From Candy to Chemotherapy: Characterizing Sensory Neuropathy Associated with Prediabetes and Paclitaxel Treatment

By

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Abstract

Peripheral neuropathy is one of the major co-morbidities associated with diabetes mellitus. Neurotoxic chemotherapies including paclitaxel also display significant risk of development of peripheral neuropathy. It is hypothesized that paclitaxel might also affect sensory neurons innervating the pancreas parenchyma, including the islets, disrupting insulin production. Overall, we evaluated several different aspects of somatic and visceral neuropathy in both animal models and human patients. In mice, we evaluated the effects of paclitaxel treatment on C57BL/6 mice as well as the use of ketogenic diet as a neuroprotective agent. We showed that paclitaxel caused mechanical allodynia as well as a reduction in pancreatic islet innervation. Paclitaxel also caused high fat diet-fed mice to develop increased weight gain and insulin instability. Consumption of a ketogenic diet prevented the paclitaxel-associated weight gain and fat deposition in male mice but did not appear to have an effect on glucose or insulin levels. In human patients, we evaluated the evaluated the lifestyle, metabolic, and epidermal changes in prediabetic patients with and without neuropathy through an ongoing clinical study. Prediabetic patients with neuropathy displayed a reduced intraepidermal nerve fiber density, higher neuropathy screening instrument scores, as well as lower LDL levels compared to prediabetic patients without neuropathy and healthy controls. Both pre-diabetes and chemotherapy lead to sensory nerve damage, and our results demonstrate that additional sensory nerve populations such as pancreatic afferents should be considered in the clinical evaluation of patients due to a more insidious presentation and long-term consequences on metabolic health.

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Table of Contents

Acceptance Page i
Abstract iii
Acknowledgements iv
Table of Contents viii
List of Figures x
Chapter 1: Introduction 1
Diabetes Mellitus 2
Diabetic and Prediabetic Neuropathy 3
Chemotherapy Induced Peripheral Neuropathy (CIPN) 5
Sensory Innervation of Pancreatic Islets 6
Ketogenic Diet as a Neuroprotective Intervention 10
Chapter 2: Paclitaxel-Induced Neuropathy Impacts Metabolism and Glucose Tolerance in Mice 15
1. Abstract 16
2. Introduction 17
3. Methods 18
4. Results and Figures 22
5. Discussion 36
Chapter 3: Elimination of Capsaicin-Responsive Sensory Neurons During Neonatal Development Affects Metabolic Parameters in Adulthood 40
1. Abstract 41
2. Introduction 42
3. Methods 42
4. Results and Figures 45
5. Discussion 52
Chapter 4: Effects of Ketogenic Diet on Paclitaxel Induced Neuropathy in Mice 53
1. Abstract 54
2. Introduction 55
3. Methods 56
4. Results and Figures 59
5. Discussion 73
Chapter 5: Characterization of Peripheral Neuropathy in Prediabetic Patients: The PACMAN Study 78

1. Abstract 79
2. Introduction 80
3. Methods 82
4. Results and Figures 86
5. Discussion 148
Chapter 6: General Discussion and Conclusions 153
References 167
Appendix A: Informed Consent 209

List of Figures

Figure 1 Neuroendocrine feedback loop between beta cells and sensory neurons 8
Figure 2 Neuroprotective Mechanisms of Ketone Bodies 12
Figure 3 Paclitaxel causes a decrease in hind paw withdrawal threshold 23
Figure 4 Paclitaxel treatment decreases pancreatic islet innervation 26
Figure 5 Paclitaxel treatment caused mild elevations in blood glucose and insulin levels ... 30
Figure 6 Paclitaxel treatment with a high fat diet caused increased weight gain and insulin variability in male mice 34
Figure 7 Neonatal Capsaicin Delays Paclitaxel-Induced Mechanical Allodynia 46
Figure 8 Neonatal Capsaicin Causes Increases in Weight, Glucose and Insulin 50
Figure 9 Paclitaxel's effect on fasting blood ketone levels 61
Figure 10 Ketogenic diet's effect on hind paw sensitivity with paclitaxel treatment 64
Figure 11 Ketogenic diet's effect on weight and body composition with paclitaxel treatment 68
Figure 12 Ketogenic diet's effect on glucose and insulin levels with paclitaxel treatment ... 71
Figure 13 Inclusion/Exclusion Criteria 87
Figure 14 Demographics 90
Figure 15 Lifestyle and Smoking History 93
Figure 16 Diabetes and Neuropathy History 96
Figure 17 Physical Measurements and Vitals 99
Figure 18 Medical History 104
Figure 19 Laboratory Analysis of Blood Draws – Glucose and Insulin 108
Figure 20 Laboratory Analysis of Blood Draws – Lipid Panel 111
Figure 21 Laboratory Analysis of Blood Draws – Hemoglobin & Hematocrit 114
Figure 22 IPAQ: Job-Related Physical Activity 117
Figure 23 IPAQ: Transportation-Related Physical Activity 120
Figure 24 IPAQ: Housework, House Maintenance, and Caring for Family 123
Figure 25 IPAQ: Recreation, Sport, and Leisure-Time Physical Activity 126
Figure 26 IPAQ: Time Spent Sitting 129
Figure 27 Pittsburgh Sleep Quality Index (PSQI) Results 132
Figure 28 Pittsburgh Sleep Quality Index (PSQI) Trouble Sleeping Factors 135
Figure 29 Brief Pain Inventory for Diabetic Neuropathy (BPI-DPN) 139
Figure 30 Michigan Neuropathy Screening Instrument (MNSI) and Utah Early Neuropathy Scale (UENS) Results 143
Figure 31 Skin Biopsy Histology Analysis 146

Chapter 1: Introduction

Diabetes Mellitus

Diabetes mellitus is a metabolic disorder defined by hyperglycemia due to decreased insulin production or insulin sensitivity in peripheral tissues. Globally, prevalence of diabetes has increased over the past 20 years to epidemic proportions, beyond what was previously estimated by the International Diabetes Foundation [1]. Currently 13% of all adults in the United States suffer from diabetes, with the elderly population disproportionately affected at 26.8% over 65 years old [2]. Epidemiological trends and predictions forecast that rates of diabetes will continue to rise at an alarming rate over the next several decades [3-5].

There currently exists several treatment options for both Type 1 and Type 2 diabetes with the treatment goals of lower duration and associated morbidity of both acute and chronic hyperglycemia [6, 7]. Type 1 diabetes largely requires insulin replacement therapy with both long acting and short acting insulin regimens. Type 2 diabetes treatment begins with conservative management including diet and exercise. The most common first line therapy is the widely prescribed biguanide, metformin [8, 9]. After which there exists a plethora of other medications which act on the beta cell's insulin secretion pathway, or the peripheral tissues to help sensitize them to the effects of endogenous insulin. Insulin secretagogues include older sulfonylurea drugs such as glimepiride or glipizide that block the ATP-dependent potassium channel in beta cells leading to increase insulin release [10]. Additional oral medications that promote release of endogenous insulin stores include GLP-1 analogues and DPP-4 inhibitors. GLP-1 analogues such as exenatide, slow gastric emptying and bind GLP-1 receptors on beta cells to increase insulin secretion via adenylyl cyclase and PKA mediated pathways [11]. DPP-4 inhibitors block dipeptidyl peptidase-4, which normally degrades endogenous GLP-1 levels [12]. Other diabetic medication includes thiazolidinediones, amylin analogues, alpha-glucosidase

inhibitors and SGLT-2 inhibitors. Thiazolidinediones, such as pioglitazone, are ligands of PPAR-gamma transcription factor increasing insulin sensitivity [13, 14]. Amylin analogues, such as pramlintide, decrease glucagon and gastric emptying [15]. Alpha-glucosidase inhibitors, such as acarbose or miglitol, prevent the breakdown of disaccharides in the brush border of enterocytes [16, 17]. SGLT-2 inhibitors, such as canagliflozin, prevent reabsorption of glucose in the proximal convoluted tubule of the nephron, allowing for increased excretion of glucose in the urine [18].

Poorly treated diabetes leads to several comorbidities including retinopathy, nephropathy, neuropathy, erectile dysfunction, and macrovascular disease [19-21]. It can lead to blindness, kidney failure, heart attacks, strokes, and lower limb amputation. In the united states diabetes has become the 7th leading cause of death, it also contributes to heart disease and stroke which are the 1st and 5th cause of death, respectively [22, 23].

Diabetic and Prediabetic Neuropathy

One of the most common complications of diabetes is the development of diabetic peripheral neuropathy (DPN) which usually presents as numbness, tingling, or pain in stocking-glove distribution. It begins in the plantar surface of the feet and ascends with increasing disease duration and severity. While incidence rates vary depending on diagnostic criteria, up to 50% of diabetic patients will develop DPN during their lifetime [24, 25]. Between 10-26% of DPN patients will develop pain symptoms with their neuropathy including burning, stabbing, or electrical pain [26]. The etiology of this neuropathic pain remains poorly understood [27]. Many pre-diabetic patients develop neuropathy before they reach the clinical threshold for the diagnosis of overt type 2 diabetes, in fact, up to 20% already have symptoms of neuropathy at

time of diagnosis [28-32]. In addition, over 25% of patients with idiopathic neuropathy were subsequently found to have impaired glucose metabolism or prediabetes [33-36]. Prediabetic neuropathy and diabetic neuropathy share similar clinical features, as they likely have similar underlying mechanisms, although symptoms of prediabetic neuropathy are generally milder [28, 37]. Once developed, diabetic peripheral neuropathy is largely irreversible, and recommended treatment options remain limited to treating the underlying hyperglycemia to prevent progression and supportive analgesia for the painful symptoms [38]. Current first line therapy for painful diabetic neuropathy includes anticonvulsants (pregabalin or gabapentin), SNRIs or TCAs [39-41].

According to evidence-based guidelines by the American College of Neurology, American Association of Neuromuscular and Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation, pregabalin has been considered effective for the treatment of painful diabetic neuropathy (Level A), while venlafaxine, duloxetine, gabapentin among others were deemed less efficacious (Level B) [42]. Current medications do not prevent or reverse neuropathic changes or provide adequate pain relief for many patients, and many have substantial side effects [43-45].

As the exact mechanism for diabetic neuropathy remains unknown, and likely multifactorial, targeted treatments remains elusive [39, 46-48]. Molecular pathways already identified that are implicated in diabetic neuropathy include: polyol pathway, hexosamine pathway, PKCs signaling, oxidative stress, methylglyoxal modification, AGEs pathway, PARP pathway, MAPK pathway, NF-κB signaling, hedgehog pathways, TNF-α signaling, cyclooxygenase pathway, interleukins, lipoxygenase pathway, nerve growth factor, Wnt pathway, autophagy, and GSK3 signaling among others [46, 49]. This underscores the need for

additional studies to better understand the disease process and cellular changes that occur during diabetic neuropathy to allow for additional new targets and interventions.

To better understand changes that occur in prediabetic neuropathy, we created a clinical study to evaluate the **p**rediabetic **a**xonal **c**hanges in **m**etabolic syndrome **a**nd **n**europathy, abbreviated the "PACMAN" study. In this study we recruited and evaluated prediabetic patients with neuropathy, not due to another neurologic condition. We also evaluated prediabetic patients without symptoms of neuropathy as well as healthy patients to serve as negative controls. We were able to assess behavioral differences among these three groups as well as lab values that might demonstrate novel risk factors or associations with development of prediabetic neuropathy. Furthermore, through a skin biopsy we were able to assess intraepidermal nerve fiber density, and other histological changes present in prediabetics who develop neuropathy. This study will be discussed in greater detail in Chapter 5: Characterization of Peripheral Neuropathy in Prediabetic Patients: The PACMAN Study.

Chemotherapy Induced Peripheral Neuropathy (CIPN)

Among the many different forms of chemotherapy available, several belong to "neurotoxic" classes that frequently develop peripheral neuropathy. The most important of these include the vinca-alkaloids, platinating agents, and Taxanes [50]. Paclitaxel is a commonly used Taxane drug for the treatment of breast, ovarian, lung cancer, and Kaposi's sarcoma [51]. It exerts is antineoplastic effect through microtubule stabilization, preventing spindle apparatus dynamic changes necessary for mitosis from occurring [52]. The neurotoxic side effects of this medication occur frequently and are often dose-limiting, complicating the treatment of the underlying cancer. Approximately 30-40% of patients receiving neurotoxic chemotherapy will

develop CIPN, most commonly as a sensory peripheral neuropathy [53, 54]. Furthermore, many patients may develop chronic long-term neuropathy persisting after cancer treatment, that leads to increased morbidity with limited treatment options [55]. Symptoms have been reported up to 11 years following treatment with neurotoxic chemotherapy [56]. Treatment for CIPN largely consist of pharmacologic neuropathic pain management. Currently, duloxetine is the only treatment of CIPN recommended by the American Society of Clinical Oncology and has shown evidence of benefit in randomized controlled trials [57, 58]. However, many different pharmacologic treatments have been used for CIPN including venlafaxine, gabapentin, pregabalin, topical menthol, baclofen, amitriptyline, ketamine, capsaicin patch, cannabinoids, and some physicians still advocate for opioid analgesia [59-61].

Sensory Innervation of Pancreatic Islets

The pancreas is largely an exocrine organ with the majority of its tissue parenchyma consisting of pancreatic ducts and acini to aid in the secretion of pro-digestive enzymes. The endocrine component comprises approximately 1-2% of the total pancreas mass and is isolated in small islands of cells called islets of Langerhans [62]. Within the islets there are different subtypes of cells including: insulin producing beta cells, glucagon producing alpha cells, somatostatin producing delta cells, pancreatic polypeptide producing gamma cells, and ghrelin producing epsilon cells [63-65]. These islets are heavily innervated by sensory neurons as well as autonomic neurons [66, 67]. It is well established that insulin secretion from beta cells is influenced by efferent autonomic input, while the role that sensory neurons play on insulin homeostasis has largely been overlooked [68, 69]. The sensory neurons in the pancreas contain neuropeptides in their nerve terminals such as substance P and CGRP [70, 71]. While these

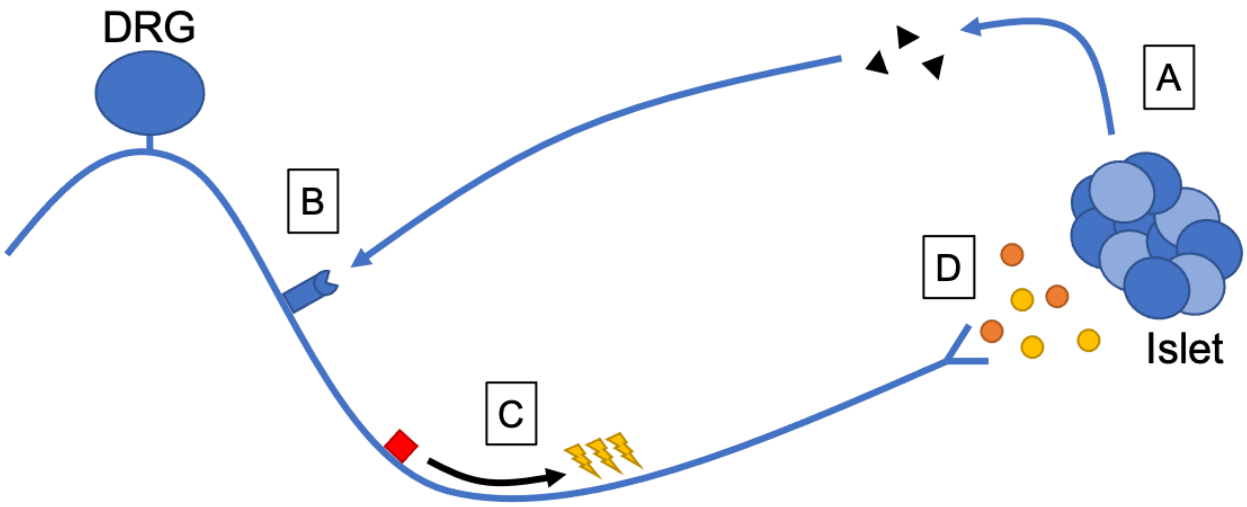
neuropeptides have been shown to play a role in inflammation and pancreatitis, they may also have a role on pancreatic islet physiology including beta cell function [72-77]. Recently it has been shown that neuropeptides might preserve beta cells in the pancreatic islets in type 1 and 2 diabetes [78].

There exists a theoretical neuroendocrine feedback loop where sensory neurons innervating the pancreatic islets express insulin receptors [79] (Fig 1). When the beta cells of the islets secret insulin it activates the sensory neurons in an antidromic fashion to secret neuropeptides such as CGRP and substance P to exert negative feedback onto the islets and help maintain tight insulin control [80]. This may occur via TRPV1 sensitization as TRPV1, insulin receptor, and neuropeptides have been shown to colocalize within the pancreatic sensory neuron [81, 82]. As a potential validation of this pathway, we have previously shown that a genotypic mouse line with a selective sensory neuron insulin receptor knockout (SNIRKO) leads to a hyperinsulinemic state after several weeks, otherwise these mice are phenotypically normal, without any gross developmental delays [79]. Chapter 3 addresses whether elimination of these peptidergic sensory neurons during neonatal development leads to alterations in glucose and insulin regulation in adulthood.

Figure 1 Neuroendocrine feedback loop between beta cells and sensory neurons

Diagram of the sensory neurons innervating the pancreatic islets. Beta cells release insulin (A), through a glucose stimulated intracellular mechanism, which binds to insulin receptors present on the sensory neuron's cell membrane (B). Through cell signaling processes, which may involve TRPV1 sensitization (C), neuropeptides including substance P and CGRP are secreted from the nerve terminals to act on beta cells decreasing further insulin secretion (D). Insulin represented by black triangles. TRPV1 receptor represented by red box. Action potential represented by lightning bolts. Substance P and CGRP represented by orange and yellow circles.

Figure 1



Ketogenic Diet as a Neuroprotective Intervention

Ketones, such as beta-hydroxybutyrate and acetoacetate, have been shown to be neuroprotective and promote neurite outgrowth [83-85]. In the embryological state ketones were the primary biochemical fuel source for developing neurons and as such might retain that physiological niche in the adult [86, 87]. Ketones are naturally occurring endogenous molecules, derived from acetyl-CoA, that arise as a metabolite of beta-oxidation of fatty acids, usually during states of starvation, extreme long-distance exercise, or simply the lack of carbohydrate availability [88-90].

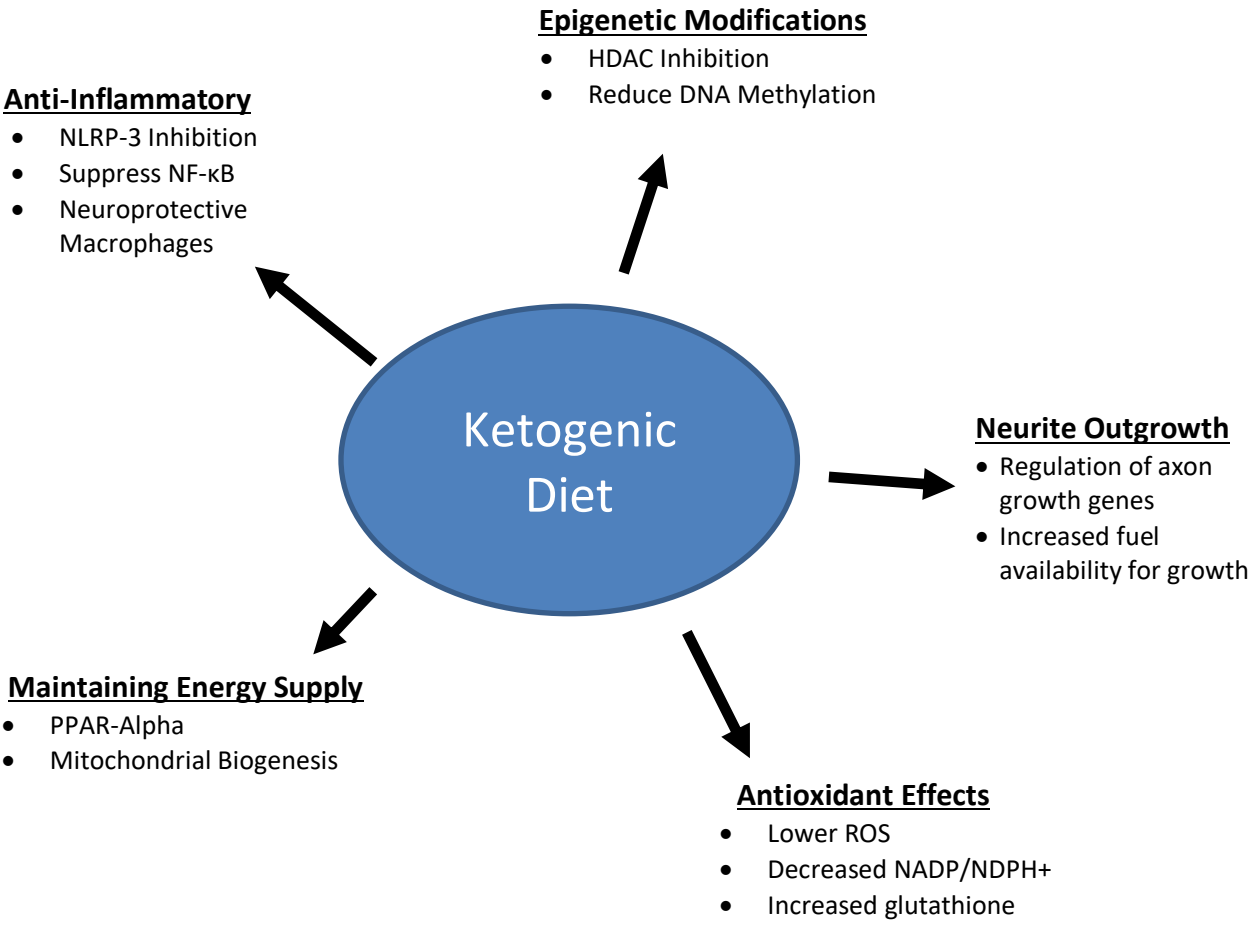
Ketones, in the contexts of diabetes, are usually associated with the severe metabolic condition of diabetic keto acidosis (DKA) [91]. However, at lower concentrations they have been shown to provide protection for diabetic peripheral neuropathy. We have previously shown in our lab that ketones prevented and even reversed intraepidermal nerve fiber changes seen in type 2 diabetic mice on a high fat diet [85]. We further showed in *in-vitro* experiments that ketones with or without glucose had significant neurite outgrowth compared to DRG cultures supplemented with glucose alone. A ketogenic diet has been implemented in several other neurodegenerative diseases as a possible therapeutic intervention. It is already a widely used diet that has gained recent popularity in society for its reputation in preventing obesity [92-95]. It may be possible that it could ameliorate the effects of other neuropathy etiologies such as chemotherapy-induced peripheral neuropathy. Although consensus has not been reached on the exact mechanism by which ketone bodies provide a neuroprotective effect, recent research has provided several possible theories [96]. First of all, several of the proposed mechanisms by which a ketogenic diet is neuroprotective are similar to those activated by caloric restriction [83, 97, 98]. Typical adherence to a ketogenic diet in patients is associated with caloric restriction as

part of the dietary protocol or simply by the unpleasant taste of several ketogenic foods [99, 100]. The ketogenic diet has been shown to reduce oxidative stress, help maintain a neural metabolic fuel source, reduce inflammatory signaling pathways and cause epigenetic changes, among other mechanisms (Fig 2). The ketogenic diet has been shown to lower reactive oxygen species (ROS) in isolated mitochondria via activation of mitochondrial uncoupling proteins [101]. It has further been that beta-hydroxybutyrate reduces NADP+/NADPH ratio, increases glutathione levels, and decreases the concentration of semiquinone [102]. A ketogenic diet can improve metabolic efficiency of the cell. This has been shown through increased ATP concentrations as well as mitochondrial biogenesis [103, 104]. Ketone bodies have been shown to bind HCA2 expressed on immune cells such as microglia, dendritic cells, and macrophages, and its subsequent activation helps induced a neuroprotective subset of macrophages [105]. Ketones have also been shown to suppress NF-KB signaling [106] and the NLRP-3 inflammasome [107-109]. Ketone bodies also function as inhibitors of histone deacetylase, which has been shown to play a role in neuropathic pain states [110] [111]. The ketogenic diet has also shown to decrease DNA methylation through adenosine signaling [112, 113]. We have shown that a ketogenic diet promotes neurite outgrowth; however, the mechanism of increased nerve growth remains unclear [85].

Figure 2 Neuroprotective Mechanisms of Ketone Bodies

Summary of various mechanisms and pathways by which a ketogenic diet (ketone bodies) may ameliorate the effects of peripheral neuropathy such as in diabetic peripheral neuropath or chemotherapy-induced peripheral neuropathy.

Figure 2


Epigenetic Modifications
HDAC Inhibition
Reduce DNA Methylation
Anti-Inflammatory
NLRP-3 Inhibition
Suppress NF-κB
Neuroprotective Macrophages
Ketogenic Diet
Neurite Outgrowth
Regulation of axon growth genes
Increased fuel availability for growth
Maintaining Energy Supply
PPAR-Alpha
Mitochondrial Biogenesis
Antioxidant Effects
Lower ROS
Decreased NADP/NDPH+
Increased glutathione

Conclusion

Sensory neurons play a key role in providing the central nervous system with information regarding our surroundings. In the case of small nociceptive c-fibers, they relay pain information that helps protect the human body from injury due to mechanical forces, temperature, or chemical irritants. When pathology arises in the sensory neurons signal transduction of this crucial information is impaired or sometimes amplified causing unnecessary and even chronic pain states. The following chapters discuss experiments focusing on neuropathic damage secondary to chemotherapy as well as prediabetes or impaired glucose tolerance. Although these two forms of neuropathy have different etiologies and disease progression, both diabetes and CIPN reduce the small pain fibers of the epidermis in humans and mice animal models and may have more overlap than previously acknowledged [114-118].

Chapter 2: Paclitaxel-Induced Neuropathy Impacts Metabolism and Glucose Tolerance in Mice

1. Abstract

Paclitaxel chemotherapy is known to cause a treatment-limiting peripheral neuropathy primarily affecting the lower extremities, however less well understood are its effects on visceral sensory and autonomic afferents including those that innervate the pancreas. Pathology affecting pancreatic sensory neurons has been shown to deregulate insulin secretion leading to increased susceptibility to hyperglycemia. C57BL/6 male and female mice were exposed to intraperitoneal paclitaxel injections and monitored for metabolic changes over several weeks. A 12 mg/kg dose of paclitaxel was found to be sufficient to induce lasting mechanical allodynia and resulted in a significant decrease in pancreatic islet innervation of PGP 9.5- and tyrosine hydroxylase-positive axons. Paclitaxel exposure alone did not cause robust changes in fasting glucose or serum insulin levels. However, a cohort of paclitaxel treated mice was subsequently switched to a high fat diet, which caused increased weight gain and pronounced insulin instability. Our data validates the importance of pancreatic afferents in insulin homeostasis and demonstrates their susceptibility to chemotherapeutic insult.

2. Introduction

Paclitaxel, originally isolated from the bark of the Pacific yew *Taxus brevifolia,* is a commonly used chemotherapeutic agent in the treatment of various forms of cancer including breast, lung, and ovarian [119, 120]. A member of the taxane family, it is known to be a class of neurotoxic chemotherapy that presents with a distal sensory peripheral neuropathy (DSPN) that can become treatment limiting [54]. In fact, sensory neuropathy is one of the most commonly reported side effects and limits treatment when administered in high doses or when given in combination with other neurotoxic chemotherapeutic agents such as cisplatin.

Paclitaxel exerts its antineoplastic effects through stabilization of microtubules preventing formation of a normal mitotic apparatus, and subsequent induction of apoptosis in dividing cells [121, 122]. Its novel anti-mitotic effects allow for treatment of cancers that have developed resistance to another first-line chemotherapy. However, these intrinsic microtubule-stabilizing effects also impair axonal transport, which contributes to its neurotoxicity [123-125]. Chronic paclitaxel administration can lead to axonal degeneration and has been evidenced in sural nerve biopsies in human patients [126]. *In vitro* experiments have shown that paclitaxel-mediated axonal degeneration may adversely affect other cell processes, such as calcium-activated proteases calpains and mitochondrial dysfunction [127, 128].

Multiple clinical studies have reported development of a sensory peripheral neuropathy following paclitaxel treatment in patients [129-131]. Symptoms include numbness, tingling, other paresthesias, as well as burning pain in the distal extremities [132]. These symptoms appear one to three days following paclitaxel infusion and resolve in mild cases; however, they can progress into a chronic, debilitating neuropathy with few effective treatment options for relief. Paclitaxel has also been studied in laboratory animal models and causes a peripheral neuropathy resulting in

mechanical allodynia in multiple strains of inbred rats and mice, including C57BL/6 mice [133-139].

Elevated glycemic levels have been reported following paclitaxel treatment in rat models, as well as in patient populations [140-143]. Although it is not clear how paclitaxel affects glycemic status, we have explored the possibility of sensory neuron involvement in glycemic regulation through negative feedback on insulin-producing beta cells. Insulin receptors are expressed on sensory neurons and mice lacking sensory neuron insulin receptors display elevated insulin and impaired glucose tolerance [79]. Sensory neurons have been shown to modify beta cell insulin production and release through neuropeptide signaling [70, 79, 144, 145]. Transient receptor potential vanilloid 1 (TRPV1), expressed on sensory nerve endings, has also been implicated in regulating this negative feedback loop [146-148].

Here, we tested whether paclitaxel injection alters glycemic status in mice via damage to sensory neurons innervating pancreatic islets. We also determined the additive impact of high fat diet feeding after paclitaxel administration. These findings provide important preclinical observations that further our understanding of the long-term consequences of chemotherapy-induced neuropathy beyond sensory dysfunction and pain.

3. Methods

Animals: Eight-week-old C57BL/6 male and female mice were purchased from Charles River Laboratories (Wilmington, MA) and were used for all experiments, except the high fat diet treatment, which was only performed in male mice. While behavioral and hormonal changes occur during the different stages of the female estrous cycle, it was not tracked during these experiments. The mice were housed on 12:12 hour light/dark cycle in the research support facility at the

University of Kansas Medical Center. All mice were given ad libitum access to food and water. All animal use was in accordance with National Institutes of Health guidelines and conformed to the principles specified in a protocol approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Experimental design: Adult mice were injected with paclitaxel, once daily, for three days, then observed over the next four weeks. Weekly measurements included weight, fasting blood glucose, fasting insulin, and Von Frey filament testing. The animals were maintained on a standard chow diet during the course of the study. In a follow up experimental group, half of the mice were changed from standard chow diet to a high fat diet at four weeks post paclitaxel treatment and monitored for an additional eight weeks.

Diet: All mice were initially placed on standard chow diet (8604; Envigo, Madison, WI; 14% kcals from fat, 32% protein, and 54% carbohydrate). A cohort of mice was switched from standard chow diet to a high fat diet (07011; Envigo; 54% kcals from vegetable shortening (hydrogenated) and corn oil fat, 21% protein and 24% carbohydrate) four weeks after paclitaxel injections.

Weight and Body Composition: Mice were weighed weekly on digital laboratory scale. Body composition analysis to assess fat mass and lean mass was measured by magnetic resonance imaging using the EchoMRI-100 (EchoMRI, Houston, TX).

Paclitaxel Injections: For development and characterization of a mouse model of chemotherapy induced peripheral neuropathy, mice were dosed with 5 mg/kg or 12 mg/kg paclitaxel via intraperitoneal (IP) injection daily for three days. For all subsequent experiments 12 mg/kg paclitaxel dosing was used. Control animals received a saline-based sham injection via IP injection daily for three days. Paclitaxel (Alvogen, Pine Brook, NJ, NDC # 47781-593-07) is formulated in a cremaphor/ethanol solution to maintain drug solubility. Cremaphor is used frequently as a

formulation vehicle for poorly water soluble drugs, however, has shown capacity for biologic effects including peripheral neuropathy [149]. Therefore, to control for confounding effects, the saline sham injections were formulated with comparable cremaphor and ethanol volumes,10% (V/V) cremaphor and 10% (V/V) ethanol.

Glucose & Insulin Measurements:

Fasting Glucose: Animals were fasted for 3 hours prior to glucose evaluation. Blood was collected via tail nick and glucose levels were measured by a colorimetric glucose diagnostic assay (Sigma, St. Louis, MO).

Fasting Insulin: Animals were fasted for 3 hours prior to insulin evaluation. Blood was collected via tail nick, allowed to clot on ice for 30 minutes, spun at 3000g for 30 minutes at 4°C, and the serum supernatant was collected. The serum was stored at -80°C until analysis could be completed via Mouse Ultrasensitive ELISA (ALPCO, Salem, NH).

Intraperitoneal Glucose Tolerance Test: Animals were fasted for 6 hours prior to IP injection of glucose, dosed at 1g of glucose per kg body weight. Blood glucose levels were assayed at 0, 15-, 30-, 60-, and 120-minutes post injection. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated by the formula: HOMA-IR = (Fasting Glucose (mg/dL) * Fasting Insulin (μU/mL))/405.

Sensory Behavioral Testing: Sensory testing to quantitate paw mechanical sensitivity was performed using Von Frey monofilaments. Animals were acclimated for 30 minutes twice, prior to behavioral testing in a sound isolation chamber with ambient white noise. During acclimation and sensory testing, mice were placed in individual clear plastic chambers (11x5x3.5cm) on a wire mesh screen elevated 55cm above a table. Mechanical sensitivity was determined by the presence of a hind paw withdrawal to Von Frey monofilaments of varying sizes: 1.65 (0.0045g), 2.36

(0.02g), 2.83 (0.068g), 3.22 (0.158g), 3.61 (0.178g), 4.08 (1.20g), 4.31 (2.041g) and 4.74 (5.495g). Calculation of the 50% withdrawal threshold was performed utilizing the formula from the up-down method as previously described [150].

Tissue Dissection: Mice were euthanized by exsanguination under isoflurane anesthesia. Following euthanasia, whole pancreases were removed and fixed in 4% paraformaldehyde. After twenty-four hours, pancreases were rinsed in 1xPBS and subsequently cryoprotected in 30% sucrose solution. Then they were frozen at -20°C and sectioned at 30 μm on a LEICA CM 1950 cryostat. Non-serial sections were mounted onto Superplus microscope slides (Fisher) and stored at -20°C until use.

Immunohistochemistry: Pancreas sections were incubated for 1 hour with blocking solution containing Superblock™ (450 μl per slide, Thermo Scientific, Cat # 37515, Lot # TG269957), 1.5% normal donkey serum, 0.5% porcine gelatin, and 0.5% Triton X-100. This was followed by incubation with primary antibodies, including guinea pig anti-Insulin 1:400 (Abcam, Cat # ab7842, Lot # GR3230256-1), rabbit anti-PGP 9.5 1:1500 (Bio-Rad, Cat # 7863-0504), sheep anti-tyrosine hydroxylase (TH) 1:1000 (Abcam, Cat # ab113). The sections were then washed with 1xPBS and incubated with secondary antibodies: donkey anti-guinea pig 488 1:200 (Jackson ImmunoResearch Labs, Cat # 706-485-148, Lot # 86723), donkey anti-rabbit 555 1:1000 (Molecular Probes, Cat # A-21429, Lot # 65E1-1), and donkey anti-sheep 555 1:1000 (Jackson ImmunoResearch Labs, Cat # 713-026-147, Lot # 28402) respectively. Sections were rinsed and cover slipped. A sterile PBS solution was used as mounting media. 8-16 sections and 86-114 islets were analyzed per mouse. Fluorescent images of pancreatic tissue, at 20x magnification, were collected using a Nikon Eclipse 90i microscope and QIClick digital CCD Camera (QImaging, Surrey, BC, Canada). Nikon Elements software was used for image analysis and automated

measurement of islet area and nerve area was determined using signal thresholding. In the TH stained tissue, the pancreatic islet cross sectional area was identified by a signal intensity of insulin staining fluorescence of 1930-16383 and nerve area was identified by TH fluorescence intensity of 1146-16383. In the PGP-9.5 stained tissue, insulin fluorescent intensity was set at 2015-16383 and PGP-9.5 fluorescent intensity was set at 1146-16383. Axonal area within the islet was divided by islet area and reported as fractional area.

Statistical Analysis: All data are presented as mean ± standard error of the mean (SEM). Data were analyzed using multiple t tests, two-way ANOVA with repeated measures and Tukey multiple comparisons test. Statistical significance was defined as $P < 0.05$ and statistics were performed using GraphPad Prism version 7.00 for macOS, GraphPad Software, La Jolla California USA as well as IBM SPSS Statistics version 27, IBM Corporation, Armonk New York USA.

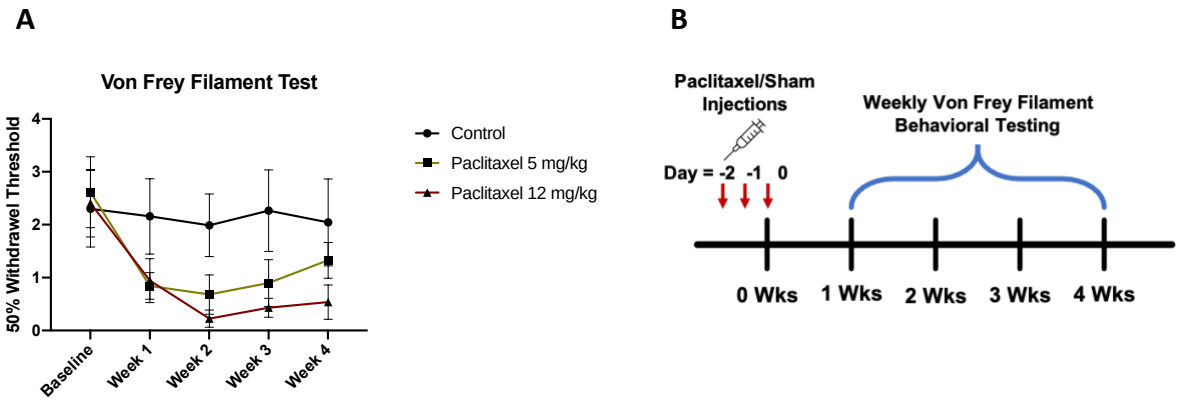
4. Results and Figures

Paclitaxel Produces Mechanical Allodynia in C57BL/6 Mice: Intraperitoneal injections of paclitaxel dosed at 5 mg/kg and 12 mg/kg caused a subsequent decreased withdrawal threshold during Von Frey filament testing over the four subsequent weeks compared to vehicle control (Fig 3A). The 12 mg/kg dosage showed greater changes in withdrawal threshold and remained at a decreased threshold throughout the 4 weeks, although neither the 5 mg/kg or 12 mg/kg dosage achieved statistical significance compared to the vehicle control group. ($p = 0.2355$, $p = 0.0970$, respectively, Fig 3B).

Figure 3 Paclitaxel causes a decrease in hind paw withdrawal threshold

Von Frey Filament testing measuring mechanical sensitivity in 8-week-old male C57BL/6 mice, via application to right hind paw. Experimental timeline. (A) Hind paw withdrawal threshold calculated over four weeks following paclitaxel exposure of 5 mg/kg and 12 mg/kg. (B) Data are means ± SEM. Repeated measures two-way ANOVA. Bonferroni post-hoc test. Control n = 4, Paclitaxel 5 mg/kg n = 5, Paclitaxel 12 mg/kg n = 5)

Figure 3

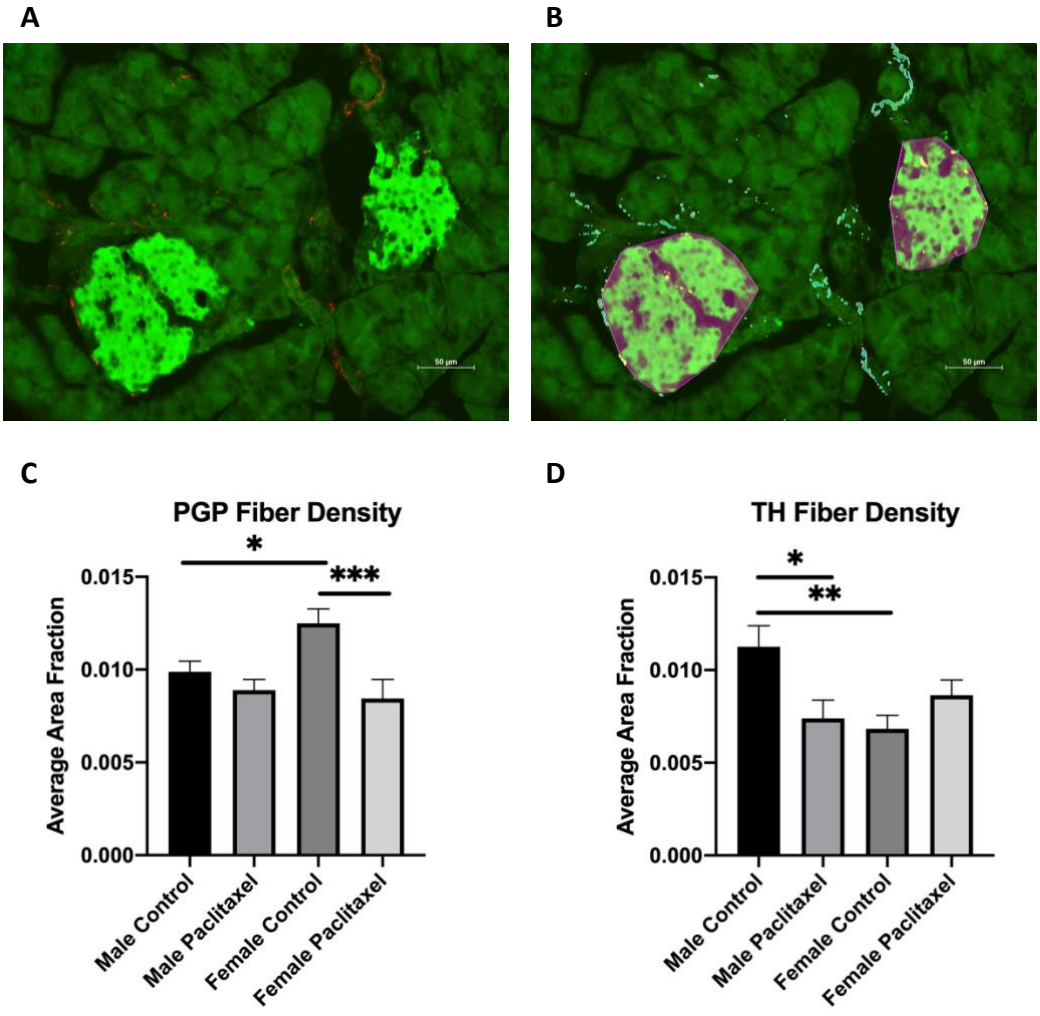

A
Von Frey Filament Test
50% Withdrawel Threshold
0
1
2
3
4
Baseline
Week 1
Week 2
Week 3
Week 4
Control
Paclitaxel 5 mg/kg
Paclitaxel 12 mg/kg
B
Paclitaxel/Sham Injections
Day = -2 -1 0
Weekly Von Frey Filament Behavioral Testing
0 Wks
1 Wks
2 Wks
3 Wks
4 Wks

Paclitaxel Decreases Sensory Fiber Innervation of Pancreatic Islets: Immunohistochemical analysis of pancreatic tissue revealed that paclitaxel exposure decreases total islet innervation in a sex dependent manner within two weeks. Female mice exposed to paclitaxel showed a significant decrease in PGP-9.5 positive fibers within pancreatic islets ($p = 0.0009$, Fig 4C) compared to control female mice. Male mice exposed to paclitaxel did not display a significant reduction in PGP9.5-positive fibers. A sex difference was also observed in the control animals alone that male mice display significantly less innervation of pancreatic islets than female mice ($p = 0.0495$). Upon analysis of autonomic innervation, determined by tyrosine hydroxylase (TH) positive staining fibers, male mice exposed to paclitaxel were found to have a significant reduction of fibers compared to control male mice ($p = 0.0175$, Fig 4D). Female mice exposed to paclitaxel did not display a significant reduction of TH positive fibers. In the control animals, female mice had significantly less TH positive fibers than male mice ($p = 0.0035$).

Figure 4 Paclitaxel treatment decreases pancreatic islet innervation

Fluorescent microscopy displaying mouse pancreatic islet in green and PGP-9.5 positive nerve fibers in red (A). Same image with superimposed islet area in pink and nerve fiber area in light blue, determined by automation criteria based on signal intensity thresholding (B). Quantification of islet innervation by PGP-9.5 positive nerve fibers (C). Quantification of islet innervation by Tyrosine Hydroxylase positive nerve fibers (D). Fiber density reported in average area fraction as ratio of nerve fiber area overlapping with islet area, divided by islet area. Data are means ± SEM. *****$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, two-way ANOVA, Tukey post-hoc test. PGP Fiber Density: Male Control n = 6 (272 islets), Male Paclitaxel n = 6 (274 islets), Female Control n = 6 (316 islets), Female Paclitaxel n = 6 (228 islets). TH Fiber Density: Male Control n = 6 (296 islets), Male Paclitaxel n = 6 (311 islets), Female Control n = 6 (334

Figure 4


A
B
C
PGP Fiber Density
Average Area Fraction
0.015
0.010
0.005
0.000
*
***
Male Control
Male Paclitaxel
Female Control
Female Paclitaxel
D
TH Fiber Density
Average Area Fraction
0.015
0.010
0.005
0.000
*
**
Male Control
Male Paclitaxel
Female Control
Female Paclitaxel

Paclitaxel Treatment Alone Does Not Dramatically Alter Glucose and Insulin Levels: Mice were weighed prior to and on a weekly basis following paclitaxel treatment. Paclitaxel treatment was not found to have a significant effect on body weight in male or female mice ($p = 0.3093$, $p = 0.5508$, respectively, Fig 5A-B). Likewise, EchoMRI measurements revealed no significant effect of paclitaxel on lean or fat mass in male mice at four weeks ($p = 0.3423$, $p = 0.1790$, respectively, data not shown). Bi-weekly fasting blood glucose and insulin levels were analyzed following paclitaxel treatment. No significant differences in fasting blood glucose levels were observed in male or female mice following paclitaxel treatment ($p = 0.0698$, $p = 0.8641$, respectively, Fig 5C-D). No differences in fasting insulin levels were observed between male paclitaxel-treated mice and control ($p = 0.7662$, Fig 5E), while female mice had significantly elevated serum insulin levels following paclitaxel treatment ($p = 0.0063$, Fig 5F). It is important to note that the changes in observed glucose and insulin in the paclitaxel-treated animals remained fairly well regulated with levels between 85 and 150 mg/dL and between 0.1 and 0.6 ng/mL, respectively.

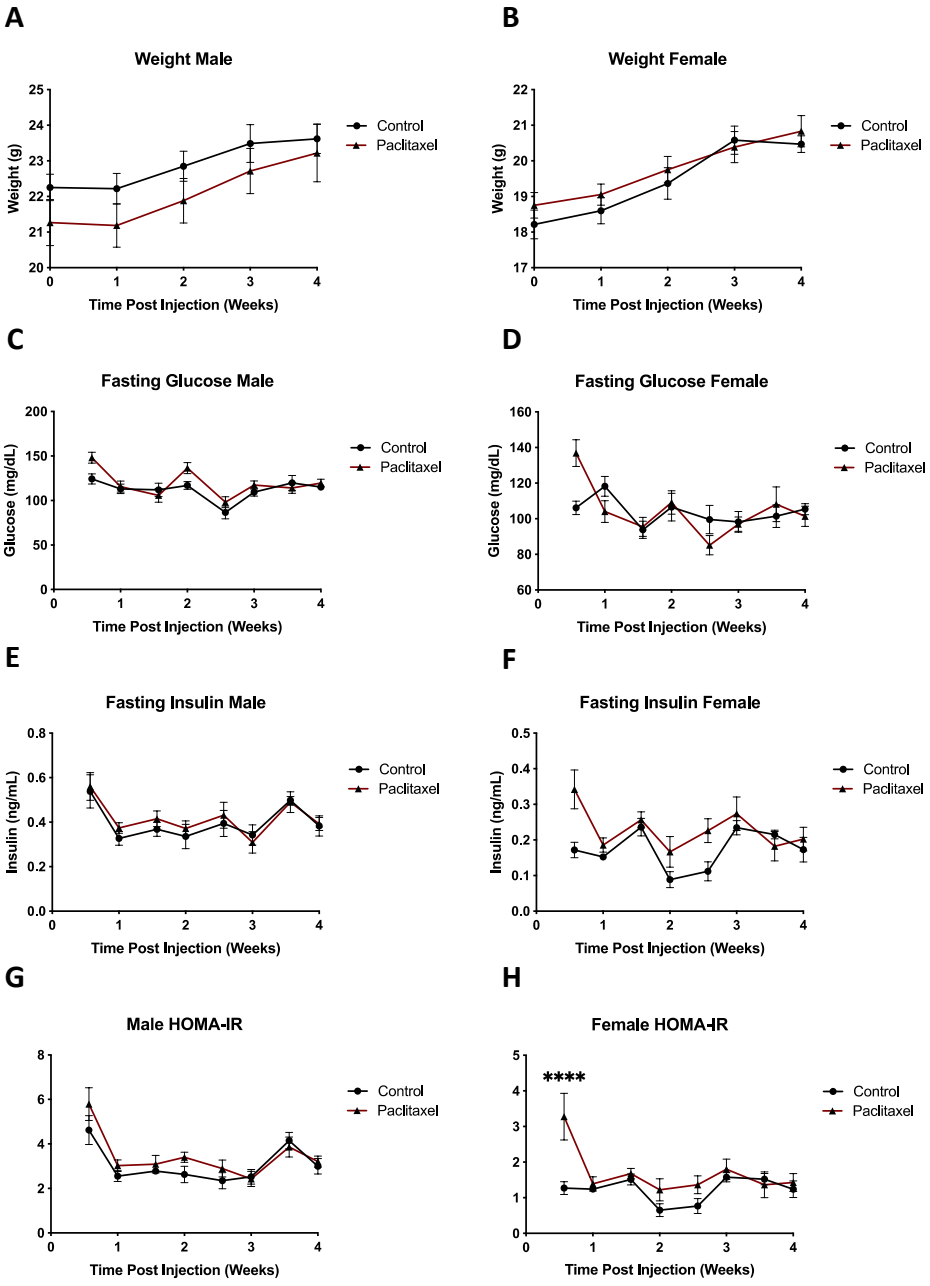
Homeostatic model assessment for insulin resistance (HOMA-IR) is a useful measure to quantify insulin resistance and beta-cell function that was first described in 1985 [151]. Its approximation, derived from fasting glucose and insulin levels, was found not to be significantly different in paclitaxel treated male mice from control animals ($p = 0.2033$, Fig 5G). In female mice however, paclitaxel treated animals displayed a significant increase in HOMA-IR ($p = 0.0010$), with a significant elevation is observed at the day four time point ($p < 0.0001$, Fig 5H). At the conclusion of the four-week observation, an intraperitoneal glucose tolerance test was performed in male mice and paclitaxel treatment resulted in a trend towards increased blood glucose, especially at the 15 minute timepoint, although not significant ($p = 0.1448$, data not shown). This

would reflect a diminution of the first phase insulin response, which is often associated with development of Type 2 Diabetes Mellitus [152, 153].

Figure 5 Paclitaxel treatment caused mild elevations in blood glucose and insulin levels

Body weight was measured weekly in male (A) and female (B) mice that were treated with paclitaxel or control solution. Biweekly fasting glucose measurements were conducted in male (C) and female mice (D). Biweekly fasting serum insulin levels were also measured in male mice (E) and female mice (F). HOMA-IR, a measure of insulin resistance, derived from fasting glucose and fasting insulin values in male mice (G) and female mice (H). Data are means ± SEM. ****$P < 0.0001$. Repeated measures two-way ANOVA. Bonferroni post-hoc test. A-B, D, F, H: n=6, both groups; C, E, G: n=15, both groups.

Figure 5


A
Weight Male
Weight (g)
Time Post Injection (Weeks)
Control
Paclitaxel
B
Weight Female
Weight (g)
Time Post Injection (Weeks)
Control
Paclitaxel
C
Fasting Glucose Male
Glucose (mg/dL)
Time Post Injection (Weeks)
Control
Paclitaxel
D
Fasting Glucose Female
Glucose (mg/dL)
Time Post Injection (Weeks)
Control
Paclitaxel
E
Fasting Insulin Male
Insulin (ng/mL)
Time Post Injection (Weeks)
Control
Paclitaxel
F
Fasting Insulin Female
Insulin (ng/mL)
Time Post Injection (Weeks)
Control
Paclitaxel
G
Male HOMA-IR
Time Post Injection (Weeks)
Control
Paclitaxel
H
Female HOMA-IR
****
Time Post Injection (Weeks)
Control
Paclitaxel

Addition of a High Fat Diet Increases Weight Gain and Increases Variability in Insulin Levels: To "challenge" the glycemic control of the paclitaxel-treated mice and to better reflect the pro-metabolic syndrome diet of patients [154, 155], a cohort of C57BL/6 male mice were placed on a high fat diet four weeks after paclitaxel or control treatment. Both paclitaxel treatment and diet were found to have a significant effect on weight gain ($p = 0.0125$, $p < 0.0001$, respectively, Fig 6A). Paclitaxel-treated mice fed a high fat diet gained significantly more weight than either control mice on a high fat diet (week 5, $p = 0.0190$) or paclitaxel-treated mice that remained on a chow diet (week 5-12, $p = 0.0422$, 0.0287, 0.0074, 0.0058, 0.0164, 0.0059, 0.0040, 0.0005 respectively). Control mice on a high fat diet also gained significantly more weight than control mice on chow diet (week 8, 10-12, $p = 0.0284$, 0.0314, 0.0079, 0.0062 respectively).

Both paclitaxel treatment and diet were found to have a significant effect on blood glucose levels ($p = 0.0405$, $p < 0.0001$, respectively, Fig 6B). The paclitaxel-treated mice on a high fat diet also showed significantly elevated blood glucose levels compared to the paclitaxel-treated mice on chow diet (week 6, 8, 9, $p = 0.0344$, 0.0431, 0.0148, respectively, Fig 6B). Control mice on a high fat diet also displayed significantly increased blood glucose levels compared to control mice on chow diet (week 9, 10, 12, $p = 0.0371$, 0.0153, 0.0170 respectively). However, there was no significant difference in blood glucose measurements between control- and paclitaxel-treated mice on the high fat diet.

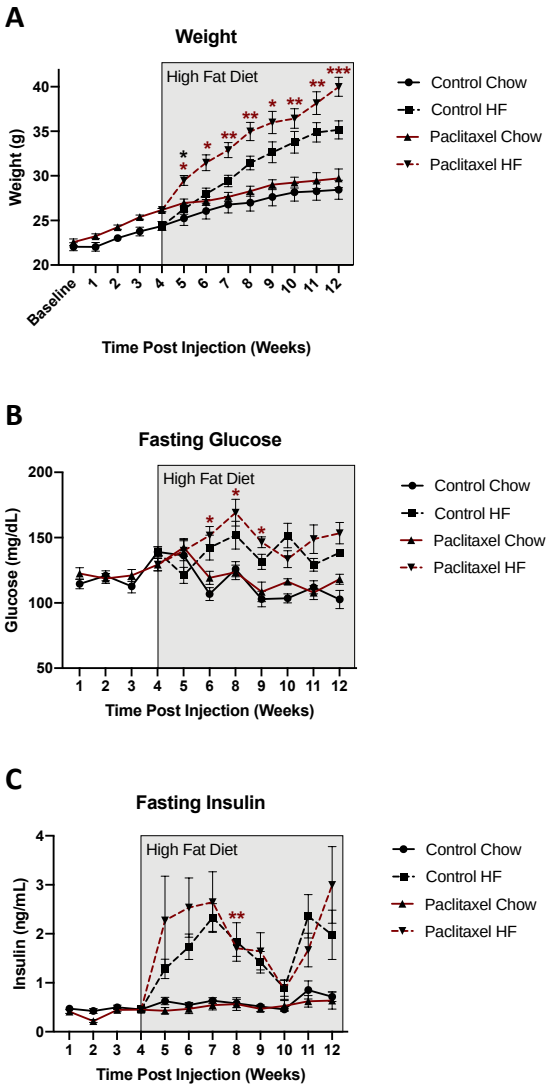
Diet was found to have a significant effect on insulin levels ($p < 0.0001$) while the effect of paclitaxel treatment did not reach significance ($p = 0.5186$, Fig 6C). High fat diet feeding induced hyperinsulinemia in both control- and paclitaxel-treated mice, relative to their chow-fed counterparts, during weeks six through eight (Control Chow vs Control HF: week 6 $p = 0.0330$, week 7 $p = 0.0093$; Paclitaxel 12 mg/kg Chow vs Paclitaxel 12 mg/kg HF: 8 $p = 0.0041$; Fig 6C).

It is important to mention that while the paclitaxel treated mice on a high fat diet did not show significant difference from the control mice on high fat diet, the individual variability of insulin levels within these two groups has a striking difference. In evaluating the standard error of mean of insulin levels, the paclitaxel treated mice on high fat diet have rapid onset of high levels of insulin variation following onset of the diet, while the control animals on high fat diet steadily become more variable following onset of the diet through week eight. Compared to these groups, the paclitaxel chow and control chow remained much more tightly controlled with less variation.

Figure 6 Paclitaxel treatment with a high fat diet caused increased weight gain and insulin variability in male mice

Weekly weight measurements before and after addition of high fat diet depicted by shaded box (A). Weekly fasting glucose measurements before and after addition of high fat diet (B). Weekly fasting insulin measurements of male mice before and after addition of high fat diet (C). Data (A-C) are means ± SEM. ***P* < 0.05, ****P* < 0.01, *****P < 0.001. Repeated measures three-way ANOVA. Bonferroni post-hoc test. A-C: n=5-6.

Figure 6


A
Weight
High Fat Diet
Weight (g)
Time Post Injection (Weeks)
Baseline
Control Chow
Control HF
Paclitaxel Chow
Paclitaxel HF
B
Fasting Glucose
High Fat Diet
Glucose (mg/dL)
Time Post Injection (Weeks)
Control Chow
Control HF
Paclitaxel Chow
Paclitaxel HF
C
Fasting Insulin
High Fat Diet
Insulin (ng/mL)
Time Post Injection (Weeks)
Control Chow
Control HF
Paclitaxel Chow
Paclitaxel HF

5. Discussion

In this study, 12 mg/kg paclitaxel treatment, administered once daily over three consecutive days, was sufficient to produce hindpaw mechanical allodynia in C57BL/6 mice for at least four weeks. Paclitaxel caused a reduction in sensory innervation of pancreatic islets in female mice and a decrease in tyrosine hydroxylase positive neurons with within the islets in male mice. Paclitaxel treatment alone did not cause robust changes in glucose levels or insulin homeostasis in mice fed a standard chow diet. However, the small changes observed do suggest a hyperglycemic effect of paclitaxel in male mice and a hyperglycemic and hyperinsulinemic effect of paclitaxel in female mice. Introducing a high fat diet four weeks after Paclitaxel treatment resulted in significantly increased weight gain, fasting blood glucose levels, and hyperinsulinemia with increased variability within the group.

The paclitaxel treatment changes in mechanical sensitivity observed through Von Frey filament testing have been previously reported in the literature [156-160]. Whereas in this study the metabolic effects on glycemic control due to paclitaxel appear minimal and acutely reversible, it may be possible that the neurotoxic effects require a longer timeframe to manifest with overt glycemic increases. Paclitaxel is typically given to patients IV over three hours every three weeks for months to years [161]. This increase exposure time would likely increase risk for neuropathic symptoms, compared to the three-injection designed mouse model. It is also important to realize that many patients receiving chemotherapy may have other comorbidities predisposing them to development of type 2 diabetes such as sedentary lifestyle and genetics. There exists consistent evident that increased body fat is associated with increased risk of several forms of cancer as well as diabetes [162-164].

It was shown in the sensory nerve insulin receptor knockout mouse (SNIRKO) model that serum insulin levels showed increase variability prior to significant hyperinsulinemia compared to control mice [79]. Since paclitaxel treated animals fed high fat diet also showed increased insulin variability relative to controls, it is possible that the same neuroendocrine feedback loop is being affected, and that eventually those animals would display hyperinsulinemia as well if the study was continued longitudinally. Consistent with this idea, the SNIRKO mice only began to display elevations in insulin at 29 weeks of age.

It is important to address that in the immunofluorescence analysis of pancreatic islets that the female controls displayed a significant increase in average area fraction of PGP-9.5 staining of the islet compared to male controls. The male controls however, showed a significant increase in the average area fraction of TH staining of the islets compared to the female controls. This is the first time this sexual difference has been shown to our knowledge.

A clinically relevant possibility exists that if the decrease in fiber innervation of pancreatic islets can be correlated with fiber innervation the epidermis of the lower extremities following treatment with neurotoxic chemotherapy such as paclitaxel, then it may be possible to use a peripheral skin biopsy as a biomarker of pancreatic innervation. Lower leg skin biopsies are already a commonly used, reliable procedure to quantify peripheral neuropathy [165, 166]. This correlation is very testable in animal models and would provide some insight into commonalities in peripheral and visceral degeneration in peripheral neuropathy.

The high fat diet was administered to serve as a metabolic challenge to the mice. Indeed, high fat diet is a frequently used mouse model to study the metabolic disorders of human obesity including impaired glucose tolerance and type 2 diabetes [154, 155, 167]. It was hypothesized that paclitaxel's neurotoxic effect on pancreatic innervation may not be severe enough to elicit

metabolic changes on a normal chow diet. However, when the neuroendocrine system was additionally stressed by the consumption of a high fat diet it is possible that paclitaxel treatment could push the insulin homeostasis over the edge. Furthermore, many cancer patients receiving chemotherapy may not change their diet during and/or after treatment and a high fat diet in mice may better reflect the high fat "western" diet.

Patients treated with paclitaxel often complain of tingling, burning, or painful sensations in their distal limbs, which has prompted the vast majority of preclinical studies on paclitaxel-induced sensory neuropathy to be focused almost exclusively on innervation changes in the distal extremities [131, 168]. Although the pancreas is highly innervated by putative nociceptive neurons, it is unclear whether diminished beta cell innervation would evoke pancreatic pain [169, 170]. Most analyses of disease-related changes in the sensory innervation of the pancreas, including that of the pancreatic islets, has been performed in biopsies from patients with chronic pancreatitis and pancreatic cancer, which show increased innervation that correlates with reported pain scores [171-176]. Limited evidence of hyperglycemia following paclitaxel treatment suggests that chemotherapeutic neuropathy may impact the pancreas. Although there currently exists a relative paucity of clinical literature on the diabetogenic effects of paclitaxel chemotherapy [141], this may be an oversight due to the high incidence of diabetes in the general population, especially at elevated ages, when cancer occurs at a higher incidence. For example, breast cancer, which is commonly treated with paclitaxel, has a peak incidence in the 6^{th} decade of life [177, 178]. To further validate the incidence of post-paclitaxel hyperglycemia, it may be necessary to conduct additional retrospective cohort studies of patient populations receiving paclitaxel treatment and evaluate their glycemic status post-treatment.

In conclusion, this study shows that paclitaxel causes pathologic changes to pancreatic sensory innervation in addition to causing a peripheral neuropathy manifesting behavioral changes such as allodynia. Importantly, this work suggests that paclitaxel might have the potential to deregulate physiologic neuroendocrine feedback loops intrinsic to the pancreatic innervation affecting glycemic control.

Chapter 3: Elimination of Capsaicin-Responsive Sensory Neurons During Neonatal Development Affects Metabolic Parameters in Adulthood

1. Abstract

Mice pups treated with neonatal capsaicin injections develop systemic loss of TRPV1-positive sensory neurons. Through this animal model, we evaluated paclitaxel's effect on C57BL/6 mice. 2-day old mice were given subcutaneous capsaicin injections and allowed to mature into adult animals. Mice were subsequently given paclitaxel or sham injections and monitored for the following weeks. Neonatal capsaicin treatment delayed the development of mechanical allodynia related to paclitaxel treatment during behavioral testing of paw mechanical sensitivity. Neonatal capsaicin treatment in male mice led to robust increases in weight, glucose and insulin levels, irrespective of paclitaxel treatment status. Female animals showed more moderate trends of increase glucose and insulin levels following neonatal capsaicin treatment. While neonatal capsaicin provides some protection from paclitaxel-induced peripheral neuropathy, it appears to have had no effect on metabolic changes caused by paclitaxel treatment. However, neonatal capsaicin appears to exert an independent effect on metabolic parameters in C56BL/6 mice, which were precipitated by paclitaxel or sham vehicle control injections.

2. Introduction

Capsaicin is the pungent ingredient in chile peppers and is the major agonist of the transient receptor potential cation channel subfamily V member 1 (TRPV1) [179, 180]. When given parenterally, neonatal capsaicin exposure has long been shown as a reliable method to remove TRPV1 sensory afferents systemically via apoptosis [181-183]. It is generally well tolerated; however, a few physiological changes have been observed including increased circadian body temperature [184, 185]. In adult animals, capsaicin treatment provides a milder more transient effect on TRPV1 neurons through increased signaling, followed by a period of decreased sensitivity [186-188]. Based upon this finding, the pharmaceutical industry has developed a topical 8% capsaicin patch to provide local analgesia for up to 12 weeks [189, 190].

The neonatal capsaicin treatment has been verified to effectively reduce sensory neurons innervating the pancreas in several studies [191-194]. In healthy C57BL/6 mice, capsaicin treatment has previously been shown to lead to a significant decrease in blood glucose response to a glucose tolerance test [194]. It has also been shown to increase insulin sensitivity in adult rats [195]. However, neonatal capsaicin exposure has never been evaluated in conjunction with paclitaxel treatment. We decided to employ this technique in our paclitaxel CIPN mouse model to evaluate whether it would alter paclitaxel's effects. Our hypothesis is that by removing TRPV1-positive neurons innervating the pancreatic islets, mice would display an altered response to the metabolic effects of paclitaxel chemotherapy.

3. Methods

Animals: Pregnant female mice were from Charles River Laboratories (Wilmington, MA) and their litters were used for all experiments. Both male and female animals were evaluated to assess

sex specific differences. While behavioral and hormonal changes occur during the different stages of the female estrous cycle, it was not tracked during these experiments. The mice were housed on 12:12 hour light/dark cycle in the research support facility at the University of Kansas Medical Center. All mice were given ad libitum access to food and water. All animal use was in accordance with National Institutes of Health guidelines and conformed to the principles specified in a protocol approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Experimental design: Neonatal mice pups were injected subcutaneously with 50 mg/kg capsaicin in neck scruff. The mice pups were left alone and allowed to mature into 8-week-old adult mice. All animals survived during this maturation time. Adult mice were injected with paclitaxel or sham vehicle control, once daily, for three days, then observed over the next four weeks. Weekly measurements included weight, fasting blood glucose, fasting insulin, and Von Frey filament testing. The animals were maintained on a standard chow diet during the course of the study.

Diet, Weight and Body Composition: All mice were initially placed on standard chow diet (8604; Envigo, Madison, WI; 14% kcals from fat, 32% protein, and 54% carbohydrate). Mice were weighed weekly on digital laboratory scale.

Paclitaxel Injections: Mice were dosed with 12 mg/kg paclitaxel via intraperitoneal (IP) injection daily for three days. Control animals received a saline-based sham injection via IP injection daily for three days. Paclitaxel (Alvogen, Pine Brook, NJ, NDC # 47781-593-07) is formulated in a cremaphor/ethanol solution to maintain drug solubility. Cremaphor is used frequently as a formulation vehicle for poorly water soluble drugs, however, has shown capacity for biologic effects including peripheral neuropathy [149]. Therefore, to control for confounding effects, the

saline sham injections were formulated with comparable cremaphor and ethanol volumes,10% (V/V) cremaphor and 10% (V/V) ethanol.

Glucose & Insulin Measurements:

Fasting Glucose: Animals were fasted for 3 hours prior to glucose evaluation. Blood was collected via tail nick and glucose levels were measured by a colorimetric glucose diagnostic assay (Sigma, St. Louis, MO).

Fasting Insulin: Animals were fasted for 3 hours prior to insulin evaluation. Blood was collected via tail nick, allowed to clot on ice for 30 minutes, spun at 3000g for 30 minutes at 4°C, and the serum supernatant was collected. The serum was stored at -80°C until analysis could be completed via Mouse Ultrasensitive ELISA (ALPCO, Salem, NH).

Sensory Behavioral Testing: Sensory testing to quantitate paw mechanical sensitivity was performed using Von Frey monofilaments. Animals were acclimated for 30 minutes twice, prior to behavioral testing in a sound isolation chamber with ambient white noise. During acclimation and sensory testing, mice were placed in individual clear plastic chambers (11x5x3.5cm) on a wire mesh screen elevated 55cm above a table. Mechanical sensitivity was determined by the presence of a hind paw withdrawal to Von Frey monofilaments of varying sizes: 1.65 (0.0045g), 2.36 (0.02g), 2.83 (0.068g), 3.22 (0.158g), 3.61 (0.178g), 4.08 (1.20g), 4.31 (2.041g) and 4.74 (5.495g). Calculation of the 50% withdrawal threshold was performed utilizing the formula from the up-down method as previously described [150].

Statistical Analysis: All data are presented as mean ± standard error of the mean (SEM). Data were analyzed using multiple t tests, two-way ANOVA with repeated measures and Tukey multiple comparisons test. Statistical significance was defined as $P < 0.05$ and statistics were

performed using GraphPad Prism version 7.00 for macOS, GraphPad Software, La Jolla California USA as well as IBM SPSS Statistics version 27, IBM Corporation, Armonk New York USA.

4. Results and Figures

A cohort of C57BL/6 mice was given 50 mg/kg capsaicin subcutaneously in two-day old pups and allowed to mature into 8-week-old adults before paclitaxel treatment was performed as previously described (Fig 7A).

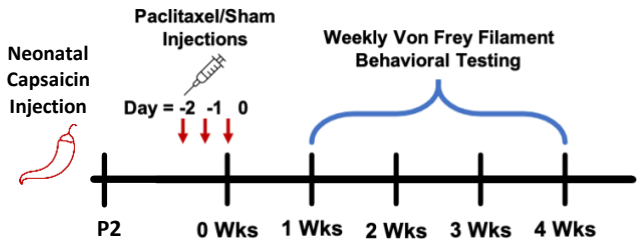
Behavioral testing using Von Frey filaments was performed weekly following paclitaxel injections to evaluate mechanical sensitivity. Paclitaxel-treated mice that received neonatal capsaicin exposure had significantly higher withdrawal thresholds compared to paclitaxel only treated animals at weeks 1 and 2 ($p = 0.0247$ and $p = 0.0028$, respectively, Fig 7B). Paclitaxel-treated mice that received neonatal capsaicin exposure did not have significant changes compared to control or capsaicin control-treated mice throughout the 4 weeks evaluated. It appears that the neonatal capsaicin treatment provided some protection from paclitaxel-induced allodynia at least 2 weeks following paclitaxel treatment.

Figure 7 Neonatal Capsaicin Delays Paclitaxel-Induced Mechanical Allodynia

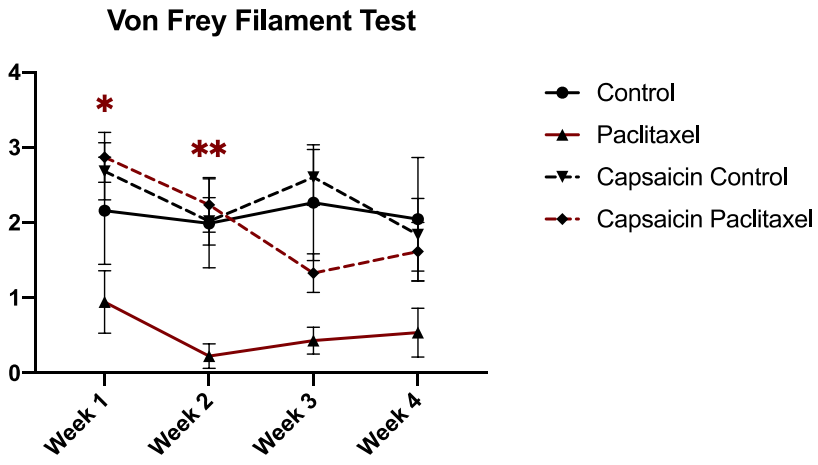
Timeline of experiment with neonatal capsaicin injection occurring at day 2 (P2), and 0 weeks represents time of paclitaxel injections when mouse is approximately 8 weeks old (A). Weekly Von Frey filament testing in male mice (B). Data are means ± SEM. *****$P < 0.05$, ** $P < 0.01$, two-way ANOVA with repeated measures, Tukey post-hoc test. n = 4-8.

Figure 7

A


Paclitaxel/Sham
Injections
Weekly Von Frey Filament
Behavioral Testing
Neonatal
Capsaicin
Injection
Day = -2 -1 0
P2
0 Wks
1 Wks
2 Wks
3 Wks
4 Wks

B


Von Frey Filament Test
4
3
2
1
0
*
**
Week 1
Week 2
Week 3
Week 4
Control
Paclitaxel
Capsaicin Control
Capsaicin Paclitaxel

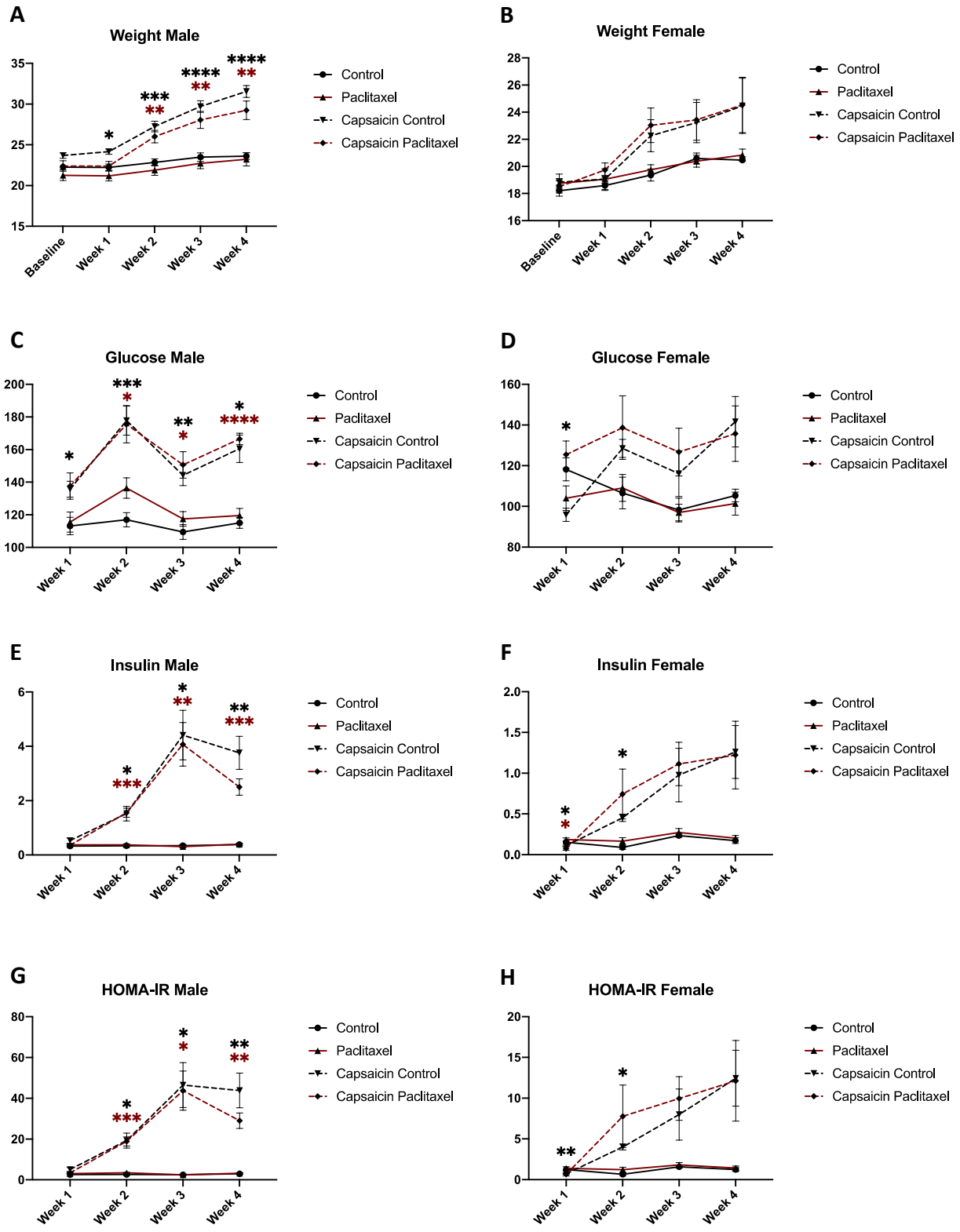
Weight, fasting glucose, fasting insulin, and calculated HOMA-IR were measured weekly in male and female mice. In male mice, the capsaicin control mice showed significantly elevated body weights compared to the control mice for all 4 weeks after paclitaxel injections ($p = 0.0214$, $p = 0.0007$, $p < 0.0001$, $p < 0.0001$, respectively, Fig 8A). The male capsaicin paclitaxel mice also showed significantly higher body weights compared to the paclitaxel mice for weeks 2-4 ($p = 0.0062$, $p = 0.0050$, $p = 0.0050$, respectively). In the female mice there was a significant source of variation based on treatment however no significant difference was observed between the different groups during any given week ($p = 0.0421$, Fig 8B). In male mice, the capsaicin control mice showed significantly elevated fasting glucose levels compared to the control mice for all 4 weeks after paclitaxel injections ($p = 0.0250$, $p = 0.0005$, $p = 0.0028$, $p = 0.0031$, respectively, Fig 8C). The male capsaicin paclitaxel mice showed significantly higher fasting glucose levels compared to the paclitaxel mice for weeks 2-4 ($p = 0.0494$, $p = 0.0185$, $p <0.0001$, respectively). In female mice, the capsaicin control mice also showed significantly elevated fasting glucose levels compared to the control mice, but just for the first week ($p = 0.0426$, Fig 8D). In male mice, the capsaicin control mice showed significantly elevated fasting insulin levels compared to the control mice for weeks 2-4 ($p = 0.0114$, $p = 0.0121$, $p = 0.0037$, respectively, Fig 8E). The male capsaicin paclitaxel mice also showed significantly elevated fasting insulin levels compared to the paclitaxel mice for weeks 2-4 ($p = 0.0006$, $p = 0.0091$, $p = 0.0009$). In female mice, the capsaicin control mice showed significantly increased fasting insulin levels compared to the control mice for weeks 1-2 ($p = 0.0455$, $p = 0.0160$, respectively, Fig 8F). The female capsaicin paclitaxel mice showed significantly elevated fasting insulin levels compared to the paclitaxel mice just at week 1 ($p = 0.0447$). In male mice, the capsaicin control mice showed significantly higher HOMA-IR values compared to the control mice for weeks 2-4 ($p = 0.0111$, $p = 0.0212$, p

= 0.0082, respectively, Fig 8G). The male capsaicin paclitaxel mice also showed significantly elevated HOMA-IR values compared to the paclitaxel mice for weeks 2-4 ($p = 0.0009$, $p = 0.0145$, $p = 0.0011$, respectively). In female mice, the capsaicin control mice showed significantly elevated HOMA-IR values compared to the control mice at weeks 1-2 ($p = 0.0089$, $p = 0.0112$, respectively, Fig 8H).

Figure 8 Neonatal Capsaicin Causes Increases in Weight, Glucose and Insulin

Observed metabolic parameters following neonatal capsaicin and paclitaxel treatment. Weekly weight measurements in male mice (A). Weekly weight measurements in female mice (B). Weekly fasting glucose measurements in male mice (C). Weekly fasting glucose measurements in female mice (D). Weekly fasting insulin measurements in male mice (E). Weekly fasting insulin measurements in female mice (F). Weekly HOMA-IR scores in male mice (G). Weekly HOMA-IR scores in female mice (H). Data are means ± SEM. ***P* < 0.05, ** *P* < 0.01, ****P* < 0.001, *****P* < 0.0001, two-way ANOVA with repeated measures, Tukey post-hoc test. Males weight n = 6-8. Males glucose, insulin and HOMA-IR n = 8-15. All female data n = 3-6.

Figure 8


A
Weight Male
B
Weight Female
C
Glucose Male
D
Glucose Female
E
Insulin Male
F
Insulin Female
G
HOMA-IR Male
H
HOMA-IR Female
Control
Paclitaxel
Capsaicin Control
Capsaicin Paclitaxel
Baseline
Week 1
Week 2
Week 3
Week 4

5. Discussion

In summary, while paclitaxel did not appear to have any significant effect on weight, glucose, insulin or HOMA-IR in male or female animals, the neonatal capsaicin demonstrated a robust effect in male mice and a lesser, yet still significant, effect in female mice on these metabolic parameters. This gender discrepancy may be due to low sample size of capsaicin treated female mice. In order to expedite the availability of pups for neonatal capsaicin injections, pregnant dams were ordered from Charles River and the litter pups were used for the study. However, the litters generated were not evenly split between male and female mice. Of the litters that dropped, there were 17 males and only 6 females available for the study.

The results of the neonatal capsaicin treatment were unexpected, that it provided some protection in the observed paclitaxel-induced allodynia, while at the same time causing weight gain and increasing fasting glucose and insulin. Whether or not these effects can be attributed specific alterations in the sensory neurons innervating the pancreas is not possible as neonatal capsaicin exerts a wide-spread systemic effect on all TRPV1 sensory afferents [196]. A possible explanation for the increased withdrawal threshold observed in the capsaicin paclitaxel treated mice could be the direct effects of the capsaicin on the intraepidermal nerve fibers themselves. Neonatal capsaicin has shown a decrease in the IENFD in previous studies [197]. Another interesting finding from this study is that while paclitaxel treatment appears to have no effect on capsaicin treatment induced changes in weight, fasting glucose, and insulin, the alterations related to capsaicin exposure appear to differentiate following the paclitaxel/sham injection. Most of the animals had similar values at baseline, prior to paclitaxel treatment, but soon afterwards developed significant changes. Subsequent experiments with more targeted approach such as local capsaicin injection, or nerve ablations, are warranted.

Chapter 4: Effects of Ketogenic Diet on Paclitaxel Induced Neuropathy in Mice

1. Abstract

Paclitaxel chemotherapy is a widely used cancer treatment associated with a dose dependent neurotoxicity. It has been highly associated with development distal sensory neuropathy that may become treatment limiting. Mice treated with paclitaxel develop decreased withdrawal thresholds as well as metabolic changes that may be associated with sensory nerve damage to the pancreatic afferents. Ketogenic diets consist of very low carbohydrate content and have shown increasing promise in providing neuroprotection for a variety of neurological disorders. In these experiments, a ketogenic diet was employed as a potential neuroprotective intervention in a mouse model of paclitaxel CIPN. Male and female C57BL/6 mice were fed a ketogenic diet prior to treatment of intraperitoneal paclitaxel and observed for the subsequent weeks to evaluate changes in metabolic and mechanical sensitivity. The ketogenic diet improved the decreased withdrawal threshold seen in paclitaxel-treated mice. It also prevented the paclitaxel-associated weight gain and fat deposition in male mice but did not appear to have an effect on glucose or insulin levels. The ketogenic diet overall appears to ameliorate some of the neurotoxic and metabolic effects of paclitaxel treatment in the mouse animal model and might be an easily implemented translational intervention in paclitaxel treated patients during their infusion treatments to reduce the associated neurotoxic morbidity.

2. Introduction

Paclitaxel is frequently used chemotherapeutic used in the treatment of several forms of malignancy including breast, lung, and ovarian cancers [51]. It is frequently administered intravenously in a cremaphor-diluted form as a dosage of 175 mg/m^2 over the course of three hours every three weeks [198]. Paclitaxel, however, displays significant potential toxicities including myelosuppression, hypersensitivity reaction and a dose-related peripheral neuropathy [199-203]. Approximately 40% of patients receiving paclitaxel chemotherapy will develop peripheral neuropathy following treatment [131].

Mouse models of paclitaxel-induced peripheral neuropathy have been well documented in the literature [204-210]. Paclitaxel is frequently administered as multiple intraperitoneal injections over the course of several days as opposed to infusions in clinical patients. The mice subsequently develop a mechanical allodynia, demonstrated through decreased withdrawal threshold in von Frey filament testing. However, to our knowledge no literature exists on the effects of a ketogenic diet in the context of paclitaxel-induced peripheral neuropathy in the mouse animal model.

A ketogenic diet was first employed as a neuroprotective treatment in the management of epilepsy patients over 100 years ago [83, 98, 211]. The diet mimics metabolic conditions associated with starvation, elevating levels of blood ketones including beta-hydroxybutyrate and acetoacetate. Neurons primarily use glucose as an energy substrate for biochemical metabolism however they may also use ketones as an alternate fuel source [212-214]. During embryological development the central nervous system is developing quickly, and ketone bodies help support the large energy demands in addition to glucose [86, 87]. Although once thought to only be associated with the morbidity of type 1 diabetic ketoacidosis (DKA), ketone body research has

exploded over the last half century. Interesting that at high levels present in DKA ketone bodies have been shown to be neurotoxic [212, 215]. It now is thought to be beneficial for multiple neurodegenerative diseases including Alzheimer's, Parkinson's, diabetic neuropathy, and many other conditions [107, 108, 216-219]. While the mechanisms by which a ketogenic diet is neuroprotective are poorly understood, it may result from improved mitochondrial function to resist metabolic challenges. Another mechanism postulated by which ketones may be neuroprotective is the establishment of an anti-inflammatory state [110, 220, 221]. Long-term ketogenic diet has been associated with reduced inflammatory mediators through down-regulation of NLRP3 inflammasome expression and reducing the production of isoprostanes [109, 222].

Our lab has previously shown that a ketogenic diet was able to significantly reverse mechanical allodynia and restore intraepidermal nerve fiber density loss seen in diabetic peripheral neuropathy, while being unable to reverse metabolic changes [85]. We hypothesize that a ketogenic diet's neuroprotective effects extend to the peripheral nervous system and might prove therapeutic for other peripheral neuropathies, including chemotherapy-induced peripheral neuropathy. We have shown that mice fed a ketogenic diet display reduced mitochondrial reactive oxygen species in their peripheral nerves compared to control fed mice [223]. We hypothesize that a ketogenic diet will ameliorate the metabolic changes as well as the peripheral neuropathy symptoms induced by paclitaxel treatment in mice. These findings should deepen our understanding of paclitaxel-induced peripheral neuropathy and provide a potential avenue for an easily implemented dietary intervention for patients suffering neuropathy secondary to their cancer treatment.

3. Methods

Animals: Eight-week-old C57BL/6 male and female mice were purchased from Charles River Laboratories (Wilmington, MA) and used for all experiments. The estrous cycle was not tracked in the female mice during these experiments. The mice were housed on 12:12 hour light/dark cycle in the research support facility at the University of Kansas Medical Center. All mice were given ad libitum access to food and water for the duration of the experiments. All animal use was in accordance with National Institutes of Health Guidelines and conformed to the principles specified in a protocol approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Overview of Experimental Design and Timeline: Mice were placed on a ketogenic diet or remained on standard chow diet, for two weeks prior to the intraperitoneal injections of paclitaxel or saline/cremaphor control. Following the injections, the mice were observed for the next six weeks to evaluate metabolic changes and monitor mechanical sensitivity. After the six weeks all mice were euthanized.

Paclitaxel Administration: In this mouse model of paclitaxel induced peripheral neuropathy, mice were dosed with 12 mg/kg paclitaxel (Alvogen, Pine Brook, NJ, NDC # 47781-593-07) via intraperitoneal (IP) injection daily for three consecutive days. Control animals received a saline-based sham injection via IP injection daily for three days. The saline control was formulated in a cremaphor and ethanol-based solution comparable to the paclitaxel vehicle to minimize any confounding effects of the vehicle formulation, especially since cremaphor has been shown to have some effect on peripheral neuropathy development [149, 224, 225].

Diet: Mice were placed on a standard chow diet (8604; Envigo, Madison, WI; 14% kcals from fat, 32% protein, and 54% carbohydrate) or ketogenic diet (96355; Envigo; 90.5% kcals from vegetable shortening (hydrogenated) and corn oil fat, 9.2% protein, and 0.3% carbohydrate).

Weight and Body Composition: Mice were weighed on a digital laboratory scale. Body composition analysis to assess fat mass and lean mass was measured by magnetic resonance imaging using the EchoMRI-100 (EchoMRI, Houston, TX).

Glucose & Insulin:

Fasting Glucose: Animals were fasted for 3 hours prior to glucose evaluation. Blood was collected via tail nick and glucose levels were measured by a colorimetric glucose diagnostic assay (Sigma, St. Louis, MO).

Fasting Insulin: Animals were fasted for 3 hours prior to insulin evaluation. Blood was collected via tail nick, allowed to clot on ice for 30 minutes, spun at 3000g for 30 minutes at 4°C, and the serum supernatant was collected. The serum was stored at -80°C until analysis could be completed via Mouse Ultrasensitive ELISA (ALPCO, Salem, NH).

Blood Ketones: Blood ketones were measured from a tail nick blood collection using a Precision Xtra ketone monitoring system (Abbott, #9881465, Abbott Park, IL), mice were fasted for 3 hours prior to blood collection.

Von Frey Filament Testing: Sensory testing to quantitate paw mechanical sensitivity was performed using Von Frey monofilaments. Animals were acclimated for 30 minutes twice, prior

to behavioral testing in a sound isolation chamber with ambient white noise. During acclimation and sensory testing, mice were placed in individual clear plastic chambers (11x5x3.5cm) on a wire mesh screen elevated 55cm above a table. Mechanical sensitivity was determined by the presence of a hind paw withdrawal to Von Frey monofilaments of varying sizes: 1.65 (0.0045g), 2.36 (0.02g), 2.83 (0.068g), 3.22 (0.158g), 3.61 (0.178g), 4.08 (1.20g), 4.31 (2.041g) and 4.74 (5.495g). Calculation of the 50% withdrawal threshold was performed utilizing the formula from the up-down method as previously described [150].

Statistical Analysis: All data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA and two-way ANOVA with repeated measures. Post-hoc analysis was performed via Tukey multiple comparisons test. Statistical significance was defined as $P < 0.05$ and statistics were performed using GraphPad Prism version 7.00 for macOS, GraphPad Software, La Jolla California USA as well as IBM SPSS Statistics version 27, IBM Corporation, Armonk New York USA.

4. Results and Figures

Paclitaxel Increases Blood Ketone Levels in Male and Female Mice:

Paclitaxel treatment caused a significant increase in blood ketone levels in male mice fed a chow diet compared to sham injected mice fed a chow diet ($p = 0.0424$, Fig 9A). In male mice fed a ketogenic diet, paclitaxel also caused a significant increase in blood ketone levels compared to sham injected mice also fed a ketogenic diet ($p = 0.0219$). The effect of ketogenic alone did not appear to cause an increase in observed blood ketones ($p = 0.9994$ in the male sham mice, $p = 0.9994$ in the male paclitaxel mice).

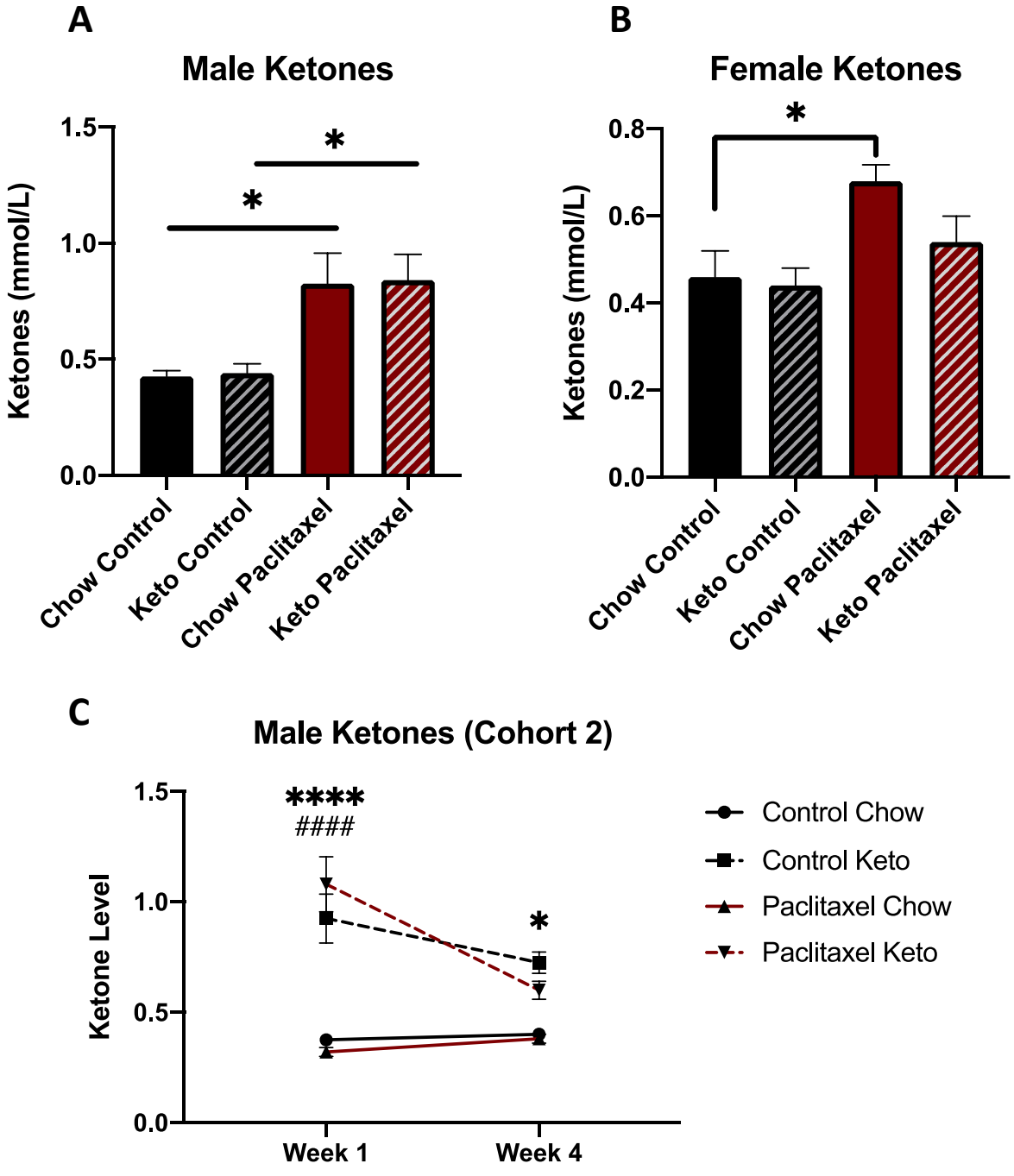
Paclitaxel treatment also caused a significant increase in blood ketone levels in female mice fed a chow diet compared to sham injected mice fed a chow diet ($p = 0.0326$, Fig 9B). However, in female mice fed a ketogenic diet, paclitaxel did not cause a significant increase in blood ketone levels compared to sham injected mice also fed a ketogenic diet ($p = 0.5170$). Similar to the male mice, the effect of ketogenic diet alone did not appear to cause an increase in observe blood ketones ($p = 0.9920$ in the female sham mice, $p = 0.2434$ in the male paclitaxel mice).

A second cohort of male mice was repeated with the same experimental design and ketone levels were found to be significantly elevated in control ketogenic diet mice compared to control chow mice ($p < 0.0001$, Fig 9C) at one week following paclitaxel or control injections. Ketone levels were also found to be significantly elevated in paclitaxel ketogenic diet mice compared to control ketogenic diet mice ($P < 0.0001$). This time the effect of paclitaxel alone in ketogenic or chow mice did not cause significant changes to blood ketone levels ($p = 0.9383$, $p = 0.3834$ respectively). At four weeks following injections, only control ketogenic diet mice remained significantly elevated compared to control chow mice ($p = 0.0290$).

Figure 9 Paclitaxel's effect on fasting blood ketone levels

Blood ketone levels measured in mice four weeks following treatment prior to euthanasia. Blood ketone levels in male mice. (A) Blood ketone levels in female mice. (B) Blood ketone levels in 2nd cohort of male mice (C). Data are means ± SEM. ***P* < 0.05, one-way ANOVA (A-B) and two-way ANOVA with repeated measures (C). Post-Hoc analysis performed via Tukey multiple comparisons test (male and female treatment groups n = 4-5).

Figure 9


A
Male Ketones
Ketones (mmol/L)
1.5
1.0
0.5
0.0
*
*
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel
B
Female Ketones
Ketones (mmol/L)
0.8
0.6
0.4
0.2
0.0
*
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel
C
Male Ketones (Cohort 2)
Ketone Level
1.5
1.0
0.5
0.0
****
####
*
Week 1
Week 4
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto

Ketogenic Diet Prevents Paclitaxel-Associated Increase in Mechanical Allodynia:

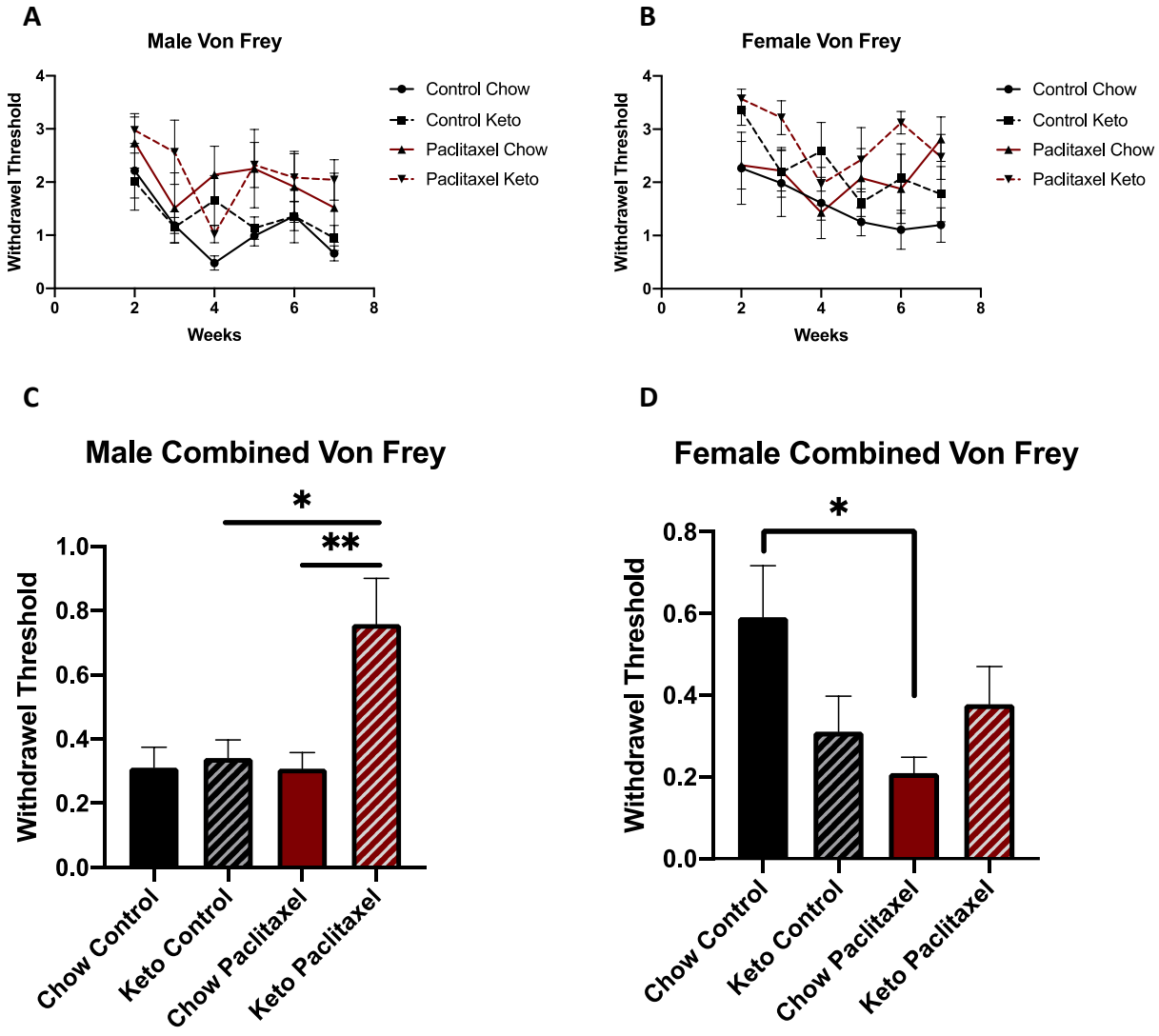
Weekly behavioral testing of hind paw sensitivity in male mice did not display any significant source of variation based on treatment among the four groups: control chow diet, control ketogenic diet, paclitaxel chow diet and paclitaxel ketogenic diet ($p = 0.0566$, Fig 10A). The weekly behavioral testing of hind paw sensitivity in female mice, however, did display a significant source of variation based on treatment among the four groups ($p = 0.0418$, Fig 10B). Upon post-hoc analysis no significance differences were found at any given time point during the study.

When the behavioral testing of hind paw sensitivity in male mice was performed by core facility experts at the end of the study, the paclitaxel ketogenic diet mice displayed significantly increased withdrawal thresholds compared to the chow paclitaxel mice ($p = 0.0092$, Fig 10C). The paclitaxel ketogenic diet mice also had significantly higher withdrawal thresholds compared to the control ketogenic diet mice ($p = 0.0107$). The control ketogenic diet mice's mechanical sensitivity was not significantly different from the control chow diet mice ($p = 0.9958$). When the behavioral testing was repeated on the female animals the paclitaxel ketogenic diet did not show a significant difference from the paclitaxel chow diet mice but is trending in that direction ($p = 0.5638$, Fig 10D). However, a significant decrease in mechanical sensitivity was observed in the paclitaxel chow mice compared to the control chow mice, similar to what has been observed in our previous paclitaxel experiments ($p = 0.0270$).

Figure 10 Ketogenic diet's effect on hind paw sensitivity with paclitaxel treatment

Behavioral testing of hind paw sensitivity in mice performed weekly with paclitaxel injections occurring at "week 2." Weekly behavioral testing of hind paw sensitivity in the male mice. (A) Weekly behavioral testing of hind paw sensitivity in female mice. (B) Repeated behavioral testing of hind paw sensitivity performed by behavior core facilities prior to euthanasia on male mice. (C) Repeated behavioral testing of hind paw sensitivity in performed by behavior core facilities prior to euthanasia on male mice. (D) Data are means ± SEM, $*P < 0.05$, $**P < 0.01$. Weekly behavioral testing of hind paw sensitivity analyzed by two-way ANOVA with repeated measures and Tukey multiple comparisons test (A-B). Repeated behavioral testing of hind paw sensitivity analyzed by one-way ANOVA with Tukey multiple comparisons test (C-D) (male and female treatment groups n = 4-5).

Figure 10


A
Male Von Frey
Withdrawel Threshold
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
B
Female Von Frey
Withdrawel Threshold
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
C
Male Combined Von Frey
Withdrawel Threshold
*
**
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel
D
Female Combined Von Frey
Withdrawel Threshold
*
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel

Ketogenic Diet Reduced Paclitaxel-Associated Weight Gain and Body Fat Composition:

Although no significant source of variation, based on treatment, in weight change was seen among the four groups in the male mice, the paclitaxel chow mice trended towards increased weight gain compared to the other groups ($p = 0.1437$, Fig 11A). This trend towards increase weight gain was absent in the paclitaxel ketogenic diet mice. Ketogenic diet alone in control mice did not cause a trend towards increase weight gain compared either. No significant source of variation, based on treatment, in weight change was seen among the four groups in the female mice as well ($p = 0.5785$, Fig 11B). Overall, the different treatment groups appeared closely associated in weight gain, with no individual group trending towards significance in the female mice tested.

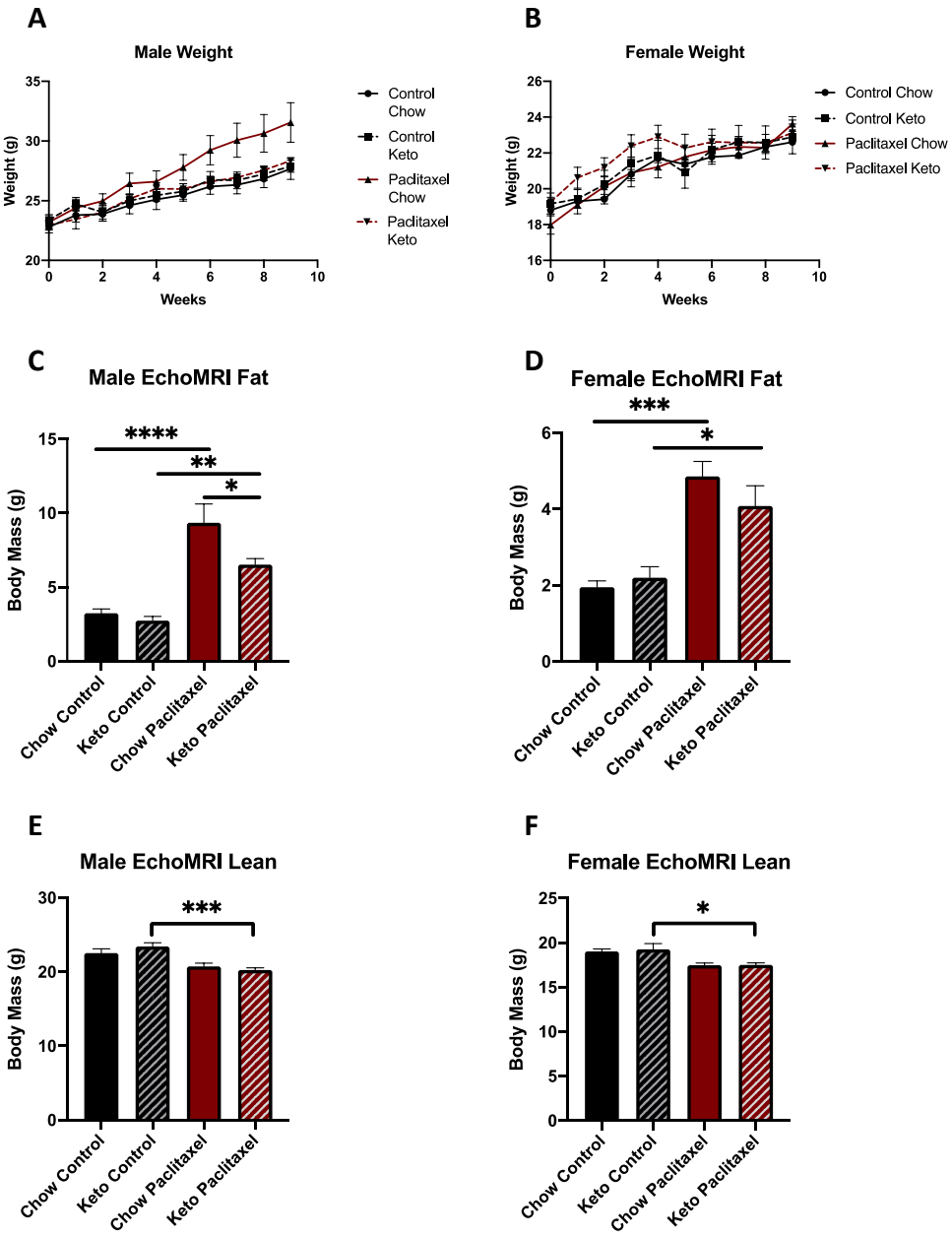
During echoMRI testing of the male mice at the end of the study, the paclitaxel chow mice displayed significant increase in fat mass compared to the control chow mice ($p < 0.0001$, Fig 11C). The control ketogenic diet mice did not have significant increase in fat mass compared to the control chow mice ($p = 0.9436$). The paclitaxel ketogenic diet mice had significantly decreased fat mass compared to the paclitaxel chow diet, but significantly more than the control ketogenic diet mice ($p = 0.0319$, $p = 0.0027$ respectively). In the echoMRI testing of female mice, we observed that the paclitaxel chow mice had significant increase in fat mass compared to the control chow mice ($p = 0.0002$, Fig 11D). The control ketogenic diet mice did not have significant increase in fat mass compared to the control chow mice ($p = 0.9645$). The paclitaxel ketogenic diet mice had significantly increased fat mass compared to the control ketogenic mice ($p = 0.0116$). However, in the female mice the paclitaxel ketogenic diet mice, a significant decrease in fat mass compared paclitaxel chow mice was not observed ($p = 0.4604$).

EchoMRI testing of the male mice revealed a significant decrease in lean body mass in the paclitaxel ketogenic diet mice compared to the control ketogenic diet mice ($p = 0.0008$, Fig 11E). All other treatment groups revealed no significant difference in lean body mass in the male mice. EchoMRI testing of the female mice also revealed a significant decrease in lean body mass in the paclitaxel ketogenic diet mice compared to the control ketogenic diet mice ($p = 0.0277$, Fig 11F).

Figure 11 Ketogenic diet's effect on weight and body composition with paclitaxel treatment

Weight measurements were taking weekly throughout the experiment with the paclitaxel injections occurring at "week 2." Weight measurements of male mice. (A) Weight measurements of female mice. (B) Echo MRI data was collected once at the end of the experiment prior to euthanasia. Echo MRI fat mass of male mice. (C) Echo MRI fat mass of female mice. (D) Echo MRI lean mass of male mice. (E) Echo MRI lean mass of female mice. (F) Data are means ± SEM. *****$P < 0.05$, ******$P < 0.01$, *******$P < 0.001$, ********$P < 0.0001$, weight data analyzed by two-way ANOVA with repeated measures and Tukey multiple comparisons test. Echo MRI data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Male and female treatment groups n = 4-5)

Figure 11


A
Male Weight
Weight (g)
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
B
Female Weight
Weight (g)
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
C
Male EchoMRI Fat
Body Mass (g)
****
**
*
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel
D
Female EchoMRI Fat
Body Mass (g)
***
*
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel
E
Male EchoMRI Lean
Body Mass (g)
***
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel
F
Female EchoMRI Lean
Body Mass (g)
*
Chow Control
Keto Control
Chow Paclitaxel
Keto Paclitaxel

Ketogenic Diet and Paclitaxel Treatment do not Alter Blood Glucose or Insulin Levels

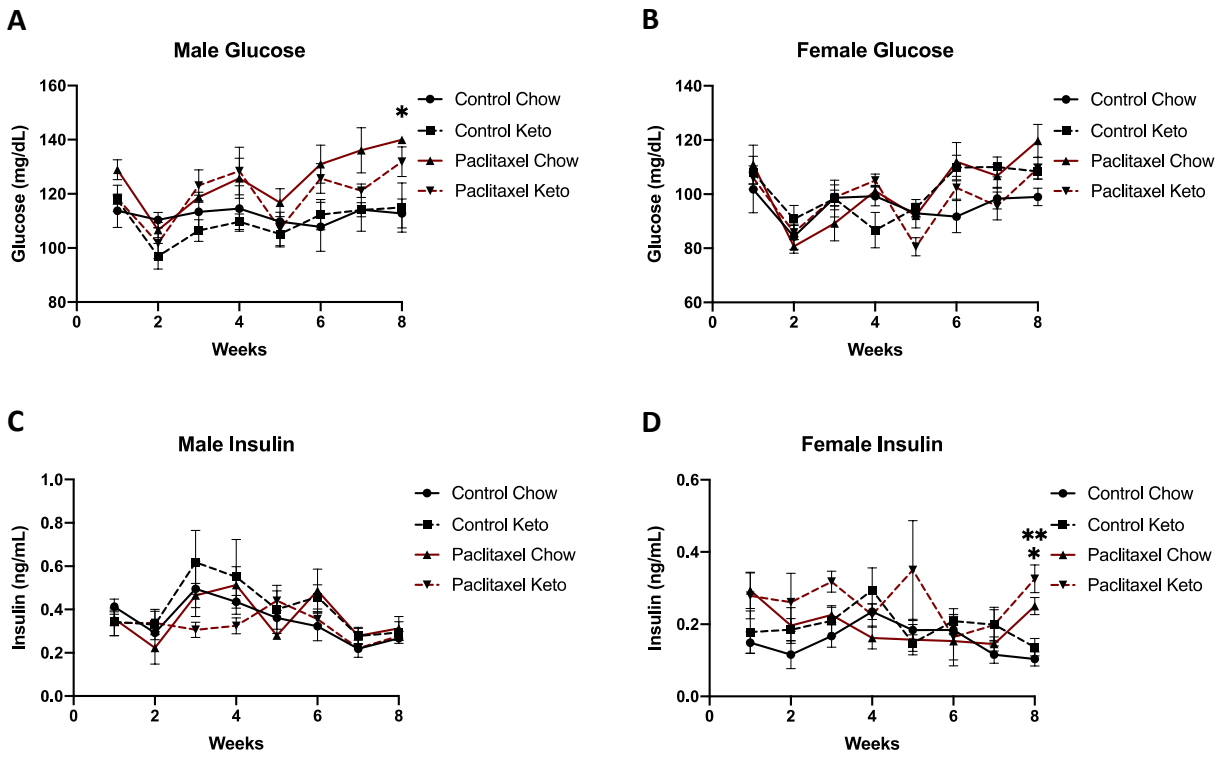
Weekly measurement of male fasting blood glucose did not display any significant source of variation based on treatment among the four groups: control chow diet, control ketogenic diet, paclitaxel chow diet and paclitaxel ketogenic diet ($p = 0.0852$, Fig 12A). During post-hoc analyses, the paclitaxel chow mice were found to have significantly elevated fasting blood glucose compared to the control chow mice at week 8 ($p = 0.0374$). This is consistent with previous studies on paclitaxel treatment on male mice causing transient increases in fasting blood glucose during the weeks following the injections. Weekly measurement of female fasting blood glucose did not display any significant source of variation based on treatment among the four groups either ($p = 0.2883$, Fig 12B).

Weekly measurement of male fasting insulin levels did not display any significant source of variation among the four groups ($p = 0.6371$, Fig 12C). Weekly measurement of female fasting insulin levels, however, did display a significant source of variation among the four groups ($p = 0.0398$, Fig 12D). At week 8 paclitaxel chow mice had significantly increased fasting insulin levels compared to control chow mice ($p = 0.0065$). Also, paclitaxel ketogenic diet mice had significantly increased fasting insulin levels compared to control ketogenic diet mice ($p = 0.0181$). While paclitaxel ketogenic diet mice trended toward lower fasting insulin levels it was not significantly different from the paclitaxel chow mice ($p = 0.4041$).

Figure 12 Ketogenic diet's effect on glucose and insulin levels with paclitaxel treatment

Blood samples were taken weekly for glucose and insulin throughout the experiment with the paclitaxel injections occurring at "week 2." Weekly fasting glucose values for male mice (A). Weekly fasting glucose values for female mice (B). Weekly fasting insulin values for male mice (C). Weekly fasting insulin values for female mice (D). Data are means ± SEM. ***P* < 0.05. Data analyzed by two-way ANOVA with repeated measures and Tukey multiple comparisons test. (Male and female treatment groups n = 4-5)

Figure 12


A
Male Glucose
Glucose (mg/dL)
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
*
B
Female Glucose
Glucose (mg/dL)
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
C
Male Insulin
Insulin (ng/mL)
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
D
Female Insulin
Insulin (ng/mL)
Weeks
Control Chow
Control Keto
Paclitaxel Chow
Paclitaxel Keto
**
*

5. Discussion

In this experiment a ketogenic diet was employed as a potential therapeutic treatment for mice receiving paclitaxel chemotherapy. At first, it appeared that paclitaxel treatment itself lead to elevated blood ketones. However, in the second cohort of mice the ad libitum ketogenic diet was able to independently induce a state of ketosis in the mice, defined as blood ketone levels greater than 0.5 mmol/L [226-228]. Behavioral testing of hind paw sensitivity revealed increased mechanical sensitivity in paclitaxel treated mice that was ameliorated by the ketogenic diet, with greater efficacy in the male mice. Male mice trended towards increase weight gain with the paclitaxel treatment, but this trend was reversed with the addition of the ketogenic diet which appeared more tightly associated with the control mice. This effect was not observed in female mice. EchoMRI analysis revealed an increase in fat mass following paclitaxel treatment in both male and female animals. A ketogenic diet was able to prevent some of this fat mass increase, at least in the male animals. Both male and female animals should a decrease in lean mass with paclitaxel treatment in ketogenic diet mice compared to chow mice. Lastly, while paclitaxel and ketogenic diet don't appear to cause robust changes to glucose or insulin levels, there were isolated time points at which paclitaxel caused elevations in blood glucose in male chow mice or insulin in female chow mice that were not trending to the same extent in ketogenic diet mice.

Surprisingly, consumption of a ketogenic diet did not increase blood ketone levels in the mice in the first cohort. Multiple sources in the literature have shown that mice fed a ketogenic diet develop elevated blood ketone levels [229-233]. It was decided to repeat the experiment with a second male cohort to verify this unexpected result in our mice. The second cohort of male mice showed a robust increase in blood ketone levels in the ketogenic diet compared to the chow diet in both the paclitaxel and control mice at one week post treatment. Both of these

groups blood ketone levels appeared to decrease over time. It has been shown that on long-term ketogenic diet, blood ketones tend to peak within the first couple weeks, but then decrease over time, likely due to keto-adaptation [234]. It may be possible that in the first cohort of mice, since blood ketones were only measured at 5 weeks that they had already keto-adapted to the point where significance was lost. It is possible that paclitaxel treatment itself is able to increase blood ketone levels as it has been shown to cause mitochondrial dysfunction which may alter ketone metabolism [235-237]. This may explain the elevated ketone levels in the mice treated with paclitaxel, irrespective of diet, however this effect was not recapitulated in the 2nd cohort of mice. Furthermore, there exists no evidence in the literature that paclitaxel has caused ketosis, independent of a ketogenic diet.

The behavioral testing of hind paw sensitivity was not able to replicate previous findings in our lab of sustained decrease in withdrawal threshold in paclitaxel-treated chow mice. Possible explanations for this discrepancy may include noise disruptions/construction that was ongoing in the animal facility during the behavioral testing, also this cohort of mice had several animals that fought to the point of requiring separation. For example, at week 4 there was is a large dip in withdrawal threshold, noticeable in several treatment groups of male and female mice. Due to this change in variation compared to previous experiments it was decided to have the behavioral core facilities repeat the behavioral testing of hind paw sensitivity. When the core facilities performed the behavioral testing at the end of the experiment, male paclitaxel treated mice on a ketogenic diet showed increased withdrawal thresholds compared to paclitaxel treated mice on a chow diet which supports the hypothesis that a ketogenic diet may be neuroprotective. This trend was observed in female mice as well but did not reach significance. One point of concern is that the male control chow mice had a low withdrawal threshold, not significantly different from the

paclitaxel treated mice. We have previously seen that control chow mice should not experience a decrease in withdrawal threshold from their baseline which was averaged above 2.0 (see Fig 3A). The female mice, however, did show a significant difference in the control chow mice compared with the paclitaxel chow mice similar to previous findings [238-240].

Male paclitaxel chow mice displayed a trend towards increased weight gain compared to control animals, that might have reached significance had the study been longer. While not significantly different, it is interesting to see the paclitaxel ketogenic diet mice weight remain much closer to control and chow fed animals. This trend towards increased weight with paclitaxel treatment appears to be only in male animals as the female weight data does not appear to be diverging from the control animals. In humans, paclitaxel has been associated with increased weight gain in both breast and lung cancer patients [241, 242]. These studies show that at least in humans, paclitaxel-associated weight gain is observed in both male and females. In mice however, there may be a sex-related difference in weight gain caused by paclitaxel treatment. The echoMRI analysis showed that paclitaxel effectively caused a two-fold increase in body fat composition. Ketogenic diet was able to significantly reduce this robust change in male mice, but only trend towards reduction in female mice. In both male and female mice, paclitaxel ketogenic diet mice had significantly lower lean body mass compared to control ketogenic diet mice, and a similar trend exists in the chow mice. In a prospective study of breast cancer patients treated with chemotherapy it was shown that they developed increased fat mass and decreased lean mass following treatment [243]. The cause for these changes in addition to weight gain have been unclear, possibly attributed to the cancer's metabolic effects, additional treatment with prednisone, adjuvant tamoxifen therapy or radiation therapy. Based on our findings in this study, these effects may be attributable to paclitaxel alone, or in combination with another

drug/intervention. However, there have also been additional studies taxane treated breast cancers that saw no change in subcutaneous, visceral, or intramuscular adipose tissue [244]. The robust increase in fat body mass following paclitaxel exposure provides another risk factor for development of insulin resistance beyond a neurotoxic etiology in chemotherapy patients as central obesity, increased body fat and waist circumference are closely associated with development of diabetes and increased morbidity and mortality [245-250].

While the weekly fasting glucose values did not reveal robust changes with paclitaxel treatment or ketogenic diet, they did show an isolated significant increase at week 8 in the male mice treated with paclitaxel. As there are many mechanisms that play a role in insulin secretion and sensitivity as well as glycemic status, it may be that paclitaxel-related pathology is simply not enough to deregulate glycemic homeostasis. More significant increases in fasting glucose may simply take more time to develop following paclitaxel treatment. As with the SNIRKO mice, it took 29 weeks before development of hyperinsulinemia [79]. The female paclitaxel chow mice displayed a significant increase in insulin at week 8 as well as the paclitaxel ketogenic diet mice compared to controls. While the glucose and insulin data does not show robust changes due to paclitaxel treatment it does show isolated points of significant increase and that the ketogenic diet, at least in males, does not.

Overall, it appears that in most parameters evaluated where paclitaxel had an adverse effect, the addition of a ketogenic diet ameliorated the pathological change or at least did not have an additive effect. The ketogenic diet did not appear to cause significant pathological changes in the control mice. This provides additional evidence that a ketogenic diet is a well-tolerated intervention, that may provide some beneficial effects in the context of paclitaxel-induced neuropathy and associated metabolic changes. Being a dietary intervention, the

ketogenic diet would not require any FDA approval and could be immediately implemented in the clinical setting for evaluation in human chemotherapy patients. Ketogenic diets are generally well tolerated and becoming increasingly popular in overweight populations, those with clinical conditions as well as healthy individuals [251-255]. Furthermore, it is easier than ever before for patients to maintain a ketogenic diet due to the recent increase in awareness, initiation protocols, teaching kitchens, premade ketogenic food options, and ways to calculate and administer the diet [256-258].

Chapter 5: Characterization of Peripheral Neuropathy in Prediabetic Patients: The PACMAN Study

1. Abstract

Prediabetes is associated with the development of peripheral neuropathy in some patients prior to the diagnosis of type 2 diabetes mellitus. Understanding the progression of neuropathy is important as rates of prediabetes continue to rise. In this current ongoing clinical study, patients are recruited into one of three groups based on metabolic status and presence of neuropathy: healthy (H), prediabetes without neuropathy (PD-N), or prediabetes with neuropathy (PD+N). Patients with prediabetes as well as healthy controls were recruited and enrolled into the study, primarily from the KU Frontiers patient database. During the course of two clinical visits, subjects provided demographic information, health history, social/lifestyle history, responded to various questionnaires, provided blood samples for evaluation of metabolic health, as well as provided a lower leg skin biopsy to evaluate intraepidermal nerve fiber density (IENFD). To date, 72 subjects have been screened and 22 subjects have completed the study. Prediabetic subjects with neuropathy were significantly older and more likely to be male in this study population. Prediabetic subjects with neuropathy have significantly decreased IENFD (H = 11.03, PD-N = 11.94, PD+N = 4.27 fibers/mm, $p < 0.5$) compared to healthy or prediabetes subjects without neuropathy. Also, prediabetic subjects with neuropathy have significantly lower LDL levels than healthy or prediabetic subjects without neuropathy H = 97.0, PD-N = 124.3, PD+N = 84.0, $p < 0.5$), suggesting that LDL levels may play a role in neuropathy in prediabetes. Our studies suggest that unlike rodents, subjects with prediabetes have IENFD loss across all epidermal fiber types.

2. Introduction

Prediabetes is recognized as an impaired glucose tolerance state with higher levels of blood glucose, but without crossing the thresholds established for diagnosis of type 2 diabetes mellitus. It was formally established a sperate diagnosis in 1997 by the American Diabetes Association (ADA) with specific cut off criteria of fasting glucose [259]. The specific criteria for prediabetes diagnosis are: HbA1c (5.7-6.4%), fasting glucose (100-125 mg/dl), or oral glucose tolerance (140-199 mg/dl) [260]. There exists a consensus that in this early state of impaired glucose metabolism, that the disease course is reversible as significant pathology has not occurred to the same state as type 2 diabetes [261-264]. However, prediabetes does place a patient at increased risk for development of type 2 diabetes [265]. Prediabetes is also associated with a constellation of symptoms called metabolic syndrome or syndrome X [266-268]. The associated comorbidities include: obesity, hypertension, dyslipidemia, and is largely considered a prothrombotic and proinflammatory state [269]. The most common treatment for prediabetes consists of lifestyle modification counseling including diet and exercise to reduce obesity [270]. In addition many physicians may start patients on a low dose metformin [271]. While prediabetes may seem like a benign condition that can be conservatively managed, it is widespread among the US population affecting more than one-third of adults and appears to be increasing at an alarming rate [272-275].

While peripheral neuropathy is commonly recognized in diabetic patients, it may be occurring early in the disease progression during the prediabetic state [276]. Many patients diagnosed with cryptogenic sensory peripheral neuropathy are found to be prediabetic [28, 31]. Neuropathy in prediabetic patients usually presents as a length dependent polyneuropathy that appears in a stocking glove distribution [277]. It includes symptoms such as numbness, tingling,

burning, hyperalgesia or allodynia that usually first present in the feet [278]. These symptoms cause increased morbidity and can lead to major changes in their quality of life. Prediabetic and diabetic neuropathy leads to increased risk for long term sequalae including diabetic foot ulcers, osteomyelitis, and subsequent lower extremity amputation [24, 279]. Acutely, this peripheral neuropathy can affect gait, balance, activities of daily living (ADLs), shoe comfort, and reduce sleep quality [280-286]. Prediabetic neuropathy like diabetic neuropathy remains largely symptomatic as the only disease-modifying treatment remains limited to treated the underlying hyperglycemia to prevent disease progression [287]. Pain related symptoms are treated with calcium channel agonists, tricyclic antidepressants, or selective serotonin/norepinephrine reuptake inhibitors as well as many other second line medications including capsaicin creams and lidocaine patches [38, 288, 289].

Several risk factors exist in the literature for the development of peripheral neuropathy in prediabetic and diabetic patients, including hyperglycemia duration, age, BMI, waist circumference, smoking, lower socioeconomic status and presence of retinopathy [290-293].

In type 2 diabetes-associated peripheral neuropathy there exist a reliably observed decrease in IENFD, as well as subsequent decrease in axon regeneration [294-298]. Our lab has previously shown that mouse models of diabetes also show a decreased IENFD [298-300]. There does not exist robust evidence in the clinical literature that prediabetic peripheral neuropathy is also associated with a significant decrease in IENFD [301, 302]. One recent study has suggested that no difference exists between prediabetic patients skin biopsy IENFD and normal healthy patients [303].

Our hypothesis is that prediabetic peripheral neuropathy in human patients also shows a reduction in the intraepidermal nerve fiber density, similar to overt diabetic peripheral

neuropathy. Furthermore, it is expected that the development of painful neuropathic symptoms in prediabetic neuropathy will be associated with a change in the peptidergic/nonpeptidergic ratio of nerve fiber free endings in the epidermis. We have previously shown that, in a mice model of prediabetes, peptidergic and nonpeptidergic are damaged differentially [298]. We also sought to find additional risk factors or protective factors that might elucidate why only a subset of prediabetic patients develop neuropathy, allowing for more targeted management of patients before their disease has not progressed to a more chronic, irreversible state [304].

3. Methods

Experimental design: Study design consisted of two separate clinical visits, with the first visit occurring at the University of Kansas Clinical and Translational Science Unit (CTSU) and the second visit at the Landon Center on Aging neurology clinic. Patients were compensated $50 per visit to cover transportation expenses and time lost due to study participation. Patients were informed that their participation was completely voluntary and that they may withdraw from the study at any time. The study was approved by the University of Kansas Medical Center Institutional Review Board (# STUDY00004259).

Patient Recruitment: Patients were recruited primarily through telephone contacting and informational mailers from the KU Frontiers Research Participant Registry. This database contains more than 60,000 patients from the KU health system and the community who have previously agreed to participate in research studies [305]. Informational flyers were also posted around campus research facilities and high traffic areas, and with IACUC approval, shared on social media.

Visit 1: The first visit was completed by the study coordinator with the clinical components, including blood draws, performed by the dedicated nursing staff at the CTSU. The following paperwork and forms were completed:

- Written informed consent was obtained following review of the IRB-approved Research Consent Form with the study subject (Appendix A).
- Inclusion/exclusion criteria was verified with the patient (Fig 13).
- Recruitment source information regarding how the participant found out about the study and the mode of their initial contact was obtained.
- Demographical information was obtained.
- Personal and family history of diabetes and neuropathy were obtained.
- Social and lifestyle history including a detailed smoking history were obtained.
- A general medical history categorized by body system was obtained.
- The following questionnaires were obtained:
    - Pittsburgh Sleep Quality Index (PSQ)
    - International Physical Activity Questionnaire (IPAQ)

The clinical component of the first visit included obtaining physical measurements including: heart rate, blood pressure, height, weight, and waist circumference. Fasting laboratory collection first required a point of care glucose test to make sure the patient was not hyperglycemic prior to performing the oral glucose tolerance test (OGTT). Initial blood draws for laboratory analysis included a 4 mL heparin tube for fasting plasma glucose and a lipid panel, a 6 mL SST tube for insulin, a 4 mL EDTA tube for HbA1c, and a 4 mL EDTA tube for hematocrit and hemoglobin

levels. The OGTT consisted of the patient drinking a 75 mg bottle of Trutol in less than 5 minutes, having blood draws at 60 minutes and 120 minutes collected in a 4 mL heparin tube and a 6 mL SST tube for glucose and insulin, respectively. Any adverse effects during the visit were documented and Dr. Pasnoor was informed and consulted as the health care provider overseeing the study.

Visit 2: The second visit was completed by the study coordinator and Dr. Pasnoor from the Department of Neurology at the Landon Center on Aging. The following questionnaires were completed:

- Michigan Neuropathy Screening Instrument (MNSI)
- Brief Pain Inventory for Diabetic Neuropathy (BPI-DPN)
- Utah Early Neuropathy Scale (UENS)

A 3 mm punch biopsy was obtained on the distal lower leg, proximal to the lateral malleolus by Dr. Pasnoor, following local application of lidocaine for anesthesia. The wound was covered by a sterile bandage and the subject was informed of how to maintain good wound hygiene to prevent subsequent infection.

Group Assignment: Subjects were assigned to one of three groups: Healthy, Prediabetes without Neuropathy, and Prediabetes with Neuropathy. Prediabetes status was determined by the 1st visit lab results in concordance with the established ranges outlined by the American Diabetes Association (ADA) guidelines: HbA1c (5.7-6.4%), fasting glucose (100-125 mg/dl), or oral

glucose tolerance (140-199 mg/dl) [260]. Neuropathy status was determined by Dr. Pasnoor following her neuropathy assessment in the 2nd visit.

Skin Biopsy Processing: The skin biopsy was placed in Zamboni fixative for 1 hour by the study coordinator, documenting the time of biopsy collection, time of fixation, and time of transport to cryoprotection. Following the 1 hour of fixation the biopsy was washed in PBS and placed in 30% sucrose for cryopreservation for 24 hours. Then the biopsy was washed in PBS before being frozen on dry ice and stored at -20°C.

Biopsy Sectioning and Analysis: Biopsies were sectioned at 30 μm on a LEICA CM 1950 cryostat. Non-serial sections were mounted onto Superplus microscope slides (Fisher) and stored at -20°C until use. Biopsy sections were incubated for 1 hour with blocking solution containing Superblock™ (450 μl per slide, Thermo Scientific, Cat # 37515, Lot # TG269957), 1.5% normal donkey serum, 0.5% porcine gelatin, and 0.5% Triton X-100. This was followed by incubation with primary antibodies, including anti-TrkA, anti-PGP 9.5, anti-langerin/CD207. The sections were then washed with 1xPBS and incubated with secondary antibodies. Sections were rinsed and cover slipped. A sterile PBS solution was used as mounting media. 3 separate regions of 3 non-serial sections were analyzed per subject and average values were calculated as the result of these 9 measurements. Fluorescent images of dermal-epidermal junction, at 20x magnification, were collected using a Nikon Eclipse 90i microscope and QIClick digital CCD Camera (QImaging, Surrey, BC, Canada). Nikon Elements software was used for image analysis including fiber and cell counting as well as measurement of dermal-epidermal junction width.

All cell counts and fiber density was reported as cells/mm or fibers/mm. Subject identifiers were removed from the slides and researcher was blinded during biopsy analysis and quantification.

Statistical Analysis: All data are presented as mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA with Tukey multiple comparisons test and chi-squared analysis. Statistical significance was defined as $P < 0.05$ and statistics were performed using GraphPad Prism version 7.00 for macOS, GraphPad Software, La Jolla California USA as well as IBM SPSS Statistics version 27, IBM Corporation, Armonk New York USA.

4. Results and Figures

Figure 13 Inclusion/Exclusion Criteria

Specifications for targeted patient population eligibility of the study, summarizing criteria for inclusion in, and exclusion from, the study. These criteria were used during the patient recruitment communication and reviewed with the study coordinator prior to the first clinical visit.

Figure 13

Inclusion Criteria	Exclusion Criteria
Age 45-75	Skin conditions, circulatory insufficiency, or open wounds in the leg
Males and females	Stroke or other significant nervous system pathology
Any racial or ethnic group	Lidocaine allergy
Healthy (non-diabetic/non-neuropathic)	Difficulty with blood clotting due to Coumadin (Warfarin) use or blood clotting disorder
Prediabetes without Neuropathy History	Body weight > 350 lbs
Prediabetes with Neuropathy History	History of anemia or vitamin B12 deficiency
	History of chemotherapy treatment
	Donated blood in the last 12 weeks or a whole blood transfusion in the last 6 months
	Clinical anemia (hematocrit <32 for women or <36 for men)

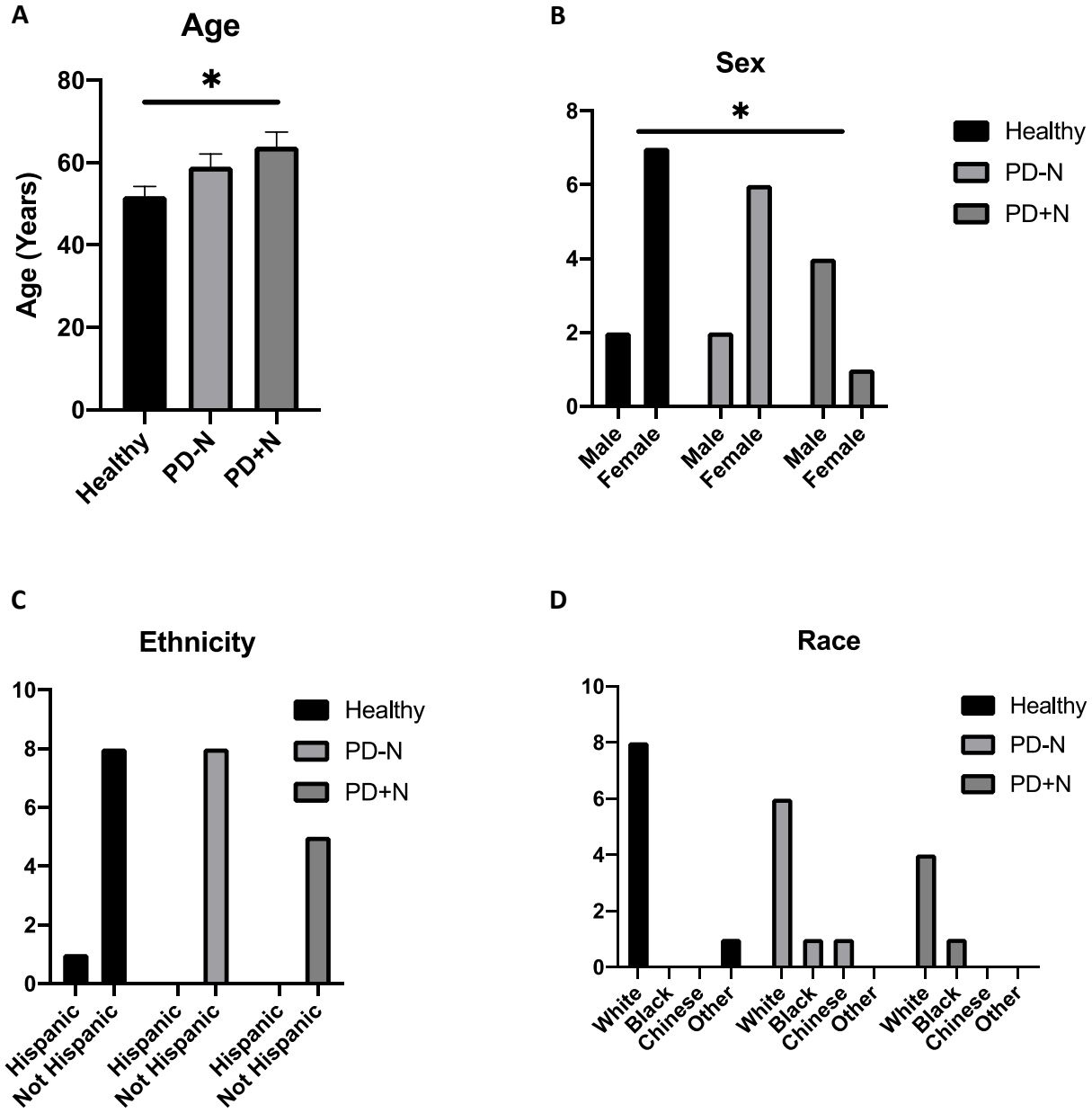
Prediabetes with Neuropathic Symptoms are Older and More Likely to be Male:

Demographic data was collected from the patients showed that prediabetic patients with neuropathy were significantly older than healthy patients, but not from prediabetic patients without neuropathy ($p = 0.0411$, $p = 0.6550$ respectively, Fig 14A). Healthy patients showed no significant difference in average age from prediabetic patients without neuropathy ($p = 0.2184$). Recruited prediabetic patients with neuropathy were significantly more likely to be male than the healthy patients, but not significantly different from prediabetic patients without neuropathy ($p = 0.0363$, $p = 0.0530$ respectively, Fig 14B). Healthy patients and prediabetic patients without neuropathy were not different in terms of male/female distribution ($p = 0.8928$). There were no significant differences in ethnic composition between the three groups ($p = 0.3311$, $p = 0.4392$, $p = 1.0000$, Fig 14C). There were also no significant differences in racial composition between the three groups ($p = 0.3564$, $p = 0.9431$, $p = 0.6881$, Fig 14D)

Figure 14 Demographics

Demographical information collected from patients who completed the clinical study. Age of patients at time of first visit (A). Reported sex of the patient (B). Reported ethnicity of the patient (C). Reported racial group of the patient (D). Data are means ± SEM. ***P* < 0.05, age data analyzed by one-way ANOVA with Tukey multiple comparisons test. Sex, ethnicity and racial data are reported as frequencies and analyzed by chi-square analysis. (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 14


A
Age
Age (Years)
80
60
40
20
0
*
Healthy
PD-N
PD+N
B
Sex
8
6
4
2
0
*
Male
Female
Male
Female
Male
Female
Healthy
PD-N
PD+N
C
Ethnicity
10
8
6
4
2
0
Hispanic
Not Hispanic
Hispanic
Not Hispanic
Hispanic
Not Hispanic
Healthy
PD-N
PD+N
D
Race
10
8
6
4
2
0
White
Black
Chinese
Other
White
Black
Chinese
Other
White
Black
Chinese
Other
Healthy
PD-N
PD+N

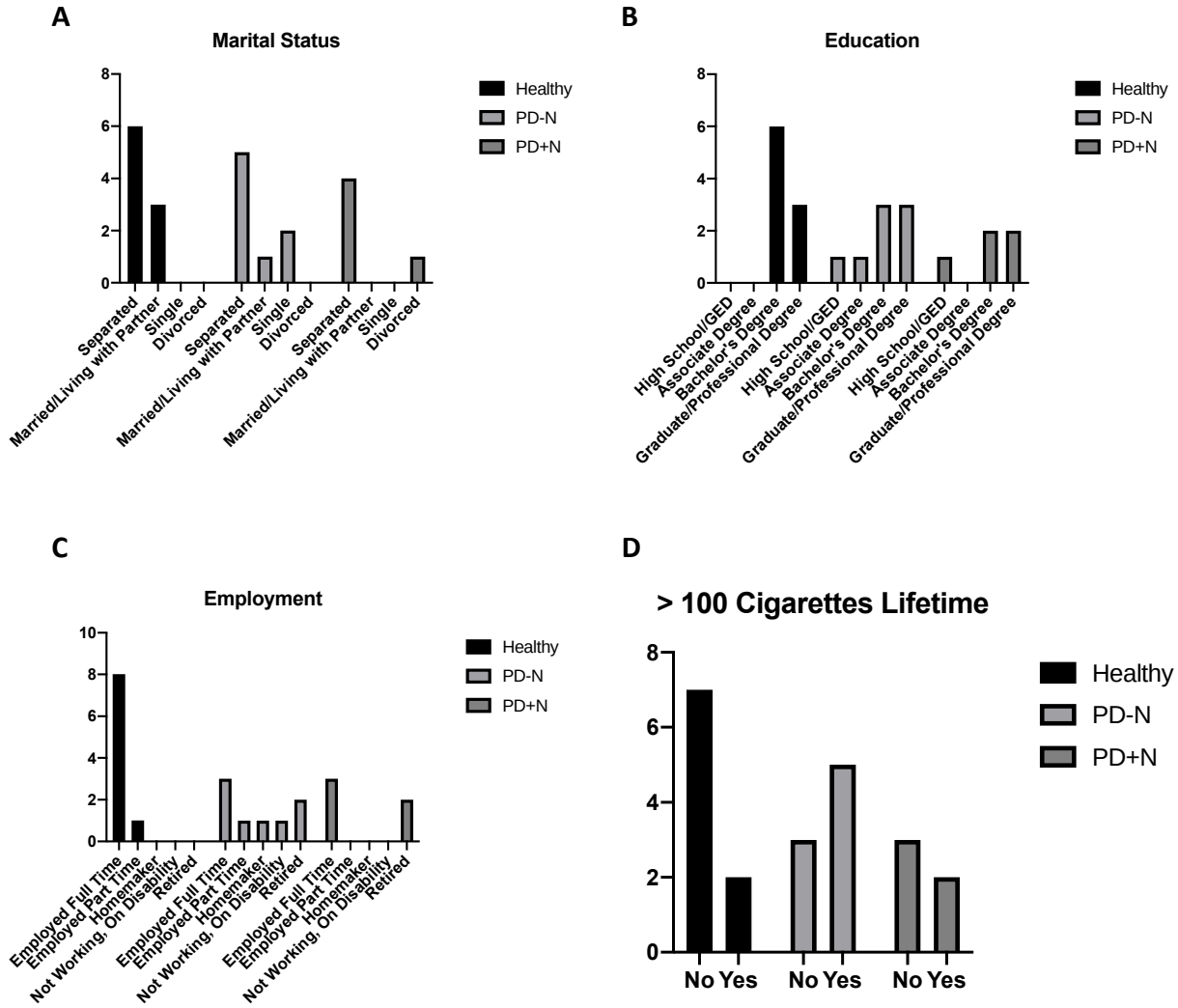
Lifestyle and Reported Smoking History were not Significantly Different Among Groups:

There was no significant difference observed in marital status among the three groups ($p = 0.2184$, $p = 0.1698$, $p = 0.3066$, Fig 15A). There was also no significant difference observed in educational level or degree obtained among the three groups ($p = 0.3992$, $p = 0.3263$, $p = 0.8620$, Fig 15B). There was no significant difference observed in employment status among the three groups ($p = 0.1822$, $p = 0.1056$, $p = 0.6559$, Fig 15C). There was no significant difference observed in whether or not the patient had smoked greater than 100 cigarettes during their lifetime among the three groups ($p = 0.0921$, $p = 0.4805$, $p = 0.4285$, Fig 15D).

Figure 15 Lifestyle and Smoking History

Lifestyle history information collected from patients who completed the clinical study. Reported marital status of patient (A). Reported highest degree obtained by patient (B). Reported employment status of patient (C). Reported lifetime smoking status of patient (D). Data are reported as frequencies and analyzed by chi-square analysis. (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 15


A
Marital Status
Healthy
PD-N
PD+N
Separated
Married/Living with Partner
Single
Divorced
B
Education
High School/GED
Associate Degree
Bachelor's Degree
Graduate/Professional Degree
C
Employment
Employed Full Time
Employed Part Time
Homemaker
Not Working, On Disability
Retired
D
> 100 Cigarettes Lifetime
No
Yes

Prediabetics with Neuropathy More Likely to Have Previous Prediabetes Diagnosis, but Prediabetes without Neuropathy More likely to have Family History of Neuropathy:

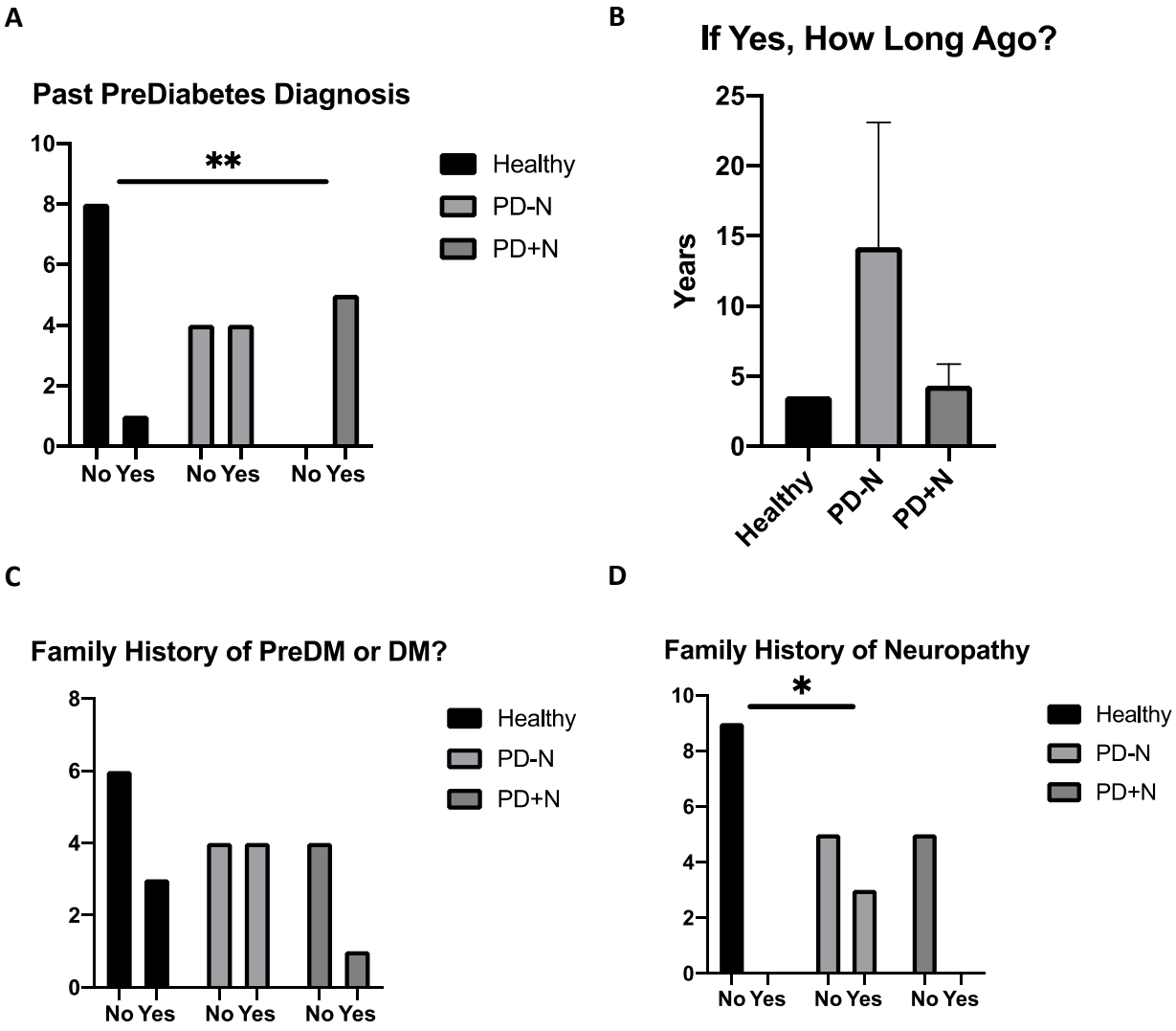
Prediabetic patients with neuropathy were significantly more likely to report a past diagnosis of prediabetes than healthy patients in the study ($p = 0.0013$, Fig 16A). They were not significantly more likely to report a past diagnosis of prediabetes than the prediabetes without neuropathy group ($p = 0.0574$). Prediabetes patients without neuropathy showed no significant difference compared to healthy patients either ($p = 0.0790$). While prediabetic subjects without neuropathy reported having the most years since receiving the prediabetic diagnosis, there was no significant difference among the three groups ($p = 0.4684$, Fig 16B).

When asked about a family history of prediabetes or diabetes there was no significant difference in the reported data among the three groups ($p = 0.4858$, $p = 0.5967$, $p = 0.2794$, Fig 16C). The prediabetic patients without neuropathy were significantly more likely to have a positive family history of neuropathy compared to healthy patients ($p = 0.0429$, Fig 16D). Prediabetic patients with neuropathy did not have a significant increase in family history of neuropathy compared to health patients or to prediabetic patients without neuropathy ($p = 1.0000$, $p = 0.1185$).

Figure 16 Diabetes and Neuropathy History

Diabetes and neuropathy history collected from patient. Whether or not the patient had a previous diagnosis of prediabetes (A). If yes, how many years ago was that diagnosis made? (B). Family history of either prediabetes or diabetes (C). Family history of neuropathy (D). Data are reported as frequencies and analyzed by chi-square analysis (A, C-D). ***P* < 0.05, ****P* < 0.01 Data are means ± SEM, analyzed by one-way ANOVA with Tukey multiple comparisons test (B). (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 16


A
Past PreDiabetes Diagnosis
**
Healthy
PD-N
PD+N
No Yes
No Yes
No Yes
B
If Yes, How Long Ago?
Years
Healthy
PD-N
PD+N
C
Family History of PreDM or DM?
Healthy
PD-N
PD+N
No Yes
No Yes
No Yes
D
Family History of Neuropathy
*
Healthy
PD-N
PD+N
No Yes
No Yes
No Yes

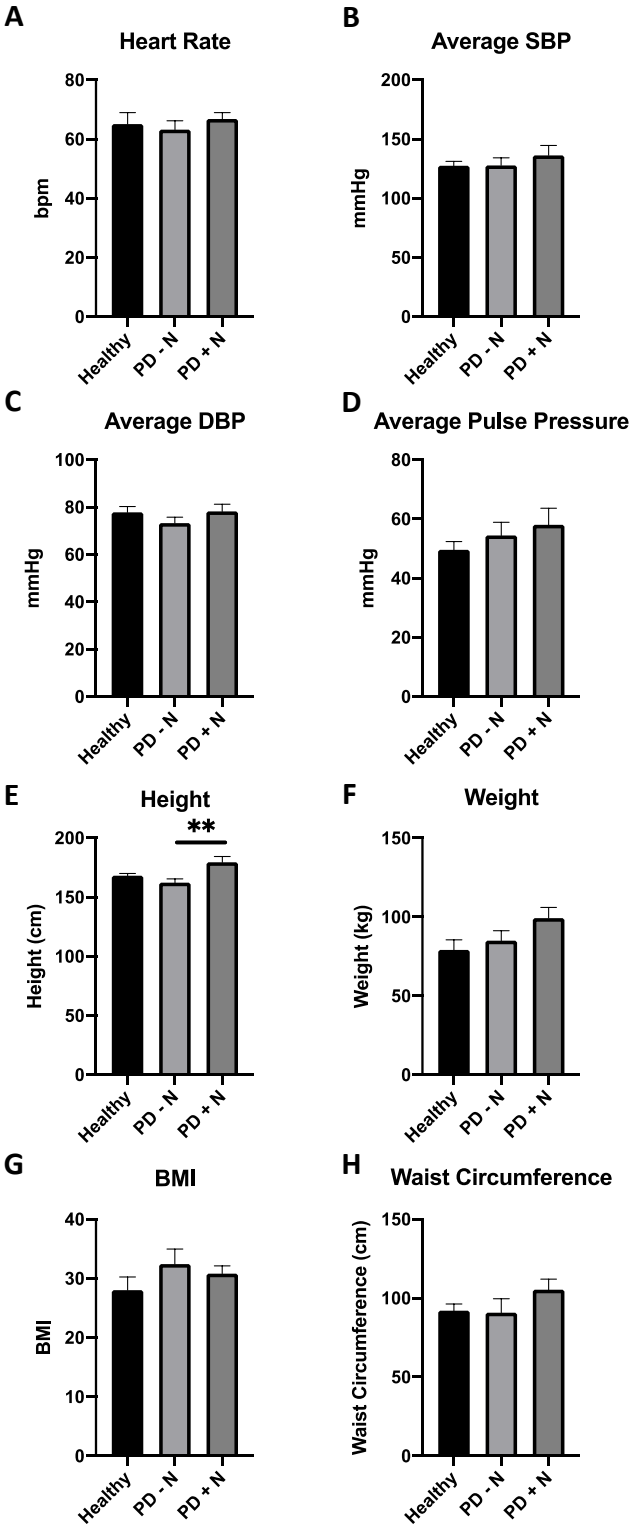
Prediabetics with Neuropathy are Taller than those without Neuropathy:

When heart rate was analyzed by the nursing staff at the CTSU, no significant difference was observed among the three groups ($p = 0.7962$, Fig 17A). When systolic blood pressure was measured via sphygmomanometer, no significant difference was observed among the three groups ($p = 0.5795$, Fig 17B). When diastolic blood pressure was measured via sphygmomanometer, no significant difference was observed among the three groups ($p = 0.3612$, Fig 17C). No significant difference was observed in calculated pulse pressure among the three groups ($p = 0.3865$, Fig 17D). There was a significant difference observed in height between the three groups ($p = 0.0078$, Fig 17E). Prediabetic patients with neuropathy were significantly taller than prediabetic patients without neuropathy ($p = 0.0058$). There was no significant difference in height observed between healthy patients and prediabetics with or without neuropathy (0.0661, 0.3547 respectively). There was no significant difference observed in weight among the three groups ($p = 0.1571$, Fig 17F). There was no significant difference observed in calculated BMI among the three groups ($p = 0.3649$, Fig 17G). There was no significant difference observed in measured waist circumference among the three groups ($p = 0.4235$, Fig 17H).

Figure 17 Physical Measurements and Vitals

Physical measurements and vitals collected by nursing staff at CTSU. Heart rate observed on patient via the radial artery (A). Average systolic blood pressure (SBP) taken from two independent readings on the patient (B). Average diastolic blood pressure (DBP) taken from two independent readings on the patient (C). Average pulse pressure (PP) calculated by formula: PP = SBP – DBP (D). Height measured on patient (E). Weight measured on patient (F). BMI calculated from heigh and weight by the following formula: BMI = Weight *10000/(Height)^2 (G). Waist circumference measured on patient (H). Data are means ± SEM. ****P* < 0.05. (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 17


A
Heart Rate
bpm
80
60
40
20
0
Healthy
PD - N
PD + N
B
Average SBP
mmHg
200
150
100
50
0
Healthy
PD - N
PD + N
C
Average DBP
mmHg
100
80
60
40
20
0
Healthy
PD - N
PD + N
D
Average Pulse Pressure
mmHg
80
60
40
20
0
Healthy
PD - N
PD + N
E
Height
**
Height (cm)
200
150
100
50
0
Healthy
PD - N
PD + N
F
Weight
Weight (kg)
150
100
50
0
Healthy
PD - N
PD + N
G
BMI
BMI
40
30
20
10
0
Healthy
PD - N
PD + N
H
Waist Circumference
Waist Circumference (cm)
150
100
50
0
Healthy
PD - N
PD + N

Presence of System Specific Medical Histories were not Significantly Different Among Groups:

Medical history was reviewed for each organ system in an itemized fashion. The subject reported whether or not they had received a diagnosis for the given organ system and then gave a brief history of the diagnosis. There was no significant difference observed in the presence of a dermatologic diagnosis among the three groups ($p = 0.1758$, Fig 18A). Healthy patients' dermatologic diagnoses included dermatitis and negative mole biopsy, while prediabetics with neuropathy dermatologic diagnoses included two patients with history of melanoma. There was no significant difference observed in the presence of a head, ears, eyes, nose, and throat (HEENT) diagnosis among the three groups ($p = 0.3698$, Fig 18B). Healthy patient's HEENT diagnoses included tonsillectomies; prediabetics without neuropathy included hearing loss, sinusitis, headaches, and glaucoma; and prediabetics with neuropathy included vocal cord polyps and tonsillectomies. There was no significant difference observed in the in the presence of a respiratory diagnosis among the three groups ($p = 0.4297$, Fig 18C). Healthy patient's respiratory diagnoses included asthma and obstructive sleep apnea; prediabetics without neuropathy included sleep apnea and COPD; prediabetics with neuropathy included asthma, sleep apnea, and shortness of breath.

There was no significant difference observed in the presence of a cardiovascular diagnosis among the three groups ($p = 0.7831$, Fig 18D). Healthy patient's cardiovascular diagnoses included pre-eclampsia, patent foramen ovale, and hypertension; prediabetics without neuropathy included tachycardia, hypertension, heart palpitations, coronary artery disease, atrial fibrillation; prediabetics with neuropathy included hypertension and hyperlipidemia.

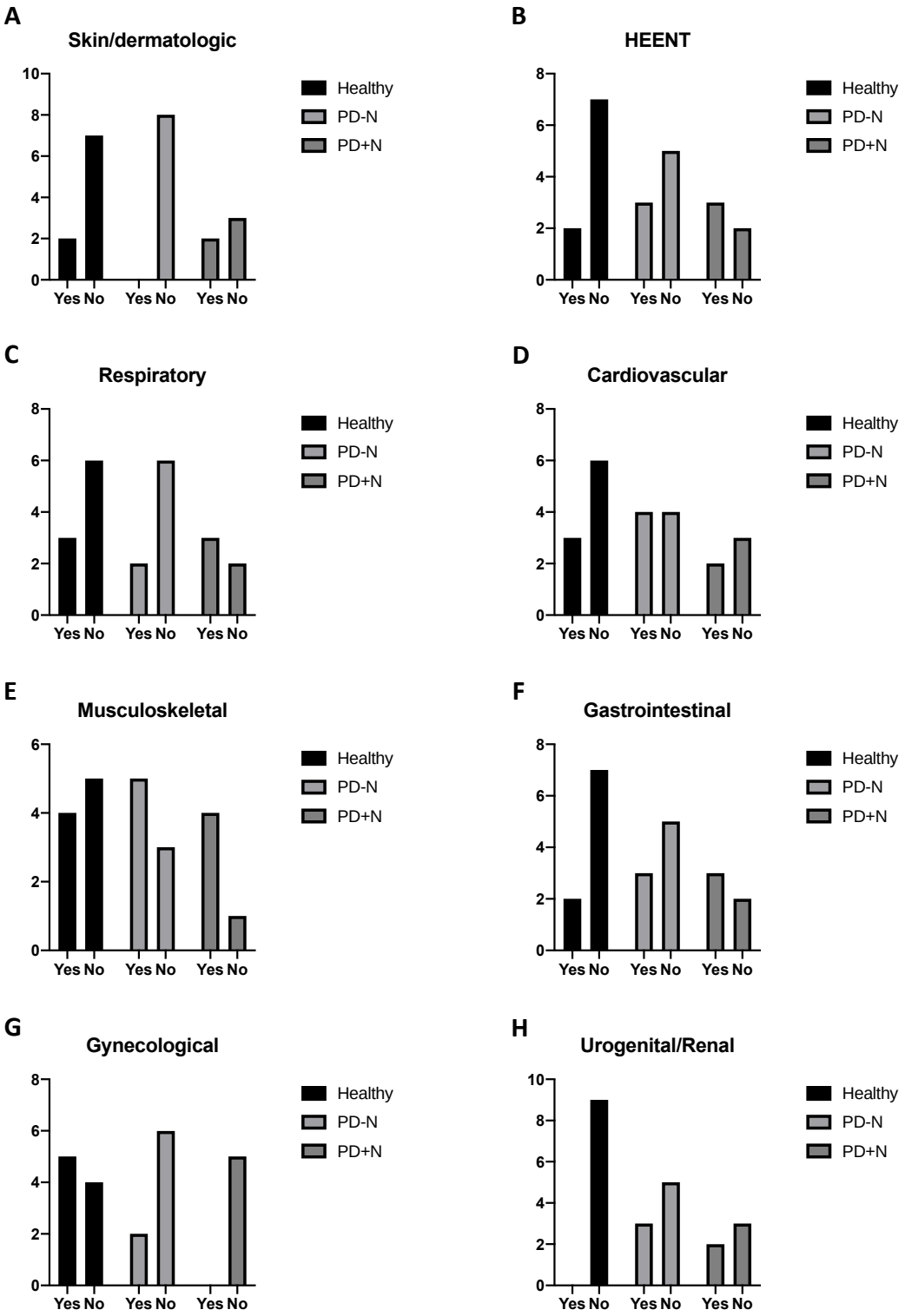
There was no significant difference observed in the presence of a musculoskeletal diagnosis among the three groups ($p = 0.4187$, Fig 18E). Healthy patient's musculoskeletal diagnoses included osteoporosis, osteoarthritis, shoulder dislocation and wrist fracture; prediabetics without neuropathy included fibromyalgia, degenerative disc disease, arthritis and rotary cuff repair; prediabetics with neuropathy included bunion removal, torn ACL repair, trigger finger release, spinal stenosis, knee replacement, and gout. There was no significant difference observed in the presence of a gastrointestinal diagnosis among the three groups ($p = 0.3698$, Fig 18F). Healthy patient's gastrointestinal diagnoses included colitis; prediabetics without neuropathy included irritable bowel syndrome, gastroesophageal reflux disease (GERD), nodule removed from stomach and constipation; prediabetics with neuropathy included GERD, hemorrhoid removal and clostridium difficile. There was no significant difference observed in the presence of a gynecological diagnosis among the three groups ($p = 0.0888$, Fig 18G). Healthy patient's gynecological diagnoses included hysterectomy, menopause, and polyps; prediabetics without neuropathy included partial hysterectomy. There was no significant difference observed in the presence of a urogenital/renal diagnosis among the three groups ($p = 0.1059$, Fig 18H). Prediabetics without neuropathy's urogenital/renal diagnoses included urinary urgency, incontinence, and enlarged prostate; prediabetics with neuropathy included kidney failure, benign prostatic hyperplasia, prostate cancer, and hydronephrosis. There was no significant difference observed in the presence of an endocrine/metabolic diagnosis among the three groups ($p = 0.5985$, Fig 18I). Healthy patient's endocrine/metabolic diagnoses included hypothyroid and hyperlipidemia; prediabetics without neuropathy included hypothyroid and hyperlipidemia; prediabetics with neuropathy included hyperlipidemia. There was no significant difference observed in the presence of a neurological diagnosis among the three groups ($p = 0.3541$, Fig

18J). Healthy patient's neurological diagnoses included migraines. There was no significant difference observed in the presence of a hematopoietic/lymphatic diagnosis among the three groups ($p = 0.4693$, Fig 18K). Healthy patient's hematopoietic/lymphatic diagnoses included immune thrombocytopenic purpura. There was no significant difference observed in the presence of a psychiatric/mental health diagnosis among the three groups ($p = 0.5614$, Fig 18L). Healthy patient's psychiatric/mental health diagnoses included sleep disorder, insomnia, depression and generalized anxiety; prediabetics without neuropathy included anxiety; prediabetics with neuropathy included depression. There was no significant difference observed in the presence of another diagnosis among the three groups ($p = 0.3998$, Fig 18M). There was no significant difference observed in the presence of an allergic/intolerance diagnosis among the three groups ($p = 0.7983$, Fig 18N). Healthy patient's allergic/intolerance diagnoses included seasonal allergies, rodents, cats, dust mites, and nickel; prediabetics without neuropathy included seasonal allergies, peanuts, cabbage, acetaminophen and tetracyclines; prediabetics with neuropathy included seasonal allergies and codeine.

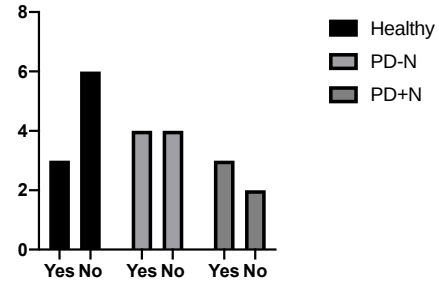
Figure 18 Medical History

Systems based medical history collected from patient by study coordinator at the CTSU clinical visit. Reported dermatologic medical history (A). Reported head, ears, eyes, nose, and throat medical history (B). Reported respiratory medical history (C). Reported cardiovascular history (D). Reported musculoskeletal medical history (E). Reported gastrointestinal medical history (F). Reported gynecological medical history (G). Reported urogenital and renal medical history (H). Reported endocrine and metabolic medical history (I). Reported neurological medical history (J). Reported hematopoietic and lymphatic medical history (K). Reported psychiatric or mental health medical history (L). Reported other medical history (M). Reported allergic and intolerance medical history (N). Data are reported as frequencies and analyzed by chi-square analysis (A-N). (Healthy n = 9, PD-N n = 8, PD+N n = 5)

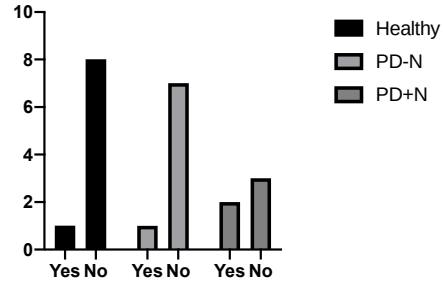
Figure 18


A
Skin/dermatologic
B
HEENT
C
Respiratory
D
Cardiovascular
E
Musculoskeletal
F
Gastrointestinal
G
Gynecological
H
Urogenital/Renal
Healthy
PD-N
PD+N
Yes No
Yes No
Yes No

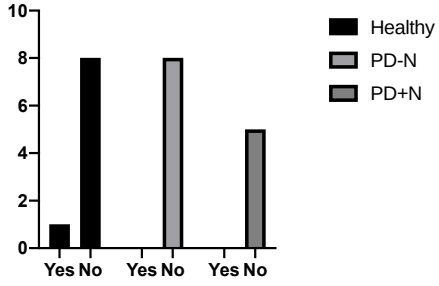
I


Endocrine/Metabolic
8
6
4
2
0
Yes No Yes No Yes No
Healthy
PD-N
PD+N

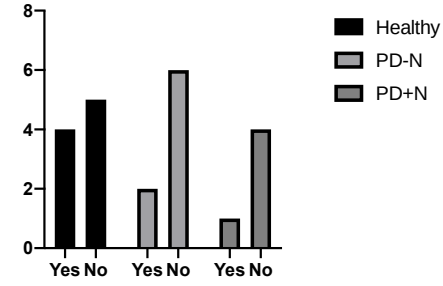
J


Neurological
10
8
6
4
2
0
Yes No Yes No Yes No
Healthy
PD-N
PD+N

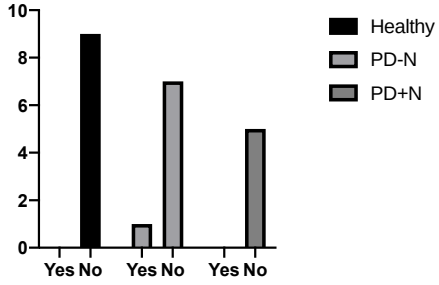
K


Hematopoetic/Lymphatic
10
8
6
4
2
0
Yes No Yes No Yes No
Healthy
PD-N
PD+N

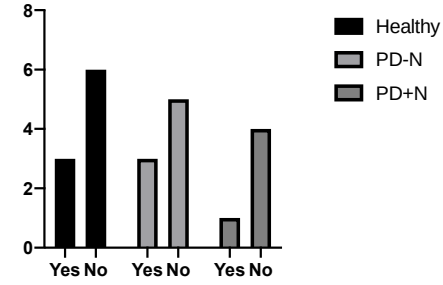
L


Psychiatric/Mental Health
8
6
4
2
0
Yes No Yes No Yes No
Healthy
PD-N
PD+N

M


Other
10
8
6
4
2
0
Yes No Yes No Yes No
Healthy
PD-N
PD+N

N


Allergic/Intolerance
8
6
4
2
0
Yes No Yes No Yes No
Healthy
PD-N
PD+N

Prediabetic Patients Displayed Relative Hyperglycemia During Fasting and OGTT:

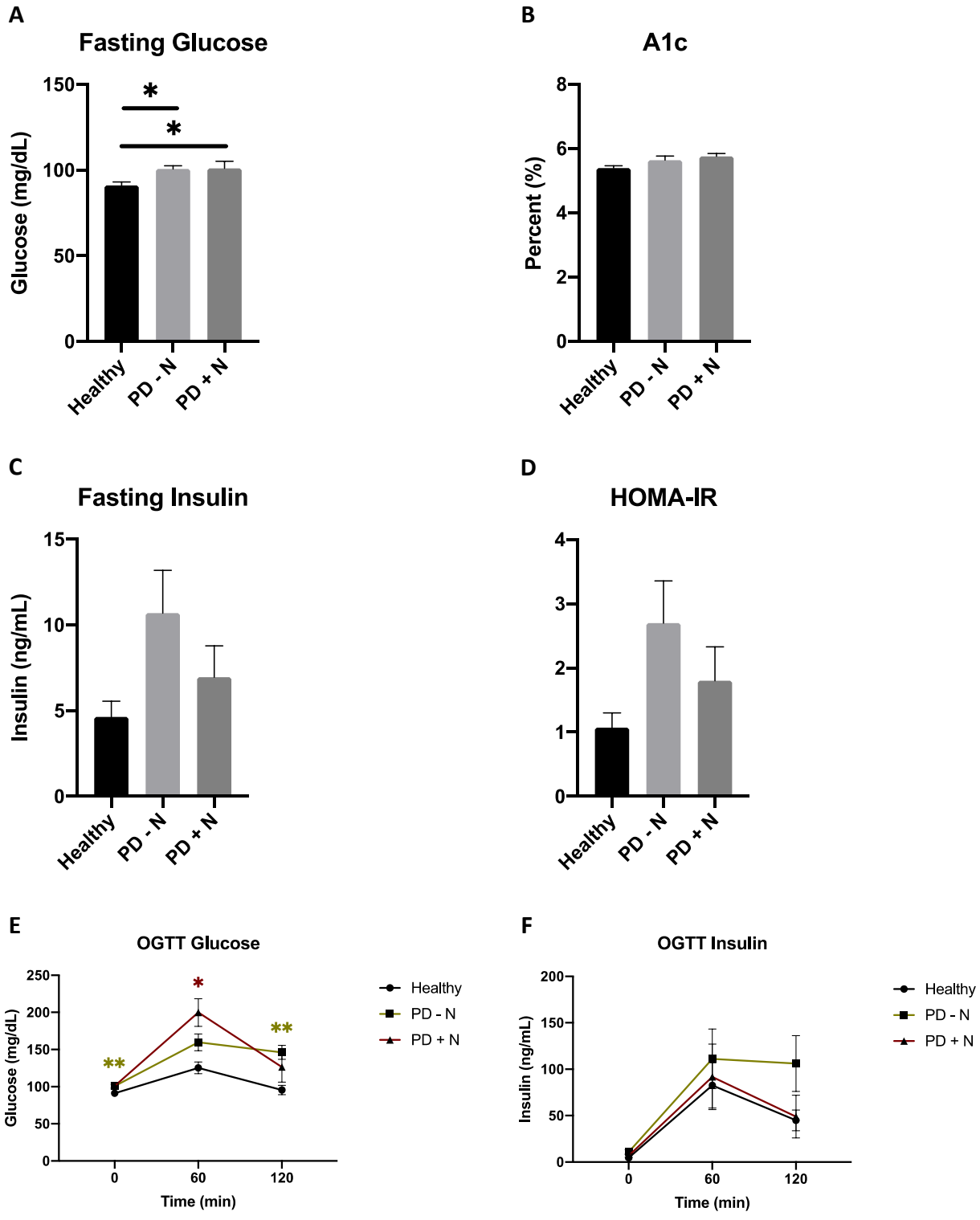
Prediabetic patients without neuropathy exhibited significantly higher fasting blood glucose compared to healthy patients ($p = 0.0192$, Fig 19A). Prediabetic patients with neuropathy also displayed significantly higher fasting blood glucose compared to healthy patients ($p = 0.0349$). Prediabetic patients with and without neuropathy did not have significantly different fasting blood glucose levels ($p = 0.9944$). No significant difference was observed in hemoglobin A1c measurements among the three groups ($p = 0.0500$, Fig 19B). No significant difference was observed in fasting insulin among the three groups ($p = 0.0829$, Fig 19C). No significant difference was observed in HOMA-IR, a measure of insulin resistance, among the three groups ($p = 0.0787$, Fig 19D).

During the oral glucose tolerance test prediabetic patients without neuropathy were found to have significantly elevated glucose at time point 0 minutes and 120 minutes ($p = 0.0079$, $p = 00.17$ respectively, Fig 19E). Prediabetic patients with neuropathy had significantly elevated glucose at time point 60 minutes ($p = 0.0281$). During the oral glucose tolerance test insulin level did not vary significantly among the three patient groups ($p = 0.3154$, Fig 19F).

Figure 19 Laboratory Analysis of Blood Draws – Glucose and Insulin

Results of laboratory analysis from blood draws completed on patient. Fasting glucose value of patient (A). Hemoglobin A1c value of patient (B). Fasting insulin value of patient (C). Homeostatic Model Assessment for Insulin Resistance calculated by the formula: HOMA-IR = Fasting Glucose * Fasting Insulin / 22.5 (D). Glucose values recording throughout the oral glucose tolerance test (E). Insulin values recorded throughout the oral glucose tolerance test (F). Data are means ± SEM. ***P* < 0.05, ****P* < 0.01. Data analyzed via one-way ANOVA and two-way ANOVA with repeated measures. Post-hoc analysis performed via Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 19


A
Fasting Glucose
Glucose (mg/dL)
150
100
50
0
*
*
Healthy
PD - N
PD + N
B
A1c
Percent (%)
8
6
4
2
0
Healthy
PD - N
PD + N
C
Fasting Insulin
Insulin (ng/mL)
15
10
5
0
Healthy
PD - N
PD + N
D
HOMA-IR
4
3
2
1
0
Healthy
PD - N
PD + N
E
OGTT Glucose
Glucose (mg/dL)
250
200
150
100
50
0
*
**
**
0
60
120
Time (min)
Healthy
PD - N
PD + N
F
OGTT Insulin
Insulin (ng/mL)
200
150
100
50
0
0
60
120
Time (min)
Healthy
PD - N
PD + N

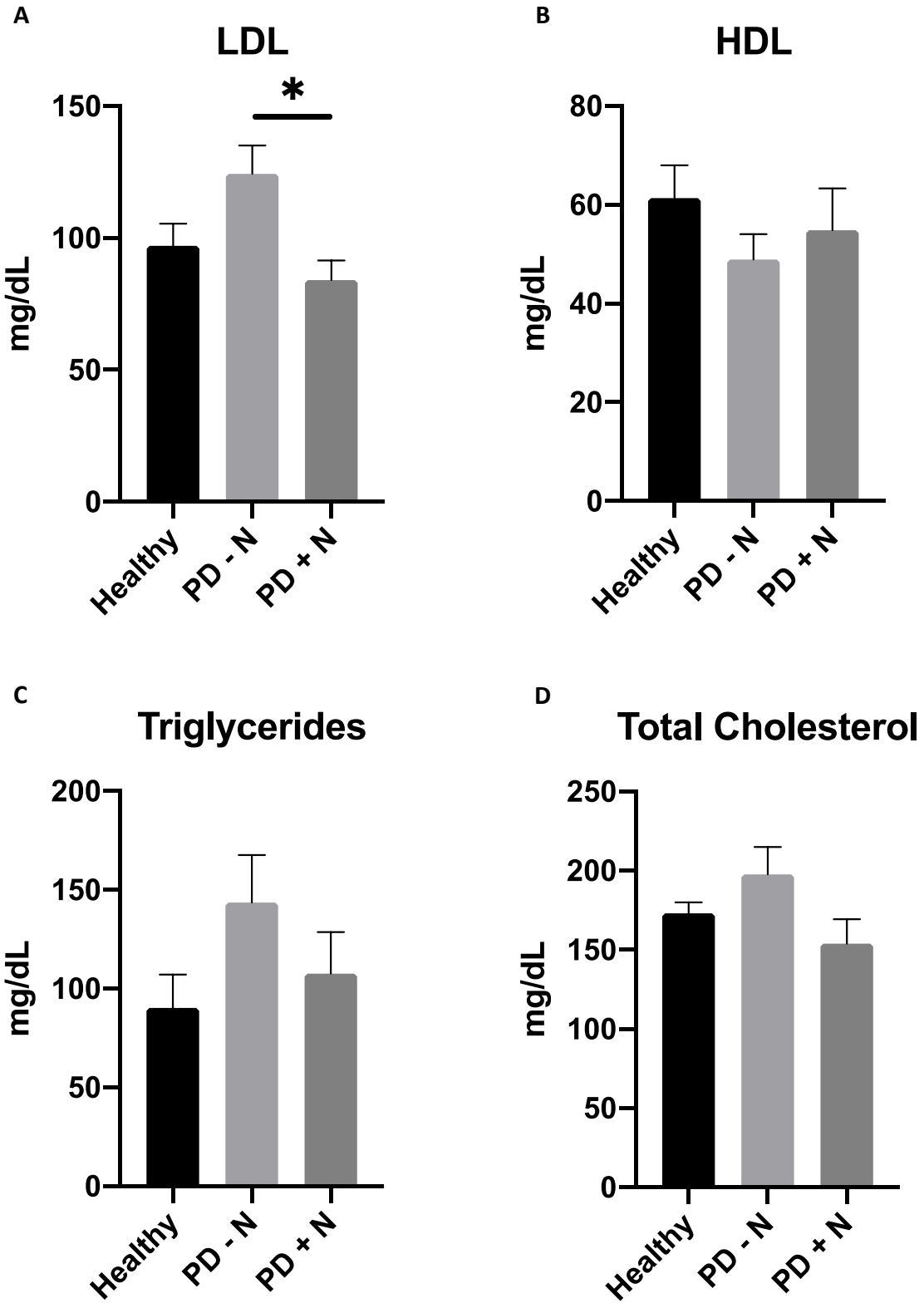
Neuropathy is Associated with Lower LDL Levels in Prediabetic Patients:

Patients were fasting during for 12 hours prior to the lipid panel blood test. Prediabetic patients without neuropathy had significantly higher levels of LDL compared to prediabetic patients with neuropathy ($p = 0.0341$, Fig 20A). Healthy patients displayed no significant difference in LDL levels compared to prediabetics with or without neuropathy ($p = 0.6465$, $p = 0.1030$ respectively). There was no significant difference observed in HDL levels between the three groups ($p = 0.3765$, Fig 20B). There was no significant difference observed in triglyceride levels between the three groups ($p = 0.1755$, Fig 20C). There was also no significant difference observed in total cholesterol levels among the three groups ($p = 0.1180$, Fig 20D).

Figure 20 Laboratory Analysis of Blood Draws – Lipid Panel

Results of laboratory analysis of blood lipid panel. Low density lipoprotein value of patient (A). High density lipoprotein value of patient (B). Triglycerides values of patient (C). Total cholesterol value of patient (D). Data are means ± SEM. ***P* < 0.05. Data analyzed by Data analyzed via one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 20


A
LDL
*
150
100
50
0
mg/dL
Healthy
PD - N
PD + N
B
HDL
80
60
40
20
0
mg/dL
Healthy
PD - N
PD + N
C
Triglycerides
200
150
100
50
0
mg/dL
Healthy
PD - N
PD + N
D
Total Cholesterol
250
200
150
100
50
0
mg/dL
Healthy
PD - N
PD + N

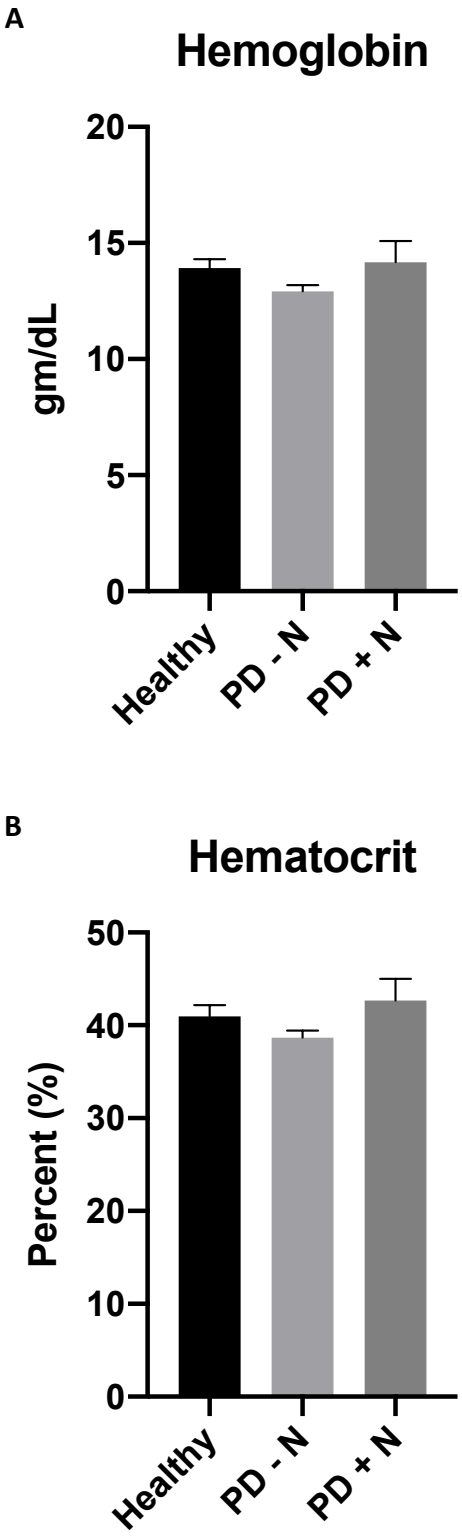
Prediabetes with or without Neuropathy has no Effect on Erythrocyte Status:

There was no significant difference observed in hemoglobin between the three groups ($p = 0.1791$, Fig 21A). There was also no significant difference observed in hematocrit between the three groups ($p = 0.1592$, Fig 21B).

Figure 21 Laboratory Analysis of Blood Draws – Hemoglobin & Hematocrit

Results of laboratory analysis of hemoglobin and hematocrit values. Hemoglobin value of patient (A). Hematocrit value of patient (B). Data are means ± SEM. Data analyzed via one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 21


A
Hemoglobin
gm/dL
20
15
10
5
0
Healthy
PD - N
PD + N
B
Hematocrit
Percent (%)
50
40
30
20
10
0
Healthy
PD - N
PD + N

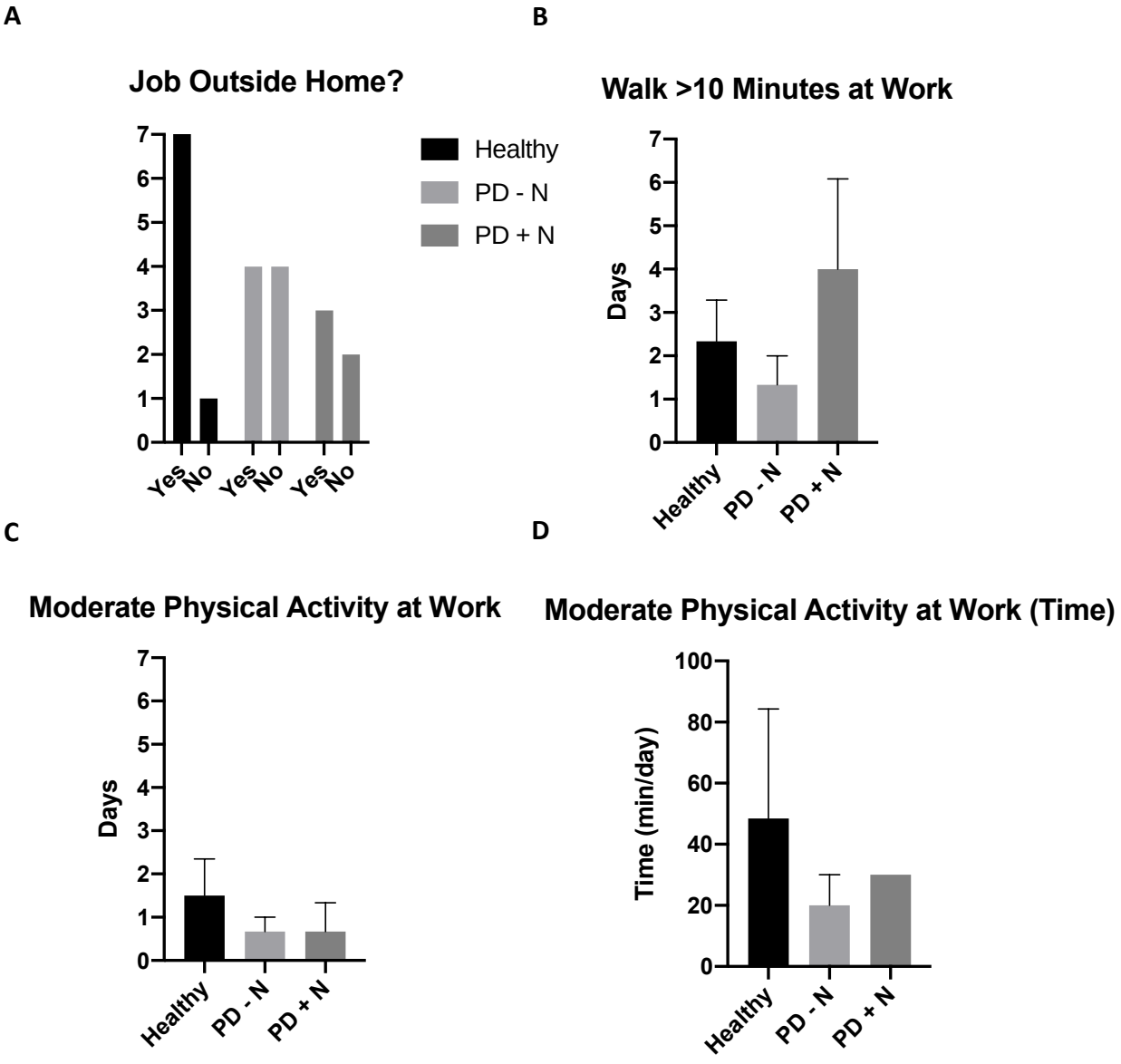
Prediabetics with or without Neuropathy are not More Sedentary at Work:

Subjects completed the International Physical Activity Questionnaire (IPAQ) to evaluate whether their physical activity status at their job correlated with their prediabetes or neuropathy status. The IPAQ is a widely used questionnaire that has shown to be reliable and valid in diverse study populations of adults [306-308]. There was no significant difference observed in the responses of the subjects when asked whether or not they had a job outside their home ($p = 0.0790$, $p = 0.2069$, $p > 0.9999$, Fig 22A). There was no significant difference observed in the responses of the subjects when asked if they walked more than ten minutes per day at work ($p = 0.4462$, Fig 22B). There was no significant difference observed in the responses of the subjects when asked how many days per week they performed moderate physical activity at work ($p = 0.6959$, Fig 22C). There was no significant difference observed in the responses of the subjects when asked how much time they spent per day performing moderate physical activity at work ($p = 0.8360$, Fig 22D).

Figure 22 IPAQ: Job-Related Physical Activity

Responses to the International Physical Activity Questionnaire: Job-Related Physical Activity section, completed at CTSU clinical visit. Whether or not the subject worked a job outside their own home? (A). Days per week the subject walked more than 10 minutes while at work (B). Days per week the subject performed moderate physical activity at work, defined as: “activities that take moderate physical effort and make you breathe somewhat harder than normal” (C). Minutes per day the subject performed moderate physical activity at work. Data are reported as frequencies and analyzed by chi-square analysis (A). Data are means ± SEM, analyzed by one-way ANOVA with Tukey multiple comparisons test (B-D). (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 22


A
Job Outside Home?
Healthy
PD - N
PD + N
Yes
No
B
Walk >10 Minutes at Work
Days
Healthy
PD - N
PD + N
C
Moderate Physical Activity at Work
Days
D
Moderate Physical Activity at Work (Time)
Time (min/day)

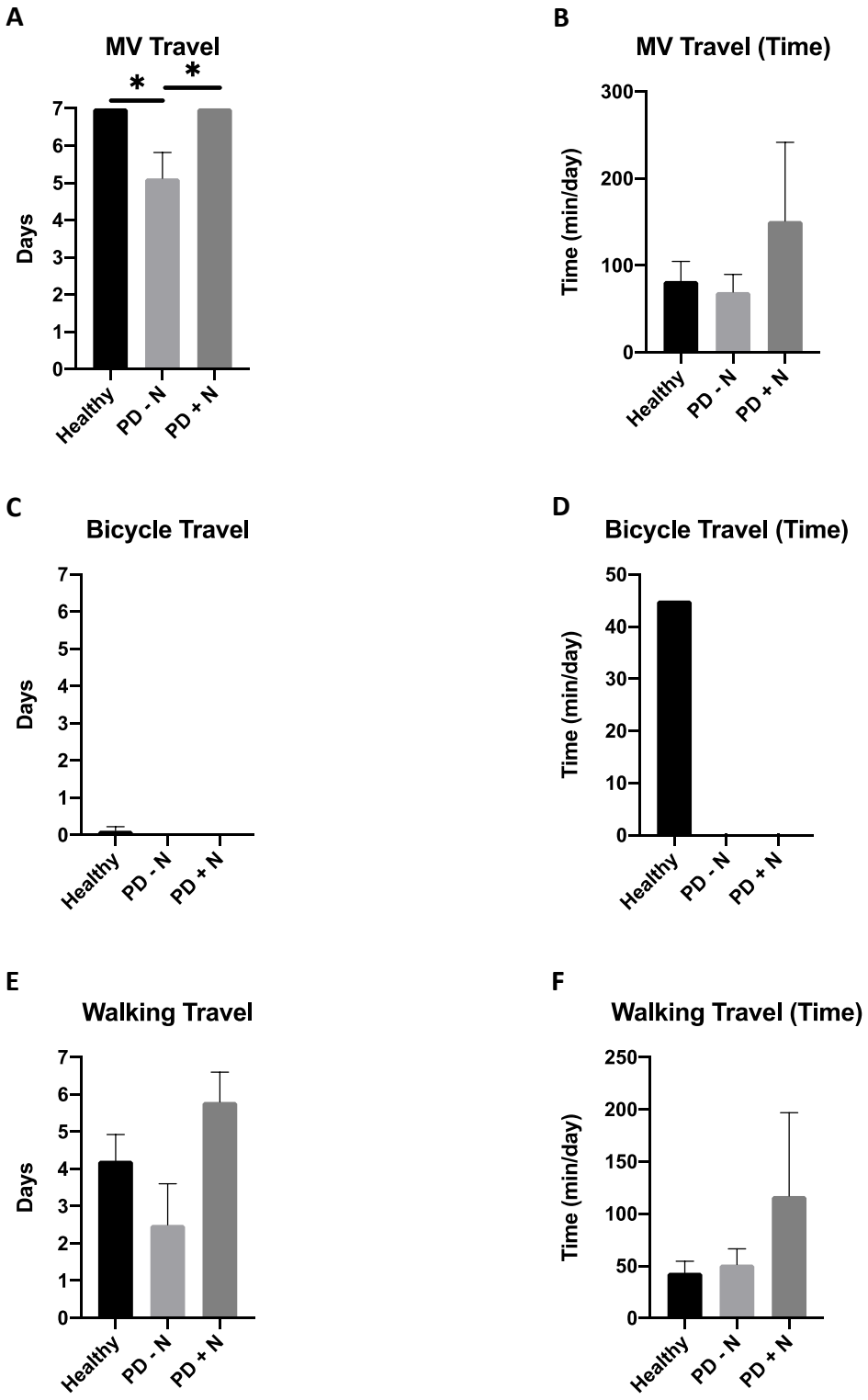
Prediabetics with Neuropathy Spent More Days per Week Traveling by Motor Vehicle:

Subjects completed the transportation portion of the International Physical Activity Questionnaire (IPAQ) to evaluate whether their transportation preferences correlated with their prediabetic or neuropathy status. A significant difference was observed in the number of days spent traveling by motor vehicle between the prediabetics with neuropathy and the prediabetics without neuropathy. The prediabetics spent significantly more days per week traveling by motor vehicle ($p = 0.0316$, Fig 23A). However, healthy patients also were observed to spend significantly more days per week traveling by motor vehicle than prediabetics without neuropathy ($p = 0.0113$). There was no significant difference observed in time spent per day traveling by motor vehicle between the three groups ($p = 0.4044$, Fig 23B). However, prediabetics with neuropathy trended towards spending more time per day traveling by motor vehicle. Only one patient reported bicycle travel greater than 10 minutes, therefore significant difference in bicycle transportation could not be determined (Fig 23C-D). It is noted that the one individual who reported bicycle travel was in the healthy subjects group. No significant difference was observed in the number of days spent walking more than 10 minutes at a time ($p = 0.4435$, Fig 23E). There was also no significant difference observed in the time spent walking per day among the three groups ($p = 0.3922$, Fig 23F).

Figure 23 IPAQ: Transportation-Related Physical Activity

Responses to the International Physical Activity Questionnaire: Transportation-Related Physical Activity section, completed at CTSU clinical visit. Days per week the subject traveled in a motor vehicle such as a train, bus, car, or tram (A). Minutes per day the subject spent traveling in a motorized vehicle (B). Days per week the subject bicycled for at least 10 minutes at a time to go from place to place (C). Minute per day the subject spent bicycling from place to place (D). Days per week the subject walked for at least 10 minutes at a time to go from place to place (E). Minutes per day the subject spent walking from place to place (F). Data are means ± SEM. ***P* < 0.05. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 23


A
MV Travel
*
*
Days
Healthy
PD - N
PD + N
B
MV Travel (Time)
Time (min/day)
Healthy
PD - N
PD + N
C
Bicycle Travel
Days
Healthy
PD - N
PD + N
D
Bicycle Travel (Time)
Time (min/day)
Healthy
PD - N
PD + N
E
Walking Travel
Days
Healthy
PD - N
PD + N
F
Walking Travel (Time)
Time (min/day)
Healthy
PD - N
PD + N

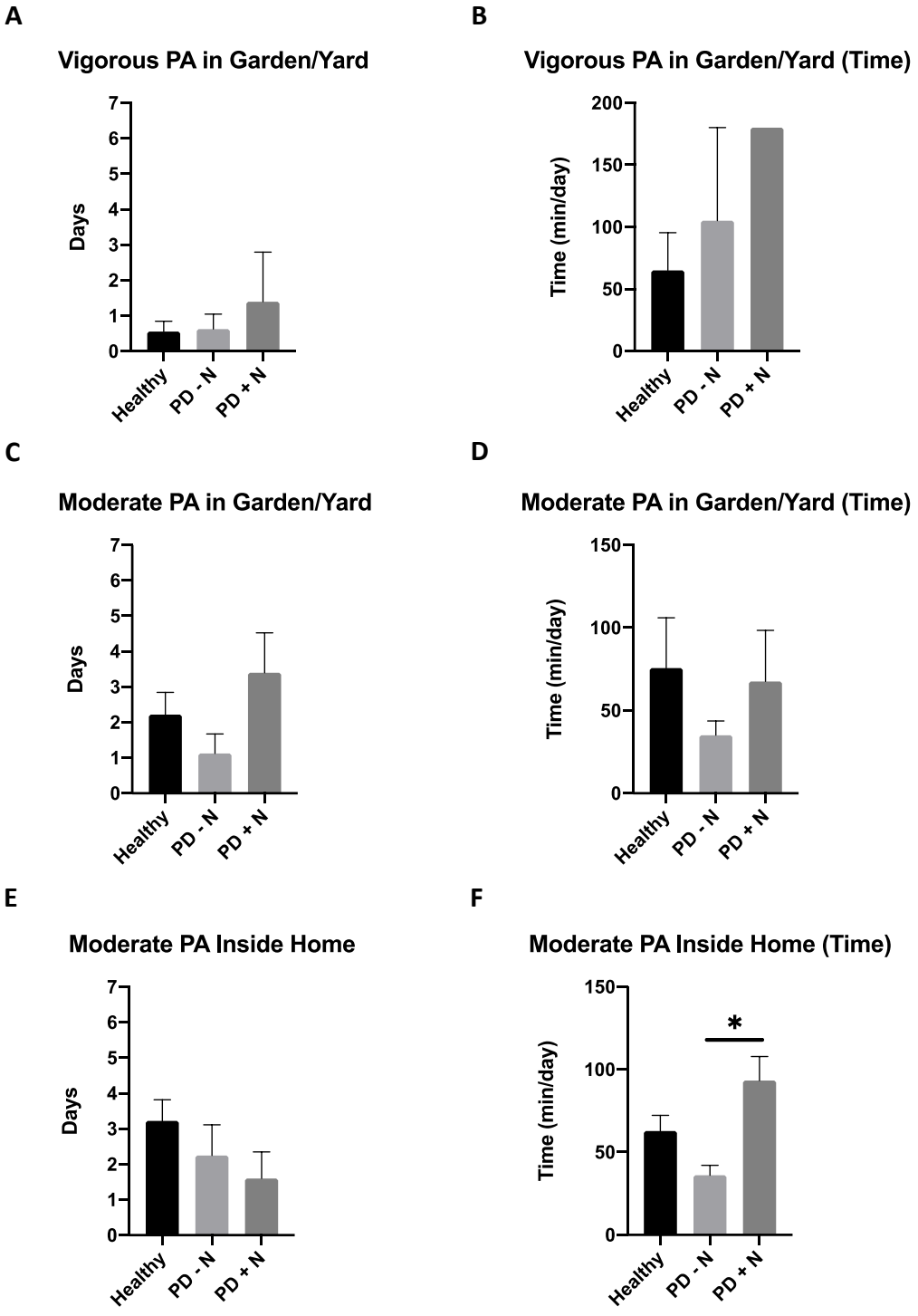
Prediabetics with Neuropathy Spent More Time per Day Performing Moderate Physical Activity Inside the Home:

Subjects completed the "Housework, House Maintenance, and Caring for Family" portion of the International Physical Activity Questionnaire (IPAQ) to evaluate whether their physical activity in the home setting preferences correlated with their prediabetic or neuropathy status. There was no significant difference observed in days per week the subjects spent performing at least 10 minutes of vigorous activity at a time in the garden or yard among the three groups ($p = 0.6499$, Fig 24A). There was no significant difference observed in the time spent per day performing vigorous activity in the garden or yard among the three groups ($p = 0.4939$, Fig 24B). There was no significant difference observed in days per week the subjects spent performing at least 10 minutes of vigorous activity at a time in the garden or yard among the three groups ($p = 0.1373$, Fig 24C). There was no significant difference observed in the time spent per day performing vigorous activity in the garden or yard among the three groups ($p = 0.6119$, Fig 24D). There was also no significant difference observed in the days per week the subject spent performing at least 10 minutes of moderate activity at a time inside the home ($p = 0.3462$, Fig 24E). However, prediabetics with neuropathy reported spending significantly more time per day performing moderate activity inside the home than prediabetics without neuropathy ($p = 0.0173$, Fig 24F).

Figure 24 IPAQ: Housework, House Maintenance, and Caring for Family

Responses to the International Physical Activity Questionnaire: Housework, House Maintenance, and Caring for Family section, completed at CTSU clinical visit. Days per week the subject performed vigorous physical activity in the garden or yard, defined by: “activities that take hard physical effort and make you breathe much harder than normal” (A). Minutes per day the subject performed vigorous physical activity in the garden or yard (B). Days per week the subject performed moderate physical activity in the garden or yard, defined by: “activities that take moderate physical effort and make you breathe somewhat harder than normal” (C). Minutes per day the subject performed moderate physical activity in the garden or yard (D). Days per week the subject performed moderate physical activity inside the home. Minutes per day the subject performed moderate physical activity inside the home. Data are means ± SEM. ***P* < 0.05. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 24


A
Vigorous PA in Garden/Yard
Days
0
1
2
3
4
5
6
7
Healthy
PD - N
PD + N
B
Vigorous PA in Garden/Yard (Time)
Time (min/day)
0
50
100
150
200
Healthy
PD - N
PD + N
C
Moderate PA in Garden/Yard
Days
0
1
2
3
4
5
6
7
Healthy
PD - N
PD + N
D
Moderate PA in Garden/Yard (Time)
Time (min/day)
0
50
100
150
Healthy
PD - N
PD + N
E
Moderate PA Inside Home
Days
0
1
2
3
4
5
6
7
Healthy
PD - N
PD + N
F
Moderate PA Inside Home (Time)
Time (min/day)
0
50
100
150
*
Healthy
PD - N
PD + N

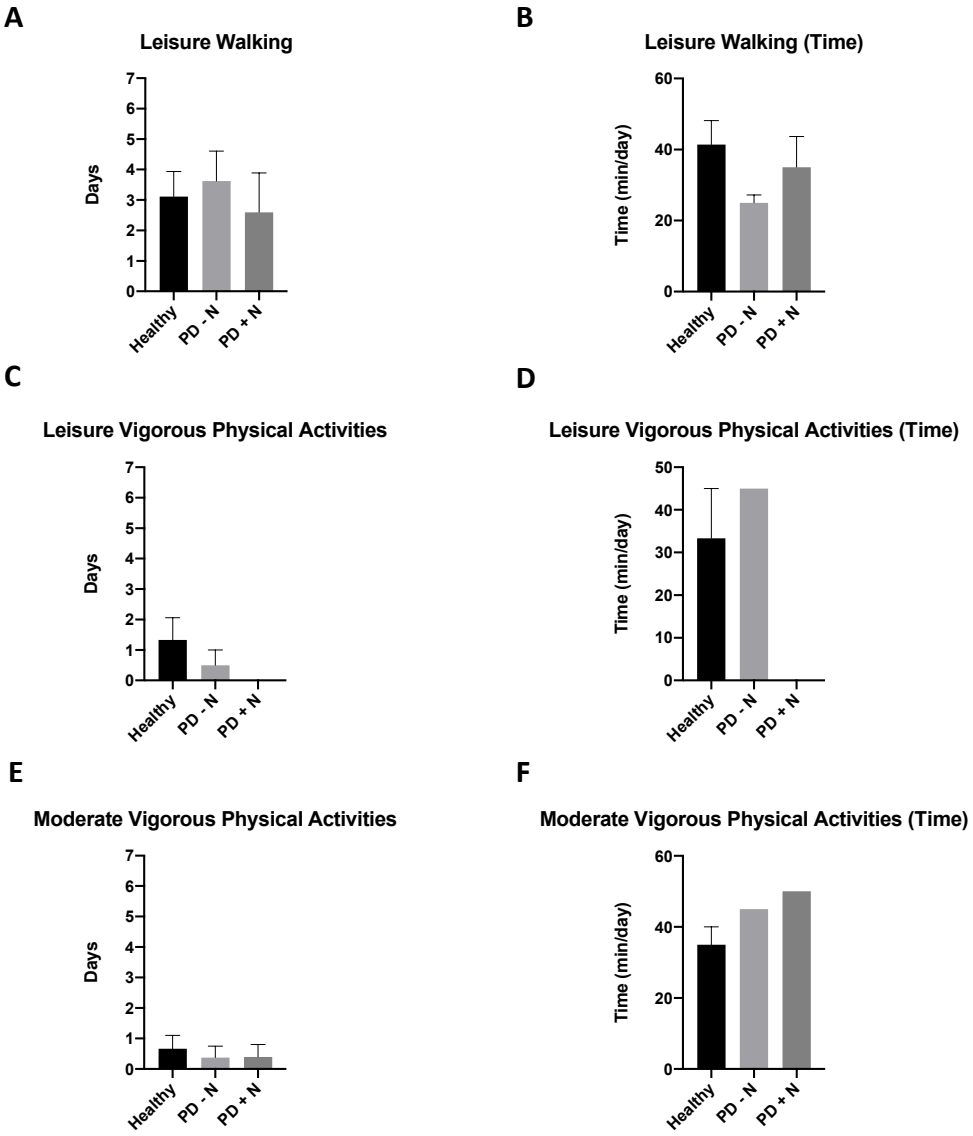
Prediabetics with or without Neuropathy do not Spend Less Time in Sports, Recreation, or Leisure-Time Physical Activities:

Subjects completed the "Recreation, Sport, and Leisure-Time Physical" portion of the International Physical Activity Questionnaire (IPAQ) to evaluate whether their physical activity during these activities correlated with their prediabetic or neuropathy status. There was no significant difference observed in reported days per week spent walking during leisure time among the three groups ($p = 0.7958$, Fig 25A). There was also no significant difference observed in time per day spent walking during leisure time among the three groups ($p = 0.1607$, Fig 25B). There was no significant difference observed in days per week spent in vigorous physical activities during leisure time among the three groups ($p = 0.3339$, Fig 25C). There was also no significant difference observed in time per day spent in vigorous physical activities during leisure time among the three groups ($p = 0.4146$, Fig 25D). There was no significant difference observed in days per week spent in moderate physical activities during leisure time among the three groups ($p = 0.8524$, Fig 25E). There was also no significant difference observed in time per day spent in moderate physical activities during leisure time among the three groups ($p = 0.4286$, Fig 25F).

Figure 25 IPAQ: Recreation, Sport, and Leisure-Time Physical Activity

Responses to the International Physical Activity Questionnaire: Recreation, Sport, and Leisure-Time Physical section, completed at CTSU clinical visit. Days per week the subject walked at least 10 minutes during leisure-time (A). Minutes per day the subject walked during leisure-time (B). Days per week the subject performed vigorous physical activities during leisure-time (C). Minutes per day the subject performed vigorous physical activities during leisure-time (D). Days per week the subject performed moderate physical activities during leisure-time (E). Minutes per day the subject performed moderate physical activities during leisure-time (F). Data are means ± SEM. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 25


A
Leisure Walking
Days
Healthy
PD - N
PD + N
B
Leisure Walking (Time)
Time (min/day)
C
Leisure Vigorous Physical Activities
D
Leisure Vigorous Physical Activities (Time)
E
Moderate Vigorous Physical Activities
F
Moderate Vigorous Physical Activities (Time)

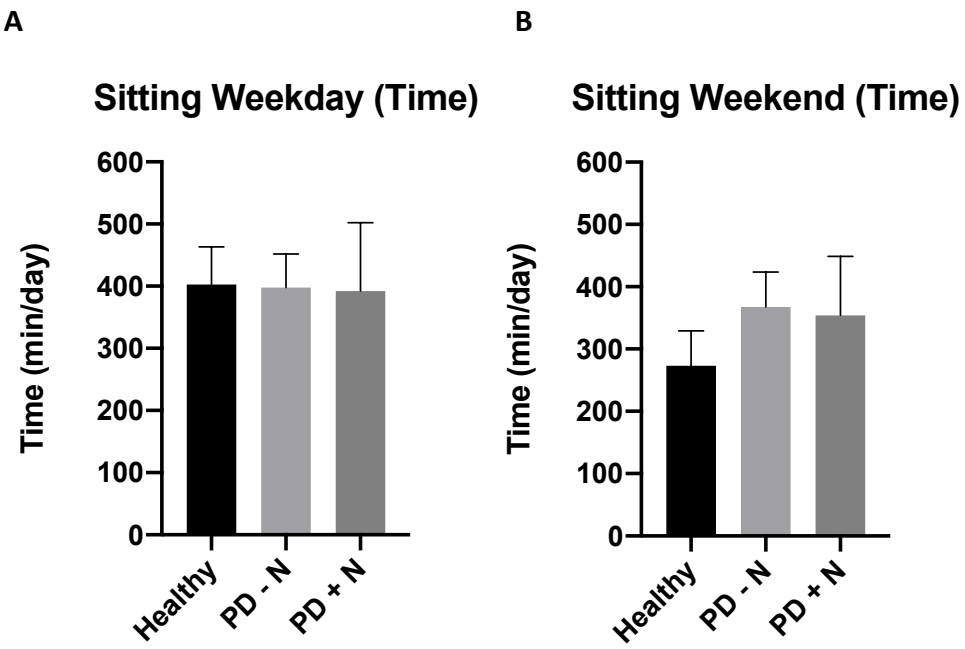
Prediabetics with or without Neuropathy do not Spend More Time in Sitting:

Subjects completed the "Time Spent Sitting" portion of the International Physical Activity Questionnaire (IPAQ) to evaluate whether their weekly sitting correlated with their prediabetic or neuropathy status. There was no significant difference observed in time spent sitting per weekday among the three groups ($p = 0.9946$, Fig 26A). There was also no significant difference in time spent sitting per day on the weekend between the three groups ($p = 0.5809$, Fig 26B).

Figure 26 IPAQ: Time Spent Sitting

Responses to the International Physical Activity Questionnaire: Time Spent Sitting section, completed at CTSU clinical visit. Minutes per day spent sitting on a weekday (A). Minutes per day spent sitting on a weekend (B). Data are means ± SEM. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 26


A
Sitting Weekday (Time)
Time (min/day)
600
500
400
300
200
100
0
Healthy
PD - N
PD + N
B
Sitting Weekend (Time)
Time (min/day)
600
500
400
300
200
100
0
Healthy
PD - N
PD + N

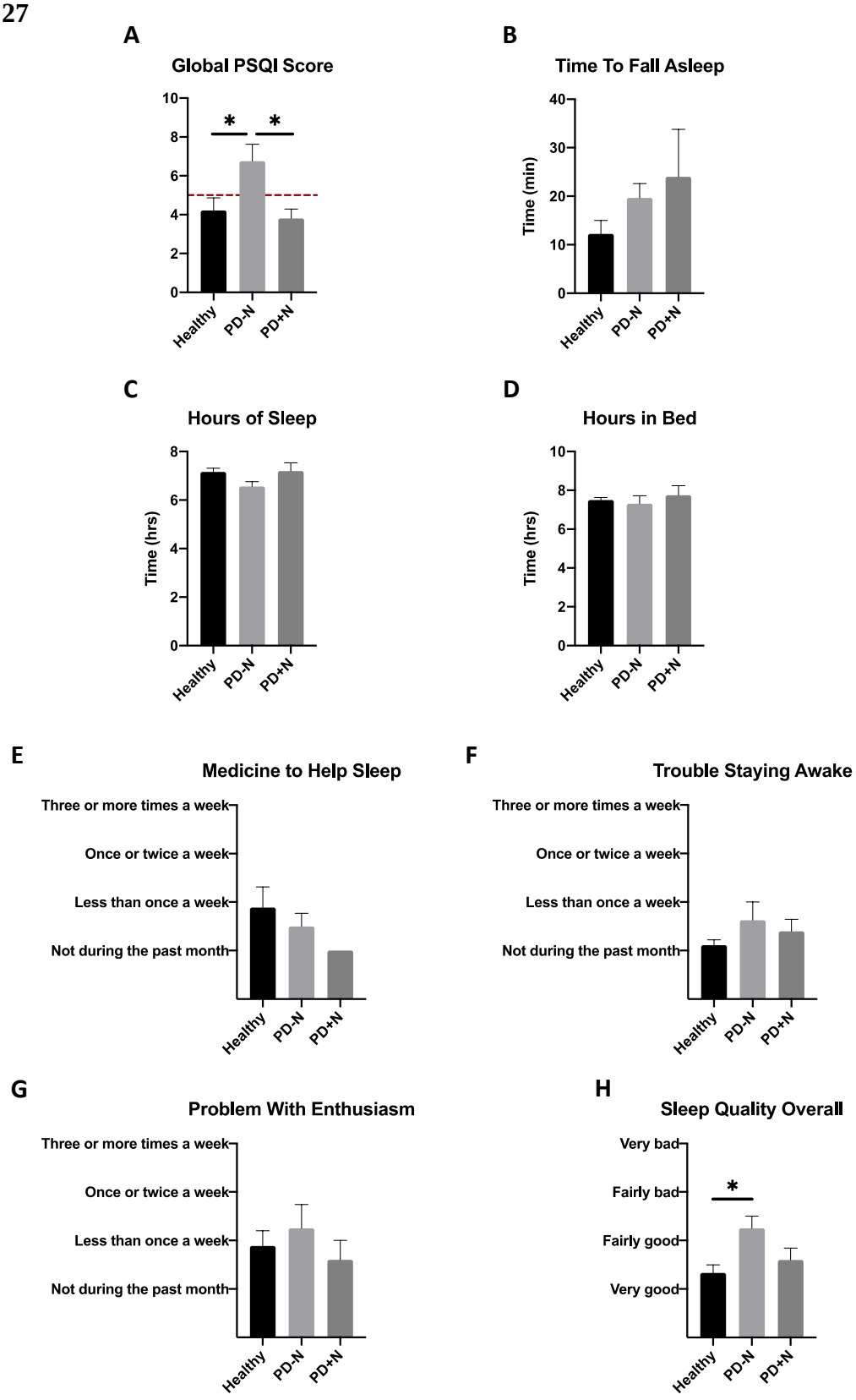
Prediabetics without Neuropathy Report Higher Levels of Sleep Dysfunction:

Study subjects completed The Pittsburgh Sleep Quality Index (PSQI) to evaluate their sleep quality parameters. The PSQI is a commonly used as a screening tool for sleep dysfunction in human patients that has shown strong validity and reliability [309, 310]. Prediabetics without neuropathy displayed significantly higher global PSQI scores than prediabetics with neuropathy and healthy subjects ($p = 0.0484$, $p = 0.0471$ respectively, Fig 27A). Prediabetics with neuropathy, however, did not demonstrate significantly different global PSQI scores than healthy subjects ($p = 0.9261$). There was no significant difference observed in time reported to fall asleep among the three groups ($p = 0.2529$, Fig 27B). There was no significant difference observed in reported hours of sleep or total hours in bed between the three groups ($p = 0.0776$, $p = 0.7078$ respectively, Fig 27C-D). There was no significant difference observed in whether or not medicine was needed to help sleep between the three groups ($p = 0.2609$, Fig 27E). There was no significant difference observed in whether or not the subject had trouble staying awake during the day between the three groups ($p = 0.3613$, Fig 27F). There was no significant difference observed in having problems with enthusiasm ($p = 0.5859$, Fig 27G). Prediabetics without neuropathy reported significantly worse sleep quality overall than healthy patients ($p = 0.0131$, Fig 27H).

Figure 27 Pittsburgh Sleep Quality Index (PSQI) Results

Responses to the Pittsburg Sleep Quality Index (PSQI), completed at CTSU clinical visit. Calculated global PSQI score (A). Reported time to sleep initiation (B). Reported total hours of daily sleep (C). Reported total time spent in bed daily (D). Reported use of pharmacologic sleep aids (E). Reported difficulty of staying awake (F). Reported problems with enthusiasm (G). Reported overall sleep quality (H). Data are means ± SEM. ***P* < 0.05, dashed red line represents cut-off value. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 27


A
Global PSQI Score
10
8
6
4
2
0
*
*
Healthy
PD-N
PD+N
B
Time To Fall Asleep
Time (min)
40
30
20
10
0
Healthy
PD-N
PD+N
C
Hours of Sleep
Time (hrs)
8
6
4
2
0
Healthy
PD-N
PD+N
D
Hours in Bed
Time (hrs)
10
8
6
4
2
0
Healthy
PD-N
PD+N
E
Medicine to Help Sleep
Three or more times a week
Once or twice a week
Less than once a week
Not during the past month
Healthy
PD-N
PD+N
F
Trouble Staying Awake
Three or more times a week
Once or twice a week
Less than once a week
Not during the past month
Healthy
PD-N
PD+N
G
Problem With Enthusiasm
Three or more times a week
Once or twice a week
Less than once a week
Not during the past month
Healthy
PD-N
PD+N
H
Sleep Quality Overall
Very bad
Fairly bad
Fairly good
Very good
*
Healthy
PD-N
PD+N

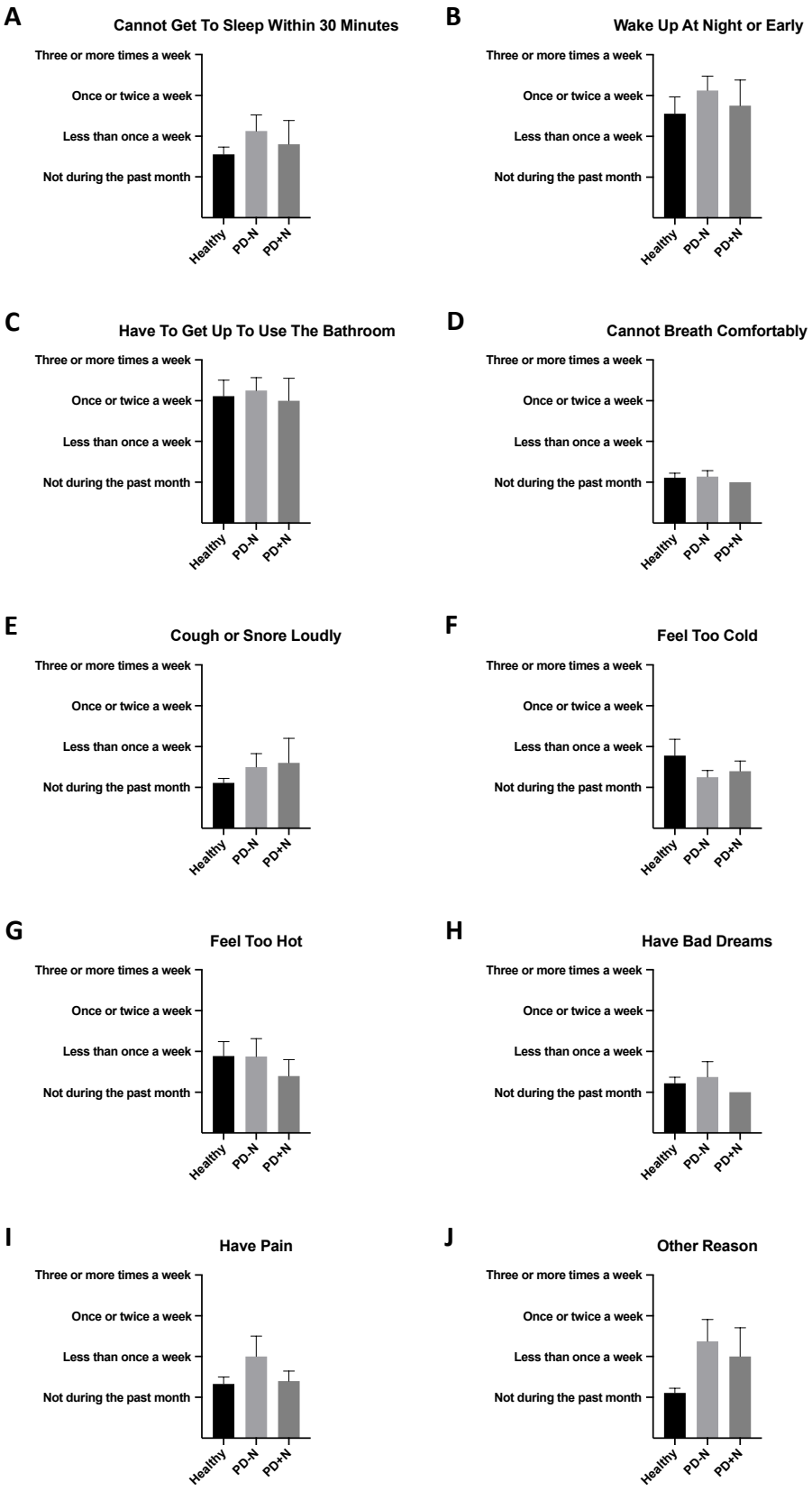
Prediabetics with and without Neuropathy do not Report Increased Frequency of a Specific Reason for Trouble Sleeping:

In subsection 5 of the PSQI subjects reported on frequency of causal factors that may contribute to trouble sleeping. No significant difference was observed in being able to initiate sleep within 30 minutes between the three groups ($p = 0.4949$, Fig 28A). No significant difference was observed in waking up at night or early in the morning between the three groups ($p = 0.6004$, Fig 28B). No significant difference was observed in having to get up at night to use the bathroom between the three groups ($p = 0.9182$, Fig 28C). No significant difference was observed in being able to breath while sleeping between the three groups ($p = 0.7251$, Fig 28D). No significant difference was observed in coughing or snoring loudly between the three groups ($p = 0.5206$, Fig 28E). No significant difference was observed in being too cold at night between the three groups ($p = 0.4515$, Fig 28F). No significant difference was observed in being too hot at night between the three groups ($p = 0.6941$, Fig 28G). No significant difference was observed in having bad dreams between the three groups ($p = 0.6528$, Fig 28H). No significant difference was observed in having pain at night between the three groups ($p = 0.3318$, Fig 28I). No significant difference was observed any other reasons between the three groups ($p = 0.0883$, Fig 28J).

Figure 28 Pittsburgh Sleep Quality Index (PSQI) Trouble Sleeping Factors

Responses to the Pittsburg Sleep Quality Index (PSQI): Question 5 components, completed at CTSU clinical visit. Reported difficulty initiating sleep withing 30 minutes (A). Reported waking up at night or early in the morning (B). Reported having to get up to use the bathroom (C). Reported not being able to breath comfortably (D). Reported coughing or snoring loudly (E). Reported feeling too cold (F). Reported feeling too hot (G). Reported having bad dreams (H). Reported having pain (I). Reported other reason for trouble sleeping (J). Data are means ± SEM. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 28


A
Cannot Get To Sleep Within 30 Minutes
B
Wake Up At Night or Early
C
Have To Get Up To Use The Bathroom
D
Cannot Breath Comfortably
E
Cough or Snore Loudly
F
Feel Too Cold
G
Feel Too Hot
H
Have Bad Dreams
I
Have Pain
J
Other Reason
Three or more times a week
Once or twice a week
Less than once a week
Not during the past month
Healthy
PD-N
PD+N

Prediabetics with Neuropathy Report Higher Levels of Pain on BPI-DPN

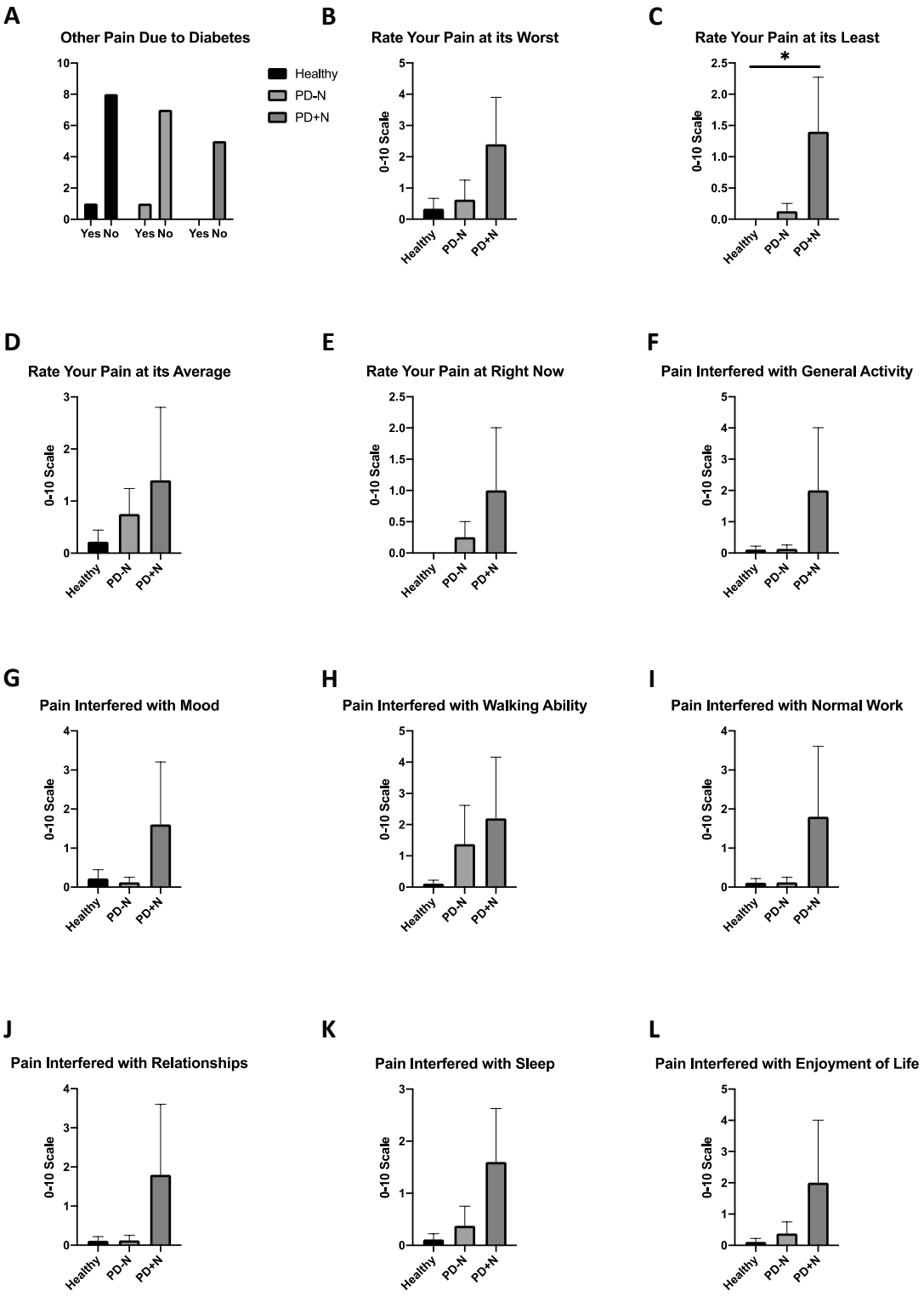
The Brief Pain Inventory for Diabetic Neuropathy is a widely used screening instrument for evaluation of pain in the clinical setting, in this case adapted for diabetic peripheral neuropathy [311-314]. No significant difference was observed in whether pain beyond normal daily pain, due to diabetes, was reported between the three groups ($p = 0.7200$, Fig 29A). No significant difference was observed in reported pain intensity at its worst between the three groups ($p = 0.1821$, Fig 29B). Prediabetics with neuropathy reported higher intensity of pain at its least compared with healthy subjects ($p = 0.0340$, Fig 29C). Prediabetics without neuropathy did not report significantly different pain at its least compared to prediabetics with neuropathy or healthy patients ($p = 0.0621$ and 0.9579, respectively). No significant difference was observed in reported pain intensity at its average between the three groups ($p = 0.4801$, Fig 29D). No significant difference was observed in reported pain intensity at the current moment between the three groups ($p = 0.2898$, Fig 29E). No significant difference was observed in whether pain interfered with general activity between the three groups ($p = 0.2298$, Fig 29F). No significant difference was observed in whether pain interfered with mood between the three groups ($p = 0.2845$, Fig 29G). No significant difference was observed in whether pain interfered with walking ability between the three groups ($p = 0.4256$, Fig 29H). No significant difference was observed in whether pain interfered with normal work between the three groups ($p = 0.2357$, Fig 29I). No significant difference was observed in whether pain interfered with relations with other people between the three groups ($p = 0.2357$, Fig 29J). No significant difference was observed in whether pain interfered with sleep between the three groups ($p = 0.1201$, Fig 29K). No significant difference was observed in whether pain interfered with enjoyment of life between the three groups ($p = 0.2907$, Fig 29L). Prediabetics with neuropathy generally trended towards

reporting higher intensity pain or greater pain interference, although not reaching significance. Furthermore, none of the healthy patients reported taking medication for pain due to diabetes, two prediabetics without neuropathy reported taking aspirin as needed and a muscle relaxer, respectively. One prediabetic patient reported taking gabapentin for diabetic pain.

Figure 29 Brief Pain Inventory for Diabetic Neuropathy (BPI-DPN)

Responses to the Brief Pain Inventory for Diabetic Neuropathy (BPI-DPN), completed at the department of neurology clinical visit. Reported pain beyond normal daily pain due to diabetes (A). Rating pain at its worse intensity (B). Rating pain at its least intensity (C). Rating pain at its average intensity (D). Rating pain as it feels presently (E). Rating how much pain has interfered with general activities (F). Rating how much pain has interfered with mood (G). Rating how much pain has interfered with walking ability (H). Rating how much pain has interfered with normal work (I). Rating how much pain has interfered with relationships (J). Rating how much pain has interfered with sleep (K). Rating how much pain has interfered with enjoyment of life (L). Data are reported as frequencies and analyzed by chi-square analysis (A). Data are means ± SEM, measured in intensity scale 0-10 with 0 being no pain and 10 being pain as bad as you can imagine (B-E). Data measured in inference scale 0-10 with 0 being does not interfere and 10 being completely interferes (F-L). ***$P < 0.05$. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 29


A
Other Pain Due to Diabetes
Healthy
PD-N
PD+N
Yes No
Yes No
Yes No
B
Rate Your Pain at its Worst
0-10 Scale
Healthy
PD-N
PD+N
C
Rate Your Pain at its Least
*
0-10 Scale
Healthy
PD-N
PD+N
D
Rate Your Pain at its Average
0-10 Scale
Healthy
PD-N
PD+N
E
Rate Your Pain at Right Now
0-10 Scale
Healthy
PD-N
PD+N
F
Pain Interfered with General Activity
0-10 Scale
Healthy
PD-N
PD+N
G
Pain Interfered with Mood
0-10 Scale
Healthy
PD-N
PD+N
H
Pain Interfered with Walking Ability
0-10 Scale
Healthy
PD-N
PD+N
I
Pain Interfered with Normal Work
0-10 Scale
Healthy
PD-N
PD+N
J
Pain Interfered with Relationships
0-10 Scale
Healthy
PD-N
PD+N
K
Pain Interfered with Sleep
0-10 Scale
Healthy
PD-N
PD+N
L
Pain Interfered with Enjoyment of Life
0-10 Scale
Healthy
PD-N
PD+N

Prediabetics with Neuropathy Demonstrate Significantly Higher Scores on Neuropathy Screening Questionnaires:

The Michigan Neuropathy Screening Instrument includes two separate assessments: a 15 question self-administered questionnaire and a lower extremity examination performed by a health professional [315, 316]. The self-assessment questions include topics such as: numbness, burning pain, sensitivity, muscle cramps, prickling feelings, pain from bedsheets, temperature discrimination, open sores, etc., and is considered abnormal with a score of $\geqslant$ 7. The lower extremity examination includes inspection and assessment of vibratory sensation and ankle reflexes, with an abnormal score of $\geqslant$ 2.5. There was no significant difference observed in the MNSI 1 self-assessment questionnaire score between the three groups ($p = 0.1422$, Fig 30A). However, during the MNSI 2 examination prediabetics with neuropathy scored significantly higher on the assessment than healthy subjects ($p = 0.0034$, Fig 30B). There was no significant difference observed between prediabetics without neuropathy and prediabetics with neuropathy or healthy subjects ($p = 0.0918$ and $p = 0.2175$ respectively).

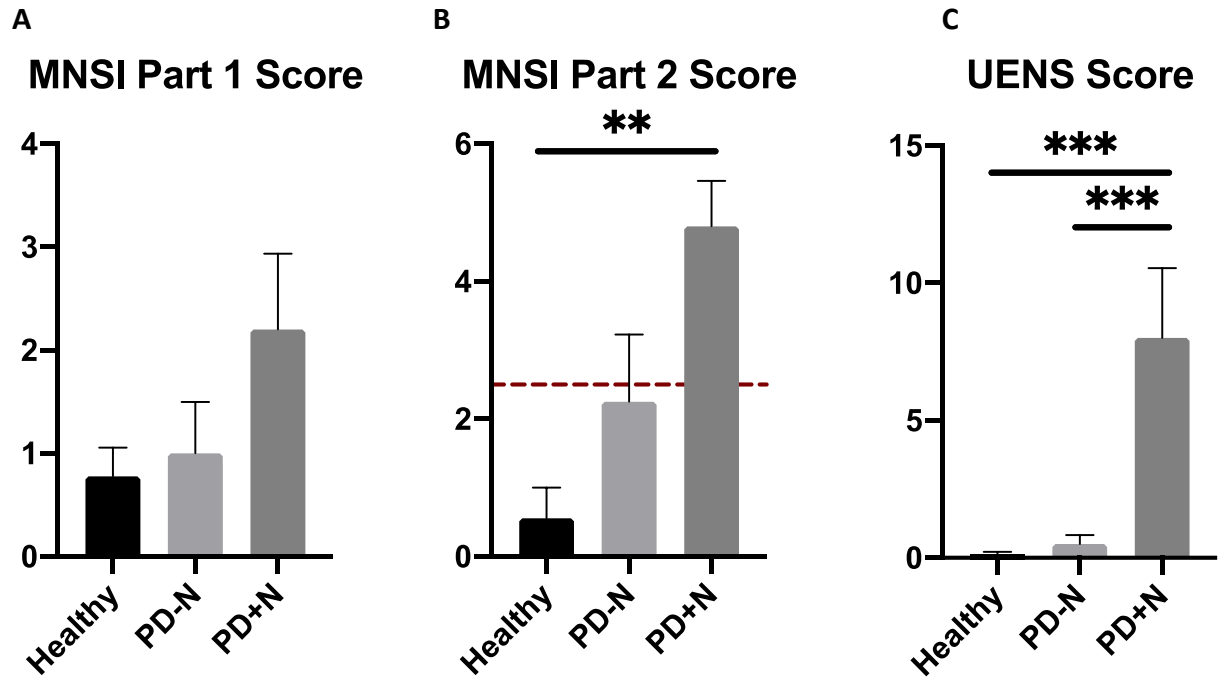
The Utah Early Neuropathy Scale (UENS) was given to patients to evaluate their neuropathy status. The UENS has been shown to be a reliable examination scale specific to early sensory predominant polyneuropathy [317, 318]. It has been shown to more closely correlate with in small-fiber neuropathy than other neuropathy screening instruments. The UENS encompasses the following physical exam assessments: bilateral great toe extension strength, bilateral pin sensation, bilateral allodynia in feet, bilateral presence and duration of vibratory sensation in the great toe as well as joint position, bilateral deep tendon reflexes of the ankles. Prediabetics in this study received a significantly higher total UENS score than prediabetics without neuropathy or healthy patients ($p = 0.0003$, $p = 0.0001$ respectively, Fig 30C).

Prediabetics without neuropathy did not have a significantly different total UENS score than healthy patients ($p = 0.9523$).

Figure 30 Michigan Neuropathy Screening Instrument (MNSI) and Utah Early Neuropathy Scale (UENS) Results

Responses to the Michigan Neuropathy Screening Instrument (MNSI) and Utah Early Neuropathy Scale (UENS), completed at the department of neurology clinical visit. Calculated score for the MNSI part 1 self-assessment questionnaire (A). Calculated score for the MNSI part 2 physical evaluation (B). Calculated score for the UENS (C). Data are means ± SEM. ****P* < 0.01, *****P* < 0.001, red dashed line represents cut-off value. Data analyzed by one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5).

Figure 30


A
MNSI Part 1 Score
4
3
2
1
0
Healthy
PD-N
PD+N
B
MNSI Part 2 Score
**
6
4
2
0
Healthy
PD-N
PD+N
C
UENS Score
***
***
15
10
5
0
Healthy
PD-N
PD+N

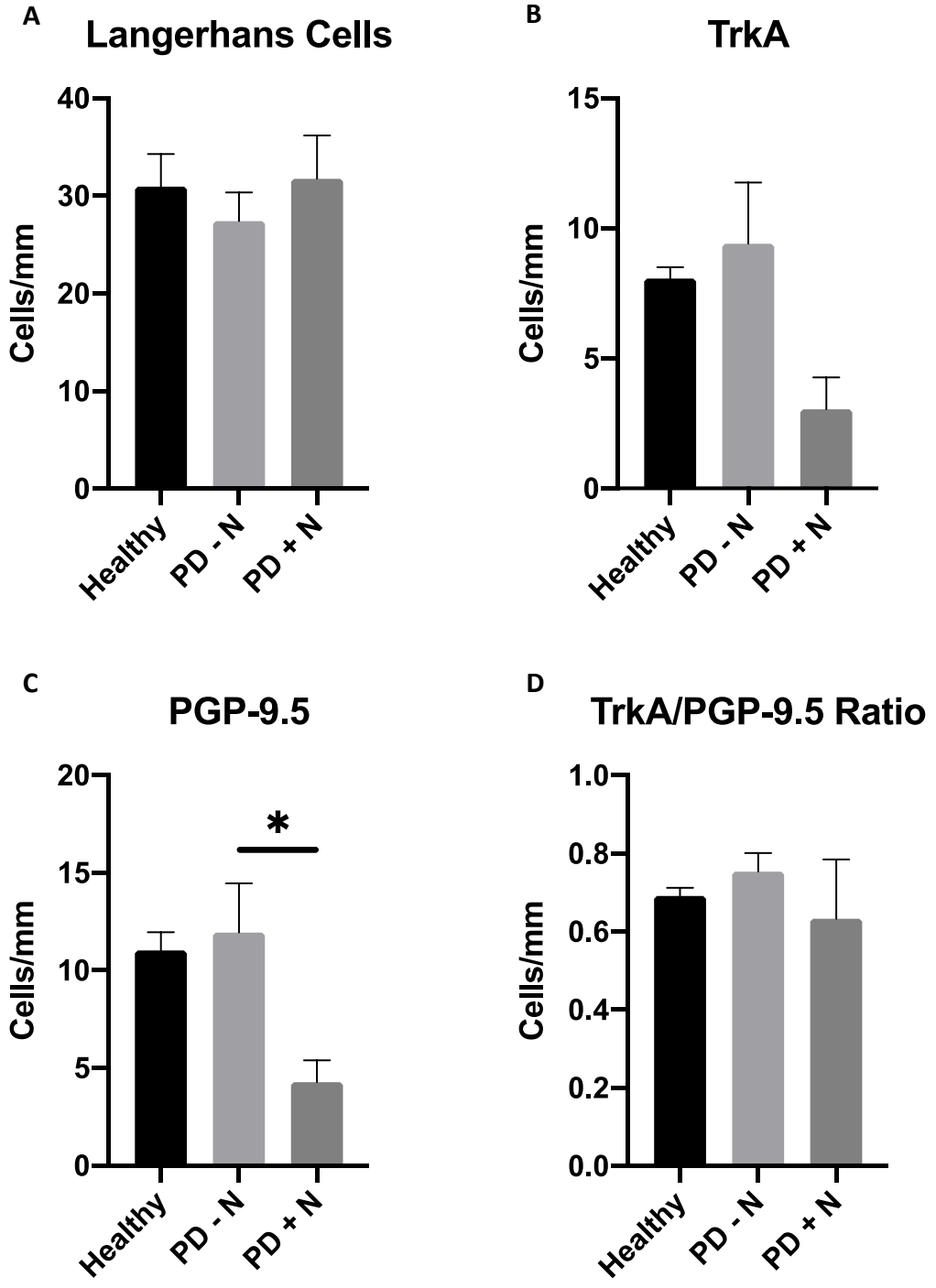
Prediabetic Patients with Neuropathy had Lower Intra Epidermal Nerve Fiber Density:

Skin biopsy analysis was performed blinded in randomized batches. There was no significant difference observed in the Langerhans cell density among the three groups ($p = 0.6673$, Fig 31A). There was also no significant difference observed in the peptidergic TrkA-positive IENFD among the three groups ($p = 0.0838$, Fig 31B). However, there was significant difference observed in PGP 9.5-postive IENFD among the three groups ($p = 0.0345$, Fig 31C). Prediabetic patients with neuropathy displayed a significant reduction in PGP 9.5-postive IENFD compared to prediabetics without neuropathy ($p = 0.0359$). Healthy patients did not reveal significant changes in their IENFD compared to prediabetics with or without neuropathy ($p = 0.0922, 0.9412$ respectively). The calculated TrkA/PGP 9.5 ratio was not determined to be significantly different among the three groups ($p = 0.6018$, Fig 31D). Although not quantified, structural changes were noted in the dermal-epidermal junction of some prediabetic patients with neuropathy including the flattening of rete pegs and the relative absence of blood vessels in the superficial dermal tissue.

Figure 31 Skin Biopsy Histology Analysis

Results of immunofluorescent microscopic analysis of skin biopsy harvested from non-glabrous tissue superior to the lateral malleolus. Langerhans cell density along epidermal border (A). TrkA-positive intraepidermal nerve fiber density (B). PGP 9.5-positive intraepidermal nerve fiber density (C). TrkA/PGP 9.5 Ratio (D). Data are means ± SEM. ***P* < 0.05. Data analyzed via one-way ANOVA with Tukey multiple comparisons test. (Healthy n = 9, PD-N n = 8, PD+N n = 5)

Figure 31


A
Langerhans Cells
Cells/mm
40
30
20
10
0
Healthy
PD - N
PD + N
B
TrkA
Cells/mm
15
10
5
0
Healthy
PD - N
PD + N
C
PGP-9.5
Cells/mm
20
15
10
5
0
*
Healthy
PD - N
PD + N
D
TrkA/PGP-9.5 Ratio
Cells/mm
1.0
0.8
0.6
0.4
0.2
0.0
Healthy
PD - N
PD + N

5. Discussion

In this clinical observational study healthy patients were compared to prediabetics with and without neuropathy via a battery of questionnaires, blood tests, neurological assessments, and skin biopsy analysis. While prediabetics with neuropathy were more likely to be older and male in our patient cohort, all other demographical, lifestyle, and smoking history was not found to be different between the three groups. Due to the recruitment difficulty of prediabetics with neuropathy we were not able to adjust our study population to control for differences in age and gender. It is acknowledged that age has an independent effect of intraepidermal nerve fiber density and is a risk factor for prediabetes and neuropathy, therefore may be a confounding variable [319-321]. Prediabetics with neuropathy were more likely to have a previous diagnosis of prediabetes. Many patients report that their physicians do not tell them that they are prediabetic. It has been repeatedly shown in the literature that primary care providers have not proven reliable in diagnostic identification and management of the prediabetic population and that many prediabetic patients are not aware of their diagnosis [270, 322-324]. Having additional symptoms such as early onset peripheral neuropathy may drive healthcare providers to pursue the etiology such as glycemic status and share prediabetic diagnoses.

Physical measurements were not different among the groups except that prediabetics with neuropathy were found to be taller in our cohort. It has been proposed that diabetic peripheral neuropathy is a length dependent process with neurons containing the longest axons being preferentially damaged first, hence the stocking glove distribution of the long sensory axons in the extremities [325, 326]. Therefore, one explanation for why some patients develop neuropathy during the prediabetes phase, and others do not, might simply be increase axon length in taller individuals. It was interesting to see in our study cohort that BMI and waist circumference were

not different between the healthy subjects and the prediabetics, as these have been shown by others to be correlated [327]. However, our "healthy" subjects still had a higher BMI than national average. The ongoing obesity epidemic has preferentially afflicted the Midwest and south with higher average BMI's and rates of obesity [328, 329].

Prediabetics had increased fasting blood glucose compared to healthy subjects but did not have significantly increased HbA1c values, although they were trending towards higher values. It has been shown in the literature that HbA1c is a less sensitive test and will delay diagnosis compared to other measures of hyperglycemia [330-332]. This likely reflects the early time course of prediabetes, and that the glycemic instability is fluctuating in and out of borderline hyperglycemic ranges. It is also apparent by the significant changes in the fasting glucose and OGTT glucose values compared the non-significance of the fasting insulin and OGTT insulin values that glycemic control starts becomes dysregulated before large alterations in measured insulin levels.

Prediabetics with neuropathy were found to have lower levels of LDL. While LDL cholesterol traditionally is considered to play a pathological role in the development of atherosclerosis, peripheral artery disease and coronary artery disease, it may have a protective role in neuropathic alterations [333, 334]. This neurologic role may be highlighted by the literature reports of statin-induced neuropathy [335-338]. Statins are a widely used HMG-CoA reductase inhibitors that block the rate-limiting step of cholesterol synthesis and are first line therapy for lowering LDL levels [339]. It has been shown that LDL particles may lead to neurite outgrowth during *in vitro* experiments [340, 341]. While the cardiovascular benefits of lowering LDL levels still may outweigh the risks of increased neuropathic changes, it might be prudent to evaluate the patient's neurologic status.

The results of the IPAQ sections were largely non-significant between the three groups, however we observed that prediabetics without neuropathy spent less days per week traveling by motor vehicle and prediabetics with neuropathy spent more time performing moderate physical activity in the home. There was also a trend towards prediabetics with neuropathy doing more activities outside the house, walking at work, and as a form of transportation. This might be explained by patients making deliberate lifestyle modifications in response to the neuropathic symptoms. Patients are less likely to make lifestyle modifications if their prediabetes remains largely asymptomatic. In light of the recent COVID pandemic, results of the IPAQ may have changed dramatically as society adapted to the closure of gyms, recreational events, workplace, and other public areas [342]. These measures, including quarantines, have led to unhealthy diets, decreased physical activity and increased rates of sedentary lifestyle with large segments of the population becoming furloughed or working from home [343-346].

The results of the PSQI revealed that prediabetics without neuropathy had a higher global PSQI score as well as higher reported levels of overall impaired sleep quality, however the results were unable to elucidate any specific reason or cause for the impaired sleep quality. Poor sleep quality has been shown to be associated with prediabetes and glycemia in adults [347-349]. We expected the prediabetics with neuropathy to also report higher PSQI scores, however in our study population they did not, which may be due to small sample size.

The results of the BPI-DPN revealed that prediabetics with neuropathy had higher levels of pain at its least intensity, in other words when their chronic pain is at its best. Prediabetics with neuropathy trended towards higher levels of pain in almost all components of the BPI-DPN, while not reaching significance. This shows how prediabetic peripheral neuropathic pain can affect almost all daily activities and could significantly decrease the patient's quality of life.

In our study there was no significant difference reported in the MNSI self-assessment, while prediabetics with neuropathy only trended towards a higher reported overall score. The MNSI part 2, healthcare provider assessment, reliably showed the prediabetics with neuropathy scoring above the 2.5 cut-off value as well as being significantly higher than the healthy subjects. The UENS also showed the prediabetics with neuropathy scoring significantly higher than prediabetics without neuropathy as well as healthy patients. Thus, the UENS for our population appears to be the most sensitive screening test for neuropathy in prediabetic patients.

PGP 9.5, a marker of total nerve fibers, significantly decreased in prediabetics with neuropathy [350]. Here we show that even in the early reversible stages of prediabetes, there is objective nerve fiber loss in the epidermis. However, peptidergic-selective nerve changes in IENFD were not observed as we previously reported in mice [298]. Humans might have different mechanism for glycemic related neuropathy than mice [351]. Humans do have a significantly lower baseline IENFD in healthy states than the mice hindpaw [196, 352]. Langerhans cells do not increase in number in the epidermis in our prediabetics with neuropathy as has previously been reported in diabetic peripheral neuropathy [353-355]. An additional target for future evaluation in the skin is the recently discovered Schwann cell projections that extend into the epidermis and closely interact with the “free” nerve endings [356]. These Schwann cell projections have been shown to play an essential role in physiological role in nociception and have not yet been evaluated in the context of prediabetic peripheral neuropathy.

This article reflects a “progress report” of mid-study results and subsequent conclusions. The prediabetic axonal changes in metabolic syndrome and neuropathy (PACMAN) will continue with patient recruitment, especially targeting prediabetics with neuropathy to increase study power. Due to current study sample size, we are unable to sub-stratify prediabetics with

neuropathy into painful predominant phenotype and painless phenotype subgroups to analyze for variation in IENFD fiber type. Studying patients in the prediabetic population has proven extremely difficult with regards to patient recruitment and eligibility. Trying to reach this specific cohort is challenging and reveals the widespread prevalence of the diabetes epidemic. Based on the ADA criteria for diagnosis of prediabetes many patients who had previously been diagnosed with prediabetes have already progressed above the upper threshold and developed overt type 2 diabetes [260]. Furthermore, many patient's physicians never told them that they had prediabetes or impaired glucose tolerance.

Future directions include implementation a ketogenic diet in human subjects with prediabetic neuropathy to evaluate whether or not it can reverse IENFD as well as neuropathy screening scores such as MNSI and UENS. We have previously shown ketogenic diet to ameliorate and rescue the reduction in IENFD in diabetic mice [85]. This presents a unique situation to be able to intervene in the early prediabetic phase of glycemic disease to evaluate neuropathy treatment before the patient experiences the multisystem morbidities associated with chronic diabetes [357, 358].

Overall, we have shown that prediabetics with neuropathy demonstrate a reduction in IENFD density, report higher neuropathy screening questionnaire scores and pain scores, and maintain lower levels of LDL. Generally, the three groups studied (healthy, prediabetics without neuropathy, and prediabetics with neuropath) appeared quite similar with few significant differences in lifestyle, activity level, or laboratory analysis. While prediabetes may remain a relatively benign diagnosis, serving to warn providers and patient of coming disease, neuropathic changes in the peripheral are already beginning to establish themselves in the epidermis in a subset of patients.

Chapter 6: General Discussion and Conclusions

Diabetic Peripheral Neuropathy and Paclitaxel-Induced Peripheral Neuropathy:

While glycemic related peripheral neuropathy and paclitaxel/chemotherapy-induced peripheral neuropathy have different etiologies and risk factors, they may have significant overlap in their neurotoxicity to the peripheral nervous system. Mitochondrial stress/dysfunction has been proposed as a major mediator neurodegeneration in both diabetic peripheral neuropathy and paclitaxel-induced peripheral neuropathy [235, 359, 360]. Further support of this is treatment of neuropathic pain is the same regardless of whether it developed secondary to diabetes or to paclitaxel treatment. Many of the mainstay mediations used to treat paclitaxel induced neuropathy are also those with demonstrated efficacy in diabetic neuropathy [361]. These first line agents include anticonvulsants (Pregabalin or Gabapentin) SNRIs or TCAs [39].

Chapter 2 Discussion: Paclitaxel-Induced Neuropathy Impacts Metabolism and Glucose Tolerance in Mice

In our paclitaxel CIPN mouse model, we demonstrated that 12 mg/kg of paclitaxel, given via IP injection, caused a mechanical allodynia of the hindpaw. It also caused minor metabolic changes that were pronounced by the addition of a high fat diet metabolic challenge. Creating a mouse model of paclitaxel-induced neuropathy will allow us to better understand this prevalent form of CIPN seen in cancer patient's treatment and recovery.

A primary limitation of this study was that it was performed in otherwise healthy C57BL/6 mice, which impairs its translationally relevant findings to human subjects. No patient would ever be expose to a toxic chemotherapy regimen unless they had a diagnosis of malignancy that warranted such treatment. This experiment would need to be repeated in a tumor graft model such as a breast cancer xenograft to better understand paclitaxel's effect on the

nervous system [362, 363]. Compared to normal cells, tumor cells upregulate their glucose metabolism and uncouple glycolysis from the mitochondrial tricyclic acid (TCA) cycle, instead diverting to increased lactate fermentation [364, 365]. This metabolic phenotype is known as the Warburg Effect and has implications on cancer growth, host immunological response, and altering the tumor microenvironment [366, 367]. Furthermore, cancer has been shown to exacerbate chemotherapy induced sensory neuropathy in prior studies [368-370].

An additional limitation that could be improved in this study would be to perform the experiment in metabolic cages that could assess mice activity levels. While the paclitaxel appears to make the mice gain weight and have changes in metabolic parameters evaluated, this may be secondary to decreased activity and energy expenditure. As mice developed allodynia, it may cause them discomfort to move around the cage, leading to increase sedentariness.

Chapter 3 Discussion: Elimination of Capsaicin-Responsive Sensory Neurons During Neonatal Development Affects Metabolic Parameters in Adulthood

In this study, we demonstrated that neonatal capsaicin treatment delayed paclitaxel-induced mechanical allodynia but appears to have a paclitaxel-independent effect on weight, glucose, and insulin levels. The major limitation of this study was the use of a systemic treatment that affects all TRPV1-postive neurons in nearly every organ system, including alterations in the central nervous system [371, 372]. The use an intervention with such broad effects makes it difficult to establish the source of these observed metabolic changes. Using more targeted treatment that only affects pancreatic afferents would strengthen our hypothesis that this neuron population serves an important physiological function in providing negative feedback onto insulin producing beta cells. Future studies with greater specificity for this neuron population

might include, intra-pancreatic injection of capsaicin, thoracic rhizotomy, or optogenetic manipulation of the pancreatic afferents.

As mentioned previously, an interesting finding of this study was that the effects of neonatal capsaicin did not appear to manifest until after the intraperitoneal injections were performed at 8 weeks. The paclitaxel and vehicle control injections did not display significant difference from each other but both groups only appeared to diverge from the groups not treated with neonatal capsaicin until after these injections. Cremaphor/ethanol solution which was present in both the vehicle control as well as the paclitaxel formulation has also been shown to have a neurotoxic effect on peripheral nerves in some studies [373, 374]. A future study should collect more metabolic data in the weeks following the neonatal capsaicin treatment through maturation to ascertain whether neonatal capsaicin treated animals display metabolic variation prior to paclitaxel injections, since this study only took samples immediately prior to injections at 8 weeks and following paclitaxel treatment.

Another limitation in this study was the small sample size of female animals compared to male animals. This was due to chance as equal gender prevalence of litters could not be guaranteed, but the female cohort may need to be repeated to be sufficiently powered to see if similar effects exist in females due to neonatal capsaicin treatment.

Chapter 4 Discussion: Effects of Ketogenic Diet on Paclitaxel Induced Neuropathy in Mice

Through the use of a ketogenic diet in paclitaxel treated mice we observed increased withdrawal thresholds in paclitaxel ketogenic diet mice compared to paclitaxel chow mice. We confirmed that ketogenic diet prevented the trend in increased weight gain caused by paclitaxel in male mice and prevented the associated fat mass increase seen in paclitaxel animals.

One limitation of this study is that we did not measure quantity of diet consumed or caloric intake of the different groups. Much of the metabolic changes observed in the ketogenic diet fed animals may be due to lower levels of food consumption. It was noted that there were significantly increased levels of fighting in the male animals of this cohort. The cause behind this increased fighting could not be elucidated, but many animals had to be separated into individual cages due to fighting and subsequent injury. This group was the first cohort we ordered after the COVID-19 pandemic-associated research shutdowns, and we wondered if the increased mice aggression could be related to staffing/procedural changes at the breeding facility. This could be a possible explanation for some discrepancies in the data such as why the Von Frey filament data in control animals and paclitaxel treated animals was different from our previous studies.

An additional future study to be performed would be to create a ketogenic diet rescue cohort where mice are started on chow diet through the paclitaxel treatment and switched to a ketogenic diet two weeks following the IP injections. In diabetic neuropathy studies performed previously in the lab ketogenic diet exerted a protective effect when given as a prevented treatment or as a rescue to the peripheral neuropathy [85].

Chapter 5 Discussion: Characterization of Peripheral Neuropathy in Prediabetic Patients: The PACMAN Study

Our findings from this study so far show that prediabetics with neuropathy display a reduced IENFD of all axon fiber types. Peripheral neuropathy screening instruments are able to detect prediabetics with histological findings of peripheral neuropathy. Additionally, lower LDL levels were associated with neuropathy status in prediabetic patients.

One limitation of this study was patient recruitment of prediabetic patients with neuropathy. As only a small subset of prediabetic patients have been reported to develop neuropathy it made finding and successfully recruiting this patient population challenging [29]. Additionally, the onset and lasting impact of the current COVID-19 pandemic has made patient recruitment more difficult as for a time all clinical trials were suspended, and afterwards many patients expressed hesitance at coming into a clinical setting.

As stated prior the PACMAN study, examining the changes in prediabetic patients who develop neuropathy, will continue with patient recruitment and subsequent reanalysis of data. With increasing sample size, and thereby power, we hope to identify additional risk factors for the development of neuropathy in prediabetic patients. As we have shown that prediabetics with neuropathy display a reduced IENFD, the next step would be to test treatment options in this population including a ketogenic diet and exercise. We have shown both prevention of neuropathy in mouse models of diabetes as well as rescue of diabetes associated IENFD reduction [85]. To our knowledge a ketogenic diet has not been evaluated in the context of prediabetic patients with peripheral neuropathy. Prediabetic patients with neuropathy could be enrolled in a short pilot study of 1-2 months and placed on a ketogenic diet. A skin biopsy along with neuropathy screening questionaries (MNSI, UENS, etc.) could be completed at the beginning and end of the study, with the hypothesis that IENFD would increase within several weeks along with decreased reported symptoms of neuropathy. While exercise is commonly recommended as a first line therapy for prediabetes as well as diabetes, few studies have evaluated exercises effect on diabetic peripheral neuropathy [114, 375]. There has been one report of diabetic without neuropathy having an increase in IENFD following a moderately intense aerobic and resistance exercise program [376]. A similar short-term longitudinal study

could be performed in prediabetic patients with neuropathy to evaluate changes in IENFD and peripheral neuropathy symptoms. Although technically more difficult, a longitudinal clinical study of prediabetic patients with neuropathy might prove insightful as disease progression can better be measured than in the reported study which serves as more of a cross sectional snapshot of disease status at a point in time. Both a ketogenic diet and exercise could be readily implemented as treatment options for prediabetic patients as they would not require any regulatory oversight or FDA approval, have been studied previously as interventions for other forms of peripheral neuropathy and are generally well-tolerated and could be tailored to individual patient circumstances [377-380].

As we still have additional skin biopsy samples from patients, future histologic imaging of these groups could include the presence of the recently discovered nociceptive Schwann cell appendages in the epidermis [356]. A novel histological application is the use tissue clearing and stacked confocal imaging for 3D analysis of the intraepidermal nerve fibers to allow for better analysis of previously overlapping structures in the traditional two-dimensional imaging [381-383]. Our lab has recently gained expertise in this technique and could apply it to this prediabetic patient population to gain increase clarity and insight on structural changes occurring in the sensory neuron population in the periphery. Furthermore, application of this technique might reveal more reliable findings on the association of reduced IENFD and associated local microvascular changes in the superficial dermal layer [384-386].

Additional Future Experiments:

A future *in-vitro* study to strengthen our hypothesis that paclitaxel damages the sensory neurons innervating pancreatic islets, would be to culture extracted mouse islets. Training has

already been accomplished in the surgical technique of performing the necessary abdominal laparotomy: reflecting the bowl to the left to identify the common bile duct (CBD), cross-clamping the ampulla of vater, then injecting collagenase in an anterograde fashion into the CBD distal to the bifurcation of the cystic duct, followed by removal of the engorged pancreas tissue for subsequent digestion and extraction. The following experimental design would be to add paclitaxel to the islet culture and then measure glucose stimulated insulin secretion (GSIS), then the experiment would be repeated with the islets being co-cultured with DRGs. Previous reports of embryonic DRG and islet cell co-cultures demonstrate a reduced GSIS from beta cells, demonstrating that sensory nerve activity inhibits the insulin response [387]. We hypothesize that adding paclitaxel would cause reverse this observed decrease in GSIS in the islet-DRG co-culture via sensory neuron pathology.

Additional clinical studies in patients with translational applications have also been considered. It is well known that pancreatic pain localizes to the upper abdomen and radiates to the back [346, 388, 389]. Typically, this kind of pancreatic pain occurs in pancreatitis or pancreatic cancer as both show an upregulation in sensory afferents within the pancreatic parenchyma [390, 391]. Patients with pancreatic pain could be evaluated to see if they have changes in insulin secretion or glycemic regulation. However, it is acknowledged that chronic pancreatitis patients can develop a type 3c diabetes from auto destruction of pancreatic tissue, including the islets themselves being replaced with fibrotic tissue [392-394]. Intractable pancreatic pain can be difficult to treat and often pain medications are not enough to maintain adequate analgesia, thus requiring a surgical approach [395, 396]. Both celiac plexus blocks with local anesthetics and celiac plexus neurolysis via chemical ablation have been performed [397-399]. Therefore, both pancreatitis patients as well as pancreatic cancer patients present unique

populations to study the role of sensory innervation of islets. Both the increase in innervation during development of the chronic pain state, as well as the effects of rapid denervation following surgical intervention on the celiac plexus can be evaluated.

Conclusion:

Overall, we have shown that paclitaxel causes a peripheral neuropathy in mice and that it may cause early fluctuations in insulin levels as well as glycemic parameters especially in states of metabolic challenge such as a high fat diet. A ketogenic diet did improve mechanical allodynia to some extent, and improved metabolic markers in our paclitaxel animal model. We have also further confirmed that prediabetes does also cause neuropathy and that prediabetics with symptoms of neuropathy, not due to any other cause, have an objectively lower IENFD. Both prediabetes and chemotherapy use are extremely common conditions affecting large proportions of society, disproportionally affecting older individuals [400, 401]. While peripheral neuropathy may seem lower on the triage latter of clinical management compared to the more acute complications of either glycemic disease or cancer/chemotherapy treatment. Development of peripheral neuropathy presents a huge disease burden on patients with few available treatments. My father is a manager at a running shoe store, and regrettably a large proportion of his customers are neuropathy patients (both diabetic and chemotherapy-induced) seeking relief from pain and discomfort in their feet. For many peripheral neuropathy patients, the discomfort simply becomes unbearable. As patient life expectancy increases due to improved treatment of acute disease, additional focus will turn to better management of chronic conditions such as peripheral neuropathy. Also, as growing evidence accumulates that a neuroendocrine feedback loop regulating insulin secretion does exist, and that sensory neurons play a role to some extent

in this feedback, future research might find a new targeted therapy to improve insulin secretion and glycemic control.

In conclusion, to summarize the key findings from this dissertation, I have shown that damage to peripheral neurons leads to negative changes in systemic metabolism. This occurs to a very minor extent in paclitaxel induced neuropathy and more robustly in neonatal ablation of capsaicin-sensitive neurons. Furthermore, a ketogenic diet may improve neuropathy that may be independent of its actions on systemic metabolism. Patients with prediabetes develop neuropathy and our study will help phenotype these patients. Our studies suggest that closer examination of dyslipidemia in prediabetic neuropathy is required, and that axon loss in patients with prediabetes may not be identical to axon loss in mice. We previously showed that the decreased withdrawal threshold seen in diabetic mice models of peripheral neuropathy correlates positively (and significantly) with peptidergic nerve fiber density but not with non-peptidergic nerve fibers [298]. Therefore, we determined that lower levels of peptidergic neurons is associated with increased mechanical allodynia in diabetic mice models.

As the PACMAN study evaluated a myriad of different potential risk factors for development of prediabetic neuropathy in human patients, it may be necessary to return to genetic animal models to be able to tease out the relative contribution of each component. Furthermore, it may be the combination of several different risk factors that predisposes the prediabetic patient to neuropathic symptoms. The translational application of such a model could be in the form of risk factor questionnaire that asks patients about their possible lifestyle risk factors, vital signs, and a few targeted symptoms, from which a calculated risk for development similar to how an atherosclerotic cardiovascular disease (ASCVD) risk is calculated for patients.

Knowing one's neuropathy risk factor would be valuable to clinicians knowing which medications to prescribe as well as lifestyle interventions to implement. With such a large data set of multiple potential risk factors it may also be beneficial to apply machine learning algorithms to better analyze the data and optimize predictive analytics.

A future study that could provide increase insight into this proposed neuroendocrine feedback loop would be through evaluation of an in-vitro co-culture of pancreatic islets and dorsal root ganglion. Through a cell culture one could measure glucose induced insulin secretion of islet beta cells, then administer paclitaxel treatment to the culture and re-evaluate whether the beta cells can secret insulin to the same extent. I would hypothesize that paclitaxel would impair the DRG neurons to a greater extent in the in-vitro model, as this system would limit the number of potentially confounding factors and alternate homeostatic adjustments present in the animal model. During my PhD training I gained experience in islet isolation and extraction from pancreatic islets but did not have time to complete these culturing experiments.

From the neonatal capsaicin mice experiments we can validate that the TRPV1 neurons are important for insulin signaling and that this proposed neuroendocrine feedback loop may play a greater role than previously anticipated. The onset of hypinsulinemia was much quicker and more robust than previously reported in our labs work with the SNIRKO mice [79]. Another way to increase specificity to determine where the robust effect of the capsaicin treated animals is occurring to alter their metabolic state is to perform regional ablation of TRPV1+ neurons. Through targeted injections of capsaicin, we could ablate only the splanchnic neurons innervating the pancreas or we could target the nodose ganglion afferents, as opposed to

eliminating all TRPV1+ neurons in a neonatal model. In fact, it has been shown that mice neonates express a higher predominance of TRPV1+ fibers during early development, then some of those fibers switch and become non-peptidergics, therefore it is possible that the effect we observed in the neonatal capsaicin treated animals was due to more than just ablation of peptidergic afferents.

A translational application of the neonatal capsaicin treatment would be the genetic condition known as CIPA or congenital insensitivity to pain with anhidrosis. Individuals with this condition have a mutation in NTRK1, a gene that encodes for the receptor for nerve growth factor. This leads to a systemic loss of sensory and sympathetic neurons. Interestingly it has been shown in studies that CIPA patients demonstrate decrease first phase insulin response [402]. As this patient population is better characterized it may become evident that they are at increased risk for development of overt diabetes and other metabolic pathologies.

It is interesting that in the PACMAN study we have observed that the prediabetics with neuropathy had a significantly lower LDL level than the prediabetics without neuropathy. There is increasing evidence in the literature that lower lipids are associated with diabetic peripheral neuropathy and are related to neuropathic changes such as increased pain, lower nerve conduction velocities, and presence of nerve lesions [403]. However, maintaining lower LDL levels is a core tenet and standard of cardiovascular healthcare to prevent atherosclerotic heart disease.

One potential mechanism by which the ketogenic may be exerting a neuroprotective effect is through upregulation of autophagy. It has been shown that increase levels of autophagy increase neuron metabolic health and may reduce neuropathic symptoms. Dorsal root ganglions must support extremely long neurite outgrowths of over a meter, in the case of the lower extremities. Our lab is also investigating the effects of heat shock proteins in mice as well as human patient through another clinical trial where we are warming patients core body temperature up through immersion in a hot tub to attempt to upregulate heat shock proteins. This may be another possible therapeutic intervention with translational potential for diabetic or chemotherapy induced peripheral neuropathy.

An additional point of discussion is the difference between painless peripheral neuropathy and painful peripheral neuropathy. It is often believed that patients will develop more painful phenotypes early on in their disease progression of peripheral neuropathy. However, as the neuron damage becomes more sever eventually the afferent signal ceases all together and the patient develops decreased sensitivity. Due to difficulty in patient recruitment, complicated by the presence of the COVID-19 pandemic, we have not been able to stratify our prediabetic patients with neuropathy based on presence or absence of pain due to low numbers. We hope to be able to continue to recruit additional patients and be able to stratify the data between these two unique populations. With significant subject numbers of prediabetic with painful neuropathy and prediabetics with painless neuropathy we might be able to then see a significant difference in peptidergic nerve fiber ratio that was not seen previously in our study.

While prediabetic neuropathy and chemotherapy induced peripheral neuropathy likely differ in several underlying pathogenic mechanisms, they may have more in common than previously acknowledged. Through my experience with paclitaxel in mouse animal models, as well as my work in our clinical study characterizing prediabetic patients with neuropathy, I learned valuable insights about both disease processes. I furthermore had the privilege of being able to work in a basic science laboratory while also gaining training in the conduct of clinical research, both of which provided impactful experience in my training as a future physician-scientist. I learned how to frame clinical questions from the basic science findings we observed in the mice, and simultaneously I saw clinical differences in my patient populations in the PACMAN study that generated discussions about potential animal studies or genetic models that we could perform in the lab. I believe that these last four years in the Neuroscience Graduate Program provided excellent training for me to learn basic and clinical science in peripheral neuropathy research, and the skills and knowledge obtained will be immensely valuable for a future career as an MD/PhD.

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Appendix A: Informed Consent

RESEARCH CONSENT FORM
Epidermal Axon Changes in Patients with Prediabetes
Protocol #0004259

Participant Name:_____ Unique Code:_____

INTRODUCTION
You are being asked to join a research study. You are being asked to take part in this study because you are either healthy (no diabetes and no nerve damage in your feet and legs), have prediabetes *but* no nerve damage in your feet and legs, or because you have prediabetes *and* nerve damage in your feet and legs. You do not have to participate in this research study. The main purpose of research is to create new knowledge for the benefit of future patients and society in general. Research studies may or may not benefit the people who participate.

Research is voluntary, and you may change your mind at any time. There will be no penalty to you if you decide not to participate, or if you start the study and decide to stop early. Either way, you can still get medical care and services at the University of Kansas Medical Center (KUMC).

This consent form explains what you have to do if you are in the study. It also describes the possible risks and benefits. Please read the form carefully and ask as many questions as you need to, before deciding about this research.

You can ask questions now or anytime during the study. The researchers will tell you if they receive any new information that might cause you to change your mind about participating.

This research study will take place at the University of Kansas Medical Center (KUMC) with Wright, PhD as the researcher. About 90 people will be in the study at KUMC.

BACKGROUND
People with prediabetes can have damage to the nerves in the legs and feet. This can worsen if patients progress to full diabetes. The nerve damage can cause pain, numbness, and problems with walking. Doctors and researchers do not understand how prediabetes damages these nerves and causes pain. There are very few treatments available for people with nerve damage to improve the nerves of the legs and the feet. This study may help researchers understand how nerves are damaged in patients with prediabetes by allowing them to see how prediabetes changes specific proteins in the nerves. By understanding how these nerves change during the prediabetes to diabetes progression, researchers hope to identify ways in which prediabetes can hurt nerves. This may help researchers design better treatments to halt or reverse the pain.

PURPOSE

By doing this study, researchers will compare the nerves of healthy individuals to people with prediabetes to analyze the damage that prediabetes causes to the nerves.

PROCEDURES

If you are eligible and decide to participate in this study, your participation will involve two study visits over two weeks. You will be asked to come to the Clinical Research Center for your first visit, which will last approximately three hours. The second visit will take place about one week later at the KU Landon Center on Aging and should last only one hour. Below are descriptions of the procedures that will be completed at each visit:

Visit 1 – To prepare for the visit, you must not have anything to eat or drink (other than water) for 10 hours before arriving for your appointment. You also must not participate in vigorous physical activity for 24 hours before the visit. Please take any medications you normally take in the morning.

- You will first review the Informed Consent Form and discuss it with research staff. If you decide that you want to participate, you will be asked to sign the form.
- You will be asked about your current health, medical history, and any medications you may be taking.
- You will have your height, weight, blood pressure, and heart rate measured.
- Before we collect any blood samples, we will administer a point of care (POC) glucose finger stick to ensure that you blood sugar is within a safe range to continue.
- You will have blood collected, while fasting, for the following tests: HbA1c, insulin lipid panel, hematocrit and hemoglobin (about 4 teaspoons of blood total).
- After fasting blood collection is done, you will have an oral glucose tolerance test (OGTT), which measures how your body uses glucose. You will drink a 10 oz. glucose drink and blood will be collected 60 minutes after you finish the drink and 120 minutes after (about 2 teaspoons of blood per draw).
- After the OGTT is complete, we will administer a POC glucose finger stick to ensure that your blood sugar is within a safe range before you leave.
- You will be asked to complete demographic and lifestyle questionnaires.
- After the completion of Visit 1 activities, subject will be asked about any symptoms related to hypoglycemia or hyperglycemia, and will be discharged only if they do not have any of these symptoms.

During this visit, we will find out if you meet the study requirements and are eligible to continue on to Visit 2. If the results of your blood tests meet the study criteria for diabetes you will not proceed to Visit 2. We will refer you to your healthcare provider for additional testing and treatment. With your permission, we can provide the results of your laboratory tests to your healthcare provider.

Visit 2 – You will not need to fast for this visit.

- You will be asked to complete a series of questionnaires about your past and current pain status. This will provide us with information about your perception of pain.
- A physician (Dr. Mamatha Pasnoor or a colleague) will review your tests from the first visit, examine your legs and feet, and ask you several questions about any pain you may experience. This will help determine whether you have nerve damage in your legs.
- The physician will then perform a skin biopsy for immunocytochemical analysis. Your skin will be cleansed with an antiseptic solution and you will be given a local anesthetic called Lidocaine to numb a small area of skin just above the ankle (approximately an inch in diameter). You might feel a small pinprick and a brief burning sensation for a few seconds. A small device, called a punch, is used to remove a paper-thin, circular piece of skin that is approximately 3 mm in diameter. Antibiotic ointment and a small Band-Aid will be placed over the wound. While a skin biopsy is a standard procedure commonly used by dermatologists and neurologists, in this case it is being done solely for research purposes, and not because you need to have it done as part of your medical care.

RISKS

Blood Collection and Oral Glucose Tolerance Test

You will be asked to fast overnight for blood collection. You may feel faint or dizzy from not eating. The study visit will be scheduled in the morning when possible, and you will be provided with a snack immediately following testing. We will also administer a POC glucose finger stick to ensure that your blood sugar is within a safe range before you leave.

There may be slight discomfort during the blood draw, and there is the possibility of a small bruise at the skin area of the blood draw. There is a small risk of skin infection at the area of the blood draw. There is also a very small possibility of vein inflammation. There is also a very small possibility of anemia from repeated blood collections.

During the oral glucose tolerance test, there is a very small risk of developing symptoms of low blood sugar level, which include rapid heartbeat, sweating, and headache. Nausea and vomiting are also rare, but known, risks of the oral glucose tolerance test.

Skin Biopsy

The skin biopsy procedure is not painful. However, when the Lidocaine is injected, you might feel a small pinprick and a brief burning sensation for a few seconds from the anesthesia. A small percentage of patients can have an allergic reaction to the Lidocaine. If an allergic reaction to the Lidocaine occurs, it will be treated immediately with medicines in the clinic. Following the biopsy there will probably be some soreness. There is a risk that the biopsy site will bleed or become infected. People with diabetes are at an increased risk of infection, but even in diabetic patients, the risk for infection is

low. The biopsy will leave a small scar that will fade in intensity over time.

Questionnaires
Some of the questions related to your medical history or pain status may result in feelings of discomfort or embarrassment. You may decline to answer any questions at any time.

Other Risks
There is potential loss of privacy. We will protect your information by labeling your research records with a code, and keeping the key to the code in a password protected database. There may be other risks of the study that are not yet known.

NEW FINDINGS STATEMENT
You will be told about anything new that might change your decision to be in this study. You may be asked to sign a new consent form if this occurs.

BENEFITS
You are not expected to benefit from this study. Researchers hope that the information from this research study may be useful in the treatment of patients with nerve damage in their legs from prediabetes.

ALTERNATIVES
Participation in this study is voluntary. Deciding not to participate will have no effect on the care or services you receive at the University of Kansas Medical Center.

COSTS
There will be no cost to you for participating in the study.

PAYMENT TO SUBJECTS
You will be paid $50 for each study visit ($100 total) to help with the cost of transportation or other expenses due to your participation in this study.

You will be given a ClinCard, which works like a debit card. You will be paid $50 after each study visit is completed. The money will be available within 1 business day. You can use the ClinCard at the ATM or at a store. No one at KUMC will know where you spent the money. If your card is lost or stolen, please call (866) 952-3795.

The KUMC Research Institute will be given your name, address, social security number, and the title of this study to allow them to write checks for your study payments. Study payments are taxable income. A Form 1099 will be sent to you and to the Internal Revenue Service if your payments are $600 or more in a calendar year.

Your personal information will be kept on a secure computer. It will be removed from the computer after the study is over and the money on the card has been used. Your information will not be shared with other businesses. It will be kept completely

confidential.

IN THE EVENT OF INJURY

If you have a serious side effect or other problem during this study, you should immediately contact Dr. Douglas Wright at (913) 588-6918. If it is after 5:00 p.m., a holiday or a weekend, you should call (913) 588-4852. A member of the research team will decide what type of treatment, if any, is best for you at that time.

If you have a bodily injury as a result of participating in this study, treatment will be provided for you at the usual charge. Treatment may include first aid, emergency care and follow-up care, as needed. Claims will be submitted to your health insurance policy, your government program, or other third party, but you will be billed for the costs that are not covered by the insurance. You do not give up any legal rights by signing this form.

INSTITUTIONAL DISCLAIMER STATEMENT

If you think you have been harmed as a result of participating in research at the University of Kansas Medical Center (KUMC), you should contact the Director, Human Research Protection Program, Mail Stop #1032, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160. Under certain conditions, Kansas state law or the Kansas Tort Claims Act may allow for payment to persons who are injured in research at KUMC.

CONFIDENTIALITY AND PRIVACY AUTHORIZATION

The researchers will protect your information, as required by law. Absolute confidentiality cannot be guaranteed because persons outside the study team may need to look at your study records. The researchers may publish the results of the study. If they do, they will only discuss group results. Your name will not be used in any publication or presentation about the study.

Your health information is protected by a federal privacy law called HIPAA. By signing this consent form, you are giving permission for KUMC to use and share your health information. If you decide not to sign the form, you cannot be in the study.

The researchers will only use and share information that is needed for the study. To do the study, they will collect health information from the study activities and from your medical record. You may be identified by information such as name, address, phone, date of birth, social security number, or other identifiers. Your health information will be used at KU Medical Center by Dr. Wright, members of the research team, the University of Kansas Hospital Medical Record Department, KU Hospital Laboratory, the KUMC Research Institute, the KUMC Human Subjects Committee and other committees and offices that review and monitor research studies. Your date of birth will also be shared with Quest Diagnostics laboratories in order to accurately analyze laboratory results. No other identifiers, such as your name, will be shared with Quest Diagnostics. Study records might be reviewed by government officials who oversee research, if a regulatory review takes place.

All other study information that is sent outside KU Medical Center will have your name and other identifying characteristics removed, so that your identity will not be known. Because identifiers will be removed, your health information will not be re-disclosed by outside persons or groups and will not lose its federal privacy protection.

Your permission to use and share your health information remains in effect until the study is complete and the results are analyzed. After that time, researchers will remove personal information from study records.

QUESTIONS

Before you sign this form, Dr. Wright or other members of the study team should answer all your questions. You can talk to the researchers if you have any more questions, suggestions, concerns or complaints after signing this form. If you have any questions about your rights as a research subject, or if you want to talk with someone who is not involved in the study, you may call the Human Subjects Committee at (913) 588-1240. You may also write the Human Subjects Committee at Mail Stop #1032, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160.

SUBJECT RIGHTS AND WITHDRAWAL FROM THE STUDY

You may stop being in the study at any time. Your decision to stop will not prevent you from getting treatment or services at KUMC. The entire study may be discontinued for any reason without your consent by the investigator conducting the study.

You have the right to cancel your permission for researchers to use your health information. If you want to cancel your permission, please write to Dr. Wright. The mailing address is Douglas Wright PhD, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. If you cancel permission to use your health information, you will be withdrawn from the study. The research team will stop collecting any additional information about you. The research team may use and share information that was gathered before they received your cancellation.

CONSENT

Dr. Wright or the research team has given you information about this research study. They have explained what will be done and how long it will take. They explained any inconvenience, discomfort or risks that may be experienced during this study.

By signing this form, you say that you freely and voluntarily consent to participate in this research study. You have read the information and had your questions answered. ***You will be given a signed copy of the consent form to keep for your records.***

_____
Print Participant's Name

_____ _____ _____
Signature of Participant Time Date

_____
Print Name of Person Obtaining Consent

_____ _____
Signature of Person Obtaining Consent Date