MITOCHONDRIAL HAPLOGROUPS IN OBESE PATIENTS PREDISPOSED TO NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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Date: July 23, 2014

Summer Semester 2014
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DEDICATION

This dissertation is dedicated to my lovely nephew “Little Strawberry”.

ACKNOWLEDGEMENT

I would like to express my special thanks to Dr. Ancha Baranova for her support, encouragement, and mostly being my advisor. I also wish to express my sincere gratitude to Dr. Rohini Mehta who patiently helped me throughout my project. I place on record, very special thanks to Dr. Mike Estep for valuable advises in lab techniques and Dr. Aybike Birerdinc for professional guidance and creating a friendly environment. Thanks to my friend Kameron Tavakolian for his assistance in the project. I also would like to thank Maria Keaton and Katherine Doyle for their great advices in Inova lab. I am so grateful to Debby Kermer, research consultant, in Fenwick library and Lei Wang for helping me with data analysis. My special thanks to Dr. Alessandra Luchini, committee member, and Dr. Zobair Younossi, Vice President for Research at Inova Health System, for providing this great opportunity for students to participate in studies. Finally, I would like to express my very special thanks to my dear friend Shadi and my parents for their support and encouragement.

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LIST OF ABBEREVIATIONS

BMI……Body Mass Index
CR-region……..Control Region
CRS……...……..Cambridge Reference Sequence
ddNTP……..dideoxy Nucleotide Triphosphate
FFA…….…..Free Fatty Acid
HCC…….Hepatocellular Carcinoma
HDL……..High Density Lipoprotein
HVS……Hyper Variable Segment
LDL……..….Low Density Lipoprotein
MDB……Mallory-Denk Body
MetS…….……Metabolic Syndrome
mtDNA……..Mitochondrial DNA
NAFLD……..non-Alcoholic Fatty Liver Disease
NASH…….……non-Alcoholic Steatohepatitis
OXPHOS…….Oxidative Phosphorylation
rCRS…….……..revised Cambridge Reference Sequence
SNP……..…….Single Nucleotide Polymorphism
T2D…….…….Type 2 Diabetes

ABSTRACT

MITOCHONDRIAL HAPLOGROUPS IN OBESE PATIENTS PREDISPOSED TO NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

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George Mason University, 2014

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Liver diseases are considered a significant health problem worldwide. Non-alcoholic fatty liver disease (NAFLD), which is widely considered the hepatic manifestation of the metabolic syndrome, is a complex multifactorial disease trait where environment and genetic variations interact to determine the wide spectrum of disease progression. One of the key challenges is to predict the progression of NAFLD. Our central hypothesis is that certain mitochondrial genotype or genotypes may serve as an indicator of increased susceptibility to the progressive course of NAFLD. In this study, the association of sequence variations (haplogroups) in the control loop of mitochondria was investigated. A total of 115 cases of morbidly obese patients with liver biopsy results were included (average BMI=48.0±11.1; White=80.2%; African-American=17.4%; Females=75.6%). All these patients also had metabolic syndrome. Among 86 profiled individuals, 64 (74.4%) were diagnosed with NAFLD. After extracting DNA from whole

blood cells and amplifying 1122 base pairs of control loops by PCR and Sanger sequencing, all samples have been categorized into one of 11 haplogroups (H, L, K, U, J, T, M, W, N, C, and X). The most common haplogroup in this study (34.9%) was H haplogroup, while other haplogroups including L (15.1%), K (13.95%), U (11.6%), J (11.6%), T (7.0%), M, W, N, C, and X (1.2% each) were less common. The presence of NAFLD was found to be associated significantly with non-L haplogroups (H, K, T, C, U, M, N, J, W, X) (80.84% vs 38.4%) (Fisher's test, two tailed P= 0.003). Within the major haplogroups, the prevalence of NAFLD varied: H (80.00%), L (38.46%), K (83.33%), J and U (80.00% each) (Fisher's test two tailed P= 0.068). Moreover, within L haplogroups, L3 shows the least tendency to develop NAFLD. Further investigations are warranted to assess why in individuals with East African L3 haplogroup the susceptibility to NAFLD is reduced.

1: INTRODUCTION

1.1 Obesity and Metabolic Syndrome.

Obesity and overweight are defined as accumulation of excessive fat and determined by body mass index (BMI). In adults, BMI > 25 kg/m2 is considered overweight, while individuals with BMI over 30 kg/m2 are defined as obese. In 2009, mean BMI in the United States was 28.5±0.5 for more than 20 years old males and 28.4±0.7 for more than 20 years old females (World Health Organization, 2014).

Obesity has doubled worldwide since 1980, according to the World Health Organization report. In 2008, 1.4 billion adults were overweight or obese, and 40 million children under the age of 5 were obese or overweight in 2012 (World Health Organization, 2014). Both obesity and overweight are associated with proportional increases in all-cause mortality and are major risk factors for cardiovascular disease, stroke, type 2 diabetes (T2D), hypertension, and certain types of cancers especially colon and hormonally related cancers.

Metabolic syndrome, a common metabolic disorder, has a significant association with obesity. In most countries, up to 20% to 30% of the adult population have the metabolic syndrome (MetS). The criteria for defining MetS vary, but in most guidelines it includes rise in fasting plasma glucose, triglyceride, blood pressure, visceral obesity, and decrease in HDL-cholesterol level (Scaglione et al., 2010). MetS raises the cardiovascular

disease risk twice and type 2 diabetes by about 5-fold (Grundy et al., 2008; Liu et al., 2014). Additionally, both obesity and metabolic syndrome are associated with liver steatosis and non-alcoholic steatohepatitis (NASH) (Younossi, 2008).

1.2 Non-Alcoholic Fatty Liver Disease: Definition.

In 1980, non-alcoholic fatty liver disease (NAFLD) was described for the first time by Ludwig and colleagues as a liver disease that histologically mimics alcoholic hepatitis without a history of excessive drinking (Ludwig et al., 1980). NAFLD is defined by accumulation of fat, mainly triglycerides, in the liver when the fat weight exceeding 5% to 10% of the liver weight and there are no evidence of secondary fat accumulation due to excessive alcohol consumption, viral infection, hereditary disorders, or use of steatogenic medications (Chalasani et al., 2012; Neuschwander-Tetri and Caldwell, 2003). The threshold for alcohol consumption varies by study; in many centers up to 20-40 gram per day in men and 20 gram per day in women or roughly the equal amount of two glasses of wine per day is acceptable, whereas other investigators cited a criterion of 10 gram per day or less (Neuschwander-Tetri and Caldwell, 2003). NAFLD includes a wide range of liver damage from mild steatosis to non-alcoholic steatohepatitis (NASH) which is defined as steatosis with or without inflammation, ballooning degeneration and/or presence of Mallory-Denk bodies. NASH can ultimately can progress to fibrosis followed by cirrhosis and hepatocellular carcinoma (HCC) (Kneeman et al., 2012).

1.3 Non-Alcoholic Fatty Liver Disease: Prevalence.

Liver diseases are considered a significant health problem both in United States and worldwide (Erickson et al., 2009; Tiniakos et al., 2010). NAFLD is recognized as the leading cause of chronic liver disease in the United States and worldwide (Erickson, 2009; Tiniakos et al., 2010). The prevalence rate of NAFLD is reported as between 10-35% of the general population. Because one-third of the population are obese, the prevalence of NAFLD is likely to be about 30% in the US (Vernon et al., 2011). Obese individuals, type 2 diabetes patients and males are at greater risk (Lazo et al., 2013). The NAFLD prevalence varies by ethnicity; it is noted to be 45% in Hispanics, 33% in Caucasians, and 24% in Blacks (Angulo, 2007; Pan and Fallon, 2014). Mexican-Americans are at especially high risk (Lazo et al., 2013).

It is also important to note that NAFLD is lately being ascribed as the hepatic manifestation of the metabolic syndrome (Medina-Santillán et al., 2013; Younossi, 2008). The progression of NAFLD to non-alcoholic steatohepatitis (NASH) is more common in patients with metabolic syndrome (Yki-Järvinen, 2014). Based on the NHANES data, 2.6% of the US population may have NASH; many of these patients are non-obese (Younossi et al., 2012).

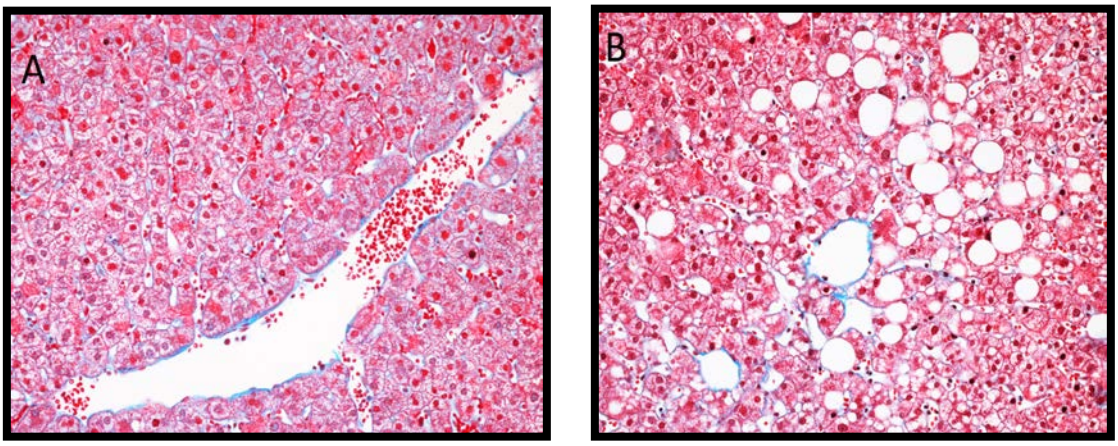
1.4 Non-Alcoholic Fatty Liver Disease: Histopathology.

NAFLD covers a histological spectrum of liver disease that starts with accumulation of small and large droplets of fat and can progress to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. As its name implies, NAFLD begins with fat gathering into the hepatocytes, a lesion termed steatosis (Brunt and Tiniakos, 2010). The minimum criterion requirement for histological diagnosis of

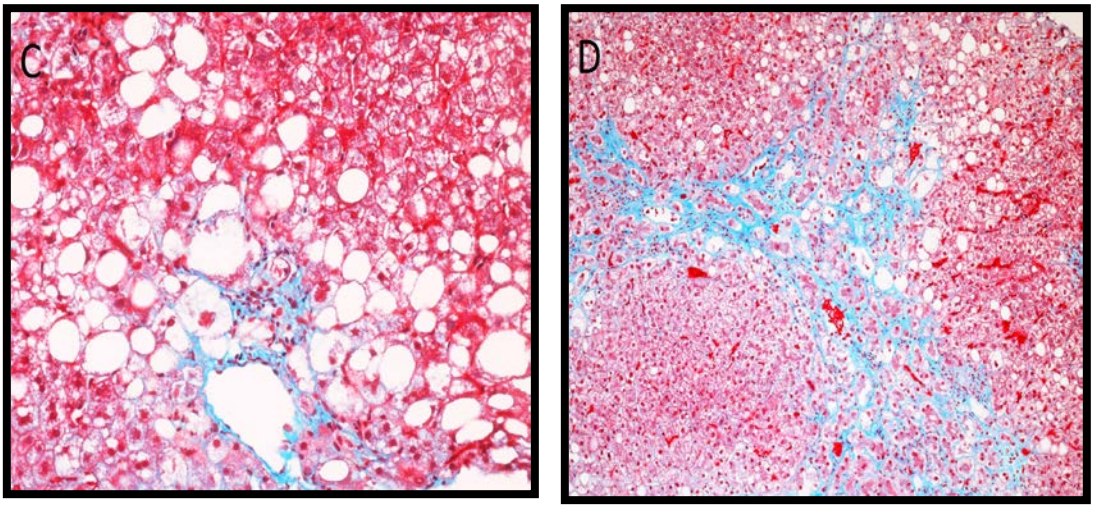
NAFLD is more than 5% steatotic hepatocytes in a liver biopsy (Bondini et al., 2007). The intracytoplasmic fat can create several features. A large macrovesicular fat droplet displace the nucleus and remaining cell contents to the cell periphery; in some cases when in addition to large fat droplets, numerous small lipid droplets are present in the cytoplasm, mixed steatosis (microsteatosis and microsteatosis) occurs. Rarely, pure microsteatosis may be seen which requires special stains such as Oil-Red-O for the histological assessment (Tiniakos et al., 2010). In addition, other features such as lipogranulomas, and mild portal and lobular inflammation may also be seen in NAFLD (Brunt and Tiniakos, 2010). Steatosis is evaluated quantitatively and reported according to the percentage of liver parenchyma occupied by steatotic hepatocytes: 0%-33% mild, 33%-66% moderate, and >66% severe steatosis (Brunt and Tiniakos, 2010; Kleiner et al., 2005).

The minimal histological criteria for diagnosis of NASH include steatosis, hepatocyte injury, and lobular inflammation. In NASH, hepatocyte injury can occur in the form of ballooning, apoptosis, or lytic necrosis. In hepatocellular ballooning liver cell appears enlarged due to a large lipid droplet with rarefied pale cytoplasm, and hyperchromatic nucleus (Caldwell et al., 2010). Alterations of the intermediate filament cytoskeleton with reduced or lost keratin 8 (CK8) and 18 (CK18) can be detected by immunostaining and may serve as an objective marker for histological recognition of early stages of hepatocyte ballooning degeneration (Lackner et al., 2008). In NASH, hepatocyte injury leads to hepatocyte apoptosis and its elevation correlates to disease severity (Feldstein et al., 2003). Lobular inflammation is usually mild and inflammatory

cells such as lymphocytes, eosinophils, and neutrophils are present. Portal inflammation is also mild with mostly chronic inflammatory infiltrates. The “chicken wire” fibrosis pattern in NAFLD is characteristic for this pathology and is due to collagen deposition along the sinusoids and around the hepatocytes. Other histological lesions may include Mallory-Denk bodies (MDBs), megamitochondria, and glycogenated nuclei (Brunt and Tiniakos, 2010). Mallory-Denk bodies are perinuclear eosinophilic inclusions that consist of keratin 8, keratin 18, ubiquitin, and chaperones. Mallory-Denk bodies can be observed in steatohepatitis with different etiologies (Hanada et al., 2008). Round or needle-shaped giant mitochondria with crystalline inclusions, known as megamitochondria, are seen in 5% to 15% of liver cells in NASH patients (Le et al., 2004). Another supportive factor of NASH is the presence of glycogenated vacuolated nuclei in periportal liver cells (Tiniakos et al., 2010).



Figures 1A and 1B. A. Normal liver tissue. Note the lack of lipid vesicles and fibrosis in trichrome stained specimen. B. Steatosis without steatohepatitis. Moderate cytoplasmic fat globules are present without fibrosis. ***Reprinted with permission of Dr. Zachary Goodman.***



Figures 1C and 1D. C. Steatohepatitis with minimal fibrosis. Hepatocyte ballooning, inflammation, and minimal fibrosis (intensified in blue by trichrome stain) are visible. D. Steatohepatitis with advanced fibrosis. *Reprinted with permission of Dr. Zachary Goodman.*

1.5 Non-Alcoholic Fatty Liver Disease: Pathogenesis.

NAFLD is considered a multifactorial disease where host genetic factors and environment interact to produce disease phenotype and determine disease progression (Al-Serri et al., 2012). Among environmental factors, Western type of diet and sedentary life are most important. Visceral obesity is strongly associated with NAFLD due to the release of pro-inflammatory cytokines (IL-6, IL-1, TNF-α) and hormones (leptin, resistin) into circulation, and increased lipolysis with overflow of free fatty acids (FFAs) to the liver. This flow impairs intracellular pathway of lipid metabolism and induces steatogenic transformation of hepatocytes. In addition, increased synthesis of triglyceride and β-oxidation of FFAs reduce antioxidant defenses and elevate oxidative stresses. Consequently, functional and morphological alterations in hepatocytes generates signal

which activates apoptotic/necrotic pathways in liver parenchyma. Persistence of this active signal culminates in hepatic fibrosis (Grattagliano et al., 2012).

The role of mitochondria in NAFLD is critical, as these organelles are contributing to both oxidative stress and to cell death. Mitochondria may serve as mediator of liver injury at later stages of NAFLD. In patients with more advanced forms of NAFLD, an ultrastructural changes with a decreased ability to synthesize ATP in mitochondria are observed; these change are accompanied be overall decreases in the levels of mitochondrial DNA and the activity of respiratory complexes (Grattagliano et al., 2012; Kenney et al., 2013).

Variation in individual susceptibility to progressive disease and the ethnic differences in the prevalence of the disease suggest that genetic factors play a significant role in pathogenesis of NAFLD. A number of candidate genes associated with NAFLD have been identified (Anstee and Day, 2013; Hernaez, 2012). About 50% of individuals with NAFLD carry at least one variant (G) allele at polymorphic position *rs738409* in the open reading frame of *PNPLA3* gene that encodes patatin-like phospholipase domain-containing 3 protein (Romeo et al., 2008). The *rs738409* (G) variant is associated with higher liver fat content even after adjustment for BMI, diabetes status, and alcohol use (Romeo et al., 2008). Later studies showed that *rs738409* (G) variant of *PNPLA3* is a loss-of-function mutation that affects the secretion of apoB-containing lipoproteins and promotes intracellular lipid accumulation in the liver by reducing the lipidation of VLDL (Pirazzi et al., 2012). Interestingly, in individuals with PNPLA3 variant, NAFLD is not accompanied by features of metabolic syndrome (Shen et al., 2014). Observed variation

in individual susceptibility to progressive disease and ethnic studies suggest that genetic factors play a significant role in pathogenesis of NAFLD. NAFLD is commonly associated with visceral obesity, but not all obese individuals develop NAFLD. Additionally, the mechanisms influencing or predisposing individuals to progressive form of NAFLD such as NASH and/or fibrosis are unclear.

1.6 Mitochondria.

Mitochondria are membrane-bounded organelles that convert organic molecules into energy by aerobic metabolism (Snustad and Simmons, 2012). These organelles multitask in the cell environment, with their main function being production of adenosine triphosphate (ATP) (Grattagliano et al., 2012). Mitochondria almost certainly have developed from prokaryotic cells. According to endosymbiotic theory, mitochondria were probably free living bacteria and that were engulfed by another cell via phagocytosis around 1.45 billion years ago (Martin and Mentel, 2010). These prokaryotes contributed their genomes and the ability of oxidative phosphorylation. Mitochondrial genetic system includes both the DNA and the macromolecules needed for transcription and translation (Snustad and Simmons, 2012). However, modern mitochondria are only semi-autonomous as they utilize a number of proteins encoded by nuclear genes, synthesized in cytoplasm and imported into mitochondria.

Importantly, sperm cells do not contribute their mitochondrial genomes to resultant zygotes. The mitochondrial genotype is determined by the oocyte only, thus, being inherited maternally. Number of copies of mtDNA is considerably different in

various cell types. During mitosis, tens to hundreds of mitochondria separate randomly to two daughter cells (Strachan and Read, 2011).

Human mtDNA is a highly polymorphic circular double-stranded molecule of a 16,569 nucleotide pairs. It replicates unidirectionally and encodes for 37 genes including 13 oxidative phosphorylation (OXPHOS) protein subunits, 2 ribosomal RNAs and 22 transfer RNAs, one for each of the 22 types of mitochondrial tRNA (Figure 2). Mitochondrial DNA overall base content is 44% (G+C) with a differing base composition in the strands: the rich in cytosine strand is known as light strand (L), while the heavy (H) strand is rich in guanine. The repeat synthesis of a short segment of H strand produces a third DNA strand that called 7S DNA. The 7S DNA strand can displace H strand and create the control region/displacement loop (CR/D-loop) region, that harbors the major promoters (Strachan and Read, 2011) (Figure 2). The mtDNA lacks histones and has poor DNA repair systems. Furthermore, it functions in close proximity to the highly mutagenic oxygen radicals, thus, being at a greater risk of mutational damage and experiencing much faster evolution rate compared to nuclear DNA.

1.7 Mitochondria D Loop.

The control region/displacement loop (CR/D-loop) region is the most variable region in the mitochondrial genome. It does not code for proteins or RNAs, while being critically important in mtDNA replication and transcription. The most polymorphic nucleotide sites within this loop are concentrated in three ‘hypervariable segments’ HVS-I , HVS-II and HVS-III. These sites evolve at a rate much faster than average (Orekhov et al., 1999; Wilkinson-Herbots et al., 1996; Stoneking, 2000). Both germline and somatic

mtDNA mutations occur preferentially at hypervariable sites, supporting the view that hypervariable sites are indeed mutational hotspots (Stoneking, 2000).

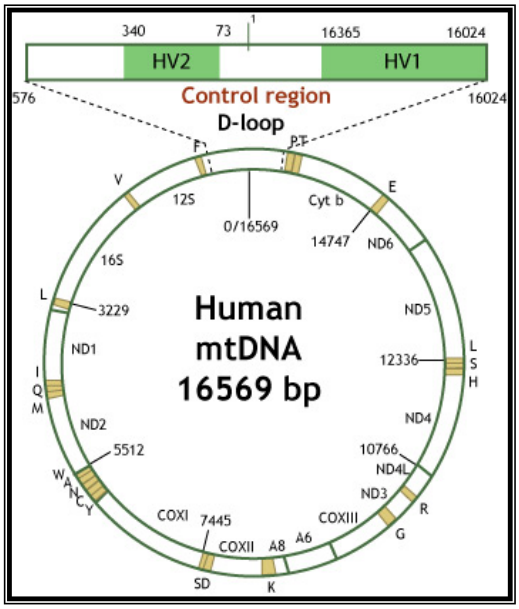


Figure 2. Organization of mitochondrial DNA *Reprinted with permission from* ("Forensic Applications," 2007).

In 1981, the first human mitochondrial DNA (mtDNA) sequence, the Cambridge Reference Sequence (CRS), was published. Subsequent sequencing of other individual's mitochondrial genomes revealed substantial differences to the CRS. The average number of base pair differences between two human mitochondrial genomes is estimated to be from 9.5 to 66 (Zeviani et al., 1998). The lack of recombination and relatively high mutation rate compared to nuclear DNA has resulted in the accumulation of a wide range of neutral, population-specific base substitutions in mtDNA along radiating maternal

lineages, of which specific combinations constitute the so-called mitochondrial haplogroups. Mitochondrial DNA represents one of the most informative systems for inter- and intra-specific study of human genetic diversity. The sequencing of mtDNA is commonly performed for genealogical and forensic purposes. Since mtDNA is transmitted maternally, it is often used to trace individual's maternal lineage. Notably, more than half of these substitutions (transitions/trasnversions/indels) are located in the hypervariable segment I (HVS-I) (Orekhov et al., 1999). About 99% of mitochondrial variation in the United States Caucasian population may be classified into one of the following ten haplogroups (H, I, J, K, M, T, U, V, W, X) (Allard et al., 2002).

It is important to stress that the control region of mitochondrial DNA contains origin of mtDNA replication and promoters for its transcription. Thus, inter-individual differences in the control region may alter the functional capacity of mitochondria. Although most mitochondrial base substitutions are believed to be neutral and population-specific, same variants in mtDNAs exert varying influences on the outcomes of diseases (Brandstätter et al., 2003; Moreno-Loshuertos et al., 2006; Pello et al., 2008; Pichaud et al., 2012). Thus, having on or another mitochondrial haplogroup may serve as predisposing or protective factor for a given disease. For instance, mtDNA haplogroup N9a were shown to confer resistance against type 2 diabetes in Asians (Fuku et al., 2007), while haplogroups A and M7a are a genetic risk for coronary atherosclerosis in the Japanese elderly (Sawabe et al., 2011).

1.8 SPECIFIC AIMS

The profiling of mitochondrial DNA polymorphisms is extremely informative systems for research into human genetic diversity. The existence of hypervariable sites (sites that evolve at a rate much faster than average) in the non-coding human mtDNA control region has been well documented. At these hypervariable sites, both germline and somatic mtDNA mutations may occur; these sites are indeed preferred by the mutations. This observation supports the view that hypervariable sites are indeed mutational hotspots (Stoneking, 2000). Given the role of mitochondria in metabolic pathways, variation in mitochondrial sequence may predispose to certain metabolic diseases, in particular, to NAFLD. It is evident that the functional defects in mitochondrial DNA could directly contribute to the progression of NAFLD through a decay of mitochondrial oxidative capacity.

The mitochondrial genome is present at high copy numbers in each cell, and is thus relatively easy to obtain enough DNA from limited amount of non-invasively collected biological material (i.e. buccal swabs). The control region of mitochondrial DNA contains origin of mtDNA replication and promoters for transcription, hence, the variations in control region may have functional importance. Even if variations in the control region are neutral polymorphisms, they may be haplotypically linked to truly functional variants within other loci in mtDNA, hence, they might be used in predisposition assays, for example, as indicators of increased susceptibility to progressive NAFLD. This may serve as a step towards NAFLD management. Additionally, the mitotyping may help overcome problems of population stratification in frame of Personalized Medicine paradigm.

This study was performed in pursuing two **Specific Aims** outlined below:

1. To investigate the association of sequence variations (haplogroups and indels) in HVI and HVII region of control loop of mitochondria with NAFLD in morbidly obese patients with metabolic syndrome.

2. To investigate the association between mitochondrial DNA sequence variants and the degree of hepatic steatosis, the presence of NASH and the severity of fibrosis in patients with NAFLD.

2: METHODS AND MATERIALS

2.1 Specific Aims and Samples.

The goal of this study was to investigate the association of sequence variations (haplogroups) in control loop of mitochondria in patients with metabolic syndrome and biopsy proven NAFLD and control group. This study was conducted in Betty and Guy Beatty Center for Integrated Research at Inova Fairfax Hospital, Falls Church, Virginia. Patients' total number was 115 with average BMI=48±11.1; which 113 patients were obese with BMI>30 and 2 patients overweight with 25<BMI>30. All patients in this study fulfilled the criteria for metabolic syndrome. Namely, three or more of the five following features were present in each of profiled patients: total cholesterol level of 200 milligrams per deciliter (mg/dl) of blood or greater, low density lipoprotein (LDL) level of 130 mg/dl or greater, triglyceride level of 150 mg/dl or greater, high density lipoprotein (HDL) of less than 50 mg/dl in women or less than 40 mg/dl in men, and BMI greater than 30.

Pre-existing samples were obtained from patients who underwent bariatric surgery and who also had clinical liver biopsy results and had whole blood, serum, plasma, fat, stomach, muscle and liver tissues previously collected and archived. This specimen and data collection was approved under IRB protocol "Translational Research in Chronic disease (#05.047)".

All of the liver samples after staining with hematoxylin-eosin or trichrome were processed and graded by a single pathologist based on predetermined grading system. According to liver biopsy results the patients were grouped into two cohorts: patients with normal liver biopsy were referred to as No NAFLD patients (N=27); patients with liver biopsy reflecting presence of NAFLD (steatosis, NASH) were grouped as NAFLD (N=82); and patients with non-NAFLD liver disease (N=6) such as obstructive biliary disease or chronic hepatitis due to undetermined etiology formed the third cohort.

Each liver biopsy was accompanied by whole blood sample that was retrieved from -80 degree storage freezer and utilized in this protocol. These samples and corresponding clinical data have been de-identified in compliance with HIPAA regulations.

2.2 DNA Extraction from Whole Blood Cells.

Total DNA was extracted from whole blood cells according to Spin Protocol (QIAamp®).

Before extraction, whole blood samples were kept in room temperature for 30-45 minutes to be thawed. Meanwhile, heating block was set at 56º C for step 4 (incubation) and 2 ml of molecular grade water were prepared at 95º C for steps 11 and 14 (washing DNA from columns). Ethanol (99%, 5 ml) was prepared for step 6; the buffers AW1, AW2, AL and proteinase K were visually inspected for precipitation.

In a 1.5 ml microcentrifuge tube, an aliquot of QIAGEN proteinase K (20 µl) was mixed by pipetting with 200 µl of blood sample to ensure homogenous solution. In the next step, Buffer AL (200 µl) was added to the sample, mixed by pulse-vortex for 15

seconds and incubated at 56°C for 10 minutes. After incubation, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. In this step, 200 µl 99% ethanol was added to the sample, and mixed again by pulse-vortex for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was again briefly centrifuged to remove drops from the inside of the lid. This mixture from step 6 was applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim and centrifuged at 8000 rpm for 1 minute. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded. The QIAamp Mini spin column was opened and 500 µl Buffer AW1 was added without wetting the rim then centrifuged at 8000 rpm for 1 minute. Next, the QIAamp Mini spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded. Then, the QIAamp Mini spin column was opened and 500 µl Buffer AW2 was added without wetting the rim and centrifuged at full speed (13,200 rpm) for 3 minutes. In this step, the QIAamp Mini spin column was placed in a new 2 ml collection tube and discarded the old collection tube with the filtrate then centrifuged at full speed for 1 minute. Later, the QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and discarded the collection tube containing the filtrate. The QIAamp Mini spin column was opened and 50 µl of preheated molecular grade water (95ºC) was added. Then, it was incubated at room temperature for 5 minutes and after incubation, centrifuged at 8,000 rpm for 1 minute. For the second time, the QIAamp Mini spin column was opened 50 µl of molecular grade water (95ºC) was added and incubated at room temperature for 5 minutes. Then, it was centrifuged at 8,000 rpm for 1 minute.

The final extracted DNA yield was 100 µl. After any DNA extraction, the yield concentration and purity was checked by UV Spectrophotometry and DNA was randomly controlled by Agarose Gel Electrophoresis. Finally, extracted DNA was kept in -20C for short term storage and in -80C for long term storage.

2.3 DNA Quantification and Quality Assessment by Spectrophotometry and Agarose Gel Electrophoresis.

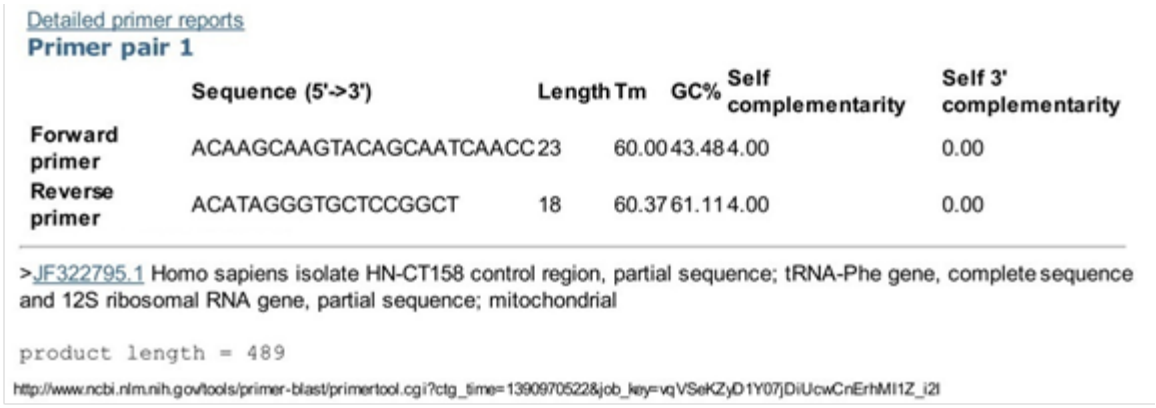
For each DNA sample, an aliquot of 10 µl was diluted in 90 µl molecular grade water to make 100 µl of solution with 1:10 DNA concentration. Then, 100 µl diluted DNA applied to spectrophotometer (GeneQuant 1300); and the absorbance of DNA samples were measured at 230, 260, and 280 nm wavelengths. The ratios of absorbance at 260nm and 280nm (A260/A280) were used as a measure of contamination. DNA maximum absorption is at 260nm and any protein contamination can change the A260/A280 ratio significantly (Green and Sambrook, 2012). In this experiment, DNA samples with ratio between 1.7 and 2.0 were considered pure enough to use as sequencing templates.

Additionally, to assess the integrity of extracted DNA agarose gel electrophoresis was carried out. Agarose powder was measured to make a 40 ml gel of 1% agarose gel. Then, 40 ml 1 X TAE buffer (electrophoresis buffer) was prepared. In the next step, the buffer was added to agarose powder in Erlenmeyer flask and was heated in a microwave. After heating, the flask was swirled until the agarose dissolved completely and there wasn't any crystal. When the molten gel had reached 45-50ºC, 2 µl of ethidium bromide were added to a final concentrations of 0.5 µg/ml. The gel solution was mixed thoroughly

by gentle swirling to avoid air bubbles. Then, the warm agarose solution was poured into the cast and put the comb and the gel was allowed to set completely (30-45 minutes). After agarose gel cooled a bit, the cast was placed in the electrophoresis tank and electrophoresis buffer was poured on top of the gel (1 mm above the gel) and the comb was removed. Then, 5 µl of PCR product was added to 1 µl of loading dye (*Thermo Scientific*®) and 4µl molecular grade water to reach the total volume of 10µl and it was spun for 10-15 seconds. In this step, the samples were slowly loaded into the slots and one of the slots was loaded with 10µl *MassRuler Low Range DNA Ladder* (*Thermo Scientific*®). The electrical lead was attached and 100 volt for 90 minutes was applied. Then, the electrical current was turned off and the gel was examined and the image was captured. Finally, the gel was inspected for any degradation and smearing as an indicator of poor DNA quality.

2.4 Primer Design and Optimization.

Previously reported specific primers for the control region of the mitochondria were used, Table 1, (primer sets: AA', BB', CC', DD') (Orekhov et al., 1999; Wang et al., 2013a). Primer set EE' was designed later, by NCBI Primer BLAST Software (Figure 3. Use of this primer was necessary to fill up the gap between areas of control region sequenced with previously described primers and to assemble a high-quality continuous sequence for haplogroup analysis. Primers were custom synthesized by Invitrogen Life Technologies.



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ACAAGCAAGTACAGCAATCAACC	23	60.00	43.48	4.00	0.00
Reverse primer	ACATAGGGTGCTCCGGCT	18	60.37	61.11	4.00	0.00

>JF322795.1 Homo sapiens isolate HN-CT158 control region, partial sequence; tRNA-Phe gene, complete sequence and 12S ribosomal RNA gene, partial sequence; mitochondrial

product length = 489

http://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1390970522&job_key=vqVSeKZyD1Y07jDiUcwCnErhMI1Z_i2I

Figure 3. NCBI Primer Blast result for set EE' primers.

Table 1. Forward and reverse primers for mitochondrial control region. Abbreviations L and H refer to Light and Heavy strands of mtDNA, respectively.

Region	Sequence	Product Length
A: H16401 A': L15997	5′-TGATTTCACGGAGGATGGTG-3′ 5′-CACCATTAGCACCCAAAGCT-3′	443bp
B: H340 B': L073	5'-CTGTTAAAAGTGCATACCGCCA-3' 5'-CTCACGGGAGCTCTCTCCATGC-3'	401bp
C: H574 C': L438	5'-GGTGATGTGAGCCCGTCTAA-3' 5'-GCTTCTGGCCACAGCACTTA-3'	323bp
D: H16365 D': L16024	5'-GAGGATGGTGGTCAAGGGAC-3' 5-CACCATTAGCACCCAAAGCT-'3'	433bp
E: H119 E': L16201	5'-ACATAGGGTGCTCCGGCT-3' 5'-ACAAGCAAGTACAGCAATCAACC-3'	489bp

2.5 Amplification of mtDNA Control Region by PCR.

Mitochondrial control region was amplified by 4 sets of primers in order to assemble a sequence with 1122 bp length of human mtDNA according to Cambridge Reference Sequence (CRS). The PCR reaction was set up for 25 µl, containing 12.5 µl *HotStar Taq Master Mix Kit* (Qiagen®), 2.5 µl (250 nM) of each primer, 1µl (5-15 ng) of DNA (template), and 6.5µl molecular grade water. PCR amplification was conducted on the *S1000™ Thermal Cycler* (BIO-RAD). The PCR thermal condition were at 95ºC for 15 minutes, followed by 30 cycles of 95ºC for 1 minute, 50ºC for 1 minute, and 72ºC for 1 minute. After 30 cycles, it was followed by 5 minute extension at 72ºC. Final product was kept at 4ºC. 5µl of the PCR product was used for qualification and quantification by gel electrophoresis and after verification 20 µl were sent for purification and Sanger sequencing by GenScript® (Piscataway, NJ).

2.6 PCR Product Quantification and Quality Assessment by Gel Electrophoresis.

Agarose powder (1.0gr) was measured to make 2.5% agarose gel. Then, 40 ml 1 X TAE buffer (electrophoresis buffer) was prepared. In the next step, the buffer was added to agarose powder in Erlenmeyer flask and was heated in a microwave. After heating, the flask was swirled until the agarose dissolved completely and there wasn't any crystal. When the molten gel had reached 45-50ºC, 2 µl of ethidium bromide was added to a final concentration of 0.5 µg/ml. The gel solution was mixed thoroughly. Then, the warm agarose solution was poured into the cast and put the comb and the gel was allowed to set completely (30-45 minutes). After agarose gel became cool, the cast was placed in the electrophoresis tank and electrophoresis buffer was poured on top of the gel (1 mm above

the gel) and the comb was removed. Then, 5 µl of PCR product was added to 1 µl loading dye diluted by 4µl molecular grade water and spun for 10-15 seconds. In this step, the samples were slowly loaded into the slots and one of the slots was loaded with 10µl *MassRuler Low Range DNA Ladder* (*Thermo Scientific*®). The electrical lead was attached and 100 volt for 90 minutes was applied. Then, the electrical current was turned off and the gel was examined and the image was captured. Finally, the gel was inspected for any non-specific product and the products length and concentration were measured according to the MassRuler ladder (Figures 4 and 5).

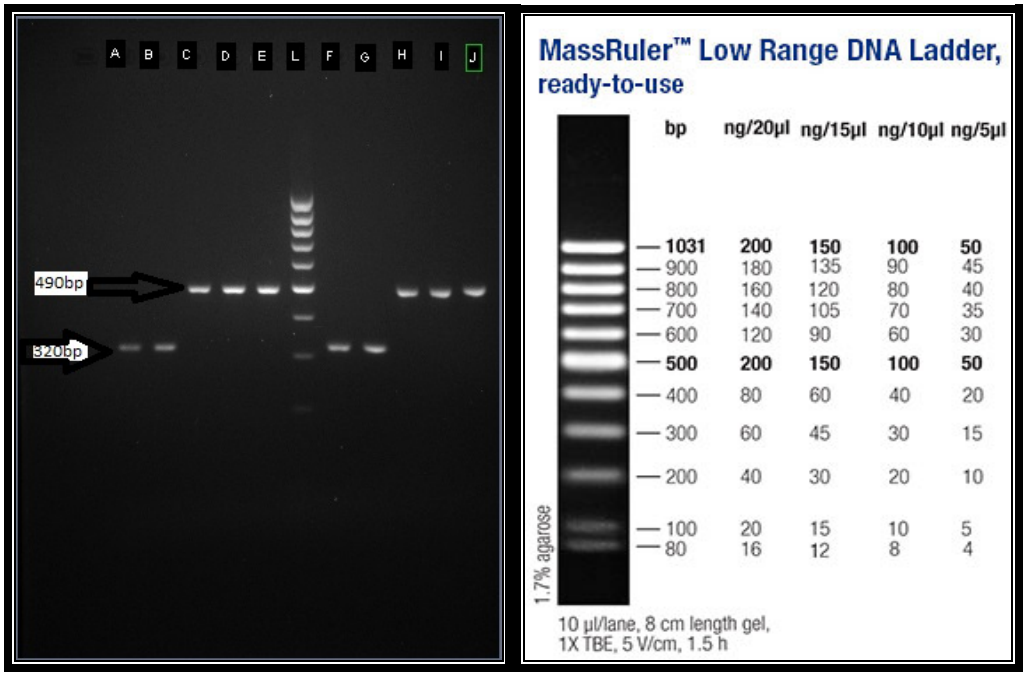


Figure 4. PCR product length and quantity measurement. For instance, product C with 490 bp length and concentration of ~80-100ng/10µl and product A with 320 bp length and concentration of ~ 30-40ng/10µl.

Figure 5. MassRuler Ladder. *Reprinted with permission* (Thermo Scientific®).

2.7 Sanger Sequencing.

A final volume of 20 µl of each PCR product samples with the concentration of 10-20 ng/µl was added to tubes and 10 µl of (5 µM) each primer (forward and reverse) were shipped by FedEx® to GenScript® (Piscataway, NJ) for PCR products purification and Sanger sequencing. Sanger sequencing employs PCR reaction with fluorescently labeled dideoxynucleotide triphosphate (ddNTPs) as a supplement to standard deoxynucleotides. In this way, one can generate labeled oligonucleotides that differ in their length by 1 nucleotide. By using ddNTPs, four populations of fragments are generated to terminate at every nucleotide of the template DNA strand. Then, the nucleotide sequence of entire template strand could be derived from the order of the bands, or in high-throughput sequencing runs, from chromatographic peaks colored by four different fluorescent dies (Green and Sambrook, 2012).

2.8 Sequence Validation, Quality Control, and Assembling.

Any raw sequencing data contain certain proportion of errors and requires editing. To clean up raw sequencing data and to assemble resultant sequences, several procedures were applied sequentially, including editing, trimming, and sequence comparing. In initial sequence processing step, both forward and reverse sequences' chromatograms were manually inspected using Chromas Lite® (Applied Biosystems Version 2.1.1) (Figure 6) and the nucleotide sequences provide by base calling feature were edited if necessary. In almost all cases, the sequences adjacent to the sequencing primer, about 30-40 bp in size, were trimmed due to low confidentiality of base calling and low intensity of chromatographic peaks.

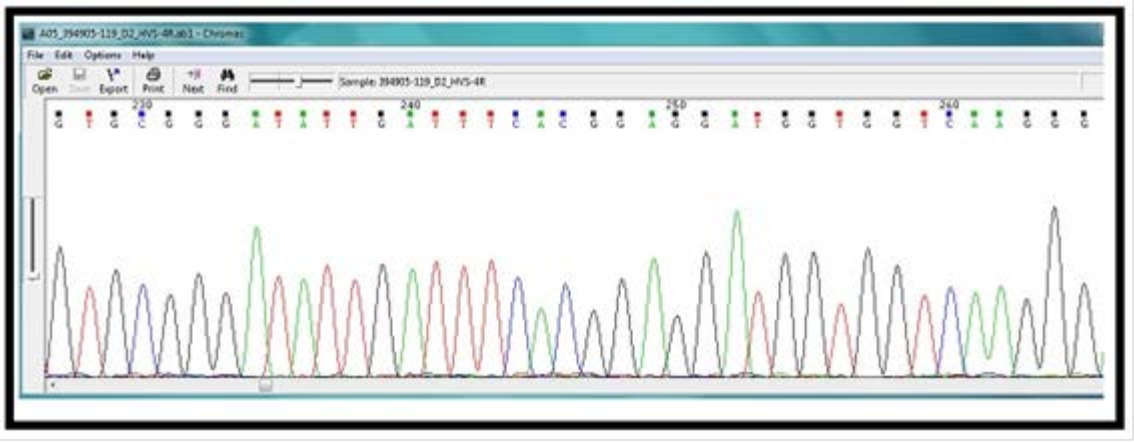


Figure 6. Typical Chromatogram visualized using Chromas Lite. A 40-bp sequence frame is shown.

In the next analytic step, forward and reverse sequences were compared to each other (Figure 7) using Clustal Omega Multiple Sequence Alignment software (European Bioinformatics Institute), followed by consensus editing if it was required. At final step, four forward and four reverse sequences from each DNA sample were uploaded in Sequencher® (version 5.2.4), followed by sequence end trimming, and consensus editing to reach confidentiality of > 99% for each base. Then, these 8 sequences were aligned to target fragment of human mtDNA Cambridge Ref. Seq. (CRS) with the length of 1122 bp, in order to produce one aligned 1122 bp long-sequence for each analyzed DNA sample (Figure 8).

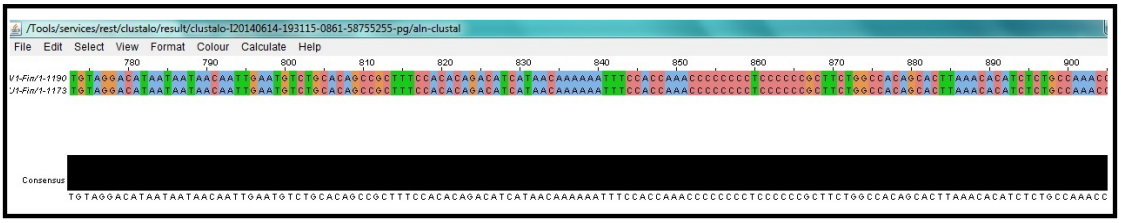


Figure 7. A screen shot of forward and reverse sequences alignment performed using Clustal Omega software.

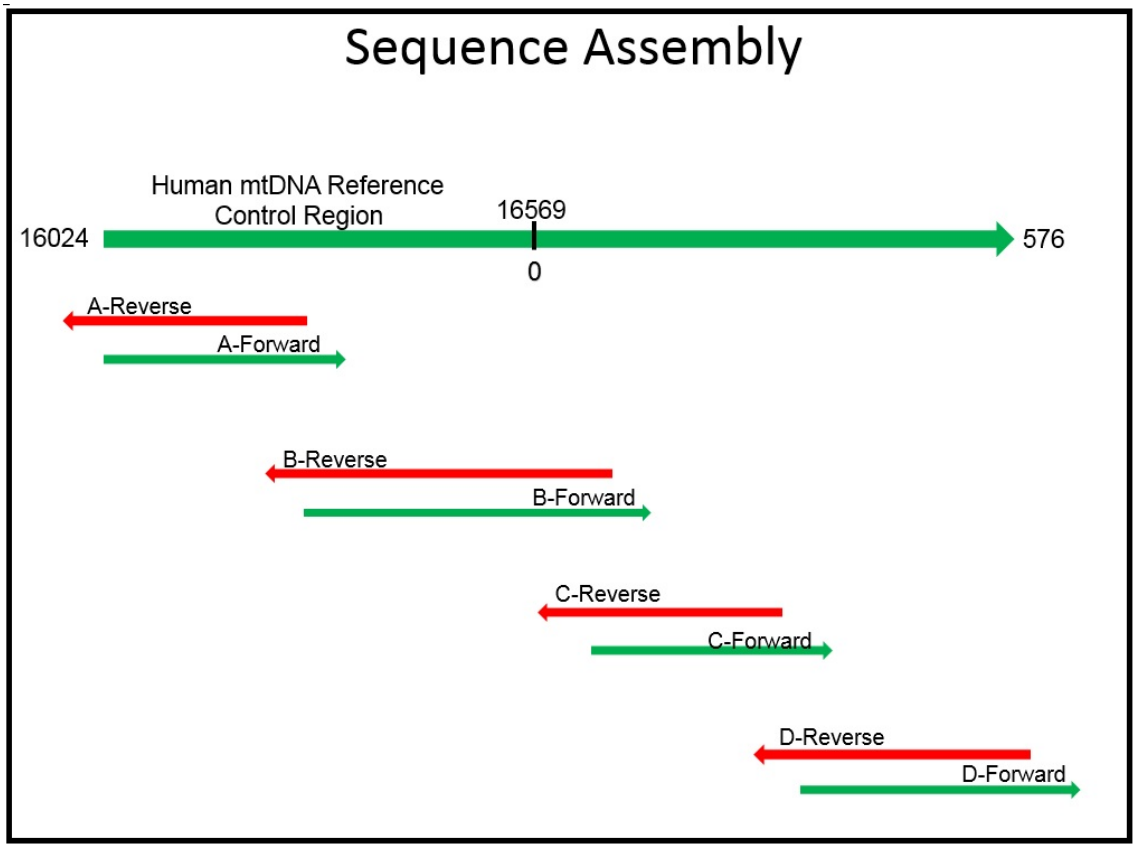


Figure 8. A scheme of the alignment of experimentally obtained DNA sequences to target fragment of human mtDNA Cambridge Ref. Seq. (CRS) with the length of 1122 bp.

2.9 Defining Mitochondrial Haplotypes by MitoTool.

MitoTool software is commonly used for handling human mtDNA sequence data and for determining haplogroups and other variants that deviate from the revised Cambridge Reference Sequence (rCRS) (Fan and Yao, 2011). In order to define haplogroups, MitoTool requires a minimum of 501 bp of control region sequence.

In the attempt to sort obtained sequences by their respective haplogroups, the primer pairs BB' and CC' (Table 1) were used to amplify sequences C and D (Figure 8), that correspond to 3'part area of the control region of human mtDNA. After assembling patient-specific sets of 501 bp sequences and uploading them MitoTool, it was found that MitoTool correctly determines the mitochondrial haplotypes in about 50% of assembled alignments, while the rest of alignments remain not classified due to detection ambiguity. Therefore, the primers AA' and EE' (Table 1) were designed to amplify adjacent 5' sequences A and B, respectively, and expand assessed area of control region (Figure 8). The resultant sequences were covering the entire control region and had trimmed lengths of 1122 bp. When expanded DNA sequences were uploaded into MitoTool, about 90-95% of the sequences were correctly classified into haplogroups (Figure 9).

This study started with 113 DNA samples. When samples with ambiguous haplogroup results, low-quality sequences, and sequences form patients with non-NAFLD liver diseases were excluded from the study, a set of 86 individual DNA sequences remained. As the last step in evaluating the sequences from different patients, a Clustal Omega multiple alignment tool was employed to visualize sequence variants in the entire cohort of 86 patients.

Sample	Variant	Match	Missing
A3	73, 152, 263, 309+C, 315+C, 16145, 16176A, 16223, 16390, 16519	N1b1b	
B3	73, 150, 263, 315+C, 16192, 16311	U5b2a1a1	
D3	73, 150, 152, 263, 295, 309+C, 315+C, 489, 16069, 16126, 16193, 16278, 16355, 16356	J2b1a	
E3	73, 146, 152, 263, 315+C, 16224, 16311, 16519	K2a	
F3	263, 309+CC, 315+C, 16172, 16311	HV6	
G3	73, 146, 263, 309+CC, 315+C, 458, 16126, 16218, 16287, 16294, 16296, 16304, 16519	T2b6b	
K3	73, 114, 263, 315+C, 497, 16224, 16234, 16311, 16519	K1a1b1a	K1a1b1a:16093
L3	263, 315+C, 456, 523-524d, 16304	H5	
N3	73, 195, 263, 315+C, 497, 524+ACAC, 16093, 16224, 16311, 16519	K1a4a1a1; K1a4a1a3; K1a14; K1a15; K1a16; K1a26	
O3	73, 150, 152, 263, 295, 309+C, 315+C, 489, 16069, 16126, 16145, 16193, 16278, 16344	J2b1a	

Figure 9. A screenshot of mitotyping evaluation by MitoTool software.

2.10 Statistical Analysis.

To assess correlation of histopathological assessment with haplotypes, Fisher's test analysis was carried out (http://www.physics.csbsju.edu/stats/contingency.html). The patients were divided into haplogroups and compared with each other in the two groups: case vs control. Three such comparisons were carried out NAFLD vs No NAFLD; NAFLD vs non-NAFLD and Non-NAFLD vs No NAFLD. To assess the impact of low sample numbers on Fisher's test results, haplogroups with low sample number were collapsed into 1 category and analysis was carried out.

3: RESULTS

3.1 Primer Validation.

Previously reported specific primers for the control region of the mitochondria were used (primer sets: AA', BB', CC', DD') (Orekhov et al., 1999; Wang et al., 2013a). Primer EE' was designed later to fill the gap between primers AA' and BB'. Primer pairs AA' and DD' amplified HVS1 region, while primer pairs BB' and CC' amplified HVS2 and HVS3 regions of the control region respectively (Table 1). Initial test of these primers using PCR conditions as reported by the authors (Orekhov et al., 1999; Wang et al., 2013) resulted in non-specific products. In order to eliminate non-specific products while using these primers (Figure 9), DNA samples (templates) were diluted (1:10) with molecular grade water and PCR protocol was altered from 95ºC for 1 minute, **58ºC** for 1 minute, 72ºC for **30 second**, followed by final 7 minute extension at 72ºC, to different protocol as follows: 95ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute for a total of 30 cycles, followed by final 5 minute extension at 72ºC (Orekhov et al., 1999). This resulted in reduction in non-specific products in primer sets AA', BB', and CC' but not in primer DD'.

In order to further validate DD', an optimization of primer concentration approach was tested. The secondary aim of this optimization was to increase the product yield for downstream sequencing. When 500 nM concentrations of the primer were used in the

final PCR reaction mix, no changes in product yield were observed. On the contrary, amounts of non-specific products increased substantially, especially in case of primer BB' (Figure 10).

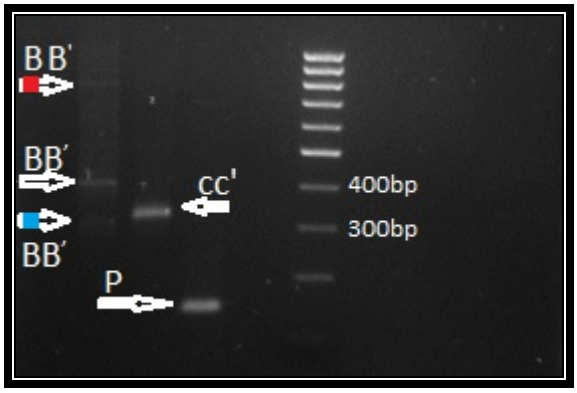


Figure 10. Primer validation gel electrophoresis. The product obtained using primer pair BB' (white arrow) is co-amplified with h higher molecular weight product (red arrow) and lower molecular weight product (blue arrow), both non-target. P: positive control, CC' primer product.

After these alterations, while the outputs of PCR amplification for primer pairs AA', BB', and CC' substantially approved gave improved results (Figure 11), primer pair DD' (Wang et al., 2013) failed to yield clear bands due to non-specific amplification.

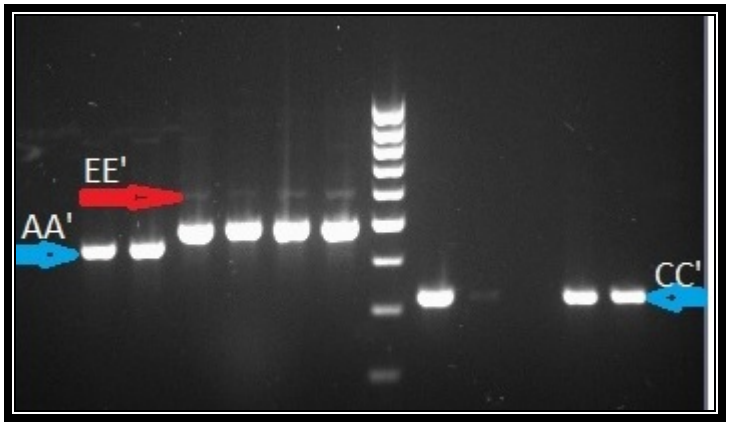


Figure 11. Gel electrophoresis analysis of amplified DNA samples with both specific and non-specific products present. The red arrow points to non-specific products with ~600 bp length. The AA' and CC' arrows point to specific products with 443 bp and 323 bps respectively. Primers used: AA', CC', EE'.

3.2 Patients Demographics.

From a total of 115 patients with metabolic syndrome (BMI=48±11.1), 113 cases were obese with a BMI>30 and 2 were overweight with 25<BMI>30 (Table 2). Out of entire cohort, 71.3% (N=82) cases were diagnosed with NAFLD, 23.48% (N=27) with no liver disease, and 5.22% (N=6) with non-NAFLD types of liver disease.

Table 2. Cohort Demographics.

Demographic/Clinical Feature	Average & Percentage (N=115)
Age	44.26 ± 11.04
BMI	48.0 ± 11.1
Female	74.78% (86)
Male	25.22% (29)
No Liver Disease (Group 1)	23.48% (27)
Non-NAFLD Liver Disease (Group 2)	5.22% (6)
Non-NASH NAFLD (Group 3)	34.78% (40)
NASH (Group 4)	36.52% (42)

3.3 Sequence Analysis and Haplogroup Assignment.

The first step in haplogroup assignment was assessing the quality of sequence data of the forward and reverse sequences for all the three primer pairs (AA', BB', CC'). This assessment was done by visualizing the sequences on a chromatogram by Chromas Lite® (Applied Biosystems Version 2.1.1). The next step was aligning the forward and reverse sequences to obtain a consensus sequence for each primer pair. Finally, two consensus regions sequenced from primer pairs BB' and CC' were aligned to obtain a continuous segment of 548 bp of the D loop (spanning HVS2-HVS3). The third primer pair, AA', provided the sequence for 443bp region of the D loop that resides within HVS1. Out of the 115 samples, 16 samples were eliminated because of low signal quality and/or poor consensus between forward and reverse sequence as revealed during alignment attempts (Figures 12 and 13).

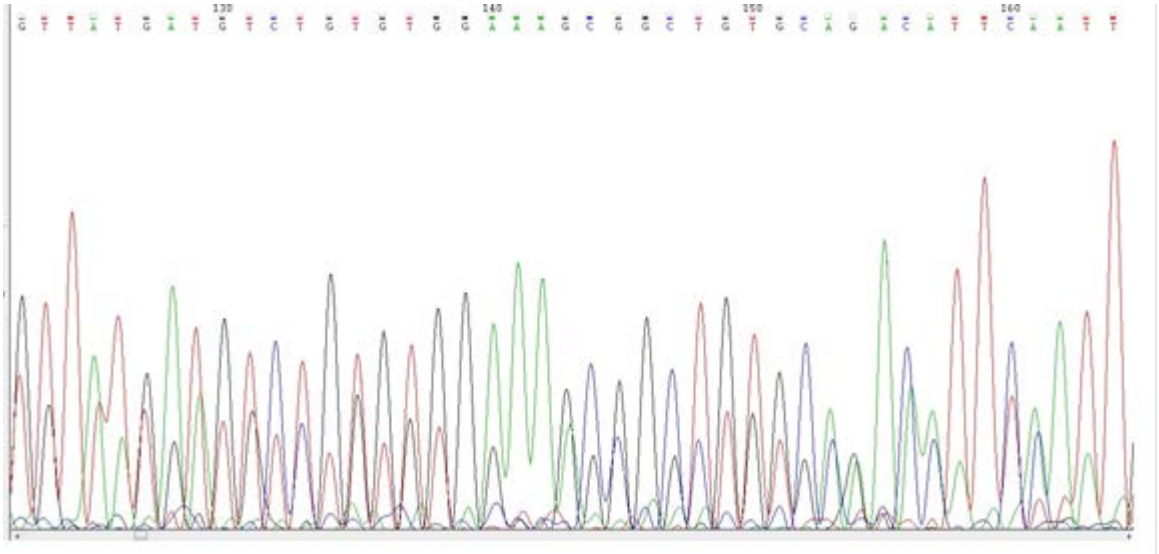


Figure 11. Example of chromatogram that corresponds to poor sequencing quality.

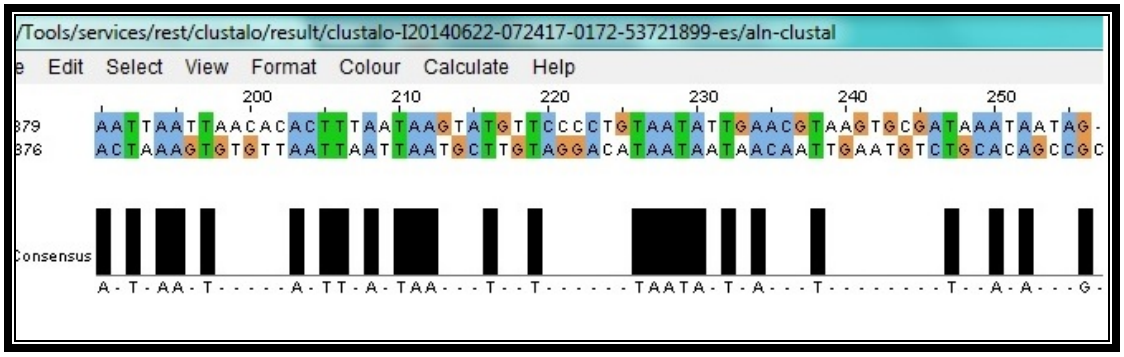


Figure 12. An alignment of sequences that originated from forward and reverse sequencing primers (example).

3.4 Histopathological Grading and Failed Sequencing.

It is possible that at least in some DNA samples the low quality of obtained sequence may be explained by the presence of a proportion of mutant mtDNA clone or clones within cellular population of mtDNA molecules. It is also possible that non-resolved samples were characterized by heteroplasmy that is defined as the presence of a mixture of more than one type of a mitochondrial DNA (mtDNA) within a cell or individual. Heteroplasmy is a known beneficent contributor to the severity of many mitochondrial diseases (Rose et al., 2007). Moreover, mtDNA samples of centenarians were shown to display a higher than average degree of heteroplasmy (Rose et al., 2007).

To explore whether NAFLD may be associated with overall detriment in mtDNA quality or with beneficial effects of heteroplasmy, the liver biopsy results of the 16 samples excluded from further study for the low quality of mtDNA sequences were re-examined. Among these 16 samples, nine samples (56.25%) were assigned high

pathological grade (grade≥3), 4 samples (25%) were identified as steatotic, while 3 samples had minimal or no steatosis (18.75%, grade<2).

It is highly plausible that patients with severe NAFLD (NASH, fibrosis and/or cirrhosis) may have mutations or large insertion/deletion in the control region of mitochondrial DNA. To test for possible association, the Fisher's tests for both 2x2 and 2x3 contingency tables were performed. However the test had not shown that the difference was statistically significant (two-tailed P= 0.0896 for Group 3 vs Group 4 comparison in 2x2 contingency table; two-tailed P= 0.24 for entire 2x3 table), an interesting trend was evident (Table 3). To further verify this hypothesis, the mtDNA samples will need to be re-extracted and resequenced.

Table 3. Analysis of an association between overall quality of the sequence and pathological grading. For the purpose of this comparison, patients with non-NAFLD types of liver disease were excluded (N=6).

Group	High Quality Sequences	Low Quality Sequences
No Liver Disease (Group 1)	25	2
non-NASH NAFLD (Group 2)	35	4
NASH (Group 3)	33	9

3.5 Mitotype Assignment using HVS2-HVS3 region sequences.

For haplogroup assignment, we started with HVS2-HVS3 region only as it contains relatively more variants than other regions of D loop. It was expected that the analysis of HVS2-HVS3 region will be sufficient for initial haplogroup assignment. A total of 99 sequences amplified using primer pairs BB' and CC' (501) were uploaded into MitoTool. This analysis allowed us to determine the mitochondrial haplotypes in 56% of the samples, while the rest of the samples remained not assigned due to insufficient amount of nucleotide variants within individual sequences (Figure 13).

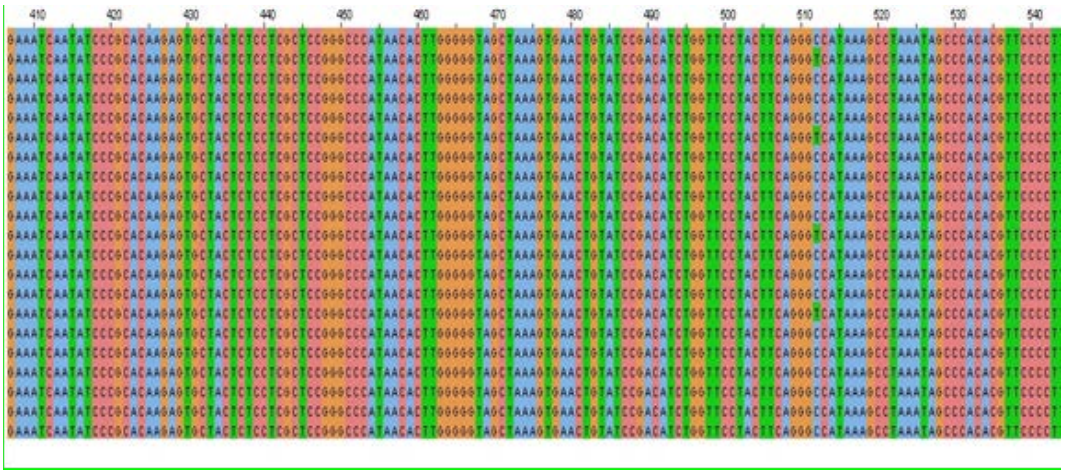


Figure 13. Multiple sequence alignment of 25 samples that cannot be resolved using an analysis of HVS2-HVS3 region only.

3.6 Mitotype Assignment using extended sequenced region.

To overcome mitotype assignment problem described above, the sequences covering D loop were extended by designing an additional primer pair. This primer pair (EE')

(Table 1) was designed to span the area from HVS1 into HVS2 (Segment B of Figure 8). Primer EE' was validated in a similar fashion as other primer pairs to obtain specific product amplification and high product yield (Figure 9). The forward and reverse sequences from primer EE target region were then aligned to obtain a consensus sequence. Finally all the consensus sequences were multi-aligned to obtain a contiguous 1122 bp region spanning the entire D loop. With a sequence that covers the entire control region (1122 bp), MitoTool could resolve mitotypes in 92% of samples.

3.7 Non-resolved mitotypes.

A total of 7 DNA samples were not assigned to unique haplogroup even after sequence extension to 1122 bp. In all seven cases, MitoTool recognized the ambiguous haplogroup as R0, HV, or H cluster. The ambiguity in the assignment is related to insufficient amount of variation in these mtDNA samples. In order to overcome this problem, it may be required to either sequence the entire mtDNA or perform at least one more extension step.

3.8 A study of association of haplogroups with NAFLD and its progressive form, NASH.

The cohorts were grouped based on grading as described in methods section 2.1. For association studies, 6 patients with liver disease but not NAFLD etiology (non-NAFLD such as obstructive biliary disease or chronic hepatitis due to undetermined etiology) were excluded. Further, 16 samples with low quality sequences, and 7 samples with ambiguous haplogroup assignment were eliminated. This resulted in a total of 86 mtDNA samples available for association studies. Among these 86 patients, 64 patients

(74.4%) were diagnosed with NAFLD (steatosis grade > 1). A majority of patients were females (75.6%, N=65). Among females, 67.7% (N=44) had NAFLD. Among the 21 male patients (24.4%), however, there was higher incidence of NAFLD with 95.2% prevalence. Race is known confounder in association studies. In our cohort, a total of 69 patients (80.2%) were of Caucasian origin. The prevalence of NAFLD in this sub-cohort was at 81.2%, while in African-Americans sub-cohort (N=15) only 40% of patients had NAFLD.

According to sequence data analysis by MitoTool, H haplogroup (34.9%) was the most common haplogroup in this study, while other haplogroups including L (15.1%), K (13.95%), U (11.6%), J (11.6%), T (7.0%), M, W, N, C, and X (1.2% each) were less common (Figure 14). The distribution of patients with NAFLD vs No Liver disease varied among the haplogroups (Figure 15). 80.84% of the patients having NAFLD belonged to one of the following haplogroups (H, J, T, U, K, M, W, N, C, X) (Table 4), (Figure 16). In contrast, only 38.46% of patients with L haplotype had NAFLD (Fisher's test, two tailed P= 0.003) (Figure 16).

To assess the association of NAFLD with haplogroups, 5 major haplogroups (H, L, K, J, U) were examined by Fisher's test where NAFLD cohort (Group3 and 4) was compared to No Liver disease cohort (Group 1). The result showed trending difference in prevalence of NAFLD in patients with L haplotype and other haplotypes (each 80.00%), K (83.33%), and L (38.46%) (Two tailed P= 0.068) (Figure 15). Notably, due to small sample size, some haplogroups (M, W, N, C, X) were represented by only 1 patient each, (Table 4) which would limit the Fisher's test results. It is important to note that the entire

L haplogroup (N=13) was African-Americans, but not all African-Americans carried L haplotype (84.2% with L haplogroup vs 15.8% with non L haplogroups). However, there were no trend in relative prevalence of pathological grade 3 (non-NASH NAFLD) and grade 4 (NASH) among five major haplogroups (H, L, J, K, and U) (Fisher's test, two tailed P= 0.711).

For further association studies, all non-L haplogroups were pooled together and compared to L haplogroup (Figure 16). Notable, 80.84% of patients with haplogroups H, J, T, U, K, M, W, N, C, and X, and 38.46% of patients with L haplogroup were diagnosed with NAFLD (Fisher's test, two tailed P= 0.003).

Haplogroup L2 was the most common among L mega haplogroup (N=7). Interestingly, among these seven cases, only two patients were diagnosed with NAFLD. In L3 haplogroup, the second most common haplogroup of L mega family, no cases of NAFLD were registered. Sub-Saharan L3 haplogroup is considered the youngest African haplogroup that was a definitive haplogroup in population that dispersed out of Africa (Wallace, 2005). It would be interesting to examine if L3 haplogroups directly contributes its protective effect against NAFLD (Table 5).

Table 4. Haplotype classification by pathological grading of NAFLD.

Haplotype	Grade >1/NAFLD	Grade 1/No Liver Disease	Percentage of total population (N)
J	8	2	11.6%(10)
T	4	2	6.9%(6)
L	5	8	15.1%(13)
H	24	6	34.9%(30)
U	8	2	11.6%(10)
K	10	2	14.0%(12)
M	1	0	1.2%(1)
W	1	0	1.2%(1)
N	1	0	1.2%(1)
C	1	0	1.2%(1)
X	1	0	1.2%(1)
Total	64	22	86

Table 5. The prevalence of NAFLD within L haplogroup mega family.

SUBHAPLOGROUP	SAMPLES	NAFLD %
L0	1	0%
L1	2	100%
L2	7	28.6%
L3	5	0%
L	1	100%

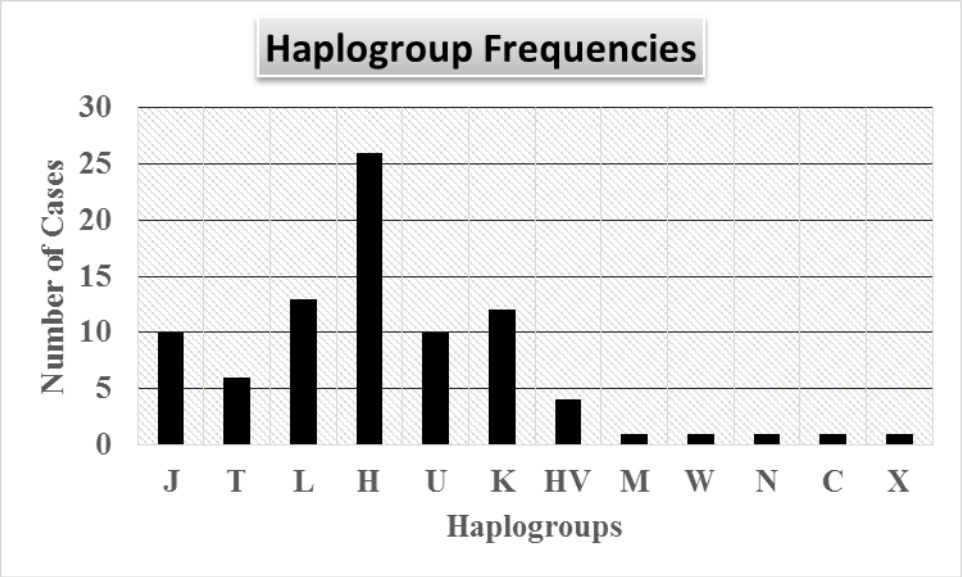


Figure 14. The frequencies of major haplogroups.

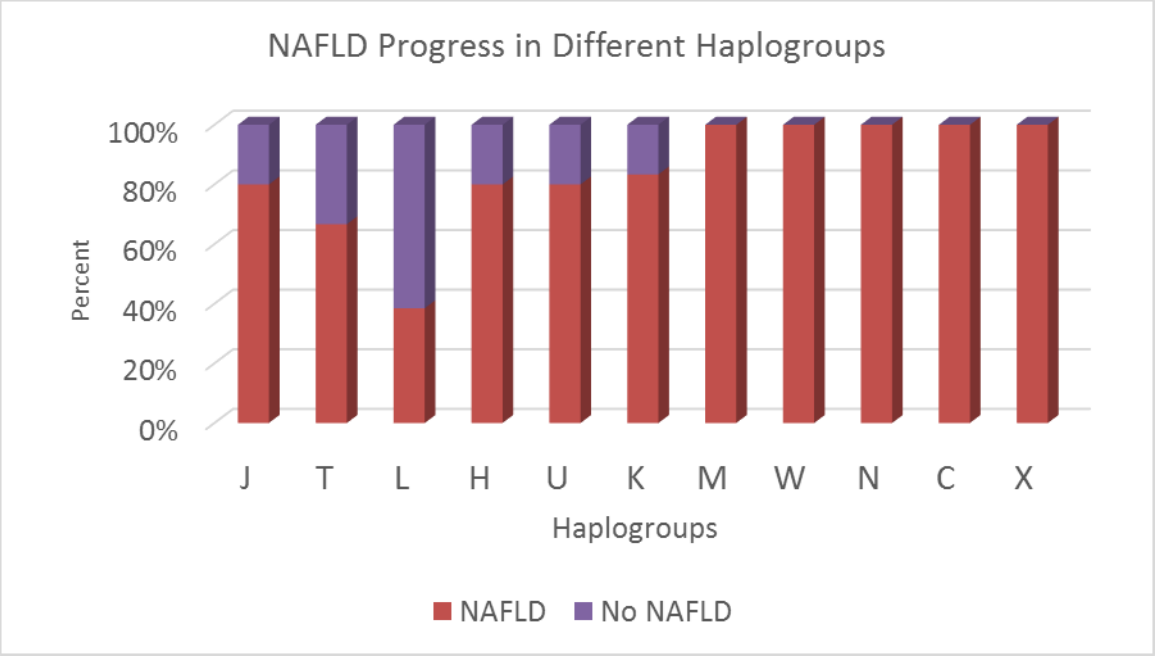


Figure 15. The prevalence of NAFLD in each identified haplogroup.

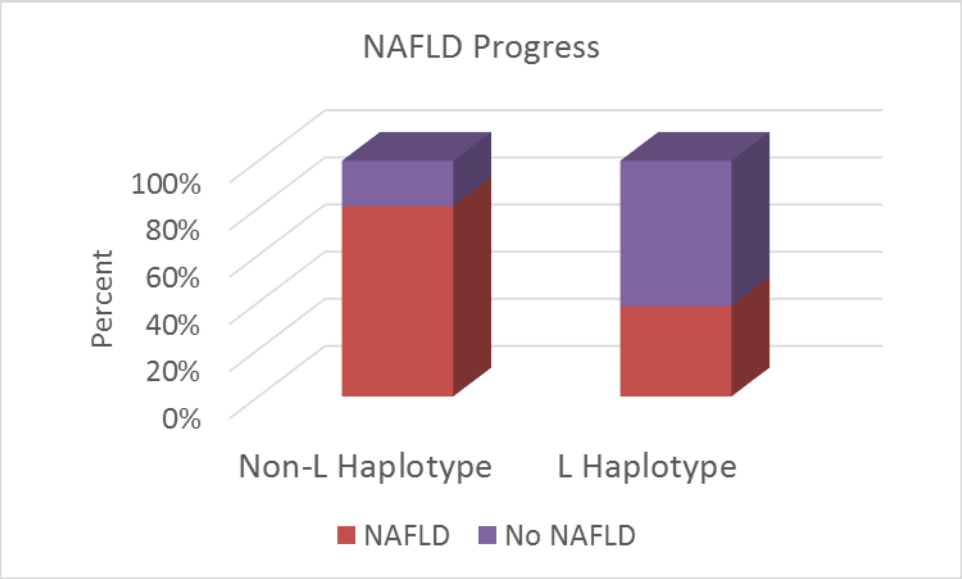


Figure 16. The prevalence of NAFLD in L haplogroup as compared to Non-L haplogroups (Fisher's test, two tailed P= 0.003).

4: DISCUSSION

According to World Health Organization report, obesity poses significant threat to global health and well-being. The insulin resistance that is a hallmark of metabolic syndrome is a manifestation of a pattern of metabolic disturbances related to central obesity and resultant systemic inflammation (McKeigue, 1996; Schmidt and Duncan, 2003). Consequently, non-alcoholic fatty liver disease (NAFLD), which is the most common manifestation of metabolic syndrome, became a significant health problem and the leading cause of chronic liver disease worldwide (Bedogni et al., 2005). Unfortunately, there is no definite, non-invasive diagnostic test that can reveal NAFLD in early stages to stop the disease progression. Therefore, identifying susceptible patients is a vital task.

In this study, we tried to find out if there is any correlation between NAFLD and specific mitochondrial genotype. Nucleus and mitochondria are the only DNA storage organelles in mammalians. Mitochondrial DNA inheritance is exclusively maternal, in contrast to nuclear DNA with both paternal and maternal copy. Mitochondrial DNA is extremely redundant, its copy number typically ranges from a few hundreds to a few thousands per cell (Alexeyev et al., 2013). Because mitochondria have poor DNA repair system and lack histones, it accumulates mutations faster than nuclear DNA. High mutation rate in mtDNA contributes to a variety of human pathologies such as

mitochondrial diseases, diabetes, cardiovascular disease, and cancer. In addition, reduction in mtDNA copy number can also disturb its normal function or cause pathology. For instance, it was found that adipocytic lipogenesis correlates to mtDNA (Kaaman et al., 2007).

A mitochondrial haplogroup is defined as a unique combination of variants (SNPs). Most variants in mtDNA are located in the control region with 1122 bp length. Mitochondrial Eve refers to the matrilineal most recent common ancestor of recent humans. Mitochondrial Eve originated from Africa and it is the origin of all African (L1, L2, L3) whereas all non-African haplogroups were originated from L3 haplogroup that further gave rise to nine other European haplogroups (H, I, J, K, T, U, V, W, and X), while Asian populations belong to A, B, C, D, and E haplogroups (Lin et al., 2012). Our study shows that obese individuals with mtDNA of L haplogroup, especially these of East-African origin, are somewhat protected from developing NAFLD as compared to other haplogroups, including H haplogroup, that is the most common in Caucasians. More studies with larger cohorts are needed to figure out what causes the relative protective effects of L haplogroup in its carriers toward NAFLD. One possible explanation would be that L haplogroup of mtDNA may be relatively more resistant to oxidative stress. It is known that mtDNA can be damaged by deletions, insertions, and single nucleotide substitutions. Visceral obesity is associated with an increase in pro-inflammatory cytokines and enhanced lipolysis with overflow of free fatty acids (FFAs) to the liver. FFAs excess flow to the liver impair intracellular pathway of lipid metabolism that induces steatogenic transformation of hepatocytes. In parallel, increasing

synthesis of triglyceride and β-oxidation of FFAs reduce antioxidant defenses and elevate oxidative stresses all over the body. It is possible that mtDNA of certain mitochondrial haplogrous is more susceptible to damage by oxidative agents or by other mechanisms. Defects or polymorphisms in mitochondrial DNA could contribute to the generation of NAFLD through a progressive decay of mitochondrial oxidative capacity.

5: CONCLUSION

Variation in mitochondrial sequence may serve as a predisposing factor for progressive NAFLD. In this study the association of haplogroups in D- loop of mitochondria and NAFLD was investigated. In our cohort, NAFLD prevalence was higher in men (95.2%) than women (67.7%); and in Whites (80.2%) than African-Americans (40.0%). NAFLD was found to be associated significantly with non-L haplogroups (H, K, T, C, U, M, N, J, W, X) (80.84% vs 38.4%); and among L haplogroups, L3 was least likely to develop liver steatosis. Further investigation needs to be carried out to assess if L haplogroup protects individuals against NAFLD and/or its potential progression to NASH and if there is a correlation between mitochondrial degradation and severe NAFLD.

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