THE EFFECTS OF CHEMOTHERAPEUTIC OR ANTIRETROVIRAL DRUGS ON MITOCHONDRIAL FUNCTION

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DEDICATION

To my parents, Marlene Anderson and the late Robert A. Anderson, Sr. for all of their love and support; to my husband, Brian Keating, for always being there for me through this long journey.
ACKNOWLEDGEMENTS

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ABSTRACT

Chemotherapeutic drugs such as anthracyclines and antiretroviral drugs such as nucleoside reverse transcriptase inhibitors (NRTIs) have been in use for decades and are highly effective drugs. Unfortunately, drugs that affect tumor or viral DNA can also affect mitochondrial DNA, leading to deleterious side effects such as cardiomyopathy, metabolic disorders, or peripheral neuropathy. There is a dearth of information as to how these drugs affect mitochondrial function in certain populations and how much drug exposure must occur before these changes manifest (short-term versus life-long treatment). In this dissertation, we examined the long-term effects of treatment with an anthracycline, doxorubicin (DOX), and an adjuvant therapy, dexrazoxane (DEX), on mitochondrial function in childhood survivors of acute lymphoblastic leukemia (Chapter 2). We also examined the long-term mitochondrial effects of combination antiretroviral treatment (cART) in perinatally HIV-infected adolescent populations with and without insulin resistance (Chapter 3). Additionally, we performed a cross-sectional analysis on adult populations from Thailand who were HIV-infected and cART naïve, HIV-infected with an NRTI induced symptomatic peripheral neuropathy (PN), or HIV negative on mitochondrial parameters as well as measures of epidermal nerve fiber densities (ENFD), antioxidant levels, and oxidative stress. Finally, we performed a longitudinal analysis of the HIV-infected, drug naïve group on measures of ENFD, antioxidant levels, and oxidative stress at three time points: before cART treatment, during treatment with a stavudine (d4T) based regimen, and after switching to a zidovudine (ZDV) based regimen (Chapter 4). We hypothesized that in each study drug treatment would affect mitochondrial DNA numbers and oxidative phosphorylation enzyme activities. In the final study, we also hypothesized that the group with PN would have lower ENFD, and more mitochondrial dysfunction and oxidative stress than the groups without PN. Finally, we hypothesized that treatment with the mitochondrially toxic d4T would induce more ENFD loss, mitochondrial dysfunction, and oxidative stress than treatment with the less mitochondrially toxic ZDV in the HIV-infected group without PN. We observed across these three studies that drugs that affect tumor cell or viral DNA replication can alter mitochondrial function either through alterations to the DNA, observed as lower copy
numbers per cell, or through disturbances in the oxidative phosphorylation system, measured as changes in complex I or IV enzyme activities. We also found in the final study those with d4T-induced PN have greater mitochondrial dysfunction and oxidative stress than those who are HIV-infected and drug naïve. Finally, we determined that short-term d4T treatment induces similar mitochondrial alterations and oxidative stress as those seen in the HIV infected group with PN, which can be ameliorated through a drug regimen switch to ZDV. Taken together, the results from these studies indicate that these drugs, while very effective, are inducing systemic mitochondrial changes and research in this area should continue in order to determine the full extent of the mitochondrial alterations. This information could one day lead to equally effective treatments that are more mitochondrial friendly, improving patient quality of life.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>O2-</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>“D” drug</td>
<td>Dideoxy-type antiretroviral drug</td>
</tr>
<tr>
<td>3TC</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>8-oxo-deoxyguanine</td>
</tr>
<tr>
<td>ABS</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AMP</td>
<td>Adolescent master protocol</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>cART</td>
<td>Combination antiretroviral therapy</td>
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<tr>
<td>CI</td>
<td>Complex I</td>
</tr>
<tr>
<td>CIV</td>
<td>Complex IV</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variance</td>
</tr>
<tr>
<td>D4T</td>
<td>Stavudine</td>
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<tr>
<td>DEX</td>
<td>Dexrazoxane</td>
</tr>
<tr>
<td>DFCI</td>
<td>Dana Farber Cancer Institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENFD</td>
<td>Epidermal nerve fiber density</td>
</tr>
<tr>
<td>Fe3+</td>
<td>Iron (III) ion</td>
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<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
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<tr>
<td>FTC</td>
<td>Emtricitabine</td>
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<td>GLUT4</td>
<td>Glucose transporter type 4</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>GPO</td>
<td>Government Pharmaceutical Organization</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
</tr>
<tr>
<td>HR</td>
<td>High risk</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescent intensity</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>mtRNA</td>
<td>Mitochondrial RNA</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NtRTI</td>
<td>Nucleotide reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NVP</td>
<td>Nevirapine</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>Protein gene product 9.5</td>
</tr>
<tr>
<td>PHACS</td>
<td>Pediatric HIV/AIDS Cohort Study</td>
</tr>
<tr>
<td>P$_i$</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PN</td>
<td>Peripheral neuropathy</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>PRX</td>
<td>Peroxiredoxin</td>
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<tr>
<td>PRX2</td>
<td>Peroxiredoxin 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOD1 (Cu/Zn)</td>
<td>Superoxide dismutase 1 (copper/zinc)</td>
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<tr>
<td>SOD2 (Mn)</td>
<td>Superoxide dismutase 2 (manganese)</td>
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<tr>
<td>SOD3 (Cu/Zn)</td>
<td>Superoxide dismutase 3 (copper/zinc)</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
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<tr>
<td>TDF</td>
<td>Tenofovir</td>
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<tr>
<td>TOP</td>
<td>Topoisomerase</td>
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<tr>
<td>TP</td>
<td>Triphosphorylated</td>
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<tr>
<td>TRX</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TRX1</td>
<td>Thioredoxin 1</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZDV</td>
<td>Zidovudine</td>
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</table>
CHAPTER 1.

Introduction
1.1. MITOCHONDRIAL FUNCTION

1.1.1. The Mitochondrion

The mitochondrion is a ubiquitous, double membrane organelle found in almost all eukaryotic cells except red blood cells. They have multiple cellular functions including apoptosis, fatty acid β-oxidation, citric acid cycle, calcium storage, and energy production (Figure 1.1) [1-3]. Energy production occurs in the inner membrane, which contains four protein complexes that accept and transport electrons donated from the citric acid cycle, creating a proton gradient that flows from the intermembrane space into the center, or matrix, of the mitochondrion. The four protein complexes are of the electron transport chain are: NADH dehydrogenase (I), citrate synthase (II), cytochrome b oxidoreductase (III), and cytochrome c oxidase (IV). Electrons enter the chain through complexes I and II and travel through complexes III and IV. From there, most of these electrons will finally reduce the terminal electron acceptor, oxygen, into water, though an occasional electron will slip out of the transport chain prior to complex IV and form reactive oxygen species (ROS; Figure 1.1). During electron transport, protons are released from complexes I, III, and IV into the intermembrane space which produces a more positively charged environment as opposed to the negatively charged matrix. This charge differential creates the proton motive force, which allows a fifth protein complex, adenosine triphosphate (ATP) synthase, to combine adenosine diphosphate (ADP) and an inorganic phosphate (P_i) into ATP [2,4]. This important ability, termed oxidative phosphorylation (OXPHOS), is what earns the mitochondrion the name “powerhouse” of the cell, producing about 90% of the ATP found in most cells [2]. All cells require ATP, but some tissues in the body have higher energy demands than others and are thus more dependent upon ATP. These tissues include the heart, the brain, and skeletal muscles [2,4].

Mitochondria are very unique organelles in that they contain their own small, circular double stranded DNA. This DNA is tethered to the inner membrane in the matrix and is replicated by the mitochondrial DNA enzyme polymerase γ [4-6]. Each mitochondrion contains anywhere from 2-10 copies of DNA, each of which encodes 37 of the roughly 80 genes that are necessary for mitochondrial function: 13 oxidative phosphorylation polypeptides, 22 transfer RNAs, and 2 ribosomal RNAs. The remaining
respiratory proteins, and all of the genes required for mitochondrial structure, transcription, translation, and biogenesis are encoded in the nuclear DNA (Figure 1.2) [4-7].

1.1.2. Mitochondrial DNA

Mitochondrial DNA (mtDNA) are located in the mitochondrial matrix where they can be exposed to newly formed ROS such as superoxide (Figure 1.1). When the circular mtDNA molecules are not actively replicating, they are supercoiled into nucleoids. Though mtDNA does not have histones like nuclear DNA does, it is surrounded by other proteins, which offer the DNA some protection from ROS. During replication, the mtDNA are uncoiled and are thus susceptible to damage from ROS [8]. ROS can oxidatively modify mtDNA, causing lesions such as 8-oxo-deoxyguanaine (8-oxo-dG) and other mutations or deletions [8,9]. While many of the mtDNA mutations and deletions that occur are innocuous and cause little to no disruption in mitochondrial function, modifications can also occur in important gene sequences such as those that code for OXPHOS. Increased mtDNA damage in these sequences can lead to improper OXPHOS polypeptide transcription and translation, disrupting OXPHOS activity and decreasing ATP production. This disruption of the OXPHOS transport chain can also increase electron slippage, amplifying the generation of superoxide and other ROS. The proliferation of ROS can increase mtDNA damage and OXPHOS disruption leading to a vicious cycle that results in mitochondrial dysfunction [2,7].

MtDNA molecules within a mitochondrion do not replicate at the same time and are thus exposed to ROS at different rates, incurring differing amounts of modifications. This results in a state of heteroplasmy [7,10]. If the amount of dysfunctional mitochondria in a tissue or system reaches a threshold of more dysfunctional than functional mitochondria, however, signs of ATP depletion can manifest. Those tissues with the highest energy demands, such as the heart and central nervous system, are the first ones affected [2,10,11]. Mitochondrial dysfunction has been linked to the development of many diseases including metabolic disorders such as insulin resistance and diabetes [12,13], and neuronal dysfunction and loss as seen in peripheral neuropathies [4,14,15].
1.1.3. Oxidative Stress

The majority of cellular oxygen consumption occurs in the electron transport chain in the mitochondria where oxygen functions as the terminal electron acceptor. This process also produces 90% of the endogenous ROS through slippage of approximately 1-3% of the electrons from OXPHOS protein complexes I and III. Electrons that prematurely slip out of the electron transport chain into the mitochondrial matrix can incompletely reduce oxygen, generating free radicals such as superoxide (\(\bullet O_2^-\)) or hydroxyl (\(\bullet OH\)). These ROS have very short half-lives, making them highly reactive molecules that can oxidize lipids, nucleotides, and proteins resulting in damaged cell membranes and DNA (Figure 1.1) [9,11,16]. Furthermore, primary ROS such as superoxide can generate secondary radicals through interactions with other molecules. For example, when superoxide is reduced by antioxidants, oxygen and hydrogen peroxide (\(H_2O_2\)) are formed. \(H_2O_2\) is also highly reactive and can combine with free radicals to generate further ROS such as hydroxyl. These hydroxyl radicals have a very short half-life and are more highly reactive than \(H_2O_2\), oxidizing lipids to form highly reactive lipid peroxides such as malondialdehyde (MDA). Thus, the production of just one type of ROS can set off a chain reaction leading to the formation of many ROS [16]. While these molecules can be hazardous in large quantities, production of ROS in low to moderate amounts is a normal cellular process and these molecules play important roles in cell signaling and defenses against foreign particles such as bacteria [16,17].

In order to maintain an appropriate amount of ROS, cells possess powerful antioxidants enzymes such as superoxide dismutases (SOD), catalase, and thioredoxins and peroxiredoxins (TRXs and PRXs) that can reduce ROS to non-toxic molecules. In humans, SOD has three forms: SOD1 copper/zinc (Cu/Zn) found in the cytoplasm, SOD2 manganese (Mn) found in the mitochondrion, and SOD3 (Cu/Zn) found in the extracellular matrix. SOD dismutates superoxide radicals into oxygen and \(H_2O_2\) [9,11,17,18]. \(H_2O_2\) is further reduced into water and oxygen by cellular enzymes such as catalase and PRXs. When PRX reduces \(H_2O_2\), the enzyme becomes oxidized and requires an electron donor, such as TRX, in order to be reduced into its active form again [17,18].

During normal physiological function, a delicate balance is maintained between ROS and antioxidant levels, called the redox state. An increase in ROS levels however,
can unbalance the redox state. When this occurs, antioxidant enzyme production can be increased in order to defend against the increased ROS production. If the increased ROS generation continues, however, antioxidant defenses can be overwhelmed. This culminates in a state of oxidative stress with the resultant oxidatively modified DNA, proteins, and lipids [9,16]. Oxidative stress has been implicated in the development of many diseases and plays a large role in the aging process [7,16].

Figure 1.1. Mitochondrial oxidative phosphorylation, ROS production, and apoptosis. This figure shows the main functions of the mitochondria such as OXPHOS, ATP production, ROS generation from electron slippage out of OXPHOS, and the initiation of apoptosis through the release of cytochrome c oxidase [11].
Figure 1.2. Mitochondrial DNA. This diagram shows the mitochondrial genome, a small circular, double stranded DNA, each of which encodes 37 genes: 13 oxidative phosphorylation polypeptides, 22 transfer RNAs, and 2 ribosomal RNAs. Letters in each ring represent proteins encoded by that gene segment: ND 1-6 genes code for OXPHOS complex I (NADH dehydrogenase), Cytb for complex III (cytochrome b), COI-III for complex IV (cytochrome c oxidase), and ATP 6 and 8 for complex V (ATP synthase). The D loop is the non-coding control region containing the Heavy (H) and Light (L) strand promoters and the H strand origin of replication [5].

1.2. DRUG-INDUCED MITOCOHDRIAL DYSFUNCTION

1.2.1. Drugs can affect mitochondrial function

The organelle known as the mitochondrion may have come to reside inside eukaryotic cells when an alpha-proteobacterium was engulfed by a eukaryotic progenitor, creating a symbiotic relationship [2,6,7]. Mitochondria are similar to their bacterial ancestors in that they are composed of two membranes and contain their own small circular DNA as well as the machinery needed to replicate it. These similarities to bacteria may be the reason that mitochondria are susceptible to the effects of certain antibiotics that block microbial ribosomes as these drugs can target mitochondrial translation [6]. Furthermore, drugs that can block nuclear or viral DNA replication, such
as chemotherapeutic or antiviral drugs, have been shown to block mtDNA replication as well [6,19,20]. The benefit of reducing mortality from human immunodeficiency virus (HIV) or cancer, outweighs the risks of developing drug-induced mitochondrial dysfunction and the morbidities that can result, therefore the use of these drugs will continue. Knowledge of how these drug promote mitochondrial dysfunction is important for the development of new classes of drugs that are mitochondrially friendly, but still potent against the primary disease.

1.2.2. Anthracyclines

Anthracyclines such as danorubicin and doxorubicin are widely used antibiotic anticancer drugs that were first isolated in the 1960’s from the actinobacterium Streptomyces. Because they are among the most effective anticancer treatments available, these drugs are still widely in use today to treat many solid tumors like carcinomas and sarcomas and hematological cancers such as leukemias and lymphomas [21]. Pediatric cancer survival rates have improved greatly from 30% in the 1960’s to around 80% currently and much of this improvement comes from almost half of childhood survivors of cancer having been treated at least once with an anthracycline [21,22]. Unfortunately, the biggest drawback to the use of anthracyclines clinically is the development of a progressive, dose-dependent, dilated cardiomyopathy that can culminate in irreversible congestive heart failure and death [21-23]. Studies have determined that not only are childhood cancer survivors 8 times more likely than the general population to die from cardiovascular disease [24], but that the clinical onset is delayed for many years after the last anthracycline treatment was given [25]. For example, thirty-year pediatric cancer survivors exposed to anthracyclines are 15 times more likely to develop cardiomyopathy as compared to their non-exposed siblings [22].

Extensive studies have determined that anthracycline-induced cardiotoxicity is dose dependent and that the risk increases exponentially at doses of 450 to 550 mg/m² of body surface area. Thus, current cumulative pediatric doses are typically less than 550mg/m² [21,25-27]. Even at lower doses, however, cardiac stress tests in anthracycline-treated participants show impaired heart contractility and heart tissue biopsies show cell loss, compensatory hypertrophy, and mitochondrial swelling
illustrating that any amount of anthracyclines can damage cardiac cells. These results suggest that there is no safe dose for these drugs [21,27].

1.2.3. The effect of anthracyclines on cancer cells

Research demonstrates that anthracyclines exert their anticancer effects by damaging cancer cell DNA in several ways: by inducing DNA strands breaks, DNA intercalation, and increasing ROS production. Each of these methods generates nuclear DNA damage that diminishes DNA transcription and RNA translation, effecting cellular dysfunction and leading to cell death [21,25].

Anthracyclines can induce DNA strand breaks by acting on special enzymes called topoisomerases (TOP). TOPs modulate the shape of DNA by transiently cleaving one (TOP type I) or both DNA strands (TOP type II) at the phosphate backbone to relax any supercoils that form in the unwinding process during replication or transcription. TOPs accomplish this process without introducing any changes to the DNA sequence [21,28]. Anthracyclines can disrupt this process by intercalating their planar rings between the DNA nucleotide base pairs and preventing the type II TOPs from detaching from the DNA, which reduces religation and increases cleavage. This TOP “poisoning” leads to double stranded breaks, mutations, and deletions that ultimately trigger cell death [21,28]. Anthracyclines have the highest affinity for TOPs type II α and β, located in the nucleus and mitochondria, respectively. Type II α is highly expressed in rapidly dividing cells such as cancer cells, which may be the reason that anthracyclines can effectively target the nuclear DNA of these cells [21]. Anthracyclines can also alter tumor DNA directly by intercalating at an estimated one molecule per five base pairs, or by covalently forming adducts with 5’-GC-3’ bases. Intercalation can cause large-scale deletions from the genome and adducts can induce double strand breaks, both of which can trigger cell death. As intercalation occurs much more frequently than adduct formation, it is this mechanism that is more likely to exert the chemotoxic effect [21].

Another way in which anthracyclines such as doxorubicin can alter tumor DNA is by increasing cellular generation of ROS by forming complexes with iron (Fe³⁺). Anthracyclines have a high affinity for Fe³⁺ and drug-iron complexes can easily form. When these complexes encounter oxygen, they can cycle between oxidation states of Fe²⁺
and Fe³⁺, creating superoxide radicals and H₂O₂ which damage tumor cell DNA and trigger cell death [21,25,29]. As anthracyclines can effectively induce tumor cell death using several different mechanisms, they are highly useful agents in cancer treatment.

1.2.4. The effect of anthracyclines on mitochondria

The usefulness of anthracyclines is hindered by the results from longitudinal research linking exposure to these drugs and the development of late-onset cardiomyopathy [22]. It has been hypothesized that because anthracyclines can alter DNA in tumor cells, they can also alter DNA in heart tissue. Pharmacological studies on anthracyclines have observed that high levels of these drugs accumulate in the liver, white blood cells, bone marrow, and heart tissue [21]. Once inside cells, anthracyclines accumulate primarily in both the nucleus and the mitochondria at higher levels than those found in the plasma [21]. Microscopic structural studies of cardiac tissue in anthracycline treated participants found unhealthy, swollen mitochondria containing disrupted cristae [21]. Furthermore, preclinical studies on anthracycline exposure observed disrupted OXPHOS, particularly in complexes I and IV [30,31], and changes in the mtDNA in the form of mutations, deletions, and reduced copy numbers per cell in heart tissue [25,32]. Energy-demanding tissues such as the heart can be the first to show signs of mitochondrial dysfunction and ATP depletion [2,7]. Thus, the effect of anthracyclines on mitochondria may be the key to understanding how these drugs trigger late-onset cardiomyopathy.

As previously mentioned, if important mtDNA sequences are mutated or deleted, OXPHOS and ATP production can be compromised as a result. In studies on rodents, mtDNA mutations and reduced copy numbers have been seen in heart tissue after short-term treatment with the anthracycline doxorubicin. In one of these studies, skeletal muscle was also examined, but no effect of doxorubicin treatment was found demonstrating that the effect on mtDNA is specific to heart mitochondria [33]. The affinity of anthracyclines to heart tissue may be due in part to one of the components of the inner mitochondrial membrane, cardiolipin. Cardiolipin is a phospholipid, similar in composition to its counterpart found in the membranes of bacteria, which is found in high concentrations in cardiac cell mitochondria [6,21]. Anthracyclines have a high affinity for
cardiolipin and cardiolipin in turn, may facilitate accumulation of these drugs in the inner mitochondrial membrane where they can associate with the iron contained in the OXPHOS complexes. These iron-anthracycline complexes can generate ROS, which can affect the mtDNA by generating lesions or mutations, eventually compromising OXPHOS and ATP production. The same mechanisms by which anthracyclines affect tumor DNA, may play a role in the development of cardiac cell mitochondrial dysfunction.

It is known that anthracyclines can acutely alter cardiac mtDNA, but clinical signs and symptoms of drug-induced cardiomyopathy typically manifest years after treatment. As mentioned previously, under normal physiological circumstances, mtDNA mutations occur frequently owing to the genomes’ close proximity to OXPHOS generated ROS. Mitochondrial diseases resulting from these mutations, however, are slow to develop because a threshold of dysfunctional mitochondria must first be reached [2,7]. If the initial anthracycline exposure alters the mtDNA of cardiac cells, then OXPHOS would be altered in some cells, causing increased ROS production. Greater ROS generation would intensify the rate of mtDNA mutations and deletions, further altering OXPHOS in a vicious cycle, ending in ATP depletion. Over time, the cells containing mutated mtDNA would surpass the bioenergetic threshold (i.e., more impaired than unimpaired mitochondria), and a disease state such as cardiomyopathy would ensue (Figure 1.3) [2,11,21]. This threshold hypothesis may explain why anthracycline-induced cardiomyopathy may not manifest for more than 20 years after the last anthracycline exposure.
Figure 1.3. The effect of anthracyclines on mitochondria. Anthracyclines can induce damage to mtDNA and the OXPHOS electron transport chain (ETC) as well as increase ROS production, leading to a vicious cycle of mitochondrial damage [21].

1.2.5. Antiretroviral drugs

The first cases of human immunodeficiency virus (HIV) were seen in the U.S. in 1981 and to date, over 20 million people have died worldwide. HIV is treated with a regimen called combination antiretroviral therapy (cART), consisting of several drugs that inhibit viral replication. With the discovery of antiretroviral drugs in 1987, HIV infection became a chronic, treatable condition and today about 34 million people globally are living with HIV [34,35]. If cART is initiated before advanced stages of HIV disease, it can reduce the loss of immune cells and prevent immunodeficiency [36]. Unfortunately, while cART can extend the life span of infected individuals, it does not fully restore them back to health. Plasma viral RNA concentrations can be reduced to undetectable levels, but the virus is not fully eradicated due not only to its ability to rapidly mutate, but also because it can maintain reservoirs of infected cells, especially in the central nervous system [34,36]. In addition, a high level of adherence to cART is required for maintaining undetectable viral loads and discontinuation of treatment for long periods of time can cause a rebound. Proper adherence, however, can be difficult to maintain owing to the considerable cost, the lack of access to treatment, or the severe side effects [34,36].
In developing countries, lack of access to treatment as well as the prohibitive cost of cART can make HIV infection a death sentence. In developed countries like the U.S., participants have better access to treatment but the expense can be considerable. A one-month supply of a typical three-drug regimen of cART can cost upwards of $1200-$1400 [36]. In addition to the cost, taking three different medications at different dosages every day can be difficult to maintain. Although there are now 3-in-1 pills available (e.g., Atripla) [37], making adherence more feasible, the cost is relatively similar to the multiple pill treatments ($1465 for a one-month supply of Atripla versus $1479 for three medications) [38]. Regardless of cost, cART adherence rates in the U.S. are relatively poor with one study showing 62% of 3,000 participants self-reporting 100% adherence [39] and another study that analyzed data from electronic medication bottle cap monitoring found only 30% of 81 participants were able to maintain 95% adherence of their protease inhibitor regimen [40].

The final roadblock to proper cART adherence is the development of side effects such as cardiovascular disease, metabolic disorders such as insulin resistance and diabetes, and peripheral neuropathies [12,20,41]. First generation cARTs such as stavudine and zidovudine were linked to severe adverse effects such as lactic acidosis, hepatic failure, and skeletal myopathy [41,42]. Even with reduced doses of these drugs, side effects such as peripheral neuropathy can persist [43,44]. Second generation drugs such as tenofovir have shown fewer severe side effects than the first generation drugs, but some participants can develop renal impairments and loss of bone mineral density. Long-term data on these newer drugs is still being collected [20,36,37].

1.2.6. The effect of cART on HIV

Antiretroviral drugs reduce HIV viral loads by blocking a step in the virus replication cycle and they are classified based on which part of the cycle they interrupt. Some of the typically prescribed drug types include: nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). NRTIs are structural analogs of the DNA nucleosides thymidine, adenosine, guanosine, or cytidine. NRTIs can be recognized by replication enzymes such as DNA polymerases and viral
reverse transcriptase (RT), which can use them as substrates in DNA replication. These analogs differ from native nucleotides, however, because they lack the 3’ OH group of deoxyribose which would normally form the 3’-5’ phosphodiester bond in a replicating DNA strand. Thus, when NRTIs are placed into the replicating viral DNA strand by RT, they prevent the addition of further nucleotides, causing premature termination of replication. RT lacks the internal enzyme repair mechanisms that human cellular DNA polymerases have, and so does not remove the analogs. NRTIs can also interfere with replication by competitively binding to RT, blocking its activity. These drugs require three intracellular phosphorylation steps, making them most effective in dividing cells. NRTIs include: stavudine (d4T), zidovudine (ZDV), emtricitabine (FTC), and lamivudine (3TC) [37,41,45,46]. A similar class of drugs to the NRTIs is the nucleotide reverse transcriptase inhibitors (NtRTIs). The difference between NRTIs and NtRTIs is that the NtRTIs are able to inhibit viral replication with one less intracellular phosphorylation step than NRTIs require to be recognized by RT, making these drugs effective in dividing and non-dividing cells. A commonly prescribed NtRTI is tenofovir (TDF) [37,47]. Non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as efavirenz or nevirapine, block replication in a different manner than NRTIs and NtRTIs by directly binding to RT near the active site and impairing its polymerization activity [48]. The final drug class, protease inhibitors, does not act on RT; instead it prevents the viral enzyme protease from cleaving newly synthesized viral polypeptide chains, preventing their conversion into infectious proteins. PIs include ritonavir and lopinavir [37,47].

As RT is highly error prone when transcribing RNA into DNA and does not have proof reading or enzyme repair mechanisms to correct these errors, viral mutations occur frequently, making it difficult to eradicate the virus. In the past, fighting HIV with one drug that employed a single mechanism of action was met with drug resistance. Currently, a multi-drug treatment regimen, cART, is used to prevent this resistance by blocking viral replication through more than one mechanism of action. cART typically consists of a combination of three drugs: one NNRTI and two NRTIs [49]. The ultimate goal of antiretroviral treatment is to decrease plasma viral loads below the level of detection by preventing viral replication [41,50]. While cART can decrease HIV replication and extend patient’s lives, these regimens must be administered long-term,
leading to a major downside of cART treatment – side effects. Such complications from long-term treatment include anemia, rash, bone loss, insulin resistance, and peripheral neuropathy. Certain NRTIs such as d4T and didanosine, in addition to inducing lipoatrophy, dyslipidemia, and peripheral neuropathy, can also induce life-threatening side effects such as cardiomyopathy, lactic acidosis, and hepatic steatosis [20,47].

1.2.7. The effect of cART on the mitochondria

NRTIs can inhibit cellular DNA polymerases making them very effective at blocking viral DNA replication, but this mechanism also plays a role in the development of serious side effects that mimic many signs and symptoms observed in mitochondrial diseases [20,47]. There are five principal human cellular DNA polymerases: α, β, σ, ε, and γ. Polymerases (pol) α, β, σ, ε, are located in the nucleus and polymerase γ is found in the mitochondria. All of these polymerases have the ability to incorporate either native nucleotides or NRTI analogs. Research has shown that, not only do NRTIs inhibit DNA polymerases, they also inhibit some polymerases more strongly than others as follows: RT >> pol-γ > pol-β > pol-α = pol-ε [20,46]. Further investigation has determined that DNA pol-γ has similar affinities for nucleotide analogs and native nucleotides, incorporating one as often as the other into the replicating mtDNA strand. In addition, while mtDNA does have the ability to repair incorporated analogs through the exonuclease activity of DNA pol-γ, certain NRTIs such as d4T or ZDV can inhibit this mechanism. The direct inhibition of mtDNA pol-γ by NRTIs, the affinity of mtDNA for NRTI analogs, and the inhibition of repair mechanisms can all contribute to the loss of mtDNA replication. Furthermore, some NRTIs such as ZDV or d4T have been shown to increase ROS production and decrease ATP levels in cells in vitro [51,52]. Decreases in mtDNA replication and abundance can cause decreases in OXPHOS and increased ROS production, leading to decreased ATP production and mitochondrial dysfunction. This mechanism of NRTI-induced toxicity is known as the mitochondrial polymerase γ hypothesis (Figure 1.4) [19]. NRTI-induced mitochondrial dysfunction has been documented in HIV negative cells as well as in animals and humans [51-56]. In HIV negative, NRTI exposed humans, mtDNA depletion and changes in OXPHOS have been observed in different tissues and cells including adipose tissue [55] and peripheral blood.
mononuclear cells (PMBCs) [56,57]. Populations exposed to d4T, didanosine, and ZDV, can exhibit symptoms such as myopathy, lactic acidosis, metabolic disorders, and peripheral neuropathy. These symptoms are similar to those experienced by patients with inherited mitochondrial disorders [41]. In HIV positive participants, it was found that those who exhibited better adherence to their NRTI regimens had higher serum levels of ROS and lower total antioxidant levels than those participants who were less adherent or NRTI naïve [58]. NRTIs such as d4T have been shown to be more toxic to mitochondria than other NRTIs such as ZDV and lamivudine (3TC). This may be due to the activity of pol-γ; it has a higher affinity for d4T than for native nucleotides, with only a moderate affinity for ZDV and other NRTIs [59,60]. As such, d4T more strongly induces mtDNA chain termination, leading to mitochondrial alterations. As knowledge of d4T toxicity has grown, its use in developed countries has declined. Switching to less toxic NRTIs has been shown to improve mitochondrial parameters such as mtDNA copies per cell and to return the physical structure of the mitochondria to normal with intact membranes and clearly defined cristae [61,62].
Figure 1.4. The effects of NRTIs on mitochondrial function. This figure shows the progressive inhibitory effects of NTRI-induced chain termination on mitochondrial DNA and other downstream pathways. Once NRTIs are activated through intracellular phosphorylation, they can inhibit mitochondrial polymerase γ, leading to depletion of mtDNA, which then affects transcription and translation of mtDNA encoded OXPHOS polypeptides, ending in reduced ATP production. Furthermore, disruption of OXPHOS can lead to increased electron slippage and ROS generation, which can overwhelm the antioxidant system and result in oxidative stress. Decreased ATP production and increased oxidative stress can lead to the development of cellular and metabolic disorders [19].
1.3. CONTINUED USE OF MITOCHONDRIALLY TOXIC DRUGS

Until equally effective drugs are produced that have fewer or no mitochondrial toxicities, physicians will continue to employ anthracyclines and NRTIs as the treatments of choice in the fight against cancer and HIV. In the meantime, understanding the toxic effects of these drugs on mitochondria will allow physicians to better tailor patient dosage to reduce potential side effects as much as possible. This knowledge will also allow doctors to screen for the mitochondrial changes that may precede the development of side effects. Early detection of severe side effects may increase patients’ quality of life and, in some cases, could even save lives.

1.4. SUMMARY

In summary, while research has come a long way toward increasing the lifespan of those diagnosed with cancer or infected with HIV, these diseases have yet to be eradicated. The drugs used to save the lives of patients with these diseases are potent, but the adverse effects these drugs are associated with can decrease the quality of those lives, and in the case of anthracyclines, could even cause death. Antiretrovirals and chemotherapeutics exert their effects on HIV and cancer DNA. Mitochondrial DNA, however, is not spared from these effects and this discovery has lead to the current hypothesis on the development of severe side effects after treatment with these drugs. It is hypothesized that cells that require the most ATP, such as cardiac and neuronal cells, are the first to suffer from the drugs’ effects. When enough altered mtDNA accumulates in the mitochondria that ATP production is severely reduced, severe side effects such as cardiomyopathy and peripheral neuropathy manifest (Figure 1.5). Chapter 2 will explore the long-term mitochondrial effects of doxorubicin treatment in childhood cancer survivors with an emphasis on whether adjuvant treatment with dexrazoxane is mitochondrially protective. Chapter 3 will evaluate the long-term mitochondrial effects of combination antiretroviral treatment on the development of metabolic disorders in perinatally HIV-infected preadolescents and adolescents. The purpose of these two studies is to determine how these drugs affect mitochondrial function in the long-term.

Previous studies have shown that even short-term treatment with certain antiretroviral drugs can affect mitochondrial function in HIV-negative patients [55-57]
and that the effects of HIV and cART on mitochondria may be additive [63,64]. Also, mitochondrial dysfunction has been linked to changes in peripheral nerve fibers and the development of peripheral neuropathy (PN). PN rates in HIV positive, cART treated patients can be upwards of 40% [47,65-67]. In Chapter 4, we will examine differences in mitochondrial function, oxidative stress, and nerve fiber densities in participants with and without HIV, and in HIV positive participants with and without PN, to determine what factors may contribute to the development of PN. Examining changes in epidermal nerve fiber densities (ENFD) is a validated predictor of PN development [68,69], and as such, will be our surrogate marker for changes in the larger peripheral nerves. Furthermore, we will observe how different cART regimens affect mitochondrial function and ENFD in those with HIV. We will also compare the HIV positive participants with and without PN to better understand what factors other than HIV (e.g., oxidative stress, mitochondrial function, etc.) may induce changes in ENFD. Finally, longitudinal assessments of a cART naïve, HIV positive cohort as they progress through treatment with different cART regimens may help uncover the mechanisms of short-term drug-induced mitochondrial changes and how they affect ENFD.
Figure 1.5. Hypothesized pathways of mitochondrial dysfunction induced by anthracyclines such as DOX, and antiretroviral NRTI drugs such as d4T. This figure illustrates how two different classes of drugs can exert similar effects on mitochondrial function through disruption of mtDNA by blocking the actions of polymerase γ or topoisomerase II. This can affect OXPHOS, and can in turn increase oxidative stress and decrease ATP production. DOX can also affect mtDNA through the formation of DOX/iron complexes, increasing production of ROS, and leading to mutations and deletions in the mitochondrial genome (modified from [19]).
CHAPTER 2.
The Long-Term Effects of Doxorubicin and Dexrazoxane on Mitochondrial Function in Childhood Acute Lymphoblastic Leukemia Survivors
2.1. ABSTRACT

Doxorubicin (DOX)-treated survivors of childhood cancer have an increased risk of developing progressive, irreversible cardiomyopathy. This myopathy is caused at least in part by DOX-induced mitochondrial dysfunction from deletions or mutations in the mitochondrial genome and/or by increasing ROS production. One response to mitochondrial dysfunction is to increase the number of mitochondrial DNA copies per cell (mtDNA) to maintain OXPHOS and ATP production. Dexrazoxane (DEX), an iron chelator, may reduce the DOX-induced ROS production and prevent mitochondrial dysfunction when given in conjunction with DOX. Patients with high-risk acute lymphoblastic leukemia (ALL) treated on DFCI Childhood ALL protocols from 1987-2005 from multiple sites were eligible for this study. These patients had received DOX alone or DOX plus DEX (DOX/DEX) during their chemotherapy treatment. MtDNA copies per cell and mitochondrial oxidative phosphorylation (OXPHOS) enzyme activities were examined in peripheral blood mononuclear cells (PBMCs). MtDNA copies per cell and mitochondrial OXPHOS enzyme activities of complexes I, NADH dehydrogenase (CI), and IV cytochrome c oxidase (CIV), activities were measured by qRT-PCR and thin layer chromatography and immunoassays, respectively.

Sixty-four participants provided samples at a median follow-up of 7.8 (2.9-30.2) years post-treatment and 58% of them had been treated with DOX/DEX. Median cumulative DOX dose was 300mg/m². A significant difference was observed between the groups on mtDNA copies per cell (p=0.001, adjusted p=0.015). No significant differences were found between the groups on CI or CIV enzyme activities. The results from this study demonstrate that DOX-treated survivors have increased mtDNA copies per cell, which does not occur under concurrent DEX treatment. The increase in mtDNA copies may have been a compensatory response to altered mtDNA. Increased copy numbers may have been able to augment mitochondrial function, precluding OXPHOS enzyme activity differences between the groups. While there was no observable effect of DOX on OXPHOS activities, it is possible that the compensatory increase in mtDNA copy number could eventually fail, leading to changes in mitochondrial function. Future longitudinal studies should further explore this effect.
2.2. INTRODUCTION

2.2.1. Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL), also known as acute lymphocytic leukemia, is a cancer of the immature B and T cells in the bone marrow. These abnormal lymphoid progenitor cells, called lymphoblasts, are morphologically and immunophenotypically similar to B- and T-lineage progenitor cells. The main difference is that lymphoblasts have genetic alterations that cause them to quickly proliferate and replace the bone marrow and normal white blood cells over time [70]. ALL is referred to as “acute” as the course of this disease progresses rapidly and can be fatal from within a few weeks to a few months [71]. The ALL immunophenotype the patient is diagnosed with depends on the white blood cell lineage from which the cancer cells arise and can be determined through flow cytometry. B-cell lineage ALL is the most commonly occurring type, followed by T-cell lineage. Determination of the immunophenotype is very important as it establishes the prognosis and the most appropriate treatment. Factors that lead to an improved prognosis are: younger age, white blood cell count of less than 50,000 cells/µL at diagnosis, B-cell lineage, and faster response to chemotherapy [70,72].

Over 60% of the 6,000 new ALL cases that are diagnosed each year in the U.S. are patients younger than 20 years of age with a peak incidence rate around 2 to 5 years of age [72]. Diagnosis is slightly more common in males than in females. ALL symptoms include weakness, fatigue, anemia, bone or joint pain, frequent infections, and enlarged lymph nodes and/or spleen. Once the diagnosis has been made, a treatment course that occurs over a 2 to 2.5 year period begins [70,72]. The chemotherapy course for most ALL patients contains 2 to 3 different types of drugs. High-risk ALL patients (having an initial white blood cell count above 50,000 cells/µL, having a T-cell lineage, or being outside the typical age range of 2 to 5 years) can receive up to 4 or more chemotherapy drugs. Most ALL patients will receive an anthracycline as part of their chemotherapy course [71,72].

2.2.2. Doxorubicin in the treatment of ALL

Anthracyclines such as doxorubicin (DOX) are widely used antitumor drugs. DOX has been in use since it’s discovery in the 1960’s and due to its efficacy, it is still
widely used today to treat many cancers including childhood leukemias and lymphomas [21]. The biggest drawback of DOX treatment has been the development of a progressive, dose-dependent, cardiomyopathy [21,24,26] that may not become clinically evident for up to 30 years after the last exposure [26].

The current hypothesis as to why DOX treatment leads to the development of cardiomyopathy is that DOX induces mitochondrial dysfunction. Extensive studies on non-drug induced, inherited mtDNA mutations provide a strong link between metabolic disorders, peripheral neuropathies, cardiomyopathies and mitochondrial dysfunction [2,11]. Studies examining the effect of DOX on cardiac cells and tissue from treated animals have shown structural mitochondrial defects such as swelling, DNA defects such as reductions in mtDNA copy numbers, increased deletions [25,33] and higher levels of mitochondrial 8-oxodeoxyguanosine adducts (8-oxodG; a measure of mitochondrial oxidative stress) [73], as well as functional changes such as reduced cytochrome c oxidase activity [25,33,74]. In addition, higher levels of ROS such as malondialdehyde and superoxide were observed which could further damage mtDNA, proteins, and lipids [25,27,33]. While some changes in cardiac mitochondria can be observed after one dose of DOX in preclinical studies, typically the onset of any signs of myocardial damage occurs after several weeks [21,25,33]. This delay in clinical signs in animals mimics late-onset cardiomyopathy development in humans and may be due to a characteristic effect observed in mitochondrial disorders where a maximum threshold of dysfunctional mitochondria must be reached before signs and symptoms of disease appear [1,2].

2.2.3. Adjuvant DOX treatment with dexrazoxane

Since the discovery of DOX-induced cardiomyopathy, researchers have been searching for adjuvant treatments that would delay or prevent the cardiotoxic effects of DOX without decreasing its chemotherapeutic efficacy. As mentioned in Chapter 1, DOX can combine with free iron and generate ROS. Heart tissue may be more susceptible to DOX-induced oxidative insults as it has a high concentration of cardiolipin, which may facilitate accumulation of these drugs in the inner mitochondrial membrane where they can associate with the iron contained in the OXPHOS complexes. Furthermore, heart tissue contains lower levels of catalase and glutathione peroxidase
relative to other tissues, affording it less protection from DOX-induced ROS [21,29]. Thus, it was originally proposed that antioxidants would offer the best mitochondrial protection in cardiac tissue by reducing the superoxide and other ROS catalyzed by iron-DOX complexes.

Various antioxidants have been tested in conjunction with DOX in preclinical and clinical studies; however, minor cardioprotection was afforded in the preclinical studies, which was highly dependent on the species tested, and no protection was observed in humans [27,29,75]. More recent research has focused on an adjuvant drug called dexrazoxane hydrochloride (DEX). DEX is structurally similar to ethylenediaminetetraacetic acid (EDTA), and like EDTA, it chelates iron. DEX can bind iron that is free, loosely bound, or already complexed with DOX and this mechanism may allow DEX to prevent formation of DOX-induced ROS [27,29,76]. Preclinical studies in rats with DEX and DOX showed that adjuvant DEX treatment prevented DOX-induced mitochondrial dysfunction in the heart by maintaining OXPHOS activity and mtDNA integrity. Furthermore, DEX treatment in these animals did not interfere with DOX kinetics nor did it reduce its chemotoxic effects [32], mirroring results seen in other rodent models as well as in pigs and dogs. Additionally, no harmful effects of DEX treatment have been observed in animal models [27].

In several clinical trials of adult cancer patients receiving DOX, DEX administration was well tolerated with mild side effects. Furthermore, DEX treatment concurrent with DOX chemotherapy significantly reduced the rate of cardiomyopathy development without altering over-all survival rates [75]. Since then, the Food and Drug Administration approved DEX for adult patients being treated with anthracycline-based chemotherapies to reduce the incidence of cardiomyopathy [27,77]. Unfortunately, DEX treatment is rarely prescribed for pediatric cancer patients unless they are at high risk for cardiomyopathy. Physicians have typically cited concerns over potential toxicity or secondary malignancy development as to why they are reluctant to prescribe it to pediatric patients. For example, in a retrospective study of over 8,000 pediatric ALL patients in the U.S. who had received anthracycline-based chemotherapy between the years 1999 and 2009, only 2.4% were given DEX [78]. Recent research on DEX treatment concurrent with DOX in high-risk ALL patients followed for several years after
treatment showed that DEX adjuvant treatment reduced DOX-induced cardiotoxicity through improvement of left ventricular wall thickness and improved function, markedly in females (who tend to be at higher risk), without diminishing the antitumor effect of DOX [22].

Though this research indicates that DEX treatment provides some protection against DOX-induced cardiotoxicity, it does not delve into the mechanisms by which it does so. As mentioned previously, DOX exerts its effect not only on cancer cells, but also on cardiac cells, likely through mitochondrial dysfunction. In preclinical studies, DEX treatment prevents this mitochondrial dysfunction. Little is known, however, about the long-term mitochondrial effects of DOX or DOX plus DEX treatment in pediatric patients. Due to the late onset of DOX-induced cardiomyopathy, it is important to follow up patients for many years after the treatment has concluded.

**2.2.4. ALL protocol 95-01**

The participants in this study were enrolled by the Dana-Farber Cancer Institute (DFCI) Childhood ALL Consortium. This consortium has been conducting large multi-institutional pediatric ALL clinical trials since 1981 [79,80]. The DFCI ALL Consortium Protocol 95-01 enrolled ALL participants from 1996 until 2000 to test methods by which treatment-related morbidity could be reduced without reducing antitumor efficacy. Two groups of participants were examined: standard-risk and high-risk (HR). Patients participated in studies designed to determine whether certain adjuvant therapies could reduce anthracycline-induced toxicities. HR participants were randomized to one of two groups: DOX only treatment, or DOX treatment combined with DEX treatment. These HR participants were followed up over the course of several years to observe changes in cardiac structure and function and several measures in the circulating blood relating to cardiac health (published elsewhere) and mitochondrial health [22,79,80] ¹.

The aim of this study was to measure mitochondrial DNA (mtDNA) copies per cell and the oxidative phosphorylation (OXPHOS) enzyme activities of Complex I (CI;

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¹ All clinical assessments were completed and laboratory specimens collected by our collaborators in the DFCI/ALL consortium. The consortium consists of nine institutions headed by the Dana-Farber Cancer Institute and the principle investigator, Dr. Stephen Sallan.
NADH dehydrogenase) and Complex IV (CIV; cytochrome c oxidase) in peripheral blood mononuclear cells (PBMCs) of DOX and DOX/DEX treated HR childhood survivors of ALL. Quantification of mtDNA copies per cell was chosen because mtDNA depletion from mutations or deletions can lead to the development of diseases [1,59]. CI and CIV of the OXPHOS electron transport chain were chosen as they are located at the beginning and the end of the transport chain and both complexes are encoded exclusively in the mitochondrial DNA as opposed to the nuclear DNA [1]. The hypothesis of this study was that DOX only treated participants would have higher mtDNA copies per cell due to DOX-induced alterations. This increase would be a compensatory clonal expansion to try to maintain translation of functional OXPHOS proteins at levels needed for normal OXPHOS activity. This higher number of mtDNA copies per cell, however, may not provide enough functional copies of OXPHOS proteins, leading to decreased OXPHOS enzyme activities.

2.3. MATERIALS AND METHODS

2.3.1. Study population

A total of 64 participants enrolled in the multi-center Dana-Farber Cancer Institute Childhood ALL Consortium Protocol between the years 1987 and 2005 were assessed. This protocol was a pediatric clinical trial for children under the age of 18 years who had been recently diagnosed with ALL. Participants were enrolled in the study if they met all of the following eligibility criteria: previously diagnosed with high risk ALL, registered and treated on DFCI/ALL Consortium protocols, no prior relapse (in first remission) and no prior allogeneic stem cell transplant, at least 4 years of age from date of diagnosis of ALL, and no history of secondary malignancies treated with radiation or chemotherapy. These participants were considered high risk by fulfilling one or more of the following criteria: an initial white blood cell count of 50 x 10⁹/L or higher, age at diagnosis younger than 1 year or older than 10 years, presence of lymphoblasts in cerebrospinal fluid, presence of an anterior thoracic cavity mass, or having a T cell versus a B cell immunophenotype. Standard risk participants had received lower doses of DOX and thus, having a lower risk of cardiac effects during study follow up, were not eligible for this study. Written, informed consent was obtained from a parent or guardian if the
participant was 18 years of age or younger or from participants if they were over 18 years of age, following approval of the study by the Institutional Review Boards at all multi-center sites.

2.3.2. PBMC isolation

Blood samples were drawn into EDTA and heparin containing vacutainers at each site a median of 7.8 years after DOX or DOX/DEX treatment was complete. PBMCs were isolated within 8 hours via Ficoll gradient (GE Healthcare, Waukesha, WI) and platelets were removed by two PBS washes and centrifugation at 300 RCF. The PBMCs were then pelleted and frozen at -80°C.

2.3.3. MtDNA quantification by real-time PCR

For measurement of mtDNA copies/cell, quantitative real time polymerase chain reaction (qRT-PCR) is a rapid method of quantification and was conducted as previously described by our lab [61]. For each participant sample, PBMC pellets were thawed in a 37°C water bath and 50µl of cold 1X phosphate buffered saline was immediately added to re-suspend the pellets. Forty microliters was removed from each sample for later protein extraction and the remaining 10µl was added to 190µl of 1X phosphate buffered saline. DNA was then isolated from PBMC pellets using a DNAeasy kit (Qiagen, Inc., Valencia, CA) and total DNA purity and quantity in ng/µL was determined using a Micro Volume UV-Vis Spectrophotometer (NanoDrop Products, Wilmington, DE). Isolated DNA was stored at 4°C.

A control plasmid containing the 90bp mtDNA NADH dehydrogenase subunit II (ND2) and the 98bp Fas Ligand housekeeping gene was serially diluted to prepare a standard curve. The genomic primers, GenDIR (GGC TCT GTG AGG GAT ATA AAG ACA) and GenREV (CAA ACC ACC CGA GCA ACT AAT CT) were specific for the region of the genome encoding for Fas Ligand. The mitochondrial primers, mtREV (CCG GAG AGT ATA TTG TTG AAG AG) and mtDIR (CAC AGA AGC TGC CAT CAA GTA), amplify a region of mtDNA encoding for ND2. Each well of the plate contained a total reaction volume of 20µL consisting of: SYBR Green Master Mix (FastStart Taq DNA polymerase, reaction buffer, dNTP mix, MgCl₂; LifeTechnologies, Carlsbad, CA),
1µM of each primer, and 5ng of DNA from each sample. The cycling conditions for PCR consisted of: pre-incubation with a target temperature of 95°C for 5 minutes followed by amplification over the course of 37-40 cycles at 95°C for 10 seconds (denaturing), 58°C for 10 seconds (annealing), and 72°C for 10 seconds (extension) per cycle. When amplification was complete, a melt curve analysis was run under the following conditions: 95°C for 5 seconds and 65°C for 1 minute followed by a final cooling cycle of 40°C for 30 seconds. All standards and samples were run in duplicate and the results were analyzed using Version 1.5.0 LightCycler 480 software (Roche, Indianapolis, IN). Absolute mtDNA copies per cell were calculated by dividing the mean mtDNA values by the mean genomic DNA values and then multiplying this number by 2 (there are two copies of nuclear-encoded genes in each cell). Laboratory staff were blind to participant group classification.

2.3.4. Oxidative phosphorylation activity by thin layer chromatography and immunoassay

The mitochondrial OXPHOS enzyme activities of Complex I (CI; NADH dehydrogenase) and Complex IV (CIV; cytochrome c oxidase) were quantified using OXPHOS thin layer chromatography and immunoassay (Abcam, Cambridge, MA) as described previously [81]. Frozen PBMC pellets were thawed in a 37°C water bath and 50µl of cold 1X PBS was immediately added to resuspend the pellets. Ten microliters was removed from each sample for later DNA extraction and 210µl of buffer A (1.5% lauryl maltoside, 25mM HEPES, 100mM NaCl; Abcam, Cambridge, MA) with 1X protease inhibitor cocktail (PI; Thermo Fischer Scientific, Waltham, MA) was added to the remaining 40µl. Samples were kept on ice and vortexed briefly every 5 minutes for 20 minutes. The samples were then centrifuged at 14,000 x g for 20 minutes at 4°C to remove cellular debris. Finally, lysates were flash frozen and stored at -80°C.

Prior to testing participant samples, standard curves were performed using control protein lysate from HeLa cells to determine the optimum amount of protein lysate to load for this assay. HeLa protein lysate was extracted and quantified as mentioned above to create a 2-fold serial dilution from 0 to 20µg. Values obtained from the standard curve were plotted using PRISM Version 5.0d (GraphPad Software, Inc., San Diego, CA). The
most linear section of the curve was divided into thirds and the point that was 2/3 from the bottom of this section, estimated at 10µg, was considered the optimum protein amount for this study.

The frozen protein lysate extracts were thawed and the total protein content of each sample was determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL) using a serial dilution of bovine serum albumin as a standard curve for quantification. A control sample of HeLa protein lysate was included in each dipstick assay for quality control. Ten micrograms of total sample and control protein lysate was loaded into the microplate wells. The dipsticks, which have a nitrocellulose membrane with a line of immobilized monoclonal antibody (mAb) at one end and an absorbent pad at the other end, were immersed into the protein lysate. The lysate was absorbed by the wicking pad and as it moved across the mAb line by capillary action, the target antigen, if present in the protein lysate, was immunocaptured (Figure 2.1). Thirty microliters of a washing buffer (50mM TRIS, 150mM NaCl) was added to remove any unbound proteins from the dipsticks. Then they were immersed in 300µl of an activity buffer containing a substrate and an electron acceptor. For CI, the activity buffer contained NADH and nitrotetrazolium blue (NBT). The CI enzymes oxidized NADH and the oxidized NAD+ reduced NBT, forming a purple precipitate at the mAb line (Figure 2.1). For CIV, the activity buffer contained reduced cytochrome c and di-amino benzidinetetrachloride (DAB). The CIV enzymes oxidized reduced cytochrome c and the oxidized cytochrome c oxidized DAB, forming a red precipitate at the mAb line (Figure 2.1). The signal intensity (optical density; OD) of the precipitate was proportional to the activity of the enzyme complex and was quantified using a portable immunochromatography reader (ICA-1000; Hamamatsu, Japan). All participant and control samples were run in duplicate. Participant OD values were normalized by dividing the sample OD by 10µg and then multiplying by 1000 (OD/µg x 10³). Laboratory staff were blind to participant group classification.
2.3.5. Statistical analysis

A Wilcoxon Rank-Sum test was used to compare continuous measures between groups. A model for the continuous outcome measures of mtDNA copies per cell and CI and CIV enzyme activities was based on the maximum likelihood variance component estimation to test for differences between treatment groups (DOX vs. DOX/DEX). Participants were grouped by age as either <10 years or ≥10 years of age. The baseline participant characteristics such of sex, age (<10 years or ≥10 years), T cell phenotype, initial white blood cell counts, and red blood cell contamination in PBMC samples were controlled for. Cumulative dose was also included in the modeling in a subset of the data but those participants dosed in mg/kg were excluded from analysis. As the distributions were skewed, the values for mtDNA copies per cell and CI and CIV enzyme activities were log10 transformed for modeling. Significance was set at a p value of 0.05 and all statistics were performed using SAS 9.2 software.

2.4. RESULTS
2.4.1. Participant characteristics

Sixty-four participants provided PBMC samples at a median of 7.8 (range=2.9-30.2) years since ALL diagnosis. Two samples did not contain sufficient mtDNA for qRT-PCR analysis and six and five samples did not contain sufficient protein levels for CI and CIV enzyme activity analysis, respectively. Approximately half of the participants were female (48%) and most were in the age group <10 years (64%). The majority of participants had leukemia originating in B cells (89%) versus in T cells (11%). All participants received DOX treatment with 27 participants receiving DOX only (42%) and
37 participants receiving DOX concomitantly with DEX (58%). The cumulative DOX dose was reported for 50 of the 64 participants with a median of 300 mg/m² (range=120-382; Table 2.1).

**Table 2.1. Participant Characteristics and ALL and DOX Profiles.**

<table>
<thead>
<tr>
<th></th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total N</strong></td>
<td>64</td>
</tr>
<tr>
<td><strong>Years to sample from treatment, median (range)</strong></td>
<td>7.8 (2.9, 30.2)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (52)</td>
</tr>
<tr>
<td>Female</td>
<td>31 (48)</td>
</tr>
<tr>
<td><strong>Age in years, median (range)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;10 yrs.</td>
<td>41 (64)</td>
</tr>
<tr>
<td>≥10 yrs.</td>
<td>23 (36)</td>
</tr>
<tr>
<td><strong>T-cell immunophenotype</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (11)</td>
</tr>
<tr>
<td>No</td>
<td>57 (89)</td>
</tr>
<tr>
<td><strong>Initial WBC count x10⁹/L, median (range)</strong></td>
<td>32.2 (1.3, 354.0)</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>27 (42)</td>
</tr>
<tr>
<td>DOX/DEX</td>
<td>37 (58)</td>
</tr>
<tr>
<td><strong>Cumulative DOX dose mg/m², median (range)</strong></td>
<td>300 (120, 382)*</td>
</tr>
</tbody>
</table>

* N=50 reported

2.4.2. Higher mitochondrial DNA copies per cell in DOX versus DOX/DEX treated participants

The DOX only treated group had significantly higher numbers of mtDNA copies per cell (n=27; median=1106, range=144-6747) than the DOX/DEX treated group (n=35; 310, 15-1859; p=0.001, adjusted p=0.015; Table 2.2, Figure 2.2). Drug treatment was independently associated with mtDNA copies per cell after sex, age, immunophenotype, initial white blood cell counts, and visible red blood cell contamination (as seen with the naked eye) in PBMC samples were controlled for (p=0.001; Table 2.3).
2.4.3. No difference between DOX and DOX/DEX treatment groups on CI or CIV enzyme activities

No significant differences were found between the DOX only and the DOX/DEX treatment groups on CI or CIV enzyme activities (Table 2.2). Drug treatment was not associated with CI or CIV enzyme activities.

Table 2.2. Mitochondrial Parameters for the DOX Only and DOX/DEX Groups.

<table>
<thead>
<tr>
<th></th>
<th>DOX only Median (range)</th>
<th>DOX/DEX Median (range)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtDNA copies per cell</td>
<td>27 1106 (144-6747)</td>
<td>35 310 (15-1859)</td>
<td>0.001</td>
</tr>
<tr>
<td>CI activity (OD/µg x 10^3)</td>
<td>25 10.5 (5.0-31.3)</td>
<td>33 11.7 (5.0-41.4)</td>
<td>0.97</td>
</tr>
<tr>
<td>CIV activity (OD/µg x 10^3)</td>
<td>25 9.8 (5.0-20.1)</td>
<td>34 8.1 (4.7-23.4)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Figure 2.2. MtDNA copies per cell in the DOX only and the DOX/DEX groups. The mtDNA copies per cell of the DOX only group (represented in blue) were significantly higher compared to the DOX/DEX group (represented in red) at a median of 7.8 years since last DOX treatment. The black lines in each group of dots indicate the median.
Table 2.3. Drug Treatment Associations, Controlling for Visible Red Blood Cell Contamination in PBMC Samples.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(DOX alone vs. DOX/DEX)</td>
<td>0.46</td>
<td>0.13</td>
<td>0.001</td>
</tr>
<tr>
<td>Sex (Male vs. Female)</td>
<td>0.11</td>
<td>0.14</td>
<td>0.45</td>
</tr>
<tr>
<td>Age (&lt;10 years vs. ≥10 years)</td>
<td>0.02</td>
<td>0.14</td>
<td>0.89</td>
</tr>
<tr>
<td>Immunophenotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T-cell vs. B-cell)</td>
<td>-0.18</td>
<td>0.21</td>
<td>0.41</td>
</tr>
<tr>
<td>Visible RBC contamination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(No vs. Yes)</td>
<td>0.02</td>
<td>0.14</td>
<td>0.89</td>
</tr>
<tr>
<td>Initial WBC count</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.0006</td>
<td>0.001</td>
<td>0.60</td>
</tr>
</tbody>
</table>

2.5. DISCUSSION

In this study, high risk childhood ALL survivors who had been treated with the chemotherapy agent DOX had significantly more mtDNA copies per cell in their PBMCs than ALL participants who had been treated with DOX combined with the iron chelator DEX. Mitochondrial OXPHOS enzyme activities for CI and CIV, however, did not differ significantly between the two groups.

DOX treatment when combined with the adjuvant DEX has been shown in multiple preclinical and clinical studies to provide protection to cardiac cells from DOX-induced cardiomyopathy without reducing its potent antitumor effect [27,32,75]. Physicians, however, rarely administer DEX with DOX treatment to pediatric patients [75,78]. In this study, we have shown that in pediatric ALL participants treated with DOX alone, mtDNA copies per cell in PBMCs were three times higher than in participants treated with DOX and DEX. It may be the case that DOX-exposed mitochondria are undergoing clonal expansion to compensate for DOX-induced deletions or mutations to maintain OXPHOS and ATP levels. This would also explain why there was no loss of OXPHOS enzyme activities in the DOX only group. In two previous
studies, Lebrecht et al. [25,33] found that DOX exposed rats had higher mtDNA mutations and deletions in cardiac tissue than controls. While the DOX treated rats in these studies had lower mtDNA copies per cell than the controls, our results may differ due to the timing of the studies. The effects of the DOX treatment in the rats were observed within a few weeks of the last DOX exposure, whereas our participant sample had last been exposed to DOX more than seven years in the past. While we did not measure mutations and deletions in our population, it is possible that mtDNA copies were initially decreased by DOX damage and then later increased through clonal expansion in order to maintain steady OXPHOS levels. Further follow up is be needed to determine if this compensatory system would eventually break down, leading to a loss of OXPHOS activity.

In this study, mtDNA alterations did not occur in the DOX/DEX treated participants. Preclinical and clinical studies have shown that DEX treatment with DOX decreases the risk of DOX-induced cardiovascular events [22,32,75]. The participants enrolled in this study were sampled from a larger cohort where cardiac function was examined after DOX or DOX/DEX treatment [22]. While effects of DEX on cardiac function were mild and more significant in the females, generally there was an improvement in cardiac outcomes. Furthermore, there was no increased risk of secondary neoplasms in the DEX treated group nor did DEX treatment lessen the antitumor effect of DOX [22,80]. These results combined with our own findings suggest that DEX adjuvant treatment in pediatric high risk ALL patients can decrease the risk of DOX-related cardiac events as well as offer systemic mitochondrial protection as observed in PBMCs. Unfortunately, DEX treatment in pediatric patients is still largely controversial. In a large retrospective study examining records of pediatric ALL patients from 1999-2009, only 2.5% of 2,500 patients received DEX adjuvant treatment. Physicians typically stated concerns over secondary neoplasm development [78]. Encouragingly, hospitals located geographically closer to the Dana-Farber Cancer Institute, which conducted one of the aforementioned studies using DEX treatment in pediatric ALL patients, were more likely to treat with DEX than those institutions located further away [78]. It is possible that over time, DEX treatment will become more widespread as more physicians become aware of the published data.
This study had several limitations. One limitation was the type of sample acquired from these participants. Many studies on DOX-induced cardiotoxicity have measured cardiac outcomes or cardiac tissue. We examined systemic changes in PBMC mitochondria in place of tissue specific changes in the heart, as it would not be possible to acquire cardiac mitochondria from pediatric participants. Studies have shown that mitochondrial parameters measured in different tissues tend to correlate well [55,82], so it is possible that what we observed in PBMC mitochondria may reflect what is occurring in cardiac mitochondria. Our results in PBMCs also show that DOX treatment may be altering bone marrow stem cells in these participants as PBMCs have a turn over rate of 3-4 days [34] and these participants’ cells were collected more than seven years after their last DOX treatment. This issue deserves further study to determine for how long this effect can occur and how DOX could ultimately alter tissues other than in the heart.

A further limitation of the study was the limited amount of PBMCs acquired from the participants. While there were enough mitochondria in the PBMC pellets to measure mtDNA copy numbers and OXPHOS enzyme activities, there was not enough sample left over to measure markers indicative of mitochondrial numbers per cell. It is possible that the increased mtDNA numbers were due to increases in the number of mitochondria present in the PBMCs. A study by Lebrecht et al. [33] found higher citrate synthase activity in cardiac cells of DOX treated rats; mtDNA copies per cell, however, were lower. An assay measuring the mitochondrial proteins citrate synthase or porin would have indicated if there were increased numbers of mitochondria in the PBMCs of our participants, but these assays require larger amounts of mitochondrial protein than we were able to isolate from the pellets and further samples were not available from the repository. As the participants were pediatric, there was general concern over drawing large volumes of blood. Future studies should take this into consideration when collecting participant samples.

The final limitations of our study were the small sample size and study design. The lack of significant differences found between the DOX and DOX/DEX groups on OXPHOS enzyme activities may be attributable to low power to detect such differences. It is also possible that changes in OXPHOS enzyme activities may become more obvious at further follow up time points. This study was designed as a small, cross-sectional pilot
study, and as such, it did not examine a large number of participants nor did it contain a longitudinal component. On account of the results of this pilot study, a large longitudinal study is currently underway to examine similar effects in the larger cohort from which these participants were sampled. The follow up component and larger sample size may allow us to examine not only changes in OXPHOS activities but also whether increases in mtDNA copy number are related to higher numbers of mitochondria in the PBMCs.

These findings suggest that there are changes in mitochondrial function in DOX treated pediatric ALL survivors that are abrogated by DEX treatment. Further evaluation is required to uncover the relationship of DOX treatment and PBMC mitochondrial dysfunction. Longitudinal studies that follow DOX and DOX/DEX treated ALL survivors over time will allow for a better understanding of the changes that may occur in mitochondrial function parameters.
CHAPTER 3.
Mitochondrial Dysfunction in a Perinatally HIV-Infected, Pediatric Population on cART

Author’s Note: I have previously contributed to the writing of a version of this work and this work has been published in a peer-reviewed journal [81].
3.1. ABSTRACT

Abnormalities in mitochondrial function have been linked to metabolic disorders such as insulin resistance and diabetes. In adults receiving combined antiretroviral therapy (cART) for HIV infection, metabolic disorders occur at a higher rate than in the general population. Recently, similarly high rates have been observed in perinatally HIV-infected, pediatric participants on cART. Multiple studies have linked mitochondrial dysfunction to metabolic disorders. Furthermore, there is much information in the literature linking cART and mitochondrial impairment. This indicates that HIV positive, pediatric populations on cART may have mitochondrial dysfunction. A case control study in HIV-infected pediatric participants receiving cART was conducted comparing mitochondrial function in cases with confirmed insulin resistance (IR) with a matched sample of control participants without IR. Participants were matched on age, race, Tanner stage of development, and body mass index (BMI) z-scores. Mitochondrial function was assessed by measuring mitochondrial DNA (mtDNA) copies per cell and the oxidative phosphorylation (OXPHOS) enzyme activities of Complex I (NADH dehydrogenase) and Complex IV (cytochrome c oxidase) in peripheral blood mononuclear cells (PBMCs). The IR cases showed a trend toward lower mtDNA copies/cell (median=158, IQR=96, 443) than the controls (median=199, IQR=80, 421). In both groups of children, however, the fasting serum glucose levels were inversely correlated with CI enzyme activity, particularly in cases (controls: r=-0.72, p=0.003; cases: r=-0.47, p=0.03), which may indicate an initial compensatory response in the mitochondria. These results show that IR in HIV-infected, pediatric population on cART is associated with mitochondrial dysfunction. Future longitudinal studies are needed to better clarify the relationship between cART and mitochondrial dysfunction, especially as this relationship may be altered as these children advance into puberty.
3.2. INTRODUCTION

3.2.1. Perinatal HIV infection

Of the 34 million people currently living with HIV, almost half of them are women of childbearing age and as a result, there are roughly 3.3 million children living with HIV infection acquired through their mothers [35,83]. An HIV positive pregnant woman can perinatally transmit the virus to her child through vertical transmission. This can occur during pregnancy (25% of cases), vaginal delivery (60-70% of cases), or from breastfeeding (about 10% of cases) [84,85]. Guidelines have since been put in place recommending that antiretroviral drugs, typically zidovudine (AZT/ZDV; this drug crosses the placenta), be given as early during the pregnancy as possible [85]. Studies have shown that the lower the mothers’ HIV viral load before birth, the lower the chances are of vertical transmission, confirming that cART should be initiated as soon as possible during pregnancy [86]. After the birth, newborns are also given antiretroviral drug treatment for up to six months [83,85]. Since these guidelines were instituted, perinatal transmission rates have dramatically decreased from 20% to 2% or less [56,83].

While perinatal transmission rates have decreased dramatically, those who were born HIV positive before guidelines were put in place are now adolescents who face a lifetime of cART in order to maintain undetectable viral loads. With long-term cART comes side effects such as various metabolic disorders. In adults, the association of cART and metabolic disorders are well documented and certain cART drugs such as NRTIs are well known culprits in the development of lipodystrophy, dyslipidemia, insulin resistance, and cardiovascular disease [20,46,47,87]. The long-term metabolic effects of cART exposure in perinatally HIV-infected children are only now beginning to be understood.

3.2.2. Insulin resistance

Under normal physiological conditions, as blood glucose levels increase, pancreatic beta cells release insulin. Adipocytes and myocytes have insulin receptors, which respond to the insulin signal by activating insulin receptor substrate-1 (IRS-1), which activates phosphoinositide 3-kinase (PI3K), which in turn activates protein kinase B (AKT), causing a translocation of glucose transporter 4 (GLUT4) transporters to the
cell membrane to take up the glucose [88,89]. Insulin resistance (IR) occurs when these cells begin to lose the ability to respond to insulin, likely through inhibition of GLUT-4, IRS-1, or PI3K [89,90]. Blood glucose levels do not decrease, and as a result, pancreatic beta cells release more insulin [89,91,92]. Thus the signs of IR are high fasting serum insulin levels (10 IU/mL or above) and normal (70-100mg/dL) to impaired fasting glucose levels (above 100mg/dL) [93,94].

There are few methods to determine IR with the gold standard being the euglycemic hyperinsulinemic clamp [95]. Currently, the fasting homeostatic model assessment of insulin resistance score (HOMA-IR) is typically used. This score is calculated by measuring the fasting glucose and insulin levels in the blood and using these values in the following equation: (fasting insulin (µU/mL) x fasting glucose (mmol/L))/22.5) [96]. In adults, a HOMA-IR score of ≥2.6 indicates IR [97]. In children, a standard HOMA-IR score is more difficult to define due to the changes in insulin sensitivity that occur during puberty. This as well as age, sex, and weight must be taken into consideration when determining an adolescent HOMA-IR cutoff [98].

IR is a major component of metabolic syndrome, linking it to obesity, diabetes, dyslipidemia, high blood pressure, and heart disease [88]. IR is a symptom of type II diabetes and studies show that it can occur 10-20 years before the onset of frank diabetes [99]. Though populations with IR may not yet have diabetes, they are at risk for a host of other metabolic diseases. The risk of developing IR in the general population depends on genetic factors as well as age, obesity, stress, and lack of physical exercise [89].

### 3.2.3. cART and insulin resistance

Treatment with antiretroviral therapy has greatly reduced the number of deaths due to HIV and AIDS (WHO), but cART is linked with the development of metabolic disorders including dyslipidemia and the increased risk of cardiovascular disease. Furthermore, higher rates of impaired glucose homeostasis such as insulin resistance and diabetes have been observed in those on cART [12,88,92]. In adults without HIV, the prevalence of IR is about 17% [75]. In HIV-infected adults receiving cART, however, IR occurs at a higher rate of 25-30% [75,100]. Surprisingly, rates of IR in HIV-infected pediatric populations on cART are around 15% [100,101]. While it is difficult to find
exact estimates of the rate of IR in HIV negative adolescents, the type II diabetes rate in those under the age of 20 years is 0.3% versus 11.3% in adults reflecting the fact that age is a large risk factor for metabolic disorders [102]. The prevalence of IR in pediatric HIV positive populations is concerning due to their young age as they may be at increased risk to progress to type II diabetes as they advance into puberty compared to adolescents without HIV [87].

While those with HIV infection are at higher risk of IR than the general population, the exact roles that HIV and cART play in the development of IR are not completely understood. HIV infection per se may not exert strong effects on insulin sensitivity with some studies showing that HIV infected, cART naïve populations have normal insulin function [88]. This suggests that the main culprit behind IR in these participants may be cART. In fact, when HIV negative adults were given an NRTI for a one-month period, insulin sensitivity in their muscle tissue significantly decreased as compared to those receiving a placebo [103]. This effect could be a result of NRTIs, which are known to affect mtDNA replication leading to decreased mtDNA copies and decreased OXPHOS levels (as described in detail in Chapters 1 and 4). NRTI-induced mitochondrial dysfunction has been documented in HIV negative cells as well as in animals and humans [51-53,55,56]. In HIV negative, NRTI exposed humans, mtDNA depletion and changes in OXPHOS have been observed in different tissues and cells including adipose tissue [55] and peripheral blood mononuclear cells (PMBCs) [56,57]. Thus, mitochondrial changes precipitated by cART could lead to the development of metabolic disorders such as IR in HIV positive populations.

3.2.4. Mitochondria and insulin resistance

The possible mechanisms of mitochondrial dysfunction in altered glucose homeostasis may include disruption of mtDNA replication, which would lead to impaired OXPHOS and ATP production. This mechanism was elucidated in a study using conplastic strains of Brown Norway and spontaneously hypertensive rats. Conplastic strains have the nuclear genome of one strain and the mitochondrial genome of another (Jackson labs) allowing for changes in the mtDNA sequences without affecting the nuclear sequence. Naturally occurring mtDNA sequence variations led to decreased
cytochrome c oxidase enzyme activity and protein quantity in liver mitochondria in the altered strain as compared to the unaltered strain. Furthermore, decreased ATP levels in skeletal muscle and impaired fasting glucose tolerance in these animals were also observed [104]. Though these animals did not develop frank diabetes from mitochondrial dysfunction, they exhibited impaired glucose tolerance, which is a risk factor for diabetes. These results link spontaneous mtDNA sequence variations to the development of metabolic dysfunction. Recent research on IR in clinical populations also points to an association with mitochondrial dysfunction [12,89,91,99]. In a study examining muscle biopsies from an adult population with IR, it was observed that there were fewer mitochondria. In addition, these mitochondria were swollen or fractured, and had decreased OXPHOS enzymes and lower levels of ATP production [89,99,105,106]. A similar study in IR young adults found a 30% decrease in ATP production in muscle cells compared to controls with normal insulin sensitivity [99]. Though signs of mitochondrial impairment in glucose homeostasis disorders have been seen mainly in adipose tissue or skeletal muscle cells in preclinical and clinical studies [91,99,106,107] dysfunction has also been observed in PBMCs from adults with type II diabetes. In these cells, mtDNA levels [108,109] and OXPHOS levels were lower than in controls [110]. These studies highlight that alterations in mitochondrial function are linked to dysfunctional glucose homeostasis.

3.2.5. Insulin resistance in HIV-infected children

The long-term effects of cART on mitochondrial function are of great importance when considering HIV infected pediatric populations, as they will face a lifetime on cART. Many of these HIV-infected children on cART show signs of metabolic disorders early in life [111]. In the Pediatric HIV/AIDS Cohort (PHACS) study, HOMA-IR scores indicating IR occurred at a rate of 15% in this perinatally HIV-infected, population on cART. IR in this population was also associated with the use of certain types of cART, implicating antiretrovirals in the etiology of IR [100]. Another study examining HIV-infected children on long-term cART that focused on mitochondrial parameters observed lower mtDNA copies per cell in PBMCs [112]. It appears likely that cART exposure in pediatric populations could lead to mitochondrial dysfunction and metabolic alterations.
Metabolic dysfunction such as IR and diabetes can increase the risk of stroke and come with a myriad of debilitating effects [88]. There are notably few studies in the scientific literature, however, examining mitochondrial function in, HIV-positive children on cART who have already developed IR.

3.2.6. The Pediatric HIV/AIDS Cohort Study

The Pediatric HIV/AIDS Cohort Study (PHACS) was a prospective study created to investigate the effects of cART treatment and HIV-infection in perinatally HIV-infected preadolescents and adolescents. A portion of these participants was enrolled into a further cohort called the Adolescent Master Protocol (AMP) between March 2007 and December 2009. Children from 15 sites across the U.S. and Puerto Rico were eligible for enrollment into AMP if they were born to HIV-infected mothers, are HIV-positive, aged 7-16 years, and were previously enrolled in another longitudinal cohort study such as: the International Maternal Pediatric Adolescent AIDS Clinical Trial protocols (designed to evaluate the long-term effects of in utero and postnatal HIV infection and cART exposure) and the Women and Infants Transmission Study (a longitudinal study of HIV-infected pregnant women and their infants).

In the AMP protocol, participants had clinical, laboratory, and body composition assessments to track metabolic changes, neurodevelopmental parameters, growth, HIV disease status, and other outcomes. Demographic information, clinical and family history, and detailed cART history were routinely collected. Blood samples including PBMCs were collected annually and stored for future studies.²

The aim of this study was to measure mitochondrial DNA (mtDNA) copies per cell and the oxidative phosphorylation (OXPHOS) enzyme activities of Complex I (CI; NADH dehydrogenase) and Complex IV (CIV; cytochrome c oxidase) in peripheral blood mononuclear cells (PBMCs) of perinatally HIV-infected, cART treated children with and without insulin resistance. CI and CIV of the OXPHOS electron transport chain were chosen as they are located at the beginning and the end of the transport chain and

² All clinical assessments were completed and laboratory specimens collected by our collaborators in the PHACS network. The network consists of multiple institutions including the PHACS coordinating center at Tulane University headed by Dr. Russell Van Dyke.
are encoded on the mitochondrial DNA. The hypothesis of this study was that HIV-infected children with IR would have lower mtDNA copies per cell and decreased OXPHOS enzyme activities as compared to those without IR.

3.3. MATERIALS AND METHODS

3.3.1. Study population

A total of 42 children with perinatally acquired HIV infection enrolled in the multi-center U.S.-based Adolescent Master Protocol (AMP) of the Pediatric HIV/AIDS Cohort (PHACS) were assessed. Participants provided clinical, laboratory, and body composition assessments at preset intervals to monitor HIV disease status, metabolic and neurodevelopmental parameters, and growth. Clinical data included: antiretroviral treatment history (current and lifetime), HIV viral load, T cell subsets, weight, height, and body mass index (BMI; weight (kg)/height$^2$ (m$^2$)) expressed as z-scores. Participants were then assessed for IR, as defined by fasting HOMA-IR (fasting insulin (µU/mL) x fasting glucose (mmol/L)/22.5), depending on Tanner stage of development. A HOMA-IR score of $\geq$ 2.5 in participants who were Tanner stage 1 and $> 4.0$ in Tanner stage 2 or higher were classified as IR. The participants’ parent or legal guardian provided informed written consent. The Institutional Review Boards at all multi-center sites approved this study.

3.3.2. PBMC isolation

Blood samples were drawn into EDTA and heparin containing vacutainers at each site during annual participant wellness examinations. Peripheral blood mononuclear cells (PBMCs) were isolated within 8 hours via Ficoll gradient (GE Healthcare, Waukesha, WI) and platelets were removed by two PBS washes and centrifugation at 300 RCF. The PBMCs were then pelleted and frozen at -80°C.

3.3.3. MtDNA quantification by real-time PCR

Blood samples were drawn into EDTA and heparin containing vacutainers at each site. Peripheral blood mononuclear cells (PBMCs) were isolated within 8 hours via Ficoll gradient (GE Healthcare, Waukesha, WI) and platelets were removed by two PBS washes
and centrifugation at 300 RCF. The PBMCs were then pelleted and frozen at -80°C. For measurement of mtDNA copies/cell, quantitative real time polymerase chain reaction (qRT-PCR) is a rapid method of quantification and was conducted as previously described by our lab [61]. For each participant sample, PBMC pellets were thawed in a 37°C water bath and 50µl of cold 1X phosphate buffered saline was immediately added to resuspend the pellets. Forty microliters was removed from each sample for later protein extraction and the remaining 10µl was added to 190µl of 1X phosphate buffered saline. DNA was then isolated from PBMC pellets using a DNAeasy kit (Qiagen, Inc., Valencia, CA) and total DNA purity and quantity in ng/µL was determined using a Micro Volume UV-Vis Spectrophotometer (NanoDrop Products, Wilmington, DE). Isolated DNA was stored at 4°C.

A control plasmid containing the 90bp mtDNA NADH dehydrogenase subunit II (ND2) and the 98bp Fas Ligand housekeeping gene was serially diluted to prepare a standard curve. The genomic primers, GenDIR (GGC TCT GTG AGG GAT ATA AAG ACA) and GenREV (CAA ACC ACC CGA GCA ACT AAT CT) were specific for the region of the genome encoding for Fas Ligand. The mitochondrial primers, mtREV (CCG GAG AGT ATA TTG TTG AAG AG) and mtDIR (CAC AGA AGC TGC CAT CAA GTA), amplify a region of mtDNA encoding for ND2. Each well of the plate contained a total reaction volume of 20µL consisting of: SYBR Green Master Mix (FastStart Taq DNA polymerase, reaction buffer, dNTP mix, MgCl₂; LifeTechnologies, Carlsbad, CA), 1µM of each primer, and 5ng of DNA from each sample. The cycling conditions for PCR consisted of: pre-incubation with a target temperature of 95°C for 5 minutes followed by amplification over the course of 37-40 cycles at 95°C for 10 seconds (denaturing), 58°C for 10 seconds (annealing), and 72°C for 10 seconds (extension) per cycle. When amplification was complete, a melt curve analysis was run under the following conditions: 95°C for 5 seconds and 65°C for 1 minute followed by a final cooling cycle of 40°C for 30 seconds. All standards and samples were run in duplicate and the results were analyzed using Version 1.5.0 LightCycler 480 software (Roche, Indianapolis, IN). Absolute mtDNA copies per cell were calculated by dividing the mean mtDNA values by the mean genomic DNA values and then multiplying this number by 2 (there are two copies of nuclear-encoded genes in each cell).
3.3.4. Oxidative phosphorylation activity by thin layer chromatography and immunoassay

The mitochondrial OXPHOS enzyme activities of Complex I (Cl; NADH dehydrogenase) and Complex IV (CIV; cytochrome c oxidase) were quantified using OXPHOS thin layer chromatography and immunoassay (Abcam, Cambridge, MA) as described previously [81]. Frozen PBMC pellets were thawed in a 37°C water bath and 50µl of cold 1X PBS was immediately added to resuspend the pellets. Ten microliters was removed from each sample for later DNA extraction and 210µl of buffer A (1.5% lauryl maltoside, 25mM HEPES, 100mM NaCl; Abcam, Cambridge, MA) with 1X protease inhibitor cocktail (PI; Thermo Fischer Scientific, Waltham, MA) was added to the remaining 40µl. Samples were kept on ice and vortexed briefly every 5 minutes for 20 minutes. The samples were then centrifuged at 14,000 x g for 20 minutes at 4°C to remove cellular debris. Finally, lysates were flash frozen and stored at -80°C.

Prior to testing participant samples, standard curves were performed using control protein lysate from HeLa cells to determine the optimum amount of protein lysate to load for this assay. HeLa protein lysate was extracted and quantified as mentioned above to create a 2-fold serial dilution from 0 to 20µg. Values obtained from the standard curve were plotted using PRISM Version 5.0d (GraphPad Software, Inc., San Diego, CA). The most linear section of the curve was divided into thirds and the point that was 2/3 from the bottom of this section, estimated to 10µg, was considered the optimum protein amount for this study.

The frozen protein extracts were thawed and the total protein content of each sample was determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL) using a serial dilution of bovine serum albumin as a standard curve for quantification. A control sample of HeLa protein lysate was included in each dipstick assay for quality control. Ten micrograms of total sample and control protein lysate was loaded into the microplate wells. The lysate was absorbed by the wicking pad and as it moved across the mAb line by capillary action, the target antigen, if present in the protein lysate, was immunocaptured. Thirty microliters of a washing buffer (50mM TRIS, 150mM NaCl) was added to remove any unbound proteins from the dipsticks. Then they
were immersed in 300µl of an activity buffer. The signal intensity (OD) of the precipitate was proportional to the activity of the enzyme complex and was quantified using a portable immunochromatography reader (ICA-1000; Hamamatsu, Japan). All participant and control samples were run in duplicate. Participant OD values were normalized by dividing the sample OD by 10µg and then multiplying by 1000 (OD/µg x 10³). Laboratory staff were blind to participant group classification.

3.3.5. Statistical analysis

Spearman correlations were conducted to examine relationships between mitochondrial markers and fasting glucose, insulin, and HOMA-IR score in cases and controls separately. Interquartile ranges (IQR) were used to summarize the characteristics of cases and controls, including CD4+ T cell percent, HIV viral load (copies/mL), antiretroviral history, CDC disease stage, and sociodemographics. Comparisons between the cases and controls on these clinical characteristics were performed in an unmatched analysis using Chi-square or Fisher’s exact test for categorical variables and Wilcoxon test for continuous variables. The difference between each IR case and matching control was calculated and the mean (95% confidence intervals) of the differences across matched pairs was evaluated for each mitochondrial marker. A one-sample t-test was performed to examine the probability of there being no difference between matched cases and controls. Significance was set at a p value of 0.05. All data analyses were performed in SAS.

3.4. RESULTS

3.4.1. Clinical assessments

Twenty-one IR participants were matched to 21 HIV-infected controls without IR based on age at the time of sample collection (±12 months), sex, race, Tanner stage (± one Tanner stage difference in participants with Tanner stage > 1), and BMI z-score (±0.5 z-score difference). Two case-control pairs were matched using all criteria except for Tanner stage, because these cases did not have this information documented on their clinical data forms. By examining the age of these two participants, it was determined that they would likely have completed puberty based on normative data, and thus the
criteria requiring a higher cut-off for the HOMA-IR scores (>4.0) was used to categorize them.

Eighty-one percent of the participants were African American with a median age of 13 years. There were an almost equal number of males and females (Table 3.1). As IR cases and controls were matched on age, race, sex, BMI z-score and Tanner stage, there were no significant differences between the groups on these variables (Table 3.1). All participants were receiving cART at the time of the PBMC collection. The majority of the participants were currently taking an NRTI regimen with a median lifetime NRTI exposure of 10 years. There were no significant differences on HIV disease status/severity between IR cases and controls (Table 3.1).

There were no significant differences between the IR cases and the controls in the use of different types of antiretroviral drugs that have been reported to be associated with cardiovascular disease risk or metabolic changes in HIV-positive participants (e.g., NRTIs and PIs; Table 3.2). There were no significant differences in current or past use of cART between IR cases and controls, though the power to detect such differences was limited (data not shown).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th></th>
<th>Case Control Status</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (N=21)</td>
<td>Case (N=21)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>11 (52%)</td>
<td>11 (52%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Male</td>
<td>10 (48%)</td>
<td>10 (48%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (Min, Max)</td>
<td>13.54 (8.72, 16.20)</td>
<td>13.45 (8.50, 16.02)</td>
<td>0.71</td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>11.46, 14.35</td>
<td>11.38, 14.04</td>
<td></td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black or African American</td>
<td>17 (81%)</td>
<td>17 (81%)</td>
<td>0.45</td>
</tr>
<tr>
<td>White</td>
<td>3 (14%)</td>
<td>1 (5%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (5%)</td>
<td>3 (14%)</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hispanic or Latino</td>
<td>3 (14%)</td>
<td>3 (14%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Not Hispanic or Latino</td>
<td>18 (86%)</td>
<td>18 (86%)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI Z-score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (Min, Max)</td>
<td>1.24 (-1.16, 2.21)</td>
<td>1.28 (-1.37, 2.47)</td>
<td>0.88</td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>0.06, 1.58</td>
<td>0.06, 1.67</td>
<td></td>
</tr>
<tr>
<td><strong>Tanner stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3 (14%)</td>
<td>5 (24%)</td>
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</tr>
<tr>
<td>2</td>
<td>4 (19%)</td>
<td>2 (10%)</td>
<td></td>
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<tr>
<td>3</td>
<td>4 (19%)</td>
<td>4 (19%)</td>
<td>0.86</td>
</tr>
<tr>
<td>4</td>
<td>6 (29%)</td>
<td>4 (19%)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4 (19%)</td>
<td>4 (19%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0 (0%)</td>
<td>2 (10%)</td>
<td></td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (Min, Max)</td>
<td>1.80 (0.20, 4.00)</td>
<td>5.95 (2.60, 24.80)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>1.20, 3.00</td>
<td>4.95, 8.05</td>
<td></td>
</tr>
<tr>
<td><strong>Fasting insulin value</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mcU/mL)</td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Median (Min, Max)</td>
<td>8.60 (1.20, 21.70)</td>
<td>29.70 (10, 88)</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>5.50, 13.00</td>
<td>23.45, 40.20</td>
<td></td>
</tr>
<tr>
<td><strong>Fasting glucose value</strong></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (Min, Max)</td>
<td>82 (69, 96)</td>
<td>87 (57, 114)</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>75, 90</td>
<td>82.50, 94.50</td>
<td></td>
</tr>
<tr>
<td><strong>CD4 percent</strong></td>
<td></td>
<td></td>
<td>0.91</td>
</tr>
<tr>
<td>Median (Min, Max)</td>
<td>34.50 (13, 51.4)</td>
<td>33.1 (14.35, 46.00)</td>
<td></td>
</tr>
<tr>
<td>Q1, Q3</td>
<td>31, 36.2</td>
<td>26.85, 40.5</td>
<td></td>
</tr>
<tr>
<td><strong>CD4 percent &lt; 15%</strong></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>No</td>
<td>20 (95%)</td>
<td>20 (95%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (5%)</td>
<td>1 (5%)</td>
<td></td>
</tr>
<tr>
<td><strong>HIV RNA &lt;400 copies/mL</strong></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>No</td>
<td>8 (38%)</td>
<td>8 (38%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 (62%)</td>
<td>13 (62%)</td>
<td></td>
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</tbody>
</table>
Table 3.2. Antiretroviral Treatment Status between Cases and Controls.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case Control Status</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (N=21)</td>
<td>Case (N=21)</td>
</tr>
<tr>
<td>CDC class</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A - Mildly symptomatic</td>
<td>6 (29%)</td>
<td>10 (48%)</td>
</tr>
<tr>
<td>B - Moderately symptomatic</td>
<td>6 (29%)</td>
<td>4 (19%)</td>
</tr>
<tr>
<td>C - Severely symptomatic</td>
<td>4 (19%)</td>
<td>6 (29%)</td>
</tr>
<tr>
<td>N - Not symptomatic</td>
<td>5 (24%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>PI-current use</td>
<td>No</td>
<td>6 (29%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>15 (71%)</td>
</tr>
<tr>
<td>PI-ever use</td>
<td>No</td>
<td>1 (5%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>20 (95%)</td>
</tr>
<tr>
<td>PI-lifetime use (months)</td>
<td>Median (Min, Max)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>78.25 (22.36, 141.67)</td>
<td>98.39 (43.99, 128.98)</td>
</tr>
<tr>
<td></td>
<td>Q1, Q3</td>
<td>54.10, 103.79</td>
</tr>
<tr>
<td>NNRTI-current use</td>
<td>No</td>
<td>15 (71%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6 (29%)</td>
</tr>
<tr>
<td>NNRTI-ever use</td>
<td>No</td>
<td>11 (52%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>10 (48%)</td>
</tr>
<tr>
<td>NNRTI-lifetime use (months)</td>
<td>Median (Min, Max)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60.20 (9.07, 113.92)</td>
<td>44.32 (14.24, 111.36)</td>
</tr>
<tr>
<td></td>
<td>Q1, Q3</td>
<td>30.67, 86.37</td>
</tr>
<tr>
<td>NRTI-current use</td>
<td>No</td>
<td>2 (10%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>19 (90%)</td>
</tr>
<tr>
<td>NRTI-ever use</td>
<td>Yes</td>
<td>21 (100%)</td>
</tr>
<tr>
<td>NRTI-lifetime use (months)</td>
<td>Median (Min, Max)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>117.11 (22.36, 164.26)</td>
<td>128.98 (98.07, 173.49)</td>
</tr>
<tr>
<td></td>
<td>Q1, Q3</td>
<td>101.49, 153.04</td>
</tr>
<tr>
<td>Not on ARV</td>
<td>Yes</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>ZDV-current use</td>
<td>Yes</td>
<td>4 (19%)</td>
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<tr>
<td>ZDV-ever use</td>
<td>Yes</td>
<td>17 (81%)</td>
</tr>
<tr>
<td>d4T-current use</td>
<td>Yes</td>
<td>5 (24%)</td>
</tr>
<tr>
<td>d4T-ever use</td>
<td>Yes</td>
<td>16 (76%)</td>
</tr>
</tbody>
</table>

*Fisher’s exact test or Wilcoxon test used as appropriate.
**The p value could not be calculated as all participants have used NRTIs.
ZDV=Zidovudine
d4T=Stavudine
3.4.2. Lower mtDNA copies per cell in the IR cases versus the non-IR control group

MtDNA copies per cell were lower in the in IR cases (median=158; 25th IQR=96, 50th IQR=443) than in controls (median=199; 25th IQR=80, 50th IQR=421) with a difference of -39.9 in a matched analysis (p=0.098; Table 3.3, Figure 3.1). There were one case and one control with mtDNA copies per cell of higher than 400 copies per cell (421 and 443, respectively). Using a boxplot, the distribution of the mtDNA values was plotted in the cases and the controls separately. The values above 400 copies per cell were more than 3 IQR from the box in the cases and more than 1.5 IQR in the controls. When the data was analyzed without these two outliers included, the mean difference between cases and controls was -40.5 in a matched analysis (p=0.044; Table 3.3).

Examination of the correlations between fasting insulin, fasting glucose, HOMA-IR score, and mtDNA copies per cell showed no associations (data not shown).

3.4.3. No differences between the IR cases versus the non-IR control group on CI or CIV enzyme activities

No significant differences were found between the IR cases and the controls on CI or CIV enzyme activities (Table 3.3). Examination of the correlations between fasting insulin, fasting glucose, HOMA-IR score, and mitochondrial enzyme activities revealed that serum glucose was inversely related to CI enzyme activity in controls (controls: r=-0.47, p=0.03), with a greater correlation observed in the IR cases (r=-0.72, p=0.0003). When the case and control with the outlier mtDNA copy numbers and their matched pairs were excluded, these correlations remained significant (cases: r=-0.70, p=0.0008; controls: r=-0.44, p=0.05). Serum glucose was not associated with CIV enzyme activity. In addition, there was no association between insulin or HOMA-IR score and CI or CIV enzyme activity (data not shown).
Figure 3.1. Differences in PBMC mtDNA copies per cell between cases and controls. The diamond represents the median in each group without the outliers and the line represents the median with the outliers included. The error bars represent the 25th and 75th IQR without the outliers.
3.5. DISCUSSION

In this study, we showed that HIV-infected children on cART with IR show signs of mitochondrial dysfunction as compared to HIV-infected children on cART without IR matched on age, race, Tanner stage of development and BMI z-score. There was no difference between the groups on exposure to NRTIs or other cART drugs. The IR cases showed a trend toward lower mtDNA copies/cell as compared to the controls. In both groups of children, however, the fasting serum glucose levels were inversely correlated with CI enzyme activity, particularly in cases.

There is a dearth of studies examining mitochondrial function and genetic markers in HIV-infected, children on cART with IR. In this matched case-control study, there were differences in mtDNA copies per cell in HIV-infected, children on cART with and without IR. With lower mtDNA copies per cell in the IR cases, it would be expected that the OXPHOS CI and CIV enzyme activities, encoded by mtDNA, would also be significantly lower. Neither of the OXPHOS enzyme activities were significantly lower in the IR cases and, though not statistically significant, the median levels of both OXPHOS enzyme activities were actually higher. Higher OXPHOS CI and CIV enzyme activities in the IR cases may be an initial compensatory response in the mitochondria to higher circulating insulin levels. It is possible that as participants progress into frank diabetes, mtDNA copies per cell may further decrease, causing this compensatory mechanism to fail and thus leading to the observable decreases in OXPHOS enzyme activities [108-110]. Longitudinal studies in this pediatric cohort are needed to determine whether this will occur.

There was a significant negative correlation between fasting glucose levels and CI enzyme activity. Changes in OXPHOS have also been observed in adult type II diabetic skeletal muscle CI activity [106]. While the fasting serum insulin levels of the cases in this study were high, their fasting serum glucose levels fell within the normal range, as they have not yet progressed to full diabetes. The negative correlation between glucose and CI enzyme activity is similar to the OXPHOS impairment that has been seen in diabetes [106,109,110]. As blood glucose levels increase, the initial compensatory activity of CI in response to the high levels of insulin begins to fail. A comparable matched study in HIV-infected children with and without type II diabetes may better
explore this progression. It has been determined in several studies that cART plays a role in altering the function of the cellular glucose transporter, GLUT4 [88]. Furthermore, the role of mitochondrial OXPHOS impairment leading to the development of IR has also been established [89]. Though a clear link between cART-associated mitochondrial dysfunction and IR has been shown in adults, this same link has yet to be observed in HIV-infected, children on cART. A larger study would better evaluate whether such an association exists.

This study was limited by the small sample size. The matched design, however, was an advantage that offered better control of potential participant confounds. A further limitation of this study was the small PBMC volumes available which restricted the variety of the mitochondrial assays that could be performed. Larger sample volumes in future studies will allow for a more comprehensive assessment of the mitochondrial effects. PBMCs are not usually considered the ideal tissue in which to observe mitochondrial effects due to the smaller number of mitochondria per cell. Other types of tissue such as muscle, which contain large numbers of mitochondria per cell, would be prohibitively difficult to obtain in children. Previous studies have demonstrated that mitochondrial RNA and OXPHOS protein levels in PBMCs correlate with levels in subcutaneous adipose tissue and could also correlate with other cells [55,82].

In this pilot study, there were no HIV-negative children with IR enrolled as controls. A control group would be helpful in further determining if the mitochondrial changes in this population are related to cART. It was hypothesized that IR and mitochondrial dysfunction could be associated with cART in HIV-infected children, but the exposure to cART between IR cases and controls did not differ. This may have been due to the small sample size limiting the power to detect differences in individual antiretroviral drug types between the two groups. Further factors such as family history of diabetes, other risk factors for metabolic diseases, or chronic inflammation from HIV infection could also alter the progression to IR in some children and not in others. Further research is necessary to better define the roles of cART and other clinical characteristics in the development of IR in HIV-infected children.

These findings suggest that there are changes in mitochondrial function in HIV-infected children with IR. Further evaluation is required to uncover the relationship of
mitochondrial dysfunction and metabolic changes in HIV-infected children. In addition, the role of different types of cART in the development of pediatric metabolic dysfunction requires further study. Longitudinal studies that follow HIV-infected children with IR over time will allow for a better understanding of the changes that may occur in mitochondrial function parameters as these children on long-term cART advance into puberty.
CHAPTER 4.
The Effects of HIV and cART on Mitochondrial Function, Epidermal Nerve Fibers, and Oxidative Stress in an Adult, Thai Population
4.1. ABSTRACT

HIV infection and NRTI treatment are both associated with the development of peripheral neuropathy (PN). The aim of this study was to measure mitochondrial DNA (mtDNA) copies per cell and the oxidative phosphorylation (OXPHOS) enzyme activities in peripheral blood mononuclear cells (PBMCs), levels of five antioxidants and one measure of ROS in plasma (oxidative stress measures), and epidermal nerve fiber density (ENFD) in distal and peripheral skin from HIV positive (+) and negative (-) participants in Thailand. We hypothesized that HIV+ participants on long-term stavudine treatment (d4T; >6 months) who have confirmed symptomatic PN (HIV+/PN) would have altered mitochondrial parameters, lower distal ENFD, and higher oxidative stress than HIV- participants or HIV+ participants without PN who are cART naïve and after the HIV+ group receives short-term d4T treatment (6 months). Furthermore, HIV+, cART naïve participants would show signs of mitochondrial dysfunction, distal ENFD loss and oxidative stress at study entry as compared to HIV- participants. A cART regimen containing d4T would increase mitochondrial dysfunction, distal ENFD loss and oxidative stress in the HIV+ participants, while switching to a less mitochondrially toxic regimen containing zidovudine (ZDV) would improve mitochondrial parameters and distal ENFD and decrease oxidative stress. ENFDs were significantly longer in the HIV- group, with no differences between the two HIV+ groups and no changes over time with different cART regimens in the HIV+ group. MtDNA copies per cell did not differ between cohorts and increased only after 72 weeks of cART in the HIV+ group. OXPHOS activity was significantly altered in both of the HIV+ groups but only CIV was altered after 72 weeks of cART. The HIV+/PN group had the highest levels of several antioxidants, followed by the HIV+ group. D4T treatment induced higher antioxidant levels in the HIV+ group, which were reduced after switching to a ZDV-based regimen. TBARS did not differ between the three groups but decreased in the HIV+ group after the switch to ZDV. The results from this study indicate that d4T induces changes in mitochondrial function and oxidative stress that may induce PN. Switching to a ZDV-based regimen ameliorates some of these mitochondrial and oxidative stress effects in a population without PN.
4.2. INTRODUCTION

4.2.1. Peripheral neuropathy and epidermal nerve fiber densities

Peripheral neuropathy (PN) is the length dependent degeneration of peripheral neurons, called nerve fibers. Fiber loss typically begins in the longest nerves of the body such as those that enervate the feet and hands. Approximately 15% of people in the U.S. over the age of 40 have some type of PN and this incidence increases with age. Other factors related to the increased risk of PN are increased height, diabetes, and HIV infection [113,114]. Symptoms of PN include pain, burning, tingling or numbness in stocking-glove distribution, beginning in the feet and moving into the lower legs and hands. While the symptoms can be treated, there is no treatment to reverse nerve cell degeneration. PN typically begins with the loss of the small, unmyelinated sensory fibers in the skin. These fibers begin as nerve bundles leaving the dorsal root ganglion near the spinal column where they eventually enter the dermis, losing their myelination and terminating in the epidermis as free nerve endings [113]. These unmyelinated nerve fibers account for 90% of epidermal nerve fibers and transmit sensations of pain and temperature to the brain. They also play an autonomic role by enervating sweat glands. Unmyelinated epidermal nerve fiber endings are less efficient than myelinated fibers, with a slow conduction velocity of 2m/sec compared to 20m/sec in myelinated fibers [43,69,113,115].

Patients with peripheral neuropathy can have loss of small fibers without any signs or symptoms of neuropathy. This can make diagnosis difficult as nerve conduction studies measuring the speed of the electrical conduction of motor or sensory nerves are not an accurate measure of small fiber loss since these unmyelinated fibers tend to be “invisible” to this test [43]. More recently, skin punch biopsies and immunohistochemical staining have become the gold standard for diagnosis of small fiber loss. These biopsies require minimal equipment and are small and relatively painless for the patient. To acquire the tissue sample, a skin punch of 3-4mm in size is taken from the upper (peripheral) or lower (distal) leg, fixed, and stained with an antibody to protein gene product 9.5 (PGP 9.5), a panaxonal marker found in neurons. The stained skin slices are examined under a microscope and the number of unmyelinated nerve fibers per length of epidermis is counted (Figure 4.1) [69,113,114,116]. Studies have shown that HIV
positive participants have fewer epidermal nerve fibers than HIV negative participants, even without showing any clinical signs or symptoms of neuropathy [68,116,117], and that distal leg epidermal nerve fiber density (ENFD) is a valid predictor of small, unmyelinated nerve fiber damage and PN risk in HIV infection [68].

**Figure 4.1. Epidermal nerve fibers stained with PGP 9.5.** The blue arrows indicate healthy, intact epidermal nerve fibers in a 50µM section of epidermis [116].

Neurons, whether in the central or peripheral nervous system, require large amounts of energy to maintain the membrane gradients needed to conduct action potentials (electrical signals) and to release their chemical messengers called neurotransmitters from the terminals. This energy, in the form of adenosine triphosphate (ATP), comes from small organelles called mitochondria [118]. As neurons are highly dependent upon ATP for conducting action potentials and releasing neurotransmitters, the majority of their mitochondria are located in the axons and terminals [119]. Unmyelinated axons, consuming 2.5 to 10-fold more energy than myelinated axons [14], critically rely on mitochondria for survival. Loss of mitochondrial function and ATP production can lead to neuronal dysfunction and death as seen in length dependent PNs [118]. When skin punch biopsies from human subjects with and without PN were fixed and fluorescently stained for nerve axons and OXPHOS CIV proteins within the axonal mitochondria, biopsies from participants with PN showed lower amounts of CIV proteins [14]. While mitochondrial alterations and loss of nerve fibers are associated, the exact relationship of these variables is still unclear [14,43,68].
4.2.2. HIV epidemiology and molecular structure

The first cases of human immunodeficiency virus (HIV) were seen in the U.S. in 1981 and to date, over 20 million people have died worldwide. It was discovered in 1984 that HIV infection is the cause of AIDS and that this virus could be contracted from exposure to infected blood, vaginal secretions, semen, saliva, and breast milk [34,120]. HIV infection typically occurs through sexual intercourse, exposure to infected blood from transfusions or needle sharing by drug users, or from vertical transmission during childbirth (mother to child transmission) [34,120]. There are two strains of HIV, HIV-1, and HIV-2, which is a less virulent form of the virus. HIV-2 infection is typically confined to Western Africa, and thus most of the HIV infections that occur globally are caused by the HIV-1 strain [34]. HIV is a retrovirus, meaning the virus contains in place of DNA, RNA, which is reverse transcribed into DNA by a special enzyme. The virus and its RNA is surrounded by an envelope protein complex that is derived from the host cell membranes helping to mask it from detection by the host’s immune system. The viral envelope contains glycoproteins gp41 and gp120, which are essential for infection. HIV protein gp120 has a high affinity for the glycoprotein CD4, found on macrophages, monocytes, dendritic cells and T helper cells, all of which are essential components of a healthy immune system. Once gp120 is bound to CD4 and a co-receptor, either CCR5 or CXCR4, the envelope protein, gp41, fuses with the membrane of the host cell and allows for entry of viral RNA and enzymes. Each viral particle contains two identical, positive stands of RNA that code for nine genes, encoding enzymes such as protease, integrase, and reverse transcriptase. Three of these genes are required for the transcription and production of new virions (gag, pol and env) and the remaining genes encode for proteins that regulate infection and replication. Inside the infected cell, the viral RNA is synthesized to complementary DNA by the RNA dependent, DNA polymerase reverse transcriptase (RT). The resulting viral DNA is integrated into the host DNA by integrase, where it is translated into proteins. These nascent proteins are then cleaved by protease and reconstructed. Finally, the host cell membrane envelops the viral proteins, displaying gp41 and gp120, and the new virus buds off from the cell [34,121]. Over time, the virus can kill many CD4+ T cells, causing immunodeficiency. The average healthy person has a CD4+ T cell count of around 500 to 1,000 cells/mm³. An HIV positive person is
considered to have progressed to AIDS once their CD4 T cell counts have dropped below 200 cells/mm$^3$ and is then at great risk for opportunistic infections and malignancies [34]. There is no cure for or vaccine against HIV.

4.2.3. Combination antiretroviral therapy

In 1987, the FDA approved the first antiretroviral therapy (ART), azidothymidine (AZT, also known as zidovudine or ZDV). From that point forward, HIV infection became a chronic, treatable condition with the number of deaths from HIV dropping drastically by 1995 [120]. Today, there are about 34 million people globally living with HIV infection [34,35]. HIV is treated with a regimen called combination antiretroviral therapy (cART), consisting of several drugs that inhibit viral replication. Some of the most typically prescribed drugs include: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). WHO guidelines recommend that HIV positive patients begin cART treatment once CD4 cell counts are at or below 350 cells/mm$^3$. Those who are pregnant or very young may begin treatment sooner [35].

As mentioned previously in Chapter 1, the ability of NRTIs to inhibit all cellular DNA polymerases makes them very effective at blocking viral DNA replication, but this mechanism also plays a role in the development of mitochondrial dysfunction by blocking the action of mitochondrial polymerase $\gamma$ [19]. Unlike NNRTIs, once NRTIs enter cells through non-facilitated passive diffusion or carrier-mediated transport, they must first be triphosphorylated (TP) before they can act on DNA polymerases. Cellular kinases determine the final NRTI-TP concentrations. Cellular kinase expression can vary by tissue with different tissues expressing kinases at different levels. Also, cells express different kinases depending on whether they are quiescent or proliferative. Furthermore, proliferating cells have affinities for different NRTIs at different stages of mitosis (resting or active) and kinases are expressed at different levels during these stages [37,41,46]. Because of these cellular differences, the mitochondria in some cells or tissues may be affected more strongly by NRTIs than others leading to selective tissue toxicity [122].

Once NRTIs have been triphosphorylated, they can inhibit viral replication through incorporation by DNA polymerases as described previously. Certain NRTIs such
as d4T have been shown to be mitochondrially toxic and their use in developed countries has declined [47]. As such, it is plausible to assume that the side effects associated with these drugs have also declined. It is true that the prevalence of some of the life threatening side effects seen with d4T treatment has decreased over time, but the prevalence of one side effect, peripheral neuropathy, has remained unchanged [47,65]. In a large, multicenter study, Ellis et al., [65] examined over 1500 HIV positive participants for signs of PN in order to update the risk factors associated with current drug regimens now that dideoxy-type ART or “D” drugs (d4t, didanosine, and zalcitabine) have been phased out. Results indicated that age and lower nadir CD4 cell count are still risk factors for PN along with past use of a “D” drug. Surprisingly, current cART use is still a risk factor for PN even without current “D” drug use. The prevalence of neuropathy in this cohort, 57%, is similar to rates seen in previous studies in developed countries [65]. This result was replicated in a large-scale longitudinal study of over 2,000 participants [43]. While the use of “D” drugs declined over the period of follow-up, PN prevalence remained stable, even when participants showed improved viral control and immune function after they began cART. Better viral suppression and immune recovery were observed in HIV positive patients on cART, but PN rates remained stable indicating that HIV infection and NRTIs may not affect the function of these neurons and their mitochondria [42].

4.2.4. HIV and peripheral neuropathy

As the rates of PN are unchanged after NRTI treatment is induced [65], it is likely that HIV infection and NRTI treatment may both contribute to the development of this disease [15,68]. It has been hypothesized that, first, HIV infection itself promotes peripheral nerve damage through induction of mitochondrial dysfunction and reduced ATP production [57,123]. HIV infection alone has been observed to induce the loss of peripheral nerve axons [117,124] possibly due to mitochondrial dysfunction leading to activation of mitochondrially activated (intrinsic) apoptosis at the axonal level. This process occurs separately from the cell body as cell body loss does not occur as frequently [57,119,123,125]. This axonal loss may be due to the action of HIV viral proteins, which have been shown to disrupt mitochondrial function in neurons. For
example, the HIV proteins gp120 and tat have been observed to sensitize neurons to oxidative stress, promoting neuronal apoptosis [126,127]. Disruption of mtDNA and/or OXPHOS, as mentioned in Chapter 1, can escalate ROS generation leading to a state of oxidative stress, where the elevated quantity of ROS can overwhelm antioxidant levels. Both tat and gp120 can increase oxidative stress in neurons. It has been noted that tat exposure can disrupt mitochondrial membrane potential and inactivate the OXPHOS protein cytochrome c oxidase (CIV) in vitro in human and mouse brain cells [123]. In another study, tat exposure was shown to increase levels of malondialdehyde (MDA), a mutagenic lipid peroxidation product, in the brains of rats [128]. Finally, gp120 exposure has been observed to decrease mitochondrial levels of SOD in vitro in rat cerebellar neurons [129]. These studies demonstrate the ability of HIV proteins to disrupt neuronal mitochondrial function and increase ROS production, which can then lead to a state of oxidative stress. These very mechanisms may be behind the neuronal loss that occurs in HIV PN.

Once HIV infected patients are placed on cART regimens, viral loads and viral proteins decrease. At this point, however, ART drugs such as NRTIs may further promote mitochondrial dysfunction by disrupting mtDNA replication as previously described, inflicting more axonal damage. Now the HIV damaged neurons have a reduced threshold to the NRTI induced mitochondrial dysfunction and they degenerate (Figure 4.2) [15]. This additive effect has been shown in mice expressing the viral protein gp120. With only gp120 expression, no changes were seen in the murine peripheral axons. However, when these mice were also given an NRTI, didanosine, loss of unmyelinated peripheral nerve axons and behavioral changes associated with peripheral neuropathy (i.e., hypersensitivity to heat) were observed [63]. Similar findings were observed in a study examining the effects of feline immunodeficiency virus (FIV) alone and in combination with didanosine, with the loss of small fibers observed in FIV infected felines only after administration of the NRTI [64]. The results from these studies indicate that small peripheral nerve fiber loss is more likely to occur after exposure to both HIV and NRTIs.
4.2.5. HIV and Thailand

The first reported HIV case in Thailand occurred in 1984, and as of 2012, more than 1 million Thai individuals have been infected [130]. In order to more effectively combat the HIV epidemic in a cost effective manner, the Thai government pharmaceutical organization (GPO) procured a license to override the pharmaceutical companies’ patents on cART drugs and produce generic equivalents [130]. By 2011, over half of HIV infected Thai adults were receiving cART [131]. Unfortunately, as of 2008, 94% of these treated participants were receiving a “D” drug such as d4T [67]. “D” drugs like d4T are still frequently found as part of cART regimens in developing African countries and Asian countries such as Thailand due to their lower cost [66,67]. As mentioned in the previous section, the additive effect of combining HIV infection and “D” drug treatment can increase the risk of developing PN. In a developing country such as Nigeria, rates of PN were around 37% for HIV infected, cART naïve participants and 42% for those on cART. When the cART regimen included d4T, the PN rates increased to over 70% [66]. In Thailand, one study found the same rates of probable PN in HIV infected participants on cART and cART naïve participants (around 30% for both). The majority of these participants on cART were currently being treated with a regimen containing a “D” drug [67].
Our research group recently measured ENFD in Thailand by examining an HIV infected, cART naïve cohort (N=150). Participants in this cohort were eligible to enroll only if they had no signs or symptoms of PN and were not on cART. On average, the ENFD length in the distal leg (near the ankle) was 21.0 fibers/mm. ENFD correlated negatively with PBMC mitochondrial OXPHOS enzyme activities showing a relationship between small fiber loss and mitochondrial function [68]. As of yet, the effect of cART on ENFD in this group remains to be studied. Additionally, the relationship of oxidative stress to mitochondrial function and ENFD has not yet been examined in this group. Finally, because a cohort with symptomatic PN was also recruited, a comparison of mitochondrial and oxidative stress measures between this group and the non-peripheral neuropathy cohort may better illuminate the mechanisms of small fiber loss.

The aim of this study was to measure mitochondrial DNA (mtDNA) copies per cell and the oxidative phosphorylation (OXPHOS) enzyme activities of Complex I (CI; NADH dehydrogenase) and Complex IV (CIV; cytochrome c oxidase) in peripheral blood mononuclear cells (PBMCs), levels of five antioxidants and one measure of ROS in plasma (oxidative stress measures), and ENFD in distal and peripheral skin from HIV positive and negative adults in Thailand. The hypothesis of this study was that HIV positive participants on long-term d4T treatment (>6 months) who have confirmed symptomatic peripheral neuropathy (HIV+/PN) would have altered mitochondrial parameters, lower distal ENFD, and higher oxidative stress than HIV negative (-) participants or HIV positive (+) participants without PN who are cART naïve. Furthermore, HIV+, cART naïve participants would show signs of mitochondrial dysfunction, distal ENFD loss and oxidative stress at study entry as compared to HIV negative participants. A d4T containing cART regimen would increase mitochondrial dysfunction, distal ENFD loss, and oxidative stress in the HIV+ participants, while switching these participants to a less mitochondrially toxic drug such as ZDV would improve mitochondrial parameters and distal ENFD and decrease oxidative stress. Finally, short-term d4T treatment (6 months) in the HIV+ cART naïve cohort would alter mitochondrial and oxidative stress parameters such that they would be more similar to the HIV+/PN group on long-term d4T.
4.3. MATERIALS AND METHODS

4.3.1. Study populations and clinical assessments: The 003 cohort

Of the 150 participants who were enrolled in a clinical trial comparing the safety and efficacy of three antiretroviral treatment regimens (003 cohort) and 75 participants enrolled in the cross-sectional assessment of long-term stavudine (d4T) exposure (014 cohort), the PBMCs and plasma of a total of 48 participants were assessed. All participants were adults enrolled from one of two sites in central Thailand: the Queen Savang Vadhana Memorial Hospital in Chonburi and the Thai Red Cross AIDS Research Centre in Bangkok.

All participants enrolled into the 003 cohort were HIV-infected, antiretroviral drug-naïve and were randomized (1:1:1) to one of three drug arms for a period of 72 weeks. Arm 1 received stavudine (d4T; 30mg), lamivudine (3TC; 150mg), and nevirapine (NVP; 200mg twice daily) for 24 weeks, followed by zidovudine (ZDV; 250mg), 3TC (150mg), and NVP (200mg twice daily) for 48 weeks. Arm 2 received ZDV, 3TC, and NVP for 72 weeks. Arm 3 received tenofovir (TDF; 300mg) and emtricitabine (FTC; 200mg) once daily with NVP (200mg) twice daily for 72 weeks. These drugs were produced in generic form by the Thai government. While d4T use is being phased out in Thailand, it was prescribed to participants in this study for no longer than 24 weeks to improve CD4 cell counts in this group before they were placed on ZDV. The reasoning behind this regimen design is that if ZDV treatment begins at low CD4 cell counts, there is an elevated risk for the development of anemia [37,47]. Thus the short-term d4T period of 24 weeks was chosen to ensure a maximum improvement in CD4 cell counts while minimizing the side effects of d4T. Because ZDV treatment may cause anemia, randomization to drug arms was performed centrally using a block design stratified by participants’ entry hemoglobin levels (<10 or ≥10g/dL).

The inclusion criteria were: age ≥18 years, documented HIV infection, CD4+ T-cell count <350 cells/mm³, Thai national identity card, understanding of the study and ability to sign the consent forms, no history of allergy to anesthetic medications, and no prior exposure to antiretroviral drug therapies. Women with past exposure to antiretroviral drugs during pregnancy were included if the exposure was at least three months prior to study enrollment. Participants were excluded for abnormal laboratory
values (absolute neutrophil count <750 cells/mm\(^3\), hemoglobin <8.0g/dL, serum alanine aminotransferase greater than five times the upper limit of normal or serum creatinine greater than two times the upper limit of normal), any active AIDS-defining illness, other active medical illnesses, current use of immunomodulation therapy or experimental therapy, current pregnancy or breastfeeding, the presence of an active malignancy, or co-infection with hepatitis B.

Participants were examined for PN during the initial screening process using the AIDS Clinical Trials Group/Neurology and Neurologic AIDS Research Consortium methodology. Participants were determined to have possible PN if they had any of the following signs or symptoms: absent or diminished ankle reflex, or diminished vibratory, pin or temperature sensation, or contact allodynia. Any participants who presented with symptomatic or asymptomatic PN were excluded from enrollment in the 003 cohort as one of the drugs prescribed in this study, d4T, is associated with the development of PN.

HIV status including CD4 cell counts, HIV RNA levels, as well as general medical information such as height, weight, and BMI were obtained. Participants were assessed at weeks 0 (study entry), 24, and 72 and blood samples were obtained. Viable PBMCs and plasma were isolated and stored at -80°C. Phanuphak et al., [132] have previously reported on the safety and efficacy of these drug regimens in this cohort. ³

4.3.2. Study populations and clinical assessments: The 014 cohort

Participants in the 014 cohort study were enrolled into one of 4 groups: Group 1 included 25 HIV-infected participants currently on a d4T based cART regimen who had a diagnosis of symptomatic PN (HIV+/PN). Group 2 included 25 HIV-infected participants on long-term d4T treatment who had no signs or symptoms of PN. Group 3 included 50 HIV-negative participants who did not have signs or symptoms of PN to serve as controls to the HIV positive participants in both the 014 and the 003 cohorts (HIV-). Group 4 included 25 HIV-infected participants on long-term d4T treatment with a diagnosis of

³ All clinical assessments were completed and laboratory specimens collected by our collaborators in the SEARCH study at two institutions in Thailand, the Thai Red Cross AIDS Research Center and the Queen Savang Vadhana Memorial Hospital, headed by Dr. Jintanat Ananworanich.
asymptomatic PN. Only Groups 1 and 3, HIV+/PN and HIV-, were examined in this study.

The inclusion criteria for the HIV+/PN and HIV- groups were: age ≥18 years, Thai national identity card, understanding of the study and ability to sign the consent forms, no history of allergy to anesthetic medications, no treatment with concomitant medications (except for d4T), no conditions known to cause PN other than HIV (e.g. diabetes), not currently pregnant. Additionally, participants were included in the HIV+/PN group if HIV infection was documented with an HIV RNA by PCR of < 50 copies/mL and the participant was currently on d4T treatment. Furthermore, participants were included in this group if they had evidence of symptomatic PN that began after receiving d4T treatment, such as absent or diminished ankle reflex, or diminished vibratory, pin or temperature sensation, or contact allodynia. Participants enrolled in the HIV- group were required to be HIV seronegative, without any signs or symptoms of peripheral neuropathy.

HIV status including CD4 cell counts, HIV RNA levels (in the HIV+/PN group only), as well as general medical information such as height, weight, and BMI were obtained from both groups. All participants were assessed at the initial screening visit and at study entry (week 0) and blood samples were obtained. Viable PBMCs and plasma were isolated and stored at -80°C. The 003 and 014 cohort studies received IRB approval from four Institutional Review Boards: Queen Savang Vadhana Memorial Hospital Chulalongkorn University, University of Hawaii, and University of California San Francisco. All informed consents were completed and obtained in Thai language from all participants.

4.3.3. Epidermal nerve fiber density acquisition, measurement, and quantification

Epidermal nerve fiber densities of the lower leg and upper thigh were determined using methods previously described [68]. The area of skin was biopsied by injecting with 1% lidocaine to numb the area. Using sterile techniques, a 4mm skin punch biopsy was taken from the skin of the lower leg near the ankle (distal) and from the upper lateral thigh (proximal) at each time point. Skin samples were processed on site and sent to the Cutaneous Nerve Laboratory at Johns Hopkins for immunostaining and fiber
quantification. The biopsies were fixed and stained with an antibody to protein gene product (PGP) 9.5, a marker specific to and ubiquitously found in neurons. Sections were cut to 50µM thick and the number of unmyelinated nerve fibers per length of epidermis was counted to determine the density. ENFD was expressed as fibers per mm of epidermis.

4.3.4. Plasma and PBMC isolation

Blood samples were drawn into EDTA and heparin containing vacutainers at each site. The samples were spun at 1000 x g at 4°C for 10 minutes to separate the plasma from the red and white blood cells. Multiple collection tubes containing plasma from one subject were pooled together before being aliquoted into cryovials and frozen at -80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from the same aliquots within 8 hours via Ficoll gradient (GE Healthcare, Waukesha, WI) and platelets were removed by two PBS washes and centrifugation at 300 RCF. The PBMCs were then pelleted and viably frozen in media at -80°C.

4.3.5. MtDNA quantification in PBMCs by real-time PCR

For measurement of mtDNA copies/cell, quantitative real time polymerase chain reaction (qRT-PCR) is a rapid method of quantification and was conducted as previously described by our lab [61]. For each participant sample, viable PBMCs were thawed in a 37°C water bath, spun for 15 minutes at 300 RCF for 15 minutes to pellet the cells. The freezing media was removed and 500µl of cold 1X phosphate buffered saline was immediately added resuspend the cells and 400µl was removed from each sample for later protein extraction. DNA was extracted from remaining 100µl containing viable PBMCs using the QuickGene DNA tissue kit S (Fujifilm Corporation, Tokyo, Japan). Total DNA purity and quantity in ng/µL was determined using a Micro Volume UV-Vis Spectrophotometer (NanoDrop Products, Wilmington, DE) and the isolated DNA was stored at 4°C. qRT-PCR was performed using a LightCycler FastStart DNA Master Plus SYBR Green I (Roche Applied Sciences, Indianapolis, IN) with a LightCycler Carousel-Based System.
A control plasmid containing the 90bp mtDNA NADH dehydrogenase subunit II (ND2) and the 98bp Fas Ligand housekeeping gene was serially diluted to prepare a positive control containing $1 \times 10^6$ copies of DNA per µL. The genomic primers, GenDIR (GGC TCT GTG AGG GAT ATA AAG ACA) and GenREV (CAA ACC ACC CGA GCA ACT AAT CT) were specific for the region of the genome encoding for Fas Ligand. The mitochondrial primers, mtREV (CCG GAG AGT ATA TTG TTG AAG AG) and mtDIR (CAC AGA AGC TGC CAT CAA GTA), amplify a region of mtDNA encoding for ND2. Each glass capillary contained a total reaction volume of 20µL consisting of: SYBR Green Master Mix (FastStart Taq DNA polymerase, reaction buffer, dNTP mix, MgCl$_2$; LifeTechnologies, Carlsbad, CA), 1µM of each primer, and 30ng of DNA from each sample. The cycling conditions for PCR consisted of: pre-incubation with a target temperature of 95°C for 5 minutes followed by amplification over the course of 30-36 cycles at 95°C for 3 seconds (denaturing), 58°C for 5 seconds (annealing), and 72°C for 5 seconds (extension) per cycle. When amplification was complete, a melt curve analysis was run under the following conditions: 65°C, with the temperature increasing 0.1°C/s until 95°C was reached. All standards and samples were run in duplicate and the results were analyzed using version 4.0 LightCycler software (Roche, Indianapolis, IN). Absolute mtDNA copies per cell were calculated by dividing the mean mtDNA values by the mean genomic DNA values and then multiplying this number by 2 (there are two copies of nuclear-encoded genes in each cell).

4.3.6. Oxidative phosphorylation activity in PBMCs by ELISA

The mitochondrial OXPHOS enzyme specific activities of Complex I (CI; NADH dehydrogenase) and Complex IV (CIV; cytochrome $c$ oxidase) were quantified using OXPHOS activity enzyme-linked immunosorbent assays (ELISA; Abcam, Cambridge, MA). Frozen PBMC pellets were thawed in a 37°C water bath and 400µl of cold 1X PBS was immediately added to resuspend the pellets. To this, 200µl of buffer A (1.5% lauryl maltoside, 25mM HEPES, 100mM NaCl; Abcam, Cambridge, MA) with 1X protease inhibitor cocktail (PI; Thermo Fischer Scientific, Waltham, MA) was added to the sample. Samples were kept on ice and vortexed briefly every 5 minutes for 20 minutes.
The samples were then centrifuged at 14,000 x g for 20 minutes at 4°C to remove cellular debris. Finally, lysates were flash frozen and stored at -80°C.

The frozen protein extracts were thawed and the total protein content of each sample was determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL) using a serial dilution of bovine serum albumin as a standard curve for quantification. Prior to testing participant samples, standard curves were performed using control protein lysate from PBMCs (Astarte Biologics, Redmond, WA) to determine the optimum amount of protein lysate to load for this assay. PBMC protein lysate was extracted and quantified as mentioned above to create a serial dilution from 0 to 100µg for Complex I and from 0 to 50µg for Complex IV. Values obtained from the standard curve were plotted using PRISM Version 5.0d (GraphPad Software, Inc., San Diego, CA). The most linear section of the curve was divided into thirds and the point that was 2/3 from the bottom of this section, estimated to 75µg for Complex I and 25µg for Complex IV, was considered the optimum protein amount for this study. Buffer A was used as a blank and PBMC protein lysate was used as a positive control.

Each well of the microplate was coated with capture antibodies for the target protein complex (I or IV). The protein lysates were incubated in the wells for 3 hours at 27°C to allow for the capture of the target protein. The wells were rinsed to remove any unbound proteins and 200µL of activity buffer containing a substrate and an electron acceptor was added to each well. For the CI microplate, the activity buffer contained NADH and a dye. The CI enzymes oxidized NADH and the oxidized NAD+ then reduced the dye, producing an increase in absorbance values. For the CIV microplate, the activity buffer contained reduced cytochrome c, which was oxidized producing a decrease in absorbance values. The plates were read in a SpectraMax 190 absorbance microplate reader (Molecular Devices LLC, Sunnyvale, CA). CI plates were read at 450nm at 30 second intervals for 30 minutes at 27°C and CIV plates were read at 550nm at 1 minute intervals for 40 minutes at 30°C. All controls and samples were run in duplicate.

The specific activity of each sample and control was calculated by first averaging the replicates together. These average values were then plotted linearly over time and the raw slope value for each control and sample was calculated using a linear regression.
These raw slope values were then divided by the amount of protein loaded per well in µg (75µg for CI and 25µg for CIV) to obtain the adjusted slope values. These values were multiplied by one million for the final absorbance value. CI specific activity in each well was proportional to the increase in absorbance at 450nm over time and was expressed as the change in absorbance per minute per amount of sample loaded in the well (((OD/µg/minutes)*1,000,000). CIV specific activity in each well was proportional to the decrease in absorbance at 550nm over time and was expressed as the change in absorbance per minute per amount of sample loaded in the well (((OD/µg/minutes)*1,000,000).

4.3.7. Protein levels of catalase, superoxide dismutases 1 and 2, thioredoxin 1, and peroxiredoxin 2 in plasma by multiplexing

The Luminex XMap Multiplex allows for high throughput measurement of markers of metabolic processes, cell signaling pathways, and antioxidant levels in cell and tissue lysates or in plasma using paramagnetic antibody capture microspheres and a laser detection system. Each microsphere is internally color coded with fluorescent dyes to create different sets of beads for different analytes, allowing the Milliplex XMap Oxidative Stress panel (EMD Millipore, Billerica, MA) to simultaneously measure the levels of five antioxidant analytes in each sample. The antioxidants measured were: catalase, peroxiredoxin 2 (PRX2), thioredoxin 1 (TRX1), and superoxide dismutases 1 (SOD; cytoplasmic) and 2 (mitochondrial).

A HepG2 derived protein lysate (EMD Millipore, Billerica, MA) was serially diluted to prepare a standard curve of median fluorescent intensity (MFI) values. A total of 25µL of plasma samples and standards was loaded to each well to which the antibody coated microsphere beads were added. The samples and beads were incubated for 2 hours at 27°C to allow for capture of the analytes of interest. Samples were washed to remove unbound proteins and then incubated with biotinylated detection antibody microspheres for 1 hour at 27°C. Finally a reporter molecule containing Streptavidin conjugated with a fluorescent reporter, phycoerythrin (PE), was added. The plate was then placed into the Luminex 200 laser detection system (Luminex Corp, Austin, TX) where the microspheres were illuminated by two lasers, red and green. The red laser excited the microsphere
internal dye causing it to fluoresce, identifying the microsphere bead set. The green laser then excited the Streptavidin-PE fluorescent dye, identifying the captured analyte. Finally, high-speed digital-signal processors quantified the results based on the intensity of the fluorescent signals from the samples as compared to those of the standard curve using Luminex xPonent version 3.1 (Luminex Corp, Austin, TX). Final results determined using the Milliplex Analyst software version 5.1 (VigeneTech Inc., Carlisle, MA). All standards and samples were run in duplicate and all analytes were expressed as pg/mL.

4.3.8. Thiobarbituric acid reactive substances assay for malondialdehyde quantification in plasma

ROS are highly reactive molecules that can alter lipids, membranes, and DNA. Lipid peroxidation is a by-product of ROS activity and can be easily measured using the thiobarbituric acid reactive substances assay (TBARS), a well-established lipid peroxidation assay. This assay can test serum, urine, tissue and cellular lysates or plasma for malondialdehyde (MDA), which is a naturally occurring product of lipid peroxidation. MDA forms adducts to thiobarbituric acid (TBA) at high temperatures (90-100°C) under acidic conditions. The formation of this adduct creates a red colored product that can be read colorimetrically in a standard plate reader.

Purified MDA (Cayman Chemical, Ann Arbor, MI) was serially diluted to prepare a standard curve from 0 to 50µM. A total of 100µL of participant plasma or prepared standard was loaded into a 15mL conical tube followed by 100µL of sodium dodecyl sulfate (SDS; Cayman Chemical, Ann Arbor, MI). Four milliliters of color reagent (2.1mL of acetic acid, 2.1mL of sodium hydroxide, and 265.5g of TBA per sample; Cayman Chemical, Ann Arbor, MI) was added to each sample or standard. The tubes were then placed in boiling water (90-100°C) for 60 minutes and quickly cooled on ice for 10 minutes to halt the reaction. The cooled samples were spun at 1600 x g for 10 minutes to remove any precipitates and 1mL was carefully removed and placed into a 1.5mL microtube. These aliquots were stored at 27°C for 20 minutes to reduce cloudiness. One hundred and fifty microliters of each sample and standard was placed into each well of a microplate and read in a SpectraMax 190 absorbance microplate
reader (Molecular Devices LLC, Sunnyvale, CA) at 535nm. To calculate the MDA concentration, the absorbance value (ABS) of the blank standard (0uM) was subtracted from each standard and sample for a corrected ABS value. The corrected ABS values of the standards were then plotted linearly as a function of MDA concentration and the slope of the line and the y intercept were calculated. These values were used to calculate the MDA uM concentration of the samples by subtracting the y intercept from the corrected ABS and then dividing by the slope. All samples and standards were assayed in duplicate.

4.3.9. Statistical analyses

A Kruskal-Wallis ANOVA on Ranks for non-parametric analysis was used to assess differences between the HIV+, HIV+ with PN, and HIV- participants on antioxidant levels, TBARS levels, mtDNA copies/cell, OXPHOS enzyme activities, and ENFD at week 0, followed by a Dunn's post-test to correct for multiple comparisons. Spearman’s correlations were used to examine the relationships between antioxidant levels, TBARS levels, mtDNA copies/cell, OXPHOS enzyme activities, and ENFD within each participant group at week 0 and within the HIV+ participant group at the additional time points of 24 and 72 weeks. A Mann-Whitney U Rank Sum test for non-parametric analysis was employed to examine differences between the long-term d4T treatment group (HIV+/PN) and the short-term d4T treatment group (HIV+ at 24 weeks). This same test was also employed to examine the effects of NRTI treatment over time (0 vs. 24, 24 vs. 72, and 0 vs. 72 weeks) in the HIV+ participants. Any ANOVA tests with a p value <0.2 were further examined with Mann-Whitney U Rank Sum tests. Analyses with a p value of 0.05 were considered significant and p values between 0.051-0.1 were considered a trend. All analyses were run on SigmaPlot version 12.4.

4.4. RESULTS

4.4.1. Participant characteristics and clinical assessments

Of the 48 participants examined, 24 were enrolled in the 003 cohort from Arm 1 (HIV+, cART naïve) and 36 were enrolled in the 014 cohort: 24 participants from Group 1 (HIV- controls) and 24 from Group 3 (HIV+ with symptomatic PN). Participants were chosen from each cohort through random number generation. Participant characteristics
are presented in Table 4.1. The three participant groups were similar in terms of BMI. There were differences between the 014 HIV+/PN and HIV- groups on years of age at study entry with the HIV+/PN group (mean=39.0, SD=6.5) being older than the HIV- (mean=33.0, SD=5.2; p=0.002). The 003 HIV+ group had significantly lower CD4 cell counts at study entry (mean=165.5, SD=96.0) than the 014 HIV+/PN group (mean=480.0, SD=213.6; p<0.001). This difference in CD4 cell counts was expected as the 014 HIV+/PN group had been diagnosed with HIV an average of 5.9 years ago and had been on d4T treatment for an average of 4.3 years (SD=2.5), while the 003 HIV+ group entered the study cART naïve and had been diagnosed with HIV an average of 1.9 years ago (SD=2.3; Table 4.1). After 72 weeks of cART treatment the 003 HIV+ group had a self-reported cART adherence rate of over 95% [132], which was confirmed through pharmacological tests on plasma inter- and intracellular drug levels (unpublished data from Dr. Peter Anderson).

Table 4.1. SEARCH Participant Characteristics at Study Entry (Week 0).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SEARCH Cohort</th>
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<tr>
<td></td>
<td>HIV+, cART naïve (N=24)</td>
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<tr>
<td></td>
<td>HIV+, symptomatic PN (N=24)</td>
</tr>
<tr>
<td></td>
<td>HIV- controls (N=24)</td>
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<td>CDC Classification</td>
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<td>A, n (%)</td>
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<td>Mean age years (SD)</td>
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<tr>
<td>Mean BMI (SD)</td>
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<td>22.2 (2.9)</td>
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<td>480.0 (213.6)^</td>
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<tr>
<td>Mean time since HIV dx, years (SD)</td>
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<td>5.9 (4.0)^</td>
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<tr>
<td>Mean years d4T tx (SD)</td>
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</tr>
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<td>4.3 (2.5)</td>
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* p=0.002
^ p<0.001
4.4.2. Distal and peripheral ENFD differed primarily by HIV status

Significant differences were observed in distal ENFD between the HIV- group (N=24) and the HIV+ groups with (N=17) and without (N=24) PN. The HIV- group had longer mean distal ENFD (29.9 fibers/mm, SD=11.2) than the HIV+ group (20.9 fibers/mm, SD=6.3; p=0.001) or the HIV+/PN group (21.8 fibers/mm, SD=8.6; p=0.02). The HIV- group (N=24) also had higher peripheral ENFD (47.1 fibers/mm, SD=12.3) than the HIV+ group (N=23; 35.7 fibers/mm, SD=8.0; p<0.001). There were no significant differences on either distal or peripheral ENFD between the HIV+ and HIV+/PN (peripheral; 40.6, SD=14.7) groups. Peripheral ENFD were typically around 60% longer than distal ENFD within each group (p<0.001). Not all of the collected ENFD specimens were examined in the HIV+/PN group due to lack of available funding. In addition, one peripheral ENFD specimen in the HIV+ group was not in acceptable condition for measurement. Results are presented in *Figure 4.1.*
Figure 4.1. Distal and peripheral ENFD from HIV-, HIV+, and HIV+/PN participants at study entry. Bar graph depicting distal ENFD (top) in HIV- (blue, N=24), HIV+/drug naïve (purple, N=24), and HIV+/PN on d4T treatment (green, N=17) groups at study entry. Bar graph depicting peripheral ENFD (bottom) in HIV- (blue, N=24), HIV+/drug naïve (purple, N=23), and HIV+/PN on d4T treatment (green, N=17) groups at study entry.
4.4.3. PBMC mtDNA copies per cell did not differ between the groups

Next we examined mitochondrial parameters to determine if there were differences between the cohorts. The mean PBMC mtDNA copies per cell did not differ significantly between the HIV- (241.8, SD=59.4), HIV+ (230.2, SD=78.5), or HIV+/PN (233.5, SD=130.9) groups at study entry (N=24 in all groups).

4.4.4. PBMC mitochondrial OXPHOS enzyme activities differed by group

Mean PBMC complex I specific activity was significantly higher in the HIV- group (N=24; 35.2, SD=4.2) than in the HIV+ (N=22; 34.2, SD=14.1; p=0.04) and HIV+/PN groups (N=19; 16.7, SD=4.9; p<0.001). Also, mean CI activity was significantly higher in the HIV+ group as compared to the HIV+/PN group (p<0.001; Figure 4.2).

Mean complex IV specific activity was significantly higher in the HIV- group (N=24; 70.1, SD=6.8) than in the HIV+ group (N=22; 55.1, SD=10.2; p<0.001). There were no significant differences between the HIV- and the HIV+/PN groups (N=23; 81.5, SD=32.6). The HIV+ group, however, had significantly lower CIV activity than the HIV+/PN group (p=0.004; Figure 4.2). The HIV+ and HIV+/PN cohorts have a sample sizes less than 24 for CI and CIV activities as the amount of mitochondrial proteins extracted from several of the PBMC samples were lower than the amount required to successfully run the assays. Specific enzyme activity is expressed as (OD/µg/minutes)*1,000,000.

4.4.5. Plasma antioxidant levels were higher in those with HIV or HIV and PN

The differences observed in mitochondrial OXPHOS CI and CIV enzyme activities between the groups indicate mitochondrial dysfunction. This dysfunction can lead to an increase in oxidative stress, which has been implicated in the development of PN. We measured plasma levels in pg/mL of several antioxidants, one of which is mitochondrial specific (SOD2). Mean SOD2 levels were significantly higher in the HIV+ (50.0, SD=11.7; p<0.001) and HIV+/PN groups (71.3, SD=10.9; p<0.001) as compared to the HIV- group (36.9, SD=8.6). Also, mean levels of catalase were significantly higher in the HIV+ (1006.1, SD=498.9; p<0.001) and HIV+/PN groups (1382.2, SD=476.7;
p<0.001) as compared to the HIV- group (606.1, SD=288.2; Figure 4.3). Mean SOD1 levels, however, were only significantly higher in the HIV+/PN group (120.7, SD=129.4) as compared to the HIV- group (67.7, SD=99.7; p=0.04) with only a trend toward higher SOD1 level in the HIV+ group (74.4, SD=55.0) as compared to the HIV- group (p=0.14). Finally, mean PRX2 levels were significantly higher in the HIV+ (103.2, SD=116.2; p=0.002) and HIV+/PN groups (229.2, SD=154.4; p<0.001) as compared to the HIV-group (39.1, SD=48.4; Figure 4.4). TRX1 levels were not significantly different between the HIV- (177.3, SD=80.6), HIV+ (234.8, SD=112.3), or the HIV+/PN groups (260.0, SD=144.2).

Mean levels of catalase, SOD2, and PRX2 were significantly higher in the HIV+/PN than in the HIV+ group (p<0.001 for all; Figures 4.3 and 4.4). One data point in the HIV+ group was not analyzed due to a high coefficient of variance (CV) between the replicates (>50%) and lack of sample available to perform a retest, resulting in an N of 23. The HIV- and HIV+/PN groups have an N of 24.
Figure 4.2. Complex I and IV enzyme activities from HIV-, HIV+, and HIV+/PN participants at study entry. Bar graph depicting CI activity (top) in HIV- (blue, N=24), HIV+/drug naïve (purple, N=22), and HIV+/PN on d4T treatment (green, N=19) groups at study entry. Bar graph depicting CIV activity (bottom) in HIV- (blue, N=24), HIV+/drug naïve (purple, N=22), and HIV+/PN on d4T treatment (green, N=23) groups at study entry. Specific enzyme activity is expressed as (OD/µg/minutes)*1,000,000.
Figure 4.3. Mean plasma levels of SOD2 and catalase were higher in the HIV+ and HIV+/PN groups as compared to the HIV- group. Bar graphs depicting SOD2 (top) and catalase (bottom) in HIV- (blue, N=24), HIV+/drug naïve (purple, N=23), and HIV+/PN on d4T treatment (green, N=24) groups at study entry.
Figure 4.4. Mean plasma levels of PRX2 were higher in the HIV+ and HIV+/PN groups as compared to the HIV- group and SOD1 levels were significantly higher in the HIV+/PN as compared to the HIV- group. Bar graphs depicting PRX2 (top) and SOD1 (bottom) in HIV- (blue, N=24), HIV+/drug naïve (purple, N=23), and HIV+/PN on d4T treatment (green, N=24) groups at study entry.
4.4.6. Plasma TBARS levels did not differ between the groups

Mitochondrial dysfunction and increased antioxidant levels can indicate increased ROS production. As such, we measured plasma TBARS levels as an indicator of ROS levels. Mean plasma TBARS levels were not significantly different between the HIV- (4.7µM, SD=1.0), HIV+ (5.6µM, SD=1.7), or HIV+/PN (5.6µM, SD=1.5) groups at study entry (N=24 for all groups).

4.4.7. Distal ENFD inversely correlated with plasma SOD2 in HIV+ and HIV+/PN

In the HIV+/PN group, there was a significant correlation with plasma SOD2 increasing as distal ENFD decreased (N=17; r=-0.65, p<0.001). In the HIV+ group, a similar correlation was observed but was only a trend (N=23; r=-0.31, p=0.15). Results are shown in Figure 4.5.
Figure 4.5. As distal ENFD decreased, plasma SOD2 levels increased in the HIV+ and HIV+/PN groups. Filled circles indicate HIV+, drug naïve samples (N=23; r=-0.31, p=0.15) and open circles indicate HIV+/PN samples (N=17; r=-0.65, p<0.001).

4.4.8. Distal and peripheral ENFD did not differ between HIV+ and HIV+/PN at 6 months

Treatment with d4T is a known risk factor for the development of PN. As the HIV+, drug naïve group was on d4T treatment from week 0 to week 24 of the study, we compared ENFD in this short-term d4T treatment (6 months) group with the long-term d4T treatment (mean of 4 years) HIV+/PN group. There were no significant differences on distal or peripheral ENFD between the HIV+/PN group (distal=21.8 fibers/mm, SD=8.6; peripheral=40.6 fibers/mm, SD=14.7) and the HIV+ group after short-term d4T treatment (distal=21.8 fibers/mm, SD=9.4; peripheral=36.9 fibers/mm, SD=9.6).
4.4.9. PBMC mtDNA copies per cell did not differ between HIV+ and HIV+/PN at 6 months

We also compared mitochondrial parameters in this short-term d4T treatment (6 months) group with the long-term d4T treatment (mean of 4 years) HIV+/PN group. Mean PBMC mtDNA copies per cell were still not significantly different between the HIV+/PN group (233.5, SD=130.9) and the HIV+ group after short-term d4T treatment (245.8, SD=91.1).

4.4.10. Significant differences in PBMC mitochondrial OXPHOS enzyme activities between the HIV+/PN group at entry and the HIV+ group at 6 months d4T treatment

Mean PBMC complex I specific activity was still significantly higher in the HIV+ group after 6 months on d4T treatment (N=23; 34.3, SD=8.5) than in the HIV+/PN group (N=19; 16.7, SD=4.9; p<0.001). Mean CIV activity was still significantly lower in the HIV+ group after 6 months on d4T treatment (N=23; 70.1, SD=11.0) than in the HIV+/PN group (N=23; 81.5, SD=32.6; p=0.03; Figure 4.6). This difference in CIV activity, however, is less significant at this time point than it was at entry when the HIV+ group was drug naïve (N=22; 55.1, SD=10.2; p=0.004; Figure 4.2) indicating that CIV activity may have increased in the HIV+ group after d4T treatment, causing this group to more closely resemble the HIV+/PN group.

4.4.11. Plasma antioxidant levels were higher in HIV+/PN than in HIV+ at 6 months

As mentioned in Chapter 1, mitochondrial dysfunction can lead to increased oxidative stress. As the OXPHOS enzyme activities in the HIV+ group after 6 months of d4T treatment more closely resemble those of the HIV+/PN group, we next measured oxidative stress levels to see if they would become more similar between the groups. Mean plasma SOD2 differences lost significance after short-term d4T treatment, becoming only a trend toward higher levels in the HIV+/PN (71.3, SD=10.9) than in the HIV+ group (66.3, SD=15.0; p=0.10). Mean TRX1 levels, however, decreased in the HIV+ group after short-term d4T treatment, becoming significantly lower in the HIV+ group after short-term d4T treatment (N=24; 173.2, SD=87.4) than in the HIV+/PN on
long-term d4T (N=24; 260.0, SD=144.2; p=0.03; data not shown). While differences in SOD1 levels remained non-significant between the groups (HIV+; 72.4, SD=50.8 and HIV+/PN; 120.7, SD=129.4), higher catalase and PRX2 levels in the HIV+/PN group (catalase; 1382.2, SD=476.7 and PRX2; 229.2, SD=154.4) as compared to the HIV+ group (catalase; 1257.9, SD=426.0 and PRX2; 196.1, SD=133.5) were no longer significant after short-term d4T treatment.

4.4.12. Plasma TBARS did not differ between HIV+ and HIV+/PN at 6 months

Mitochondrial dysfunction and increased antioxidant levels can indicate increased ROS production, however, there were still no significant differences on mean plasma TBARS levels between the HIV+/PN group (5.6µM, SD=1.5) and the HIV+ group (5.9µM, SD=1.0) after receiving short-term d4T treatment.
Figure 4.6. The HIV+/PN group on long-term d4T treatment (mean of 4 years) still had lower PBMC CI OXPHOS enzyme activities than the HIV+ group after short-term d4T (6 months). CIV activities were still higher in the HIV+/PN group, but the difference was less significant after short-term d4T treatment. Bar graphs depicting CI enzyme activity (top) and CIV enzyme activity (bottom) in HIV+ on short-term d4T treatment (purple, CI and CIV N=23) and HIV+/PN on long-term d4T treatment (green, CI, N=19; CIV, N=23). Specific enzyme activity is expressed as (OD/µg/minutes)*1,000,000.
4.4.13. Distal and peripheral ENFD did not change with cART treatments

After observing that short-term d4T treatment in the HIV+ group affected their mitochondrial and oxidative stress parameters such that they were more similar to the HIV+/PN group on long-term d4T, we focused on the changes induced in the HIV+ group as they transitioned from drug naïve to d4T exposed over the course of 24 weeks. In addition, after 24 weeks of d4T, this group was switched to ZDV for 48 weeks, further allowing us to separate the effects of general cART treatment from those induced by d4T or ZDV alone. This part of the study was designed to measure the short-term effects of different cART drugs on ENFD, mitochondrial parameters, and oxidative stress in a cART naïve, HIV+ group without signs or symptoms of PN at study entry. The mean distal and peripheral ENFD did not significantly change from weeks 0 (distal=20.9 fibers/mm, SD=6.3; peripheral=35.7 fibers/mm, SD=8.0) to 24 (distal; 21.8 fibers/mm, SD=9.4 and peripheral; 36.9 fibers/mm, SD=9.6), 24 to 72 (distal; 18.9 fibers/mm, SD=8.9 and peripheral; 37.5 fibers/mm, SD=10.0), or 0 to 72.

4.4.14. PBMC mtDNA copies per cell increased during cART treatment in the HIV+ group

While ENFD did not change measurably within our treatment timeframe, it was possible that there were measurable changes in the mitochondrial parameters. Mean PBMC mtDNA copies per cell in the HIV+ group increased significantly from week 24 (24 weeks of d4T; N=24; 245.8, SD=91.1) to week 72 (d4T to ZDV switch; 48 weeks on ZDV; N=23; 295.0, SD=84.8; p=0.03) and from week 0, (no drug treatment; N=24; 230.2, SD=78.5) to week 72 (72 weeks of cART treatment; p=0.009; Figure 4.7). There were no significant differences between weeks 0 and 24 (24 weeks of d4T). One data point was dropped at 72 weeks (989 copies per cell) as it was >2 standard deviations above the mean. When statistical tests were run with and without the outlier the results remained significant (weeks 24 to 72 with the outlier, p=0.02 versus p=0.03 without the outlier; weeks 0 to 72, p=0.005 versus p=0.009 without the outlier).
Figure 4.7. Mean PBMC mtDNA copies increased from week 24 to 72, corresponding to the d4T to ZDV switch, and from week 0 to week 72 of cART. Bar graphs depicting mtDNA copies per cell in HIV+ participants without cART at week 0 (orange, N=24), after 24 weeks of a d4T based cART regimen (week 24; red, N=24), and after switching to a ZDV based regimen for 48 weeks (week 72; grey, N=23).

4.4.15. PBMC mitochondrial OXPHOS enzyme activities increased from 0 to 72 weeks in the HIV+ group

Mitochondrial DNA copies per cell increased with cART treatment in general, but there was no significant change from weeks 0 to 24, coinciding with d4T treatment. Next, we measured OXPHOS enzyme activities to examine whether the changes seen in mtDNA copies at certain time points were mirrored in changes in OXPHOS activities. There were no significant differences in mean PBMC CI OXPHOS enzyme activity between weeks 0 (34.2, SD=14.1), 24 (34.3, SD=8.5), or 72 (38.2, SD=12.4). Mean CIV OXPHOS enzyme activity, however, increased significantly from week 0 (N=24; 55.1, SD=10.2) to week 72 (N=24; 66.6, SD=13.1; p=0.001; data not shown), but there were no significant differences between 0 and 24 weeks (61.9, SD=11.0) and 24 and 72 weeks.
4.4.16. Plasma antioxidant levels changed with treatment in the HIV+ group

In general, cART improved PBMC mitochondrial parameters in the HIV+ group without any significant detrimental effect of d4T treatment. In the previously mentioned results, the HIV+ group became more similar to the HIV+/PN in terms of antioxidant levels after d4T treatment. Therefore, we measured systemic antioxidant levels over the treatment time points to determine if d4T, ZDV, or general cART treatment affect oxidative stress. Mean plasma catalase levels increased significantly from week 0 (1006.1, SD=498.9) to week 24 (1257.9, SD=426.0; p=0.01) and decreased from week 24 to week 72 (980.8, SD=388.7; p=0.02). Mean SOD2 levels increased from week 0 (50.0, SD=11.7) to week 24 (66.3, SD=15.0; p<0.001) and decreased from week 24 to week 72 (52.5, SD=12.9; p=0.002). Similarly, mean PRX2 levels increased from week 0 (103.2, SD=116.2) to week 24 (196.1, SD=133.5; p=0.002) and decreased from week 24 to week 72 (100.8, SD=78.1; p=0.003). There was no significant difference from week 0 to week 72 in catalase, SOD2, or PRX2 levels. There was a trend toward a decrease on mean TRX1 levels from week 0 (234.8, SD=112.3) to week 24 (173.2, SD=87.4; p=0.052) and from week 0 to week 72 (175.6, SD=83.6; p=0.06), but no significant difference from weeks 24 to 72. There were no significant changes in mean SOD1 levels between weeks 0 (74.4, SD=55.0), 24 (72.4, SD=50.8), or 72 (54.3, SD=48.7). All mean antioxidant levels are presented in pg/mL. Results are presented in Figures 4.8 and 4.9.

4.4.17. Plasma TBARS decreased from 24 to 72 weeks in the HIV+ group

Differences in antioxidant levels were seen with different drug regimens, with changes occurring after d4T treatment. ZDV treatment generally returned antioxidants to pretreatment levels. Thus, we examined next if ROS levels were also affected by different drug regimens. There was a significant decrease in TBARS levels from weeks 24 (N=24; 5.9µM, SD=1.0) to 72 (N=23; 5.1µM, SD=1.2; p=0.03), coinciding with the d4T to ZDV switch. Although TBARS levels appeared to increase from weeks 0 (N=24; 5.6µM, SD=1.7) to 24, it was not significant. There was also no significant difference between weeks 0 and 72. One data point was dropped at 72 weeks (9.2µM) as it was >2 standard deviations above the mean. When statistical tests were run with the outlier, the
significance became a trend \((p=0.06\text{ with the outlier})\). Results are presented in *Figure 4.10*. 
Figure 4.8. Mean plasma levels of catalase and SOD2 increased from week 0 to 24, corresponding to d4T treatment. Levels decreased from week 24 to 72, corresponding to the switch from d4T to ZDV. Bar graphs depicting catalase (top) and SOD2 (bottom) in HIV+ participants without cART at week 0 (orange, N=23), after 24 weeks of a d4T based cART regimen (week 24; red, N=24), and after switching to a ZDV based regimen for 48 weeks (72 weeks; grey, N=24).
Figure 4.9. Mean plasma levels of PRX2 increased from week 0 to 24, corresponding to d4T treatment and decreased from week 24 to 72, corresponding to the switch from d4T to ZDV. Mean TRX1 levels decreased from week 0 to weeks 24 and 72.

Bar graphs depicting PRX2 (top) and TRX1 (bottom) in HIV+ participants without cART at week 0 (orange, N=23), after 24 weeks of a d4T based cART regimen (week 24; red, N=24), and after switching to a ZDV based regimen for 48 weeks (week 72; grey, N=24).
Figure 4.10. Mean plasma TBARS levels decreased from week 24 to 72, corresponding to the switch from d4T to ZDV. Bar graph depicting TBARS levels in HIV+ participants without cART at week 0 (orange, N=24), after 24 weeks of a d4T based cART regimen (week 24; red, N=24), and after switching to a ZDV based regimen for 48 weeks (week 72; grey, N=23).

4.4.18. Plasma TBARS levels inversely correlate with PBMC mtDNA copies per cell after 72 weeks of treatment

A significant correlation was seen at 72 weeks of cART with plasma TBARS levels increasing as PBMC mtDNA copies per cell decreased (N=22; r=-0.55; p=0.008; Figure 4.11). One data point was dropped from the TBARS data (9.2µM) and one data point was dropped from the mtDNA copies per cell data (989 copies per cell) as both were >2 standard deviations above the means. When statistical tests were run with and without the outliers the results remained significant (with the outliers, N=24; r=-0.60; p=0.002).
Figure 4.11. As plasma TBARS levels increased, PBMC mtDNA copies per cell decreased after 72 weeks of cART. Filled circles indicate the HIV+ data points after 72 weeks of cART (N=22; r=-0.55; p=0.008). Outlier values were removed.

4.5. DISCUSSION

4.5.1. Summary

In this short-term, cross sectional study we have shown that adult HIV+, cART naïve participants without peripheral neuropathy from Thailand have lower distal and peripheral ENFD than similar participants who are HIV-. In addition, while mtDNA copies per cell did not differ between the two groups, the HIV+ participants have lower mitochondrial OXPHOS enzyme activities in complexes I and IV than the HIV- participants. Finally, the HIV+ group had significantly higher levels of the antioxidants catalase, SOD2, and PRX2 than the HIV- group, while ROS levels, measured as plasma TBARS, did not differ between the groups. These results illustrate the effects of HIV alone on ENFD, mitochondrial parameters, and oxidative stress.
In this component of the study we also observed that HIV+ participants without signs or symptoms of PN have similar distal and peripheral ENFD to HIV+ participants who have been diagnosed with symptomatic PN. While mtDNA copies per cell did not differ between these two groups, there were differences on OXPHOS enzyme activities. The HIV+/PN group had lower CI activity than the HIV+ group. In CIV activity, however, the HIV+/PN group was higher than the HIV+/PN group. Furthermore, the HIV+/PN group had significantly higher antioxidant levels of catalase, SOD2, and PRX2 than the HIV+ group. Finally, there were no differences between the two groups on TBARS levels. Additionally, we compared the HIV+/PN group, who have been on d4T treatment for an average of 4 years, to the HIV+ group after 24 weeks of d4T treatment. Treatment with d4T changed the CIV enzyme activity and levels of several antioxidants in the HIV+ group such that they became more similar to the HIV+/PN group. The only new finding was that the HIV+ group had significantly lower TRX1 levels as compared to the HIV+/PN group.

Finally, in the longitudinal component of this study, we followed the HIV+ group over time to examine the changes that occurred with cART regimens that differed by NRTI (d4T or ZDV). We observed changes induced by d4T treatment that appeared to be reversed by switching to a ZDV-based regimen. The most significant changes occurred in the antioxidant levels with elevated levels of SOD2, catalase and PRX2 from weeks 0 to 24 (d4T treatment) that decreased from weeks 24 to 72 (the switch to ZDV treatment). Also, TBARS levels significantly decreased from week 24 to 72, corresponding with the d4T-to-ZDV-based regimen switch.

4.5.1. ENFD differences between the HIV-, HIV+, and HIV+/PN groups

In our cohorts, the HIV+, drug naïve group had significantly lower distal and peripheral ENFD than the HIV- cohort. HIV infection has been shown to decrease ENFD [15] and several studies have observed this effect in animals. For instance, after cats were infected with FIV, peripheral nerve fibers lost axonal density and axons began to die back [64]. Also, when peripheral nerves are exposed to HIV viral proteins in vivo in rodents, distal ENFD decreases as the axons die back [133]. Other studies have seen this in HIV infected humans, where HIV+ populations have lower ENFD than HIV- populations.
Thus, it has been postulated that HIV infection alone can affect the peripheral nerve fibers. Our findings between the HIV- and HIV+ cART naïve cohorts confirm this hypothesis.

While our HIV- controls had significantly higher distal ENFD than either the HIV+ or HIV+/PN groups, the two HIV+ groups did not differ on distal or peripheral ENFD. ENFD is a validated predictor of PN risk that is unaffected by disease severity and has a diagnostic efficiency of 88% [68,137,138]. Yet our HIV+ group that has been diagnosed with symptomatic PN has similar distal and peripheral ENFD as the HIV+ group that does not have evidence of PN. Similar observations have been made in studies examining HIV+ participants on antiretroviral treatments with and without PN, showing that distal and peripheral ENFDs are similar between HIV+ participants with asymptomatic or symptomatic PN and HIV+ participants without PN [135,136]. In one study looking at participants without HIV who had PN, mitochondrial dysfunction in the nerve terminals was observed in the form of reduced OXPHOS CIV, even in “borderline” PN participants who still had ENFD within the non-PN, or normal, range [14]. These results highlight the fact that fiber loss is not always indicative of PN and that mitochondrial dysfunction can precede fiber loss.

In addition to HIV alone inducing dysfunction and loss of small nerve fibers, exposure to dideoxy or “D” drugs such as d4T, is a known risk factor for PN development [20,41,115,139,140]. Our PN cohort has been on d4T treatment for an average of 4 years. As part of the enrollment criteria for this study, the development of symptomatic PN had to have occurred as a result of d4T treatment and not HIV alone (i.e., signs and symptoms of PN occurred shortly after d4T treatment began). Though our participants were treated long-term with d4T and had developed PN as a result, ENFD did not differ from the HIV+ drug naïve group. In a study by Mallon et al., [55], adipocytes from HIV- participants who were treated for 6 weeks with d4T showed altered mitochondrial RNA transcripts as early as 2 weeks after treatment. Thus, we decided to also compare the ENFDs of the HIV+/PN group with the HIV+ group after they had been treated with d4T for 6 months to examine any possible short-term effects of d4T. ENFD still showed no changes in the HIV+ group after short-term treatment.
In general, our three SEARCH cohorts differ from those examined in previous ENFD studies. The participants from Thailand enrolled in our cohorts were typically around 64 inches in height, whereas the average American height ranges from 64.5 inches for females to 70 inches for males [141]. While height influences the risk of PN development, with taller persons being at greater risk [68], results on whether height is associated with ENFD are mixed with more studies citing no association [68,113,142]. No associations of weight and ENFD have been observed [68,113,142]. In addition, normative reference ranges for ENFD for a U.S. based, HIV- population that was 30-39 years old and predominantly white were 11.9 fibers/mm for distal and 17.8 fibers/mm for peripheral [113]. Similar values were seen in other studies with larger participant age ranges (20-71 years [142]; 26-80 years, distal ENFD only [143]).

Our Thai HIV- cohort participants, who are in their mid-thirties, have distal and peripheral ENFD values more than twice the U.S. normative values, at 29.9 and 47.1 fibers/mm, respectively. Their shorter stature may play a role in this discrepancy. It is possible that general life style differences such as diet and exercise or genetic differences could have contributed to the longer ENFDs observed in our study. Though the SEARCH cohorts had longer ENFD than other published cohorts, their distal and peripheral ENFD correlated strongly (p<0.001 for both groups) and their peripheral ENFDs were 62-66% longer than their distal ENFD, which mirrors findings in other study cohorts [68,113,142].

4.5.2. MtDNA copies per cell in the HIV-, HIV+, and HIV+/PN groups

As mentioned in the above section, mitochondrial alterations may precede fiber loss. Therefore we examined mitochondrial parameters such as mtDNA copies per cell. In this study PBMC mtDNA copies per cell did not differ significantly between the HIV+ cART naïve group and the HIV- group, similar to previous results obtained in our lab [144]. These results are also similar to findings published from other laboratories that examined mtDNA in PBMCs from HIV+, cART naïve participants and HIV- controls [82,145]. As it has been previously postulated that NRTI treatments such as d4T can block mtDNA pol γ activity and thus reduce mtDNA copies per cell [19,20,41,46], we hypothesized that the HIV+/PN would have lower mtDNA copies as they have been on long-term d4T treatment. MtDNA copies per cell, however, did not differ between the
HIV+/PN group and the HIV+ or HIV- groups, indicating that any mitochondrial dysfunction occurring in these PN participants is not due to the loss of mtDNA. It is possible that the mitochondria in the PN group have been able to compensate for any increases in mtDNA deletions through clonal expansion, and thus we observed similar amounts of mtDNA. In a study by Lehmann et al [119], peripheral nerves taken from HIV+ participants with confirmed PN showed increased levels of a common mtDNA deletion as compared to HIV+ participants without PN or HIV-, PN free controls. It is not possible to say for certain as we did not measure mtDNA deletions or mutations.

We also compared the HIV+ group after short-term d4T treatment to the HIV+/PN long-term d4T cohort to ascertain whether 6 months of d4T treatment would cause a measurable change in mtDNA copies per cell. We did not find any differences at the 6-month treatment time point. It may be that we did not see differences in mtDNA copies per cell between these groups because we measured this parameter in PBMCs. In a study by Mallon et al., [55] PBMCs and adipose tissue taken from HIV- subjects on NRTI treatment for 6 weeks did not show decreased levels of mtDNA. There were, however, decreases in adipose tissue mitochondrial RNA (mtRNA) expression. This group hypothesized that because ATP and ADP concentrations can affect mtRNA transcription, that the accumulation of phosphorylated NRTIs in the mitochondria may also decrease mtRNA transcription [55]. As we did not analyze mtRNA levels in this study, we cannot say if this was the case and future research should examine mtRNA levels in these cohorts. Although the mtDNA copy numbers were similar between the three cohorts, dysfunction could have occurred elsewhere in the mitochondria.

4.5.3. OXPHOS differences between the HIV-, HIV+, and HIV+/PN groups

Reductions in OXPHOS have been seen in PBMCs from HIV+ participants who were either ART naïve [146] or ART treated [112] as compared to HIV- participants. Typically these reductions occur primarily in CIV. In vitro studies with the HIV protein tat have also observed this effect on CIV in murine heart, liver and brain as well as in rodent neuronal cell mitochondria [123,147]. In our study, the HIV+ cART naïve group had reductions in both CI and CIV enzyme activities in PBMCs as compared to the HIV- controls, paralleling results from previous studies in our research group. This reduction in
CI and CIV enzyme activities occurred without a reduction in mtDNA copy numbers demonstrating that HIV infection is affecting the mitochondrial OXPHOS RNA, altering the protein complexes themselves, or even altering a system upstream of the OXPHOS chain such as glycolysis or the citric acid cycle.

The HIV+/PN group exhibited a different effect in the CI and CIV enzyme activities with lower CI activity than the HIV- or HIV+ groups and higher CIV activity than the HIV+ group. Previous studies examining mitochondrial changes in PN have primarily seen effects in CIV alone [14,68,119,123]. One factor that may have caused our results to differ could be that our PN participants have been on long-term d4T treatment. Seminal studies examining the effects of NRTI exposure in E. patas monkeys showed OXPHOS alterations in brain, heart tissue, and skeletal muscle mitochondria. More specifically, drug-induced reductions in OXPHOS activity were observed in CI with an increase in the activities of CII (citrate synthase) and CIV [148,149]. While the d4T exposure in our PN group did not affect mtDNA copy numbers, it could have altered the mtRNA, such that CI activity was reduced. The subsequent increase in CIV activity, paralleling that seen in the studies by Ewings et al [148] and Gerschenson et al [149], may be a compensatory response to the loss of CI activity. The protons in the mitochondria that create the gradient needed for ATP production only enter the intermembrane space through CI, III, and IV [6,150]. If CI is unable to move protons, than CII activity increases to compensate. This increased flow of electrons through CII requires the activity of CIV to be upregulated in order to maintain a steady pace of ATP production [150]. As mtRNA levels were not analyzed in this study, we cannot confirm that mtRNA alterations are the cause of the OXPHOS disturbances.

Upon comparison of the HIV+ group after 6 months of d4T treatment to the HIV+/PN on OXPHOS activities, CI activity was still significantly higher and CIV activity was still significantly lower in the HIV+ group after 6 months on d4T treatment than in the HIV+/PN group. This difference however is less significant at this time point than it was when the HIV+ group was cART naïve at study entry. This indicates that CIV activity may have increased in the HIV+ group after d4T treatment, causing their PBMC mitochondrial function to more closely resemble that of the HIV+/PN group.
4.5.4. Oxidative stress differences between the HIV-, HIV+, and HIV+/PN groups

In response to altered OXPHOS enzyme activities, we expected to see elevated antioxidant levels and increased production of ROS in the HIV+ group as compared to the HIV- group as OXPHOS disturbances are known to increase electron slippage, leading to increased ROS generation [4,6]. We observed that the HIV+ group had higher antioxidant levels of SOD2, catalase, and PRX2 than the HIV- group. This is contradictory to previous studies on cART naïve HIV+ populations where lower levels of SOD have been observed [151] and these levels tend to decline with increasing severity of HIV disease [152]. Our results may differ from these studies because most of the previous observation on SOD levels were performed over a decade ago when more HIV infected participants were progressing to AIDS and had a greater severity of disease. In addition, the isoform of SOD examined was rarely specified and was most likely SOD3, or extracellular SOD [151,153]. Each isoform of SOD is an important cellular antioxidant. SOD2, however, has been shown to be extremely important for mitochondrial health in murine knockout and knockdown studies [154,155]. A complete knockout of SOD2 in mice produces a phenotype that is embryonic lethal with severe OXPHOS deficiencies seen in the heart and liver. This phenotype is much more severe than those seen in animal knockouts of SOD1 or SOD3, demonstrating the importance of SOD2 [155]. Furthermore, dorsal root ganglia neurons (peripheral neurons) from mice that were bred to under express SOD2 were found to have higher levels of superoxide production and decreased neuronal growth [154]. We have seen altered OXPHOS in the HIV+ participants in this study, which could increase electron slippage and ROS generation. As SOD2 is a mitochondrial specific antioxidant, an upregulation in SOD2 levels would be expected to occur in response to OXPHOS dysfunction.

In this study, catalase levels were also higher in the HIV+ group as compared to the HIV- group. This is similar to what was seen in an older study where blood catalase levels were higher in HIV+ participants and increased with increasing severity of HIV disease [156]. Finally, in our study, PRX2 levels were higher in the HIV+ group. Unfortunately, there is little information in the literature on any of the PRX enzyme isoforms and HIV infection. The fact that these three particular enzymes were all elevated in the HIV+ group could be a result of the particular ROS they detoxify. SOD2 catalyzes
the dismutation of superoxide into H$_2$O$_2$ and oxygen. As H$_2$O$_2$ can combine with other molecules to create further ROS, it also must be reduced into oxygen and water. This is accomplished by either catalase or PRX2 [16]. If elevated levels of SOD2 are generated in response to increased generation of superoxide, then there would also be a higher level of H$_2$O$_2$ produced, requiring more catalase and PRX2 to reduce it.

As mentioned previously, altered OXPHOS could increase the amount of ROS produced. Antioxidant levels were increased in the HIV+ group and this indicates that this is occurring in response to elevated ROS. In this study, we examined plasma TBARS as a marker of lipid peroxidation, but we did not observe any differences between the HIV- and HIV+ groups. In other studies, blood lipid peroxidation levels in HIV+ participants were elevated [157,158] and ROS levels increased with increasing disease severity [153,157,159]. The participants examined in those studies, however, were on cART treatment and thus their results are not comparable to the findings from our drug naïve HIV+ group. Furthermore, the TBARS assay only measures thiobarbituric reactive lipid peroxidation products. It is possible that levels of other types of lipid peroxidation products such as F2-isoprostanes or other ROS were increased in our cohort and future studies should further assess ROS levels.

In our HIV+/PN group, antioxidant levels were higher than either the HIV- or HIV+ groups for SOD2, catalase, and PRX2. Additionally, we found that as distal ENFD decreases, plasma SOD2 levels increase in the HIV+/PN group with a trend in for the same result in the HIV+ group. SOD2 is known to be very important for normal functioning of not only mitochondria, but also in maintaining neuronal health [129,154]. SOD2 levels have been shown to increase in rodent peripheral nerves in vivo after acute damage [160,161]. On the other hand, exposure of rodent neurons in vitro to HIV viral proteins such as gp120 without cART has been shown to decrease SOD2 [129]. Our results may differ from those of Saha et al., due to cART treatment. cART drugs, especially “D” drugs such as d4T, are known to induce oxidative stress through mitochondrial dysfunction [19,20,41,46,129]. More recent studies are finding that any cART use, regardless of current or past “D” drug exposure, is still a risk factor for PN development [65]. Furthermore, participants who are more adherent to their cART regimens have been observed to have higher levels of oxidative stress than those who are
less adherent [58]. Our HIV+/PN group has been on d4T for several years. Self-reported cART adherence rates in our HIV+ cohort during drug treatment were 95% and were confirmed through pharmacological testing (unpublished data from Dr. Peter Anderson) making it likely that adherence in the HIV+/PN group is equally high. We know from previous studies that HIV proteins can disrupt neuronal mitochondrial function and increase ROS production, which can then lead to a state of oxidative stress [123,128]. Once participants are placed on cART regimens, viral loads and viral proteins levels decrease. It has been postulated that cART drugs such as NRTIs may induce more axonal damage, further promoting mitochondrial dysfunction such that the peripheral nerves that were previously exposed to HIV would then have a reduced threshold to the NRTI induced mitochondrial dysfunction [15]. As mentioned in Chapter 1, this additive effect has been shown in mice expressing the viral protein gp120. Peripheral axon damage and loss was not observed until these mice were also given an NRTI [63]. Similar findings were seen in a study examining the effects of FIV alone and in combination with an NRTI where small nerve fiber loss was only observed in FIV infected felines after administration of the NRTI [64]. These results indicate that small peripheral nerve fiber oxidative stress and the corresponding fiber loss is more likely to occur after exposure to both HIV and NRTIs. Our HIV+/PN group had higher levels of several antioxidants than the HIV+ drug naïve cohort, adding further credence to the additive HIV and NRTI exposure hypothesis.

To further explore the effects of short-term versus long-term d4T exposure in these cohorts, we also compared the HIV+ group after 6 months of d4T treatment to the long-term treated HIV+/PN group. Differences between these two groups in plasma SOD2 lost significance after short-term d4T treatment, becoming only a trend toward higher levels in the HIV+/PN. In addition the significantly higher catalase and PRX2 levels in the HIV+/PN group as compared to the HIV+ were no longer significant after the HIV+ group was treated with d4T. These results indicate that the antioxidant status of the HIV+ group after short-term d4T exposure became more similar to the HIV+/PN group on long-term d4T.

Finally, TRX1 levels, which had not been significantly different between the two cohorts previously, became significantly lower in the HIV+ group after short-term d4T
treatment as compared to the HIV+/PN group. One study examining plasma TRX1 levels determined that HIV+ populations have higher amounts than HIV- populations [162].

The TRX1 enzyme has several important roles including reducing H$_2$O$_2$, reducing the oxidized form of PRX2, and enhancing transcription factor binding to target nuclear DNA sequences [163]. In addition, TRX1 also plays a role in the development of inflammatory processes, although the exact mechanisms are unknown. For example, elevated levels of plasma TRX1 are also seen in human populations with rheumatoid arthritis, hepatitis C, and asthma [164]. HIV infection is strongly associated with inflammatory processes and HIV+ humans or animals in animal HIV models show increased plasma levels of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 1 beta (IL-1β) [165-167] as well as inappropriate activation of T-cells, monocytes, and macrophages [165,167,168].

Treatment with cART reduces viral loads and inflammation to some extent [34,168], and thus, we saw a reduction in TRX1 levels in our HIV+ cohort after 6 months of d4T treatment. The HIV+/PN group may also have had a decrease in TRX1 shortly after beginning d4T treatment, showing an initial return to health with increases in CD4 cell counts. At some point during their treatment, however, their inflammation and TRX1 levels may have increased again and this could have coincided with the development of PN. There is much debate in the literature on HIV PN as to whether inflammatory processes occur first and lead to peripheral fiber loss, or if the fiber loss occurs first prompting an inflammatory response [15,115,145]. As monocytes are part of the PBMC population, and as we saw mitochondrial changes in the PBMCs of our HIV+ and HIV+/PN cohorts, inappropriate activation through HIV infection could lead these circulating cells to induce inflammation, leading to changes in oxidative stress. Perhaps this could even influence the health of peripheral nerve fibers once these monocytes become macrophages and infiltrate the surrounding tissue. As we did not examine the HIV+/PN cohort until many years into their treatment and we did not measure inflammation, it is difficult to say with any certainty if that was the case.

We also observed that after 6 months of d4T treatment in the HIV+ group, that TBARS levels were slightly elevated as compared to the HIV+/PN group, but these results did not reach significance. It is possible that ROS were higher at an earlier or later
time point in d4T treatment than at the 6-month time point we examined in this part of the study.

4.5.5. ENFD changes over time with different cART regimens in the HIV+ group

We observed that short-term d4T treatment in the HIV+ group altered their mitochondrial and oxidative stress parameters such that these outcomes were more homologous to the HIV+/PN group on long-term d4T. We then examined the changes induced in the HIV+ group as they transitioned from cART naïve to a d4T-based regimen over the course of 24 weeks. In addition, after 24 weeks of d4T exposure, this cohort was switched to a ZDV-based regimen for 48 weeks. This longitudinal component of the study allowed us to better separate the effects of general cART treatment from those induced by d4T or ZDV alone on ENFD, mitochondrial parameters, and oxidative stress in a cART naïve, HIV+ group without signs or symptoms of PN at study entry.

We determined that there were no significant changes in ENFD over time in the HIV+ group, regardless of the cART regimen. As we had seen no differences between, the HIV+ and HIV+/PN groups at entry or at the 6 month treatment time point for the HIV+ group, we did not expect to see any changes over the 72 weeks of cART treatment. While “D” drugs such as d4T, are known risk factor for PN development [20,41,115,140], it is possible that any changes in ENFD could occur after 6 months of treatment. However, our HIV+/PN cohort has been on d4T for around 4 years on average and their ENFDs were not significantly lower and thus, it is likely that other measurable changes occurred in the nerve fibers of the HIV+ group during cART exposure such as mitochondrial dysfunction or oxidative stress.

4.5.6. Changes in mtDNA copies per cell over time with different cART regimens in the HIV+ group

Treatment with d4T- and ZDV-based cART regimens did not specifically affect mtDNA copies per cell in the HIV+ group. Changes were seen from week 0 to week 72, however, indicating a general effect of cART treatment in improving mitochondrial function. The pol γ hypothesis states that mtDNA is affected by disruption of replication by NRTI treatment [19,20,41,46]. In our cohort, mtDNA copies per cell did not decrease
after d4T or ZDV treatment and in fact increased after 72 weeks of cART. As changes in adipose tissue mitochondrial RNA have been observed in as little as 2 weeks after NRTI exposure and after 6 weeks of exposure in PBMCs [55], we expected that PBMC mtDNA would be diminished after 24 weeks of d4T treatment. As mentioned in the cross-sectional discussion, there could be elevated mutations or deletions that we did not measure, for which clonal expansion could compensate. In addition, as mtDNA changes can occur rapidly [55], it is possible that mtDNA copies per cell were significantly altered by d4T at an earlier treatment time point (2 or 12 weeks) and that 48 weeks of ZDV treatment allowed the mtDNA to recover from the effects of HIV and d4T. For example, a study conducted in our lab on the effects of switching from a long-term d4T-based regimen to a short-term ZDV-based regimen found that adipose tissue mtDNA copies per cell recovered after 48 weeks of ZDV treatment [62]. Long-term d4T treatment as observed in our HIV+/PN group may have more detrimental effects on mtDNA copies per cell; this cohort has significantly lower mtDNA copies per cell at study entry (N=24; 233.5, SD=130.9) as compared to the HIV+ group after 72 weeks of cART (N=23; 295.0, SD=84.8; p=0.01).

We may not have observed changes in mtDNA in the cross-sectional or longitudinal studies simply due to our choice of sample type. NRTIs may have a stronger effect on mtDNA in adipose tissue, for example, rather than in PBMCs. NRTIs such as d4T are known to cause lipoatrophy [37,47] and many studies have observed changes in mtDNA copy numbers induced by NRTI treatment in these cells [55,62,82]. The differences in mtDNA copy numbers in PBMCs and adipose tissue after NRTI exposure could be due to differences in the cellular kinases that phosphorylate these drugs. Levels and types of these enzymes can vary by cell type leading to selective tissue toxicity [37,41,46]. As observed in the above cross-sectional study, lack of significant changes in mtDNA copy numbers does not mean that other mitochondrial parameters such as OXPHOS were not affected by the different regimens.
4.5.7. Changes in OXPHOS over time with different cART regimens in the HIV+ group

In this cohort, CI enzyme activity was not changed over time by cART treatment, regardless of the drug regimen. There was also no effect of regimen on CIV activity; however, activity levels did increase from week 0 to week 72 of cART treatment, mirroring the effects seen in mtDNA copies per cell. This may be an effect of an initial return to mitochondrial health as HIV viral loads diminish and CD4 cells counts improve. Also, it is possible that CIV activity was significantly altered by d4T at an earlier treatment time point than we measured and that the regimen switch allowed CIV activity to recover as discussed in the mtDNA section above. Or, it may be that long-term d4T treatment as observed in our HIV+/PN group may have eventual detrimental effects on OXPHOS that could have occurred after the 72 week time point. For example, the HIV+/PN cohort still had significantly higher CIV OXPHOS (N=23; 85.1, SD=32.6) at study entry as compared to the HIV+ group after 72 weeks of cART (N=22; 66.6, SD=13.1; p=0.003), though the significance is less strong than it was when the HIV+ cohort had been on a d4T-based regimen for 6 months (p<0.001). A longer follow up period may be necessary to further examine this effect.

4.5.8. Changes in oxidative stress over time with different cART regimens in the HIV+ group

The alterations to mitochondrial function in this cohort after cART were minor and indicated a gradual return to health. In this study, we also measured changes in antioxidant levels over time as the HIV+ group was placed on cART treatment to determine if the same effect would be found. Plasma levels of SOD2, catalase, and PRX2 rose from week 0 to week 24, coinciding with the initiation of the d4T-based regimen, and fell from week 24 to week 72, coinciding with the switch from the d4T- to the ZDV-based regimen. While we did not observe mitochondrial changes in DNA or OXPHOS during the d4T treatment, antioxidant levels were greatly increased. Again, this may be a result of the additive effects of some “D” drugs with HIV. In that case, it is possible that the initial HIV infection is increasing ROS generation, thus diminishing antioxidant enzyme levels and inducing a state of oxidative stress. Then the d4T treatment allows
antioxidant levels to increase and reduce the amount of ROS in an initial return to health. Or it may be that d4T treatment is further elevating the ROS levels, creating the aforementioned additive effect, and antioxidant levels are increasing as a result. For instance, the HIV+/PN group has been on d4T for several years and they exhibited higher antioxidant levels than the HIV+ group, even after short-term d4T treatment. When we examined TBARS levels, ROS levels were significantly lower in the HIV+ group from weeks 24 to 72, coinciding with switch from d4T- to the ZDV-based regimen. There was a slight increase from 0 to 24 weeks, but this result was not significant. It appears that the ZDV-based regimen (weeks 24 to 72) allowed the ROS levels to decrease. ZDV has also been linked to mitochondrial dysfunction and oxidative stress, but d4T exposure seems to exert a much stronger effect [46]. The effects of NRTIs on mitochondria and oxidative stress can differ depending on the tissue examined, with many stronger effects observed in adipose tissue as opposed to PBMCs [55,82,144]. Changes in ROS levels could be more apparent in other cell or tissue types. In addition, it is possible that ROS were significantly elevated at a d4T treatment time point that we did not examine. Finally, there are many other types of ROS that we did not measure.

Additionally, we found a significant negative correlation between plasma TBARS levels and PBMC mtDNA copies per cell at week 72 of cART treatment. As ROS levels increase, oxidation of mtDNA could be elevated causing more mutations and deletions [8], eventually causing lower levels of mtDNA copies per cell. Lipid peroxides resulting from ROS redox reactions such as malondialdehyde are highly mutagenic and can easily form DNA adducts [169,170], further affecting mtDNA numbers. We had observed that at week 72 of cART treatment, TBARS levels were lower than at week 24 of d4T treatment and thus, it is possible that the lower TBARS levels occurred because of the switch from d4T to ZDV, allowing mtDNA copies per cell to rebound.

4.5.9. Limitations and conclusions

The findings of this study emphasize the effects that HIV infection and cART have on mitochondrial function and oxidative stress. They also illustrate that alterations to mitochondrial function affects the development of PN. This study did have several limitations. Examining the nerve fiber mitochondrial structure microscopically for
evidence of swelling or undefined cristae for instance may have provided us with important information on the axonal mitochondrial health. Unfortunately, once the nerve fiber samples were sent to John’s Hopkins University for staining and counting, we were not able to retrieve them for further testing, and therefore, we could not examine the nerve fibers. Also, our measurements of mitochondrial dysfunction and oxidative stress were systemic – from the PBMCs and plasma of our populations. Thus, we cannot say for certain that the changes seen systemically reflect the changes seen in the peripheral nerve fibers.

Another limitation is that this was a clinical study that had been conducted previously and as such there were limited amounts of samples with which to conduct experiments beyond what had been proposed in the original grant. Viable PBMC samples were in short supply whereas plasma samples had been largely untested. Therefore, our choice of assays was also limited. Examining other ROS such as H₂O₂ or super oxide, for instance, may have provided us with more information as to the oxidative state of these cohorts, but without viable cells, these assays are not feasible. It is also possible that we did not see significant differences between our cohorts on measures such as mtDNA or ROS due to our small sample size. The HIV+/PN cohort had only 25 patients enrolled, and thus, the number of patients we could examine in this cohort was restricted. Furthermore, we may have had more robust results had we examined other time points in our longitudinal cohort. For example, plasma and PMBC samples were also collected at weeks 2, 12, 36, and 48 of the 72-week study. Changes induced by d4T may have been more evident in the HIV+ group at earlier time points. The ENFDs, however, were only assessed at weeks 0, 24, and 72 in each of these cohorts and thus it would have been more difficult to correlate the systemic changes at other time points to those occurring in the ENFDs.

Finally, our study lacked an HIV- cohort that was placed on different cART regimens, which would have allowed us to better tease out the effects of cART versus HIV infection on mitochondrial function and oxidative stress. While such a cohort was enrolled and followed over time in the larger SEARCH study, we decided to focus our resources on a true positive cohort (HIV+/PN) and a true negative cohort (HIV-) in comparison to our HIV+ drug naïve cohort. Future studies could further examine
mitochondrial function and oxidative stress in this HIV-, drug treated group to better understand what effects cART has on ENFD and mitochondrial function without the additive effects of HIV. Also, examining the HIV+ group that was asymptomatic for PN could better elucidate how the symptomatic and asymptomatic PN cohorts differ from one another.

From the results of this study, we better understand the changes in mitochondrial function and oxidative stress that are occurring in those participants with HIV who have developed d4T-induced PN. We have also gained a better understanding of the effects of HIV infection and different cART treatments on these outcomes. Hopefully, future studies will be able to better clarify how these systemic changes are related to changes in the peripheral nerves. In addition, if we can determine exactly when these changes occur in cART treatment, physicians may be able to prevent PN development through well-timed drug regimen changes. Finally, clarification of the underlying mechanisms of PN development may help researchers determine the best treatments.
CHAPTER 5.
Discussion
5.1. ABROGATION OF DOX-INDUCED MITOCHONDRIAL FUNCTION BY DEX

DOX treatment is known to increase the risk of later cardiomyopathy development by inducing mtDNA damage in myocytes [21]. This disease does not manifest for many years as a threshold of damaged mtDNA must be surpassed before the signs and symptoms of energy depletion manifest [1,2]. We have seen in this study that DOX exposure in childhood also has long-term effects on white blood cell mtDNA, possibly through permanent alterations to bone marrow stem cells. These DNA alterations, however, have not yet led to measurable changes in OXPHOS in our participants indicating that a compensatory mechanism is prevailing. Treatment with the iron chelator DEX abrogated DOX effects on mtDNA copy numbers in PBMCs, possibly through a reduction in the iron-DOX complexes that can induce oxidative stress. Oxidative stress was not measured in these participants with or without DEX treatment, so it is difficult to ascertain if this was the mechanism behind the abrogation.

Another potential question that has not been addressed is whether there are trans-generational effects of DOX exposure on mtDNA. MtDNA is maternally inherited and maternal mutations or deletions can also be inherited as seen in certain genetic mitochondrial diseases (e.g., Leber’s hereditary optic neuropathy and myoclonus epilepsy and ragged red fibers) [2,7]. The participants in our study were observed anywhere from 3 to 30 years after treatment and this means that many of these participants are of, or will soon reach, child-bearing age. We have shown in this study that the alterations to PBMC mtDNA are still present many years after DOX treatment without DEX adjuvant therapy. It is possible that the female participants in the DOX only group could have a higher risk of passing on any germ cell mtDNA mutations or deletions that occur from DOX exposure. MtDNA mutates at a higher rate than nuclear DNA [1,2] but due to threshold effects, simply having mtDNA mutations does not guarantee that mitochondrial disease phenotypes will develop. For example, the rate of the most common pathogenic mtDNA mutations in the general population is 1 in 200 while the rate of mtDNA diseases has a minimum prevalence of 1 in 5,000 [2]. Studies in mouse models of mtDNA mutations have shown that the ovaries in mice harboring mtDNA mutations select against those oocytes harboring the most deleterious mutations, preventing them from being fertilized.
The more mild mtDNA mutations, however, can still be transmitted to the next generation. Such germ line mtDNA mutations can increase the risk of developing certain types of cancer [1], possibly causing a vicious cycle. While DOX is known to induce mutations in female germ cells [171], it is unknown what effect this could have on the progeny of DOX treated survivors of ALL. In a murine study, female mice treated with one DOX dose were able to conceive and bear relatively normal offspring, though the DOX treated dams reached menopause sooner than controls due to loss of oocytes. The most distressing effects were observed several generations out from the DOX-treated progenitors with increased neonatal deaths and brain malformations seen in generations 4 and 6. That would equate to more than 70 years after the first DOX treatments were ever used in humans. Currently, more than 40 years after DOX was first used as a chemotherapeutic drug, no human transgenerational effects have been observed [171].

Another question brought about by this research study is the fate of DEX adjuvant treatment in pediatric populations. While this is not the first study to show that combination DEX treatment with DOX in pediatric ALL patients can reduce the deleterious effects of DOX [22,23,80], DEX is still not widely used as an adjuvant in pediatric patients. The major concern with DEX use is that it would reduce the chemotoxic effect of DOX, elevating the risk of secondary neoplasm development (SND) [78]. While the exact chemotoxic mechanisms of DOX are not well understood, one of the ways in which DOX may kill tumor cells is through ROS generation. If this mechanism is diminished by DEX adjuvant treatment, it is possible that DOX may be less effective. SND after DOX and DEX treatment has been examined in multiple cohorts, both adult and pediatric, and the risk of SND was negligible in all but one study [23].

A further concern is the fact that DEX is a metal chelator. Chelators sequester specific redox active metal ions (e.g., iron, copper, and zinc), rendering them inactive [172]. Many metal ions have important roles in cells. For example, some are important cofactors for various enzymes such as copper in the cytochrome c oxidase protein complex, manganese in mitochondrial SOD, magnesium in DNA replication, and zinc in some transcription factor binding motifs. Iron also has many important roles including heme synthesis [172,173]. While chelation therapy can be beneficial for heavy metal
poisoning, the use of this therapy for diseases such as cancer and heart disease is generally not recommended due to the risk of severe side effects such as anemia, heart arrhythmias and kidney dysfunction [172,173]. Our study participants, however, were on short-term DEX treatment that was combined with DOX treatment as recommended by the FDA [77], and after several years of follow-up, our DEX treated cohort did not show higher rates of SND than the DOX only group and participants tolerated DEX treatment well. Regardless of the apparent safety and efficacy of DEX adjuvant treatment in pediatric ALL cohorts, it may still take time for treatment guidelines to change.

5.2. MITOCHONDRIAL DYSFUNCTION AND IR IN PERINATAL HIV INFECTION

cART is associated with the development of various metabolic diseases including impaired glucose homeostasis disorders such as insulin resistance and diabetes [12,88,92]. These associations have been observed in adults and more recently, in perinatally infected HIV adolescents who have been on cART for the majority of their lives [75,100,101]. The metabolic changes seen in HIV infected populations have been linked to cART-induced mitochondrial function, specifically from thymidine analogs such d4T and ZDV [19,20]. In this study we observed that IR, HIV positive adolescents showed a trend toward lower mtDNA copies per cell in their PBMCs as compared to a similar non-IR cohort. These alterations in mtDNA, however, did not result in disturbances in OXPHOS activities. While not significant, CI and CIV median activities were higher in the IR group. This is a normal response to changes in glucose levels. As systemic glucose levels increase, glycolysis and the citric acid cycle activities would be amplified, leading to more electrons ready to enter OXPHOS. This buildup of electrons would require a subsequent amplification in CI and CIV activities [150,174]. OXPHOS augmentation is a short-term solution to changes in glucose homeostasis and this process can’t continue indefinitely, especially if there is a loss of mtDNA copies per cell. Studies on diabetes have shown that with higher glucose levels, ROS levels increase [175]. Higher activities of OXPHOS translate to more electron slippage, which can generate higher levels of ROS and these ROS can damage the mtDNA, decreasing OXPHOS capabilities [6,11]. In fact, CI enzyme activities in our study correlated negatively with
serum glucose levels, which indicates that any compensatory mechanism currently working to keep OXPHOS at normal levels may soon fail. The longer these children are IR, the higher their risk of developing further mitochondrial dysfunction and frank diabetes.

In this study, there were no differences between the groups on cART exposure. While certain types of cART, such as NRTIs, are known to effect metabolic parameters [19,20,46], we did not see that effect in this study. It may be that we simply did not examine a large enough population to see a measurable effect of cART in this study. It could be possible that other factors such as inflammation from HIV infection and family history of diabetes also play a role in determining who will develop IR in these cohorts. It is also possible that more significant mitochondrial changes from HIV infection and cART exposure could be observed in cells that are involved in glucose uptake such as skeletal muscle and adipose tissues and that the systemic changes we observed in PBMCs are milder in comparison. Further research is needed in this area to determine how much of a role cART treatment plays in altering glucose homeostasis in these children.

Finally, HIV positive populations on cART are known to have a greater risk of developing not only IR, but also diabetes [100,176,177]. Diabetes is a known risk factor for other diseases such as PN, possibly through the induction of oxidative stress [175,178]. It is difficult to determine the exact risk of PN development in diabetic HIV positive patients, however, as they are typically excluded from PN studies [43]. In one study on PN in HIV infection in which diabetic PN participants were included, the diabetic participants had a poorer prognosis for recovery of degenerated small fibers than those without diabetes [43]. While small fiber loss and PN were not examined in this cohort, uncontrolled glucose homeostasis combined with HIV infection and NRTI exposure, could be a triple threat, placing these participants at greater risk for the development of PN as well as a host of other diseases.

5.3. THE ROLES OF MITOCHONDRIAL FUNCTION, OXIDATIVE STRESS, HIV, AND cART IN PN

In this study, we observed that an HIV positive cohort with d4T-induced symptomatic PN had altered OXPHOS and higher antioxidant levels as compared to
cohort(s) without PN that were HIV negative or HIV positive and cART naïve. These results confirm that mitochondrial dysfunction and oxidative stress are associated with PN development. These results, however, do not explain why the PN rates in HIV positive patients in Thailand (around 30%) are lower than those seen in Nigeria (40-70%, depending on cART regimen) and the U.S. (over 50%) [65-67]. Past treatment with “D” drugs such as d4T are associated with greater risk of PN, but current cART use is still a risk factor for PN even without current “D” drug use [65]. Thus, regardless of whether “D” drugs have been phased out in any country in question, PN is still a risk of HIV infection and cART treatment. The fact that mitochondrial dysfunction is associated with PN may offer a clue as to why the prevalence differs by country. Single nucleotide polymorphisms in mtDNA (haplotypes) are concentrated by geographical region, resulting in haplogroups (groups of people who share similar haplotypes) [1]. Haplotypes have been shown to influence predisposition to certain diseases and studies have determined that certain European and African haplogroups have increased risk of developing HIV related PN [1,179,180] while other haplogroups were shown to have some protection against cART-induced side effects such as lipodystrophy [1]. This mitochondrial genetic phenomenon may explain why rates for HIV related PN are higher in parts of Africa and the U.S. and lower in Thailand [63-65].

In addition to haplotypes, another question that arises is how adherence rates could affect PN development. As mentioned in Chapter 1, cART adherence rates in the U.S. are relatively poor [39,40] whereas the adherence rates in our SEARCH cohort were fairly high (over 95%). In HIV cART treatment there is an “adherence paradox” where high cART adherence can increase drug-induced side effects. These side effects then can lead to reductions in patient drug adherence, causing disease progression [181]. In a study on HIV PN in Bangkok, Sithinamsuwan et al, [67] found that symptoms of PN were associated with lower HIV viral loads, meaning participants with better cART adherence, and thus greater exposure to these drugs, had more symptoms of PN [67]. As this was a cross-sectional study, subsequent changes in adherence due to PN symptoms were not measured. These results demonstrate the delicate balance that has to be maintained between keeping viral loads low and patient quality of life high. Regimen switches may be one way to maintain this balance. Currently, d4T and ZDV are slowly being phased
out of use in Thailand in favor of TDF, which is not a thymidine analog and is less mitochondrial toxin. TDF has not traditionally been prescribed in developing countries due to its higher cost [35]. The Thai GPO, however, has been generating a generic formula of Atripla and will soon phase out d4T and ZDV [35,130]. While the side effects of TDF are less severe than those of d4T or ZDV, TDF exposure can cause adverse renal effects and bone density loss [37,47]. The loss of bone density is associated with lower BMI and lower CD4 cell counts [182]. In the SEARCH HIV+ drug naïve cohort, one of the main reasons a randomized group of these participants were placed on d4T and then switched to ZDV is that ZDV can cause anemia, especially if the treatment is started with low CD4 cell counts. D4T does have serious long-term effects, but in the short-term it is very efficient at reducing viral loads and increasing CD4 cell counts, even more so than ZDV as was seen in the recently published study on the safety and efficacy of these cART regimens in the HIV+ cohort [132]. Also, as we saw in Chapter 4, switching from a d4T- to a ZDV-based regimen after 6 months improved d4T-affected mitochondrial and oxidative stress parameters. Our SEARCH HIV+ participants had fairly low BMIs and CD4 cell counts (21.6 and 165.5 cells/mm³, respectively) at study entry, putting them at greater risk for bone mineral loss if placed on a TDF-based regimen. It is possible that by placing these participants on a d4T-based regimen for short period of time to lower viral loads and increase CD4 counts, that any long-term d4T effects and the TDF-associated bone loss can be avoided. Further research is needed to determine if this would be a feasible approach in this population.

Finally, in this study on HIV, cART, and PN, we had expected to see the same additive effect that has been observed in animal studies, where HIV infection induces neuronal mitochondrial dysfunction and increases oxidative stress. Then, once cART treatment begins, viral loads and viral proteins decrease, but cART itself promotes further mitochondrial dysfunction, inflicting more axonal damage. The neurons, with a reduced threshold to the cART-induced mitochondrial dysfunction, begin to degenerate. Our results showed this in terms of oxidative stress in the HIV+ group as they went from cART naïve to a d4T-based regimen. They entered the study with higher oxidative stress than the HIV- group and this stress increased after d4T treatment. We did not, however, observe this effect in the ENFD. Instead we found that HIV+/PN participants on d4T did
not show significant loss of ENFD as compared to HIV+ drug naïve participants without PN, indicating that HIV alone plays a greater role in the initial loss of ENFD than d4T in these cohorts. We did see not only higher oxidative stress but also mitochondrial dysfunction systemically in both HIV+ cohorts with the highest levels of dysfunction and oxidative stress in the HIV+/PN cohort. This demonstrates that although HIV infection can induce mitochondrial dysfunction, adding d4T clearly has a stronger affect. It may be that the loss of ENFD combined with mitochondrial dysfunction and oxidative stress must occur before PN becomes symptomatic. A study in rat models of PN caused by diabetes, NRTIs, or chemotherapeutics, found that inhibiting any one of the protein complexes in the mitochondrial OXPHOS chain reduced neuropathic pain symptoms [183]. It stands to reason that the higher levels of CIV activities observed in our HIV+/PN group could be mediating PN symptoms. Further research is needed in this area to better understand the roles of ENFD, OXPHOS, and oxidative stress in the development of symptomatic PN.

5.4. CONCLUSIONS

We have seen across these three studies that drugs that affect tumor cell or viral DNA replication can alter mitochondrial function either through alterations to the DNA, observed as lower copy numbers per cell, or through disturbances in the OXPHOS system, measured as changes in CI or CIV enzyme activities. While we had originally hypothesized that mtDNA damage would lead to altered OXPHOS, we have observed changes in mtDNA without significant changes in OXPHOS in Chapters 2 and 3, and in Chapter 4 we observed altered OXPHOS without measurable changes in mtDNA copy numbers. These results highlight the fact these drugs may be affecting processes other than mtDNA replication by changing DNA transcriptional regulation or RNA translation processes, altering the OXPHOS protein complexes themselves, or by affecting other upstream mitochondrial systems such as the citric acid cycle or glycolysis. In addition, the studies in Chapters 2 and 3 demonstrated the long-term effects of exposure to anthracyclines or cART. In Chapter 4, the study showed not only the long-term effects of d4T treatment (HIV+/PN cohort) on mitochondrial and oxidative stress parameters but also the short-term effects that can be measured after only 6 months of d4T exposure.
(HIV+ after 24 weeks of d4T). Furthermore, we observed how quickly d4T can affect mitochondrial function and oxidative stress in HIV positive drug naïve participants and how these effects can be reversed by cART regimen switching. Finally, we demonstrated that the additive theory of HIV and cART inducing ENFD loss as seen in animal models, occurred not with ENFD, but with oxidative stress in our human populations. We instead raised some interesting questions about the role of OXPHOS in symptomatic PN development.

In general we did observe drug-induced mitochondrial alterations in these populations, though not always in the way we expected. The changes in mtDNA copy numbers in the pediatric populations did not appreciably affect OXPHOS functioning. The reverse was seen in our adult populations where no significant changes were seen in mtDNA copy numbers, but OXPHOS was altered. This may reflect mitochondrial changes that occur with aging. Mitochondria are believed to play a major role in the aging process [1,11] and the different effects observed in our populations may reflect differences in mitochondrial age. It may be that younger mitochondria with fewer age induced mutations or deletions and more efficient OXPHOS can better tolerate the toxic effects of these drugs than can older mitochondria. This is one of the proposed hypotheses behind why distal peripheral nerves may acquire more HIV and cART induced mitochondrial damage than proximal nerves [119]. It is difficult to say with any certainty if aging mitochondria were a factor in our studies as we did not measure aging related mutations or deletions in our populations. Longitudinal follow up of our pediatric populations into adulthood may shed more light on this prospect. Regardless, these drugs, while very effective, are inducing systemic mitochondrial changes and research in this area should continue in order to determine the full extent of the mitochondrial alterations. This information could one day lead to equally effective treatments that are more mitochondrial friendly, improving patient quality of life.

5.5. FUTURE DIRECTIONS

The changes we observed in our populations were systemic in nature as the samples we examined were either PMBCs or plasma. It may be important also to measure any changes in tissues. For example, in DOX exposure, it may be of interest to further
examine bone marrow cells for mitochondrial changes as the alterations we observed occurred in PBMCs many years after the last DOX exposure. In terms of IR in perinatally infected HIV positive populations, examining the mitochondria in the muscle and/or fat tissue may yield more significant results than those seen in our population samples as these tissues have insulin receptors and are responsible for the uptake of glucose in the body. In the HIV positive participants with and without PN, measuring changes in the peripheral nerve cell mitochondria may better explain the systemic results that we obtained. Perhaps non-human primate models of NRTI exposure on PN or IR could provide further clues by giving us access to tissues and cells that it would be difficult if not impossible to obtain from our human participants (e.g., muscle or fat biopsies from pediatric participants) though this type of research can be prohibitively expensive.

Furthermore, measures of oxidative stress could be useful in both DOX and cART exposed populations, as was performed in our SEARCH cohorts. This could allow future studies to determine what role, if any, oxidative stress plays in the disease processes induced by these drug types. We know that DOX may kill tumor cells in part through ROS formation [21] and that DEX may decrease DOX-induced ROS formation [21,75]. We also know that oxidative stress plays a role in PN development [119,184] as well as in diabetes induced complications such as PN, stroke, and cardiovascular disease [88,175,178]. As we saw alterations in the PBMC mitochondria in both the DOX exposed and the cART exposed pediatric populations, it would be interesting to see if these drug-induced alterations had also produced a state of oxidative stress and if the DEX treatment would abrogate this effect.

Finally, future studies could further examine the role of genetics in drug-induced mitochondrial dysfunction. Genetic profiles of the DOX exposed patients may help determine who is more susceptible to cardiac cell mitochondrial dysfunction and thus, cardiomyopathy development. Similarly, studying the mitochondrial genetic profiles of HIV positive IR populations could provide better information on why certain patients may be more likely to develop IR while on a cART regimen. In general mitochondrial genetics play a role in the development of many interesting phenotypes from longevity and athletic performance to diabetes, heart disease and cancer [1,11]. Across all three of these studies on drug induced mitochondrial dysfunction, determining the mitochondrial
genetics of our cohorts may give us a better understanding of why some populations are more susceptible to the adverse effects of chemotherapeutics or cART regimens than others.
REFERENCES


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leukocytes from type 2 diabetic patients. *Free Radical Biology & Medicine, 50*; 1215-21.


112. Moren, C. N.-J., A.; Garrabou, G; Catalan, M; Rovira, N; Tbias, E; Cardellach, F; Miro, O; Fortuny, C. (2012). Mitochondrial evolution in HIV-infected children receiving first- or second-generation nucleoside analogues. *Journal of Acquired Immune Deficiency Syndrome, 60*; 111-16.


146. Miro, O. L., S; Martinez, E; Pedrol, E; Milinkovic, A; Deig, E; Garrabou, G; Casedemount, J; Gatell, J; Cardellach, F. (2004). Mitochondrial effects of HIV infection on the peripheral blood mononuclear cells of HIV-infected patients who were never treated with antiretrovirals. *Clinical Infectious Diseases*, 39; 710-16.


count is associated with greater bone mineral density loss after ART initiation. *Clinical Infectious Diseases, 57* (10), 1483-8.
