L-carnitine as a Probe to Assess Mitochondrial Drug Toxicity

by

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Dedication

Para ti Papi, te extrañaré y recordaré siempre

To my dad, I miss you every day.

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Abstract

Adverse drug reactions (ADRs) are an inherent risk of any drug therapy regimen. Despite significant advances in medical sciences and constant improvements in the drug development process, ADRs still represent a major healthcare concern for patients and clinicians as well as a burden on the healthcare system. Recently, mitochondrial metabolites have become of interest because of their potential use as signaling molecules for mitochondrial health. From both a patient and healthcare perspective, there are benefits in identifying mitochondria-specific metabolites that could be clinically employed to probe and assess mitochondrial related alterations in metabolism. The mechanisms mediating the pharmacokinetics and disposition of mitochondrial substrates and metabolites can be used to identify increased toxicity risk specifically due to mitochondrial dysfunction caused by ADRs.

With this rationale, this dissertation looked to (1) apply a database mining strategy to identify candidate mitochondrial metabolites that could be clinically employed to identify individuals at increased risk of mitochondrial-related ADRs, (2) to demonstrate how an L-carnitine challenge can be useful as a metabolic stress biomarker to identify individuals at increased risk of mitochondrial-related ADRs, and (3) to establish differences between male and female CFZ treated mice and associated interactions with L-carnitine.

To achieve this, a database screening strategy with an *a priori* established criteria was employed to identify candidate mitochondrial metabolites that could be clinically useful to identify increased risk of mitochondrial-related ADRs. Four publicly available databases were used and allowed for a systematic process of elimination, which led to a concise list of clinically

feasible human metabolite candidates for use as functional tracers of mitochondrial metabolic health. L-carnitine was identified as a candidate mitochondrial metabolite.

We then hypothesized that an intravenous dose of exogenous L-carnitine- an “L-carnitine challenge test”- could identify individuals at risk for mitochondrial-related ADRs by provoking variation in subsequent measurement of serial L-carnitine and/or acetylcarnitine blood concentrations. To test this, an established animal model of mitochondrial drug toxicity was used in which male C57BL/6 mice were treated with clofazimine (CFZ) by its addition to their chow for 8-weeks. Following CFZ treatment, mice were injected with a high dose (1,000 mg/kg) of L-carnitine. Metabolic functions were tracked, and urine and blood samples were assayed for L-carnitine and acetylcarnitine concentrations using a quantitative LC/MS analysis. The L-carnitine challenge test identified CFZ-dependent differences in whole blood acetylcarnitine concentration. This finding supports and substantiates the potential of the challenge test as a “probe” to identify drug-related toxicological manifestations.

Lastly, we looked to further establish L-carnitine feasibility for clinical use in both male and female, and to aid in further closing the knowledge gap of sex-related differences in ADR incidence, since females are found to experience ADRs at a higher rate than males. The “L-carnitine challenge test” was once again used, this time in both male and female mice. The results from this project showed that the L-carnitine “challenge test” induced differences in whole blood acetylcarnitine concentrations in both CFZ-treated male and female mice. This dissertation serves as a starting point in the development of an “L-carnitine challenge” test a clinically useful probe to identify drug-related toxicological manifestations, with the goal of potentially reducing ADRs incidences during lifesaving drug treatment.

Chapter 1
Introduction

1.1 Mitochondria

Mitochondria are organelles found in almost all eukaryotic cells. They are often referred to as the "powerhouses" of the cell because they are responsible for producing the majority of the cell's energy in the form of adenosine triphosphate (ATP). Aside from their main function of generating energy for the cell through cellular respiration, mitochondria are intricately involved in many metabolic pathways, such as fatty acid oxidation (FOA), the synthesis of lipids, amino acids, and nucleotides, intermediary metabolism, steroid metabolism as well as the regulation of calcium levels in the cell. They play a role in other cellular processes such as cell growth, differentiation, and death. They also have their own DNA and the ability to reproduce independent of the cell. They are essential for both energy metabolism and the management of catabolic and anabolic processes, and a variety of signaling pathways[1–3]. For all these reasons, healthy, fully functional mitochondria are vital for metabolic homeostasis.

Because mitochondria are complex organelles and are involved in many different functions, there are many different sites of action, mechanisms, and pathways in which they can be targeted or affected[4,5] (**Figure 1-1**). Mitochondria can be directly affected by the inhibition of mitochondrial DNA (mtDNA) transcription of electron transport chain (ETC) complexes, and by the impairment of mtDNA replication and thus mitochondrial protein synthesis[5–7]. Depletion of carnitine and Coenzyme A (CoA) can also induce alterations in mitochondrial function leading to impaired ATP synthesis and uncoupling of oxidative phosphorylation. A number of

exogenous molecules have also been found to interfere with one or more of the complexes in the respiratory chain, affecting the ETC **(Figure 1-1)**[8,9]. These off-target interactions with pathways, mechanisms, structural components and functional features in mitochondria can and lead to toxicity[8,10] and then result in adverse drug reactions (ADRs).

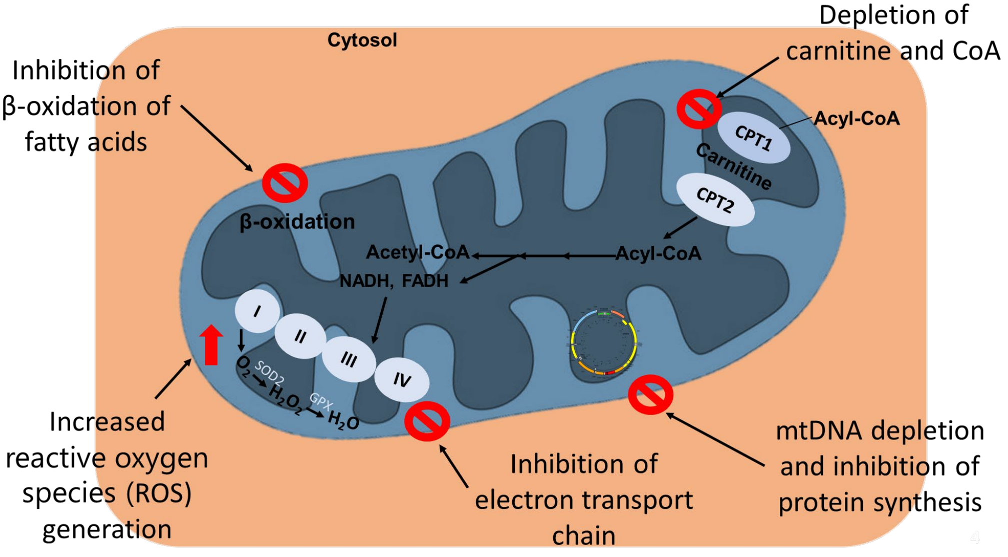

Figure 1-1 Sites of action, mechanisms and pathways that can be altered in mitochondria that lead to dysfunction.

1.2 Adverse Drug Reactions

Adverse drug reactions (ADRs) are defined by the World Health Organization as, "one that is noxious, is unintended, and occurs at doses normally used in man."[11] It is important to note that this definition does not include therapeutic failures, drug abuse, errors in drug administration, or complications arising from patient non-compliance. Typically, ADRs are considered to be an inherent risk of medication use, yet they pose a major burden on the United States' health care system[12–14]. ADRs are among the top six leading cause of morbidity and

mortality in the nation, accounting for as many as 6% of all hospitalizations, and costing as much as $150 billion per year[15–18]. As evidenced by their high frequency of occurrence in hospitals and general practices, ADRs are an important clinical issue and a major healthcare concern. For the healthcare service industry, ADRs result in increased hospitalization rates, prolonged hospital stays, and sometimes clinical investigation. For the drug development industry, ADRs are a challenging obstacle that is often encountered when bringing a drug product to the market.

The United States Food and Drug Administration (FDA) withdraws many approved drug products from the market every year because of ADRs[19–21]. During drug development, randomized controlled trials serve as the gold standard for determining the efficacy and safety of compounds but often fall short in the detection of mitochondrial-related ADRs. This is because most clinical trials are performed in standardized settings on participants that may not be representative of the real-world patient population. For example, females and elderly patients are often underrepresented in clinical trials and individuals receiving certain concomitant drug therapies may be excluded from participation. Another major limitation of clinical trials stems from the duration of drug treatment; many mitochondrial ADRs manifest only after continuous long-term drug exposure, something which many trials do not adequately account for when using relatively short follow-up times.[14,22]

Adverse drug reactions often result from unwanted drug interactions unrelated to the drug's intended, primary mechanism of action. The scientific premise of my thesis work was based on the findings that even though most drugs are designed to directly activate or inhibit specific molecular targets involved in pathological processes, many ADRs are toxicological manifestations of off-target drug interactions affecting the structure and metabolic function of

mitochondria.[4,23,24] These interactions then result in metabolic stress which can be toxicologically reflected in lack or loss of drug efficacy or in a drug-induced injury.[25–27]

Presently, there are no clinically utilized specific biomarkers of mitochondrial toxicity, and such testing is not required by the FDA as part of the drug approval process.[6,25] To date, assessment of drug-induced mitochondrial alterations has been retrospective, meaning that once a drug starts showing signs of toxicity, it is examined for potential mitochondrial liabilities.

1.2.1 ***Drug-induced mitochondrial alterations***

Drug-induced alterations in mitochondria can be caused by a wide range of drugs and lead to a variety of side effects. Drugs of all different classes and with different pharmacological mechanisms of actions have been found to alter mitochondria metabolic function; these include antiepileptic drugs, antivirals, antibiotics, and some cancer drugs **(Table 1-1)**.

Table 1-1 Examples of drugs known to cause alterations in mitochondrial metabolic function

Drug Name	Valproic Acid (VPA)	Clofazamine (CFZ)	Zidovudine (ZDV)	Cisplatin (CSP)	Propofol	Cyclosporine (CyA)
Chemical Structure						
Drug Class	antiepileptic	antibiotic	antiviral	anticancer	anesthetic	immunosuppressant
Mechanism of Pharmacological Action	Increases brain concentrations of gamma-aminobutyric acid (GABA), blocks voltage-gated ion channels, and inhibits histone deacetylase	Inhibits mycobacterial growth and binds preferentially to mycobacterial DNA	Inhibits HIV's reverse transcriptase (RT) via DNA chain termination	Binds to genomic DNA in the cell nucleus to form cross-links which triggers cytotoxic processes	Increases GABA-mediated inhibitory function in the CNS	Exact mechanism unknown, but thought to inhibit production and release of interleukin-2

All of the different pathways, mechanisms and sites of action discussed above can be unintentionally targeted and affected by drugs. Some examples of these interactions include zidovudine (ZDV), a nucleotide reverse transcriptase inhibitor (NRTI) used in the treatment of HIV as an integral component of highly active antiretroviral therapy.[28] ZDV undergoes intracellular triphosphorylation and inhibits viral replication by incorporating into the viral DNA strand, impeding viral RNA-dependent DNA polymerase (reverse transcriptase)[28]. The clinical utility of many NRTIs, like ZDV and stavudine, is constrained due to their association with ADRs during chronic therapy at high doses. They have been found to gradually reduce mitochondrial function in various tissues by preventing mitochondrial replication via inhibition of the polymerase that replicates mtDNA.[25,28,29]

Another example of a medication that interacts with mitochondria is cisplatin (CSP). CSP is an effective anticancer drug, used for the treatment of testicular, lung, and ovarian cancers. Although it is well tolerated, its clinical use is limited by its side effects; nephrotoxicity, cytotoxicity, and cardiomyopathy[30]. In recent years, several studies have demonstrated that CSP-induced cytotoxicity is closely related to increased reactive oxygen species generation, and that this increase alters the mitochondrial membrane potential and damages the respiratory chain, which ultimately triggers the apoptotic process[31].

Table 1-2 Drugs with FDA Black Box Warnings for hepatotoxicity and cardiovascular toxicity that are attributable to mitochondrial liabilities

Hepatotoxicity Black Box Warning				Cardiovascular Toxicity Black Box Warning			
Antiviral	Antibiotic	Anti-Cancer	Central Nervous System	Anthracyclines	NSAIDs	Anti-Cancer	Diabetes
Abacavir	Isoniazid	Flutamide	Divalproex Sodium	Daunorubicin	Ibuprofen	Tamoxifen	Pioglitazone
Zidovudine	Ketoconazole	Tamoxifen	Nefazodone	Doxorubicin	Celecoxib	Arsenic Trioxide	Rosiglitazone
Tenofovir			Valproic Acid	Epirubicin	Diclofenac		
Lamivudine				Idarubicin	Indomethacin		
Didanosine					Naproxen		

Drug-induced alterations in mitochondria can lead to a wide range of adverse events, including fatigue, muscle weakness, cognitive impairment, and changes in mood. These are worsened with many pathological states including cancer[32], sepsis[33], obesity[34], and diabetes. In severe cases, drug-induced mitochondrial dysfunction can lead to organ failure and death. It has also been found that some of drugs used in the treatment of these conditions can themselves impair mitochondrial function. More often than market withdrawal, adverse events prompt a Black Box Warning from the FDA, for inclusion in the patient insert[25]. Although often triggered by toxicity to liver, heart, kidney, or central nervous system, other mechanisms such as drug–drug interactions also elicit such warnings. Some examples of these are included in **Table 1-2**. Importantly, mitochondrial toxicity has been described for many members of different drug classes.

In summary, there is sufficient evidence that numerous medications induce alterations mitochondrial function and metabolism that result in adverse effects or toxicities. Knowledge of these adverse drug-mitochondrial interactions may inform the design of prospective testing and guide clinicians in identifying "at risk" patients. This study sought to identify candidate

mitochondrial metabolites that could be used to identify individuals at increased risk of mitochondrial-related ADRs.

1.3 Biomarkers of Adverse Drug Reactions

There has been significant recent interest and progress in the development of new, robust biomarkers for the prediction, diagnosis, and prognostication of ADRs. This includes the use of predictive genomic biomarkers to reduce the burden of ADRs and biomarkers that can be used to modify drug dose. For example, the use of thiopurine methyltransferase (TPMT) testing before prescribing azathioprine. Azathioprine is a potentially effective medicine in the treatment of inflammatory diseases, but its use is restricted by dose limiting ADRs. Testing for TPMT deficiency (enzyme responsible for the metabolism of azathioprine) has allowed for the reduction of life-threating ADRs like myelosuppression or bone marrow suppression[35]. Another example is the use of human leukocyte antigen (HLA) biomarkers which are used to identify susceptibility to immune-mediated injuries to major organs such as skin, liver, and bone marrow from a variety of drugs[36].

Another example of the potential use of biomarkers is represented by the carnitine pool, comprised of L-carnitine and its acylated fatty-acid derivatives (ACs). The carnitine pool is recognized for facilitating FAO in mitochondria and peroxisomes[37,38]. The carnitine pool represents a group of mitochondrial-derived metabolites, the blood concentrations of which generally reflect disorders of long-chain FAO, also known as the inborn errors of metabolism[39–41]. The Health Resources and Services Administration of the U.S. Department of Health and Human Services recommends that neonatal screening includes tests for inborn errors of metabolism such as L-carnitine uptake or transport defects. L-carnitine and acylcarnitine levels are not routinely

measured outside of this neonatal screening. Consequently, the utility of L-carnitine and acylcarnitines as metabolic biomarkers has predominately centered around the implications of this screening and the significant clinical impact of found defects.[42,43] This information supports the idea of considering mitochondrial metabolites as potential probes of mitochondrial metabolism health.

1.3.1 Mitochondria Metabolites

As discussed, mitochondria are essential organelles that coordinate several metabolic processes producing metabolites required for cell survival and proliferation. Within these complex functions, mitochondria evolved several mechanisms of communication with the host, some of which are critical for cell homeostasis. Some of the most important correspondence occurs via mitochondrial metabolites. Mitochondrial metabolites, such as ATP and nicotinamide adenine dinucleotide (NADH), are molecules that are involved in the energy metabolism within cells. They are produced and consumed by the mitochondria as part of the process of cellular respiration, which generates the energy required for various cellular functions. There is a growing interest in the use of mitochondrial metabolites as biomarkers of various diseases and conditions, including ADRs. Studies have suggested that changes in the levels of these molecules may be associated with ADRs, and that they may be useful for identifying at risk patients or for monitoring the progression of the disease. For example, some studies have found that changes in the levels of ATP, NADH and nicotinamide adenine dinucleotide phosphate (NADPH) may be associated with drug-induced liver injury and other forms of toxicity[44]. Additionally, research has suggested that blood levels of certain mitochondrial metabolites may be associated with the development of drug-induced mitochondrial alterations, which can lead to a variety of adverse effects.

More recently, it has become clear that mitochondrial metabolites, normally considered as intermediates of energy generation, can act as signaling molecules by promoting regulatory post-translational modifications on proteins, or via affecting chromatin structure and function.[45,46] This role of mitochondrial metabolites is the basis for considering them as potential biomarkers or probes of mitochondrial metabolism alterations. As discussed, mitochondrial impairment by drugs may involve interference with many different pathways and mechanism, all of which can lead to ADRs. Thus, identifying specific metabolites of mitochondrial metabolism alterations can help stratify patients at risk of mitochondrial toxicity and potentially avoid downstream ADRs.

1.4 L-Carnitine

L-carnitine **(Figure 1-2)** is a small, polar molecule de novo synthesized from two amino acids, lysine and methionine, but is largely acquired by dietary intake of animal products such as red meats, dairy, poultry, and fish[37,47]. The carnitine pool mostly resides in the skeletal muscle but is also found in the blood, liver, kidney, brain, and heart. The plasma and tissue concentrations are heavily conserved, which allows for the detection of small perturbations. The normal plasma levels consist of 83% L-carnitine and 17% acylcarnitines, with acetylcarnitine representing 75% of the acylcarnitines[37]. L-Carnitine is eliminated from the body mainly via urinary excretion. The compounds are highly regulated through reabsorption (98%) in the renal tubules and distribution in the tissues via the sodium-dependent organic cation/carnitine transporter (OCTN) family[37,38]. L-carnitine is FDA approved for the treatment of carnitine deficiency and is available as an intravenous (IV) formulation (Carnitor®, Leadiant Biosciences, Gaithersburg, MD USA).

L-carnitine Acetylcarnitine

Figure 1-2 L-carnitine and its most abundant metabolite, acetylcarnitine

1.4.1 L-carnitine function and metabolism

L-carnitine's primary function is to facilitate the transport of fatty acids across the inner mitochondrial membrane, making them available for mitochondrial β-oxidation **(Figure 1-3)**[40,48]. In the carnitine shuttle system long-chain fatty acids (LCFA) are imported into the mitochondria for oxidation. LCFA are converted to acyl-CoA via acyl-CoA synthase. Then the enzyme, carnitine palmitoyltransferase (CPT) 1 produces acylcarnitines from acyl-CoA and free carnitine. Carnitine-acylcarnitine translocase (CACT) moves acylcarnitine across the inner mitochondrial membrane as carnitine is exported out. CPT2 converts the acylcarnitine back into acyl-CoA and free carnitine. Acyl-CoA is then available for β-oxidation that produces one molecule of acetyl-CoA per cycle of oxidation, which enters the TCA cycle. The cycle provides the necessary electron donors to feed into the ETC, thus powering oxidative phosphorylation.

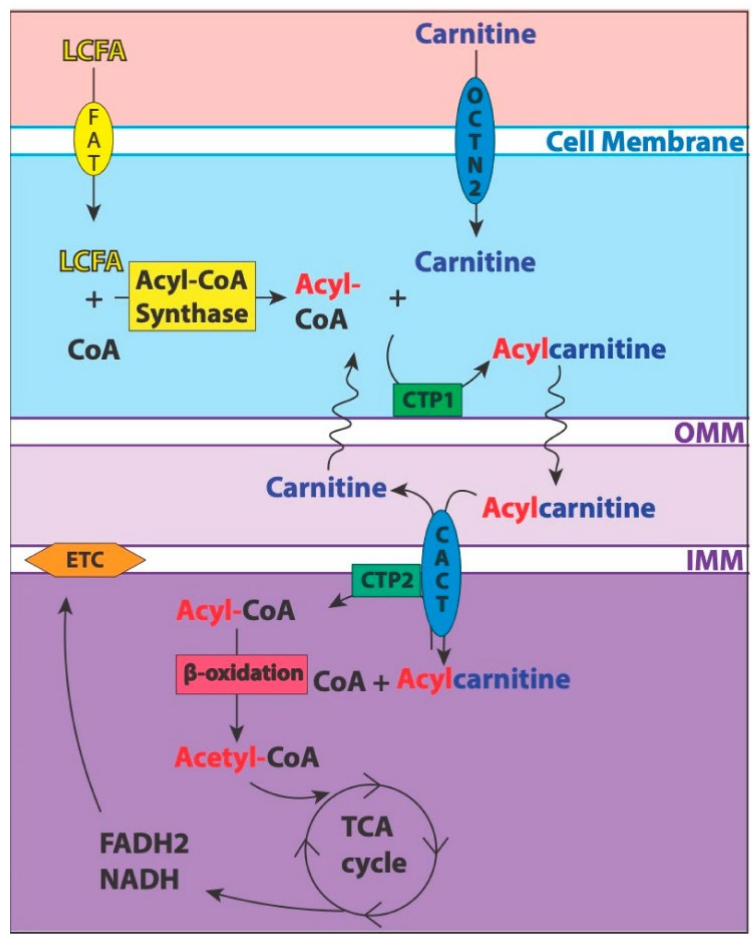

Figure 1-3 The Carnitine Shuttle. The figure highlights the major components of the carnitine shuttle system used to import long-chain fatty acids (LCFA) into the mitochondria for oxidation. LCFA are converted to acyl-CoA via acyl-CoA synthase. Then the enzyme, carnitine palmitoyltransferase (CPT) 1 produces acylcarnitines from acyl-CoA and free carnitine. Carnitine-acylcarnitine translocase (CACT) moves acylcarnitine across the IMM as carnitine is exported out. CPT2 converts the acylcarnitine back into acyl-CoA and free carnitine. Acyl-CoA is then available for β-oxidation that produces 1 molecule of acetyl-CoA per cycle of oxidation, which enters the TCA cycle. The cycle provides the necessary electron donors to feed into the electron transport chain (ETC), thus powering oxidative phosphorylation.

The total body carnitine pool is comprised of L-carnitine, acetylcarnitine, short-chain (C3–C5), medium-chain (C6–C12), and long-chain (C14–C20) acylcarnitines[37]. Alterations in blood concentrations of L-carnitine and acylcarnitines are usually reported as changes from baseline, or as a ratio between the summed value of all measured acylcarnitines (or individual acylcarnitines) to free L-carnitine. Since L-carnitine is much higher in abundance relative to the acylcarnitines,

an acetylcarnitine/L-carnitine ratio in the blood exceeding 0.4 is thought to represent disturbed mitochondrial metabolism[37]. Additionally, ratios of ACs and other metabolites, such as free fatty acids, have also been used to assess different metabolic pathways[47].

Blood concentrations of acetylcarnitine reflect intracellular levels and the regulation of acetyl-CoA and free CoA via carnitine acetyl-CoA transferase[37,49]. Increased or decrease in production of acetyl-CoA represents a critical mechanism for buffering the metabolic status between fed (glucose oxidation) and fasted (fat oxidation) states, referred to as metabolic flexibility[50]. Therefore, persistent changes in blood concentrations of acetylcarnitine over time may represent a signal of metabolic inflexibility.

1.5 Clofazimine

Clofazimine (**Figure 1-4**) is a weakly basic, very lipophilic, phenazine compound that is FDA approved antibiotic[51]. It has been used effectively against leprosy for over 40 years, curing over 14 million people worldwide, which makes it to be a part of World Health Organization's list of essential medications[52]. CFZ is highly lipophilic and is characterized by an unusually long elimination half-life (up to 70 days), which is associated with extensive accumulation of the drug in the body[53,54]. The major side effect of orally administered CFZ is a strong red skin pigmentation, observed in more than 94% of patients, as well as the formation of intracellular crystal-like drug inclusions (CLDIs) inside macrophages. The formation of these CLDIs leads macrophages to produce multiple membranes to sequester these CLDIs[55]. Patients may also experience ichthyosis or gastrointestinal symptoms, but at the normal dosage of 50 to 100 mg/day, no other major side effects have been reported[56,57].

While CFZ is well tolerated, in addition to its adverse impact on mitochondrial function[58], it imposes considerable metabolic stress on the host secondary to induction of a catabolic state[59–61].

For these reasons CFZ was selected as the mito-toxic drug of choice to evaluate the potential use of a mitochondrial metabolite as a probe of mitochondrial metabolic health.

Figure 1-4 Clofazimine

1.6 Rationale

Significant advances in medical sciences have been made but ADRs still represent a major healthcare concern for individual patients and clinicians as well as an economic burden on hospitals and pharmaceutical companies attempting to develop new drug candidates. ADRs are an inherent risk of any drug therapy regimen, each of which is differentially governed by specific factors such as genotype and health status of a patient, the compound's pharmacokinetic parameters (e.g., volume of distribution, clearance, half-life, etc.), dose and frequency of administration. Recently, mitochondrial metabolites have become of study interest because of their potential use as signaling molecules for mitochondrial health. From both a patient and healthcare perspective, there are potential benefits in identifying mitochondria-specific metabolites that could be clinically employed to probe and assess mitochondrial related alterations in metabolism.

1.7 Central Hypothesis

The mechanisms mediating the pharmacokinetics and disposition of mitochondrial substrates and metabolites can be used to identify individual's increased toxicity risk specifically due to mitochondrial dysfunction caused by adverse drug reactions.

1.8 Specific Aims

1.8.1 Specific Aim 1

Specific aim 1 looked to apply a database mining strategy to identify candidate mitochondrial metabolites that could be clinically employed to identify individuals at increased risk of mitochondrial-related ADRs.

1.8.2 Specific Aim 2

Specific Aim 2 looked to demonstrate how an L-carnitine challenge can be useful as a metabolic stress biomarker to identify individuals at increased risk of mitochondrial-related ADRs.

1.8.3 Specific Aim 3

Establish differences between male and female CFZ treated mice and associated interactions with L-carnitine.

1.9 References

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Chapter 2

Database Screening as a Strategy to Identify Endogenous Candidate Metabolites to Probe and Assess Mitochondrial Drug Toxicity

Part of this chapter was adapted from the following publication:

McCann, M. R.; **George De la Rosa, M. V.**; Rosania, G. R.; Stringer, K. A. L-Carnitine and Acylcarnitines: Mitochondrial Biomarkers for Precision Medicine. *Metabolites* **2021**, *11* (51). https://doi.org/10.3390/metabo11010051.

2.1 Abstract

Adverse drug reactions (ADRs) are considered an inherent risk of medication use, and some ADRs have been associated with off-target drug interactions with mitochondria. Metabolites that reflect mitochondrial function may help identify patients at risk of mitochondrial toxicity. We employed a database screening strategy with an *a priori* established criteria to identify candidate mitochondrial metabolites that could be clinically useful to identify individuals at increased risk of mitochondrial-related ADRs. Four publicly available databases were used and allowed for a systematic process of elimination, which led to a concise list of clinically feasible human metabolite candidates for use as functional tracers of mitochondrial metabolic health. L-carnitine, its acetylated metabolite, acetylcarnitine and other acylcarnitines are mitochondrial biomarkers used to detect inborn errors of metabolism. The identification of L-carnitine as the candidate mitochondrial metabolite supports the usefulness of a database strategy for the discovery of candidate metabolite biomarkers of drug toxicity.

2.2 Introduction

Adverse drug reactions (ADRs) often result from drug effects unrelated to the drug's primary mechanism of action. Even though ADRs are considered an inherent risk of medication use, they can have detrimental consequences to patients and pose a major burden on the United States healthcare system[1–3]. Most drugs are designed to directly activate or inhibit specific molecular targets involved in pathological processes, yet many ADRs have been found to be toxicological manifestations of off-target drug interactions affecting the structure and function of cellular organelles, such as mitochondria[4–6]. Healthy, fully functional mitochondria are vital for metabolic homeostasis because they are essential for both energy metabolism and the management of catabolic and anabolic processes; they also participate in a variety of signaling pathways[7–9]. Presently, there are no clinically utilized specific biomarkers of mitochondrial toxicity, and such testing is not required by the FDA as part of the drug approval process[10,11]. This is the case despite that numerous investigations have shown that mitochondrial dysfunction is a major mechanism of drug-induced injury[11,12].

Nevertheless, a number of studies have demonstrated gene-mitochondrial disease associations that have led to the development of early clinical diagnosis assays which are used for routine newborn testing to identify inborn errors in metabolism[13,14]. Existing databases of centralized information, such as Gene Expression Omnibus (GEO), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG), could be used to accelerate and improve the discovery of potential targets, biomarkers, and tracers that could aid in the assessment of mitochondrial-related ADRs[15–17]. The strategy of database screening has been used in various fields of study including to identify cancer-associated gene products and has resulted in the discovery of a prostate cancer-related gene, PAGE-1/GAGE-B19, a Ewing's sarcoma-associated

gene, XAGE-1[18], and a number of differentially expressed transcripts in breast cancer[19] and glioblastoma multiforme[20]. It has also been valuable for the identification of biomarkers of different diseases such as endometriosis[21]. However, these strategies have not been used to identify candidate metabolic biomarkers of mitochondrial-related ADRs.

To address this need, we developed a well-defined methodological approach to identify mitochondria-specific metabolites that could be clinically employed to probe and assess mitochondrial drug toxicity. We proceeded to identify a candidate metabolic biomarker that met all *a priori* criteria, which would be subsequently followed by *in vivo* evaluation in a mouse model to corroborate the database screening algorithm and establish its feasibility for clinical use.

2.3 Methods:

2.3.1 Database screening methodology

The study was carried out following an *a priori* algorithm (**Figure 2-1**) which was specifically designed to identify mitochondrial-related metabolites that could be clinically employed to probe and assess mitochondrial drug toxicity and direct the development of an in vivo and clinically-relevant "challenge test". A set of criteria was established for each step of the workflow and each database that was screened; candidate metabolites were included or excluded from further consideration based on these criteria. For the methodology, we used four different databases in sequential order for the initial portion of the analysis: The National Center for Biotechnology Information's (NCBI) Gene database (https://www.ncbi.nlm.nih.gov/gene), The Braunschweig Enzyme Database (BRENDA) (https://www.brenda-enzymes.org/), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/), and the Universal Protein Resource (UNIPROT) database (https://www.uniprot.org/). The resulting

candidates were then subjected to evaluation by three criteria that were assessed using Drug Bank (https://go.drugbank.com/), the FDA Orange Book (https://www.accessdata.fda.gov/scripts/cder/ob/index.cfm), and the United States Pharmacopeia (USP; https://www.usp.org/). The rationale for the use of each database, the sequence of evaluation and the inclusion/exclusion criteria for each candidate are outlined in the following section.

2.3.2 The National Center for Biotechnology Information's (NCBI) Gene Database

The NCBI Gene database was selected as the starting point for our strategic database screening methodology because it integrates gene-specific information from multiple data sources which includes, NCBI Reference Sequence (RefSeq), organism databases and Gene Ontology. This allowed access to many different types of gene-specific data connected to the record. Mitochondrial genetic information has been previously used to establish causes of diseases, for example associations have been observed between mitochondrial haplogroups, a population that shares similar mtDNA sequence changes or polymorphism[22], and predisposition to Parkinson's disease[23], multiple sclerosis[24], Leber hereditary optic neuropathy[25], type 1 and type 2 diabetes, cardiomyopathies and stroke-like episodes[26]. To search this database, the word "mitochondrial" was used as the initial search word, since the focus of our investigation was mitochondrial related metabolites. All non-mitochondrial related genes were excluded for the next steps. The NCBI Gene database also collects taxonomic group information from the NCBI Taxonomy database[27]. Each of the identified mitochondrial genes were categorized based on taxonomic domain, (eukaryotes, bacteria, viruses, and archaea), using the taxonomy tool, to identify genes exclusively present in eukaryotes. It was important to make this distinction because the focus of the work was on mitochondrial genes and mitochondria are known to be

present only in eukaryotic cells. Duplicates (the same gene present in different organisms) were removed from the dataset.

2.3.3 BRENDA Database: Substrates and Products of Eukaryote Mitochondrial Genes

BRENDA is a comprehensive relational database on functional and molecular information of enzymes, based on the primary literature[28]. BRENDA is an important tool for biochemical and medical research because it covers information on properties of all classified enzymes, including data on substrates/products, structure, and stability. BRENDA was used in the second step of the methodology to search for the substrate and products information of the identified mitochondrial genes. This step was taken to eliminate any protein structural components and focus on the substrate and products (e.g., metabolites).

2.3.4 KEGG Database: Separations of Small Molecules and Structural Components

KEGG was used as the third step to categorize the identified metabolites as small molecules using a molecular weight cutoff of ≤ 1000 daltons. Small molecules were of interest since the inner mitochondrial membrane favors uncharged, low molecular weight and lipophilic substances.

2.3.5 UNIProt Database: Specific Gene Location

The last step in the workflow was to verify whether transformation of the remaining candidate metabolites was exclusively mediated by mitochondria. To confirm this, the UNIPROT database was used. This database provides a resource for protein sequences and functional information. To search the gene location, we used gene names in the UNIPROT search tab. As a result, the gene location was explained at the subcellular level and the organelles where the gene was present, highlighted in yellow. The gene products information was also

verified with proper substrates, products and enzyme name, reaction, and pathways information in detail. Only genes that were exclusively present in mitochondria were considered further.

2.3.6 Application of final criteria: Physiochemical and pharmacokinetic properties of selected compounds

The final metabolite candidates were evaluated for clinical applicability using the following criteria: 1) availability of peer-reviewed literature documenting physiologic blood concentrations and pharmacokinetic data in both mice and humans, 2) an FDA approved formulation for intravenous use and 3) a commercially available enzyme-linked immunosorbent assay or published LC/MS analysis protocol for quantification in blood. The databases used for the application of these criteria were: Drug Bank (https://go.drugbank.com/), the FDA Orange Book (https://www.accessdata.fda.gov/scripts/cder/ob/index.cfm) and the US Pharmacopeia (https://www.usp.org/). We needed this information to be able to perceive and measure any changes in the candidate metabolite levels during the challenge test. We needed the compound to be available as an FDA approved formulation, so it could be safely used and be in compliance with animal use and care guidelines. Even though there are many available assays and LC/MS protocols can be developed, having a commercially available assay or an established LC/MS protocol for the selected compound would also facilitate the development of the "challenge test".

2.3.7 Literature Evaluation

A comprehensive evaluation of published research was used to rank the relevance of the final selected compounds as biomarkers based on their known association with ADR's, mitochondrial function, and known animal and human physiological blood concentrations **(Table 2-1)**.

Table 2-1 Literature evaluation criteria for three finalist compounds

Compound	trimethylglycine	L-carnitine	quinol
Databases searched between 08-2021 and 01-2022.	PubMed (https://pubmed.ncbi.nlm.nih.gov/) Science Direct (https://www.sciencedirect.com/) Google Scholar (https://scholar.google.com/)		
Search Terms (identified from results of database search strategy)	"Trimethylglycine & mitochondria", "trimethylglycine" & adverse drug reaction", "trimethylglycine & biomarker", "trimethylglycine & pharmacokinetics", "betaine & mitochondria", "betaine" & adverse drug reaction", "betaine & biomarker" "betaine & pharmacokinetics"	"L-carnitine & mitochondria", "L-carnitine" & adverse drug reaction", "L-carnitine & biomarker" "L-carnitine & pharmacokinetics"	"quinol & mitochondria", "quinol" & adverse drug reaction", "quinol & biomarker" "quinol & pharmacokinetics", "hydroquinone & mitochondria", "hydroquinone" & adverse drug reaction", "hydroquinone & biomarker" "hydroquinone & pharmacokinetics"
Article Type	Research Articles, Reviews, Systematic reviews, Clinical Trials, Case reports, Technical Reports		
Species Included	Human, mice		
Language	English		
Year range	1980- present		

2.4 Results:

A systematic algorithm based on rational, *a priori* criteria was developed for discovering mitochondrial-specific, candidate metabolites **(Figure 2-1 and Table 2-2)**. Initially, 32,798 genes were identified by the NCBI Gene database; 30,652 were categorized as eukaryotes genes, 1,348 as bacterial, 521 as archaea and 277 as viral **(Figure 2-2)**. Only the 30,652 genes categorized as eukaryote were considered further. After screening for the "eukaryote" mitochondrial genes, use of the GeneID reduced the list of relevant mitochondrial genes from 30,652 to 2,682 which also accounted for the removal of duplicate entries. This step also

permitted the building of a dataset with a gene symbol, gene description and gene synonyms for all similar genes that were present in the original list.

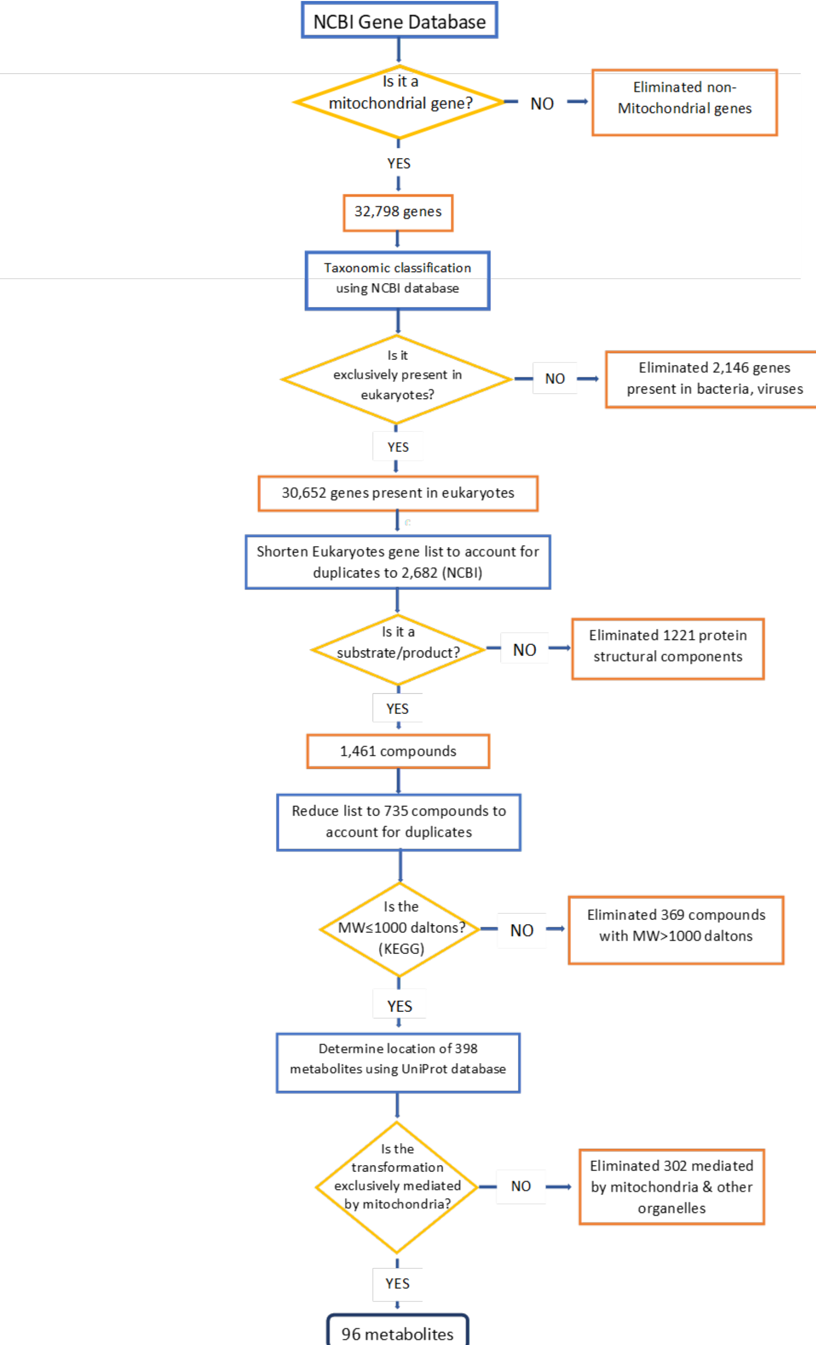


Figure 2-1 Methodology workflow diagram of the databases used to search for the candidate mitochondrial metabolites. The final 96 candidate metabolites were subjected to additional evaluation criteria which included assessment of physiochemical and pharmacokinetic properties and feasibility for clinical use. The workflow follows unified modeling language and includes the established a priori criteria used for each step.

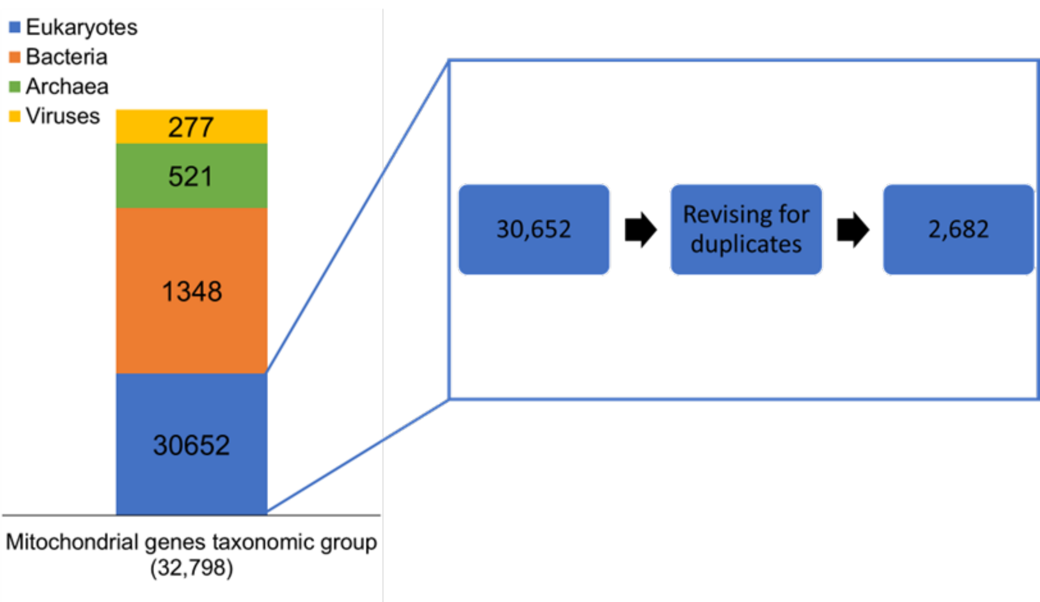


Figure 2-2 Mitochondrial genes taxonomy distribution. Data reduction was achieved by further categorizing genes as eukaryote, bacterial, archaea, or virus, the eukaryote genes of interest were assessed for redundancy based on the gene description and duplicates were removed.

Use of the BRENDA database identified the remaining 2,682 genes as products and substrates (1,461 compounds); the other 1,221 genes were eliminated from further consideration since they were found to be protein structural components. The 1,461 gene metabolites were combined into one data set and all the duplicate compounds, genes that were found to be encoded for the same enzyme and substrate/product, were removed. This step resulted in 735 metabolite candidates. We then used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to categorize metabolite candidates as small molecules (MW $\leq$ 1000 daltons) 24,25 which reduced the remaining 735 candidates to 398. From the 398 remaining candidate mitochondrial metabolites, the Universal Protein Resource (UNIPROT) database confirmed that 96 were exclusively mitochondrial metabolites. The results from each step of the database screening methodology and data reduction steps are summarized in **Figure 2-1**.

Table 2-2 Candidate Mitochondrial Metabolites Identified from Database Screening

Metabolite	HMDB ID*
(20R,22R)-20,22-dihydroxy-cholesterol	N/A
(2E)-2-enoyl-CoA	N/A
(2E,4E)-2,4-dienoyl-CoA	HMDB0301033
(2E,6E)-farnesyl diphosphate	HMDB0000961
(3R)-3-hydroxy-2-oxo-4-phosphooxybutanoate	HMDB0006801
(3S)-3-hydroxyacyl-CoA	N/A
(6S)-6beta-hydroxy-1,4,5,6-tetrahydronicotinamide-adenine dinucleotide phosphate	N/A
(E)-but-2-enoyl-CoA	HMDB0002009
(S)-1-pyrroline-5-carboxylate	HMDB0001301
[(1->3)-beta-D-glucosyl] n	N/A
1,2-diacyl-3-O-(beta-D-galactosyl)-sn-glycerol	N/A
1,2-diacyl-3-O-[alpha-D-galactosyl-(1->6)-beta-D-galactosyl]-sn-glycerol	N/A
2,3-dehydroacyl-CoA	N/A
2-acetolactate	HMDB0006833
2-dehydropantoate	HMDB0304068
2'-deoxyribonucleoside 5'-triphosphate	N/A
2-oxobutanoate	HMDB0000005
3-(methylsulfanyl)-L-aspartate89- [ribosomal protein S12]	N/A
3-methylcrotonoyl-CoA	HMDB0001493
3-methylglutaconyl-CoA	HMDB0001057
3-oxo acid	N/A
3-oxoacyl-CoA	HMDBP09209
3-phosphooxypyruvate	HMDB0001024
4 beta-D-galactopyranose	HMDB0246552
4-aminobutanoate	HMDB0000112
4-hydroxy-2-oxoglutarate	HMDB0002070
4-methylpentanal	HMDB0001318
4-phosphooxy-L-threonine	HMDB0006802
5-aminolevulinate (methyl 5-aminolevulinate)	HMDB0015127
5'-deoxyadenosine	HMDB0001983
acetyl-CoA	HMDB0001206
acylglycerol	HMDBP08704
acyl-sn-glycerol 3-phosphate	HMDBP04526
arachidonate	HMDB0001043
beta-amyrin	HMDB0249098

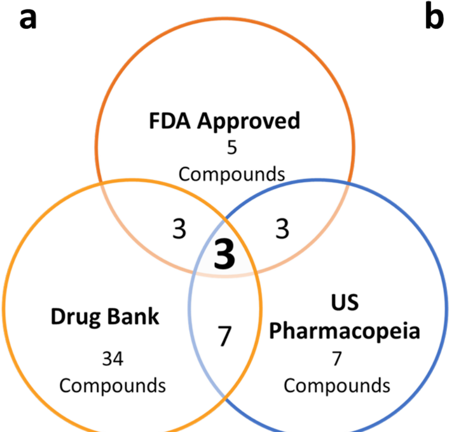
β-D-galactopyranosyl-(1-4)- β -D-galactopyranosyl-(1-6)- β-D-galactopyranosyl-(1-3)- β -D-galactopyranose	N/A
betaine aldehyde	HMDB0001252
butanoyl-CoA	HMDB0304279
cardiolipin	HMDB0010226
CDP-diacylglycerol	HMDB0006968
coenzyme Q10 (Ubidecarenone)	HMDB01072
coproporphyrinogen III	HMDB0001261
dolichyl beta-D-mannosyl phosphate	HMDB0012218
dolichyl phosphate	HMDB0006353
ferricytochrome c	HMDB0012947
FMNH2	HMDB0001142
glycerone phosphate	HMDB0001473
isopentenyl diphosphate	HMDB0001120
itaconate	HMDB0002092
L-ascorbate	HMDB0000044
L-carnitine	HMDB0000062
L-galactono-1,4-lactone	HMDB0304401
L-glutamate	HMDB0000148
L-glutamate 5-semialdehyde	HMDB0002104
L-methionine	HMDB0000696
L-ornithine	HMDB0000214
L-phenylalanine	HMDB0000159
L-Threonine	HMDB0000167
Margaroylglycine	HMDB0013246
O-acetyl-(*R*)-carnitine	N/A
O-acetylcarnitine	HMDB0000201
O-acetyl-L-serine	HMDB0003011
O-hexadecanoyl-(*R*)-carnitine	HMDB0000222
O-phospho-L-serine (Dexfosfoserine)	HMDB0000272
oxidized rubredoxin	N/A
phosphatidate	N/A
phosphatidylethanolamine	HMDB0008828
phosphatidylglycerol	HMDB0010570
phosphatidyl-L-serine	HMDB0014291
phosphocreatine	HMDB0001511
plantacyanin	HMDB0303586
polysulfide	N/A
pregnenolone	HMDB0000253

prostaglandin H2	HMDB0001381
protoheme IX	HMDB0003178
protoporphyrin IX	HMDB0000241
protoporphyrinogen IX	HMDB0001097
quinol	HMDB0002434
reduced coenzyme Q10	N/A
reduced rubredoxin	N/A
retinal	HMDB0001358
retinoate	HMDB0001852
sn-glycerol 3-phosphate (3-Phosphoglycerol)	HMDB0000126
succinate semialdehyde	HMDB0001259
succinyl-CoA	HMDB0001022
sulfur- (sulfur carrier)	N/A
tetrahydrofolate (Tetrahydrofolic acid)	HMDB0001846
Thymidine monophosphate	HMDB0001227
trans-2(or 3)-enoyl-CoA	HMDB0003944
trans-2,3-dehydroacyl-CoA	N/A
trimethylglycine	HMDB0000043
ubiquinol	HMDB0001304
ubiquinone	HMDB0001072
UDP-glucose	HMDB0000286

* Human Metabolome Database identification number

Of the final 96 compounds, seven were found in the US Pharmacopeia, 34 compounds were in Drug Bank and only five were found to be FDA approved for human use. Three of these compounds were in all three databases **(Figure 2-3a)**, which made them finalist candidates: trimethylglycine (betaine; HMDB0000043), L-carnitine (HMDB0000062), and quinol (hydroquinone; HMDB0002434; **Figure 2-3**). **Figure 2-3b** shows the physiochemical and pharmacokinetic properties of the three final compounds.

a



b

	Trimethylglycine	L-Carnitine	Quinol
Chemical Structure			
Molecular Weight (g/mol)	117.15	161.20	110.11
Human Metabolome Database (HMDB)	HMDB0000043	HMDB0000062	HMDB0002434
HBD	0	1	2
HBA	2	3	2
LogP	-4.9	-2.9	0.59
Vol of Distribution (L/kg)	1.3	0.3	–
Half-life (h)	14	17.4	18.7
Clearance (L/h)	0.084	4	–

Figure 2-3 Final metabolite candidates following application of a database screening methodology. a) Venn diagram shows the number of the final 96 compounds that were present in at least one of the three databases searched for physiochemical and pharmacokinetic properties: the FDA Orange Book (https://www.fda.gov/drugs/drug-approvals-and-databases/orange-book-data-files), Drug Bank (https://go.drugbank.com/) and the US Pharmacopeia (https://www.usp.org/). Only three compounds were present in all three databases. b) Table of relevant physicochemical and pharmacokinetic properties of the three final candidate metabolites. HBD = Hydrogen Bond Donor; HBA = Hydrogen Bond Acceptor; LogP represents the logarithm (base 10) of the partition coefficient (P), the ratio of the compound's organic (oil)-to-aqueous phase concentrations; Vol = volume. Chemical structures in Figure 2b were created in ChemDraw V20.0 (PerkinElmer Informatics, Waltham, MA USA).

Accordingly, L-carnitine was selected as the most clinically feasible candidate as it is available as an FDA approved formulation for intravenous injection (Carnitor®, Leadiant Biosciences, Gaithersburg, MD; USA). L-carnitine is converted to acetylcarnitine via carnitine acetyltransferase (EC 2.3.1.7) in mitochondria, acetylcarnitine is also the most abundant acylcarnitine, which made it the metabolite of choice for further testing as a metabolic tracer of mitochondrial metabolism.

2.5 Discussion

As reported herein, use of a database screening and process of elimination strategy, led to the identification of 96 candidate mitochondrial metabolites that could be measured in blood to provide useful readouts of drug-induced changes in mitochondrial function. Further selection

based on clinical relevance and *in vivo* evaluation revealed L-carnitine as a highly feasible candidate for clinical use based on *a priori* criteria. This result supported by literature evaluation, corroborated the usefulness of the database approach for strategically identifying those endogenous metabolites that could serve as candidate biomarkers for detecting changes in metabolism induced by drugs that target a specific organelle. Of noteworthy significance, the existing information that was publicly available in databases allowed for a systematic process of elimination, which led to a concise list of clinically feasible human metabolite candidates for use as functional tracers of mitochondrial metabolic health.

Mitochondria possess diverse structural components and functional features which can be targeted by a compound and lead to toxicity[29,30]. These interactions can be caused by different types of drugs and occur in different sites and pathways. These targets include inhibition of mitochondrial DNA (mtDNA) transcription of electron transport chain (ETC) complexes and enzymes required for any of the steps of glycolysis and β-oxidation, the depletion of L-carnitine and Coenzyme A (CoA), uncoupling of oxidative phosphorylation, and interference with one or more of the complexes in the respiratory chain, affecting the ETC. Early signals of many of these processes are difficult to detect in the blood.

While assessment of ADR-associated mitochondrial dysfunction is not currently part of the drug approval process, a blood assay that could be used to report drug-induced changes in mitochondrial function could be valuable. Mitochondria contain their own genome which can affect the probability of ADRs but the mitochondrial genome is not easily accessible in the clinical situation[30,31]. Rather, a circulating metabolite like L-carnitine, that could reflect the underlying cellular and molecular mechanisms that predisposes individuals to unintended perturbation of mitochondrial metabolic function by a medication, would be most clinically

achievable and feasible. To this end, the algorithm developed herein could be clinically used to probe and assess a range of different adverse drug effects on the metabolic status of other organelles in addition to mitochondria **(Figure 2-1 and Table 2-2).**

The identified three final compounds were all mitochondrial metabolites. To pinpoint the most ideal candidate, we assessed information about the pharmacokinetic and physiochemical properties from different databases **(Figure 2-3)**. L-carnitine is FDA approved for the treatment of L-carnitine deficiency and is available as an intravenous (IV) formulation (Carnitor®, Leadiant Biosciences, Gaithersburg, MD USA). Trimethylglycine, also known as betaine, is available as an FDA approved oral formulation (CYSTADANE®, Recordati Rare Diseases Inc, Lebanon, NJ, USA) that is used for the treatment of homocystinuria to decrease high homocysteine blood levels; currently, there is no IV formulation. As such, trimethylglycine was removed as a candidate. Quinol (hydroquinone) is an aromatic organic phenol which participates in the mitochondria Q cycle. This is the process by which the electrons are transferred from ubiquinol to cytochrome c, which results in the net movement of protons across the inner mitochondrial membrane[32]. Even though quinol has a critical role in the electron transport chain, as a therapeutic, it is used in combination with fluocinolone and tretinoin as an FDA-approved topical treatment for melasma; it is not available in a formulation for injection.

Having selected L-carnitine as the metabolite of choice and knowing that there are different mechanisms by which a drug can impact mitochondrial function; an extensive literature review was conducted focusing on drugs attributed to disruptions in mitochondria metabolism, specifically L-carnitine and acetylcarnitine. Severe impairment of mitochondrial fatty acid oxidation is associated with an accumulation of fatty acid derivatives such as acetylcarnitine, acylglycine esters, and dicarboxylic acids in plasma and urine[33]. Specifically, this literature

evaluation looked to further support the selection of L-carnitine as a result of the database screening strategy and focused on how the resulting changes in L-carnitine and/or acetylcarnitine levels after drug administration can be used as indicators of these metabolic disruptions, thereby providing a new method for therapeutic drug monitoring.

We focused on the six drugs (valproic acid (VPA), clofazimine (CFZ), zidovudine (ZDV), cisplatin (CSP), propofol and cyclosporine (CyA)) previously shown in **Table 1-1** that are known to cause alterations in the metabolic function of the mitochondria but are not representative of all drugs with known mitotoxic effects. The specific effects of these drugs on the L-carnitine pool are characterized and summarized in **Table 2-3**.

2.5.1 Valproic Acid

Valproic acid (VPA) is a widely used antiepileptic agent to treat myoclonic, atonic, and absence seizure disorders in children and adults[34,35]. VPA is generally well tolerated and safe relative to the conditions it is used to treat. Some adverse events include gastrointestinal disturbances, sedation, transient effects on coagulation, and hepatotoxicity[35]. VPA-induced hepatotoxicity can be characterized into four distinct subtypes: (1) a transient elevation in liver transaminases, (2) reversible hyperammonemia, (3) toxic hepatitis, and (4) a Reye-like syndrome[36,37]. The exact mechanism of VPA hepatotoxicity is unknown, but different theories have been proposed and three of these are related to VPA and L-Carnitine[37].

A serious consequence of VPA administration is the depletion of body L-carnitine storage. Since VPA is a short-chain fatty acid that requires L-carnitine for oxidation, it combines with L-carnitine within the mitochondria via carnitine acyltransferases. The resulting ester, valproylcarnitine, is then transported out of the mitochondria and eliminated in the urine, thus depleting body L-carnitine stores[37,38]. L-carnitine deficiency due to VPA is the proposed

mechanism for hyperammonemia and the development of VPA-induced hyperammonemic encephalopathy (VHE), as shown in **Figure 2-4**[39]. This occurs when the VPA metabolites trap mitochondrial free CoA so that L-carnitine cannot be restored through the action of CPT2. The reduction of L-carnitine from this mechanism then results in decreased β-oxidation of VPA and shifts the metabolism toward ω-oxidation, producing a toxic metabolite 2-propyl-4-pentenoic acid (4-en-VPA). This metabolite reduces ammonia elimination through inhibition of carbamoyl-phosphate synthase I, the first enzyme in the urea cycle, resulting in increased ammonia levels[40].

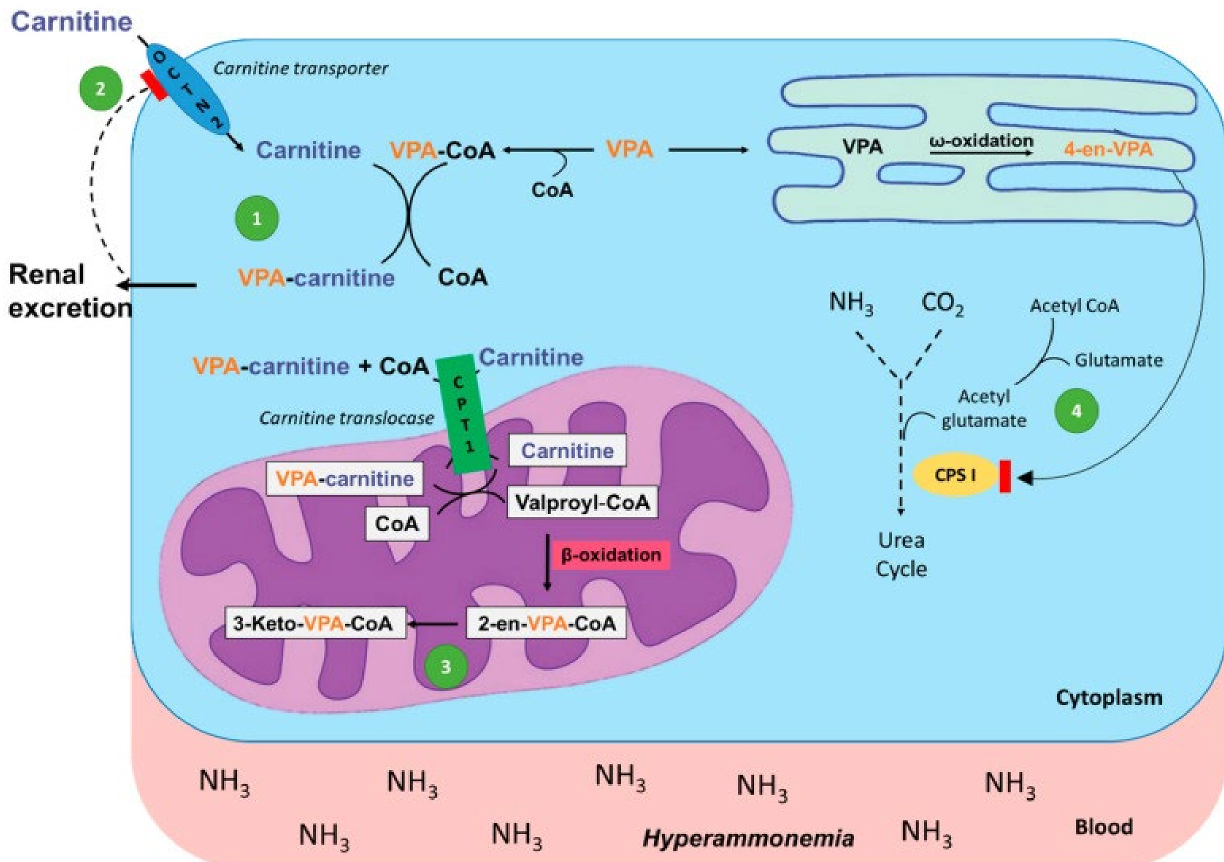


Figure 2-4 Mechanism of valproic acid (VPA)-induced hyperammonemia. 1) VPA is attached to coenzyme A (CoA) and transferred to LC creating VPA-carnitine, which is then renally excreted. 2) VPA-carnitine inhibits an ATP-dependent carnitine transporter, effectively blocking LC entry into the cell. 3) Other VPA metabolites, 2-en-VPA and 3-Keto-VPA, trap mitochondrial free CoA so that it cannot participate in ATP production, further inhibiting the ATP-dependent carnitine transporter. 4) The decrease in free CoA and LC results in decreased formation of N-acetylglutamate, which is a necessary co-factor

for carbamoylphosphate synthetase I (CPS I), the primary enzyme of the first steps of the urea cycle. This leads to the accumulation of ammonia as it can no longer be incorporated into urea and excreted, resulting in hyperammonemia.

There are several studies of the impact of VPA on L-carnitine levels both *in vitro* and *in vivo*[41–43]. One of these studies measured the concentration of L-carnitine in serum, red blood cells, muscle, liver, and urine in VPA-treated rats. The study found that the mean serum and muscle L-carnitine concentrations decreased and the mean pooled acylcarnitines concentration increased relative to control animals[42]. Another study found that after injecting mice with VPA, hepatic concentrations of free CoA and L-carnitine decreased, and the ratio of acetylcarnitine (AC)/L-carnitine (LC) increased[43]. The authors also observed significantly increased medium-chain acylcarnitines and decreased long-chain acylcarnitines in the plasma of the VPA treated mice. These changes in L-carnitine levels observed in rats and mice are in agreement with the aforementioned VPA mechanism that leads to L-carnitine depletion, and the consequential increase in the AC/LC ratio reflecting mitochondrial dysfunction.

In humans, a study by Opala et al., assessed the effect of VPA and other antiepileptic drug (OAD) treatments on the plasma L-carnitine pool in pediatric patients[37]. Both, the VPA monotherapy and polytherapy groups had significantly lower L-carnitine levels compared to the healthy controls. The AC/LC ratio was also higher than in the VPA monotherapy and polytherapy groups, when compared to the control subjects. The authors observed that the L-carnitine pool of the OAD (polytherapy with no VPA) group did not differ from the healthy subjects, which reinforce the theory that VPA depletes the L-carnitine pool.

It seems clear that VPA toxicity may be identified by measuring the disturbances of the L-carnitine pool. Unfortunately, L-carnitine and acetylcarnitines are not routinely measured, other than in neonatal screenings as previously stated. By having information about specific levels of the acylcarnitines and the AC/LC ratio could provide critically important information

and a more individual picture of each patient that could impact clinical decision-making about prescribing, dosing, and monitoring VPA. Including routine monitoring of L-carnitine and acetylcarnitines blood levels as part of the VPA treatment could serve as early indicators of ADRs and aid in the prescription of L-carnitine supplementation as a means to mitigate VPA-induced L-carnitine deficiency.

2.5.2 Clofazimine

Clofazimine (CFZ) is a FDA-approved, weakly basic, red-pigmented, phenazine antibiotic that is included in the WHO List of Essential Medications as part of the standard treatment for leprosy[44]. CFZ is highly lipophilic and is characterized by an unusually long pharmacokinetic half-life of up to 70 days, which is associated with extensive accumulation of the drug in the body[45,46]. While CFZ is well tolerated, it imposes a considerable metabolic burden and interferes with mitochondrial function[47–49].

In a study of the metabolic consequences of chronic (8-week) CFZ administration in mice, L-carnitine was found to be one of many metabolites associated with one-carbon metabolism that was altered by CFZ treatment[50]. Notably, L-carnitine was found to be highly elevated in the animals that exhibited the most severe metabolic disruption in response to drug treatment. Furthermore, L-carnitine was the only urine metabolite that decreased after 2 weeks of CFZ treatment and remained low in the metabolically stressed animals, one of the earliest detected changes in metabolism[50].

The CFZ-induced changes in L-carnitine levels can be explained, in part, by the requirement of methionine, lysine, and ascorbate for the de novo synthesis of L-carnitine, which is a primary source of L-carnitine in mice. Since CFZ treatment also caused a modest reduction in body weight despite an increase in the amount of food consumed (catabolism), disruption in

carnitine homeostasis could also have occurred by an enhanced utilization of fat stores for energy production.

CFZ has a known influence on mitochondrial metabolism because it accumulates within the organelle, so it is not unexpected that long-term exposure resulted in perturbed L-carnitine concentrations in mice. There are no known clinical studies of CFZ treatment and metabolism. However, the CFZ-induced perturbation in mice may be useful to better understand the mechanism of CFZ associated toxicity. Moreover, monitoring of L-carnitine levels in CFZ-treated patients may prove to be useful as an indicator of pending mitotoxic effects of the drug. This could be especially useful when initiating CFZ treatment and monitoring its therapeutic effect.

2.5.3 Zidovudine

Zidovudine (ZDV) is a nucleotide reverse transcriptase inhibitor (NRTI) used in the treatment of HIV as an integral component of highly active antiretroviral therapy[51]. The clinical utility of many NRTIs, like ZDV is constrained due to their association with ADRs during chronic therapy at high doses. They have been found to gradually reduce mitochondrial function in various tissues by preventing mitochondrial replication via inhibition of the polymerase that replicates mtDNA[11,51].

Several *in vitro* and *in vivo* studies have alluded to possible mechanisms that contribute to ZDV-induced myopathy through the impairment of skeletal muscle mitochondria. Data suggest that the mechanism of ZDV-induced mitochondrial toxicity may be caused by L-carnitine deficiency[51]. A ZDV-induced reduction in the amount of L-carnitine may be a major factor in mitochondrial alterations that lead to the accumulation of lipid droplets in the cytoplasm of muscle cells[52,53]. These findings have been corroborated in an *in vitro* study in which L-carnitine

treatment of C2C12 cells, a myoblastic cell line, prevented the dose-dependent ZDV-induced inhibition of cell growth[53]. These data suggest that lipid accumulation is due to depletion of L-carnitine rather than mtDNA depletion or other forms of mitochondrial dysfunction. The study also investigated the mechanism by which ZDV treatment leads to cellular reduction in L-carnitine. It was shown that ZDV reduced the transport of L-carnitine across the plasma membrane by noncompetitive inhibition of the sodium-dependent transport of L-carnitine. These data suggest that ZDV may directly interact with OCTN2, the primary L-carnitine transporter, interfering with the necessary renal reabsorption of L-carnitine. This mechanism would potentiate total body L-carnitine deficiency and warrants further pharmacometabolomic study to confirm.

A clinical study of patients with ZDV-induced myopathic symptoms of varying severity concluded that ZDV-induced muscle mitochondrial impairment resulted in a reduction in muscle L-carnitine levels, most likely due to a decrease in L-carnitine uptake by the muscle[52]. There are two possible explanations for the decrease of L-carnitine in the muscle: (1) reduced energy within the muscle cell can cause a shift toward the glycolytic pathway that results in excess cytoplasmic acetyl-CoA. This may esterify L-carnitine, which is then exported out of the muscle and is eliminated in the urine. The other being (2) muscle uptake of L-carnitine from the blood is compromised by a shortage of energy within the cytosol due to deficiency of the mitochondrial respiratory chain enzymes, cytochrome c oxidase and reductase[52]. Both scenarios reveal that reduced muscle L-carnitine caused by mitochondrial impairment results in a shortage of energy of the muscle fibers, which manifest clinically in different degrees of myopathy and muscle weakness.

In aggregate, studies to date demonstrate that ZDV causes dysregulation of the L-carnitine pool leading to reduced intracellular levels. The studies above focused on skeletal muscle, but this dysregulation in L-carnitine metabolism may be found in other tissues and cause other ZDV-induced ADRs such as hepatic lipid accumulation (hepatic steatosis). This poses a major clinical concern for a medically vulnerable population such as HIV patients. Tracking the blood L-carnitine pool, and additionally muscle L-carnitine levels, may be an important monitoring parameter. It would permit the early identification of metabolic descent before it presents clinically, as myopathy, a known ADR of ZDV. This could also lead to the early use of L-carnitine supplementation to ameliorate myopathy symptoms.

2.5.4 Cisplatin

Cisplatin (CSP) is an effective anticancer drug used for the treatment of testicular, lung, and ovarian cancers. Although it is well-tolerated, the clinical use is limited by its side effects; nephrotoxicity, cytotoxicity, and cardiomyopathy[54]. In recent years, several studies have demonstrated that CSP-induced cytotoxicity is closely related to increased reactive oxygen species generation and that this increase alters the mitochondrial membrane potential and damages the respiratory chain, which ultimately triggers the apoptotic process[55–57].

It is well documented that CSP therapy is associated with increased excretion of a number of vital endogenous substances including L-carnitine[58,59]. A study of CSP on plasma L-carnitine concentration and urinary excretion showed a 30% increase in total L-carnitine plasma concentration upon treatment that normalized within 7 days after cessation of treatment[58]. These changes were associated with a tenfold increase in renal L-carnitine excretion. Similar findings were made in other studies, one of which was conducted in pediatric cancer patients[59,60]. In the pediatric cohort, there was a strong inverse correlation between fatigue and blood L-carnitine

levels after a week of chemotherapy. In aggregate, CSP-induced derangement in L-carnitine was attributed to a combination of cytotoxicity that caused a release of L-carnitine from damaged tissues, and impaired renal reabsorption mechanisms, as well as inhibition of the cellular uptake of L-carnitine caused by a change in OCTN2 activity.

The use of CSP is well known for its associated toxicities, some of which have dose-limiting implications. The use of pharmacometabolomics as an approach to monitor changes in the L-carnitine pool could potentially help bring about a precision medicine strategy to guide CSP dosing or even therapeutic changes, especially when attempting to control fatigue after chemotherapy. Longitudinal monitoring of urine LC levels could aid in the proper use of LC supplementation to reduce ADR severity.

2.5.5 Propofol

Propofol (2,6-diisopropylphenol) is a c-aminobutyric acid (GABA) receptor agonist that is supplied as an injectable lipid emulsion for intravenous use. It has become the most commonly used intravenous anesthetic due to its favorable pharmacokinetic and pharmacodynamic profile[61,62]. Propofol has been associated with a number of serious ADRs such as metabolic acidosis, cardiac asystole, myocardial failure, rhabdomyolysis, and death[63–65]. Propofol infusion syndrome (PRIS) is defined as metabolic acidosis (lactic acidosis) with a base deficit > 10 mmol/L in at least 1 occasion, arrhythmias, heart failure, renal insufficiency, hepatomegaly, and rhabdomyolysis following the infusion of propofol[66,67].

There is *in vitro* evidence that suggests impaired mitochondrial function is a mechanism by which PRIS syndrome occurs[66,68–70]. Studies on isolated rat liver mitochondria have demonstrated an uncoupling type of effect of propofol on mitochondrial function, which may be the result of an increase in proton permeability of the inner mitochondrial membrane[71,72].

Another study suggests that ATP production may be impaired by propofol[73]. Propofol was also found to impair the mitochondrial ETC in isolated heart samples from guinea pigs[69].

When looking at the effects of propofol and PRIS in humans, various pediatric case studies have shown changes in serum and plasma levels of acylcarnitines. A study of a child sedated with propofol reported raised serum levels of C3-DC, C5, creatine kinase, troponin T, triglyceride, lactate, and myoglobinaemia[67]. Propofol caused a disruption of mitochondrial fatty-acid oxidation and long-term infusion was associated with an increase in C3-DC, which inhibits CPT I, a critical enzyme involved in the L-carnitine shuttle. Consequently, the entry of long-chain acylcarnitine esters in muscle tissue is impaired. Similarly, a case study with a propofol sedated patient that developed various ADRs exhibited abnormality in acylcarnitine metabolism[74]. Plasma samples revealed increased levels of acetyl and hydroxyl-butyryl species, an elevation of fatty acylcarnitine intermediates, especially medium-chain unsaturated and dicarboxylic species. In a third case study, where propofol infusion was administered to treat recurrent seizures, daily plasma samples showed that as the propofol infusion continued daily, C4 rose significantly above the normal range (1.0 µmol/L)[75].

In the above-mentioned cases, it is hypothesized that propofol interferes with the diffusion of medium- and short-chain fatty acids into the mitochondria and inhibits the respiratory chain (at complex II), resulting in a rise in blood levels of C5, C4, and/or C2[74,75]. Based on these studies, elevated C2, C3-DC, C4, or C5 imply impaired metabolism of branched chain amino acids (BCAA) and/or metabolic inflexibility, therefore, supporting their use as potential early biomarkers of the onset of PRIS that could be incorporating into a therapeutic monitoring strategy for propofol[67,68,74,75].

2.5.6 Cyclosporine

Cyclosporine (CyA) is used as an immunosuppressive agent following organ transplantation and as treatment of several autoimmune diseases[76]. Its use is limited due to its associated side effects, especially nephrotoxicity but it remains widely used as an immunosuppressant. Animal studies suggest that ROS is implicated in chronic CyA nephrotoxicity. CyA increases ROS generation and lipid peroxidation in renal tissue affecting renal function and favoring interstitial fibrosis[77]. CyA produces an increase in ROS within the mitochondria, leading to inner membrane cardiolipin oxidation and impairment of the membrane potential[77].

A study of long-term (20 weeks) CyA treatment assessing urine, blood, liver, kidney, and pancreatic concentrations of acid-soluble L-carnitine in diabetic rats found that CyA treatment caused changes in L-carnitine and myo-inositol concentrations in biologic fluids and certain tissues[78]. Diabetic rats excreted significantly higher concentrations of L-carnitine in the urine, except when treated with CyA. CyA prevented the urinary loss of L-carnitine, which caused a decrease in the measured levels of L-carnitine in urine. Similarly, CyA treatment significantly decreased L-carnitine levels in the blood. Conversely, CyA treatment resulted in an increase on the hepatic concentration of L-carnitine but did not affect the L-carnitine levels in the pancreas or kidney. A study with cadaveric kidney transplant patients being administered either azathioprine (AZA) or CyA showed significantly elevated levels of total L-carnitine, and short-chain acylcarnitines in serum when patients were treated with CyA and prednisone and compared to AZA-treated[79].

Treatment with CyA induced changes to L-carnitine and short-chain acylcarnitines, which may suggest a drug-induced effect on the reabsorption and/or BCAA metabolism. While future studies are warranted to interrogate the mechanisms to pinpoint the specific metabolic

pathways involved, measurements of L-carnitine and short-chain acylcarnitines could provide information on the status of mitochondrial function of CyA-treated patients.

Table 2-3 Drugs that influence L-carnitine and acylcarnitines levels in the body

Drug	Proposed Mechanism	Subject	Biospecimen	Drug-induced Alterations in Carnitine/Acylcarnitine Levels
Valproic Acid	• VPA depletes LC by forming valproylcarnitine that is renally eliminated	1. Rats 2. Mice 3. Humans (Pediatric)	1. Serum, muscle, and urine 2. Whole liver 3. Plasma	1. Decreased LC (serum, muscle); increased pooled ACs and AC/LC ratio (serum, muscle); increased ACs (urine) 2. Decreased LC; increased AC/LC ratio 3. Decreased LC, increased AC/LC ratio
Clofazimine	• CFZ stimulates FAO, decreases urine excretion of LC precursors (methionine, ascorbate)	1. Mice	1. Urine, whole blood	1. Decreased LC (urine); increased LC (whole blood)
Zidovudine	• Non-competitively inhibits OCTN2, the primary carnitine transporter • Disruptions to mitochondrial respiratory enzymes	1. C2C12 cells 2. Humans	1. Cells 2. Muscle biopsy	1. Decreased LC 2. Decreased LC
Cisplatin	• Cytotoxicity → cell death → release of LC into blood • Impaired uptake and reabsorption via OCTN2	1. Humans (Various cancers) 2. Humans (Various cancers)	1. Plasma, urine 2. Serum, urine	1. Increased LC (plasma); increased renal excretion of LC (urine) 2. Increased LC (serum, urine)
Propofol	• Disruptions to the mitochondrial ETC • Increased permeability of IMM • Inhibition of respiratory complexes	1. Humans 2. Humans 3. Humans	1. Serum 2. Plasma 3. Plasma	1. Increased C3-DC, C5 2. Increased MCAC 3. Increased C4

Cyclosporine	• CyA induced nephrotoxicity impacts carnitine reabsorption • ROS generation impairs mitochondrial membrane potential	1. Rats 2. Humans	1. Urine, blood, liver, kidney, pancreas 2. Serum	1. Decreased LC (urine, blood); increased LC (liver); no change (kidney, pancreas) 2. Increased LC, SCAC

SCAC= short-chain acylcarnitines, MCAC= medium-chain acylcarnitines, LCAC= long-chain acylcarnitines

2.6 Conclusion

This study, using a database search strategy, aimed to identify candidate mitochondrial metabolites that could be clinically useful to identify individuals at increased risk of mitochondrial-related ADRs. Mitochondrial impairment by drugs may involve interference with many different pathways and mechanisms, all of which can lead to unexpected ADRs. The results of the database screening and process of elimination strategy pointed to L-carnitine as the principal candidate mitochondrial metabolite. Targeted measurements of carnitine and acetylcarnitine may be informative for the identification of metabolic ADRs of drug-induced mitochondrial dysfunction during treatment with a number of different drugs including the examples discussed in this chapter. Drug-induced mitotoxic signals that could be detected in advance of a clinical phenotype may be particularly useful for therapeutic drug monitoring and the avoidance of ADRs.

2.7 References

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Chapter 3

L-Carnitine Challenge as a Metabolic Stress Biomarker to Identify Risk of Mitochondrial-Related ADRs

3.1 Abstract

We identified L-carnitine as a candidate metabolite from a database screening strategy employed to find mitochondrial metabolites that could be clinically useful to risk stratify individuals for mitochondrial-related ADRs. L-carnitine and its acetylated metabolite, acetylcarnitine and other acylcarnitines have been used to detect inborn errors of metabolism. We hypothesized that an intravenous dose of exogenous L-carnitine- an L-carnitine challenge test- could identify individuals at risk for mitochondrial-related ADRs by provoking variation in subsequent measurement of serial L-carnitine and/or acetylcarnitine blood concentrations. To test this hypothesis, we used an established animal model of mitochondrial drug toxicity in which male C57BL/6 mice were treated with clofazimine (CFZ) by its addition to their chow for 8-weeks. Following CFZ treatment, mice were injected with a high dose (1,000 mg/kg) of L-carnitine, "challenge test". CFZ-induced changes in weight were consistent with previous work and reflect CFZ-induced changes in metabolism. The L-carnitine challenge test identified CFZ-dependent differences in whole blood acetylcarnitine concentration. This finding supports and substantiates the potential of the challenge test as a "probe" to identify drug-related toxicological manifestations.

3.2 Introduction

The database screening and process of elimination strategy methodology discussed in the previous chapter led us to identify L-carnitine as a candidate metabolic biomarker that met all *a priori* criteria and that could potentially be clinically employed to probe and assess mitochondrial drug toxicity. This selection was further supported by an extensive literature evaluation that described how the resulting changes in L-carnitine and/or acetylcarnitine blood levels after drug administration can be used as indicators of metabolic disruptions[1].

L-carnitine is an endogenous compound that is found in all mammalian species[2] and forms the principal component of what is referred to as the 'carnitine pool', this pool also includes short-, medium-, and long-chain esters collectively referred to as 'acylcarnitines'[2,3]. The primary role of endogenous L-carnitine is to facilitate the transport of fatty acids across the inner mitochondrial membrane, making them available for β-oxidation[3,4]. L-carnitine is also believed to be important for acting as an acyl group acceptor in order to maintain sufficient cellular levels of free coenzyme A (CoA) and it may act as an osmoprotectant in organs such as the kidney and as a general cell membrane stabiliser[2,4,5].

L-carnitine and acylcarnitines, including acetylcarnitine are established biomarkers of mitochondrial metabolism used to screen neonates for a series of genetic disorders affecting fatty acid oxidation, known as the inborn errors of metabolism[6–9]. However, despite the growing evidence that shows their clinical utility outside of these disorders, L-carnitine and acylcarnitines are not routinely measured beyond this screening. Measurements of the carnitine pool have been used to identify the disease and prognosticate mortality among disorders as well as identify subjects experiencing adverse drug reactions from various medications[1].

Blood and tissue concentrations of L-carnitine are heavily conserved, which allows for the detection of small perturbations. The normal plasma levels consists of 83% L-carnitine and 17% acylcarnitines, with acetylcarnitine representing 75% of the acylcarnitines, making it the most abundant of the L-carnitine metabolites[3]. Elimination of L-carnitine and acylcarnitines from the blood occurs as primarily via urinary excretion[4,10]. After glomerular filtration, 98–99% of L-carnitine is reabsorbed by in the proximal tubule[3,4]. Proximal tubular reabsorption of L-carnitine is carried out by OCTN2, an organic cation transporting protein that has a high affinity for L-carnitine.

Several studies have investigated the pharmacokinetics of exogenous L-carnitine administered for supplementation or for therapeutic use[2,11,12]. Exogenously administered L-carnitine is rapidly eliminated by the kidneys such that plasma concentrations are normalized within 12–24 hours after an intravenous dose.[13] After a 2g dose is administered, about 70–90% is recovered unchanged in urine within 24 hours[11,12]. The remainder of the dose is incorporated into tissue as L-carnitine or other components of the carnitine pool, including the various acylcarnitines[13]. The renal clearance of L-carnitine has been found to increase within increasing dose[11,12]. L-carnitine has also been found to have a role in moderating the ratio of free CoA to acyl-CoA, a function that is particularly important under conditions of stress[14,15].

In addition to meeting *a priori* criteria, L-carnitine was selected as the most clinically feasible because it is available as an FDA approved formulation for intravenous injection (Carnitor®, Leadiant Biosciences, Gaithersburg, MD; USA). L-carnitine is converted to acetylcarnitine via carnitine acetyltransferase (EC 2.3.1.7) in mitochondria (**Figure 3-1**), acetylcarnitine is also the most abundant acylcarnitine, which made it the metabolite of choice for further testing as a metabolic tracer of mitochondrial metabolism.

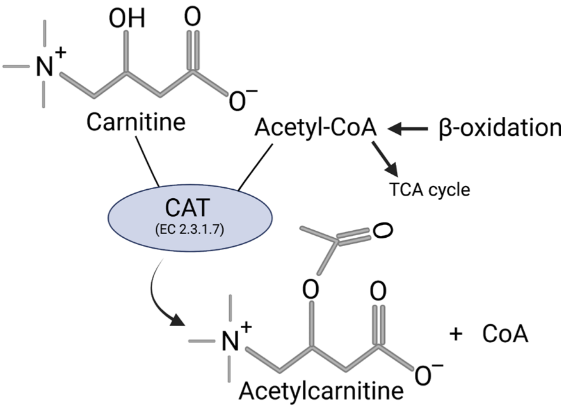

Figure 3-1 Generation of acetylcarnitine from L-carnitine occurs via carnitine acetyltransferase (CAT; EC 2.3.1.7). In mitochondria, the production of acetylcarnitine is catalyzed by CAT from L-carnitine and acetyl-CoA, a product of beta-oxidation and substrate for the tricyclic acid (TCA) cycle. In the setting of a surplus of L-carnitine as would occur from an L-carnitine "challenge test", and a reduced supply of acetyl-CoA secondary to clofazimine-induced metabolic stress (e.g., catabolism), the production of acetylcarnitine may be reduced compared to a non-catabolic state. Figure created by BioRender.com.

CFZ was selected as the *in vivo* assay system to test the usefulness of L-carnitine for its potential clinical use. CFZ is an FDA-approved, weakly basic, red-pigmented, phenazine antibiotic that is included in the WHO List of Essential Medications as part of the standard treatment for leprosy[16,17]. It is highly lipophilic and is characterized by an unusually long elimination half-life (up to 70 days), which is associated with extensive accumulation of the drug in the body[16,18]. Over the years CFZ has been studied not only for its role in leprosy treatment, but also for its potential repurposing and use in other conditions such as multidrug-resistant tuberculosis and SARS-CoV-2[19–21]. While CFZ is well tolerated, in addition to its adverse impact on mitochondrial function[18], it imposes considerable metabolic stress on the host secondary to induction of a catabolic state[22–25] (**Figure 3-2**).

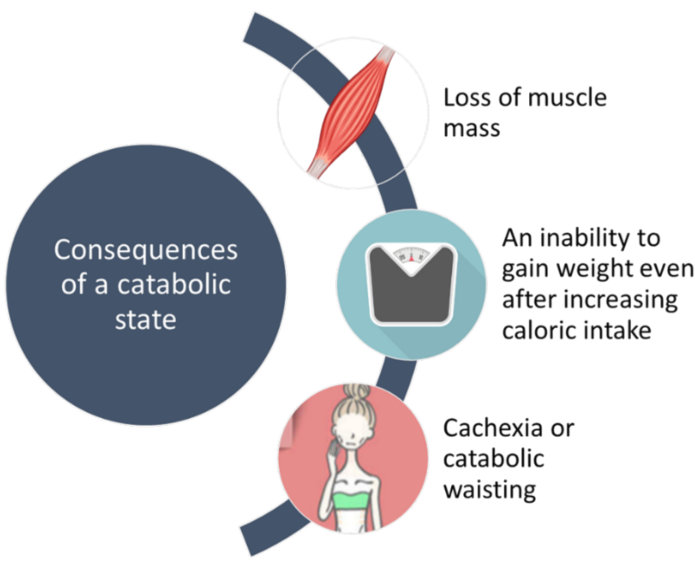

Figure 3-2 Consequences and manifestations of a catabolic state

To further corroborate the results from the database screening algorithm and establish L-carnitine feasibility for clinical use, an *in vivo* evaluation in a mouse model called the "L-carnitine challenge" was carried out.

3.3 Methods

3.3.1 In vivo L-carnitine Challenge

The main candidate metabolite identified by our database strategy was evaluated using a mouse model of mitochondrial drug toxicity. The animal protocol was approved by the University of Michigan's Institutional Animal Care and Use Committee (protocol number PRO00009404) and animal care was provided in accordance with the NIH Guide for the Care and Use of Laboratory Animals. We also complied with the ARRIVE guidelines[26].

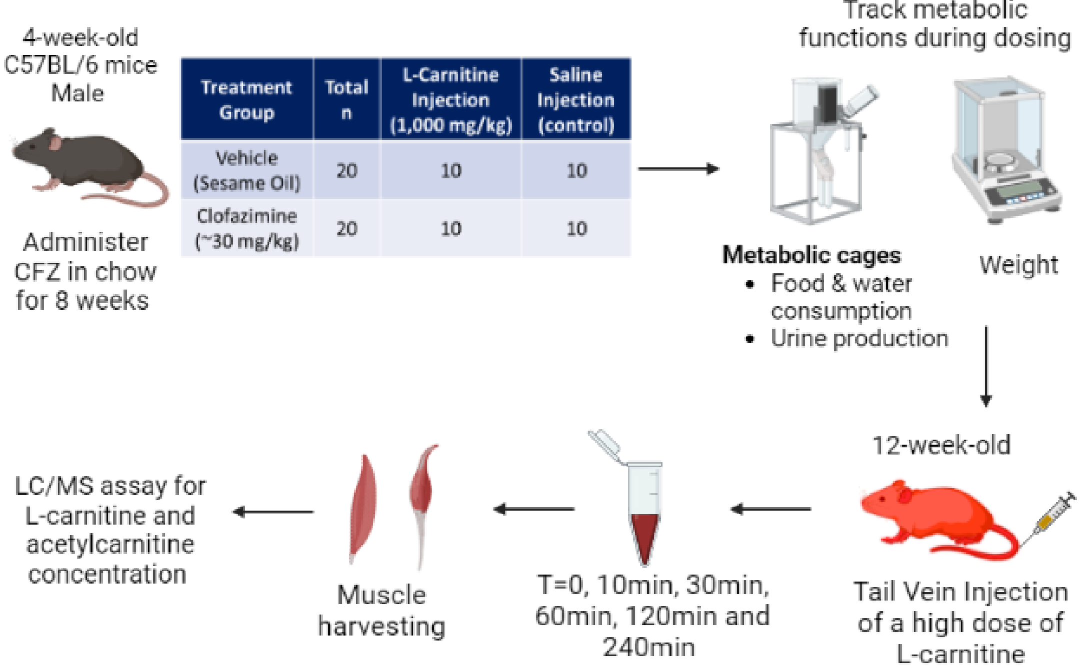

Figure 3-3 In vivo L-carnitine challenge test methodology and treatment groups. Figure created with BioRender.com

An L-carnitine "challenge test" was used to provoke drug-related mitochondrial toxicological manifestations. (**Figure 3-3**) Male C57BL/6 mice were treated with CFZ, an FDA approved medication known to cause mitochondrial dysfunction[25], by its addition to chow for 8-weeks (~40 mg/kg) as previously described[25]. Following CFZ treatment, mice were injected with a high dose (1,000 mg/kg) of L-carnitine, referred to as the "challenge test". Metabolic functions were tracked, including weight, food and water consumption, and urine production. The amount of L-carnitine and acetylcarnitine in urine is shown in mole fraction. These values were calculated by dividing the amount of L-carnitine or acetylcarnitine recovered after 24h in urine by the starting dose of the L-carnitine challenge injected. Pooled urine samples (5 mice/per sample) were collected at 24h using metabolic cages (Techniplast®). Whole blood samples were collected via the saphenous vein (BL, 10min, 30min, 60min, 120min) and the retro-orbital plexus

by removal of the eye while under anesthesia (inhaled isoflurane) for the terminal timepoint (240min) then flash frozen in liquid nitrogen. At the study termination, mice were euthanized according to IACUC guidelines (Policy on Human Care and Use of Laboratory Animals Approved Animal Welfare Assurance Number, D16–00072 (A3114–01)). Sodium-heparin preserved whole blood and centrifuge-clarified urine samples were stored (-80°C) until the time of assay. L-carnitine and acetylcarnitine concentrations were measured using a quantitative liquid chromatography–mass spectrometry (LC/MS) assay.

3.3.2 Quantification of L-Carnitine and acetylcarnitine in blood and urine

To determine L-carnitine and acetylcarnitine concentrations in mouse blood and urine, water (490 µL) was added into 10 µL blood or urine. Internal standard solution (5 µL; L-carnitine-13C, D3 and acetylcarnitine-D3, 5 µg/mL in acetonitrile, Thermo Fisher Scientific) was added to the diluted blood or urine samples and mixed for 30 min at room temperature. Finally, 150 µL of acetonitrile was added to precipitate macromolecules. The mixture was vortexed for 10 min and centrifuged (3000 g for 10 min at 4°C). The supernatant was transferred to the autosampler vials for LC–MS/MS analysis.

To construct calibration curves for L-carnitine and acetylcarnitine, 5 µL internal standard solution was added into 100 µL of 12 nonzero mixed standards, which were prepared in water, and mixed for 30 min at room temperature. Then, 150 µL of Acetonitrile was added into 50 µL of the mixture. After vortexed and centrifuged with the conditions for samples, the standard solutions were injected to LC-MS/MS. By plotting the peak area ratio of L-carnitine or acetylcarnitine to the internal standard versus the sample concentration. The concentration range evaluated was from 1 to 5000 ng/ml. Quality control solutions were prepared from separate weighted powder to the concentration of 10, 250, 2500ng/ml in water. Then, quality control

samples were obtained from mixing of a diluted blood sample with above solution at 1:1 (v/v). Quality control samples were run before, in the middle and after the samples to evaluate the accuracy and intra-batch precision of the developed method.

3.3.3 Liquid Chromatography Conditions

L-carnitine and acetylcarnitine concentrations (ng/ml) were determined by the LC–MS/MS method developed and validated for this study. The LC–MS/MS method consisted of a Shimadzu LC-20AD HPLC system (Kyoto, Japan), and chromatographic separation of the tested compound was achieved using an Agilent Poroshell 120 EC-C18 column (3.0 x 100 mm, 2.7 µm) at 25 °C. Five microliters of the supernatant was injected. The flow rate of gradient elution was 0.35 ml/min with mobile phase A (10mM ammonium formate and 0.1% formic acid in purified deionized water) and mobile phase B (0.1% formic acid in acetonitrile). An Sciex QTOF X500R mass spectrometer equipped with an electrospray ionization source (ABI-Sciex, Toronto, Canada) in the positive-ion high resolution multiple reaction monitoring (HRMRM) mode was used for detection. Protonated molecular ions and the respective ion products were monitored at the transitions of m/z 162.11 > 103.0364 for L-carnitine, 204.12 > 85.0250 for acetylcarnitine, 166.14 > 103.0380 for L-carnitine-13C, D3 and 207.14 > 85.0255 for acetylcarnitine-D3. We adjusted the instrument settings to maximize analytical sensitivity and specificity of detection. Data were processed with software SCIEX OS (version 2.1.6).

LC–MS/MS analysis was performed by using an X500R QTOF (SCIEX). Components were separated on a 3.0 x 100mm EC-C18 column with 2.7µm particle size (Agilent). The mobile phase delivered at 0.35 mL/min was a mixture of (A) 10mM Ammonium formate and 0.1% formic acid in purified deionized water and (B) 0.1% formic acid in acetonitrile, using the

following gradient elution: 0.01-1.50 min (1%B), 3.00-4.50 min (95%B), and 4.60-6.60 min (1%B).

3.3.4 Data processing and statistical analysis

GraphPad Prism Version 9.3.1 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. All data are presented as mean ± SD. Significance between different treatment groups, vehicle treated vs CFZ treated, was assessed by two-tailed unpaired Student's t-test for the urine mole fraction, weight, food consumption, water consumption, urine volume and muscle mass. Significance between different treatment groups in whole blood L-carnitine and acetylcarnitine concentrations and acetylcarnitine/L-carnitine ratio over time was assessed by a mixed-effects model with a Šidák multiple comparison correction. Statistical difference was considered at $p < 0.05$.

3.4 Results

3.4.1 In vivo L-Carnitine challenge

L-carnitine was evaluated to assess the validity of our database strategy and its feasibility for clinical use as a marker of mitochondrial-related ADR using CFZ-treated mice as a model. These results show how CFZ treatment causes host perturbation of metabolic functions, observed in weight, food and water consumption, urine production and loss of skeletal muscle mass. The results from these experiments are depicted in **Figures 3-4, 3-5 and 3-6**.

The first indication of CFZ induced metabolic perturbations is evident by changes in mouse body weight (**Figure 3-4**). During the 8-week treatment CFZ treated mice exhibit significant weight loss when compared to the vehicle treated mice (**Figure 3-4a**). This difference

can be further observed in **Figure 3-4b**, where CFZ-treated mice weighed on average (SD) less (22.6+1.21g) than vehicle treated (25.7+1.08g) at the end of the 8-week treatment.

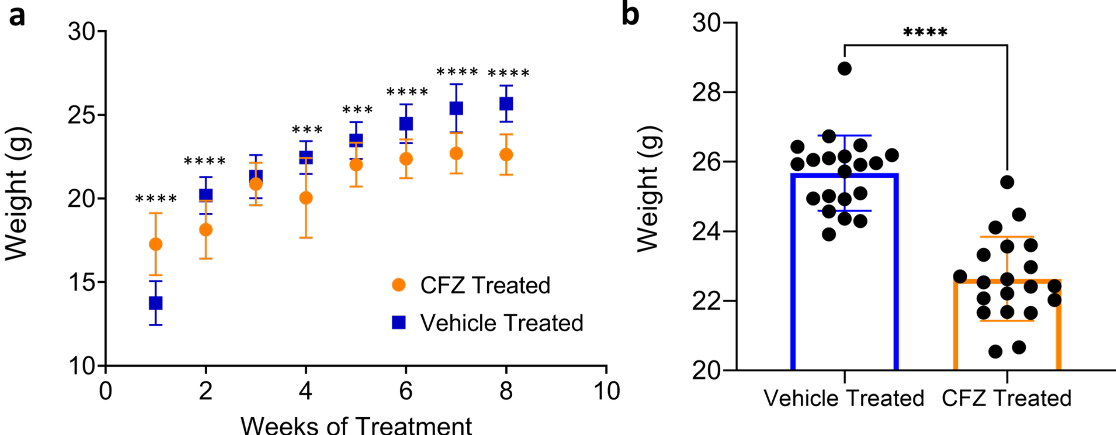

Figure 3-4 Change in body weight over 8-weeks of clofazimine (CFZ) treatment. a) Weight (g) over the 8-week treatment of vehicle treated (blue) and CFZ treated (orange) mice. b) CFZ-treated mice weighed on average (SD) less (22.6+1.21g) than vehicle treated (25.7+1.08g) at the end of the 8-week treatment. (****p=<0.0001). Data are the mean (SD) of 20 mice/group.

Despite the differences in weight, both vehicle and CFZ treated mice consumed the same amount food per day (3.0 ± 0.28 g, p=0.58) (**Figure 3-5a**). However, CFZ treated mice consumed on average less water (2.6mL ±0.07) than vehicle treated (4.0mL ±0.60) mice (**p=0.007) (**Figure 3-5b**), which in turn led to CFZ treated mice having a lower average (SD) urine production over 24h (4.2mL ±1.05) than the vehicle treated (6.4mL ±0.60) mice (*p=0.022) (**Figure 3-5c**).

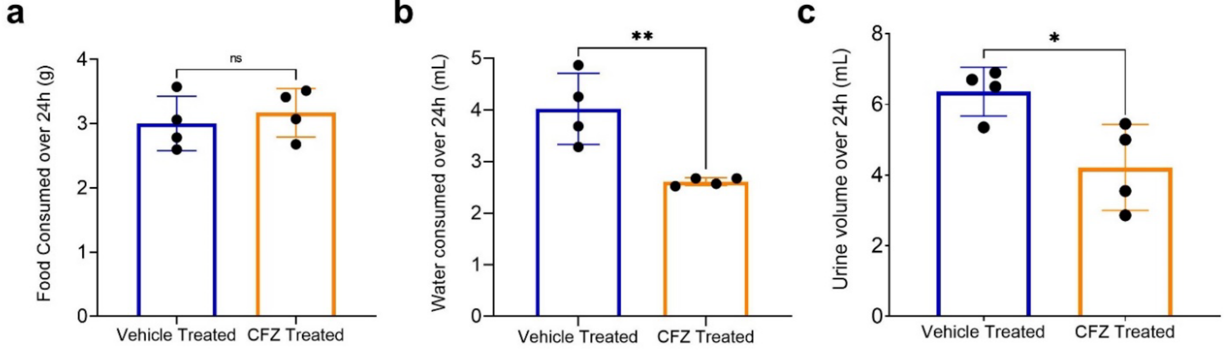

Figure 3-5 Metabolic functions of vehicle treated (blue) and clofazimine (CFZ)-treated (orange) mice. a) In both groups, food consumption over 24h did not differ (3.0g ±0.42 vs 3.2±0.37 CFZ, p=0.58). b) CFZ treated mice consumed on average less water (2.6mL ± 0.07) than vehicle treated (4.0mL ±0.60) mice (**p=0.007) c) CFZ treated mice produced on average less urine (4.2mL ±1.05) than vehicle treated (6.4mL ±0.60) mice (*p=0.022). Data are the mean (SD) of 4 metabolic cages of 5 mice/group.

Another indication of metabolic alteration was reflected in changes in skeletal muscle mass. In both the gastrocnemius and the quadricep muscle CFZ treated, and vehicle treated mice exhibited similar muscle masses when normalized by total body weight (**Figure 3-6**).

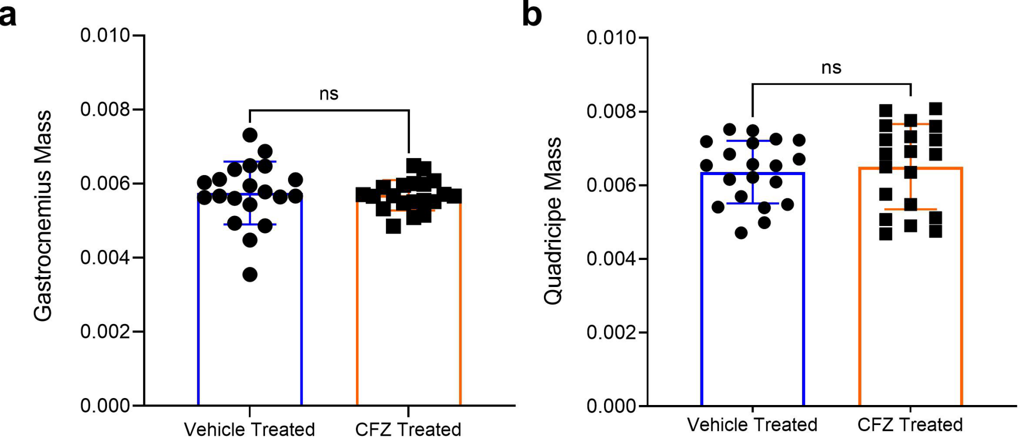

Figure 3-6 Muscle mass of vehicle treated (blue) and clofazimine (CFZ)-treated (orange) mice. a) Gastrocnemius muscle mass in CFZ and vehicle treated mice normalized by weight was the same (0.006, p=0.76) b) No difference was observed between CFZ treated (0.007) and vehicle treated (0.006) mice in quadricep muscle mass (p=0.65). Data are mean (SD) of 20 mice/group normalized by total body weight.

In urine, the amount of both L-carnitine and its primary metabolite, acetylcarnitine (HMDB0000201), was not different between the two treatment groups (**Figure 3-7**).

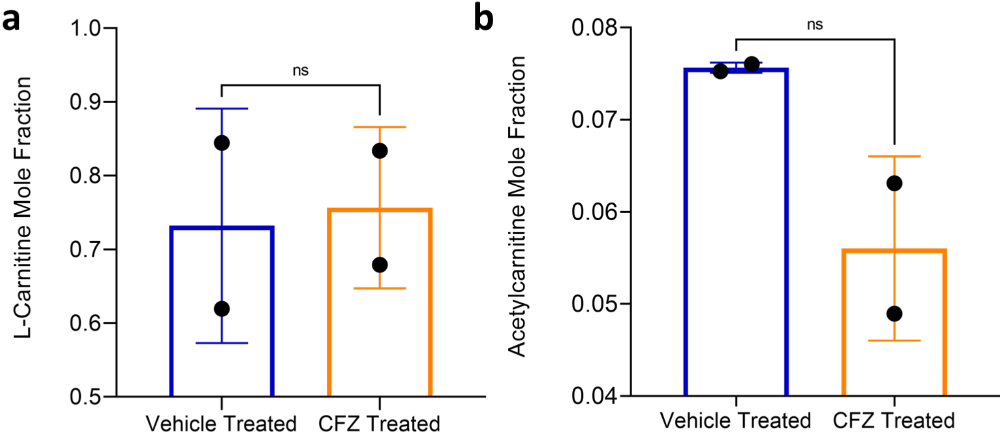

Figure 3-7 The mole fraction of L-carnitine and acetylcarnitine in the urine 24h after a bolus IV L-carnitine injection were not changed by clofazimine (CFZ) treatment. a) The urine mole fraction of L-carnitine in CFZ (orange) and vehicle treated (blue) mice was 0.73 and 0.76, respectively (p=0.87). b) The urine mass ratio of acetylcarnitine in CFZ treated and vehicle treated mice was 0.6 and 0.76, respectively (p=0.11). Data are the mean (SD) of two metabolic cages each housing 5 mice/group. Mole fraction of L-carnitine and acetylcarnitine was calculated by dividing the amount of L-carnitine or acetylcarnitine recovered in urine collected over 24h following the administration of the L-carnitine challenge dose.

Following tail vein injection of L-carnitine (1000 mg/kg), vehicle and CFZ treated mice had similar L-carnitine levels in whole blood (**Figure 3-8a**). However, the mean (SD) whole blood acetylcarnitine concentrations were lower in CFZ treated mice at 30min (*p=0.0138), 60min (**p=0.0024), and 120min (*p=0.0465) compared with vehicle treated mice. Pre-treatment and levels at 10min (p=0.379) were not different (**Figure 3-8b**).

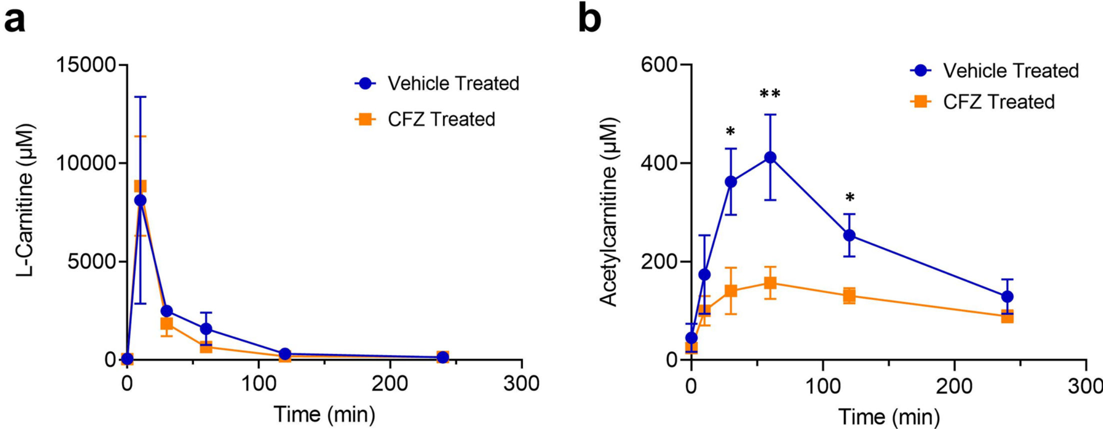

Figure 3-8 Whole blood L-carnitine and acetylcarnitine concentrations (μM) in clofazimine (CFZ) and vehicle treated mice following the L-carnitine "challenge test" (1000mg/kg). a) Following tail vein injection of L-carnitine, vehicle and CFZ treated mice had similar L-carnitine levels in whole blood. b) Following tail vein injection, CFZ treated mice had lower acetylcarnitine levels at 30min (*p=0.0138), 60min (**p=0.0024), and 120min (*p=0.0465) compared with vehicle treated mice. Pre-treatment levels and at 10min (p=0.379) the difference was not significant. Data are the mean (SD) of 10 mice/group.

Post-carnitine injection, the CFZ-treated mice tended to have a lower blood acetylcarnitine to L-carnitine ratio (a reflection of carnitine homeostasis), but this was not statistically significant (**Figure 3-9**).

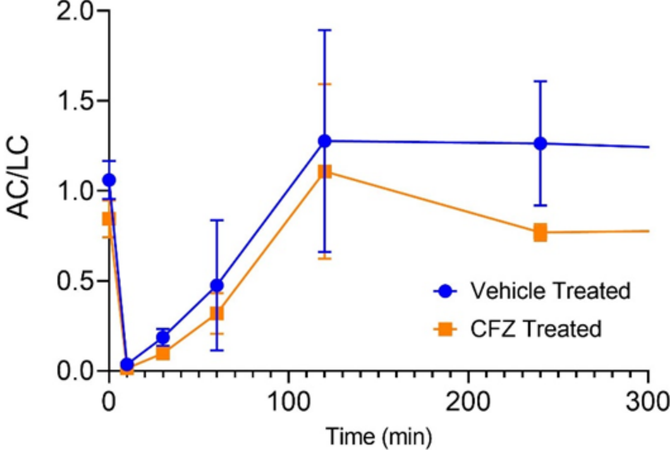

Figure 3-9 Acetylcarnitine/L-carnitine ratio of whole blood concentration measurements at different timepoints for vehicle treated (blue) and CFZ treated mice (orange).

3.5 Discussion

While CFZ is well tolerated, but in addition to its known adverse impact on mitochondrial function[18], it imposes considerable metabolic stress on the host secondary to induction of a catabolic state[22–25]. The results of these metabolic alterations are evident (**Figures 3-4, 3-5 and 3-6**) with observed changes in weight, skeletal muscle mass and metabolic functions. These findings are consistent with our previous reports[25] and are consistent with manifestations of a catabolic state.[27]

Following 8-weeks of CFZ treatment we subjected mice to the L-carnitine challenge test (1,000 mg/kg given as a single IV injection). Although we did not detect a CFZ-induced change in whole blood concentrations of L-carnitine, the-L-carnitine challenge test induced differences in whole blood acetylcarnitine concentrations in CFZ-treated mice. Specifically, in CFZ treated mice, acetylcarnitine concentrations were significantly lower when compared to vehicle treated mice (**Figure 3-8b**). Typically, high dose L-carnitine supplementation leads to a proportional increase in blood levels of both L-carnitine and acetylcarnitine, the primary acetylated form of L-carnitine[28,29]. L-carnitine and the L-carnitine shuttle are essential for the transport of long-chain fatty acids into the mitochondria. In normal, healthy individuals, skeletal muscle stores 97% of total body L-carnitine, while L-carnitine in blood accounts for only 0.1% of total body L-carnitine[30]. In the tissue compartment (including skeletal muscle) the generation of acetylcarnitine relies on the availability of two substrates- L-carnitine and acetyl-CoA (**Figure 3-1**). The enzyme, CAT (EC 2.3.1.7), converts them to acetylcarnitine and CoA. A possible explanation for the decline in acetylcarnitine production in response to L-carnitine supplementation in CFZ-treated mice is a reduction in the availability of acetyl-CoA for

acetylcarnitine generation secondary to CFZ-induced catabolism. High energy demand leads to consumption of acetyl-CoA leaving less reserve available for the production of acetylcarnitine[31].

Acetyl-CoA is a key molecule in the production of energy, the biosynthesis of other compounds, and the regulation of several metabolic pathways[31]. It plays a central role in metabolism, particularly in the process of cellular respiration. Acetyl-CoA is formed from the breakdown of carbohydrates, fats, and certain amino acids, and is the starting point for several metabolic pathways, including the TCA cycle and the synthesis of fatty acids. Acetyl-CoA is also used to generate cholesterol and other lipids, it is also a key intermediate in the process of ketogenesis, to produces ketone bodies that can be used as an alternative energy source for the brain and other tissues when glucose is not available[32]. Overall, acetyl-CoA is a versatile and important molecule that plays a critical role in the metabolism of cells. Emerging evidence reveals that cells monitor the levels of acetyl-CoA as a key indicator of their metabolic state, through distinctive protein acetylation modifications dependent on this metabolite[31]. A CFZ-induced catabolic state, may lead to high demand of acetyl-CoA for all of its different functions, many of which may have a priority over acetylcarnitine production, leading to a reduction in acetylcarnitine despite a surplus of L-carnitine. This finding highlights the importance of measuring acetylcarnitine. A number of studies have relied on the measurement of acetylcarnitine and acylcarnitines as markers for different disease states and drug-induced ADRs[1]. The differences induced in acetylcarnitine blood levels by the L-carnitine “challenge test” in CFZ versus vehicle treated mice point to the feasibility of its use as a “probe” to identify drug-related mitochondrial and medication-induced toxicological manifestations.

We did consider other explanations for reduced levels of acetylcarnitine following L-carnitine supplementation in CFZ-treated mice. These included CFZ induced nephrotoxicity. This explanation is unlikely since CFZ-induced nephrotoxicity would be expected to influence L-carnitine blood levels, not just acetylcarnitine since it is also primarily renally excreted[2,13]. There is also no clinical or experimental evidence that CFZ causes nephrotoxicity. CFZ has been found to lack the nephrotoxicity and neurotoxicity associated with other drugs[33]. Notably, we have previously shown that the rate of urine creatinine excretion, as a measure of renal function, was not different between the CFZ treated and vehicle treated groups.[25]

Another possible explanation that we considered for reduced levels of acetylcarnitine in CFZ-treated mice was the possible CFZ-induced inhibition of the enzyme CAT. Although a direct measurement of enzyme activity was not conducted, we looked in the literature for known CAT inhibitors and their structural activity relative to CFZ. There are some know CAT inhibitors, such as mildronate[34] and 3-keto-4-pentenoyl-CoA[35]. Neither show similar structural activity to CFZ. Mildronate inhibits CAT in a competitive manner through binding to the carnitine binding site because the bound conformation of mildronate closely resembles that of carnitine except for the orientation of the trimethylammonium group[34]. This leads us to believe that CFZ is not inhibiting the CAT enzyme.

We acknowledge that there are limitations to this study. Clofazimine is only one of many drugs that can affect mitochondrial function through a variety of different mechanisms. We acknowledge that the interaction between other mitochondriotoxic drugs and L-carnitine/acetylcarnitine transport, metabolism and disposition pathways could be very different from those that occur during CFZ treatment. This study was also limited to mice of the same genetic background. It therefore does not fully account for all the variation in L-carnitine and

acetylcarnitine transport, metabolism and disposition that may characterize an entire population with different genetics, ages, and exposures to different environmental conditions that might lead to a more varied response to either CFZ or to an L-carnitine challenge test.

Despite these limitations, our prospective study identified functional biomarkers of mitochondrial toxicity that could be used to stratify individuals at increased ADR risk[36]. The findings described here serve as a starting point in the development of a test to probe the relationship between baseline metabolic stress and drug-related mitochondrial toxicity that could help determine which patients may be at risk. To further refine these measurements, a more detailed assessment of the distribution of L-carnitine and acetylcarnitine in response to the L-carnitine challenge test, could also be elaborated using an isotope labeled L-carnitine analog. Such a metabolic tracer could yield additional information about drug-induced changes to endogenous L-carnitine and acetylcarnitine stores, transport, and utilization.

3.6 Conclusions

Clinical use of an L-carnitine challenge test with subsequent measurement of mitochondrial metabolites like acetylcarnitine, could be an important early step to identify occult medication-induced mitochondrial toxicity. Targeted measurements of carnitine and acetylcarnitines may be informative for the identification of metabolic ADRs of drug-induced mitochondrial dysfunction during treatment with a number of different drugs including the examples discussed in this chapter. Drug-induced mitotoxic signals that could be detected in advance of a clinical phenotype may be particularly useful for therapeutic drug monitoring and the avoidance of ADRs.

3.7 References

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Chapter 4
L-Carnitine Challenge Test as an Indicator of Sex-Related Predisposition for Adverse Drug Reactions

4.1 Abstract

Physiologic differences between male and females affect drug activity in different ways, including pharmacokinetics, pharmacodynamics, and the predisposition to adverse drug reactions (ADRs). Females experience ADRs nearly twice as often as males, yet the role of sex as a biological factor in the generation of ADRs is poorly understood. Some ADRs are associated with off-target drug interactions with mitochondria. Metabolites that reflect mitochondrial function may help identify mitochondrial toxicity risks and help elucidate the sex related differences of ADRs predispositions. Growing evidence has revealed the potential use of L-carnitine and acetylcarnitine as mitochondrial biomarkers of ADRs, beyond their current use to screen neonates for inborn errors of metabolism. This research looks to establish an L-carnitine challenge test to identify risk of mitochondrial-related ADRs by provoking variation in L-carnitine and/or acetylcarnitines blood levels, and to better understand the source of male and female differences in ADRs susceptibility. Male and female C57BL/6 mice were treated with either the vehicle (sesame oil) or with the metabolic altering drug, clofazimine (CFZ) by its addition to their chow for 8-weeks. Following CFZ treatment, mice were injected with a high dose (1,000 mg/kg) of L-carnitine, "challenge test". Metabolic functions were tracked, including weight, food, and water consumption. Urine and blood samples were assayed for L-carnitine and

acetylcarnitine concentrations using a quantitative LC/MS analysis. Additional whole blood metabolite concentrations were measured using NMR. L-carnitine-induced similar differences in whole blood acetylcarnitine concentration in both male and female CFZ-treated mice, indicating no apparent sex-related differences in mitochondrial metabolism related to long-term CFZ treatment. Although CFZ treated male and female mice exhibited similar levels of measured whole blood metabolites, indicating no apparent sex-related differences, these levels varied significantly between CFZ, and vehicle treated mice regardless of sex. This finding suggests that metabolomics could lead to better understanding of the mechanisms/pathways that are affected and may contribute to ADRs.

4.2 Introduction

Pharmacological response and effects are influenced by multiple factors, from biological to environmental. Sex differences have been described in pharmacokinetics and contribute to the interindividual variation in drug disposition, therapeutic response, and drug toxicity between male and females[1–3]. These variations may be due to sex differences in reproductive physiology, hormone levels, and genetic polymorphisms[4]. The impact of sex differences on pharmacological response is a field of study with growing interest, partly because these differences may contribute to differences in the frequency and severity of ADRs observed between male and females. Studies have suggested that female patients experience more frequent and more severe ADRs than males[5–7], however, evidence for sex differences in the incidence of ADRs is still limited. It has also been suggested that there is a female predisposition to the number and type of ADRs with different drug classes[8]. Collectively, this evidence supports the notion that sex may influence drug disposition and susceptibility to ADRs.

Historically, women have been underrepresented in clinical trials. Most clinical trials have been performed in young white male participants and women were excluded due to hormonal fluctuations and the possibility of being or becoming pregnant[9,10]. This also applied to preclinical trials in which most animals were male, with resulting in research finding predominantly only applicable to males.[6,11] Clinical trials are now required to enroll more diverse participants as a result of the introduction of the ICH guideline in 1997, which recommends including participants who are representative of the user population[12]. Despite the immense progress made with equality in human research, women are still at greater risk of harm then men by FDA-approved medications.

CFZ is an antimycobacterial agent that has been widely used as part of the standard treatment of leprosy since the 1960s[13,14]. More recently, we have learned that the extensive accumulation of CFZ in the body is associated with the induction of considerable metabolic burden and interference with mitochondrial function[15]. However, these consequences have mainly been studied in male humans and mice. Most CFZ studies that have included female mice have been focused on the repurposing of the drug as part of a treatment regimen for drug-resistant tuberculosis[16–18], with attempt to further understanding of sex-related alterations in mitochondrial metabolism.

Some studies have looked at sex-specific differences in mitochondrial function in response to different drug treatments and disease states[19–23]. One example evaluating the observed higher incidence of Alzheimer's in females than males, aimed to identified sex-specific differences in mitochondrial function in Alzheimer’s patients by correlating mitochondrial function in peripheral mononuclear blood cells (PBMCs) and brain energy metabolites[23]. Sex-associated differences in mitochondrial function were detected in both PBMCs and brain metabolites[23].

Another area of predominant focus for this type of research is central nervous system (CNS) injury. Mitochondrial metabolic dysfunction is a common feature of CNS injury. Evidence suggests males predominantly utilize proteins while females predominantly use lipids as a fuel source within mitochondria and that these differences may significantly affect cellular survival following injury[22]. Another study investigated whether sex-specific differences in acute hepatotoxicity could be observed *in vitro* by comparing hepatotoxic drug effects in male and female primary human hepatocytes[24]. Significant sex-related differences were found after exposing primary human hepatocytes to known hepatotoxicans drugs like diclofenac, acetaminophen, chlorpromazine, or verapamil. Differences were observed in mitochondrial injury, nuclear condensation, and plasma membrane permeability with female primary hepatocytes showing an overall higher sensitivity than males[24].

As reported in the previous chapters, L-carnitine was identified as a known candidate mitochondrial metabolite that could be clinically useful to identify increased risk of mitochondrial-related ADRs. We previously reported that CFZ-treated male mice exhibited lower concentration levels of whole blood acetylcarnitine, an L-carnitine metabolite, than vehicle treated animals following an L-carnitine challenge test. With the knowledge that males and females can exhibit different responses to drug treatment, we looked to answer the question: Do females treated with a mitochondrial toxicant medication, manifest a similar disruption in acetylcarnitine levels following L-carnitine supplementation as male mice? To further corroborate the results from the previous study, establish L-carnitine feasibility for clinical use in both male and female, and aid in closing the knowledge gap of sex-related differences in ADR incidence, we conducted an *in vivo* evaluation of the "L-carnitine challenge test" in male and female mice.

4.3 Methods

4.3.1 In vivo L-carnitine Challenge Test

L-carnitine was evaluated using a mouse model of mitochondrial drug toxicity. The animal protocol was approved by the University of Michigan's Institutional Animal Care and Use Committee (protocol number PRO00009404) and animal care was provided in accordance with the NIH Guide for the Care and Use of Laboratory Animals. We also complied with the ARRIVE guidelines[25].

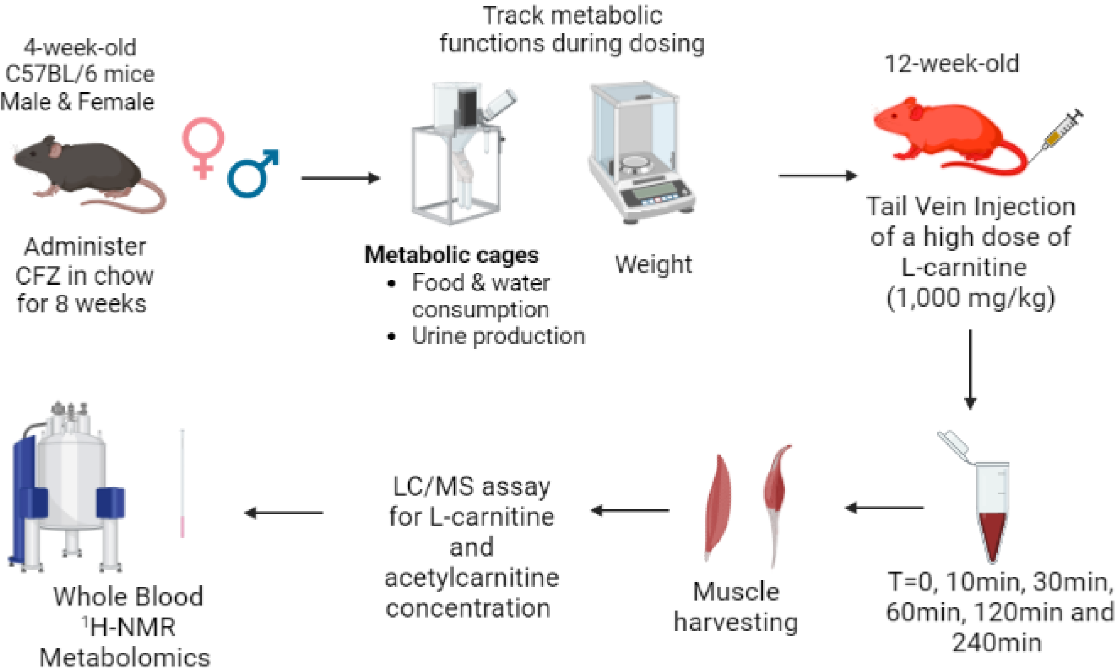

Figure 4-1 *In vivo* L-carnitine challenge test methodology. Created with BioRender.com

An L-carnitine "challenge" was used to provoke drug-related mitochondrial toxicological manifestations (**Figure 4-1**). Male and female C57BL/6 mice were treated with CFZ, an FDA approved medication known to cause mitochondrial dysfunction[15], by its addition to chow for 8-weeks (~40 mg/kg) as previously described[15]. Following CFZ treatment, mice received a single,

intravenous high dose (1,000 mg/kg) of L-carnitine, referred to as the "challenge test". Metabolic functions were tracked, including weight, food and water consumption, and urine production. The amount of L-carnitine and acetylcarnitine in urine was measured and calculated as the mole fraction. This was done by dividing the amount of L-carnitine or acetylcarnitine recovered after 24h in urine by the starting dose of the administered L-carnitine. Pooled urine samples (5 mice/per sample) were collected at 24h using metabolic cages (Techniplast®). Whole blood samples were collected (BL, 10min, 30min, 60min, 120min) via the saphenous vein and at the terminal timepoint (240min) by retro-orbital plexus following removal of the eye while under anesthesia (inhaled isoflurane); blood samples were immediately flash frozen in liquid nitrogen. At study termination, mice were euthanized in accordance with IACUC guidelines (Policy on Human Care and Use of Laboratory Animals Approved Animal Welfare Assurance Number, D16–00072 (A3114–01)). Sodium-heparin preserved whole blood and centrifuge-clarified urine samples were stored (-80°C) until the time of assay. L-carnitine and acetylcarnitine concentrations were measured using a quantitative liquid chromatography–mass spectrometry (LC/MS) assay.

4.3.2 Quantification of L-carnitine and acetylcarnitine in blood and urine

Quantitation of L-carnitine and acetylcarnitine was conducted by the University of Michigan Pharmacokinetics Core. To determine L-carnitine and acetylcarnitine concentrations in mouse blood and urine, water (490 µL) was added into 10 µL blood or urine. Internal standard solution (5 µL; L-carnitine-13C, D3 and acetylcarnitine-D3, 5 µg/mL in acetonitrile, Thermo Fisher Scientific) was added to the diluted blood or urine samples and mixed for 30 min at room temperature. Finally, 150 µL of acetonitrile was added to precipitate macromolecules. The mixture was vortexed for 10 min and centrifuged (3000 g for 10 min at 4°C). The supernatant

was transferred to the autosampler vials for LC–MS/MS analysis. Tissue samples were homogenized (Precellys tissue homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France) with the addition of 80% N-dimethylformamide (DMF)-PBS solution with a ratio of 5:1 volume (mL) to weight of tissue (g). Then, the tissue homogenization was diluted 50 times with water and treated using the same procedure as that for the blood samples to extract the compound for LC–MS/MS analysis.

To construct calibration curves for L-carnitine and acetylcarnitine, 5 µL internal standard solution was added to 100 µL of 12 nonzero mixed standards, which were prepared in water, and mixed for 30 min at room temperature. Then, 150 µL of acetonitrile was added into 50 µL of the mixture. The samples were vortexed and centrifuged and supernatants and internal standards were injected into LC-MS/MS. By plotting the peak area ratio of L-carnitine or acetylcarnitine to the internal standard versus the sample concentration. The concentration range for each compound evaluated was from 1 to 5000 ng/ml. Quality control solutions were prepared from separate weighted powder to the concentration of 10, 250, 2500ng/ml in water. Then, quality control samples were obtained from mixing a diluted blood samples with above solution at 1:1 (v/v). Quality control samples were run before, in the middle and after the samples to evaluate the accuracy and intra-batch precision of the developed method.

4.3.3 Liquid Chromatography Conditions

L-carnitine and acetylcarnitine concentrations (ng/ml) were determined by the LC–MS/MS method developed and validated for this study. The LC–MS/MS method consisted of a Shimadzu LC-20AD HPLC system (Kyoto, Japan), and chromatographic separation of the tested compound was achieved using an Agilent Poroshell 120 EC-C18 column (3.0 x 100 mm, 2.7 µm) at 25 °C. Five microliters of the supernatant was injected. The flow rate of gradient elution

was 0.35 ml/min with mobile phase A (10mM ammonium formate and 0.1% formic acid in purified deionized water) and mobile phase B (0.1% formic acid in acetonitrile). An Sciex QTOF X500R mass spectrometer equipped with an electrospray ionization source (ABI-Sciex, Toronto, Canada) in the positive-ion high resolution multiple reaction monitoring (HRMRM) mode was used for detection. Protonated molecular ions and the respective ion products were monitored at the transitions of m/z 162.11 > 103.0364 for L-carnitine, 204.12 > 85.0250 for acetylcarnitine, 166.14 > 103.0380 for L-carnitine-13C, D3 and 207.14 > 85.0255 for acetylcarnitine-D3. We adjusted the instrument settings to maximize analytical sensitivity and specificity of detection. Data were processed with software SCIEX OS (version 2.1.6).

LC–MS/MS analysis was performed by using an X500R QTOF (SCIEX). Components were separated on a 3.0 x 100mm EC-C18 column with 2.7µm particle size (Agilent). The mobile phase delivered at 0.35 mL/min was a mixture of (A) 10mM Ammonium formate and 0.1% formic acid in purified deionized water and (B) 0.1% formic acid in acetonitrile, using the following gradient elution: 0.01-1.50 min (1%B), 3.00-4.50 min (95%B), and 4.60-6.60 min (1%B).

4.3.4 Nuclear Magnetic Resonance (NMR) Spectroscopy Metabolomics

Whole Blood Sample Processing

Whole blood samples were thawed in an ice water bath before being measured and transferred to a 2mL microcentrifuge tube using a glass serological pipet. 1mL of a 1:1 methanol:chloroform mixture was added for a 1:1:1 sample:methanol:chloroform ratio. Samples were then sonicated for 2 minutes, incubated at -20°C for 20 minutes, and then centrifuged at 13400 x g for 30 minutes at 4°C. The aqueous supernatant was collected post-centrifugation and added to a 5mL microcentrifuge before being snap frozen in liquid nitrogen and dried by

lyophilization. Post-lyophilization, samples were resuspended in a 50mM phosphate buffer in D2O (deuterium oxide/deuterated water). After being resuspended, samples were vortexed to fully resuspend all solids, and then measured and transferred to labeled cryotubes using a 1mL glass serological pipet. If the sample volume was less than 500uL, D2O was added to the sample to bring its volume up to 500uL. 50uL of DSS-d6 of known concentration was then added to all samples to act as an internal standard for NMR. Samples were then frozen and stored at -80C until time of spectra acquisition.

NMR Spectral Acquisition

Spectra were acquired at the University of Michigan's Biochemical NMR Core Laboratory on a Varian (now Agilent, Inc., Santa Clara, CA) 500MHz NMR spectrometer with a VNMRS console operated by host software VNMRJ 4.0. Spectra were recorded using 32 scans of a proton-proton-NOESY pulse sequence (commonly called a METNOESY pulse sequence)[26]. Spectra were acquired at room temperature (295.45+/-0.3K) using a 5-mm Agilent "One-probe." The pulse sequence is as follows: A 1s recovery delay, which includes a 990ms saturation pulse of 80Hz induced field strength empirically centered on the water resonance, 2 calibrated 90degree pulses, a mixing time of 100ms, a final 90degree pulse, and an acquisition period of 4s. Optimal excitation pulse widths were obtained by using an array of pulse lengths as previously described[15].

NMR Spectral Analysis

NMR spectra of serum and WB were analyzed with Chenomx NMR Suite 8.2 (Edmonton, AB, Canada) software. The Processor module was used to phase shift, baseline correct and excise water from each spectrum as previously described[26]. Compounds were then identified and quantified using the profiler module of the software, which allows metabolites to

be quantified relative to an internal standard of known concentration[26]. Data was scaled to correct for differences in initial sample volume before analysis.

4.3.5 Data processing and statistical analysis

GraphPad Prism Version 9.5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis. All data are presented as mean (±SD). Significance between different treatment groups, male vs female and vehicle treated vs CFZ treated, was assessed by ordinary one-way ANOVA with a Šidák multiple comparison test for the urine mole fraction, weight, food consumption, water consumption and urine volume. Muscle mass comparisons were assessed by unpaired two tailed student t-test after correcting for total body mass. Significance between male and females and different treatment groups in whole blood L-carnitine and acetylcarnitine concentrations and acetylcarnitine/L-carnitine ratio over time was assessed by a mixed-effects model with a Šidák multiple comparison correction. NMR metabolite concentrations between treatment groups were assessed by unpaired two tailed student t-test with Welch's correction. Statistical difference was considered at $p < 0.05$.

4.4 Results

4.4.1 In vivo L-Carnitine challenge

L-carnitine was evaluated to assess its feasibility for clinical use as a marker of mitochondrial-related ADRs in male and females using CFZ-treated mice as a model. Results in **Figures 4-2 to 4-6** show the effects of CFZ treatment in host metabolic functions, observed in weight loss, food and water consumption, urine production and loss of skeletal muscle mass.

As we have previously shown, the first indication of CFZ induced metabolic perturbations was change in total body weight of the mice (**Figure 4-2**). Even though females

overall weigh less than the male counterparts, they start around the same weight but follow individual growth curves. During the 8-week treatment both male and female CFZ treated mice (orange) exhibit significant weight loss when compared to the vehicle treated (blue) mice (**Figure 4-2 a and b**). This difference can be further observed in **Figure 4-2c**, where CFZ-treated male mice exhibited a higher weight loss percent on average (SD) (10.75±6.42%) than CFZ treated female mice (7.31±5.11%) at the end of the 8-week treatment.

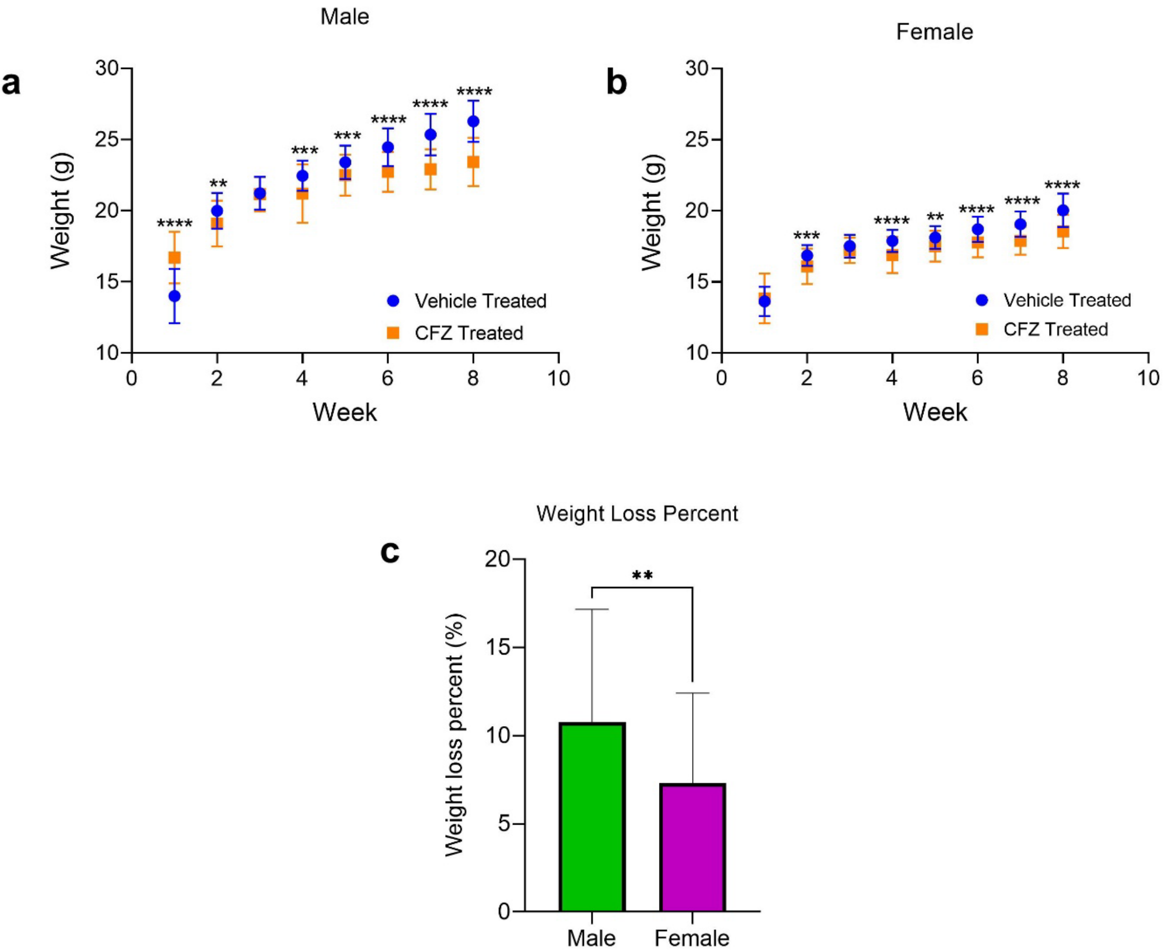

Figure 4-2 Changes in total body weight over 8-week treatment period. **a)** Male mice weight over the 8-week treatment of vehicle treated (blue) and CFZ treated (orange) mice. CFZ treated mice (23.42±1.70g) weighed, on average, less than vehicle treated mice (26.28±1.45g) at the end of treatment (****p<0.0001). **b)** The weight of female mice over the 8-week treatment of vehicle treated (blue) and CFZ treated (orange) mice. CFZ treated female mice (18.55±1.18g) weighed, on average, less than vehicle treated mice (20.04±1.17g) at the end of treatment (****p<0.0001). **c)** CFZ-treated males (green)

had a greater percent weight loss than CFZ treated females (purple) (**p= 0.004). Data are the mean (SD) of 50 mice/group.

When comparing food consumption between all treatment groups, no significant difference was observed between the four treatment groups (**Figure 4-3**).

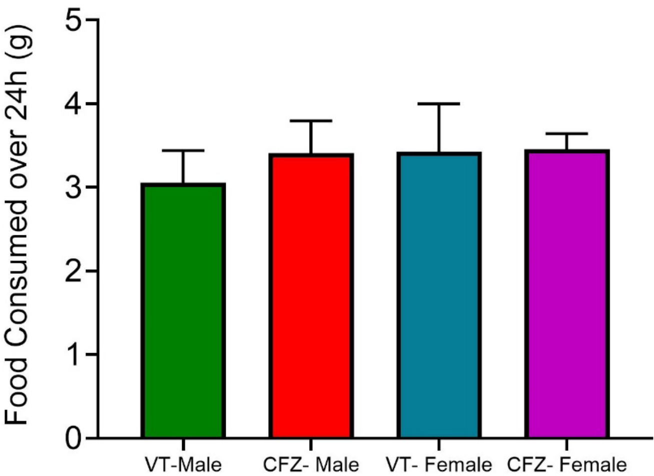

Figure 4-3 Food consumption over 24h study period. Vehicle treated male (green) mice consumed on average (mean±SD) the same amount of food (3.05±0.38g) as CFZ treated males (red) (3.40±0.39g) and as vehicle treated females (blue) (3.43±0.57g) and CFZ treated females (purple) (3.45±0.19g). No difference was found in the amount of food consumed between the four groups. VT-Male vs CFZ-Male (p=0.22), VT-Male vs VT Female (p=0.18), CFZ-Male vs CFZ-Female (p=0.99), and VT-Female vs CFZ-Female (p=0.99). Data are the mean (SD) of 5 mice/group.

Water consumption in CFZ-treated females was lower when compared to vehicle treated females (****p<0.0001). All other groups exhibited no difference in the amount of water consumed over 24h (**Figure 4-4**).

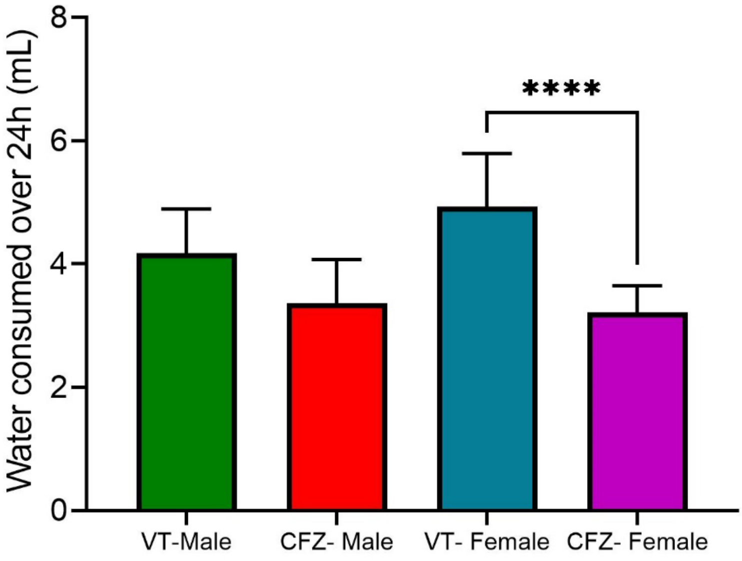

Figure 4-4 Water consumption over 24h study period. Vehicle treated male (green) mice consumed on average (mean±SD) the same amount of water (4.2 ± 0.72mL) as CFZ treated males (red) (3.4±0.71mL) and as vehicle treated females (blue) (4.9±0.86mL) and CFZ treated females (purple) (3.2±0.44mL). Only vehicle treated females and CFZ-treated females exhibited a significant difference (****$p<0.0001$). No difference was found in the amount of water consumed between the rest of the groups. VT-Male vs CFZ-Male ($p=0.06$), VT-Male vs VT Female ($p=0.08$), and CFZ-Male vs CFZ-Female ($p=0.98$). Data are the mean (SD) of 10 cages of 5 mice/group.

Another metabolic function tracked during the study was the amount of urine produced over 24h (**Figure 4-5**). There was no observed difference in the amount of urine produced by male vehicle treated and male CFZ treated mice. In females, CFZ treatment resulted in less urine production than in vehicle treated. When comparing male and females from both treatment groups, both CFZ and vehicle treated female mice produced less urine than male mice.

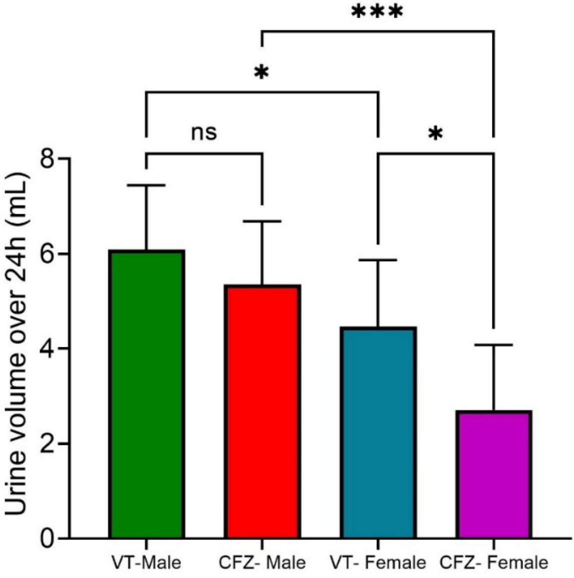

Figure 4-5 Urine production over 24h study period. Male CFZ (red) and vehicle treated (green) mice produced similar urine volumes (5.4±1.33mL vs 6.1 ±1.35mL, p=0.66). CFZ treated female mice (purple) produced less urine (2.7 ±1.37mL) than vehicle treated (4.5 ±1.40mL) mice (*p=0.03). CFZ treated female mice produced less urine (2.71 ±1.37mL) than CFZ-treated males (5.4 ±1.33mL) mice (***p=0.0004). Vehicle treated female mice (blue) produced less urine (4.5 ±1.40mL) than vehicle treated male (6.1±1.35mL) mice (*p=0.05). Data are the mean (SD) of 10 cages of 5 mice/group.

We also considered another indication of metabolic alteration that could be influenced by CFZ treatment, skeletal muscle mass. Skeletal muscle (quadricep) mass loss, normalized by body weight, occurred to a similar degree in both male and female mice (**Figure 4-6a**). However, male mice exhibited a higher muscle mass loss than female mice in the gastrocnemius (**Figure 4-6b**).

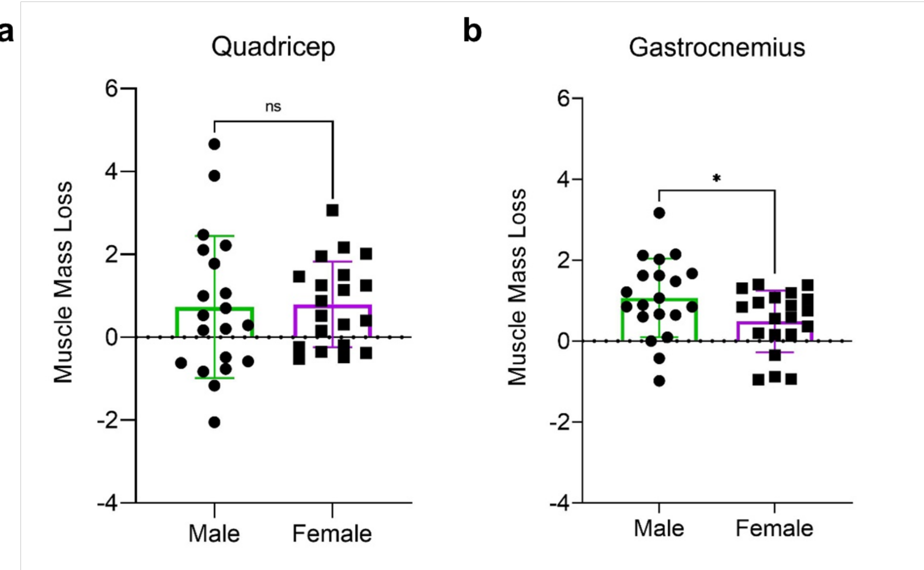

Figure 4-6 Change in skeletal muscle mass normalized to total body weight. **a)** Quadricep muscle mass loss was similar for both male (green) and female (purple) mice (p=0.88). **b)** Male mice (green) exhibited a greater loss in the gastrocnemius muscle (1.07 ± 0.97g), than female (purple) mice (0.49 ± 0.76g, *p=0.042). Data are mean (SD) of 20 mice per group. Muscle mass loss was calculated by normalizing the muscle mass of each individual mouse by total body weight and calculating the weight difference between vehicle treated and CFZ treated mice.

Since muscle mass loss in the gastrocnemius was found to be different between male and female mice, the concentration of both L-carnitine (**Figure 4-7**) and acetylcarnitine (**Figure 4-8**) in the gastrocnemius muscle was measured. No difference was observed in the amount of L-carnitine or acetylcarnitine in the gastrocnemius of both CFZ and vehicle treated male and female mice.

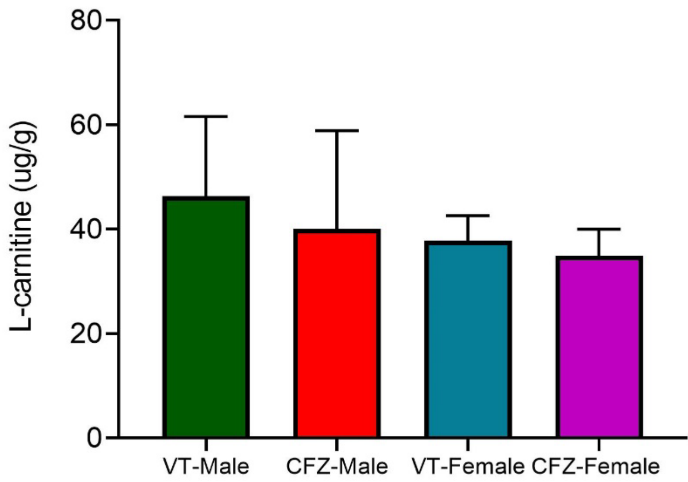

Figure 4-7 L-carnitine (ug/g protein) concentration in gastrocnemius muscle after carnitine challenge dose (1,000mg/kg). All four groups exhibited similar amounts of L-carnitine in muscle, vehicle treated males (green) (46.32 ±15.24 ug/g), CFZ-treated males (red) (40.07 ±18.80 ug/g), vehicle treated females (blue) (37.77 ±4.82 ug/g), and CFZ-treated females (purple) (34.93± 5.1 ug/g). VT-Male vs CFZ-Male (p=0.72), VT-Male vs VT Female (p=0.45), CFZ-Male vs CFZ-Female (p=0.84), and VT-Female vs CFZ-Female (p=0.98). Data are the mean (SD) of 10 mice/group.

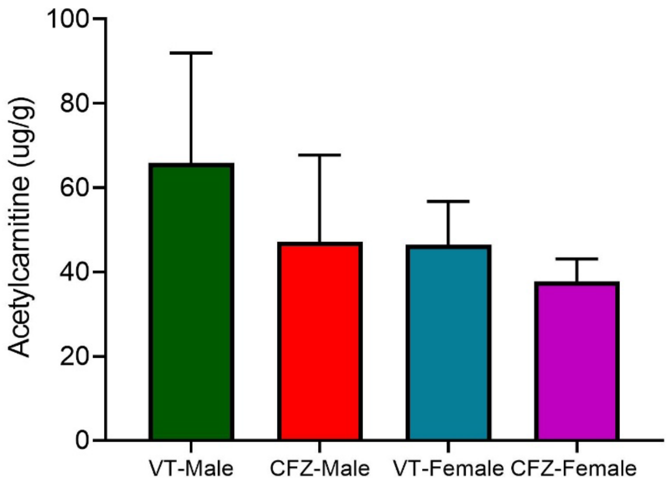

Figure 4-8 Acetylcarnitine (ug/g protein) concentration in gastrocnemius muscle after carnitine challenge dose (1,000mg/kg). All four groups exhibited similar amounts of L-carnitine in muscle, vehicle treated males (green) (65.88 ±26.10 ug/g), CFZ-treated males (red) (47.15 ±20.61 ug/g), vehicle treated females (blue) (46.44 ±10.28 ug/g), and CFZ-treated females (purple) (37.75 ±5.31 ug/g). VT-Male vs CFZ-Male (p=0.09), VT-Male vs VT Female (p=0.07), CFZ-Male vs CFZ-Female (p=0.67), and VT-Female vs CFZ-Female (p=0.73). Data are the mean (SD) of 10 mice/group.

In urine, the amount of L-carnitine was not different between the two treatment groups (**Figure 4-9**) in both male and females. Only vehicle treated females exhibited a lower amount of L-carnitine in urine when compared to vehicle treated males (*p=0.03). However, the amount of acetylcarnitine recovered in urine was higher in both vehicle treated male and females when compared to CFZ treated (**Figure 4-10**) mice. The amount of acetylcarnitine was significantly higher in vehicle treated males compared to vehicle treated females.

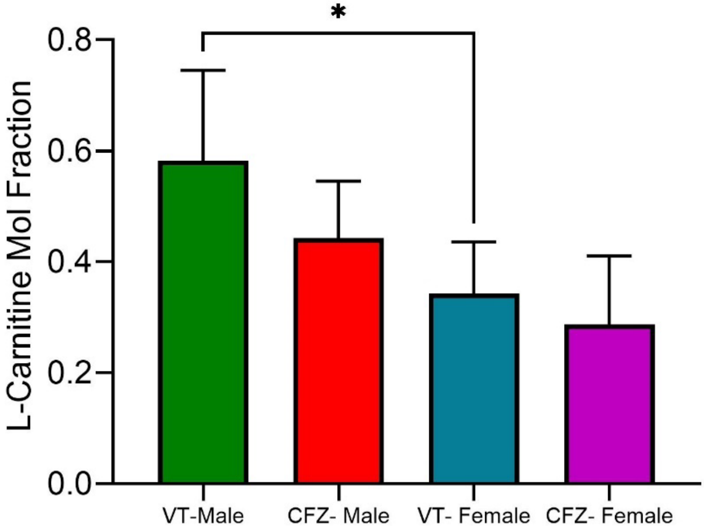

Figure 4-9 Amount of L-carnitine recovered (mole fraction) in urine after an L-carnitine challenge dose (1,000mg/kg). The L-carnitine in the urine 24h after a bolus IV L-carnitine injection was not changed by clofazimine (CFZ) treatment in male mice, CFZ (red) and vehicle treated (green) mice was 0.44 and 0.58, respectively (p=0.32). In female mice, the amount of L-carnitine recovered in urine was not changed by CFZ treatment; it was 0.29 and 0.34 in CFZ (purple) and vehicle treated (blue) mice, respectively (p=0.93). Urine L-carnitine in CFZ treated male (red) and female (purple) mice was 0.44 and 0.29, respectively (p=0.23). Urine L-carnitine in vehicle treated male (green) and female (blue) mice was 0.58 and 0.34, respectively (*p=0.03). Mole fraction of L-carnitine was calculated by dividing the amount of L-carnitine recovered in urine collected over 24h following the administration of the L-carnitine challenge dose. Data are the mean (SD) of five metabolic cages each housing 5 mice/group.

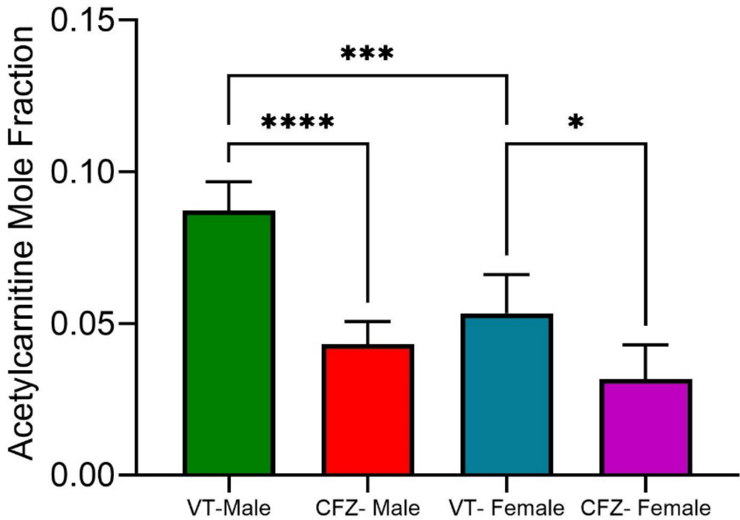

Figure 4-10 Amount of acetylcarnitine recovered (mole fraction) in urine after L-carnitine challenge dose (1,000mg/kg). CFZ treated male mice (red) had less acetylcarnitine in urine (0.04) than vehicle treated mice (0.09, ****p<0.0001). In females, the amount of acetylcarnitine recovered in urine was less in CFZ treated (0.03) than vehicle treated mice (0.05, *p=0.02) Acetylcarnitine in CFZ treated male (red) and female (purple) mice was 0.04 and 0.03, respectively (p=0.34). Acetylcarnitine in vehicle treated male (green) and female (blue) mice was 0.09 and 0.05, respectively (***p=0.0004). Mole fraction of acetylcarnitine was calculated by dividing the amount of acetylcarnitine recovered in urine collected over 24h following the administration of the L-carnitine challenge dose. Data are the mean (SD) of five metabolic cages each housing 5 mice/group.

Following tail vein injection of L-carnitine (1000 mg/kg), both male and female vehicle and CFZ treated mice had similar L-carnitine levels in whole blood (**Figure 4-11**).

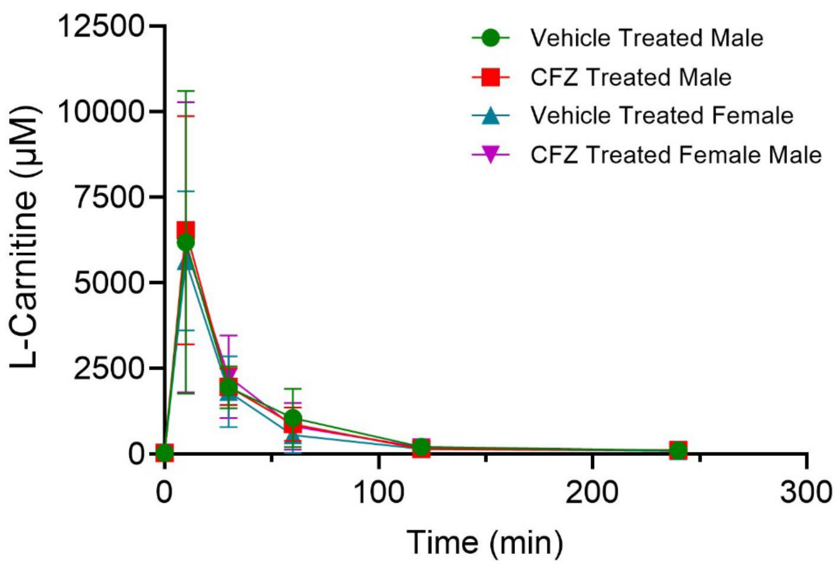

Figure 4-11 Whole blood L-carnitine concentrations (µM) in CFZ and vehicle treated male and female mice following tail vein injection of an L-carnitine “challenge test” (1000mg/kg). No difference was observed between any of the treatment groups. Data are the mean (SD) of 10 female and male mice per time point.

The mean (SD) whole blood acetylcarnitine concentrations in males was lower in CFZ treated male mice (red) at 10min (*p=0.05), 60min (***p=0.0002), and 120min (*p=0.02) compared with vehicle treated (green) mice (**Figure 4-12a**). A similar trend was observed with acetylcarnitine whole blood levels in female mice, where CFZ treated female mice (purple) had lower acetylcarnitine levels at BL (*p=0.05), and 30min (**p=0.008) (**Figure 4-12b**). Whole blood acetylcarnitine concentration following tail vein injection of L-carnitine (1000 mg/kg), within male and female vehicle and CFZ treated mice, were similar for both groups (**Figure 4-12c and d**). The mean (SD) whole blood acetylcarnitine concentrations showed a trend of being lower in CFZ treated male mice when compared to female mice (**Figure 4-12d**) but this difference was not significant.

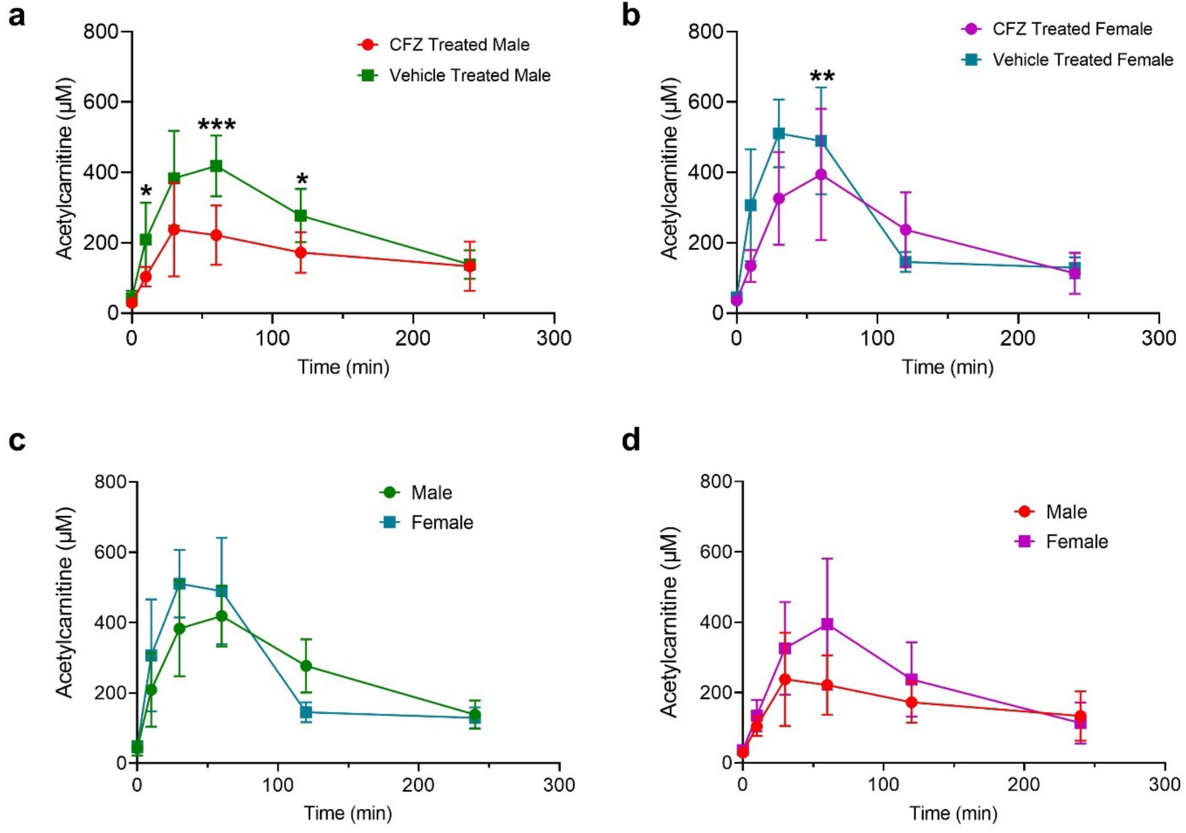

Figure 4-12 Whole blood acetylcarnitine concentrations (µM) in male and female mice following tail vein injection of an L-carnitine "challenge test" (1000mg/kg). **a)** CFZ treated male mice had lower acetylcarnitine levels at 10min (*p=0.05), 60min (***p=0.0002), and 120min (*p=0.02) compared with vehicle treated mice. **b)** CFZ treated female mice had lower acetylcarnitine levels at BL (*p=0.05), and 30min (**p=0.008), at 10min (p=0.06) and 60min (0.85) the difference was not significant. **c)** Vehicle treated male and female mice had similar acetylcarnitine levels. **d)** CFZ treated male and female mice had similar acetylcarnitine levels. Data are the mean (SD) of 10 female and male mice per time point.

Post-L-carnitine injection, CFZ-treated male mice tended to have a lower blood acetylcarnitine to L-carnitine ratio (a reflection of carnitine homeostasis), but this was not statistically different (**Figure 4-13a**). In females, the same trend was observed at all timepoints except 120min, where CFZ treated mice had a higher acetylcarnitine to L-carnitine ratio, but none of these differences were statistically different (**Figure 4-13b).**

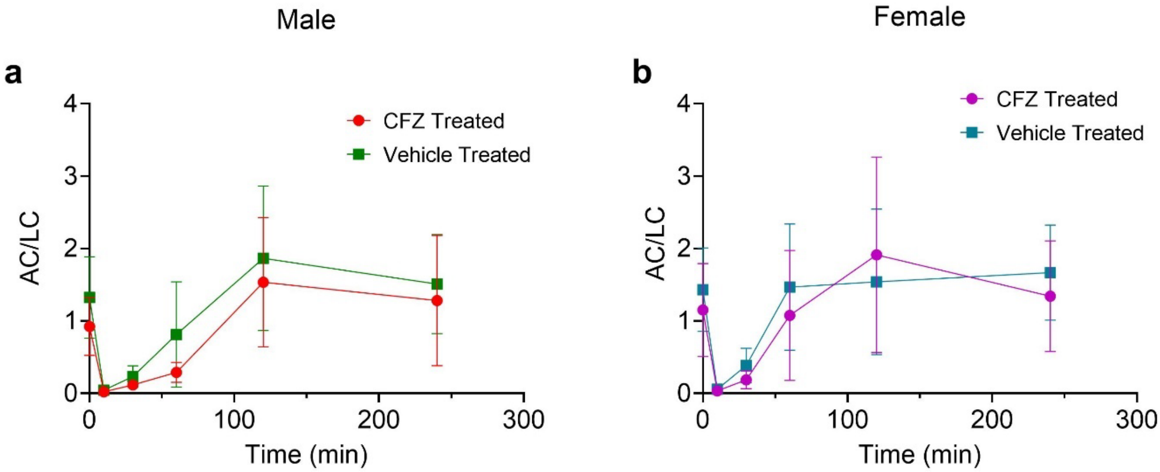

Figure 4-13 Acetylcarnitine/L-carnitine ratio of whole blood concentration measurements at different timepoints for vehicle treated male (green), CFZ treated male (red), vehicle treated female (blue) and CFZ treated female (purple).

4.4.2 NMR Metabolomics

A total of 45 metabolites were detected and quantified in whole blood samples that were available from 8-week CFZ (n=19) and vehicle-treated male mice (n=14), and CFZ (n=18) and vehicle treated (n=10) female mice, respectively. Of the total whole blood samples, CFZ treated male mice (n=9), vehicle treated male mice (n=4), CFZ treated female mice (n=9), and vehicle treated female mice (n=2) were collected after L-carnitine challenge injection (1,000 mg/kg), the remaining were from mice that did not receive an L-carnitine challenge and are referred to as unchallenged. In unchallenged, 8-week CFZ treated male and female mice, there were no sex-related changes in 44 of the 45 blood metabolite concentrations (**Figure 4-14**); ATP levels were higher in male than females (*p=0.05). In L-carnitine challenged mice, there were no differences in whole blood metabolite levels between CFZ-treated male and female mice (**Figure 4-15**). Since no sex-related differences were found and no differences were observed between

challenged and unchallenged mice, further analysis was conducted only based on CFZ treatment, regardless of sex.

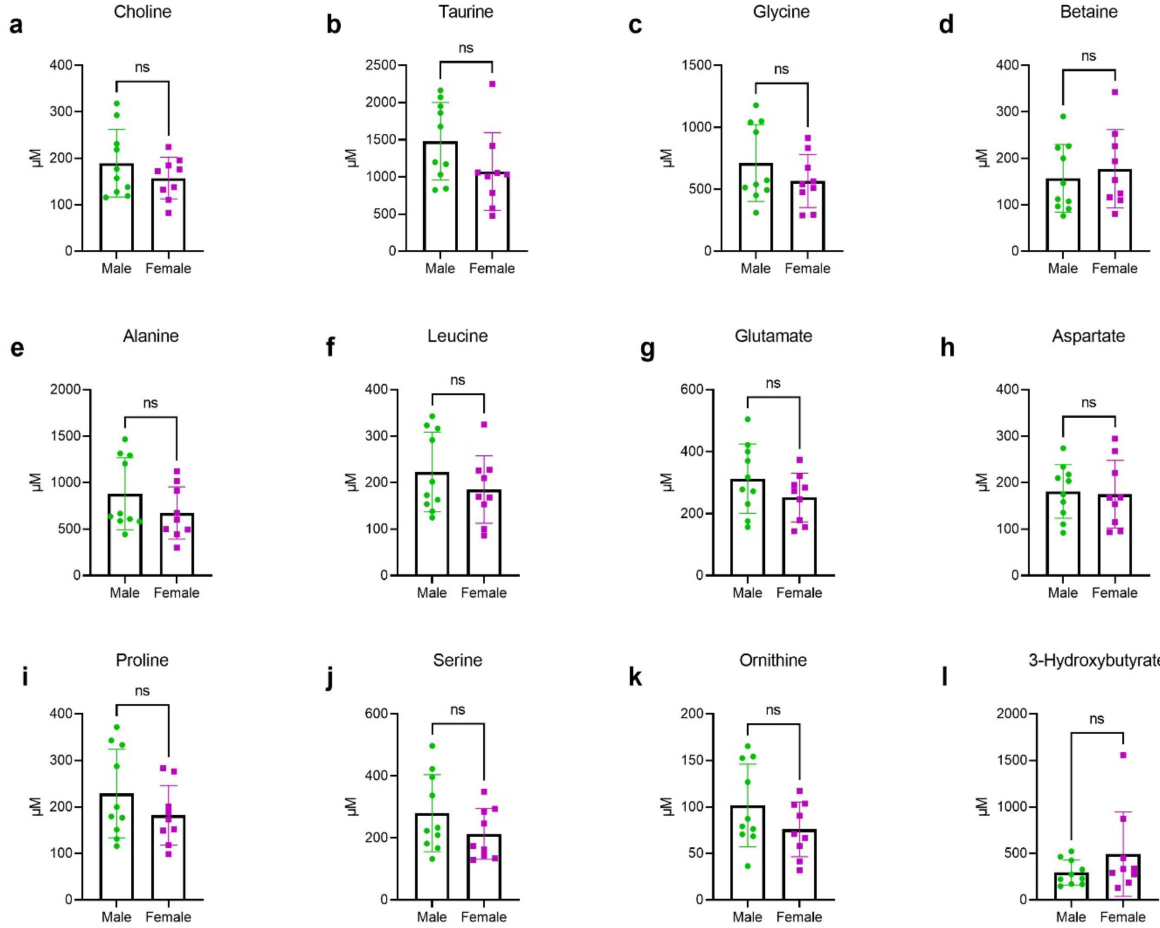

Figure 4-14 Whole blood metabolites concentrations after 8-weeks of CFZ treatment in unchallenged male and female mice. (**a**) choline (KEGG ID: C00114; p=0.27); (**b**) taurine (KEGG ID: C00245; p=0.11); (**c**) glycine (KEGG ID: C00037; p=0.26); (**d**) betaine (KEGG ID:C00719; p=0.57); (**e**) alanine (KEGG ID: C00041; p=0.21); (**f**) leucine (KEGG ID: C00123; p=0.32); (**g**) glutamate (KEGG ID: C00025; p=0.19); (**h**) aspartate (KEGG ID: C00049; p=0.85); (**i**) proline, (KEGG ID: C00148; p=0.23); (**j**) serine (KEGG ID: C00065; p=0.19); (**k**) ornithine, a product of the urea cycle (KEGG ID: C00077; p=0.16); (**l**) the ketone body, 3-hydroxybutyrate (3-OHB; KEGG ID: C01089; p=0.21). Data are the mean (SD) of 10 and 9 CFZ-treated male and female mice respectively.

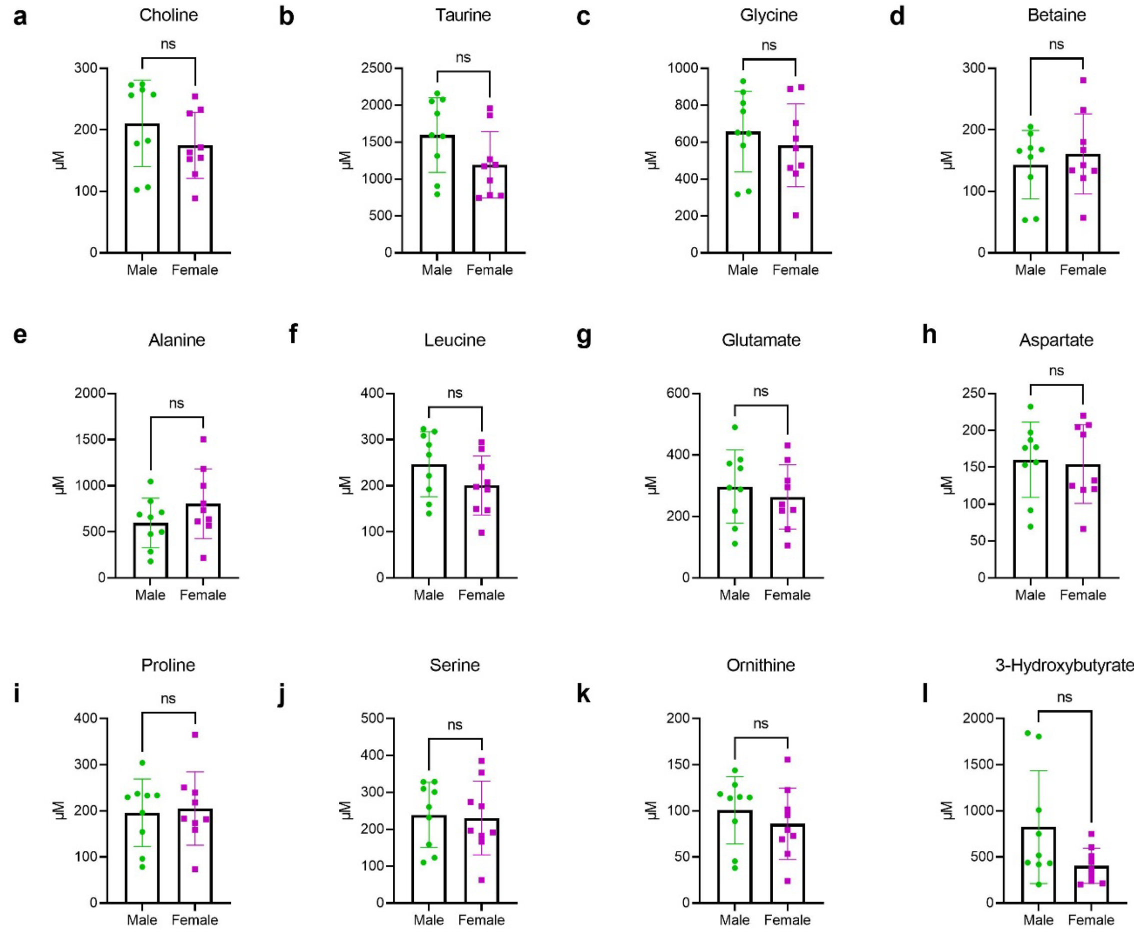

Figure 4-15 Whole blood metabolites concentrations after 8-weeks of CFZ treatment in L-carnitine challenged male and female mice. (**a**) choline (KEGG ID: C00114; p=0.24); (**b**) taurine (KEGG ID: C00245; p=0.09); (**c**) glycine (KEGG ID: C00037; p=0.49); (**d**) betaine (KEGG ID:C00719; p=0.55); (**e**) alanine (KEGG ID: C00041; p=0.19); (**f**) leucine (KEGG ID: C00123; p=0.17); (**g**) glutamate (KEGG ID: C00025; p=0.53); (**h**) aspartate (KEGG ID: C00049; p=0.82); (**i**) proline, (KEGG ID: C00148; p=0.80); (**j**) serine (KEGG ID: C00065; p=0.85); (**k**) ornithine, a product of the urea cycle (KEGG ID: C00077; p=0.42); (**l**) the ketone body, 3-hydroxybutyrate (3-OHB; KEGG ID: C01089; p=0.066). Data are the mean (SD) of 9 CFZ-treated male and female mice.

In CFZ-treated mice of the total 45 detected metabolites, 33 exhibited an increase, 3 decreased with CFZ treatment and 9 remained unchanged. There were alterations in most blood metabolite concentrations, including choline, serine, glycine and betaine (**Fig. 4-16a-f**); these were all increased compared to vehicle-treated mice.

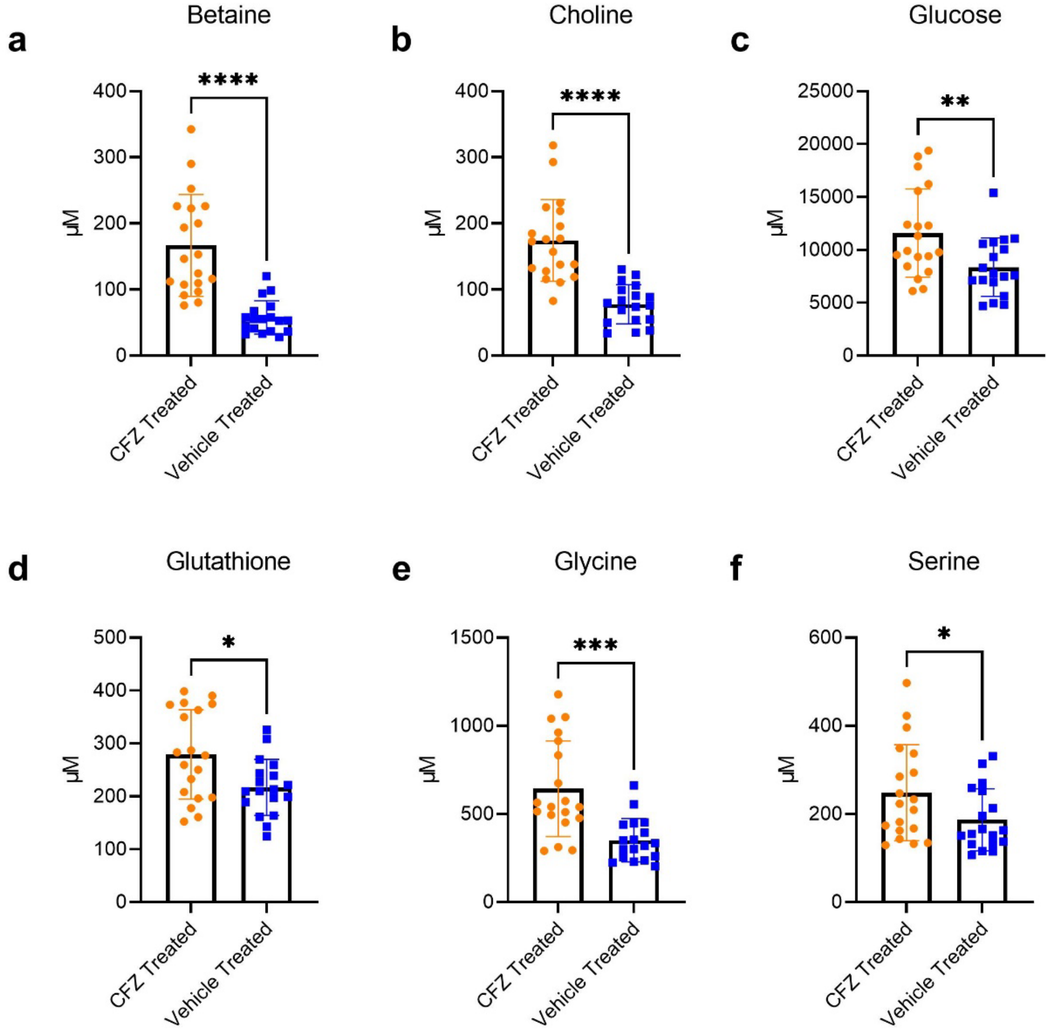

Figure 4-16 Whole blood metabolites concentrations after 8-weeks of CFZ or vehicle treatment. (**a**) betaine (KEGG ID:C00719; ****p<0.0001) (**b**) choline (KEGG ID: C00114; ****p<0.0001); (**c**) glucose (KEGG ID: C00031; **p=0.009); (**d**) glutathione (KEGG ID C00051; *p=0.011); (**e**) glycine (KEGG ID: C00037; ***p=0.0003); (**f**) serine (KEGG ID: C00065; *p=0.05). Data are the mean (SD) of 19 CFZ-treated and 18 vehicle treated mice.

Additionally, to the metabolites listed above, another set of essential and non-essential amino acids also exhibited an increase after 8 weeks of CFZ treatment (**Fig. 4-17**).

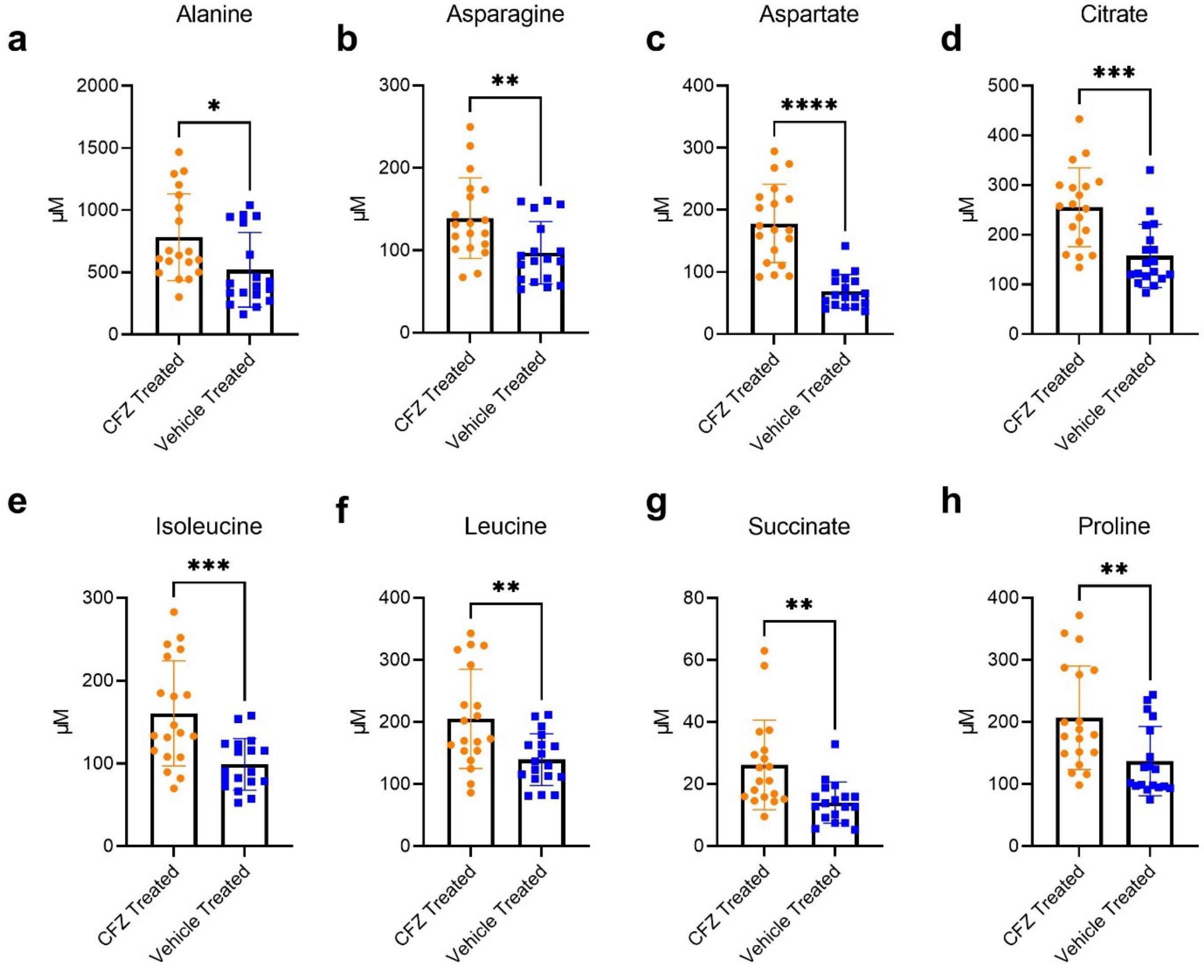

Figure 4-17 Whole blood metabolites concentrations after 8-weeks CFZ or vehicle treatment. (**a**) alanine (KEGG ID: C00041;* p=0.02); (b) asparagine (KEGG ID: C00152; **p=0.006) ; (**c**) aspartate (KEGG ID: C00049; ****p<0.0001); (**d**) citrate (KEGG ID C00158; ***p=0.0002); (**e**) isoleucine (KEGG ID C00407; ***p=0.0008); (**f**) leucine (KEGG ID C00123; **p=0.004); (**g**) succinate (KEGG ID C00042; **p=0.003); (**h**) proline, (KEGG ID: C00148; **p=0.005). Data are the mean (SD) of 19 CFZ-treated and 18 vehicle treated mice.

4.5 Discussion

Despite that CFZ is well tolerated, it imposes considerable metabolic stress on the host secondary to induction of a catabolic state[15,27–29] and adversely impacts mitochondrial function[30]. The results of these metabolic alterations are evident (**Figures 4-2 to 4-6)**, with the observed changes in weight, muscle mass and metabolic functions, including food and water consumption.

All these observations are consistent with our previous findings[15] and with the known manifestations of a catabolic state[31], loss of muscle mass and an inability to gain weight even after increasing caloric intake, as explained in the previous chapter. The differences previously observed in CFZ treated male mice, were also evident but not remarkably different in CFZ-treated females. The main sex-related finding of CFZ treatment was weight loss (**Figure 4-2c**) in which males had a greater percent loss of total body weight and mass of the gastrocnemius muscle than female mice (**Figure 4-6**). Because of the observed difference in gastrocnemius muscle mass loss, the concentration of both L-carnitine (**Figure 4-7**) and acetylcarnitine (**Figure 4-8)** in the gastrocnemius muscle was measured. No difference was observed in the amount of L-carnitine or acetylcarnitine in the gastrocnemius of both CFZ and vehicle treated male and female mice.

Following 8-weeks of CFZ treatment we subjected mice to the L-carnitine challenge test (1,000 mg/kg given as a single IV injection). Although we did not detect a CFZ-induced change in the amount of L-carnitine in urine in most groups, vehicle treated females showed a lower amount of L-carnitine in urine when compared to vehicle treated males (**Figure 4-9**). The amount of acetylcarnitine in urine was lower in CFZ treated male and female mice when compared to vehicle treated animals (**Figure 4-10**). This result is most likely attributable to the impact of CFZ on acetylcarnitine metabolism. As stated in the previous chapter, the generation of acetylcarnitine relies on the availability of L-carnitine and acetyl-CoA, a reduction in the availability of acetyl-CoA for acetylcarnitine generation secondary to CFZ-induced catabolism can be attributed to the low levels of acetylcarnitine. The high energy demand of a catabolic state leads to consumption of acetyl-CoA leaving less reserve available for the production of acetylcarnitine[32]

Similarly, in whole blood we did not detect a sex-related CFZ-induced change in concentrations of L-carnitine (**Figure 4-11**). The mean (SD) whole blood acetylcarnitine concentration trended lower in CFZ treated male mice when compared to CFZ-treated females (**Figure 4-12d**) but this difference was not significant. The observed trend seems to indicate that there are no apparent sex-related differences induced by CFZ treatment or identified by the L-carnitine challenge test.

In CFZ-treated male mice, the L-carnitine challenge test induced differences in whole blood acetylcarnitine concentrations. Specifically, acetylcarnitine concentrations were lower when compared to vehicle treated mice (**Figure 4-12a**), supporting the findings of the previous chapter but with a bigger sample size, corroborating the potential use of an L-carnitine challenge test as a probe of metabolic ADRs. In females, the same was observed to a lesser extent, with CFZ -treated mice exhibiting lower acetylcarnitine concentrations than vehicle treated (**Figure 4-12b**). The observed difference in CFZ and vehicle treated mice in both male and females, indicate that the CFZ-induced alterations in metabolism present in a similar manner regardless of sex. Collectively, these findings support that CFZ-induced high energy demand leads to consumption of acetyl-CoA leaving less reserve available for the production of acetylcarnitine. This indicates that at least for the drug CFZ, no further considerations with regards to sex need to be made when developing the L-carnitine challenge test as a probe of mitochondrial metabolic health.

To further evaluate CFZ treatment in male and females, we conducted whole blood NMR metabolomics and compared metabolite concentrations (µM) of unchallenged (no L-carnitine challenge) male and female mice to L-carnitine challenged male and female mice. Of the total 45 metabolites detected, 44 of the concentrations were not different between male and females

(**Figure 4-14**). The only metabolite that was found to different in male than female mice was ATP (*p=0.05). The L-carnitine challenge did not alter whole blood metabolite levels between CFZ-treated male and females supporting that there is no CFZ-induced sex-related differences that are influenced by the L-carnitine challenge (**Fig. 4-15**).

Having established that there is no CFZ-induced sex-related differences that are influenced by the L-carnitine challenge, and to further evaluate the CFZ-induced high energy demand, our metabolomics analysis was focused on the effects of CFZ treatment on the host metabolism.

Differences were observed in several metabolites' levels between vehicle and CFZ-treated mice (**Fig. 4-16 & 4-17**). Of the 45 detected metabolites, 33 showed an increase with CFZ treatment, 3 decreased and 9 remained unchanged. These results observed in CFZ-treated mice are supported by a previous study in which CFZ treated mice exhibited higher levels choline, glycine, serine and betaine, all integral to one-carbon metabolism (**Fig. 4-16 & 4-18**). One-carbon metabolism encompasses a complex metabolic network involving the folate and methionine cycle, it integrates carbon units from amino acids, including serine and glycine, and generates diverse outputs, such as the biosynthesis of lipids, nucleotides and proteins and the substrates for methylation reactions.[33] These two cycles also link with the trans-sulfuration pathway, which plays a critical role in the regulation of the redox status by producing glutathione. One-carbon metabolism is critical for the maintenance of genomic stability through nucleotide metabolism as well as for the epigenetic control of DNA and histones.[15,33] (**Figure 4-18**). CFZ treated mice exhibited higher levels of betaine, choline, glucose, glutathione, glycine, serine and taurine, all metabolites involved in one-carbon metabolism.

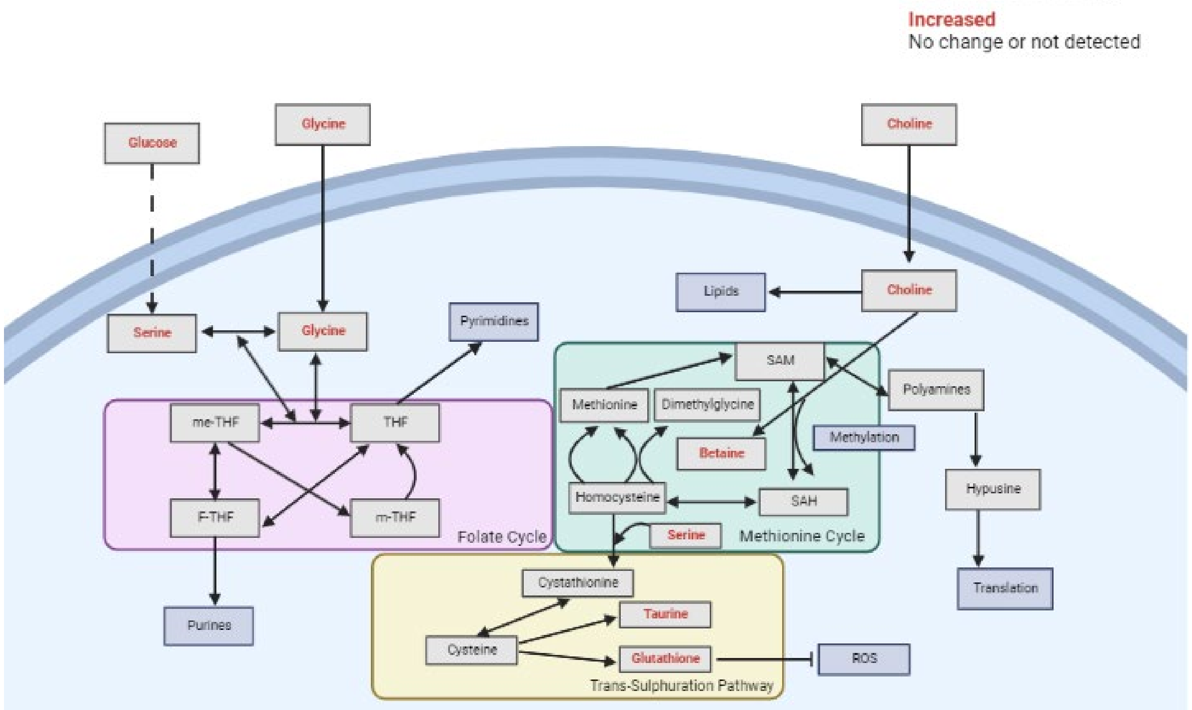

Figure 4-18 One-carbon metabolism with metabolic changes induced in the ^{1}H-NMR detected whole blood (WB) metabolomes after 8-weeks of clofazimine (CFZ) treatment. Metabolites that increased with CFZ treatment are shown in red. THF= tetrahydrofolic acid; me-THF=N5N10-methylene-tetrahydrofolic acid; m-THF=N5-methyl-tetrahydrofolicacid; F-THF=N10-formyl-tetrahydrofolic acid; SAM=S-adenosylmethionine; SAH=S-adenosyhomocysteine; ROS= reactive oxygen species. Created with BioRender.com based on Konno M., et al., 2017.[34]

In addition to the amino acids involved in one-carbon metabolism, the results suggest a CFZ-induced reorientation in amino acid metabolism, a phenomenon that was previously describe[15]. The whole blood levels of alanine, asparagine, aspartate, citrate, isoleucine, leucine, proline, and succinate were increased in CFZ treated mice **(Fig. 4-17)**. This suggests that CFZ altered host amino acid metabolism. Overall, the CFZ-induced increase in WB essential and non-essential amino acids observed implicate an increased requirement for amino acid driven biosynthetic processes such as protein and membrane synthesis that are likely required for the macrophage-mediated CFZ sequestration response[15].

We acknowledge that there are limitations to this study. As previously mentioned, CFZ is only one of many drugs that can affect mitochondrial metabolic function through a variety of different mechanisms. We acknowledge that the interaction between other mitochondriotoxic drugs and L-carnitine/acetylcarnitine transport, metabolism and disposition pathways could be very different from those that occur during CFZ treatment. This study was also limited by an absence of detailed evaluation of the CAT enzyme activity after CFZ treatment, quantification of enzyme activity could help us elucidate if there is any form of CAT inhibition caused by CFZ that could also explain the low acetylcarnitine levels observed. The ^{1}H-NMR whole blood metabolomics study was also limited to the terminal timepoint after 8-weeks of treatment. Sampling at earlier timepoints, after 1 or 2 weeks of treatment CFZ treatment, could identify earlier indications of shifts in metabolism associated with CFZ bioaccumulation.

Despite these limitations, the findings described here corroborate our previous findings for the potential development of a test to probe the relationship between baseline metabolic stress and drug-related mitochondrial toxicity that could help determine ADR risk regardless of sex. Additionally, the sex-related differences were not differentiated by an L-carnitine challenge test. To further refine these measurements, a more detailed assessment of the distribution of L-carnitine and acetylcarnitine in response to the challenge test, could be elaborated using an isotope labeled L-carnitine analog, along with more extensive metabolite evaluation.

4.6 Conclusions

CFZ treatment did not induce any sex-related differences in metabolism that was not further provoked by the L-carnitine "challenge test" revealed as measured by whole blood acetylcarnitine concentration in CFZ-treated mice. These findings serve as an indication of the potential use of the challenge test as a "probe" to identify latent drug-related mitochondrial

toxicological manifestations in both males and females. The found differences in NMR measured metabolite concentrations could lead to better understanding of the mechanisms/pathways that contribute to ADRs in both males and females. NMR metabolomics identified the broad metabolic consequences of CFZ and the limited scope of the L-carnitine challenge test.

4.7 References

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Chapter 5
Conclusions and Future Outlook

Despite significant advances in medical sciences and constant improvements in the drug development process, ADRs still represent a major healthcare concern for patients and clinicians as well as an economic burden on hospitals and pharmaceutical companies attempting to develop new drug candidates. ADRs are an inherent risk of any drug therapy regimen, each of which is differentially governed by specific factors such as genotype and health status of a patient, the compound's pharmacokinetic parameters (e.g., volume of distribution, clearance, half-life, etc.), dose and frequency of administration. ADRs found to be toxicological manifestations of off-target drug interactions affecting the structure and function of cellular organelles, such as the mitochondria, are of significant interest since presently, there are no clinically utilized specific biomarkers of mitochondrial toxicity, and the current FDA drug approval process can miss these manifestations since they can be subtle, not readily apparent or might appear long-term.

To address this need, within this dissertation, we have explored (1) the application of a database mining strategy to identify endogenous candidate metabolites to probe and assess mitochondrial drug toxicity, (2) the use of an L-carnitine challenge test as a metabolic stress biomarker to identify risk of mitochondrial-related ADRs, and (3) the possible application of the L-carnitine challenge test as an indicator of sex-related predisposition for ADRs.

The first project aimed to identify candidate mitochondrial metabolites that could be clinically useful to identify increased risk of mitochondrial-related ADRs, using a database

search strategy. The results of the database screening and process of elimination strategy pointed to L-carnitine as a highly feasible candidate for clinical use based on *a priori* criteria. This result further supported by literature evaluation, corroborated the usefulness of the database approach for strategically identifying those endogenous metabolites that could serve as candidate biomarkers for detecting changes in metabolism induced by drugs that target a specific organelle. Of noteworthy significance, the existing information that was publicly available in databases allowed for a systematic process of elimination, which led to a concise list of clinically feasible human metabolite candidates for use as functional tracers of mitochondrial metabolic health.

Targeted measurements of carnitine and acetylcarnitines were revealed to be potentially informative for the identification of metabolic ADRs of drug-induced mitochondrial dysfunction during treatment with a number of different drugs including the examples discussed in this dissertation. Drug-induced mitotoxic signals that could be detected in advance of a clinical phenotype may be particularly useful for therapeutic drug monitoring and the avoidance of ADRs altogether.

The limited number of databases used for the mining strategy and the number of *a priori* identified criteria could be considered a limitation to this study. There are multiple databases that could be explored to further identify candidates based on different criteria than the ones utilized here. One noteworthy characteristic is that this workflow methodology could be modified based on the needs of the user, by establishing a different set of *a priori* criteria. To further advance this methodology, artificial intelligence (AI) and automation tools could aid and build upon the database screening methodology strategy. As AI capabilities are becoming more accessible to non-professional users in and outside of science, it allows for the potential to screen multiple databases at a time, faster and efficiently.

Despite these limitations, this project identified L-carnitine as a functional biomarker of mitochondrial toxicity that could be used to stratify individuals at increased ADR risk. The findings described here served as a starting point in the development of a test to probe the relationship between baseline metabolic stress and drug-related mitochondrial toxicity that could help determine which patients may be at risk.

The second project looked to further corroborate the results from the database screening algorithm and to establish L-carnitine feasibility for clinical use using an *in vivo* evaluation in a mouse model called the "L-carnitine challenge". The most significant finding from this project was that the L-carnitine challenge test induced differences in whole blood acetylcarnitine concentrations in CFZ-treated mice. Specifically, in CFZ treated mice, acetylcarnitine concentrations were significantly lower when compared to vehicle treated mice. The decline in acetylcarnitine production in response to L-carnitine supplementation in CFZ-treated mice was attributed to a reduction in the availability of acetyl-CoA for acetylcarnitine generation secondary to CFZ-induced catabolism. The high energy demand of a CFZ-induced catabolic state leads to an increase consumption of acetyl-CoA leaving less reserve available to produce acetylcarnitine.

This project revealed that clinical use of an L-carnitine challenge test with subsequent measurement of mitochondrial metabolites like acetylcarnitine, could be an important early step to identify occult medication-induced mitochondrial toxicity. Targeted measurements of carnitine and acetylcarnitines may be informative for the identification of metabolic ADRs of drug-induced mitochondrial dysfunction during treatment with several different drugs including the examples previously discussed in chapter 2. Drug-induced mitotoxic signals that could be

detected in advance of a clinical phenotype may be particularly useful for therapeutic drug monitoring and the avoidance of ADRs.

To further refine these measurements, the use of an isotope labeled L-carnitine analog could be elaborated for a more detailed assessment of the distribution of L-carnitine and acetylcarnitine in response to the L-carnitine challenge test. Such a metabolic tracer could yield additional information about drug-induced changes to endogenous L-carnitine and acetylcarnitine stores, transport, and utilization. This study was also limited to mice of the same genetic background. It therefore does not fully account for all the variation in L-carnitine and acetylcarnitine transport, metabolism and disposition that may characterize an entire population with different genetics, ages, and exposures to different environmental conditions that might lead to a more varied response to either CFZ or to an L-carnitine challenge test.

The third project was conducted to (1) further corroborate the results from the database screening methodology study, (2) to further establish L-carnitine feasibility for clinical use in both male and female, and (3) to aid in further closing the knowledge gap of sex-related differences in ADR incidence, since females have been found to experience ADRs at a higher rate than males. The “Carnitine challenge test” was once again carried out for *in vivo* evaluation in a mouse model. The results from this project showed that CFZ treatment did not induce any sex-related differences in metabolic functions that was not further provoked by the L-carnitine “challenge test”. While the L-carnitine “challenge test” once again induced differences in whole blood acetylcarnitine concentrations in CFZ-treated mice in both male and females, further supporting our previous finding on the potential of this challenge test as a probe of mitochondrial metabolism health. The L-carnitine “challenge test” revealed no sex-related differences in mitochondrial function as evidenced by whole blood acetylcarnitine concentration in CFZ-

treated mice, this is an important finding since it serves as an indication of the potential use of the challenge test as a “probe” to identify latent drug-related mitochondrial toxicological manifestations not only in males, but also in females.

While the effects of long-term CFZ treatment have been studied in males multiple times, the differences observed in whole blood acetylcarnitine concentrations between male and females indicate that after long-term CFZ treatment the L-carnitine challenge test did not alter whole blood metabolite levels between CFZ-treated male and females supporting that there is no CFZ-induced sex-related differences that are influenced by the L-carnitine challenge test. The differences observed in NMR metabolite concentrations between CFZ-treated and vehicle treated mice regardless of sex could lead to better understanding of the mechanisms/pathways that contribute to ADRs. The results from NMR metabolomics identified the broad metabolic consequences of CFZ in one-carbon metabolism and amino acid metabolism.

Although this study sheds some light in the effects of long-term CFZ treatment in both male and females, it was limited in the evaluation of ^{1}H-NMR whole blood metabolomics to the terminal timepoint after 8-weeks of treatment. Sampling at earlier timepoints, after 1 or 2 weeks of treatment CFZ treatment, could identify earlier indications of shifts in metabolism associated with CFZ bioaccumulation and macrophage sequestration. Once again, the use of an isotope labeled L-carnitine analog could be elaborated for a more detailed assessment of the distribution of L-carnitine and acetylcarnitine in response to the L-carnitine challenge test in both male and females.

In full, this dissertation has (1) developed the potential application of a database mining strategy to identify endogenous candidate metabolites to probe and assess mitochondrial drug toxicity, (2) proposed the use of an L-carnitine challenge test as a metabolic stress biomarker to

identify risk of mitochondrial-related ADRs, and (3) explored the potential application of the L-carnitine challenge test as an indicator of sex-related predisposition for ADRs. All of this with the goal of potentially reducing ADRs incidences during lifesaving drug treatment.