AMERICAN UNIVERSITY OF BEIRUT

IMPACT OF INTERMITTENT FASTING ON PREDIABETES-INDUCED NEUROPATHY: INSIGHTS ON A NOVEL MECHANISTIC PATHWAY

By

MAYA MHAMAD DANNAWI

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, cell biology and physiological sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
June 2019

AMERICAN UNIVERSITY OF BEIRUT

IMPACT OF INTERMITTENT FASTING ON PREDIABETES-INDUCED NEUROPATHY: INSIGHTS ON A NOVEL MECHANISTIC PATHWAY

by

Maya Mhamad Dannawi

Approved by:

Dr. Assaad A. Eid, Associate Professor — Advisor
Department of Anatomy, Cell Biology,
and Physiological Sciences

Dr. Abdo Jurjus, Professor — Member of Committee
Department of Anatomy, Cell Biology,
and Physiological Sciences

Dr. Hala Darwish, Associate Professor — Member of Committee
Hariri School of Nursing

Dr. Omar Obeid, Professor — Member of Committee
Department of Nutrition and Food Sciences
Faculty of Agricultural and Food Sciences

Date of thesis defense: June 21, 2019

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name:

Dannawi	Maya	Mhamad
Last	First	Middle

X Master's Thesis Master's Project Doctoral Dissertation

I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

X I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes

after: **One ---- year from the date of submission of my thesis, dissertation, or project.**

Two ---- years from the date of submission of my thesis, dissertation, or project.

X Three ---- years from the date of submission of my thesis, dissertation, or project.

Signature Maya Dannawi Date: 26/6/2019

ACKNOWLEDGEMENTS

Accomplishing the work at hand was not an easy task but going through this journey was pleasant because of the people that I was surrounded with. I cannot emphasize in the strongest of terms how grateful I am for my advisor, parents, and colleagues. Thank you Dr. Eid, for believing in me and allowing me to be an independent thinker and worker capable of paving my scientific paths. Mom, dad, Reina, and Dina you were always there, and I trust you will always be. Thank you Dr. Fred for your vital input and dedication to helping us. Lab-ees, your support throughout my work and our endless conversations are pillars to my lab experience. Thank you AUB for this experience for the endless Lab/Jafet nights, broken gym promises, and myriads of coffee cups. Thank you Sara and Marina for being the ones I shared those moments with and for preventing homesickness by being my family in Beirut.

AN ABSTRACT OF THE THESIS OF

Maya Mhamad Dannawi for Master of Science
Major: Physiology

Title: IMPACT OF INTERMITTENT FASTING ON PREDIABETES-INDUCED NEUROPATHY: INSIGHTS ON A NOVEL MECHANISTIC PATHWAY

Background: Prediabetes, also known as intermediate hyperglycemia, is an alarming precursor of type 2 diabetes mellitus, which is fraught with complications. Peripheral neuropathy is one major complication that is a cause of disability worldwide. A growing body of literature links prediabetes, obesity and metabolic syndrome to the risk of both diabetic peripheral neuropathy (DPN) and cryptogenic sensory peripheral neuropathy (CSPN). Lifestyle changes are key in the management of prediabetes. Recent data suggest that intermittent fasting is the cornerstone in the management of obesity, however, its role in prediabetic complications is not well described. Furthermore, the mechanism of action by which intermittent fasting exert its metabolic role remains to be poorly investigated.

Reactive oxygen species production (ROS) has been described as the final common signaling pathway orchestrating the onset and development of complications. Moreover, increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes and its different complications. Cytochromes P450 monooxygenases, especially those involved in the epoxygenase pathways controlling EETs production, are considered to be a negative regulator of ROS production. Nevertheless, this role has not been fully interrelated to peripheral neuropathy.

Aim: In this study we investigated the influence of intermittent fasting on prediabetes-induced peripheral neuropathy. Furthermore, we explored the cellular and molecular mechanistic pathways by which intermittent fasting exerts its effect.

Methods: C57/BL6 mice were recruited and prediabetes was induced by high calories/high fat diet feeding. After 8 weeks of high calories/high fat diet feeding, mice were exposed to a protocol of alternate day fasting. They were divided as follows:
[1]control mice (14.1 kcal% fat), [2]control prediabetic mice (60 kcal% fat), prediabetic mice that underwent an alternate day fasting protocol with different types of diet; either with [3](14.1 kcal% fat), [4](30 kcal% fat), [5](60 kcal% fat). Body mass composition (fat, lean and free fluids), behavioral (sensory and motor), molecular and biochemical tests were performed.
In parallel, and in order to assess the role of dyslipidemia *in vitro*, mouse Schwann cells MSC80 were treated with saturated palmitate, unsaturated Oleate or with 10mM of glucose for 24 hours.

Results: High-fat diet consumption lead to neurophysiological defects and sensorimotor abnormalities paralleled by a reconditioning of peripheral myelin gene expression, and an increase in NADPH-induced ROS generation that is due to an alteration in the epoxygenase pathway as assessed by the decrease in CYP1a1/1a2 expression reflecting the decrease in EETs production. Intermittent fasting regulates NADPH-derived ROS production, restores the homeostatic effect of the CYPs enzymes producing EETs, prevents prediabetes-associated neurophysiological and sensorimotor defects and reinstates peripheral myelin gene expression. Furthermore, body mass composition analysis showed significant variations in the parameters between prediabetic and treated mice. To further confirm the role of the falling-off in EETs production in prediabetes-induced peripheral neuropathy, [5]prediabetic mice were treated with AUDA, an sEH inhibitor, that increases physiological availability of EETs. Our data using AUDA mimicked the beneficial effect observed with the intermittent fasting protocol. Of interest, results of the cultured SCs treated with either, palmitate, oleate or 10mM glucose in the presence or absence of AUDA mirrored the *in vivo* data.

Conclusion: Intermittent fasting and EETs bioavailability proved to have a protective effect in reversing the pathogenesis of peripheral neuropathy in the prediabetic state. Taken together, our results suggest a novel interventional modality in the management of diabetes and its associated complications.

CONTENTS

LIST OF ILLUSTRATIONS

Figure Page

LIST OF ABBREVIATIONS

ADF	Alternate day fasting
O	Oleate
P	Palmitate
15 mM G	15 mM Glucose
AUDA	12-(3-adamantan-1-yl-ureido) dodecanoic acid
IF	Intermittent fasting
CYP 450/ CYP	Cytochromes P450
ADA	American Diabetes Association
DPN	Diabetic peripheral neuropathy
ROS	Reactive oxygen species
EET	Epoxy-ecosatetraenoic acids
HETE	Hydroxy-eicosatetraenoic acids
MSC80	Mouse Schwann Cells 80
DM/ T2DM	Diabetes mellitus/ Type two diabetes mellitus
HFD	High fat-diet
HbA1c	Hemoglobin A1c
NADPH	Nicotinamide adenine dinucleotide phosphate
PNS	Peripheral nervous system
MPZ	Myelin Protein Zero

CHAPTER I

INTRODUCTION

A. Prediabetes

Diabetes mellitus (DM) is among the prime instigators of death universally. [1] Prediabetes, also known as intermediate hyperglycemia, is an alarming precursor of diabetes mellitus and specifically type 2 diabetes mellitus. [2, 3] In the past decade, prediabetes has gained significant attention because emerging evidence highlighted the pivotal role it plays. Actually, it was estimated that every year around 5-10% of prediabetic patients become diabetic. [4, 5] Moreover, according to the American Diabetes Association (ADA), up to 70% of patients with prediabetes will eventually develop diabetes. [6]

1. Epidemiology

About 84 million adults in the states are currently facing prediabetes; one in three adults in the United States. About 90% of these adults are oblivious that they are affected with prediabetes and are unknowingly setting themselves up for all the consequences that this disease entails. The incidence of diabetes is evidently growing at rapid rates globally. In the US alone, up to 1.5 million new diabetic patients are diagnosed annually. These increases are matching the rapid increases in the prevalence of obesity. [7-9]

2. *Etiology and risk factors*

There are many factors that could significantly increase the risk of developing prediabetes. That includes: age, ethnicity (African American, Latin America, Native American, or Asian/Pacific Islander), sex (males), obesity (especially a body mass index greater than 25 kg/m^2), a high blood pressure, polycystic ovarian syndrome, family history of diabetes, improper lifestyle and physical inactivity. [3, 10]

3. *Diagnosis*

American diabetes association (ADA) describes prediabetes to be associated with a fasting plasma glucose of 100-125 mg/dl and/or a plasma glucose concentration of 140-199 mg/dl following a 75 g oral glucose tolerance test and/or an HbA1c of 5.7-6.4%. [11]

4. *Symptoms*

The majority of prediabetic patients do not exhibit symptoms related to diabetes other than high levels of blood glucose [10], although these levels do not reach diabetes thresholds. [12] Thus, individuals exhibiting some of the risk factors should be monitored regularly. Other possible symptoms that could manifest in a minority of the patients include: increased appetite, sudden weight loss/ weight gain, weakness and fatigue, sweating, slow healing of bruises, blurred vision, recurrent skin infections, gum bleeding... [10]

5. *Glucose Homeostasis*

Glycemia is under a highly homeostatic control. Constantly, the concentration of glucose in the bloodstream represents a balance between the entry and exit of glucose from the blood circulation via cellular metabolism or excretion. **(Figure 1)** Dysregulation results in increased glucose levels. All of this is subject to multiple regulatory mechanisms, with insulin and glucagon playing the fundamental roles. [13]

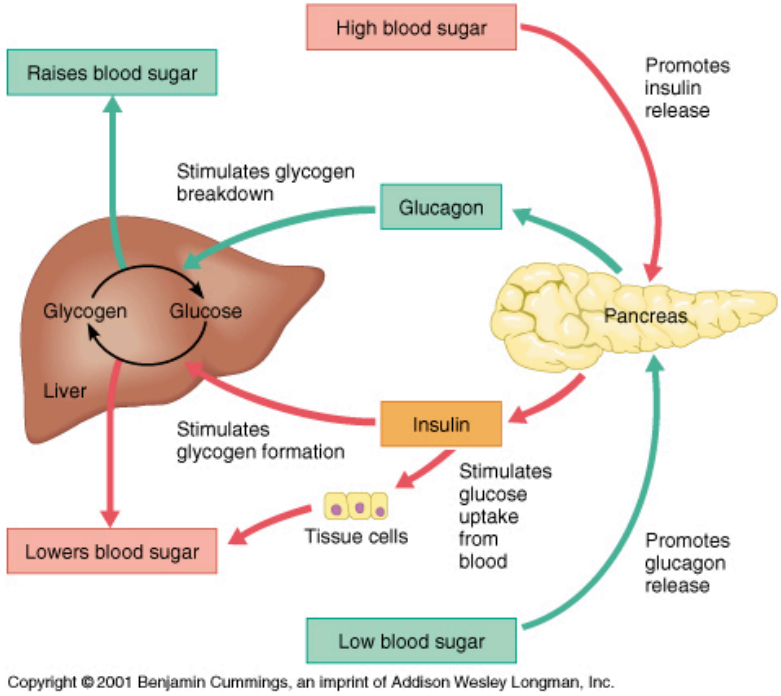


Figure 1: Glucose and Glycogen homeostatic pathways: Role of insulin and glucagon, Benjamin Cummings, 2001

6. *Pathophysiology*

Insulin deficiency, insulin resistance, and defective glucose sensing at the level of pancreatic beta cells are the central pathophysiological determinants that together initiate and intensify the hyperglycemic state in a diabetic condition. [14]

Hyperglycemia stimulates an excessive release of insulin by the pancreatic beta cells, causing a high exposure to insulin. Thus, the insulin receptors, responsible for opening glucose channels that drive glucose into the cells, will eventually become desensitized and their response will decrease. This reduction exacerbates the hyperglycemic state, prolongs the metabolic disturbances and leads to the development of not only type 2 diabetes but also to the development of metabolic syndrome. [15-19]

Moreover, prediabetes is mainly associated with dyslipidemia. Increasing evidence has shown an association of obesity, dyslipidemia specifically, with the prediabetic cellular and molecular injuries irrelevant to hyperglycemia. [16] This will largely be the focus of our study.

These pathophysiological events represent a preliminary phase leading to a metabolic cascade which potentially has deleterious consequences if not adequately addressed.

7. Reversion to normoglycemia: management and treatment

Following lifestyle and drug-based interventions among prediabetic individuals, numerous studies have confirmed a significant decrease in the risk of developing diabetes. [20-22] Prediabetes may also be reverted completely back to normoglycemia. In a population based observational study in England, up to 80% of the participants with impaired fasting glucose at the start of the study had normal fasting glucose after being followed up for 10 years. [4]

According to the research in the field, lifestyle interventions are considered the cornerstone in preventing diabetes. However, some anti-diabetic and anti-obesity drugs have been shown to also play a pivotal role in delaying the onset of diabetes or even

reversing prediabetes. [6, 20] Moreover, some surgical mediations, namely bariatric surgeries, have presented promising effects on improving the prediabetic state. [23]

In summary, the rate of progression from prediabetes to type 2 diabetes became a serious and a debilitating problem. The complications associated to type 2 diabetes (presented in the below sections) start at the prediabetic stage. Thus, preventative interventions at the prediabetic stage become a compelling public health priority.

B. Diabetes Mellitus

Diabetes is a heterogeneous metabolic disease characterized by hyperglycemia; manifested mainly due to a chronic decrease or insufficiency of insulin in the body. [18]

1. Diagnosis

American diabetes association (ADA) defines diabetes to be associated with a fasting plasma glucose of =/> 126 mg/dL, and/or a plasma glucose concentration of =/>11.1 mmol/L following a 75 g oral glucose tolerance test and/or an HbA1c of =/> 6.5%. [11]

2. Epidemiology

According to the 2014 National Diabetes Statistics Report, an estimated 29.1 million people, or 9.3% of the population, have diabetes mellitus (Type I or II) in the United States. [24] According to the World Health Organization, approximately 347 million people suffered from diabetes in 2008. This number is estimated to double by 2030.

3. Types of Diabetes Mellitus

According to the ADA, diabetes is sub-grouped into 4 types. Type 1 diabetes, also termed as juvenile diabetes, is mainly an autoimmune disease caused by a total destruction of pancreatic β-cells and usually affects adolescents. [25] Type 2 diabetes mellitus is manifested by a gradual loss of pancreatic β-cells causing first an increase in insulin which is thought to be as a defense mechanism; this is followed by a gradual decrease in the insulin bioavailability, coupled with an increase in insulin resistance. The third type of diabetes mellitus is gestational diabetes which usually occurs during the second or third trimester of pregnancy. [26] Diabetes can also be induced by other specific triggers, namely drug or chemical toxicity, genetic disorders, endocrinopathies, insulin receptor disorders and in association with pancreatic exocrine disease. [27]

C. Diabetes-induced complications

The burden of the diabetic complications is significantly increasing in parallel with the growing diabetes pandemic. The main instigators and prevention strategies remain elusive. While exogenous insulin and other medications can control many aspects of diabetes, numerous complications affecting the vascular system, kidneys, retina, lens, nerves, and skin are common and are extremely costly in terms of longevity and quality of life. [28]

1. Conventional complications: Microvascular and macrovascular

Diabetes mellitus is usually associated with “conventional” microvascular and macrovascular complications affecting several organ systems. The former includes retinopathy, nephropathy and neuropathy, recently known as the “triopathy”. Whereas

the macrovascular complications comprise peripheral artery disease, accelerated cardiovascular impairments, and strokes. [29] **(figure 2)**

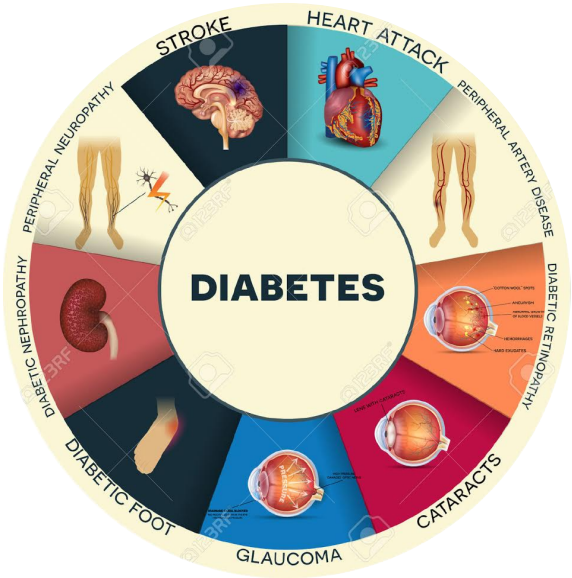


Figure 2: Conventional diabetic complications

2. Non-conventional complications

Among the "non-conventional" manifestations associated with diabetes, chronic complications include dementia, cancer, sexual dysfunction, and depression. [30-32]

D. Neuropathy

Neuropathy is a heterogenous disease with disparate functional and pathobiological mechanisms, mostly associated with diabetes. It may affect both distal and proximal nerves comprising small unmyelinated and large myelinated fibers. [33] This disorder is divided into central, peripheral and autonomic neuropathy. [34] Furthermore, it can be sub-grouped depending on the mechanism of the injury into focal mononeuropathy caused by a lesion of a single nerve or polyneuropathy. [35]

1. Diabetic peripheral neuropathy

Diabetic peripheral neuropathy, which is the main focus of our study, is a common disease in the field of neurology, yet its etiology has not been well elucidated. It is the most common type of neuropathy targeting 50-70% of diabetic patients. [36]

Moreover, several studies confirmed that patients with prediabetes have an elevated risk of polyneuropathy even before the onset of frank diabetes, and that these patients have an elevated risk of metabolic syndrome or its associated abnormalities. [37]

a. Pathophysiology

Nervous affiliations throughout the entire body may be affected in DPN depending on the duration and severity of the disease. Diabetic peripheral neuropathy adopts a "stocking-glove" pattern, where loss of sensation progresses in a distal-to-proximal fashion.

Clinical manifestations are predominantly sensory and can be classified as positive such as tingling, burning, or stabbing pain and negative including sensory loss, weakness, and numbness. Motor symptoms are not as common and occur later with the disease progression. [36, 38] [33]

Diabetic peripheral neuropathy firstly affects the small diameter thinly myelinated Aδ fibers alongside the unmyelinated C fibers, which convey impulses related to temperature perception and pain. With the progression of the neuropathy, degeneration of the larger myelinated nerve fibers results in a loss of ankle reflexes, sensory ataxia, and reduced proprioception. Ultimately, the risk of limb amputations increases as diseased tissue develops from ulcer formation and infection. [39]

b. Management

Unfortunately, to date, there is no specific treatment for diabetic neuropathy. The management of the disease is limited to analgesic agents that mainly alleviate the associated pain. [40]

c. Dyslipidemia-induced diabetic peripheral neuropathy

Although diabetes mellitus is well known to cause peripheral neuropathy and numerous studies reported hyperglycemic related mechanisms of nerve damage, recent clinical studies have revealed an association of obesity with peripheral neuropathy irrelevant to hyperglycemia. [41] A growing body of literature links prediabetes, obesity and metabolic syndrome. High fat fed mice, a model of prediabetes and dyslipidemia, has been shown by numerous studies to develop a form of neuropathy without overt hyperglycemia. [41-48] These facts confirm a possibility that some conditions associated with obesity constitute serious risk factors of neuropathy.

Palmitate, the most abundant saturated fatty acid in the human plasma and the adipose tissues, can be used to mimic dyslipidemia and obesity *in vitro*. [49] It was shown to be increased significantly in obese and diabetic patients as compared with healthy individuals. [50-52] Palmitate has also been implicated in dysregulation and apoptosis in different types of cells including pancreatic beta cells, podocytes, retinal cells, cardiac cells, endothelial cells, and most relevant for our study, Schwann cells. [53] [54-57] However, its implication in prediabetes has not been identified yet, specifically in prediabetes-induced peripheral neuropathy.

E. Saturated and unsaturated fatty acids in prediabetes and dyslipidemia

The mechanisms by which palmitate is involved in cell damage and diabetic neuropathy are still poorly investigated. While saturated fatty acids, such as palmitate,

are involved in cellular dysregulations leading to apoptosis [53, 54, 56, 57], many recent studies have reported that monounsaturated fatty acids, including oleate, are considered chief moderators that may counteract the deleterious effects of saturated fatty acids. [55, 58-65]

Increasing evidence have shown that palmitate induces oxidative stress [53, 54, 58, 61, 66], mediating injury and apoptosis of cells in the nervous system [57, 60, 67]. Therefore, palmitate can hypothetically be used in studies as a potent mediator of diabetic neuropathy. On the other hand, oleate may act as a potential beneficial therapeutic agent that reverses the adverse effects of palmitate. [55, 60-62, 64, 65]

F. Intermittent fasting as a potential management for prediabetes

Lifestyle changes are key in the management of prediabetes. Recent data suggest that intermittent fasting is the cornerstone in the management of metabolic disorders and obesity. [68-71] However, its role in prediabetes and prediabetic complications is not described.

1. Overview

Intermittent fasting is a form of abstinence from food for a certain period of time depending on the specific regimen followed. It has long been adapted either for religious or for social motives. However, it gained significant scientific exposure for the favorable outcomes it has shown in the past years.

Strict diets and calorie restriction regimens have long been used by people, however due to the increased burden of this regimen on people and the significant limitations and restrictions associated with it, intermittent fasting has been slowly

stealing the spotlights and gaining people's appreciation. [70, 72] There are various protocols of intermittent fasting depending on the time point.

2. *Types of Intermittent Fasting*

a. Alternate day fasting

Alternate day fasting is one of the most popular and commonly followed approaches of intermittent fasting. It consists of a fast day where subjects abstain from food for an entire day, this is followed by an ad libitum food access regimen the next day. To reduce stress markers, some studies allow a minimal calorie intake, 25% of the regular calorie consumption, on the fast day. This type has been suggested to be highly effective in weight loss and considered a positive influence on metabolic biomarkers and cardiovascular risk factors. [73]

b. Time restricted feeding

Time restricted feeding, sometimes referred to as the 16:8 fasting regimen, is another popular form of intermittent fasting. It consists of having a fixed timeframe between fasting and ad libitum food access every day. The fasting period usually varies between 3-10 hours depending on the sex, age, and the subject's will power. Animal studies have showed that time restricted feeding was associated with significant enhancements in body mass index, lipid profile enhancements, glucose and insulin homeostasis. [74-76]

c. 5:2 fasting

This regimen consists of having 2 fasting days with none to minimum energy intake, while feeding ad libitum the other days of the week. [69]

d. Religious fasting

Theoretically speaking, religious fasting regimens are considered the oldest forms of intermittent fasting and the basis of its presence scientifically. There are many forms depending on the religion. This type has been studied clinically and experimentally and it proved to have profitable metabolic and health outcomes. [69]

3. Intermittent fasting in obesity and diabetes

It is of utmost importance to prevent the progression of prediabetes into diabetes, as its complications are debilitating. Increasing evidence has shown that intermittent fasting might serve as a therapeutically convenient approach for diabetes. Studies have shown that intermittent fasting was successful in restoring pancreatic beta cells, enhancing health metabolic parameters, decreasing cardiovascular disease risk factors and ameliorating diabetic complications. [77-83]

The mechanism of action by which intermittent fasting exert its metabolic role has not yet been well elucidated. Further studies are needed to assess its role in prediabetes and in the possibility of completely reversing the metabolic parameters and the associated complications.

4. Intermittent fasting and diabetic complications

Intermittent fasting has been shown to ameliorate the diabetic state and to play a role in decreasing the severity of the diabetic complications. Some studies have shown that intermittent fasting was successful in alleviating the neuropathic phenotype in a mouse model and preventing the progression of type one diabetic nephropathy. [84, 85] Moreover, a study confirmed that intermittent fasting was able to prevent diabetic

retinopathy. [86] However, its role and mechanisms of action in neuropathy is still not well described.

5. Intermittent fasting and oxidative stress

The effect of intermittent fasting on oxidative stress has not been explained explicitly before. However, few studies have tackled this relation and proved some beneficial effects of intermittent fasting in an oxidative-stress state. [87-90]

G. Oxidative stress

Human body is constantly exposed to various reactions and pathways that could lead to the production of reactive species entitled free radicals: reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chlorine species (RCS). The transfer of their free unpaired electron triggers an oxidation of cellular machinery. In order to preserve the homeostatic state of our bodies, endogenous or exogenous antioxidant systems that counterbalances and confronts the injurious outcomes of such species exist. Any disparity between the quantity of the free radicals and the antioxidants lead to the phenomenon of "oxidative stress". [91] Thus, oxidative stress develops when the organism fails to detoxify the free radicals that are produced during metabolic activity. This could play a major role in the development of chronic and degenerative diseases such as cancer, autoimmune disorders, neurodegenerative diseases and diabetes. [92]

1. *Underlying pathogenic mechanisms in diabetes*

Reactive oxygen species production (ROS) has been described as the final common signaling pathway orchestrating the onset and development of diabetic complications. Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes and its different complications. It is believed that in the onset and progression of the various diabetic complications, free radicals lead to the damage of lipids, proteins, DNA, and other macromolecules, affecting cell signaling pathways, eventually leading to cell death. [93]

Free radicals are produced excessively in diabetes by glucose oxidation, non-enzymatic glycation of the cellular matrix; lipids and proteins, and the subsequent oxidative degradation of glycated proteins. Abnormal levels of free radicals and the associated decline of the antioxidative defense mechanisms can lead to cell apoptosis, increased lipid peroxidation, and the development of insulin resistance. **(figure 3)** All of this is considered a prime trigger for the development of the diabetic complications. [94]

2. *Reactive oxygen species*

Reactive Oxygen Species (ROS) are oxygen-containing chemically active molecules that are byproducts of ongoing cellular reactions. The most abundant ones are peroxides, superoxide, and hydroxyl radical. Dyslipidemia and hyperglycemia have been shown to disrupt the oxidant-antioxidant equilibrium by instigating additional

ROS production. [95-97] Subsequently, the identification of cellular sources of ROS is central to understanding its related pathobiology in diabetes.

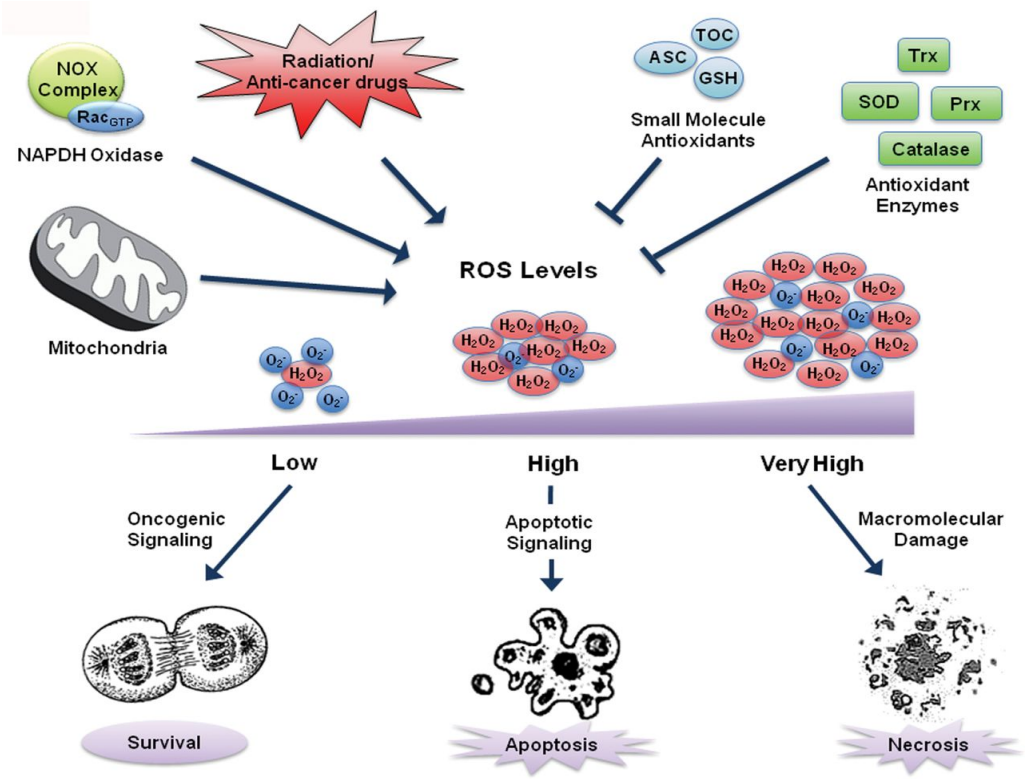


Figure 3: Reactive oxygen species sources and pathways, *Krishna et al. 2011*

3. *Sources of reactive oxygen species*

Intracellular glucose metabolism is associated with ROS production via glucose autoxidation, mitochondrial oxidative phosphorylation, and the production of advanced glycation end products. Additionally, a number of enzymes have been implicated in diabetic-induced ROS production and are reported to include nicotinamide adenine dinucleotide phosphate oxidase (7 distinct isoforms), nitric oxide synthase, lipoxygenases, cyclooxygenases, xanthine oxidase, and notably cytochrome P450 monooxygenases. [98] **(figure 4)**

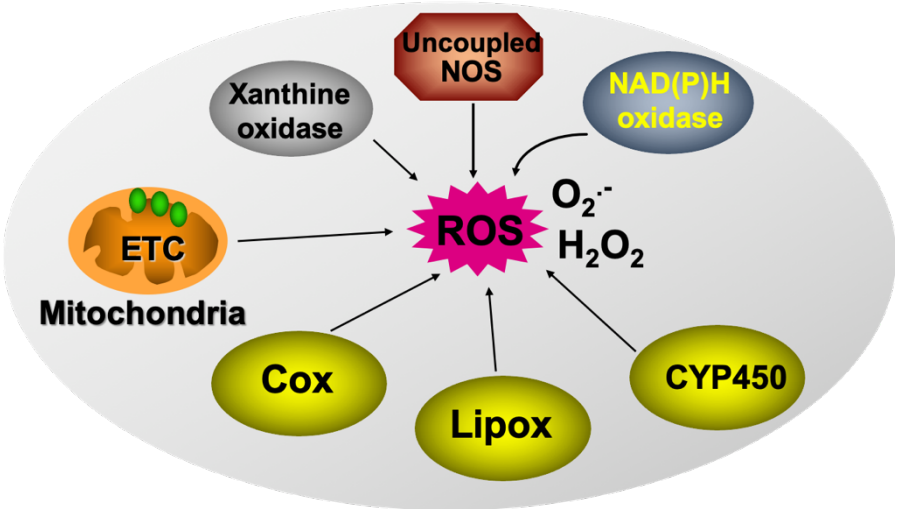


Figure 4: Sources of ROS, Eid et al, 2014

4. Oxidative stress in diabetic peripheral neuropathy

Oxidative stress has been reported to be associated with diabetic complications and specifically peripheral neuropathy. Schwann cells are the regenerating key factors in peripheral neuropathy, and they have been reported to be impaired in diabetes through hyperglycemic and oxidative stress pathways. [99, 100] However, the relation between prediabetes induced-neuropathy, and oxidative stress is not elucidated yet. [101-105]

H. Cytochromes P450

The Cytochromes P450 monooxygenases are a fundamental source of reactive oxygen species production. Cytochromes P450, which have a pigment (P) that has a 450-nm spectral peak when reduced and bound to carbon monoxide, are a large family of hemoproteins. They are mainly responsible of the oxidation or reduction of endogenous molecules (hormones, cholesterol, saturated and unsaturated fatty acids eicosanoids, steroids, acids, and vitamins), and exogenous molecules (environmental

chemicals, pollutants and drugs). Mainly, this metabolism leads to a successful detoxification of many compounds. However, sometimes it can lead to the production of many harmful molecules associated with many diseases such as cancer, birth defects and diabetes. [106]

Cytochromes P450 have been described to be bound to membranes of both the mitochondria and the endoplasmic reticulum and to be associated with redox reactions. [107] Furthermore, CYPs are reported to be major sources of free radicals, mainly reactive oxygen species, in numerous tissues [108-110] with implications in diabetic complications. [111, 112] In humans, approximately 60 CYP genes have been identified; any polymorphism associated with a gene is associated with enzyme dysfunction and a range of disorders. [113]

1. CYP450 involvement in arachidonic acid metabolism

Arachidonic acid, a 20-carbon chain polyunsaturated fatty acid, is an integral constituent of the biological cell membrane. It's associated with a lot of metabolic pathways and signaling cascades involved in treatment and diagnosis of a lot of diseases including neuropathy. [114-119] Cytochromes p450 are one of the enzymes involved in the arachidonic acid metabolism. Arachidonic acid release is induced by the action of phospholipase A2 on membrane phospholipids. Free arachidonic acid may be metabolized through three separate pathways by either the cyclooxygenases, lipoxygenases or CYP450 monooxygenases. CYP isoforms, involved in the monooxygenase pathway, may be classified as epoxygenases or hydroxylases. These enzymes convert arachidonic acids into expoxyecosatetraenoic acids (EET) or hydroxy-eicosatetraenoic acids (HETE) respectively. [120] **(figure 5)**

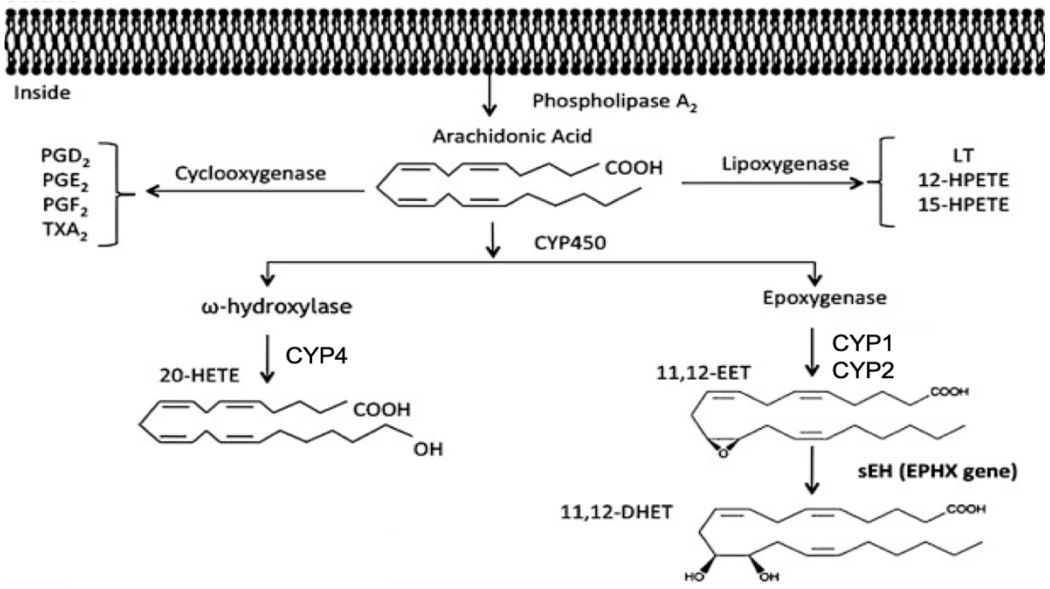


Figure 5: Arachidonic Acid metabolism, Konkel et al. 2011

2. *Different families and subfamilies of CYP450*

The human cytochrome P450 (CYP) superfamily comprises around 60 genes. Cytochrome P450 proteins are conveniently arranged into families and subfamilies on the basis of percentage amino-acid sequence identity. To date, no studies have explicitly described the fluctuations of CYPs in obesity linked to neuropathy. Some studies showed how the CYP450 appear to be isozyme-specific. However, the effect of obesity on the majority of the cytochromes P450 isozymes is inconclusive and further investigations are needed. [121]

3. *Cytochromes epoxygenases:*

Epoxygenases are a group of the CYP450 enzymes that participate in the metabolism of some polyunsaturated fatty acids, primarily arachidonic acid, to epoxide products: the eicosatrienoic acid epoxides (EETs). These CYP epoxygenases are sub-

grouped into 2 subfamilies the CYP1 and CYP2 isozymes. [107, 113] It is noteworthy to mention that, in injurious cases, EETs are converted excessively into a 20 times less metabolically active form dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolases sEH.

Cytochromes P450 monooxygenases, especially those involved in the epoxygenase pathways controlling EETs production, are considered to be a negative regulator of ROS production. Nevertheless, this role has not been fully interrelated to peripheral neuropathy.

4. ***Cytochromes hydroxylases:***

The ω-hydroxylation (addition of a hydroxyl moiety at the terminal ω-carbon) of arachidonic acids produces 20-hydroxyeicosatetraenoic acid (20-HETE) and is catalyzed by ω - hydroxylases of the CYP4A and CYP4F subfamilies. [107, 113]

5. ***Role of CYP450 in prediabetes and diabetic complications***

Many experiments have reported the variations of EETs and HETEs in diabetes. We and others have previously shown that in diabetes the bioavailability of EETs (product of the action of epoxygenases: CYP1 or CYP2) decreases while HETEs (product of the action of hydroxylases: CYP4) increase. It has been also established that oxidative stress followed by these fluctuations could play a key role in the pathogenesis of the diabetic complications. [112, 122]

Moreover, some studies highlighted beneficial effects of EETs in diabetes. One study in 2016 reported that elevated levels of EETs managed to reduce insulin resistance, decrease the beta cells destruction, and thus control insulin sensitivity in diabetes. [116]

6. *Soluble epoxide hydrolase inhibiton*

The inhibition of soluble epoxide hydrolase has gained significant attention in the past decade. Metabolically stable drugs that inhibit sEH started to emerge in the field of diabetic neuropathy and confirmed promising outcomes in restoring the homeostatic balance in CYP450 mechanisms and ROS production. AUDA (12-(3-adamantan-1-yl-ureido) dodecanoic acid) inhibits the enzyme soluble epoxide hydrolase that converts EETs to DHETs; increasing the bioavailability of EETs. **(figure 6)** This increase is thought to play a major role in stabilizing the negative consequences of oxidative stress-related nerve damages. Many experimental studies have reported that targeting and inhibiting sEH had promising therapeutic effects on cardiovascular pathology, inflammations, diabetic nephropathy and diabetic neuropathy. [114, 115, 117-119, 123]

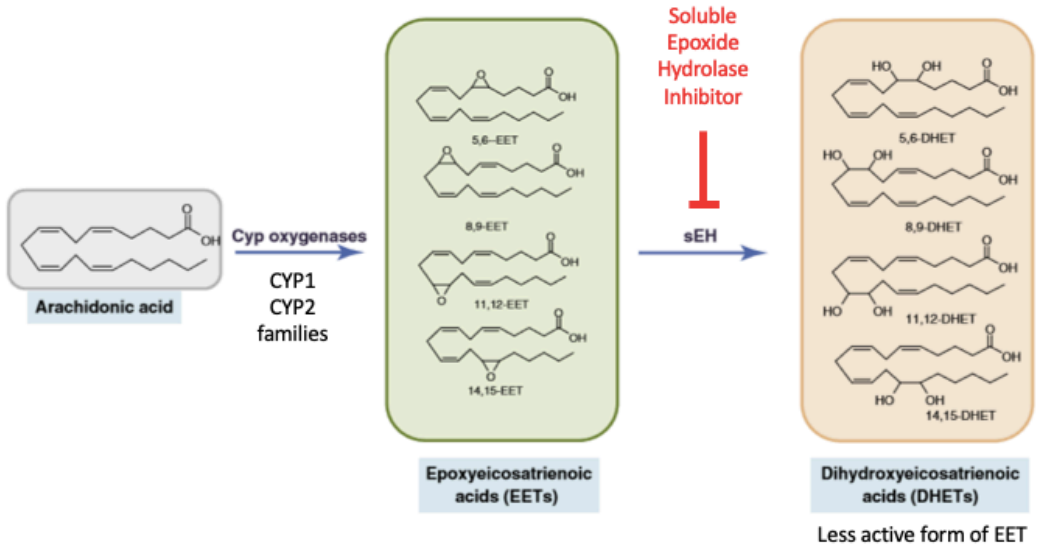


Figure 6: Role of soluble epoxide hydrolase inhibitors, *Pillarisetti et al. 2015*

CHAPTER II

HYPOTHESIS AND AIM OF THE STUDY

Peripheral neuropathy may be more common in patients with prediabetes than previously thought and early interventions may be warranted in this patient population. Thus, understanding the mechanistic pathway correlating prediabetes and peripheral neuropathy is of great importance to improve the quality of life of patients and to design novel therapeutic intervention approach. Lifestyle modification (dietary approach and exercise) as well as drug-based interventions are known to play a major role in reducing the burden of prediabetic complications. However, what constitute the best approach in lifestyle modification and what is the most suitable pharmacological intervention in prediabetes management are not well identified.

In this study, we aim to explore if prediabetes induces sensorimotor deficits and investigate the role of CYP450 in prediabetes induced-neuropathy in cultured cells (*in vitro*) and experimental animal models (*in vivo*). Moreover, we want to assess the effect of intermittent fasting on the observed homeostatic/ functional changes in prediabetes-induced neuropathy and to correlate the change in EETs bioavailability with prediabetes-induced neuropathy.

We hypothesize that intermittent fasting may reverse some of the prediabetes-associated complications specifically peripheral neuropathy. Furthermore, we suspect that the beneficial effect of intermittent fasting is due to a decrease in oxidative damage (Figure 7).

Antioxidant therapy alone has been insufficient in halting the progression of Diabetic peripheral neuropathy. Subsequently, targeted therapy based on identifying specific cellular sources of reactive oxygen species (ROS), is of great importance. Amongst the various sources of ROS, Cytochromes P450 emerged as major family of enzymes responsible for the production of superoxide (O2•−) and hydrogen peroxide (H2O2), but it's role in the progression peripheral neuropathy is not yet identified. ***In this study we also hypothesize, that intermittent fasting regulates the homeostatic levels of cytochromes P450 enzymes, especially those involved in the epoxygenase pathway (EET production), controlling thus, indirectly, the production of ROS, and protecting the peripheral nerves. (figure 7)***

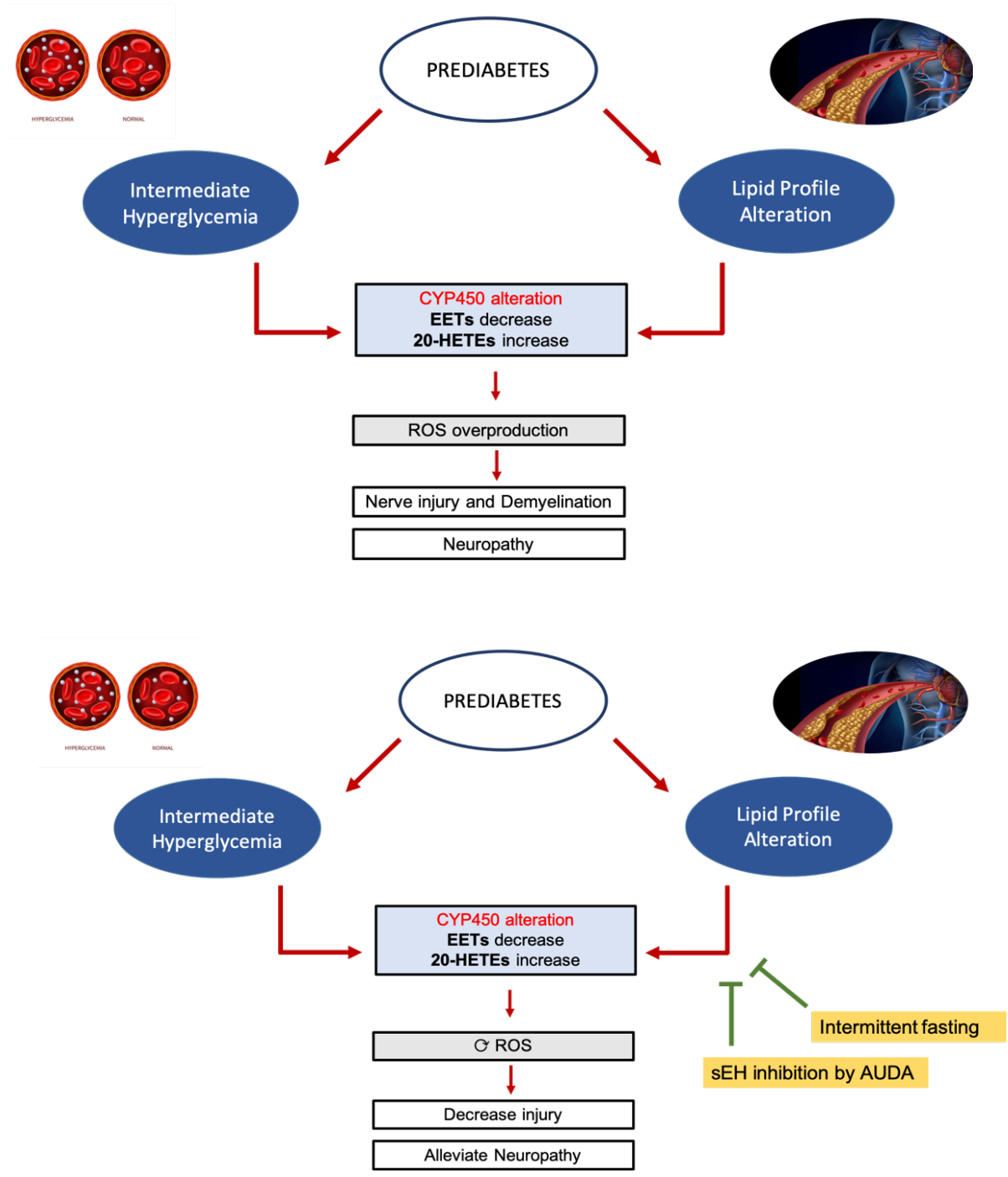


Figure 7: Hypothesis and aim of the study

CHAPTER III

MATERIALS AND METHODS

A. Protein extraction and Lowry assay

To extract proteins, cultured MSC80 cells or mouse sciatic nerves were lysed using cold phosphate buffered saline (PBS) and then they were centrifuged at 1,400 rpm for 5 minutes at 4°C and the supernatant was removed. Next, we added over the pellet a RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 100 mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol (NP40), 1% of the protease and phosphatase inhibitors and 1mM phenylmethylsulfonyl fluoride. The lysates were left to rotate overnight at 4^{o}C. The next day, they were sonicated for 20 cycles 30s/cycle at 4°C and then centrifuged at 13,600 rpm for 30 minutes at 4°C. Protein concentration in the supernatants was measured using the Lowry Protein Assay.

B. Western Blot

For immunoblotting, 20-40 µg of proteins were separated on 12-15% polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The blots were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline for one hour and then incubated overnight with rabbit polyclonal anti-CYP4a1/4a2/4a3 (1:500, Abcam),

mouse polyclonal anti-CYP 1a1/1a2 antibody (1:500, Detroit R&D), rabbit polyclonal anti-MPZ (1:500, Abcam) and mouse polyclonal anti-HSC70 (1:1000, Abcam) which was considered our housekeeping gene. The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:10000, Bio-Rad). Bands were visualized by the stain-free chemidoc imaging system (Bio-Rad Laboratory, CA, USA). Densitometric analysis was performed using Image J software.

C. NADPH oxidative activity assay

NADPH oxidase activity was measured in sciatic nerves and in Schwann cells. Proteins were extracted from sciatic nerves using cooled mortar and pestle by smashing the frozen nerve and suspending the remnants in the lysis buffer (20 mM KH2PO4 (pH 7.0), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). Cultured MSC80 cells were washed twice with ice-cold PBS and scraped from the plate on ice using the lysis buffer as well. To start the assay, 10-30 μg of homogenates were added to 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin (behaving as the electron acceptor), and 100 μM NADPH (acting as the substrate for the NADPH oxidase). Photon emission expressed as relative light units (RLU) was measured every 30 s for 5 minutes in a luminometer. Superoxide production was expressed as relative light units/min/mg of protein. Protein content was measured using the Bio-Rad protein assay reagent.

D. *In vivo:* Animal experimental design

All animal work was conducted according to the institutional guidelines and approved by the Institutional Animal Care and Use Committee. All animals were kept in a temperature-controlled room and on a 12/12 - dark/light cycle and had standard chow (containing 14.1% fat) and water access. Body weight and Blood glucose levels changes were monitored once a week via tail vein punctures and a glucometer.

Thirty male 6-weeks-old C57BL/6J mice were divided into 6 groups (n=5/group): **1)** Control group, that were fed normal chow for 18 weeks (duration of the study) (Control), **2)** Prediabetic group fed a high fat diet of 60% fat (HFD-60) for 18 weeks, (Prediabetic), **3)** Prediabetic group fed a high fat diet of 60% fat for 8 weeks followed by an alternate day fasting protocol (ADF) combined with dietary fat reversal (14.1% fat intake) for 10 weeks (Prediabetic + ADF 14.1% fat), **4)** prediabetic group fed a high fat diet of 60% fat for 8 weeks followed by an alternate day fasting protocol combined with dietary fat reversal (30% fat intake) for 10 weeks, (Prediabetic + ADF 30% fat), **5)** prediabetic group, that were fed a HFD-60 for 8 weeks then underwent a protocol of ADF while keeping the 60 % fat intake for 10 weeks, (Prediabetic + ADF 60% fat) **6)** Prediabetic group, that were fed a HFD-60 for 8 weeks then were treated with AUDA (20 mg/Kg – oral gavage) while keeping the 60 % fat intake for 10 weeks, (Prediabetic + ADF 60%).

Prior to sacrifice, sensory-motor dysfunction was assessed using the Raised Beam Walking Test and Plantar AnalgesiaTest.

Table 1: Animal work experimental design

<table>
<tr><td>Birth</td><td>Control</td><td>Prediabetic</td><td>Prediabetic +
ADF (14.1%)</td><td>Prediabetic +
ADF(30%)</td><td>Prediabetic +
ADF (60%)</td><td>Prediabetic +
AUDA</td></tr>
<tr><td>6 weeks</td><td rowspan="3">Normal chow</td><td colspan="5">Normal chow
14.1 % fat</td></tr>
<tr><td>8 weeks</td><td rowspan="2">HFD
60% fat</td><td colspan="4">HFD
60% fat</td></tr>
<tr><td>10 weeks
(Treatment)</td><td>ADF/
Normal chow
14.1% fat</td><td>ADF/
HFD
30 % fat</td><td>ADF/
HFD
60 % fat</td><td>AUDA/
HFD
60% fat</td></tr>
</table>

E. Alternate day fasting

The specific diet for each group was removed every other day around 10 a.m. for 24 hours, during which access to water only was allowed *ad libitum*. The next day, food was provided; and mice had unrestricted access to food for the following 24 hours.

F. Diet Composition

In order to prepare our different types of diet we used 8604 diet which is a fixed formula, non-autoclavable diet, designed to support growth and reproduction of rodents. It consists of the ingredients that are simplified below. **(figure 8)** Moreover, to make a high fat diet we used Ghee from Mazola vegetables, its ingredients are also elucidated below. **(figure 9)**

Macronutrients		
Crude Protein	%	24.3
Fat (ether extract) [a]	%	4.7
Carbohydrate (available) [b]	%	40.2
Crude Fiber	%	4.0
Neutral Detergent Fiber [c]	%	12.4
Ash	%	7.4
Energy Density [d]	kcal/g (kJ/g)	3.0 (12.6)
Calories from Protein	%	32
Calories from Fat	%	14
Calories from Carbohydrate	%	54
Minerals		
Calcium	%	1.4
Phosphorus	%	1.1
Non-Phytate Phosphorus	%	0.7
Sodium	%	0.3
Potassium	%	1.0
Chloride	%	0.5
Magnesium	%	0.3
Zinc	mg/kg	80
Manganese	mg/kg	100
Copper	mg/kg	25
Iodine	mg/kg	2
Iron	mg/kg	300
Selenium	mg/kg	0.34
Amino Acids		
Aspartic Acid	%	2.3
Glutamic Acid	%	4.1
Alanine	%	1.4
Glycine	%	1.3
Threonine	%	0.9
Proline	%	1.6
Serine	%	1.6
Leucine	%	1.9
Isoleucine	%	1.0
Valine	%	1.1
Phenylalanine	%	1.1
Tyrosine	%	0.9
Methionine	%	0.4
Cystine	%	0.4
Lysine	%	1.4
Histidine	%	0.6
Arginine	%	1.5
Tryptophan	%	0.3

Vitamins		
Vitamin A [e, f]	IU/g	12.6
Vitamin D_3 [e, g]	IU/g	2.4
Vitamin E	IU/kg	120
Vitamin K_3 (menadione)	mg/kg	40
Vitamin B_1 (thiamin)	mg/kg	27
Vitamin B_2 (riboflavin)	mg/kg	8
Niacin (nicotinic acid)	mg/kg	63
Vitamin B_6 (pyridoxine)	mg/kg	13
Pantothenic Acid	mg/kg	21
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.05
Biotin	mg/kg	0.38
Folate	mg/kg	3
Choline	mg/kg	2530
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.1
C18:1ω9 Oleic	%	0.9
C18:2ω6 Linoleic	%	1.9
C18:3ω3 Linolenic	%	0.2
Total Saturated	%	0.9
Total Monounsaturated	%	1.1
Total Polyunsaturated	%	2.1
Other		
Cholesterol	mg/kg	50

Figure 8: Dietary composition of the normal chow (Envigo)

Nutrition facts of Mazola Vegetable Ghee (per 14 grams)	
Calories:	120
Calories from fat:	120
Total fat:	14 g
Saturated:	4 g
Trans-fat:	1 g
Polyunsaturated:	6 g
Monounsaturated:	4 g
Cholesterol:	0 mg
Sodium:	0 mg
Total Carbohydrate:	0 g
Sugar:	0 g
Protein:	0 g

Figure 9: Dietary composition of Ghee used to make a high fat diet (Mazola)

G. Drug administration

AUDA was dissolved in methylcellulose and DMSO (5%). It was administered orally with a gavage needle at a 20 mg/Kg dose daily for 10 weeks with the same diet (HFD-60). It is noteworthy to mention that in order to decrease the variables that could affect our results, the control group and the control prediabetic group were treated with the dissolving solution (methylcellulose + 5% DMSO) which was also administered by oral gavage.

H. Raised Beam Walking test

Motor coordination and balance assessment is performed via the Raised beam walking test [124]. Animals were placed on a platform with a rod of 1.2 cm diameter, 100 cm length and around 70 cm above a flat surface. At one end of the rod, we set a secure platform to house the animal. First, the mice were allowed to adapt, and they were trained for 2 days to cross. On the third day, videos were taken, and the time acquired to cross the beam, the speed, the number of stops and the number of

faults/slips were recorded for analysis. Each measurement was performed 3 times/animal.

I. Hind Paw Withdrawal test

Thermal algesia and pain perception were assessed for this study using the Hind paw withdrawal test [125]. The IITC plantar Analgesia meter was used and set up according to the manufacturer's protocol. The test is characterized by a heating beam set at an idle intensity of 2% and active intensity of 25% with a cut-off time set at 20 seconds. The platform onto which animals were placed for acclimation was set at 32 °C. The heating beam was targeted at the hind paw of animals and the time to sense the heat and withdraw their paws was recorded for analysis. Each measurement was performed 7 times/animal.

J. Body mass composition analysis

Fat mass, free Body fluid and lean mass values from the mice were documented non-invasively prior to sacrifice using the minispec body composition analyzer LF110 (Bruker). It uses a magnetic resonance technology and is based on Time Domain NMR. It acquires and analyzes TD-NMR signals from all protons in the entire sample volume to provide the components of interest.

K. Sacrifice and organs harvesting

Mice were sacrificed following a 10-week treatment at the age of 6 months; blood glucose levels were recorded the same day. Fasting blood glucose was measured the day before to minimize stress before the sacrifice. At sacrifice, we collected blood directly

from the heart after the mice were deeply anesthetized. Sciatic nerves were flash frozen in liquid nitrogen and stored at -80 °C for proteins and RNA extraction for further molecular studies.

To harvest the sciatic nerves of these mice, each mouse was pinned to a dissecting board in a supine position. 70% ethanol was used to disinfect the skin. Using a razor blade, a deep cut was made at the base of the tail along the vertebral column. Both hind limbs were held together in position using one hand and the base of the tail was held with the other. To expose the sciatic nerves, the tail was pulled away from the hind limb at the point of the cut. The part of the nerve that runs along the lumbo-sacral region was exposed and appears as a thick whitish cord. This step was performed with caution because excessive pulling force could thin or break it, or even distort the histological morphology of the myelin. [126]

L. Lipid profile analysis

Non-Esterified Fatty Acids, total Cholesterol levels and Triglyceride levels were measured from the serum collected from mice at sacrifice.

M. HbA1c measurement

HbA1c done using the variant II Hemoglobin Testing System (BIORAD) on blood samples collected from the mice models at sacrifice.

N. ROS generation: Detection of superoxide via HPLC

The HPLC-based assay allows separation of superoxide-specific EOH from the nonspecific ethidium. Briefly, quantification of DHE, EOH, and ethidium

concentrations was performed by comparison of integrated peak areas between the obtained and standard curves of each product under chromatographic conditions identical to those described above. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was monitored by ultraviolet absorption at 370 nm. The results are expressed as the amount of EOH produced (nmol) normalized for the amount of DHE consumed (i.e., initial minus remaining DHE in the sample; μmol).

O. *In Vitro:* Cell culture experimental design

Mouse Schwann cells (MSC80) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) containing 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 1% Penicillin/Streptomycin (PS) (Sigma-Aldrich). Cells were incubated at 37 °C with 5% CO2 until confluency was reached. For experimental work, cells were serum starved with 2% FBS and 1% PS for 12 hours and then were treated with a series of supplements depending on the condition. We had 14 different conditions that were divided as follows. There were 7 main conditions that were either pre-treated or not with 1 μM AUDA (synthesized at AUB, chemistry department, laboratory of Professor Kamal Bou Hadir) for 1 hour: Control (Ctrl) incubated with 5mM glucose and considered euglycemic, or with 127 μM Oleate (O) (Sigma-Aldrich), or with 127 μM Palmitate (P) (Sigma-Aldrich), or with 15 mM Glucose, or a combination of Palmitate and Oleate, or a combination of Palmitate and 15 mM Glucose, or a combination of Palmitate and Oleate and 15 mM Glucose. The cells were exposed to the different conditions for 24 hours before harvesting. **(figure 8)**

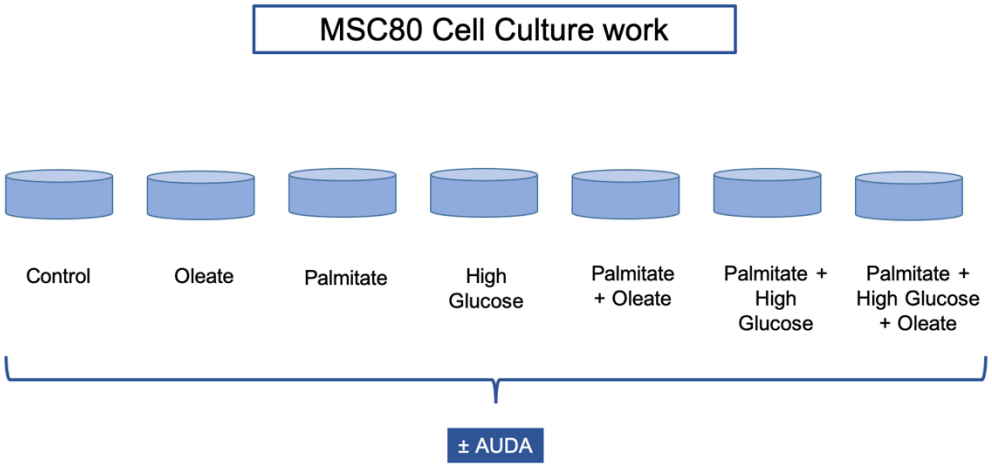


Figure 10: Cell culture experimental design

P. Statistical analysis

Results are expressed as mean ± SE from multiple independent experiments. Statistical significance is assessed by one-way ANOVA and subgroup analysis were done using the Fisher method.

* $p<0.05$ *vs.* control and # $p<0.05$ *vs.* prediabetic.

CHAPTER IV

RESULTS

A. *In vivo* Experiments

1. Metabolic parameters

Prediabetes is associated with a wide-range of alterations in the metabolic parameters. Changes in glucose levels and lipid metabolism were evaluated in prediabetic mice (high calories/60% fat food regimen) as well as in prediabetic mice that underwent a protocol of alternate day fasting (ADF) combined to a food regimen with different fat percentages (60% or dietary reversal regimen of 30% fat, 14.1% fat). Our data show that alternate day fasting reduces the weight gain induced by high calories/60% fat feeding. This was associated with a decrease in the random blood glucose levels along with a reduction in the prediabetes-increased HbA1c levels **(Figure 11-13)**. Furthermore, total cholesterol (TC) levels, triglyceride (TG) levels, and levels of the FFA, released from triglyceride by the actions of lipoprotein lipase and hepatic lipase (NEFA), are elevated in blood of prediabetic mice. ADF whether combined to a 14.1%, 30% or 60% high fat diet intake reduces the levels of TC, TG and NEFA. Of interest, treatment of the prediabetic mice on 60% fat with AUDA did not show any effect on the measured metabolic parameters. **(Figure 12)**

Metabolic Parameters	Control	Prediabetic	Prediabetic + ADF (14.1%)	Prediabetic + ADF(30%)	Prediabetic + ADF (60%)	Prediabetic + AUDA
Body weight (g)	27.5 ± 0.23	**34.3 ± 3.01 ***	24.6 ± 1.11 #	28.6 ± 2.21 #	29.2 ± 2.05 #	**32.7 ± 1.98**
Glucose (mg/dL)	151 ± 15.7	**200 ± 11.7 ***	144 ± 9.3 #	157 ± 5.04 #	171 ± 8.66	**182 ±13.48**
HbA1c (%)	4.16 ± 0.1	**4.82 ± 0.11 ***	4.58 ± 0.09	3.82 ± 0.39 #	4.06 ± 0.14 #	**4.4 ± 0.11**

Figure 11: Metabolic Parameters (n=5)

Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control; # p<0.05 vs prediabetic

Lipid Profile	Control	Prediabetic	Prediabetic + ADF (14.1%)	Prediabetic + ADF(30%)	Prediabetic + ADF (60%)	Prediabetic + AUDA
Non-esterified Fatty Acids (mM)	0.68 ± 0.03	**1.18 ± 0.05 ***	0.66 ±0.05 #	0.74 ± 0.05 #	0.72 ± .003 #	1.18 ± 0.05
Total Cholesterol (mM)	2.2 ± 0.1	**6.36 ± 0.14 ***	3.34 ± 0.05 #	3.52 ± 0.13 #	4.38 ± 0.19 #	**5.84 ± 0.1 #**
Triglyceride levels (mM)	0.48 ± 0.03	**0.97 ± 0.04 ***	0.38 ± 0.03 #	0.52 ± 0.06 #	0.62 ± .003 #	**0.95 ± 0.04**

Figure 12: Lipid Profile Analysis (n=5)

Data are expressed as mean± standard error of mean (SEM). * p<0.05 vs control; # p<0.05 vs prediabetic

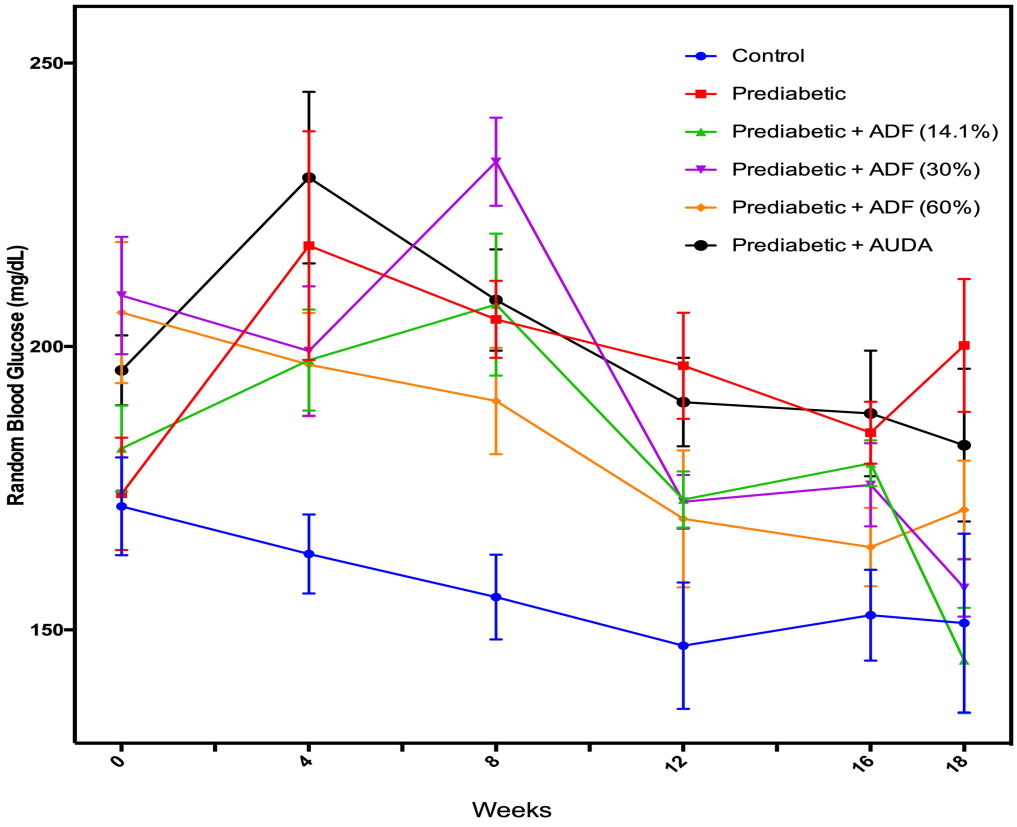


Figure 13: Random Blood Glucose levels at the beginning of study, week4, week8, week12, week16, and week18 (prior to sacrifice) n=5

2. Food intake

Throughout the study, we recorded the average amount of food consumed by the different groups of mice per week. Our data show no statistically significant changes in the amount of food consumed between the control mice and the mice that underwent an ADF protocol with dietary fat reversal to 14.1% fat or 30% fat. Moreover, we did not see significant changes between the amount of food consumed between prediabetic mice and prediabetic mice that underwent an ADF protocol with the same high fat diet regimen (60% fat). Food intake of prediabetic mice and prediabetic mice that underwent an ADF protocol with 60% fat was significantly increased in comparison with the control littermates. In addition, prediabetic mice that underwent an ADF protocol with dietary fat reversal showed significant decrease in food consumption in comparision to the prediabetic mice. **(Figure 14)**

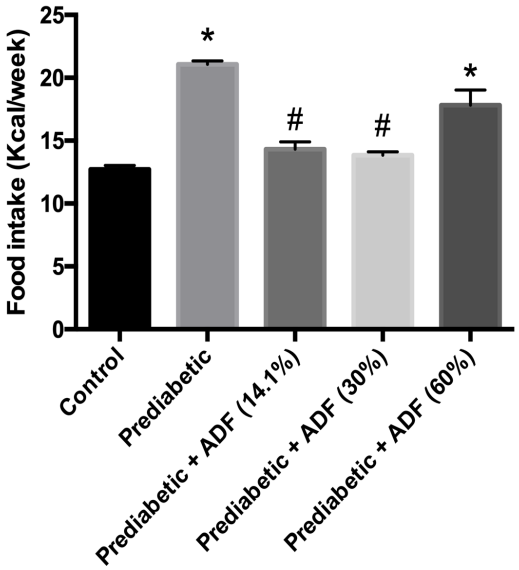


Figure 14: Average of food intake of mice per week (g)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; # $p<0.05$ vs prediabetic

3. Energy Efficiency

Energy efficiency was calculated as weight gained for 100 Kcal of food consumed throughout the study. Our results suggest that the total energy efficiency was significantly decreased in the prediabetic mice managed with ADF with dietary fat reversal to 14.1% fat and with prediabetic mice managed with ADF that were maintained on a high fat diet regimen with 60% fat. **(figure 15)**

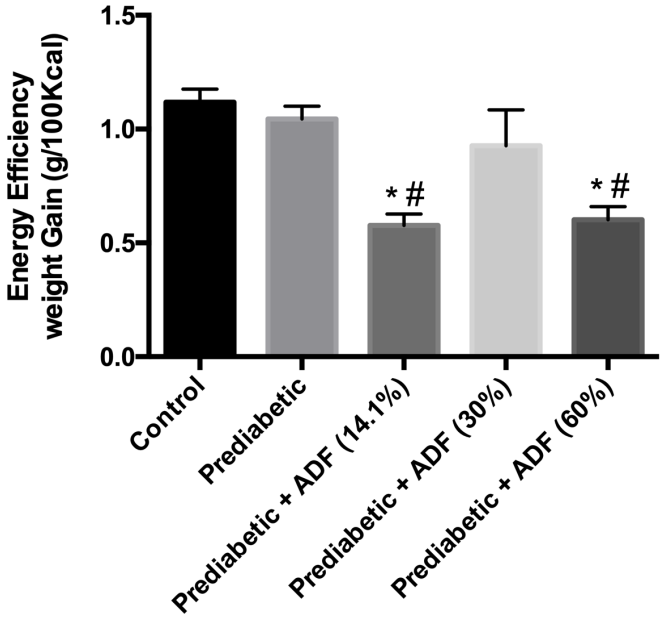


Figure 15: Total energy efficiency throughout the study (weight gain in g per 100 Kcal consummed) (n=5)

4. Body mass composition

Body composition assessment upon high fat feeding show a significant increase in body weight and fat mass percentage in the prediabetic group when compared to their control littermates (**Figure 16-A, 16-B**). In contrast, lean mass percentage was reduced in the prediabetic group of mice when compared to the control, while the percentage of free fluids did not differ between the two groups (**Figure 16-C, 16-D**). Interestingly, when prediabetic mice followed the alternate day fasting protocol combined to a dietary restriction intake of fat, 14.1% or 30% or were kept on 60% fat, body weight and fat percentage were reduced, while the lean mass percentage was increased. Furthermore, the percentage of free fluids was only decreased in the prediabetic mice that had a dietary intake reversal to 14.1% of fat accompanied by ADF (**Figure 16-D**). As for the prediabetic mice that were treated with AUDA, we did not observe any change in the body mass composition parameters when compared to the prediabetic mice group (**Figure 16**)

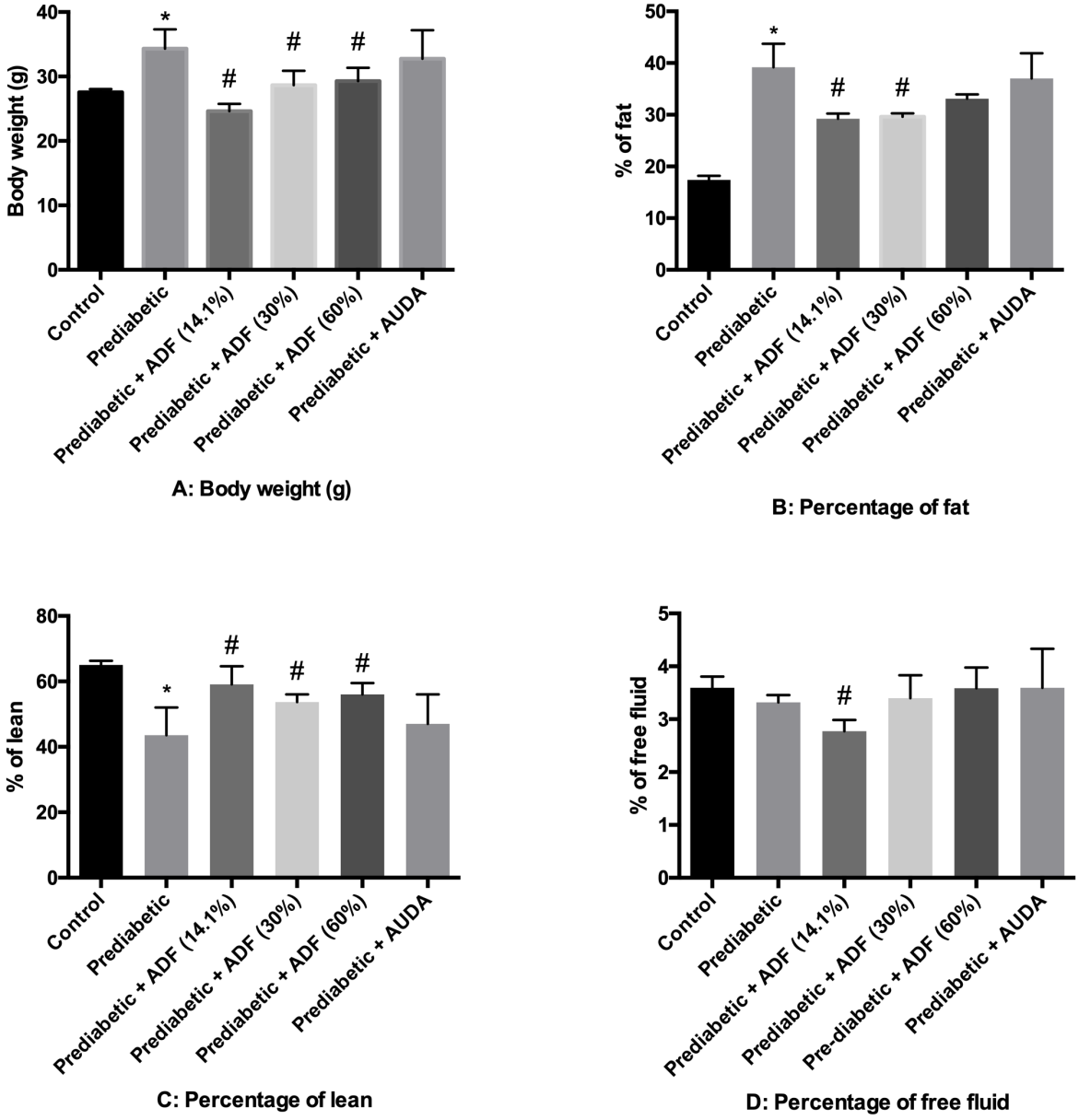


Figure 16: Body mass composition (n=5)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; # $p<0.05$ vs prediabetic

5. Plantar algesia test

Increasing evidence have shown that prediabetes is associated with the development of sensory deficits reflecting nerve injury and damage in the peripheral nervous system. [41-48] We used the hind paw withdrawal test to assess thermal algesia in the prediabetic mice as well as the prediabetic mice that underwent a 24 hours alternate day fasting protocol with different fat percentages (14.1%, 30%, and 60%). The results of this test show that prediabetic mice took a significantly longer time to sense the heat of the beam and withdraw their paws. By contrast, prediabetic mice on ADF regimen with either 14.1%, 30% or 60% fat intake had a significantly lower latency suggesting that the ADF restored thermal algesia. Of interest, increases in the physiological availability of EETs, using the pharmacological soluble epoxide hydrolase inhibitor AUDA, mimicked the effect of ADF associated with or without dietary fat reversal **(Figure 17)**

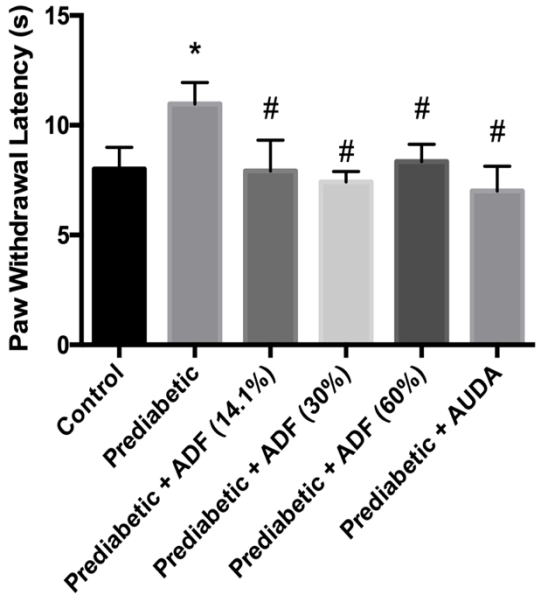


Figure 17: Plantar analgesia test (n=3)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; #

6. Beam walking test

Peripheral neuropathy is also associated with motor dysfunctions. To further confirm the severity of prediabetes-induced peripheral neuropathy, we used the beam walking test. The data from the test showed a relatively longer period of time for the prediabetic mice to cross the beam with an increased tendency to slip in comparison to their controls that seemed to cross with minimal setbacks. Notably, prediabetic mice that were subject to ADF regimen with (60% fat) or with ADF with dietary fat reversal (14.1% or 30% fat) show a significant improvement in their assessed motor dysfunction and performed similar to the control group **(Figure 18)**. A similar pattern was observed in mice treated with AUDA (**Figure 18)**.

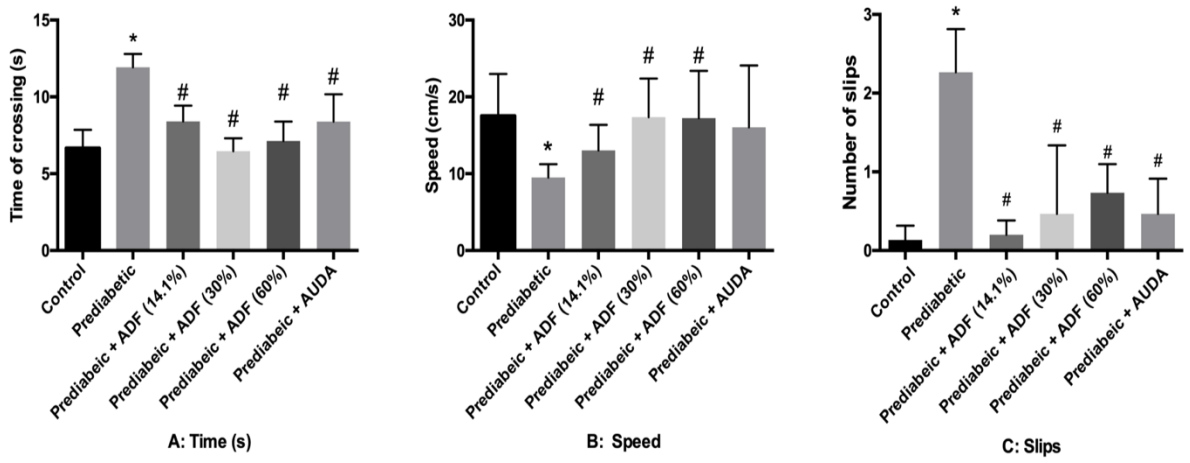


Figure 18: Beam walking Test (n=5)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; # $p<0.05$ vs prediabetic

7. Myelin Protein Alteration

Myelin Protein Zero (P0) is the predominant protein (60%) of the PNS produced solely by Schwann cells. Defects in P0 expression have been implicated in neurological disorder such as in demyelinating neuropathies. [35]

A significant reduction of MPZ was observed in sciatic nerves isolated from the prediabetic mice. Intriguingly, alternate day fasting or soluble epoxide hydrolase inhibition by AUDA reversed this effect and restored MPZ protein to levels compared to that of the control littermates. **(Figure 19)**

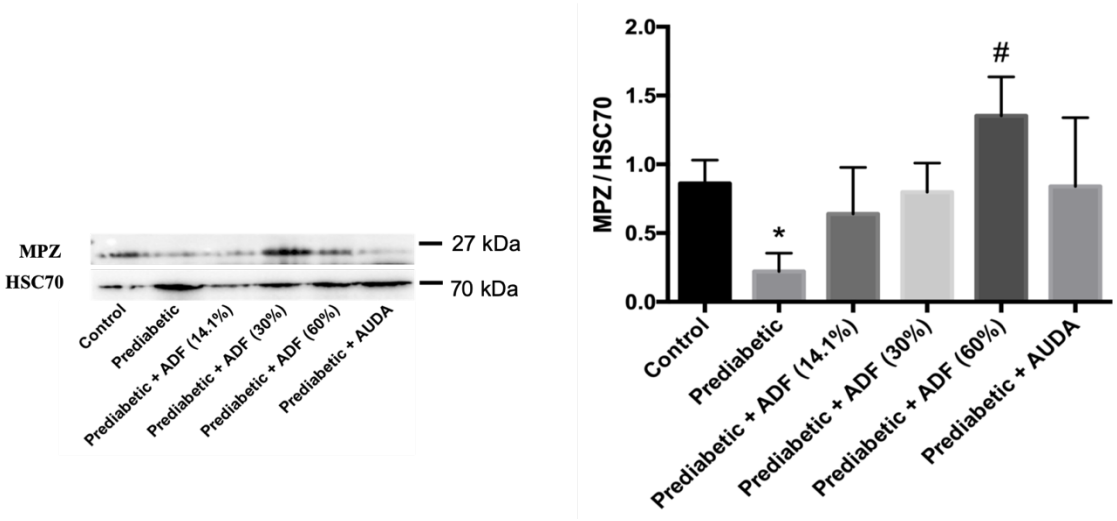
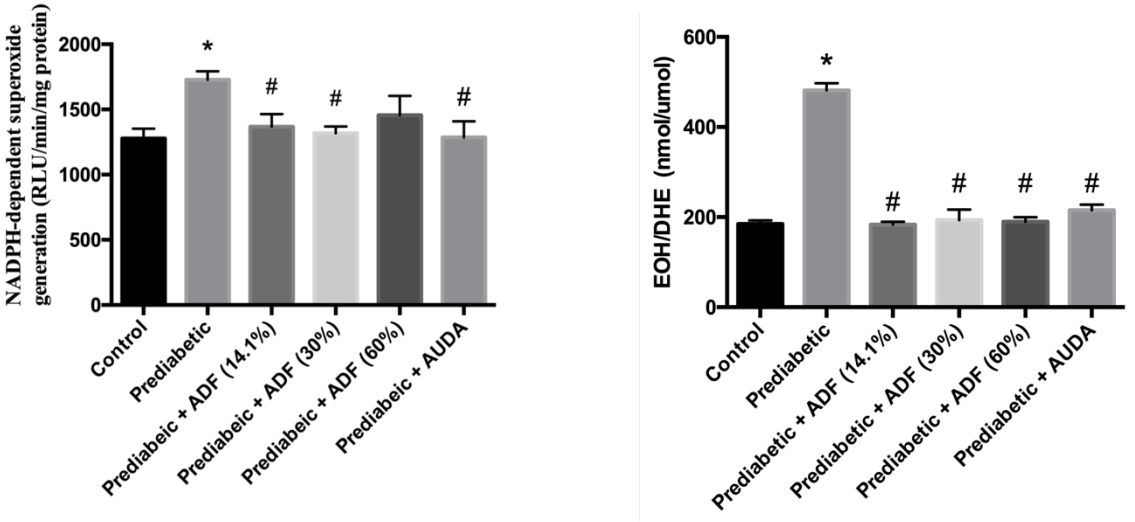


Figure 19: Myelin Protein Zero expression in Sciatic nerves (n=3)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; # $p<0.05$ vs prediabetic

8. ROS generation

ROS production was shown by our group and others to be associated with diabetes induced microvascular complications [112, 122]. To determine if intermittent fasting regulates ROS production through a NADPH-dependent mechanism, we assessed superoxide production in the sciatic nerves isolated from the different groups of mice (Figure 16-B). Our data show that superoxide generation and NADPH oxidase activity are markedly increased in the prediabetic mice **(Figure 20)**. These observations are reversed with the intermittent fasting regimen whether accompanied with dietary fat reversal (14.1% or 30% fat intake) or not (60% fat intake) **(Figure 20).** In parallel experiments, our data show prediabetic mice on a 60% fat diet have a decrease in ROS production and NADPH oxidase activity when treated with the sEH inhibitor, AUDA, suggesting that one of the major sources of ROS in this prediabetes-induced peripheral injury is cytochrome P450.



A: NADPH oxidase activity assay (n=5) **B: ROS production by HPLC test (n=3)**

Figure 20: ROS production in sciatic nerves (n=5)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; # $p<0.05$ vs prediabetic

9. Cytochromes P450 alterations in prediabetes-induced peripheral neuropathy

CYP reactions in vivo require the cofactor NADPH as the source of electrons and an additional enzyme, cytochrome P450 reductase. Our laboratory has previously identified CYPs as a major source of ROS generation. However, the role of CYPs in neuropathy or prediabetes-induced neuropathy is not yet described. Our data show that CYP1A1/1A2 a major cytochromes P450 subfamily of the epoxygenase pathway (EETs production) is significantly reduced in the isolated sciatic nerves of the prediabetic mice when compared to their control littermates. This decrease in the protein expression of CYP1a1/1a2 is partially reversed with the alternate day fasting regimen accompanied with dietary fat reversal (14.1% or 30% fat intake) and significantly restored with 60% fat intake (Figure 15). As expected, treatment with AUDA, restored the homeostatic expression of CYP1A1/1A2 protein **(Figure 21)**.

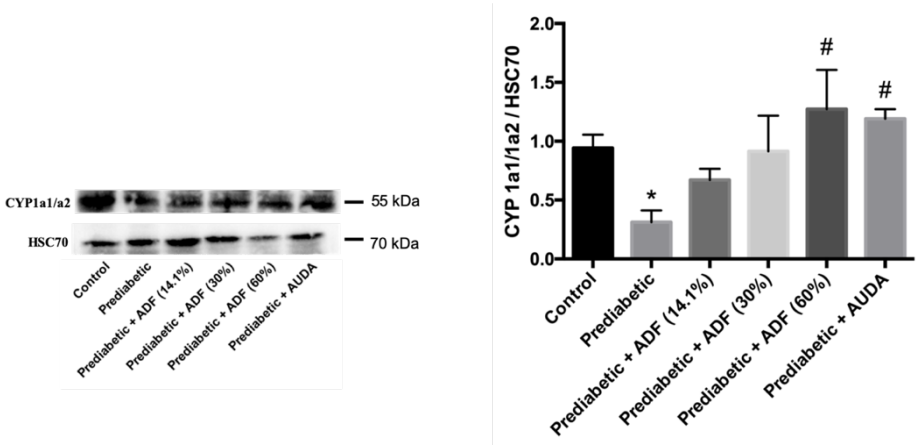


Figure 21: CYP1a1/1a2 expression in Sciatic nerves (n=5)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; # $p<0.05$ vs

B. *In vitro* Experiments

To further confirm our *in vivo* data, *in vitro* experiments were designed to simulate the prediabetes state in cultured Schwann cells and examine the associated CYP450 alterations. For that , MSC80s were treated with saturated palmitate, unsaturated oleate, 15mM of glucose, or their combinations in the presence or absence of the sEH inhibitor (increases in EETs physiological levels), AUDA.

1. Myelin Protein alteration

Myelin alteration is a crucial process to maintain peripheral nerve integrity [35]. Our preliminary data show that Myelin protein zero (MPZ) protein expression , a major structural component of the myelin sheath in the peripheral nervous system, is altered in the cultured MSC80 in the presence of palmitate or 15mM of glucose but not in the presence of oleate suggesting a form of reconditioning of peripheral myelin gene expression in the prediabetic state **(figure 22).**

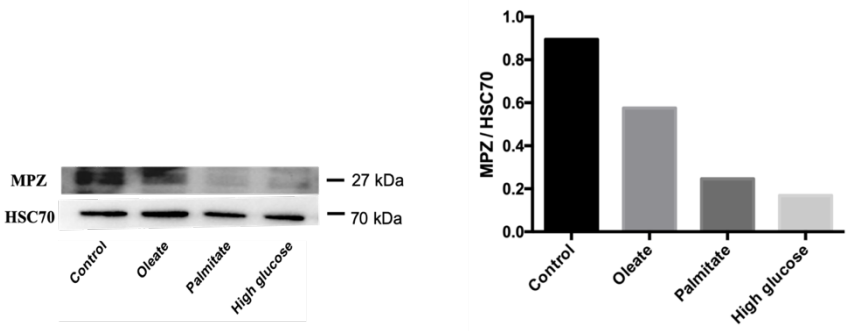


Figure 22: Myelin Protein Zero expression in MSC80s (n=1)

2. NADPH oxidase activity assay analysis

Superoxide anion generation reflected by the NADPH oxidase assay () show that cultured MSC80 cells have an increase in superoxide anion production when treated with palmitate, a combination of palmitate and oleate or a combination of palmitate, oleate and 15mM of glucose. However, treatment with either oleate or 15mM of glucose alone did not affect the ROS production as assessed by the NADPH oxidase activity **(Figure 23).** Of interest, treatment with AUDA reduced the observed increase in superoxide anion generation that was illustrated in the palmitate, the combination of palmitate and oleate or the combination of palmitate, oleate and 15mM glucose treatments. **(Figure 24)** Taken together, these data suggest that the saturated FFA palmitate is the driving component leading to ROS generation **(Figure 23, 24).**

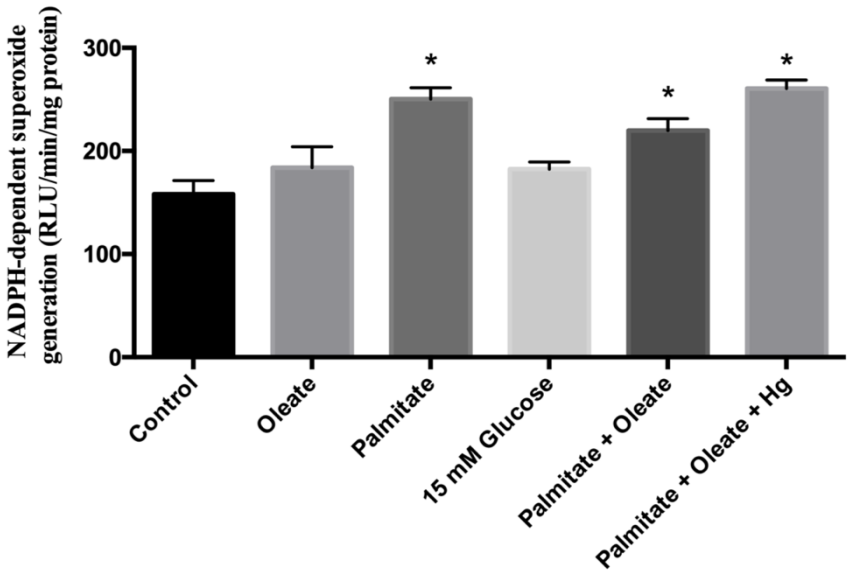


Figure 23: Effect of free fatty acids and 15 mM Glucose on ROS production (n=3)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control

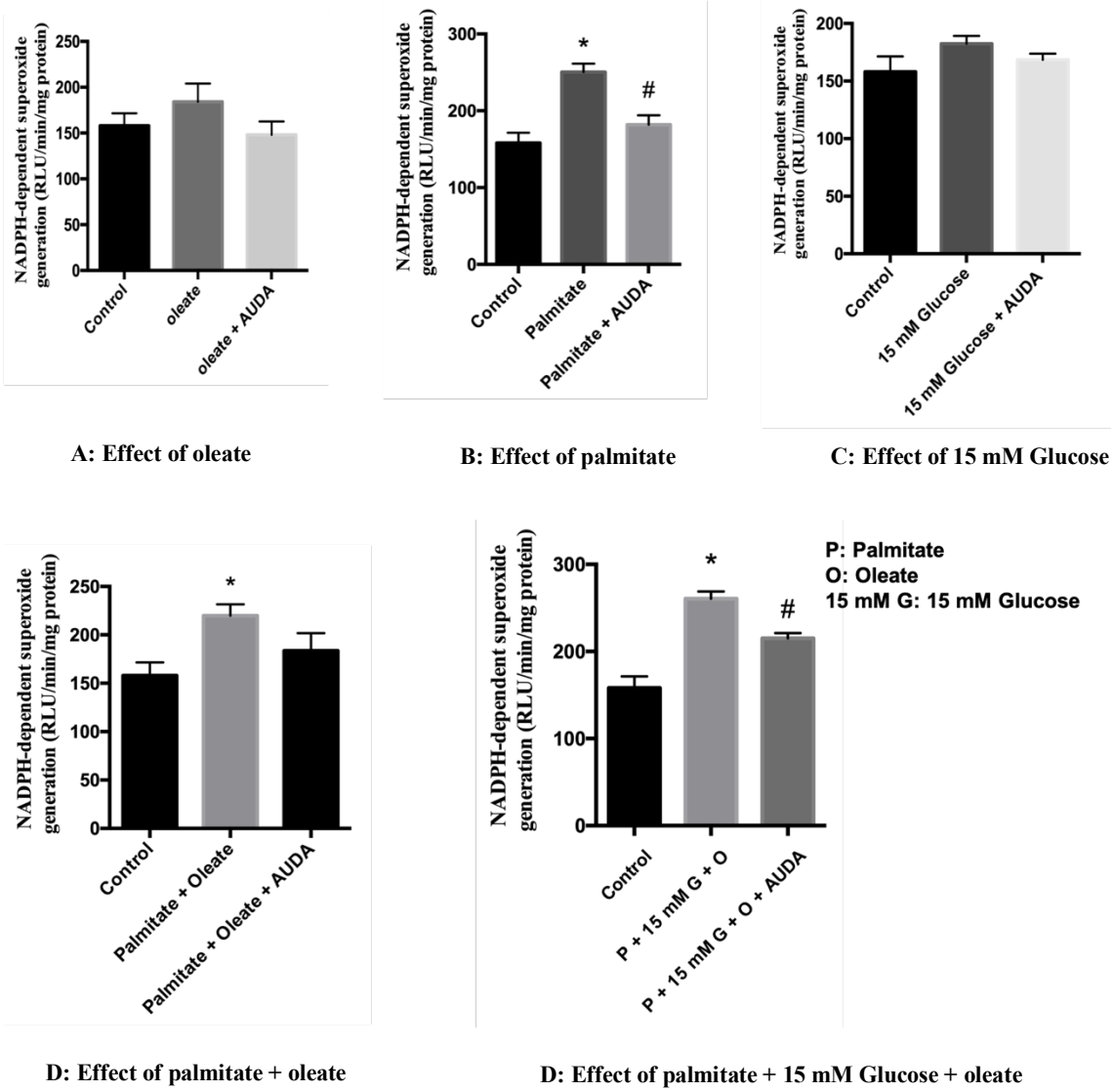


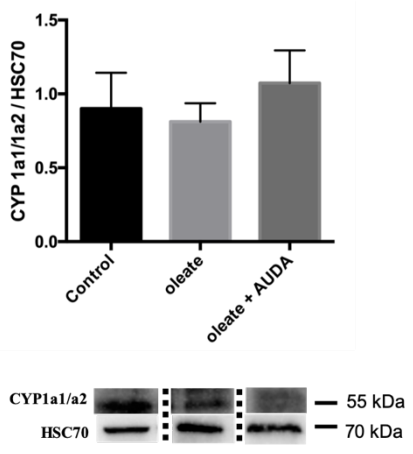
Figure 24: Effect of AUDA on ROS production in MSC80 (n=3)

Data are expressed as mean± standard error of mean (SEM). * $p<0.05$ vs control; # $p<0.05$ vs associated condition without AUDA treatment

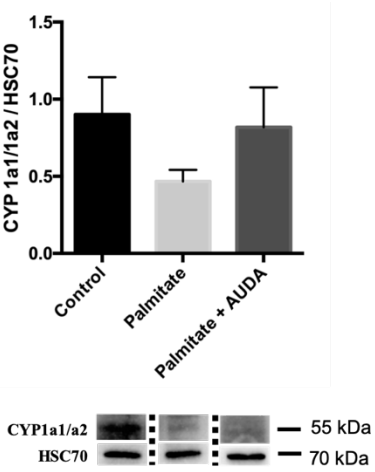
3. Cytochromes P450 1A1/1A2 alterations in prediabetes-induced peripheral neuropathy

Our animal experiments data suggest that Cytochromes P450 1A1/1A2 alterations in prediabetes orchestrate the production of reactive oxygen species and induces peripheral nerve damage. Of interest, immortalized mice Schwann cells in vitro, show a decrease in Cytochromes P450 1A1/1A2 protein expression (suggesting a decrease in EETs availability) when exposed to 24 hours of palmitate, a combination of palmitate and oleate, or a combination of palmitate, oleate and 15 mM of glucose. No changes were observed when cultured MSC80 were exposed to 24 hours of oleate. (**Figures 23**). Furthermore, the preincubation with AUDA in the presence of the different treatments (oleate, palmitate, 15mM glucose or their combinations) restored, to a certain extent, the homeostatic levels of the cytochrome P450 1A1/1A2 protein expression.

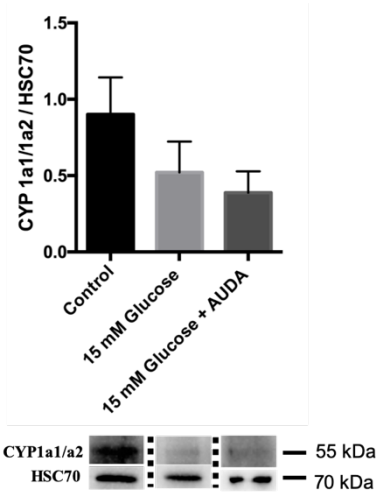
Taken together, our in vitro data suggest that prediabetes-induced myelin alteration could be controlled by the epoxygenase pathway that is in turn monitoring the ROS production in this system. **(Figure 25).**



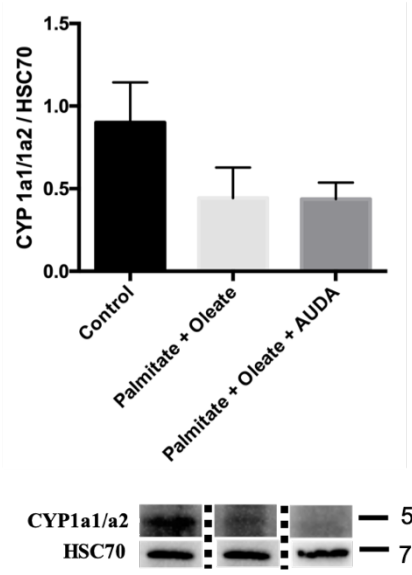
A: Effect of oleate



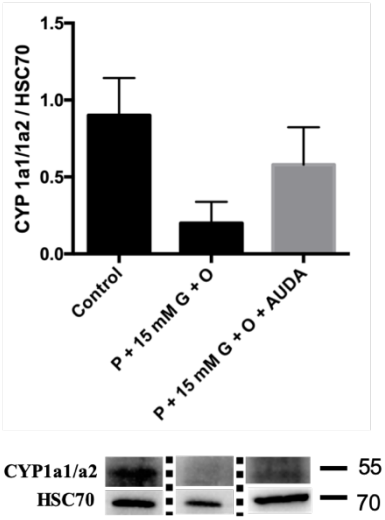
B: Effect of palmitate



C: Effect of 15 mM Glucose



D: Effect of palmitate + oleate



P: Palmitate
O: Oleate
15 mM G: 15 mM Glucose

D: Effect of palmitate + 15 mM Glucose + oleate

Figure 25: CYP 1a1/1a2 expression in MSC80 (n=3)

CHAPTER V

DISCUSSION

Prediabetes is associated with dyslipidemia, intermediate hyperglycemia and increased levels of HbA1c. [127] Our mice exhibited blood glucose concentrations and HbA1c levels that fit the diagnosis criteria for prediabetes. Intermittent fasting was successful in reversing these levels to values comparable to that of the controls. However, AUDA did not reverse blood glucose levels which remained relatively high till the sacrifice. This is actually explained by the fact that AUDA is not described as a hypoglycemic drug suggesting that the improvements associated with AUDA are attributed to its physiological action in the CYP450 pathways rather than the effect of normalization of blood glucose levels.

Lipid profile analysis show significant increases in the levels of Non-Esterified fatty acids, Total cholesterol and Triglycerides levels in the prediabetic state, confirming the manifestation of dyslipidemia. These levels were restored when mice underwent a protocol of alternate day fasting with 60% fat and dietary reversal with 14.1% and 30% fat.

In line with these observations, body mass composition analysis showed a similar pattern. Body weight was significantly increased in our prediabetic model. Interestingly, all mice treated with alternate day fasting whether with a low-fat (14.1%), an intermediate-fat (30%) or a high-fat diet (60%) exhibited significant decreases in

body weight when compared to the prediabetic mice. ADF could successfully maintain this weight reduction even when the diet was sustained at 60% fat content.

The percentage of fat mass levels were also significantly increased in the prediabetic state. This increase was restored upon management with ADF implicating that the beneficial effects of intermittent fasting are not really prompted by the limitation of food intake and its associated weight decrease but rather to its action on physiological pathways. The fact that prediabetic mice treated with ADF with a high-fat diet (60%) showed a decrease in fat mass percentage in comparison with the prediabetic mice, taking into consideration that on average the 2 groups consumed the same quantity of food of the same diet per week**,** implicates that intermittent fasting increased significantly fat metabolism. Prediabetes significantly decreased the percentage of lean mass and it was restored upon management with ADF. This provides evidence that intermittent fasting may be effective for maintaining lean mass during weight loss. In summary, ADF was associated with a decrease in percentage of fat mass and an increase in percentage of lean mass, suggesting that on fast days the body breakdowns fat first for energy and stores lean mass as a defense mechanism even with a high-fat diet.

Moreover, prediabetes can be associated with nerve injury and damage. This was validated in our study. The peripheral nervous system comprises a meshwork of nerves, glial cells and ganglia outside the brain and spinal cord. It plays a crucial role in relaying motor and sensory inputs from the central nervous system to the rest of the body. In the Plantar Analgesia Test, it is expected that mice with no peripheral nerve injury are capable of thermal sensation and withdrawal of the paw prior to tissue damage, while those with peripheral nerve injury lack sensation. [48] Indeed, the results

of the plantar analgesia test in our study test show that prediabetic mice took a significantly longer time to sense the heat of the beam and withdraw their paws compared to the controls, which was significantly restored upon management with alternated day fasting and sEH inhibition by AUDA.

As for the Beam Walking Test, which we used to assess motor function and balance, the performance of prediabetic mice was poor relative to control mice; this was reversed after intermittent fasting and sEH inhibition by AUDA. The findings of the two tests imply impaired sensorimotor dysfunctions within the peripheral nerves. This is in line with what was previously reported in literature that prediabetes could also induce peripheral nerve injury with overt clinical manifestations. [41-48]

Schwann cells are the myelinating cells found in the peripheral nervous system. They are involved in the conduction of impulses along the axons of nerves (including the sciatic nerves), nerve development, and regeneration, as well as providing trophic support for the neurons. [35] MPZ expression, an omnipotent myelin protein in the PNS, was shown to be significantly downregulated *in vivo* (sciatic nerves) and our preliminary data showed a trend of decrease *in vitro* (MSC80s). This supports the idea of the presence of serious alterations in the myelination process in the prediabetic state.

Given together, our results show that our animal and cell culture models exhibited prediabetes and a form of nerve damage, myelination dysregulations and sensorimotor deficits representing peripheral neuropathy. Intermittent fasting and sEH inhibition by AUDA were successful in reversing these molecular and phenotypic changes.

On the other hand, growing evidence in the literature suggests a solid implication of oxidative stress and increased ROS production as prime instigators of

diabetic complications. [91, 93, 94] To ensure the liability of the oxidative stress pathway, we measured ROS generation levels by the HPLC test and NADPH oxidase activity assay. ROS production and NADPH oxidase activity were both significantly increased in the prediabetic state and reduced upon treatment. Moreover, numerous studies in the field reported a considerable alteration in the arachidonic acid metabolism, mainly the CYP450 pathways. Our previous work in the lab linked diabetic nephropathy with a decrease in EETs (product of CYP1 and CYP2 subfamilies) and an increase in HETEs (product of CYP4 subfamilies). [112, 122] Findings from our study were novel in suggesting a significant decrease in CYP1a expression in prediabetic-induced neuropathy models. Alternate day fasting with dietary reversal to 14.1 or 30% fat or a protocol of alternate day fasting with 60% fat were successful in reversing these molecular disturbances and restoring homeostatic balances. This entails that ADF succeeded in attenuating the alterations associated with ROS production which alleviated PNS injury and nerve damage. To further confirm the key role of EETs, prediabetic mice were treated with AUDA, an sEH inhibitor, that increases physiological availability of EETs. sEH inhibition has been previously described to reverse some of the molecular and phenotypic alterations associated with oxidative stress in neuropathy. [117-119] Our data using AUDA mimicked the beneficial effect observed with the intermittent fasting protocol sustaining its role in a novel mechanistic pathway that involves CYP450 enzymes.

Of interest, results of our cultured MSC80s treated with saturated or unsaturated fatty acids and glucose mirrored the *in vivo* data. Unsaturated fatty acids have been shown by numerous studies to be a major toxic free fatty acid in the human tissues and to have protective effects against the deleterious consequences of saturated fatty acids.

[56, 57, 60, 61, 64, 65] In fact, our study offered evidence that oleate prevented palmitate-induced Schwann cell injury and the associated ROS production. These results suggest that unsaturated fatty acids are the prime fatty acids involved in the pathogenesis of prediabetes and the associated disturbances.

Taken together, these findings showed that prediabetes increased ROS production through NADPH upregulation and CYP1a downregulation. Dyslipidemia-induced ROS generation and EET reduction resulted in injury in the peripheral nervous system manifested by abnormal sensorimotor response. Alternate day fasting with a high-fat diet of 60% fat or with dietary reversal to 14,1% or 30% fat show beneficial effect on the pathogenesis of prediabetes and reversed the associated damages. This suggests that the action of fasting itself (and not the limitation of food intake) was successful in restoring and normalizing the **deleterious** consequences of prediabetes.

CHAPTER VI

CONCLUSION

To sum up, our study successfully described a novel pathway in the pathogenesis of prediabetic neuropathy. Dyslipidemia and intermediate hyperglycemia altered the CYP50 pathway by decreasing EETs bioavailability. These alterations might serve as plausible instigator of ROS overproduction. Moreover, our results suggest that oxidative stress is one of the final common signaling pathways orchestrating myelination alterations leading to nerve injury and damage in a prediabetic state. Furthermore, our study confirmed that upon management with intermittent fasting, we are restoring metabolic, molecular and phenotypic alterations back to homeostatic balance.

REFERENCES

1. Organization, W.H., *Top 10 causes of death worldwide.* http://www.who.int/mediacentre/factsheets/fs310/en/, 2017.
2. Schwarz, P.E., *[Prediabetes: high risk factor for diabetes mellitus type 2? - Successful prevention with early interventions for lifestyle modification].* Dtsch Med Wochenschr, 2012. **137**(37): p. 1788.
3. Tabak, A.G., et al., *Prediabetes: a high-risk state for diabetes development.* Lancet, 2012. **379**(9833): p. 2279-90.
4. Forouhi, N.G., et al., *Incidence of Type 2 diabetes in England and its association with baseline impaired fasting glucose: the Ely study 1990-2000.* Diabet Med, 2007. **24**(2): p. 200-7.
5. Nathan, D.M., et al., *Impaired fasting glucose and impaired glucose tolerance: implications for care.* Diabetes Care, 2007. **30**(3): p. 753-9.
6. Li, G., et al., *The long-term effect of lifestyle interventions to prevent diabetes in the China Da Qing Diabetes Prevention Study: a 20-year follow-up study.* Lancet, 2008. **371**(9626): p. 1783-9.
7. Anothaisintawee, T., et al., *The Relationship Among Morningness-Eveningness, Sleep Duration, Social Jetlag, and Body Mass Index in Asian Patients With Prediabetes.* Front Endocrinol (Lausanne), 2018. **9**: p. 435.
8. DiBonaventura, M., et al., *Obesity in Germany and Italy: prevalence, comorbidities, and associations with patient outcomes.* Clinicoecon Outcomes Res, 2018. **10**: p. 457-475.
9. Granados, A., et al., *Association of abdominal muscle composition with prediabetes and diabetes: The CARDIA study.* Diabetes Obes Metab, 2019. **21**(2): p. 267-275.
10. Alvarez, S. and A.M. Algotar, *Prediabetes*, in *StatPearls*. 2019: Treasure Island (FL).
11. American Diabetes, A., *2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2019.* Diabetes Care, 2019. **42**(Suppl 1): p. S13-S28.
12. WHO, *Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia : report of a WHO/IDF consultation.* 2006.
13. Ferrannini, E., A. Gastaldelli, and P. Iozzo, *Pathophysiology of prediabetes.* Med Clin North Am, 2011. **95**(2): p. 327-39, vii-viii.
14. Tabak, A.G., et al., *Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study.* Lancet, 2009. **373**(9682): p. 2215-21.
15. Bergman, M., *Pathophysiology of prediabetes and treatment implications for the prevention of type 2 diabetes mellitus.* Endocrine, 2013. **43**(3): p. 504-13.

16. Gourgari, E., E. Spanakis, and A.S. Dobs, *Pathophysiology, risk factors, and screening methods for prediabetes in women with polycystic ovary syndrome.* Int J Womens Health, 2016. **8**: p. 381-7.
17. Kabadi, U.M., *Major Pathophysiology in Prediabetes and Type 2 Diabetes: Decreased Insulin in Lean and Insulin Resistance in Obese.* J Endocr Soc, 2017. **1**(6): p. 742-750.
18. Mathis, D., L. Vence, and C. Benoist, *beta-Cell death during progression to diabetes.* Nature, 2001. **414**(6865): p. 792-8.
19. Weir, G.C. and S. Bonner-Weir, *Five stages of evolving beta-cell dysfunction during progression to diabetes.* Diabetes, 2004. **53 Suppl 3**: p. S16-21.
20. Diabetes Prevention Program Research, G., et al., *10-year follow-up of diabetes incidence and weight loss in the Diabetes Prevention Program Outcomes Study.* Lancet, 2009. **374**(9702): p. 1677-86.
21. Investigators, D.T., et al., *Effect of rosiglitazone on the frequency of diabetes in patients with impaired glucose tolerance or impaired fasting glucose: a randomised controlled trial.* Lancet, 2006. **368**(9541): p. 1096-105.
22. Tuomilehto, J., et al., *Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance.* N Engl J Med, 2001. **344**(18): p. 1343-50.
23. Sjostrom, L., et al., *Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery.* N Engl J Med, 2004. **351**(26): p. 2683-93.
24. *National diabetes statistics report: estimates of diabetes and its burden in the United States.* http://www.cdc.gov/diabetes/pubs/statsreport14/national-diabetes-report-web.pdf, 2014.
25. Atkinson, M.A. and N.K. Maclaren, *The pathogenesis of insulin-dependent diabetes mellitus.* N Engl J Med, 1994. **331**(21): p. 1428-36.
26. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2014. **37 Suppl 1**: p. S81-90.
27. *American Diabetes Association: clinical practice recommendations 1997.* Diabetes Care, 1997. **20 Suppl 1**: p. S1-70.
28. Brownlee, M., *Biochemistry and molecular cell biology of diabetic complications.* Nature, 2001. **414**(6865): p. 813-20.
29. Sattar, N. and D. Preiss, *Diabetic microvascular complications as simple indicators of risk for cardiovascular outcomes and heart failure.* Lancet Diabetes Endocrinol, 2016. **4**(7): p. 555-6.
30. Hazuda, H.P., et al., *Long-term Association of Depression Symptoms and Antidepressant Medication Use With Incident Cardiovascular Events in the Look AHEAD (Action for Health in Diabetes) Clinical Trial of Weight Loss in Type 2 Diabetes.* Diabetes Care, 2019. **42**(5): p. 910-918.
31. Qi, J., et al., *Cancer risk among patients with type 2 diabetes: A real-world study in Shanghai, China.* J Diabetes, 2019.
32. Reus, G.Z., et al., *Relationship of Oxidative Stress as a Link between Diabetes Mellitus and Major Depressive Disorder.* Oxid Med Cell Longev, 2019. **2019**: p. 8637970.

33. Aaron I. Vinik, M.D., Ph.D., *Diabetic Sensory and Motor Neuropathy.* N Engl J Med, 2017.
34. Tesfaye, S., et al., *Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments.* Diabetes Care, 2010. **33**(10): p. 2285-93.
35. John D England, A.K.A., *Peripheral neuropathy.* Lancet, 2004.
36. Pop-Busui, R., et al., *Diabetic Neuropathy: A Position Statement by the American Diabetes Association.* Diabetes Care, 2017. **40**(1): p. 136-154.
37. Stino, A.M. and A.G. Smith, *Peripheral neuropathy in prediabetes and the metabolic syndrome.* J Diabetes Investig, 2017. **8**(5): p. 646-655.
38. Vaeggemose, M., et al., *Magnetic Resonance Neurography Visualizes Abnormalities in Sciatic and Tibial Nerves in Patients With Type 1 Diabetes and Neuropathy.* Diabetes, 2017. **66**(7): p. 1779-1788.
39. O'Brien, P.D., et al., *ER stress in diabetic peripheral neuropathy: A new therapeutic target.* Antioxid Redox Signal, 2014. **21**(4): p. 621-33.
40. Forbes, J.M. and M.E. Cooper, *Mechanisms of diabetic complications.* Physiol Rev, 2013. **93**(1): p. 137-88.
41. Smith, A.G., et al., *Lifestyle intervention for pre-diabetic neuropathy.* Diabetes Care, 2006. **29**(6): p. 1294-9.
42. Callaghan, B.C. and E.L. Feldman, *Measuring neuropathy in obese populations: Challenge accepted. Reply to "Diagnostic accuracy of neuropathy tests in obese population remains elusive".* Clin Neurophysiol, 2018. **129**(7): p. 1504-1505.
43. Callaghan, B.C., et al., *Diabetes and obesity are the main metabolic drivers of peripheral neuropathy.* Ann Clin Transl Neurol, 2018. **5**(4): p. 397-405.
44. Callaghan, B.C., et al., *Better diagnostic accuracy of neuropathy in obesity: A new challenge for neurologists.* Clin Neurophysiol, 2018. **129**(3): p. 654-662.
45. Feldman, E.L., et al., *New Horizons in Diabetic Neuropathy: Mechanisms, Bioenergetics, and Pain.* Neuron, 2017. **93**(6): p. 1296-1313.
46. Grisold, A., B.C. Callaghan, and E.L. Feldman, *Mediators of diabetic neuropathy: is hyperglycemia the only culprit?* Curr Opin Endocrinol Diabetes Obes, 2017. **24**(2): p. 103-111.
47. Hinder, L.M., et al., *Dietary reversal of neuropathy in a murine model of prediabetes and metabolic syndrome.* Dis Model Mech, 2017. **10**(6): p. 717-725.
48. O'Brien, P.D., et al., *Juvenile murine models of prediabetes and type 2 diabetes develop neuropathy.* Dis Model Mech, 2018. **11**(12).
49. Mittendorfer, B., et al., *What does the measurement of whole-body fatty acid rate of appearance in plasma by using a fatty acid tracer really mean?* Diabetes, 2003. **52**(7): p. 1641-8.
50. Basu, A., et al., *Systemic and regional free fatty acid metabolism in type 2 diabetes.* Am J Physiol Endocrinol Metab, 2001. **280**(6): p. E1000-6.
51. Isley, W.L., W.S. Harris, and J.M. Miles, *The effect of high-dose simvastatin on free fatty acid metabolism in patients with type 2 diabetes mellitus.* Metabolism, 2006. **55**(6): p. 758-62.
52. Reaven, G.M., et al., *Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM.* Diabetes, 1988. **37**(8): p. 1020-4.

53. Xu, S., et al., *Palmitate induces ER calcium depletion and apoptosis in mouse podocytes subsequent to mitochondrial oxidative stress.* Cell Death Dis, 2015. **6**: p. e1976.
54. Cacicedo, J.M., et al., *Palmitate-induced apoptosis in cultured bovine retinal pericytes: roles of NAD(P)H oxidase, oxidant stress, and ceramide.* Diabetes, 2005. **54**(6): p. 1838-45.
55. Maedler, K., et al., *Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function.* Diabetes, 2003. **52**(3): p. 726-33.
56. Staiger, K., et al., *Saturated, but not unsaturated, fatty acids induce apoptosis of human coronary artery endothelial cells via nuclear factor-kappaB activation.* Diabetes, 2006. **55**(11): p. 3121-6.
57. Suzuki, J., et al., *Palmitate induces apoptosis in Schwann cells via both ceramide-dependent and independent pathways.* Neuroscience, 2011. **176**: p. 188-98.
58. Fu, D., J. Lu, and S. Yang, *Oleic/Palmitate Induces Apoptosis in Human Articular Chondrocytes via Upregulation of NOX4 Expression and ROS Production.* Ann Clin Lab Sci, 2016. **46**(4): p. 353-9.
59. Kien, C.L., et al., *Dietary intake of palmitate and oleate has broad impact on systemic and tissue lipid profiles in humans.* Am J Clin Nutr, 2014. **99**(3): p. 436-45.
60. Kwon, B., H.K. Lee, and H.W. Querfurth, *Oleate prevents palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory signaling in neuronal cells.* Biochim Biophys Acta, 2014. **1843**(7): p. 1402-13.
61. Lee, H., J.Y. Lim, and S.J. Choi, *Oleate Prevents Palmitate-Induced Atrophy via Modulation of Mitochondrial ROS Production in Skeletal Myotubes.* Oxid Med Cell Longev, 2017. **2017**: p. 2739721.
62. Palomer, X., et al., *Palmitic and Oleic Acid: The Yin and Yang of Fatty Acids in Type 2 Diabetes Mellitus.* Trends Endocrinol Metab, 2018. **29**(3): p. 178-190.
63. Pardo, V., et al., *Opposite cross-talk by oleate and palmitate on insulin signaling in hepatocytes through macrophage activation.* J Biol Chem, 2015. **290**(18): p. 11663-77.
64. Plotz, T., et al., *The monounsaturated fatty acid oleate is the major physiological toxic free fatty acid for human beta cells.* Nutr Diabetes, 2017. **7**(12): p. 305.
65. Salvado, L., et al., *Oleate prevents saturated-fatty-acid-induced ER stress, inflammation and insulin resistance in skeletal muscle cells through an AMPK-dependent mechanism.* Diabetologia, 2013. **56**(6): p. 1372-82.
66. Nakamura, S., et al., *Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria.* J Biol Chem, 2009. **284**(22): p. 14809-18.
67. Mayer, C.M. and D.D. Belsham, *Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons: rescue of resistance and apoptosis through adenosine 5' monophosphate-activated protein kinase activation.* Endocrinology, 2010. **151**(2): p. 576-85.

68. Aksungar, F.B., et al., *Comparison of Intermittent Fasting Versus Caloric Restriction in Obese Subjects: A Two Year Follow-Up.* J Nutr Health Aging, 2017. **21**(6): p. 681-685.
69. Patterson, R.E., et al., *Intermittent Fasting and Human Metabolic Health.* J Acad Nutr Diet, 2015. **115**(8): p. 1203-12.
70. Patterson, R.E. and D.D. Sears, *Metabolic Effects of Intermittent Fasting.* Annu Rev Nutr, 2017. **37**: p. 371-393.
71. Santos, H.O. and R.C.O. Macedo, *Impact of intermittent fasting on the lipid profile: Assessment associated with diet and weight loss.* Clin Nutr ESPEN, 2018. **24**: p. 14-21.
72. Stockman, M.C., et al., *Intermittent Fasting: Is the Wait Worth the Weight?* Curr Obes Rep, 2018. **7**(2): p. 172-185.
73. Ganesan, K., Y. Habboush, and S. Sultan, *Intermittent Fasting: The Choice for a Healthier Lifestyle.* Cureus, 2018. **10**(7): p. e2947.
74. Greenhill, C., *Benefits of time-restricted feeding.* Nat Rev Endocrinol, 2018. **14**(11): p. 626.
75. Moro, T., et al., *Effects of eight weeks of time-restricted feeding (16/8) on basal metabolism, maximal strength, body composition, inflammation, and cardiovascular risk factors in resistance-trained males.* J Transl Med, 2016. **14**(1): p. 290.
76. Rothschild, J., et al., *Time-restricted feeding and risk of metabolic disease: a review of human and animal studies.* Nutr Rev, 2014. **72**(5): p. 308-18.
77. Barnosky, A.R., et al., *Intermittent fasting vs daily calorie restriction for type 2 diabetes prevention: a review of human findings.* Transl Res, 2014. **164**(4): p. 302-11.
78. Corley, B.T., et al., *Intermittent fasting in Type 2 diabetes mellitus and the risk of hypoglycaemia: a randomized controlled trial.* Diabet Med, 2018. **35**(5): p. 588-594.
79. Furmli, S., et al., *Therapeutic use of intermittent fasting for people with type 2 diabetes as an alternative to insulin.* BMJ Case Rep, 2018. **2018**.
80. Grajower, M.M. and B.D. Horne, *Clinical Management of Intermittent Fasting in Patients with Diabetes Mellitus.* Nutrients, 2019. **11**(4).
81. Olansky, L., *Strategies for management of intermittent fasting in patients with diabetes.* Cleve Clin J Med, 2017. **84**(5): p. 357-358.
82. Wei, S., et al., *Intermittent administration of a fasting-mimicking diet intervenes in diabetes progression, restores beta cells and reconstructs gut microbiota in mice.* Nutr Metab (Lond), 2018. **15**: p. 80.
83. Zubrzycki, A., et al., *The role of low-calorie diets and intermittent fasting in the treatment of obesity and type-2 diabetes.* J Physiol Pharmacol, 2018. **69**(5).
84. Madorsky, I., et al., *Intermittent fasting alleviates the neuropathic phenotype in a mouse model of Charcot-Marie-Tooth disease.* Neurobiol Dis, 2009. **34**(1): p. 146-54.
85. Tikoo, K., et al., *Intermittent fasting prevents the progression of type I diabetic nephropathy in rats and changes the expression of Sir2 and p53.* FEBS Lett, 2007. **581**(5): p. 1071-8.

86. Haluzik, M. and M. Mraz, *Intermittent Fasting and Prevention of Diabetic Retinopathy: Where Do We Go From Here?* Diabetes, 2018. **67**(9): p. 1745-1747.
87. Faris, M.A., et al., *Impact of ramadan intermittent fasting on oxidative stress measured by urinary 15-f(2t)-isoprostane.* J Nutr Metab, 2012. **2012**: p. 802924.
88. Hu, Y., et al., *Postoperative intermittent fasting prevents hippocampal oxidative stress and memory deficits in a rat model of chronic cerebral hypoperfusion.* Eur J Nutr, 2019. **58**(1): p. 423-432.
89. Wegman, M.P., et al., *Practicality of intermittent fasting in humans and its effect on oxidative stress and genes related to aging and metabolism.* Rejuvenation Res, 2015. **18**(2): p. 162-72.
90. Zhang, J.J., et al., *Effect of alternate-day fasting therapy combined with Linggui Zhugan Decoction () on hepatic oxidative stress in hyperlipidemic rat.* Chin J Integr Med, 2015.
91. Asmat, U., K. Abad, and K. Ismail, *Diabetes mellitus and oxidative stress-A concise review.* Saudi Pharm J, 2016. **24**(5): p. 547-553.
92. Pham-Huy, L.A., H. He, and C. Pham-Huy, *Free radicals, antioxidants in disease and health.* Int J Biomed Sci, 2008. **4**(2): p. 89-96.
93. Maritim, A.C., R.A. Sanders, and J.B. Watkins, 3rd, *Diabetes, oxidative stress, and antioxidants: a review.* J Biochem Mol Toxicol, 2003. **17**(1): p. 24-38.
94. Baynes, J.W., *Role of oxidative stress in development of complications in diabetes.* Diabetes, 1991. **40**(4): p. 405-12.
95. Cameron, N.E. and M.A. Cotter, *Effects of antioxidants on nerve and vascular dysfunction in experimental diabetes.* Diabetes Res Clin Pract, 1999. **45**(2-3): p. 137-46.
96. King, G.L. and M.R. Loeken, *Hyperglycemia-induced oxidative stress in diabetic complications.* Histochem Cell Biol, 2004. **122**(4): p. 333-8.
97. Scott, J.A. and G.L. King, *Oxidative stress and antioxidant treatment in diabetes.* Ann N Y Acad Sci, 2004. **1031**: p. 204-13.
98. Niedowicz, D.M. and D.L. Daleke, *The role of oxidative stress in diabetic complications.* Cell Biochem Biophys, 2005. **43**(2): p. 289-330.
99. Eckersley, L., *Role of the Schwann cell in diabetic neuropathy.* Int Rev Neurobiol, 2002. **50**: p. 293-321.
100. Lupachyk, S., et al., *Triglyceride, nonesterified fatty acids, and prediabetic neuropathy: role for oxidative-nitrosative stress.* Free Radic Biol Med, 2012. **52**(8): p. 1255-63.
101. Feldman, E.L., *Oxidative stress and diabetic neuropathy: a new understanding of an old problem.* J Clin Invest, 2003. **111**(4): p. 431-3.
102. Greene, D.A., et al., *Glucose-induced oxidative stress and programmed cell death in diabetic neuropathy.* Eur J Pharmacol, 1999. **375**(1-3): p. 217-23.
103. Russell, J.W., et al., *High glucose-induced oxidative stress and mitochondrial dysfunction in neurons.* FASEB J, 2002. **16**(13): p. 1738-48.
104. Vincent, A.M., et al., *Sensory neurons and schwann cells respond to oxidative stress by increasing antioxidant defense mechanisms.* Antioxid Redox Signal, 2009. **11**(3): p. 425-38.

105. Vincent, A.M., et al., *Oxidative stress in the pathogenesis of diabetic neuropathy.* Endocr Rev, 2004. **25**(4): p. 612-28.
106. Ingelman-Sundberg, M., *Genetic susceptibility to adverse effects of drugs and environmental toxicants. The role of the CYP family of enzymes.* Mutat Res, 2001. **482**(1-2): p. 11-9.
107. Rendic, S. and F.J. Di Carlo, *Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors.* Drug Metab Rev, 1997. **29**(1-2): p. 413-580.
108. Bondy, S.C. and S. Naderi, *Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species.* Biochem Pharmacol, 1994. **48**(1): p. 155-9.
109. Fleming, I. and R. Busse, *Vascular cytochrome P450 in the regulation of renal function and vascular tone: EDHF, superoxide anions and blood pressure.* Nephrol Dial Transplant, 2001. **16**(7): p. 1309-11.
110. Puntarulo, S. and A.I. Cederbaum, *Production of reactive oxygen species by microsomes enriched in specific human cytochrome P450 enzymes.* Free Radic Biol Med, 1998. **24**(7-8): p. 1324-30.
111. Eid, A.A., et al., *Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases.* Diabetes, 2009. **58**(5): p. 1201-11.
112. Eid, S., et al., *Involvement of renal cytochromes P450 and arachidonic acid metabolites in diabetic nephropathy.* J Biol Regul Homeost Agents, 2013. **27**(3): p. 693-703.
113. Nebert, D.W. and D.W. Russell, *Clinical importance of the cytochromes P450.* Lancet, 2002. **360**(9340): p. 1155-62.
114. Chen, G., et al., *Genetic disruption of soluble epoxide hydrolase is protective against streptozotocin-induced diabetic nephropathy.* Am J Physiol Endocrinol Metab, 2012. **303**(5): p. E563-75.
115. Chiang, C.W., et al., *Genetic deletion of soluble epoxide hydrolase attenuates inflammation and fibrosis in experimental obstructive nephropathy.* Mediators Inflamm, 2015. **2015**: p. 693260.
116. Huang, H., J. Weng, and M.H. Wang, *EETs/sEH in diabetes and obesity-induced cardiovascular diseases.* Prostaglandins Other Lipid Mediat, 2016. **125**: p. 80-9.
117. Wagner, K., et al., *Soluble epoxide hydrolase inhibition alleviates neuropathy in Akita (Ins2 Akita) mice.* Behav Brain Res, 2017. **326**: p. 69-76.
118. Wagner, K., et al., *Comparative efficacy of 3 soluble epoxide hydrolase inhibitors in rat neuropathic and inflammatory pain models.* Eur J Pharmacol, 2013. **700**(1-3): p. 93-101.
119. Wagner, K., et al., *Soluble epoxide hydrolase inhibition is antinociceptive in a mouse model of diabetic neuropathy.* J Pain, 2014. **15**(9): p. 907-14.
120. Capdevila, J., et al., *The oxidative metabolism of arachidonic acid by purified cytochromes P-450.* Biochem Biophys Res Commun, 1981. **101**(4): p. 1357-63.
121. Kotlyar, M. and S.W. Carson, *Effects of obesity on the cytochrome P450 enzyme system.* Int J Clin Pharmacol Ther, 1999. **37**(1): p. 8-19.
122. Eid, S., et al., *20-HETE and EETs in diabetic nephropathy: a novel mechanistic pathway.* PLoS One, 2013. **8**(8): p. e70029.

123. Pillarisetti, S. and I. Khanna, *A multimodal disease modifying approach to treat neuropathic pain--inhibition of soluble epoxide hydrolase (sEH).* Drug Discov Today, 2015. **20**(11): p. 1382-90.
124. Luong, T.N., et al., *Assessment of motor balance and coordination in mice using the balance beam.* J Vis Exp, 2011(49).
125. Dirig, D.M., et al., *Characterization of variables defining hindpaw withdrawal latency evoked by radiant thermal stimuli.* J Neurosci Methods, 1997. **76**(2): p. 183-91.
126. Bala, U., et al., *Harvesting the maximum length of sciatic nerve from adult mice: a step-by-step approach.* BMC Res Notes, 2014. **7**: p. 714.
127. Bansal, N., *Prediabetes diagnosis and treatment: A review.* World J Diabetes, 2015. **6**(2): p. 296-303.