Sex-Differences in Taxane Cancer Treatment Toxicities

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by

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Dedication

To

Stuart Stivers

for your love, patience, and support

&

All who paved the way

If I have seen further, it is by standing on the shoulders of giants.

Isaac Newton

Henrietta's were different: they reproduced an entire generation every twenty-four hours, and they never stopped. They became the first immortal human cells ever grown in a laboratory.

Rebecca Skloot

The Immortal Life of Henrietta Lacks

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A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.

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Marie Skłodowska Curie, for imprinting in me scientific curiosity and a desire to learn since I was three years old. For unknowingly making my scientific contributions possible as a radiobiologist, cancer researcher, and woman scientist. Thank you for sharing a special connection with me.

Image 1: Marie Curie in her laboratory 1905 © Bettmann/CORBIS

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Publications

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Abstract

To this day, most preclinical research and clinical use of chemo and radiation cancer therapies do not take sex-differences into consideration, despite women having higher risk of adverse treatment toxicities and making up a larger survivor population than men. Additionally, evolving advances in cancer treatments continue to increase clinical success and there remains a growing unmet need to characterize the long-term toxic effects of frontline therapies, used in both women and men, on cancer survivors' quality of life.

To address these needs, a series of experiments examined the sex-specific effects of paclitaxel, a commonly used taxane family chemotherapeutic, and thoracic radiotherapy treatments on rodents' cognitive and/or cardiac function. Female and male Wild Type (WT) mice were treated with paclitaxel (150 and 300 mg/kg) administered weekly over 6 weeks or exposed to 19 Gy cardiac irradiation. Systemic, behavioral, and cardiovascular endpoints were examined to assess paclitaxel-induced toxicity and cardiac function and histology were used to assess thoracic radiotherapy-induced toxicity.

Interestingly, female WT mice exhibited enhanced tolerance compared to male WT mice in both treatment regimens. To gain more understanding of the observed female-specific protection, RhoB-deficient and aged female WT mice (22 months) were used. RhoB GTPase deficiency was interrogated for the possible impact of sex-hormones in cancer therapy toxicity (RhoB proteins being associated with estrogen receptors in females and cardiac fibrosis in males). In female mice, RhoB deficiency and advanced age had no impact on paclitaxel-induced neurocognitive impairment, but RhoB deficiency did compromise survival after radiotherapy.

Conversely, RhoB deficiency reduced paclitaxel-induced neurotoxicity in males and extended male survival after radiation.

In summary, this study revealed novel sexually dimorphic toxicities for commonly used taxane and radiation treatments, mediated in-part through age and/or RhoB-dependent pathways, which are associated with estrogen function in females and immune function in both sexes. This study provides additional data to the growing preclinical and clinical body of literature revealing sex-differences in disease progression and the critical roles of sex-hormones in immune response mechanisms. Therefore, further research is justified to elucidate estrogen and androgen-dependent mechanisms involved in the sex- and organ-specific toxicities observed after taxane and radiation therapies in preclinical and clinical research.

Chapter 1 Introduction

1.1 Significance

From the advent of modern-day biomedical research in the mid-20th century to the groundbreaking molecular biology studies of the past few decades, most research has not taken sex-differences into consideration (Check Hayden, 2010; Kim, Tingen and Woodruff, 2010; Zucker and Beery, 2010; Beery and Zucker, 2011). This stems from a historic preference of favoring male models, in part due to mainstream logic that sex does not impact fundamental biological function (Cahill, 2014), but also due to female sex-hormone fluctuations contributing to larger variations in biological endpoints, such as cognitive function, necessitating increased sample size and higher study costs (Keitt, Fagan and Marts, 2004; Becker *et al.*, 2005; Hausmann, 2017). In addition, past US congressional guidelines limited women in early-stage clinical trials due to fear of causing possible reproductive harm in trial participants, frequently leading to complete removal of women from any part of clinical studies (Mazure and Jones, 2015). As a result, from decades of sex-bias in pre-clinical and clinical studies, women have suffered from adverse drug reactions of FDA approved drug compounds 1.5 to 1.7-fold more than men U.S. General Accounting Office, 1992; Rademaker, 2001; Drug safety: Most Drugs Withdrawn in Recent Years Had Greater Health Risks for Women, 2001; Parekh et al., 2011). Fortunately, in acknowledging the consequences of such skewed biological research, the NIH implemented a policy in 2016 mandating the consideration of sex as a biological variable in NIH-funded biomedical research (NIH Policy on Sex as a Biological Variable, Office of Research on Women's Health; Arnegard et al., 2020). In accordance with this new policy and due to my own

scientific interest in the context of environmental toxicology, I dedicated my dissertation research to studying sex-differences in chemotherapy paclitaxel toxicity.

Through the course of my thesis, I provide background on the taxane family of chemotherapeutics with an emphasis on sex-specific biological mechanisms, state the hypothesis and experimental rational behind my dissertation research, and provide analysis of my experimental findings. By the end of the thesis, I provide new insight into sex-specific paclitaxel toxicity and propose updated hypotheses and future research directions. Fundamentally and most importantly, through this thesis, I hope to have demonstrated the importance and necessity of pursuing sex-specific research for the advancement of medicine.

1.2 Current Knowledge of Sex-Differences in Chemotoxicity

Little or no biomedical research pertains to characterizing sex-differences after paclitaxel exposure, despite paclitaxel being the subject of tens of thousands of scientific publications spanning over 50 years and one of the most widely used antineoplastic agents for men and women today. As a commonly used and extensively studied compound, there is an unmet need to conduct basic sex-dependent animal research on systemic paclitaxel exposure. Such research is important to provide insight into clinically observed sex-disparities in chemotherapeutic efficacies and adverse phenotypes (Tran *et al.*, 1998; Rademaker, 2001; Anderson, 2005; Bren, 2005; Jeffrey Wang and Ying Huang, 2007; Schmetzer and Flörcken, 2012; Kim, Lim and Moon, 2018; Haupt *et al.*, 2021), as well as providing further context into cancer incidence, susceptibility, and survivorship, where women typically have more favorable diagnoses and outcomes than men (Cook *et al.*, 2011; Haupt *et al.*, 2021). In addition, sex is still rarely considered when administering chemotherapeutic doses in the clinic (Islam *et al.*, 2017).

In women, chemotherapy dose is more frequently decreased during treatment compared to men due to greater acute normal tissue toxicity, such as nausea, vomiting, or neuropathic pain (Davidson *et al.*, 2019; Wagner *et al.*, 2019). Increased sensitivity in women to chemotherapy adverse reactions is likely due to lower drug clearance rates compared to men (Kim, Lim and Moon, 2018; Wagner *et al.*, 2019) and such predictable pharmacokinetic sex-discrepancies brings into question the validity of current body surface area chemotherapy dosing standards (Wagner, 2020).

Fortunately, continued advances in cancer biology and oncological treatment modalities, such as precision stereotactic radiotherapy and targeted small molecule therapies, continues to increase survivorship for both sexes (Torre *et al.*, 2016). However, with longer progression-free survival outcomes comes a growing concern about the long-term toxicities affecting the quality-of-life (QOL) in cured and/or surviving patients. Evidence suggests that cranial radiotherapy and systemic chemotherapeutic agents cause long-term changes to cognitive function in humans and rodent models (Ahles and Saykin, 2002; Jansen *et al.*, 2005; Wefel and Schagen, 2012; Koppelmans *et al.*, 2013). Additionally, there are no known treatments to prevent or mitigate these effects, that often cause permanent changes in a variety of cognitive domains, spanning learning, memory, social behavior, and pain, significantly impacting patient QOL. Female cancer survivors are the most susceptible population to long-term cancer treatment-induced cognitive dysfunction, in part, due to higher survival rates for women compared to men with the same cancer diagnosis and quality of care (Cook *et al.*, 2011) but also due to increased sensitivity to adverse chemotherapy toxicities (Davidson *et al.*, 2019;

Wagner *et al.*, 2019). Thus, there is a genuine need to further study the short- and long-term impact of systemic anti-cancer therapies at the normal tissue level.

For this reason, I have investigated sex-effects of paclitaxel chemotherapy exposure in healthy female and male mice. After six weekly intraperitoneal injection exposures of chemotherapeutic relevant levels of paclitaxel in mice, I examined cognition through behavioral testing and assessed post-treatment tissue toxicity. Drawing from paclitaxel and taxane oncology literature and my own experimental results, I provide original data and analysis contributing to advances in the basic understanding of paclitaxel-induced normal tissue toxicity and pharmacology.

1.2.1 Paclitaxel and Taxane Background

Taxanes, starting with the discovery of paclitaxel in 1962, have an interesting and notable research and translational history that has been well reviewed (Wiernik *et al.*, 1987; Walsh and Goodman, 2002; Renneberg, 2007; Weaver, 2014; Kampan *et al.*, 2015). In brief, paclitaxel, initially given the generic name of taxol upon isolation from the American Northwestern Pacific yew tree, *Taxus brevifolia Nutt. (Taxaceae*), was the only compound developed into clinical use from a 20-year collaborative effort of a nationwide plant-screening program for anticancer agents by the National Cancer Institute (NCI) and US Department of Agriculture (Wani *et al.*, 1971). The anti-neoplastic effects of paclitaxel were observed in animal cells in the 1970s (Wani *et al.*, 1971; Fuchs and Johnson, 1978; Schiff, Fant and Horwitz, 1979; Schiff and Horwitz, 1980), eventually leading to successful tumor regression in clinical trials by the 1980s (Wiernik *et al.*, 1987; McGuire *et al.*, 1989). Due to limitations of public commercialization at the time, the NCI and US congress transferred the rights of paclitaxel from

public to private property of Bristol-Myers Squibb in 1991, despite concerns over granting proprietary rights for a naturally derived compound discovered through public funding (United States Congress, House Committee on Small Business, Subcommittee on Regulation, Business Opportunities, 1992). The Federal Drug Administration (FDA) approved Bristol-Myers Squibb's Taxol® (generic term renamed to paclitaxel) as a chemotherapeutic treatment for ovarian cancer in 1992, breast cancer in 1994, and lung cancer in 1999 (Tuma, 2003). Paclitaxel has since become a frontline neoadjuvant and adjuvant treatment for many cancers and efficacy has led the way to the development of similar antimitotic compounds, known as the taxane family of chemotherapeutics. Of the family, docetaxel and cabazitaxel are also approved for clinical use, most notably for castration-resistant prostate cancers.

Taxanes are classified by their microtubule-stabilizing activity, inhibiting microtubule dynamics by preventing disassembly and cellular mitosis (further explained in *Mechanisms of Action*) (Wani *et al.*, 1971; Schiff, Fant and Horwitz, 1979). Taxanes are poorly soluble in water and require solvents to prevent crystallization during administration. Docetaxel is sufficiently dissolved in Tween 80 and ethanol, but paclitaxel requires Cremaphor EL, which is a powerful solvent known to induce adverse side-effects in patients through acute cytotoxicity. The novel nanoparticle albumin-bound formulation of paclitaxel (nab-paclitaxel) enables solubility into cells without Cremaphor EL. Semi-synthetic taxanes, docetaxel and cabazitaxel, were developed to address initial concerns over Pacific yew environmental protection as well as adverse reactions and resistance to paclitaxel neoadjuvant treatments (Yared and Tkaczuk, 2012). Docetaxel, derived from the fast-growing European yew tree, *Taxus baccata*, was approved by the FDA in 1999 for breast cancer and in 2004 for prostate cancer. Docetaxel has longer

intracellular drug retention and greater affinity to β-tubulin than paclitaxel and differs in microtubule assembly effects and potency (Eisenhauer, 1995). Cabazitaxel was developed to reduce affinity to the ATP-binging cassette (ABC) drug efflux pump, MDR1/p-glycoprotein, which is a primary mechanism of resistance for paclitaxel and other chemotherapeutics (Yared and Tkaczuk, 2012). Cabazitaxel is also more potent than paclitaxel and has shown effectiveness in docetaxel-resistant tumors. It was approved by the FDA in 2010 as a second line treatment for docetaxel-resistant prostate cancer (Paller and Antonarakis, 2011). Although docetaxel and cabazitaxel have higher potency than paclitaxel, paclitaxel is still the most commonly prescribed taxane. As a result, the majority of literature reviewed studies paclitaxel, but the fundamental mechanisms of microtubule stabilization shared by all taxanes cause qualitatively similar effects and symptoms.

1.3 Translational and Clinical Research

Although little pre-clinical research exists comparing taxane sex-effects, decades of clinical use documents sex-specific pharmacological differences after taxane chemotherapy administration. This is of particular concern for oncologists, as chemotherapy administration is based on body surface area instead of pharmacology and contributes to sex-differences in therapy toxicities, frequently leading to adjusted treatment doses (Wagner, 2020). This section will highlight the major sex-specific clinical observations during taxane treatments and corresponding pre-clinical literature providing insight into biological mechanisms behind sex-differences. The section also covers basic *Pharmacokinetics* as well as pharmacodynamically focused *Adverse Drug Reactions* and *Neurotoxicity*, which account for taxanes' dose-limiting toxicities and quality of life outcomes.

1.3.1 Pharmacokinetics

One of the most prominent sex-differences in pharmacokinetics is faster elimination of almost all chemotherapies (and many drugs) in men compared to women (Tanaka, 1999; Anderson, 2005; Kim, Lim and Moon, 2018). This is thought to reduce men's risk of adverse drug reactions, but also brings to question whether each sex regularly receive their maximum tolerated dose, which is critical for optimizing curative intent (Özdemir *et al.*, 2018; Wagner *et al.*, 2019; Wagner, 2020). A retrospective population analysis of solid tumor patients receiving paclitaxel infusions (n=168) found that on average male patients had 20% higher maximal elimination capacity compared to female patients (Joerger *et al.*, 2006). Paclitaxel is known to bind to plasma proteins extensively and non-specifically and, therefore, has negligible red blood cell transport (Schrijvers, 2003), suggesting little impact of sex in hematocrit transport activity (Murphy, 2014). Sex-differences in pharmacokinetics may impact drug disposition and important treatment factors such as intracellular taxane concentrations in normal and metastatic tissue (Maloney *et al.*, 2020).

1.3.2 Adverse Drug Reactions

Clinical taxane use has been associated with more adverse drug reactions than other chemotherapies, particularly for breast cancer patients. The first group of breast cancer patients to receive paclitaxel (n=55 studied) upon approval in 1999 demonstrated significantly higher rates of adverse acute toxicities compared to non-taxane treated patients (n=83), including arthralgia/myalgia (45% vs 26%) and ataxia (20% vs 5%) (Thornton *et al.*, 2008). Paclitaxel-induced toxicities also seem to be more common in women, as one retrospective

analysis of non-small lung cancer patients receiving paclitaxel reported more adverse drug reactions in women (77%) compared to men (66%, p=0.0004). However, in these lung cancer patients, women demonstrated longer progression-free survival than men (Hazard Ratio (HR) 0.83, p=0.02) (Wheatley-Price *et al.*, 2010). Such data supports sex-specific trends in overall cancer toxicities as well as cancer outcomes (Haupt *et al.*, 2021). Women are more likely to suffer from more serious adverse drug toxicities, compared to men after most types of chemotherapy exposures (Schmetzer and Flörcken, 2012; Cristina *et al.*, 2018; Davidson *et al.*, 2019), but benefit from better survival outcomes (Siegel, Miller and Jemal, 2017; Miller *et al.*, 2019). Current understanding in sex-dimorphisms in disease suggest sex-specific immune modulation plays a critical role in cancer treatment-induced normal tissue toxicities and survival outcomes (Klein and Flanagan, 2016; Roved, Westerdahl and Hasselquist, 2017; Márquez *et al.*, 2020), thereby warranting further investigation into the context of taxane treatments.

The most acute taxane-associated adverse drug reactions are hypersensitivity reactions (HSRs), including flushing, hemodynamic alterations, dyspnea, musculoskeletal/neuropathic pain, and gastrointestinal issues (Weiss *et al.*, 2016). All chemotherapeutics can induce HSRs varying in severity and degree, but taxane-induced HSRs typically manifest during the first or second infusion (Castells *et al.*, 2008; Limsuwan and Castells, 2010). Immune response, involving Immunoglobulin E (IgE) and/or IgG mast cell signaling, mediates hypersensitivity reactions, but the impact of sex on mechanisms have yet to be characterized in pre-clinical or clinical literature. Fortunately, patients are successfully treated with premedication of corticosteroids and antihistamines, but poor measures exist to predict patient risk, severity, and optimal treatment strategy for taxane-induced HSRs. This is in part due to solvent and taxane

moiety-dependent variability in HSR mechanisms, but sex has also been considered as a risk factor due to pharmacokinetic differences of steroids and taxanes elimination rates and frequent observations of HSRs in breast and ovarian cancer patients (Picard and Castells, 2014). In fact, a recent medical record analysis of 3,181 Stanford Cancer Institute patients receiving paclitaxel or docetaxel associated female sex with increased risk of overall HSRs (HR 1.26) and gynecology oncology patients had increased risk of overall (HR 1.34) and high-grade HSRs (HR 2.34) (Lansinger *et al.*, 2021). As mentioned before, such data supports other trends of higher adverse toxicities in women. Due to the fact that HSRs are easily treatable and transient (only associated with infusions) little pre-clinical research has explored sex-specific mechanisms. However, such research could provide critical insight into fundamental sex-differences in immune response that contribute to the development of other toxicities, such as taxane dose-limiting and quality of life-impairing neurotoxicity.

1.3.3 Neurotoxicity

Most chemotherapeutic agents, including taxanes, have limited blood brain barrier (BBB) penetration and are readily purged from the brain through membrane-bound p-glycoprotein (p-Gp) pumps (Kemper *et al.*, 2003; Balayssac *et al.*, 2005). However, certain taxane preparations, like cabazitaxel, have greater BBB permeability (Cisternino *et al.*, 2003) or contain solvents associated with additional BBB permeability, such as paclitaxel in Cremophor EL, which inhibits hippocampal cell proliferation in rodents (Huehnchen *et al.*, 2017). Dorsal root ganglia and peripheral nerves are subject to chemotherapeutic agent toxicity through blood-nerve barrier permeability which, due to lymphatic and p-Gp absence in nerves, likely causes the commonly observed chemotherapy-induced peripheral neuropathic toxicities

(Montague and Malcangio, 2017). Although such distinguishing features of the central nervous system are thought to exist independently of sex, the majority of such basic research has utilized male rodents and therefore sex-differences in the aforementioned neuronal characteristics cannot be entirely ruled out.

Clinical manifestations of taxane-induced neurotoxicity predominantly include peripheral neuropathy and cognitive dysfunction (Costa *et al.*, 2020). Limited comparisons of sex-differences in the neurotoxicity of taxane recipients exist, but as most clinical data documents women (predominantly breast cancer survivors), broader comparisons are confounded, and suggests the preponderance of taxane-induced neurotoxic phenotypes burden women.

1.3.3.1 Peripheral Neuropathy

Taxane-induced peripheral neuropathy (PN)-associated pain is the most concerning and critical clinical observation affecting patients' immediate and long-term quality of life (Scripture, Figg and Sparreboom, 2006; Augusto *et al.*, 2008; Tofthagen, McAllister and Visovsky, 2013; Costa *et al.*, 2020). Since the elimination of chemotherapy-induced neutropenia through the implementation of preventative granulocyte colony-stimulating factor treatment (Sarosy *et al.*, 1992), PN-associated pain is the most common reason for taxane treatment dose reductions (Seretny *et al.*, 2014). Although most cancer patients receiving taxanes develop PN (Costa *et al.*, 2020), no comprehensive analysis of clinically observed sex-differences exists. In an analysis of 219 American breast cancer survivors treated with adjuvant paclitaxel, 97% developed PN and 60% developed chronic PN one year following the cessation of treatment cessation (Tanabe *et al.*, 2011). On the other hand, in 82 prostate cancer patients who received

docetaxel, only 32% developed PN (Nakai *et al.*, 2020). Although docetaxel is thought to induce less PN than paclitaxel (Costa *et al.*, 2020), these data further highlight the distinct clinical sex-differences in taxane treatment strategy and PN outcomes that produce a greater burden on female cancer survivors (Winters-Stone *et al.*, 2017; Kober *et al.*, 2018). Moreover, only non-pharmacological PN pain management strategies exist for taxane recipients (Weimer, 2003; Windebank and Grisold, 2008; Costa *et al.*, 2020). One reason for unsuccessful pharmacological approaches may be due to the lack of mechanistic insight regarding fundamental sex-differences in taxane-induced PN. However, in recent years growing pre-clinical evidence suggests that taxane-induced PN manifests through distinct neuroimmune mechanisms in female and male rodents and may provide valuable insight into understanding and treating sex-differences in taxane-induced PN as well as other toxicities.

Based on pre-clinical studies primarily using male models (Mogil, 2012), glial cells and proinflammatory immune responses from innate immune cells are implicated in peripheral neuropathy (Vallejo *et al.*, 2010), including taxane-induced pain (Boyette-Davis *et al.*, 2011). Candidate mechanisms, such as toll-like receptor (TLR) 4 activation (Li *et al.*, 2014), extensively studied in male rodents have only recently been examined in females. As a result, sex-specific findings in the field of pain have been categorical, illustrating that microglia, and their associated signaling molecules, drive neuropathic pain only in the male sex (Sorge *et al.*, 2015; Rosen, Ham and Mogil, 2017; Gregus *et al.*, 2021).

Supporting this, one recent study found that TLR9 inhibition only attenuated paclitaxel-induced mechanical allodynia (touch induced pain) in males and not female mice. However, interestingly TLR9 antagonism reduced paclitaxel-induced pain in female nude mice, lacking T

and B cells (Luo, Huh, *et al.*, 2019), adding to evidence that females preferentially utilize humoral immunity, but also have the ability to recruit innate immunity-dependent microglia when adaptive immunity is unavailable (Sorge *et al.*, 2015).

Sex hormones and sex organs drive sex-specific immunity (Klein and Flanagan, 2016). In one study examining pain thresholds and proinflammatory cytokine receptor levels in the dorsal root ganglion of female rats exposed to paclitaxel, ovariectomies significantly increased pain thresholds and decreased receptor expression levels after exposure, compared to females with 17β-estradiol and progesterone replacement (Y. Wang *et al.*, 2018), suggesting that paclitaxel-induced neuropathic pain in females is sex hormone dependent.

Sex-differences in stress phenotypes (Bale and Epperson, 2015) is a growing field of research as well as clinical risk factor and comorbidity of taxane-induced PN (Kober *et al.*, 2018). In a study examining the neuroendocrine stress axis in rats, paclitaxel-induced hyperalgesia was significantly more attenuated in female rats, compared to males, following β2-adrenergic receptor reduction through targeted intrathecal antisense oligodeoxynucleotide administration (ODN). By contrast, ODN decreasing glucocorticoid receptors attenuated paclitaxel-induced pain in males, but not females. Additionally, the study found that neonatal handling prevented paclitaxel-induced PN in male but not female rats, providing evidence of distinct sex-specific neuroendocrine mechanisms related to paclitaxel-induced pain (Ferrari *et al.*, 2020).

Based on these recent findings and current understanding in sex-differences in immune function, female-specific pain is likely due to humoral immune responses, such as T and B cell activation, and may require novel treatment approaches and a more sophisticated

understanding of mechanisms in both sexes (Parvathy and Masocha, 2015; Lopes *et al.*, 2017; Luo, Gu, *et al.*, 2019). These data also bring in to question other possible impacts of sexually dimorphic immune responses in taxane-induced toxicities. Divergent preferential immune function between the sexes, defined as females utilizing more adaptive immunity while males rely more heavily of innate immunity, is becoming a dominant theme in pharmacology and etiology (Klein and Flanagan, 2016; Natri *et al.*, 2019; Haupt *et al.*, 2021) and, therefore, perhaps it should be the starting point of studying sex as a biological variable in drug and disease research.

1.3.3.2 Cognitive Dysfunction

Chemotherapy-induced cognitive dysfunction, also known as 'chemobrain,' is of growing clinical concern due to detrimental impacts on long-term quality of life in increasing populations of aging cancer survivors (Argyriou *et al.*, 2011; Myers, 2012; Wefel and Schagen, 2012; Dietrich and Kaiser, 2016; Loh *et al.*, 2016; Janelsins *et al.*, 2017; Vitali *et al.*, 2017; Li and Caeyenberghs, 2018). As outlined in the above *Peripheral Neuropathy* section, existing literature of pre-clinical rodent studies comparing both sexes suggest greater taxane-induced peripheral neuropathic pain in females than males. But paradoxically, recent (albeit limited) literature comparing paclitaxel-induced cognitive dysfunction in both sexes demonstrates female-specific resistance (Liang *et al.*, 2020). 16 mg/kg paclitaxel (dissolved in Cremophor EL and ethanol) intraperitoneal exposures of C57BL6 mice induced cognitive dysfunction in prefrontal cortex-associated Novel Object Recognition task and anxiety-like behavior in Elevated Plus Maze in males, but not females. Such data seem contrary to the cognitive impairment reported with adjuvant taxane treatments in women.

The first group of breast cancer patients to receive the FDA approved paclitaxel (n=55 studied) not only demonstrated significantly higher rates of adverse acute toxicities (mentioned in *Adverse Drug Reactions* section), but also significantly more mental distress and reduced mental quality of life compared to women who received other chemotherapy ($p<0.023$). In addition, this study observed longer emotional recovery for paclitaxel treated patients, that required an average of 2 years, compared to 6-12 months for patients not receiving taxanes (Thornton *et al.*, 2008). In a recently published study comparing sexes, a small population of nasopharyngeal carcinoma survivors receiving adjuvant docetaxel, cisplatin, and fluorouracil chemotherapy, found that female sex was associated with cognitive dysfunction ($p=0.039$), manifesting in 50% of women (8 of n=16) and only 20% of men (10 of n=50) (Wang *et al.*, 2020). A 2017 longitudinal study of breast cancer survivors comparing adjuvant chemotherapies with and without taxanes (n=51) demonstrated short- and long-term cognitive impairment in attention and executive function after all treatments, with a more pronounced impact on short-term verbal learning and speed measures in the taxane group (Cerulla *et al.*, 2017). Another analysis of breast cancer survivors shortly after and 1 year after adjuvant chemotherapy (majority with paclitaxel, up to 8 patients without) demonstrated acute (65%, 24 of n=37) and long-term (61%, 17 of n=28) cognitive decline (Wefel *et al.*, 2010). A recent female-only rodent study supports these clinical data of adjuvant therapy-induced cognitive impairment in women. Brown et. al. characterized diminished hippocampal-dependent cognitive behavior and compromised dendritic architecture and signaling proteins in female hippocampal tissue after adjuvant docetaxel, doxorubicin, and cyclophosphamide therapy (Brown *et al.*, 2021).

In perhaps the only clinical analysis of cognitive functioning after taxanes in males, 65+ year old castrate-resistant prostate cancer patients treated with adjuvant therapies, including docetaxel, did not experience significant cognitive impairment measured by the Montreal Cognitive Assessment (Alibhai *et al.*, 2020). In contrast, an analysis comparing 65+ year old early-stage breast cancer survivors, all treatments (radiotherapy with or without doxorubicin ± docetaxel) demonstrated significant impacts on cognitive decline in 49% of patients, with the oldest patients (70-81 years) most sensitive to docetaxel associated decline (p=0.05) (Lange *et al.*, 2016).

Although little research investigates sex-specific burdens of chemotherapy-induced cognitive dysfunction in cancer survivors, it is interesting to point out that almost all clinical studies assessing taxane-induced cognitive outcomes are in breast cancer survivors, while most pre-clinical rodent studies examine outcomes in males (Fardell *et al.*, 2013; Seigers *et al.*, 2014; Callaghan and O'Mara, 2015; Huehnchen *et al.*, 2017; Panoz-Brown *et al.*, 2017). The larger amount of female-specific clinical data is in part due to the total of 8.7 million women cancer survivors in the U.S (over 600,000 more survivors than men) of which 3.8 million are breast cancer survivors (Miller *et al.*, 2019). In addition, the larger proportions of women suffering from adverse chemotherapy effects compared to men undoubtably impacts stress and depression and, therefore, cognitive function and quality of life during and after recovery. The diversity in clinical patient data (e.g. cancer type, adjuvant treatments, age, etc.) is not always comparable to highly-controlled pre-clinical studies and likely accounts for the discrepancy in taxane-induced cognitive dysfunction phenotypes presenting in women, but less frequently in female rodents.

1.4 Basic Research

'Mechanism of action' in cancer therapy refers to an agent's primary antineoplastic mechanism, often antimitotic in nature. Twentieth century chemotherapy pre-clinical research almost exclusively studied cancer cells and scientists were predominantly concerned with the 'mechanisms of action' directly related to inhibiting cancer growth. In the 21st century, as survival rates and treatment efficacies increased, quality-of-life outcomes became more important and a growing issue for cancer survivors, increasing biomedical research interests in normal tissue effects. Due to the non-specific nature of chemotherapeutics, it is important to consider how mechanisms of action impact normal tissue toxicity and sex.

It is interesting to note that virtually all basic and pre-clinical *in vivo* cancer drug research done in the 1900s, such as with paclitaxel, utilized female HeLa cervical adenocarcinoma cell lines, the first and most widely used immortalized human cell line derived from 31-year-old Henrietta Lacks' aggressive cervical cancer in 1951 (*Significant Research Advances Enabled by HeLa Cells – Office of Science Policy*, no date; Wani *et al.*, 1971). Although no other cell line has contributed more to foundational biological understanding and biomedical advances, its sex was rarely considered or examined as an experimental variable. Despite little consideration of sex in basic research, clinical data provides justification for such research. As discussed in earlier in *Translational and Clinical Research*, women have better treatment outcomes across most cancer types and sex-differences are readily observed in adverse drug effects, drug disposition, and pharmacokinetics, which undoubtably impacts chemotherapy antineoplastic efficacy and normal tissue toxicity. The following sections draw from fundamental research (not considering sex) of taxane antineoplastic activity and sex-

specific cancer data to highlight potential sex-specific mechanisms of toxicity relevant to all, if not, most tissue.

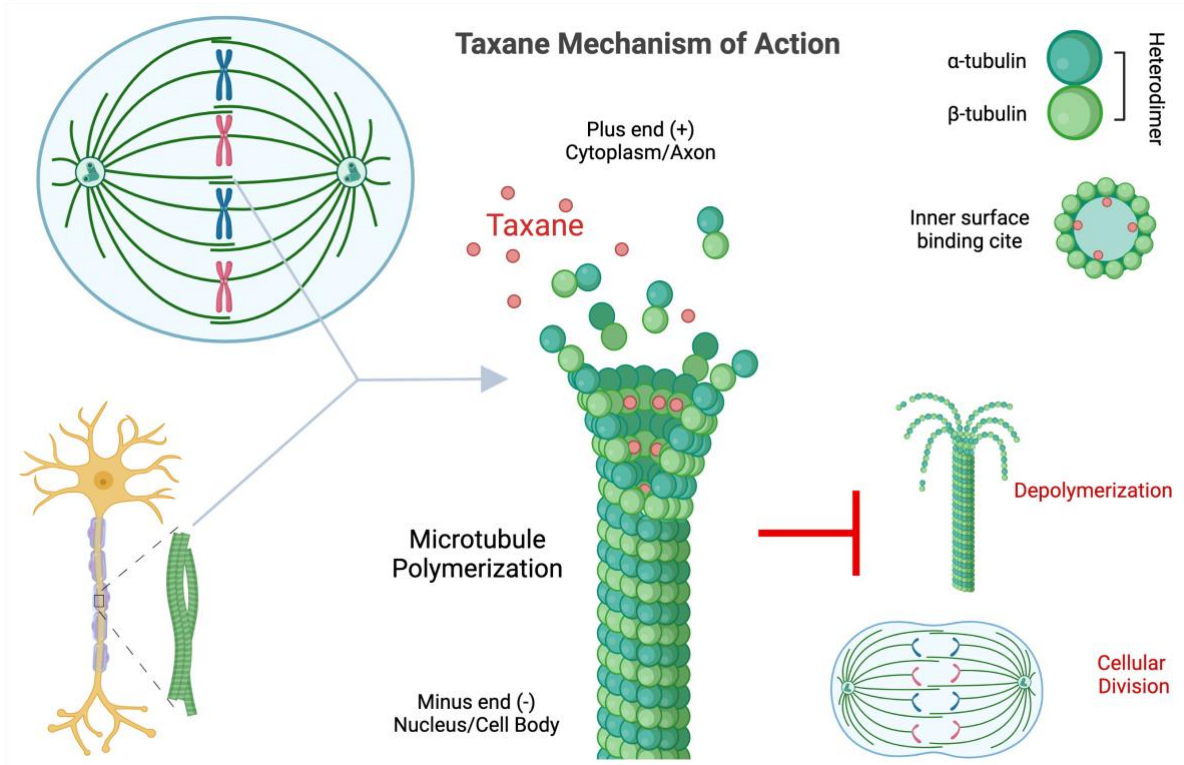


Image 2. Known taxane mechanism of action. Taxanes bind to the inner surface of microtubule filaments, on the β-tubulin globular protein of the heterodimer, protofilament tubule arrangement. Taxanes prevent microtubule depolymerization, preventing normal microtubule dynamics for cellular activities, impacting cytoskeletal structure and microtubule function, such a mitosis and microtubule-dependent cellular transport. Created with BioRender.

1.4.1 Mitotic Arrest

After initial observations of paclitaxel-induced Kb HeLa-derived cell growth inhibition, published in 1971 (Wani *et al.*, 1971), tumor drug researcher, Susan Horwitz, was the first to characterize the mechanism of paclitaxel-induced mitotic arrest with her graduate student, Peter Schiff (Weaver, 2014). Their landmark 1979 publication identified paclitaxel as a microtubule assembly promoting poison, unlike previously identified microtubule poisons which prevent microtubule polymerization. In the words of Horwitz, microtubules behave like "paralyzed cytoskeleton[s]" (Horwitz and Goldman, 2015) in the presence of β-tubulin bound paclitaxel. Paclitaxel reduces the critical concentration of tubulin subunits necessary for microtubule polymer formation, increasing the percentage of α- and β-tubulin heterodimers assembled, and inhibits mitosis through failure of metaphase depolymerization. They observed that paclitaxel-induced microtubule growth was even resistant to cold and calcium depolymerization treatments (Schiff, Fant and Horwitz, 1979). The observation of paclitaxel-induced polymerization and "parlay[sis]" of microtubules causing mitotic arrest was confirmed in a variety of cell and animal models, in both cancerous and non-cancerous tissue (Schiff and Horwitz, 1980; de Brabander *et al.*, 1981; Milas *et al.*, 1995; Shi, Orth and Mitchison, 2008). For decades, the dominant perception of paclitaxel's tumor treatment efficacy was through the inhibition of metaphase bipolar spindle depolymerization and mitotic checkpoint-dependent mitotic arrest (Waters *et al.*, 1998; Sudo *et al.*, 2004). However, investigators have also observed taxane-induced apoptosis unassociated with mitotic arrest with formidable cancer regression in low-proliferating tumors, suggesting alternative cytotoxic pathways critical in taxane antineoplastic activity (Milas *et al.*, 1995; Milross *et al.*, 1996; Shi, Orth and Mitchison,

2008; Komlodi-Pasztor *et al.*, 2011; Mitchison, 2012). In fact, drugs designed to exclusively inhibit mitosis have shown limited anti-neoplastic success and fail to replace microtubule poison chemotherapeutics (Shi, Orth and Mitchison, 2008; Chakravarty *et al.*, 2011; Mitchison, 2012; Florian and Mitchison, 2016). Advances in characterizing taxane-induced microtubule dysfunction beyond mitotic arrest suggest alternative cytotoxic mechanisms of action may be the preponderance of taxane anti-cancer activity.

1.4.2 Microtubule Dynamic Dysfunction

Although the primary antineoplastic mechanism of taxanes was initially thought to involve mitotic arrest through microtubule dysfunction, current scientific consensus also attributes taxane tumoral regression efficacy to additional, perhaps more potent, microtubule-dependent cytotoxic mechanisms (refer to pior section on *Mitotic Arrest*). Due to the importance of microtubule function in a range of cellular activity, most notably mitotic spindle assembly, cytoskeletal structure, and cytoplasmic cellular cargo migration (Gundersen and Cook, 1999; Etienne-Manneville, 2010), it is hypothesized that taxanes also elicit antineoplastic activity through mitotic checkpoint-independent cell death (Milross *et al.*, 1996; Komlodi-Pasztor *et al.*, 2011; Zasadil *et al.*, 2014) and cellular transport disruption (Darshan *et al.*, 2011; Carbonaro *et al.*, 2012; Thadani-Mulero, Nanus and Giannakakou, 2012). In fact, early paclitaxel research demonstrated aberrant microtubule dynamics and toxicity at doses insufficient to induce mitotic arrest (Jordan *et al.*, 1993, 1996; Torres and Horwitz, 1998; Ikui *et al.*, 2005). In a notable recent study, adjuvant paclitaxel-treated human breast tumor biopsies revealed that intratumoral drug concentrations were insufficient to induce mitotic arrest and tumor regression efficacy was attributed to increased multipolar spindle formations, implicating p53-

independent, chromosome missegregation-induced cellular death (Zasadil *et al.*, 2014). Such data suggest that taxanes can produce a range microtubule-dependent aberrant phenotypes, dependent on intracellular drug concentration and phase of cell-cycle.

Understanding the impact of taxanes on microtubule-dependent nuclear trafficking and signaling is perhaps one of the most challenging phenotypes to characterize due to the extent and complexity of microtubule mediated mechanisms. However, the influence of taxanes on androgen receptor signaling has been extensively observed and studied in the field of prostate cancer and provides additional understanding into the impact of taxane-induced microtubule dysfunction and sex-specific mechanisms of toxicity.

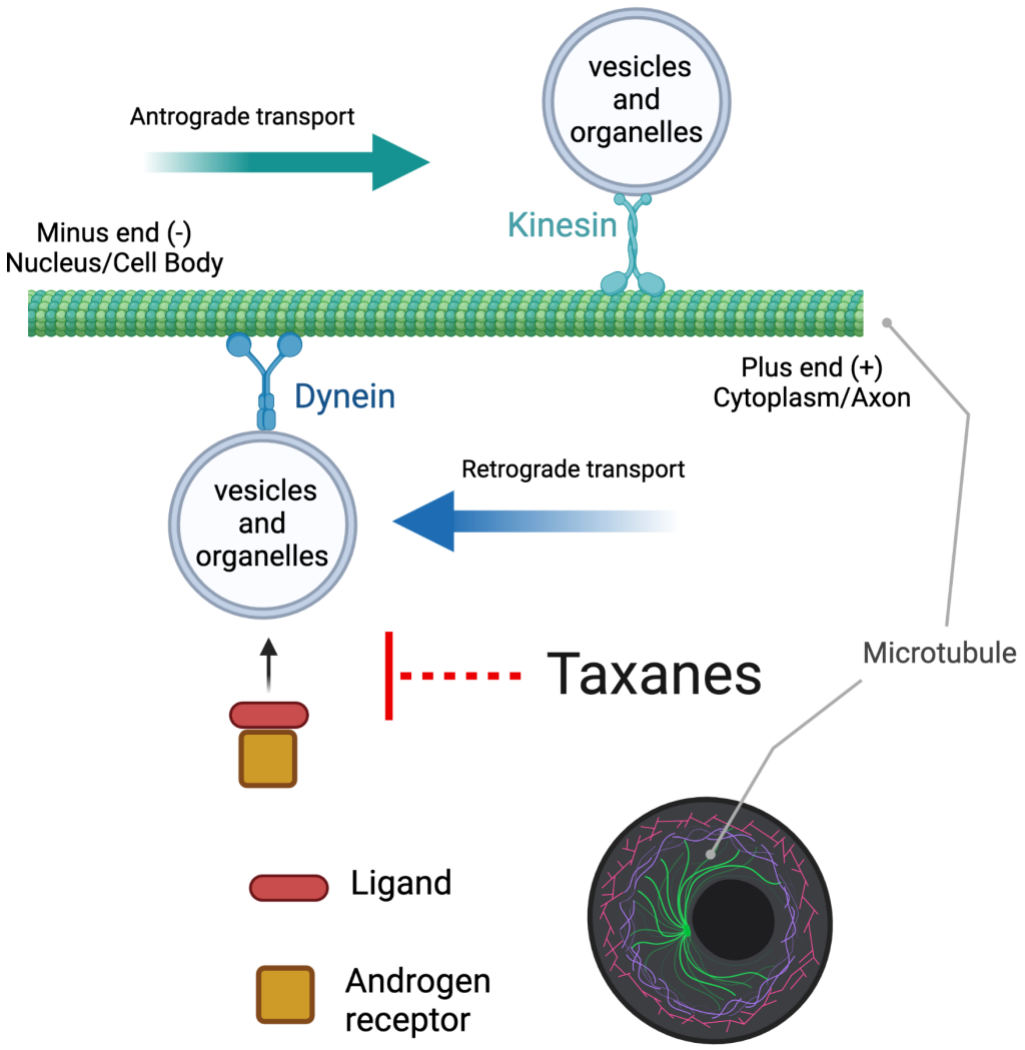


Image 3. Taxane-induced androgen receptor (AR) microtubule-dependent signaling dysfunction. In the field of prostate cancer research, taxanes are observed to interfere with ligand-mediated AR signaling by inhibiting AR binding to dynein motor protein, preventing nuclear trafficking of AR. Created with BioRender.

1.4.2.1 Androgen Receptor Signaling Dysfunction

In the field of prostate cancer research taxanes are known to inhibit androgen receptor (AR) signaling through microtubule dysfunction (Zhu *et al.*, 2010; Darshan *et al.*, 2011). AR signaling mechanisms are the cause and treatment target for prostate cancers (Feldman and Feldman, 2001; Attard, Richards and Bono, 2011; Watson, Arora and Sawyers, 2015). Androgen deprivation therapy (ADT), used to block AR or eliminate AR ligands, initially work to inhibit the growth of prostate cancers as a frontline treatment, but over time castrate-resistant prostate cancer (CRPC) occurs through loss of ADT sensitivity (Watson, Arora and Sawyers, 2015). In 2004, taxanes became the first class of chemotherapeutic drugs demonstrating improved survival in CRPC and are now used in conjunction with frontline ADT treatments, prior to the development of castration resistance (Petrylak *et al.*, 2004; Tannock *et al.*, 2004; Bono *et al.*, 2010; Antonarakis *et al.*, 2017). Although research on the effects of taxanes on AR signaling is almost exclusive to the field of prostate oncology, these male-specific observations provide context into fundamental sex-differences of systemic taxane exposures for male and female malignant and normal tissue.

Researchers have observed taxane-dependent inhibition of AR expression and activity in prostate cancer (Gan *et al.*, 2009; Kuroda *et al.*, 2009; Zhu *et al.*, 2010). Specifically, Darshan et. al. demonstrated that paclitaxel inhibits microtubule ligand-induced AR nuclear accumulation and downstream transcriptional activity (Darshan *et al.*, 2011). The same study indicated a significant correlation between AR cytoplasmic sequestration (instead of nuclear accumulation) and therapeutic efficacy in the circulating tumor cells of CRPC patients receiving taxanes. Interestingly, although not entirely understood, taxane-induced interference with AR signaling

produce antineoplastic efficacy in both naïve and castrate resistant malignancies (Watson, Arora and Sawyers, 2015). One pre-clinical research study observed that chronic AR activation, through testosterone-BSA exposure, enhanced paclitaxel microtubule disrupting dynamics, inhibited cell proliferation, and induced apoptosis in androgen sensitive and insensitive human prostate cancer cell lines (Kampa *et al.*, 2006). In the same study xenografted mouse tumors (both androgen sensitive and insensitive) decreased in mass with both testosterone-BSA and paclitaxel separately, an effect that was enhanced in combination. These data support clinical observations of taxane treatment efficacies in prostate cancers sensitive and resistant to ADT (Antonarakis *et al.*, 2017).

Androgen receptor signaling is critical for prostate cancer as well as normal male tissue development, thus providing a potential sex-specific discrepancy in taxane tissue toxicity. AR knockout (KO) mouse studies have not only demonstrated critical function of AR in male (and female) gonad and gamete development, but also in male body fat, bone, blood, and immune phenotypes (Yeh *et al.*, 2002; Venken *et al.*, 2006; Kerkhofs *et al.*, 2009; Lai *et al.*, 2012). Conditional and constitutive ARKO male mice have severe deficits in both innate and adaptive immunity, resulting in phenotypes such as thymus enlargement, immature B-cell populations, and risk of neutropenia and bacterial infections (Lai *et al.*, 2012). Although little research has investigated such ARKO phenotypes in female mice, since ARs dominate signaling function for male sex hormones (while estrogen receptors dominate female sex hormones) taxanes may induce disproportionate toxicity in male tissue. Supporting this idea, a developmentally critical Y-linked gene (found in male mice and men) modulates docetaxel sensitivity through AR signaling (Komura *et al.*, 2016). KDM5D (lysine-specific demethylase 5D) physically interacts

with nuclear AR and demethylates (deactivates) H3K4me3 transcriptional marks, which normally regulate enhanced AR transcriptional activity (possibly via a negative feedback). Accordingly, the attenuation of KDM5D expression led to increases in H3H4me3 marks in promoter regions of AR-regulated genes as well as protection against docetaxel in AR-positive prostate cancer cell lines. In addition, an Oncomine cancer database analysis revealed significantly lower KDM5D expression in CRPC patient samples with poorer cancer prognosis and treatment outcomes. Although KDM5D expression is associated with taxane sensitivity, the fundamental mechanism is still unclear. ERG (E-26 transformation-specific-related gene) is another gene implicated in AR-signaling in prostate cancer (Yu *et al.*, 2010; Chen *et al.*, 2013) through microtubule depolymerization. ERG binds to αβ-tubulin, and when overexpressed, reduces binding site availability for taxane-induced microtubule polymerization, leading to taxane resistance in prostate cancer cells and tumors (Galletti *et al.*, 2014). Such data defines taxane as AR signaling poisons, in part, due to epigenetic regulation. However, it is important to note that AR signaling can also take place through nuclear transport in a microtubule-independent fashion. The capability of tumors to preferentially shuttle AR through nuclear transport, is thought to contribute to taxane resistance in prostate cancer. The ability to circumvent microtubule transport machinery has been demonstrated by AR-V7, is a commonly found AR splice variant that lacks the hinging domain necessary to attach to the tubulin-dynein transporter molecule for minus-end (nuclear) microtubule transport (Kim *et al.*, 2021). Identification of AR-V7 splice variants in tumors is associated with advanced CRPC, taxane resistance, and reduced patient survival (Hu *et al.*, 2009; Hörnberg *et al.*, 2011; Antonarakis *et al.*, 2014; Maughan and Antonarakis, 2015; Tagawa *et al.*, 2019; Rizzo *et al.*, 2021).

1.4.2.2 Estrogen-Mediated Microtubule Dynamics

As the field of prostate cancer continues to elucidate the complex relationships between taxane-induced microtubule dysfunction and AR signaling, the role of estrogen and estrogen receptors (ER) on taxane-induced microtubule dynamics is less clear. However, a couple of studies suggest that estrogens and androgens may have opposite roles in microtubule polymerization. One study examining the roles of sex hormone exposures on tubulin polymerization induction, observed that prior incubation of tubulin proteins and cells, derived from fetal rat hippocampi, with 17β-estradiol inhibited microtubule assembly, while incubation with testosterone inhibited microtubule disassembly (Kipp and Ramirez, 2003). Other studies have also observed an AR+ dependent relationship between testosterone/androgen and microtubule polymerization (Butler, Leigh and Gallo, 2001; Kampa *et al.*, 2006), suggesting the presence of androgens may promote microtubule polymerization/assembly indirectly. On the other hand, recent evidence suggests estrogens may have a direct role in microtubule polymerization and function. Lo et. al. conducted a computational protein screen analysis that unexpectedly identified ER as a cognate receptor to the β-tubulin taxane binding site, suggesting tubulin-ER cross-reactivity. The researchers then confirmed that the taxane binding site had affinity with estrogen and selective estrogen receptor modulators (SERMs) in human epithelial cells and modify microtubule dynamics in a similar fashion as paclitaxel (Lo *et al.*, 2019). Interestingly, a separate *in vitro* study observed no synergistic or additive antimetastatic effects with the coadministration of the SERM, tamoxifen, with paclitaxel in ER+ breast cancer cells (Dougherty *et al.*, 2004). Such data suggests that ER ligands may directly interact with

tubulin and modulate microtubule dynamics, and in the presence of taxanes, attenuate taxane-induced microtubule polymerization.

In summary, the above section on *Microtubule Dynamic Dysfunction* outlined possible sex-hormone-mediated sex-differences in taxane-dependent microtubule dysfunction. Taxanes induce AR signaling dysfunction in a microtubule dependent manner that provide a sex-specific toxicity in male tissue due to androgen dominance. In females, the cross-reactivity of ER ligands at the taxane-tubulin binding site may inhibit taxane-mediated microtubule polymerization and attenuate downstream microtubule-dependent toxicity. This suggests sex-hormone mediated male-specific susceptibility and female-specific protection against taxane-induced microtubule dysfunction.

Chapter 2 Experimental Design

2.1 Translational Research Strategy

The understanding of taxanes' mechanisms of toxicity have evolved from initial 20th century studies of antineoplastic mitotic arrest to modern-day characterizations of microtubule-dependent signaling dysfunction and acute and long-term normal tissue neurotoxicity. Although significant advances have been made in elucidating mechanisms of taxane-induced toxicity in fields of prostate cancer and neuroimmunology, there remains an unmet need to characterize basic cytotoxic sex-differences after systemic taxane exposures. In an effort to fill this gap of knowledge, I have conducted experiments that compare sex-specific toxicities of chemotherapy-modeled doses of paclitaxel on cancer-free mice in the domains of neurological and cardiac function. Due to the translational relevancy of adjuvant cancer therapy exposures, additional collaborative research was conducted to separately characterize sex-specific effects of thoracic radiation exposure in cancer-free mice.

Together, these data provide additional context around fundamental biological sex-specific mechanisms of cancer therapies used in both women and men and demonstrate the need in elucidating cancer therapy toxicities for advancement of cancer treatment outcomes and personalized medicine.

2.1.1 Hypothesis I: Sex-dimorphisms in paclitaxel-induced outcomes

There are well documented preclinical and clinical data on the protective effects of estrogen in female cardiac and immune function (Horton, White and Maass, 2004; Choudhry *et al.*, 2005; Kher *et al.*, 2005; Klein and Flanagan, 2016; Roved, Westerdahl and Hasselquist, 2017;

Moulton, 2018; Shufelt *et al.*, 2018; Natri *et al.*, 2019; Márquez *et al.*, 2020). Based on this information, I hypothesize that estrogen-mediated immune and microtubule function provides a net protective effect in female-mice of reproductive age from paclitaxel-induced cytotoxicity in the domains of cognitive and cardiac function. Furthermore, the sex-specific dominance of androgens and AR signaling in male mice of reproductive age provides additional sensitivity in males to taxane-induced cytotoxicity, via microtubule-modulated AR signaling dysfunction (Darshan *et al.*, 2011).

In order to investigate the validity of the hypothesized sex-differences in taxane toxicity, I conducted experiments fulfilling the following aims of characterizing:

I. *in vivo* sex-specific systemic taxane exposure outcomes in Wild-Type female and male mice through the assessment of weight, survival, and cognitive function,

II. post-paclitaxel treatment immune phenotypes in cardiac tissue,

III. and the role of compromised estrogen activity and immune response through replicating experiments in aged Wild-Type female and RhoB deficient ($RhoB^{-/-}$) mice.

The chosen biological endpoints reflect the translational need to study the poorly understood neurotoxicity of taxane-induced cognitive dysfunction and a desire to examine a model organ known to exhibit sex-differences in a sex-hormone-dependent manner, the heart. After initial sex comparisons of paclitaxel-treated Wild-Type mice, aged female Wild-Type and RhoB deficient mice underwent the same treatments and analyses to examine the translationally relevant effect of age-induced estrogen depletion and the potential impact of RhoB inhibition on sex-hormone and immune function, respectively, in taxane-induced toxicity.

Due to limitations in acquiring reliable estradiol assay measurements from mouse serum (Haisenleder *et al.*, 2011) and well-known confounding impacts on cognitive, immune, and cardiac function of ovariectomy models (Baeza *et al.*, 2010; Medina-Contreras *et al.*, 2020), I chose translationally relevant models of endogenous estrogen through verifying the presence of estrous cycles by vaginal cytology in females. All Wild-Type and RhoB-deficient females exhibited regular estrous cycles, except for 22-month-old Wild-Type females, which had predominantly leukocyte-like endothelial cell phenotypes (data not shown). Therefore, 22-month-old females and all males were assumed to have lower endogenous estrogen than females with estrous cycles.

RhoB, of the family of Rho GTPases, was a target of interest to elucidate the role of estrogen and immune response after paclitaxel because it has been associated with the presence of ERα in breast cancer tumors and cells (Médale-Giamarchi *et al.*, 2013). Therefore, RhoB deficiency was hypothesized to attenuate female-specific protection from taxane-induced toxicity and RhoB $^{-/-}$females and males would have similar cytotoxic outcomes to Wild Type males and aged females.

In addition to examining the impact of sex, age, and RhoB genotype on the effects of paclitaxel exposures, two doses were examined among the cohorts of animals. A 300 mg/kg paclitaxel dose was initially given to Wild-Type female and male mice but required adjustment to 150 mg/kg for the remainder of the study's cohorts when males exhibited unexpected precipitous lethality. However, one cohort of RhoB $^{-/-}$ females was further investigated at 300 mg/kg, in an attempt to characterize genotype-specific high-dose protection in females.

The role of AR signaling was not investigated in this study due to the preponderance of evidence of its involvement in male cancer tissue exposed to taxanes (reviewed in section on *Androgen Receptor Signaling Dysfunction*). However, it was still hypothesized to sensitize male normal tissue to paclitaxel, which to my knowledge has not been examined, and should be verified in future experiments.

2.1.2 Hypothesis II: Sex-specific cardiac irradiation effects

Collateral damage of surrounding normal tissues of tumor targets is a risk of radiotherapy treatment. Left-sided breast cancer radiation treatment has well-documented risk of short and long-term cardiotoxicity in breast cancer patients (Taylor *et al.*, 2015). Preclinical and clinical investigations provide insight into the range of acute and chronic vascular injury persisting after radiation-induced cardiac toxicity (Boerma *et al.*, 2016). However, although sex-hormone-mediated cardioprotection of female patients is well known within the field of cardiovascular disease research (Horton, White and Maass, 2004; Choudhry *et al.*, 2005; Kher *et al.*, 2005; Shufelt *et al.*, 2018), no investigation related to the impact of sex is available after exposure to radiotherapy and sex-specific mechanisms of radiation-induced cardiac damage have yet to be characterized.

Accordingly, the impact of radiotherapy on the general health status and survival of male and female Wild Type and RhoB deficient mice was investigated along with more specific effects on cardiac function. We hypothesized that females through estrogen-mediated cellular protection pathways, are more protected from thoracic irradiation of 19 Gy X-rays compared to males. Furthermore, we hypothesized that estrogen-mediated protection (Médale-Giamarchi *et al.*, 2013) would be disrupted and, therefore, inhibited upon inactivation of RhoB GTPase

activity through RhoB deficient mouse models, and conversely protect males, possibly through the inhibition of Rho-mediated fibrosis (Bourgier *et al.*, 2005; Monceau *et al.*, 2012).

The hypotheses were tested through addressing the following experimental aims of investigating:

I. *in vivo* sex-specific thoracic 19 Gy radiation outcomes in Wild-Type female and male mice through the assessment of survival and cardiac function through echocardiography
II. and the role of compromised estrogen activity through replicating experiments in RhoB deficient mice.

Comparing the biological variables of survival and cardiac function provide a systemic long-term assessment of the role of sex differences and RhoB after cardiac irradiation. Initial characterization of sex-differences between monotherapies (paclitaxel and radiotherapy) were chosen as a starting point for accumulating data on basic mechanisms for the intention of developing hypothesis-driven research on sex-differences in translational adjuvant treatments in the future.

2.2 Materials and Methods

Materials and Methods were borrowed and slightly modified from the Chmielewski-Stivers *et al.,* 2021. Additional behavioral testing and Western Blot analysis were conducted on brain and cardiac tissue but did not produce reproducible results and are excluded from this thesis due to assay quality control issues.

2.2.1 Wild Type and RhoB$^{-/-}$ Mice

All animal procedures and necropsy were approved by the institutional animal care and use committees of University of California, Irvine (UCI) and/or University of Switzerland, Lausanne (Centre Hospitalier Universitaire Vaudois, Lusanne, CHUV). Wild type (WT) animals were either purchased from Jackson Labs (Sacramento, CA) or provided by on-campus breeding facilities from UCI and RhoB–deficient/knockout (RhoB $^{-/-}$) were provided by CHUV. All animals were housed with ad libitum standard diet and water on a 12-hr light cycle. All animal-related handling and experiments were performed during standard light hours. Animal weight was measured every week. Vaginal cytology was performed at UCI on all the female animals prior and during behavior weeks to confirm reproductive status.

2.2.2 Paclitaxel Exposures

Adult male and female mice (C56Bl6/J and RhoB $^{-/-}$), ranging from 12 to 88 weeks of age (**Table 1**), were divided into vehicle- (0.02% ethanol made in sterile saline) and paclitaxel-treated groups, and paclitaxel or 0.02% ethanol saline vehicle was administered via intraperitoneal injection once a week over 6 weeks at a dose of 150 mg/kg or 300 mg/kg (25 or 50 mg/kg each week, respectively) (paclitaxel, Biosynth Carbosynth, Compton, UK). Initial 300 mg/kg dose was calculated via animal equivalent dose (AED) (mg / kg) = Human dose (mg/kg) × K_m ratio (K_m mouse= 12.3, body surface area variable) (Nair and Jacob, 2016).

2.2.3 Irradiation Procedures

Heart irradiation was performed with a small animal imaged-guided irradiator, the X-RAD 225 Cx (Pxi Precision X-ray, North Branford, CT, USA). The isocenter was placed in the heart using cone beam computed tomography, (Precision X-Ray, North Branford,

CT, USA). Irradiation was performed with 2 beams parallel-opposed a 15 mm circular collimator (Precision X-Ray, North Branford, CT, USA) at 225 Kev, 13 mA, with a 0.3 mm copper filter. Treatment was delivered using a treatment planning system (SmART-Plan, SmART Scientific Solution, Maastricht, The Netherlands). For irradiation, anesthetized mice were immobilized in a feet-first supine position.

2.2.4 Behavioral Testing

All tests were performed in designated behavior rooms with ambient noise during light hours at the UCI McGaugh Hall Animal Vivarium. Fear extinction was recorded and analyzed using the Habitest Modular System and FreezeFrame software (Harvard Apparatus, Coulbourn Instruments, Holliston, MA). All tasks were performed and recorded by the same individual as described previously (Acharya *et al.*, 2019). Open-Field Test (OFT) was performed in a fully lit behavior room, mice were placed in an empty white arena with no habituation or prior extensive handling. Exploration and distance traveled were measured over 5 min using Ethovision XT (Noldus, Wageningen, the Netherlands) software as described previously(Dey *et al.*, 2020). Light Dark Box (LDB) was performed in a fully lit behavior room: mice were placed in an arena with an open white 'light' portion connected to a closed black 'dark' compartment. Transitions between each compartment were recorded over 10 min using Ethovision XT (Noldus) software as described previously (Acharya *et al.*, 2019).

Fear Extinction (FX) memory test (Acharya *et al.*, 2019) was performed in a dark room lit only by a red light. Mice were placed in a closed chamber and received 3 tone-associated electric shocks. Twenty-four hours later they were placed in a different chamber context, in the same room, for 3 consecutive days with no shock. For the final 'Extinction Test' day, mice were

placed in the new context and played the tone associated with the original shock, without receiving shock. Percent freezing was recorded to assess animals' ability to extinguish original fear memory.

2.2.5 Age and Testing Timeline

Animals underwent behavior testing after paclitaxel exposures at UCI. Age of animals, week of behavioral testing during study, and N animals tested are specified in **Table 1**. All animals underwent paclitaxel treatments, behavior, and necropsy at UCI, except RhoB $^{-/-}$ 300 mg/kg exposed females that underwent paclitaxel treatments at CHUV and behavior and necropsy at UCI.

Paclitaxel Group			Age at start of study			Time of behavioral task during study								
Geno type	**Sex**	**Dose (mg/kg)**	**Age (Weeks)**	*N Con*	*N PTX*	**OFT (Week)**	*N Con*	*N PTX*	**LDB (Week)**	*N Con*	*N PTX*	**FX (Week)**	*N Con*	*N PTX*
WT	F	150	**15**	11	18	**10**	11	18	**12**	7	8	**14**	8	8
	M		**14**	12	18	**11**	12	16	**13**	12	16	**14**	8	8
	F	300	**11**	14	14	**9**	14	13	**11**	14	11	**13**	8	8
	M		**13**	12	12	**-**	-	-	**-**	-	-	**8**	6	6
	F	150	**28**	7	7	**10**	7	7	**12**	7	7	**13**	7	7
			81	5	7	**10**	5	6	**12**	5	5	**13**	5	3
RhoB $^{-/-}$	F	150	**20**	10	12	**11**	10	12	**13**	10	8	**14**	8	8
	M		**20**	10	14	**10**	10	14	**12**	10	13	**13**	8	8
	F	300	**25**	12	12	**17**	12	8	**17**	12	8	**18**	8	8

Table 1: Behavior Testing Timeline. WT=Wild type animal, RhoB $^{-/-}$=RhoB–deficient animals, F=female, M=male, PTX=paclitaxel, OFT=Open Field Testing, LDB=Light Dark Box, and FX=Fear Extinction.

2.2.6 Transthoracic Ultrasound Imaging (Echocardiography)

Transthoracic ultrasound imaging was performed on radiation-exposed animals in CHUV using the MS400 (18–38 MHz) probe from Vevo 2100 color Doppler ultrasound machine (VisualSonics, Toronto, ON, Canada). Mice are lightly anesthetized with 1–1.5% isoflurane (Provet, Lyssach, Switzerland), maintaining heart rate at 500–600 beats per minute. The mice were placed in decubitus dorsal on a heated 37C platform to maintain body temperature. A topical depilatory agent was used to remove the hair, and ultrasound gel was used as a coupling medium between the transducer and the skin. The heart was imaged in the 2D mode in the parasternal long-axis view. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and the posterior wall of the left ventricle at the level of the papillary muscles. The measurements were taken in three separate M-mode images and averaged. Left ventricular Ejection Fraction (%EF) was also calculated. %EF is derived from the formula of EF (%) = [(LVDA LVSA)/LVDA] $\times$ 100.

2.2.7 Necropsy, Histology, and Tissue Analysis

Paclitaxel-exposed animals were anesthetized at UCI and radiation-exposed animals were anesthetized at CHUV, under the same conditions using isoflurane prior to euthanization via intracardiac perfusion with saline and heparin (10 U/ml, Sigma-Aldrich, St. Louis, MO). Cardiac tissue were extracted for processing and cut along the transverse axis, with upper portions stored in 70% ethanol prior to processing. Paclitaxel-exposed tissue was sent for processing at the UCI Department of Pathology & Laboratory Medicine's Experimental Tissue Resource histopathology core facility (Orange, CA), where they were paraffin embedded and

transversely cut using a scientific microtome (Thermo Fisher Scientific, Waltham, MA, USA) into 4 µm sections and underwent ethanol dehydration and CD45 (M070101-2, Agilent, Santa Clara, CA) tissue staining. Radiation-exposed cardiac tissue was paraffin embedded and transversely cut using a scientific microtome (Thermo Fisher Scientific, Illkirch, France) into 5 µm sections and underwent ethanol dehydration and H&E tissue staining at CHUV.

CD45 stained cardiac tissue slide stereology was performed using Stereo Investigator (MBF Bioscience Williston, VT) software. CD45 cells were manually counted in 11 µm thick stained left ventricle cardiac tissue under bright field 60d oil magnification (Nikon). Counting frame size of 75 µm x 75 µm in a 1000×1000 grid layout was used. Three sections were counted per animal and all counting was performed by one individual. The means for each group of estimated CD45+ cell population based on user defined section thickness were calculated using Stereo Investigator program.

2.2.8 Statistical Analysis

Statistical analyses were conducted using Prism (v8.4, GraphPad, San Diego, CA, USA). Unpaired, two-tailed tests were used to compare two groups and two-way analysis of variance (ANOVA) was used to assess for interaction and group effects of more than two groups, for behavioral testing, echocardiography, and CD45 staining stereology, if significance was found, Bonferroni's multiple comparisons test was conducted on two-way ANOVA results. Log-rank (Mantel–Cox) test was used to compare survival curves. $P \leq 0.05$ was considered statistically significant.

Chapter 3 Results

Data adapted from Chmielewski-Stivers *et al.,* 2021 with additional unpublished histology and RhoB$^{-/-}$ behavioral data.

3.1 Paclitaxel Study

3.1.1 Male-specific paclitaxel-induced lethality after 300 mg/kg paclitaxel exposures

To characterize the long-term normal tissue toxicity of chemotherapeutic doses of paclitaxel (PTX), approx. 3-month-old female and male Wild Type C57 Bl6 (WT) mice (refer to Table 1) were exposed to 300 mg/kg (50 mg/kg doses x 6 weeks) of PTX via intraperitoneal injection and monitored up to 15 weeks after first exposure. The PTX dose of 300 mg/kg, initially piloted on a group of Wild-Type (WT) female retired breeders (data not shown), unexpectedly caused precipitous lethality in WT male mice by Week 10 of the study (Log-rank Mantel-Cox test demonstrated significant differences in survival between exposure and sex, $p=0.001$) (Fig. 1A). WT males demonstrated considerably more weight loss compared to females after 300 mg/kg PTX exposures during the first 10 weeks of the study (2-way ANOVA interaction effect interaction effect, $p<0.0001$, and sex effect, $p<0.0001$) and multiple comparisons Bonferroni test demonstrated significant sex-differences in weights as early as 7, 8, 9, and 10 weeks ($p=0.014$, $p<0.0001$, $p=0.012$, $p<0.0001$, respectively) indicating greater toxicity to PTX in males compared to females (Fig. 1B). Female mice underwent the entire 15-week study and behavioral tasks, males only underwent Fear Extinction paradigm.

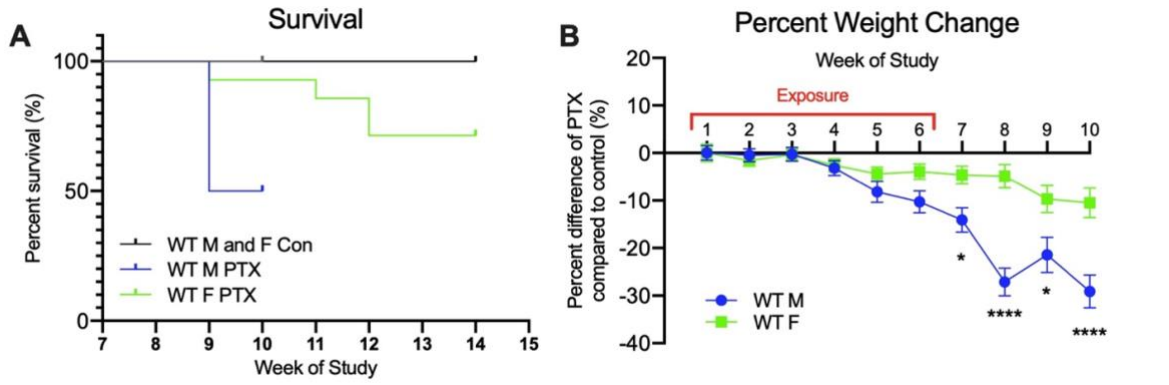


Figure 1. WT male mice experienced more lethality and weight loss after 300 mg/kg PTX compared to WT female mice. (A) Precipitous lethality was achieved 10 weeks after initial dose of PTX in male mice, females exhibited 25% lethality after 14 weeks. Log-rank (Mantel-Cox) test comparison of survival curves significantly different between sexes (p=0.001). **(B)** WT males more sensitive to weight loss after 300 mg/kg PTX exposure. Significant interaction effect (p<0.0001) and sex effect (p<0.0001) for weight change in male WT animals compared to females. 2-way ANOVA Bonferroni's multiple comparisons test labeled as *p<0.05, ****p<0.0001. Chart uses mean ± s.e.m.

At 300 mg/kg, behavioral deficits were demonstrated in both male and female exposure groups. Acquisition of conditioned fear was not impaired in exposed or control female cohorts that demonstrated a gradual decrease in freezing behavior over extinction sessions (Fig. 2A). In males, 300 mg/kg exposure did not impair fear acquisition, but failed to gradually decrease freezing behavior over extinction sessions (Fig. 2B). Both females and males exposed to 300 mg/kg experienced compromised fear extinction during fear extinction tests (2-way ANOVA sex effect $p<0.0001$ and treatment effect $p<0.0001$), as well as significantly more freezing between control vs. exposed females ($p=0.0041$), control vs. exposed males ($p=0.0041$), control females vs. control males ($p=0.0002$), and exposed females vs. males ($p=0.0002$) according to 2-way ANOVA multiple comparisons, Bonferroni analysis (Fig. 2C). Unfortunately, due to significant male-specific toxicity to PTX at 300 mg/kg exposure, males were sacrificed 10 weeks post first exposure, prior to conducting additional behavioral testing after fear extinction. Female animal continued behavioral testing and demonstrated significant differences in distance traveled during Open Field Testing, suggesting PTX may have impacted locomotor activity of animals (unpaired t-test, $p<0.0001$) (Fig. 2D). In addition, PTX exposed females transitioned fewer times between the light and dark compartments during Light Dark Box testing, suggesting possible increased anxiety-like behavior after 300 mg/kg PTX exposure in female WT animals (unpaired t-test, $p=0.0041$) (Fig. 2E).

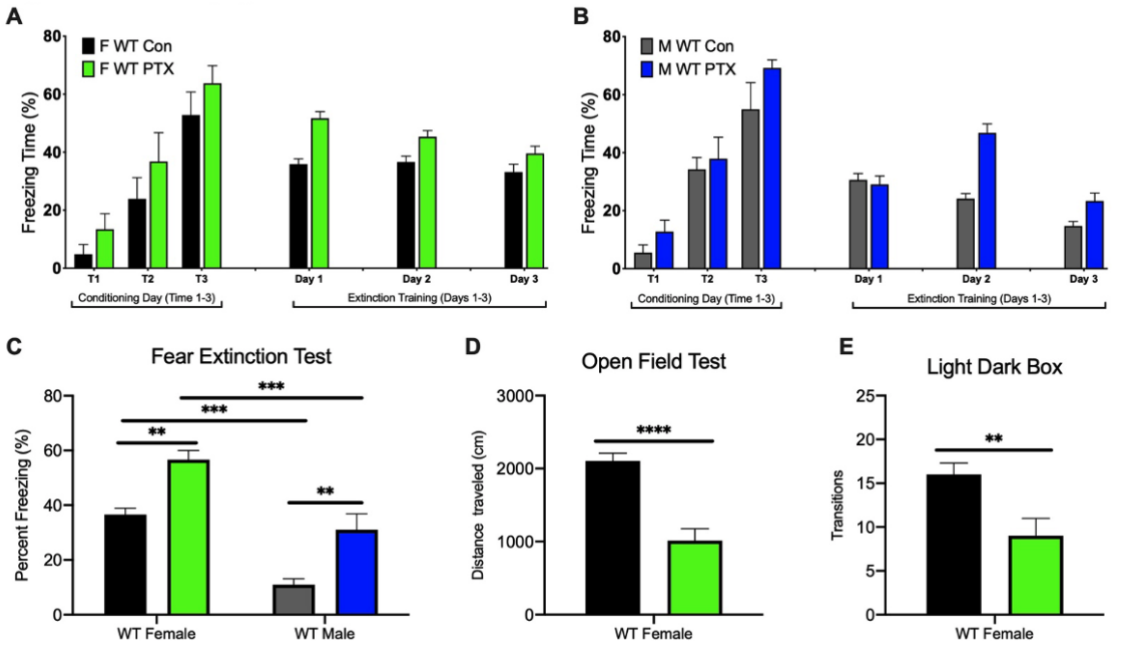


Figure 2. Female and male WT 300 mg/kg PTX exposed animals show deficits in fear extinction memory and WT females display increased anxiety. (A) PTX administration in WT female mice did not impair the acquisition of conditioned fear (three tone-shock pairings). All mice showed a graduate decrease in freezing behavior over extinction sessions (tone only) (n=8). **(B)** PTX administration in WT male mice did not impair the acquisition of conditioned fear (three tone-shock pairings). Controls, but not exposed mice showed a graduate decrease in freezing behavior over extinction sessions (tone only) (n=6). **(C)** PTX exposure compromised fear extinction abolishment on test day (2-way ANOVA sex effect P<0.001 and treatment effect P<0.0001; females n=8, males n=6). **(D)** PTX exposure significantly decreased spontaneous exploration behavior in female WT mice (control n=14, exposed n=12). **(E)** Exposed females exhibited significantly more anxiety behavior (control n=14, exposed n=10). **(A-E)** Graphs designate mean ± s.e.m; **(A-C)** *P* value for significant interaction, 2-way ANOVA: *P* value derived from multiple comparisons 2-way ANOVA, Bonferroni test: *p<0.05, **p<0.01, ****p<0.0001. **(D-E)** *P* value was derived from unpaired t-test **p<0.01, ***p<0.001, ****p<0.0001.

3.1.2 Female-specific neurotoxic and systemic protection after 150 mg/kg paclitaxel exposures

In order to characterize cognitive function and systemic toxicity at a tolerable, non-lethal dose of PTX, new cohorts of female and male WT mice (refer to Table 1) were exposed to 150 mg/kg (25 mg/kg doses x 6 weeks) of PTX via intraperitoneal injection and monitored for 15 weeks after first exposure. In male mice (n=18) the 150 mg/kg dose of PTX resulted in a 20% reduction in survival whereas in females (n=18) lethality was not observed (Fig. 3A). Enhanced toxicity of PTX was also evident in males that lost significantly more weight compared to females over the course of the 15-week study (2-way ANOVA interaction effect interaction effect, $p=0.042$, and sex effect, $p<0.0001$). At specific times, multiple comparisons Bonferroni test demonstrated significant sex-differences in weights at weeks 13, 14, and 15 ($p=0.016$, $p=0.0030$, $p=0.014$, respectively) indicating greater toxicity to PTX in males compared to females after 150 mg/kg PTX exposure (Fig. 3B).

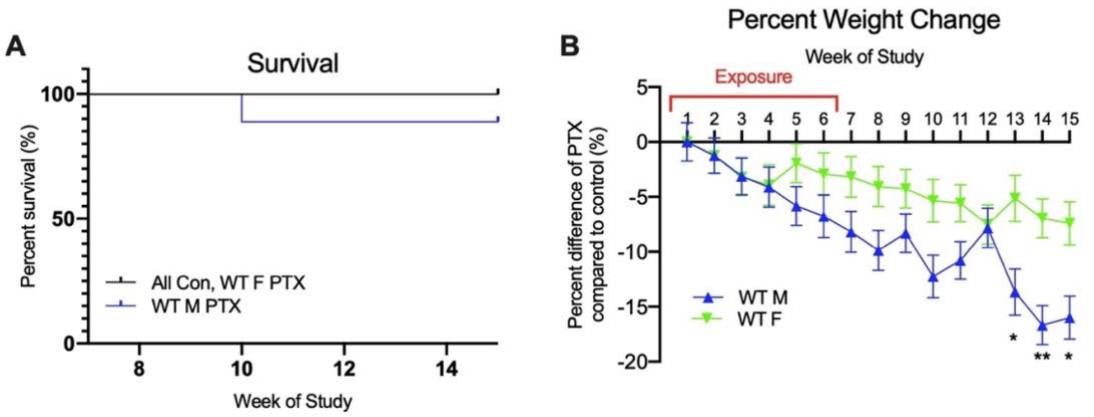


Figure 3. Sex-specific weight loss in WT males retained after 150 mg/kg PTX exposure. (A) 20% of WT males died after 150 mg/kg PTX exposure while all females lived. No significance in survival curve Log-rank (Mantel-Cox) test. **(B)** WT males remain more sensitive to PTX-induced weight loss than WT females at 150 mg/kg. Significant interaction effect ($p=0.042$) and sex effect ($p<0.0001$) for weight change in male WT animals compared to females. 2-way ANOVA Bonferroni's multiple comparisons test labeled as *$p<0.05$, **$p<0.01$. Chart uses mean ± s.e.m.

At this same PTX dose, behavioral testing demonstrated further a greater sensitivity in WT male compared to females. Acquisition of conditioned fear was not impaired in exposed or control female cohorts that demonstrated a gradual decrease in freezing behavior over extinction sessions (Fig. 4A). Similarly, PTX exposure (150 mg/kg) did not impair fear acquisition in males, but compared to controls, exposed males did not exhibit the gradual decrease in freezing behavior over extinction sessions (Fig. 4B). Importantly, a fear extinction test conducted 72 h after training demonstrated a significant impairment in extinction memory in males, but not females (2-way ANOVA interaction effect $p=0.0026$ and treatment effect $p=0.046$), as well as significantly more freezing in exposed males compared to exposed females and control males (multiple comparisons, Bonferroni test, $p=0.047$ and $p=0.0041$, respectively) (Fig. 4C). There was no effect of PTX exposure on distance traveled in males or females during Open Field Testing, suggesting PTX had no significant effect on locomotor activity of animals (Fig. 4D). However, PTX exposed males transitioned fewer times between the light and dark compartments during Light Dark Box testing, suggesting increased anxiety-like behavior after 150 mg/kg PTX exposure in male, but not female WT animals (2-way ANOVA interaction effect $p=0.028$ and sex effect $p=0.0083$; multiple comparisons Bonferroni test WT male control vs. exposed, $p=0.011$, and WT female exposed vs. WT male exposed, $p=0.0042$) (Fig. 4E).

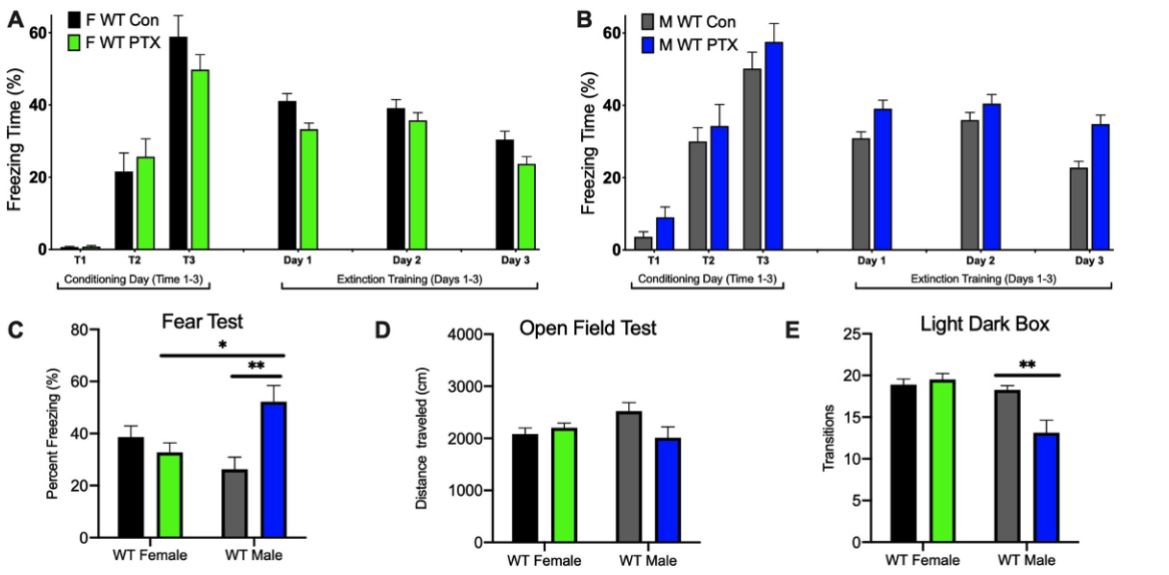


Figure 4. Sex-specific WT male deficits in fear extinction memory and anxiety behavior after 150 mg/kg PTX exposure. (A) PTX administration in WT female mice did not impair the acquisition of conditioned fear (three tone-shock pairings). All mice showed a graduate decrease in freezing behavior over extinction sessions (tone only) (n=8). **(B)** PTX administration in WT male mice did not impair the acquisition of conditioned fear (three tone-shock pairings). Controls, but not exposed mice showed a graduate decrease in freezing behavior over extinction sessions (tone only) (n=8). **(C)** WT females, but not males, successfully abolished fear extinction memory (2-way ANOVA interaction effect p=0.0026 and treatment effect p=0.0457, n=8). **(D)** PTX exposure had no impact on spontaneous exploration (females: control n=12, exposed n=18; males: control n=12, exposed n=16). **(E)** Exposed males exhibited significantly more anxiety behavior (2-way ANOVA interaction effect p=0.0280 and sex effect p=0.0083; females: n=8; males: control n=12, exposed n=16). **(A-E)** Graphs designate mean ± s.e.m; **(A-E)** *P* value derived from multiple comparisons 2-way ANOVA, Bonferroni test: *p<0.05, **p<0.01.

3.1.3 Age increases susceptibility to paclitaxel-induced mortality and weight loss, but not cognitive disfunction, in WT females

In an effort to further investigate female WT protection against PTX, we exposed groups of 9 to 11 and 22-month-old female WT mice to 150 mg/kg (25 mg/kg x 6 weeks, i.p.) of PTX. We hypothesized that younger females of reproductive age, 9- to 11-month-old, would remain protected from PTX due to estrogen generated by functioning ovaries, while 22-month-old females would have greater susceptibility to PTX-induced toxicity due to reproductive senescence characterized by depleted ovarian-produced endogenous estrogen.

13 weeks after initial paclitaxel exposure 40% of 22-month-old female mice died (n=7), while no control or 9- to 11-month-old exposed females died (Log-rank, Mantel-Cox test, survival curves significantly different, $p=0.010$) (Fig. 5A). In addition, 22-month-old females lost significantly more weight than 9- to 11-month-old animals over the course of 19 weeks (2-way ANOVA, significant age effect, $p<0.0001$, n=5 for 9- to 11-month-olds and n=7 for 22-month-olds, Bonferroni's multiple comparisons week 17 significant difference, $p=0.018$) (Fig. 5B).

Four months after first exposures, all animals underwent fear and anxiety behavior testing. Similarly to 6 month-old WT female testing results (refer to Fig. 1), acquisition of conditioned fear was not impaired in exposed or control 9 to 11 month-old WT females and demonstrated a gradual decrease in freezing behavior over extinction sessions (Fig. 5C). In addition, fear acquisition or the gradual decrease in freezing behavior over extinction sessions was not impaired in 22-month-old control or exposed females (Fig. 5D). Interestingly, fear extinction tests demonstrated no significant difference freezing behavior across all female

groups (2-way ANOVA, n=5 for 9- to 11-month-olds and n=7 for 22-month-olds), suggesting aged females are not susceptible to PTX-induced fear extinction dysfunction (Fig. 5E). Open Field Testing also demonstrated no significant difference in distance traveled between exposure or age, suggesting paclitaxel did not compromise locomotor activity (2-way ANOVA n=5 for 9- to 11-month-olds and n=7 for 22-month-olds) (Fig. 5F). No difference in Light Dark Box transition behavior also suggested aged WT females were not susceptible to PTX-induced anxiety (2-way ANOVA n=5 for 9- to 11-month-olds and n=7 for 22-month-olds) (Fig. 5G).

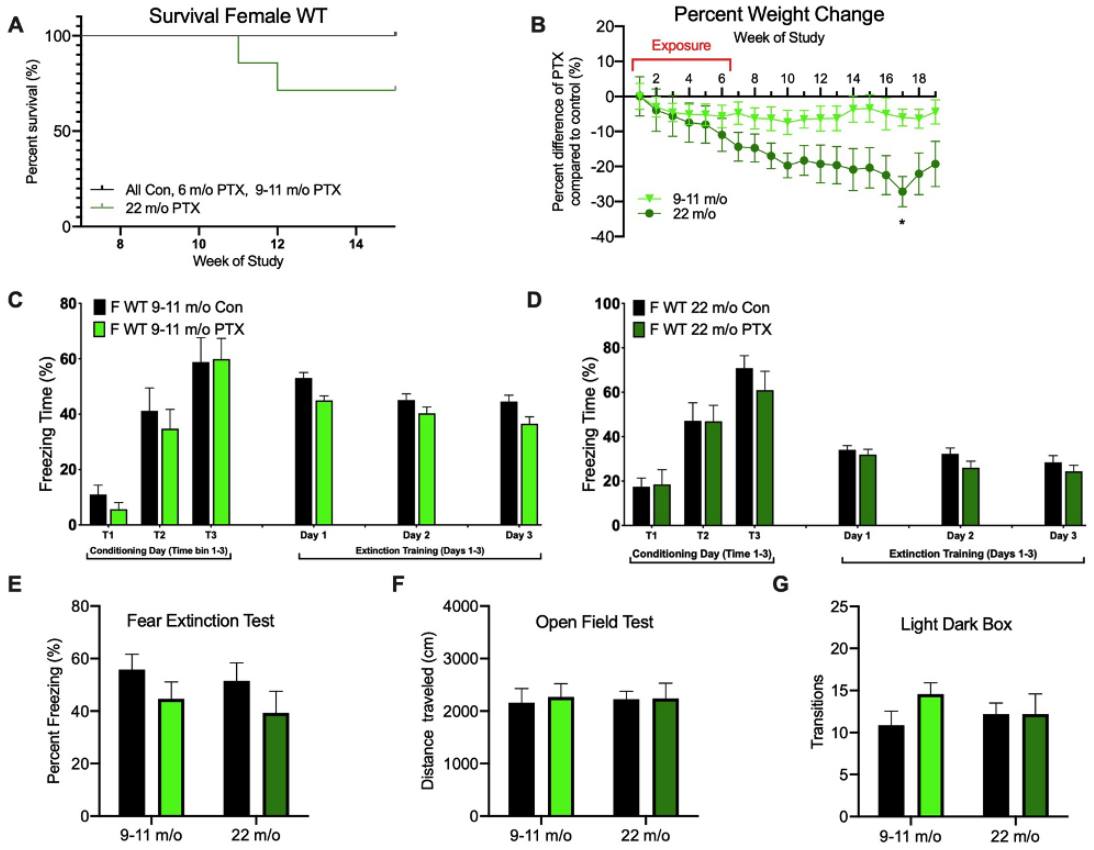


Figure 5. Aging WT females sensitive to PTX-induced lethality and weight loss, but not cognitive dysfunction after 150 mg/kg. (A) 30% of 22-month-old WT females died by week 13 of study. Log-rank (Mantel-Cox) test comparison of survival curves significantly different (p=0.0100). **(B)** Significant age effect (p<0.0001) for weight change in WT females. 2-way ANOVA Bonferroni's multiple comparisons test labeled as *p<0.05. **(C)** PTX administration in younger, 9 to 11-month-old, WT female mice did not impair the acquisition of conditioned fear (three tone-shock pairings). All mice showed a graduate decrease in freezing behavior over extinction sessions (tone only) (n=7). **(D)** PTX administration in aging, 22-month-old, WT female mice did not impair the acquisition of conditioned fear (three tone-shock pairings). Mice showed no change in freezing behavior over extinction sessions (tone only) (n=5). **(E)** No difference in fear extinction memory in young and aged WT females after treatment (9-11 m/o n=7, 22 m/o n=5). **(D)** PTX exposure had no impact on spontaneous exploration (9-11 m/o n=7, 22 m/o n=5). **(E)** PTX exposure did not induce a change in anxiety (9-11 m/o n=7, 22 m/o n=5). **(B-G)** Graphs designate mean ± s.e.m.

3.1.4 RhoB deficiency attenuates sex-differences in paclitaxel-induced toxicity

As mentioned earlier, RhoB was a target of interest to elucidate the possible role of sex-hormones on paclitaxel toxicity because RhoB expression has been associated ERα expression in breast cancer (Médale-Giamarchi *et al.*, 2013). I exposed female and male mice deficient of RhoB activity to 150 m/kg (25 mg/kg x 6 weeks, i.p.) PTX. RhoB deficiency was hypothesized to attenuate female-specific protection from taxane-induced toxicity and RhoB $^{-/-}$ females and males would have similar cytotoxic outcomes to WT males and aged females.

After 150 mg/kg PTX, 11% of WT males (n=18), 7.1% of RhoB deficient males (n=14), and 14% RhoB deficient females (n=14) died within 15 weeks of first exposure of PTX, while no WT females died (Fig. 6A). We observed a (close to significant) trend of increased weight loss in RhoB deficient females compared to WT females over 15 weeks compared (2-way ANOVA, genotype effect p=0.051) (Fig. 6B). In contrast, there was no difference in weight loss between WT and RhoB deficient males (2-way ANOVA, genotype effect, p=0.99) (Fig. 6C).

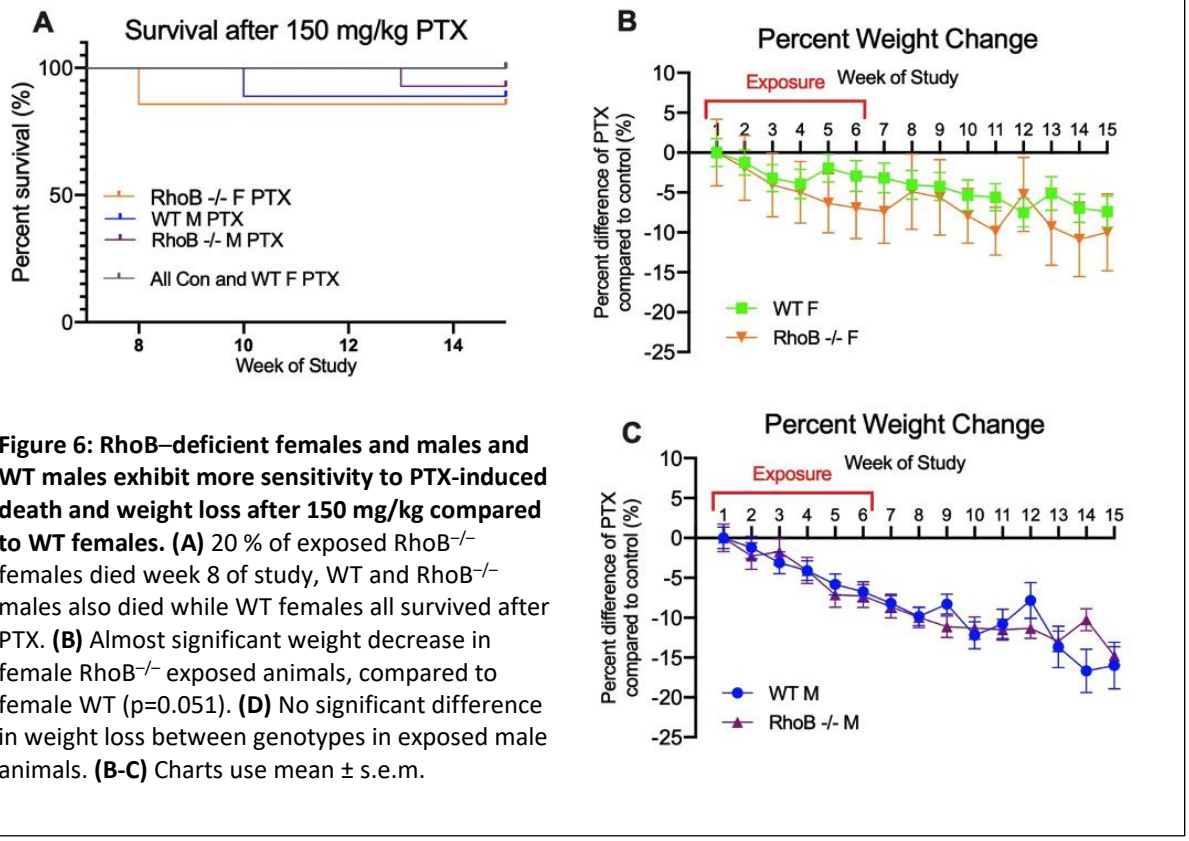


Figure 6: RhoB–deficient females and males and WT males exhibit more sensitivity to PTX-induced death and weight loss after 150 mg/kg compared to WT females. (A) 20 % of exposed $RhoB^{-/-}$ females died week 8 of study, WT and $RhoB^{-/-}$ males also died while WT females all survived after PTX. **(B)** Almost significant weight decrease in female $RhoB^{-/-}$ exposed animals, compared to female WT (p=0.051). **(D)** No significant difference in weight loss between genotypes in exposed male animals. **(B-C)** Charts use mean ± s.e.m.

RhoB–deficient animals demonstrated no significant cognitive dysfunction after 150 mg/kg PTX exposures, but males demonstrated trends in impairment. Acquisition of conditioned fear was not impaired in exposed or control female cohorts, but both also did not demonstrate a decrease in freezing behavior over extinction sessions (Fig. 7A). Similarly, PTX exposure did not impair fear acquisition in RhoB–deficient males, but compared to controls, exposed males exhibited more freezing behavior over extinction sessions, with no gradual decrease over training days (Fig. 7B). Fear extinction testing exhibited no significant difference in freezing between exposures or sexes (Fig. 7C). Distance traveled in males or females during Open Field Testing significantly varied between sexes, but not between control and exposed groups of the same sex, suggesting PTX had a sex-dependent effect on locomotor activity in animals (2-way ANOVA, sex effect $p=0.0007$ and exposure effect $p=0.034$; female: control n=10, exposed n=12, male: control n=10, exposed n=14) (Fig. 7D). No significant difference in traveling between light and dark compartments was seen across sexes or exposures during Light Dark Box testing (Fig. 7E). However, it may be interesting to note that RhoB–deficient animals have fewer transitions compared to age-matched WT animals (Fig. 7E), and this might signify a genotype-specific sensitivity to anxiety-like behavior in $RhoB^{-/-}$ animals.

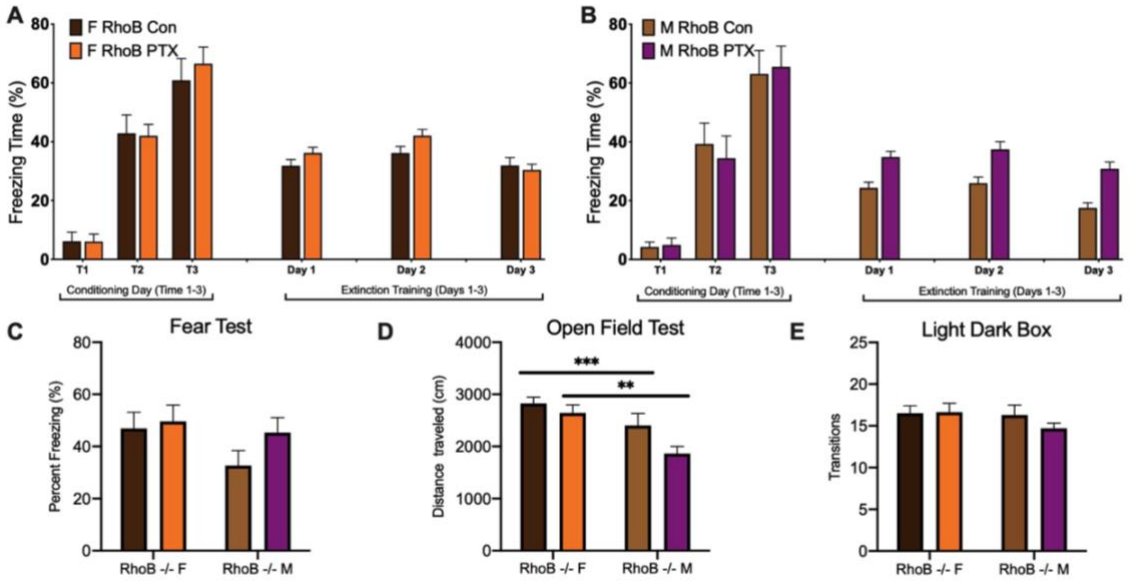


Figure 7: RhoB deficiency dampens male-specific sensitivity to paclitaxel-induced neurotoxicity at 150 mg/kg. (A) PTX administration in RhoB deficient female mice did not impair the acquisition of conditioned fear (three tone-shock pairings). Mice showed no change in freezing behavior over extinction sessions (tone only) (n=8). **(B)** PTX administration in RhoB deficient male mice did not impair the acquisition of conditioned fear (three tone-shock pairings Mice showed no change in freezing behavior over extinction sessions (tone only) (n=8). **(C)** No difference in fear extinction memory in RhoB deficient animal groups after treatment or between sex (n=8). **(D)** PTX exposure had no impact on spontaneous exploration, but males explored less than females 2-way ANOVA sex effect p=0.0007 and exposure effect p=0.0339; female and male: control n=10, exposed n=14. *P* value derived from multiple comparisons 2-way ANOVA, Bonferroni test: **p<0.01, ***p<0.001. **(E)** PTX exposure did not induce a change in anxiety behavior (females: control n=10, exposed n=8; males: control n=10, exposed n=13). **(A-E)** Graphs designate mean ± s.e.m.

3.1.5 RhoB deficiency protects females from long-term paclitaxel-induced toxicity at 300 mg/kg

RhoB–deficient females were also tested at the higher dose of 300 mg/kg (50 mg/kg x 6 weeks) PTX to further characterize the role of RhoB in female-specific protection. Interestingly at 300 mg/kg PTX exposure, RhoB deficiency extended survival for females by about 3 weeks, with 29% of WT females dying by Week 12 and 33% of $RhoB^{-/-}$ females dying by Week 15 (Log-rank Mantel-Cox test comparison of survival curves, p=0.026) (Fig. 8A). Similarly, more weight loss was observed in WT females compared to RhoB–deficient females over the course of 14 weeks (2-way ANOVA, genotype effect, p=0.0049), suggesting a toxicity threshold for WT females and protection against long-term toxicity with RhoB deficiency (Fig. 8B). High-dose PTX exposure did not impair fear acquisition in RhoB–deficient females, but compared to controls, exposed females exhibited more freezing behavior over extinction sessions, with no gradual decrease over training days, similar to RhoB–deficient males at 150 mg/kg (Fig. 8C). No difference was observed in fear testing, open field exploration, or light-dark box transitioning, suggesting no paclitaxel-induced cognitive impairment at high doses in $RhoB^{-/-}$ females (Fig. 8D-F).

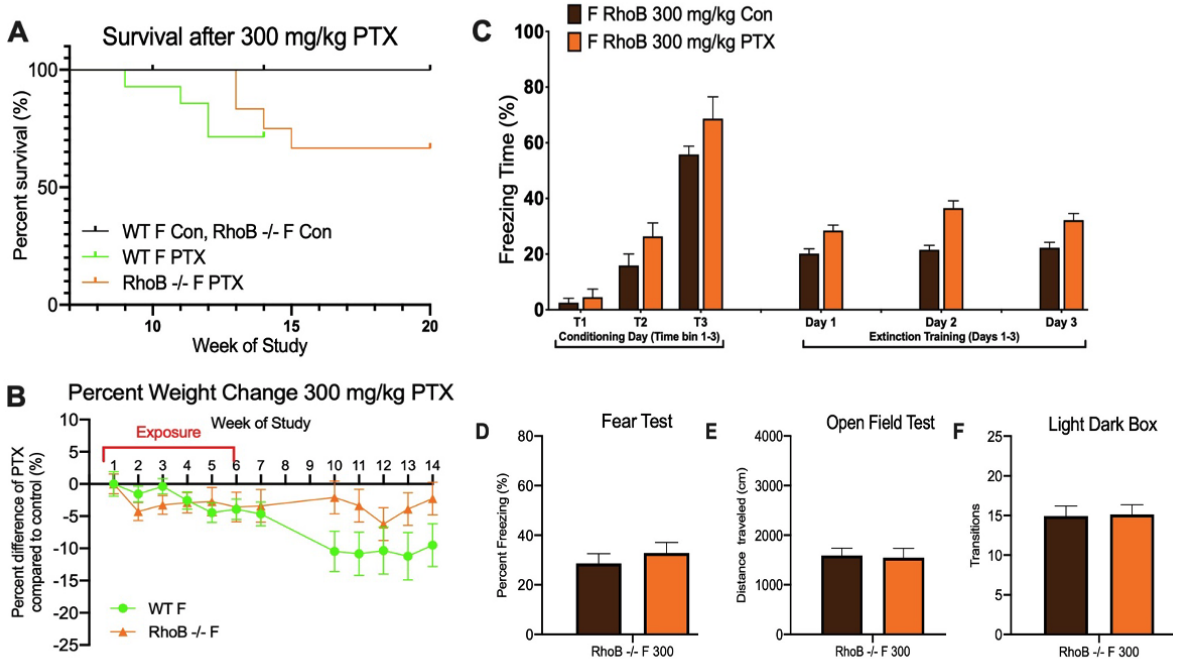


Figure 8: RhoB deficiency protects against long-term toxicity in females exposed to 300 mg/kg. (A) 29% of WT females die by week 12, and 33% of RhoB–deficient females die by week 15 of study. Log-rank (Mantel-Cox) test comparison of survival curves significantly different (p=0.026). **(B)** RhoB–deficient females lost less weight than WT females after 300 mg/kg PTX. 2-way ANOVA significant genotype effect (p=0.0049). **(C)** PTX administration in RhoB–deficient female mice did not impair the acquisition of conditioned fear (three tone-shock pairings). Mice showed no change in freezing behavior over extinction sessions (tone only). (n=8). **(D-F)** No significant differences (unpaired t-test) in fear extinction memory, spontaneous exploration, or anxiety behavior in RhoB deficient females after 300 mg/kg PTX (FT: n=8; OFT: control n=12, exposed n=8; LDB: control n=12, exposed n=9). **(C-F)** Graphs designate mean ± s.e.m.

3.1.6 Sex, age, and RhoB-dependent leukocyte infiltration in cardiac tissue after paclitaxel exposure

Due to hypothesized involvement of sex-hormone-dependent impact on paclitaxel-induced toxicity, further investigation was pursued to elucidate immune response profiles in cardiac tissue of exposed mice. Cardiac tissue is associated with sex-hormone-dependent differences in function and protection, therefore I hypothesized that females would demonstrate more protection and RhoB deficiency would mitigate it, due to attenuation of sex-hormone and immune response. Interestingly, young WT females exhibited higher baseline levels of leukocyte common antigen, CD45, in controls compared to exposed and there was no significant change in WT males (2-way ANOVA interaction effect p=0.0033) (Fig. 9A & 10). When comparing across age, WT female controls exhibited a U curve of CD45 positive cells, with significant age (p=0.0035) and interaction (p=0.0036) effects (2-way ANOVA) (Fig. 9B & 10). RhoB deficiency seemed to attenuate sex-dimorphisms after 150 mg/kg paclitaxel exposure, seen in WT animals, but still demonstrated an overall sex effect (p=0.027) (2-way ANOVA) (Fig. 9C & 11). At 300 mg/kg RhoB–deficient females recruited significantly more CD45 positive cells to cardiac tissue (unpaired t-test, p=0.0031) (Fig. 9D & 11).

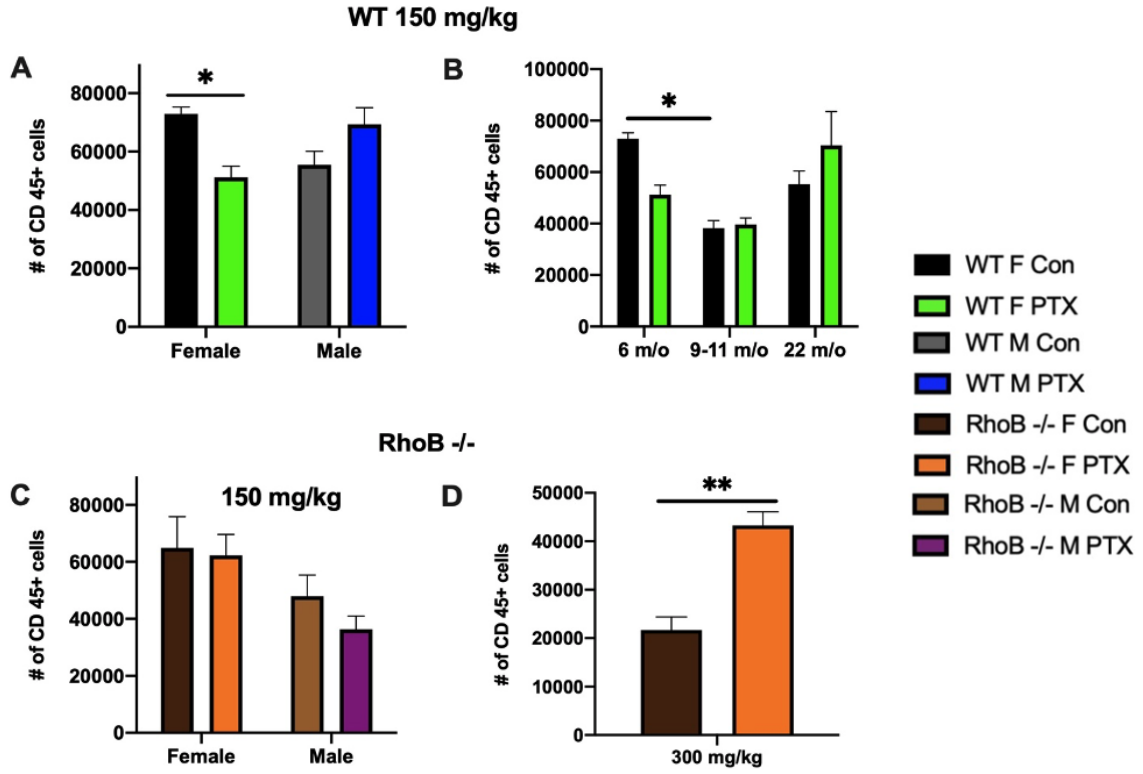


Figure 9: Sex, age, and RhoB impact paclitaxel-induced immune cell infiltration in cardiac tissue. A) WT Females have significantly fewer CD45+ cells in the left ventricle cardiac tissue after PTX. Males have no significant change from PTX exposure. 2-way ANOVA interaction effect p=0.0033, multiple comparisons, Bonferroni test: *p<0.05. **B)** Age-dependent variations in baseline CD45+ cell types, U-like baseline ageing curve. 2-way ANOVA, interaction effect p=0.036 and age effect p=0.0035, multiple comparisons, Bonferroni test: *p<0.05. **C)** Control and exposed RhoB–deficient females have larger levels of CD 45+ cells than males, however, there is no change from PTX exposure. 2-way ANOVA sex effect p=0.027, no multiple comparisons effect. **D)** Significantly more CD45+ cell recruitment following 300 mg/kg exposure in RhoB–deficient females. Unpaired t-test, p=0.0031. **(A-D)** N=3, 3 cardiac sections per animal analyzed. Graphs designate mean ± s.e.m.

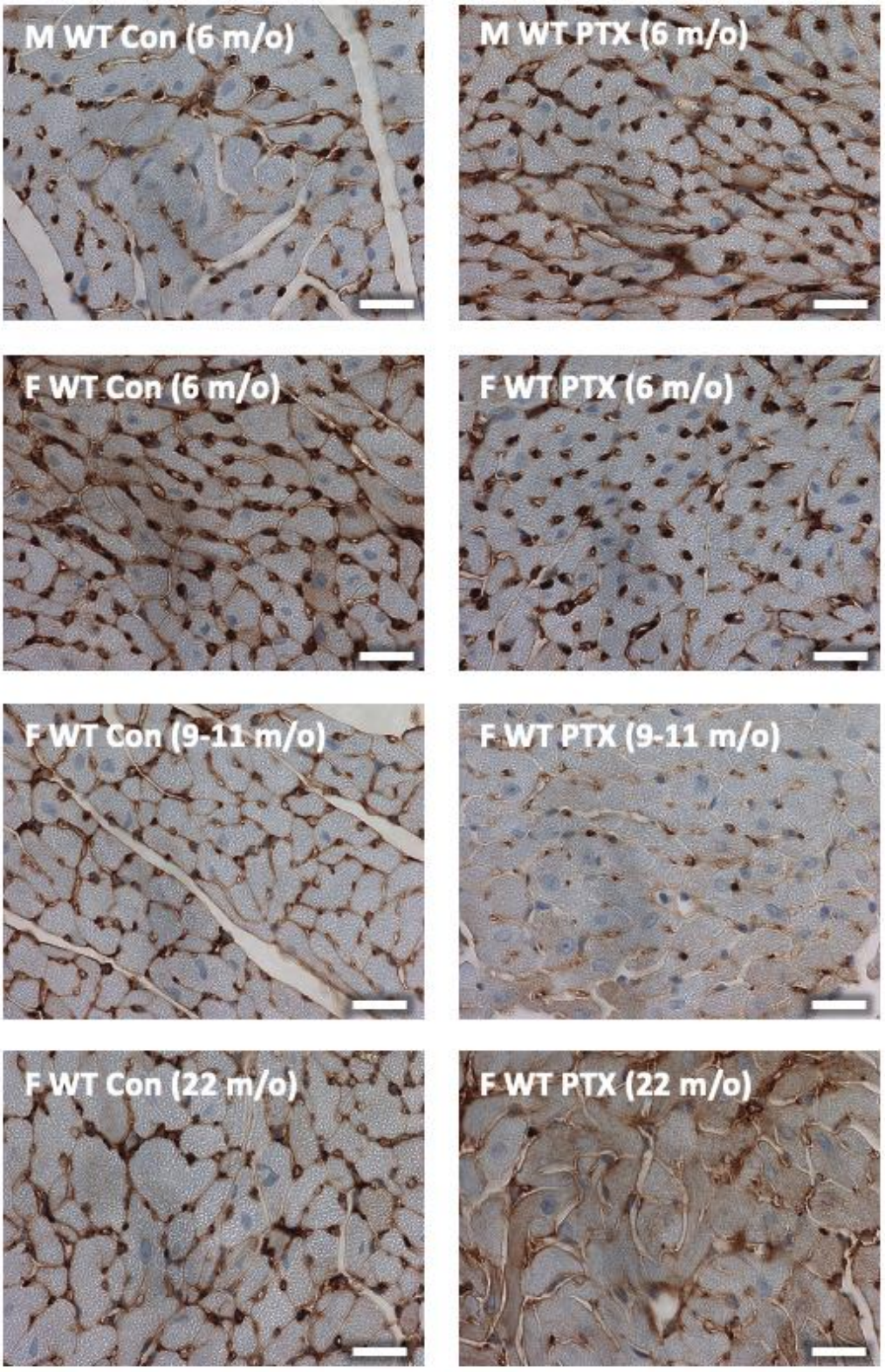


Figure 10: Representative CD45 staining of Wild Type animals exposed to 150 mg/kg paclitaxel. Left ventricle images taken at 60X, bars represent 30 µm.

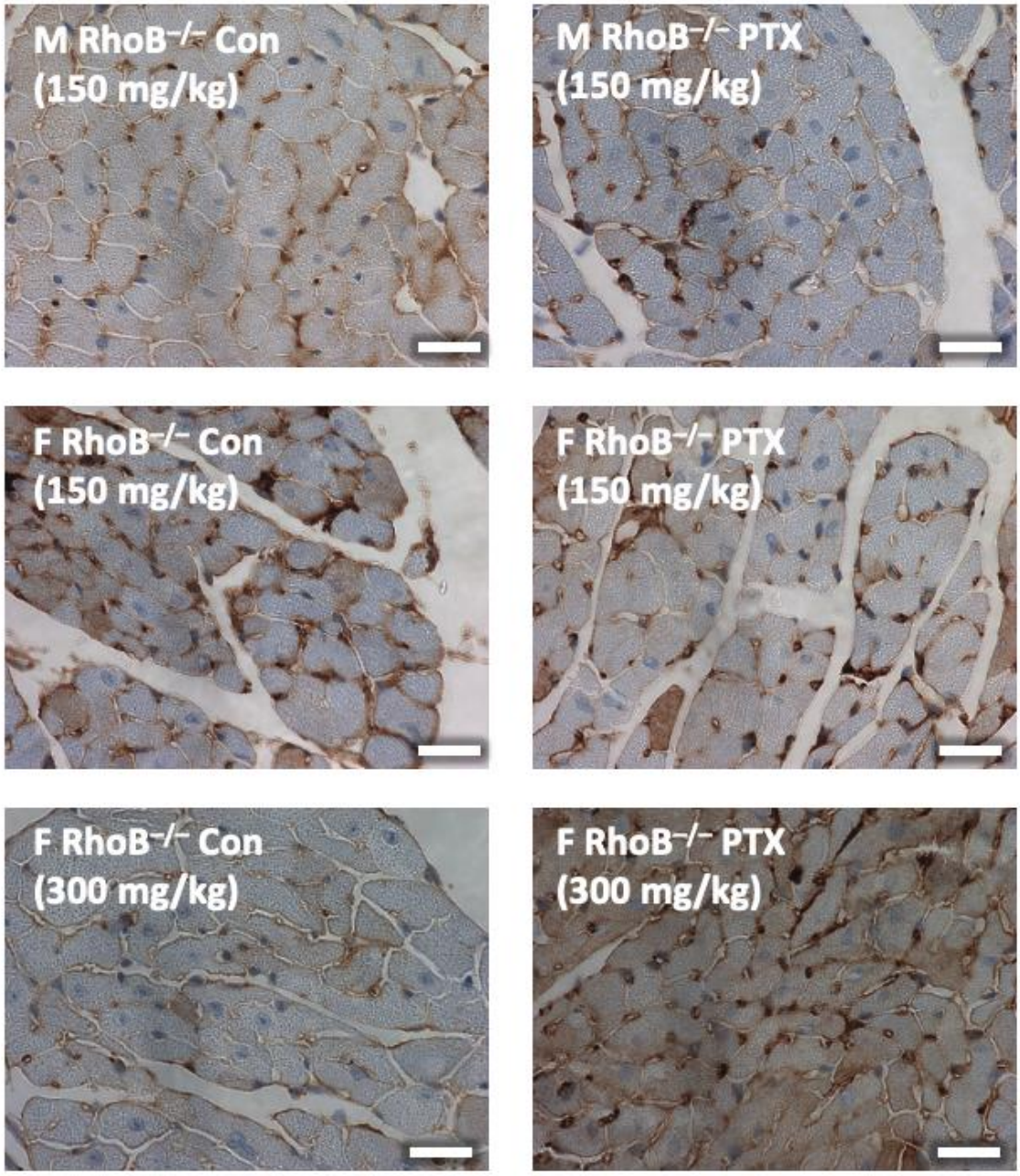


Figure 11: Representative CD45 staining of RhoB–deficient animals exposed to 150 mg/kg or 300 mg/kg paclitaxel. Left ventricle images taken at 60X, bars represent 30 µm.

3.2 Radiotherapy Study

The following section has been borrowed and directly extracted from the Results section and Figures 2 and 5 of Chmielewski-Stivers *et al.,* 2021.

3.2.1 Female-specific protection against cardiotoxic effects of radiotherapy

To further characterize sex differences following anticancer therapies, a single high dose of 19 Gy local irradiation, known to elicit cardiotoxicity, was given to adult WT male and female mice. Long-term cardiotoxicity was investigated 53 weeks post-radiotherapy, where 75% of males compared to only 20% of females died (log-rank (Mantel–Cox) test, p=0.0021), showing a female-specific protection from cardiac irradiation (Fig. 12A).

Echocardiography was used to monitor heart function in animals. In WT females, cardiac function monitored by the ejection fraction (%EF) was similar in control and irradiated animals (data not shown), whereas irradiated WT males exhibited a 25% and 15% decrease in %EF at 20- and 50-weeks post-radiotherapy, respectively, as compared to age-matched controls (p=0.003) (Fig. 12B). Histopathological analysis confirmed the functional results obtained by ultrasound. No significant structural alteration of cardiac ventricles was found in females until 50 weeks post-radiotherapy (Fig. 12C). On the contrary, a dramatic disruption of ventricular structure associated with cardiomyocyte atrophy was observed 20 weeks post-irradiation. This pathogenic pattern worsened 50 weeks post-radiotherapy. Large patches of scar tissue infiltrating the septum and right ventricle were observed, indicative of delayed and massive death of cardiomyocytes. Resultant replacement amyloidosis/fibrosis altered proper

contractility of the cardiac muscle and caused loss of cardiac function as measured by ultrasound (Fig. 12C).

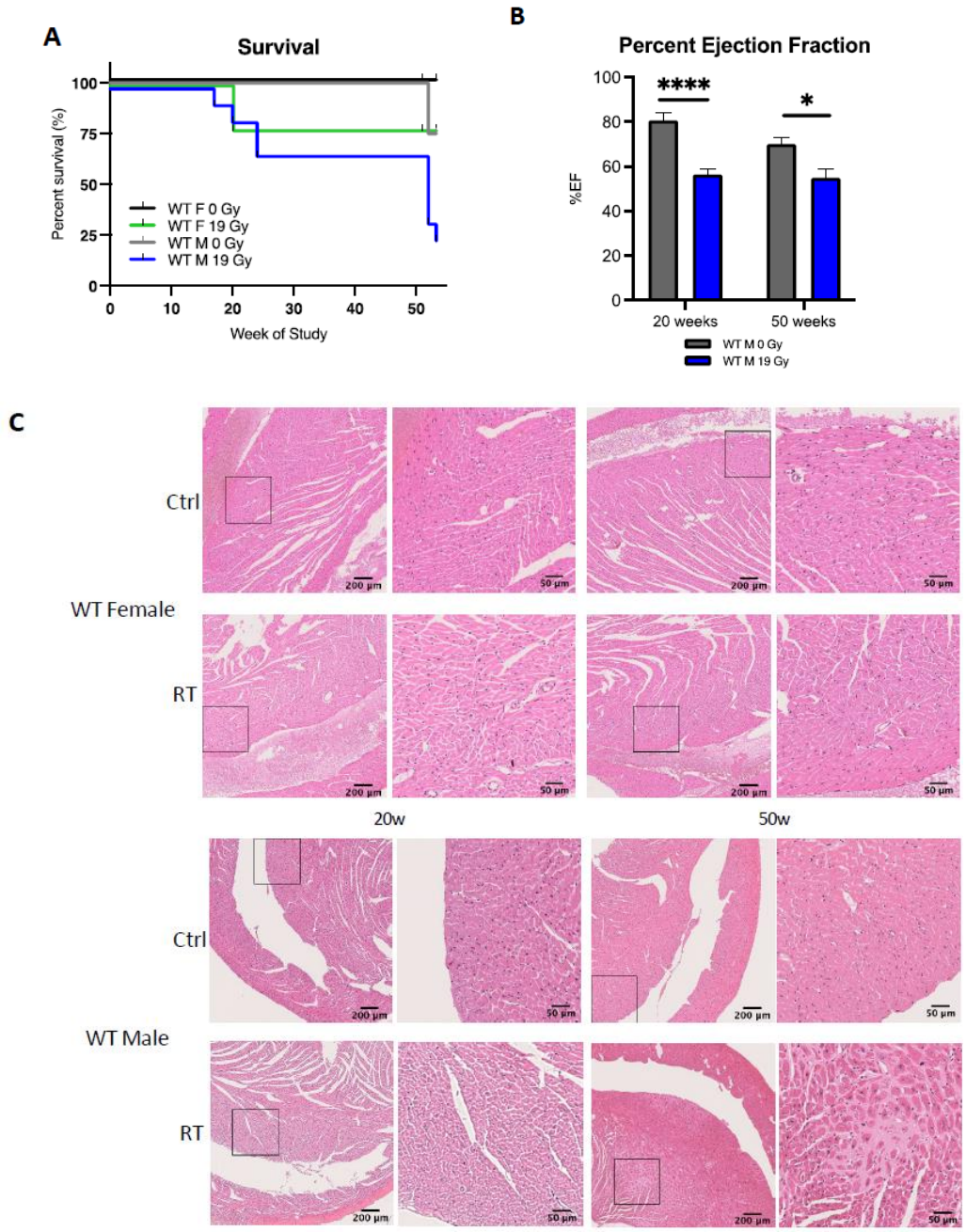
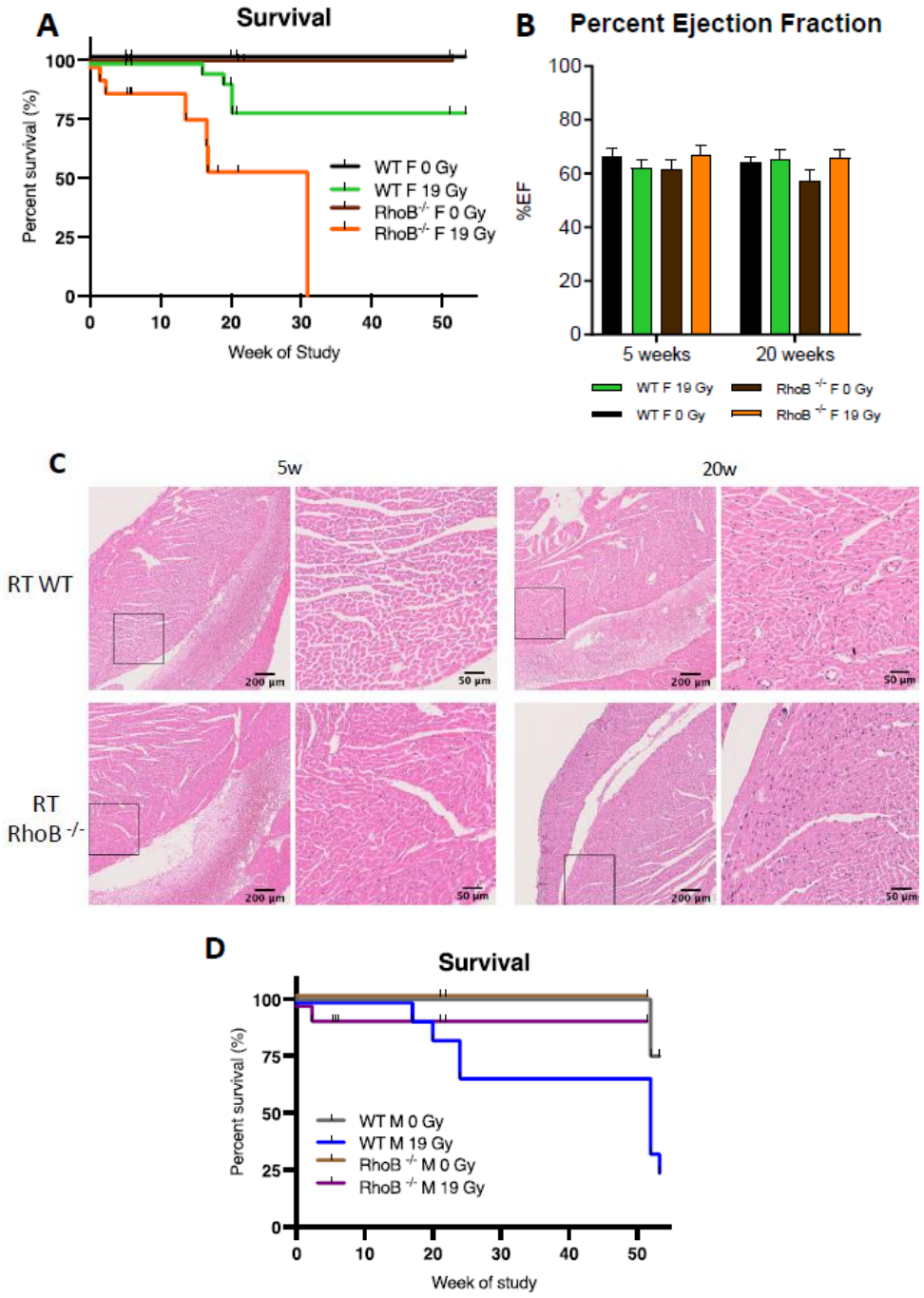


Figure 12: Wild-type females are protected from cardiotoxicity induced by radiotherapy (19 Gy). (**A**) Survival curves show that 77% of WT males died (n=9), while 25% of WT females (n=12) were alive 50 weeks post-19 Gy thoracic irradiation. Log-rank (Mantel–Cox) test p=0.0021. (**B**) Significant change in %EF after exposure was shown in WT males (p=0.0003). Multiple comparisons measure significant changes after 19 Gy at 20 weeks (p<0.0001) and 50 weeks (p=0.0127). Graphs designate mean ± s.e.m; p-value derived from multiple comparisons mixed-effect model (REML), Bonferroni test: * p<0.05 and **** p<0.0001. (**C**) Representative images of H&E-stained cardiac tissue showed no alteration of cardiac structure 20 weeks post-radiotherapy in females and males with a sex-specific evolution in time. Alterations of the cardiac structure were observed in males 50 weeks post-radiotherapy with larges patches of replacement amyloidosis/fibrosis deposition and scarring tissue associated with immune infiltration, whereas in females, cardiac structure remained quasi-normal.

3.2.2 RhoB deficiency triggers opposite effects in female and male mice exposed to radiotherapy

In females, estrogen is known to be a major cardioprotective factor, which provides the rationale for exploring whether RhoB$^{-/-}$ would decrease the female-specific protection against 19 Gy radiation-induced cardiotoxicity observed in WT female mice. Indeed RhoB$^{-/-}$ females exhibited an increased sensitivity to radiotherapy exposure with death occurring at early time points, reaching (lethal dose) LD_{50} at 16 weeks and an LD_{100} at 30 weeks, while 80% of WT females survived to the end of the study 50 weeks post-irradiation (Fig. 13A).
While alterations in cardiac function were not identified by ultrasound (Fig. 13B, unpaired t-test p=0.0231), histological analyses did reveal enhanced infiltration of leukocytes in cardiac ventricles in RhoB$^{-/-}$ females 20 weeks post-radiotherapy (Fig. 13C). Interestingly, in males, the reverse was observed, as RhoB$^{-/-}$ males were more tolerant to radiotherapy whereas 75% of the WT males died post-radiotherapy (Fig. 13D). Further, no alteration of cardiac function was found in RhoB$^{-/-}$ males by ultrasound (data not shown), and no histologic damage or fibrosis infiltration was detected (Fig. 13E). Importantly, these results identified for the first time a sex-specific difference in response to radiotherapy, with RhoB as a major molecular determinant. In females, RhoB might mediate cardioprotection after irradiation via activation of the estrogen pathway, whereas in males, RhoB might mediate cardiotoxicity via activation of fibrogenic pathways (Bourgier *et al.*, 2005; Monceau *et al.*, 2012).



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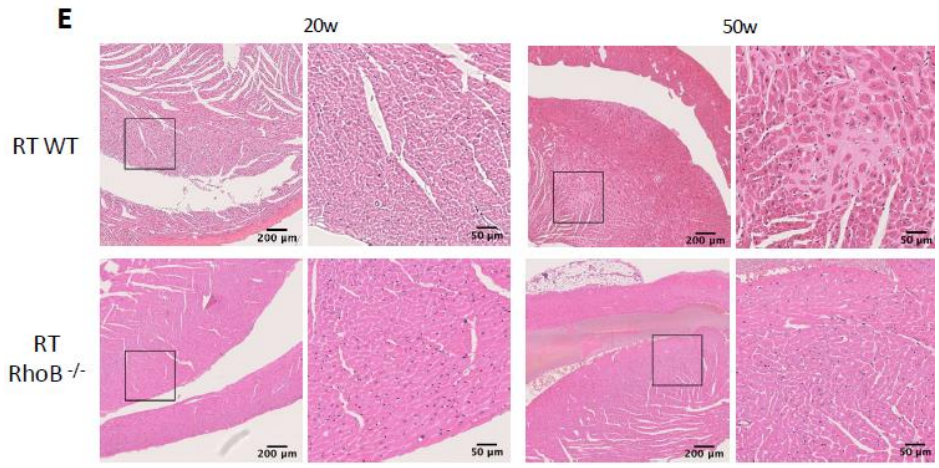

Figure 13: RhoB deficiency reverses cardioprotection in females exposed to radiotherapy. (**A**) RhoB-deficient females reached LD100 after 19 Gy thoracic at 30 weeks post-irradiation, whereas WT females exhibit 80% survival. Significant change between RhoB$^{-/-}$ 0 Gy (n=14), RhoB$^{-/-}$ 19 Gy (n=18), WT 0 Gy (n=32), and WT 19 Gy (n=32), and female mice exposure survival curves ($p<0.0001$) in log-rank (Mantel–Cox) test. (**B**) No change in %EF was observed in WT and RhoB$^{-/-}$ animals 5- and 20-weeks post-RT. Graph designates mean ± s.e.m. (**C**) H&E staining analysis showed no alteration of cardiac structure 5 weeks post-irradiation and enhanced leukocyte infiltration at 20 weeks. (**D**) RhoB-deficient males (n=15) were protected from radiation-induced toxicity as compared to WT males (n=9). (**E**) Representative images of H&E-stained cardiac tissue showed no alteration of cardiac structure 20- and 50-weeks post-irradiation in WT and RhoB$^{-/-}$ males.

Chapter 4 Discussion

(The following *Discussion* section has been extracted from Chmielewski-Stivers *et al.,* 2021 *Discussion* and *Conclusion*)

Recently, sex-related differences in cancer incidence have increasingly been recognized and attributed to regulation at the genetic and molecular level and to sex hormones such as estrogen. Surprisingly, and despite the emergence of tailored treatments and personalized medicine, the impact of sex on therapeutic outcome and tolerance to treatment to date has been poorly investigated. These investigations are of utmost relevance since sex-specific differences may not only affect tumor biology but also the pharmacokinetics and dynamics of drugs and response to local treatment such as radiotherapy. In this context, the present experimental study was designed to investigate the impact of sex following two classical anticancer treatments, i.e., paclitaxel and radiotherapy. Both are widely used and administered according to the type and grade of cancer, but importantly, not according to the sex of the patient. Taking thoracic malignancies as a paradigm, the impact of paclitaxel on neurocognition and of radiotherapy on cardiac function were investigated in two distinct cohorts of female and male mice. Interestingly, our results are the first to show that female mice are protected from paclitaxel-induced neurotoxicity as well as from radiotherapy-induced cardiotoxicity. We also identified RhoB as an organ and sex-specific molecular determinant. Neuroprotection was not RhoB-mediated in either sex, whereas cardioprotection was RhoB-mediated in an opposite manner between female and male mice.

Accumulating evidence supports sex-related response to chemotherapeutic agents with differences in efficacy and toxicity (Kim, Lim and Moon, 2018). For instance, Joerger et al.

reported significant variation in the pharmacodynamics of paclitaxel in female patients with solid tumors compared to male patients. Females exhibited 20% lower elimination of paclitaxel than male patients did, with their peripheral compartments saturated at lower plasma concentrations (0.83 female vs. 1.74 mmol/L male) and paclitaxel elimination was slower (1 h female vs. 0.5 min male) (Joerger *et al.*, 2006). Enhanced sensitivity of female patients to paclitaxel is further supported by the high number of female patients exhibiting severe leukopenia upon combined treatment with paclitaxel and carboplatin (Yamamoto *et al.*, 2008; Schmetzer and Flörcken, 2012). Conversely, female patients diagnosed with lung carcinoma who were treated with paclitaxel combined with carboplatin showed longer median progression-free survival (PFS) rate than male patients did (Yamamoto *et al.*, 2008; Schmetzer and Flörcken, 2012).

Our present investigation focused on the adverse neurocognitive consequences of paclitaxel as neurotoxicity is known to be one of the more prominent side effects of paclitaxel that can have a dramatic impact on a patient's quality of life. This question was investigated using a series of cognitive tests to interrogate locomotor activity, anxiety-like behavior, and fear extinction as a higher-order measure of cognitive flexibility. The five-day context-dependent fear extinction task used examined the ability to acquire and then extinguish a tone-associated fear memory. Whereas the ability to acquire a fear memory is predominantly amygdala mediated (Maren and Quirk, 2004), long-term (four day) extinction recruits medial prefrontal cortex activity (Phelps *et al.*, 2004), engaging in translationally relevant executive function necessary for managing quality-of-life in cancer therapy recipients.

Present data indicate that female mice were more tolerant than males to higher doses of paclitaxel, selected to be comparable with dosages used in breast cancer patients (Nair and Jacob, 2016). Our results are consistent with a recent study by Liang et al., who found that at a lower peripheral neuropathy-inducing dose of paclitaxel (16 mg/kg), female C57B6 mice exhibited protection against behavioral deficits compared to males but both sexes showed mechanical pain hypersensitivities, indicative of peripheral neuropathy (Liang *et al.*, 2020). Liang et. al. also explored the molecular signature and found altered gene expression related to neurotransmission suggesting dysfunction in the medial prefrontal cortex (mPFC), known to be critical in both positive and negative regulation of extinction memory (Quirk, 2002). The other reported mechanisms of paclitaxel-induced neurotoxicity involved axonal/neuronal mitochondrial dysfunction, altered calcium homeostasis and calcium channel expressions, changes in peripheral nerve excitability, including altered expression and function of ion channels, immune dysfunction, and neuroinflammation at the level of axons, dorsal root ganglia, and within the spinal cord (Zajączkowska *et al.*, 2019), as well as direct effects on brain tissues (Gangloff *et al.*, 2005; Ferrari-Toninelli *et al.*, 2008; James *et al.*, 2008; Ferris *et al.*, 2019).

In the present study, we chose a more physiological and functional approach to investigate the contribution of female hormones. While the majority of published studies have focused on the contribution of testosterone (Liang *et al.*, 2020), the role of female hormones has been less investigated. Notwithstanding, chemotherapeutic agents including paclitaxel are known to accelerate menopause and induce drops in hormone levels associated with cognitive impairments. The precise mechanisms linking estrogen to cognition are however complex, as

estrogen supplementation has been shown to improve cognitive function (Bender *et al.*, 2001; Jenkins *et al.*, 2005). Wang et. al., for example, observed alternative downstream estrogen receptor-mediated signaling activity depending on endogenous or exogenously administered estrogen facilitating memory-dependent long-term potentiation in female rat brain tissue (W. Wang *et al.*, 2018). Based on the foregoing, we decided to implement a genetic approach through the use of RhoB–deficient mice that disrupts estrogen signaling and thought to use aged females with naturally lower levels of female hormones. Interestingly, data indicated that improved performance on select cognitive tasks in female mice was not dependent on RhoB-dependent estrogen signaling or female hormones. Using either model (RhoB–deficient or aged female mice), neurocognitive impairments observed after Paclitaxel treatment were not enhanced. While the exact mechanisms associated with this enhanced neurologic tolerance will require further investigation, immune regulation as suggested by Liang et al., or increased resistance of oligoprogenitor cells (OPC) responsible for maintaining the state of CNS myelination are plausible possibilities.

In addition to neurological side effects, cardiotoxicity of various cancer therapies defines another major concern in the field of oncology. Specifically, cardiac damage induced by radiotherapy remains a critical dose-limiting factor (Darby *et al.*, 2005; Schultz-Hector and Trott, 2007; Andratschke *et al.*, 2011; Bouillon *et al.*, 2011) despite recent advancements in treatment planning and image-guided radiation therapy. Additionally, as the number of long-term cancer survivors is increasing, complications emerge that can dramatically impair quality of life. Whereas over the past four decades, research has enhanced our understanding of the patho-physiological, cellular, and molecular processes governing radiation-induced cardiac toxicity

(Boerma *et al.*, 2016), the impact of sex remains relatively undefined and under investigated (Schlaak *et al.*, 2020). In clinical and preclinical studies, proper comparisons between female and male patients have never been done as they have not included corrections for the volume of irradiation (Schlaak *et al.*, 2020), and only a few preclinical studies have ever included both sexes. Until now, experimental studies on radiation-induced cardiotoxicity were mainly conducted in male mice (Gabriels *et al.*, 2012; Monceau *et al.*, 2013, 2014; Seemann *et al.*, 2013), largely based upon assumptions coming from the cardiovascular field stating that females should be more resistant than males to cardiac diseases. However, cardiovascular disease in women remains understudied, underdiagnosed, and undertreated, and even now, women remain under-represented in clinical trials related to cardiac disease (Mauvais-Jarvis *et al.*, 2020). For nearly 20 years, the contribution of estrogen has been identified in the pathogenesis of heart failure because postmenopausal women have increased risk of developing cardiac diseases (Hayward, Kelly and Collins, 2000). Estrogen has been shown to attenuate the development of pressure overload-induced hypertrophy in mice (van Eickels *et al.*, 2001; Aryan *et al.*, 2020) through regulation of nitric oxide synthase (NOS) (Loyer *et al.*, 2007).

In this context, and to parallel our studies with paclitaxel, the role of RhoB deficiency in cardiac toxicity was investigated in both sexes by image-guided focal radiotherapy. Present findings revealed an enhanced tolerance of C57Bl6 female mice to radiotherapy, as compared to the male mice. Data presented here also support the role of estrogen in female tolerance (Horton, White and Maass, 2004; Choudhry *et al.*, 2005; Kher *et al.*, 2005; Shufelt *et al.*, 2018), as RhoB-deficient females having disrupted estrogen signaling through ERα are sensitized to

radiotherapy. While limited sample sizes of aged female mice precluded more comprehensive cardiac investigations in the present study, our findings with RhoB-deficient male mice found them to be protected from radiation injury.

Interestingly, our study revealed opposite effects of RhoB deficiency on cardiac outcomes in female and male mice. Whereas in females, RhoB deficiency short-circuited the protective estrogen pathway, in males it disrupted the deleterious fibrogenic pathway dependent upon RhoB/CTGF identified in our previous study (Monceau *et al.*, 2014). Consistent with these prior findings, no fibrosis deposition was observed and cardiac function was maintained. Whether this observation is specific to this strain of mouse will require further investigation, but our findings highlight the need to design future investigations to directly assess the impact of sex on organ-specific toxicities induced by anticancer therapeutic regimens.

Further work is clearly required to unravel the mechanistic basis of our findings and explore how they might impact personalized treatments in clinical practice. Neurocognitive assessments were undertaken to survey the impact of systemic paclitaxel across multiple brain regions, but the precise levels of paclitaxel crossing the blood–brain barrier into discrete regions and among the different age groups were not assessed. More importantly, the impact of combined treatment (paclitaxel + radiotherapy) was not investigated, whereas the combination is expected to enhance cardiac toxicity and possibly influence neurocognitive outcome by the release of paracrine factors. Future studies undertaking direct measurement of circulating and tissue-specific estrogen levels along with other nongenetic targeted interventions aimed at dissecting fibrogenic signaling cascades would also provide deeper

mechanistic insight into tissue remodeling processes affecting the heart and surrounding organs (e.g., esophagus and lung).

In conclusion, our results are the first to identify sex- and organ-specific responses to systemic paclitaxel administration and localized radiotherapy with enhanced tolerance in WT females. These results may have important implications for the management of cancer patients and implementation of personalized medicine in oncology. Further investigations should include female and male cohorts and the influence of combined strategies that may encompass unforeseen off-target effects.

Chapter 5 Future Directions

(The remainder of this dissertation is original to this work)

Over the course of my environmental toxicology studies, two emerging themes have shaped the way I think about health and disease. First, that immune dysfunction is the cause and/or effect of all disease (Franceschi and Campisi, 2014) and, second, that sexually dimorphic mechanisms and strategies of immune response (Klein and Flanagan, 2016; Lopes *et al.*, 2017; Natri *et al.*, 2019; Márquez *et al.*, 2020; Takahashi and Iwasaki, 2021) are perhaps among the most important difference between biological sex (other than reproduction). Case in point, sex-differences in immune function has recently come front and center in the discussion of the symptoms and disease characterization of the current pandemic.

The evolving clinical data on COVID-19 increasingly indicates sex-specific immune-dependent risks for developing severe acute and long-term chronic symptoms associated with SARS-CoV-2 infection (Brodin, 2021; Takahashi and Iwasaki, 2021). Sex-specific data from the first year of the pandemic suggests men have higher risk of developing severe symptoms, almost three times more likely of requiring intensive care (OR=1.39) (Peckham *et al.*, 2020), and have about 1.7 times higher risk of death (Scully *et al.*, 2020), compared to women. On the other hand, women have been found to suffer disproportionately from persistent respiratory symptoms and systemic pain following recovery from acute viral COVID infection. One 2020 observational study of 201 adults in the United Kingdom who had recovered from acute SARS-CoV-2, but still had persistent symptoms of respiratory and/or daily life functional impairment, was predominantly composed of women (71%), with most frequent symptoms including fatigue, breathlessness, and body aches (83%-98%) (Dennis *et al.*, 2021). Interestingly, the study

also identified that radiologically diagnosed myocarditis (heart inflammation) was significantly associated with severe post-COVID-19 syndrome ($p>0.05$). These developing data on COVID-19 pathology are consistent with our current understanding of sex-differences in immunity, most notably that females have more robust acute viral response and vaccine efficacy but are also more likely to suffer from long-term chronic immune dysfunction, compared to males, as a result of robust immune activity.

Such significant clinical observations about sex-differences during the current pandemic have highlighted and, hopefully, launched further discussions about sex-specific mechanisms of immune response and disease across scientific disciplines. This current era also provides real world context and relevancy to the sex-specific literature reviewed and experimental findings of this thesis. Furthermore, I believe it is important and useful to discuss the experimental findings in the context of rapidly evolving relevant sex-specific research that did not exist prior to the initiation of paclitaxel investigations for the purpose of suggesting future hypothesis-driven research directions.

5.1 Sex-differences in paclitaxel-induced immune response

Sex-differences in the innate and/or adaptive immune responses are perhaps the most important characterizations of disease and paramount for treating and curing the most onerous chronic conditions that impact public health today, such as neurodegenerative and age-related diseases (Acharya *et al.*, 2016; Guerrero, de Strooper and Arancibia-Cárcamo, 2021). Diversity, variability, and biological unknowns of human populations in preclinical and clinical data often challenge and provide exceptions to generalizations. Based on my current understanding, across all ages, females have greater innate and adaptive immunity compared to males, but

aging males develop greater innate immunity, while females rely more on adaptive immunity when older (Márquez *et al.*, 2020; Haupt *et al.*, 2021). As demonstrated in the 'U' curve comparing young and old WT female mouse CD45+ cells in cardiac tissue (Fig. 9 & 10), age and reproductive status-dependent regulation of immune response complicates disease phenotypes and needs further elucidation. The CD45 staining of leukocytes was not specific enough to discriminate possible lymphocyte and monocyte/adaptive and innate immunity ratios, rendering definitive histological characterizations unreliable. Future investigations into more specific cardiac immune infiltration could clarify if clinical trends of tempered adaptive immunity in aging women translates to aging female mice across different genetic backgrounds, and whether female mice have higher lymphocyte levels than males (characteristic of adaptive and humoral immunity).

5.2 Role of RhoB in immune response

Although, not initially hypothesized, a plausible explanation for the lack of sex-differences in weight, survival, and cognitive data in RhoB deficient animals after paclitaxel exposure may be due to attenuated RhoB-dependent B-cell activation in females. As initially hypothesized, RhoB-dependent cellular protection pathways, via estrogen modulation, was posited to drive sex-specific protection in females. Therefore, RhoB-deficient females were expected to mirror weight, survival, and cognitive dysfunction seen in WT males. As hypothesized, there was no difference in male WT and RhoB-deficient weight change and survival, but RhoB-deficient males did not exhibit paclitaxel-induced cognitive dysfunction (Fig. 7), therefore some alternative neuroprotective pathways may be involved. In addition, seemingly paradoxically, RhoB-deficient females exhibited more weight loss than WT females at

150 mg/kg paclitaxel doses (Fig. 6), supporting our hypothesis, but on the other hand, had better survival and less weight loss compared to WT females at 300 mg/kg paclitaxel doses (Fig. 8), not supporting our hypothesis for RhoB-dependent cellular protection and survival. Upon further literature investigation, RhoB is implicated in immune-modulation, including in macrophage function (reviewed Vega and Ridley, 2018) and endothelial cell activity (Kroon *et al.*, 2013). In addition, RhoB deficient mice (male and female) were observed to have attenuated autoimmune responses in a rheumatoid arthritis and lupus model. RhoB deficiency was observed to selectively inhibit self-antigen responses and was likely a critical component for B cell activation (Mandik-Nayak *et al.*, 2017). Therefore, if WT females preferentially engaged in (RhoB-mediated) adaptive immunity and humoral responses (i.e. B cell activation) upon paclitaxel toxic exposure (explained in *Peripheral Neuropathy* section), then RhoB deficiency may short circuit preferential adaptive immune mechanisms in females and alters peripheral neuropathy as well as the risk of developing long-term autoimmune phenotypes. Notably, at 300 mg/kg paclitaxel, much more toxic to WT males than females (Fig. 1), RhoB-deficient females had a distinct advantage in terms of weight loss, survival, and cognitive impairment compared to WT females (Fig. 8). At this dose, the humoral response may have triggered autoimmune dysfunction in WT females, while the lack of autoimmune capabilities could have protected RhoB-deficient females at high doses. As suggested before, characterizing lymphocyte to monocyte ratio in cardiac tissue (or other tissues suck as lung) between WT and RhoB-deficient animals could provide further insight into sex-specific immune responses after toxic exposures.

5.3 Sex-specific androgen receptor function in chemotherapy toxicity

As outlined earlier in *Microtubule Dynamic Dysfunction*, a developmentally critical, Y-linked gene that modulates nuclear androgen receptor (AR) signaling, KDM5D79, may be risk factor for male-specific tissue exposed to high doses of taxanes (Komura *et al.*, 2016). Based on research on taxane treatment in prostate cancer, it is clean that taxane-induced microtubule dysfunction, inhibiting AR signaling, is a dominant antimetastatic mechanism of action in prostate cancer as well as for inducing normal tissue damage in males. However, AR is also functionally important in females. In considering sex-differences, comparing male testosterone function (through AR signaling) and female estrogen function (through ER signaling) as dominant regulators of cellular function between each sex during systemic stress may provide insight into clinically observed outcomes during and after cessation of cancer treatments. For example, in other chemotherapy or toxicological exposures that do not directly inhibit AR signaling, AR may provide male-specific protection through reducing immune-mediated lethal side effects, such as neutropenia. The development of neutropenia and high-grade neutropenia during chemotherapy treatment are more common and severe in women than in men (Abdel-Rahman, 2019). In male mouse studies, castration or AR deficiency is known to cause neutropenia, with AR knockout (KO) causing a more severe phenotype than castration, suggesting AR is more important than testosterone in granulopoiesis and neutrophil function in males (Lai *et al.*, 2012). Based on this information, taxane-induced AR signaling disruption may have caused lethal neutropenia in the male mice in this study. This information also suggests that for other chemotherapeutics not affecting AR signaling, males may have reduced risk of developing neutropenia, compared to females, due to higher baseline levels of testosterone

and AR signaling function. Unfortunately, no castration or ARKO studies have examined granulopoiesis or neutrophil function in females, only that ARKO females are known to have impaired fertility (Walters *et al.*, 2009) which highlights the functional importance of AR in females. Comparing such AR responses between the sexes may inform sex-specific disparities of immune function and common phenotypes like chemotherapy-induced neutropenia.

5.4 Immune response in cancer progression and treatment

As cancer therapies evolve into the realm of personalized medicine, a one-fit-all approach must be adapted to account for sexual dimorphisms in treatment response. Immune-mechanisms associated with chemotherapy symptoms, including hypersensitivity reactions, neuropathies, and cognitive dysfunction, are known to be regulated by hormones, genetics, and epigenetics dependent on sex and age (Cook *et al.*, 2011; Kim, Lim and Moon, 2018; Özdemir and Dotto, 2019; Shin, Jung and Moon, 2019; Wang, Cowley and Liu, 2019; Wagner, 2020; Haupt *et al.*, 2021).

Interestingly, sex has emerged as a critical factor for the development, efficacy, and advancement of new targeted cancer immunotherapy modalities (Özdemir and Dotto, 2019; Wang, Cowley and Liu, 2019; Klein and Morgan, 2020; Ye *et al.*, 2020). It is hypothesized that because females have stronger baseline immune system activity, tumors with high antigenicity are eradicated quickly and metastasize less frequently in women, compared to men. Reduced tumor antigenicity could also translate to weaker responses to targeted immunotherapy. In support of this tenet, women have been shown to have better outcomes with combination therapies involving chemo/radiotherapy and immune checkpoint inhibitors combination (Wang, Cowley and Liu, 2019). Men, on the other hand, have a weaker immune response and higher

tumor antigenicity and generally show improved outcomes with immune checkpoint inhibitors (or immunotherapy) monotherapies (Wang, Cowley and Liu, 2019). This emerging hypothesis is summarized in **Image 4**, depicting possible sex-differences in cancer and cancer-therapy immune response and patient outcomes. Although this schematic does not apply to all cancer types and treatment outcomes in men and women, it does provide a framework for interpreting certain clinical data. One theme that can be drawn from this overview is the lower risk of developing cancer, higher survival outcomes, but also the increased risk of cancer treatment-induced adverse effects in women, compared to men. Sex plays a significant impact not only in the trajectory of sex-specific cancers (i.e. gynecologic, breast, and testicular), but also non-sex-specific cancers and disease, perhaps in a large part due to immune response differences in female and males.

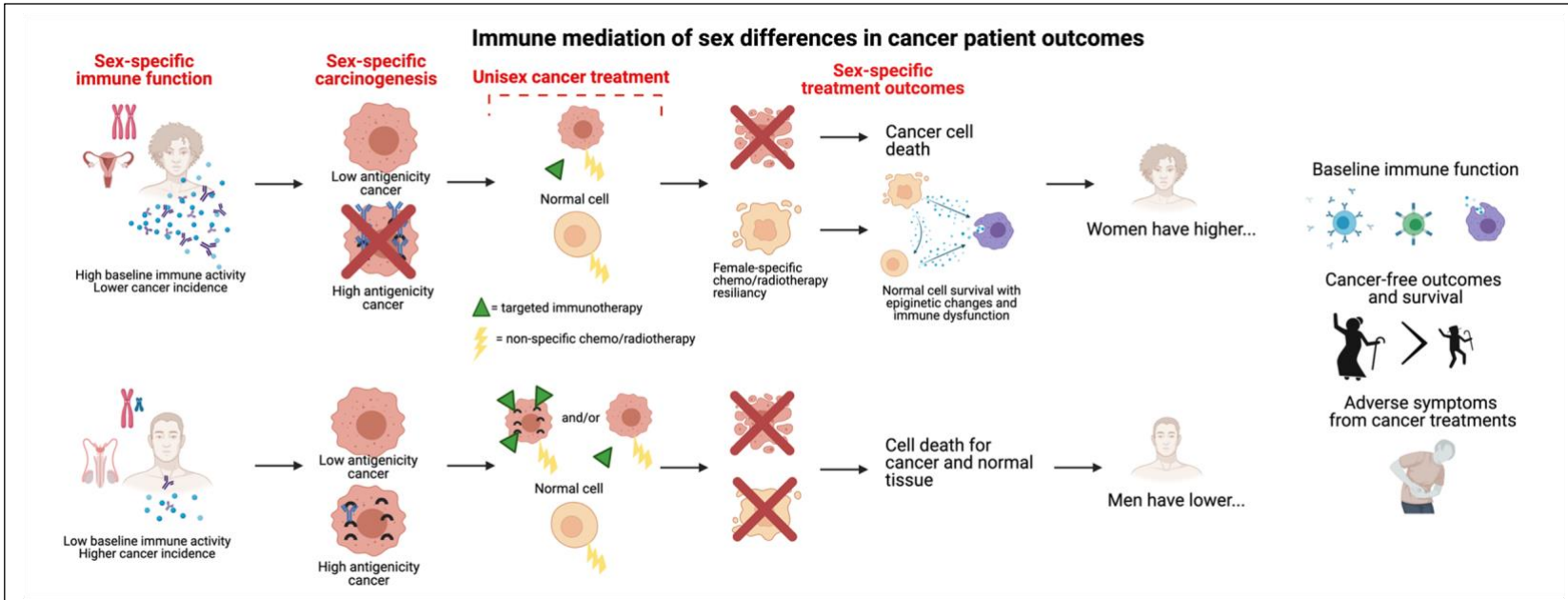

Image 4: Hypothesized immune-mediated sex-differences in cancer development and outcomes. Created with BioRender.

5.5 Conclusion

Through the course of my dissertation research and writing, I have realized that addressing sex as a biological variable as a standard for future biomedical research is paramount to future scientific advances. Although the initial hypotheses presented were not all supported by experimental results, in reviewing existing scientific literature through the lens of sex-differences, I was able to connect mechanisms studied in different fields and provide new evidence-based hypotheses for the sex-specific and RhoB-dependent experimental results of my thesis. Through this research I now hypothesize that 1) females and males have distinct paclitaxel-induced immune activation strategies, specifically, females favor humoral immunity and males favor innate immunity, 2) RhoB deficiency in females inhibits preferential humoral immunity, possibly causing greater acute paclitaxel-induced systemic toxicity, but may also prevent the development of female-specific long-term autoimmune-induced neuro and cytotoxic phenotypes, and 3) the critical function of androgen receptor signaling in males causes sex-specific toxicity through paclitaxel-induced microtubule dysfunction. Clearly, sex-hormone mediated immune response pathways account for sex-differences in normal tissue cytotoxicity of cancer therapies.

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