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The Effects of Chronological Age on Mitochondrial DNA (mtDNA) Deletion in Muscle and Blood of Older Individuals: A Maternal Line Study

GRADUATE THESIS

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By

Roy Ralph Wittock B.Sc. (Hon.) ©
Advisor: Dr. Robert Thayer
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ABSTRACT

Since Harman [1] first proposed that the mitochondria plays a role in the aging process, several lines of research have been undertaken to support his theory. According to the theory, oxygen free radicals are responsible for the age-associated decline in function at the cellular, tissue and organ levels. The mitochondria use oxygen at a high rate, but due to a dysfunction in its usage of oxygen the mitochondria releases oxygen free radicals, which exceeds the cellular antioxidant defense. The mitochondrial genome, however, is extremely susceptible to oxidative damage and DNA mutations, including deletions when compared to nuclear DNA. One of the causative agents of this deletion(s) includes oxygen free radicals, by-product of ATP (energy) production. The most frequent deletion associated with aging and diseases is known as "the common deletion" or 4977 base pair (bp) deletion. In recent years a number of studies have been able to detect mitochondrial DNA (mtDNA) deletions in various tissues from individuals over 20 years of age. However, these studies were all cross-sectional in nature and the DNA sources were all post-mitotic tissues such as muscle and brain. Mitotic tissues, such as blood were not used even though acquiring blood is usually less invasive and would be more convenient to study the aging process. Also, since aging occurs over time a longitudinal study design would be the most appropriate model for the detection and quantification of mtDNA deletion(s). It has been well documented that identical mtDNA is transmitted from mother to child at the point of conception, and therefore monitoring the incidence of mtDNA deletion(s) by employing a maternal line across several generations would mimic a longitudinal study model. Therefore, the first purpose of this study is to mimic the benefits of a longitudinal study design by employing maternal lines (3 & 4 generations) to establish mtDNA deletion(s) as a sensitive, specific and stable biomarker for studying the aging process. The second purpose of this study is to determine the differences in mtDNA deletions when comparing muscle and blood. There were 75 healthy participants with age ranging from 8 months to 99 years. The participants provided 71 blood samples and 27

muscle biopsies. Four participants (two Great grandmother: lab #s 9 & 30 and two Daughters: lab #s 8 & 63) belonged to more than one generation. Although four individuals were classified for more than one generation, their data was entered only once and analyzed accordingly. After accounting for the repeated results and the withdrawals there was a total of 80 blood sample results and 27 muscle biopsy results. Using the Polymerase Chain Reaction (PCR), mtDNA from blood and muscle samples were amplified and the proportion of total and deleted mtDNA determined using ethidium bromide staining and a gel electrophoresis analysis system. The proportion of deleted mtDNA was compared between different age cohorts and between blood and muscle samples using the following statistical methods: i) A one way analysis of variance (one way ANOVA) was used to determine the significant difference between the different age cohorts and it was observed that the age cohorts differed significantly. ii) Further, a post hoc analysis using the Tukey HSD test proved statistical significance for all the age cohorts studied. iii) The McNemar Chi-Square was used to determine the significant difference of the proportion of deletion for the matched pairs (muscle versus blood) and it was observed that there was no significant difference between the blood and muscle samples. iv) In addition, the Kappa Statistic was used to evaluate the measure of agreement on the proportion of 4977-bp deletion in blood and muscle samples, and it was observed that there was no significant agreement between the two samples. v) Further, a Chi-Square test was used to determine the significant difference of the proportion of mtDNA deletion from each sample. It was observed that the proportion of deletion in blood was statistically significant ($p < 0.05$) across four (4) age cohorts studied. Also, the proportion of deletion in muscle was statistically significant across three (3) age cohorts studied. vi) Finally, a post hoc pairwise comparison was used to locate the significant difference ($p < 0.05$) in each of the chi-square analyses and it was observed that GGM were significantly higher ($p < 0.05$) in the proportion of 4977-bp deletion for blood versus GD and GGC. Similarly, daughters (D) were significantly higher ($p < 0.05$) than their daughters (GD) and their granddaughters (GGC). Also in muscle, GGM were significantly lower ($p < 0.05$) than their

granddaughters (GD) in the proportion of 4977-bp deletion. Likewise daughters (D) were significantly lower ($p < 0.05$) than their daughters (GD). No significant difference reported in the proportion of deletions between blood and muscle samples would indicate that either blood or muscle could be used as a source for the isolation, detection and quantification of mtDNA deletions. However, it must be noted that in blood samples there was a trend toward greater proportion of deletion as age increased but for muscle samples, the reverse was true. We reported for the first time the use of a maternal line model to explain the effects of time on mtDNA deletion and aging. In addition, from a technical standpoint, we were able for the first time to isolate, elute and quantify mtDNA from both blood and muscle samples that has been isolated, eluted and quantified from blood presence that were stored on FTA Cards. Finally, in our research we report for the first time the existence of the deletion in all infants in the absence of any known mitochondrial disorder suggesting factors other than chronological age may be responsible for this deletion.

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April, 2002

Roy Ralph Wittock

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INTRODUCTION

Considerable research has been conducted in an attempt to explain the factors that cause aging. The multifaceted decline of functions during human aging supports the view that aging is the result of multiple underlying causes. One of the most prominent hypothesis employed to explain aging is the Error Accumulation Hypothesis, first proposed by Harman in 1956 [1] and states that as cells age they accumulate errors or mutations, some of which have been identified as mitochondrial DNA (mtDNA) deletions. The mitochondrion has been cited as the organelle implicated in this accumulation of errors. However, little research has been conducted employing a longitudinal study design to support the Error Accumulation Hypothesis and show the relationship between the mitochondria and the aging process over time. Also, there has been limited work performed on isolating, detecting and quantifying these deletions in a mitotic tissue such as blood.

The following introduction will discuss the main function of the mitochondria within the cell, its vulnerability, and the main factors resulting in its damage leading to severe consequences for the cell. This introduction also looks at the existing research designs and the types of tissue samples used for analyzing error accumulations in the mitochondria. In addition, several studies have been used to explain the relationship between the mitochondria and the aging process. These studies have attempted to explain the mechanism surrounding the Error Accumulation Hypothesis and how over time the mitochondria could play a key role in the aging process. This overview will also examine the evidence and discuss its application in attempting to elucidate the molecular basis of aging.

Aging

Aging is defined as the progressive accumulation of various changes with time both at the molecular and cellular levels, resulting in an increased susceptibility of humans to diseases and

ultimately death. [2] However, the exact causes and the molecular mechanisms surrounding the aging process remain unclear. Presently, there are three popular hypotheses attempting to explain the aging process, all of which seem to have an impact on the mtDNA: There is Programmed Cell Death or apoptosis, [3] which is cellular degradation, initiated by a genetic factor or certain chemical or morphological changes within the mitochondria. [3] In addition, there is the Telomere Hypothesis [4] which proposes that DNA sequences within the nucleus of the cell shorten during cell division and over time could lead to the loss of functionally important regions, which could ultimately affect the mitochondrial genome. [4] Finally, there is the Error Accumulation Hypothesis [1], which is the major focus of this review. This hypothesis suggests that the mitochondria, because of its constant production of energy in the form of adenosine triphosphate (ATP) and a very harmful by-product, oxygen free radicals, the structures within the mitochondria are exposed to the harmful effect of the free radicals. [5] Since the mtDNA is located in the matrix, [6] where there is a complex mixture of soluble enzymes in close proximity that catalyze the reactions involved in respiration, it becomes highly vulnerable to oxidative damage. [5] In addition to mtDNA damage, ultra structural and physiological changes such as swelling and rupture of the outer mitochondrial membrane destroy the integrity of the entire mitochondrion. [7,8,9,10] With time, the cells accumulate increasing levels of damage or mutations within macromolecules such as proteins, lipids and DNA, [1] resulting from continuous exposure to oxygen free radicals. Accumulation of these mutations over time diminishes their capacity to generate ATP, thereby disrupting cells, tissues and the whole organism's ability to function efficiently. [11] Consequently, there is age- associated impairment of cells and tissues resulting in aging characteristics, a variety of diseases and subsequent death. [12] Considering the multiple factors contributing to the aging process and its complexity, why should a single organelle, the mitochondrion, play such an important role in the aging process? The following section will address this question.

The Mitochondrion

Key Function of Mitochondria

The mitochondrion is the chief site for energy (ATP) production. [5] It produces 90% of the cell's ATP through the coupling of oxidative phosphorylation to mitochondrial respiration in human and other animal cells. [5] The driving force for cellular activity is the conversion of chemical bond energy to mechanical energy. [13] The most efficient path for ATP regeneration is through oxidation of the local stores of glycogen and fat in the body. [13] Built into the folding or *cristae* of the inner mitochondrial membranes are a series of molecules that serve in electron transport during oxidation. This electron-transport chain consists of flavoprotein, coenzyme Q and a group of iron-containing pigments called cytochromes. [14] These molecules of the electron transport system are fixed in position within the inner-mitochondrial membrane in such a way that they can collect electrons from reducing agents such as NADH and $FADH_2$ and transport them in a defined sequence and direction for the phosphorylation of (adenine di-phosphate) ADP to form ATP. [14] At the very last step of this oxidation process, the cytochromes will donate electrons to oxygen where the oxygen, an electron acceptor becomes reduced and water is formed. [13]

The Size of the Mitochondrial Genome

The mitochondria have their own DNA, which generally accounts for less than 1% of the total cellular DNA. [15] MtDNA is the only extra-chromosomal DNA in human cells and it exists as a 16, 569-base pair (bp) double-stranded closed circular molecule that is packed into nucleoid structures within the matrix, [6] of the mitochondrion. [5] The relatively small size of the mitochondrial genome made it possible for it to be sequenced and published in 1981. [5] It revealed that the entire mitochondrial genome, with the exception of the D-loop, encodes either a key respiratory chain protein or a crucial tRNA or rRNA molecule. [16] It further revealed that mtDNA carries the information for 37 genes that encodes 22tRNAs, 2rRNAs and 13 subunits of the oxidative phosphorylation complex. [5] The rRNA and tRNA molecules are used in the

machinery that synthesizes the 13 polypeptides. [5] The 13 polypeptides are subunits of the protein complexes in the inner mitochondrial membrane, including cytochrome b and subunits of NADH dehydrogenase, cytochrome c oxidase, and ATP synthase. [5] Although the nuclear DNA encodes the majority of enzymes involved in oxidative phosphorylation, the 13 sub-units derived from the mtDNA are essential for normal electron transport chain activity and ATP production. [5] Therefore, a symbiosis exists: the cell is dependent on the mitochondria for energy production, and the mitochondria are dependent on the cell for the majority of its structural and enzymatic proteins necessary for adequate function. [17] The nuclear genome is also involved in the regulation of mitochondrial activity, including the expression and replication of mtDNA. [18,19]

Maternal Inheritance of mtDNA

Mitochondrial DNA (mtDNA) is inherited maternally, [5,20] and the DNA transmission from mother to child is of high fidelity [21] (Figure A-1, page 65). Through the oocyte cytoplasm, the mother transmits her mtDNA to her offspring. The contribution of sperm to the mtDNA in a fertilized egg is insignificant. [22] Technically the child should have the identical mtDNA as the mother at the time of conception, therefore differences seen in mtDNA deletions within a maternal line could be the result of aging or environmental factors. In fact, Parsons et al. [21] reported that in the transmission of mtDNA, there is only one base substitution in every 33 (1/33) generations.

Susceptibility of mtDNA

Another feature of the mitochondria that makes it of interest when studying the aging process is the fact that its DNA has been shown to be very susceptible to damage. The mtDNA is naked [5] in that unlike nuclear DNA the genome does not bind to histones or other specific DNA-binding proteins that normally serve to protect nuclear DNA. As well, it is located near the inner membrane of the mitochondria (the matrix), [11] which is continually exposed to high levels of

oxygen free radicals generated through oxidative phosphorylation or extrinsic sources within the environment. [23,24] In addition, the compactness of the mitochondrial genome (no introns or intervening sequences between coding regions) increases the possibility that mutation will affect functionally important regions. [12,25] More over, it replicates more frequently than nuclear DNA and lacks proof reading or efficient DNA repair systems. [26,27,28] However the mitochondrial DNA polymerase, the enzyme that replicates mitochondrial DNA (DNA polymerase gamma) has some editing capabilities. [29] This is a common feature of DNA polymerases, which permits them to remove incorrect bases that have been added to the strand being synthesized (3'-5' exonuclease activity) and replace them with the correct one. [29] A wide variety of specific oxidative lesions in mtDNA (including abasic sites, thymine and cytosine lesions, 8-hydroxy-deoxyguanosine, and others) can all be repaired efficiently and therefore, suggests that a base excision repair mechanism also operate in the mitochondria. [29] In addition, there appears to be a functional alkyltransferase repair pathway within mitochondria. [29] However, there is evidence to suggest that the mechanism of nucleotide excision and repair systems which remove dimers do not function properly in mitochondria when compared to similar nuclear repair pathways. [29] Even with the existing repair mechanisms in operation, there remains a 10-fold greater mutation rate in mtDNA than in nuclear DNA, presumably due to the high level of oxidative damage. [13]

Oxygen Free Radical Formation

Like most manufacturing processes, the energy production system in oxidative phosphorylation produces toxic waste in the form of reactive intermediate oxygen species or oxygen free radicals, which have been shown to be very destructive to the mitochondrion. [1,31] These intermediates may be defined as any species capable of independent existence and possessing one or more unpaired electron(s). [32] Approximately 2-5% of the molecular oxygen that enters the mitochondria is thought to escape complete reduction to water. This reduction process has been calculated to account for 95 to 98% of the total oxygen consumption without any oxygen intermediates being

produced. [33] This small fraction (2 to 5%) of the oxygen consumed by cells, can be utilized in an alternative univalent pathway for the reduction of oxygen in which oxygen free radicals are produced. [13] Some examples of free radicals, which exist in the mitochondria are the hydroxyl (OH^-) ion, superoxide anion (O_2^-, an oxygen-centered radical), peroxyl (RO_2^-, radical intermediates arising as a result of lipid oxidation), and nitric oxide (NO^-). In addition, some nonradical oxygen-derived species are hypochlorous acid (HOCL), hydrogen peroxide (H_2O_2), singlet oxygen ($^-O_2$), and ozone (O_4). When these molecules are generated in cells, they aggressively attack and degrade nucleic acids as well as a variety of membranous structures, [32] and many other molecules. [34] Among all the reactive species, the hydroxyl radical may be formed either through reduction in the iron catalyzed Fenton reaction or through Haber-Weiss reaction. [13] This free radical is the most reactive chemical species in the biological world. [35] To counter the negative impact of the reactive species, living organisms have developed a sophisticated antioxidant system to cope with oxygen free radical generation. [36] The antioxidant system includes chemical compounds such as vitamin E and C, β-carotene, and glutathione, as well as a series of enzymes specializing in the removal of these free radicals from the body or reducing them to more stable species. [36] There is, however, a subtle balance, which exists between the generation of free radicals and the ability of the antioxidant system to manage. [35] When factors such as, chronological age, tobacco smoke and environmental toxins shift the balance in favor of excess free radical generation, it leads to deletions occurring in mtDNA. [10]

Age Association with the "common deletion"

The most prevalent deletion associated with aging has been shown to be the 4977-bp deletion or the common deletion. [37] It was demonstrated to span 4977-bp with 13-bp repeats located at both ends of the 8470 and 13, 447 nucleotide positions (np) which are presumable hotspots for the generation of the deletion, [38,39,40] and is detected predominantly in elderly humans. [41,42,43] Hayakawa et al. [44], suggested that the proportion of mtDNA deletion is increased exponentially

with age, estimating that the increase could be as high as a 1000. [45] In addition, multiple mtDNA deletions have been detected and the number of deletions in individuals over 70 has been reported to be significantly higher than those under the age of 40 years. [46] Several other researchers [47,48,49] have investigated the age association with the common deletion, observing the 4977-bp deletion in the brain tissue of two older yet normal subjects. Similarly, Cortopassi and Arnheim [50] demonstrated that brain and heart mtDNA of normal adults over age 21 contained very low levels of the 4977-bp deletion, but importantly, the amount of deleted mtDNA increased with age. Later, Yen et al. [42] reported the 4977-bp deletion in the liver mtDNA of normal human subjects in their mid 30's. In support of these results, Corral-Debrinski et al. [51] revealed that the 4977-bp deletion started to appear in human heart tissue at age 40. Further evidence to support the 4977-bp deletion as an aging deletion was the presence of the deletion in various tissues of individuals over 40 years of age. [12] The deletion was also shown to increase with age in skeletal muscle [52,53,54,55] and various other tissues [56,57,58] but was undetectable in fetal tissues such as heart and brain. [59] A more recent investigation conducted by von Wurmb et al. [55], reported no deletion specific fragments in fetal blood or in individuals under 20 years old. It is interesting to note that the age onset of the mtDNA deletions varied greatly with individuals in that a specific type of deletion may appear quite early in some individuals, but does not occur in others until very late in life. [59] The variability observed in the 4977-bp deletion suggests that a longitudinal study or a similar design is necessary to study mtDNA deletion over time. As can be seen in research conducted by Wei [59] a cross sectional study approach does not effectively account for the variability that is prevalent in mtDNA deletions among different individuals.

Disease Association with the "common deletion"

In addition to the common deletion, there have been more than 80 large-scale deletions identified in various tissues of humans and these deletions have been linked to specific mitochondrial diseases. [47] Although the concept of mitochondrial disease(s) was first introduced [60] as early as

1962, it was only in 1988 that two pioneering reports showed an association of mtDNA mutations with encephalomyopathies. [47,61] In recent years a variety of chronic degenerative diseases that involve brain, heart, muscle, kidney and endocrine glands have been shown to result from mutations in mtDNA. [62] The first pathogenic mtDNA mutations identified were associated with rare syndromes such as Leber's Hereditary Optic Neuropathy (LHON), Myoclonic Epilepsy, Ragged-Red Fiber disease (MERRF), Kearns-Sayre Syndrome (KSS) and, Chronic Progressive External Ophtalmoplegia (CPEO). [63,64] In addition, to the diseases associated with mtDNA deletion(s), mitochondria have also been shown to induce apoptosis [65] and play a non-specific role in some carcinogenic processes. [59] Since there is a clear link between mitochondrial dysfunction, specific human diseases and apoptosis, the relatively small size of the mitochondrial genome becomes an attractive target for genetic analysis. [5]

Apoptosis

The association between aging and apoptosis marks one of the most important recent developments in aging research. Apoptosis, or programmed cell death, is another prominent hypothesis used to better explain the aging process [3] and it suggests that mitochondria play a central role in the regulation of apoptosis. Similar to the Error Accumulation Hypothesis, apoptosis results in an age- associated impairment of cells and tissues resulting in aging characteristics, a variety of diseases and subsequent death. [12] The two hypotheses are not mutually exclusive, but rather complementary. In apoptosis, this form of cellular destruction is characterized by specific morphologic and biochemical changes [66] such as the degradation of chromatin. [67,66] This degradation of materials in the mitochondrion over time will diminish its capacity to generate ATP, thereby disrupting cells, tissues and the whole organism's ability to function efficiently. [11] In support of the role of the mitochondria in apoptosis, several major arguments have been presented that implicates the mitochondria as playing a major role in the process of apoptosis:

- The mitochondria undergo major changes in membrane integrity as a result of mtDNA deletion. These changes include swelling and rupture of the outer mitochondrial membrane and the subsequent release of pre-apoptotic proteins including cytochrome C (a key component in the induction of apoptosis) [68]. The release of mitochondrial cytochrome c leads to the cytosolic assembly of the apoptosome-a caspase activation complex involving apoptosis protease-activating factors (APAF) and caspase-9 which are both required for the activation of caspases and endonucleases in the apoptotic process. [68] (Caspases are a family of cysteine proteases implicated in biochemical and morphological changes in cells).
- Also, the mitochondria have large multi-conductance channels (permeability transition [PT] pores) that play a role in apoptosis. These PT pores are multi-protein complexes that participate in a variety of functions particularly the regulation of transmembrane potential (a proton gradient across the inner mitochondrial membrane generated during electron transport and ATP production). [15] In addition, the anti-apoptotic oncoprotein Bcl-2 (a nuclear encoded mitochondrial protein) [65,69] stabilizes mitochondrial membrane integrity and prevents opening of the PT pore. Changes in membrane integrity and the PT pores cause the disruption of the mitochondrial inner transmembrane potential leading to subsequent decrease in ATP production, cellular dysfunction and subsequent death. [15]
- Finally, oxidative stress imposed on cells as a result of oxidative phosphorylation can have a profound effect on the onset or progression of apoptosis. [15,70] Intriguingly, apoptosis induced by hydrogen peroxide formed during oxidative stress can be stopped or reversed by over expression of Bcl-2 [65,69] leading to the depletion of the Bcl-2 and the loss of anti-apoptotic capabilities within the mitochondrion.

Mitochondrial involvement is probably not required for apoptosis, however, mitochondrial processes may be important in the apoptotic pathway. [15] The above arguments have proven to be quite compelling in supporting the role of the mitochondria as critical to the aging process.

Blood and the "Common Deletion"

It was once believed that blood cells, like fetal cells, do not survive long enough to accumulate mtDNA mutations, and for this reason blood samples have often been used as a negative control. [43,42] Since blood cells from patients with mitochondrial abnormalities show rather low levels of mutated mtDNA, it has been proposed that frequent cell division in hematopoetic precursors select against the survival of cells containing defective mitochondria. [47] This intra cellular selection cannot take place in post-mitotic tissues like muscle and nerve and hence the high deletion rates in post-mitotic tissues. However, arguing against the occurrence of 4977-bp deletion exclusively in post-mitotic cells is the observation that more than 99% of peripheral blood lymphocytes subsist in the G_0 phase [22], which would imply that the deletion could be easily detected in blood. Further, it has been demonstrated recently, that about 40% of circulating lymphocytes have a life span that exceeds 6 months, and some even persist for over 30 years. [71] As a consequence there is enough time for these cells to accumulate mitochondrial alterations, which should be detectable using sensitive PCR techniques. [55] In addition, there is evidence that cells containing deleted or mutant mtDNA replicate efficiently and the cellular selection favoring the normal or wild type mtDNA may not be a rapid process. [63] The mutant mtDNA is smaller than the wild type mtDNA so it replicates faster, hence the advantage. [63] In addition, the ratio of mtDNA in both the wild type and the mutant type (heteroplasmy) will affect the functional ability of the cell. [63]

In an attempt to explain the association between specific tissues and their accumulation of the 4977-bp deletion, researchers have been able to demonstrate the presence of the 4977-bp deletion in well-differentiated tissues such as muscle and brain of older adults. [56,57,58] Small amounts of this deletion have also been detected in mitotic tissue such as the blood of patients with certain disease conditions. [73,74,75] With further research von Wurmb et al. [55] detected the 4977-bp deletion in blood of healthy persons. [42,76,43] Prior to this research Gattermann et al. [25] was the only

group to detect deletion-specific fragments in some blood and bone marrow samples of hematologically normal patients by using nested Polymerase Chain Reaction (PCR). In contrast, using an automated detection system (fluorogenic probes labeled with fluorescent dyes) [57], were unable to demonstrate any accumulation of the 4977-bp deletion with increasing age in whole venous blood, isolated human platelets and peripheral blood mononuclear cells, as was observed for several other tissues. [57] Therefore, the combination of limited research and conflicting results necessitated a study to address the question whether blood could be used to detect the 4977-bp deletion associated with aging. This is relevant as the acquisition of blood samples is less invasive and may serve as a reliable source for the study of the 4977-bp deletion with age. Also, developments in DNA analysis technologies such as PCR, using a more sensitive polymerase (Deep-Vent [exo-] DNA Polymerase) and a staining technique (ethidium bromide) along with more accurate detection systems (digital gel electrophoresis analysis) may make it much easier and more accurate to isolate and detect mtDNA deletion in mitotic tissues.

Research Design and the "Common Deletion"

Previously, researchers have used cross-sectional studies to examine the effect of time on mtDNA deletion. [70] However, since aging occurs over time, a longitudinal study design would be more effective in examining the effects of chronological age on mtDNA deletion and aging. It has been well documented that identical mtDNA is transmitted maternally via germ cells at the point of conception [5,20], and research evidence supports somatic expression of mtDNA deletion [77] that is, mtDNA deletion are not transmitted via germ cells. [78] This would imply that any mtDNA deletion seen in an individual would be a result of aging or environmental factors and not as a result of germ line mutation. Therefore, a research study using a maternal line extending over 3 or 4 generations would mimic the strengths of a longitudinal study design and overcome the limitations of cross sectional studies and more accurately determine the effects of time on

mtDNA deletion. Only a few studies, have used this approach to examine the 4977-bp deletion and aging.

Statement of the Problem

As revealed by the research literature there is an association between chronological age and the 4977-bp age associated mtDNA deletion. Also there is little doubt that the mitochondria play a pivotal role in the aging process. However, few, have examined mtDNA deletion overtime using maternal lines across different generations nor have they been able to detect mtDNA deletions in healthy individuals under the age of 20 years. Therefore, the first purpose of this study is to mimic the benefits of a longitudinal study design by employing maternal lines (3 or 4 generations) to establish mtDNA deletion(s) as a sensitive, specific and stable biomarker for studying the aging process, providing evidence to substantiate the Error Accumulation Hypothesis. Several researchers have demonstrated that mtDNA deletion(s) can be detected in muscle, a post-mitotic tissue using improved DNA analysis techniques, but only a few attempts have been made to isolate, detect and quantify mtDNA deletion(s) from blood, a more convenient mitotic tissue. Thus, the second purpose of this study is to determine the differences in mtDNA deletions when comparing muscle and blood.

Hypotheses

There are four null hypotheses in this study; the first ($H_{o\,1}$) null hypothesis states that the four age cohorts are similar and the second ($H_{o\,2}$) null hypothesis states that the proportion of 4977-bp deletion in a sample of blood will be equal to the proportion of 4977-bp deletion in a sample of muscle. The third ($H_{o\,3}$) null hypothesis states that the proportion of 4977-bp deletion in a sample of blood across four (4) age cohorts of i) Great grandmother (GGM), ii) Daughter (D), iii) Grand daughter (GD) and iv) Great grandchild (GGC) is equal and the fourth ($H_{o\,4}$) null hypothesis

states that the proportion of 4977-bp deletion in a sample of muscle across three (3) age cohorts of i) GGM, ii) D and iii) GD is equal.

METHODS

Participants

This study investigated the maternal line of 21 families (GGM, D, GD and GGC) each consisting of between three and four generations with only the last generation comprising males. There were 75 healthy participants ranging in age from 8 months to 99 years. The group provided 71 blood samples and 27 muscle biopsies (Figure A-2, page 66). Four participants (two Great grandmother: lab #s 9 & 30 and two Daughters: lab #s 8 & 63) belonged to more than one generation. Although four individuals were classified for more than one generation, their data was entered only once and analyzed accordingly. After accounting for the repeated results and the withdrawals there was a total of 80 blood sample results and 27 of muscle biopsy results (Table 1).

Table 1. Descriptive data on participants donating muscle and blood samples [♀] (1-30)

Family number	Generation	Age (years) [♂]	Lab number	Tissue	
				Muscle	Blood
01	GGC	24	1/85	Y	Y
01	GD	49	2/86	Y	Y
01	D	69	3/87	Y	Y
01	GGM	91	4	N	Y
03	GGC	4	5	N	Y
03	GGC	13	6	N	Y
03	GD	30	7/88	Y	Y
03	D	49	8/89	Y	Y
03	GGM	69	9	N	Y
04	GGC	2	10	N	Y
04	GD	42	11/90	Y	Y
04	D	69	12/91	Y	Y
04	GGM	91	13/92	Y	Y
05	GGC	5	14	N	Y
05	GD	24	15/93	Y	Y
05	D	52	16/94	Y	Y
05	GGM	74	17/95	Y	Y
06	GGC	5	18	N	Y
06	GD	35	19/96	Y	Y
06	D	63	20/97	Y	Y
06	GGM	83	21/98	Y	Y
07	GGC	8 months	22	N	Y
07	GD	19	23/99	Y	Y
07	GD	19	24/100	Y	Y
07	D	42	25/101	Y	Y
07	GGM	73	26/102	Y	Y
08	GGC	9	27	N	Y
08	GD	36	28	N	Y
08	D	58	29/103	Y	Y
08	GGM	80	30	N	Y

Table 1. (Continued) Descriptive data on participants donating muscle and blood samples[9] (31-57)

Family number	Generation	Age (years)[Φ]	Lab number	Tissue	
				Muscle	Blood
09	GGC	3	31	N	Y
09	GD	38	32/104	Y	Y
09	D	63	33/105	Y	Y
09	GGM	99	34/106	Y	Y
10	GD	18	35	N	Y
10	D	38	36	N	Y
10	GGM	69	37	N	Y
11	GGC	3	38	N	Y
11	GD	26	39/107	Y	Y
11	D	47	40/108	Y	Y
11	GGM	76	41/109	Y	Y
12	GD	24	42/110	Y	Y
12	D	49	43	Y	Y
12	GGM	69	44	N	Y
13	GGC	10 months	45	N	Y
13	GD	28	46	N	Y
13	GD	31	47	N	Y
13	D	48	48	N	Y
13	GGM	69	49	N	Y
14	WITHDREW		50		
14	WITHDREW		51		
14	WITHDREW		52		
14	WITHDREW		53		
15	GGC	15	54	N	Y
15	GD	33	55	N	Y
15	D	59	56/111	Y	Y
15	GGM	80	57	N	Y

Table 1. (Continued) Descriptive data on participants donating muscle and blood samples[φ] (58-84)

Family number	Generation	Age (years)[Φ]	Lab number	Tissue	
				Muscle	Blood
16	GD	30	58	N	Y
16	D	53	59	N	Y
16	GGM	78	60	N	Y
17	GGC	5	61	N	Y
17	GD	24	62	N	Y
17	D	49	63	N	Y
17	GGM	69	64	N	Y
18	GGC	4	65	N	Y
18	GD	26	66	N	Y
18	D	49	67	N	Y
18	GGM	69	68	N	Y
19	GD	20	69	N	Y
19	D	36	70	N	Y
19	GGM	69	71	N	Y
20	GGC	10 months	72	N	Y
20	GD	20	73	N	Y
20	D	44	74	N	Y
20	GGM	62	75	N	Y
21	GGC	29	76	N	Y
21	GD	49	77	N	Y
21	D	68	78	N	Y
22	GGC	23	79	N	Y
22	GD	58	80	N	Y
22	D	82	81	N	Y
23	GGC	8	82	N	Y
23	GD	46	83	N	Y
23	D	66	84	N	Y

[φ] GGM = Great grandmother, D = Daughter, GD = Granddaughter, GGC = Great grandchild.
[Φ] Unless indicated explicitly otherwise Y = present and N = absent

This study received the approval of the Lakehead University Ethics Committee and all the participants signed an informed consent (Appendices C-1 and C-2). Also each participant completed the Canadian National Public Health Survey [79] and the results obtained from the

survey were used to exclude individuals who were highly trained endurance athletes and those suffering from chronic, systemic or degenerative disorders (Appendix C-3). In addition, a selection criterion (Appendix C-4) was designed to exclude any participants that might have any factors other than chronological age that could have influenced the quantity of deleted mtDNA.

Procedure

Sample Collection

A blood sample (< 1ml) was collected from each of the participants (Appendix B-1). The samples were obtained from a finger prick and stored on a FTA Card™ (Life Technologies) following the manufacturer's instructions (Appendix B-3). Also, approximately 5-25mg of muscle biopsy tissue [80] was taken from the vastus lateralis muscle of 27 out of the 71 participants and stored at -80°C for subsequent use (Figure A-3, page 67). See (Appendices B-2 and B-3).

DNA Preparation

The muscle biopsy tissue was homogenized and emulsified in a buffer solution (600 mM NaCl, 15mM Tris, pH 7.5) and a 100 µl of the total volume applied to each circle on a labeled FTA Card™ (Life Technologies) following the manufacturer's instructions. At the time of homogenization all fat and connective tissue were removed from the samples and each sample was weighed. To remove blood, the muscle samples were washed several times in Tris buffer (pH 7.5). Following the wash, the samples were minced with a scalpel and brought up to 20 volumes with respect to their original weight. The muscle samples were homogenized on ice using a disposable hand held homogenizer (1.5 ml Disposable Pellet Pestle), [Kimble/Kontes, Vineland, New Jersey USA]. Total DNA (nuclear and mitochondrial) was extracted from both the blood and muscle samples using the FTA purification Protocol (Life Technologies) according to the manufacturer's instructions. Total DNA was then eluted from the FTA Card™ (following

modifications to the manufacturer's instructions) by adding 100µl of TE to the tubes containing 2 to 3 purified paper punches and the samples incubated at 4°C for 24hrs. The concentration of total DNA in each sample was determined by spectrophotometry (GeneQuant II RNA/DNA Calculator, Pharmacia Biotech [Biochrom] Ltd., Cambridge, England) and then adjusted to 1 ng/µl (Appendix B-4).

Oligonucleotides, Standards and Controls

HPLC purified oligonucleotide primers were chemically synthesized by Operon Technologies, Inc. (Almeda, Ca), using nucleotide sequences obtained from the literature. [5] The sequences of the synthesized oligonucleotides and the sizes of PCR products amplified are shown in Table 2. In addition to negative controls (TE buffer: 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) that was used in the DNA purification and elution stages), our PCR analysis for mtDNA deletions always included positive controls (DNA from a patient known to carry the common deletion). The size standard pBR322 DNA- *Msp* 1 Digest (New England BioLabs Inc.) was used to determine the location of the DNA band of interest and also as a reference during the quantification phase.

Table 2. Oligonucleotide primers used for PCR amplification of the 4977-bp deleted mtDNA and total mtDNA

Primer pair	Amplified Position 5'- 3'	The length of amplified product (bp)
T_1T_2 *	1257 - 1433	177
D_1D_2 ᵠ	8416 - 13519	127

T_1 (1257 - 1279) 5'-TATACCGCCATCTTCAGCAAAC-3'
T_2 (1433 - 1411) 5'-TACTGCTAAATCCACCTTCGAC-3'
D_1 (8416 - 8437) 5'-CCTTACACTATTCCTCATCACC-3'
D_2 (13519 - 13498) 5'-TGTGGTCTTTGGAGTAGAAACC-3'

* The primer set T_1T_2 was used for the determination of total mtDNA
ᵠ The primer set D_1D_2 was used for the determination of deleted mtDNA

The primer pair (T_1: nucleotide position 1257 and T_2: nucleotide position 1433) were used to amplify the 177-bp fragment from the 12s region, a highly conserved area [5] of the mitochondrial

genome which is known to be free of mutations, specifically deletions, and therefore should always be present for detection and amplification. This was used to represent total mtDNA or a positive control. The primer pair (D_1: nucleotide position 8416 and D_2: nucleotide position 13519) was used to amplify the deletion-specific 127-bp fragment from a conserved region [5] flanking the 4977-bp deletion indicating the presence of the 4977-bp mtDNA deletion (Figure A-4, page 68).

Polymerase Chain Reaction (PCR)

Two separate PCR's were performed on each sample, amplifying the two desired sets of DNA fragments. Each PCR reaction was performed in a 25 µl reaction mixture containing; template DNA, one pair of primers (T_1T_2 or D_1D_2) and each deoxyribonucleoside triphosphate (dNTP), $MgSO_4$, thermopol reaction buffer, Deep-Vent (exo-) DNA Polymerase and Bovine Serum Albumin (BSA) all obtained from New England BioLabs Inc. The volume and concentration of the template DNA, primers, dNTPs, $MgSO_4$, buffer, polymerase and BSA are listed in Table 3.

Table 3. The volume and concentration of the different reagents required for a 25 µl PCR reaction mixture.

Reagent	**Concentration of Stock Solution**	**Concentration per Reaction**	**Volume per Reaction**
Buffer	10X	1X	2.5 µl
$MgSO_4$	100 mM	1X (2 mM)	0.5 µl
BSA	10X	1X	0.25 µl
dNTPs	100X (100 mM)	1X (1 mM)	0.25 µl
Polymerase	2000 U/ml	0.5 units	0.25 µl
Primers	20 µM	0.2 µM	0.5 µl
ddH_2O	N/A	N/A	15.75 µl
Template DNA		1ng	5.0 µl
Total			**25 µl**

Samples were amplified in a PTC-225 DNA Engine Tetrad Thermocycler (MJ Research, Inc., Waltham, Massachusetts). The first cycle consisted of a hot start at 95°C for 5 min followed by 30 cycles consisting of a denaturation step at 94°C for 30s, annealing at 60°C for 1min, and primer extension at 72°C for 30 sec, with a final extension for 10 min at 72°C (Appendix B-5).

Polymerase Sensitivity

Mitochondrial DNA comprises less than 1% of the total DNA [81] and the highest mean concentration of any particular mutation such as the 4977-bp deletion may be maximally about 1 % in any particular tissue homogenate. [82] Therefore, the amplification of any fragment of the mtDNA representing the 4977-bp deletion will require a very sensitive PCR polymerase. Deep-Vent (exo-) DNA Polymerase was chosen because when compared with other polymerases, it was more sensitive in detecting low concentrations (as low as 10 fg) of DNA fragments. [83] According to Hilali et al. [83], the use of this Polymerase might prove to be a universal PCR method in amplifying very low levels of DNA.

Detection System

Following amplification, for each participant, 4µl of the total and 4µl of the deleted PCR product (alternating between total and deleted samples) were loaded in separate lanes on the same gel (6% polyacrylamide gel). Also, 4µl of the size/reference standard (pBR322 DNA- *Msp* 1 Digest) and 4µl of the appropriate control were loaded in separate lanes. The gel was allowed to run at 140V, 500mA for 45 min (Appendix B-6). After separation, the DNA bands were visualized by transillumination under UV light after staining with ethidium bromide [50µg/ml], (Figure A-5, page 69). See (Appendix B-7).

Quantification of DNA Bands

Quantification of amplification products was performed using the Kodak Electrophoresis Documentation and Analysis System [EDAS] (Eastman Kodak Company, Rochester, NY) and image analysis software (Kodak 1D Image Analysis Software). Each gel contained 4µl of the size standard pBR322 DNA- *Msp* 1 Digest, at a concentration of 400ng. The standard produces 16 bands ranging from 76-bp to 622-bp. When calculating the band mass of each sample, the software determines total area (the leading and trailing edges) of each band and then sums the intensity of each pixel within the band rectangle (Appendix B-7). During these calculations, the software determines the intensity of the background of the image and adjusts the results to calculate the true band intensity. Using the band intensity measured for each standard band in the standard lane, 1D analysis software models the data using different functional forms: power, exponential.2nd and 3rd order polynomials to generate a fitted curve (Figure 1).

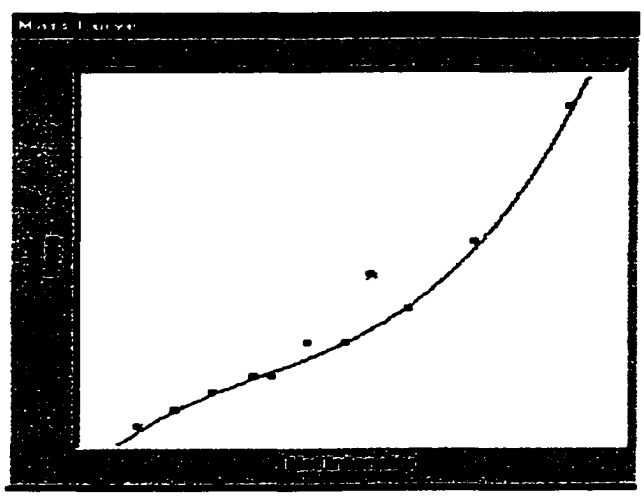

Figure 1. A standard fitted curve of the mass versus the net intensity of each standard band generated by the Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY)

To determine the mass in nanograms (ng) of total and deleted bands in each sample the true band intensity for each sample is plotted against the standard fitted curve. The 177-bp fragment from the highly conserved region of the mitochondrial genome was used to represent total mtDNA,

whereas the 127-bp fragment represented deleted mtDNA (4977-bp). Following this, a ratio (mass of deleted fragment divided by mass of total fragment) was determined for each sample. To avoid false positive results and address the issue of template or reagent contamination, each PCR reagent along with blank FTA Card punches and negative controls (TE: 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) were simultaneously processed along side FTA Card punches containing sample. In addition, selected fragments of the 12s region (highly conserved region of mtDNA) and the Hypervariable Region 2, (HV2) of the template mtDNA were sequenced (ABI Prism 310 Genetic Analyzer, PE Applied Biosystems, Foster City California) to confirm the presence of single template DNA and to establish a maternal line relationship. The results suggested that each sample contained only single template DNA indicating that there was no contamination and the 12s and HV2 regions of the mtDNA of all the members in each maternal line were identical, indicating that members within each maternal line were clearly related. Finally, to address the issue of reproducibility the entire methodology was repeated several times under the same conditions and the results were the same (Appendix B-9).

Statistical Analysis

The statistical analysis was conducted using the following six step process:

i) Since each age cohort represented a generation a one way analysis of variance (ANOVA) was used to determine the significant difference between the different age cohorts. ii) Further, to determine the source of the significant difference between the age cohorts a post hoc Tukey HSD test was used. iii) Considering that we sampled both blood and muscle from each patient, the McNemar Chi-Square test was used to determine the significance of the difference for the matched pairs (muscle versus blood). iv) In addition, the kappa statistic, described by Montelpare and McPherson [84], was used to determine the extent to which muscle and blood samples were similar or in agreement. v) A chi square test was used to determine the significance of proportion from several independent samples (blood and muscle) in different age cohorts. This technique

was described by Fleiss [85] and is commonly used to evaluate designs in which proportion of events comprise the data. vi) Finally a post hoc pairwise comparison was used to locate the cause of the significant difference within each chi-square for blood and muscle samples. Additional material further outlining the methodology used in this research is shown in (Appendix B).

RESULTS

In this study, the use of a unique research design, optimization of the PCR protocol, [using longer primers, a more sensitive polymerase (Deep-Vent [exo-] DNA Polymerase) and targeting shorter amplification fragments], we were able to effectively determine the proportion of the 4977-bp deletion in both blood and muscle samples from participants of different age cohorts, some as young as 8-months old. Moreover, the fact that each child in the study presented the deletion was significant and totally unexpected. The detection of this deletion may be partly explained by the method used to isolate, elute and quantify mtDNA from blood and muscle samples stored on FTA Cards. In addition, the high sensitivity of Deep-Vent [exo-] DNA Polymerase and its ability to detect DNA template at very low template concentration may have played a critical role in the ability to amplify the 4977-bp deletion in blood samples.

Further, we employed a maternal line for the first time to mimic a longitudinal study design and effectively investigate the effect of chronological age on mtDNA deletion. This unique research design consisted of four (4) age cohorts [Great Grand mother (GGM), Daughter (D), Granddaughter (GD) and Great grandchild (GGC)]. Each age cohort was revealed to be statistically significant ($p < 0.05$) from each other. Our results demonstrated that in blood samples there was an increased proportion of deletion as age increases, but for muscle samples, the reverse was true. We believe that the combination of an improved methodology and the use of a maternal line model enabled us to present some of the most relevant findings to date

attempting to explain the role of the 4977-bp deletion in the aging process. The results are outlined in the following section.

Statistical Significance between Age Cohorts

The 21 maternal lines provided a fairly large sample size (n = 71) and a wide distribution of ages across the cohorts (Table 4). The age ranged from 8 months old to 99 years old and enabled us to successfully investigate the effects of time on mtDNA deletion extending almost ten decades.

Table 4. Distribution of ages (years)[Φ] among generations included in the study[•]

Variable	GGM	D	GD	GGC
Mean	79.6	52.2	31.5	8.6
Minimum	62	36	19	1
Maximum	99	82	58	29
Standard deviation	10.8	9.6	11.1	8.7
Sample size	18	21	23	18

[•]GGM = Great grandmother, D = Daughter, GD = Granddaughter, GGC = Great grandchild.
[Φ]Unless indicated explicitly otherwise

Since the maternal line design was based primarily on the analysis of mtDNA deletion across four age cohorts it was necessary to ensure that all the age cohorts were significantly different from each other. Therefore a one way analysis of variance (one way ANOVA) was used to test for significance. The results of the analysis of the one way ANOVA shown in Table 5 indicated that there was significance difference ($p < 0.05$) across the four age cohorts studied.

Table 5. ANOVA summary for the different age cohorts

ANOVA Summary Table				
Source	**SS**	**df**	**MS**	**F**
Between-Group	40670.37	3	13556.79	133.3
Within-Group	6815.33	67	101.72	
Total	47485.7	70		
*** $p < 0.05$**	**F = 133.3**	**Critical Value = 2.51**		
For there to be statistical significance the F value must be greater the critical value				

To test for significant difference across the different age cohorts a post hoc Tukey HSD test was used to locate the source of significant difference. The results shown in Table 6 indicated that all the age cohorts were significantly different ($p < 0.05$) from each other.

Table 6. Summary of the Tukey HSD Post Hoc Test

Comparison of Mean	**Comparison of Mean**	**Mean Difference**
Gen $_1$ vs. Gen $_2$	79.64 – 52.20	27.40 *
Gen $_1$ vs. Gen $_3$	79.64 – 31.57	48.07 *
Gen $_1$ vs. Gen $_4$	79.64 – 8.60	71.04 *
Gen $_2$ vs. Gen $_3$	52.20 – 31.57	20.63 *
Gen $_2$ vs. Gen $_4$	52.20 – 8.60	43.60 *
Gen $_3$ vs. Gen $_4$	31.57 – 8.60	22.97 *
*** $p < 0.05$**	**HSD = 11.37**	
Any between-group differences that exceeded HSD 11.37 are significant		

The proportion of deleted mtDNA in 71 blood samples and 27 muscle samples are reported in Table 7. However, there were four instances (two Great grandmother: lab #s 9 &30 and two Daughters: lab #s 8 & 63) where participants belonged to more than one generation. These samples were not reanalyzed statistically; instead the original results were presented according to

the number of generations that they were a part of. After accounting for the repeated results and the withdrawals there was a total of 80 blood sample results and 27 of muscle biopsy results.

Electrophoretogram of PCR Products

Following amplification, for each participant's sample (blood and muscle), 4µl of the total and 4µl of the deleted PCR product (alternating between total and deleted samples) were loaded in separate lanes on the same gel (6% polyacrylamide gel). Also, 4µl of the size/reference standard (pBR322 DNA- *Msp* 1 Digest) and 4µl of the appropriate controls were loaded in separate lanes. The results of six participants in the study are illustrated in Figure 2. The ratio of total mtDNA and deleted mtDNA provides a proportion of deleted mtDNA displayed in Table 7.

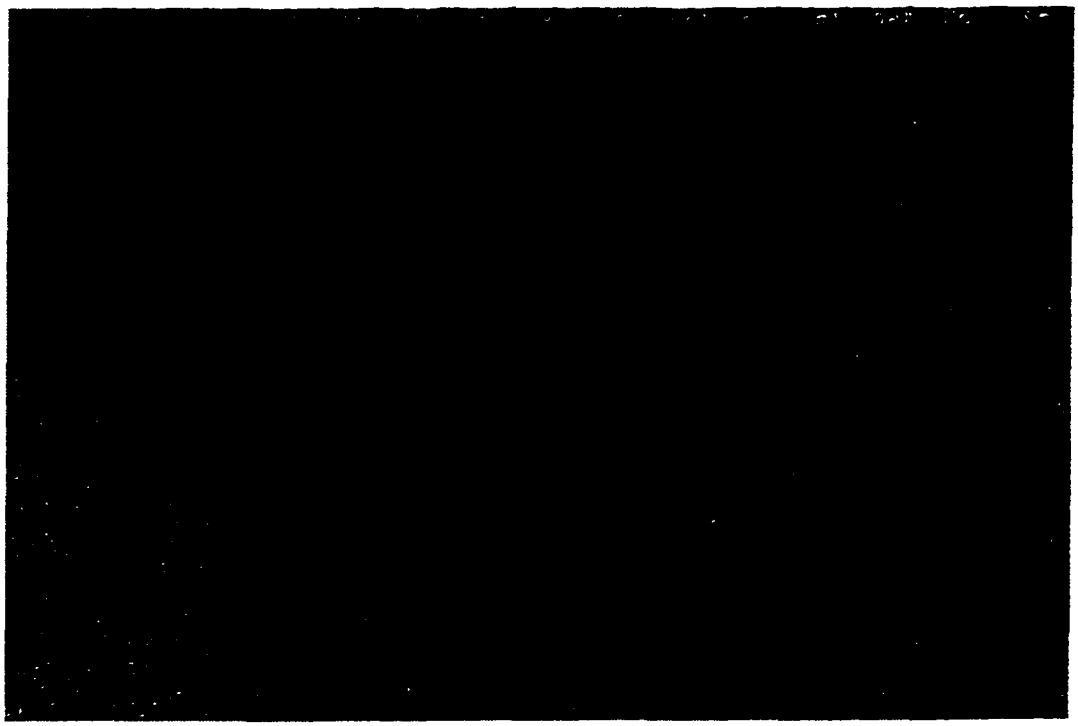

Figure 2. Electrophoretogram of PCR products. Detection of two different amplification fragments (Total mtDNA = 177-bp) and Deleted mtDNA = 127-bp) in total mtDNA from human blood using polyacrylamide gel electrophoresis (PAGE) following ethidium bromide staining. Amplification was carried out using 1 ng of template DNA (nuclear and mitochondrial DNA) from 6 individuals for 30 cycles. An aliquot of 4 µl of amplified products were separated on polyacrylamide gel and stained with ethidium bromide stain. Lane 1: 177-bp fragment (primer pair T_1/T_2) from participant # 1, Lane 2: 127-bp fragment (primer pair D_1/D_2) from participant # 1, up to Lane 11: 177-bp fragment (primer pair T_1/T_2) from participant # 6 and Lane 12: 127-bp fragment (primer pair D_1/D_2) from participant # 6. Lane 13: negative control for PCR master mix and (primer pair T_1/T_2), Lane 14: negative control for PCR master mix and (primer pair D_1/D_2). Size standard was 4 µl of pBR322 DNA- *Msp* 1 Digest, at a concentration of 400ng, with 16 bands ranging from 622-bp to 76-bp visible in order from the top to bottom respectively of lane 15.

Table 7. The proportion of deleted mtDNA in blood and muscle samples[♥] (1-30)

Family number	Generation	Age (years)[♥]	Lab number	Tissue	
				Muscle	Blood
01	GGC	24	1/85	44.6	39.3
01	GD	49	2/86	46.3	35.6
01	D	69	3/87	70.8	45.7
01	GGM	91	4	-	53.2
03	GGC	4	5	-	16.7
03	GGC	13	6	-	16.9
03	GD	30	7/88	66.3	47.0
03	D	49	8/89	42.3	38.6
03	GGM	69	9	-	49.9
04	GGC	2	10	-	32.2
04	GD	42	11/90	64.0	30.3
04	D	69	12/91	25.0	48.3
04	GGM	91	13/92	30.0	28.4
05	GGC	5	14	-	16.5
05	GD	24	15/93	51.1	28.2
05	D	52	16/94	32.1	12.5
05	GGM	74	17/95	21.4	21.2
06	GGC	5	18	-	33.7
06	GD	35	19/96	34.0	29.2
06	D	63	20/97	29.1	14.0
06	GGM	83	21/98	77.7	60.4
07	GGC	8 months	22	-	34.8
07	GD	19	23/99	41.1	34.2
07	GD	19	24/100	35.6	24.1
07	D	42	25/101	11.7	52.9
07	GGM	73	26/102	22.0	66.3
08	GGC	9	27	-	11.0
08	GD	36	28	-	78.5
08	D	58	29/103	57.9	44.0
08	GGM	80	30	-	64.3

Table 7. (Continued) The proportion of deleted mtDNA in muscle and blood samples[9] (31-57)

Family number	Generation	Age (years)[Φ]	Lab number	Tissue	
				Muscle	Blood
09	GGC	3	31	-	28.7
09	GD	38	32/104	51.3	27.5
09	D	63	33/105	28.8	38.9
09	GGM	99	34/106	20.5	62.4
10	GD	18	35	-	28.0
10	D	38	36	-	38.4
10	GGM	69	37	-	49.9
11	GGC	3	38	-	41.0
11	GD	26	39/107	65.3	36.6
11	D	47	40/108	33.4	50.0
11	GGM	76	41/109	15.8	43.2
12	GD	24	42/110	36.8	23.4
12	D	49	43	42.3	38.6
12	GGM	69	44	-	49.9
13	GGC	10 months	45	-	24.0
13	GD	28	46	-	22.0
13	GD	31	47	-	34.1
13	D	48	48	-	55.5
13	GGM	69	49	-	49.9
14	WITHDREW		50		
14	WITHDREW		51		
14	WITHDREW		52		
14	WITHDREW		53		
15	GGC	15	54	-	47.0
15	GD	33	55	-	35.0
15	D	59	56/111	14.4	45.8
15	GGM	80	57	-	64.3

Table 7. (Continued) The proportion of deleted mtDNA in muscle and blood samples[Ψ] (58-84)

Family number	Generation	Age (years)[Φ]	Lab number	Tissue	
				Muscle	Blood
16	GD	30	58	-	28.6
16	D	53	59	-	48.8
16	GGM	78	60	-	39.1
17	GGC	5	61	-	42.8
17	GD	24	62	-	35.2
17	D	49	63	-	38.5
17	GGM	69	64	-	49.9
18	GGC	4	65	-	18.8
18	GD	26	66	-	26.9
18	D	49	67	-	38.5
18	GGM	69	68	-	49.9
19	GD	20	69	-	19.7
19	D	36	70	-	42.4
19	GGM	69	71	-	42.6
20	GGC	10 months	72	-	22.0
20	GD	20	73	-	44.3
20	D	44	74	-	35.1
20	GGM	62	75	-	37.6
21	GGC	29	76	-	35.9
21	GD	49	77	-	39.6
21	D	68	78	-	46.8
22	GGC	23	79	-	22.6
22	GD	58	80	-	31.9
22	D	82	81	-	43.2
23	GGC	8	82	-	23.9
23	GD	46	83	-	24.9
23	D	66	84	-	58.0

[Ψ]GGM = Great grandmother, D = Daughter, GD = Granddaughter, GGC = Great grandchild
[Φ]Unless indicated explicitly otherwise

Proportion of Deletions in Blood versus Muscle Samples

The McNemar Chi-Square test consisted of two variables (blood and muscle) and four possible outcomes that is, the number of individuals with proportion of deleted mtDNA $<$ or $\geq$ the median proportion within each age cohorts of the two variables.

Table 8. Structure of the 2 x 2 design used in the McNemar and Kappa calculations

		BLOOD	
		<Median	*≥ Median*
MUSCLE	*<Median*	Cell "a" = 7	Cell "b" = 6
	≥Median	Cell "c" = 5	Cell "d" = 9
N = 27	***("z"critical = 1.96)*** ***p = 0.05***	**McNemar "z" test = 0.3**	**kappa statistic = 0.18**

This Chi-Square was used to test the null hypothesis that the proportions of 4977-bp deletion in a sample of blood will be equal to proportions 4977-bp deletion in a sample of muscle. The McNemar Chi-Square observed (χ^2_{obs}) was 0.3 and the chi-square critical (χ^2_{crit}) was 1.96 or $P < 0.05$. Because the χ^2_{obs} was less than the χ^2_{crit} the null hypothesis was accepted (Table 8).

After computing the McNemar χ^2 to determine the paired comparison differences in the proportion of 4977-bp deletion in blood versus muscle we evaluated the measure of agreement on the proportion of 4977-bp deletion between blood and muscle using the Kappa Statistic. The kappa statistic was 0.18 and the χ^2_{crit} was 1.96 or $P < 0.05$. Because the Kappa Statistic was less than the χ^2_{crit} the agreement existing between blood and muscle for the proportions of 4977-bp deletion was not considered to be significant.

Proportion of Deletions in Blood and Muscle Samples Across age Cohorts

When using the technique described by Fleiss [85] to compute the proportions of the 4977-bp deletion in blood and muscle samples across age cohorts, it was necessary to separate the participants into two groups. After examining a number of studies relating to the detection of the 4977-bp deletion [55,86,87,88], we concluded that in a given sample of individuals' age ranging from 0 to 100 years, the average proportion of 4977-bp deletion detected would be 30 % of the total

mtDNA. Therefore we designed two categories (< 30% or ≥30%) deleted mtDNA and assigned each participant accordingly (Table 9 and Table 10).

The Comparison of "m" Proportions from several independent samples described by Fleiss [85] was used to determine whether chronological age influenced the proportion of 4977-bp deletion from blood and muscle across the four age cohorts (GGM, D, GD and GGC). This statistic tested the second ($H_{o\,2}$) and third ($H_{o\,3}$) null hypotheses. The second null hypothesis stated that the proportion of 4977-bp deletion from a sample of blood across the four cohorts is equal. The χ^2_{obs} in blood was 11.85 and the χ^2_{crit} was 7.815. Since the χ^2_{obs} in blood was greater than the χ^2_{crit} the null hypothesis was rejected.

Table 9. The proportion of individuals with deletions ≥ 30% deleted mtDNA in blood samples of the four generations within the study (The Comparisons of m Proportions)*

Generation	# of Participants	# of Individuals with deletion ≥ 30%	Proportion of Individuals with deletions ≥ 30%
1-GGM	11	9	0.818
2-D	19	17	0.895
3-GD	23	12	0.522
4-GGC	18	8	0.444
Overall	71	46	2.679

*A 30% proportion of deleted mtDNA was used for statistical purposes to separate individuals into two categories (< 30% or ≥30%)

The significant χ^2_{obs} for both blood and muscle necessitated post hoc pairwise comparison to locate the significant difference between age cohorts. The results of the pairwise comparison for blood are shown in Table 11.

Table 10. Summary of significant difference between the age cohorts for the proportion of deleted mtDNA in blood samples*

Cohorts	Chi- Square Observed (χ^2_{obs})	Chi- Square Critical (χ^2_{crit})	Comments
GGM & D	0.38	3.841	Non significant (0.38 < 3.841)
GGM & GD	6.22	3.841	Significant (6.22 > 3.841)*
GGM & GGC	3.94	3.841	Significant (3.94 > 3.841)*
D & GD	11.01	3.841	Significant (11.01 > 3.841)*
D & GGC	8.50	3.841	Significant (8.50 > 3.841)*
GD & GGC	0.24	3.841	Non significant (0.24 < 3.841)
$\chi^2_{crit} = 3.841$	$P < 0.05$		
*For there to be statistical significance the (χ^2_{obs}) must be greater the (χ^2_{crit})			

*GGM = Great grandmother, D = Daughter, GD = Granddaughter, GGC = Great grandchild.

The results indicated that GGM differed significantly in the proportion of 4977-bp deletion for blood versus GD and GGC but not when compared to their immediate daughters (D). This finding is illustrated with the graph of proportion of deletion in blood shown in Figure 3.

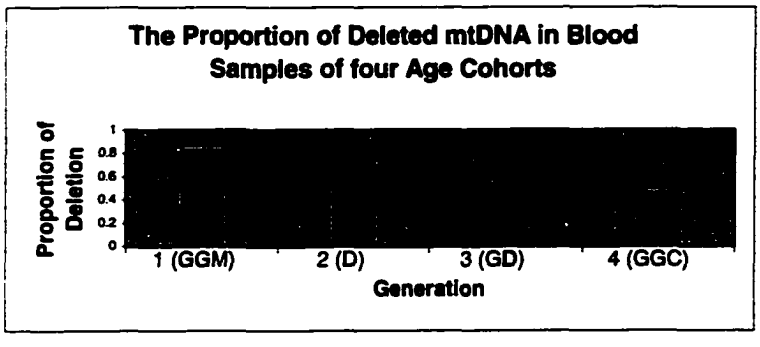

Figure 3. The proportion of individuals with deletions ≥ 30% deleted mtDNA in blood samples of the four generations within the study

The third null hypothesis stated that the proportion of 4977-bp deletion for a sample of muscle across three (3) cohorts of i) GGM, ii) D and iii) GD is equal. The χ^2_{obs} in muscle was 8.81 and

the χ^2_{crit} was 5.991. Since the χ^2_{obs} in muscle was greater than the χ^2_{crit} the null hypothesis was rejected.

Table 11. The proportion of individuals with deletions ≥ 30% deleted mtDNA in muscle samples of three (3) generations within the study (The Comparisons of m Proportions)*

Generation	# of Participants	# of Individuals with deletion ≥ 30%	Proportion of Individuals with deletions ≥ 30%
1-GGM	6	2	0.333
2-D	10	5	0.500
3-GD	10	10	1.000
Overall	26	17	1.833

*A 30% proportion of deleted mtDNA was used for statistical purposes to separate individuals into two categories (< 30% or ≥30%)

Similarly, the proportion of 4977-bp deletion for blood with daughters (D), while not significantly different ($p < 0.05$) from their mothers (GGM) was significantly different, from both their daughters (GD) and their granddaughters (GGC). Granddaughters and GGC were not significantly different from each other in their proportion of 4977-bp deletion. The results of the pairwise comparison for blood are shown in Table 11 and for muscle, shown in Table 12.

Table 12. Summary of significant difference between the age cohorts for the proportion of deleted mtDNA in muscle samples*

Cohorts	**Chi- Square Observed (χ^2_{obs})**	**Chi- Square Critical (χ^2_{crit})**	**Comments**
GGM & D	0.433	3.841	Non significant (0.433 < 3.841)
GGM & GD	7.48	3.841	Significant (7.48 > 3.841)*
D & GD	6.65	3.841	Significant (6.65 > 3.841)*
χ^2_{crit} = 3.841	***P* < 0.05**		
*For there to be statistical significance the (χ^2_{obs}) must be greater the (χ^2_{crit})			

*GGM = Great grandmother, D = Daughter, GD = Granddaughter, GGC = Great grandchild.

In muscle, only three cohorts were used for comparison of the 4977-bp deletion. The results illustrated in Figure 4 are consistent with the post hoc analysis in Table 12. GGM did not differ significantly ($p < 0.05$) from their daughters in the proportion of 4977-bp deletion. However, GGM were significantly different ($p < 0.05$) from their granddaughters (GD) in the proportion of 4977-bp deletion. However, daughters (D) who did not differ significantly ($p < 0.05$) from their mothers (GGM), differed significantly ($p < 0.05$) from their daughters (GD).

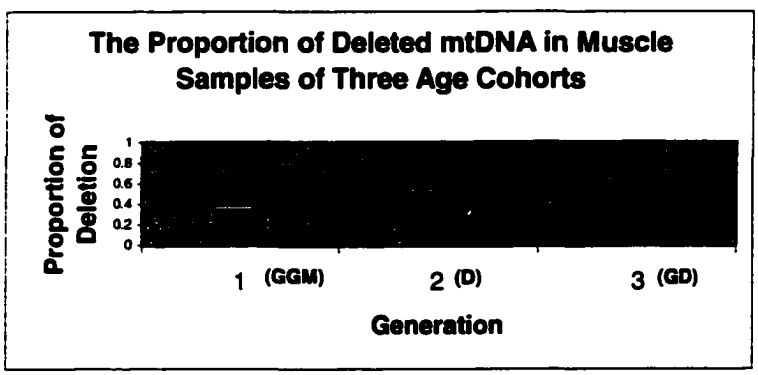

Figure 4. The proportion of individuals with deletions ≥ 30% deleted mtDNA in muscle samples of three generations within the study

DISCUSSION

In this study we investigated 21 maternal lines, and by optimizing the PCR protocol, the use of longer primers, a more sensitive polymerase, and shorter PCR products, we were able to effectively isolate and quantify the 4977-bp deletion in both blood and muscle samples from participants of different age cohorts, including 8-month-old infants. The results clearly demonstrated that there was an age-association with the 4977-bp deletion in blood samples however, we did not identify any increased accumulation of the deletion in muscle tissues with time. We revealed some compelling and interesting results in several aspects of this study and the following discussion will assist in explaining: First how we were able to successfully detect the 4977-bp deletion in blood (a mitotic tissue) and muscle (a post mitotic tissue), second the influence of chronological age on the proportion of the 4977-bp deletion and finally the influence of other factors that may have affected the presence and/or frequency of the 4977-bp deletion. It is unclear why muscle revealed a decrease in the proportion of the 4977-bp deletion as age increases. Accordingly to the literature muscle cells revealed a high proportion of the 4977-bp deletion as age increases [43], therefore, if blood and muscle samples are taken from the same individual, both samples are expected to present similar deletion patterns. Since this was not the case in our study some possible explanations might be the limitations that we encountered with our quantification procedure and the comparatively small sample size (n = 27) for the muscle samples.

Mitotic versus Post mitotic Tissue

We analyzed mtDNA from both blood and muscle samples and there was no significant difference ($p < 0.05$) found in the proportions of the 4977-bp deletion in the two samples. In addition we evaluated the measure of agreement on the proportion of 4977-bp deletion in blood and muscle samples, and it was observed that there was no significant agreement ($p< 0.05$), that is the two samples were not exactly the same.

Blood and the 4977-bp Deletion

In a previous study [43], post mitotic tissues (muscle, lung and testis) and a mitotic tissue (blood) were analyzed and the presence of the 4977-bp deletion were demonstrated in all the post mitotic tissues, but were undetectable in the mitotic tissue. [43] In addition, Meissner et al. [57] failed to demonstrate the accumulation of the 4977-bp deletion in mitotic tissues such as blood, platelets and peripheral blood mononuclear cells. More recently, several researchers [25,55] were able to detect deletion specific fragments in blood samples. Gattermann et al. [25] detected the deletion in blood of individuals in the absence of any known hematological diseases. However, patients examined in this group had undergone cardiac surgery, and it can be assumed that marked pathological changes were present due to coronary heart disease [89], which may have been accompanied by a rise in the 4977-bp deletion in the blood of these patients. As well, von Wurmb et al. [55] used both intravital blood and postmortem blood to detect the 4977-bp deletion but it might be argued that the postmortem process may have contributed to an increased proportion of 4977-bp deletion. In addition, the presence of this deletion in some of the samples may be related to certain disease conditions as some of the donors were suffering from diseases capable of inducing an increased accumulation of 4977-bp deletion in blood. [55] In our study, to eliminate the effects of disease or postmortem processes we utilized only intravital blood samples from participants free from any known disease conditions.

The fact that we were able to detect this deletion in blood samples of all the individuals (n = 71) was quite significant. Until recently it has been assumed that damaged mtDNA only occurs in post mitotic, well differentiated tissues and that blood cells, like fetal cells do not survive long enough to accumulate mtDNA deletions. [43,42] For this reason blood samples have often been used as negative controls [76,43] Further, it has been proposed that frequent cell division in hematopoetic precursors select against the survival of cells with genetically defective mitochondria. [47] As a result we investigated and compared blood (a mitotic tissue) and muscle (a post mitotic tissue)

from 27 participants to see whether mitotic tissues would yield different proportions of mtDNA deletion than post mitotic tissues. From our results we were able to demonstrate that blood samples accumulated sufficient mtDNA deletions that were detectable using our improved PCR protocol. In addition, there was no significant difference between the two types of tissues.

In support of our results Liu et al. [90] was able to detect the 4977-bp deletion in other mitotic tissues such as skin from subjects of various ages. Previously Lee et al. [43] argued that post mitotic tissues such as muscle and testis do not replicate and as a consequence present high deletion rates in these tissues. Also, there appears to be more deleted mtDNA in the tissues that turn over slowly or not at all (e.g. brain and muscle) as compared to those that turn over more rapidly (e.g., lung and blood cells). [43] Significantly, this means that cells with lower mitotic activity bear high levels of 4977-bp deletion. [43] However, we detected the 4977-bp deletion in both blood and muscle samples in the absence of any significant differences between the two samples and therefore concluded that blood may be used in lieu of muscle as a reliable source for the study of deletions in mtDNA.

Stem Cells and the 4977-bp Deletion

There has been some evidence that bone marrow stem cells (the precursor for blood cells), may contain low levels of mtDNA deletions [25], which is contrary to the proposal that frequent cell division in hematopoetic precursors select against the survival of cells containing genetically defective mitochondria. [47] These cells would not proliferate long enough and would therefore eliminate mutated mitochondrial genomes. [25] However, clinical and experimental evidence has provided for selection against proliferating cells harboring deletions of mtDNA [91], that is, there is a progressive loss of deleted mtDNA during invitro cell proliferation. The presence of the 4977-bp deletion in blood of 8-month-old infants in our study raises the question whether mitotic tissues are able to accumulate detectable amounts of mtDNA deletions.

If proliferating cells expressing the 4977-bp deletion have a growth disadvantage, how does one explain the presence and persistence of this common deletion in normal adult bone marrow, and more importantly its presence in 8-month-old infants in the present study? First, it must be recognized that in contrast to post-mitotic tissues, mtDNA mutations in the bone marrow can only persist if they occur in self-renewing stem cells. [25] Second, it is unlikely that mutations of mtDNA will affect the survival of stem cells, because such cells spend most of their time in the resting phase (Go) of the cell cycle. [25] Only after initiation of cell division with its consequent increase in the cell's energy do the cells become susceptible to mitochondrial defects. [55] Since blood cells originate from the bone marrow, which is a mitotic tissue, we believe that in our study blood cells from the bone marrow maintained sufficient mtDNA deletions for their detection. In addition, it must be noted that although cells containing deleted mtDNA may grow slowly the deleted ones may replicate more efficiently, due to their smaller size. In addition, the cellular selection favoring normal mitochondrial genomes may not be a rapid process. [63] The presence of the 4977-bp deletion in bone marrow stem cells may explain why the deletion persists in blood cells long enough for detection in all our 71 blood samples.

However, arguing against the occurrence of the 4977-bp deletion exclusively in resting cells is the observation that more than 99% of peripheral blood lymphocytes subsist in the Go phase [22], which would mean that the deletion should be easily detectable in blood. The fact that we were able to detect the 4977-bp deletion in all blood samples (n = 71) was a strong endorsement of the argument expressed by Gerdes et al. [22] In addition, it has been observed that about 40% of circulating lymphocytes have a life span that exceeds 6 months and a number may in fact persist for over 30 years. [71] This should provide sufficient time for these cells to accumulate mitochondrial alterations that are detectable using sensitive PCR assay and may help to explain the recent detection of the 4977-bp deletion in blood by ourselves and others. [25,55]

Muscle and the 4977-bp Deletion

Researchers have demonstrated that tissues with high oxygen consumption (brain, heart, and skeletal muscle) have significantly higher levels of mtDNA deletions than most other tissues. [72] Furthermore, the 4977-bp deletion has been shown to be present in well-differentiated cells with low mitotic rate, such as brain and muscle cells [37] In this study, the fact that we were able to detect the 4977-bp deletion in all muscle samples (n = 27) proved to be consistent with studies conducted by other researchers [37,43,55] in identifying muscle as a reliable source for the study of mutations in mtDNA.

In spite of recent evidence that the 4977-bp deletion may be detected in mitotic tissues, most researchers have still only been able to demonstrate the presence of the 4977-bp deletion in well-differentiated tissues. [37,43,55] This poses the question as to why there have only been only a few successful attempts to isolate and detect the 4977-bp deletion in mitotic tissues.

Improved Methodology

It might be argued that the failure of other groups to detect the 4977-bp deletion in blood of non-diseased participants may be attributed to technical problems such as the method of DNA isolation, amplification and detection of the amplified fragment. By employing a variety of technical approaches, we developed for the first time a simple methodology for collecting, storing and isolating DNA for PCR amplification. This method involved the dry storage of DNA followed by a protocol for elution and quantification of the DNA. It has been firmly established that the most common method for demonstrating the 4977-bp deletion involves the amplification of deletion-specific fragments and subsequent detection of the PCR products on ethidium bromide stained agarose gels [92,42,43]. However, employing this methodology limits detection at the nanogram level. [93] Fortunately, Bassam et al. [94] developed a technique that is more sensitive for the detection of nucleic acid at the picogram level, by specific reduction of silver ions.

Further modification of the PCR technique was achieved by von Wurmb et al. [55], as they optimized the PCR protocol by the use of longer primers, and visualization of amplification products on silver-stained PCR gels enabling them to detect the 4977-bp deletion in a high percentage of blood samples from subjects representing a wide spectrum of ages. Similar to von Wurmb et al. [55], we employed longer primers to enhance detection of the 4977-bp deletion. However, in our study we did not limit our changes to the use of longer primers as we made additional modifications to the PCR protocol.

Recent Modification to the PCR Protocol

With our methodological improvements (using longer primers, a more sensitive polymerase, and also shorter amplification fragments), to the PCR protocol we were able to detect the 4977-bp deletion successfully in blood and muscle samples at very low template concentrations using conventional ethidium bromide staining. More specifically, we employed 22-bp primers, small deletion-specific fragments (127-bp and 177-bp) along with an extremely sensitive polymerase (Deep-Vent [exo-] DNA Polymerase, New England BioLabs Inc.), which produced a higher amplification efficiency and greater yield. According to Hilali [83], Deep-Vent [exo-] DNA Polymerase, when compared with other polymerases such as Taq polymerase is the most sensitive polymerase in detecting template concentrations as low as 10 fg. Coupled with that, we employed a very sensitive quantification system (Eastman Kodak) that detected proportions of deletion as low as 11% of total mtDNA (Table 7). The detection of the deletion in blood samples from asymptomatic individuals was very important as it provides a less invasive technique to allow researchers to gain further insights into mutations in the mitochondrial genome.

Chronological Age and the 4977-bp Deletion

In this study with the use of a maternal line model, we investigated the effect of chronological age on the proportion of the 4977-bp deletion in all participants. According, to the Error

Accumulation Hypothesis [1], the accumulation of mtDNA deletions will increase over time. In support of this hypothesis [44], revealed that the proportion of mtDNA with deletions increased exponentially with age. Furthermore, with increasing age there was the appearance of multiple deletions; the number of deletions in those over 70 was significantly higher ($p < 0.05$) than those under age 40 [46]. There still persists the controversy that aging is the cause of the 4977-bp deletion. However, some of these studies were limited by sample size, research design and methodology. Several recent studies [51,95], have presented hypoxemia as a possible alternative to aging as the causative factor resulting in the 4977-bp deletion. In our study we confirmed that aging was not the sole factor causing the 4977-bp deletion since the proportion of mtDNA deletion in the blood of daughters (X age = 52.2) was not significantly ($p < 0.05$) lower than that of great grandmothers (X age = 79.6). This result is contrary to the work of several researchers [47,48,49], as they investigated the age-association with the common deletion and observed that the 4977-bp deletion increased with age. However, an association of the 4977-bp deletion exclusively with aging is not consistent with our finding as we detected the 4977-bp deletion in asymptomatic infants as young as 8-months-old and as well, there was no significant difference ($p < 0.05$) in the proportion of deletion between the infants (GGC) and their mothers (GD).

In support of our findings, Pallotti et al. [96] argued against the 4977-bp deletion as an aging phenomenon as he observed that point mutations in the mtDNA did not accumulate significantly ($p < 0.05$) with age. Furthermore it has been suggested that mtDNA deletion detected in "normal" individuals may be inherited or generated pre- or post-partum. [97] Few researchers have been able to demonstrate the 4977-bp deletion in very young children. It has been suggested that mtDNA deletions in normal adults as well as children may be inherited. This conclusion was based on detection of the 4977-bp deletion in individuals as young as 3 years [97], however, there were only two 3-year-olds in this study and one sample was from an autopsy case and the other was affected by a mitochondrial disorder. As mentioned previously it has been suggested that

samples from autopsy cases and those affected by an underlying disease may elevate the proportion of mtDNA deletions. [25,55] This may help to explain why Tengan et al. [97] were able to detect this deletion in 3-year-olds. However, supporting the work of Tengan et al. [97], Chen et al. [98] were able to detect the 4977-bp deletion in oocytes. Apparently, the genetic bottleneck that has been proposed to filter defective mtDNA molecules seems to precede the mature oocyte stage [99], indicating that if a deletion is present in the oocyte, it will be transmitted to the embryo. [97] Consequently, many of these mtDNA deletions would be generated during early life, but may only be detected with accumulation over several years. [97] This provides a possible explanation why we were able to detect the 4977-bp deletion in infants as young as 8-months-old using our more sensitive detection methodology than previously employed.

In our study it was observed that the proportion of deletion in blood was statistically significant ($p < 0.05$) across four (4) age cohorts studied and the proportion increased as age increased. Likewise, the proportion of deletion in muscle was statistically significant ($p < 0.05$) across the three (3) age cohorts studied, however, muscle samples did not show an increase in the proportion of 4977-bp deletion with an increase in age possibly due to methodological limitations and or the small sample size of the blood samples. Furthermore, pairwise comparison was used to locate the significant difference between age cohorts for both muscle and blood and the results demonstrated that only certain age cohorts contributed to the significant difference ($p < 0.05$) [tables 10 & 12]. It was observed that GGM were significantly higher ($p < 0.05$) in the proportion of 4977-bp deletion for blood versus GD and GGC. Similarly, daughters (D) were significantly higher ($p < 0.05$) than their daughters (GD) and their granddaughters (GGC). Also in muscle, GGM were significantly lower ($p < 0.05$) than their granddaughters (GD) in the proportion of 4977-bp deletion. Likewise daughters (D) were significantly lower ($p < 0.05$) than their daughters (GD).

As a consequence of the variability of the age-associated mtDNA deletions, Wei [59] established four criteria for associating mtDNA with aging. First, the deletion should only be present in appreciable amounts in the mtDNA of elderly human subjects; second, the incidence and abundance of the deletion should be increased with age of the subject; third, the deletion should be detectable in asymptomatic elderly subjects. Finally, the scope of the deletion must be distinctively revealed by the primer-shift PCR method.

Our results differed significantly from the "Wei criteria" in establishing the 4977-bp deletion as truly an aging deletion. We observed mtDNA deletions in substantial amounts in children as well as in elderly participants. In our research we report the existence of the deletion in all infants (n = 18) in the absence of any known mitochondrial disorder. The results indicated that GGM differed significantly in the proportion of 4977-bp deletion for blood versus GD and GGC but not when compared to their immediate daughters (D). Similarly, the proportion of 4977-bp deletion for daughters (D), while not significant from their mothers (GGM) was significantly different from their daughters (GD) and their granddaughters (GGC). Granddaughters and GGC were not significantly different in their proportion of 4977-bp deletion.

In muscle the GGM did not differ significantly from their daughters in the proportion of 4977-bp deletion. However, GGM were significantly different from their granddaughters (GD) in the proportion of 4977-bp deletion. Likewise daughters who again did not differ significantly from their mothers (GGM) in the proportion of 4977-bp deletion differed significantly from their own daughters (GD). Therefore, our results do not support the 4977-bp as solely an age-associated event due to the fact that there was the presence of the deletion in children as young as 8-months of age. Therefore, failure of our results to comply with two of the "Wei criteria" raises the possibility that other factors may contribute to the presence of the 4977-bp deletion.

Alternative Factors Affecting the 4977-bp Deletion

Our results do not support the 4977-bp deletion as primarily an aging deletion and therefore may not serve as a sensitive biomarker of the aging process. This deletion seems to be quite ubiquitous and it could be argued that maternal inheritance, certain mitochondrial diseases or environmental factors such as tobacco smoke may have contributed to the 4977-bp deletion.

Maternal Line Transmission of the 4977-bp Deletion

Importantly, it has been well documented that the identical mtDNA is transmitted from mother to child at the point of conception and is of high fidelity. [21] In support of this, Chen et al. [97] argued that age related deletions are not transmitted to the embryo because there is a genetic "bottleneck", which would filter the mtDNA molecule. Our research design was based on the premise that a homogeneous set of mtDNA would be transmitted from mother to child and any differences seen in the mtDNA profile of offspring within a generation would be as a result of age or environmental factors. However, arguing against the genetic "bottleneck" were the recent findings of, female mice harboring heteroplasmic mtDNA point mutations that transmitted a variable amount of hetroplasmic mtDNA to their offspring. [100] In addition, it has recently been reported that women harboring mtDNA deletions appear to transmit them to their offspring. [100]

Further evidence to support the maternal passage of information pertinent to mtDNA deletions is the presence of the 4977-bp deletion in very young children with mitochondrial disorders. For example, the expression of the 4977-bp deletion was evident in a family in which the mother had progressive external ophtalmoplegia (PEO), and her 4-year-old son had Pearson-marrow pancreas syndrome. [86] Additional proof for maternal transmission of mtDNA deletions was the recent report by Bai and Seidman [101], who identified the 4977-bp deletion in lymphocytes of a pedigree of a family suffering from bilateral sensor neural hearing loss (SNHL); interestingly this deletion was present in a 6-month-old with SNHL. In the present study, we identified the 4977-bp

deletion in asymptomatic 8-month-old infants. It has been suggested that the heteroplasmic presence of the 4977-bp deletion in mother and child may be due to stochastic segregation of the heteroplasmic mtDNA in the oocytes, coupled with a "bottleneck" at which only few of the thousands of mtDNA in every oocyte are selected to populate the organism. [102,86] Significantly, individuals born with mutant mtDNA would start with a lower OXPHOS level compared to those with a normal genotype and with increasing age as their residual OXPHOS capacity declines it may exceed organ specific energetic thresholds resulting in the earlier onset of disease. [101]

Disease and the 4977-bp Deletion

Although the concept of mitochondrial disease(s) was first introduced [60] as early as 1962, it was only in 1988 that two pioneering reports showed an association of mtDNA mutations with certain disease conditions. [47,61] In recent years a variety of chronic degenerative diseases have been demonstrated to result from mutations in mtDNA. [62] Therefore, it was absolutely necessary to ensure that all our participants were free from any known disease condition by completing an extensive health survey. The results revealed that all the participants appeared to be free of any known mitochondrial disease.

Smoking and the 4977-bp Deletion

Exogenous factors such as tobacco smoke have been demonstrated to influence the level of deleted mtDNA.[103,104] Lui et al. [105] demonstrated that the 4977-bp deletion occurred more frequently in the hair follicles of smokers than those of non-smokers. As well, a more recent study by Lee et al. [106] revealed that the frequency of deletions in human lung tissue was both age and smoke dependent. Several studies have shown that oxygen free radicals can also be produced exogenously as a component of tobacco smoke. Kiyosawa et al. [107] indicated that cigarette smoking induces oxidative DNA damage in peripheral blood cells in a relatively short period of time. In our study, the health survey revealed that either indirect or direct tobacco smoke affected

43 participants. As a result, twelve (12) third and fourth generations and 17 families overall were affected by either indirect or direct tobacco smoke. The cause of mtDNA deletion by tobacco smoke has been attributed to the elevated levels of oxygen free radicals. [107] Therefore, it is possible that the presence of the 4977-bp deletion in very young children (GGC) in our study may in fact be due to the substantial number of smokers associated with the maternal lines and the transmission of the 4977-bp deletion from mother to child.

In summary, we have developed a methodology to successfully collect, store and isolate mtDNA that allowed for the quantification and detection of the 4977-bp mtDNA deletion in both blood and muscle samples. Importantly, there were no significant differences, in the frequency of 4977-bp deletion, between the two samples. This is significant in that it will allow researchers to study aging, mitochondrial disorders and mutations in a sample of blood. In addition, we were the first to demonstrate substantial amounts of this deletion in asymptomatic infants as young as 8-month old. Significantly, individuals born with mutant mtDNA would start with a lower OXPHOS level compared to those with a normal genotype and with increasing age as their residual OXPHOS capacity declines it may exceed organ specific energetic thresholds resulting in the earlier onset of disease. [101] The overall observation of the common deletion in all four generations provides important support for maternal passage for not only point mutations but also large-scale deletions. Lastly, with our modified methodology (the use of a maternal line and improved PCR conditions) we have provided important information concerning a better understanding of the nature of the 4977-bp in the human mitochondrial genome.

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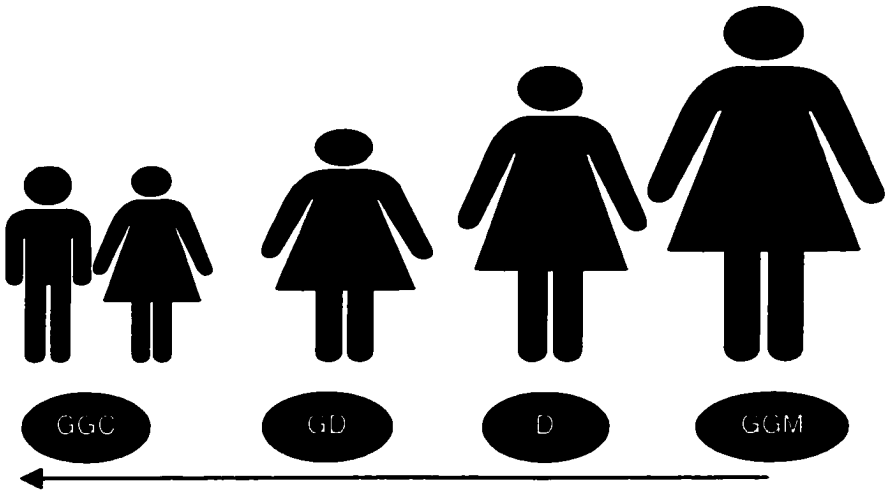
APPENDIX A

ADDITIONAL FIGURES

The Maternal Line



Key

GGM=Great Grandmother, **D**=Daughter, **GD**= Granddaughter, **GGC**=Great grandchild

- Mother passes identical mtDNA to child
- Differences seen in mtDNA deletions within generations would be because of age or environmental factors

Figure A-1. The maternal transmission of mtDNA across four generations

Sample & Generation Outline

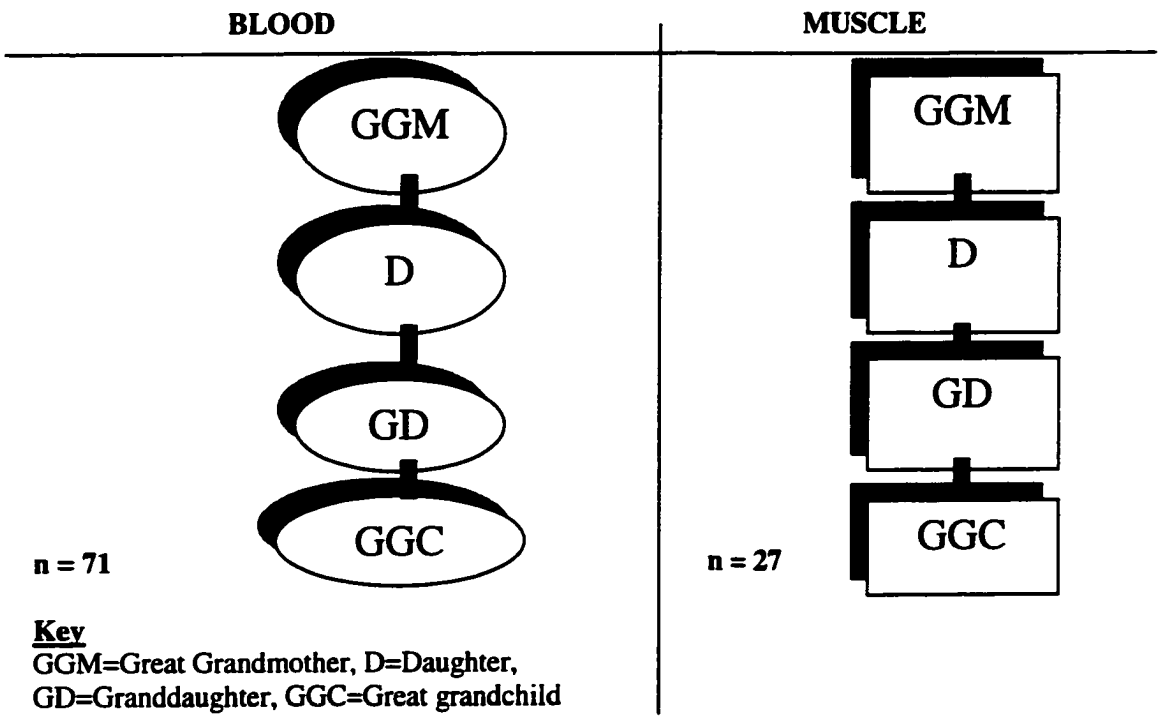


Figure A-2. A schematic representation of the sample and generation outline

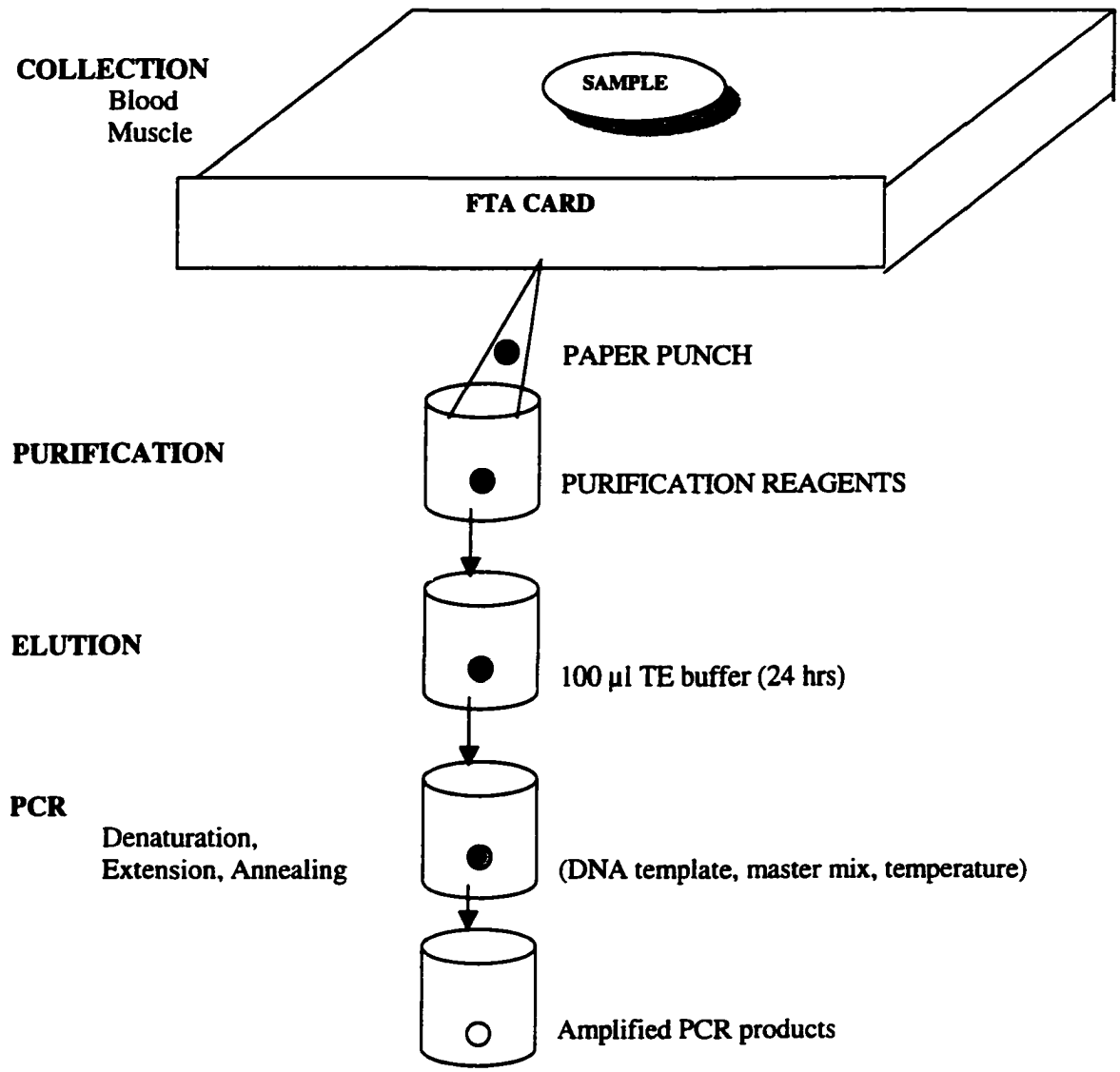
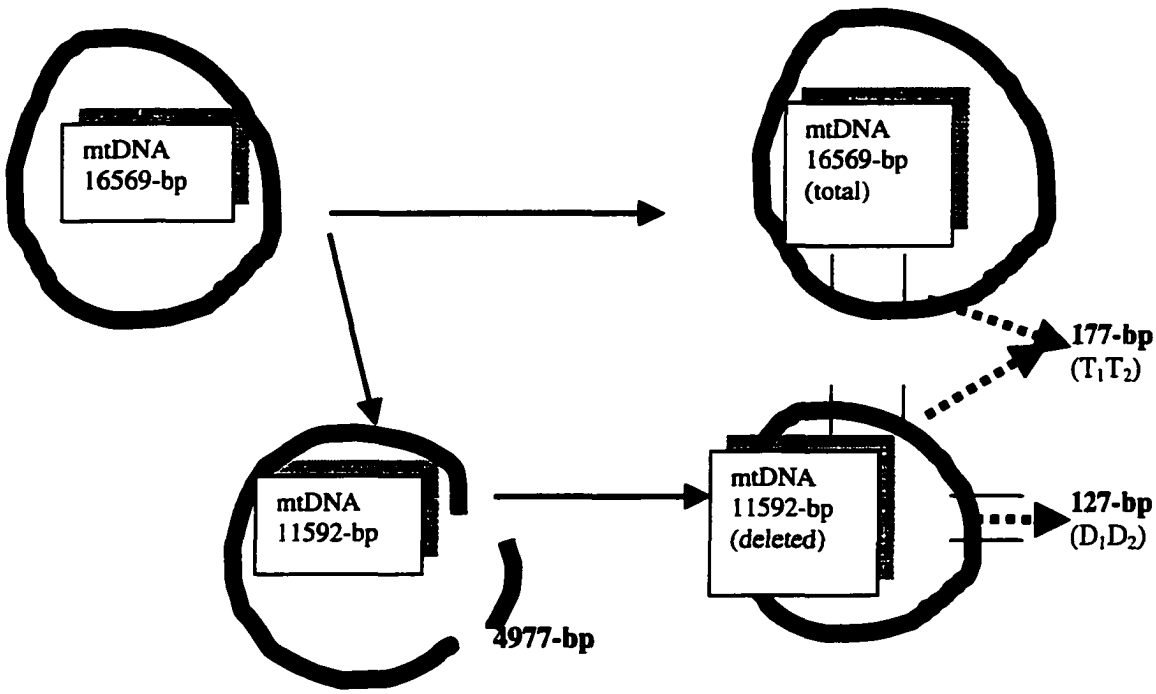


Figure A-3. An outline of the collection, storage and PCR amplification of mtDNA

The 4977-bp deletion



Legend
mtDNA=mitochondrial DNA
127-bp=deleted mtDNA
177-bp=total mtDNA
T_1T_2=primer pair (total)
D_1D_2=primer pair (deleted)

Figure A-4. Schematic diagram of formation of the 4977-bp deletion and the fragments used to represent total mtDNA and deleted mtDNA. Also shown are the primers and their region of amplification

Detection and Quantification

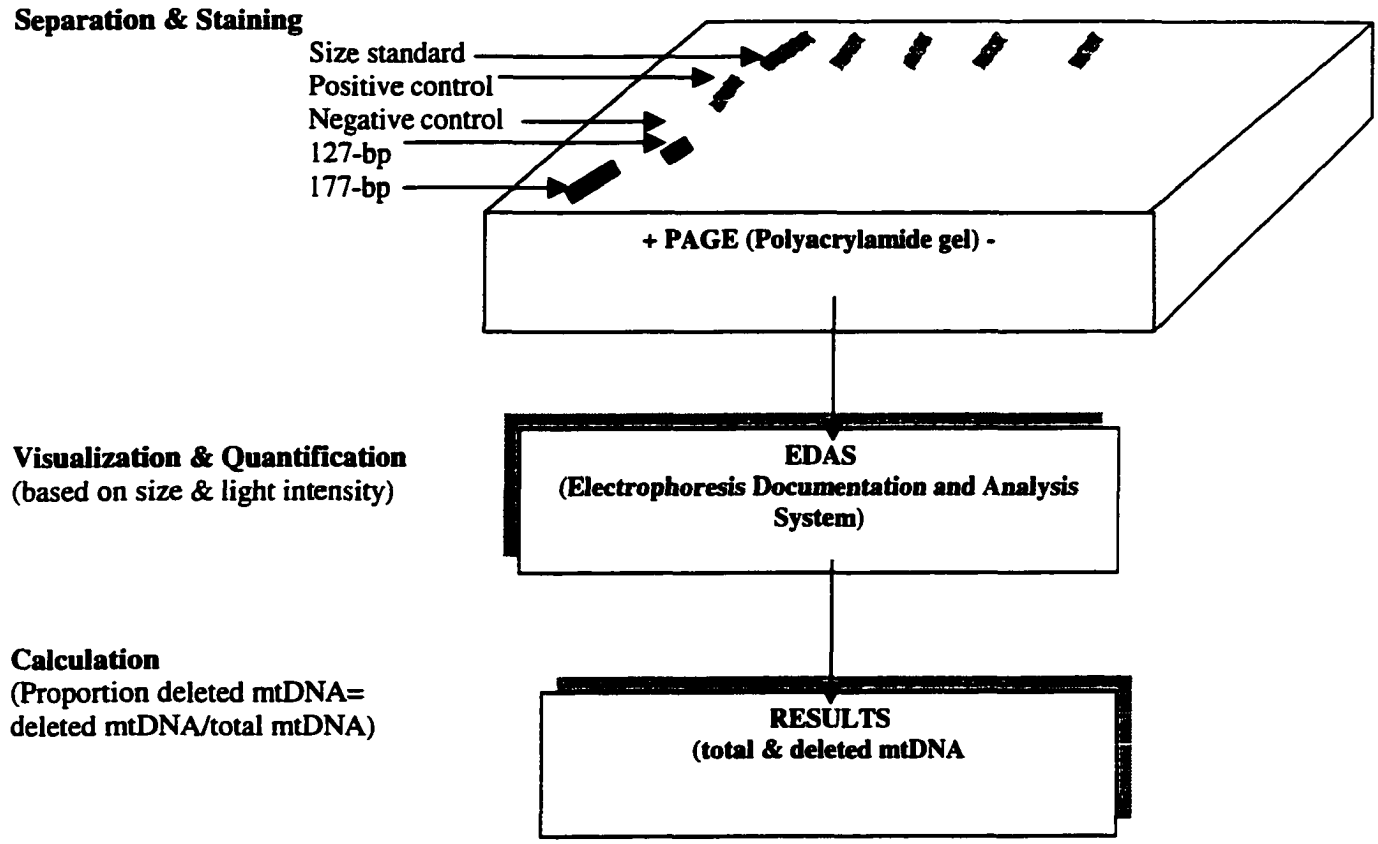


Figure A-5. A representation of the separation and quantification of mtDNA (total and deleted).

APPENDIX B

ADDITIONAL METHODOLOGY

APPENDIX B-1

BLOOD SAMPLING GUIDELINES AND PROCEDURES

Materials Needed

- Lancet pen
- Lancet
- Alcohol swab
- Elastoplast
- FTA card

Procedure

- Label FTA card
  - Name, age and date of collection (represented by a code)
  - E.g. first initial, last initial and a #
- Loading lancet pen
  - Remove tip of the pen
  - Twist-off the end of a lancet
  - Insert the plastic end in the lancet pen leaving the needle exposed
  - Replace the tip of the pen
- Wash hands with soap and water
- Massage a selected finger (index) optional
  - Gently stroking towards the tip
- Clean finger
  - Using alcohol swab
- Firing lancet pen
  - Pull-back the black end of the pen (listen for the click)
  - Position the tip of the pen on the tip of the finger
  - Press the black button
- Blood collection
  - Position the finger over the FTA card & gently stroke
  - As the blood settles on the finger, apply to FTA card
- Clean-up

- Use alcohol swab to apply pressure to the finger while removing excess blood
- Apply adhesive bandage to finger

- Lancet disposal
  - Remove tip of pen
  - While holding black sides of the pen, pull-back the top end of the pen (lancet falls out)
- Ensure that all materials containing blood are properly cleaned and disposed of.

APPENDIX B-2

MUSCLE SAMPLING GUIDELINES AND PROCEDURES

Materials needed

- Biopsy needles (sterilized)
- 3 cc syringes
- 22 gauge needles
- 2 µl tubes
- Scalpel
- Gauze
- Elastoplast
- Xylocaine
- Bentadine

Procedure

- Label 2 µl tubes
    - Name, age and date of collection (represented by a code)
    - E.g. first initial, last initial and a #
- Sterilize skin of the front of the quadriceps muscle with alcohol or bentadine solution
- Anaesthetize skin and subcutaneous tissue with 2% xylocaine (avoiding contact with muscle)
- Incise skin and deep fascia with scalpel blade
- Insert the outer cannula of the needle in the muscle
- Insert the obturator in the cannula
- Insert the trocar in the cannula
- Press muscle bulk into needle side-window
- Cut off sample by gently rotating the trocar within the obturator and a hook (small opening) on the trocar will take the actual biopsy
- Remove needle and use trocar to evacuate specimen from within the obturator
- Close wound and seal skin with elastoplast
- Place specimen into a labeled tube and store at -80°C.

- At the time of homogenization remove all fat and connective tissue from the samples and then record the weight
- To remove blood, wash the muscle samples several times in Tris buffer
- Following the wash, chop the samples with a scalpel and bring up to 20 volumes with respect to their original weight
- Homogenize each sample on ice using a disposable hand held homogenizer (1.5 ml Disposable Pellet Pestle), [Kimble/Kontes, Vineland, New Jersey, USA]
- Emulsify the muscle homogenate in a NaCl solution (600 mM NaCl, 15mM Tris, pH 7.5) and apply the total volume to each circle on a labeled FTA Card
- Ensure that all materials containing muscle and blood are properly cleaned and disposed of.
- Precautions were taken when handling all body specimens (Appendix B-8).

**The muscle biopsy technique was reintroduced by Bergstrom.* [80]

APPENDIX B-3

STORAGE OF SAMPLE

Description of FTA Card

FTA Cards are designed for the convenient collection, storage, and subsequent purification of blood samples for DNA analysis, data banking, and genomics. In this research we have also used the FTA Cards to collect and store muscle samples homogenized in a buffer solution. The specimen area is constructed from FTA paper, which is impregnated with a proprietary formulation. The card is constructed of materials that will not affect the DNA analysis of samples. A cover sheet protects the samples and allows the card to stand on its own while drying.

Application of sample

Spot sample directly on the FTA Card. Allow drying 1 hour to overnight before processing further

Caution

Universal Precautions should be used when handling any potentially infectious human-source blood products

APPENDIX B-4

DNA PURIFICATION

Extraction of DNA for PCR Amplification (Life Technologies)

Procedure

- Using a clean 1 to 3 mm punch, remove 2-3 punches from the middle of the FTA Card and place them into an amplification tube with a minimum capacity is 300 µl
- Add 200 µl of FTA Purification Reagent to each tube
- Cap each tube and vortex 1-2 seconds at low speed
- Allow the tubes to sit for 5 min at room temperature with a second brief vortex halfway through the incubation
- After the 5-min incubation, vortex for a third time and then carefully remove as much of the reagent as possible
- Repeat steps 2 through 4 an additional two times for a total of three washes with the FTA Purification reagent
- After FTA Purification Reagent has been removed for the third time, add 200 µl of TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- Cap each tube and vortex 1-2 seconds at low speed
- Allow the tubes to sit for 5 min at room temperature with a brief vortex half way through the incubation
- Pour off the TE and replace with an additional 200 µl of TE
- Cap each tube and vortex 1-2 seconds at low speed
- Allow the tubes to sit for 5 min at room temperature with a brief vortex half way through the incubation
- Pour off the TE and allow the FTA paper punch to completely air dry
    - This will require approximately 1 hour at room temperature
    - Alternatively, placing the tube with the punch at 60°C for 30 min can accelerate the drying
- After processing, the immobilized DNA is fixed and stored on the FTA Card. Total DNA can now be eluted from the FTA Card™ (following some modifications to the manufacturer's instructions) by adding 100µl of TE to the tubes containing 2 to 3 purified paper punches and the samples incubated at 4°C for 24hrs.

- The concentration of total DNA in each sample can be determined by spectrophotometry (GeneQuant II RNA/DNA Calculator, Pharmacia Biotech [Biochrom] Ltd., Cambridge, England) and then adjusted to 1 ng/µl
- The eluted DNA is now ready for amplification
    - It is recommended that 25µl reaction volumes be used

APPENDIX B-5

PCR AMPLIFICATION

Materials Needed

- Purified template DNA
- Size standard and controls
- Master mix
    - dNTPs
    - Buffer
    - BSA
    - ddH_2O
    - $MgSO_4$
- Primers
- Polymerase
- Mineral oil

Procedure

- Combine 5 µl of purified template DNA, or control at a concentration of 1ng/µl and 20 µl of master mix (PCR reagents)
- Add each sample to the appropriately labeled PCR micro plates
- The appropriate volumes and concentrations of each component of the master mix is prepared for a 25 µl reaction volume containing, 0.5 µl of one pair of primers (T_1T_2 or D_1D_2] [20 µM] and 0.25 µl of each deoxyribonucleoside triphosphate (dNTP) [0.25 µM each], 0.5 µl of $MgSO_4$ [1X], 2.5 µl thermopol reaction buffer [1X], 0.25 µl Deep-Vent (exo-) DNA Polymerase [0.5 units] also, 0.5 µl of Bovine Serum Albumin (BSA) [1X] (See table 3)
- Amplify samples in a PTC-225 DNA Engine Tetrad Thermocycler (MJ Research, Inc., Waltham, Massachusetts). The first cycle consists of a hot start at 95°C for 5 min followed by 30 cycles consisting of a denaturation step at 94°C for 30s, annealing at 60°C for 1min, and primer extension at 72°C for 30 sec, with a final extension for 10 min at 72°C
- Add a drop of mineral oil to prevent precipitation
- Cover the plate quickly and place in the thermocycler which provides the appropriate temperature and time for DNA amplification to take place

APPENDIX B-6

DNA SEPARATION

Procedure

- Arrange an electrophoretic chamber using 6% polyacrylamide gel and 1X TBE buffer
    - Peel the tape off the bottom of the cassette
    - Gently pull the comb out of the cassette. Put the comb aside so it can be used to separate the cassette plates at the end of the run
    - Mount the cassette(s) into the electrophoresis apparatus so the printed side faces the outer (anode) buffer chamber. If running only one gel, mount an appropriate buffer dam
    - Fill the buffer chambers with appropriate amounts of running buffer
    - Use a pipette to wash the sample wells with 1X running buffer, displacing any air bubbles in the wells
- Combine 4µl of 6X running buffer and 4µl of each sample (total or deleted)
- Combine 4µl of 6X running buffer and 4µl of each control
- For each participant, load 4µl of each mixture (alternating total and deleted samples) to separate lanes on the gel (use printed lane markings as guides)
- Load 4µl of the size/reference standard (pBR322 DNA- *Msp* 1 Digest) and 4µl of the appropriate control in separate lanes
- Attach the electrophoresis apparatus to the power supply and run the gel(s) at a constant voltage of 130 V, 500A for 45 min or until the dye front is near the bottom of the gel(s).
- When the run is complete, shut off power and remove gel(s).
- Place the cassette(s) on a flat surface with the notched (well-side) facing up. Starting near the top, work your way around the cassette using the end of the comb to separate the two plates. Carefully remove the short plate. The gel will adhere to either the short or long plate.
- Hold the plate with the gel over an open container. If the gel is adhered to the larger plate carefully insert a flat edged device (comb) through the plate's slot and gently push out the bottom of the gel; allow the gel to peel away and gently drop into the container. If the gel is adhered to the smaller plate carefully use the comb or a spatula to loosen one lower corner of the gel; allow the gel to peel away and gently drop into the container

APPENDIX B-7

PRODUCT VISUALIZATION AND ANALYSIS

Procedure

- Stain gel for 15-20 min in an ethidium bromide solution.
- Visualize PCR products using a transilluminator where the stain that is bound to DNA will fluoresce
- Quantify DNA bands using the Kodak Electrophoresis Documentation and Analysis System [EDAS]. The system captures electrophoretic images and allows you to quantify DNA bands based on the intensity information for each band
- EDAS software is able to determine the mass in nanograms (ng) of total mtDNA and deleted mtDNA in each sample
- Determine the proportion of deletion in each sample by calculating the ration of total to deleted mtDNA

APPENDIX B-8

PRECAUTIONS

Since these procedures involve the handling of fresh, human tissues, precautions must be taken to minimize the risk of infection with HIV, hepatitis B and other agents. Therefore: -

- Wear protective clothing
- Cover working area with disposable material
- Wash hands properly
- Wipe with a disinfectant such as hypochlorite after use
- Sterilize instruments after use
- Dispose of all human remain safely
- Label specimens accurately and store safely

APPENDIX B-9

PCR QUALITY CONTROL

To address the issue of PCR saturation

- Tubes labeled (1-5) with the same samples were ran at 26, 28, 30, 32 & 34 cycles
- 95°C 5 min- Hot start, 94°C 30s- Denaturation, 60°C 1 min- Annealing, 72°C 30s- Extension, 72°C 10 min- Final extension.
- After quantification, the highest cycle that showed incremental changes in DNA band concentration levels was used as the amount of cycles in the study. This addressed the concern of saturation (Figure B-1).

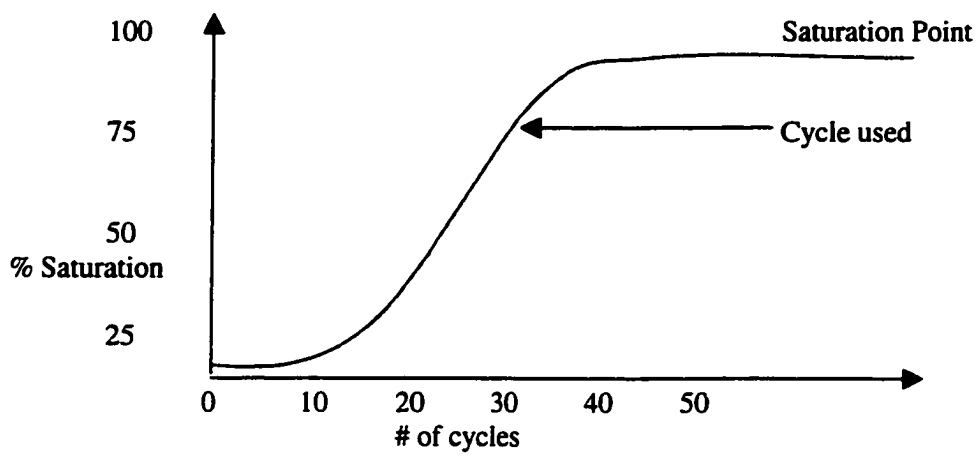


Figure. B-1. Graph showing PCR saturation and how the number of cycles used in the study was derived. This graph shows that as the number of PCR cycles (x-axis) increases the % saturation (y-axis) of the PCR increased. The reaction is within its linear phase at approximately 15-35 cycles. However after about 40 cycles the graph plateaus and the PCR saturation point is reached. For the purpose of this study 30 cycles was used, that is within the linear phase of the reaction and not at the saturation point. This is the optimal number of cycles that has demonstrated the best PCR product yield and choosing the reaction cycles before saturation will ensure that quantities of different PCR products can be best determined before the end of the reaction.

To address the issue of primer specificity

- A primer switch was done
    - The pair of primers (D_1D_2) that is designed for deleted mtDNA (127-bp) was used in an attempt to amplify total mtDNA (177-bp)

- There was no amplification. This addressed the concern of primer specificity (Figure B-2)

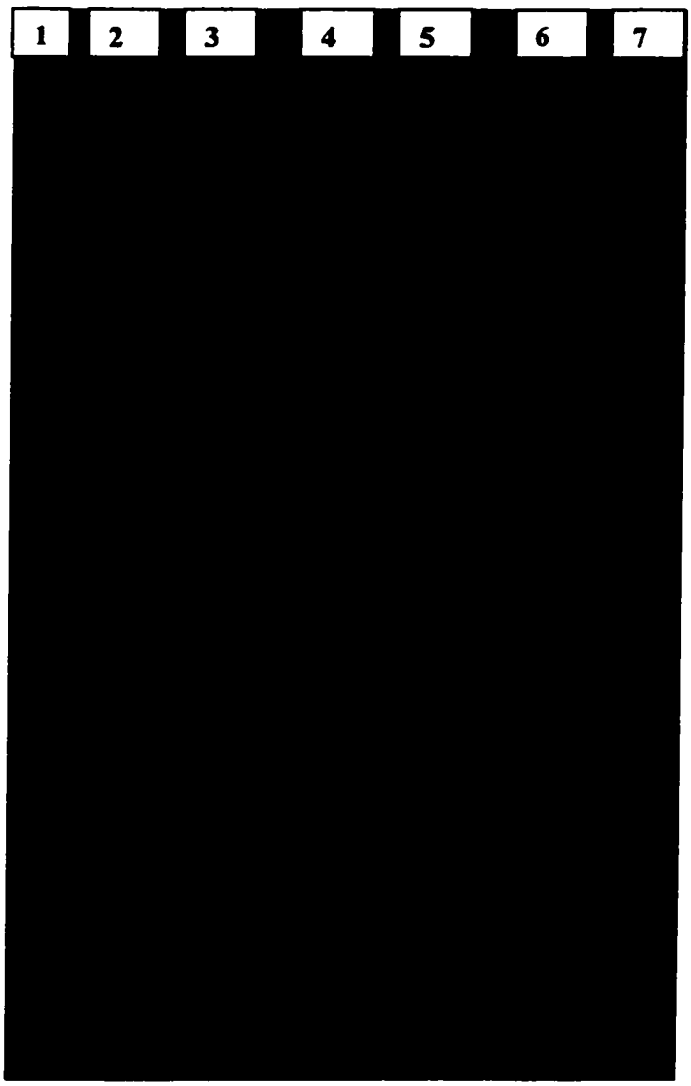


Figure B-2. Electrophoretogram of the PCR products amplified from mtDNA with the 4977-bp deletion. Lane 1 indicates the 177-bp (total mtDNA) amplified by primer pairs T_1T_2. Lane 2 is deleted mtDNA amplified by primer pairs T_1T_2. Lane 3 is total mtDNA amplified by primer pairs D_1D_2. Lane 4 indicates the 127-bp (deleted mtDNA) amplified by primer pairs D_1 D_2. Lane 5 indicates the positive control of blood from an 85-year-old male. A multiplex PCR (177-bp [total mtDNA] and the 127-bp [deleted mtDNA] amplified by the two primer pairs T_1T_2& D_1D_2. Lane 6 indicates the negative control of ddH_2O and PCR master mix. Lane 7 indicates the size/reference standards (pBR322 DNA- Msp I Digest). This proves that the primers were specific for the desired amplification fragments.

To address the issue of contamination and relationship within generations

- Reagents and template contamination
    - Negative controls were processed with each PCR
    - Each PCR reagent was used to replace template DNA as negative controls
    - Blank FTA Card punches were simultaneously processed along side FTA punches with sample to look for contamination of FTA Cards
    - Selected fragments of the 12s region (highly conserved region of mtDNA) and the Hypervariable Region 2, (HV2) of the template mtDNA were sequenced to confirm the presence of single template DNA and to establish a maternal line relationship.
    - The results suggested that each sample contained only single template DNA indicating that there was no contamination and the 12s and HV2 regions of the mtDNA of all the members in each maternal line were similar suggesting that members' within reach maternal lines were related.

To address the issue of reproducibility

The entire methodology was repeated several times under the same conditions and the results were similar.

APPENDIX C

ADDITIONAL MATERIALS

APPENDIX C-1

LETTER OF INFORMED CONSENT

For participants in the following research study:

The Effects of Chronological on Mitochondrial DNA (mtDNA) Deletion in Muscle and Blood of Older Adults (Using mtDNA Deletion as a Measure of Aging).

Principal Investigator: Roy Wittock B.Sc. (Hon)
Supervisor: R. Thayer Ph.D.
Lakehead University
Thunder Bay, Ontario P7B 5E1
(807) 346-7815

I _____, consent to participate in a study which will require completing the National Public Health Survey which will provide the researchers with information concerning both my health and smoking status. In addition, I agree to provide 1 ml (one drop) of blood and 5-25 mg of muscle tissue (one grain of rice) in order to determine the *Effects of Chronological Age on Mitochondrial DNA* (mtDNA) Deletion *on the Aging Process.* I understand that the blood sample and the muscle biopsy will be used to review the mitochondrial genome and to detect mtDNA deletion if present.

In order to investigate the effects of time (chronological age) on these deletions we will have to undertake a longitudinal study approach rather than a cross sectional study approach. There is sufficient evidence suggesting that there will be an increased amount of mtDNA deletion with chronological age. These deletions have been associated with certain aging characteristics as well as certain age-associated diseases.

It has been well established that the mitochondrion is inherited maternally that is, the mtDNA of the child is identical to the mother at the point of conception. Therefore, changes in the child's mtDNA profile could be as a result of chronological age or some environmental factors such as tobacco smoke. Essentially children are "mitochondrically identical' to their mother. Using the mother/child relationship in this study will provide the attributes of a longitudinal study design.

Dr. _____ and _____ from the research team have explained to me the entire laboratory procedure. A qualified experienced laboratory Phlebotomist will collect onto a FTA Card less than 1 µl of whole blood from a finger prick using a lancet pen. For a brief period after the procedure however, there may or may not be a burning sensation similar to the sensation felt from a needle prick.

The muscle biopsy technique was reintroduced by Bergstrom [80] in 1962 and has been used extensively in the area of biochemistry since that time. An experienced medical doctor will take the muscle biopsy. In this procedure, using aseptic conditions, a small area of the biceps muscle will be injected with a local anesthetic (2-3 ml of xylocaine hydrochloride). Approximately 5 to

25 mg of muscle tissue will be taken using a small biopsy needle and placed onto a FTA Card. There exists the possibility of a hypersensitivity reaction to the local anesthetic, in which slight discomfort could persist for 1 to 2 hours following the procedure. Furthermore, there exists the possibility of slight muscle soreness for a few days following the muscle biopsy procedure. In addition, if samples are taken in a highly innervated and vascularized area, there exist the possibility of nerve damage and arterial bleeding. However, the area that will be sampled in this study reduces the probability to almost nil. I am aware that the entire procedure will take approximately 5 minutes. Proper care and hygiene offered by the laboratory setting will eliminate any possibility of contamination or discomfort. All materials used in the collection of the blood and muscle (including the actual muscle biopsy) will be disposed as follows:

- All materials will be placed in a biohazards waste collection unit
- Collection units will be placed in cardboard boxes
- Cardboard boxes are to be wrapped in plastic and sealed with tape
- Boxes will then be collected and disposed of by BFI Medical Waste Systems

My participation is voluntary and I have the right to withdraw from the study at anytime. I am also aware that all the risks and benefits of engaging in this study have been outlined clearly. Results of the study may appear in a publication however; your identity will remain confidential at all times. Confidentially will be assured by the use of a numbering system for identification and names will not be used. After the completion of the research, the results will be stored securely for at least seven (7) years and a summary of the project will be made available to me upon request. I understand that the Research Ethics Board of Lakehead University has approved this research.

_____

Signature of Participant Date

_____

Signature of Witness Date

I have explained the nature of the study to the participant and believe he/she has understood it

_____

Signature of Researcher Date

APPENDIX C-2

COVER LETTER

Dear Sir/Madam:

I am a graduate student in the School of Kinesiology at Lakehead University. I have also completed an Honors Bachelor of Science B.Sc. (Hon.) degree in Medical laboratory Sciences.

I am conducting a molecular study of *the Effects of Chronological Age on Mitochondrial DNA (mtDNA) Deletion in Muscle and Blood of Older Adults (Using mtDNA Deletion as a Measure of Aging).* This study is being conducted under the direction and supervision of Dr. Robert Thayer, Professor in the School of Kinesiology. Upon successful completion of this research I will partially fulfill the requirements of the Masters of Science (MSc.) degree in Applied Sports Science with a specialization in Gerontology.

With the knowledge and experience I gather from this study as well as the completion of my degree, I will be able to contribute to the development and advancement of Medical Science. With a specialization in Gerontology, I will be well aided to make new discoveries or clarify existing ideas pertaining to the effects of Chronological Age on the aging process. Your participation in this study will allow me to provide a greater understanding and help to elucidate the molecular mechanism underlying the aging process.

There are several different theories attempting to explain the aging process. One of the more prominent theories is called *Error Accumulation.* This theory rest on the assumption that as cells get older they accumulate errors called mitochondrial DNA (mtDNA) deletions. In an attempt to elucidate the mechanism underlying the aging process, the mitochondrial genome in older cells are studied and compared to younger cells. The mitochondrion contains its own genetic material, a small, circular molecule containing 16,569-bp (base pairs). The mitochondrial genome, however, is extremely susceptible to DNA deletion when compared to nuclear DNA. The most frequent deletion is known as "the common deletion" or 4977-bp deletion. The causative agents of this deletion include oxygen free radicals, a normal byproduct of ATP production. It has been shown in existing literature that there is a strong association between mtDNA deletion, chronological age and the aging process in post mitotic tissues such as muscle and brain. However, there are several limitations to these existing research studies for example, they have relied on data obtained from cross-sectional studies and not on longitudinal studies. In order to address these limitations, a longitudinal study model will be used. The model is designed out of the fact that there is maternal transmission of mtDNA. Therefore, the attributes of a longitudinal study design will be realized by investigating the mtDNA deletion pattern of five (5) different maternal lines ranging from Great grandmother (GGM) to Great grandchild (GGC) [4 generations].

A qualified experienced laboratory Phlebotomist will collect and store, onto a FTA Card less than 1 ml of whole blood from a finger prick using a lancet pen. For a brief period after the procedure however, there may or may not be a burning sensation similar to the sensation felt from a needle prick.

The muscle biopsy technique was reintroduced by Bergstrom [80] in 1962 and has been used extensively in the area of biochemistry since that time. An experienced medical doctor will collect the muscle biopsy. The medical personnel will be Rob Petrella MD, PhD., an adjunct professor to the School of Kinesiology and Medical Director, Center for Activity and Aging, University of Western Ontario. In this procedure, using aseptic conditions, a small area of the biceps muscle will be injected with a local anesthetic (2-3 ml of xylocaine hydrochloride). Approximately 5 to 25 mg of muscle tissue will be taken using a small biopsy needle then stored onto a FTA Card. The method of analysis will be a modified version of the polymerase chain reaction (PCR) techniques (a standardized laboratory technique used to identify and quantify the mtDNA deletions in blood and muscle samples). Electrophoresis and densitometry will be used for the final detection and quantification of the mtDNA deletion.

There exists the possibility of a hypersensitivity reaction to the local anesthetic, in which slight discomfort could persist for 1 to 2 hours following the procedure. Furthermore, there exists the possibility of slight muscle soreness for a few days following the muscle biopsy procedure. In addition, if samples are taken in a highly innervated and vascularized area, there exist the possibility of nerve damage and arterial bleeding. However, the area that will be sampled in this study reduces the probability to almost nil. The entire procedure will take approximately 5 minutes. Proper care and hygiene offered by the laboratory setting will decrease the risk of contamination or discomfort. All materials used in the collection of the blood and muscle (including the actual muscle biopsy) will be disposed as follows:

- All materials will be placed in a biohazards waste collection unit
- Collection units will be placed in cardboard boxes
- Cardboard boxes are to be wrapped in plastic and sealed with tape
- Boxes will then be collected and disposed of by BFI Medical Waste Systems

Your participation is voluntary and you have the right to withdraw from the study at anytime. You will be made aware of all the risks and benefits of engaging in this study. Furthermore, all terminology and concepts related to the study will be explained to you. Results of the study may appear in a publication however, your identity will remain confidential at all times. Confidentiality will be assured by the use of a numbering system for identification and names will not be used. After the completion of the research, the results will be stored securely for at least seven (7) years and it will be made available to you upon request. You understand that the Research Ethics Board of Lakehead University has approved this research.

I thank in you in advance for your interest in this research. Should any questions or concerns arise, please contact myself using the information provided below.

Sincerely yours,

Roy Ralph Wittock, B.Sc. (Hon)
School of Kinesiology, Lakehead University
(807) 346-7815 (Office)
(807) 345-4043 (Home)
E-mail: rrwittock@mail.lakeheadu.ca

APPENDIX C-3

HEALTH PROMOTION SURVEY

Statistics Canada / Statistique Canada

Confidential when completed

Health Promotion Survey — HPS-2

1: ☐☐☐ - ☐☐☐ - ☐☐☐☐ (Telephone Number) 6: ☐☐☐☐☐ (Sequence number)

Collected under the authority of the Statistics Act, Revised Statutes of Canada, 1985, Chapter S10.

Interviewer Name: _____

SECTION A: PERCEPTIONS OF HEALTH

A1. First I would like to ask you a few questions about your health.

In general, compared to other people your age, would you say your health is...

1 ○ Excellent?
2 ○ Very good?
3 ○ Good?
4 ○ Fair?
5 ○ Poor?

A2. Would you describe your life as...

1 ○ Very stressful?
2 ○ Somewhat stressful?
3 ○ Not very stressful?
4 ○ Not at all stressful?

A3. The next questions are about your current physical condition.

How tall are you without shoes?

1 ☐☐ feet ☐☐ inches or 2 ☐☐☐ centimetres

A4. How much do you weigh?

3 ☐☐☐ pounds or 4 ☐☐☐ kilograms

A5. How much would you like to weigh?

5 ☐☐☐ pounds or 6 ☐☐☐ kilograms

7 ○ Same as A4

8 ○ Don't know

A6. Are you limited in the kind or amount of activity you can do because of a long term illness, physical condition or health problem? By long term I mean a condition that has lasted or is expected to last more than 6 months.

1 ○ Yes 2 ○ No ⟶ *Go to B1*

A7. Are your activities limited...

	Yes	No / Don't Know	Not Applicable
a) At home?	01 ○	02 ○	03 ○
b) At work or school?	04 ○	05 ○	06 ○
c) In other activities (such as leisure time pursuits or transportation to or from work)?	07 ○	08 ○	09 ○

A8. How well do you feel you are coping with this limitation? Would you say...

1 ○ Very successful?
2 ○ Somewhat successful?
3 ○ Not very successful?
4 ○ Not at all successful?
5 ○ Don't know

A9. How important is each of the following in coping with your limitation? Is it "Very important", "Somewhat important" or "Not at all important"?

	Very important	Somewhat important	Not at all important	Don't know or Not Applicable
a) Medical treatment you received?	01 ○	02 ○	03 ○	04 ○
b) Your family or friends?	05 ○	06 ○	07 ○	08 ○
c) Your general state of health?	09 ○	10 ○	11 ○	12 ○
d) Your own determination?	13 ○	14 ○	15 ○	16 ○
e) Prayer or spiritual help?	17 ○	18 ○	19 ○	20 ○

SECTION B: IMPROVING HEALTH

B1. Do you believe any of the following would help you to improve your health and well-being?

	Yes	No / Don't Know	Not Applicable
a) A more secure income?	01 ○	02 ○	03 ○
b) Moving to another neighbourhood or community?	04 ○	05 ○	06 ○
c) A change in job or business?	07 ○	08 ○	09 ○
d) Spending more time with family or close friends?	10 ○	11 ○	12 ○
e) Exercising more or being more physically active?	13 ○	14 ○	15 ○
f) Losing weight?	16 ○	17 ○	18 ○
g) Stop smoking?	19 ○	20 ○	21 ○
h) Cut down on drinking?	22 ○	23 ○	24 ○
i) Reduce drug use or medications?	25 ○	26 ○	27 ○
j) Taking better care of teeth?	28 ○	29 ○	30 ○
k) Learning to relax more and worry less?	31 ○	32 ○	33 ○

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SECTION C: BLOOD PRESSURE & CHOLESTEROL

C1. **The next few questions are about heart health. In your opinion, what are the main causes of heart disease?**

(MARK ALL THAT APPLY, DO NOT READ)

(IF RESPONDENT SAYS, "LIFESTYLE" OR "DIET", PROBE FOR SPECIFICS)

- 01 ○ Don't know
- 02 ○ Smoking
- 03 ○ Lack of exercise
- 04 ○ Eating fatty foods/Cholesterol
- 05 ○ Being overweight
- 06 ○ Poor diet
- 07 ○ Eating too much salt (sodium)
- 08 ○ Stress
- 09 ○ Family medical history
- 10 ○ High blood pressure
- 11 ○ High blood cholesterol
- 12 ○ Too much alcohol
- 13 ○ Other *(specify)*

C2. **When did you last have your blood pressure checked?**

- 1 ○ Within the last 6 months
- 2 ○ 7–12 months
- 3 ○ 13–24 months
- 4 ○ More than 2 years
- 5 ○ Don't know
- 6 ○ Never → *Go to C6*

C3. **Have you ever been told by a doctor, nurse or other health professional that you have high blood pressure?** ***(FOR WOMEN, ADD:*** **"except when you were pregnant".)**

- 1 ○ Yes
- 2 ○ No
- 3 ○ Don't know

} *Go to C6*

C4. **Are you doing anything to control your blood pressure?**

- 4 ○ Yes
- 5 ○ No → *Go to C6*

C5. **What are you doing?** ***(MARK ALL THAT APPLY, DO NOT READ)***

- 01 ○ Medication/pills
- 02 ○ Quit smoking
- 03 ○ Exercise regularly
- 04 ○ Losing weight or maintaining weight loss
- 05 ○ Reduce salt intake
- 06 ○ Other diet change
- 07 ○ Relaxation
- 08 ○ Reduce alcohol use
- 09 ○ Other *(specify)*
- 10 ○ Don't know

C6. **Were you ever told by a doctor, nurse or other health professional that your blood cholesterol was high?** ***(THIS WOULD REQUIRE A BLOOD SAMPLE)***

- 1 ○ Yes
- 2 ○ No
- 3 ○ Don't know/ Can't remember

} *Go to D1*

C7. **Are you doing anything to control your cholesterol?**

- 4 ○ Yes
- 5 ○ No → *Go to D1*

C8. **What are you doing?** ***(MARK ALL THAT APPLY, DO NOT READ)***

- 1 ○ Losing weight or maintaining weight loss
- 2 ○ Reduce cholesterol in diet
- 3 ○ Eat less fatty foods
- 4 ○ Other change in diet
- 5 ○ Exercise regularly
- 6 ○ Control stress and fatigue
- 7 ○ Take prescribed medication
- 8 ○ Other *(specify)*

SECTION D: EXERCISE

D1. **The next few questions are about exercise. By exercise we mean vigorous activities such as aerobics, jogging, racquet sports, team sports, dance classes, or brisk walking.**

How many times per week, on average, do you exercise? ***(DO NOT READ)***

- 1 ○ Daily
- 2 ○ 5–6 times a week
- 3 ○ 3–4 times a week
- 4 ○ 1–2 times a week
- 5 ○ Less than once a week
- 6 ○ Never
- 7 ○ Don't know

} *Go to D4*

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D2. When you do this exercise, how much time are you actually active? Would it usually be....

1O Less than 15 minutes?

2O Between 15 and 30 minutes?

3O More than 30 minutes?

D3. Do you feel that you get as much exercise as you need or less than you need?

4O As much as needed

5O Less than needed

6O Don't know

D4. I am going to read four sentences describing daily routines or activities. Tell me which one best describes your usual situation.
(MARK ONLY ONE)

1O 1. You sit during the day and do not walk about very much.

2O 2. You stand or walk about quite a lot during the day, but do not have to carry or lift things very often.

3O 3. You lift or carry light loads, or you have to climb stairs or hills often.

4O 4. You do heavy work or carry very heavy loads.

SECTION E: SMOKING

E1. The next few questions are about smoking.

Have you ever smoked cigarettes?

1O Yes 2O No ⟶ *Go to E6*

E2. At the present time do you smoke cigarettes?

3O Yes 4O No ⟶ *Go to E6*

E3. Do you usually smoke cigarettes every day?

5O Yes ⟶ How many per day? ☐☐ cigarettes

6O No

E4. In your day to day activities, do you find restrictions placed on where or when you can smoke?

7O Yes 8O No ⟶ *Go to E6*

E5. Have these restrictions affected how much you smoke each day?

1O Yes ⟶ How so? 2O Less each day

3O More each day

4O Tried to quit

5O No, about same

6O Don't know

E6. How many of the people living in your household smoke cigarettes daily? *(IF SMOKER, ADD:* "including yourself")

☐☐ people
(If none, enter 00)

E7. Do you ever feel unpleasant effects from the cigarette smoke of others?

7O Yes 8O No

SECTION F: ALCOHOL

F1. Now I would like to ask some questions about alcohol consumption.

In the next questions when we use the word drink it means:

- One bottle of beer or glass of draft
- One small glass of wine
- One shot or mixed drink with hard liquor

Have you ever taken a drink? (beer, wine, liquor or other alcoholic beverage)

1O Yes 2O No ⟶ *Go to G1*

F2. In the past 12 months, have you taken a drink? (beer, wine, liquor or other alcoholic beverage)

3O Yes 4O No ⟶ *Go to G1*

F3. In the past 12 months, how often on average did you drink alcohol? Was it...

1O Every day?

2O 4–6 times a week?

3O 2–3 times a week?

4O Once a week?

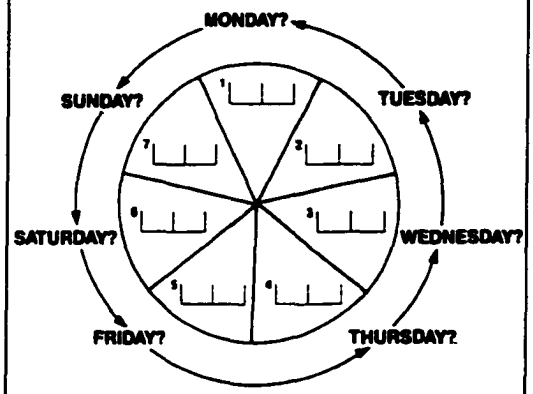
5O Once or twice a month?

6O Less often than once a month?

F4. **Thinking back over the last 7 days, starting with yesterday, how many drinks did you have on each day?**

Did not have any drinks in the past 7 days... 1○ ⟶ *Go to F5*

How many drinks did you have on...



F5. **I'm going to read you a few statements about the reasons why people drink. For each, tell me if it is a reason you drink. Do you drink...**

	Yes	No	Don't Know
a) To be sociable or to add to the enjoyment of meals?	01○	02○	03○
b) To feel good or get in a party mood?	04○	05○	06○
c) To overcome stress or when sad, lonely or depressed?	07○	08○	09○

SECTION G: DRUGS

G1. **Now I would like to ask you about your use of medicines, pills and other drugs.**

In the past 12 months have you used...

	Yes	No
a) Tranquilizers such as valium?	01○	02○
b) Diet pills or stimulants?	03○	04○
c) Anti-depressants?	05○	06○
d) Codeine, demerol or morphine?	07○	08○
e) Sleeping pills?	09○	10○
f) ASA (Aspirin) or other pain reliever?	11○	12○

G2. **Have you ever used...**

			Have you used it in the past 12 months? Yes	No
a) Marijuana or hashish?	01○	Yes	02○	03○
	04○	No		
b) Cocaine or crack?	05○	Yes	06○	07○
	08○	No		
c) LSD (acid)?	09○	Yes	10○	11○
	12○	No		
d) Amphetamines (speed)?	13○	Yes	14○	15○
	16○	No		
e) Heroin?	17○	Yes	18○	19○
	20○	No		

SECTION H: ROAD SAFETY PRACTICES

H1. **The next questions are about road safety.**

How often do you use seatbelts when you ride in a car? *(READ RESPONSES)*

1○ **Always?**

2○ **Most of the time?**

3○ **Sometimes?**

4○ **Rarely or never?**

H2. **Have you driven an all terrain vehicle (ATV) or snowmobile in the last 12 months?**

1○ Yes ⟶ **How often did you wear a helmet?**

- 2○ Always
- 3○ Most of the time
- 4○ Sometimes
- 5○ Rarely or never

8○ No

H3. ***INTERVIEWER CHECK ITEM:***

- *If F1 or F2 is "No"* 7○ ⟶ *Go to I1*
- *Otherwise* ..... 8○ ⟶ *Go to H4*

H4. **In the past 30 days, how many times have you driven a motor vehicle within two hours of drinking any amount of alcohol?**

☐☐ *(If none, enter 00)*

88○ Don't drive

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SECTION I: NUTRITION

I1. **The next questions are about nutrition.**

In the last 7 days, on how many days did you have the following as part of your breakfast?

a) **Just coffee, tea or nothing at all** ..... [1] ☐
(IF ANSWER IS 7 GO TO I2)

b) **Eggs, bacon, ham or other meat** ..... [2] ☐

c) **Bread, toast, pastries, pancakes or cereals** ..... [3] ☐

d) **Fruit or juice** ..... [4] ☐

e) **Cheese, milk or other dairy products (other than in your coffee or tea)** ..... [5] ☐

I2. **In your opinion, what are the two best ways for people to lose weight?**

(DO NOT READ — PROBE FOR SECOND CHOICE)
(IF ANSWER IS "TO DIET", ASK TO EXPLAIN)

[01] ○ Increase physical activity/exercise
[02] ○ Eat less sweets and sugar
[03] ○ Eat fewer calories
[04] ○ Don't eat between meals (snacks)
[05] ○ Skip meals
[06] ○ Eat less food (generally)
[07] ○ Eat more fruits and vegetables
[08] ○ Eat foods low in fat
[09] ○ Eat a balanced or nutritious diet
[10] ○ Other *(specify)*

I3. **Are you now trying to lose weight?**

[1] ○ Yes
[2] ○ No

I4. **Do you consider yourself...**

[3] ○ Overweight?
[4] ○ Underweight?
[5] ○ Just about right?
} *Go to J1*

I5. **Would you say you are...**

[6] ○ Very overweight?
[7] ○ Somewhat overweight?
[8] ○ Only a little overweight?

SECTION J: SOCIAL RELATIONSHIPS

J1. **The next few questions are about relationships and helping one another.**

In the past 30 days, have you helped care for a relative or friend who was suffering from a physical or mental health problem?

[1] ○ Yes
[2] ○ No
[3] ○ Don't know/Not sure

J2. **In the past 30 days, have you experienced a physical or mental health problem for which you received some care from a relative or friend?**

[4] ○ Yes
[5] ○ No
[6] ○ Don't know/Not sure

SECTION K: WORKPLACE

K1. **The next few questions are about your employment status.**

Which of the following best describes your main activity during the last 12 months? Were you mainly...

[1] ○ Working at a job or business? → *Go to K3*
[2] ○ Looking for work? → *Go to K2*
[3] ○ A student?
[4] ○ Retired?
[5] ○ Keeping house?
[6] ○ Other
→ *Go to L1*

K2. **Did you have a job or business at any time during the past 12 months?**

[7] ○ Yes
[8] ○ No → *Go to L1*

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K3. How many weeks did you work at a job or business during the last 12 months? (include vacation, illness, strikes, lock-outs and maternity/paternity leave)

☐☐ Weeks

K4. For whom do (did) you work?

1○ Self-employed

INTERVIEWER: If more than one job reported, list main job.

K5. What kind of business, industry or service is (was) that?

K6. What kind of work do (did) you do?

K7. In total, about how many people work in your business or company at all its locations?

1○ 1 to 19

2○ Between 20 and 99

3○ Between 100 and 499

4○ More than 500

5○ Don't know

INTERVIEWER: K8 to K12 applies to all jobs

K8. Have any of the following caused you excess worry or stress at work in the last six months?

	Yes	No Don't Know	Not Applicable
a) Unreasonable deadlines?	01○	02○	08○
b) Duties are not clear?	04○	05○	08○
c) Not enough influence over what you do and when you do it?	07○	08○	08○
d) No feedback on how you're doing?	10○	11○	12○
e) Conflicts with other people at work?	13○	14○	15○

K9. In the last year, how many days were you away from work because you were sick, injured or disabled?

☐☐☐ days 999○ Don't know

K10. Did you regularly work evening or night shifts?

1○ Yes 2○ No

K11. Did you regularly work on Saturday or Sunday?

3○ Yes 4○ No

K12. Do you know if the following programs are available at your place of work?

	Yes	No Don't Know	Not Applicable
a) Programs to improve health such as physical activity, nutrition or smoking cessation?	01○	02○	03○
b) Safety or accident prevention programs?	04○	05○	06○
c) Psychological, drug or alcohol counseling?	07○	08○	09○

SECTION L: WOMEN'S HEALTH

L1. *INTERVIEWER CHECK ITEM:*

Respondent is: 1○ *Female* → *Go to L2*

2○ *Male* → *Go to M1*

L2. The next questions are about preventive health practices for women.

How often do you perform breast self-examination? Would you say...

3○ At least once a month?

4○ Once every 2-3 months?

5○ Less often?

6○ Never?

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L3. Have you ever had a mammogram, that is, a breast X-ray?

1 ○ Yes → When was the last time?
- 2 ○ Less than 12 months ago
- 3 ○ 1 to 2 years ago
- 4 ○ More than 2 years ago
- 5 ○ Don't know

8 ○ No

7 ○ Don't know

L4. Have you ever had a PAP smear?

1 ○ Yes → When was the last time?
- 2 ○ Less than 12 months ago
- 3 ○ 1 to 3 years ago
- 4 ○ More than 3 years ago
- 5 ○ Don't know

8 ○ No

7 ○ Don't know

L5. Have you ever given birth?

1 ○ Yes 2 ○ No → *Go to L8*

L6. In what month and year was your last child born?

3 [ ][ ] Month 4 19[ ][ ] Year

L7. Did you breast-feed your last child?

1 ○ Yes → How long did you breast-feed your last child?
- 2 ○ Less than 1 month
- 3 ○ 1 — 2 months
- 4 ○ 3 — 4 months
- 5 ○ 5 — 6 months
- 6 ○ 6 or more months

7 ○ No

8 ○ Tried/not successful

L8. I would like to ask you a few personal questions about sexual behaviour because of its importance to personal health and social problems. Once again, please be assured that anything you tell me will remain confidential.

Now, we would like to know your age when you first had sexual intercourse. This is important information because it has some bearing on health in later years. Do you remember how old you were?

[ ][ ] Age

97 ○ Never
96 ○ Refused to answer } *Go to L11*

L9. In the past 12 months, have you had sexual intercourse?

1 ○ Yes

2 ○ No
3 ○ Refused to answer } *Go to L11*

L10. In the past 12 months, with how many partners did you have sexual intercourse?

[ ][ ] partners

96 ○ Refused to answer

L11. I would now like your opinion on some ways for people in general to prevent getting a sexually transmitted disease.

After I read each one, tell me if you think it is "Very effective", "Somewhat effective", or "Not at all effective" for preventing sexually transmitted diseases:

	Very effective	Somewhat effective	Not at all effective	Don't know how effective	Don't know method
a) A condom?	01 ○	02 ○	03 ○	04 ○	05 ○
b) A diaphragm?	06 ○	07 ○	08 ○	09 ○	10 ○
c) Spermicidal jelly or foam?	11 ○	12 ○	13 ○	14 ○	15 ○
d) Ask if partner has a sexually transmitted disease?	16 ○	17 ○	18 ○	19 ○	20 ○
e) Sex only with regular partner?	21 ○	22 ○	23 ○	24 ○	25 ○
f) No sex at all?	26 ○	27 ○	28 ○	29 ○	30 ○

L12. What do you think your chances are of getting a sexually transmitted disease? Would you say they are...

1 ○ High?

2 ○ Medium?

3 ○ Low?

4 ○ None?

---

5 ○ Don't know

6 ○ Already have an STD

L13. Due to what you know about sexually transmitted diseases, have you changed your sexual behaviour in the past 12 months?

7 ○ Yes

8 ○ No → *Go to N1*

L14. Have you...

	Yes	No
a) Had sexual intercourse with only one partner?	1 ○	2 ○
b) Used condoms for protection?	3 ○	4 ○
c) Been more careful in selecting sexual partners?	5 ○	6 ○
d) Anything else? *(specify)*	7 ○	8 ○

INTERVIEWER: *Go to N1*

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SECTION M: MEN'S HEALTH

M1. I would like to ask you a few personal questions about sexual behaviour because of its importance to personal health and social problems. Once again, please be assured that anything you tell me will remain confidential.

Now, we would like to know your age when you first had sexual intercourse. Do you remember how old you were?

☐☐ Age

97 ○ Never
98 ○ Refused to answer
} *Go to M4*

M2. In the past 12 months, have you had sexual intercourse?

1 ○ Yes
2 ○ No
3 ○ Refused to answer
} *Go to M4*

M3. In the past 12 months, with how many partners did you have sexual intercourse?

☐☐ partners

96 ○ Refused to answer

M4. I would now like your opinion on some ways for people in general to prevent getting a sexually transmitted disease.

After I read each one, tell me if you think it is "Very effective", "Somewhat effective", or "Not at all effective" for preventing sexually transmitted diseases:

	Very effective	Somewhat effective	Not at all effective	Don't know how effective	Don't know method
a) A condom?	01 ○	02 ○	03 ○	04 ○	05 ○
b) A diaphragm?	06 ○	07 ○	08 ○	09 ○	10 ○
c) Spermicidal jelly or foam?	11 ○	12 ○	13 ○	14 ○	15 ○
d) Ask if partner has a sexually transmitted disease?	16 ○	17 ○	18 ○	19 ○	20 ○
e) Sex only with regular partner?	21 ○	22 ○	23 ○	24 ○	25 ○
f) No sex at all?	26 ○	27 ○	28 ○	29 ○	30 ○

M5. What do you think your chances are of getting a sexually transmitted disease? Would you say they are...

1 ○ High?
2 ○ Medium?
3 ○ Low?
4 ○ None?

---

5 ○ Don't know
6 ○ Already have an STD

M6. Due to what you know about sexually transmitted diseases, have you changed your sexual behaviour in the past 12 months?

7 ○ Yes
8 ○ No → *Go to N1*

M7. Have you...

	Yes	No
a) Had sexual intercourse with only one partner?	1 ○	2 ○
b) Used condoms for protection?	3 ○	4 ○
c) Been more careful in selecting sexual partners?	5 ○	6 ○
d) Anything else? *(specify)*	7 ○	8 ○

SECTION N: DENTAL HEALTH

N1. Next I would like to ask you some questions about your teeth.

Do you have one or more of your natural teeth?

1 ○ Yes
2 ○ No → *Go to N5*

N2. Have you seen a dentist in the past 12 months?

3 ○ Yes
4 ○ No
5 ○ Don't know
} *Go to N4*

N3. During this time, did you see a dentist for...

	Yes	No
a) A dental checkup or cleaning?	01 ○	02 ○
b) A filling or extraction? (non-emergency)	03 ○	04 ○
c) Any periodontal treatment? (gum treatment)	05 ○	06 ○
d) Orthodontic treatment? (braces)	07 ○	08 ○
e) Crown or bridge work?	09 ○	10 ○
f) A dental emergency?	11 ○	12 ○

N4. How often do you usually brush your teeth? *(DO NOT READ)*

1 ○ Twice or more a day/after every meal
2 ○ Once a day
3 ○ A few times a week
4 ○ Once a week
5 ○ A few times a month
6 ○ Once a month
7 ○ Rarely/Never
8 ○ Don't know

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N5. Are you covered by dental insurance?

[1] ○ Yes

[2] ○ No

[3] ○ Don't know

SECTION O: HOME AND ENVIRONMENTAL ISSUES

O1. The next questions are about home and environmental issues.

Do you, or others in your household...

	Yes	No Don't know	Not applicable
a) Own a smoke alarm that works?	01 ○	02 ○	03 ○
b) Own a first-aid kit?	04 ○	05 ○	06 ○
c) Have a household member trained in first aid?	07 ○	08 ○	09 ○
d) Own a fire extinguisher that works?	10 ○	11 ○	12 ○
e) Read nutrition labels on packages to make food choices?	13 ○	14 ○	15 ○
f) Check that the water heater thermostat does not exceed 50°C or 120°F? (scalding)	16 ○	17 ○	18 ○
g) Recycle papers, bottles, cans, etc.?	19 ○	20 ○	21 ○
h) Compost fruit and vegetable waste?	22 ○	23 ○	24 ○
i) Buy products made of recycled materials?	25 ○	26 ○	27 ○

O2. During the past 12 months, how much do you think that environmental pollution has affected your health? Would you say...

[1] ○ Very much?

[2] ○ A fair amount?

[3] ○ Not very much?

[4] ○ Not at all?

[5] ○ Don't Know

SECTION P: GOVERNMENT ACTION ON HEALTH PROMOTION ISSUES

P1. I will now read a list of health topics. For each I'd like your opinion about how important you feel it is for the government to deal with each topic.

Tell me on a scale of 1 to 10; with 1 being "not at all important" and 10 being "extremely important", how important do you feel it is for the government to deal with...

Not at all important — Extremely important

1 2 3 4 5 6 7 8 9 10

		Don't know
a) Drug use?	01 ☐	[11] ○
b) Smoking?	02 ☐	[11] ○
c) Alcohol problems?	03 ☐	[11] ○
d) Child health?	04 ☐	[11] ○
e) Eating habits?	05 ☐	[11] ○
f) Mental health?	06 ☐	[11] ○
g) Accident prevention on the road?	07 ☐	[11] ○
h) Accident prevention at work?	08 ☐	[11] ○
i) Exercise or physical activity?	09 ☐	[11] ○
j) Environmental pollution?	10 ☐	[11] ○
k) AIDS?	11 ☐	[11] ○
l) Other sexually transmitted diseases?	12 ☐	[11] ○
m) Dental health?	13 ☐	[11] ○
n) Heart disease?	14 ☐	[11] ○

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SECTION Q:
HEALTH INTENTIONS — PAST AND FUTURE

Q1. **Did you do something to improve your health in the past 12 months?**

[1] ○ Yes

[2] ○ No ⟶ *Go to Q4*

Q2. **What is the single most important change you have made in the past 12 months to improve your health?** *(DO NOT READ, MARK ONLY ONE)*

[01] ○ Increased exercise, sports or physical activity
[02] ○ Lost weight
[03] ○ Changed diet or eating habits
[04] ○ Quit smoking/reduced amount smoked
[05] ○ Reduced drug/medication use
[06] ○ Drank less alcohol
[07] ○ Managed or reduced blood pressure
[08] ○ Managed or reduced cholesterol
[09] ○ Managed or reduced stress
[10] ○ Changed physical environment
[11] ○ Received medical treatment
[12] ○ Changed sexual behaviour or reduced risk of STD's
[13] ○ Improved dental hygiene
[14] ○ Other *(specify)*

Q3. **Did any of the following help you to make this change?**

	Yes	No Don't know	Not Applic-able
a) Support from family and friends	[01] ○	[02] ○	[03] ○
b) Increased knowledge of health risks	[04] ○	[05] ○	[06] ○
c) Changes in legislation or by-laws	[07] ○	[08] ○	[09] ○
d) New policy or program at school or work	[10] ○	[11] ○	[12] ○
e) Change in life situation (eg. marital status, employment, moving home, etc.)	[13] ○	[14] ○	[15] ○
f) Advice or support of health professional(s)	[16] ○	[17] ○	[18] ○
g) Self-help or mutual aid group (eg. AA, Weight Watchers)	[19] ○	[20] ○	[21] ○
h) Other people setting an example	[22] ○	[23] ○	[24] ○
i) Changes in social values	[25] ○	[26] ○	[27] ○
j) Commercial products or services	[28] ○	[29] ○	[30] ○
k) Prayer or spiritual guidance	[31] ○	[32] ○	[33] ○

Q4. **Considering the health topics we've discussed in this questionnaire, is there anything you intend to change to improve your health in the next year?** *(DO NOT READ, MARK ALL THAT APPLY)* *(PROBE:* **Anything else?)**

[01] ○ Nothing
[02] ○ Increase exercise, sports or physical activity
[03] ○ Lose weight
[04] ○ Change diet or eating habits
[05] ○ Quit smoking/reduce amount smoked
[06] ○ Reduce drug/medication use
[07] ○ Drink less alcohol
[08] ○ Manage or reduce blood pressure
[09] ○ Manage or reduce cholesterol
[10] ○ Learn to manage or reduce stress
[11] ○ Change physical environment
[12] ○ Receive medical treatment
[13] ○ Change sexual behavior or reduce risk of STD's
[14] ○ Improve dental hygiene
[15] ○ Other *(specify)*

SECTION R:
CLASSIFICATION QUESTIONS

R1. **Now a few general questions.**

What is your postal code?

☐☐☐ ☐☐☐

[1] ○ Don't know

R2. **What is the highest grade or level of education you have ever attended or ever completed?** *(MARK ONLY ONE)*

[01] ○ No schooling
[02] ○ Some } Elementary
[03] ○ Completed }
[04] ○ Some } Secondary
[05] ○ Completed }
[06] ○ Some } Community college, technical college, CEGEP or nurse's training.
[07] ○ Completed }
[08] ○ Some } University (eg. B.A., M.A., Ph.D.) or teacher's college
[09] ○ Completed }
[10] ○ Other education or training

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R3. What is the month and year of your birth?

[1] ☐☐ Month [2] 19☐☐ Year

R4. What language do you speak most often at home?

[3]○ English
[4]○ French
[5]○ Italian
[6]○ Chinese
[7]○ German
[8]○ Other *(specify)*

R5. Canadians belong to many ethnic or cultural groups such as Inuit, Irish, Scottish, French or Chinese. To which ethnic or cultural groups do YOU belong? *(ACCEPT MULTIPLE RESPONSES, DO NOT PROBE)*

[01]○ English
[02]○ French
[03]○ Scottish
[04]○ Irish
[05]○ German
[06]○ Ukrainian
[07]○ Italian
[08]○ Dutch
[09]○ Canadian
[10]○ Other *(specify)*

R6. What is your current marital status? Are you...

[1]○ Single (Never married)?
[2]○ Married (and not separated), or living common-law?
[3]○ Separated?
[4]○ Divorced?
[5]○ Widowed?

R7. Are there any children under 15 years old old living in your household?

[1]○ Yes → How many are...
- 5 years old or less? [2]☐
- 6 to 11 years old? [3]☐
- 12 to 14 years old? [4]☐

[5]○ No

R8. What is your best estimate of the total income of all household members from all sources in 1989 before taxes and deductions? Was the total household income...

- Less than $20,000 [01]○→
  - Less than $10,000? [05]○→
    - Less than $5,000? [09]○
    - $5,000 or more? [10]○
  - $10,000 or more? [06]○→
    - Less than $15,000? [11]○
    - $15,000 or more? [12]○
- $20,000 or more [02]○→
  - Less than $40,000? [07]○→
    - Less than $30,000? [13]○
    - $30,000 or more? [14]○
  - $40,000 or more? [08]○→
    - Less than $60,000? [15]○
    - $60,000 to $79,999? [16]○
    - $80,000 or more? [17]○

[03]○ No income
[04]○ Don't know

SECTION S: DATA SHARING AGREEMENT

S1. Statistics Canada is conducting this survey jointly with Health and Welfare Canada and the provincial ministry responsible for health promotion in Alberta. The information collected will be kept confidential and used only for statistical purposes.

DO YOU AGREE TO SHARE YOUR ANSWERS WITH THESE MINISTRIES?

YES [1]○ NO [2]○

THANK YOU VERY MUCH FOR YOUR ASSISTANCE!

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APPENDIX C-4

SELECTION CRITERIA FOR PARTICIPANTS

Each potential participant was given a copy of the Health Promotion Survey to read and become familiar with the document. At a later date a research assistant contacted the potential participant by telephone and completes the survey. Using the Health Promotion Survey, along with previous research outlining diseases associated with mtDNA deletions, smoking definitions from the World Health Organization and guidelines for determining levels of physical activity from Statistics Canada, the research assistant selected each participant based on the following criteria.

1. Three (3) or four (4) generations
    a. Maternal line (females for the first three [3] generations)
2. Can include one or more members of the same generation (e.g. twins) based on position within the generation
3. Can be from any geographic location
4. Can be from any nationality or race
5. Must not be suffering from any known diseases Ψ
6. Must not be participating in any high endurance physical activity on a regular basis!

ΨMajor illnesses as defined in the literature [6,42,47] and which would have a direct relationship with mitochondrial DNA (mtDNA) deletion.

!Physical activity as mentioned in the literature [79], referring to activity that exceeds 10 hrs per week also excluding professional athletes. Moderate leisure-time physical activity refers to reporting physical activities requiring at least medium energy expenditure (i.e., at least 1.5 kilocalories per day, based on the duration and independently established energy demand of the activity) and reporting activities at least 12 times per month, where the duration of each session is at least 15 min. Only recreational activities are considered for this measure (for example, physical activities at work or while commuting to and from work is not included).

For the purposes of the study, smoking was defined as follows: Non-smokers as mentioned in the literature [108], is someone who, at the time of the survey, does not smoke at all. *A non-smoker* can be divided further into three categories.

B1. *Ex-smokers* are people who were formerly daily smokers but currently do not smoke at all.

B2. *Never smokers* are those who have never smoked at all, or have never been daily smokers and have smoked less than 100 cigarettes in their lifetime.

B3. *Ex-occasional* are formerly occasional, but never daily, smokers and who smoked more than 100 cigarettes in their lifetime and who does not now smoke at all.

BIOGRAPHICAL SKETCH

Roy Ralph Wittock was born in St. Catherine, Jamaica, on July 9, 1972. He also received his elementary and high school education in St. Catherine, Jamaica. In May 1995, he graduated with a diploma in Medical Technology from the University of Technology in Kingston, Jamaica. In May 1998, he graduated with a Bachelor of Science degree in Medical Laboratory Sciences (Hon) from Lakehead University in Thunder Bay, Ontario. In January 1999, he entered the School of Kinesiology Graduate Studies also at Lakehead University in Thunder Bay, Ontario. From January 1999 to April 2002, he served as a graduate teaching assistant in the School of Kinesiology and the Department of Biology at Lakehead University while studying for the Master of Science degree (MSc.) in Kinesiology with a specialization in Gerontology. His area of research deals with the effects of chronological age on mitochondrial DNA deletion. He will receive his MSc. degree Kinesiology, specialization in Gerontology, in May of 2002.