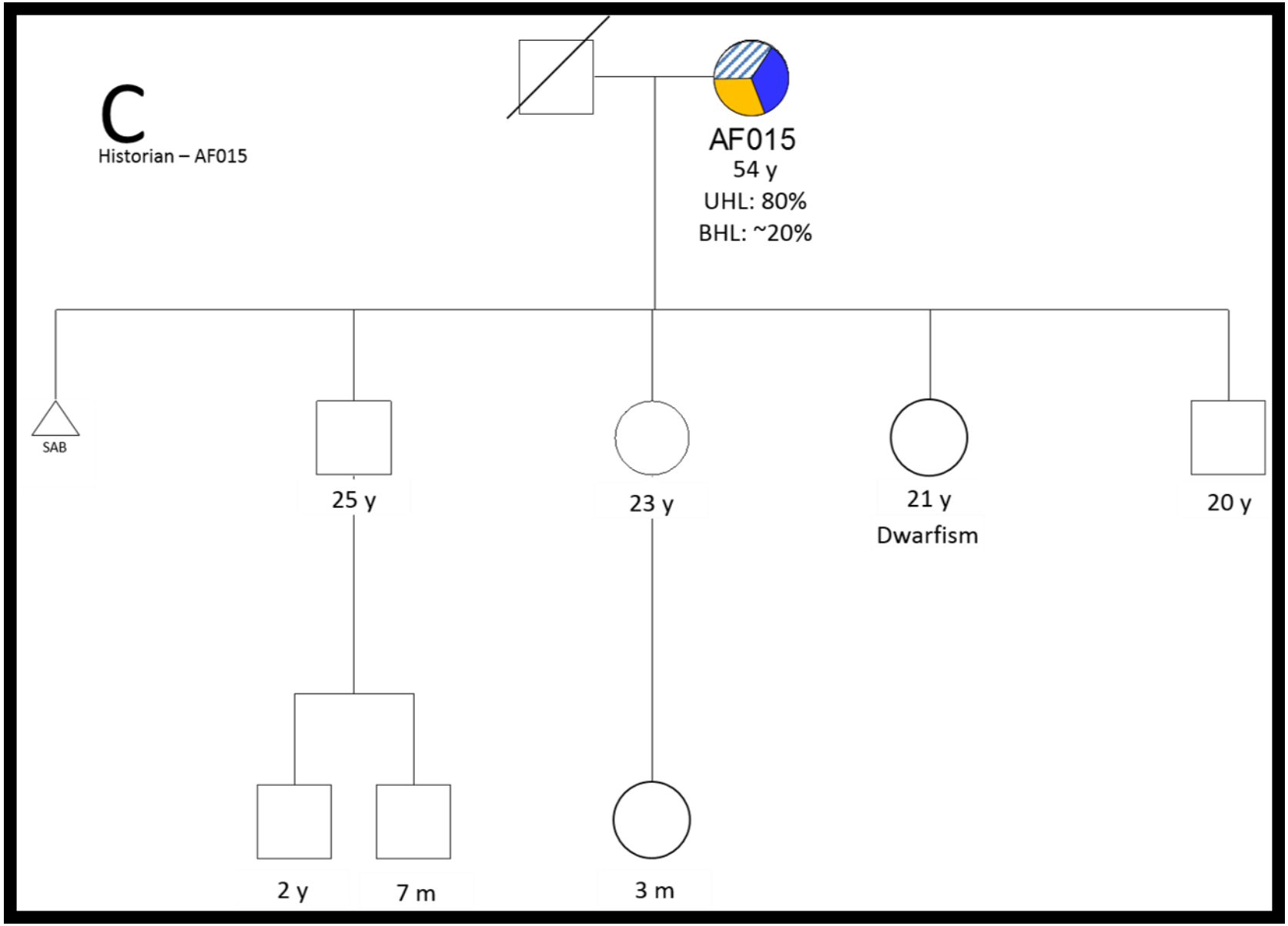

Figure 12. Family C

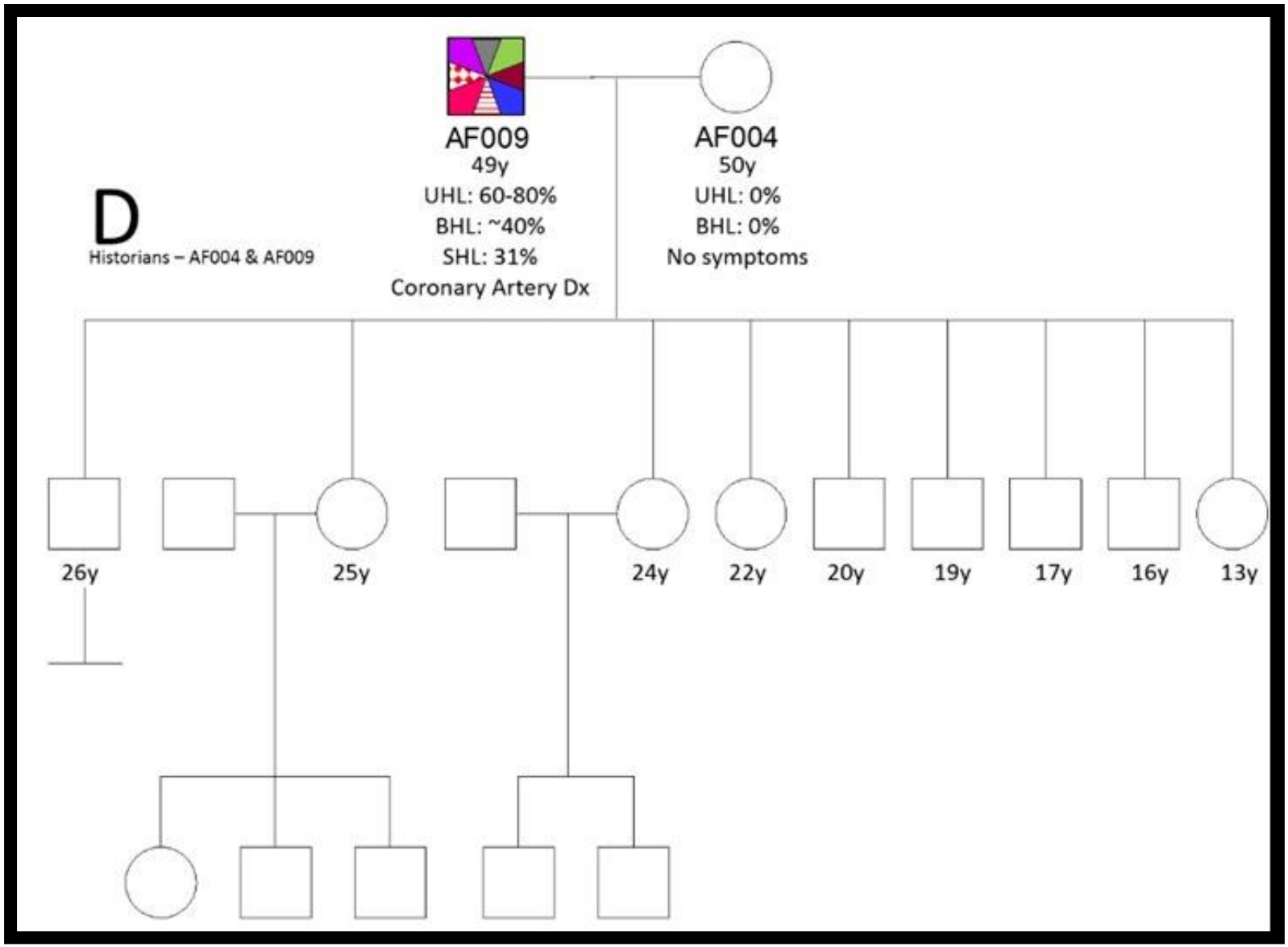

Figure 13. Family D

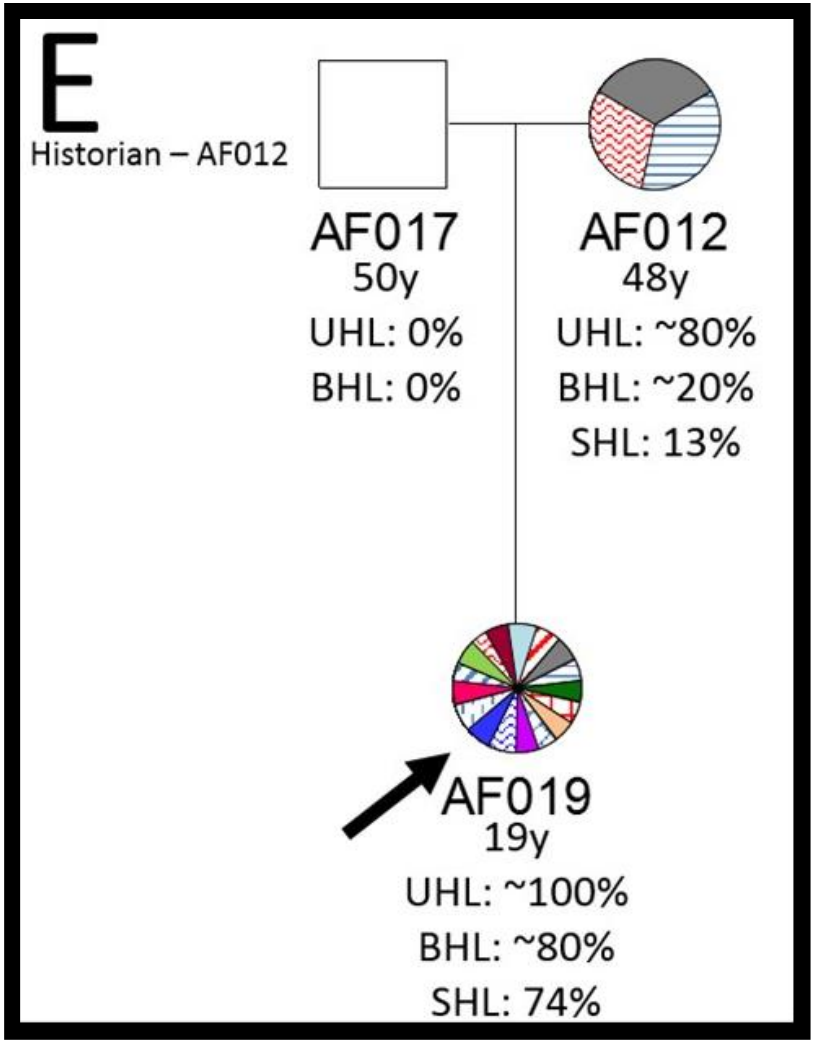

Figure 14. Family E

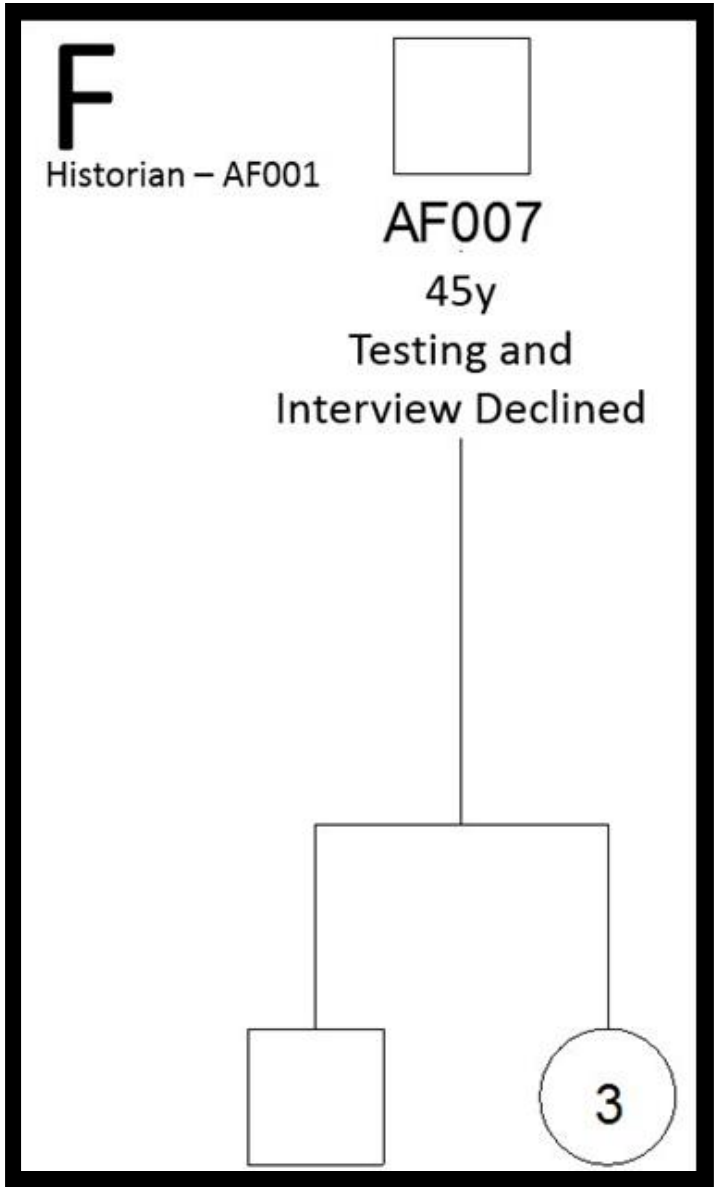

Figure 15. Family F

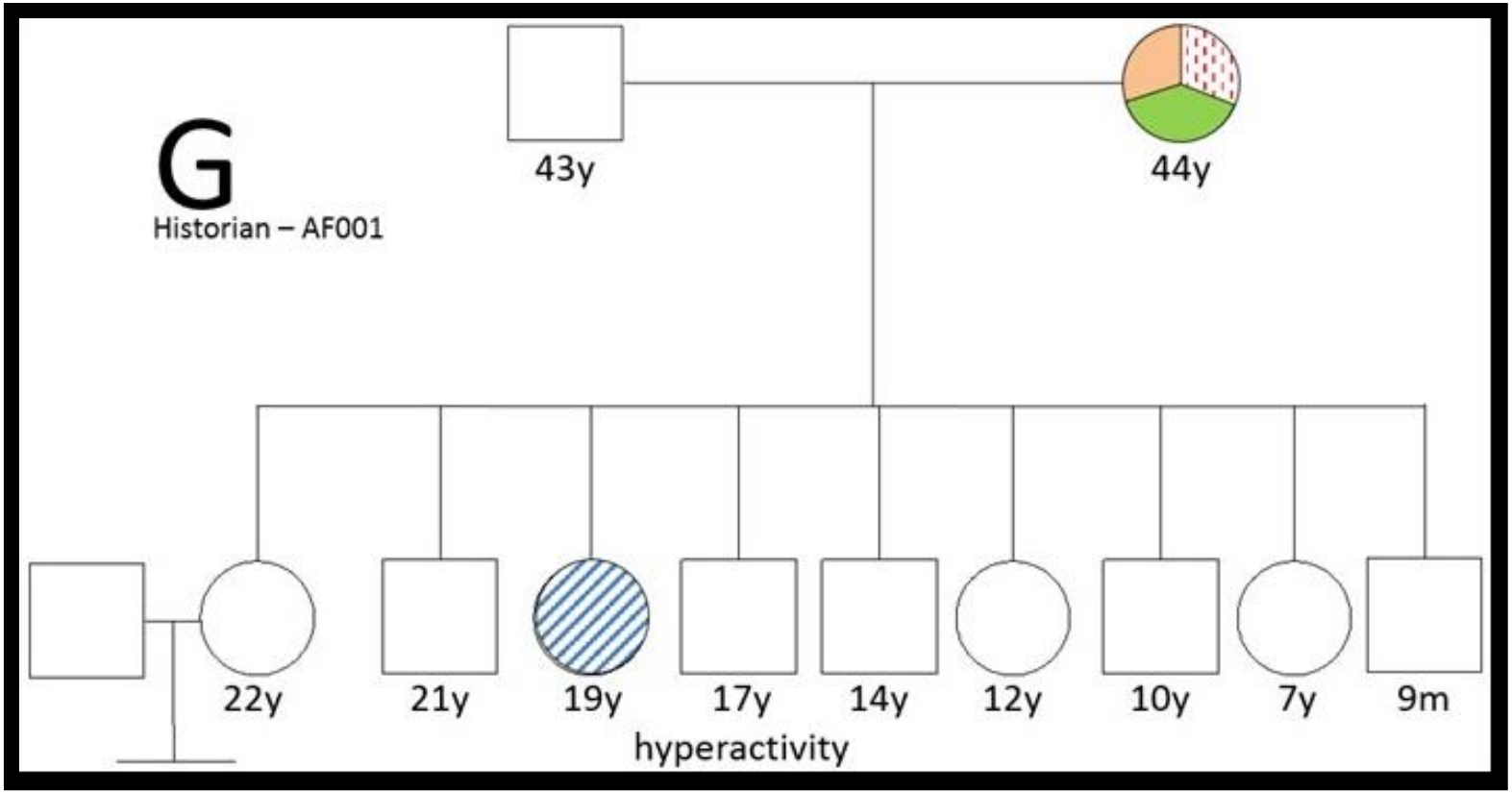

Figure 16. Family G

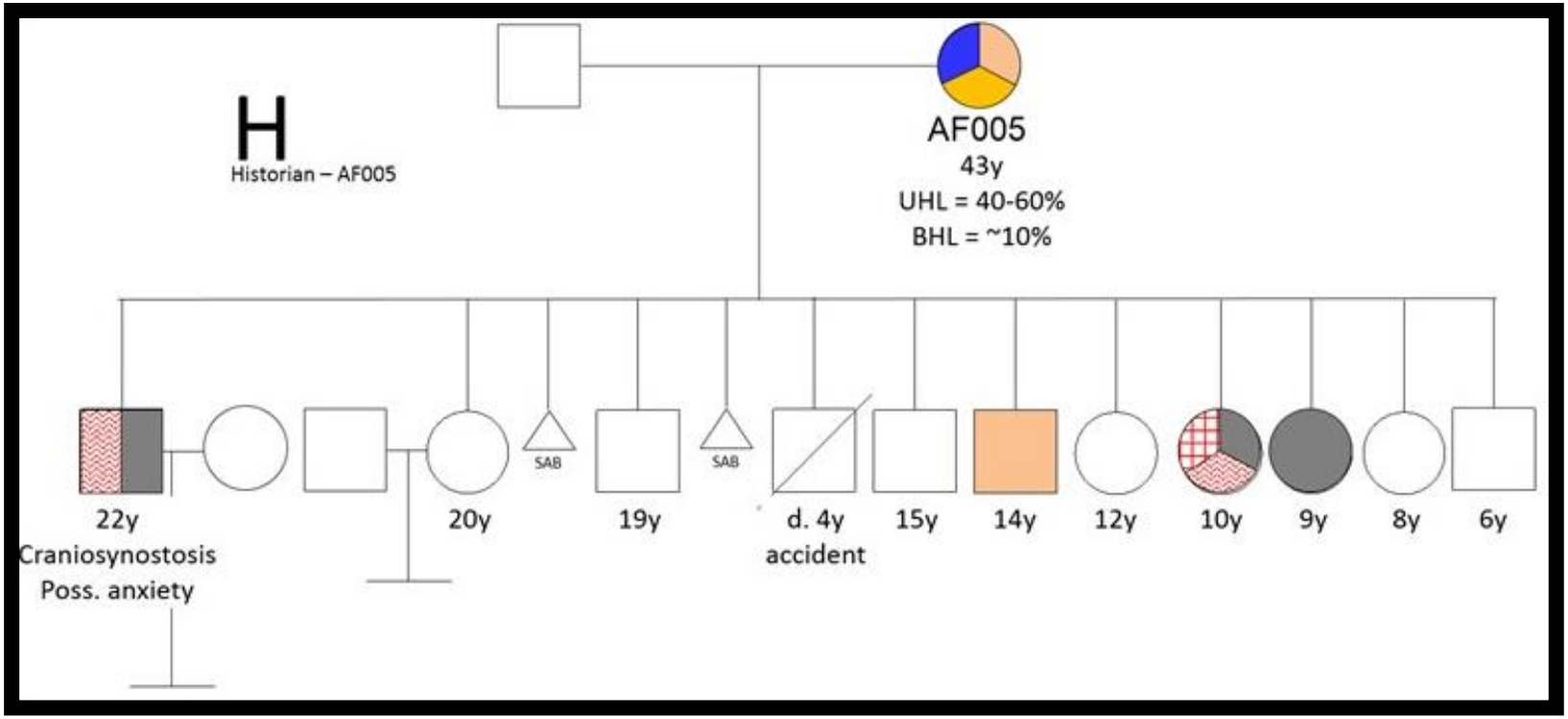

Figure 17. Family H

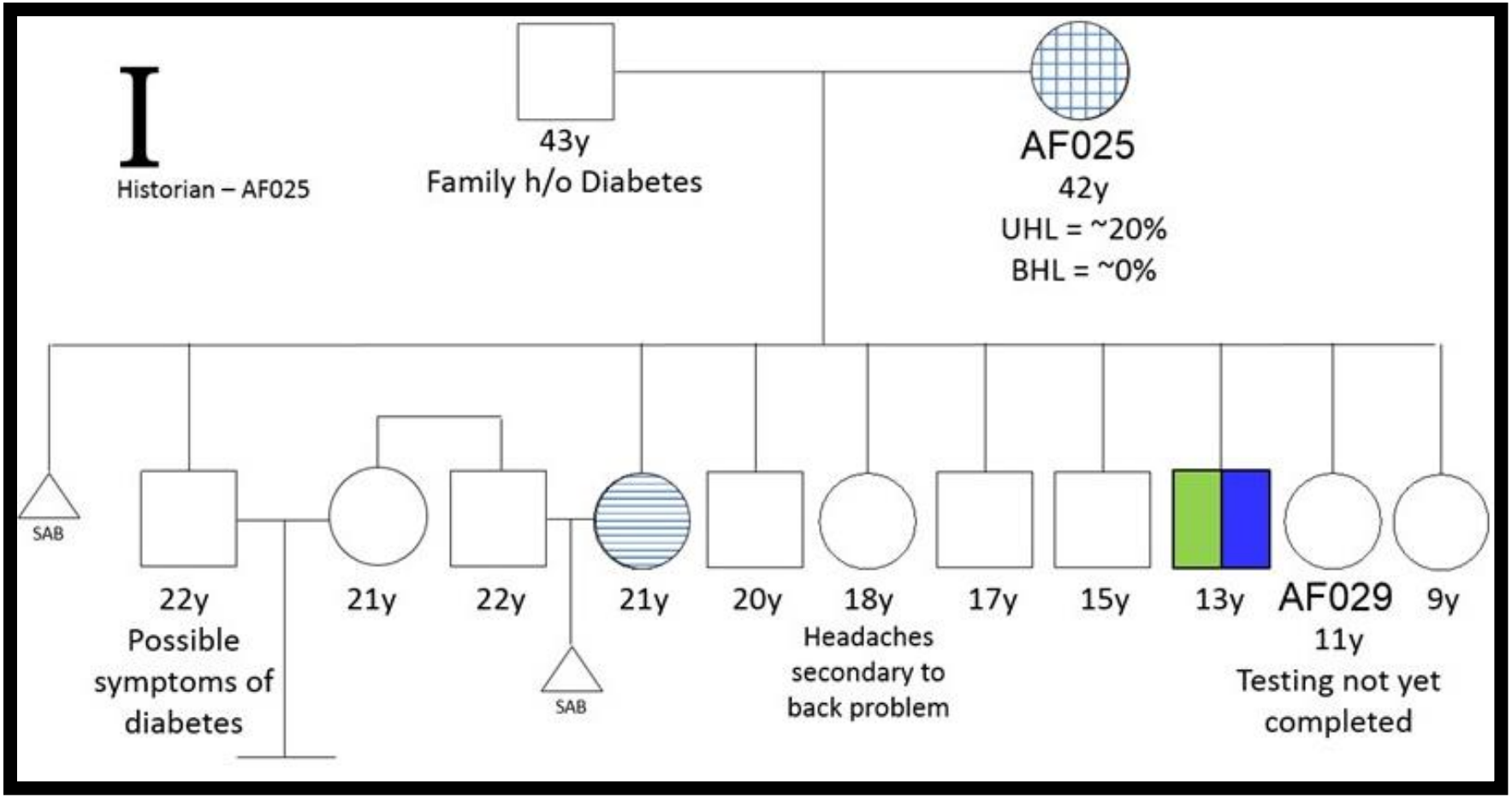

Figure 18. Family I

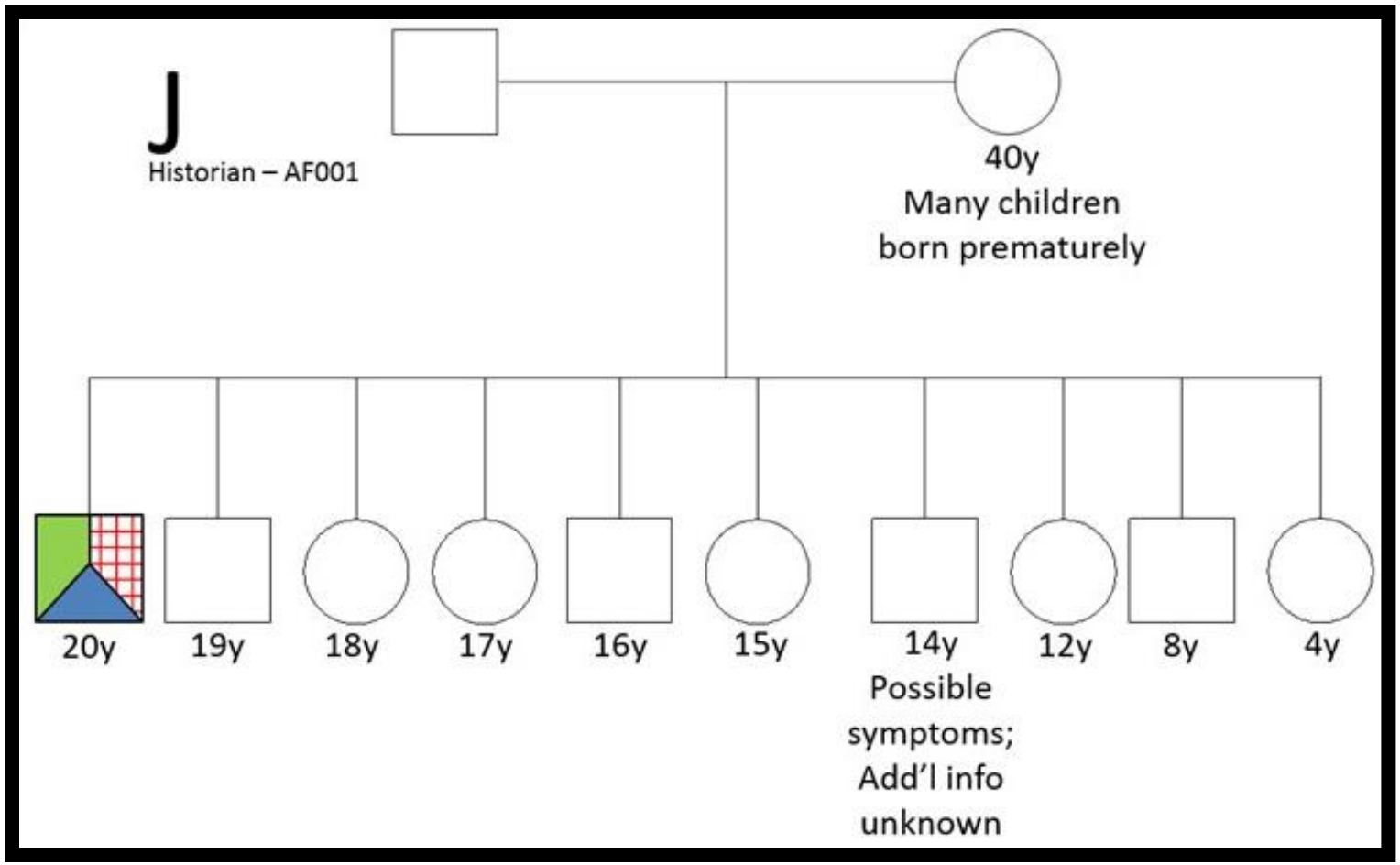

Figure 19. Family J

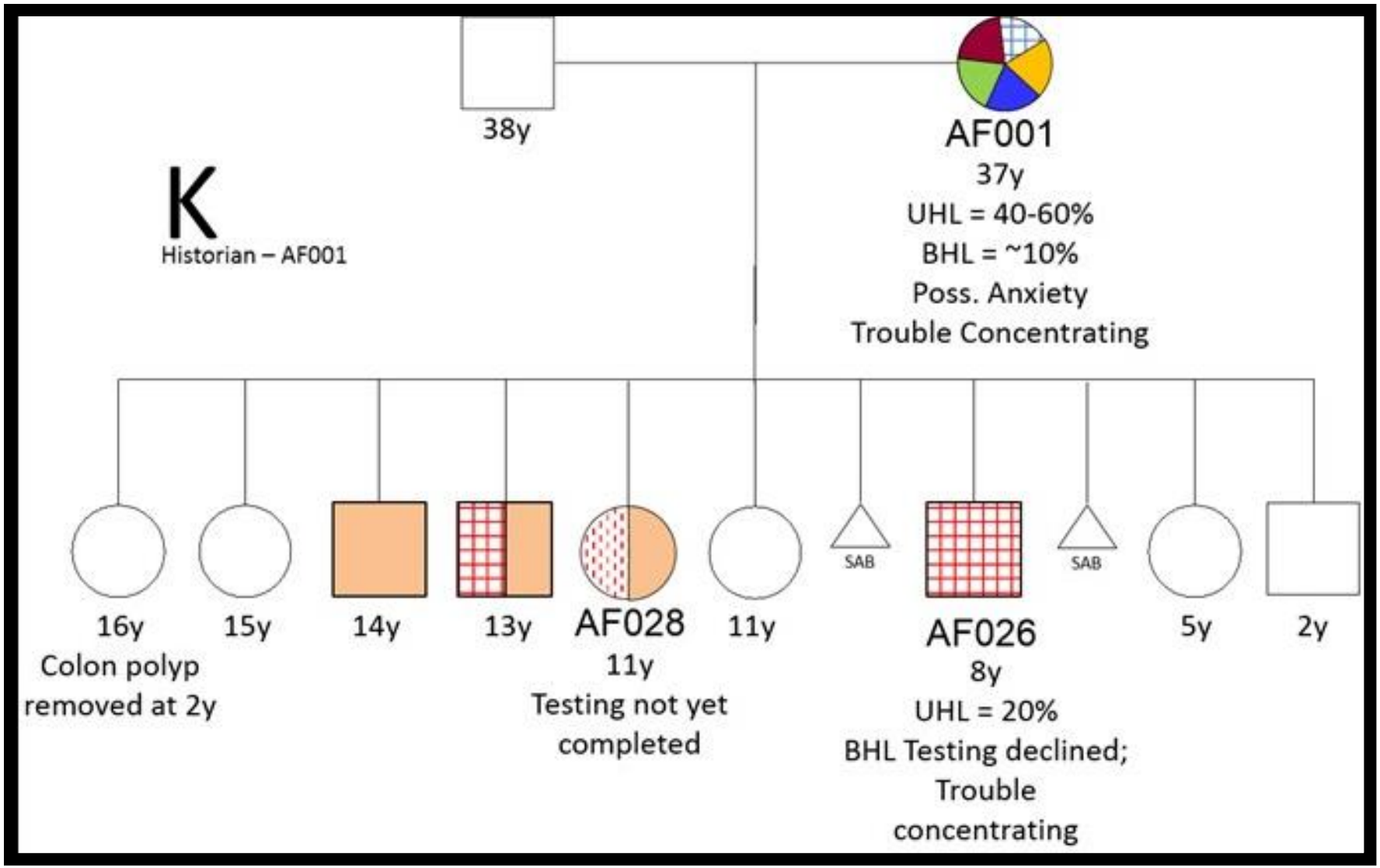

Figure 20. Family K

6.4 RESULTS DISCLOSURE VISIT

During Study Visit Two, or the results disclosure visit, multiple anecdotal interactions independently experienced by investigators qualitatively suggested that participants retained a substantial amount of information from the education intervention done during Study Visit One. The independent teams of investigators disclosing results each had the impression that information reviewed and communicated had been well-retained by the majority of participants with whom it was shared. One participant, AF005, even indicated that she had retained the educational intervention handout provided at the first study visit. Another participant, AF025 expressed clear understanding of maternal inheritance and an understanding of the implications of her results for treatment and early recognition of symptom development.

7.0 DISCUSSION

7.1 EDUCATIONAL INTERVENTION

A significant portion of study planning involved designing the educational intervention and associated questionnaires. Program planning was utilized to develop the concept of the educational intervention composed of both an oral and visual component. This concept was developed through the lens of genetic counseling, as clinical genetic counselors typically use visual aids along with an oral description of health education concepts for patients. Following the development of the educational intervention, a questionnaire was designed, keeping in mind the participant audience, to measure the efficacy of the educational intervention. A discussion of the educational intervention including discussion of the results of questionnaire responses, the format of the educational intervention, limitations and future directions of this part of the study, and an evaluation of this method compared to the genetic counseling that took place later in the study follows.

7.1.1 Questionnaire Results

The aim of the educational intervention was to provide members of an at-risk Amish community with information about mitochondrial disease in general and MELAS in particular. Questionnaires were used as a tool to measure whether or not this educational intervention was successful. The categories of answers within the questionnaire included the following: correct answers, incorrect answers, skipped answers, and "Uncertain" answers. These categories were compared to one

another using proportions in order to negate the biasing impact of the skipped questions on the statistical analysis. These proportions were compared in the pre- and post-educational intervention questionnaires to determine if there was a statistically significant difference in the answers before and after the educational intervention. A major limitation of this evaluation of statistical significance was the small number of participants, which resulted in large standard errors (SEs). This ultimately resulted in a lack of statistically significant differences among answers before and after the educational intervention. However, upon further evaluation, trends were identified that would allow for more qualitative comment on the educational intervention's efficacy. While the questionnaires did not provide statistically significant evidence of increased understanding of MELAS and mitochondrial disease, upon returning to the community to disclose results of testing, participants anecdotally appeared to demonstrate clear understanding of the basic concepts relayed during the educational intervention. This was not confirmed through quantifiable evidence, however, investigators thought this to be true based on the interactions between participants and disclosing investigators at study visit two.

7.1.1.1 Analysis of Key Questions

Due to use of McNemar's Test for statistical analysis, the consulting statistician noted that populating the post-education questionnaires with the answers from the pre-education questionnaire, as was requested by two study participants, would diminish the integrity of the statistical analysis. Therefore, the questionnaires labelled Q003 and Q007 were removed from inclusion in data analysis. Key questions that investigators thought best represented understanding of the underlying concepts being queried in each section of the questionnaire were selected prior to analysis of the questionnaires. These key questions for each section are shown in Table 3. For section one, this was the concept of basic genetics; for section two, this was the concept of

autosomal recessive inheritance. For section three, this was the concept of mitochondrial inheritance. In section four, each of the four key questions targeted a different concept related to MELAS. Question 13 focused on maternal inheritance; question 14 focused on delayed onset of symptoms; question 15 dealt with identification of symptoms related to MELAS, and question 16 pertained to targeted clinical variability and the bottleneck effect as they are related to maternal inheritance.

This educational intervention was designed with the presumption that there was some pre-existing participant understanding of basic genetics including autosomal recessive inheritance. It was thought that the increased prevalence of genetic disorders in the Amish community would have predisposed this audience to exposure to information about basic genetics. Question number one was included to provide an assessment of this presumption. However, inadequate responses to the question limited analysis of this presumption. When analyzing this question, the McNemar's Test calculation could not be completed because there was no change in the number of respondents who answered correctly from the pre-education questionnaire to the post-education questionnaire, which means there was more than one discordant value equal to zero. While this is not statistically significant data from which conclusions can be drawn, it is possible that the lack of difference in respondent data is due in combination to the expectation that there was an underlying foundational understanding of basic genetics, and the educational intervention did not aim to provide a better understanding of basic genetics. Rather, the intervention was designed to utilize an existing foundational understanding of basic genetics, and this expectation could be supported by the lack of difference in respondent data.

Analysis of the key question from section two about autosomal recessive inheritance showed no difference between the proportion of correct answers in the pre-education questionnaire

responses and the proportion of post-education questionnaire responses. However, more detailed evaluation of the responses to this question provided valuable insight. Inspection of the pre-educational intervention questionnaires shows that the number of participants who were familiar with autosomal recessive inheritance by name was lower than expected. This may indicate that an educational intervention designed to explain maternal inheritance without using autosomal recessive inheritance as a foundation may prove more successful for members of this population. However, it should also be noted that through interaction during the oral presentation of the educational intervention, participants demonstrated an understanding of the risks of having a child with an autosomal recessive condition to a couple who previously had an affected child as being 25%, which supported a prior understanding of this inheritance pattern. Additionally, participants verbally confirmed understanding of this inheritance pattern but commented that they had not heard of it referred to as being "autosomal recessive." Perhaps because of this, the use of autosomal recessive inheritance as a foundation may have been more effective if it was discussed using terminology with which participants were familiar.

The third section of the questionnaire primarily focused on mitochondrial disease as a class of disorders. As with the results of the key question from section two, the responses to the key question from section three showed there was no difference between the proportions of correct answers in the pre-education questionnaire responses and the post-education questionnaire responses. More detailed inspection of responses to this question does not provide clarity about trending.

The fourth section of the questionnaire specifically discussed MELAS. As with the results of the key question from sections two and three, the responses to the key question from section four showed there was no difference between the proportions of correct answers in the pre-

education questionnaire responses and the post-education questionnaire responses. More detailed inspection of responses to this particular question does not provide clarity about trending. However, when using the information in Figure 10 to compare the proportions of correct answers in the entire section in the pre-education questionnaire responses and the post-education questionnaire responses, there is an increase in the proportion of correct answers after the educational intervention. Using the information in Table 5, which shows the number of correct answers for the key questions in section four, it is also evident that an increased number of correct answers was seen for all of the section four key questions in the post-educational intervention questionnaire as opposed to the pre-educational intervention one. This is, again, not a statistically significant increase, likely due to the small sample size, but it does help to identify a trend in the responses. One possible explanation for the higher number of correct answers on post-education questionnaires may be that the respondents found the MELAS component of the educational intervention more meaningful, and therefore, this information was better-retained.[61] Further, the index patient's family had reached out to maternal relatives and had spoken to them about the diagnosis of MELAS in their family, which means that the participants may have had more interest in information about MELAS in particular.

It was also noticed that there was a higher number of correct answers to the key questions about basic maternal inheritance in the pre-education questionnaires than the other key questions. While not a statistically significant difference, one possible explanation for this is that the respondents were a biased population regarding that question. As most participants were related through the maternal lineage to the index patient, their presence indicated a general understanding that maternal relationships are involved in the inheritance of mitochondrial disorders.

7.1.2 Format of Educational Intervention

When designing the educational intervention, investigators decided the best presentation method would be to use a printed, visual handout paired with verbal presentation. This approach was based on characteristics of the target audience. These primarily included community characteristics, such as lack of access and exposure to electronics and limited schooling and thus a lower literacy rate, particularly scientific and genetic literacy.

It has been reported that acute comprehension problems arise when individuals with a history of low literacy are presented with medical or health-related information.[62; 63] The handout component of the educational intervention was designed to serve as a pictorial representation of the information being presented orally in order to overcome this potential barrier posed by the lower literacy and scientific education levels of this isolated Amish community. An example of steps taken to provide effective visual communication includes the use of color-coding, which has been shown to improve processing and integration of presented information.[64; 65] Several of the diagrams in the handout used color-coding to emphasize different concepts. For example, making the mutant mtDNA red throughout allowed for consistency of reference and understanding throughout the presentation. Additionally, color-coding was also utilized to more basically describe features associated with encephalomyopathy. The verbal delivery of the majority of the information in the intervention meant that anyone with a lower literacy level did not have to depend on their reading skills to follow the presentation. The verbal presentation also allowed for interaction between the participants and the presenter. The participant feedback that resulted from this interaction led to additional review of concepts deemed difficult by the community in order to ensure participant understanding of the information.

7.1.3 Benefits, Limitations, and Future Modifications

Working with an Amish community to achieve study aims has proven beneficial in several ways. While participating in research may sometimes be a burden, this community was very welcoming to the study team. The index patient's family provided access to their home for both study visit meetings. The team was graciously welcomed and shared in traditional Amish hospitality. Additionally, through investigator interactions with participants, it was clear that members of the extended family cared for one another and understood the implications of MELAS testing not just for themselves but also for their immediate and extended family members. Working with an Amish community also had benefits that were directly related to study outcomes. Participants in this community are generally knowledgeable about family and medical history information pertaining to themselves, their children, and more extended family relations, thus enriching the pedigree construction in aim two. Before interviews, participants were also able to aid in construction of a broader pedigree to more clearly elucidate family relationships for investigators. Development of the educational intervention was challenging as the team recognized the typical limits to schooling in this community and limited exposure to advanced technology. However, this was ultimately beneficial in many ways. Moving forward, the educational intervention and associated questionnaires are on track to being useful in the general population due to being limited to an eighth grade reading level. They can also be more easily adapted to other languages and formats because of the simplicity of design and limited number of words.

As discussed above, members of the Amish community have limited schooling, with most community members not participating in formal education beyond the eighth grade, and limited exposure to biology and other sciences. The presentation handout was designed to address these factors. However, upon providing each participant with his or her own copy of this handout, it

seemed that the educational goals of the study could have been more successfully achieved through a different method. During the presentation, rather than following along with their own handouts, participants instead focused their attention on the presenter's handout. It is possible that having their own copy was cumbersome or distracting rather than beneficial for some. It did appear that when attention was focused on a specific small detail on the handout , participants seemed to turn to their own handouts more often. However, this need to focus also resulted in flipping of pages to find the correct figure. Based on this observation, one possible alternative way to implement the use of the handout would be to use one or two large, poster-sized copies of the handout as a single visual aid rather than providing individual copies to each participant. This might allow participants to focus more on the information being presented and reduce the need to look at smaller details up close because of the increased size of the presenter copy of the handout. Further, a personal copy of the handout could then be provided for reference to each participant at the end of the educational session.

An additional barrier to understanding for some participants was their underlying medical condition. Some individuals suffered from significant hearing loss, which led to an inability to follow along with verbal component of the presentation. At least one participant had loss of cognitive function due to strokes and chronic illness, which limited the ability to understand both the handout and the verbal presentation. Other ailments, including poor vision, resulted in trouble utilizing the visual aids.

This educational intervention was shared with physicians, genetic counselors, genetic counseling students, lay-people, and other healthcare professionals. All these people provided feedback on the efficacy of the intervention, and modifications were made accordingly to both the handout and the verbal component of the intervention. Due to time constraints and concern for

biasing the potential study population, the intervention was not presented to anyone from the study population. However, given the Study Visit One experience with the educational intervention in the study population, this educational intervention could have benefitted from being presented to a group of Amish community members unfamiliar with mitochondrial disease prior to being finalized. This would have provided additional insight and input that could not be garnered through sharing the presentation with "English" individuals. As previously noted, it was thought by investigators that this community would be familiar with autosomal recessive genetic conditions and the autosomal recessive inheritance pattern. In actuality, based on both the results of the questionnaires and on interactions with participants during the educational intervention, it seems the participants with whom the study team primarily communicated may not have had significant exposure to these conditions and at the least, had not heard of them referred to as such. As previously discussed, including a discussion of autosomal recessive inheritance was meant to provide a framework for thinking about inheritance in general and a lead-in to explaining the maternal inheritance pattern of MELAS. However, as the analysis of responses to the pre-education questionnaire shows, as presented, this was likely not an effective foundation in this community for thinking about inheritance patterns and understanding maternal inheritance. While current study data does not provide evidence for or against utilization of this method, based on feedback from the participations, re-designing the intervention so that it focuses solely on maternal inheritance before re-implementing this educational intervention may prove beneficial.

While no statistically significant data resulted from the analysis of the questionnaire data, there were data trends that suggested improvements in understanding about MELAS after the educational intervention. However, throughout the data analysis, it was evident that additional questionnaire piloting would have increased the utility of this questionnaire as a determinant of

the efficacy of this educational intervention. While piloting was completed, no one from an Amish community was given the opportunity to participate in this activity. This limitation is similar to what was encountered with the educational intervention. Given the nature of the relationship between the study team and the community, it was thought that obtaining substantial feedback on either the questionnaire or the educational intervention was not a realistic option due to limited contact with community members other than the index family. Potential for biasing study participants was also a concern. In retrospect, it may have been beneficial to approach someone from the community but outside the index family to obtain feedback regarding the questionnaire prior to its administration.

7.1.4 Evaluating Increased Understanding of Educational Intervention through Genetic Counseling

There were two different types of interactions during which information about MELAS was presented to study participants: an educational intervention and a genetic counseling session. Comparisons between these two roles, first as a presenter providing education to a group and second as a provider of genetic counseling to one participant or nuclear family at a time, are helpful when thinking about approaches to patient education. The genetic counseling session provided a better platform for gauging participant understanding, which allowed for adjustments in counseling style, word choice, and use of visual aids. This was more difficult to achieve in a larger group setting because gauging the individual responses from several people at once posed a different set of challenges than doing so one-on-one. One possible reason for it being more challenging to assess participant needs in the group setting is that participants may have been more willing to ask specific questions that pertained to themselves or their own personal understanding

in the one-on-one session when they felt the information would be kept private and confidential. An additional possibility is that participants did not have time to process the information from the educational session immediately following the educational intervention, and may have used the handout and discussion among group members in the interim to provide clarification or additional insight into the information. Ultimately, there is no way to determine if the increased understanding observed anecdotally by investigators during study visit two was a result of the educational intervention, communication among participants, a more individualized genetic counseling session, or a combination of these and other factors. Fortunately, during study visit two, it seemed to investigators that participants left the genetic counseling sessions with an understanding of what their test result meant for themselves and their families.

7.2 ONE-ON-ONE INTERVIEW SESSIONS

The information collected from the one-on-one interviews was used to construct a detailed extended family pedigree. This pedigree depicts all family relationships and clinical features present in these family members, as reported by participants. Information about family members who were discussed by more than one of the participants was confirmed as being the same from both sources before being added to the pedigree. Overlap of information, however, was an uncommon occurrence.

7.2.1 Interview Session Format, Limitations, and Future Modifications

In each interview, the participant was asked a series of questions about his or her health and was then questioned about the health of his or her family members, keeping in mind the same symptoms about which they were questioned. This is standard practice when taking a targeted pedigree, and all interviewers had previous experience taking targeted pedigrees.[58] In general, participants were well-informed about the health histories of their immediate family members. However, when participants were answering questions about members of their extended family including cousins, nieces, nephews, and grandchildren, they sometimes were uncertain about exactly which relatives had experienced which symptoms. During these times when participants were providing information of which they were unsure, there was no way for interviewers to quantify the level of certainty of all information provided or a previously-defined method of including information about which participants were unsure. In cases where the participant had expressed uncertainty, it was left to the discretion of the interviewer to determine whether or not to record that information, and if so, how to express the lower level of certainty associated with only that piece of information. There are several possible outcomes of this uncertainty. One outcome may have been under-recording of clinical features found in the community. Another may be that it could have resulted in investigators excluding information about which participants were unsure. Retrospectively, an attempt was made to clarify any questionable information in the pedigree with the investigator who recorded the information. Attempts were also made to clarify any uncertain medical information at the second study visit. To avoid this concern in the future, it would be useful to adjust all forms used by investigators when interviewing participants to allow for a space to include a lower confidence level in a particular piece of information provided to the investigator. Finally,

including a predetermined way to record this information into the pedigree may provide clarity to investigators when evaluating for trends in the participant population as a whole.

7.3 MUTATION FREQUENCY AND CLINICAL FEATURES IN THE COMMUNITY

The HRM profiling method was selected for analysis of heteroplasmy in this population due to its cost-efficiency, rapid turnaround time, and proven ability to detect the mutation of interest. It had also been demonstrated in previous work that the HRM profiling method could detect mtDNA heteroplasmy for the m.3243A>G mutation as low as 5%, which was desirable as investigators were unsure of the clinical presentation and the expected heteroplasmy levels for participants. For those participants' samples that returned negative results using this testing, but in whom investigators felt there may be low levels of heteroplasmy due to relationships with affected or mutation-carrying individuals, additional testing could be performed that would detect heteroplasmy levels as low as 1%. However, this was not necessary in this study as all individuals in whom investigators expected to detect heteroplasmy had levels greater than 5%. The only two participants in whom heteroplasmy was not detected using this method were related to the extended family through marriage, and were thus not expected to have heteroplasmy unless there were additional kindreds with MELAS in the community. This is notable with regard to the larger aim of ascertaining frequencies of the common MELAS mutation in the general northwestern Pennsylvania Amish community. However, due to limitations to access of the community and the small sample size of participants for the study, it is not possible to utilize data from this study to determine the frequency of this mutation in the community. Rather, this work will require more

extensive community engagement, which may follow after a more long-standing relationship between the community and investigators has been established. One gratifying outcome of this project has been the establishment of an Amish Genetics Clinic in Mercer County sponsored by the Children's Hospital of Pittsburgh.

The pedigrees portray the array of clinical features with which members of this family are affected. As is consistent with a maternal inheritance pattern, all participants who were related through the maternal lineage to the index patient had some detectable level of heteroplasmy, but as is shown in the pedigrees, the clinical presentation of this mutation varies dramatically in this community. The reported clinical features in this community correlate with previously published features of MELAS and general mitochondrial disease.[15; 16] As expected, individuals with higher levels of heteroplasmy had more symptoms of MELAS. For example, the index patient, AF019 experienced the most symptoms of any of the family members, and she had a urine heteroplasmy level of approximately 100%. Other family members with lower levels of heteroplasmy, such as AF025, showed fewer or no symptoms. Also as expected, it was noted that individuals with intermediate levels of heteroplasmy, in the 40-70% range, had dramatic variability in symptoms. Some individuals in this range, such as AF012 had fewer features of the condition, while others, such as AF009 had several symptoms of MELAS. One potential underlying cause of this clinical variability may be the different levels of heteroplasmy in different tissues. For example, a seemingly less affected individual may have higher levels of heteroplasmy in tissues that do not require significant amounts of energy to be produced, such as the skin, which may result in the individual not appearing to have many symptoms. If, however, an individual has high levels of heteroplasmy in a tissue with high energy demands, such as the brain or muscles, this individual might display more classic symptoms of MELAS. During interviews, investigators questioned

participants about their healthcare as related to symptoms of MELAS. Few participants sought medical care for any of the symptoms, and for those who did, records could not be reviewed due to participants generally not having enough information to complete a release for medical information form. Because investigators did not perform physical exams and because, largely, participants did not have significant prior screening or imaging for many features of MELAS, such as cardiac or neurological imaging, investigators are not able to definitively rule out the potential presence of some clinical features in all participants. Additional clinical follow-up is planned.

7.3.1 Heteroplasmy Testing in Different Sample Types

For this study, samples collected included blood spots and urine samples from all but one participant, who declined to have blood drawn. This allowed for comparison between the results of testing in the two sample types. Due to the logistical difficulty of collecting a third sample type, cheek mucosal samples were excluded from this study, although inclusion of that sample would have provided additional data for comparison of efficacy of mutation detection in different tissues. All cases showed higher heteroplasmy in DNA derived from urine samples than in DNA derived from blood samples (Table 8). As previously noted, the mutational load in blood leukocytes is depleted over time. The majority of participants were adults, giving ample time for that depletion to have occurred. This is supported by data from analysis of the children's samples, as analysis of both their blood and urine sample heteroplasmy levels provided similar results to one another. These results are consistent with previous findings that compared the heteroplasmy levels in different sample types.[19; 20]

7.4 PUBLIC HEALTH IMPLICATIONS

The prevalence of mitochondrial disease is estimated to be approximately 1 in 5,000 individuals.[48] However, mitochondrial conditions are often difficult to recognize and diagnose. In order to make a diagnosis, clinicians must first be able to categorize the patient's phenotype as falling within the ever-increasing spectrum of recognized symptoms associated with mitochondrial disease, then they must navigate the variety of diagnostic laboratory tests available to provide sufficient evidence to diagnose the etiology of these symptoms.[66] The novel diagnosis of mitochondrial disease in an Amish community by physicians at the Children's Hospital of Pittsburgh of UPMC is anecdotal support of this concept. It was not until a patient had a severe complication of the condition, a stroke, and thus was referred to a tertiary care center for management and evaluation, that the constellation of symptoms in the patient and family were recognized as potentially due to a mitochondrial disorder and diagnostic testing was done. As demonstrated through this extended pedigree, many family members are affected with individual or less severe symptoms that were not previously recognized as being associated with mitochondrial disease; they were considered to be isolated findings by the patients' health care providers. More specifically, in this Amish family, 13 of 15 tested family members, and in fact 13 of 13 blood relatives, all except five of whom were previously not known to have mitochondrial disease were found to have MELAS and can now seek medical intervention. MELAS was clearly under-recognized based on this study in this Amish community. There are patient populations with features of mitochondrial disease, such as diabetes, hearing loss, and cardiovascular clinics. Given the fact that many individuals who suffer from mitochondrial disease are misdiagnosed with other conditions, screening those populations who have mild features of mitochondrial disease, like the participants of this study did, may provide an underlying diagnosis of mitochondrial disease that could guide treatment and

management and provide information about prognosis and early symptom recognition.[12; 66; 67] While it is not possible to predict the percent of these patients who would have mitochondrial disease, based on the work done through this study on one community, it is possible that patients would be found who could benefit from therapeutic intervention aimed at treating the symptoms of mitochondrial disease.

7.4.1 Disease Awareness

Many studies have cited the benefits of raising awareness and providing education about a disorder, and how that can lead to earlier diagnosis and treatment and therefore better outcomes.[68-72] There are organizations such as the United Mitochondrial Disease Foundation that strive to promote both "research and education about the diagnosis, treatment, and cure of mitochondrial disorders."[73] These organizations can serve as a resource to the general population about the latest news and information relating to mitochondrial disease research and information. Organizations such as these could be utilized in a public health effort to educate and to promote awareness the general population about mitochondrial disease.

One aim of this study was to design an educational intervention to promote awareness and understanding of MELAS and mitochondrial disease in general. While that intervention was designed to serve the Amish community, or, at least, a community with relatively lower literacy, different needs may or may not be required in the general population. Given the overall recommendation to develop materials for general patient education at a sixth to eighth grade reading level and the content of the handout, it would provide a foundation for general population educational materials.[74; 75] Several components of the existing intervention could be utilized and modified to achieve the goal of educating the general public, including information about the

structure and function of the mitochondria, the mitochondrial genome, maternal inheritance, and clinical features associated with mitochondrial disease.

7.4.2 Integration of the Educational Intervention in the General Population

A major modification that will facilitate adaptation of this intervention for use in the general population will be to re-format it for distribution, either online or in the form of a pamphlet or brochure that can be easily distributed. It will not be feasible to provide an oral presentation to all recipients of the information, although any printed or online version could also be used in conjunction with individualized genetic counseling. In cases where genetic counseling is not possible, the intervention could be adapted online to include audio files containing this information or in print by using more descriptive diagrams. There has been considerable research completed in recent years focusing on the types of health information consumers are obtaining online.[76-80] The conclusions drawn from this research indicate that consumers are turning more and more to the Internet for credible, trustworthy, accurate health information. However, some of this research has also shown that health information consumers struggle with identifying which sources of information are indeed credible, trustworthy, and accurate.[78; 80] Providing health information consumers with access to this information online, in clinicians' offices, and in other information repositories such as state and federal departments of health or through appropriate condition-specific support organizations may provide the general population with access they would have otherwise lacked, along with validation of the accuracy of the materials themselves.

This study can serve as a model for investigation of an effective educational intervention for the general population. One limitation of measuring the efficacy of the educational intervention designed in this study, as noted, is the lack of pre-testing of the questionnaire used as a measuring

tool. Therefore, before integrating a modified educational intervention into a larger group, pilot testing to determine its efficacy would be advantageous. A questionnaire can be developed that is modeled on studies that have used questionnaires aimed at measuring efficacy of health information resources in the general population. In addition to performing additional testing to confirm the efficacy of the questionnaire as a measuring tool, the questionnaire would have to be utilized in more expansive research in larger study populations representative of the general population to determine the efficacy of the new proposed educational intervention. Ideally, this research would also include significant involvement at each step of the process from an expert in questionnaire design and analysis specifically aimed at evaluating the efficacy of education for members of the general population.

7.4.3 Public Health Impact of Education about Mitochondrial Disease in the Amish Community

As seen at the Clinic for Special Children, as community awareness of the presence of genetic disorders increased, so did the utilization of services at the clinic, which has ultimately resulted in earlier diagnosis and treatment for patients affected with genetic disorders.[2; 3; 34] Furthermore, one of the significant community effects of this earlier diagnosis and treatment has been reduction in the financial burden of healthcare.[2] Better prognoses for children with these disorders have also resulted from earlier diagnosis and treatment.[3] Similarly, it is hoped that earlier diagnosis of mitochondrial disease yields improved outcomes for identified patients.[55] The magnitude of improvements is only tempered by uncertainties about the prevalence of mitochondrial disease in Amish communities. In this community alone, in addition to MELAS, a patient unrelated to this index family has been diagnosed with MELAS/Leigh Overlap syndrome caused by a mtDNA

mutation in the ND5 subunit of respiratory chain complex I.[81] Additional patients affected by mitochondrial disease may be identified in Northwestern Pennsylvania and in Lancaster County, PA among those Amish patients who have yet to receive a specific diagnosis.

7.4.4 Public Health Competencies

Throughout the work of this study, several public health competencies were developed and utilized. Primarily, this study revolved around cross-cutting competencies including communication and informatics, diversity and culture, leadership, public health biology, and program planning.[82] Within each of these cross-cutting competencies, several skills were used or developed during the process of this study.

When considering communication and informatics, a primary aim in this study was achieved through demonstrating effective written and oral skills for communicating with different audiences within the context of professional public health activities. This competency was developed through the creation of the educational intervention, the study visits, and through the writing of the related thesis document. Skills developed and practiced within the competency of diversity and culture were the use of basic concepts and skills involved in culturally appropriate community engagement and consideration of situations where culture-specific needs could result in more effective modifications or adaptations of a health intervention. The former occurred during and leading up to the study visits when study team members targeted study activities directly toward the Amish community being visited. The latter took place in a more reflective manner after the completion of the study when determining the benefits and limitations of particular interactions during study visits and communication that took place before visits.

Leadership skills developed particularly focused on articulation of an achievable mission and vision as well as engaging in dialogue and learning from others to advance public health goals. These skills have been constantly developing and improving throughout the entirety of this study beginning with the development of a thesis proposal and an IRB application and continuing through the data-gathering and study visits until now with the development of this thesis document.

Public health biology was paramount in this study as one of the focal points was explaining how genetics and genomics affect disease processes and public health policy and practice. It was an objective that through providing an educational intervention about MELAS and mitochondrial disease, many members of a community increased their understanding of these concepts. aLater reflection about the future directions of this work after data analysis has provided an opportunity to consider how the findings of this study may inform changes in public health policy and practice.

Finally, programming planning was a significant component of the process of this study. Of particular relevance was explaining, defining and differentiating among goals, measurable objectives, related activities, and expected outcomes for this study and potential subsequent public health programs. Another particularly relevant competency is differentiating between qualitative and quantitative evaluation methods in relation to their strengths, limitations, and appropriate uses. These two concepts coalesced in the development of the educational intervention and related questionnaires and the data analysis and interpretation that followed. Afterward, considerations were made toward explaining how the findings of a program evaluation can be used, particularly as it relates to the educational intervention and its implementation in the general population.

8.0 CONCLUSIONS AND FUTURE DIRECTIONS

8.1 CONCLUSIONS

After the identification of previously unrecognized mitochondrial disease in a northwestern Pennsylvania Amish community, this study was designed with three specific aims. The first was to characterize the incidence and clinical features of the common MELAS mutation in the northwestern Pennsylvania Amish community. This aim was met in the extended family of the index patient; however, there are plans to continue gathering incidence information among other members of the whole community. While additional testing and characterization will continue, this study has validated the first hypothesis, i.e. that there is a higher incidence of the m.3243A>G mutation in the *MT-TL1* gene in this community than was clinically recognized before this study. Through testing, thirteen individuals tested positive for this mutation, whereas prior to the study, there were only five individuals noted. The second aim was to construct a detailed extended pedigree of the index family and other key kinships in the community. This aim was met partially through completion of the pedigree in the extended family of the index patient. Patient ascertainment and pedigree development will continue as additional community members are recruited to consent to the study protocol through interaction with clinicians at the Hermitage Hospital Clinic. An IRB amendment will be required for this process. Finally, the third aim was to develop and assess the efficacy of an educational intervention designed to increase understanding of MELAS and mitochondrial disease in this community. In conjunction with this aim, the second hypothesis stated that this educational intervention would increase understanding of MELAS and mitochondrial disease in this community. While the questionnaires administered did not provide

statistically significant evidence of this, upon returning to the community to disclose results of testing, participants demonstrated increased understanding of the basic concepts relayed during the educational intervention. This was not confirmed through quantifiable evidence, however, qualitative impressions through counseling sessions and conversation suggested increased knowledge and understanding of MELAS, its symptoms, and mitochondrial inheritance.

8.2 FUTURE DIRECTIONS

This study was completed to meet part of the requirement for a Master's degree, and thus, out of necessity, there were several limitations placed on its scope. There are several ongoing and planned future studies that build on the presented work. These include outreach to the community at large to determine how prevalent mitochondrial disease, and MELAS in particular, is in this and other Amish communities. This work would also include a comparison of the prevalence of mitochondrial disease in other Amish communities outside of the northwestern Pennsylvania region to the prevalence found in the study population. It could also expand to include general population screening for mitochondrial diseases in individuals from the general population with mild phenotypes. Further elucidation of the pedigree for this kindred and other northwestern Pennsylvania Amish kindreds, and potential familial links to other Amish communities is planned and may lead to additional outreach to other Amish communities. Ultimately, further studies could be completed to link this information about family and community relationships to existing databases of Amish genealogy, which could be utilized for other studies in the future. Finally, a larger whole genome screen of random population members will serve to identify genetic risk in

the community more broadly, alerting care providers to possible diagnoses and leading to more expedient diagnoses and prompt therapy.

8.2.1 Community Preferences for Health Education

In this study, two major methods of communicating health education were utilized with participants. The first occurred during Study Visit One and was a group setting educational session. The second was private interviews between an individual or nuclear family and a healthcare professional. Through both interactions, the aim was to provide participants with information about MELAS including its clinical features and inheritance pattern. Even though both methods had the same aim, during the group educational intervention, this information was presented in a more general way, and in the interviews, it was more personalized to the particular participant including sharing of participant test results. Based on investigators' perceptions of patient understanding, the educational intervention did provide participants with basic information about the condition and inheritance pattern. However, it was evident at the time of disclosure that participants still had questions related more specifically to themselves. This two-tiered approach to information provision parallels what has been done in other settings. For example, in prenatal counseling, genetic counselors use group sessions for providing general information and one-on-one sessions for individualized counseling. However, it would be interesting to clarify with the community that this approach is efficacious for them.

8.2.2 Additional MELAS Heteroplasmy Testing in This Community

Analysis of samples collected in the extended family of the index patient in this Amish community led to the identification of many individuals with heteroplasmy. This knowledge, along with the occurrences of large families, raises concern about additional community members being at risk for having unidentified heteroplasmy. Therefore, use of urine heteroplasmy testing as a screening tool for individuals in the community who may or may not be directly related to the index family, could identify individuals who are in need of specific clinical follow-up. If this additional testing takes place, particular attention should be given to gathering pedigree information to clarify the relationships among all individuals found to have heteroplasmy for the purpose of identifying other at-risk community members.

8.2.3 General Population Screening for Mitochondrial Disease in Individuals with Mild Phenotypes

The results of this study confirm that there are individuals in this Amish community with previously unrecognized m.3243A>G mutations. As discussed previously, it is similarly suspected that some individuals in the general population who have mild symptoms or limited family history of features seen in MELAS may be living with unrecognized mtDNA mutations. There was significant variability in clinical presentations of individuals with mutations in this community, which supports the under-diagnosis of individuals with MELAS. Identifying populations with a mild MELAS or mitochondrial disease phenotype, such as those with conditions thought to be isolated, through clinics such as hearing loss, diabetes, and cardiovascular clinics might lead to identification of populations where mitochondrial disease is under-diagnosed. Because

undiagnosed mitochondrial disease has been discovered in one population, it is possible that similarly undiagnosed individuals may exist in the general population.

8.2.4 Extended Community Pedigree Construction

The construction of a comprehensive pedigree of the Amish communities in Northwestern Pennsylvania can provide guidance for identifying families and individuals at risk for certain inherited conditions. While this study yielded an adequately constructed pedigree of one extended family, it provides limited information on the pedigrees of those who are related to the index family through marriage. Because of this, additional community pedigree construction would be required to determine if there are more families at risk for being heteroplasmic for the m.3243A>G MELAS or other mitochondrial mutations. In addition, further testing would provide information to members of those families who are at risk for passing the MELAS mutation to their children if the mutation is identified. Constructing this extended pedigree may also provide additional information on the genealogy of this community and may help link it to previously-completed genealogy maps.

8.2.5 Whole Exome Sequencing and the Amish

Population-based whole exome sequencing (WES) or whole genome sequencing (WGS) has not been reported in any Amish community in the United States. A collection of WES or WGS information about members of this community could lead to further identification of disease-associated genes in Amish communities. It could be used to supplement existing ancestral information for Amish communities, reinforcing links among many Amish communities in the

United States, which allows for the collection and preservation of this historic knowledge.[67; 83-85] WES or WGS in Amish communities could provide information about genetic sequences that are identical by descent and unique to this population, which may contribute to other advances in genetic medicine benefitting themselves, other Amish communities, the general population, and the field of genetic medicine.[83; 85] Particularly in the absence of MELAS or other mitochondrial disorders and when individuals are exhibiting symptoms of disease, this may provide a diagnosis, which could be used to guide future management and treatment as well as provide prognosis information.

WES or WGS in this and other Amish communities might provide information about the different founder populations of Amish settlements. Identifying these links between communities might, in turn, provide information about conditions for which specific settlements or populations are more at risk. This information could lead to more specific offering and appropriate utilization of services based on projected needs.

APPENDIX A: IRB APPROVAL LETTER

The IRB approved this study on November 13, 2014 per this letter.

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: Gerard Vockley, MD, PhD

From: Aviva Katz, MD, Vice Chair

Date: 11/13/2014

IRB#: PRO14040237

Subject: Investigation of the Common MELAS Mutation in the Northwestern Pennsylvania Amish Community: mutation frequency and effectiveness of an educational intervention

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At its full board meeting on 9/17/2014, the University of Pittsburgh Institutional Review Board, Committee G, reviewed the above referenced research study and approved it pending minor modifications. Your responses to these comments have been reviewed and the research submission, in its currently modified form, adequately addresses the concerns of the IRB and is therefore approved.

Please note the following information:

The IRB has approved the waiver for the requirement to obtain a written informed consent for pre-education questionnaire..

This study has been approved under 45 CFR 46.404 for the inclusion of children. The IRB has determined that the written permission of one parent is sufficient.

The risk level designation is Minimal Risk.

Approval Date: 11/13/2014
Expiration Date: 9/16/2015

For studies being conducted in UPMC facilities, no clinical activities can be undertaken by investigators until they have received approval from the UPMC Fiscal Review Office.

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. Refer to the IRB Policy and Procedure Manual regarding the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

APPENDIX B: INITIAL CONTACT LETTER

This letter was sent to the index family as the first step of correspondence regarding this study.

Children's Hospital of Pittsburgh of UPMC

Division of Medical Genetics
Gerard Vockley, M.D., Ph.D.
Chief

One Children's Place
4401 Penn Avenue
Pittsburgh, PA 15224

Ph: (412) 692-5070
Fx: (412) 692-6472

July 3, 2014

[redacted]

Dear [redacted],

It has been a pleasure to provide ongoing clinical care to [redacted] over the past several years for [redacted] diagnosis of MELAS. Based on our wish to provide more complete care to members of your family and community, Dr. Goldstein and I would like to undertake a clinical project to learn how often this condition occurs in the Amish of Mercer County. This project would be conducted with Dr. Holmes Morton director of The Clinic for Special Children in Strasburg, Pennsylvania. In his care of other Amish communities, Dr. Morton has not previously identified a patient with MELAS, and is interested in learning more about this condition in the Amish.

The studies we are proposing involve gathering a detailed family history and medical histories on members of your extended family. We also would like to collect blood and possibly other samples such as saliva and urine to look for the genetic change that causes MELAS. We would appreciate having an opportunity to speak with you on the telephone to discuss these studies, and to share with you the details of what would be involved for your family. If you are interested in participating in this project, please send a return letter or call my assistant Lori Andrews at (412) 692-7775, so she can schedule a time for us to speak. We look forward to hearing back from you at your earliest convenience.

Regards,

Gerard Vockley, M.D., Ph.D.
Professor of Pediatrics and Human Genetics
University of Pittsburgh
School of Medicine
Graduate School of Public Health

APPENDIX C: LETTER OF PERMISSION FROM COMMUNITY DEACON

This is a letter from Deacon Albert Kuhns, who provided permission for the study to be conducted in the Amish community of interest.

Gerard Vockley, MD, PhD
Amy Goldstein, MD
Medical Genetics
Children's Hospital of Pittsburgh of UPMC
4401 Penn Avenue
Pittsburgh, PA 15224

Dear Drs. Vockley and Goldstein,

My name is Albert Kuhn, and I am the Deacon of the South District, Mercer County Amish community. Mr. Enos Yoder and I have had the opportunity to discuss your proposed study that you want to conduct in our community. This study will look at the frequency of MELAS, an inherited medical condition in the Mercer County Amish. I understand that the study involves asking community members to consent to receive education about MELAS and to be asked about what they have learned. They will also be asked to provide blood and/or urine samples for genetic testing for this condition and to have genetic test results returned to them when they are available from your laboratory. I understand that participation in this study is completely voluntary and a decision not to participate will have no effect on future care for any community members at any UPMC facility or with any UPMC physician.

I am willing to have you come into our community to speak with members of Mr. Yoder's extended family and other community members about your study to see if they are willing to participate. For those who are willing, the study can be accomplished during that visit to our community, or at follow-up visits, as needed. We will be able to provide you with a meeting place within our community to conduct these visits.

Sincerely,

Albert Kuhn
Deacon, South District, Mercer County

APPENDIX D: PRE-EDUCATIONAL SESSION QUESTIONNAIRE

This pre-education questionnaire was provided in a booklet format to participants.

Background Knowledge about Mitochondrial Disorders

Please read each question carefully and circle the letter corresponding to the choice you think best answers the question.

1. **An inherited, or genetic, disorder can be passed to a child by which of the following people?**
    a. His or her parents
    b. His or her aunt/uncle
    c. I am unsure of the correct answer.
    d. I have never heard of an inherited, or genetic, disorder.

    *If you chose "D," please go directly to **QUESTION 4.***

2. **Which of the following are true of inherited disorders?**
    a. An inherited condition can be passed from one person to another like a virus.
    b. A person does not have an inherited disorder if they do not have symptoms at birth.
    c. A child is born with an inherited disorder.
    d. I am unsure of the correct answer.

3. **What are our genes?**
    a. The instructions for how our bodies are made
    b. Something found inside every cell of our bodies
    c. Both of the Above
    d. I am unsure of the correct answer.

4. **A carrier of an autosomal recessive disorder is:**
    a. Someone who shows symptoms of an autosomal recessive disorder.
    b. Someone who does not show symptoms of a disorder, but whose children can have it.
    c. I am unsure of the correct answer.
    d. I have never heard of an autosomal recessive disorder.

*If you chose "D," please go directly to **QUESTION 8.***

5. **A child can only have an autosomal recessive disorder if which of the following is true?**
    a. Both of the child's parents are carriers of the disorder
    b. The child has a sibling with the disorder
    c. The child has a parent with the disorder
    d. I am unsure of the correct answer.

6. **Which of the following are true of autosomal recessive disorders?**
    a. Only females can pass on and inherit an autosomal recessive disorder.
    b. Only males can pass on and inherit an autosomal recessive disorder.
    c. Both men and women can pass on and inherit autosomal recessive disorders.
    d. I am unsure of the correct answer.

7. **Can a couple who has one child with an autosomalrecessive disorder have a child without that disorder?**
    a. Yes
    b. No
    c. I am unsure of the correct answer.

8. **A mitochondrial disorder can be passed to a child by which of the following people?**
    a. Either the mother or the father
    b. The mother
    c. A sibling
    d. I am unsure of the correct answer.
    e. I have never heard of a mitochondrial disorder.

*If you chose "E," please go directly to **QUESTION 12**.*

9. **What is heteroplasmy?**
    a. A measure of the amount of non-working mitochondrial DNA in an individual.
    b. A predictor of whether or not an individual will develop symptoms of a mitochondrial disorder.
    c. Both of the above are true.
    d. I am unsure of the correct answer.

10. **What do mitochondria do for the body?**
    a. They make energy.
    b. They keep your joints from hurting.
    c. They help your hair grow.
    d. I am unsure of the correct answer.

11. **Is a mitochondrial disorder inherited the same way as an autosomal recessive disorder?**
    a. Yes
    b. No
    c. I am unsure of the correct answer.

12. **Is MELAS is a mitochondrial disorder?**
    a. Yes
    b. No
    c. I am unsure of the correct answer.
    d. I have never heard of MELAS.

*If you chose answer "D," please go directly to **PAGE 8**.*

13. **MELAS can be passed to a child by which of the following people?**
    a. The father
    b. The mother
    c. Either parent
    d. A sibling
    e. I am unsure of the correct answer.

14. Which of the following is true of MELAS?

a. A child who has any level of MELAS-causing mitochondrial changes will show symptoms of MELAS.
b. A child who has MELAS will show symptoms at birth.
c. A child who has MELAS may not show all the symptoms of MELAS.
d. I am unsure of the correct answer.

15. Which of these symptoms is seen in patients with MELAS?

a. Headaches
b. Muscle weakness
c. Hearing Loss
d. All of the above
e. I am unsure of the correct answer.

16. In general, do women with MELAS-causing mitochondrial changes pass these changes on to all of their children?

a. Yes
b. No
c. I am unsure of the correct answer.

If you *are someone or know someone who has a mitochondrial disorder*, please answer the questions beginning on PAGE 7. Please answer the questions by checking the box that corresponds to your feelings.

If *are not someone and do not know someone who has a mitochondrial disorder*, please turn to PAGE 10.

Due to having mitochondrial disease yourself or due to someone in your family having mitochondrial disease, how often do you…

Feel depressed, isolated, sad, or lonely?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel weepy or tearful?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel frustrated, angry, or bitter?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel unable to talk to others about this condition?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel embarrassed because of this condition?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel worried by others' reaction to you because of this condition?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑

Feel this condition has interfered with your personal life?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel unable to join in family activities?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel dependent on family members?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Have problems making close personal relationships?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Lack support in the way you need from your partner?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Lack support in the way you needed from your family or close friends?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel that people do not understand the condition?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑

Feel you are unable to participate in community activities?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel you are unable to take part in routine daily activities?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel this condition has interfered with your community life?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑

Thank you for taking part in this questionnaire. Your answers are valuable and appreciated.

10

APPENDIX E: ADULT INFORMED CONSENT FORM

This is the consent form signed by adult participants.

Children's Hospital of Pittsburgh of UPMC

One Children's Hospital Drive
4401 Penn Avenue
Pittsburgh, PA 15224
www.chp.edu

CONSENT FOR AN ADULT TO ACT AS A PARTICIPANT IN A RESEARCH STUDY

STUDY TITLE:	Investigation of the common MELAS mutation in the Northwestern Pennsylvania Amish Community: mutation frequency and effectiveness of an educational intervention
PRINCIPAL INVESTIGATOR:	Gerard Vockley, M.D., Ph.D. 4401 Penn Avenue Pittsburgh, PA 15224 Telephone: 412-692-5070
CO-INVESTIGATORS:	Steven Dobrowolski, Ph.D. Amy Goldstein, M.D. Lina Gonzalez, M.D Catherine Walsh Vockley, M.S., L.C.G.C. Afifa Irani, B.S.
RESEARCH FUNDED BY:	Children's Hospital of Pittsburgh of UPMC, Division of Medical Genetics

You are being asked to be a part of a research study to gather information about a disorder called MELAS, or **M**itochondrial **E**ncephalomyopathy, **L**actic **A**cidosis, and **S**troke-like episodes.

Before you can agree to be in this study, you must read and sign this form. This form will give you more information about this study. Please ask as many questions as you need to before you decide if you want to be in the study.

Why is this study being done?

MELAS has not been found to be present in any Amish communities until recently. It is possible that it occurs in more people in Northwestern Pennsylvania than is recognized by healthcare providers. The main objectives of this study are 1) to educate the community members about MELAS and mitochondrial disease, and to gauge the effectiveness of this education through surveys; and 2) to identify and characterize the occurrence of the common gene change that causes MELAS in this community while constructing family trees showing relationships among affected family and community members.

What is MELAS?

MELAS is a rare disorder that is inherited through families from one generation to the next. MELAS is caused by a problem with the mitochondria, tiny organs in the body that produce 90% of the body's energy. MELAS may affect people in different ways. Some of the health problems seen in people with MELAS may include feeling tired or having low energy, having muscle pain

or weakness, hearing loss, learning problems, seizures, strokes, blindness, nausea/vomiting, and chronic diarrhea. This disorder can occur in children or adults. MELAS does not have a cure. Instead, treatment focuses on managing the symptoms in the affected individual. Doctors use supplements, vitamins and medications to manage these symptoms. When this management is no longer helpful, supportive or comfort-care is provided.

Who is being asked to participate in this study?
All members of the Northwestern Pennsylvania Amish community are being asked to participate in this study.

What will my participation in this study involve?
During the study, you first will be asked to complete a written questionnaire to record your current knowledge about MELAS and mitochondrial disease. You may not know all of the information that is presented in this questionnaire. This will be followed by an educational session in which you will learn about MELAS and mitochondrial disease. After this session, you will be asked to complete the same questionnaire again in order to see if the educational session is successful in increasing knowledge about MELAS and mitochondrial disease. You will then be interviewed about your family and medical history. You will also have blood drawn and provide buccal (cheek swab) and urine samples to be used to test for a genetic change or mutation known to cause MELAS. These activities will all take place in your community, and the anticipated maximum length of the first visit is approximately four hours. If all these activities cannot be completed for all study participants in one day, we will return to your community at a pre-determined date to finish these activities. You will be asked to provide permission for us to review your medical records, and this review will be done by study team members upon receipt of records.

Once the samples have been tested, you will be invited to learn the results of the studies by meeting with one of the physicians or genetic counselors when we return to the community on a scheduled date. Only your own genetic test results will be shared with you. Additional information will be provided about MELAS and about resources and services for patients with this condition, if appropriate. This results disclosure will require approximately 60 minutes.

The study will last from the time you fill out the questionnaire until the time at which we are able to return your results to you. This may take several months.

What are the possible risks of my participation in this study?
There are possible risks, side effects, and discomforts associated with participation in this study.

- *Blood draws:* The risks associated with blood draw include pain or discomfort at the site of the draw, potential bruising at that site, and rarely, fainting. As sterile, one-time-use equipment will be used for the blood draw, risk of infection is minimal. Care will be taken to avoid these potential risks and discomforts. Four teaspoons of blood will be drawn. For those weighing less than 14 pounds, the amount of blood drawn will be calculated according to weight.

- *Urine sample:* There is no known risk to participating in urine sample collection except that you may be embarrassed or shy about providing the sample.
- *Buccal swab*: The risk associated with a buccal swab is irritation to the inside of the cheek.
- *Loss of confidentiality:* The risk of allowing us to use your samples and certain limited health information is a potential loss of privacy. We will protect your privacy by labelling your samples and information with only a code, and keeping the key to the code in a password-protected database.
  Since your genetic information is being used in this study, accidental sharing of this information with people other than the members of this research team may affect your ability to get health insurance or to obtain certain jobs. This may also affect your decisions about having children. This may also have a negative impact on family relationships and may result in other people thinking differently of you or your family based on this information. To minimize these risks, non-clinical genetic information and medical information will only be recorded in files marked with codes, not your name.
- *Genetic testing:* There is a possibility of learning life-altering genetic information including the potential for future heritable disorders that cannot be treated or prevented. There is the possibility of being given inaccurate information due to a falsely negative result. This can happen when an individual has only a very low amount of genetic change that cannot be detected by the test. A diagnosis will be managed with the appropriate counseling and support and by providing you with information about available treatment.

A federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against you based on your genetic information. This law generally will protect you in the following ways:

- Health insurance companies and group health plans may not request your genetic information that we get from this research.
- Health insurance companies and group health plans may not use your genetic information when making decisions regarding your eligibility or premiums.
- Employers with 15 or more employees may not use your genetic information that we get from this research when making a decision to hire, promote, or fire you or when setting the terms of your employment.

Be aware that this GINA does not protect you against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

What are the possible benefits of my participation in the study?
You will be educated regarding MELAS and mitochondrial disease. It is unlikely that you will receive any direct benefit as a result of your participation in the study unless you have undiagnosed MELAS that is detected through the study.

While your participation may not directly benefit you, information gathered through the study will be used for improving our knowledge and treatment of MELAS and mitochondrial disease in Amish communities. This knowledge may benefit patients with MELAS or mitochondrial disease in the future by allowing for early identification of affected individuals and early start of therapies.

Do I have to take part in this study?
You do not have to be in this study. Your participation in this study is completely voluntary. It is okay if you decide you do not want to be in the study. Also, if you do start the study, you can choose to stop the study at any time. If you do not want to be in the study, you can still learn about MELAS and be tested for it through your own doctors. Your doctors will still care for you in the same manner even if you do not want to be in this study.

Whether or not you choose to participate in this study will have no effect on you or your current or future medical care at any UPMC affiliated health care provider. Whether or not you choose to participate in this study will have no effect on your current or future relationship with the University of Pittsburgh.

Will I or the community be charged for my participation in the study?
There will be no costs to you or your community to participate in this study.

Will I be paid for my participation in the study?
You will not receive any payment for participating in this study.

Who will know about my participation in this study?
Every effort will be made to keep your decision about whether or not to participate private. Any information from your medical records that is placed into this study will be kept as confidential (private) as possible. In addition, you will not be identified by name in any publication of the results of research studies involving the use of your medical record information unless you sign a separate consent form giving your permission.

What parts of my medical record information will be placed into the study files?
Review of your medical records will focus on past and current, medical information from hospital and other medical facilities. With your permission, we will obtain information concerning any diagnosis, health and family history, and results of any physical exams, tests of urine, blood, buccal tissue, other tissues, and any other tests, including results of genetic tests. This information about you will be made available to members of the research team, for an indefinite period of time. We are reviewing your records because we are looking for any features related to mitochondrial disorders that may not have been previously recognized. When reviewing medical records, only findings potentially related to mitochondrial disease and MELAS will be recorded in the study files. Medical information that does not apply to this study will not be recorded. This information will be collected from your UPMC hospital records and, if applicable, other hospital and private physician records. The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your

identifiable medical information) related to your participation in this study for an indefinite period of time. It is the University of Pittsburgh's policy to have research data maintained for at least seven years after final reporting and publication of the project. The University of Pittsburgh Research Conduct and Compliance representatives may monitor this study, and as the result of this monitoring may have access to your identifiable information.

What will happen to my sample(s) after this study is finished?
After this study is completed, your sample(s) will be kept and stored for future analysis. They will be stored without any identifying information, so your sample(s) will not be associated with your name or personal medical information. The sample(s) may be shared with other individuals who may be interested in studying inherited diseases, but the sample(s) will only be shared without this identifying information

Who will have access to my identifiable medical record information used in the study?
Access to your identifiable medical record information contained within this study will be limited to the principle investigator, co-investigators, and research staff in the Children's Hospital of Pittsburgh of UPMC Divisions of Medical Genetics and Neurology. A current, complete listing of these individuals will be provided to you upon your written request. In unusual cases, the researchers may be required to release your identifiable medical record information from the study in response to an order from a court of law.

May I withdraw, at a future date, my consent for my participation in this study?
You may withdraw your consent for your participation in the study at any time. Your decision to withdraw from the study would result in stopping collection and entry of new information about you into the study. Withdrawal can include opting out of disclosure of your genetic test results. However, any information collected from your medical records before the date that you formally withdraw your permission will not be destroyed; it will be retained and may still be used for the purposes of the study.

To formally withdraw your permission for participation in the study, you should provide a written and dated notice of this decision to the principal investigator of the study at the address listed on the first page of this consent form.

You will be withdrawn from the study by research staff if you choose not to provide a sample.

Who can I contact if I have any questions?
You may ask the doctor or any other study staff members any questions about the study. You may also write a letter or call Dr. Vockley at the address or telephone number listed on the first page of this consent form.

If you want to talk to someone else who does not work on the study, you can call the Human Subjects Protection Advocate of the IRB Office, University of Pittsburgh, at 1-866-212-2668. You can discuss problems, concerns, and questions; get information; offer input; or discuss situations in the event that the research team is unavailable.

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Children's Hospital of Pittsburgh of UPMC

One Children's Hospital Drive
4401 Penn Avenue
Pittsburgh, PA 15224
www.chp.edu

VOLUNTARY CONSENT

The above information has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by a qualified individual or by the investigator(s) listed on the first page of this consent document at the telephone number(s) given. I understand that I may always request that my questions, concerns or complaints be addressed by a listed investigator.

I understand that I may contact the Human Subjects Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668) to discuss problems, concerns, and questions; obtain information; offer input; or discuss situations in the event that the research team is unavailable.

By signing this form, I agree to participate in this research study and provide authorization to share my medical records with the research team. A copy of this consent form will be given to me.

_____ _____

Participant's Signature Date

_____

Participant's Name (Print)

The above-named individual is unable to provide direct consent for study participation because

_____

Therefore, by signing this form, I give my consent for his/her participation in this research study.

_____ _____

Representative's Name (Print) Representative's Relationship to Participant

_____ _____

Representative's Signature Date

_____ _____

Witness Signature Date

University Of Pittsburgh Institutional Review Board
Approval Date: 11/13/2014
Renewal Date: 9/16/2015
IRB #: PRO14040237

Children's Hospital of Pittsburgh of UPMC

One Children's Hospital Drive
4401 Penn Avenue
Pittsburgh, PA 15224
www.chp.edu

VERIFICATION OF EXPLANATION

I certify that I have carefully explained the purpose and nature of this research study to the above-named participant in appropriate language. He/she has had an opportunity to discuss it with me in detail. I have answered all his/her questions and he/she has provided affirmative agreement (i.e., assent) to participate in this study.

_____ _____

Investigator's Signature Date

CERTIFICATION OFINFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise. I further certify that no research component was begun until after this consent form was signed.

_____ _____

Printed Name of Person Obtaining Consent Role in Research Study

_____ _____

Signature of Person Obtaining Consent Date

University Of Pittsburgh Institutional Review Board
Approval Date: 11/13/2014
Renewal Date: 9/16/2015
IRB #: PRO14040237

APPENDIX F: CHILD INFORMED CONSENT FORM

This is the consent form signed by parents to provide consent for their child participant.

Children's Hospital of Pittsburgh of UPMC

One Children's Hospital Drive
4401 Penn Avenue
Pittsburgh, PA 15224
www.chp.edu

CONSENT FOR A CHILD TO ACT AS A PARTICIPANT IN A RESEARCH STUDY

STUDY TITLE:	Investigation of the Common MELAS Mutation in the Northwestern Pennsylvania Amish Community: Mutation frequency and effectiveness of an educational intervention
PRINCIPAL INVESTIGATOR:	Gerard Vockley, M.D., Ph.D. 4401 Penn Avenue Pittsburgh, PA 15224 Telephone: 412-692-5070
CO-INVESTIGATORS:	Steven Dobrowolski, Ph.D. Amy Goldstein, M.D. Lina Gonzalez, M.D Catherine Walsh Vockley, M.S., L.C.G.C. Afifa Irani, B.S.
RESEARCH FUNDED BY:	Children's Hospital of Pittsburgh of UPMC Division of Medical Genetics

Your child is being asked to be a part of a research study to gather information about a disorder that affects some individuals in your community. The condition is called MELAS, or **M**itochondrial **E**ncephalomyopathy, **L**actic **A**cidosis, and **S**troke-like episodes.

Before you can agree to have your child in this study, you must read and sign this form. This form will give you more information about this study. Please ask as many questions as you need to before you decide if you want your child to be in the study.

Why is this study being done?

MELAS has not been found to be present in any Amish community until recently. It is possible that it occurs in more people in Northwestern Pennsylvania than is recognized by healthcare providers. The main objectives of this study are 1) to educate the community members about MELAS and mitochondrial disease, and to gauge the effectiveness of this education through surveys; and 2) to identify and characterize the occurrence of the common gene change that causes MELAS in this community while constructing a family tree showing relationships among affected family and community members.

What is MELAS?

MELAS is a rare disorder that is inherited through families from one generation to the next. MELAS is caused by a problem with the mitochondria, tiny organs in the body that produce 90% of the body's energy. MELAS may affect people in different ways. Some of the health problems seen in people with MELAS may include feeling tired or having low energy, having muscle pain or weakness, hearing loss, learning problems, seizures, strokes, blindness, nausea/vomiting, and

chronic diarrhea. This disorder can occur in children or adults. MELAS does not have a cure. Instead, treatment focuses on managing the symptoms in the affected individual. Doctors use supplements, vitamins and medications to manage these symptoms. When this management is no longer helpful, supportive or comfort-care is provided.

Who is being asked to participate in this study?
All members of the Northwestern Pennsylvania Amish community are being asked to participate in this study.

What will my child's participation in this study involve?
During the study, your child first will be asked to take a written questionnaire to record his/her current knowledge about MELAS and mitochondrial disease. This will only be done if the child is 14 years or older. This will be followed by an educational session in which your child will learn about MELAS and mitochondrial disease. After this session, your child will be asked to take the same survey in order to assess the effectiveness of the educational session. Again, this will only be done if your child is 14 years or older. You and your child will then be interviewed about your child's family and medical history. Your child will also have blood drawn and provide buccal (cheek swab) and urine samples to be used to test for a genetic change or mutation known to cause MELAS. These activities will all take place in your community, and the anticipated maximum length of the first visit is approximately four hours. If all these activities cannot be completed for all study participants in one day, we will return to your community at a pre-determined date to finish these activities. You will be asked to provide permission for us to review your child's medical records, and this review will be done by study team members upon receipt for records.

Once the samples have been tested, you and your child will be invited to learn the results of the studies by meeting with one of the physicians or genetic counselors when we return to the community on a scheduled date. Only your own child's genetic test results will be shared with you. Additional information will be provided about MELAS and about resources and services for patients with this condition, if appropriate. This results disclosure will require approximately 60 minutes.

The study will last from the time your child fills out the questionnaire until the time at which we are able to return his/her results to you. This may take several months.

What are the possible risks of my child's participation in this study?
There are possible risks, side effects, and discomforts associated with participation in this study.

- *Blood draws:* The risks associated with blood draw include pain or discomfort at the site of the draw, potential bruising at that site, and rarely, fainting. As sterile, one-time-use equipment will be used for the blood draw, risk of infection is minimal. Care will be taken to avoid these potential risks and discomforts. Four teaspoons of blood will be drawn. For those weighing less than 14 pounds, the amount of blood drawn will be calculated according to weight.

- *Urine sample:* There is no known risk to participating in urine sample collection except that your child may be embarrassed or shy about doing the sample.
- *Buccal swab*: The risk associated with a buccal swab is irritation to the inside of the cheek.
- *Loss of confidentiality:* The risk of allowing us to use your child's samples and certain limited health information is a potential loss of privacy. We will protect your child's privacy by labelling your child's samples and information with only a code, and keeping the key to the code in a password-protected database. Since your genetic information is being used in this study, accidental sharing of this information with people other than the members of this research team may affect your child's ability to get health insurance or to obtain certain jobs. This may also affect your child's decisions about having children. This may also have a negative impact on family relationships and may result in other people thinking differently of your child or family based on this information. To minimize these risks, non-clinical genetic information and medical information will only be recorded in files marked with codes, not your child's name.
- *Genetic testing:* There is a possibility of learning life-altering genetic information including the potential for future heritable disorders that cannot be treated or prevented. There is the possibility of being given inaccurate information due to a falsely negative result. This can happen when an individual has only a very low amount of genetic change that cannot be detected by the test. A diagnosis will be managed with the appropriate counseling and support and by providing you with information about available treatment.

A federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against your child based on his/her genetic information. This law generally will protect your child in the following ways:

- Health insurance companies and group health plans may not request your child's genetic information that we get from this research.
- Health insurance companies and group health plans may not use your child's genetic information when making decisions regarding your child's eligibility or premiums.
- Employers with 15 or more employees may not use your child's genetic information that we get from this research when making a decision to hire, promote, or fire your child or when setting the terms of your child's employment.

Be aware that this GINA does not protect your child against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

What are the possible benefits of my child's participation in the study?

Your child will be educated regarding MELAS and mitochondrial disease. It is unlikely that your child will receive any direct benefit as a result of his/her participation in the study unless he/she has undiagnosed MELAS that is detected through the study.

While your child's participation may not directly benefit your child, information gathered through the study will be used for improving our knowledge and treatment of MELAS and

mitochondrial disease in Amish communities. This knowledge may benefit patients with MELAS or mitochondrial disease in the future by allowing for early identification of affected individuals and early start of therapies.

Does my child have to take part in this study?
Your child does not have to be in this study. Your child's participation in this study is completely voluntary. It is okay if you decide you do not want your child to be in the study. Also, if your child does start the study, you can choose to stop the study at any time. If you do not want your child to be in the study, you can still learn about MELAS and be tested for it through your doctors. Your doctors will still care for your child in the same manner even if you do not want your child to be in this study.

Whether or not you provide your permission for your child's participation in this study will have no effect on you or your child's current or future medical care at any UPMC-affiliated health care provider, or your child's current or future relationship with the University of Pittsburgh.

Will my child, the community, or I be charged for my child's participation in the study?
There will be no costs to you or your community to participate in this study.

Will I be paid for my child's participation in the study?
You will not receive any payment for participating in this study.

Who will know about my child's participation in this study?
Every effort will be made to keep your decision about whether or not to allow your child to participate private. Any information from your child's medical records that is placed into this study will be kept as confidential (private) as possible. In addition, your child will not be identified by name in any publication of the results of research studies involving the use of your child's medical record information unless you sign a separate consent form giving your permission.

What parts of my child's medical record information will be placed into the study files?
Review of your child's medical records will focus on past and current, medical information from hospital and other medical facilities. We will obtain information concerning any diagnosis, health and family history, and results of any physical exams, tests of urine, blood, buccal tissue, other tissues, and any other tests, including results of genetic tests. This information about your child will be made available to members of the research team, for an indefinite period of time. We are reviewing your child's records because that we are looking for any features related to a mitochondrial disorder that may not have been previously recognized. When reviewing medical records, only findings potentially related to mitochondrial disease and MELAS will be recorded in the study files. Medical information that does not apply to this study will not be recorded. This information will be collected from your child's UPMC hospital records and, if applicable, other hospital and private physician records. The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your child's identifiable medical information) related to your child's participation in this study for an indefinite period of time. It is the University of Pittsburgh's policy to have research data

maintained for at least seven years after final reporting and publication of the study, and for children's records to be maintained until they are at least 23 years old. The University of Pittsburgh Research Conduct and Compliance representatives may monitor this study, and as the result of this monitoring may have access to your identifiable information.

What will happen to my child's sample(s) after this study is finished?
After this study is completed, your child's sample(s) will be kept and stored for future analysis. They will be stored without any identifying information, so your child's sample(s) will not be associated with his/her name or personal medical information. The sample(s) may be shared with other individuals who may be interested in studying inherited diseases, but the sample(s) will only be shared without this identifying information.

Who will have access to my child's identifiable medical record information used in the study?
Access to your child's identifiable medical record information contained within this study will be limited to the principle investigator, co-investigators, and research staff in the Children's Hospital of Pittsburgh of UPMC Divisions of Medical Genetics and Neurology. A current, complete listing of these individuals will be provided to you upon your written request. In unusual cases, the researchers may be required to release your identifiable medical record information from the study in response to an order from a court of law.

May I withdraw my child, at a future date, my consent for my child's participation in this study?
You may withdraw your consent for your child's participation in the study at any time. Your decision to withdraw from the study would result in stopping collection and entry of new information about your child into the study, including disclosure of your genetic test results. However, any information collected from your child's medical record before the date that you formally withdraw your permission will not be destroyed; it will be retained and may still be used for the purposes of the study.

To formally withdraw your permission for participation in the study, you should provide a written and dated notice of this decision to the principal investigator of the study at the address listed on the first page of this consent form.

Your child may be withdrawn from the study by research staff if your child refuses to provide a sample.

Who can I contact if I have any questions?
You or your child may ask the doctor or any other study staff members any questions about the study. You may also write a letter or call Dr. Vockley at the address or telephone number listed on the first page of this consent form.

If you want to talk to someone else who does not work on the study, you can call the Human Subjects Protection Advocate of the IRB Office, University of Pittsburgh, at 1-866-212-2668. You can discuss problems, concerns, and questions; get information; offer input; or discuss

situations in the event that the research team is unavailable.

****

VOLUNTARY CONSENT
The above information has been explained to me, and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by a qualified individual or by the investigator(s) listed on the first page of this consent document at the telephone number(s) given. I understand that I may always request that my questions, concerns or complaints be addressed by a listed investigator.

I understand that I may contact the Human Subjects Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668) to discuss problems, concerns, and questions; obtain information; offer input; or discuss situations in the event that the research team is unavailable.

_____
Child's Name

I understand that, as a minor, (age less than 18 years), the above-named child is not permitted to participate in this research study without my consent. Therefore, by signing this form, I give my consent for his/her participation in this research study and provide authorization to share his/her medical records with the research team. A copy of this consent form will be given to me.

_____	_____
Parent's Name (Print)	Relationship to Participant
_____	_____
Parent's Signature	Date

Children's Hospital of Pittsburgh of UPMC

One Children's Hospital Drive
4401 Penn Avenue
Pittsburgh, PA 15224
www.chp.edu

ASSENT:

I certify that I have carefully explained the purpose and nature of this research study to the child-subject in age appropriate language. He/she has had an opportunity to discuss it with me in detail. I have answered all his/her questions and he/she has provided affirmative agreement (i.e., assent) to participate in this study.

_____ _____

Investigator's Signature Date

_____

Investigator's Printed Name

This research has been explained to me, and I agree to participate.

_____ _____

Signature of Child-Subject Date

_____

Printed name of Child-Subject

CERTIFICATION OF INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise. I further certify that no research component was begun until after this consent form was signed except those components for which a waiver to consent was obtained.

_____ _____

Printed Name of Person Obtaining Consent Role in Research Study

_____ _____

Signature of Person Obtaining Consent Date

University Of Pittsburgh Institutional Review Board
Approval Date: 11/13/2014
Renewal Date: 9/16/2015
IRB #: PRO14040237

Children's Hospital of Pittsburgh of UPMC
One Children's Hospital Drive
4401 Penn Avenue
Pittsburgh, PA 15224
www.chp.edu

CONSENT FOR CONTINUED PARTICIPATION

I am currently participating in a research study entitled "Investigation of the common MELAS mutation in the Northwestern Pennsylvania Amish Community: mutation frequency and effectiveness of an educational intervention". Consent for my participation in this research study was initially obtained from my parents. I have now reached the age of 18 years and am able to provide direct consent for continued participation in this research study. I have had a chance to review the original consent document that my parents signed on my behalf and understand the research procedures that I am being asked to participate in during the remainder of the study.

I understand that I have the right to withdraw from the study at any time and that my decision to do so will not affect my care at any UPMC affiliated health care provider or affect my relationship with the University of Pittsburgh.

The above information has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions, voice concerns or complaints about any aspect of this research study during the course of this study, and that such future questions, concerns or complaints will be answered by a qualified individual or by the investigator(s) listed on the first page of this consent document at the telephone number(s) given. I understand that I may always request that my questions, concerns or complaints be addressed by a listed investigator. I understand that I may contact the Human Subjects Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668) to discuss problems, concerns, and questions; obtain information; offer input; or discuss situations in the event that the research team is unavailable.

By signing this form I agree to continue my participation in this research study.

_____ _____
Participant's Signature Date

CERTIFICATION OF INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions, concerns or complaints as they arise. I further certify that no research component of this protocol was begun until after this consent form was signed.

_____ _____
Printed Name of Person Obtaining Consent Role in Research Study

_____ _____
Signature of Person Obtaining Consent Date

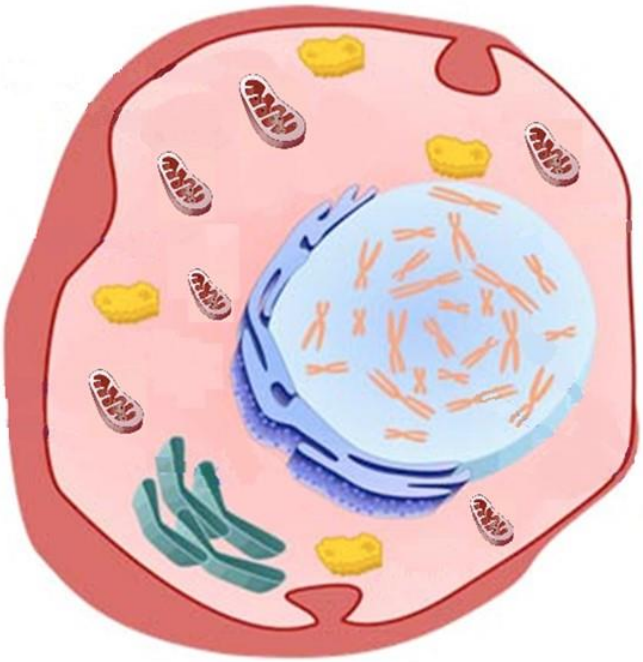
University Of Pittsburgh Institutional Review Board
Approval Date: 11/13/2014
Renewal Date: 9/16/2015
IRB #: PRO14040237

APPENDIX G: EDUCATIONAL INTERVENTION HANDOUT

This was the handout used as a visual aid for the educational intervention. Each participant had a copy on which to follow along with the presenting investigator.

Mitochondrial Disorders
in an Amish Community

The Cell



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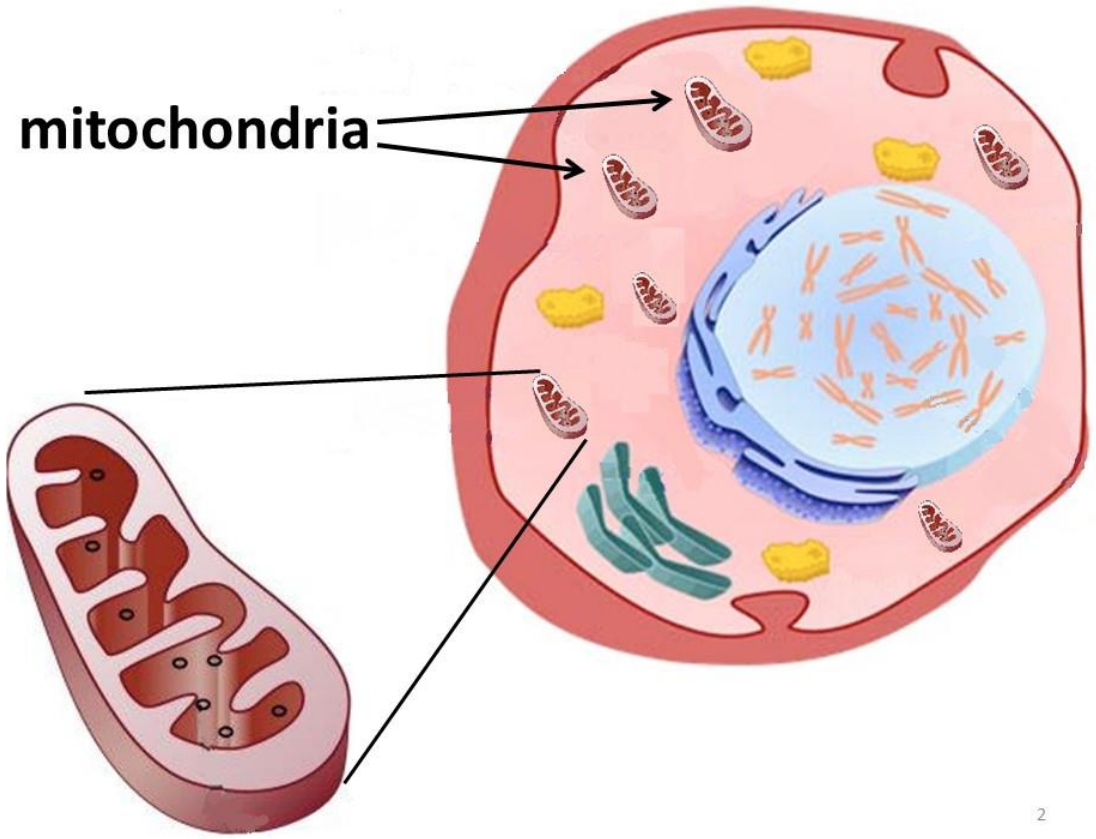
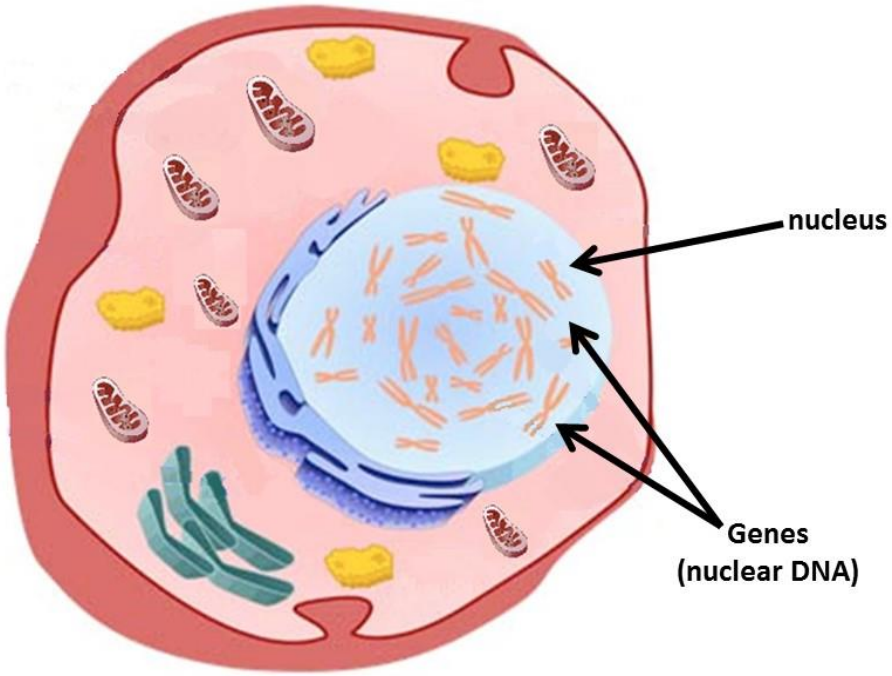


2

Mitochondrial Disorders

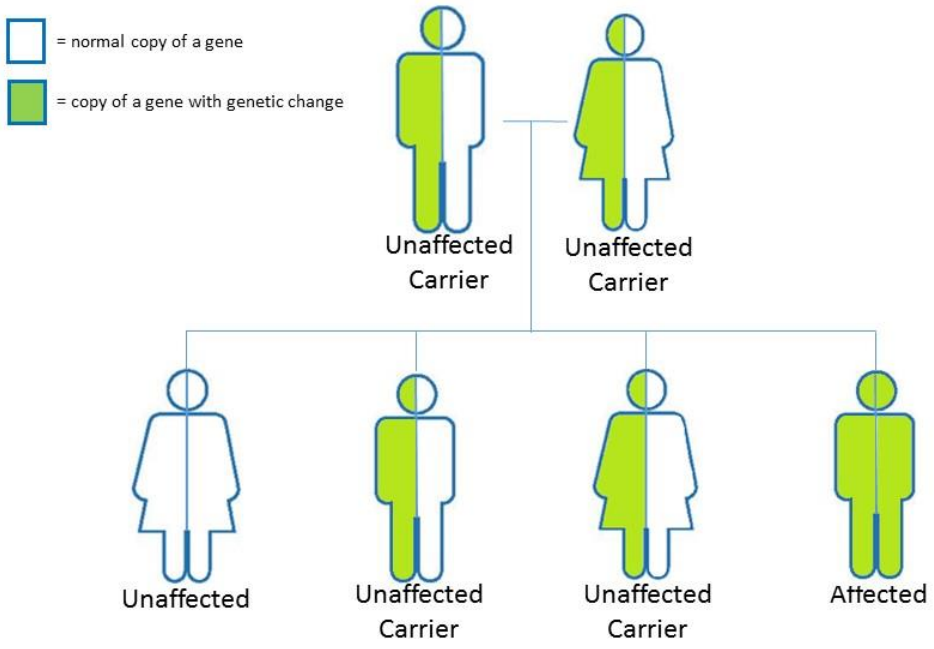
Organ	What happens to the body
Brain	Hearing loss, learning problems, seizures, stroke, dementia
Eyes	Drooping eyelids, blindness
Heart	Enlarged heart, irregular heartbeat, heart failure
Muscle	Cramps, pain, weakness, low energy
Digestive Tract	Heartburn, chronic diarrhea, nausea/vomiting
Kidneys	Kidney failure
Liver	Liver failure
Pancreas	Diabetes

3



4

Autosomal Recessive Inheritance



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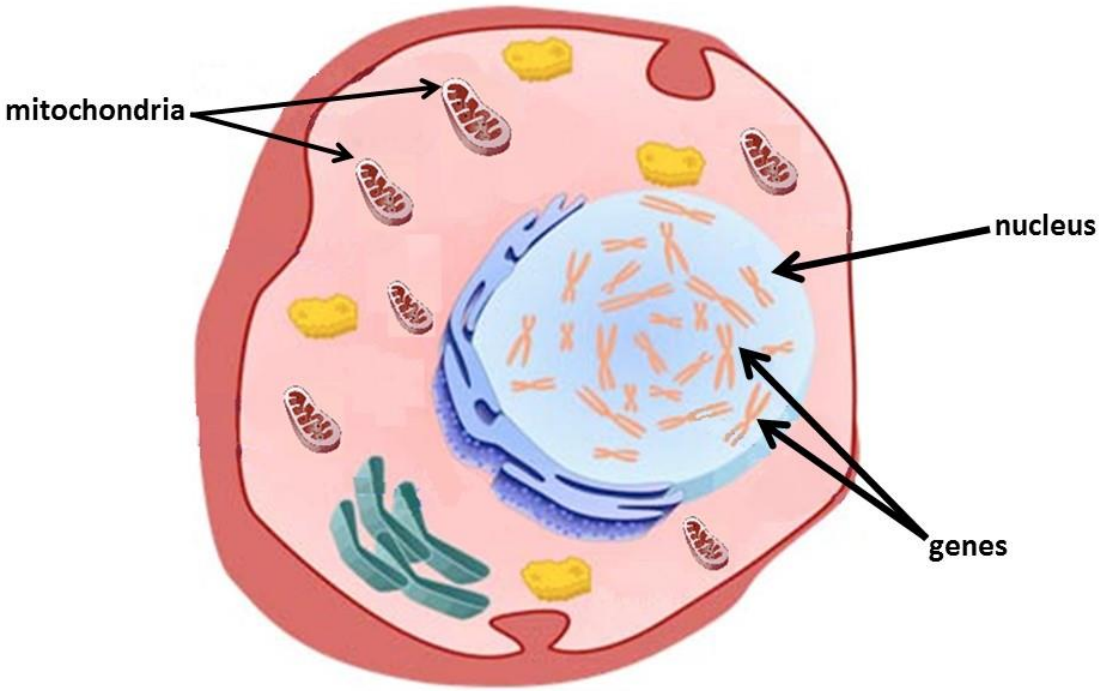
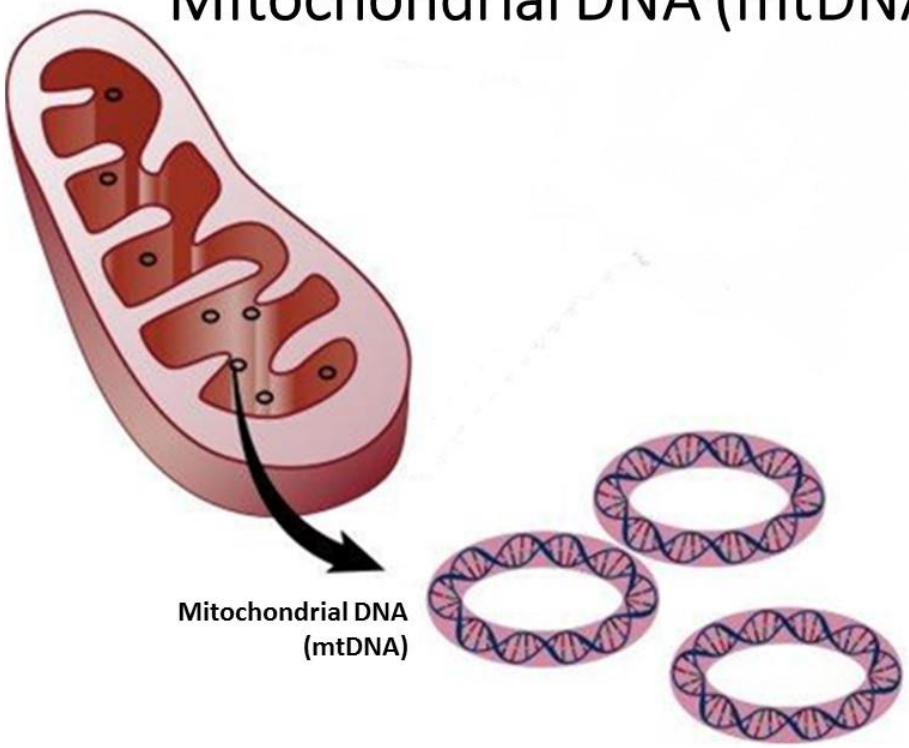


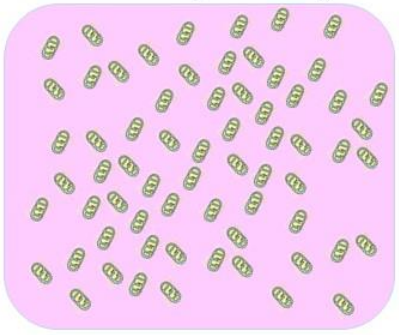
6

Mitochondrial DNA (mtDNA)



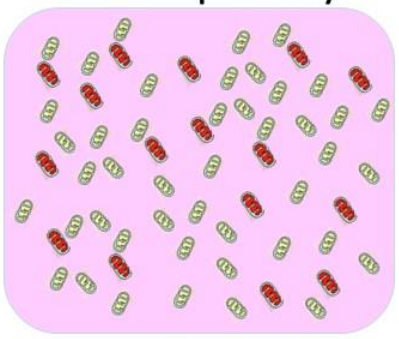
7

Homoplasmy



A single type of mtDNA

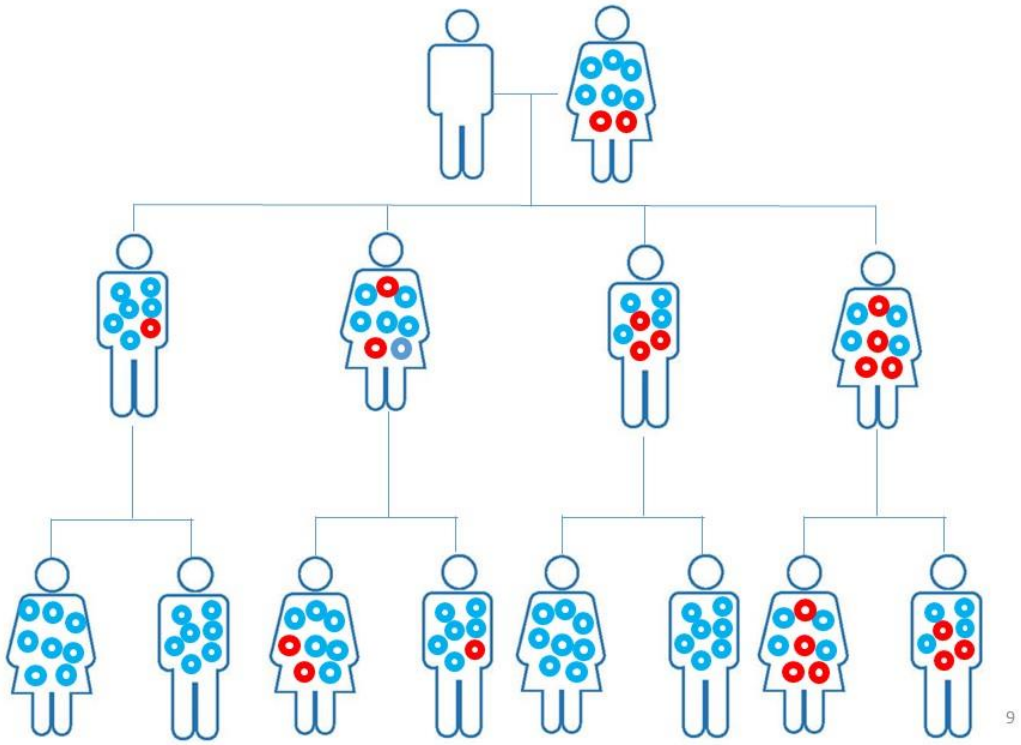
Heteroplasmy



More than one type of mtDNA

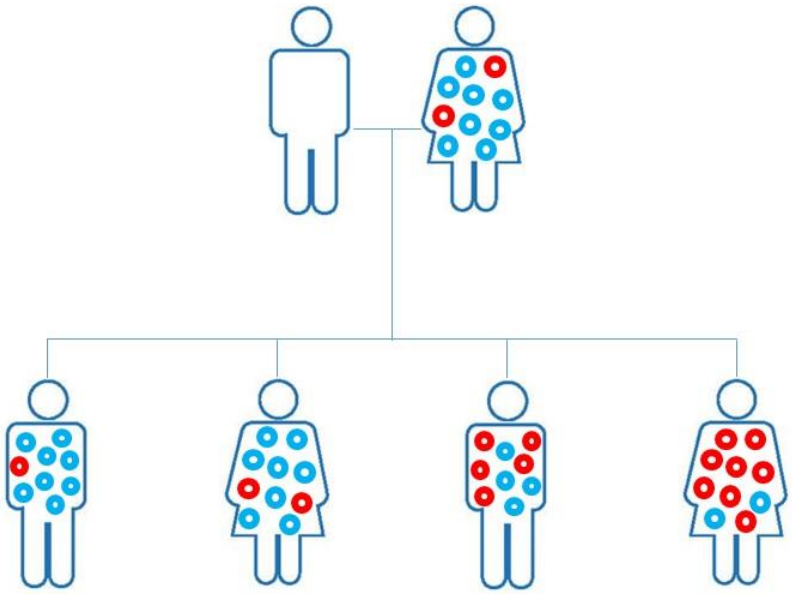
8

Mitochondrial Inheritance



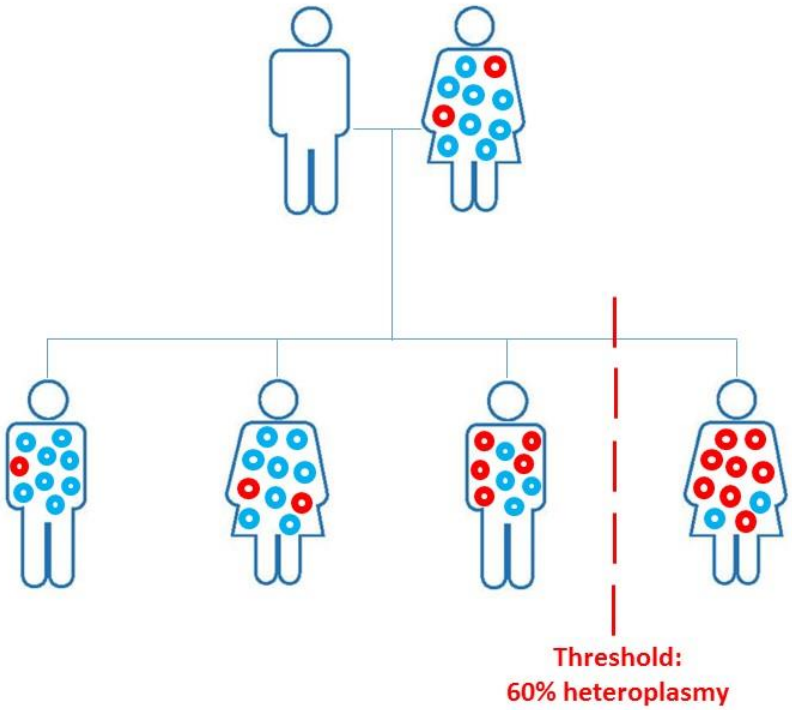
9

Mitochondrial Inheritance



10

Mitochondrial Inheritance



11

M = **M**itochondrial

E = **E**ncephalomyopathy

L = **L**actic

A = **A**cidosis

S = **S**troke-like Episodes

12

encephalomyopathy

CEPH = Brain

MYO = Muscle

PATHY = Disorder

13

Lactic Acidosis

- High amount of acid in the blood due to stress on the muscles
- Can cause:
  - Low energy (fatigued or tired)
  - Muscle weakness, cramping, or pain
  - Nausea/vomiting

Stroke-Like Episodes

- Very uncommon symptom in MELAS
- Gradual onset of symptoms:
  - Gradually have more headaches
  - Decline of brain functions
    - Memory loss
    - Personality changes
    - Decreased awareness
    - Inability to think clearly
  - May develop seizures over time

15

More about MELAS

- Other symptoms include:
  - Diabetes
  - Hearing loss
- Symptoms can begin at any age or stage of life

16

APPENDIX H: POST-EDUCATIONAL SESSION QUESTIONNAIRE

This is the post-educational questionnaire distributed to participants after the educational intervention.

Updated Knowledge about Mitochondrial Disorders

Please read each question carefully and circle the letter corresponding to the choice you think best answers the question.

1. **An inherited, or genetic, disorder can be passed to a child by which of the following people?**
    a. His or her parents
    b. His or her aunt/uncle
    c. I am unsure of the correct answer.
    d. I have never heard of an inherited, or genetic, disorder.

    *If you chose "D," please go directly to **QUESTION 4.***

2. **Which of the following are true of inherited disorders?**
    a. An inherited condition can be passed from one person to another like a virus.
    b. A person does not have an inherited disorder if they do not have symptoms at birth.
    c. A child is born with an inherited disorder.
    d. I am unsure of the correct answer.

3. **What are our genes?**
    a. The instructions for how our bodies are made
    b. Something found inside every cell of our bodies
    c. Both of the Above
    d. I am unsure of the correct answer.

4. **A carrier of an autosomal recessive disorder is:**
    a. Someone who shows symptoms of an autosomal recessive disorder.
    b. Someone who does not show symptoms of a disorder, but whose children can have it.
    c. I am unsure of the correct answer.
    d. I have never heard of an autosomal recessive disorder.

*If you chose "D," please go directly to **QUESTION 8.***

5. **A child can only have an autosomal recessive disorder if which of the following is true?**
    a. Both of the child's parents are carriers of the disorder
    b. The child has a sibling with the disorder
    c. The child has a parent with the disorder
    d. I am unsure of the correct answer.

6. **Which of the following are true of autosomal recessive disorders?**
    a. Only females can pass on and inherit an autosomal recessive disorder.
    b. Only males can pass on and inherit an autosomal recessive disorder.
    c. Both men and women can pass on and inherit autosomal recessive disorders.
    d. I am unsure of the correct answer.

7. **Can a couple who has one child with an autosomalrecessive disorder have a child without that disorder?**
    a. Yes
    b. No
    c. I am unsure of the correct answer.

8. **A mitochondrial disorder can be passed to a child by which of the following people?**
    a. Either the mother or the father
    b. The mother
    c. A sibling
    d. I am unsure of the correct answer.
    e. I have never heard of a mitochondrial disorder.

*If you chose "E," please go directly to **QUESTION 12**.*

9. **What is heteroplasmy?**
    a. A measure of the amount of non-working mitochondrial DNA in an individual.
    b. A predictor of whether or not an individual will develop symptoms of a mitochondrial disorder.
    c. Both of the above are true.
    d. I am unsure of the correct answer.

10. **What do mitochondria do for the body?**
    a. They make energy.
    b. They keep your joints from hurting.
    c. They help your hair grow.
    d. I am unsure of the correct answer.

11. **Is a mitochondrial disorder inherited the same way as an autosomal recessive disorder?**
    a. Yes
    b. No
    c. I am unsure of the correct answer.

12. **Is MELAS is a mitochondrial disorder?**
    a. Yes
    b. No
    c. I am unsure of the correct answer.
    d. I have never heard of MELAS.

*If you chose answer "D," please go directly to **PAGE 8**.*

13. **MELAS can be passed to a child by which of the following people?**
    a. The father
    b. The mother
    c. Either parent
    d. A sibling
    e. I am unsure of the correct answer.

14. Which of the following is true of MELAS?

a. A child who has any level of MELAS-causing mitochondrial changes will show symptoms of MELAS.
b. A child who has MELAS will show symptoms at birth.
c. A child who has MELAS may not show all the symptoms of MELAS.
d. I am unsure of the correct answer.

15. Which of these symptoms is seen in patients with MELAS?

a. Headaches
b. Muscle weakness
c. Hearing Loss
d. All of the above
e. I am unsure of the correct answer.

16. In general, do women with MELAS-causing mitochondrial changes pass these changes on to all of their children?

a. Yes
b. No
c. I am unsure of the correct answer.

If you *are someone or know someone who has a mitochondrial disorder*, please answer the questions beginning on PAGE 7. Please answer the questions by checking the box that corresponds to your feelings.

If *are not someone and do not know someone who has a mitochondrial disorder*, please turn to PAGE 10.

If you have already answered the questions in this section during the pre-educational survey, please do not answer them again.

Due to having mitochondrial disease yourself or due to someone in your family having mitochondrial disease, how often do you…

Feel depressed, isolated, sad, or lonely?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel weepy or tearful?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel frustrated, angry, or bitter?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel unable to talk to others about this disorder?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel embarrassed because of this disorder?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑

Feel worried by others' reaction to you because of this disorder?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel this disorder has interfered with your personal life?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel unable to join in family activities?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel dependent on family members?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Have problems making close personal relationships?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Lack support in the way you need from your partner?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑

Lack support in the way you needed from your family or close friends?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel that people do not understand the disorder?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel you are unable to participate in community activities?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel you are unable to take part in routine daily activities?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑
Feel this disorder has interfered with your community life?	Never ❑	Rarely ❑	Sometimes ❑	Often ❑	Always ❑

Thank you for taking part in this questionnaire. Your answers are valuable and appreciated.

10

APPENDIX I: MELAS-RELATED SYMPTOMS CHECKLIST

This list of symptoms associated with MELAS and mitochondrial disease was used by investigators to question historians about the presence of these symptoms in themselves and their family members.

Check the box & Circle the specific issue the patient experiences:

- ☐ Developmental Delays/learning problems
- ☐ Dementia (trouble remembering things, decreased awareness of their surroundings)
- ☐ Psychiatric concerns (worrying or feeling sad often, differences in their behavior or personality)
- ☐ Headaches/Migraines
- ☐ Seizures/Convulsions/staring spells
- ☐ Strokes
- ☐ Fainting
- ☐ Decreased vision/Loss of vision/blindness
- ☐ Ptosis (drooping eyelids)
- ☐ Hearing loss/deafness
- ☐ Excessive tiredness/fatigue
- ☐ Short stature/trouble gaining weight
- ☐ Muscle weakness, cramping, pain
- ☐ GI issues: chronic diarrhea, heartburn, nausea/vomiting
- ☐ Low muscle tone (loose or floppy muscles)
- ☐ Heart problems/Cardiomyopathy (has the heart been checked?)
- ☐ Diabetes (trouble digesting sugars) (features such as frequent urination, excessive thirst, increased hunger, weight loss, tingling in extremities)
- ☐ Trouble getting pregnant/multiple miscarriages
- ☐ Any other related concerns:

_____
_____
_____
_____
_____
_____

APPENDIX J: FOLLOW-UP LETTER FOR STUDY VISIT TWO

This letter was sent to the index family on December 5, 2014 requesting contact to coordinate Study Visit Two for results disclosure and genetic counseling regarding the completed heteroplasmy testing

Children's Hospital of Pittsburgh | of UPMC

Division of Medical Genetics
Gerard Vockley, M.D., Ph.D.
Chief

One Children's Place
4401 Penn Avenue
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Ph: (412) 692-5070
Fx: (412) 692-6472

[redacted]

December 5, 2014

Dear Mr. & Mrs. [redacted],

Thank you for welcoming us into your home last month. We found the visit to be a great success, and we enjoyed the opportunity to meet so many members of your extended family.

As you know, the next step of our project involves sharing the results of testing that was done with those family members who chose to provide samples. We wanted to ask you about the best way to coordinate this step. We have considered two options. The first is to again meet at your home or that of another family member and have your family gather to individually receive their results. If you chose to do this, we can provide some funding to traveling family members to help offset their expense. If hosting so many people is difficult, or it is too great a burden to travel during winter weather, we are also able to meet families and individuals in smaller groups at their own home or one near to them, discussing test results with families at each of those locations. This, of course, could only be done if there are family members who would be comfortable with several of us coming into their home.

In addition to discussing test results at our next visit, we will be able to make arrangements to help individuals provide samples if they were unable to do so at our last meeting. We will come prepared for this possibility, so family members don't have to decide in advance if they want to join the study,

It might be easiest to start to plan this next visit over the telephone. Please send a return letter or call my assistant Lori Andrews at (412) 692-7775, so she can schedule a time for us to speak. We look forward to hearing back from you at your earliest convenience. Thank you again for your assistance with planning this study.

Sincerely,

Gerard Vockley, M.D., Ph.D.
Professor of Pediatrics and Human Genetics
University of Pittsburgh
School of Medicine
Graduate School of Public Health

APPENDIX K: SECOND FOLLOW-UP LETTER FOR STUDY VISIT TWO

This letter was sent on January 9, 2015 to follow up from the first letter sent in December because that letter went unanswered.

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School of Medicine
Professor of Human Genetics
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Chief of Medical Genetics
Children's Hospital of Pittsburgh

Mr. & Mrs. [redacted]
[redacted]

January 9, 2015

Dear Mr. & Mrs. [redacted]

I am not certain whether you received my recent letter, so wanted I to write again to ask if you have had time to give thought to planning for our next meeting. As you know, the next step of our project involves sharing the results of testing that was done with those family members who chose to provide samples. In the previous letter I sent, I mentioned that we wanted to ask you about the best way to coordinate this step.

By way of review, we have considered two options for this visit. The first is to again meet at your home or that of another family member and have your family gather to individually receive their results. If you choose to do this, we can provide some funding to traveling family members to help offset their expenses. However, we also thought that if hosting so many people is difficult, or it is too great a burden to travel during winter weather, we are also able to meet families and individuals in smaller groups at their own home or one near to them, discussing test results with families at each of those locations. This, of course, could only be done if there are family members who would be comfortable with several of us coming into their home.

In addition to discussing test results at our next visit, we will be able to make arrangements to help individuals provide samples if they were unable to do so at our last meeting. We will come prepared for this possibility, so family members do not have to decide in advance if they want to join the study.

It might be easiest to start to plan this next visit over the telephone. Please send a return letter or call my assistant Lori Andrews at (412) 692-7775, so she can schedule a time for us to speak. We look forward to hearing back from you at your earliest convenience. Thank you again for your assistance with planning this study.

Regards,

Jerry Vockley, M.D., Ph.D.

APPENDIX L: RESPONSE LETTER REGARDING STUDY VISIT TWO

This letter was sent in response to a missed phone call from the index family, during which the patriarch requested that heteroplasmy results for all participants be mailed in individual sealed envelopes to the index family for distribution to extended family members at their next gathering.

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Jerry Vockley, MD, PhD
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Graduate School of Public Health
Chief of Medical Genetics
Children's Hospital of Pittsburgh

January 20, 2015

Mr. [redacted],

I'm sorry I missed your recent phone call. We are still very much interested in planning a return visit to Mercer to return results of our research testing on your extended family. Unfortunately, according to our approved protocol, we need to do this in person. If early spring is better for you and your family, we can certainly schedule something then. Would March be too early? If we can come then, it would give Afifa time to use the additional information to complete her project in time for graduation. Otherwise, please let me know when would be convenient. As I mentioned in my last letter, we can help pay for transportation for family members to come to a common meeting. Also, we do not necessarily need to have everyone together at one time. We are willing to travel to other family members' houses if that would be easier.

For your information, we are now planning to open a clinic at the UPMC Hermitage office dedicated to seeing our Amish patients with genetic disorders. It will likely be a joint clinic with Amy Goldstein in case families have neurologic issues to be addressed. I hope that we can have the clinic open by the spring as well.

Please feel free to contact me by phone or mail to discuss things further.

Regards,

Jerry Vockley, M.D., Ph.D.

APPENDIX M: RESULTS DISCLOSURE VISIT PROCEDURE OUTLINE

Results Disclosure Visit Procedure Outline

- Individuals present at each site include: MD &/or individual disclosing results (LGCG, MS GC student under LCGC or MD supervision, or MD)
- In a group setting, the following will be reviewed:
  - General review of the inheritance pattern of MELAS
    - If male, cannot pass to children; if female, can pass different H to all children, so they may present with no symptoms, few symptoms, or many symptoms, etc.
  - Review the symptoms of MELAS
  - MD will provide information regarding treatment and management of MELAS
  - Provide business cards for specialist co-investigator MDs for future questions or care coordination
- Disclosure will be made to those who had testing along with any nuclear family members the study participant wishes to be present
  - Disclosure should maintain privacy to the highest degree possible
- Information to include in disclosure:
  - The individual's heteroplasmy level (H) & that of their child, if tested:
    - If H=0%:
      - Explain that if this individual were ever to develop any of the symptoms seen in MELAS that they should not be treated as having MELAS and should instead seek care based on the presence of the symptom alone
    - If 0>H>~20% & patient seems asymptomatic and is female:
      - Explain that while she is not experiencing symptoms of MELAS, she can still pass MELAS onto her children, both daughters and sons.
        - Explain that her children may experience a variety of symptoms and that one child may be more severely affected than others. No child's presentation can provide information regarding how another child might present.
      - Explain that while she is not experiencing symptoms of MELAS now, she may develop symptoms in the future, so if she experiences any health problems that could be symptoms, she should seek care and provide her physician with information regarding her H/MELAS
    - If 0>H>~20% & patient seems asymptomatic and is male:
      - Reaffirm understanding of mitochondrial inheritance and explain that he cannot pass MELAS onto his children because they will inherit their mother's mtDNA.
    - If ~20<H<~40%, and the patient is experiencing symptoms:
      - Explain that the patient's symptoms are likely related to MELAS

        - Explain that while we cannot make clear predictions of clinical features to present later, and that those individuals with lower H may experience fewer or milder symptoms than those with higher H.
    - If H>~40% and the patient is experiencing symptoms:
        - Explain that the patient's symptoms are most likely related to MELAS
        - Explain that while we cannot make clear predictions of clinical features to present later, those individuals with lower H may experience fewer or milder symptoms than those with higher H.
    - If H>~40% and the patient is NOT experiencing symptoms:
        - Explain that the patient may have a higher likelihood of experiencing symptoms of MELAS in the future, and she/he should be monitored moving forward
        - If patient is female:
            - Explain that her children may experience a variety of symptoms and that one child may be more severely affected than others. No child's presentation can provide information regarding how another child might present.
            - Explain that while she is not experiencing symptoms of MELAS now, she may develop symptoms in the future, so if she experiences any health problems that could be symptoms, she should seek care and provide her physician with information regarding her H/MELAS
- For those who have symptoms, an MD will provide information regarding treatment and management of those symptoms
- Provide business cards for specialist co-investigator MDs for future questions or care coordination

- Coordinate sample collection and informed consent for those who want to participate in the study moving forward

APPENDIX N: TEMPLATE GENETIC COUNSELING SUMMARY LETTER

This is the template of the summary of genetic counseling letter that was sent to each participant to whom results were disclosed.

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April __, 2015
Patient Name
Patient DOB

Dear *Patient Name*,

It was very nice seeing you on March 21, 2015 to discuss the results of your genetic testing for MELAS. This was done by heteroplasmy testing performed through the research study PRO14040237 at the Children's Hospital of Pittsburgh of UPMC and the University of Pittsburgh. The first page of this letter contains a summary of your personal results and recommendations, and the following pages contain more information about MELAS and mitochondrial disease. You may want to keep this letter with your other medical records or other important documents.

- Your test results showed __% heteroplasmy, or said another way, ___% of your mitochondria have the genetic change that causes MELAS.
- As we discussed at our last meeting, we recommend the following supplements: _____ *(insert personalized recommendations here)*
- As we discussed, this information is important for other members of your family, particularly for your children. Because MELAS can be passed from a mother to her children, it may be useful to test your children to find out if they would benefit from preventative supplements or additional medical studies. This testing is important because of the broad range of symptoms we see in individuals with MELAS. If you are interested in organizing this testing, please contact us at your earliest convenience to discuss a plan. Please send a return letter to the address at the top of this letter or call my assistant [redacted] at [redacted], so she can help with scheduling. We are happy to come to your home to collect samples and review information about MELAS. If you would prefer another option, we will be available at the UPMC Hermitage Hospital in a clinic for our Amish patients with genetic disorders beginning in July. If you prefer to make an appointment at this clinic, please call our scheduler, [redacted] at [redacted].

Please feel free to call or write at any time if you have any questions or concerns regarding this information. We look forward to hearing back from you at your earliest convenience.

Regards,

Jerry Vockley, M.D., Ph.D.
Professor of Pediatrics
School of Medicine
Professor of Human Genetics
Graduate School of Public Health
Chief of Medical Genetics
Children's Hospital of Pittsburgh

Lina G. Gonzalez, M.D.
Post Doctoral Associate
Medical Genetics Department
Children's Hospital of Pittsburgh

Date
Patient Name
Patient DOB
Research Study: PRO14040237

Dear Patient Name,

It was very nice to see you on March 21, 2015 to discuss the results of your genetic testing for MELAS. This was done by heteroplasmy testing performed through the research study PRO14040237 at the Children's Hospital of Pittsburgh of UPMC and the University of Pittsburgh. This letter contains a summary of our discussion at that time. You may want to keep this letter with your other medical records and important papers.

As noted on the cover page of this letter, the highest level of heteroplasmy we found in you was __% heteroplasmy for the genetic change that causes MELAS. This genetic change is called the m.3243A>G mutation in the *MT-TL1* gene. When discussing this result, we reviewed the meaning of mitochondria and mitochondrial disorders and the inheritance pattern and symptoms of MELAS.

If you remember from our presentation and handout, we have trillions of cells in our body, and each one has a different function. There are cells that make up our muscles, our bones, our eyes, our hair, and every other part of our bodies. Within each of our cells, there are many different parts that each do a specific job for the cell. However, we focused on only one of these pieces of the cell, called mitochondria. Mitochondria are special structures found in almost every cell of our bodies. They are found in so many cells because this special structure is responsible for producing 90% of our body's energy.

The body's cells that need more energy to function, such as muscle or brain cells, have more mitochondria. When mitochondria do not produce enough energy for the cell, for example if a genetic change that causes a disorder is present in the mitochondria, this may hurt or kill the cell. If this happens to enough cells in one organ, such as the muscle or the eye, that organ may not work correctly. Each of our body's organs, like the muscle or the brain, require different amounts of energy, so they may not all be affected in the same way when mitochondria do not work correctly. Those organs that use more energy to work may be affected first or may be affected more severely. Since we know there are mitochondria in almost every cell in our bodies, there are many different ways mitochondrial disorders can affect a person.

The specific mitochondrial disorder we discussed in your family is called MELAS. It is caused by a genetic change in the mitochondrial DNA. Remember that this is the genetic material found within mitochondria. Because there are hundreds of mitochondria in the body each containing several copies of mitochondrial DNA, not all cells have the genetic change. Having a mixture of mitochondria with the genetic change and mitochondria without the change is called "heteroplasmy." The testing done on you is a measurement of your heteroplasmy, or a measurement of the amount of mitochondria with the genetic change.

Mitochondria are passed down from mothers to their children. This is called maternal inheritance. It means that if a woman has the genetic change causing MELAS, her children will also likely have some mitochondria with the same genetic change. If a woman has a low level of heteroplasmy, her children can inherit any level of heteroplasmy. This means that knowing a mother's heteroplasmy does not provide any information about her child's level of heteroplasmy. Because mitochondria are passed from mothers to their children, men who have heteroplasmy

cannot pass MELAS to their children. However, they are still at risk for all the symptoms of MELAS. Testing the children of any woman with MELAS may be useful to find out if they would benefit from preventative supplements or additional medical studies.

There are many symptoms of MELAS. Not everyone who has heteroplasmy for the MELAS-causing genetic change will have all the symptoms of MELAS. Some people may have many of the symptoms, and some people may have very few. MELAS can affect the body in many ways because it can cause symptoms in any body system that uses mitochondria.

Some of the symptoms of MELAS are related to the muscles. Hearing and vision loss can also be found in people with MELAS. Some individuals may have strokes, seizures (also called spells or convulsions), or stroke-like episodes. Delayed development, learning disorders, and being slow in school may also found in people with MELAS. Some people may have muscle weakness or pain or cramping. Others may have loose, floppy muscles or extreme tiredness, (also called fatigue). The digestive tract can also be affected, and some of these signs of MELAS include constipation, diarrhea, nausea, vomiting, or reflux. Diabetes, kidney failure, and heart problems can also be related to MELAS. There are other symptoms that could be caused by MELAS, but these are the most common ones.

There is no cure for MELAS, but management is available. These options are specific to each person and should be decided by a doctor. Details of your own management recommendations can be seen on the cover page of this letter.

Please feel free to call or write at any time if you have any questions or concerns regarding this information.

Regards,

Jerry Vockley, M.D., Ph.D.
Professor of Pediatrics
School of Medicine
Professor of Human Genetics
Graduate School of Public Health
Chief of Medical Genetics
Children's Hospital of Pittsburgh

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